A Gene Discovery Lab Manual For Undergraduates:

Searching For Genes Required To Make A Seed

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I. PIPETTING EXERCISE

Purpose: To learn how to use pipettes

Taken From: DNA Science: A First Course, Second Edition
Laboratory 1: Measurements, Micropipetting, and Sterile Techniques
p. 327-328. (ISBN 978-087969636-8)

Solutions Needed:

- Four Dye Solutions Labeled I-IV
  - Solution I: Blue
  - Solution II: Red
  - Solution III: Yellow
  - Solution IV: Green

Materials Needed:

- Set of pipettes (P-10, P-20, P-200 & P-1000)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack

PROCEDURE

A. Small Volume Pipette Exercise

This exercise simulates setting up a reaction, using a pipette with a range of 1-10 μL or 2-20 μL.

1. Use a permanent marker (sharpie) to label THREE 1.5 mL tubes **A**, **B** and **C** and **your initials**.
2. Use the table below as a **checklist** while adding solutions to each reaction tube.

Tube	Sol. I (Blue)	Sol. II (Red)	Sol. III (Yellow)	Sol. IV (Green)	Total Volume
A	4 μL	5 μL	1 μL	-	10 μL
B	4 μL	5 μL	-	1 μL	10 μL
C	4 μL	4 μL	1 μL	1 μL	10 μL

3. Set the pipette to **4 μL** and add **Solution I** to each reaction tube.
4. Use a *fresh tip* to add the appropriate volume of **Solution II** to a clean spot inside reaction tubes **A**, **B** and **C**.
5. Use a *fresh tip* to add 1 μL of **Solution III** to tubes **A** and **C**.
6. Use a *fresh tip* to add 1 μL of **Solution IV** to tubes **B** and **C**.
7. Close lids. Pool and mix reagents by using one of the following methods:
    a. Sharply tap the tube bottom on the bench top. Make sure that the drops have pooled into one drop at the bottom of the tube.

    Or

    b. Place the tubes in a microcentrifuge and apply a short, few-second pulse. Make sure that the **reaction tubes are placed** in a **balanced configuration** in the microcentrifuge rotor. *Caution: Spinning tubes in an unbalanced position will damage the microcentrifuge*.
8. A **total** of **10 μL** of reagents were added to each reaction tube. To check that the previous pipetting measurements were accurate, set the pipette to 10 μL and very carefully withdraw the solution from each tube.
    a. Is the tip just filled? What does this suggest?

    Or

    b. Is a small volume of fluid left in tube? What does this suggest?

    Or

    c. After extracting all the fluid, is an air space left in the tip end? What does this suggest? (The air can be displaced and the actual volume determined simply

by rotating the volume adjustment to push the fluid to the very end of the tip. Then, read the volume directly.)

9. If several measurements were inaccurate, repeat this exercise to obtain near-perfect results.

B. Large Volume Pipette Exercise

This exercise simulates a bacterial transformation or plasmid preparation, for which a P-1000 pipette is used. It is far easier to measure incorrectly when using a large-volume pipette. If the plunger is not released slowly, an air bubble may form or solution may be drawn into the piston.

1. Use a permanent marker to label TWO 1.5 mL microcentrifuge tubes **D** and **E** and **your initials**.
2. Use the matrix below as a **checklist** while adding solutions to each reaction tube.

Tube	Sol. I (Blue)	Sol. II (Red)	Sol. III (Yellow)	Sol. IV (Green)	Total Volume
D	100 µL	200 µL	150 µL	550 µL	1000 µL
E	150 µL	250 µL	350 µL	250 µL	1000 µL

3. Set the pipette to add the appropriate volume of Solutions I-IV to reaction tubes **D** and **E**. Follow the same procedure as for the Small Volume Pipette Exercise to add **Solutions I-IV** to each reaction tube.
4. Close lids. Pool and mix reagents by using one of the following methods:
    a. Sharply tap the tube bottom on the bench top. Make sure that the drops have pooled into one drop at the bottom of the tube.

    Or

    b. Place the tubes in a microcentrifuge and apply a short, few-second pulse. Make sure that the **reaction tubes are placed** in a **balanced configuration** in

the microcentrifuge rotor. <u>Caution:</u> *Spinning tubes in an unbalanced position will damage the microcentrifuge.*

5. A **total** of **1000 µL** of reagents were added to each tube. To check that the measurements were accurate, set the pipette to 1000 µL and very carefully withdraw the solution from each tube.
    a. *Is the tip just filled? What does this suggest?*
    Or
    b. *Is a small volume of fluid left in tube? What does this suggest?*
    Or
    c. *After extracting all the fluid, is an air space left in the tip end? (The air can be displaced and the actual volume determined simply by rotating the volume adjustment to push the fluid to the very end of the tip. Then, read the volume directly.)*
6. If several measurements were inaccurate, repeat this exercise to obtain near-perfect results.

II. SERIAL DILUTION EXPERIMENT

Purpose: To test the accuracy and precision of pipetting

Reference: Anhthu Bui

Introduction: ***Diluting*** is simply the **addition of a solution** (or plain solvent) to a **substance** in order to **decrease the concentration of the latter substance**. In this exercise, the **substance** is **DNA** and the **solution** is **TE Buffer**. By the end of this exercise, you will learn how to calculate the dilution factor and determine the accuracy of your pipetting technique as determined by gel electrophoresis and spectrophotometer readings.

Solutions Needed:

- DNA stock (known concentration)
- TE Buffer
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL 1 Kb Plus DNA ladder (Invitrogen)
- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- Pipettes (P-10 & P-20)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- NanoDrop spectrophotometer
- Kimwipes
- 250 mL Erlenmeyer flask
- 25 mL Erlenmeyer flask
- Saran wrap
- Scale
- Microwave
- 55°C water bath
- Hot hand protector
- Gel cast

- Gel comb
- Round bubble level
- Gel box
- Cables
- Electrophoresis power supply
- Plastic container for carrying the gel
- Gel document system (Bio-Rad)

PROCEDURE

A. Serial Dilution of a DNA Stock

1. Label THREE 1.5 mL microcentrifuge tubes as:

   "**Dil #1**" for dilution #1

   "**Dil #2**" for dilution #2

   "**Dil #3**" for dilution #3

2. Pipet **15 µL** of **TE buffer** solution into each microcentrifuge tube in step 1. (Use the **P-20 pipette**)
3. Vortex the **DNA stock solution** for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.
4. Pipet **5 µL** of your **DNA stock solution** into the **Dil #1** microcentrifuge tube. (Use the **P-10** or **P-20 pipette**)
5. Vortex the contents of the tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.
6. Pipet **5 µL** of DNA solution from the **Dil #1** tube into the **Dil #2** tube.
7. Vortex the contents of the **Dil #2** tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.
8. Pipet **5 µL** of DNA solution from the **Dil #2** tube into the **Dil #3** tube.
9. Vortex the contents of the **Dil #3** tube for **5 seconds**. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.

B. Determination of Pipetting Accuracy by Gel Electrophoresis *(See Appendix 1A)*

1. Label THREE microcentrifuge tubes with the letters "**A**," "**B**," "**C**" and "**D**."
2. Pipet **10 µL** of DNA solution to tubes A, B, C and D:

from	**DNA Stock**	**Dil #1**	**Dil #2**	**Dil #3**
to	Tube **A**	Tube **B**	Tube **C**	Tube **D**

3. Pipet **2 µL** of **6x loading dye** into tubes **A**, **B, C** and **D**. Mix by pipetting up and down 5 times. The total volume for each solution is **12 µL**.
4. Load the contents of tubes A, B, C and D into lanes 1, 2, 3 and 4.
5. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the **anode** (positively charged) side of the gel box. (The **anode** is on the side **opposite** the wells.) *Note: Similar to ethidium bromide, SYBR Safe DNA Gel Stain is positively charged. Therefore, it migrates towards the negative side of the gel box, from anode to cathode. (Opposite the direction of DNA migration). Remember that DNA is negatively charged; so, it migrates to the positive end of the gel box. (DNA migrates from cathode to anode).*
6. Put the lid on the gel box and connect the electrodes to the power supply (**RED** to **RED** and **BLACK** to **BLACK**). *Note: SYBR Safe gel stain is unstable in UV or bright room light. If possible, run the gel in the **dark** by either turning off the lights, covering the gel with a cardboard box or aluminum foil, or run the gel inside of a drawer. Realistically, hours of constant UV or bright room light exposure are required to cause any significant loss of signal.*
7. Record the **identity of samples** loaded on the gel.

Lane	**Sample**
1	DNA Stock
2	Dilution #1
3	Dilution #2
4	Dilution #3

8. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) has migrated one-half or two-thirds of the gel length.

   Time power supply turned ON:

   Time power supply turned OFF:

   How long was the gel run? _____ hour(s) and _____ minutes

10. After 1-2 hours of running the gel, turn **off** the power supply.
11. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 4128A2.

    Caution: *It is a **MUST** to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking.*
12. Take a picture of the gel using the Bio-Rad Gel Document System. Label the picture using the text program of the Gel Document System. (*Your TA will show you how.*)

    Alternatively: Print out the picture. Tape it to a piece of paper by putting a piece of white tape at a position immediately above the wells. Label the wells with the sample names.
13. Print out the picture. Store the labeled picture in your lab notebook.

C. Determination of Pipetting Accuracy Using a Spectrophotometer

While running the gel, determine the **concentration** of DNA solutions in the tubes labeled "DNA Stock," "Dil #1," "Dil #2" and "Dil #3" by using the **NanoDrop Spectrophotometer** (Your instructor will demonstrate how to use the instrument).

What is a spectrophotometer? (See Appendix 1B)

1. For each tube, read the concentration at least TWICE, using a fresh drop each time.
2. Record the DNA concentration (in **ng/µL**) from each tube.

Sample	Concentration (ng/µl)
DNA Stock	
Dil #1	
Dil #2	

Dil #3	

D. Questions and Summary

1. *What did you expect to see on your gel?*
2. *How is your pipetting accuracy as determined by gel electrophoresis?*
3. *Does the gel result show what you expected? If not, what might be the problem?*
4. *What is the dilution factor in this exercise?*
5. *Given the stock DNA concentration is **1 µg/µL**, what is the expected DNA concentration in tubes "Dil #1," "Dil #2" and "Dil #3?"*

   Hint: Use the equation $\mathbf{V_i \times C_i = V_f \times C_f}$ where,

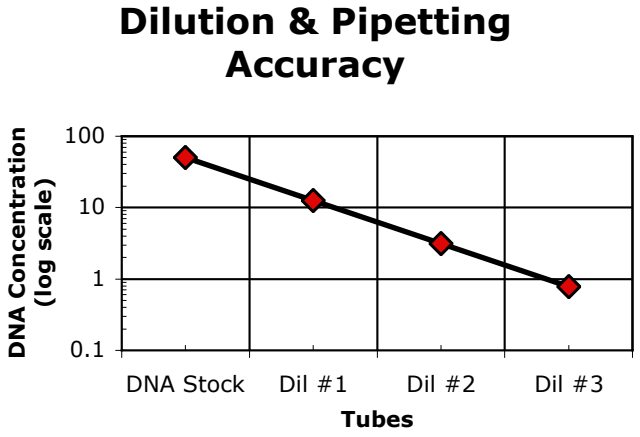
   $\mathbf{V_i}$ = **initial volume** (the volume of original DNA solution is **5 µL**)

   $\mathbf{C_i}$ = **initial concentration** (reading from the spectrophotometer; example: **1000 ng/µL**)

   $\mathbf{V_f}$ = **final volume** (the volume of Dil #1 is **20 µL**)

   $\mathbf{C_f}$ = **final concentration** (the concentration of Dil #1)

6. *Make a plot on log graph paper or Excel of the logarithm with base 2 of the expected DNA concentration (this will be your standard curve) as shown in the graph below:*
   The x-axis: Tubes (DNA stock, Dil #1, Dil #2 and Dil #3)
   The y-axis: The logarithm with base 2 of the expected DNA concentration



7. *Plot the logarithm with base 2 of the DNA concentration readings you obtained from the spectrophotometer.*
8. *How does your DNA concentration reading deviate from the expected DNA concentration?*

III. POLYMERASE CHAIN REACTION (PCR)

Purpose: To obtain a large amount of DNA molecules in a short time for other purposes, such as determination of DNA sequence or cloning into a vector for gene expression or promoter analysis.

Reference: Ex Taq DNA Polymerase Manual (Takara; see Appendix 1E)

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 µM Gene-specific Salk Forward primer
- 12 µM Gene-specific Salk Reverse primer
- 0.2 ng/µL *Arabidopsis* Columbia-0 genomic DNA
- 2 µl gene-specific PCR positive control (gene-specific PCR product made by TAs)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/µL 1 Kb Plus DNA ladder (Invitrogen)
- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- 1.5 mL sterile microcentrifuge tubes
- 0.2 mL sterile PCR tubes
- Microcentrifuge tube rack
- PCR tube rack
- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- Ice bucket, plastic container or Styrofoam box
- Microcentrifuge
- Minicentrifuge
- PCR machine
- Vortex
- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

A. Polymerase Chain Reaction (PCR)

Note: ALWAYS wear gloves and use filter tips to prevent contamination when preparing PCR reactions.

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer** and **dNTP mix** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed, put the tubes on **ice** until needed.
3. Thaw **12 µM Gene-specific Salk Forward primer** and **12 µM Gene-specific Salk Reverse primer** (corresponding to the gene to be knocked out) at room temperature as in step 2.
4. Place 0.2 ng/µL *Arabidopsis* Columbia-0 **genomic DNA** on ice.
5. Thaw 5 µL of positive control on ice.
6. Obtain **THREE** 0.2 mL sterile PCR tubes and set them on a rack for PCR tubes.
7. Write on the **lids** of the tubes the numbers **1**, **2** and **3**. Label the **sides** of the tubes with the **sample identity**, **your initials** and the **date** as follows: (*your TA will show you how to write on the tubes*)

   Tube #1: **Name of the gene**

   Tube #2: **Pos.** (Positive control containing gene-specific PCR product made by TAs)

   Tube #3: **Neg.** (Negative control containing same components as in tube #1, but **NO** genomic DNA)

   Keep the labeled PCR tubes in their rack on **ice**.
8. Obtain **ONE** 1.5 mL microcentrifuge tube and set it on a rack for 1.5 mL microcentrifuge tubes.
9. Write "**Mmix**" (for Master mix) on the lid of the tube with a black ultra-fine sharpie. Keep the tube on **ice**.
10. Briefly **vortex** each reagent. **Centrifuge** at full speed for 10 seconds. Place on **ice**.

11. Prepare Master Mix (**Mmix**) solution for **4 reactions** (**3 samples + 1 extra**) as follows:

Master Mix:

	Mmix for ONE reaction	**Mmix for 4 reactions**
Sterile water	36.75 µL	147.0 µL
10x Ex Taq buffer	5.0 µL	20.0 µL
dNTP mix	4.0 µL	16.0 µL
12 µM Gene-specific Salk Forward primer	1.0 µL	4.0 µL
12 µM Gene-specific Salk Reverse primer	1.0 µL	4.0 µL
Ex Taq DNA polymerase (5 Units/µL)	0.25 µL	1.0 µL
Total volume	**48.0 µL**	**192.0 µL**

a. Pipet the reagents in order from top down (example: water, 10x Ex Taq buffer, dNTP mix, etc.) into the **Mmix** tube.
b. After pipetting all reagents into the **Mmix** tube, close the lid of the tube. Mix the contents by vortexing at a **setting of 2-3** for **5 seconds**. Spin the tube in a microcentrifuge at FULL speed (13,200 rpm) for **10 seconds**. Put the tube back **on ice**. *Note: Do not vortex enzyme vigorously*.

12. Prepare the PCR reactions.
    a. Pipet **48 µL** of the **Mmix** solution into each PCR tube.
    b. Pipet 1-2 µL of DNA or water to tubes #1-3. (see table below)
    c. Immediately, mix the contents by **pipetting up and down at least five times.**
    d. Briefly spin tubes in the mini microcentrifuge for PCR tubes.

PCR reactions:

Components	**Tube #1 (Gene Name)**	**Tube #2 (Positive)**	**Tube #3 (Negative)**
Mmix	48 μL	48 μL	48 μL
Arabidopsis Col-0 ecotype genomic DNA (0.2 ng/μL)	2 μL	-	-
Control DNA template (Positive control)	-	1 μL	-
Sterile water (negative control)	-	1 μL	2 μL
Total volume	**50 μL**	**50 μL**	**50 μL**

13. Turn **ON** the PCR machine (MyCycler). Wait for one minute for the machine to initialize.
14. Put the PCR tubes in the wells of the 96-well hot plate of the Bio-Rad MyCycler.
15. Select the "**Protocol Library**" by pressing "**F1**."
16. Select "**HC70AL**" by pressing the yellow arrowheads surrounding the "**ENTER**" button. Select the "**HC70AL KNOCKOUT**" protocol. Press "**ENTER**."
17. The "CHOOSE OPERATION" menu will appear. Select "**VIEW PROTOCOL**." The **PCR profile** of the Knockout protocol is as follows:

    94°C 3 min
    36 cycles of 94°C 15 sec
    62°C 30 sec
    72°C 2 min
    72°C 4 min
    4°C ∞
18. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
19. Enter the **volume** of the PCR reaction. Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
20. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

1. Write the numbers **1, 2** and **3** on the lids of THREE 1.5 mL microcentrifuge tubes.
2. Arrange your THREE PCR tubes to match the order of the THREE 1.5 mL microcentrifuge tubes.
3. Pipet into each 1.5 mL microcentrifuge tube the following:

	Tube #1	**Tube #2**	**Tube #3**
6x Loading dye	3 μL	3 μL	3 μL
PCR Solution #1	25 μL	-	-
PCR Solution #2	-	25 μL	-
PCR Solution #3	-	-	25 μL

4. Mix the contents by pipetting up and down at least 5 times, or vortex and spin.
5. Load **10 μL** of **1 Kb Plus DNA ladder** in the first well of the 1% agarose gel.
6. Load **28 μL** of the sample-dye mixtures using a P-20 pipette.
7. Record the identity of the sample in each lane. Calculate the expected size of the PCR product.

Lane	**Sample**	**Expected Size (bp)**
1	1 Kb Plus DNA ladder	-
2	PCR Solution #1	
3	PCR Solution #2	
4	PCR Solution #3	-

8. Add **10 μL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
9. Run the gel at **105 volts** for 1-2 hours.

   Time power supply turned ON:

   Time power supply turned OFF:

   How long was the gel run? _____ hour(s) and _____ minutes
10. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 4128A2.

Caution: *It is a **MUST** to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking.*

9. Take a picture of the gel using the Bio-Rad Gel Document System.
10. Label the picture using the text program of the Gel Document System.
11. Print out the picture. Store the labeled picture in your lab notebook.
12. Analyze the size of the PCR product on the picture. *If the expected size is observed, proceed to Purifying PCR Products.*

What is the expected size (in bp) of the PCR product? _____ bp

What is the observed size (in bp) of the PCR product from gel electrophoresis?

_____ bp

Do you see a single band corresponding to your gene-specific PCR product?

Or

Do you see more than one band?

***Depending on the PCR results**, you can use one of the following procedures to purify the gene-specific PCR product.*

a. *If a lane on the gel contains only a **single band** corresponding to the gene-specific PCR product, then the DNA can be purified directly from the PCR solution following the **QIAquick PCR Purification Procedure** below.*

b. *If you observe more than a single band (due to contamination or mispriming), then the gene-specific PCR product must be purified from an agarose gel slice. Follow the **QIAquick Gel Extraction Procedure** below.*

IV. PURIFYING PCR PRODUCTS

QIAquick PCR Purification Procedure

Note: This procedure is used when you run 25 μL of PCR product on the gel and observe only one band.

Purpose: To purify DNA (PCR product) from free nucleotides, primers, salt and enzyme for downstream applications such as sequencing reactions.

Reference: QIAquick PCR Purification protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. #28104)
- Remaining PCR solution (~25 μL)

Materials Needed:

- Pipettes
- Filter pipet tips
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- Timer
- NanoDrop spectrophotometer
- Kimwipes

PROCEDURE

1. Write **your initials** on a 1.5 mL microcentrifuge tube.
2. Pipet ~**25 μL** of the **PCR product solution** from the PCR tube containing the gene-specific DNA fragment into the 1.5 mL microcentrifuge tube.
3. Measure the exact volume of solution.
4. Add **125 μL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR sample) to the tube in step 2. Mix by vortexing the tube for **5 seconds**.

5. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge tube rack.
6. Place a lilac **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with your **initials**.
7. Pipet the sample mixture in step 4 to the QIAquick spin column. Spin the column in the collection tube in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane*.
8. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
9. Add **750 µL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
10. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
11. Spin the column set at **FULL speed** for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min*.
12. While spinning, label a new **1.5 mL microcentrifuge** tube "**Purified PCR product**," **your initials** and the **date**.
13. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
14. Pipet **30 µL** of **Buffer EB** to the **center** of the QIAquick column membrane. Let the column sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the QIAquick membrane. Centrifuge again at FULL speed for 1 minute*.

15. Determine DNA concentration using the NanoDrop spectrophotometer.

What is the concentration of purified PCR product? _____ ng/μL

What is the size (in bp) of the PCR product from gel electrophoresis? _____ bp

QIAquick Gel Extraction Procedure

Note: This procedure is used when you run 25 μL of PCR product on the gel and observe more than a single band.

Purpose: To purify DNA (PCR product) from agarose gel for downstream applications such as sequencing reactions.

Reference: QIAquick Gel Extraction protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- Remaining PCR solutions (~25 μL)
- QIAquick Gel Extraction Kit (Qiagen, Cat. #28704)
- Isopropanol
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL 1 Kb Plus DNA ladder (Invitrogen)
- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Gel electrophoresis materials (Appendix 1A)
- Razor blade
- Metal waste container for sharp objects
- Saran wrap
- UV light box
- Scale
- Microcentrifuge

- Vortex
- 50°C water bath or heat block
- 65°C water bath or heat block
- Timer

PROCEDURE

1. Label the **lid** of a 1.5 mL microcentrifuge tube with **your initials**.
2. Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.
3. Put on a UV shield to protect your eyes and face.
4. Turn **on** the **UV box**. *Note: Turn **off** the UV box as soon as you are done excising the DNA band.*
5. Excise the desired DNA fragment from the gel using a razor blade. *Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*
6. Place the agarose slice in the 1.5 mL microcentrifuge tube.
7. Take a picture of the gel **after removing the agarose slice**. *This step serves as a record of the DNA fragment being collected.*

Note: If the desired band was faint, run the remaining 25 µL of the desired PCR solution on a new gel and excise that band as well.

   a. *Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*
   b. *Add **3 µL** of **6x loading dye** the tube of **~25 µL** PCR solution containing the gene-specific fragment.*
   c. *Load **10 µL of 1 Kb Plus DNA ladder** into the first well.*
   d. *Very slowly load the **~28 µL** sample-dye mixtures on the gel using a P-20 pipette (i.e. load ~15 µL first, then then remaining ~15 µL into the same well). Note: If you load the sample into the well too fast, the sample will be forced out of the well. Pipet slowly so that the sample sinks into the well.*
   e. *Record the identity of the sample in each lane.*

Lane	*Sample*	*Expected Size (bp)*
1	*1 Kb Plus DNA ladder*	-
2	*PCR Solution #1*	

f. *Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.*

g. *Run the gel at **105 volts** for 1.5 - 2 hours in the dark.*

h. *Take a picture of the gel.*

i. *Verify the presence of the **expected size PCR product**.*

j. *Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.*

k. *Put on a UV shield to protect your eyes and face.*

l. *Turn **on** the **UV box**. Note: Turn **off** the UV box as soon as you are done excising the DNA band.*

m. *Excise the desired DNA fragment from the gel using a razor blade. Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*

n. *Place the agarose slice in the 1.5 mL microcentrifuge tube from step 6.*

o. *Take a picture of the gel **after removing the agarose slice**. This step serves as a record of the DNA fragment being collected.*

Note: Be sure to preform steps 8-25 at room temperature.

8. Pipet **Elution Buffer** into a 1.5 mL microcentrifuge tube labeled with "**EB**" and **your initials**. Pipet **30 µL** x (number of samples +1). Warm the 1.5 mL microcentrifuge tube with elution buffer to **65°C** in a heat block. *This will be used in step 25 to remove the DNA from the membrane.*
9. Centrifuge the gel fragment at full speed for **1 minute**.
10. Estimate the **gel volume** in the microcentrifuge tube using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tube. *Note: **0.1 g** of **agarose gel** is **equivalent to 100 µL**.*

11. Add **3 gel volumes** of **Buffer QG** to the tube containing the gel slice. *For example, if the **weight** of **the agarose slice** is **0.15 g**, then its **gel volume** is **150 µL**. Therefore, add **450 µL** of **Buffer QG** to the tube.*
12. Incubate the tube at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
13. Add **1 gel volume** of **isopropanol** to the mixture and mix by **vortexing** for **5 seconds** or **inverting** the tube **5-10 times**. *This increases the yield of DNA fragments. Note: Do not centrifuge the sample at this stage.*
14. Place a **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with **your initials.** *Note: If the gel bands were faint, use one column for both gel slices. This will increase the final concentration of purified DNA.*
15. Pipet the **mixture** from **step 12** into the **spin column**. *Do NOT pipet **more than 800 µL** of the mixture into the column. If the total volume is **more than 800 µL**, repeat **steps 15-17**.*
16. Centrifuge the tube for **1 minute**. *This step allows DNA binding to the membrane.*
17. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube. *Keep collection tube for use in steps 17-19.*
18. Add **500 µL** of **Buffer QG** to the **spin column** and centrifuge at FULL speed for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
19. Add **750** of **µL Buffer PE** to the column and let the tube stand for **2-5 minutes**. Centrifuge the tube at FULL speed for **1 minute**. Discard the flow-through solution. *This step washes the column.*
20. r **two more times**.
21. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the column. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
22. While spinning the tube, label a new 1.5 mL microcentrifuge tube with "**T-DNA gel**" and **your initials.**

23. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
24. Add **30 µL** of **warm Buffer EB** (from step 8) to the **center of the column membrane**. Let the column stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. **DNA** is in the **microcentrifuge tube**.* *Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the column membrane. Centrifuge again at FULL speed for 1 minute.*
25. Determine the DNA concentration using a NanoDrop spectrophotometer.

    What is the concentration of purified PCR product? _____ ng/µL

    What is the size (in bp) of the PCR product from gel electrophoresis? _____ bp

V. SEQUENCING REACTION WITH BIG DYE V. 3

Purpose: To determine the sequence of a desired DNA fragment, such as a PCR product of the gene of interest.

References: Applied Biosystems

UCLA WebSeq website http://genoseq.ucla.edu/action/view/Sequencing

Solutions Needed:

- Applied Biosystems Big Dye version 3 (obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building; or Sigma Cat. #S3938)
- 20 μM Gene-specific Salk Forward primer
- 20 μM Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- Pipettes
- Filter pipet tips
- 0.2 mL PCR tubes or strips of 8 tubes/strip
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

Generally, **10 μL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). *Note: If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use a Master Mix (Mmix) to minimize the number of pipettings and mistakes of not adding some components into the individual reaction tubes.*

Important: *This is **NOT** a **regular** PCR reaction. Do **NOT** add **TWO** primers into a single reaction tube. **Each reaction contains only ONE primer**.*

General Components of One Reaction:

	ONE Reaction
DNA template *	**x** μL
Sterile water	**y** μL
20 μM Sequencing primer	1 μL
Big Dye v. 3	1 μL
5x Sequencing buffer	2 μL
Total volume	**10 μL**

x μL = the volume depends on **concentration** and **amount** of **DNA** (see table below)

y μL = the **remaining volume** to bring the **total volume** to **10 μL**

*** Amount of DNA template** depends on **type of DNA**:

- For **plasmid** DNA, use **800 ng**. *Note: 250 ng of plasmid DNA will work, but more DNA gives the better reads.*
- For **PCR product**, use the amount of DNA according to the table below. (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 μL. Alternatively: You may use the speed vac to concentrate your DNA.

Table: Amount of DNA to Use in a Sequencing Reaction

Size of PCR Product (bp)	**Amount of DNA Used in Reaction**
< 200	20 ng
200 - 300	50 ng
> 300	90 ng

For this exercise, there is **ONE DNA template** (the purified PCR product of the gene of interest), but there are **TWO primers**: gene-specific salk forward primer and gene-specific salk reverse primer. Therefore, it is best to prepare a **master mix** with **all components except the primers**, which will be added to the individual reaction tubes.

What is the concentration of PCR product? _____ ng/µL

What is the size of the PCR product? _____ bp

What is the amount of DNA to be used? _____ ng

What is the volume of purified DNA solution to be used? _____ µL

Sample calculations:

Size of PCR product** is **400 bp** and **its concentration** is **20 ng/µL

***Want to use 90 ng** of purified PCR product (see table above)*

*Hence, the **volume of DNA to be used** is **90 ng ÷ 20 ng/µL = 4.5 µL***

PROCEDURE

1. Get ice from the icemaker in room 4128.
2. Label the **sides** of **TWO 0.2 mL PCR tubes** with **your initials** and **primer name**. Set the tubes on a PCR tube rack sitting on ice.
3. Label the **lid** and **side** of a **1.5 mL microcentrifuge tube** "**Mmix**" and **your initials**. Set the tube on ice.
4. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: Use the information above to fill in the volume of DNA solution to be added, and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mix (Mmix) for Sequencing Reactions:

Components	**Mmix** for **ONE reaction**	**Mmix** for **3 reactions**
DNA template	**x** μL	**3x** μL
Sterile water	**y** μL	**3y** μL
Big Dye v. 3	**1** μL	**3** μL
5x Sequencing buffer	**2** μL	**6** μL
Total Volume	**9 μL**	**27 μL**

a. Mix the contents by flicking the tube five times or vortexing at a setting of 2-3 for **5 seconds**. *Note: Big Dye v. 3 contains **enzyme**, keep it on **ice** as much as possible and **do not mix vigorously**.*
b. Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
c. Set the tube back on ice.

5. Pipet **Mmix** and **gene-specific primer** into TWO labeled 0.2 mL PCR tubes.

Components	**Gene-specific Salk Forward primer**	**Gene-specific Salk Reverse primer**
Mmix	9 μL	9 μL
20 μM **Gene-specific Salk Forward** primer	1 μL	-
20 μM **Gene-specific Salk Reverse** primer	-	1 μL
Total volume	**10 μL**	**10 μL**

6. Carry out the cycling reaction using either **Applied Biosystems GeneAmp 9700**

USER: <<hc-lab>>
PROGRAM: **HC70AL BIG DYE**
The profile of the Big Dye program is:
25 cycles of 96°C 10 sec
55°C 5 sec
60°C 4 min

4°C ∞

or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
    a. Resuspend the resin by inversion or gently vortexing.
    b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
    c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
    d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
    e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
    f. Carefully transfer the spin columns to the new tubes.
    g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

        Note:

        - *Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel bed surface with the pipet tip.*
        - *For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 μL using distilled water, before application to the gel bed.*

    h. Centrifuge the columns at **3,000 rpm** for **3 minutes** at room temperature.
    i. Remove the spin columns from the microcentrifuge tubes. *The eluate contains the purified DNA.*
8. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. *Note: Use the primer name as the name of your sequence. Make sure to copy down the*

assigned file number *(example,* #106203*), which is automatically given by the computer after you enter the samples*.

9. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

VI. RETRIEVING AND ANALYZING DNA SEQUENCES

Purpose: To verify that the sequence corresponds to that of the gene of interest.

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/
    a. Enter Username: **goldberg_r**
    b. Enter Password: **embryo**
    c. Click "LOGIN."
2. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

    Example: The ***assigned file number*** is **106203**, and the gene of interest is **At5g09250**. You would see the following files:

    106203GoldR At5g09250Fw A12.ab1

    106203GoldR At5g09250Rv B12.ab1

    What are the annotations?

    106203 = assigned file number; **GoldR** = user name; **At5g09250Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.
3. Check the boxes next to the sequences to be downloaded, and click "Download selected." Alternatively, click on each filename that you want to download.
4. Open the ab1 files in the "Downloads" window using a sequence viewer program (CHROMAS on Windows, or 4PEAKS on Mac).
5. Copy DNA sequences to a Microsoft Word file. *Note: Name the files according to the name of gene of interest (for example, At5g09250).*
6. Process the DNA sequences by "BLASTN" and "BLASTX" searches. See Appendix 2. *Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Determine if the DNA sequence corresponds to the gene of interest.
8. Print out the Blast results as hard-copy records for your lab notebook.
9. Save the Blast results in the **pdf** format so that you can upload them to your webbook.

EXPERIMENT 2 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE ONE)

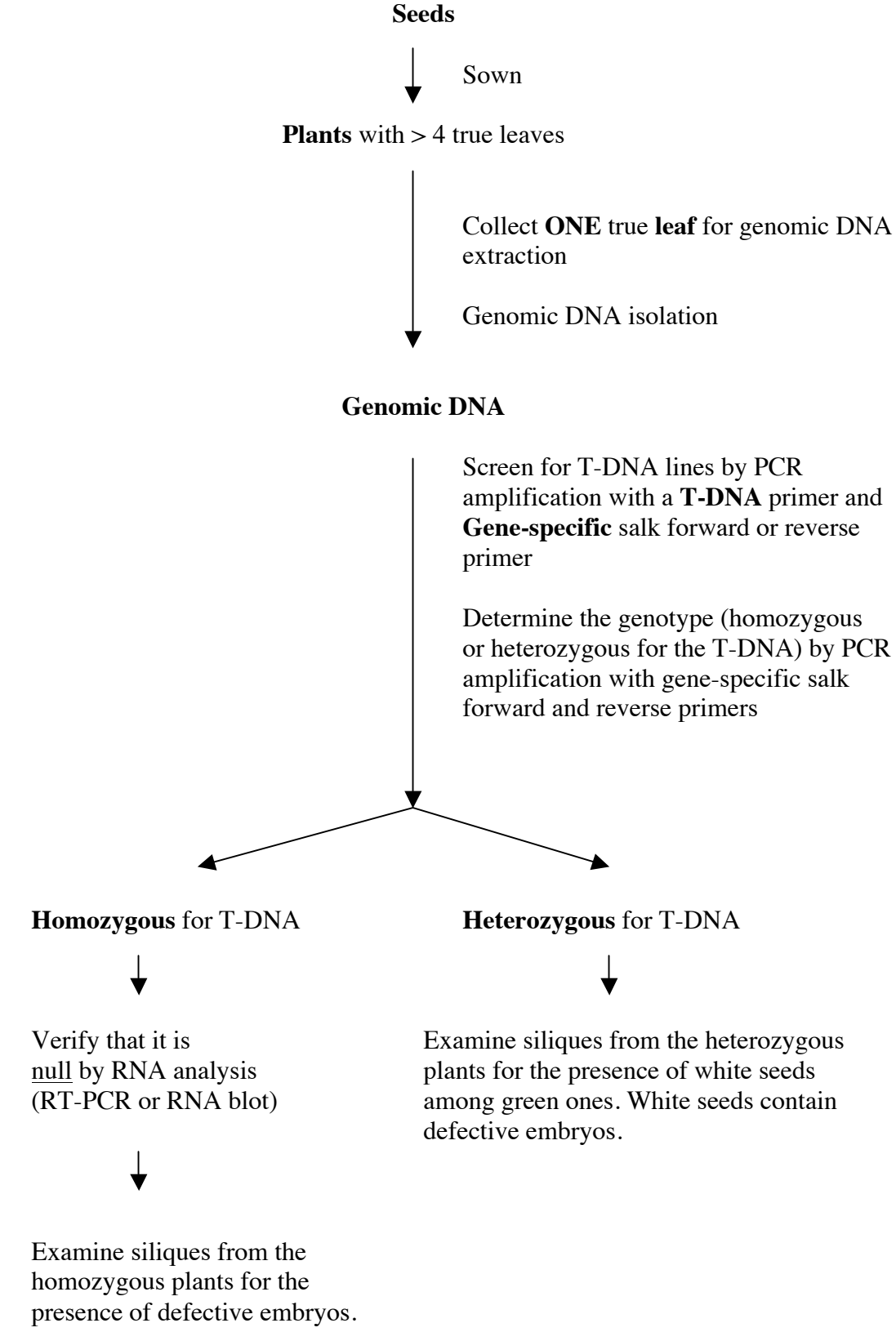
Purpose: To identify a knockout line for the gene of interest and characterize the phenotype of mutant plants.

References: University of Wisconsin - Madison Knockout Facility
Ohio State University - Arabidopsis Biological Resource Center

STRATEGY

I. **SOWING SEEDS AND GROWING PLANTS**
II. **ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS**
III. **IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES**
IV. **DETERMING THE T-DNA INSERTION SITE**

STRATEGY


Seeds
Sown
Plants with > 4 true leaves
Collect ONE true leaf for genomic DNA extraction
Genomic DNA isolation
Genomic DNA
Screen for T-DNA lines by PCR amplification with a T-DNA primer and Gene-specific salk forward or reverse primer
Determine the genotype (homozygous or heterozygous for the T-DNA) by PCR amplification with gene-specific salk forward and reverse primers
Homozygous for T-DNA
Heterozygous for T-DNA
Verify that it is null by RNA analysis (RT-PCR or RNA blot)
Examine siliques from the heterozygous plants for the presence of white seeds among green ones. White seeds contain defective embryos.
Examine siliques from the homozygous plants for the presence of defective embryos.

I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings for genomic DNA extraction.

Reference: Arabidopsis Biological Resource Center http://abrc.osu.edu/

Note: TAs sowed seeds 2-3 weeks before class started

Materials Needed:

- Tubes of knockout seeds from the Arabidopsis Biological Resource Center
- Microcentrifuge tube rack
- Sheet of white Xerox paper cut into quarters
- Black sharpie (ultra-fine or fine)
- Plastic yellow tags (5-6 tags per knockout line)
- Pointed-end forceps
- Key to the Plant Growth Center (PGC)
- BruinCard with access to PGC
- Black plastic trays (in PGC)
- Black rectangular pots in sheets (12 pots/sheet; in PGC)
- Clear plastic covers for black trays (in PGC)
- Soil (in PGC)
- One or two pairs of latex gloves

PROCEDURE

Caution: Be extremely CAREFUL with seeds. Do NOT mix up labeled tags and actual seed lines.

1. Obtain **tubes of seeds** to be grown from the cold room and put them on a microcentrifuge tube rack. *For example, S_112701 (Salk line for gene At5g11240) and wild type seeds (Columbia-0).*
2. Bring the **materials** to the **Plant Growth Center** (**PGC**).
3. At the PGC, put all of these items on **the bench** that runs along the **east wall**. This bench does NOT have any soil on it. *Note: Do NOT put tubes of seeds or plants full of mature siliques near the bench of soil because the prepared soil will be*

contaminated with these seeds, which could in turn, result in false mutant phenotypes for other people's work.

4. In the PGC, prepare ONE flat with **12 pots** of soil for every TWO knockout lines being planted.
    a. Assemble each flat as follows:
        i. Obtain a black plastic tray without holes.
        ii. Obtain a sheet of 12 rectangular plastic pots.
        iii. Obtain a clear plastic cover.
        iv. Set the sheet of 12 pots in the black plastic tray.
        v. Fill the pots loosely with soil, without compressing. Use the sifter to add a layer of fine soil. *(Soil prepared by PGC staff, Mr. Weimin Deng).*
        vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
    b. Remove **one pot** from the corner of the flat and put the soil back into the mound of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in.*
    c. Bring the flat to the bench near the sink.
    d. Use the hand brush to clean up the soil bench.
    e. Make sure that the water hose is attached to the water pipeline labeled "**fertilizer-supplemented**."
    f. Fill each flat **2/3 of the way up** the tray with "fertilizer-supplemented" water.
    g. Cover the flat with a **clear plastic cover** to prevent the growth of air-borne molds and to protect the soil from **stray *Arabidopsis* seeds**.
    h. Wait **20 minutes or until the surface of the soil appears darker** due to water seeping up from the bottom of the pots.
5. Label **11 plastic yellow tags** with a black sharpie.
    a. For **knockout lines**: **Gene name**
    Salk line #
    Date
    Pot # 1-10
    b. For **wild type**: **Columbia-0**

Date
Pot # 11

6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
7. Fold each **quarter sheet** of white paper **in half**, lengthwise.
8. Gently pour out the **knockout seeds** from the microcentrifuge tube onto one of the folded pieces of paper.
9. Bring the folded paper with seeds close to the soil of each of the first 5 pots. Lower one end of the paper near the soil surface. Use the **forceps** to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
10. Visually divide the pot into 4 quadrants, and sow a seed in each quadrant. Sow **4 seeds** per pot, for the first 5 pots. *Note: Planted seeds should not be covered with additional soil because Arabidopsis seeds need light for germination.*
11. Put the labeled tags for the **knockout line** into **each** of the **5 pots** containing knockout seeds.
12. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge tube.
13. Repeat steps 7-12 with the seeds for the next knockout line and pots #6-10. Use a **new** folded piece of white paper for each line.
14. For pot **#11**, pour out wild type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild type seed in each quadrant. **Four wild type seeds** should be sown in **pot #11.**
15. Put a **wild type labeled tag** into pot #11.
16. Cover the flat with the **clear plastic cover**. Put the flat aside.
17. After all of the lines are sown, put the flats on a metal cart and take the elevator to the lower level.
18. Put the flats on the wire racks in the cold room (the first room on the right after entering the double doors across from the elevator).

    CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil. Note: Turn the lights off when you leave the cold room.

19. Leave the flats in the cold room for **2-5 days** to **vernalize seeds.** <u>*Note:*</u> *This will eliminate any dormancy, improve the germination rate and synchronize seed germination.*
20. After 2-5 days in the cold room, put the flats on a metal cart and take the elevator to the upper level. Transfer the flats to a bench in the greenhouse room 125B.
    <u>*NOTE:*</u> *Choose a table that has no mature Arabidopsis plants bearing ripened seeds because these seeds could accidentally get in the soil of the knockout flats when the clear covers are removed.*
21. Slide the clear covers off the trays by **0.5-1 inch** so that warm air under the covers will not overheat the seedlings or create a warm environment favorable for algal and fungal growth. Sliding off the clear covers will also provide some aeration, but still maintain enough humidity for germination and also avoid seed desiccation.
22. Wait until most of the seedlings in the flats have **4 true leaves**. Then completely remove the clear covers from the flats.
23. Map the **positions of seedlings** in **each of the 11 pots** on a "Plant Layout Chart."

GENOTYPING ARABIDOPSIS PLANTS

PLANT LAYOUT CHART

Gene ID: **At__ g _____ Salk line#: _____ Date: _____**
Primers for PCR: _____
Size of PCR product: _____

Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS

Purpose: To isolate genomic DNA from seedling leaves for identifying plants containing a T-DNA insert within the gene of interest.

Recommendation:

Instead of isolating genomic DNA from all 22-24 seedlings at once, you can **start** with **6 seedlings** (**5** from the **knockout** line and **1** from **wild type**). Once you are familiar with the method of isolating genomic DNA, you can isolate genomic DNA from the remaining seedlings (including wild type), if necessary.

Materials Needed:

- Seedlings with at least 4 true leaves (knockout lines and wild type)
- Ice bucket
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Pipettes
- Pipet tips
- One or two pairs of latex gloves
- Two pairs of pointed-end forceps
- Squirt bottle of 100% ethanol solution
- Kimwipes
- Black sharpie (ultra-fine or fine)
- Pen
- Plant layout chart
- Digital camera
- Key to the Plant Growth Center
- BruinCard with access to PGC
- Extraction buffer (0.2 M Tris-HCl, pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS)
- Sterile blue micropestles
- Microcentrifuge
- Timer
- Isopropanol
- 80% ethanol solution
- SpeedVac (optional)
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain

- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution
- Gel electrophoresis materials (Appendix 1A)
- NanoDrop spectrophotometer

PROCEDURE

Attention: *You will need to **assess** the **quality** of **isolated genomic DNA** later (at **step 36**). If you plan to do this on the same day as the genomic DNA isolation, use time efficiently by preparing a 0.7% agarose gel before you start the isolation of genomic DNA (see **Appendix 1A**). While the agarose mixture cools in the 55-60°C water bath for at least 10 minutes, go to the Plant Growth Center to collect leaves. When you come back to the lab, add 10,000x SYBR Safe DNA gel stain to the agarose mixture, swirl to mix, pour the gel, and let the agarose mixture solidify in the dark.*

1. Put **SIX** sterile 1.5 mL microcentrifuge tubes on a microcentrifuge tube rack.
2. Label the lids of the tubes **1-6**.

   Tube #**1 - 5**: Seedlings #1 - 5 of **knockout** lines

   Tube #**6**: **1** Seedling/plant from **wild type** (Columbia-0)
3. Pipet **100 μL** of **Extraction Buffer** into each tube. Keep the tubes on ice. *Note: It is **not** necessary to keep tubes of **Extraction Buffer on ice** during collection of the leaf samples if genomic DNA will be isolated from samples within one hour.*
4. Gather together the following items on a plastic tray or container:
   - Bucket of ice
   - 1.5 mL microcentrifuge tubes containing 100 μL of Extraction Buffer
   - One or two pairs of latex gloves
   - Two pairs of pointed-end forceps
   - Squirt bottle of 100% ethanol solution
   - Kimwipes
   - Black sharpie (ultra-fine or fine)
   - Pen
   - Plant layout chart
   - This protocol
   - Digital camera
   - Key to the Plant Growth Center
   - BruinCard with access to PGC
5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.

6. Take pictures of the plants to document the phenotype. Take pictures of the yellow tags to identify the plants in the pictures.
7. Use the **Plant Layout Chart** to mark the **locations of the plants** and to indicate the plants that you will collect samples from by numbering them. Also make a note of any interesting phenotypes. (For example, some plants may be smaller than others.) The **order of plants** should **correspond** to the **labeled tags** that were numbered when the seeds were planted. *Note: NOT all of the seeds will have germinated.*
8. Use a piece of Kimwipes to clean the forceps with ethanol. *Note: Two sets of forceps are used per plant. The forceps must be cleaned after the collection each leaf to avoid contamination.*
9. Using forceps, remove one **small leaf** from the **plant #1**.
10. Place this leaf in microcentrifuge **tube #1** containing the Extraction Buffer.
11. Repeat steps 8-10 for the other plants.
    Note: MAKE SURE TO CLEAN THE FORCEPS BETWEEN LEAF SAMPLES!
12. Go back to the lab.
13. Homogenize or macerate the collected leaf in **tube #1** by crushing it with a **blue micropestle** until no more chunks of plant tissue are observed in the mixture. *Note: Do NOT dispose of the micropestle.*
14. Rinse the **micropestle** with **300 µL** of Extraction Buffer into the microcentrifuge tube. Put the used micropestles in a beaker labeled "used micropestles" so that they can be washed. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 µL**.
15. Vortex the **homogenate** for 5 seconds. Set the tube on ice.
16. Repeat steps 13-15 for the other tubes.
17. Centrifuge the tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**. *Note: Position the tubes in the centrifuge so that the hinge of the microcentrifuge tubes faces the outside of the microcentrifuge. This way after centrifugation you know to look for your pellet on the side of the microcentrifuge tube that has the hinge.*
18. Meanwhile, **label** a set of **microcentrifuge tubes** with **Gene Name, plant #,** "**gDNA**," **your initials** and the **date.**
19. Pipet **350 µL** of **isopropanol** to each of the new labeled tubes.

20. After centrifugation, transfer the tubes from the microcentrifuge onto a microcentrifuge tube rack. Organize tubes on the rack such that the **numbers** on the lids of the **NEW tubes match** with the **numbers** on the lids of the **tubes containing homogenates**.
21. Pipet **350 µL** of **supernatant** (**homogenate**) from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes. *Note: Use your pipet to draw off liquid from the side of the tube opposite that against which the plant material is pelleted. Start at the top and move downward as the liquid level drops. AVOID disturbing the plant debris on the bottom of the tubes as much as possible. However, it is okay if you accidentally transfer some plant debris into the isopropanol tube.*
22. Mix the isopropanol and homogenate by inverting the tubes **5-10 times**.
23. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA).*
24. Centrifuge the tubes at **room temperature** for **10 minutes** at **FULL** speed.
25. Pour or pipet each isopropanol supernatant into a waste container. *Note: DNA is now in your pellet along with RNA. Therefore, be extremely careful when pouring off the isopropanol because the pellets are sometimes loose.*
26. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert five times. *This step is to wash off any residual salt (from the extraction buffer) and isopropanol.*
27. Centrifuge the tubes at **room temperature** for **5 minutes**.
28. Pour or pipet each ethanol supernatant into a waste container. Dab the tubes on Kimwipes to remove as much ethanol as possible. *Note: Be extremely careful when pouring off the ethanol solution because the pellets are sometimes loose.*
29. Put the tubes on a microcentrifuge tube rack with their lids open, allowing the ethanol to evaporate. *Note: You may use a P-200 pipette to carefully draw off excess ethanol from the side of the tube opposite that against which the nucleic acid is pelleted. Be very careful not to pipet the pellet.*
30. Dry pellets either in a **SpeedVac** at room temperature for **5-10 minutes** (Your instructor will show you how to do this step) or by leaving on the **bench at room temperature for up to 60 minutes**.

31. After drying the pellets, resuspend each pellet by adding **200 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over a microcentrifuge tube rack **10-15 times** or **vortexing** the tubes briefly. *Note: If you vortex genomic DNA vigorously or for a long time, it will degrade.*
32. Spin the tubes in a microcentrifuge for **1 minute** to bring liquid and any contaminants to the bottom of the tubes.
33. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.
    Note: Keep DNA solutions cold as much as possible to prevent degradation of DNA because this is a crude extraction of genomic DNA, and there may be a trace amount of endonuclease present in the DNA solution.

    Attention: *At this step, you need to assess the **quality** and **quantity** of isolated genomic DNA by **gel electrophoresis** (**step 34**) and **spectrophotometer reading** (**step 35**), respectively.*
34. Analyze the **quality** of isolated genomic DNA by **gel electrophoresis**.
    *Note: First, spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*
    a. Prepare a **0.7%** agarose gel with a 20-tooth comb (0.7 g of agarose in 100 mL of 1x TAE buffer; see Appendix 1A). *Note: The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA.*
    b. Label the lids of 6 microcentrifuge tubes with **#1-6** and **your initials**, and set tubes on a microcentrifuge tube rack.
    c. Pipet **20 µL** of **isolated genomic DNA** solution into each of the labeled tubes.
    d. Add **2 µL** of **6x loading dye solution** to each tube and mix the contents by pipetting up and down 5 times.
    e. Load **10 µL** of **1 Kb Plus DNA ladder solution** into the first well.
    f. Load **20 µL** of each sample-dye mixture prepared in step d using a P-20 pipette.
    g. Record the identity of the sample in each well.

Lane	Sample	Expected Size (bp)
1	1 Kb Plus DNA ladder	-
2	Genomic DNA from Plant #1	
3	Genomic DNA from Plant #2	
4	Genomic DNA from Plant #3	
5	Genomic DNA from Plant #4	
6	Genomic DNA from Plant #5	
7	Genomic DNA from Plant #6	

Lane	Sample	Expected Size (bp)
1	1 Kb Plus DNA ladder	-
2	Genomic DNA from Plant #7	
3	Genomic DNA from Plant #8	
4	Genomic DNA from Plant #9	
5	Genomic DNA from Plant #10	
6	Genomic DNA from Plant #11	
7	Genomic DNA from Plant #12	

h. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.

i. Run the gel at **105 volts** for 1-2 hours.

Starting time:

Ending time:

j. Take a picture of the gel using the Bio-Rad Gel Documentation system.

What do you observe on the gel?

What is the size of genomic DNA?

Do you observe any other bands? What do you think they are?

35. Determine the concentration of DNA using the NanoDrop spectrophotometer. *Your instructor will demonstrate how to use the NanoDrop.*

Note: *First, spin the tubes of DNA solutions in a microcentrifuge at* ***room temperature*** *for* ***2 minutes*** *at* ***FULL*** *speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

Record the **concentration of DNA** in the solutions in the **table** below:

Sample	DNA Concentration (ng/µL)
Plant #1	
Plant #2	
Plant #3	
Plant #4	
Plant #5	
Plant #6	

Sample	DNA Concentration (ng/µL)
Plant #7	
Plant #8	
Plant #9	
Plant #10	
Plant #11	
Plant #12	

36. Dilute **5 µL** of **original DNA solutions** to a **final concentration** of **0.2 ng/µL** with **TE** buffer.

Note: *Dilution of DNA solutions will serve two purposes:*

a. Contaminants, such as carbohydrates that bind nonspecifically to nucleic acids and proteins, will be diluted out. A tiny amount of contaminants in a PCR reaction will not interfere with the amplification of the target DNA.

b. Only a small amount of Arabidopsis genomic DNA (~0.4 ng) is needed for PCR amplification.

How to make a dilution?

Use the basic formula that is widely used in general chemistry labs. That is,

$$\mathbf{V_i \times C_i = V_f \times C_f}$$

where,

$\mathbf{V_i}$ = **initial volume** (the volume of original DNA solution is **5 µL**)

$\mathbf{C_i}$ = **initial concentration** (reading from the spectrophotometer; example: **8 ng/µL**)

$\mathbf{V_f}$ = **final volume** (depends on the initial concentration)

C_f = final concentration (0.2 ng/µL)
then,

V_f = (V_i x C_i)/ C_f = (5 µL x **8 ng/µL)/(0.2 ng/µL) = 200 µL** of **total volume**

What is the volume of TE to be used in dilution?

V_{TE} = V_f - V_i = 200 µL - 5 µL = 195 µL of **TE**

a. Record the **volume of TE** and the **final volume** in the table below.

	Volume of Isolated Genomic DNA	**Volume of TE Added**	**Final Volume**
Plant #1	5 µL		
Plant #2	5 µL		
Plant #3	5 µL		
Plant #4	5 µL		
Plant #5	5 µL		
Plant #6	5 µL		

	Volume of Isolated Genomic DNA	**Volume of TE Added**	**Final Volume**
Plant #7	5 µL		
Plant #8	5 µL		
Plant #9	5 µL		
Plant #10	5 µL		
Plant #11	5 µL		
Plant #12	5 µL		

Note: If the volume of TE to be used in dilution is greater than 1.5 mL, it will not fit in a 1.5 mL microcentrifuge tube. In this case, dilute 2.5 µL of genomic DNA.

b. Label the lids and sides of microcentrifuge tubes with the following information: **0.2 ng/µL gDNA**, **plant #**, **your initials** and the **date**. Keep all tubes of DNA solutions **on ice**.
c. Spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.
d. Add the appropriate volume of TE to each newly labeled tube.
e. Add **5 µL** of **original DNA solutions** into each tube. Flick tubes to mix.
f. Spin the tubes in a microcentrifuge for **1 minute** to bring liquid and any contaminants to the bottom of the tubes.
g. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.

III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES

Purpose: To identify plants containing the T-DNA insert and to determine the genotypes of T-DNA-tagged plants (heterozygous or homozygous for the T-DNA).

STRATEGY

A. Polymerase Chain Reaction (PCR)

B. Gel Electrophoresis Analysis of PCR Product

C. Label T-DNA tagged plants

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 μM Gene-specific Salk Forward primer
- 12 μM Gene-specific Salk Reverse primer
- 12 μM LBb1.3 primer (anneals to the Left Border (LB) region of the T-DNA)
- 0.2 ng/μL genomic DNA extracted from the plants to be genotyped (including WT)
- 0.2 ng/μL genomic DNA extracted from wild type seedlings by TAs
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- 0.2 mL PCR tubes in strips of 8
- 1.5 mL microcentrifuge tubes
- Rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Ice bucket
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Microcentrifuge
- Gel electrophoresis materials (Appendix 1A)
- Key to the Plant Growth Center

- BruinCard with access to PGC
- Wooden sticks
- Tape
- Black sharpie (ultra-fine or fine)
- Pen
- Plant Layout Chart
- Phenotype Observation Record
- Digital Camera

A. Polymerase Chain Reaction (PCR)

PROCEDURE

Note: Because you do not know the orientation of the T-DNA insertion, and therefore the direction of the LBb1.3 primer, you will need to set up ***THREE*** *PCR reactions for* ***each*** *sample.*

Reaction A: ***Gene-specific Salk Forward*** *primer and* ***LBb1.3*** *primer*

Reaction B: ***LBb1.3*** *primer and* ***Gene-specific Salk Reverse*** *primer*

Reaction C: ***Gene-specific Salk Forward*** *primer and* ***Gene-specific Salk Reverse*** *primer*

Note: ALWAYS wear gloves and use filter tips to prevent contamination when preparing PCR reactions.

Note: There are ***6*** *plants to be characterized and* ***2 controls*** *(genomic DNA isolated by TA + No DNA template), prepare each* ***master mix*** *for* ***8 + 1 extra = 9 reactions****.*

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer, dNTP mix, 12 µM Gene-specific Salk Forward primer, 12 µM Gene-specific Salk Reverse primer** and **12 µM LBb1.3 primer** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed, put the tubes on **ice** until needed.

3. Spin genomic DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed. Keep on ice.
4. Label the lids and sides of **24 PCR tubes** with **A1-A8, B1-B8, C1-C8** and **your initials** and the **date**. Put them on a PCR tube rack sitting on ice.
5. Prepare a **"Reaction A" master mix** for **9 PCR reactions** in a 1.5 mL microcentrifuge tube labeled "**Mmix A**" sitting on **ice**. Pipet the reagents in order from top down (example: water, 10x Ex Taq buffer, dNTP mix, etc.) into the **Mmix A** tube.

Reaction A	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM **Gene-specific Salk Forward** primer	1.0 µL	9.0 µL
12 µM Gene-specific Salk Reverse primer	-	-
12 µM **LBb1.3** primer	1.0 µL	9.0 µL
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

6. Mix the contents of **Mmix A** by flicking the tube several times or vortexing the tube at a **setting of 2-3** for **5 seconds**. Spin the tube in a microcentrifuge at FULL speed for **10 seconds**. Put the tube back **on ice**.
7. Pipet **46 µL** of **Mmix A** into each of **8 PCR tubes** labeled **A1-A8**.
8. Pipet **4 µL** of **0.2 ng/µL genomic DNA** extracted from each of **6 seedlings** into PCR tubes A1-A6. Pipet up and down 5 times to mix the contents.
9. Pipet **4 µL** of **0.2 ng/µL genomic DNA** extracted from **wild type** (Col-0) seedlings by TAs into tube **A7**. Pipet up and down 5 times to mix the contents.
10. Pipet **4 µL** of **sterile water** into tube **A8** (**negative control** without DNA template). Pipet up and down 5 times to mix the contents.
11. Keep these PCR reactions on ice while you prepare the next master mix. Repeat steps 5-11 for Reactions B and C.

Reaction B	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM Gene-specific Salk Forward primer	-	-
12 µM **Gene-specific Salk Reverse** primer	1.0 µL	9.0 µL
12 µM **LBb1.3** primer	1.0 µL	9.0 µL
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

Reaction C	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM **Gene-specific Salk Forward** primer	1.0 µL	9.0 µL
12 µM **Gene-specific Salk Reverse** primer	1.0 µL	9.0 µL
12 µM LBb1.3 primer	-	-
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

12. Spin all PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
13. Turn **ON** the PCR machine (MyCycler). Wait for one minute for the machine to initialize.
14. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
15. Select the "**Protocol Library**" by pressing "**F1**."
16. Select "**HC70AL**" by pressing the yellow arrowheads surrounding the "**ENTER**" button. Select the "**HC70AL KNOCKOUT**" protocol. Press "**ENTER**."
17. The "CHOOSE OPERATION" menu will appear. Select "**VIEW PROTOCOL**." The **PCR profile** of the Knockout protocol is as follows:

94°C for 3 min
36 cycles of 94°C 15 sec
62°C 30 sec
72°C 2 min

72°C 4 min
4°C ∞

18. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
19. Enter the **volume** of the PCR reaction. Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
20. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

PROCEDURE

1. Prepare a **1% agarose gel** in 1x TAE buffer with a **30-tooth** comb (or 20-tooth comb depending on how many samples you have).
2. Record the identity of the sample in each lane. Use the table below as a guide.
3. Label **24 1.5 mL microcentrifuge** tubes (one for each sample) and set them on a microcentrifuge tube rack.
4. Add **3 µL** of **6x loading dye** to each tube.
5. Pipet **25 µL** of **PCR solution** to each tube. Mix the contents by pipetting up and down 5 times or by flicking the tubes. Spin briefly.
6. Load **10 µL** of **100 bp DNA ladder** in the first well.
7. Very slowly load the ~**28 µL** sample-dye mixtures on the gel using a P-20 pipette *(i.e. load ~15 µL first, then then remaining ~15 µL into the same well).*
8. Load **10 µL** of **1 Kb Plus DNA ladder** in the last well.

Lane	Sample	Primers	Expected Size (bp)
1	100 bp DNA ladder	-	-
2	Genomic DNA from Plant #1	Reaction A: **Gene-specific Salk Forward** primer and **LBb1.3** primer	
3	Genomic DNA from Plant #2		
4	Genomic DNA from Plant #3		
5	Genomic DNA from Plant #4		
6	Genomic DNA from Plant #5		
7	Genomic DNA from Plant #6		
8	Col-0 Genomic DNA (from TAs)		-
9	Sterile water		-
10	100 bp DNA ladder		
11	Genomic DNA from Plant #1	Reaction B: **LBb1.3** primer and **Gene-specific Salk Reverse** primer	
12	Genomic DNA from Plant #2		
13	Genomic DNA from Plant #3		
14	Genomic DNA from Plant #4		
15	Genomic DNA from Plant #5		
16	Genomic DNA from Plant #6		
17	Col-0 Genomic DNA (from TAs)		-
18	Sterile water		-
19	100 bp DNA ladder		
20	Genomic DNA from Plant #1	Reaction C: **Gene-specific Salk Forward** primer and **Gene-specific Salk Reverse** primer	
21	Genomic DNA from Plant #2		
22	Genomic DNA from Plant #3		
23	Genomic DNA from Plant #4		
24	Genomic DNA from Plant #5		
25	Genomic DNA from Plant #6		
26	Col-0 Genomic DNA (from TAs)		
27	Sterile water		-
28	1 Kb Plus DNA ladder	-	-

9. Add **5 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
10. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.
11. Stop the gel electrophoresis.
12. Take a picture of the gel and annotate it.
13. Analyze the data.

    Do you observe PCR fragments?

    What are the sizes of these fragments?

    Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?

    What is the orientation of the T-DNA relative to the gene?

    What are the genotypes of the 6 plants?

Note:

1. *Although the results of the PCR reactions should confirm the size of the so-called "T-DNA fragment," which contains part of the plant gene and part of the T-DNA, it is a **good scientific practice** to verify the exact location of the T-DNA insertion site.*

2. ***Depending on the results of the PCR** to genotype the plants, you can use one of the following procedures to purify the "T-DNA fragment."*
    a. *If a lane on the gel contains only a **single band** corresponding to the "T-DNA fragment," then the "T-DNA fragment" can be purified directly from the PCR solution by following the **QIAquick PCR Purification Procedure** below.*
    b. *If all lanes containing the "T-DNA fragment" also contain other bands (due to contamination or mispriming), then the "T-DNA fragment" must be purified from an agarose gel slice. Follow the **QIAquick Gel Extraction Procedure** below.*

C. Label T-DNA tagged plants

PROCEDURE

1. After determining the genotypes of the plants, make labels for the plants containing a T-DNA insert by putting a small piece of tape on a wooden stick. Write the **number** that corresponds to the **plant #** on the Plant Layout Chart and either "**homozygous for the T-DNA**" or "**heterozygous for the T-DNA**." You can also note the genotype of the plants on the Plant Layout Chart.
2. Go to the Plant Growth Center. Put the labeled wooden sticks next to the identified T-DNA tagged plants.
3. Observe T-DNA tagged plants for abnormal phenotypes. Write your observations on the **Phenotype Observation Record**. Take pictures of the plants to document the phenotype. Take pictures of the yellow tags to identify the plants in the pictures. You may take flowers back to the lab to observe the phenotype under a microscope.

PHENOTYPE OBSERVATION RECORD

Gene ID: **At__ g _____ Salk line#: _____ Date: _____**

LEAF	**Mutant**	**Wild Type**
What do the leaves look like, green or yellow, elongated or round?		
What is the range of their length in cm?		
How many leaves does each plant have?		
Is the range of leaf sizes of the mutant plant smaller or larger or similar to wild type leaves?		

STEM	**Mutant**	**Wild Type**
What is the height of the main (or longest) stem?		
What is the thickness of the stem?		
How many stems (or branches including the main and side ones) does the plant have?		

FLOWERS	**Mutant**	**Wild Type**
Do the flowers have all FOUR floral organs (green sepals, white petals, yellow anthers, green pistils)?		
How many sepals are on each flower?		
How many petals are on each flower?		
How many anthers are on each flower?		
How many pistils are on each flower?		

SILIQUES, SEEDS AND EMBRYOS	**Mutant**	**Wild Type**
How many siliques are on each plant?		
Do you see a difference in the lengths of siliques?		
How many seeds are in EACH silique?		
What is the average number of seeds in FIVE siliques?		
Do you see different COLORED seeds within a single silique?		
If yes, what colors are the seeds? How many seeds of each color?		
What stage of embryos (globular, heart, torpedo, cotyledon, mature green, or post mature green) do you see?		

IV. DETERMING THE T-DNA INSERTION SITE

Purpose: To verify the location of the T-DNA insertion site in the gene of interest indicated by the Salk Institute Genomic Analysis Laboratory website http://signal.salk.edu/

STRATEGY

A. Purifying PCR Products

B. Sequencing Reaction with Big Dye v. 3

C. Retrieving and Analyzing DNA Sequences

A. Purifying PCR Products

QIAquick PCR Purification Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe only one band.

Purpose: To purify DNA (PCR product) from free nucleotides, primers, salt, and enzyme for downstream applications such as sequencing reactions.

Reference: QIAquick PCR Purification protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- Remaining PCR solutions (~25 µL)

Materials Needed:

- Pipettes
- Filter pipet tips
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- Timer
- NanoDrop spectrophotometer

- Kimwipes

PROCEDURE

1. Write "**T-DNA**" and **your initials** on a 1.5 mL microcentrifuge tube.
2. Pipet **~25 µL** of the **PCR product solution** from the PCR tube containing the T-DNA fragment into the labeled 1.5 mL microcentrifuge tube.
3. Measure the exact volume of solution.
4. Add **150 µL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR product solution) to the tube in step 2. Mix by vortexing for 5 seconds.
5. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge tube rack.
6. Place a **QIAquick spin column** (lilac) in the provided **2 mL collection tube**. Label the lid of the column "**T-DNA**" and **your initials**.
7. Pipet the sample mixture in step 4 to the QIAquick spin column. Spin the column and collection tube in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane*.
8. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
9. Add **750 µL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
10. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
11. Spin the column set at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min.*

12. While spinning, label a 1.5 mL microcentrifuge tube "**PCR Purified T-DNA,**" **your initials** and the **date**.
13. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
14. Pipet **30 µL** of **Buffer EB** to the **center** of the QIAquick column membrane. Let the columns sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the QIAquick membrane. Centrifuge again at FULL speed for 1 minute.*
15. Determine DNA concentration using the NanoDrop spectrophotometer.

    What is the concentration of purified PCR product? _____ ng/µL

    What is the size (in bp) of the PCR product from gel electrophoresis? _____ bp

QIAquick Gel Extraction Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe more than a single band.

Purpose: To purify DNA (PCR product) from agarose gel for downstream applications such as sequencing reactions.

Reference: QIAquick Gel Extraction protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- Remaining PCR solutions (~25 µL)
- QIAquick Gel Extraction Kit (Qiagen, Cat. #28704)
- Isopropanol
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/µL 1 Kb Plus DNA ladder (Invitrogen)

- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Gel electrophoresis materials (Appendix 1A)
- Razor blade
- Metal waste container for sharp objects
- Saran wrap
- UV light box
- Scale
- Microcentrifuge
- Vortex
- 50°C water bath or heat block
- 65°C water bath or heat block
- Timer

PROCEDURE

1. Label a microcentrifuge tube "**T-DNA**" and **your initials**.
2. Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.
3. Put on a UV shield to protect your eyes and face.
4. Turn **on** the **UV box**. *Note: Turn **off** the UV box as soon as you are done excising the DNA band.*
5. Excise the T-DNA fragment from the gel using a razor blade. *Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*
6. Place the agarose slice in the 1.5 mL microcentrifuge tube.
7. Take a picture of the gel **after removing the agarose slice**. *This step serves as a record of the DNA fragment being collected.*

   Note: If the desired band is faint, you many collect all the T-DNA bands on the gel and pool them together. Alternatively, run the remaining 25 μL of the desired PCR solutions on a new gel and excise those bands as well.

a. *Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*

b. *Add **3 µL** of **6x loading dye** to each tube of **~25 µL** PCR solutions containing the "T-DNA fragment."*

c. *Load **10 µL of 1 Kb Plus DNA ladder** into the first well.*

d. ***Very slowly** load the **~28 µL** sample-dye mixtures on the gel using a P-20 pipette (i.e. load ~15 µL first, then then remaining ~15 µL into the same well).*

e. *Record the identity of the sample in each well.*

Lane	***Sample***	***Primers***	***Expected Size (bp)***
1	*1 Kb Plus DNA ladder*	-	-
2			
3			

f. *Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.*

g. *Run the gel at **105 volts** for 1.5 - 2 hours in the dark.*

h. *Take a picture of the gel.*

i. *Verify the presence of the **expected size PCR product**.*

j. *Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.*

k. *Put on a UV shield to protect your eyes and face.*

l. *Turn **on** the **UV box**. <u>Note:</u> Turn **off** the UV box as soon as you are done excising the DNA band.*

m. *Excise the desired DNA fragments from the gel using a razor blade. <u>Note:</u> Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*

n. *Place the agarose slices in the 1.5 mL microcentrifuge tube from step 6 labeled "**T-DNA**" and **your initials**.*

o. *Take a picture of the gel **after removing the agarose slices**. This step serves as a record of the DNA fragment being collected.*

Note: Be sure to preform steps 8-25 at room temperature.

8. Pipet **Elution Buffer** into a 1.5 mL microcentrifuge tube labeled with "**EB**" and **your initials**. Pipet **30 µL** x (number of samples +1). Warm the 1.5 mL microcentrifuge tube with elution buffer to **65°C** in a heat block. *This will be used in step 25 to remove the DNA from the membrane.*
9. Centrifuge the gel slice at full speed for **1 minute**.
10. Estimate the **gel volume** in the microcentrifuge tube using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tube. *Note: **0.1 g** of **agarose gel** is **equivalent to 100 µL**.*
11. Add **3 gel volumes** of **Buffer QG** to the tube containing the gel slice. *For example, if the **weight** of **the agarose slice** is **0.15 g**, then its **gel volume** is **150 µL**. Therefore, add **450 µL** of **Buffer QG** to the tube.*
12. Incubate the tube at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *Do **not** incubate at 50°C for more than 10 minutes! To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
13. Add **1 gel volume** of **isopropanol** to the mixture and mix by **vortexing** for **5 seconds** or **inverting** the tubes **5-10 times**. *This increases the yield of DNA fragments. Note: Do not centrifuge the samples at this stage.*
14. Place a **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with "**T-DNA**" and **your initials**. *Note: If the gel bands were faint, use one column for multiple gel slices. This will increase the final concentration of purified DNA.*
15. Pipet the **mixture** from **step 12** into the **spin column**. *Do NOT pipet **more than 800 µL** of the mixture into the column. If the total volume is **more than 800 µL**, repeat **steps 15-17**.*
16. Centrifuge the spin column in the collection tube for **1 minute**. *This step allows DNA binding to the membrane.*

17. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube. *Keep collection tube for use in steps 17-19.*
18. Add **500 µL** of **Buffer QG** to the **spin column** and centrifuge at FULL speed for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
19. Add **750** of **µL Buffer PE** to the column and let the tube stand for **2-5 minutes**. Centrifuge the tube at FULL speed for **1 minute**. Discard the flow-through solution. *This step washes the column.*
20. Repeat step 19 **two more times**.
21. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the column. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
22. While spinning the tube, label a new 1.5 mL microcentrifuge tube with "**Gel Purified T-DNA**" and **your initials.**
23. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
24. Add **30 µL** of **warm Buffer EB** (from step 8) to the **center of the column membrane**. Let the column stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the column membrane. Centrifuge again at FULL speed for 1 minute.*
25. Determine the DNA concentration using a NanoDrop spectrophotometer.

    What is the concentration of purified PCR product? _____ ng/µL

    What is the size (in bp) of the PCR product from gel electrophoresis? _____ bp

B. Sequencing Reaction with Big Dye v. 3

Purpose: To determine the exact location of T-DNA insertion site in the gene of interest from the Salk T-DNA knockout line.

References: Applied Biosystems

UCLA WebSeq website http://genoseq.ucla.edu/action/view/Sequencing

Solutions Needed:

- Applied Biosystems Big Dye version 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building; or Sigma Cat. #S3938)
- 20 µM LBb1.3 primer
- 20 µM Gene-specific Salk Forward primer
- 20 µM Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- 0.2 mL PCR tubes or strips of 8 tubes
- PCR tube rack
- Filter pipet tips
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

Generally, **10 µL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). *Note: If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use a Master Mix (Mmix) to minimize the number of pipettings and mistakes of not adding some components into the individual reaction tubes.*

Important: *This is **NOT** a **regular** PCR reaction. Do **NOT** add **TWO** primers into a single reaction tube. **Each reaction contains only ONE primer**.*

General Components of One Reaction:

	ONE Reaction
DNA template *	**x** μL
Sterile water	**y** μL
20 μM Sequencing primer	1 μL
Big Dye v. 3	1 μL
5x Sequencing buffer	2 μL
Total volume	**10 μL**

x μL = the volume depends on **concentration** and **amount** of **DNA**
(see table below)

y μL = the **remaining volume** to bring the **total volume** to **10 μL**

*** Amount of DNA template** depends on **type of DNA**:

- For **plasmid** DNA, use **800 ng**. *Note: 250 ng of plasmid DNA will work, but more DNA gives the better reads.*
- For **PCR product**, use the amount of DNA according to the table below. (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 μL. Alternatively: You may use the speed vac to concentrate your DNA.

Table: Amount of DNA to Use in a Sequencing Reaction

Size of PCR Product (bp)	**Amount of DNA Used in Reaction**
< 200	20 ng
200 - 300	50 ng
> 300	90 ng

For this exercise, there is **ONE DNA template** (the purified PCR product of the T-DNA fragment), but there are **TWO primers**: LBb1.3 (T-DNA) primer and gene-specific salk

primer. The gene-specific salk primer will be either forward or reverse depending on the orientation of the T-DNA Left Border (LB) relative to the gene of interest. The orientation of the T-DNA relative to the gene of interest was determined by your analysis of the genotyping PCR results. The **sequencing reaction with** the **gene-specific primer** serves **as a control for the master mix** of Big Dye and 5x Sequencing buffer. Therefore, it is best to prepare a **master mix** with **all components except** the **primers**, which will be added to individual reaction tubes.

What is the concentration of PCR product? _____ ng/µL

What is the size of the PCR product? _____ bp

What is the amount of DNA to be used? _____ ng

What is the volume of purified DNA solution to be used? _____ µL

Sample calculations:

Size of PCR product** is **400 bp** and **its concentration** is **20 ng/µL

***Want to use 90 ng** of purified PCR product (see table above)*

*Hence, the **volume of DNA to be used** is **90 ng ÷ 20 ng/µL = 4.5 µL***

PROCEDURE

1. Get ice from the icemaker in room 4128.
2. Label the **sides** of **TWO 0.2 mL PCR tubes** with **your initials**, **primer name** and **"T-DNA"** or **"WT"** allele. Set the tubes on a PCR tube rack sitting on ice.
3. Label the **lid** and **side** of a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.
4. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mix (Mmix) for Sequencing Reactions:

Components	**Mmix** for **ONE reaction**	**Mmix** for **3 reactions**
DNA template	**x** μL	**3x** μL
Sterile water	**y** μL	**3y** μL
Big Dye v. 3	**1** μL	**3** μL
5x Sequencing buffer	**2** μL	**6** μL
Total Volume	**9 μL**	**27 μL**

a. Mix the contents by flicking the tube five times or vortexing at a setting of 2-3 for **5 seconds**.
b. Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
c. Set the tube back on ice.

5. Pipet **Mmix** and **primers** into TWO labeled 0.2 mL PCR tubes.

		either	***or***
Components	**LBb1.3 primer**	**Gene-specific Salk <u>Forward</u> primer**	**Gene-specific Salk <u>Reverse</u> primer**
Mmix	**9 μL**	**9 μL**	**9 μL**
20 μM LB1 primer	**1 μL**	-	-
20 μM Gene-specific Salk Forward primer	-	**1 μL**	-
20 μM Gene-specific Salk Reverse primer	-	-	**1 μL**
Total volume	**10 μL**	**10 μL**	**10 μL**

6. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700**

USER: <<hc-lab>>
PROGRAM: **HC70AL BIG DYE**

The profile of the Big Dye program is:

25 cycles of	96°C 10 sec
	55°C 5 sec
	60°C 4 min
4°C ∞	

or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
    a. Resuspend the resin by inversion or gently vortexing.
    b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
    c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
    d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
    e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
    f. Carefully transfer the spin columns to the new tubes.
    g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

        Note:

        - *Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel bed surface with the pipet tip.*
        - *For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 µL using distilled water, before application to the gel bed.*

    h. Centrifuge the columns at **3,000 rpm** for **3 minutes** at room temperature.
    i. Remove the spin columns from the microcentrifuge tubes. *The eluate contains the purified DNA.*

8. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. *Note: Use the primer name as the name of your sequence. Make sure to copy down the* ***assigned file number*** *(example, #*106203*), which is automatically given by the computer after you enter the samples.*
9. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

C. Retrieving and Analyzing DNA Sequences

Purpose: To verify that the sequence corresponds to that of the gene of interest.

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/
    a. Enter Username: **goldberg_r**
    b. Enter Password: **embryo**
    c. Click "LOGIN."
2. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

    Example: The ***assigned file number*** is **106203**, and the gene of interest is **At5g09250**. You would see the following files:

    106203GoldR At5g09250Fw A12.ab1

    106203GoldR At5g09250Rv B12.ab1

    What are the annotations?

    106203 = assigned file number; **GoldR** = user name; **At5g09250Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.
3. Check the boxes next to the sequences to be downloaded, and click "Download selected." Alternatively, click on each filename that you want to download.
4. Open the ab1 files in the "Downloads" window using a sequence viewer program (CHROMAS on Windows, or 4PEAKS on Mac).

5. Copy DNA sequences to a Microsoft Word file. *Note: Name the files according to the name of gene of interest (for example, At5g09250).*
6. Process the DNA sequences by "BLASTN" and "BLASTX" searches. See Appendix 2. *Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Determine if the DNA sequence corresponds to the gene of interest.
8. Print out the Blast results as hard-copy records for your lab notebook.
9. Save the Blast results in the **pdf** format so that you can upload them to your webbook.

EXPERIMENT 3 – RNA ISOLATION AND RT-PCR ANALYSIS (GENE ONE)

Purpose: To determine the mRNA accumulation pattern of the gene of interest in wild type and mutant *Arabidopsis* siliques.

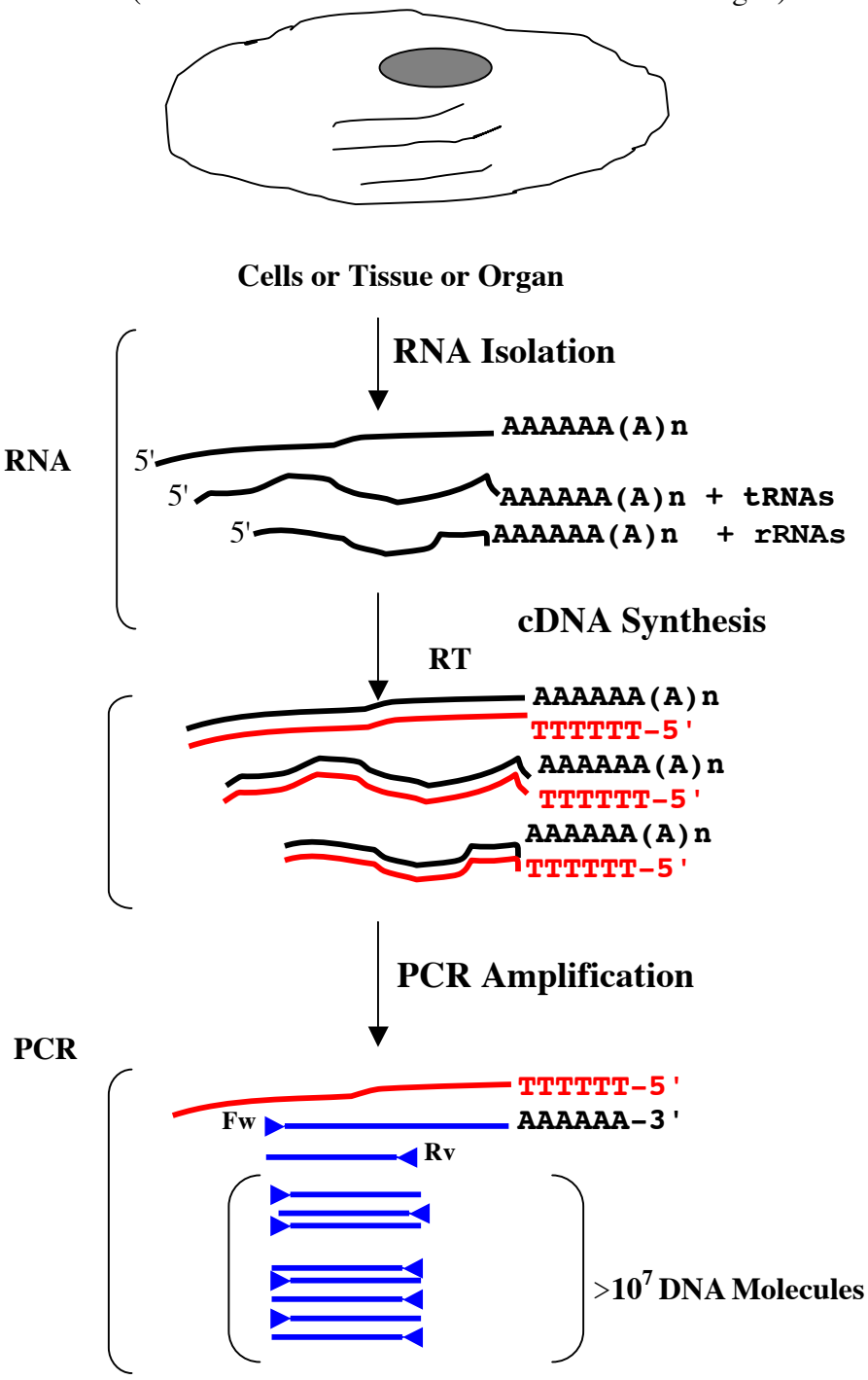
OVERVIEW OF RT-PCR STRATEGY

I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT

II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE (REVERSE TRANSCRIPTION or RT)

III. CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS

OVERVIEW OF RT-PCR (Based on RT-PCR Technical Note from Invitrogen)



I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT

Purpose: To extract total RNA from siliques to study gene expression. You will perform RT-PCR on total RNA isolated from siliques from wild type and mutant plants in order to determine if the T-DNA insertion causes a null mutation (i.e. no mRNA is expressed for the gene of interest).

References: RNeasy Plant Mini Kit Protocol (Qiagen; see Appendix 1G)

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. In: (Second Edition), *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. (ISBN 978-0879693091)

FREQUENTLY ASKED QUESTIONS

PROCEDURE

A. Grinding Tissue for RNA Extraction

B. RNA Isolation

C. Removing Contaminating Genomic DNA from Total RNA Solutions Using RNase-free DNase

D. Determining the Quality of Isolated Total RNA Before and After DNase Treatment Using Capillary Gel Electrophoresis

FREQUENTLY ASKED QUESTIONS

(Taken from Qiagen RNeasy Plant Mini Handbook June 2001; see Appendix 1G)

1. **What is the maximum amount of starting material?**

   100 mg

2. **Is the yield of total RNA the same for the same amount of starting material for different plant species?**

   No, the yield varies for different plant species.

3. **Which lysis buffer can be used for plant materials?**

   - Buffer **RLC** (Guanidine Hydrochloride) is used for endosperm and tissues containing endosperm (e.g., siliques). Although Guanidine Isothiocyanate is better at cell disruption and denaturation than Guanidine Hydrochloride, Guanidine Isothiocyanate can cause solidification of endosperm samples, making extraction of RNA impossible.
   - Buffer **RLT** (Guanidine Isothiocyanate) is used for all plant tissues except endosperm and tissues containing endosperm (e.g., siliques).

4. **Is total RNA isolated with RNeasy kit free of genomic DNA?**

   No, most (but not all) of DNA is eliminated. Therefore, if total RNA will be used for downstream application such as Reverse-transcription-PCR (RT-PCR), then DNase treatment must be carried out for the total RNA.

5. **What is the role of QIAshredder homogenizer?**

   It simultaneously **removes insoluble material** and **reduces the viscosity of the lysates** by disrupting gelatinous material.

A. Grinding Tissue for RNA Extraction

Note: ***Grinding Tissue for RNA Extraction will be carried out by the Teaching Assistants (TAs).***

Materials Needed:

- Key to the Plant Growth Center
- BruinCard with access to PGC
- Plant layout charts indicating plants homozygous or heterozygous for the T-DNA
- Ice bucket
- Kimwipes
- A squirt bottle of 100% Ethanol solution
- Forceps
- Liquid Nitrogen (from storeroom in Life Sciences Building) *Caution: It is very cold (at least -210°C). Avoid getting frostbite.*
- Dewar flask or Styrofoam box
- Diethyl Pyrocarbonate (DEPC). *Caution: DEPC is suspected to be carcinogenic and corrosive. Therefore, hand with care! DEPC inhibits RNase.*
- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Autoclaved, DEPC-treated porcelain mortar and pestle
- Autoclaved, DEPC-treated, blue micropestles
- Qiagen RNeasy Plant Mini Kit: (Cat. #74904 for 50 extractions)
- β-mercaptoethanol. *Caution: Work in the fume hood because this chemical has very bad odor.*
- Black ultra-fine sharpie
- Autoclaved, DEPC-treated spatulas
- RNase-free 14 mL disposable centrifuge tubes
- RNase-free 1.5 mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- Scale

Attention: *Before isolating RNA, use Kimwipes wetted with freshly prepared non-autoclaved DEPC treated water to clean all equipment (pipette sets, pipet stand, microcentrifuge-tube racks, micro centrifuges and rotors, test-tube racks, pens and sharpies, pipet tip boxes, microcentrifuge tube containers) to be used in isolating RNA.*

PROCEDURE

1. Get ice from the icemaker.
2. Label the white area on the side of ONE RNase-free **14 mL centrifuge tube** "**WT Siliques**." Label **SIX 1.5 mL microcentrifuge tubes** with the name of each mutant line. Chill on ice.
3. Go to the Plant Growth Center.

4. Use a piece of Kimwipes to clean the forceps with ethanol. Note: *Two sets of forceps are used per plant. The forceps must be cleaned after the collection of siliques from each line to avoid contamination.*
5. Working **quickly**, use forceps to harvest siliques from wild type *Arabidopsis* Columbia-0 siliques and siliques from each mutant line. Select siliques from a plant homozygous for T-DNA if available, otherwise use siliques from a heterozygous plant. Select siliques that contain seeds with embryos ranging from globular to torpedo stage. **Immediately**, place siliques in the chilled, labeled tubes. Note: *Clean the forceps with ethanol before collecting from a new line.*
6. Return to the lab.
7. **Immediately** chill the samples either on **crushed dry ice** or in a Dewar flask or Styrofoam box containing **liquid nitrogen** (filling up to one-third of the Styrofoam box).
8. Chill an **RNase-free** spatula in a Dewar flask containing liquid nitrogen.
9. Chill the mortar and pestle with liquid nitrogen until liquid nitrogen is not bubbling out vigorously.
10. Place **WT** siliques in the chilled mortar containing liquid nitrogen.
11. Using the pestle, grind the frozen tissue to a powder in liquid nitrogen. Note: *It is best to grind the tissue when the last drop of liquid nitrogen has just evaporated. Grind quickly. Do not let the tissue thaw. Repeat this step until there are no more chunks of tissue present.*
12. Add some liquid nitrogen to the mortar and quickly pour the tissue and liquid nitrogen to a chilled, labeled 14 mL tube set on **crushed dry ice** or in **liquid nitrogen**. You may use the chilled spatula to get the powder into the tube.
13. Lightly place the cap on top of the 14 mL tube to allow the liquid nitrogen within the tube to evaporate, but do not allow the tissue to thaw. Note: *You may also place the tube in a -70°C freezer to allow the evaporation of liquid nitrogen.*
14. For the **mutant samples**, grind the siliques in the 1.5 mL microcentrifuge tubes chilled in liquid nitrogen with the blue micropestles that have been treated with DEPC, autoclaved and chilled in liquid nitrogen. Use a new micropestle for each sample to avoid contamination. Note: *Grind quickly and place the tube back in the*

liquid nitrogen. Do not let the tissue thaw. Repeat this step until there are no more chunks of tissue present.

15. Label TWELVE 1.5 mL microcentrifuge tubes with the sample names. Set the tubes on **crushed dry ice** or in **liquid nitrogen**.
16. Use a **chilled, RNase-free spatula** to transfer a small amount **(up to 100 mg)** of **WT frozen ground material** to SIX of the new **chilled 1.5 mL microcentrifuge tubes**. Use a scale to measure, but do not let the samples thaw. Keep the new tubes on dry ice or in liquid nitrogen.
17. Use new **chilled, RNase-free spatula** to transfer a small amount **(up to 100 mg)** of **frozen ground material** for each of the six mutant lines into the new **chilled 1.5 mL microcentrifuge tubes**. Use a scale to measure, but do not let the samples thaw. Keep the new tubes on dry ice or in liquid nitrogen.
18. Store the TWELVE aliquots in the **–70°C freezer** until the RNA extraction step. Also store the remaining ground tissue for each sample in the **–70°C freezer** as a backup.
19. **On the day of RNA extraction**, prepare the **RLC lysis buffer**
    a. Determine the **total volume** (**= # of samples x 500 µL**) of **lysis buffer** needed for RNA isolation. *Note: If the total volume is greater than 1.5 mL, it is best to use a **14 mL centrifuge tube** for preparing the **lysis buffer** with **β-mercaptoethanol**.*
    b. Add **10 µL** of **β-mercaptoethanol** to every **1 mL** of **lysis buffer** in the fume hood. Mix the contents in the tube by vortexing for 5 seconds. Put the tube back on the rack.

*Note: **β-mercaptoethanol** is **toxic** and has a bad odor. It is kept in the fume hood in room 4128A2. The newly prepared lysis buffer with β-mercaptoethanol is stable 1 month after the addition of β-mercaptoethanol.*

Volume of RLC lysis buffer	Volume of β-mercaptoethanol
_____ mL	_____ µL

B. RNA Isolation

Materials Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Kimwipes
- Dewar flask or Styrofoam box
- Liquid Nitrogen (from storeroom in Life Sciences Building) <u>*Caution:*</u> *It is very cold (at least -210°C). Avoid getting frostbite.*
- Aliquots of 1.5 mL RNase-free tubes containing ~100 mg of frozen powder from ground up wild type *Arabidopsis* Columbia-0 siliques and siliques from each mutant plant (powder prepared by Teaching Assistants)
- Qiagen RNeasy Plant Mini Kit: (Cat. #74904 for 50 extractions)
- β-mercaptoethanol. <u>*Caution:*</u> *Work in the fume hood because this chemical has very bad odor.*
- Autoclaved DEPC-treated (DEPC'd) water
- P-10, P-20, P-200 & P-1000 pipettes
- RNase-free filter tips for P-10, P-20, P-200 & P-1000
- Ice bucket
- Black ultra-fine sharpie
- RNase-free 1.5 mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- Timer
- NanoDrop spectrophotometer

<u>Caution:</u>

- ***All steps of the RNeasy protocol should be carried out at room temperature.*** *During the procedure,* ***work quickly.***
- ***All centrifugation steps are carried out at 20-25°C.*** *Ensure that the centrifuge does not cool below 20°C.*
- ***Use filter pipet tips throughout the procedure.***
- ***Change GLOVES frequently*** *and keep tubes closed whenever possible.*

PROCEDURE

<u>Attention:</u> *Before isolating RNA, use Kimwipes wetted with freshly prepared non-autoclaved DEPC treated water to clean all equipment (pipette sets, pipet stand, microcentrifuge-tube racks, micro centrifuges and rotors, test-tube racks, pens and*

sharpies, pipet tip boxes, microcentrifuge tube containers) to be used in isolating RNA.

1. Locate TWO 1.5 mL microcentrifuge tubes containing a small amount **(up to 100 mg)** of **frozen ground material** from wild type or T-DNA-tagged siliques. These will be stored on dry ice or in liquid nitrogen. Quickly, **tap** the **tube** on the **bench** or the base of the vortex mixer 3-5 times to loosen the frozen powder.
2. **Immediately**, pipet **450 µL** of **RLC lysis buffer containing β-mercaptoethanol** into the 1.5 mL microcentrifuge tube containing **~100 mg** of ground **WILD TYPE** tissue. **Cap** the tube. **Immediately**, **vortex** the tube **vigorously** for at least **1 minute**. Then set the tube on a microcentrifuge tube rack. *The **lysate** should appear clear with no lumps of ground powder. (Optional) A short incubation time (1-3 minutes) at 56°C may help to disrupt the tissue, but is NOT appropriate for organs rich in starch, such as siliques or old leaves.*
3. **Repeat step 2** for the **T-DNA** sample.
4. Label the lids of TWO **QIAshredder** (**lilac**) **spin columns** placed in **2 mL collection tubes** with **your initials** and "**WT**" or "**T-DNA**."
5. Pipet the **entire volume** of **lysate** into the labeled QIAshredder spin columns.
6. Centrifuge the spin columns in the collection tubes at **FULL speed** (13,200 rpm) for **2 minutes**. *Note: Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.*
7. Meanwhile, label the lids of TWO **1.5 mL RNase-free microcentrifuge tubes** and TWO **RNeasy spin columns** (**pink**) placed **2 mL collection tubes** with **your initials** and "**WT**" or "**T-DNA**." Set the labeled tubes on a microcentrifuge tube rack at room temperature.
8. Carefully transfer the **supernatant** of the **flow-through solutions** to the **NEW labeled RNase-free 1.5 mL microcentrifuge tubes** without disturbing the cell-debris pellets in the collection tubes. Use only this supernatant in subsequent steps.

9. Add **0.5 volume** (or **225 µL**) of **room temperature 96-100% ethanol** to the **WT supernatant**. **Immediately**, mix by pipetting **up and down 10 times**. *Note: Do NOT centrifuge. Proceed immediately to step 10.*
10. Pipet the **entire volume (~650 µL, but not more than 700 µL)** of the **WT mixture** (including any precipitate that may have formed) in step 9 to the "**WT**" labeled **RNeasy spin column** (**pink**) placed in a **2 mL collection tube**. Close the lid of the tube **gently**.
11. Repeat steps 9 and 10 for the **T-DNA** mixture.
12. Centrifuge the sp**in columns** placed in a 2 mL collection tubes for **15 seconds** at **>10,000 rpm** (or FULL speed).
13. Carefully remove the **spin column** from the collection tube so that the column does not contact the flow-through. Hold the column with one hand and while **pouring** the **flow-through solution** in the collection tube into a "**waste**" **beaker**. Be sure to empty the collection tube completely. Put the column back in the collection tube. *Note: If the sample volume in step 10 is >700 µL, pipet the remaining volume of the mixture onto the RNeasy column and centrifuge as before. Discard the supernatant.*
14. Pipet **700 µL** of **Buffer RW1** to the RNeasy spin column. Close the tube gently.
15. Centrifuge for **15 seconds** at **>10,000 rpm** (or FULL speed) to wash the spin column membrane.
16. Carefully discard the flow-through as in step 13.
17. Pipet **500 µL** of **Buffer RPE** into RNeasy spin column. Close the tubes gently.
18. Centrifuge for **15 seconds** at **>10,000 rpm** (or FULL speed) to wash the spin column membrane.
19. Carefully discard the flow-through as in step 13.
20. Pipet another **500 µL** of **Buffer RPE** into the RNeasy spin column. Close the tube gently.
21. Centrifuge for **2 minutes** at **>10,000 rpm** (or FULL speed) to wash the spin column membrane. *Note: The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.*
22. Label TWO **new 2 mL collection tubes** with **your initials** and "**WT**" or "**T-DNA**."

23. Carefully transfer the columns to the **new 2 mL collection tube**s without allowing the columns to contact the flow-through. *Attention: At this point, total RNA and a small amount of genomic DNA are bound to the membrane of the pink RNeasy spin column.*
24. Discard the flow-through solution and old collection tubes.
25. Spin the columns in the new 2 mL collection tubes for **1 minute** to ensure that ethanol is removed completely from the membranes. *Caution: This step is **crucial** because if residual ethanol is still on the membrane, it will be eluted with RNA in steps 28-31.*
26. Label the lids and sides of TWO 1.5 mL RNase-free microcentrifuge tubes "**WT RNA**" or "**T-DNA RNA**," **your initial**s and the **date**.
27. Transfer the **spin columns** to these **NEW labeled tubes**.
28. Pipet **30 µL** of **RNase-free water** (supplied with the kit) or autoclaved DEPC-treated water directly onto the center of the column membrane. Close the tubes gently.
29. Wait for **1 minute** to allow the membrane to evenly absorb the water.
30. Centrifuge for **1 minute** at **>10,000 rpm** (or FULL speed) to elute RNA from the membrane.
31. Repeat **steps 28-30** with **20 µL** of **RNase-free water**. *Note: The total volume of RNA solution is about **50 µL.***
32. Mix the contents of the tubes with gentle flicking. Put tubes **on ice**. *Note: From this step on, **KEEP** RNA solutions **ON ICE** to prevent **RNA degradation**.*
33. Determine the **total volume** of **RNA solution** using a P-200 pipette. The volume should be **~48 µL**.
34. Determine **RNA concentration** and **total amount** of RNA using the NanoDrop spectrophotometer.

    Total amount of RNA = (X µg/µL) (Volume of RNA solution in µL) = **Y µg**

    *Note: **1 µg = 1,000 ng**; therefore, you need to **convert ng/µL** to **µg/µL***

Record RNA concentration and total amount of RNA

Sample	[RNA] (μg/μL)	Volume (μL)	Estimated Total Amount (μg)
Wild type siliques			
T-DNA siliques			

35. Label the lids and sides of **TWO new RNase-free microcentrifuge tubes** "**1 μL WT Silique RNA**" or "**1 μL T-DNA Silique RNA**," **your initials** and the **date**. Keep tubes **on ice**.
36. Pipet **1 μL** of the **RNA solution** into the **new labeled tubes**. These aliquots will be used to assess the quality of the RNA in **Part D.**
37. Either keep the tubes **on ice** and proceed to **Part C**, or store the RNA solutions at **-20°C for up to 1 week** or at **-70°C for up to 6 months**.

Attention: *To be safe, only **HALF** of **the volume** of the **RNA solution** is **treated with RNase-free DNase**; the remaining volume of RNA solution is kept on ice or stored in the -20°C RNA freezer until the gel electrophoresis step in part D to determine quality of RNA before DNase treatment.*

C. Removing Contaminating Genomic DNA from Total RNA Solutions Using RNase-free DNase

Reference: Turbo DNA-free kit protocol (Ambion; See Appendix 1H)

Important Note: *This protocol is suitable for **removing up to 2 μg of DNA** from up to **20 μg of RNA** in a **25-100 μL** reaction volume.*

Materials Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment

- Ice bucket
- Total RNA isolated from wild type and mutant siliques
- Ambion Turbo DNA-free kit (stored in RNA -20°C freezer; Cat. # 1907)
- Turbo DNase (included in the Ambion Turbo DNA-free kit)
- 10x Turbo DNase buffer (included in the Ambion Turbo DNA-free kit)
- DNase inactivation reagent (included in the Ambion Turbo DNA-free kit)
- Autoclaved DEPC-treated (DEPC'd) water
- Black ultra-fine sharpie
- RNase-free 1.5 mL microcentrifuge tubes
- Rack for microcentrifuge tubes
- 37°C heat block
- Timer
- Microcentrifuge
- Vortex
- P-10, P-20 & P-200 pipettes
- RNase-free filter tips for P-10, P-20 and P-200
- White Revco storage boxes
- Kimwipes
- NanoDrop spectrophotometer

PROCEDURE

1. Write down the **concentration** of the total RNA samples.

   *Note: 1 μg = 1,000 ng. Therefore, the concentration determined by the NanoDrop spectrophotometer (**ng/μL**) needs to be converted into **μg/μL**.*

	WT	**T-DNA**
RNA concentration	_____ μg/μL	_____ μg/μL

2. Determine the **volume** for **20 μg** of total RNA.

   Volume** of **20 μg RNA** = **Amount** of **RNA** ÷ **concentration** of **RNA

   *Example: If **total RNA** has a concentration of 1.25 μg/μL, then the volume of **20 μg** of **RNA** will be 20 μg ÷ 1.25 μg/μL = **16 μL***

	WT	**T-DNA**
Volume of 20 μg	_____ μL	_____ μL

3. Determine which **volume** is **smaller** for each sample: the volume of **20 µg** of total RNA **or** the volume of **half** of the isolated total RNA. **Use the smaller volume** in the DNase reaction.
4. Get ice from the icemaker.
5. Remove your total RNA samples from the -20°C RNA freezer. Thaw on ice.
6. Label TWO 1.5 mL microcentrifuge tubes with "**WT**" or "**T-DNA**," **your initials** and the **date**. Set tubes on ice.
7. Add total RNA samples, DEPC'd water, **0.1 volume** of **10x Turbo DNase buffer** and **1 µL** of **2 Units/µL Turbo DNase** (Ambion) to the labeled, chilled tubes according to the chart below. Make a master mix (Mmix) for the components that are shared in both reactions. *Note:* ***One unit** of **DNase** is defined as the amount of enzyme that degrades **1 µg of DNA** in **10 minutes** at **37°C**.*

	RNA Solution	**WT Silique RNA**	**T-DNA Silique RNA**	**Mmix for 3 Reactions**
RNA sample (up to 20 µg, but not more than half of your RNA)	**X** µL			
DEPC'd water	**Y** µL			
10x Turbo DNase buffer	**3.0** µL			
Turbo DNase (2 Units/µL)	**1.0** µL			
Total volume	**30.0** µL			

X µL = volume of RNA sample; **Y µL** = volume of DEPC'd water

*The **volume of DEPC'd water** is the difference between the total reaction volume and the sum of the volume of the other components.*

8. Mix the solutions gently by flicking the tubes. Spin briefly (**5-10 seconds**).
9. **Immediately**, store the tubes of remaining total RNA solution in a box at **-20°C for up to 1 week** or at **-70°C for up to 6 months**.
10. Incubate the DNase reactions at **37°C** in a **heat block** for **20-30 minutes**.

11. After incubation, spin tubes for **10 seconds** in a microcentrifuge to bring water condensation to the bottom of the tubes.
12. To inactivate Turbo DNase, pipet **0.1 volume** (or **3.0 µL**) of the **DNase inactivation reagent** (**WHITE slurry)** to the sample using a P-20 pipet tip. Mix well by **flicking the tube**. *Note: Make sure the slurry is WHITE. If the DNase inactivation reagent is CLEAR, vortex the mixture for a few seconds.*
13. Incubate the tube at **room temperature** for **5 minutes**. Flick the tube **2-3 times during the incubation** to re-disperse the **DNase inactivation reagent**.
14. In the meantime, label the lids and sides of **NEW RNase-free microcentrifuge tubes** "**Purified WT Silique RNA**" or "**Purified T-DNA Silique RNA**," **your initials** and the **date**.
15. Spin the tube at ~**10,000 x g (or 10,400 rpm)** for **1 minute** to pellet the **DNase inactivation reagent**.
16. **Carefully**, pipet ~**28-30 µL** of the **RNA solution** (*AVOID pipetting the PELLET!*) and transfer it into NEW labeled **RNase-free microcentrifuge tubes**. *Note: It is **okay** if a **tiny amount** of the pellet **is carried over** in the RNA solution.*
17. Keep RNA tubes **on ice**.
18. Determine the **RNA concentration** and **total amount** of RNA using the NanoDrop spectrophotometer.

    Total amount = (X µg/µL) × (volume of RNA solution) = Y µg

    *Note: **1 µg = 1,000 ng**; therefore, you need to **convert ng/µL** to **µg/µL***

Samples	**[RNA] (µg/µL)**	**Volume (µL)**	**Estimate Total Amount (µg)**
Purified WT Silique RNA			
Purified T-DNA Silique RNA			

19. Label the lids and sides of **NEW RNase-free microcentrifuge tubes** "**1 µL Purified WT Silique RNA**" or "**1 µL Purified T-DNA Silique RNA**," **your initials** and the **date**. Keep tubes **on ice**.

20. Pipet **1 µL** of the **RNA solution** into the **new labeled tubes**. These aliquots will be used to assess the quality of the purified RNA in **Part D**.
21. Put the tubes of purified RNA samples back in a box at **-20°C for up to 1 week** or at **-70°C for up to 6 months**. You may keep the **1 µL aliquots** on ice if you will proceed with **Part D.**

D. Determining the Quality of Isolated Total RNA Before and After DNase Treatment Using Capillary Gel Electrophoresis

Reference: RNA StdSens Analysis kit instruction manual (Experion, Bio-Rad)

Materials and Reagents Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Kimwipes
- Ice bucket
- 1 µL aliquots of RNA samples before and after DNase
- RNA StdSens Analysis kit (Experion, Bio-Rad)
- RNA StdSens chip (Experion, Bio-Rad)
- RNA StdSens ladder (Included in StdSens RNA Analysis kit)
- RNA StdSens filtered gel (Included in StdSens RNA Analysis kit)
- RNA StdSens gel-stain (Included in StdSens RNA Analysis kit)
- RNA StdSens loading buffer (Included in StdSens RNA Analysis kit)
- Electrode cleaner (Experion, Bio-Rad)
- Autoclaved DEPC-treated (DEPC'd) water
- Cleaning chips (Experion, Bio-Rad)
- Black ultra-fine sharpie
- RNase-free 1.5 mL microcentrifuge tubes
- Rack for microcentrifuge tubes
- 70°C heat block
- Timer
- Microcentrifuge
- Vortex
- P-10, P-20 & P-200 pipettes
- RNase-free filter tips for P-10, P-20 and P-200
- Capillary gel electrophoresis system (Experion, Bio-Rad)

Note: A single StdSens RNA chip can hold 12 RNA samples. Three students may share each chip and prepare one RNA ladder solution.

Note: Each 1 µl RNA sample should have a concentration of 5-500 ng/µl.

Note: Your instructor will prepare the gel-stain and clean the electrodes before samples are run.

PROCEDURE

1. Equilibrate Experion RNA StdSens reagents (filtered RNA gel solution, RNA loading buffer (yellow cap), gel-stain solution (amber tube)) to room temperature for at least 15 minutes. *Place the kit in a drawer or dark room to keep the gel-stain protected from light.*
2. Set a heat block to 70°C.
3. Get ice from the icemaker.
4. Locate FOUR 1 µL aliquots of RNA samples before and after DNase. Thaw RNA solutions **on ice**.
5. Spin the tubes of 1 µL RNA aliquots for **10 seconds** in a microcentrifuge. Keep RNA solutions on ice.
6. Remove a tube of **2 µL RNA ladder aliquot** from the -70°C freezer. Spin the tube in a microcentrifuge for **10 seconds** to bring down any water condensation, and keep it on ice.
7. Label a **NEW** 1.5 mL microcentrifuge tube "**RNA ladder**." Keep the tube on ice.
8. Pipet **1 µL** of **RNA ladder** into the new labeled 1.5 mL microcentrifuge tube.
9. Heat the **FOUR** tubes of **1 µL aliquots of RNA solutions** and **1 µL aliquot of RNA ladder** on a **70°C heat block** for **2 minutes**. *Note: It is okay to heat the samples for up to 5 minutes.*
10. Quench tubes **on ice** for **at least 5 minutes**.
11. Spin tubes in a microcentrifuge for **15-30 seconds**. Keep tubes **on ice**.

12. Pipet **5 µL** of **loading buffer (yellow cap)** to each RNA solution. Mix the contents by flicking the tube several times. After adding the loading buffer to all RNA solutions, spin tubes for 10 seconds. Keep the tubes **on ice**.
13. Remove an RNA StdSens chip from its plastic wrap. Using a **P-10 pipette**, pipet **9 µL of gel-stain** into the well labeled **GS** with an **orange highlight** (third well from the top). *Note: To avoid bubbles, dispense reagents into chips slowly. Always insert the pipet tip vertically and to the bottom of the chip well when dispensing liquids. Do not expel air at the end of the pipetting step. This will reduce the possibility of air bubbles becoming trapped between the reagent and the microchannels at the bottom of the chip wells.*
14. Put the chip on the **priming station**. Make sure the setting is **B1**.
15. Press the "**START**" button on the priming station. Wait for 30 seconds.
16. Open the priming station.
17. Pipet another **9 µL** of **gel-stain** to the other well labeled **GS** (second well from the top).
18. Pipet **9 µL** of **filtered gel** into the well labeled **G** (top well).
19. Pipet **6 µL** of the RNA mixtures prepared in step 12 into each sample well (1-4) and into the ladder well (labeled L). Work **quickly** to minimize sample evaporation. *Note: Each chip can hold 12 samples. Therefore, three students can share one chip. However, if there are only 4 samples, then pipet **6 µL** of loading buffer into the remaining wells (5-12). **Caution: Do NOT leave any sample well empty.***
20. Gently tap the **chip** on the **bench 3-5 times** to remove any **bubbles** present in the sample wells. Inspect the wells for the presence of bubbles. ***Note: Run the chip within 5 minutes of loading samples.***
21. Place the sample-loaded chip on the platform of the electrophoresis station and close the lid.
22. Launch the **Experion software**, select "**New Run**" and then "**RNA StdSens**."
23. Select "**Eukaryotic total RNA assay**."
24. Click the **PLAY** symbol button to begin the run. A window will pop up asking for the total number of samples loaded. Type in number of samples and the software will

avoid the wells containing only loading buffer. *The run will take up to 30 minutes for all 12 samples*.

25. While the electrophoresis is running, enter the sample information in the "**data info**" tab.
26. After the run is complete, the analyzer **beeps**. Your TA will remove the chip from the platform and discard the used chip.
27. **Your TA will immediately** place a **cleaning chip** containing **800 µL of DEPC'd water** on the platform. Close the lid of the electrophoresis system for **1 minute** to clean the electrodes.
28. Open the lid for **30 seconds** to allow water to evaporate.
29. Remove the **cleaning chip**. **Discard** the **water** and **store** the **cleaning chip** for future use.
30. Export data (electropherograms and gel images) to the desktop.
31. Copy the **exported data** on a **USB flash drive** and **upload** them onto the **HC70AL server**.
32. If there are no more runs for the day, your TA will turn off the electrophoresis system and quit the Experion software.
33. Analyze the data.

    What do you see in the picture?

    What are the RNA fragments?

    What are the sizes of RNA fragments?

    Is there any difference in brightness between different samples <u>before</u> and <u>after</u> DNase treatment?

    What is the reason for the difference?

II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE

Purpose: To generate cDNA template for PCR analysis.

Reference: iScript cDNA Synthesis Kit Instruction Manual (Bio-Rad; See Appendix 1I)

Overview:

- The **iScript reverse transcriptase** is RNase H^+, resulting in greater sensitivity than RNase H^- enzyme. **iScript** is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA.
- The enzyme is provided pre-blended with RNase inhibitor.
- The unique blend of oligo(dT) and random (6-bases, 8-bases, 10-bases) primers in the iScript Reaction Mix works exceptionally well with a wide range of targets. This blend is optimized for the production of targets <1 kb in length.
- iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

*Caution: When using **>1 µg** of total RNA, the reaction **volume** should be **scaled up** to ensure optimum synthesis efficiency. For example, use a 40 µL reaction for 2 µg.*

Note:

- *For **each** RNA sample, set up one reaction **with** Reverse Transcriptase (**+ RT**) and one reaction **without** Reverse Transcriptase (**- RT**). The -RT sample serves as a negative control for the PCR amplification step because without first strand cDNA template, there will be NO PCR product with expected size observed. However, if a PCR product is observed in the - RT sample, then RNA sample is contaminated with genomic DNA.*
- *Work with master mixes as often as possible to prevent FALSE negative results due missing components.*

Solutions Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Purified total RNA samples (after DNase, stored in -20°C RNA Freezer)
- iScript cDNA Synthesis kit (Bio-Rad, Cat. #170-8890; stored in -20°C RNA Freezer)
- iScript Reverse Transcriptase (included in iScript cDNA Synthesis kit)
- 5x iScript Reaction mix (included in iScript cDNA Synthesis kit)
- Nuclease-free water (included in iScript cDNA Synthesis kit)
- Autoclaved DEPC-treated (DEPC'd) water

Materials Needed:

- Pipettes
- RNase-free filter pipet tips
- RNase-free 1.5 mL microcentrifuge tubes
- Rack for 1.5 mL microcentrifuge tubes
- Black ultra-fine sharpie
- Ice bucket
- Microcentrifuge
- Vortex
- Timer
- 25°C heating block
- 42°C heating block
- 85°C heating block

PROCEDURE

1. Write down the **concentration** of purified total RNA samples to be used.

   *Note: 1 μg = 1,000 ng. Therefore, the concentration determined by the NanoDrop spectrophotometer (**ng/μL**) needs to be converted to **μg/μL**.*

	WT	**T-DNA**
RNA concentration	_____ μg/μL	_____ μg/μL

2. Determine the **volume** of **1 μg** of purified total RNA.

   Volume** of **1 μg RNA** = **Amount** of **RNA** ÷ **concentration** of **RNA

   *Example: If the purified **RNA** has a concentration of 0.5 μg/μL, then the volume of **1 μg** of **RNA** will be 1 μg ÷ 0.5 μg/μL = **2 μL***

	WT	T-DNA
Volume of 1 µg	_____ µL	_____ µL

3. Use the following **table** as a **guide** to fill in the volumes for purified total RNA and DEPC'd water.

Components	**RNA +RT**	**RNA -RT**
DEPC'd (or nuclease-free) water	**X** µL	**X** µL
1 µg Purified total RNA	**Y** µL	**Y** µL
+ RT Mix	5.0 µL	-
- RT Mix	-	5.0 µL
Total Reaction Volume	**20.0** µL	**20.0** µL

X µL = volume of RNA sample; **Y µL** = volume of DEPC'd water

*The **volume of DEPC'd water** is the difference between the total reaction volume and the sum of the volume of the other components.*

4. Get ice from the icemaker.
5. Set heating blocks to 25°C, 42°C and 85°C.
6. Label the lids of FOUR RNase-free 1.5 mL microcentrifuge tubes "**WT +RT**," "**WT –RT**," "**T-DNA +RT**," and "**T-DNA –RT**." Keep tubes **on ice**.
7. Thaw the tubes of **5x iScript Reaction Mix** and **nuclease-free water** at room temperature. Once the solutions are **thawed**, spin tubes in a microcentrifuge for **10 seconds**, and keep the tubes **on ice**.
8. Prepare TWO tubes for **Master mixes** (**+RT Mix** and **-RT Mix**) as follows:
    a. Determine the number of RT reactions to be set up.

    Note: ***# RT reactions = # of RNA samples** + 1 Extra*

    Example: ***# RT reactions = 3** = WT Siliques + T-DNA Siliques + 1 Extra*

    b. Label the lids of the RNase-free microcentrifuge tubes "**+RT mix**" and "**-RT mix**." Keep the tubes **on ice**.

c. Remove a tube of **iScript Reverse Transcriptase** from the **-20°C RNA freezer**. Keep the **tube on ice** at all times to prevent degradation of the enzyme in this tube.

d. Pipet the **following components** into appropriate tubes as shown below.

Components	**+RT Mix for ONE Reaction**	**+RT Mix for 3 Reactions**	**-RT Mix for ONE Reaction**	**-RT Mix for 3 Reactions**
DEPC'd (or nuclease-free) water	-	-	1 µL	3 µL
5x iScript Reaction mix	4 µL	12 µL	4 µL	12 µL
iScript Reverse Transcriptase	1 µL	3 µL	-	-
Total volume	**5 µL**	**15 µL**	**5 µL**	**15 µL**

e. Mix the contents by pipetting up and down **five times** or flicking the tubes several times.

f. Spin the tubes in a microcentrifuge for **10 seconds**. Put the tubes **on ice**.

9. Using the **+RT and -RT chart** written up in **step 3**, pipet the components into the tubes labeled in **step 6**.
10. Mix the contents in each tube by pipetting **gently** up and down **five times**. Keep tubes **on ice**.
11. Transfer all of **+RT and -RT tubes** from the ice bucket to either a heat block set to **25°C** or a rack for microcentrifuge tubes on the bench (room temperature). Incubate reaction tubes at **25°C** (or room temperature) for **5 minutes**. *This step is to allow oligo(dT) and random primers to anneal to the messenger RNA in the reactions*.
12. Incubate the tubes at **42°C** for **30 minutes** in a **heat block**. *This step is to synthesize first strand cDNAs*.
13. **After 30 minutes at 42°C**, inactivate **reverse transcriptase**, *which is known to interfere with Taq DNA polymerase in the PCR amplification step*, by heating the reactions at **85°C** for **5 minutes** in a **heat block**.
14. Chill the tubes **on ice** for at least **2 minutes**.

15. Centrifuge the tubes at room temperature for **1 minute** to bring water condensation to the bottom of the tubes. *Note: The RT reactions are ready for the PCR amplification step*.
16. Store RT reactions in a **-20°C freezer** if they are not used for PCR the same day. Otherwise, keep them **on ice** while setting up the PCR amplification step.

III. CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS

Purpose: To amplify the cDNA template corresponding to the mRNA for the gene of interest in order to determine if the T-DNA insertion causes a null mutation (i.e. no mRNA is expressed for the gene of interest).

*Note: Amplification of **tubulin** cDNA will be used as a positive control because the tubulin gene is expressed in all samples. **Control primers** are used to ensure that the absence of a gene-specific PCR product in the **+RT samples** is NOT due to technical mistakes.*

Solutions Needed:

- Reverse transcription (+RT & -RT) reactions
- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 μM Gene-specific RT Forward primer
- 12 μM Gene-specific RT Reverse primer
- 12 μM Tubulin Forward primer
- 12 μM Tubulin Reverse primer
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading buffer containing ONLY xylene cyanol
- 100 bp DNA ladder (Invitrogen)

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- Ice bucket
- 0.2 mL PCR tubes
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- PCR machine (Bio-Rad MyCycler)

- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

1. Get ice from the icemaker.
2. Determine **how many RT reactions** (including +RT's and -RT's and gene-specific primers and control primers) will be amplified.
3. Make a **table** with information such as **tube #**, **sample identity**, **+RT's/-RT's** and **primers** (see the **example** table below).

Tube #	Sample	RT	Primer set
1	WT Silique	+RT	Gene-specific RT
2	WT Silique	-RT	Gene-specific RT
3	T-DNA Silique	RT	Gene-specific RT
4	T-DNA Silique	-RT	Gene-specific RT
5 (Positive)	Genomic DNA	-	Gene-specific RT
6 (Negative)	Sterile Water	-	Gene-specific RT
7	WT Silique	+RT	Tubulin
8	WT Silique	-RT	Tubulin
9	T-DNA Silique	RT	Tubulin
10	T-DNA Silique	-RT	Tubulin
11 (Positive)	Genomic DNA	-	Tubulin
12 (Negative)	Sterile Water	-	Tubulin

4. Label the lids and sides of **TWELVE 0.2 mL PCR tubes** with the **tube number** and **your initials**. Put the labeled tubes on a PCR tube rack sitting **on ice**.
5. Label the lids and sides of **TWO 1.5 mL microcentrifuge tubes** as "**Gene-specific Mmix**" and "**Tubulin Mmix**." Put the labeled tubes **on ice**.
6. Prepare a **master mix** for each primer set for the **number of PCR reactions** being carried out **plus 1 extra** as follows:

Gene-specific Mmix Components	**Mmix for ONE Reaction**	**Mmix for 7 Reactions**
Sterile water	36.75 µL	257.25 µL
10x Ex Taq buffer	5.0 µL	35.0 µL
dNTP mix	4.0 µL	28.0 µL
12 µM Gene-specific RT Forward primer	1.0 µL	7.0 µL
12 µM Gene-specific RT Reverse primer	1.0 µL	7.0 µL
Ex Taq DNA Polymerase (5 U/µL)	0.25 µL	1.75 µL
Total Volume	**48.0 µL**	**336.0 µL**

Tubulin Mmix Components	**Mmix for ONE Reaction**	**Mmix for 7 Reactions**
Sterile water	36.75 µL	257.25 µL
10x Ex Taq buffer	5.0 µL	35.0 µL
dNTP mix	4.0 µL	28.0 µL
12 µM Tubulin Forward primer*	1.0 µL	7.0 µL
12 µM Tubulin Reverse primer*	1.0 µL	7.0 µL
Ex Taq DNA Polymerase (5 U/µL)	0.25 µL	1.75 µL
Total Volume	**48.0 µL**	**336.0 µL**

* ***Control primers*** *are used to ensure that the absence of a gene-specific PCR product in the* ***+RT samples*** *is NOT due to technical mistakes.*

7. Pipet **48 µL** of **master mix** and **2 µL** of **sample** into the appropriate labeled PCR tubes according to the **table in step 3**. The total reaction volume should be **50 µL**. Mix the contents by pipetting **gently** up and down **5 times**.
8. Carry out PCR on the Bio-Rad MyCylcer with the "**HC70AL RT PCR**" **program** containing the following profile:

94°C 3 min
40 cycles of 94°C 10 sec
62°C 30 sec
72°C 45 sec
72°C 4 min
4°C ∞

9. Prepare **100 mL** of a **2% agarose** gel in **1x TAE** buffer using a **20-tooth** comb.
10. Label TWELVE 1.5 mL microcentrifuge tubes according to the PCR reactions.
11. Add **20 µL** of **PCR solution** and **2 µL** of **6x loading dye with xylene cyanol only** to the labeled 1.5 mL microcentrifuge tubes.
12. Load **10 µL** of **100 bp DNA ladder** in the first well.
13. Load **20 µL** of each sample-dye mixture on a 2% agarose gel using a P-20 pipette.
14. Record the identity of the sample in each lane.

Lane	Tube	Sample	RT	Primer set	Expected Size (bp)
1	**-**	100 bp DNA ladder	-	-	-
2	**1**	WT Silique	+RT	Gene-specific RT	
3	**2**	WT Silique	-RT		
4	**3**	T-DNA Silique	+RT		
5	**4**	T-DNA Silique	-RT		
6	**5**	Genomic DNA	-		
7	**6**	Sterile Water	-		-
8	**7**	WT Silique	+RT	Tubulin	
9	**8**	WT Silique	-RT		
10	**9**	T-DNA Silique	+RT		
11	**10**	T-DNA Silique	-RT		
12	**11**	Genomic DNA	-		477
13	**12**	Sterile Water	-		-
14	**-**	100 bp DNA ladder	-	-	-

15. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
16. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) travels about two-thirds of the gel.

    Time power supply turned ON:

    Time power supply turned OFF:

    How long was the gel run? _____ hour(s) and _____ minutes

14. Take a picture of the gel using the Bio-Rad Gel Document System.
15. Print out the picture. Store the labeled picture in your lab notebook.
16. Analyze the data.

    How many DNA fragments do you see on the gel?

What are the sizes of the DNA fragments?

What is the size of the PCR product corresponding to the mRNA of the gene of interest?

What is the expected size of the PCR product for genomic DNA?

Is there a difference in size between the PCR products from cDNA and genomic DNA?

Is there difference in brightness between the PCR products from wild type siliques and mutant siliques?

What can you conclude about the expression of the gene of interest?

Are the RT-PCR results correlated to the GeneChip data?

EXPERIMENT 4 - IDENTIFYING FEATURES OF MUTANT SEEDS USING NOMARSKI MICROSCOPY (GENE ONE)

Purpose: To introduce **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**NIC**) microscopy technique as a tool to identify features of defective embryos in knockout mutants.

Reference: The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer's laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from Arabidopsis
    a. wild type
    b. homozygote or heterozygote mutant
- 100% ethanol
- Acetic acid
- Sterile water
- Chloral Hydrate (Cat. #C8383, Sigma-Aldrich; should be fresh)
- Glycerol (Invitrogen)
- Double-distilled water

Materials Needed:

- Pipettes
- Pipette tips (regular, non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Rulers with METRIC scale (mm)
- Plant layout chart
- Phenotype observation chart
- Fine point forceps
- 30-gauge hypodermic needles
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides
- Double-sided tape
- Dissecting microscopes (borrowed from Dr. Pei Yun Lee)
- A microscope equipped with Nomarski optical parameter (Leica CTR5000)
- Microscope camera

PROCEDURE

Each student collects the following from wild type and his/her homozygous or heterozygous mutant:

a) 5 siliques containing seeds with embryo stages of globular to torpedo.

b) 2 siliques containing seeds with mature green embryos.

Note: *Be sure to collect a wide* ***range*** *of stages. Do not collect yellow or brown siliques; these contain dry seeds.*

I. Observation of Seeds Using Light Microscopy and Fixing Seeds for Observation with Nomarski Optics

1. Prepare 5 mL of a fixative solution of ethanol: acetic acid (9:1, v/v) in a 14 mL centrifuge tube using disposable 5 mL pipets.

FIXATIVE SOLUTION	
100% ethanol	4.5 mL
Acetic acid	0.5 mL
Total volume	5.0 mL

Tightly snap the cap on the tube. **Invert** the tube to mix the contents.

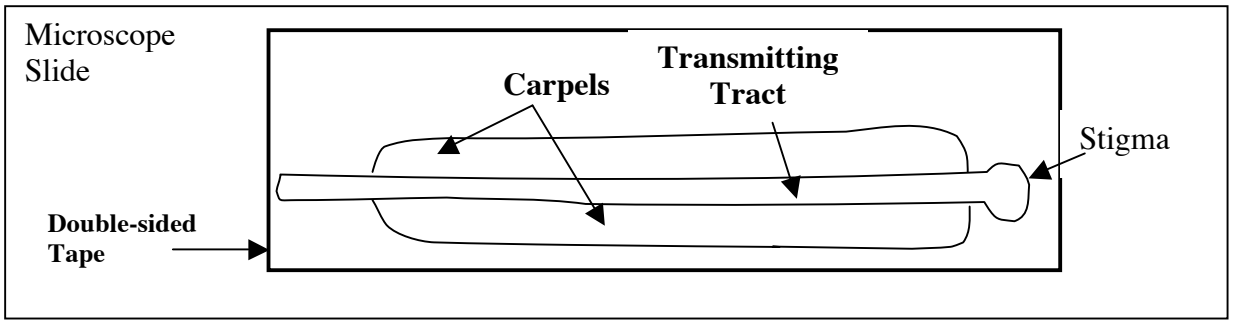
2. Pipet **1 mL** of the **fixative solution** into **FOUR** 1.5 mL microcentrifuge tubes sitting on a microcentrifuge tube rack. Label each tube in step 2 with your **initials**, the **plant #** and the **plant genotype**. *These tubes will be used in step 5.*
3. Bring the following materials to the Plant Growth Center (PGC).
   - Bucket of ice
   - Tweleve 1.5 mL microcentrifuge tubes
   - Microcentrifuge tube rack
   - Black ultra-fine sharpie
   - Ruler with METRIC scale (mm)
   - A pair of fine point forceps
   - Plant layout chart with information about plant number and the genotype of those plants
   - This protocol

- Bruincard with access to the PGC
- Key to growth chambers in the PGC

4. Measure and collect siliques according to the chart below. Place each silique in a 1.5 mL microcentrifuge tube. Write your **initials**, the **plant #**, the **plant genotype** and the **length** on the tube. Keep the tube on **ice**. *Note: Collect the **same length** of siliques for wild type and homozygous/heterozygous mutant so that you can compare them.*

Plant Genotype	**Seed Stages Collected**	**Length of Siliques Collected**	**Number of Siliques Collected**
Wild type	globular to torpedo	*0.5 - 1.0 cm*	5
Wild type	mature green	*1.0 - 1.9 cm*	2
Heterozygous or homozygous mutant	globular to torpedo	*0.5 - 1.0 cm*	5
Heterozygous or homozygous mutant	mature green	*1.0 - 1.9 cm*	2

5. Go back to the lab. **Dissect** the siliques and **observe** the seed phenotype using a dissecting microscope. *Note: Work quickly so the seeds don't dry out. You may also place a drop of water on the silique.*
    a. **Place** a **piece of double-sided tape** on a **microscope slide**. Label the microscope slide with a small piece of white tape with your **initials**, the **plant #**, the **plant genotype** and the **length**.
    b. Carefully, use **fine-point forceps** to place a silique on the tape.
    c. Under a dissecting microscope, use **fine-point forceps** to carefully place a silique on the tape and **arrange** the silique such that the **transmitting tract** is facing you (see diagram below, NOT drawn to scale).



d. With your left hand, use forceps to hold the silique on the side closest to the stem.

e. With your right hand, use a **28G** or **30G hypodermic needle attached to a 1 cc syringe** to **slice** the **carpels along each side of the transmitting tract.**

f. **Gently** peel back the carpels and stick them to the tape to reveal the seeds.

g. Observe the phenotype. Note any phenotypes that you observe on your **Screening Seeds Using Light Microscopy** chart.

In what stage of development are the seeds?

How many seeds are in the silique?

How many are green?

How many are white?

How many are brown?

What is the expected ratio of wild type seeds to mutant seeds if the mutation is seed lethal?

What is the observed ratio of wild type seeds to mutant seeds?

Are the observed results significantly different from the expected results?

Use a Chi-Square test.

$$\chi^2 = \Sigma \frac{(observed - expected)^2}{expected}$$

Probability that the deviation is due to chance alone

Degrees of Freedom	0.5	0.1	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.235	11.070	13.388	15.086	20.517

What is your null hypothesis?

How many degrees of freedom are there?

(The degrees of freedom is one less than the number of different phenotypes possible.)

What is your chi-square value?

(The chi-square statistic is a probability that indicates the chance that, in repeated experiments, deviations from the expected would be as large or larger than the ones observed in this experiment)

What is the probability that the deviation of the observed values from the expected values was a chance occurrence?

(Look up your degrees of freedom in the table. Find where your chi-square value falls in that row.)

Can you reject the null hypothesis?

If the probability is less than 0.05 (5%), reject your null hypothesis. If the probability is 0.05 (5%) or greater, then you cannot reject your null hypothesis.

h. Ask your TA to take pictures of the seeds within the siliques.

i. **Immediately**, use the fine-point forceps to transfer the cut silique into the tube with fixative solution from **step 2**.

j. Repeat steps a-i for the other siliques. *<u>Note:</u> You collected an excess of siliques so that you would have some to practice dissecting and to have a*

range of developmental stages for each genotype. However, you only need to fix FOUR siliques.

i. *Wild type, early development*
ii. *Heterozygous (or homozygous), early development*
iii. *Wild type, late development*
iv. *Heterozygous (or homozygous), late development*

6. Fix seeds and siliques in the fixative solution for at least 2 hours. *Note: It is recommended to fix the siliques **overnight** to ensure that the fixative solution penetrates the seeds and their embryos. It is okay to leave siliques in the fixative solution for up to 3 days.*
7. **Carefully**, pipet off **900 µL** of the fixative solution using a **P-1000** pipette and discard into a beaker labeled "acetic acid waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
8. **Immediately**, pipet **1 mL** of **90% ethanol** solution into the tube using a P-1000 pipette. *Note: The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
Double-distilled water	0.5 mL
Total volume	5.0 mL

9. Incubate seeds and siliques in the 90% ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*
10. Replace the 90% ethanol solution with **70% ethanol** as in steps 7 & 8.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
Double-distilled water	1.5 mL
Total volume	5.0 mL

11. Incubate seeds and siliques in the ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*

II. Observation of Seeds and Embryos Using Nomarski Optics

Note:

- ***Before observation of the seeds and their embryos, seeds must be submerged in the clearing solution. For young seeds, clearing is usually fast (~5 minutes). The older the silique, the longer it takes to clear (~ 1 hour). Seeds are ready for observation after they sink in the clearing solution.***
- ***Tissues CANNOT be stored in the CLEARING solution for more than TWO days because they will lose their structures quickly.***

1. Prepare a ***fresh*** clearing solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14 mL centrifuge tube. *Note: The TAs will prepare this solution before the lab class begins.*

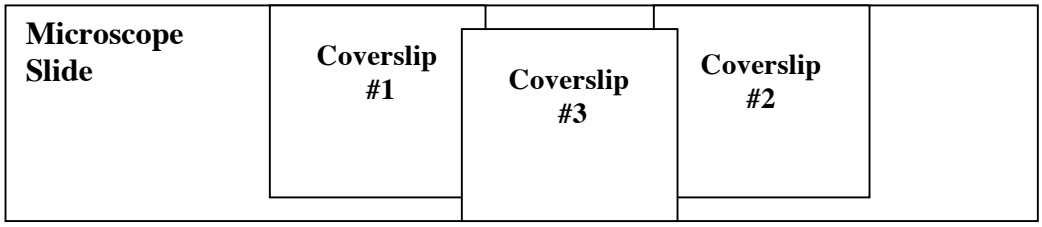
CLEARING SOLUTION

Chloral hydrate	8 g
Glycerol	1 mL
Water	2 mL
Total volume	~7 mL

2. **Carefully**, pipet off **900 µL** of the 70% ethanol solution using a **P-1000** pipette and discard into a beaker labeled "ethanol waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
3. Replace the 70% ethanol solution with **100 µL of clearing solution**.
4. Incubate seeds and siliques in the clearing solution for **5 min - 1 hour**. Wait until the seeds **sink** to the bottom of the tubes. You may lay the tube on its side so that the

silique is immersed in the clearing solution. *Note: Tissues CANNOT be stored in the CLEARING solution*.

5. Set a new glass microscope slide on the bench. Label it with your **initials**, the **plant #**, the **plant genotype** and **silique length**.
6. Use forceps to remove a silique from the clearing solution and place it on the labeled glass slide.
7. Pipet the remaining clearing solution and seeds onto the slide with the silique.
8. Carefully, place two square coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution. Avoid trapping bubbles in the solution (see diagram below).



9. Observe the seeds under Nomarski optics using the Leica CTR5000 microscope.
10. Take pictures of the embryos.

    In what stage of development are the seeds?

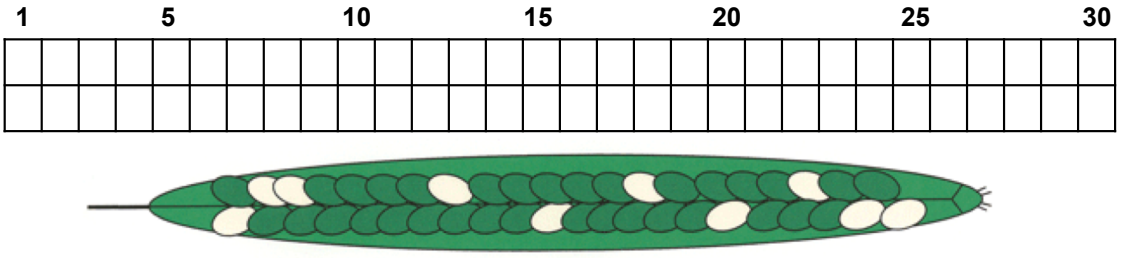
11. Repeat steps 2-10 for the remaining 3 fixed siliques.

Screening Seeds Using Light Microscopy

AGI# _____ SALK # _____ Plant #_____Genotype _____

Silique # _____ Length of Silique (cm) _____ Total Seeds _____ Total Mutant Seeds _____

<u>Instructions:</u> The grid represents the layout of the silique. Put a number in each square that corresponds to a mutant seed. Describe the seed phenotypes in the chart below. The base of the silique is defined as the region closest to the pedicel and main stem, which is at the left of the grid.



Seed	Seed Coat Color	Embryo Color	Notes
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

EXPERIMENT 5 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE TWO)

Purpose: To identify a knockout line for the gene of interest and characterize the phenotype of mutant plants.

References: University of Wisconsin - Madison Knockout Facility
Ohio State University - Arabidopsis Biological Resource Center

STRATEGY

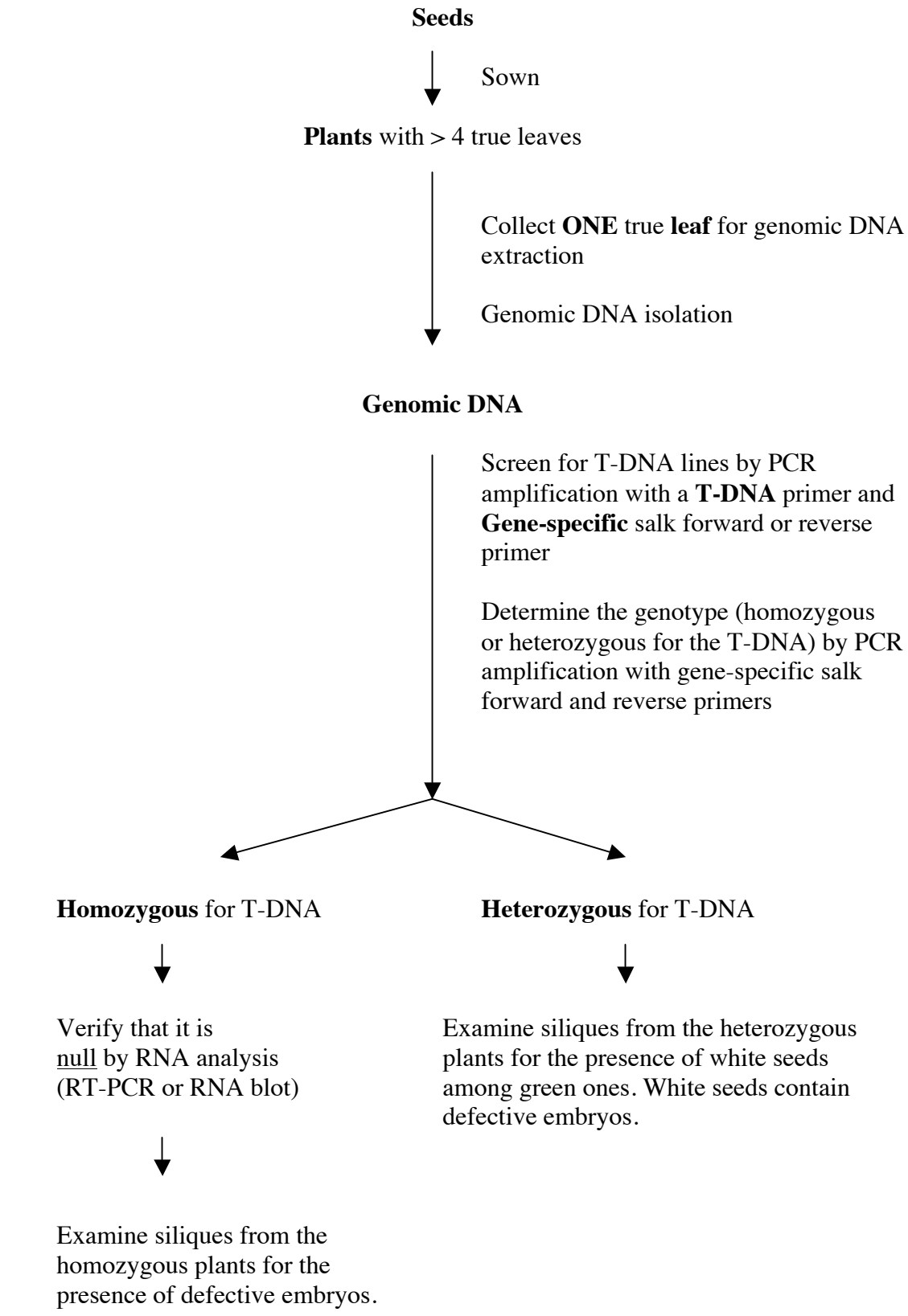
I. SOWING SEEDS AND GROWING PLANTS

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS

III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES

IV. DETERMING WHAT GENE YOU ARE STUDYING AND THE T-DNA INSERTION SITE

STRATEGY


Seeds
Sown
Plants with > 4 true leaves
Collect ONE true leaf for genomic DNA extraction
Genomic DNA isolation
Genomic DNA
Screen for T-DNA lines by PCR amplification with a T-DNA primer and Gene-specific salk forward or reverse primer
Determine the genotype (homozygous or heterozygous for the T-DNA) by PCR amplification with gene-specific salk forward and reverse primers
Homozygous for T-DNA
Heterozygous for T-DNA
Verify that it is null by RNA analysis (RT-PCR or RNA blot)
Examine siliques from the heterozygous plants for the presence of white seeds among green ones. White seeds contain defective embryos.
Examine siliques from the homozygous plants for the presence of defective embryos.

I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings for genomic DNA extraction.

Reference: Arabidopsis Biological Resource Center http://abrc.osu.edu/

Materials Needed:

- Tubes of knockout seeds from the Arabidopsis Biological Resource Center
- Microcentrifuge tube rack
- Sheet of white Xerox paper cut into quarters
- Black sharpie (ultra-fine or fine)
- Plastic yellow tags (5-6 tags per knockout line)
- Pointed-end forceps
- Key to the Plant Growth Center (PGC)
- BruinCard with access to PGC
- Black plastic trays (in PGC)
- Black rectangular pots in sheets (12 pots/sheet; in PGC)
- Clear plastic covers for black trays (in PGC)
- Soil (in PGC)
- One or two pairs of latex gloves

PROCEDURE

Caution: Be extremely CAREFUL with seeds. Do NOT mix up labeled tags and actual seed lines.

1. Obtain **tubes of seeds** to be grown from the cold room and put them on a microcentrifuge tube rack. *For example, S_112701 (Salk line for gene At5g11240) and wild type seeds (Columbia-0).*
2. Bring the **materials** to the **Plant Growth Center** (**PGC**).
3. At the PGC, put all of these items on **the bench** that runs along the **east wall**. This bench does NOT have any soil on it. *Note: Do NOT put tubes of seeds or plants full of mature siliques near the bench of soil because the prepared soil will be contaminated with these seeds, which could in turn, result in false mutant phenotypes for other people's work.*

4. In the PGC, prepare ONE flat with **12 pots** of soil for every TWO knockout lines being planted.
    a. Assemble each flat as follows:
        i. Obtain a black plastic tray without holes.
        ii. Obtain a sheet of 12 rectangular plastic pots.
        iii. Obtain a clear plastic cover.
        iv. Set the sheet of 12 pots in the black plastic tray.
        v. Fill the pots loosely with soil, without compressing. Use the sifter to add a layer of fine soil. *(Soil prepared by the PGC staff, Mr. Weimin Deng)*.
        vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
    b. Remove **one pot** from the corner of the flat and put the soil back into the mound of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in*.
    c. Bring the flat to the bench near the sink.
    d. Use the hand brush to clean up the soil bench.
    e. Make sure that the water hose is attached to the water pipeline labeled "**fertilizer-supplemented**."
    f. Fill each flat **2/3 of the way up** the tray with "fertilizer-supplemented" water.
    g. Cover the flat with a **clear plastic cover** to prevent the growth of air-borne molds and to protect the soil from **stray *Arabidopsis* seeds**.
    h. Wait **20 minutes or until the surface of the soil appears darker** due to water seeping up from the bottom of the pots.
5. Label **11 plastic yellow tags** with a black sharpie.
    a. For **knockout lines**: **Gene name**
Salk line #
Date
Pot # 1-10
    b. For **wild type**: **Columbia-0**
Date
Pot # 11

6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
7. Fold each **quarter sheet** of white paper **in half**, lengthwise.
8. Gently pour out the **knockout seeds** from the microcentrifuge tube onto one of the folded pieces of paper.
9. Bring the folded paper with seeds close to the soil of each of the first 5 pots. Lower one end of the paper near the soil surface. Use the **forceps** to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
10. Visually divide the pot into 4 quadrants, and sow a seed in each quadrant. Sow **4 seeds** per pot, for the first 5 pots. *Note: Planted seeds should not be covered with additional soil because Arabidopsis seeds need light for germination.*
11. Put the labeled tags for the **knockout line** into **each** of the **5 pots** containing knockout seeds.
12. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge tube.
13. Repeat steps 7-12 with the seeds for the next knockout line and pots #6-10. Use a **new** folded piece of white paper for each line.
14. For pot **#11**, pour out wild type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild type seed in each quadrant. **Four wild type seeds** should be sown in **pot #11.**
15. Put a **wild type labeled tag** into pot #11.
16. Cover the flat with the **clear plastic cover**. Put the flat aside.
17. After all of the lines are sown, put the flats on a metal cart and take the elevator to the lower level.
18. Put the flats on the wire racks in the cold room (the first room on the right after entering the double doors across from the elevator).
    CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil. Note: Turn the lights off when you leave the cold room.

19. Leave the flats in the cold room for **2-5 days** to **vernalize seeds.** <u>*Note:*</u> *This will eliminate any dormancy, improve the germination rate and synchronize seed germination.*
20. After 2-5 days in the cold room, put the flats on a metal cart and take the elevator to the upper level. Transfer the flats to a bench in the greenhouse room 125B.
    <u>*NOTE:*</u> *Choose a table that has no mature Arabidopsis plants bearing ripened seeds because these seeds could accidentally get in the soil of the knockout flats when the clear covers are removed.*
21. Slide the clear covers off the trays by **0.5-1 inch** so that warm air under the covers will not overheat the seedlings or create a warm environment favorable for algal and fungal growth. Sliding off the clear covers will also provide some aeration, but still maintain enough humidity for germination and also avoid seed desiccation.
22. Wait until most of the seedlings in the flats have **4 true leaves**. Then completely remove the clear covers from the flats.
23. Map the **positions of seedlings** in **each of the 11 pots** on a "Plant Layout Chart."

GENOTYPING ARABIDOPSIS PLANTS

PLANT LAYOUT CHART

Gene ID: **At__ g _____ Salk line#: _____ Date: _____**
Primers for PCR: _____
Size of PCR product: _____

Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS

Purpose: To isolate genomic DNA from seedling leaves for identifying plants containing a T-DNA insert within the gene of interest.

Recommendation:

Instead of isolating genomic DNA from all 22-24 seedlings at once, you can **start** with **6 seedlings** (**5** from the **knockout** line and **1** from **wild type**). Once you are familiar with the method of isolating genomic DNA, you can isolate genomic DNA from the remaining seedlings (including wild type), if necessary.

Materials Needed:

- Seedlings with at least 4 true leaves (knockout lines and wild type)
- Ice bucket
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Pipettes
- Pipet tips
- One or two pairs of latex gloves
- Two pairs of pointed-end forceps
- Squirt bottle of 100% ethanol solution
- Kimwipes
- Black sharpie (ultra-fine or fine)
- Pen
- Plant layout chart
- Digital camera
- Key to the Plant Growth Center
- BruinCard with access to PGC
- Extraction buffer (0.2 M Tris-HCl, pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS)
- Sterile blue micropestles
- Microcentrifuge
- Timer
- Isopropanol
- 80% ethanol solution
- SpeedVac (optional)
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain

- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution
- Gel electrophoresis materials (Appendix 1A)
- NanoDrop spectrophotometer

PROCEDURE

Attention: *You will need to **assess** the **quality** of **isolated genomic DNA** later (at **step 36**). If you plan to do this on the same day as the genomic DNA isolation, use time efficiently by preparing a 0.7% agarose gel before you start the isolation of genomic DNA (see **Appendix 1A**). While the agarose mixture cools in the 55-60°C water bath for at least 10 minutes, go to the Plant Growth Center to collect leaves. When you come back to the lab, add 10,000x SYBR Safe DNA gel stain to the agarose mixture, swirl to mix, pour the gel, and let the agarose mixture solidify in the dark.*

1. Put **SIX** sterile 1.5 mL microcentrifuge tubes on a microcentrifuge tube rack.
2. Label the lids of the tubes **1-6**.

   Tube #**1 - 5**: Seedlings #1 - 5 of **knockout** lines

   Tube #**6**: **1** Seedling/plant from **wild type** (Columbia-0)
3. Pipet **100 μL** of **Extraction Buffer** into each tube. Keep the tubes on ice. *Note: It is **not** necessary to keep tubes of **Extraction Buffer on ice** during collection of the leaf samples if genomic DNA will be isolated from samples within one hour.*
4. Gather together the following items on a plastic tray or container:
   - Bucket of ice
   - 1.5 mL microcentrifuge tubes containing 100 μL of Extraction Buffer
   - One or two pairs of latex gloves
   - Two pairs of pointed-end forceps
   - Squirt bottle of 100% ethanol solution
   - Kimwipes
   - Black sharpie (ultra-fine or fine)
   - Pen
   - Plant layout chart
   - This protocol
   - Digital camera
   - Key to the Plant Growth Center
   - BruinCard with access to PGC
5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.

6. Take pictures of the plants to document the phenotype. Take pictures of the yellow tags to identify the plants in the pictures.
7. Use the **Plant Layout Chart** to mark the **locations of the plants** and to indicate the plants that you will collect samples from by numbering them. Also make a note of any interesting phenotypes. (For example, some plants may be smaller than others.) The **order of plants** should **correspond** to the **labeled tags** that were numbered when the seeds were planted. *Note: NOT all of the seeds will have germinated.*
8. Use a piece of Kimwipes to clean the forceps with ethanol. *Note: Two sets of forceps are used per plant. The forceps must be cleaned after the collection each leaf to avoid contamination.*
9. Using forceps, remove one **small leaf** from the **plant #1**.
10. Place this leaf in microcentrifuge **tube #1** containing the Extraction Buffer.
11. Repeat steps 8-10 for the other plants.
    Note: MAKE SURE TO CLEAN THE FORCEPS BETWEEN LEAF SAMPLES!
12. Go back to the lab.
13. Homogenize or macerate the collected leaf in **tube #1** by crushing it with a **blue micropestle** until no more chunks of plant tissue are observed in the mixture. *Note: Do NOT dispose of the micropestle.*
14. Rinse the **micropestle** with **300 µL** of Extraction Buffer into the microcentrifuge tube. Put the used micropestles in a beaker labeled "used micropestles" so that they can be washed. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 µL**.
15. Vortex the **homogenate** for 5 seconds. Set the tube on ice.
16. Repeat steps 13-15 for the other tubes.
17. Centrifuge the tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**. *Note: Position the tubes in the centrifuge so that the hinge of the microcentrifuge tubes faces the outside of the microcentrifuge. This way after centrifugation you know to look for your pellet on the side of the microcentrifuge tube that has the hinge.*
18. Meanwhile, **label** a set of **microcentrifuge tubes** with **Gene Name, plant #,** "**gDNA**," **your initials** and the **date.**
19. Pipet **350 µL** of **isopropanol** to each of the new labeled tubes.

20. After centrifugation, transfer the tubes from the microcentrifuge onto a microcentrifuge tube rack. Organize tubes on the rack such that the **numbers** on the lids of the **NEW tubes match** with the **numbers** on the lids of the **tubes containing homogenates**.
21. Pipet **350 µL** of **supernatant** (**homogenate**) from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes. *Note: Use your pipet to draw off liquid from the side of the tube opposite that against which the plant material is pelleted. Start at the top and move downward as the liquid level drops. AVOID disturbing the plant debris on the bottom of the tubes as much as possible. However, it is okay if you accidentally transfer some plant debris into the isopropanol tube.*
22. Mix the isopropanol and homogenate by inverting the tubes **5-10 times**.
23. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA).*
24. Centrifuge the tubes at **room temperature** for **10 minutes** at **FULL** speed.
25. Pour or pipet each isopropanol supernatant into a waste container. *Note: DNA is now in your pellet along with RNA. Therefore, be extremely careful when pouring off the isopropanol because the pellets are sometimes loose.*
26. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert five times. *This step is to wash off any residual salt (from the extraction buffer) and isopropanol.*
27. Centrifuge the tubes at **room temperature** for **5 minutes**.
28. Pour or pipet each ethanol supernatant into a waste container. Dab the tubes on Kimwipes to remove as much ethanol as possible. *Note: Be extremely careful when pouring off the ethanol solution because the pellets are sometimes loose.*
29. Put the tubes on a microcentrifuge tube rack with their lids open, allowing the ethanol to evaporate. *Note: You may use a P-200 pipette to carefully draw off excess ethanol from the side of the tube opposite that against which the nucleic acid is pelleted. Be very careful not to pipet the pellet.*
30. Dry pellets either in a **SpeedVac** at room temperature for **5-10 minutes** (Your instructor will show you how to do this step) or by leaving on the **bench at room temperature for up to 60 minutes**.

31. After drying the pellets, resuspend each pellet by adding **200 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over a microcentrifuge tube rack **10-15 times** or **vortexing** the tubes briefly. *Note: If you vortex genomic DNA vigorously or for a long time, it will degrade.*
32. Spin the tubes in a microcentrifuge for **1 minute** to bring liquid and any contaminants to the bottom of the tubes.
33. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.
    Note: Keep DNA solutions cold as much as possible to prevent degradation of DNA because this is a crude extraction of genomic DNA, and there may be a trace amount of endonuclease present in the DNA solution.

    Attention: *At this step, you need to assess the **quality** and **quantity** of isolated genomic DNA by **gel electrophoresis** (**step 34**) and **spectrophotometer reading** (**step 35**), respectively.*
34. Analyze the **quality** of isolated genomic DNA by **gel electrophoresis**.
    *Note: First, spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*
    a. Prepare a **0.7%** agarose gel with a 20-tooth comb (0.7 g of agarose in 100 mL of 1x TAE buffer; see Appendix 1A). *Note: The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA.*
    b. Label the lids of 6 microcentrifuge tubes with **#1-6** and **your initials**, and set tubes on a microcentrifuge tube rack.
    c. Pipet **20 µL** of **isolated genomic DNA** solution into each of the labeled tubes.
    d. Add **2 µL** of **6x loading dye solution** to each tube and mix the contents by pipetting up and down 5 times.
    e. Load **10 µL** of **1 Kb Plus DNA ladder solution** into the first well.
    f. Load **20 µL** of each sample-dye mixture prepared in step d using a P-20 pipette.
    g. Record the identity of the sample in each well.

Lane	Sample	Expected Size (bp)
1	1 Kb Plus DNA ladder	-
2	Genomic DNA from Plant #1	
3	Genomic DNA from Plant #2	
4	Genomic DNA from Plant #3	
5	Genomic DNA from Plant #4	
6	Genomic DNA from Plant #5	
7	Genomic DNA from Plant #6	

Lane	Sample	Expected Size (bp)
1	1 Kb Plus DNA ladder	-
2	Genomic DNA from Plant #7	
3	Genomic DNA from Plant #8	
4	Genomic DNA from Plant #9	
5	Genomic DNA from Plant #10	
6	Genomic DNA from Plant #11	
7	Genomic DNA from Plant #12	

h. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.

i. Run the gel at **105 volts** for 1-2 hours.

Starting time:

Ending time:

j. Take a picture of the gel using the Bio-Rad Gel Documentation system.

What do you observe on the gel?

What is the size of genomic DNA?

Do you observe any other bands? What do you think they are?

35. Determine the concentration of DNA using the NanoDrop spectrophotometer. *Your instructor will demonstrate how to use the NanoDrop.*

*Note: First, spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

Record the **concentration of DNA** in the solutions in the **table** below:

Sample	**DNA Concentration (ng/µL)**
Plant #1	
Plant #2	
Plant #3	
Plant #4	
Plant #5	
Plant #6	

Sample	**DNA Concentration (ng/µL)**
Plant #7	
Plant #8	
Plant #9	
Plant #10	
Plant #11	
Plant #12	

36. Dilute **5 µL** of **original DNA solutions** to a **final concentration** of **0.2 ng/µL** with **TE** buffer.

    Note: Dilution of DNA solutions will serve two purposes:

    a. *Contaminants, such as carbohydrates that bind nonspecifically to nucleic acids and proteins, will be diluted out. A tiny amount of contaminants in a PCR reaction will not interfere with the amplification of the target DNA.*
    b. *Only a small amount of Arabidopsis genomic DNA (~0.4 ng) is needed for PCR amplification.*

    How to make a dilution?

    Use the basic formula that is widely used in general chemistry labs. That is,

    $$\mathbf{V_i \times C_i = V_f \times C_f}$$

    where,

    $\mathbf{V_i}$ = **initial volume** (the volume of original DNA solution is **5 µL**)

    $\mathbf{C_i}$ = **initial concentration** (reading from the spectrophotometer; example: **8 ng/µL**)

    $\mathbf{V_f}$ = **final volume** (depends on the initial concentration)

C_f = final concentration (0.2 ng/µL)
then,

V_f = (V_i x C_i)/ C_f = (5 µL x **8 ng/µL)/(0.2 ng/µL) = 200 µL** of **total volume**

What is the volume of TE to be used in dilution?

V_{TE} = V_f - V_i = 200 µL - 5 µL = 195 µL of **TE**

a. Record the **volume of TE** and the **final volume** in the table below.

	Volume of Isolated Genomic DNA	**Volume of TE Added**	**Final Volume**
Plant #1	5 µL		
Plant #2	5 µL		
Plant #3	5 µL		
Plant #4	5 µL		
Plant #5	5 µL		
Plant #6	5 µL		

	Volume of Isolated Genomic DNA	**Volume of TE Added**	**Final Volume**
Plant #7	5 µL		
Plant #8	5 µL		
Plant #9	5 µL		
Plant #10	5 µL		
Plant #11	5 µL		
Plant #12	5 µL		

Note: If the volume of TE to be used in dilution is greater than 1.5 mL, it will not fit in a 1.5 mL microcentrifuge tube. In this case, dilute 2.5 µL of genomic DNA.

b. Label the lids and sides of microcentrifuge tubes with the following information: **0.2 ng/µL gDNA**, **plant #**, **your initials** and the **date**. Keep all tubes of DNA solutions **on ice**.
c. Spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.
d. Add the appropriate volume of TE to each newly labeled tube.
e. Add **5 µL** of **original DNA solutions** into each tube. Flick tubes to mix.
f. Spin the tubes in a microcentrifuge for **1 minute** to bring liquid and any contaminants to the bottom of the tubes.
g. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.

III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES

Purpose: To identify plants containing the T-DNA insert and to determine the genotypes of T-DNA-tagged plants (heterozygous or homozygous for the T-DNA).

STRATEGY

A. Polymerase Chain Reaction (PCR)

B. Gel Electrophoresis Analysis of PCR Product

C. Label T-DNA tagged plants

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 μM Gene-specific Salk Forward primer
- 12 μM Gene-specific Salk Reverse primer
- 12 μM LBb1.3 primer (anneals to the Left Border (LB) region of the T-DNA)
- 0.2 ng/μL genomic DNA extracted from the plants to be genotyped (including WT)
- 0.2 ng/μL genomic DNA extracted from wild type seedlings by TAs
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- 0.2 mL PCR tubes in strips of 8
- 1.5 mL microcentrifuge tubes
- Rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Ice bucket
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Microcentrifuge
- Gel electrophoresis materials (Appendix 1A)
- Key to the Plant Growth Center

- BruinCard with access to PGC
- Wooden sticks
- Tape
- Black sharpie (ultra-fine or fine)
- Pen
- Plant Layout Chart
- Phenotype Observation Record
- Digital Camera

A. Polymerase Chain Reaction (PCR)

PROCEDURE

Note: Because you do not know the orientation of the T-DNA insertion, and therefore the direction of the LBb1.3 primer, you will need to set up ***THREE*** *PCR reactions for* ***each*** *sample.*

Reaction A: ***Gene-specific Salk Forward*** *primer and* ***LBb1.3*** *primer*

Reaction B: ***LBb1.3*** *primer and* ***Gene-specific Salk Reverse*** *primer*

Reaction C: ***Gene-specific Salk Forward*** *primer and* ***Gene-specific Salk Reverse*** *primer*

Note: ALWAYS wear gloves and use filter tips to prevent contamination when preparing PCR reactions.

Note: There are ***6*** *plants to be characterized and* ***2 controls*** *(genomic DNA isolated by TA + No DNA template), prepare each* ***master mix*** *for* ***8 + 1 extra = 9 reactions****.*

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer, dNTP mix, 12 µM Gene-specific Salk Forward primer, 12 µM Gene-specific Salk Reverse primer** and **12 µM LBb1.3 primer** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed, put the tubes on **ice** until needed.

3. Spin genomic DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed. Keep on ice.
4. Label the lids and sides of **24 PCR tubes** with **A1-A8, B1-B8, C1-C8** and **your initials** and the **date**. Put them on a PCR tube rack sitting on ice.
5. Prepare a **"Reaction A" master mix** for **9 PCR reactions** in a 1.5 mL microcentrifuge tube labeled "**Mmix A**" sitting on **ice**. Pipet the reagents in order from top down (example: water, 10x Ex Taq buffer, dNTP mix, etc.) into the **Mmix A** tube.

Reaction A	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM **Gene-specific Salk Forward** primer	1.0 µL	9.0 µL
12 µM Gene-specific Salk Reverse primer	-	-
12 µM **LBb1.3** primer	1.0 µL	9.0 µL
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

6. Mix the contents of **Mmix A** by flicking the tube several times or vortexing the tube at a **setting of 2-3** for **5 seconds**. Spin the tube in a microcentrifuge at FULL speed for **10 seconds**. Put the tube back **on ice**.
7. Pipet **46 µL** of **Mmix A** into each of **8 PCR tubes** labeled **A1-A8**.
8. Pipet **4 µL** of **0.2 ng/µL genomic DNA** extracted from each of **6 seedlings** into PCR tubes A1-A6. Pipet up and down 5 times to mix the contents.
9. Pipet **4 µL** of **0.2 ng/µL genomic DNA** extracted from **wild type** (Col-0) seedlings by TAs into tube **A7**. Pipet up and down 5 times to mix the contents.
10. Pipet **4 µL** of **sterile water** into tube **A8** (**negative control** without DNA template). Pipet up and down 5 times to mix the contents.
11. Keep these PCR reactions on ice while you prepare the next master mix. Repeat steps 5-11 for Reactions B and C.

Reaction B	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM Gene-specific Salk Forward primer	-	-
12 µM **Gene-specific Salk Reverse** primer	1.0 µL	9.0 µL
12 µM **LBb1.3** primer	1.0 µL	9.0 µL
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

Reaction C	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM **Gene-specific Salk Forward** primer	1.0 µL	9.0 µL
12 µM **Gene-specific Salk Reverse** primer	1.0 µL	9.0 µL
12 µM LBb1.3 primer	-	-
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

12. Spin all PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
13. Turn **ON** the PCR machine (MyCycler). Wait for one minute for the machine to initialize.
14. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
15. Select the "**Protocol Library**" by pressing "**F1**."
16. Select "**HC70AL**" by pressing the yellow arrowheads surrounding the "**ENTER**" button. Select the "**HC70AL KNOCKOUT**" protocol. Press "**ENTER**."
17. The "CHOOSE OPERATION" menu will appear. Select "**VIEW PROTOCOL**." The **PCR profile** of the Knockout protocol is as follows:

94°C for 3 min
36 cycles of 94°C 15 sec
62°C 30 sec
72°C 2 min

72°C 4 min
4°C ∞

18. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
19. Enter the **volume** of the PCR reaction. Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
20. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

PROCEDURE

1. Prepare a **1% agarose gel** in 1x TAE buffer with a **30-tooth** comb (or 20-tooth comb depending on how many samples you have).
2. Record the identity of the sample in each lane. Use the table below as a guide.
3. Label **24 1.5 mL microcentrifuge** tubes (one for each sample) and set them on a microcentrifuge tube rack.
4. Add **3 µL** of **6x loading dye** to each tube.
5. Pipet **25 µL** of **PCR solution** to each tube. Mix the contents by pipetting up and down 5 times or by flicking the tubes. Spin briefly.
6. Load **10 µL** of **100 bp DNA ladder** in the first well.
7. Very slowly load the ~**28 µL** sample-dye mixtures on the gel using a P-20 pipette *(i.e. load ~15 µL first, then then remaining ~15 µL into the same well).*
8. Load **10 µL** of **1 Kb Plus DNA ladder** in the last well.

Lane	Sample	Primers	Expected Size (bp)
1	100 bp DNA ladder	-	-
2	Genomic DNA from Plant #1	Reaction A: **Gene-specific Salk Forward** primer and **LBb1.3** primer	
3	Genomic DNA from Plant #2		
4	Genomic DNA from Plant #3		
5	Genomic DNA from Plant #4		
6	Genomic DNA from Plant #5		
7	Genomic DNA from Plant #6		
8	Col-0 Genomic DNA (from TAs)		-
9	Sterile water		-
10	100 bp DNA ladder		
11	Genomic DNA from Plant #1	Reaction B: **LBb1.3** primer and **Gene-specific Salk Reverse** primer	
12	Genomic DNA from Plant #2		
13	Genomic DNA from Plant #3		
14	Genomic DNA from Plant #4		
15	Genomic DNA from Plant #5		
16	Genomic DNA from Plant #6		
17	Col-0 Genomic DNA (from TAs)		-
18	Sterile water		-
19	100 bp DNA ladder		
20	Genomic DNA from Plant #1	Reaction C: **Gene-specific Salk Forward** primer and **Gene-specific Salk Reverse** primer	
21	Genomic DNA from Plant #2		
22	Genomic DNA from Plant #3		
23	Genomic DNA from Plant #4		
24	Genomic DNA from Plant #5		
25	Genomic DNA from Plant #6		
26	Col-0 Genomic DNA (from TAs)		
27	Sterile water		-
28	1 Kb Plus DNA ladder	-	-

9. Add **5 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
10. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.
11. Stop the gel electrophoresis.
12. Take a picture of the gel and annotate it.
13. Analyze the data.

    Do you observe PCR fragments?

    What are the sizes of these fragments?

    Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?

    What is the orientation of the T-DNA relative to the gene?

    What are the genotypes of the 6 plants?

Note:

1. *Although the results of the PCR reactions should confirm the size of the so-called "T-DNA fragment," which contains part of the plant gene and part of the T-DNA, it is a **good scientific practice** to verify the exact location of the T-DNA insertion site.*

2. ***Depending on the results of the PCR** to genotype the plants, you can use one of the following procedures to purify the "T-DNA fragment."*
    a. *If a lane on the gel contains only a **single band** corresponding to the "T-DNA fragment," then the "T-DNA fragment" can be purified directly from the PCR solution by following the **QIAquick PCR Purification Procedure** below.*
    b. *If all lanes containing the "T-DNA fragment" also contain other bands (due to contamination or mispriming), then the "T-DNA fragment" must be purified from an agarose gel slice. Follow the **QIAquick Gel Extraction Procedure** below.*

C. Label T-DNA tagged plants

PROCEDURE

1. After determining the genotypes of the plants, make labels for the plants containing a T-DNA insert by putting a small piece of tape on a wooden stick. Write the **number** that corresponds to the **plant #** on the Plant Layout Chart and either "**homozygous for the T-DNA**" or "**heterozygous for the T-DNA**." You can also note the genotype of the plants on the Plant Layout Chart.
2. Go to the Plant Growth Center. Put the labeled wooden sticks next to the identified T-DNA tagged plants.
3. Observe T-DNA tagged plants for abnormal phenotypes. Write your observations on the **Phenotype Observation Record**. Take pictures of the plants to document the phenotype. Take pictures of the yellow tags to identify the plants in the pictures. You may take flowers back to the lab to observe the phenotype under a microscope.

PHENOTYPE OBSERVATION RECORD

Gene ID: **At__ g _____ Salk line#: _____ Date: _____**

LEAF	**Mutant**	**Wild Type**
What do the leaves look like, green or yellow, elongated or round?		
What is the range of their length in cm?		
How many leaves does each plant have?		
Is the range of leaf sizes of the mutant plant smaller or larger or similar to wild type leaves?		

STEM	**Mutant**	**Wild Type**
What is the height of the main (or longest) stem?		
What is the thickness of the stem?		
How many stems (or branches including the main and side ones) does the plant have?		

FLOWERS	**Mutant**	**Wild Type**
Do the flowers have all FOUR floral organs (green sepals, white petals, yellow anthers, green pistils)?		
How many sepals are on each flower?		
How many petals are on each flower?		
How many anthers are on each flower?		
How many pistils are on each flower?		

SILIQUES, SEEDS AND EMBRYOS	**Mutant**	**Wild Type**
How many siliques are on each plant?		
Do you see a difference in the lengths of siliques?		
How many seeds are in EACH silique?		
What is the average number of seeds in FIVE siliques?		
Do you see different COLORED seeds within a single silique?		
If yes, what colors are the seeds? How many seeds of each color?		
What stage of embryos (globular, heart, torpedo, cotyledon, mature green, or post mature green) do you see?		

IV. DETERMING WHAT GENE YOU ARE STUDYING AND THE T-DNA INSERTION SITE

Purpose: To determine the identity of the gene you are studying, and to verify the location of the T-DNA insertion site in the gene of interest indicated by the Salk Institute Genomic Analysis Laboratory website http://signal.salk.edu/

STRATEGY

A. Purifying PCR Products

B. Sequencing Reaction with Big Dye v. 3

C. Retrieving and Analyzing DNA Sequences

A. Purifying PCR Products

QIAquick PCR Purification Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe only one band.

Purpose: To purify DNA (PCR product) from free nucleotides, primers, salt, and enzyme for downstream applications such as sequencing reactions.

Reference: QIAquick PCR Purification protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- Remaining PCR solutions (~25 µL)

Materials Needed:

- Pipettes
- Filter pipet tips
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex

- Timer
- NanoDrop spectrophotometer
- Kimwipes

PROCEDURE

1. Label the **lids** of TWO 1.5 mL microcentrifuge tubes with "**T-DNA**" or "**WT**" and **your initials**.
2. Pipet **~25 µL** of the **PCR product solution** from the PCR tubes containing the T-DNA fragment or gene-specific DNA fragment into the labeled 1.5 mL microcentrifuge tubes.
3. Measure the exact volumes of solution.
4. Add **150 µL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR sample) to the tubes in step 2. Mix by vortexing for 5 seconds.
5. Spin the tubes in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tubes. Set the tubes back on the microcentrifuge tube rack.
6. Place TWO **QIAquick spin columns** (lilac) in the provided **2 mL collection tubes**. Label the lids of the columns with "**T-DNA**" or "**WT**" and **your initials**.
7. Pipet the sample mixtures in step 4 to the QIAquick spin columns. Spin the columns in the collection tubes in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane*.
8. Discard the **flow-through solution** in the collection tubes by pouring it into a waste container. Blot the collection tubes on Kimwipes. Put the QIAquick columns back into the same collection tubes.
9. Add **750 µL** of **Buffer PE** to the QIAquick spin columns and spin at **FULL speed** for **1 minute**.
10. Discard the flow-through solution in the collection tubes by pouring it into a waste container. Blot the collection tubes on Kimwipes. Put the QIAquick columns back into the same collection tubes.

11. Spin the columns set at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min.*
12. While spinning, label TWO **1.5 mL microcentrifuge** tubes "**PCR Purified T-DNA**" or "**PCR Purified WT**," **your initials** and the **date**.
13. Transfer the **QIAquick columns** to the appropriate NEWLY labeled microcentrifuge tubes. Discard the flow-through solutions and the collection tubes.
14. Pipet **30 µL** of **Buffer EB** to the **center** of the QIAquick membranes. Let the columns sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the QIAquick membrane. Centrifuge again at FULL speed for 1 minute.*
15. Determine DNA concentration using the NanoDrop spectrophotometer.

    What is the concentration of purified PCR product? _____ ng/µL

    What is the size (in bp) of the PCR product from gel electrophoresis? _____ bp

QIAquick Gel Extraction Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe more than a single band.

Purpose: To purify DNA (PCR product) from agarose gel for downstream applications such as sequencing reactions.

Reference: QIAquick Gel Extraction protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- Remaining PCR solutions (~25 μL)
- QIAquick Gel Extraction Kit (Qiagen, Cat. #28704)
- Isopropanol
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL 1 Kb Plus DNA ladder (Invitrogen)
- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Gel electrophoresis materials (Appendix 1A)
- Razor blade
- Metal waste container for sharp objects
- Saran wrap
- UV light box
- Scale
- Microcentrifuge
- Vortex
- 50°C water bath
- Timer

PROCEDURE

1. Label the **lids** of TWO 1.5 mL microcentrifuge tubes "**T-DNA**" or "**WT**" and **your initials**.
2. Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.
3. Put on a UV shield to protect your eyes and face.
4. Turn **on** the **UV box**. *Note: Turn **off** the UV box as soon as you are done excising the DNA band.*
5. Excise the desired DNA fragments from the gel using a razor blade. *Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*
6. Place the agarose slices in the 1.5 mL microcentrifuge tubes.

7. Take a picture of the gel **after removing the agarose slices**. *This step serves as a record of the DNA fragment being collected.*

   Note: If the desired bands were faint, run the remaining 25 µL of the desired PCR solutions on a new gel and excise those bands as well.

   a. *Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*
   b. *Add **3 µL** of **6x loading dye** to each tube of **~25 µL** PCR solutions containing the "T-DNA fragment" or the "WT DNA fragment."*
   c. *Load **10 µL of 1 Kb Plus DNA ladder** into the first well.*
   d. ***Very slowly** load the **~28 µL** sample-dye mixtures on the gel using a P-20 pipette.*
   e. *Record the identity of the sample in each well.*

Lane	***Sample***	***Primers***	***Expected Size (bp)***
1	*1 Kb Plus DNA ladder*	-	-
2			
3			

   f. *Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.*
   g. *Run the gel at **105 volts** for 1.5 - 2 hours in the dark.*
   h. *Take a picture of the gel.*
   i. *Verify the presence of the **expected size PCR product**.*
   j. *Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.*
   k. *Put on a UV shield to protect your eyes and face.*
   l. *Turn **on** the **UV box**. Note: Turn **off** the UV box as soon as you are done excising the DNA band.*

m. *Excise the desired DNA fragments from the gel using a razor blade. Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*

n. *Place the agarose slices in the 1.5 mL microcentrifuge tubes (in step 6) labeled "**T-DNA**" or "**WT**" and **your initials**.*

o. *Take a picture of the gel **after removing the agarose slices**. This step serves as a record of the DNA fragment being collected.*

Note: Be sure to preform steps 8-25 at room temperature.

8. Pipet **Elution Buffer** into a 1.5 mL microcentrifuge tube labeled with "**EB**" and **your initials**. Pipet **30 µL** x (number of samples +1). Warm the 1.5 mL microcentrifuge tube with elution buffer to **65°C** in a heat block. *This will be used in step 25 to remove the DNA from the membrane.*
9. Centrifuge the gel slice at full speed for **1 minute**.
10. Estimate the **gel volume** in the microcentrifuge tubes using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tubes. *Note: **0.1 g** of **agarose gel** is **equivalent to 100 µL**.*
11. Add **3 gel volumes** of **Buffer QG** to the tubes containing the gel slices. *For example, if the **weight** of **the agarose slice** is **0.15 g**, then its **gel volume** is **150 µL**. Therefore, add **450 µL** of **Buffer QG** to the tube.*
12. Incubate the tubes at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
13. Add **1 gel volume** of **isopropanol** to the mixtures and mix by **vortexing** for **5 seconds** or **inverting** the tubes **5-10 times**. *This increases the yield of DNA fragments. Note: Do not centrifuge the samples at this stage.*
14. Place TWO QIAquick **spin columns (purple)** in TWO **2 mL collection tubes**. Label the sides of the spin columns and collection tubes with "**T-DNA**" or "**WT**" and **your initials.**

15. Pipet the **mixtures** from **step 12** into the **spin columns** (**purple**). *Do NOT pipet **more than 800 µL** of the mixture into the column. If the total volume is **more than 800 µL**, repeat **steps 15-17**.*
16. Centrifuge the tubes for **1 minute**. *This step allows DNA binding to the membrane.*
17. Discard the **flow-through solution** in the collection tubes by pouring it into a waste container. Blot the collection tubes on Kimwipes. Put the QIAquick columns back into the same collection tubes. *Keep collection tube for use in steps 17-19.*
18. Add **500 µL** of **Buffer QG** to the **spin columns** and centrifuge for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
19. Add **750** of **µL Buffer PE** to the columns and let the tubes stand for **2-5 minutes**. Centrifuge the tubes at FULL speed for **1 minute**. Discard the flow-through solution. *This step washes the column.*
20. Repeat step 19 **two more times**.
21. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the columns. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
22. While spinning the tubes, label the lids and sides of TWO new 1.5 mL microcentrifuge tubes with "**Gel Purified T-DNA**" or "**Gel Purified WT**" and **your initials.**
23. After spinning, transfer the **spin columns** to the **labeled microcentrifuge tubes**. Discard the collection tubes and flow-through.
24. Add **30 µL** of **Buffer EB** to the **center of the membranes**. Let the columns stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the QIAquick membrane. Centrifuge again at FULL speed for 1 minute.*
25. Determine the DNA concentrations using a NanoDrop spectrophotometer.

    What is the concentration of purified T-DNA PCR product? _____ ng/µL

    What is the size (in bp) of the T-DNA PCR product from gel electrophoresis? _____ bp

What is the concentration of purified WT PCR product? _____ ng/μL

What is the size (in bp) of the WT PCR product from gel electrophoresis? _____ bp

B. Sequencing Reaction with Big Dye v. 3

Purpose: To determine the identity of the gene you are studying, and to verify the location of the T-DNA insertion site in the gene of interest indicated by the Salk Institute Genomic Analysis Laboratory website http://signal.salk.edu/

References: Applied Biosystems

UCLA WebSeq website http://genoseq.ucla.edu/action/view/Sequencing

Solutions Needed:

- Applied Biosystems Big Dye version 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building; or Sigma Cat. #S3938)
- 20 μM LBb1.3 primer
- 20 μM Gene-specific Salk Forward primer
- 20 μM Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- 0.2 mL PCR tubes or strips of 8 tubes
- PCR tube rack
- Filter pipet tips
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

Generally, **10 µL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). *Note: If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use a Master Mix (Mmix) to minimize the number of pipettings and mistakes of not adding some components into the individual reaction tubes.*

Important: *This is **NOT** a **regular** PCR reaction. Do **NOT** add **TWO** primers into a single reaction tube. **Each reaction contains only ONE primer**.*

General Components of One Reaction:

	ONE Reaction
DNA template *	**x** µL
Sterile water	**y** µL
20 µM Sequencing primer	1 µL
Big Dye v. 3	1 µL
5x Sequencing buffer	2 µL
Total volume	**10 µL**

x µL = the volume depends on **concentration** and **amount** of **DNA** (see table below)

y µL = the **remaining volume** to bring the **total volume** to **10 µL**

*** Amount of DNA template** depends on **type of DNA**:

- For **plasmid** DNA, use **800 ng**. *Note: 250 ng of plasmid DNA will work, but more DNA gives the better reads.*
- For **PCR product**, use the amount of DNA according to the table below. (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 µL. Alternatively: You may use the speed vac to concentrate your DNA.

Table: Amount of DNA to Use in a Sequencing Reaction

Size of PCR Product (bp)	Amount of DNA Used in Reaction
< 200	20 ng
200 - 300	50 ng
> 300	90 ng

For this exercise, there is **ONE DNA template** (the purified PCR product of the T-DNA fragment), but there are **TWO primers**: LBb1.3 (T-DNA) primer and gene-specific salk primer. The gene-specific salk primer will be either forward or reverse depending on the orientation of the T-DNA Left Border (LB) relative to the gene of interest. The orientation of the T-DNA relative to the gene of interest was determined by your analysis of the genotyping PCR results. The **sequencing reaction with** the **gene-specific primer** serves **as a control for the master mix** of Big Dye and 5x Sequencing buffer. Therefore, it is best to prepare a **master mix** with **all components except** the **primers**, which will be added to individual reaction tubes.

What is the concentration of PCR product? _____ ng/μL

What is the size of the PCR product? _____ bp

What is the amount of DNA to be used? _____ ng

What is the volume of purified DNA solution to be used? _____ μL

Sample calculations:

Size of PCR product** is **400 bp** and **its concentration** is **20 ng/μL

***Want to use 90 ng** of purified PCR product (see table above)*

*Hence, the **volume of DNA to be used** is **90 ng ÷ 20 ng/μL = 4.5 μL***

PROCEDURE

1. Get ice from the icemaker in room 4128.
2. Label the **sides** of **TWO 0.2 mL PCR tubes** with **your initials** and **primer name**. Set the tubes on a PCR tube rack sitting on ice.
3. Label the **lid** and **side** of a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.

4. Prepare two **master mixes (Mmixs)** for **3 reactions** each (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions*.

Master Mix (Mmix) for Sequencing Reactions:

Components	**ONE reaction**	**3x Mmix** for **T-DNA allele**	**3x Mmix** for **WT allele**
DNA template	**x** μL	**3x** μL	**3x** μL
Sterile water	**y** μL	**3y** μL	**3y** μL
Big Dye v. 3	**1** μL	**3** μL	**3** μL
5x Sequencing buffer	**2** μL	**6** μL	**6** μL
Total Volume	**9 μL**	**27 μL**	**27 μL**

   a. Mix the contents by flicking the tube five times or vortexing at a setting of 2-3 for **5 seconds**.
   b. Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
   c. Set the tube back on ice.
5. Pipet **9 μL** of **WT Mmix** into each of TWO 0.2 mL PCR tubes labeled with "WT," your initials and "FW" or "RV." Add **1 μL** of **20 μM Gene-specific Salk <u>Forward</u> primer** to one tube and **1 μL** of **20 μM Gene-specific Salk <u>Reverse</u> primer** to the other tube. Mix and spin briefly.
6. Pipet **9 μL** of **T-DNA Mmix** into each of TWO 0.2 mL PCR tubes labeled with "T-DNA," your initials, "LBb1.3" and either "FW" or "RV." Add **1 μL** of **20 μM LBb1.3** to one tube and **1 μL** of either **20 μM Gene-specific Salk <u>Forward</u> primer** or **20 μM Gene-specific Salk <u>Reverse</u> primer** to the other tube. Mix and spin briefly.
7. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700**

USER: <<hc-lab>>
PROGRAM: **HC70AL BIG DYE**
The profile of the Big Dye program is:

25 cycles of	96°C 10 sec
	55°C 5 sec
	60°C 4 min
4°C ∞	

or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.

8. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
    a. Resuspend the resin by inversion or gently vortexing.
    b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
    c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
    d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
    e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
    f. Carefully transfer the spin columns to the new tubes.
    g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

        Note:

        - *Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel bed surface with the pipet tip.*
        - *For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 µL using distilled water, before application to the gel bed.*

    h. Centrifuge the columns at **3,000 rpm** for **3 minutes** at room temperature.

i. Remove the spin columns from the microcentrifuge tubes. *The eluate contains the purified DNA*.

9. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. *Note: Use the primer name as the name of your sequence. Make sure to copy down the* ***assigned file number*** *(example, #*106203*), which is automatically given by the computer after you enter the samples*.
10. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

C. Retrieving and Analyzing DNA Sequences

Purpose: To determine the identity of the gene you are studying, and to verify the location of the T-DNA insertion site in the gene of interest indicated by the Salk Institute Genomic Analysis Laboratory website http://signal.salk.edu/

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/
   a. Enter Username: **goldberg_r**
   b. Enter Password: **embryo**
   c. Click "LOGIN."
2. Find your sequence files by looking up the ***assigned file number*** and the name of the primers that you used for sequencing.

   Example: The ***assigned file number*** is **106203**, and the primer names are Gene A Fw and Gene A Rv. You would see the following files:

   106203GoldR Gene A Fw A12.ab1

   106203GoldR Gene A Rv B12.ab1

   What are the annotations?

   106203 = assigned file number; **GoldR** = user name; **Gene A Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.

3. Check the boxes next to the sequences to be downloaded, and click "Download selected." Alternatively, click on each filename that you want to download.
4. Open the ab1 files in the "Downloads" window using a sequence viewer program (FINCH TV on Windows, or 4PEAKS on Mac).
5. Copy DNA sequences to a Microsoft Word file. *Note: Name the files according to the name of gene of interest (for example, At5g09250).*
6. Process the DNA sequences by "BLASTN" and "BLASTX" searches. See Appendix 2. *Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Determine what gene your sequence corresponds to.
8. Print out the Blast results as hard-copy records for your lab notebook.
9. Save the Blast results in the **pdf** format so that you can upload them to your webbook.

EXPERIMENT 6 – RNA ISOLATION AND RT-PCR ANALYSIS (GENE TWO)

Purpose: To determine the mRNA accumulation pattern of the gene of interest in wild type and mutant *Arabidopsis* siliques.

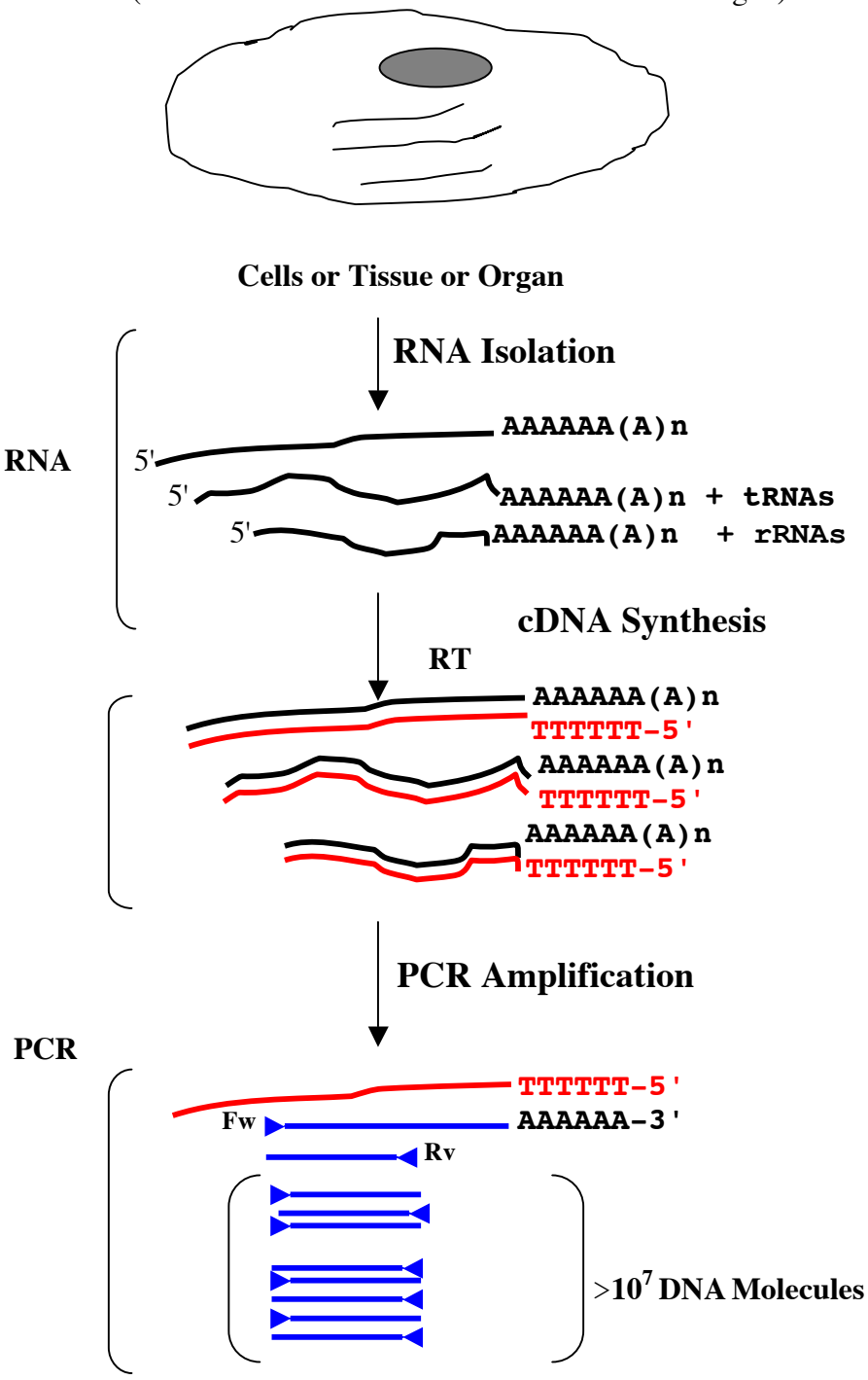
OVERVIEW OF RT-PCR STRATEGY

I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT

II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE (REVERSE TRANSCRIPTION or RT)

III. CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS

OVERVIEW OF RT-PCR (Based on RT-PCR Technical Note from Invitrogen)



I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT

Purpose: To extract total RNA from siliques to study gene expression. You will perform RT-PCR on total RNA isolated from siliques from wild type and mutant plants in order to determine if the T-DNA insertion causes a null mutation (i.e. no mRNA is expressed for the gene of interest).

References: RNeasy Plant Mini Kit Protocol (Qiagen; see Appendix 1G)

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. In: (Second Edition), *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. (ISBN 978-0879693091)

FREQUENTLY ASKED QUESTIONS

PROCEDURE

A. Grinding Tissue for RNA Extraction

B. RNA Isolation

C. Removing Contaminating Genomic DNA from Total RNA Solutions Using RNase-free DNase

D. Determining the Quality of Isolated Total RNA Before and After DNase Treatment Using Capillary Gel Electrophoresis

FREQUENTLY ASKED QUESTIONS

(Taken from Qiagen RNeasy Plant Mini Handbook June 2001; see Appendix 1G)

6. **What is the maximum amount of starting material?**

   100 mg

7. **Is the yield of total RNA the same for the same amount of starting material for different plant species?**

   No, the yield varies for different plant species.

8. **Which lysis buffer can be used for plant materials?**

   - Buffer **RLC** (Guanidine Hydrochloride) is used for endosperm and tissues containing endosperm (e.g., siliques). Although Guanidine Isothiocyanate is better at cell disruption and denaturation than Guanidine Hydrochloride, Guanidine Isothiocyanate can cause solidification of endosperm samples, making extraction of RNA impossible.
   - Buffer **RLT** (Guanidine Isothiocyanate) is used for all plant tissues except endosperm and tissues containing endosperm (e.g., siliques).

9. **Is total RNA isolated with RNeasy kit free of genomic DNA?**

   No, most (but not all) of DNA is eliminated. Therefore, if total RNA will be used for downstream application such as Reverse-transcription-PCR (RT-PCR), then DNase treatment must be carried out for the total RNA.

10. **What is the role of QIAshredder homogenizer?**

    It simultaneously **removes insoluble material** and **reduces the viscosity of the lysates** by disrupting gelatinous material.

C. Grinding Tissue for RNA Extraction

Note: ***Grinding Tissue for RNA Extraction will be carried out by the Teaching Assistants (TAs).***

Materials Needed:

- Key to the Plant Growth Center
- BruinCard with access to PGC
- Plant layout charts indicating plants homozygous or heterozygous for the T-DNA
- Ice bucket
- Kimwipes
- A squirt bottle of 100% Ethanol solution
- Forceps
- Liquid Nitrogen (from storeroom in Life Sciences Building) <u>*Caution:*</u> *It is very cold (at least -210°C). Avoid getting frostbite.*
- Dewar flask or Styrofoam box
- <u>D</u>i<u>e</u>thyl <u>P</u>yro<u>c</u>arbonate (DEPC). <u>*Caution:*</u> *DEPC is suspected to be carcinogenic and corrosive. Therefore, hand with care! DEPC inhibits RNase.*
- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Autoclaved, DEPC-treated porcelain mortar and pestle
- Autoclaved, DEPC-treated, blue micropestles
- Qiagen RNeasy Plant Mini Kit: (Cat. #74904 for 50 extractions)
- β-mercaptoethanol. <u>*Caution:*</u> *Work in the fume hood because this chemical has very bad odor.*
- Black ultra-fine sharpie
- Autoclaved, DEPC-treated spatulas
- RNase-free 14 mL disposable centrifuge tubes
- RNase-free 1.5 mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- Scale

<u>Attention:</u> *Before isolating RNA, use Kimwipes wetted with freshly prepared non-autoclaved DEPC treated water to clean all equipment (pipette sets, pipet stand, microcentrifuge-tube racks, micro centrifuges and rotors, test-tube racks, pens and sharpies, pipet tip boxes, microcentrifuge tube containers) to be used in isolating RNA.*

PROCEDURE

1. Get ice from the icemaker.
2. Label the white area on the side of ONE RNase-free **14 mL centrifuge tube** "**WT Siliques**." Label **SIX 1.5 mL microcentrifuge tubes** with the name of each mutant line. Chill on ice.
3. Go to the Plant Growth Center.

4. Use a piece of Kimwipes to clean the forceps with ethanol. Note: *Two sets of forceps are used per plant. The forceps must be cleaned after the collection of siliques from each line to avoid contamination.*
5. Working **quickly**, use forceps to harvest siliques from wild type *Arabidopsis* Columbia-0 siliques and siliques from each mutant line. Select siliques from a plant homozygous for T-DNA if available, otherwise use siliques from a heterozygous plant. Select siliques that contain seeds with embryos ranging from globular to torpedo stage. **Immediately**, place siliques in the chilled, labeled tubes. Note: *Clean the forceps with ethanol before collecting from a new line.*
6. Return to the lab.
7. **Immediately** chill the samples either on **crushed dry ice** or in a Dewar flask or Styrofoam box containing **liquid nitrogen** (filling up to one-third of the Styrofoam box).
8. Chill an **RNase-free** spatula in a Dewar flask containing liquid nitrogen.
9. Chill the mortar and pestle with liquid nitrogen until liquid nitrogen is not bubbling out vigorously.
10. Place **WT** siliques in the chilled mortar containing liquid nitrogen.
11. Using the pestle, grind the frozen tissue to a powder in liquid nitrogen. Note: *It is best to grind the tissue when the last drop of liquid nitrogen has just evaporated. Grind quickly. Do not let the tissue thaw. Repeat this step until there are no more chunks of tissue present.*
12. Add some liquid nitrogen to the mortar and quickly pour the tissue and liquid nitrogen to a chilled, labeled 14 mL tube set on **crushed dry ice** or in **liquid nitrogen**. You may use the chilled spatula to get the powder into the tube.
13. Lightly place the cap on top of the 14 mL tube to allow the liquid nitrogen within the tube to evaporate, but do not allow the tissue to thaw. Note: *You may also place the tube in a -70°C freezer to allow the evaporation of liquid nitrogen.*
14. For the **mutant samples**, grind the siliques in the 1.5 mL microcentrifuge tubes chilled in liquid nitrogen with the blue micropestles that have been treated with DEPC, autoclaved and chilled in liquid nitrogen. Use a new micropestle for each sample to avoid contamination. Note: *Grind quickly and place the tube back in the*

liquid nitrogen. Do not let the tissue thaw. Repeat this step until there are no more chunks of tissue present.

15. Label TWELVE 1.5 mL microcentrifuge tubes with the sample names. Set the tubes on **crushed dry ice** or in **liquid nitrogen**.
16. Use a **chilled, RNase-free spatula** to transfer a small amount **(up to 100 mg)** of **WT frozen ground material** to SIX of the new **chilled 1.5 mL microcentrifuge tubes**. Use a scale to measure, but do not let the samples thaw. Keep the new tubes on dry ice or in liquid nitrogen.
17. Use new **chilled, RNase-free spatula** to transfer a small amount **(up to 100 mg)** of **frozen ground material** for each of the six mutant lines into the new **chilled 1.5 mL microcentrifuge tubes**. Use a scale to measure, but do not let the samples thaw. Keep the new tubes on dry ice or in liquid nitrogen.
18. Store the TWELVE aliquots in the **–70°C freezer** until the RNA extraction step. Also store the remaining ground tissue for each sample in the **–70°C freezer** as a backup.
19. **On the day of RNA extraction**, prepare the **RLC lysis buffer**
    c. Determine the **total volume** (**= # of samples x 500 µL**) of **lysis buffer** needed for RNA isolation. *Note: If the total volume is greater than 1.5 mL, it is best to use a **14 mL centrifuge tube** for preparing the **lysis buffer** with **β-mercaptoethanol**.*
    d. Add **10 µL** of **β-mercaptoethanol** to every **1 mL** of **lysis buffer** in the fume hood. Mix the contents in the tube by vortexing for 5 seconds. Put the tube back on the rack.

*Note: **β-mercaptoethanol** is **toxic** and has a bad odor. It is kept in the fume hood in room 4128A2. The newly prepared lysis buffer with β-mercaptoethanol is stable 1 month after the addition of β-mercaptoethanol.*

Volume of RLC lysis buffer	Volume of β-mercaptoethanol
_____ mL	_____ µL

D. RNA Isolation

Materials Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Kimwipes
- Dewar flask or Styrofoam box
- Liquid Nitrogen (from storeroom in Life Sciences Building) <u>*Caution:*</u> *It is very cold (at least -210°C). Avoid getting frostbite.*
- Aliquots of 1.5 mL RNase-free tubes containing ~100 mg of frozen powder from ground up wild type *Arabidopsis* Columbia-0 siliques and siliques from each mutant plant (powder prepared by Teaching Assistants)
- Qiagen RNeasy Plant Mini Kit: (Cat. #74904 for 50 extractions)
- β-mercaptoethanol. <u>*Caution:*</u> *Work in the fume hood because this chemical has very bad odor.*
- Autoclaved DEPC-treated (DEPC'd) water
- P-10, P-20, P-200 & P-1000 pipettes
- RNase-free filter tips for P-10, P-20, P-200 & P-1000
- Ice bucket
- Black ultra-fine sharpie
- RNase-free 1.5 mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- Timer
- NanoDrop spectrophotometer

<u>Caution:</u>

- ***All steps of the RNeasy protocol should be carried out at room temperature.*** *During the procedure,* ***work quickly.***
- ***All centrifugation steps are carried out at 20-25°C.*** *Ensure that the centrifuge does not cool below 20°C.*
- ***Use filter pipet tips throughout the procedure.***
- ***Change GLOVES frequently*** *and keep tubes closed whenever possible.*

PROCEDURE

<u>Attention:</u> *Before isolating RNA, use Kimwipes wetted with freshly prepared non-autoclaved DEPC treated water to clean all equipment (pipette sets, pipet stand, microcentrifuge-tube racks, micro centrifuges and rotors, test-tube racks, pens and*

sharpies, pipet tip boxes, microcentrifuge tube containers) to be used in isolating RNA.

1. Locate TWO 1.5 mL microcentrifuge tubes containing a small amount **(up to 100 mg)** of **frozen ground material** from wild type or T-DNA-tagged siliques. These will be stored on dry ice or in liquid nitrogen. Quickly, **tap** the **tube** on the **bench** or the base of the vortex mixer 3-5 times to loosen the frozen powder.
2. **Immediately**, pipet **450 µL** of **RLC lysis buffer containing β-mercaptoethanol** into the 1.5 mL microcentrifuge tube containing **~100 mg** of ground **WILD TYPE** tissue. **Cap** the tube. **Immediately**, **vortex** the tube **vigorously** for at least **1 minute**. Then set the tube on a microcentrifuge tube rack. *The **lysate** should appear clear with no lumps of ground powder. (Optional) A short incubation time (1-3 minutes) at 56°C may help to disrupt the tissue, but is NOT appropriate for organs rich in starch, such as siliques or old leaves.*
3. **Repeat step 2** for the **T-DNA** sample.
4. Label the lids of TWO **QIAshredder** (**lilac**) **spin columns** placed in **2 mL collection tubes** with **your initials** and "**WT**" or "**T-DNA**."
5. Pipet the **entire volume** of **lysate** into the labeled QIAshredder spin columns.
6. Centrifuge the spin columns in the collection tubes at **FULL speed** (13,200 rpm) for **2 minutes**. *Note: Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.*
7. Meanwhile, label the lids of TWO **1.5 mL RNase-free microcentrifuge tubes** and TWO **RNeasy spin columns** (**pink**) placed **2 mL collection tubes** with **your initials** and "**WT**" or "**T-DNA**." Set the labeled tubes on a microcentrifuge tube rack at room temperature.
8. Carefully transfer the **supernatant** of the **flow-through solutions** to the **NEW labeled RNase-free 1.5 mL microcentrifuge tubes** without disturbing the cell-debris pellets in the collection tubes. Use only this supernatant in subsequent steps.

9. Add **0.5 volume** (or **225 µL**) of **room temperature 96-100% ethanol** to the **WT supernatant**. **Immediately**, mix by pipetting **up and down 10 times**. *Note: Do NOT centrifuge. Proceed immediately to step 10.*
10. Pipet the **entire volume (~650 µL, but not more than 700 µL)** of the **WT mixture** (including any precipitate that may have formed) in step 9 to the "**WT**" labeled **RNeasy spin column** (**pink**) placed in a **2 mL collection tube**. Close the lid of the tube **gently**.
11. Repeat steps 9 and 10 for the **T-DNA** mixture.
12. Centrifuge the sp**in columns** placed in a 2 mL collection tubes for **15 seconds** at **>10,000 rpm** (or FULL speed).
13. Carefully remove the **spin column** from the collection tube so that the column does not contact the flow-through. Hold the column with one hand and while **pouring** the **flow-through solution** in the collection tube into a "**waste**" **beaker**. Be sure to empty the collection tube completely. Put the column back in the collection tube. *Note: If the sample volume in step 10 is >700 µL, pipet the remaining volume of the mixture onto the RNeasy column and centrifuge as before. Discard the supernatant.*
14. Pipet **700 µL** of **Buffer RW1** to the RNeasy spin column. Close the tube gently.
15. Centrifuge for **15 seconds** at **>10,000 rpm** (or FULL speed) to wash the spin column membrane.
16. Carefully discard the flow-through as in step 13.
17. Pipet **500 µL** of **Buffer RPE** into RNeasy spin column. Close the tubes gently.
18. Centrifuge for **15 seconds** at **>10,000 rpm** (or FULL speed) to wash the spin column membrane.
19. Carefully discard the flow-through as in step 13.
20. Pipet another **500 µL** of **Buffer RPE** into the RNeasy spin column. Close the tube gently.
21. Centrifuge for **2 minutes** at **>10,000 rpm** (or FULL speed) to wash the spin column membrane. *Note: The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.*
22. Label TWO **new 2 mL collection tubes** with **your initials** and "**WT**" or "**T-DNA**."

23. Carefully transfer the columns to the **new 2 mL collection tube**s without allowing the columns to contact the flow-through. *Attention: At this point, total RNA and a small amount of genomic DNA are bound to the membrane of the pink RNeasy spin column.*
24. Discard the flow-through solution and old collection tubes.
25. Spin the columns in the new 2 mL collection tubes for **1 minute** to ensure that ethanol is removed completely from the membranes. *Caution: This step is **crucial** because if residual ethanol is still on the membrane, it will be eluted with RNA in steps 28-31.*
26. Label the lids and sides of TWO 1.5 mL RNase-free microcentrifuge tubes "**WT RNA**" or "**T-DNA RNA**," **your initial**s and the **date**.
27. Transfer the **spin columns** to these **NEW labeled tubes**.
28. Pipet **30 µL** of **RNase-free water** (supplied with the kit) or autoclaved DEPC-treated water directly onto the center of the column membrane. Close the tubes gently.
29. Wait for **1 minute** to allow the membrane to evenly absorb the water.
30. Centrifuge for **1 minute** at **>10,000 rpm** (or FULL speed) to elute RNA from the membrane.
31. Repeat **steps 28-30** with **20 µL** of **RNase-free water**. *Note: The total volume of RNA solution is about **50 µL.***
32. Mix the contents of the tubes with gentle flicking. Put tubes **on ice**. *Note: From this step on, **KEEP** RNA solutions **ON ICE** to prevent **RNA degradation**.*
33. Determine the **total volume** of **RNA solution** using a P-200 pipette. The volume should be **~48 µL**.
34. Determine **RNA concentration** and **total amount** of RNA using the NanoDrop spectrophotometer.

    Total amount of RNA = (X µg/µL) (Volume of RNA solution in µL) = **Y µg**

    *Note: **1 µg = 1,000 ng**; therefore, you need to **convert ng/µL** to **µg/µL***

Record RNA concentration and total amount of RNA

Sample	[RNA] (μg/μL)	Volume (μL)	Estimated Total Amount (μg)
Wild type siliques			
T-DNA siliques			

35. Label the lids and sides of **TWO new RNase-free microcentrifuge tubes** "**1 μL WT Silique RNA**" or "**1 μL T-DNA Silique RNA**," **your initials** and the **date**. Keep tubes **on ice**.
36. Pipet **1 μL** of the **RNA solution** into the **new labeled tubes**. These aliquots will be used to assess the quality of the RNA in **Part D.**
37. Either keep the tubes **on ice** and proceed to **Part C**, or store the RNA solutions at **-20°C for up to 1 week** or at **-70°C for up to 6 months**.

Attention: *To be safe, only **HALF** of **the volume** of the **RNA solution** is **treated with RNase-free DNase**; the remaining volume of RNA solution is kept on ice or stored in the -20°C RNA freezer until the gel electrophoresis step in part D to determine quality of RNA before DNase treatment.*

C. Removing Contaminating Genomic DNA from Total RNA Solutions Using RNase-free DNase

Reference: Turbo DNA-free kit protocol (Ambion; See Appendix 1H)

Important Note: *This protocol is suitable for **removing up to 2 μg of DNA** from up to **20 μg of RNA** in a **25-100 μL** reaction volume.*

Materials Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment

- Ice bucket
- Total RNA isolated from wild type and mutant siliques
- Ambion Turbo DNA-free kit (stored in RNA -20°C freezer; Cat. # 1907)
- Turbo DNase (included in the Ambion Turbo DNA-free kit)
- 10x Turbo DNase buffer (included in the Ambion Turbo DNA-free kit)
- DNase inactivation reagent (included in the Ambion Turbo DNA-free kit)
- Autoclaved DEPC-treated (DEPC'd) water
- Black ultra-fine sharpie
- RNase-free 1.5 mL microcentrifuge tubes
- Rack for microcentrifuge tubes
- 37°C heat block
- Timer
- Microcentrifuge
- Vortex
- P-10, P-20 & P-200 pipettes
- RNase-free filter tips for P-10, P-20 and P-200
- White Revco storage boxes
- Kimwipes
- NanoDrop spectrophotometer

PROCEDURE

1. Write down the **concentration** of the total RNA samples.

   *Note: 1 μg = 1,000 ng. Therefore, the concentration determined by the NanoDrop spectrophotometer (**ng/μL**) needs to be converted into **μg/μL**.*

	WT	**T-DNA**
RNA concentration	_____ μg/μL	_____ μg/μL

2. Determine the **volume** for **20 μg** of total RNA.

   Volume** of **20 μg RNA = Amount** of **RNA ÷ concentration** of **RNA

   *Example: If **total RNA** has a concentration of 1.25 μg/μL, then the volume of **20 μg** of **RNA** will be 20 μg ÷ 1.25 μg/μL = **16 μL***

	WT	**T-DNA**
Volume of 20 μg	_____ μL	_____ μL

3. Determine which **volume** is **smaller** for each sample: the volume of **20 µg** of total RNA **or** the volume of **half** of the isolated total RNA. **Use the smaller volume** in the DNase reaction.
4. Get ice from the icemaker.
5. Remove your total RNA samples from the -20°C RNA freezer. Thaw on ice.
6. Label TWO 1.5 mL microcentrifuge tubes with "**WT**" or "**T-DNA**," **your initials** and the **date**. Set tubes on ice.
7. Add total RNA samples, DEPC'd water, **0.1 volume** of **10x Turbo DNase buffer** and **1 µL** of **2 Units/µL Turbo DNase** (Ambion) to the labeled, chilled tubes according to the chart below. Make a master mix (Mmix) for the components that are shared in both reactions. *Note:* ***One unit** of **DNase** is defined as the amount of enzyme that degrades **1 µg of DNA** in **10 minutes** at **37°C**.*

	RNA Solution	**WT Silique RNA**	**T-DNA Silique RNA**	**Mmix for 3 Reactions**
RNA sample (up to 20 µg, but not more than half of your RNA)	**X** µL			
DEPC'd water	**Y** µL			
10x Turbo DNase buffer	**3.0** µL			
Turbo DNase (2 Units/µL)	**1.0** µL			
Total volume	**30.0** µL			

X µL = volume of RNA sample; **Y µL** = volume of DEPC'd water

*The **volume of DEPC'd water** is the difference between the total reaction volume and the sum of the volume of the other components.*

8. Mix the solutions gently by flicking the tubes. Spin briefly (**5-10 seconds**).
9. **Immediately**, store the tubes of remaining total RNA solution in a box at **-20°C for up to 1 week** or at **-70°C for up to 6 months**.
10. Incubate the DNase reactions at **37°C** in a **heat block** for **20-30 minutes**.

11. After incubation, spin tubes for **10 seconds** in a microcentrifuge to bring water condensation to the bottom of the tubes.
12. To inactivate Turbo DNase, pipet **0.1 volume** (or **3.0 µL**) of the **DNase inactivation reagent** (**WHITE slurry)** to the sample using a P-20 pipet tip. Mix well by **flicking the tube**. *Note: Make sure the slurry is WHITE. If the DNase inactivation reagent is CLEAR, vortex the mixture for a few seconds.*
13. Incubate the tube at **room temperature** for **5 minutes**. Flick the tube **2-3 times during the incubation** to re-disperse the **DNase inactivation reagent**.
14. In the meantime, label the lids and sides of **NEW RNase-free microcentrifuge tubes** "**Purified WT Silique RNA**" or "**Purified T-DNA Silique RNA**," **your initials** and the **date**.
15. Spin the tube at ~**10,000 x g (or 10,400 rpm)** for **1 minute** to pellet the **DNase inactivation reagent**.
16. **Carefully**, pipet ~**28-30 µL** of the **RNA solution** (*AVOID pipetting the PELLET!*) and transfer it into NEW labeled **RNase-free microcentrifuge tubes**. *Note: It is **okay** if a **tiny amount** of the pellet **is carried over** in the RNA solution.*
17. Keep RNA tubes **on ice**.
18. Determine the **RNA concentration** and **total amount** of RNA using the NanoDrop spectrophotometer.

    Total amount = (X µg/µL) × (volume of RNA solution) = Y µg

    *Note: **1 µg = 1,000 ng**; therefore, you need to **convert ng/µL** to **µg/µL***

Samples	**[RNA] (µg/µL)**	**Volume (µL)**	**Estimate Total Amount (µg)**
Purified WT Silique RNA			
Purified T-DNA Silique RNA			

19. Label the lids and sides of **NEW RNase-free microcentrifuge tubes** "**1 µL Purified WT Silique RNA**" or "**1 µL Purified T-DNA Silique RNA**," **your initials** and the **date**. Keep tubes **on ice**.

20. Pipet **1 µL** of the **RNA solution** into the **new labeled tubes**. These aliquots will be used to assess the quality of the purified RNA in **Part D**.
21. Put the tubes of purified RNA samples back in a box at **-20°C for up to 1 week** or at **-70°C for up to 6 months**. You may keep the **1 µL aliquots** on ice if you will proceed with **Part D.**

D. Determining the Quality of Isolated Total RNA Before and After DNase Treatment Using Capillary Gel Electrophoresis

Reference: RNA StdSens Analysis kit instruction manual (Experion, Bio-Rad)

Materials and Reagents Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Kimwipes
- Ice bucket
- 1 µL aliquots of RNA samples before and after DNase
- RNA StdSens Analysis kit (Experion, Bio-Rad)
- RNA StdSens chip (Experion, Bio-Rad)
- RNA StdSens ladder (Included in StdSens RNA Analysis kit)
- RNA StdSens filtered gel (Included in StdSens RNA Analysis kit)
- RNA StdSens gel-stain (Included in StdSens RNA Analysis kit)
- RNA StdSens loading buffer (Included in StdSens RNA Analysis kit)
- Electrode cleaner (Experion, Bio-Rad)
- Autoclaved DEPC-treated (DEPC'd) water
- Cleaning chips (Experion, Bio-Rad)
- Black ultra-fine sharpie
- RNase-free 1.5 mL microcentrifuge tubes
- Rack for microcentrifuge tubes
- 70°C heat block
- Timer
- Microcentrifuge
- Vortex
- P-10, P-20 & P-200 pipettes
- RNase-free filter tips for P-10, P-20 and P-200
- Capillary gel electrophoresis system (Experion, Bio-Rad)

Note: A single StdSens RNA chip can hold 12 RNA samples. Three students may share each chip and prepare one RNA ladder solution.

Note: Each 1 µl RNA sample should have a concentration of 5-500 ng/µl.

Note: Your instructor will prepare the gel-stain and clean the electrodes before samples are run.

PROCEDURE

1. Equilibrate Experion RNA StdSens reagents (filtered RNA gel solution, RNA loading buffer (yellow cap), gel-stain solution (amber tube)) to room temperature for at least 15 minutes. *Place the kit in a drawer or dark room to keep the gel-stain protected from light.*
2. Set a heat block to 70°C.
3. Get ice from the icemaker.
4. Locate FOUR 1 µL aliquots of RNA samples before and after DNase. Thaw RNA solutions **on ice**.
5. Spin the tubes of 1 µL RNA aliquots for **10 seconds** in a microcentrifuge. Keep RNA solutions on ice.
6. Remove a tube of **2 µL RNA ladder aliquot** from the -70°C freezer. Spin the tube in a microcentrifuge for **10 seconds** to bring down any water condensation, and keep it on ice.
7. Label a **NEW** 1.5 mL microcentrifuge tube "**RNA ladder**." Keep the tube on ice.
8. Pipet **1 µL** of **RNA ladder** into the new labeled 1.5 mL microcentrifuge tube.
9. Heat the **FOUR** tubes of **1 µL aliquots of RNA solutions** and **1 µL aliquot of RNA ladder** on a **70°C heat block** for **2 minutes**. *Note: It is okay to heat the samples for up to 5 minutes.*
10. Quench tubes **on ice** for **at least 5 minutes**.
11. Spin tubes in a microcentrifuge for **15-30 seconds**. Keep tubes **on ice**.

12. Pipet **5 µL** of **loading buffer (yellow cap)** to each RNA solution. Mix the contents by flicking the tube several times. After adding the loading buffer to all RNA solutions, spin tubes for 10 seconds. Keep the tubes **on ice**.
13. Remove an RNA StdSens chip from its plastic wrap. Using a **P-10 pipette**, pipet **9 µL of gel-stain** into the well labeled **GS** with an **orange highlight** (third well from the top). *Note: To avoid bubbles, dispense reagents into chips slowly. Always insert the pipet tip vertically and to the bottom of the chip well when dispensing liquids. Do not expel air at the end of the pipetting step. This will reduce the possibility of air bubbles becoming trapped between the reagent and the microchannels at the bottom of the chip wells.*
14. Put the chip on the **priming station**. Make sure the setting is **B1**.
15. Press the "**START**" button on the priming station. Wait for 30 seconds.
16. Open the priming station.
17. Pipet another **9 µL** of **gel-stain** to the other well labeled **GS** (second well from the top).
18. Pipet **9 µL** of **filtered gel** into the well labeled **G** (top well).
19. Pipet **6 µL** of the RNA mixtures prepared in step 12 into each sample well (1-4) and into the ladder well (labeled L). Work **quickly** to minimize sample evaporation. *Note: Each chip can hold 12 samples. Therefore, three students can share one chip. However, if there are only 4 samples, then pipet **6 µL** of loading buffer into the remaining wells (5-12). **Caution: Do NOT leave any sample well empty.***
20. Gently tap the **chip** on the **bench 3-5 times** to remove any **bubbles** present in the sample wells. Inspect the wells for the presence of bubbles. ***Note: Run the chip within 5 minutes of loading samples.***
21. Place the sample-loaded chip on the platform of the electrophoresis station and close the lid.
22. Launch the **Experion software**, select "**New Run**" and then "**RNA StdSens**."
23. Select "**Eukaryotic total RNA assay**."
24. Click the **PLAY** symbol button to begin the run. A window will pop up asking for the total number of samples loaded. Type in number of samples and the software will

avoid the wells containing only loading buffer. *The run will take up to 30 minutes for all 12 samples*.

25. While the electrophoresis is running, enter the sample information in the "**data info**" tab.
26. After the run is complete, the analyzer **beeps**. Your TA will remove the chip from the platform and discard the used chip.
27. **Your TA will immediately** place a **cleaning chip** containing **800 µL of DEPC'd water** on the platform. Close the lid of the electrophoresis system for **1 minute** to clean the electrodes.
28. Open the lid for **30 seconds** to allow water to evaporate.
29. Remove the **cleaning chip**. **Discard** the **water** and **store** the **cleaning chip** for future use.
30. Export data (electropherograms and gel images) to the desktop.
31. Copy the **exported data** on a **USB flash drive** and **upload** them onto the **HC70AL server**.
32. If there are no more runs for the day, your TA will turn off the electrophoresis system and quit the Experion software.
33. Analyze the data.

    What do you see in the picture?

    What are the RNA fragments?

    What are the sizes of RNA fragments?

    Is there any difference in brightness between different samples <u>before</u> and <u>after</u> DNase treatment?

    What is the reason for the difference?

II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE

Purpose: To generate cDNA template for PCR analysis.

Reference: iScript cDNA Synthesis Kit Instruction Manual (Bio-Rad; See Appendix 1I)

Overview:

- The **iScript reverse transcriptase** is RNase H^+, resulting in greater sensitivity than RNase H^- enzyme. **iScript** is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA.
- The enzyme is provided pre-blended with RNase inhibitor.
- The unique blend of oligo(dT) and random (6-bases, 8-bases, 10-bases) primers in the iScript Reaction Mix works exceptionally well with a wide range of targets. This blend is optimized for the production of targets <1 kb in length.
- iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

*Caution: When using **>1 µg** of total RNA, the reaction **volume** should be **scaled up** to ensure optimum synthesis efficiency. For example, use a 40 µL reaction for 2 µg.*

Note:

- *For **each** RNA sample, set up one reaction **with** Reverse Transcriptase (**+ RT**) and one reaction **without** Reverse Transcriptase (**- RT**). The -RT sample serves as a negative control for the PCR amplification step because without first strand cDNA template, there will be NO PCR product with expected size observed. However, if a PCR product is observed in the - RT sample, then RNA sample is contaminated with genomic DNA.*
- *Work with master mixes as often as possible to prevent FALSE negative results due missing components.*

Solutions Needed:

- 500 mL of freshly prepared 0.1% DEPC-treated water (non-autoclaved) for cleaning all equipment
- Purified total RNA samples (after DNase, stored in -20°C RNA Freezer)
- iScript cDNA Synthesis kit (Bio-Rad, Cat. #170-8890; stored in -20°C RNA Freezer)
- iScript Reverse Transcriptase (included in iScript cDNA Synthesis kit)
- 5x iScript Reaction mix (included in iScript cDNA Synthesis kit)
- Nuclease-free water (included in iScript cDNA Synthesis kit)
- Autoclaved DEPC-treated (DEPC'd) water

Materials Needed:

- Pipettes
- RNase-free filter pipet tips
- RNase-free 1.5 mL microcentrifuge tubes
- Rack for 1.5 mL microcentrifuge tubes
- Black ultra-fine sharpie
- Ice bucket
- Microcentrifuge
- Vortex
- Timer
- 25°C heating block
- 42°C heating block
- 85°C heating block

PROCEDURE

1. Write down the **concentration** of purified total RNA samples to be used.

   *Note: 1 μg = 1,000 ng. Therefore, the concentration determined by the NanoDrop spectrophotometer (**ng/μL**) needs to be converted to **μg/μL**.*

	WT	**T-DNA**
RNA concentration	_____ μg/μL	_____ μg/μL

2. Determine the **volume** of **1 μg** of purified total RNA.

   Volume** of **1 μg RNA** = **Amount** of **RNA** ÷ **concentration** of **RNA

   *Example: If the purified **RNA** has a concentration of 0.5 μg/μL, then the volume of **1 μg** of **RNA** will be 1 μg ÷ 0.5 μg/μL = **2 μL***

	WT	T-DNA
Volume of 1 µg	_____ µL	_____ µL

3. Use the following **table** as a **guide** to fill in the volumes for purified total RNA and DEPC'd water.

Components	**RNA +RT**	**RNA -RT**
DEPC'd (or nuclease-free) water	**X** µL	**X** µL
1 µg Purified total RNA	**Y** µL	**Y** µL
+ RT Mix	5.0 µL	-
- RT Mix	-	5.0 µL
Total Reaction Volume	**20.0** µL	**20.0** µL

X µL = volume of RNA sample; **Y µL** = volume of DEPC'd water

*The **volume of DEPC'd water** is the difference between the total reaction volume and the sum of the volume of the other components.*

4. Get ice from the icemaker.
5. Set heating blocks to 25°C, 42°C and 85°C.
6. Label the lids of FOUR RNase-free 1.5 mL microcentrifuge tubes "**WT +RT**," "**WT –RT**," "**T-DNA +RT**," and "**T-DNA –RT**." Keep tubes **on ice**.
7. Thaw the tubes of **5x iScript Reaction Mix** and **nuclease-free water** at room temperature. Once the solutions are **thawed**, spin tubes in a microcentrifuge for **10 seconds**, and keep the tubes **on ice**.
8. Prepare TWO tubes for **Master mixes** (**+RT Mix** and **-RT Mix**) as follows:
    g. Determine the number of RT reactions to be set up.

    Note: ***# RT reactions = # of RNA samples** + 1 Extra*

    Example: ***# RT reactions = 3** = WT Siliques + T-DNA Siliques + 1 Extra*

    h. Label the lids of the RNase-free microcentrifuge tubes "**+RT mix**" and "**-RT mix**." Keep the tubes **on ice**.

i. Remove a tube of **iScript Reverse Transcriptase** from the **-20°C RNA freezer**. Keep the **tube on ice** at all times to prevent degradation of the enzyme in this tube.

j. Pipet the **following components** into appropriate tubes as shown below.

Components	**+RT Mix for ONE Reaction**	**+RT Mix for 3 Reactions**	**-RT Mix for ONE Reaction**	**-RT Mix for 3 Reactions**
DEPC'd (or nuclease-free) water	-	-	1 µL	3 µL
5x iScript Reaction mix	4 µL	12 µL	4 µL	12 µL
iScript Reverse Transcriptase	1 µL	3 µL	-	-
Total volume	**5 µL**	**15 µL**	**5 µL**	**15 µL**

k. Mix the contents by pipetting up and down **five times** or flicking the tubes several times.

l. Spin the tubes in a microcentrifuge for **10 seconds**. Put the tubes **on ice**.

9. Using the **+RT and -RT chart** written up in **step 3**, pipet the components into the tubes labeled in **step 6**.
10. Mix the contents in each tube by pipetting **gently** up and down **five times**. Keep tubes **on ice**.
11. Transfer all of **+RT and -RT tubes** from the ice bucket to either a heat block set to **25°C** or a rack for microcentrifuge tubes on the bench (room temperature). Incubate reaction tubes at **25°C** (or room temperature) for **5 minutes**. *This step is to allow oligo(dT) and random primers to anneal to the messenger RNA in the reactions*.
12. Incubate the tubes at **42°C** for **30 minutes** in a **heat block**. *This step is to synthesize first strand cDNAs*.
13. **After 30 minutes at 42°C**, inactivate **reverse transcriptase**, *which is known to interfere with Taq DNA polymerase in the PCR amplification step*, by heating the reactions at **85°C** for **5 minutes** in a **heat block**.
14. Chill the tubes **on ice** for at least **2 minutes**.

15. Centrifuge the tubes at room temperature for **1 minute** to bring water condensation to the bottom of the tubes. *Note: The RT reactions are ready for the PCR amplification step*.
16. Store RT reactions in a **-20°C freezer** if they are not used for PCR the same day. Otherwise, keep them **on ice** while setting up the PCR amplification step.

III. CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS

Purpose: To amplify the cDNA template corresponding to the mRNA for the gene of interest in order to determine if the T-DNA insertion causes a null mutation (i.e. no mRNA is expressed for the gene of interest).

*Note: Amplification of **tubulin** cDNA will be used as a positive control because the tubulin gene is expressed in all samples. **Control primers** are used to ensure that the absence of a gene-specific PCR product in the **+RT samples** is NOT due to technical mistakes.*

Solutions Needed:

- Reverse transcription (+RT & -RT) reactions
- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 μM Gene-specific RT Forward primer
- 12 μM Gene-specific RT Reverse primer
- 12 μM Tubulin Forward primer
- 12 μM Tubulin Reverse primer
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading buffer containing ONLY xylene cyanol
- 100 bp DNA ladder (Invitrogen)

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- Ice bucket
- 0.2 mL PCR tubes
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- PCR machine (Bio-Rad MyCycler)

- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

1. Get ice from the icemaker.
2. Determine **how many RT reactions** (including +RT's and -RT's and gene-specific primers and control primers) will be amplified.
3. Make a **table** with information such as **tube #**, **sample identity**, **+RT's/-RT's** and **primers** (see the **example** table below).

Tube #	Sample	RT	Primer set
1	WT Silique	+RT	Gene-specific RT
2	WT Silique	-RT	Gene-specific RT
3	T-DNA Silique	RT	Gene-specific RT
4	T-DNA Silique	-RT	Gene-specific RT
5 (Positive)	Genomic DNA	-	Gene-specific RT
6 (Negative)	Sterile Water	-	Gene-specific RT
7	WT Silique	+RT	Tubulin
8	WT Silique	-RT	Tubulin
9	T-DNA Silique	RT	Tubulin
10	T-DNA Silique	-RT	Tubulin
11 (Positive)	Genomic DNA	-	Tubulin
12 (Negative)	Sterile Water	-	Tubulin

4. Label the lids and sides of **TWELVE 0.2 mL PCR tubes** with the **tube number** and **your initials**. Put the labeled tubes on a PCR tube rack sitting **on ice**.
5. Label the lids and sides of **TWO 1.5 mL microcentrifuge tubes** as "**Gene-specific Mmix**" and "**Tubulin Mmix**." Put the labeled tubes **on ice**.
6. Prepare a **master mix** for each primer set for the **number of PCR reactions** being carried out **plus 1 extra** as follows:

Gene-specific Mmix Components	**Mmix for ONE Reaction**	**Mmix for 7 Reactions**
Sterile water	36.75 µL	257.25 µL
10x Ex Taq buffer	5.0 µL	35.0 µL
dNTP mix	4.0 µL	28.0 µL
12 µM Gene-specific RT Forward primer	1.0 µL	7.0 µL
12 µM Gene-specific RT Reverse primer	1.0 µL	7.0 µL
Ex Taq DNA Polymerase (5 U/µL)	0.25 µL	1.75 µL
Total Volume	**48.0 µL**	**336.0 µL**

Tubulin Mmix Components	**Mmix for ONE Reaction**	**Mmix for 7 Reactions**
Sterile water	36.75 µL	257.25 µL
10x Ex Taq buffer	5.0 µL	35.0 µL
dNTP mix	4.0 µL	28.0 µL
12 µM Tubulin Forward primer*	1.0 µL	7.0 µL
12 µM Tubulin Reverse primer*	1.0 µL	7.0 µL
Ex Taq DNA Polymerase (5 U/µL)	0.25 µL	1.75 µL
Total Volume	**48.0 µL**	**336.0 µL**

* ***Control primers*** *are used to ensure that the absence of a gene-specific PCR product in the* ***+RT samples*** *is NOT due to technical mistakes.*

7. Pipet **48 µL** of **master mix** and **2 µL** of **sample** into the appropriate labeled PCR tubes according to the **table in step 3**. The total reaction volume should be **50 µL**. Mix the contents by pipetting **gently** up and down **5 times**.
8. Carry out PCR on the Bio-Rad MyCylcer with the "**HC70AL RT PCR**" **program** containing the following profile:

94°C 3 min
40 cycles of 94°C 10 sec
62°C 30 sec
72°C 45 sec
72°C 4 min
4°C ∞

9. Prepare **100 mL** of a **2% agarose** gel in **1x TAE** buffer using a **20-tooth** comb.
10. Label TWELVE 1.5 mL microcentrifuge tubes according to the PCR reactions.
11. Add **20 µL** of **PCR solution** and **2 µL** of **6x loading dye with xylene cyanol only** to the labeled 1.5 mL microcentrifuge tubes.
12. Load **10 µL** of **100 bp DNA ladder** in the first well.
13. Load **20 µL** of each sample-dye mixture on a 2% agarose gel using a P-20 pipette.
14. Record the identity of the sample in each lane.

Lane	Tube	Sample	RT	Primer set	Expected Size (bp)
1	**-**	100 bp DNA ladder	-	-	-
2	**1**	WT Silique	+RT	Gene-specific RT	
3	**2**	WT Silique	-RT		
4	**3**	T-DNA Silique	+RT		
5	**4**	T-DNA Silique	-RT		
6	**5**	Genomic DNA	-		
7	**6**	Sterile Water	-		-
8	**7**	WT Silique	+RT	Tubulin	
9	**8**	WT Silique	-RT		
10	**9**	T-DNA Silique	+RT		
11	**10**	T-DNA Silique	-RT		
12	**11**	Genomic DNA	-		477
13	**12**	Sterile Water	-		-
14	**-**	100 bp DNA ladder	-	-	-

15. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
16. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) travels about two-thirds of the gel.

    Time power supply turned ON:

    Time power supply turned OFF:

    How long was the gel run? _____ hour(s) and _____ minutes
17. Take a picture of the gel using the Bio-Rad Gel Document System.
18. Print out the picture. Store the labeled picture in your lab notebook.
19. Analyze the data.

    How many DNA fragments do you see on the gel?

What are the sizes of the DNA fragments?

What is the size of the PCR product corresponding to the mRNA of the gene of interest?

What is the expected size of the PCR product for genomic DNA?

Is there a difference in size between the PCR products from cDNA and genomic DNA?

Is there difference in brightness between the PCR products from wild type siliques and mutant siliques?

What can you conclude about the expression of the gene of interest?

Are the RT-PCR results correlated to the GeneChip data?

EXPERIMENT 7 - IDENTIFYING FEATURES OF MUTANT SEEDS USING NOMARSKI MICROSCOPY (GENE TWO)

Purpose: To introduce **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**NIC**) microscopy technique as a tool to identify features of defective embryos in knockout mutants.

Reference: The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer's laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from Arabidopsis
    a. wild type
    b. homozygote or heterozygote mutant
- 100% ethanol
- Acetic acid
- Sterile water
- Chloral Hydrate (Cat. #C8383, Sigma-Aldrich; should be fresh)
- Glycerol (Invitrogen)
- Double-distilled water

Materials Needed:

- Pipettes
- Pipette tips (regular, non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Rulers with METRIC scale (mm)
- Plant layout chart
- Phenotype observation chart
- Fine point forceps
- 30-gauge hypodermic needles
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides
- Double-sided tape
- Dissecting microscopes (borrowed from Dr. Pei Yun Lee)
- A microscope equipped with Nomarski optical parameter (Leica CTR5000)
- Microscope camera

PROCEDURE

Each student collects the following from wild type and his/her homozygous or heterozygous mutant:

a) 5 siliques containing seeds with embryo stages of globular to torpedo.

b) 2 siliques containing seeds with mature green embryos.

Note: *Be sure to collect a wide* ***range*** *of stages. Do not collect yellow or brown siliques; these contain dry seeds.*

I. Observation of Seeds Using Light Microscopy and Fixing Seeds for Observation with Nomarski Optics

1. Prepare 5 mL of a fixative solution of ethanol: acetic acid (9:1, v/v) in a 14 mL centrifuge tube using disposable 5 mL pipets.

FIXATIVE SOLUTION

100% ethanol	4.5 mL
Acetic acid	0.5 mL
Total volume	5.0 mL

Tightly snap the cap on the tube. **Invert** the tube to mix the contents.

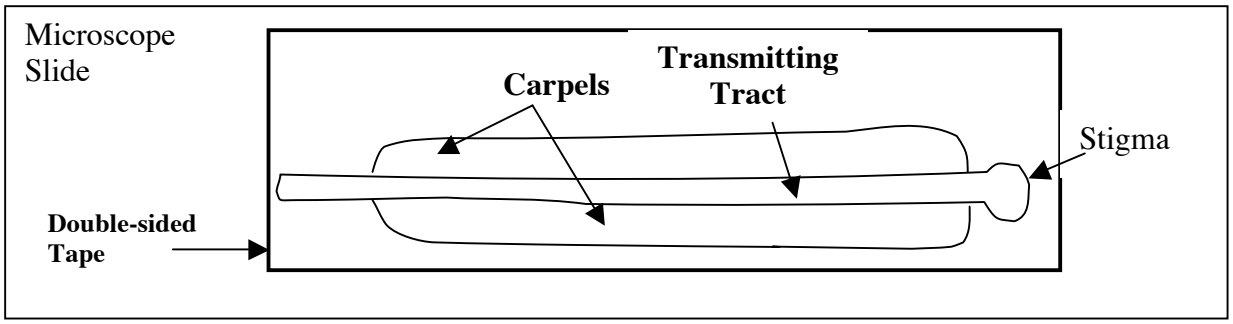
2. Pipet **1 mL** of the **fixative solution** into **FOUR** 1.5 mL microcentrifuge tubes sitting on a microcentrifuge tube rack. Label each tube in step 2 with your **initials**, the **plant #** and the **plant genotype**. *These tubes will be used in step 5.*
3. Bring the following materials to the Plant Growth Center (PGC).
   - Bucket of ice
   - Tweleve 1.5 mL microcentrifuge tubes
   - Microcentrifuge tube rack
   - Black ultra-fine sharpie
   - Ruler with METRIC scale (mm)
   - A pair of fine point forceps
   - Plant layout chart with information about plant number and the genotype of those plants
   - This protocol

- Bruincard with access to the PGC
- Key to growth chambers in the PGC

4. Measure and collect siliques according to the chart below. Place each silique in a 1.5 mL microcentrifuge tube. Write your **initials**, the **plant #**, the **plant genotype** and the **length** on the tube. Keep the tube on **ice**. *Note: Collect the **same length** of siliques for wild type and homozygous/heterozygous mutant so that you can compare them.*

Plant Genotype	**Seed Stages Collected**	**Length of Siliques Collected**	**Number of Siliques Collected**
Wild type	globular to torpedo	*0.5 - 1.0 cm*	5
Wild type	mature green	*1.0 - 1.9 cm*	2
Heterozygous or homozygous mutant	globular to torpedo	*0.5 - 1.0 cm*	5
Heterozygous or homozygous mutant	mature green	*1.0 - 1.9 cm*	2

5. Go back to the lab. **Dissect** the siliques and **observe** the seed phenotype using a dissecting microscope. *Note: Work quickly so the seeds don't dry out. You may also place a drop of water on the silique.*
    a. **Place** a **piece of double-sided tape** on a **microscope slide**. Label the microscope slide with a small piece of white tape with your **initials**, the **plant #**, the **plant genotype** and the **length**.
    b. Carefully, use **fine-point forceps** to place a silique on the tape.
    c. Under a dissecting microscope, use **fine-point forceps** to carefully place a silique on the tape and **arrange** the silique such that the **transmitting tract** is facing you (see diagram below, NOT drawn to scale).



d. With your left hand, use forceps to hold the silique on the side closest to the stem.

e. With your right hand, use a **28G** or **30G hypodermic needle attached to a 1 cc syringe** to **slice** the **carpels along each side of the transmitting tract.**

f. **Gently** peel back the carpels and stick them to the tape to reveal the seeds.

g. Observe the phenotype. Note any phenotypes that you observe on your **Screening Seeds Using Light Microscopy** chart.

In what stage of development are the seeds?

How many seeds are in the silique?

How many are green?

How many are white?

How many are brown?

What is the expected ratio of wild type seeds to mutant seeds if the mutation is seed lethal?

What is the observed ratio of wild type seeds to mutant seeds?

Are the observed results significantly different from the expected results?

Use a Chi-Square test.

$$\chi^2 = \Sigma \frac{(observed - expected)^2}{expected}$$

Probability that the deviation is due to chance alone

Degrees of Freedom	0.5	0.1	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.235	11.070	13.388	15.086	20.517

What is your null hypothesis?

How many degrees of freedom are there?

(The degrees of freedom is one less than the number of different phenotypes possible.)

What is your chi-square value?

(The chi-square statistic is a probability that indicates the chance that, in repeated experiments, deviations from the expected would be as large or larger than the ones observed in this experiment)

What is the probability that the deviation of the observed values from the expected values was a chance occurrence?

(Look up your degrees of freedom in the table. Find where your chi-square value falls in that row.)

Can you reject the null hypothesis?

If the probability is less than 0.05 (5%), reject your null hypothesis. If the probability is 0.05 (5%) or greater, then you cannot reject your null hypothesis.

h. Ask your TA to take pictures of the seeds within the siliques.

i. **Immediately**, use the fine-point forceps to transfer the cut silique into the tube with fixative solution from **step 2**.

j. Repeat steps a-i for the other siliques. *<u>Note:</u> You collected an excess of siliques so that you would have some to practice dissecting and to have a*

range of developmental stages for each genotype. However, you only need to fix FOUR siliques.

i. *Wild type, early development*
ii. *Heterozygous (or homozygous), early development*
iii. *Wild type, late development*
iv. *Heterozygous (or homozygous), late development*

6. Fix seeds and siliques in the fixative solution for at least 2 hours. *Note: It is recommended to fix the siliques **overnight** to ensure that the fixative solution penetrates the seeds and their embryos. It is okay to leave siliques in the fixative solution for up to 3 days.*
7. **Carefully**, pipet off **900 µL** of the fixative solution using a **P-1000** pipette and discard into a beaker labeled "acetic acid waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
8. **Immediately**, pipet **1 mL** of **90% ethanol** solution into the tube using a P-1000 pipette. *Note: The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
Double-distilled water	0.5 mL
Total volume	5.0 mL

9. Incubate seeds and siliques in the 90% ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*
10. Replace the 90% ethanol solution with **70% ethanol** as in steps 7 & 8.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
Double-distilled water	1.5 mL
Total volume	5.0 mL

11. Incubate seeds and siliques in the ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*

II. Observation of Seeds and Embryos Using Nomarski Optics

Note:

- ***Before observation of the seeds and their embryos, seeds must be submerged in the clearing solution. For young seeds, clearing is usually fast (~5 minutes). The older the silique, the longer it takes to clear (~ 1 hour). Seeds are ready for observation after they sink in the clearing solution.***
- ***Tissues CANNOT be stored in the CLEARING solution for more than TWO days because they will lose their structures quickly.***

1. Prepare a ***fresh*** clearing solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14 mL centrifuge tube. *Note: The TAs will prepare this solution before the lab class begins.*

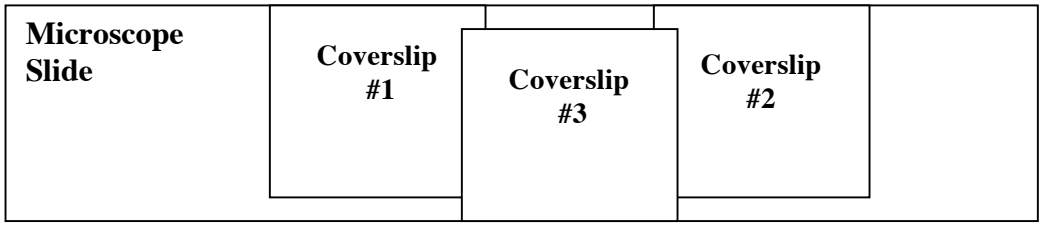
CLEARING SOLUTION

Chloral hydrate	8 g
Glycerol	1 mL
Water	2 mL
Total volume	~7 mL

2. **Carefully**, pipet off **900 µL** of the 70% ethanol solution using a **P-1000** pipette and discard into a beaker labeled "ethanol waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
3. Replace the 70% ethanol solution with **100 µL of clearing solution**.
4. Incubate seeds and siliques in the clearing solution for **5 min - 1 hour**. Wait until the seeds **sink** to the bottom of the tubes. You may lay the tube on its side so that the

silique is immersed in the clearing solution. *Note: Tissues CANNOT be stored in the CLEARING solution*.

5. Set a new glass microscope slide on the bench. Label it with your **initials**, the **plant #**, the **plant genotype** and **silique length**.
6. Use forceps to remove a silique from the clearing solution and place it on the labeled glass slide.
7. Pipet the remaining clearing solution and seeds onto the slide with the silique.
8. Carefully, place two square coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution. Avoid trapping bubbles in the solution (see diagram below).



9. Observe the seeds under Nomarski optics using the Leica CTR5000 microscope.
10. Take pictures of the embryos.

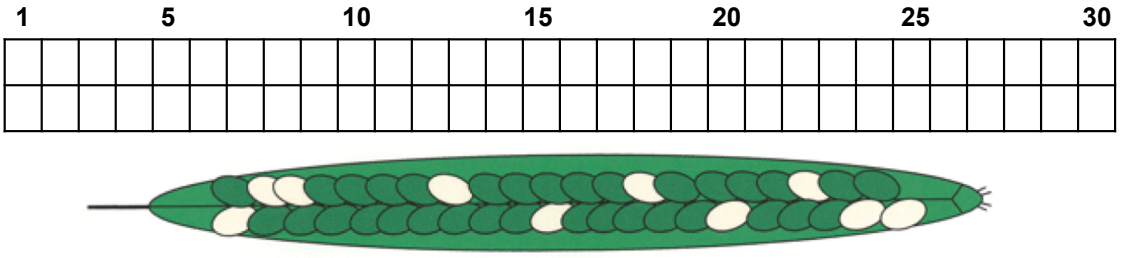
    In what stage of development are the seeds?
11. Repeat steps 2-10 for the remaining 3 fixed siliques.

Screening Seeds Using Light Microscopy

AGI# _____ SALK # _____ Plant #_____Genotype _____

Silique # _____ Length of Silique (cm) _____ Total Seeds _____ Total Mutant Seeds _____

<u>Instructions:</u> The grid represents the layout of the silique. Put a number in each square that corresponds to a mutant seed. Describe the seed phenotypes in the chart below. The base of the silique is defined as the region closest to the pedicel and main stem, which is at the left of the grid.



Seed	Seed Coat Color	Embryo Color	Notes
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

EXPERIMENT 8 – AMPLIFYING & CLONING A GENE UPSTREAM REGION (GENE TWO)

Purpose: To determine the activity of the promoter of the gene of interest at the cellular and tissue levels in *Arabidopsis* plants via the expression of the β-glucuronidase (GUS) or green fluorescent protein (GFP) gene fused to the promoter of the gene of interest.

References:

iProof High-Fidelity DNA Polymerase Kit (Bio-Rad; See Appendix 1J)

pENTR/D-TOPO Cloning Instruction Manual (Invitrogen; See Appendix 1K)

QIAprep Miniprep Handbook (Qiagen; see Appendix 1L)

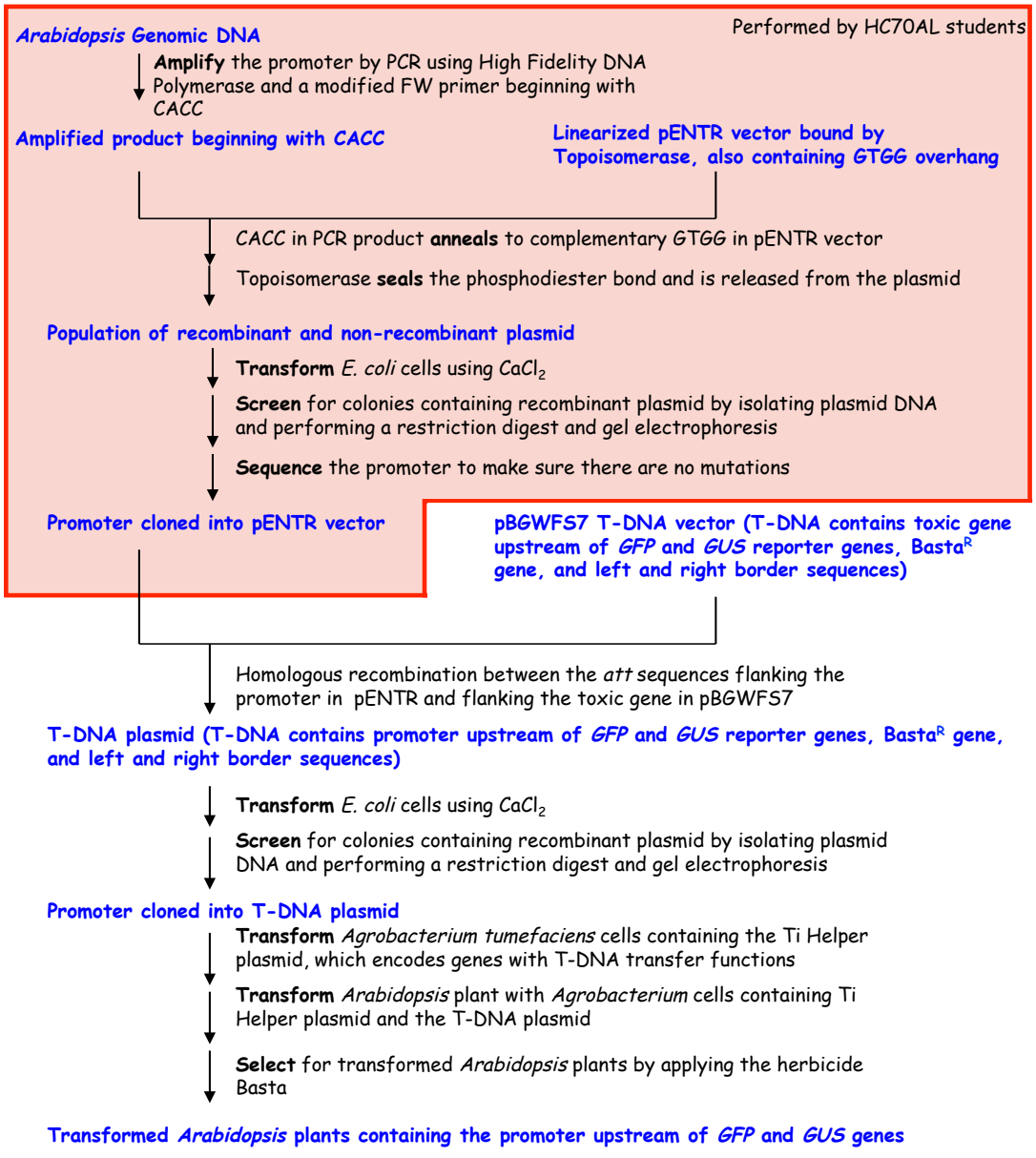
STRATEGY

I. AMPLIFICATION OF THE UPSTREAM REGION OF THE GENE OF INTEREST USING HIGH FIDELITY DNA POLYMERASE

II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

- **A. LIGATING THE PCR PRODUCT AND A pENTR/D-TOPO VECTOR**
- **B. TRANSFORMATION OF *E. COLI* CELLS WITH THE LIGATION MIXTURE**
- **C. SCREENING FOR *E. COLI* CELLS HARBORING THE RECOMBINANT PLASMID AND ISOLATING RECOMBINANT PLASMID DNA**
- **D. CONFIRMING THE AUTHENTICITY OF RECOMBINANT PLASMID DNA VIA RESTRICTION ENZYME DIGESTION**
- **E. VERIFYING THE SEQUENCE OF THE PROMOTER REGION BY SEQUENCING ANALYSIS**

STRATEGY FOR DETERMINING THE ACTIVITY OF A GENE UPSTREAM REGION



I. AMPLIFICATION OF THE UPSTREAM REGION OF THE GENE OF INTEREST

Materials Needed:

- iProof High Fidelity DNA polymerase kit (Cat.# 172-5301, Bio-Rad)
- iProof High Fidelity DNA polymerase (included in iProof High Fidelity DNA polymerase kit)
- 5x iProof HF Buffer (included in iProof High Fidelity DNA polymerase kit)
- dNTP mix (included with the Ex Taq DNA polymerase, Takara)
- Sterile water
- 12 μM Gene-specific Promoter Forward primer
- 12 μM Gene-specific Promoter Reverse primer
- High quality *Arabidopsis* genomic DNA (12 ng/μL)
- PCR product of the promoter region amplified by TAs with Ex Taq DNA Polymerase (for use as positive control)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading buffer containing ONLY bromophenol blue
- 100 bp DNA ladder (Invitrogen)

Materials Needed:

- Ice bucket
- Pipettes
- Filter pipet tips
- 0.2 mL PCR tubes
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- PCR machine (Bio-Rad MyCycler)
- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

*Note: ALWAYS wear **gloves** and use **PCR filter tips** when performing **PCR** preparation to prevent contamination.*

1. Get ice from the icemaker.

2. Thaw out tubes of **5x iProof HF Buffer** and **dNTP mix (2.5 mM each dNTP)** on a microcentrifuge tube rack for 1.5 mL microcentrifuge tubes at **room temperature** for a few minutes. Once the solutions are thawed, **vortex** for **5 seconds** to mix the contents. **Spin** tubes for **10 seconds**. Put the tubes **on ice** until needed.
3. Thaw **gene-specific promoter forward and reverse primers** corresponding to the gene of interest as in step 2.
4. Obtain **THREE 0.2 mL sterile PCR tubes** and set them on a **PCR tube rack**.
5. Label the lids and sides of the tubes with **sample name** and **date**.

   Tube #1: **Name of a gene**

   Tube #2: **Pos.** (**Positive** control for the gene of interest = PCR product of the promoter region amplified by TAs with Ex Taq DNA Polymerase)

   Tube #3: **Neg.** (**Negative** control for the gene of interest containing the same components as in tube #1, but **NO** genomic DNA)
6. Label **ONE 1.5 mL microcentrifuge tube** "**Mmix**" (for Master mix). Keep the tube on ice.
7. Prepare a **Master mix** for **4 reactions** (**3 samples + 1 extra**) (see table below)

Caution: Keep tube on ice at all times.

Note: Amplification of targets greater than 4 kb may require more DNA polymerase, but do NOT exceed 2 units of enzyme per 50 µL reaction.

Master Mix

	Mmix for 1 Reaction	Mmix for 4 Reactions	Final Concentration
Sterile water	25.5 µL	102 µL	
5x iProof HF Buffer	10 µL	40 µL	1x
dNTP mix (2.5 mM each dNTP)	4 µL	16 µL	0.20 mM
12 µM Gene-specific Promoter Forward primer	1 µL	4 µL	0.24 µM
12 µM Gene-specific Promoter Reverse primer	1 µL	4.0 µL	0.24 µM
iProof DNA polymerase (2.0 Units/µL)	0.5 µL	2 µL	
Total volume	**42 µL**	**168 µL**	

a. Pipet the reagents into the **Mmix tube** in order from the top down.

b. After pipetting all reagents into the master mix tube, close its lid. Mix the contents by vortexing at a **slow setting (**speed 2-3) for **2 seconds**. *Caution: Do **NOT** vortex a mixture with enzyme, such as DNA polymerase, **vigorously**, or for **> 5 seconds** because these two factors will **break down enzymes**, resulting in **LOW** or **NO yield** of PCR product.*

c. Spin the tube in a microcentrifuge at full speed (13,200 rpm) for **10 seconds**. Put the tube back **on ice**.

8. Prepare the PCR reactions according to the table below.

a. Pipet **42 µL** of the Mmix solution into the labeled PCR tubes.

b. Pipet **8 µL** of genomic DNA or water into tubes #1-3.

c. Mix the contents by pipetting up and down **5 times.**

PCR reactions:

	Tube #1	**Tube #2**	**Tube #3**
Mmix	**42 µL**	**42 µL**	**42 µL**
~100 ng *Arabidopsis* genomic DNA (12 ng/µL)	**8 µL**	-	-
PCR product of the promoter amplified by TAs (Positive control)	-	**1 µL**	-
Sterile water (Negative Control)	-	**7 µL**	**8 µL**
Total volume	**50 µL**	**50 µL**	**50 µL**

9. Perform PCR amplification as follows:
    a. Turn **ON** the PCR machine (MyCycler). Wait for one minute for the machine to initialize.
    b. Put the PCR tubes in the wells of the 96-well hot plate of the Bio-Rad MyCycler.
    c. Select the "**Protocol Library**" by pressing "**F1**."
    d. Select "**HC70AL**" by pressing the yellow arrowheads surrounding the "**ENTER**" button. Select the "**HC70AL IPROOF**" protocol. Press "**ENTER**."
    e. The "CHOOSE OPERATION" menu will appear. Select "**VIEW PROTOCOL**." The **PCR profile** of the iProof protocol is as follows:

PCR profile for **genomic DNA templates**

Cycling parameters		**Number of Cycles**
Activation Enzyme step	98°C for 30 seconds	**1**
Denaturation step	98°C for 10 seconds	**30**
Annealing step	63°C (or T_m+3°C) for 20 seconds	
Extension step	72°C for 2 minutes (or 15-30 seconds/kb)	
Final Extension	72°C for 5 minutes	**1**

Hold	4°C for ∞	**1**

f. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.

g. Enter the **volume** of the PCR reaction. Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*

10. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.
11. Prepare a **50 mL 1% agarose gel** in **1x TAE buffer** with a **10-tooth comb**.
12. Spin PCR tubes for **5 seconds**.
13. Label new 1.5 mL microcentrifuge tubes with **sample name**.
14. Pipet **20 µL** of PCR product into each labeled tube.
15. Add **2 µL** of **6x lower loading dye** to each tube.
16. Load **10 µL** of **1 Kb Plus DNA ladder** in the first well of the 1% agarose gel.
17. Load ~**22 µL** of each sample-dye mixture on the agarose gel.
18. Record the identity of the sample in each lane.

Lane	Tube #	Sample	Expected Size (bp)
1	-	1 Kb Plus DNA Ladder	-
2	1	Genomic DNA	
3	2	PCR product amplified by TAs	
4	3	Sterile water	-

19. Add **5 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
20. Run the gel at **~105 volts** for **1-2 hours**.

    Starting time:

    Ending time:
21. Take a **picture of the gel**.
22. Analyze the results.

II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

Materials Needed:

- Gene-specific promoter PCR product
- pENTR/D-TOPO Cloning kit (Cat.# K2400-20, Invitrogen)
- Sterile water

Materials Needed:

- Ice bucket
- Pipettes
- Filter pipet tips
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex

A. Ligating the PCR Product into the pENTR/D-TOPO Vector

1. Thaw the **pENTR/D-TOPO** vector solution **on ice**.
2. Label THREE 1.5 mL microcentrifuge tubes **pENTR + Promoter**, **pENTR Only** and **Mmix** and the **date**. Place the labeled tubes on ice.
3. Set up the **Mmix** as follows:
    a. Pipet the reagents into the **Mmix** tube.

Reagent	**pENTR + Promoter**	**pENTR Only (Negative Control)**	**Mmix for 2.3 rxns**
Sterile water	2.5 µL	2.5 µL	**5.75 µL**
Salt Solution	1.0 µL	1.0 µL	**2.3 µL**
pENTR/D-TOPO vector	0.5 µL	0.5 µL	**1.15 µL**
Freshly prepared PCR product	2.0 µL	-	-
Sterile water	-	2.0 µL	-
Total Volume	6.0 µL	6.0 µL	

    b. Mix **GENTLY** by **flicking** the tube. Do **NOT** vortex the tube!
4. Pipet **4 µL** of **Mmix** into the **pENTR + Promoter** and **pENTR Only** tubes.

5. Pipet **2 µL of freshly prepared PCR product** into the **pENTR + Promoter** tube.
6. Pipet **2 µL of sterile water** into the **pENTR Only** tube.
7. Mix **GENTLY** by **flicking** the tubes. Do **NOT** vortex the tubes!
8. Incubate the reaction for **30 minutes** at **room temperature** (22-24°C).

   Note: For most applications, 5 minutes will yield many colonies for analysis. The length of the TOPO Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. ***For large PCR products (>1 kb), increasing the reaction time will yield more colonies*** *(Taken from TOPO Cloning Manual, Invitrogen).*
9. After the ligation reaction is done, place the ligation mixture tube on ice. *Note: If you* ***don't have time****, you* ***can store*** *the* ***ligation mixture*** *at* ***–20°C overnight****.*

B. Transformation of *E. coli* Competent Cells with the Ligation Mixture

Solutions Needed:

- Ligation reactions
- pENTR/D-TOPO Cloning kit with One Shot TOP10 Competent cells (Cat.# K2400-20, Invitrogen)
- pUC19 plasmid (10 pg/µL) (Included with the TOP10 Competent cells)
- S.O.C. medium (Included with the TOP10 Competent cells)
- LB Kanamycin 50 µg/mL plates
- LB Ampicillin 100 µg/mL plates for pUC19 plasmid (control for transformation efficiency)

Materials Needed:

- Ice bucket
- Pipettes
- Pipet tips (non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- 42°C water bath
- 37°C incubator
- Orbital shaker in the 37°C incubator
- Tape

- Cell spreader
- A glass jar containing 95% ethanol solution
- Inoculating turntable
- Biological fume hood
- Parafilm

Note: Make sure to set a water bath 42°C before starting.

1. Thaw the **pUC19** control plasmid **on ice**.
2. Thaw THREE vials of **One Shot *E. coli* competent cells** for transformation **on ice** for **a few minutes**. *Note: Remove tubes of competent cells from the -70°C freezer and place them on ice just a few minutes before you need them.*
3. Label the vials number **1-3** and **your initials**.

	Vial #1	**Vial #2**	**Vial #3**
Sample Name	**pENTR + Promoter**	**pENTR Only**	**pUC19**

4. **Pipet** the **TOPO ligation mixtures** or **control plasmid DNA** (pUC19) into the vials of One Shot *E. coli* competent cells as shown below. **Mix** the contents by **flicking** each tube **gently**. *Attention: Do **NOT pipet** the mixture up and down.*

	Vial #1	**Vial #2**	**Vial #3**
pENTR + Promoter	**2 µL**	-	-
pENTR Only	-	**2 µL**	-
pUC19	-	-	**1 µL**

5. **Incubate** the cell mixture **on ice** for **10-30 minutes**.
6. **Heat-shock** the cells for exactly **30 seconds** in the **42°C water bath** without shaking.
7. Immediately, set the tubes **back on ice** for **2 minutes**.
8. **Transfer** the tubes to **a rack** for microcentrifuge tubes at room temperature.
9. In the bacterial hood, **pipet 250 µL** of room temperature **S.O.C medium** to the cell mixture. **Cap** the tube **tightly**.
10. **Shake** the tubes **horizontally** at **200 rpm** on an orbital shaker in a **37°C** incubator for **1 hour**. *Note: Attach tubes to shaker with tape.*

11. Meanwhile, **label** the **bottom** of **6 prewarmed** (37°C) plates (**4 Kanamycin** plates with no line on the side of the lids for **pENTR samples** and **2 Ampicillin** plates with ONE RED LINE on the side of the lids for **pUC19**) the following:

Plate #	Sample	Date	Initials	Volume of Cells
Kanamycin 1	pENTR + Promoter			10 μL
Kanamycin 2	pENTR + Promoter			50 μL
Kanamycin 3	pENTR Only			10 μL
Kanamycin 4	pENTR Only			50 μL
Ampicillin 1	pUC19			10 μL
Ampicillin 2	pUC19			50 μL

12. **Spread 10 μL** and **50 μL** of **each** transformation mixture on the appropriately labeled plates in the hood (TAs will show you how). *Note: Spread two volumes of cells per transformation mix in order to get at least one plate with enough colonies and well-separated colonies*.

    Plate #1: **10 μL** of transformation mixture + **40 μL** of S.O.C medium (for even spreading of a small volume)

    Plate #2: **50 μL** of transformation mixture

13. Incubate the plates in the **37°C** incubator overnight (14-16 hours).
14. **On the next day**, count the number of colonies. Seal the plates with pieces of parafilm and then store them at **4°C** (cold room or refrigerator) until the inoculation step.

    Do you expect to get the same number of colonies on the pENTR + Promoter plate as on the pENTR Only plate?

C. Screening for *E. coli* Cells Harboring the Recombinant Plasmid and Isolating Plasmid DNA

Reference: QIAprep Miniprep Handbook (Qiagen; see Appendix 1L)

Materials Needed:

- pENTR + Promoter plate with colonies

- Terrific broth (TB) medium containing 50 μg/mL Kanamycin

Materials Needed:

- 5 mL plastic pipette
- Bulb
- Black ultra-fine sharpie
- Vortex
- 37°C incubator
- Orbital shaker in the 37°C incubator
- Biological fume hood
- Sterile glass culture tubes
- Culture tube rack
- Sterile toothpicks
- Parafilm

Inoculation of a Liquid Medium with Bacterial Colonies

1. Put FOUR sterile glass tubes on a test tube rack.
2. Label the **side of each tube** with **your initials** and **number 1-4**.
3. Choose FOUR colonies and **label** them 1-4 on the agar plate.
4. Pipet **3 mL** of **Terrific Broth (TB)** medium containing **50 μg/mL Kanamycin** into **each of the 4 tubes**.
5. Inoculate the TB + Kanamycin solution in each tube with **individual** colonies by using a sterile toothpick or pipet tip to pick a single colony on the **pENTR + Promoter** plate and drop that toothpick/pipet tip into a tube.
6. Gently vortex to resuspend the colony.
7. Shake the tubes at **37°C overnight**.
    a. Transfer all 4 tubes to a **culture tube rack** on an **orbital shaker** in the **37°C incubator**.
    b. Turn the shaking **SPEED** dial (LEFT dial) to number **2** for **200 rpm**.
    c. Turn the shaking **TIME** dial (RIGHT dial) **clockwise** to the "**CONSTANT**" position.
8. Close the incubator door.
9. Wrap your plates in parafilm and store at 4°C.

10. **On the next day** (after 12-16 hours), inspect the growth of cells (appearing very cloudy) in the culture tubes. If plasmid DNA is not isolated immediately, place the culture tubes in the cold room.

Isolating Plasmid DNA

Materials Needed:

- Bacterial cultures
- QIAprep Spin Miniprep kit (Qiagen, cat. #27104)

Materials Needed:

- Ice bucket
- Pipettes
- Pipet tips (non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- NanoDrop spectrophotometer
- Kimwipes

1. Label the lids of FOUR **1.5 mL microcentrifuge tubes** with **your initials** and **number 1-4**. Set the labeled tubes on a microcentrifuge tube rack.
2. Arrange the culture tubes and labeled microcentrifuge tubes in their corresponding order. *For example, 1 to 1, 2 to 2, … , 4 to 4*.
3. Pipet **1 mL** of liquid culture into the appropriate microcentrifuge tube. Close the lids of the tubes. *Note: If the culture tubes sit in the refrigerator or cold room for more than ONE hour, vortex the tubes for 5-10 seconds to mix the contents before transferring it to the microcentrifuge tube*.
4. Spin tubes in a microcentrifuge at **FULL** speed for **30 seconds**.
5. Pour off the supernatant into a glass Erlenmeyer flask labeled "CULTURE WASTE." Dab off the extra liquid on a piece of paper towel or Kimwipes.
6. Place the tubes back on the microcentrifuge tube rack.

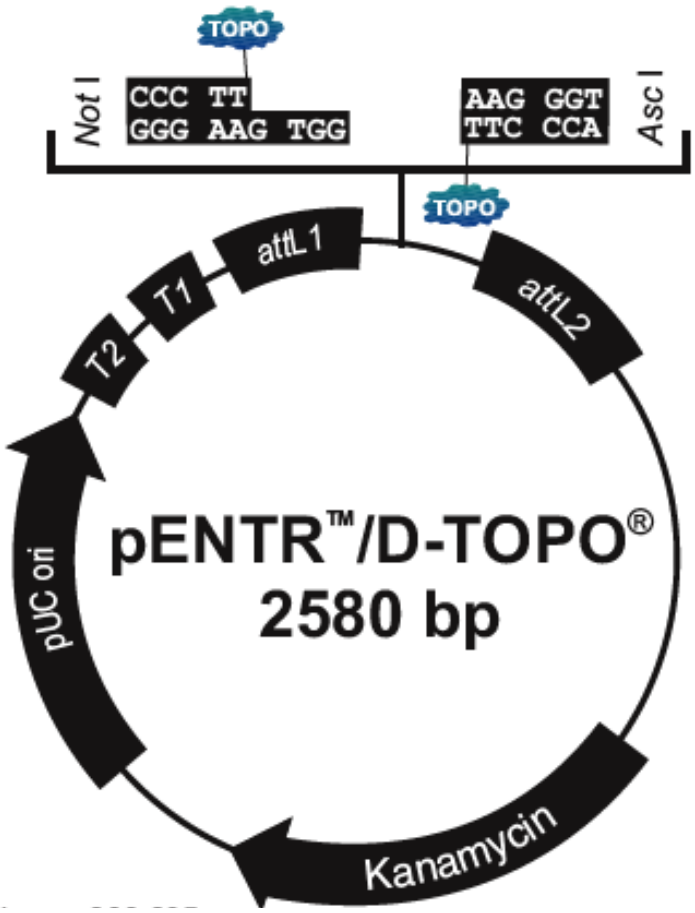
7. Pipet **another 1 mL** of liquid culture into the appropriate microcentrifuge tubes. Close the lids of the tubes.
8. Spin the tubes in a microcentrifuge at **FULL** speed for **30 seconds**.
9. Pour off the supernatant into a glass Erlenmeyer flask labeled "CULTURE WASTE." Dab off the extra liquid on a piece of paper towel.
10. Place the tubes back on the microcentrifuge tube rack.
11. Use a P-200 pipet to remove any remaining liquid from the tubes containing cell pellets. Discard the liquid into a glass Erlenmeyer flask labeled "CULTURE WASTE."
12. **Shake** or vortex **Buffer P1** (**Resuspension buffer)** to ensure that all particles are completely dissolved. *Note: Make sure that RNase A and LyseBlue are added to Buffer P1 before use*.
13. Pipet **250 µL** of **Buffer P1** (**Resuspension buffer)** to each tube. Close the lids **tightly**.
14. **Resuspend** pelleted bacterial cells by either vortexing or pipetting up and down until NO cell clumps are observed.
15. Place the tube back on the microcentrifuge tube rack.
16. Add **250 µL** of **Buffer P2** (**Lysis buffer**) to each tube. Close the lids. The solution will turn **blue**.
17. **Gently** mix by inverting the tubes **5 times** or until a homogeneously colored suspension is achieved. This step is for breaking open the bacterial cells to release their contents (chromosomal DNA, plasmid DNA, proteins, carbohydrates) into the solution. *Note:* ***Do NOT vortex*** *the contents. Vortexing can shear bacterial chromosome DNA into many tiny pieces that have the same size as the plasmid DNA.* *Note:* ***Do NOT*** *allow the lysis reaction to proceed for more than 5 min*.
18. Add **350 µL** of **Buffer N3** (**Neutralization buffer**) to tube #1. Close the lid. **Immediately**, invert the tube **5 times** to mix or until all trace of blue has gone and the suspension is **colorless**. The solution will appear cloudy. *Note:* ***Do NOT vortex*** *the mixture!*
19. Repeat step 17 for the other tubes (one by one).
20. Spin tubes in the microcentrifuge at **FULL** speed for **10 minutes**.

21. Meanwhile, label the lids of the QIAprep columns and sides of the collection tubes (**light blue**) with your **initials** and **number 1-4**. Set the columns in their collection tubes on the microcentrifuge tube rack.
22. Also, label the lids and sides of a new set of 1.5 mL tubes with the following information: **pENTR-gene name**, **number**, **your initials**, and the **date**. (Tubes will be used in step 31).
23. After 10 minutes of spinning, pour the **supernatant** from step 19 into the QIAprep column. *Caution: Make sure that the numbers on the tubes the QIAprep columns match*.
24. Spin the columns in their collection tubes at **FULL** speed for **30 seconds**.
25. Lift the column off of the collection tube and discard the flow-through liquid into a glass beaker for waste.
26. Put the column back in its collection tube.
27. Pipet **500 µL** of **Buffer PB** to each column. Spin the columns at **FULL** speed in the microcentrifuge for **30 seconds**.
28. Lift the column off of the collection tube and discard the flow-through liquid into a glass beaker for waste.
29. Pipet **750 µL** of **Buffer PE** to each column. Spin the columns at **FULL** speed in the microcentrifuge for **30 seconds**. This step is to wash residual salt and proteins from the membrane of the column. *Note: Make sure that ethanol is added to the PE buffer before use*.
30. Lift the column off of the collection tube and discard the flow-through liquid into a glass beaker for waste.
31. Spin the columns at **FULL** speed for **1 minute** to remove residual wash buffer. *Caution: If the residual wash buffer is NOT completely removed, the DNA solution will float up when the sample is loaded into the well of an agarose gel due to the presence of ethanol in the DNA solution. Also, ethanol may inhibit enzymatic activity in later steps. Note: If wash buffer remains on the column, spin for another 1-2 minutes*.

32. Transfer the QIAprep columns to the **labeled tubes (prepared in step 21)**. Discard the **collection tubes**. *Note: Make sure the numbers on the columns and microcentrifuge tubes match.*
33. Pipet **50 µL** of **Buffer EB (10 mM Tris-HCl, pH 8.5)** to the **center** of each QIAprep column. *Note: If the plasmid is >10 kb, pre-heat Buffer EB to 70°C prior to eluting DNA from the QIAprep membrane.*
34. Let the columns stand for **1 minute**. *Note: It is okay to incubate **longer than 1 minute**.*
35. Spin the tubes with columns at **FULL** speed for **1 minute**. *Steps 32-34 are for eluting plasmid DNA off the column.*
36. After spinning, discard the columns. **Save** the **eluted plasmid DNA** in the 1.5 mL microcentrifuge tubes.
37. Determine the **DNA concentration** and its **purity** using the NanoDrop spectrophotometer. Record DNA concentration.

D. Confirming the Authenticity of Recombinant Plasmid DNA Via Restriction Enzyme Digestion

Purpose: To ensure that the plasmid DNA isolated from colonies is recombinant plasmid DNA (i.e. contains the cloned promoter region), not non-recombinant DNA (i.e. the vector alone).



TOPO

Represents covalently bound topoisomerase I

Reagents and Materials Needed:

- Plasmid DNA
- Sterile water
- *AscI* restriction enzyme (New England Biolabs, 10 units/µL)
- 10x NEB Buffer 4 (supplied with *AscI*)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading buffer containing ONLY bromophenol blue
- 1 Kb Plus DNA ladder (Invitrogen)

Materials Needed:

- Ice bucket
- Pipettes
- Pipet tips (non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- 37°C water bath
- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

1. Digest **300 - 1000 ng** of plasmid DNA with the restriction enzyme ***AscI*** at **37°C** for **60 minutes**.

   Why AscI? *Check the presence of the* ***AscI*** *site in the* ***Multiple Cloning Site*** *of the pENTR/D-TOPO vector diagram on the previous page.*

Table 1. Setting up a **standard** restriction enzyme reaction

Components	**Standard Reaction**	**Final Concentration**	**Example 500 ng DNA (200 ng/μL)**
DNA	**x** μL	-----	**2.5** μL
Sterile water	**y** μL	**---**	**15.0** μL
10x NEB buffer 4	**2.0 μL**	**1x**	**2.0** μL
AscI (10 U/μL, NEB)	**0.5 μL**	**0.25 U/μL**	**0.5** μL
Total Volume	**20.0 μL**	**---**	**20.0** μL

Explanation of volumes

***x μL** = Volume of Plasmid DNA depends on the **amount** (in **ng**) of DNA to be digested and the **concentration** of plasmid DNA (in **ng/μL**)*

- ***Volume** of **10x NEB buffer** is **1/10th** the **total volume** of the reaction so that the **final concentration** of the buffer in the reaction is **1x**.*
- *The **volume** of **restriction enzyme** depends on the **amount of DNA** to be digested. Usually, **ONE Unit** of **Restriction endonuclease** (Enzyme) is defined as the amount of enzyme required to digest **1 μg** (or **1,000 ng**) of **DNA** completely in **ONE hour** under the **conditions specified for that enzyme** (**most enzymes** have an **optimal temperature of 37°C**). To ensure that DNA is completely digested after 1 hour, we use **2-10 units** of enzyme **per microgram** of DNA.*
- ***y μL** = The volume of sterile water is the remaining volume added to the reaction to bring up the total volume.*

***Note:** It is best to set up reactions with a master mix to minimize the chance of leaving out one or several components in the reactions. **The volume of NEB buffer + AscI for each reaction is fixed as 2.5 μL,** while the volume of the plasmid DNA + water is 17.5 μL. Therefore, it is best to make an **Enzyme Mix containing the buffer and AscI enzyme** (see Table 2 below) for **five reactions** (4 samples + 1 extra reaction).*

a. Label the lid of a 1.5 mL microcentrifuge tube "**Enz Mix**." Prepare the **Enzyme Mix** for the **number of plasmid DNA samples + 1 Extra reaction** (use Table 2 below). **Keep the Enzyme Mix tube on ice**. Mix the contents by flicking the tube gently. Spin the tube for 10 seconds (if necessary). Keep the tube on ice.

How many DNA samples will be digested?

Table 2. Preparation of the Enzyme Mix

Components	**Enz Mix** for **1 Reaction**	**Enz Mix** for **____Reactions**
10x NEB Buffer 4	2.0 µL	____ µL
***AscI* (10 U/µL)**	0.5 µL	____ µL
Total Volume	**2.5 µL**	____ **µL**

b. Determine the volume of plasmid DNA and the volume of water so that you have a total volume of **17.5 µL** and **fill in Table 3** below.

Table 3.

	Sample 1 _____ng/µL	**Sample 2 _____ng/µL**	**Sample 3 _____ng/µL**	**Sample 4 _____ng/µL**
300 - 500 ng plasmid DNA	_____ µL	_____ µL	_____ µL	_____ µL
Sterile water	_____ µL	_____ µL	_____ µL	_____ µL
Enzyme Mix	2.5 µL	2.5 µL	2.5 µL	2.5 µL
Total volume	**20 µL**	**20 µL**	**20 µL**	**20 µL**

c. Label the lids of 1.5 mL microcentrifuge tubes with the **sample number**, ***AscI*** and **your initials**. Keep tubes **on ice**. Set up restriction digestion reactions by pipetting the components from **Table 3** into the tubes. Mix the contents by flicking the tubes **several times**. Spin the tubes in the microcentrifuge for **10 seconds** to bring liquid to the bottom of the tubes.

d. Incubate the reactions in the **37°C water bath** for about **1 hour**.

2. In the meantime, prepare a **1% agarose gel** in **1x TAE buffer** with a **20-tooth comb**.
3. At the end of the incubation, spin tubes for **10 seconds**.
4. Add **2 µL** of **6x Loading dye** to each **restriction-digested DNA sample**. Mix the contents by pipetting the mixture or flicking the tube.
5. Load **10 µL** of **100 bp DNA Ladder** in the first well.
6. Load **20 µL** of each sample-dye mixture on the agarose gel.

   *Note: You may also load **uncut** plasmid DNA in other lanes for reference. Run 300-1000 ng of each uncut plasmid mixed with 2 µL of 6x Loading dye.*
7. Load **10 µL** of **1 Kb Plus DNA Ladder** in the first well.
8. Record the identity of the sample in each lane.

Lane	Sample	Expected Sizes (bp)
1	100 bp DNA Ladder	-
2	Plasmid #1 x AscI	
3	Plasmid #2 x AscI	
4	Plasmid #3 x AscI	
5	Plasmid #4 x AscI	
6	1 Kb Plus DNA Ladder	-
7	Plasmid #1 Uncut	-
8	Plasmid #1 Uncut	-
9	Plasmid #1 Uncut	-
10	Plasmid #1 Uncut	-

9. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
10. Run the gel at **~105 volts** for **1-2 hours**.

    Starting time:

    Ending time:
11. Take a **picture of the gel**.
12. Analyze the results.

    How many DNA fragments do you see from each plasmid DNA sample?

Which fragment corresponds to the vector?

What is the size of the vector?

Which fragment corresponds to the PCR-amplified promoter region?

What is the size of the PCR-amplified promoter region?

How many samples have the PCR-amplified promoter region?

Which plasmid DNA prep (or clone) will be used for sequencing analysis?

E. Verifying the Sequence of the Promoter Region by Sequencing Analysis

Purpose: To verify that the cloned promoter region has the **exact** sequence as the one reported in the *Arabidopsis* database.

References: Applied Biosystems

UCLA WebSeq website http://genoseq.ucla.edu/action/view/Sequencing

Solutions Needed:

- Applied Biosystems Big Dye version. 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Sigma, Cat. # S3938; also, obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- Plasmid DNA
- 20 μM M13 Forward primer
- 20 μM M13 Reverse primer
- Sterile water

Materials Needed:

- Pipettes
- Filter pipet tips
- 0.2 mL PCR tubes or strips of 8 tubes/strip
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

Generally, **10-μL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). *Note: If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use the format of Master Mix (Mmix) solution to minimize the*

number of pipettings and mistakes of not adding some components into the individual reaction tubes.

Important: *This is **NOT** a **regular** PCR reaction. Do **NOT** add **TWO** primers into a single reaction tube. **Each reaction contains only ONE primer**.*

General Components of One Reaction:

	ONE Reaction
DNA template *	**x** μL
Sterile water	**y** μL
20 μM Sequencing primer	1 μL
Big Dye v. 3 Solution	1 μL
5x Sequencing buffer	2 μL
Total volume	**10 μL**

x μL = the volume depends on **concentration** and **amount** of **DNA** (see Table below)

y μL = the **remaining volume** to bring the **total volume** to **10 μL**

*** Amount of DNA template** depends on **type of DNA**:

- For **plasmid** DNA, use **800 ng**. We found that 250 ng of plasmid DNA will work, but more DNA gives the better reads.
- For **PCR product**, use the amount of DNA according to the **table** on the next page (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 μL.

For this exercise, there is **ONE DNA template**, i.e. the plasmid containing the promoter of the gene of interest; but, there are **TWO primers**, M13 forward and M13 reverse primers. Therefore, it is best to prepare a **master mix** with **all components**, **except** the **primers**, which will be added to individual reaction tubes.

What is the concentration of the plasmid DNA? _____ **ng/μL**

What is the amount of DNA to be used? _____ **ng**

What is the volume of plasmid DNA to be used? _____ **µL**

PROCEDURE

1. Get ice from the icemaker.
2. Label the **sides** of **TWO 0.2 mL PCR tubes** with **your initials** and **primer name**. Set the tube on a PCR tube rack sitting on ice.
3. Label the **lid** and **side** of a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.
4. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mix (Mmix) for Sequencing Reactions:

Components	**Mmix** for **ONE reaction**	**Mmix** for **3 reactions**
250-500 ng of plasmid DNA	**x** µL	**x** (x 3) µL
Sterile water	**y** µL	**y** (x 3) µL
Big Dye v. 3	**1** µL	**3** µL
5x Sequencing buffer	**2** µL	**6** µL
Total Volume	**9 µL**	**27 µL**

   a. Mix the contents by flicking the tube five times or vortexing at the mixer setting of 2-3 for **5 seconds**.
   b. Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
   c. Set the tube back on ice.
5. Pipet **Mmix** and **M13 primer** into TWO labeled 0.2 mL PCR tubes.

Components	M13 Forward primer	M13 Reverse primer
Mmix	9 μL	9 μL
20 μM **M13 Forward** primer	1 μL	-
20 μM **M13 Reverse** primer	-	1 μL
Total volume	**10 μL**	**10 μL**

6. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700**

   USER: <<hc-lab>>
   PROGRAM: **HC70AL BIG DYE**
   The profile of the Big Dye program is:
   25 cycles of 96°C 10 sec
   55°C 5 sec
   60°C 4 min
   4°C ∞

   or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up sequencing reactions using DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
   a. Resuspend the resin by inversion or gently vortexing.
   b. Loosen the cap of the column a quarter turn. *This is necessary to avoid a vacuum inside the spin column*.
   c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
   d. Centrifuge at 3,000 rpm for 3 minutes at room temperature.
   e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
   f. Carefully transfer the spin columns to the new tubes.
   g. Slowly apply the sequencing reactions to the gel beds of the appropriate columns. *Note:*
      - *Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the*

sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip.

- *For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 µL using distilled water, before application to the gel bed.*

h. Spin the columns as in step d.

i. Remove the spin columns from the microcentrifuge tubes. *The eluate contains the purified DNA.*

8. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. *Note: Use the primer name as the name of your sequence. Make sure to copy down the* ***assigned file number*** *(example, #5678), which is automatically given by the Facility, after you enter the samples into the Facility computer.*
9. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

Retrieving and Analyzing DNA Sequences

Purpose: To verify that the sequence corresponds to that of the gene of interest.

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/
   a. Enter Username: **goldberg_r**
   b. Enter Password: **embryo**
   c. Click "LOGIN."
2. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

   Example: The ***assigned file number*** is **106203**, and the gene of interest is **At5g09250**. You would see the following files:

   106203GoldR At5g09250Fw A12.ab1

106203GoldR At5g09250Rv B12.ab1

What are the annotations?

106203 = assigned file number; **GoldR** = user name; **At5g09250Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.

3. Check the boxes next to the sequences to be downloaded, and click "Download selected." Alternatively, click on each filename that you want to download.
4. Open the ab1 files in the "Downloads" window using a sequence viewer program (CHROMAS on Windows, or 4PEAKS on Mac).
5. Copy DNA sequences to a Microsoft Word file. *Note: Name the files according to the name of gene of interest (for example, Promoter At5g09250).*
6. Process the DNA sequences by "BLASTN" search. See Appendix 2. *Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Determine if the DNA sequence corresponds to the upstream sequence of the gene of interest.
8. View the sequence alignment to the upstream sequence of the gene of interest.

   Are there any mutations in the promoter that you cloned?

   Do you think a mutation could affect the transcription of your reporter gene?
9. Print out the Blast result as a hard-copy record for your lab notebook.
10. Save the Blast result in the **pdf** format so that you can upload them to your webbook.

APPENDIXES
Appendix 1A

Preparation of an Agarose Gel for Gel Electrophoresis

Solutions Needed:

- DNA samples
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL DNA ladder with loading dye (Invitrogen)
- 6x Loading dye containing xylene cyanol and/or bromophenol blue dyes

Materials Needed:

- Pipettes (P-20)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- 250 mL Erlenmeyer flask
- 25 mL Erlenmeyer flask
- Saran wrap
- Scale
- Microwave
- 55°C water bath
- Hot hand protector
- Gel cast
- Gel comb
- Round bubble level
- Gel box
- Cables
- Electrophoresis power supply
- Plastic container for carrying the gel
- Gel document system (Bio-Rad)

PROCEDURE

*Note: SYBR Safe gel stain is unstable in UV or bright room light. If possible, keep the gel in the **dark** by either turning off the lights, covering the gel with a cardboard box or aluminum foil, or run the gel inside of a drawer. Realistically, hours of constant UV or bright room light exposure are required to cause any significant loss of signal.*

1. For a **1% agarose gel**, weigh out **1 gram** of agarose (powder) into a weigh boat on the scale in room 4128A2. *Note: The **percentage** of agarose in the gel reflects the amount of agarose (in **grams**) in **100 mL** of 1x TAE buffer.*
   Example: If you want to make a 0.7% agarose gel (0.7 g/100 mL, w/v), weigh out 0.7 g of agarose for 100 mL of 1x TAE buffer.
2. Carefully, pour the agarose into a 250 mL Erlenmeyer flask.
3. Measure **100 mL** of **1x TAE** buffer using a graduated cylinder, and add it to the flask in step 2.
4. Cover the flask with an inverted 25-mL Erlenmeyer flask or saran wrap. Swirl the solution to break up any lumps of agarose. *Note: The inverted flask will collect condensation from the steam produced during microwaving in step 6 below.*
5. Microwave the solution for **1-2 minute** or until the agarose granules have completely melted and the solution looks **clear**. Gently **swirl** the solution every 15 sec during microwaving to help melt the agarose evenly. *Caution: The solution gets **very hot**. Use a hot hand protector. Note: Constantly watch over the solution because if it starts boiling, it might overflow.*
6. Cool down the agarose solution for **10 min** in a **55°C water bath.**
7. While the agarose solution is cooling, prepare the gel cast with the appropriate comb. Use the round bubble level to make the gel cast level.
   *Note: **The comb depends on the number of samples to be loaded on the gel**. For example, if there are ≤ 18 samples, then use a 20-tooth comb; but, if there are ≥ 19 samples, then use a 30-tooth comb. Note: Remember that **two wells** will be for loading **DNA ladder** (on left and right sides of loaded samples).*
8. After the agarose solution has cooled down, add 10 μL of **10,000x SYBR Safe DNA gel stain** to 100 mL of agarose solution and **swirl** the flask **GENTLY** to mix. *Note: Do NOT swirl vigorously to avoid generating bubbles.*
9. Pour the **agarose/gel stain** solution into the gel cast with the appropriate gel comb. Wait **30 min** for the agarose solution to solidify. *Note: IMMEDIATELY after pouring the agarose solution, inspect the agarose solution's surface for the present of bubbles. If there are bubbles floating on the surface of the gel solution, use a pipette*

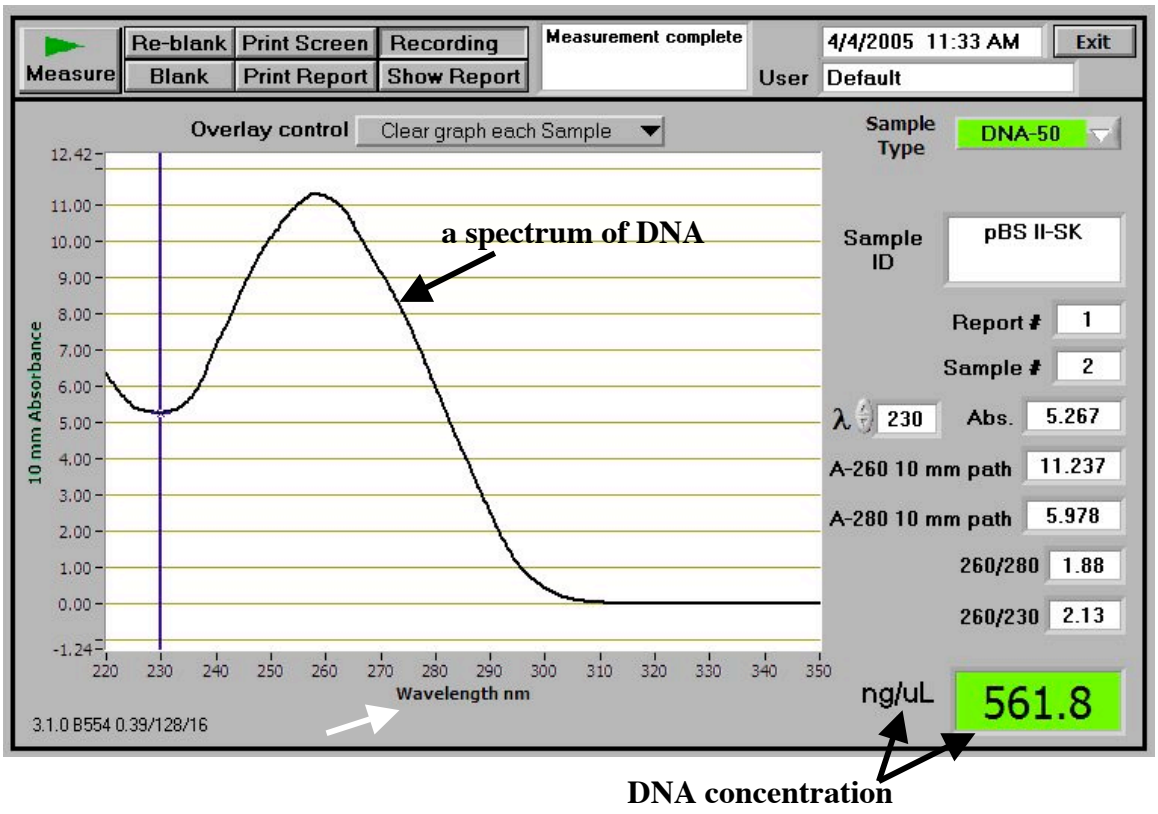
tip to pop them or move them to the sides of the gel before the gel has completely solidified.

10. Pour ~600 mL of **1x TAE** buffer into the gel box.
11. After the agarose has solidified into a gel, take out the comb by **gently** pulling it side to side and out of the gel. Put the gel in its cast into the gel box containing the 1x TAE running buffer.
12. Add **6x loading dye** to your samples. *Note: Do not use a loading dye that will travel to the same place as your DNA on the gel because the dye will obscure the DNA band. Xylene cyanol runs at ~3-4 kb, and bromophenol blue runs at ~400 bp on a 1% agarose gel.*
13. Load **10 µL** of **50 ng/µL DNA ladder with loading dye**.
14. Load samples and record the identity of the sample in each lane.
15. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (red) side of the gel box. Pipet up and down to mix. *Note: Add 5 µL of 10,000x SYBR Safe DNA gel stain for a small (50 mL) gel.*
16. Put the lid on the gel box and connect the electrodes to the power supply (**RED** to **RED** and **BLACK** to **BLACK**).
17. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) has migrated one-half or two-thirds of the gel length.
18. After 1-2 hours of running the gel, turn **off** the power supply.
19. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 4128A2. ***Caution:*** *It is a* ***MUST*** *to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking.*
20. Take a picture of the gel using the Bio-Rad Gel Document System. Label the picture using the text program of the Gel Document System. (*Your TA will show you how.*) *Alternatively: Print out the picture. Tape it to a piece of paper by putting a piece of white tape at a position immediately above the wells. Label the wells with the sample names.*
21. Print out the picture. Store the labeled picture in your lab notebook.

Appendix 1B

What is a **spectrophotometer**?

It is an **instrument** that **measures** the **amount** of **molecules absorbing a given wavelength of energy**. In this exercise, we measure the amount of DNA molecules in a given volume in the **ultraviolet wavelengths** of **200 – 280 nm** (**nm** stands for **nanometer**, which is **1 billionth** of a **meter**). The bases of DNA absorb at a wavelength of 254 nm. The absorbance of DNA molecules over the wavelength range of 220 - 350 nm is represented as a **spectrum** with a **peak** at **near 260 nm** as illustrated by the **NanoDrop spectrophotometer readout** below:



OPERATION OF NANODROP SPECTROPHOTOMETER ND-1000

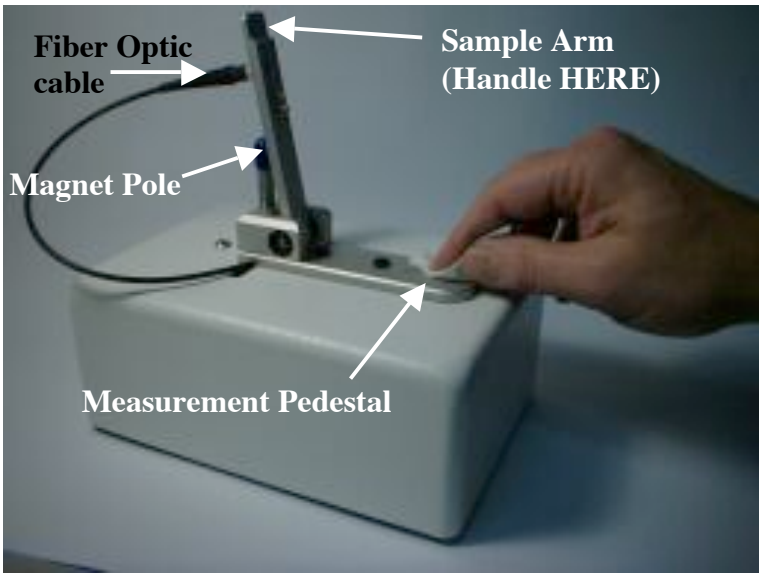
Note: The NanoDrop is powered by the laptop via the USB port.

1. **Open** the NanoDrop program by clicking the "**ND-1000 v3.7.1**" icon on the computer desktop. Wait for a few seconds for the program to open. You see the NanoDrop 1000 3.7.1 menu panel with **user field** set to **default.**
2. To read the concentration of DNA or RNA solutions, **click** on the "**Nucleic Acid**" button on the top left column.

   Note: You see the following message: "Ensure sample pedestals are clean and then load a water sample. After loading water sample, click OK to initialize instrument."
3. **Clean** the **measurement pedestals** as following:
   a) **Raise** the **sample arm** by holding its end.

   Caution: ***NEVER*** *hold the fiber optic cable when lifting and lowering the sample arm because the cord is fragile, and it is very expensive to replace it.*

   b) **Blot BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes slightly wetted with distilled water.



c) **Pipet 1.5 - 2 µL** of water onto the **measurement pedestal**.

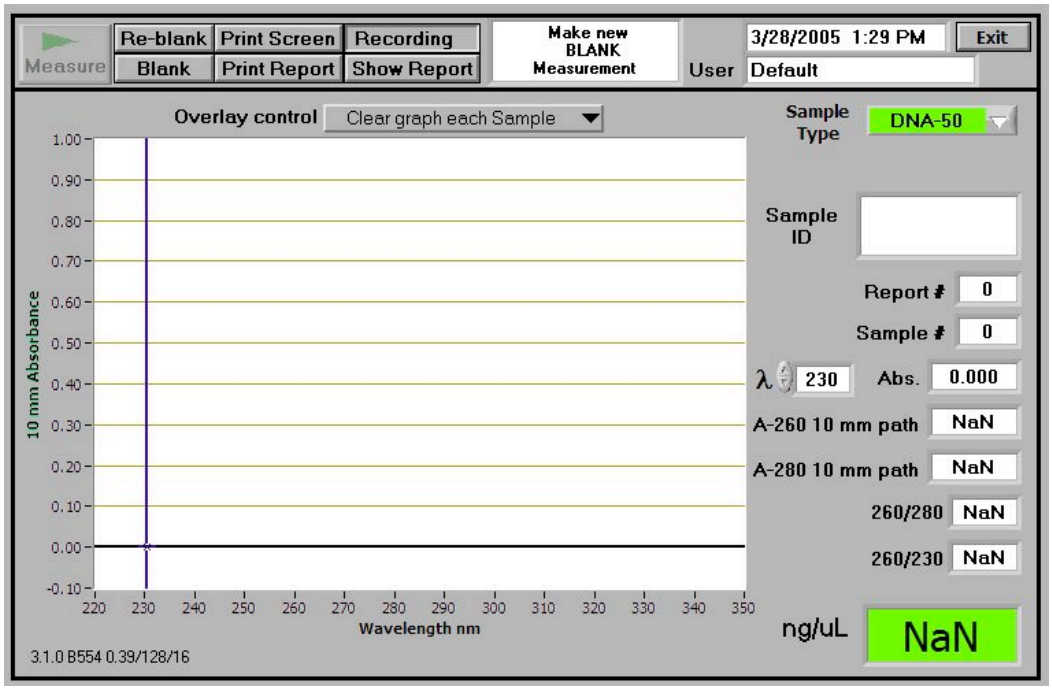
Note: Even though NanoDrop Inc. claims that the NanoDrop can read as low as 1 µL, the concentration reading is NOT consistent at this volume. Therefore, the ***minimal*** *volume for the concentration reading is* ***1.5 µL.***

d) **Slowly lower** the sample arm to its horizontal position.

Caution: *NEVER let the arm fall freely.*

e) **Click** the **OK** button.

Note: Now the NanoDrop is ***INITIALIZED.*** *You will see the* ***dialog panel*** *as shown below.*



f) Raise the sample arm to the vertical position.

g) **Wipe off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.

4. **Change SAMPLE TYPE** (if necessary) from **DNA-50** (default, for DNA) to **RNA-40** (for RNA) or **ssDNA-33** (for oligonucleotides), depending on your sample.
5. (Optional) **Change** the **OVERLAY CONTROL** field from the default setting of "CLEAR GRAPH EACH SAMPLE" to "CLEAR GRAPH ON NEW REPORT" or "ACCUMULATE UNTIL CLEAR" or "CLEAR GRAPH NOW."
6. Make a **NEW BLANK measurement.**
   a) Pipet 1.5 - 2.0 µL of either double-distilled water or TE (depending on whether your sample solution is in double-distilled water or TE) on the measurement pedestal.
   b) Lower the sample arm to the horizontal position.
   c) **Click** the **BLANK** button. *The blank was made*.
   d) After the reading is done, raise the sample arm to the vertical position.
   e) **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.
7. **Confirm** that the blank was made.
   a) Pipet 1.5 - 2.0 µL of either double-distilled water or TE (depending on whether your sample solution is in double-distilled water or TE) on the measurement pedestal.
   b) Lower the sample arm to the horizontal position.
   c) In the **SAMPLE ID** field, **type** the **identity** of the solution that you are measuring (either TE or ddH_2O).
   d) **Click** the **MEASURE** button.

      Note: After the reading is done, a ***concentration*** *(in* ***ng/µL****) and a* ***spectrum*** *of the absorbance, along with other information, are shown. The reading should be* ***less than 1 ng/µL****. If it is not, make a new blank measurement by repeating step 6.*
   e) Raise the sample arm to the vertical position.
   f) **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.

8. Measure the **samples**.
   a) In the **SAMPLE ID** field, **type** the **identity** of the sample solution.
   b) Pipet 1.5 - 2.0 μL of SAMPLE on the measurement pedestal.
   c) Lower the sample arm to the horizontal position.
   d) **Click** the **MEASURE** button to determine concentration of your sample.
   e) After the reading is done, a **sample concentration** (in **ng/μL**) and a **spectrum** of the sample, along with other information, are shown. You can either:
      i. **Save** the **window** of measured sample.
         1. Click on FILE.
         2. Choose SAVE WINDOW.
         3. Select an existing folder or create a new folder (give a name for the new folder).
         4. Type in a file name in the FILE NAME field.
         5. Click the SAVE button to save the file.
      ii. **Or**, **print** the **window** by **clicking** the **PRINT SCREEN** button.
      Note: To print the spectrum of the current sample, you MUST print it before reading the next sample. Otherwise, you need to repeat reading the sample.
   f) Raise the sample arm to the vertical position.
   g) **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.
9. **Repeat** step 8 for other samples.
10. After reading the **last sample**, **click** the **PRINT REPORT** button to print the concentrations of all samples.
11. If you are done with the NanoDrop, **click** the **EXIT** buttons.
12. **Blot BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes slightly wetted with distilled water.

Appendix 1C
100 bp DNA Ladder

Invitrogen™
life technologies

100 bp DNA Ladder

Cat. No. 15628-019 **Size: 50 µg**
Concentration: 1 µg/µl **Store at -20°C.**

Description:

The 100 bp DNA Ladder consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp. The 600 bp band is approximately 2 to 3 times brighter than the other ladder bands to provide internal orientation. This ladder is not designed for quantitation.

Storage Buffer:

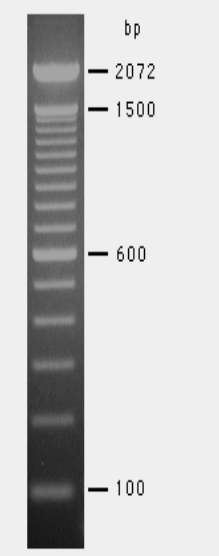
10 mM Tris-HC1 (pH 7.5)
1 mM EDTA

Recommended Procedure:

A final concentration of 20 mM NaCl is recommended for gel electrophoresis. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that the bands between 100 to 1500 bp are distinguishable. The 600 bp band must be more intense than any other band except the band at 2072 bp.



Note:

During 2% agarose gel electrophoresis with tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates near the 100-bp fragment. The 100-bp band migrates behind the bromophenol blue marker on 6% polyacrylamide gels with tris-borate (pH 8.0) as the running buffer.

Part of the 600-bp band may migrate anomalously slowly in polyacrylamide gels (1,2,3). This band may appear as an extra band near or on top of the 700-bp band.

References:

1. Hsieh, C., et al. (1991) *Mol. Gen. Genet. 225*, 25.
2. Stellwagen, N.C. (1983) *Biochemistry 22*, 6186.
3. Jordan, H. and Hartley, J. (1997) *Focus® 19*, 9.

100 bp DNA Ladder
0.5 µg/lane
2% agarose gel stained with ethidium bromide.

Cat. No. 15628-019

Appendix 1D
1 Kb Plus DNA Ladder

Invitrogen
life technologies

1 Kb Plus DNA Ladder

Cat. No. 10787-018 **Size: 250 µg**
Conc.: 1 µg/µl **Store at -20°C**.

Description:

The 1 Kb Plus DNA Ladder is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000 bp to 12,000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band contains approximately 8% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ^{32}P input; (ii) Labeling the 5′ ends with T4 polynucleotide kinase; (iii) Filling in the 3′ recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:

10 mM Tris-HCl (pH 7.5)
1 mM EDTA
50 mM NaCl

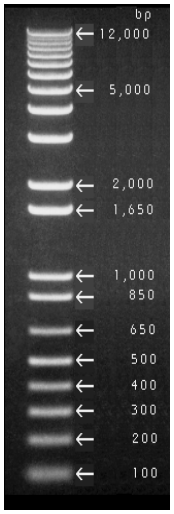
Recommended Procedure:

Invitrogen recommends the use of 10X BlueJuice™ Gel Loading Buffer (Cat. No. 10816-015) at a concentration of 2X for electrophoresis of DNA standards on agarose gels. Alternately, the DNA standard can be diluted such that the final concentration of NaCl is 20 mM. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that all bands in the ladder are distinguishable and are of approximate equal intensity by ethidium bromide staining.

Doc. Rev.: 021802



1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

5′-GATCC ——— G
G ——— CCTAG-5′
$(996\ bp)_n$

Notes:

During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

Cat. No. 10787-018

Appendix 1E
Ex Taq DNA Polymerase Manual

10XPCR Buffer,dNTP Mixture for PCR

TaKaRa Ex Taq™

Code No. RR001A
Size: 250 units

Shipping at -20˚C
Stored at -20˚C

Supplied Reagents : 10X *Ex Taq*™ Buffer
dNTP Mixture

Lot No.
Conc. : units/μl
Volume : μl
Expiry Date :

Storage Buffer:

20 mM	Tris-HCl (pH8.0)
100 mM	KCl
0.1 mM	EDTA
1 mM	DTT
0.5%	Tween®20
0.5%	Nonidet P-40®
50%	Glycerol

Unit definition: One unit is the amount of the enzyme that will incorporate10 nmol of dNTP into acid-insoluble products in 30 minutes at 74˚C with activated salmon sperm DNA as the template-primer.

Reaction mixture for unit definition:

25 mM	TAPS (pH 9.3 at 25˚C)
50 mM	KCl
2 mM	$MgCl_2$
1 mM	2-mercaptoethanol
200 μM	each dATP,dGTP,dTTP
100 μM	[α-^{32}P]-dCTP
0.25 mg/ml	activated salmon sperm DNA

Purity: Nicking activity, endonuclease and exonuclease activity were not detected after the incubation of 0.6 μg of supercoiled pBR322 DNA, 0.6 μg of λ DNA or 0.6 μg of λ-*Hin*d III digest with 10 units of this enzyme for 1 hour at 74˚C.

Applications:
For DNA amplification by Polymerase Chain Reaction (PCR).

PCR products : As most PCR products amplified with *TaKaRa Ex Taq* ™ have one A added at 3'-termini, the obtained PCR product can be directly used for cloning into T-vector. Also it is possible to clone the product in blunt-end vectors after blunting and phosphorylation of the end.

PCR test : Good performance of DNA amplification by Polymerase Chain Reaction (PCR) was confirmed by using λ DNA as the template (amplified fragment : 20 kbp).
Good performance of DNA amplification of β-globin gene by PCR was also confirmed by using human genomic DNA as the template (amplified fragment : 17.5 kbp).

PCR condition (an example)
When amplifying 1 kbp DNA fragment

98°C	10 sec	┐		98 °C	10 sec	┐	
55°C	30 sec	┘ 30 cycles	or	68 °C	1 min	┘	30 cycles
72°C	1 min	┘					

Note:Denaturation condition varies depending on an used thermal cycler and tube. It is recommended for 10-30 sec. at 94°C, or 1-10 sec. at 98°C.

General reaction mixture for PCR (total 50 μl)

TaKaRa Ex Taq ™ (5 units/μl)	0.25 μl
10X *Ex Taq* Buffer	5 μl
dNTP Mixture (2.5 mM each)	4 μl
Template	< 500 ng
Primer 1	0.2 ~ 1.0 μM (final conc.)
Primer 2	0.2 ~ 1.0 μM (final conc.)
Sterilized distilled water	up to 50 μl

Supplied 10X *Ex Taq* Buffer
Supplied Size : 1 ml/vial
Mg^{2+} concentration (10X): 20 mM
Storage : –20˚C

Supplied dNTP Mixture
Mixture of dNTP, ready for use in Polymerase Chain Reaction (PCR) without dilution.

Supplied Size	: 800 μl/vial
Concentration	: 2.5 mM of each dNTP
pH	: pH 7 ~ 9
Form	: Solved in water (sodium salts)
Purity	: ≥ 98% for each dNTP
Storage	: –20˚C

< **Cool Start Method** >
"Cool Start Method' , enables to minimize the amplification of non-specific band in PCR and achieves more accurate amplification. This is a simpler method without need for special enzyme nor additional reagents.⁺ Higher reaction specificity can be achieved by combining Hot Start PCR techniques with Taq Antibody (Code.9002A) and Cool Start method.
Protocol of Cool Start Method
1) Keep all reagents on ice until use.
2) Prepare the reaction mixture on ice.*,**
*The adding order of reagents dose not influence on results.
**The result will not be affected even when the mixture is left on ice 30 min. before thermal cycling.
3) Set a thermal cycler ready to start with the designated program.***
***No need to change PCR conditions especially for Cool Start.
4) Set the tubes in a thermal cycler and start thermal cycling immediately.
⁺JAPAN Patent 2576741 for Cool Start Method is owned by SHIMADZU CORPORATION

Note
For research use only. Not for use in diagnostic or therapeutic procedures.

Produced by TAKARA BIOTECHNOLOGY (DALIAN) CO.,LTD.
V2005.06

March 2008

QIAquick® Spin Handbook

QIAquick PCR Purification Kit
For purification of PCR products, 100 bp to 10 kb

QIAquick Nucleotide Removal Kit
For oligonucleotide (17-40mers) and DNA
(40 bp to 10 kb) cleanup from enzymatic reactions

QIAquick Gel Extraction Kit
For gel extraction or cleanup of DNA
(70 bp to 10 kb) from enzymatic reactions

QIAGEN®

Sample & Assay Technologies

Kit Contents

QIAquick PCR Purification Kits Catalog no.	(50) 28104	(250) 28106
QIAquick Spin Columns	50	250
Buffer PB*	30 ml	150 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
pH Indicator I	800 µl	800 µl
Collection Tubes (2 ml)	50	250
Loading Dye	110 µl	550 µl
Handbook	1	1

QIAquick Nucleotide Removal Kits Catalog no.	(50) 28304	(250) 28306
QIAquick Spin Columns	50	250
Buffer PN*	30 ml	140 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
Collection Tubes (2 ml)	100	500
Loading Dye	110 µl	550 µl
Handbook	1	1

QIAquick Gel Extraction Kits Catalog no.	(50) 28704	(250) 28706
QIAquick Spin Columns	50	250
Buffer QG*	2 x 50 ml	2 x 250 ml
Buffer PE (concentrate)	2 x 10 ml	2 x 50 ml
Buffer EB	15 ml	2 x 15 ml
Collection Tubes (2 ml)	50	250
Loading Dye	110 µl	550 µl
Handbook	1	1

* Buffers PB, PN, and QG contain chaotropic salts which are irritants. Take appropriate laboratory safety measures and wear gloves when handling.

Storage

QIAquick Spin Kits should be stored dry at room temperature (15–25°C). Under these conditions, QIAquick Spin Kits can be stored for up to 12 months without showing any reduction in performance and quality. Check buffers for precipitate before use and redissolve at 37°C if necessary. The entire kit can be stored at 2–8°C, but in this case the buffers should be redissolved before use. Make sure that all buffers and spin colums are at room temperature when used.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer PB contains guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

In case liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the QIAquick system.

Buffer PB

Contains guanidine hydrochloride and isopropanol: harmful, irritant, flammable. Risk and safety phrases*: R10-22-36/38. S23-26-36/37/39-46

Buffer PN

Contains sodium perchlorate and isopropanol: harmful, highly flammable. Risk and safety phrases*: R11-22. S13-16-23-26-36-46

Buffer QG

Contains guanidine thiocyanate: harmful. Risk and safety phrases*: R20/21/22-32. S13-26-36-46

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany
Tel: +49-6131-19240

* R10: Flammable. R11: Highly Flammable. R22: Harmful if swallowed. R20/21/22: Harmful by inhalation, in contact with skin and if swallowed. R32: Contact with acids liberates very toxic gas. R36/38: Irritating to eyes and skin. S13: Keep away from food, drink and animal feedingstuffs. S16: Explosive when mixed with oxidizing substances. S23: Do not breathe vapour/spray. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S36: Wear suitable protective clothing. S36/37/39: Wear suitable protective clothing, gloves and eye/face protection. S46: If swallowed, seek medical advice immediately and show the container or label.

Product Specifications

	QIAquick PCR Purification Kit	QIAquick Nucleotide Removal Kit	QIAquick Gel Extraction Kit
Maximum binding capacity	10 µg	10 µg	10 µg
Maximum weight of gel slice	—	—	400 mg
Minimum elution volume	30 µl	30 µl	30 µl
Capacity of column reservoir	800 µl	800 µl	800 µl
Typical recoveries			
Recovery of DNA	90–95% (100 bp – 10 kb)	80–95% (40 bp – 10 kb)	70–80% (70 bp – 10 kb)
Recovery of oligonucleotides (17–40mers)	0	60–80%	10–20%
Recovered			
Oligonucleotides	—	17–40mers	—
dsDNA	100 bp – 10 kb	40 bp – 10 kb	70 bp – 10 kb
Removed			
<10mers	**YES**	**YES**	**YES**
17–40mers	**YES**	no	no

Introduction

The QIAquick system, designed for rapid DNA cleanup, includes:

- **QIAquick PCR Purification Kits** for direct purification of double- or single-stranded PCR products (100 bp – 10 kb) from amplification reactions and DNA cleanup from other enzymatic reactions.
- **QIAquick Nucleotide Removal Kits** for general cleanup of oligonucleotides and DNA up to 10 kb from enzymatic reactions (e.g., labeling, dephosphorylation, restriction, and tailing).
- **QIAquick Gel Extraction Kits** for extraction of DNA fragments (70 bp – 10 kb) from standard, or low-melt agarose gels in TAE (Tris·acetate/EDTA) or TBE (Tris·borate/EDTA) buffer and DNA cleanup from enzymatic reactions.

QIAquick PCR Kits are also available in multiwell format for preparation of 8 to 96 samples (see page 37 for ordering information).

Enzymatic reaction cleanup using QIAquick Kits

The QIAquick system is suitable for fast cleanup of up to 10 µg of DNA fragments from enzymatic reactions and agarose gels (Table 1). Enzyme contamination of DNA samples can interfere with subsequent downstream applications. QIAquick Spin Kits can be used for highly efficient removal of a broad spectrum of enzymes widely used in molecular biology. In addition, QIAGEN offers the MinElute® Reaction Cleanup Kit, which is specially designed for fast and easy DNA cleanup from all enzymatic reactions. Using proven microspin technology, the MinElute Reaction Cleanup Kit delivers highly concentrated purified DNA by using an elution volume of only 10 µl (see ordering information, page 37).

Table 1. QIAquick DNA Cleanup Guide

	From solutions			From gels
	QIAquick PCR Purification Kit	QIAquick Nucleotide Removal Kit	QIAquick Gel Extraction Kit	QIAquick Gel Extraction Kit
Alkaline phosphatase	**YES**	**YES**	**YES**	**YES**
cDNA synthesis	**YES**	no	no	**YES**
DNase, nuclease digestion	**YES**	**YES**	**YES**	**YES**
Kinase:				
DNA fragments	**YES**	**YES**	**YES**	**YES**
Oligonucleotides	no	**YES**	no	no
Ligation	**YES**	**YES**	**YES**	**YES**
Nick translation	**YES**	**YES**	**YES**	**YES**
PCR	**YES**	no	no	**YES**
Random priming	**YES**	**YES**	**YES**	**YES**
Restriction digestion	**YES**	**YES**	**YES**	**YES**
Tailing:				
DNA fragments	**YES**	**YES**	**YES**	**YES**
Oligonucleotides	no	**YES**	no	no

QIAquick Kits provide high yields of pure nucleic acids, for direct use in applications such as:

- Fluorescent and radioactive sequencing
- Restriction
- Labeling
- Hybridization
- Ligation and transformation
- Amplification
- In vitro transcription
- Microinjection

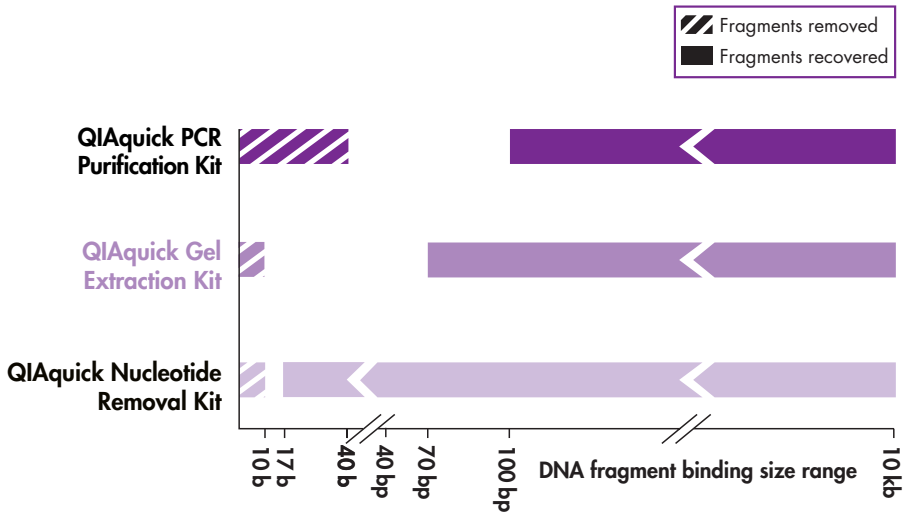


Figure 1. DNA fragment binding-size range. Recoveries of DNA fragments in the size range between "removed" and "recovered" are not defined.

Automated DNA cleanup

The QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit can be fully automated on the QIAcube. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., bind, wash, and elute) enabling purification of high-quality DNA.

The QIAcube is preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/MyQIAcube .

A detailed protocol for using QIAquick spin columns on the QIAcube is provided with the QIAcube.

Note: It is not necessary to add pH indicator I to Buffer PB when using the QIAcube.

The QIAquick Principle

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water (see page 17). QIAquick spin columns offer 3 handling options — as an alternative to processing the spin columns in a microcentrifuge, they can now also be used on any commercial vacuum manifold with luer connectors (e.g., QIAvac 6S or QIAvac 24 Plus with QIAvac Luer Adapters) or automated on the QIAcube.

Adsorption to QIAquick membrane — salt and pH dependence

The QIAquick silica membrane is uniquely adapted to purify DNA from both aqueous solutions and agarose gels, and up to 10 µg DNA can bind to each QIAquick column. The binding buffers in QIAquick Spin Kits provide the correct salt concentration and pH for adsorption of DNA to the QIAquick membrane. The adsorption of nucleic acids to silica surfaces occurs only in the presence of a high concentration of chaotropic salts (1), which modify the structure of water (2).

Adsorption of DNA to silica also depends on pH. Adsorption is typically 95% if the pH is ≤7.5, and is reduced drastically at higher pH (Figure 1). If the loading mixture pH is >7.5, the optimal pH for DNA binding can be obtained by adding a small volume of 3 M sodium acetate, pH 5.0.

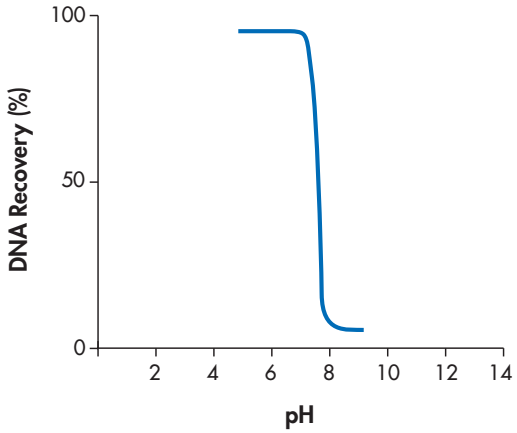


Figure 2. pH dependence of DNA adsorption to QIAquick membranes. 1 µg of a 2.9 kb DNA fragment was adsorbed at different pHs and eluted with Buffer EB (10 mM Tris·Cl, pH 8.5). The graph shows the percentage of DNA recovery, reflecting the relative adsorption efficiency, versus pH of adsorption.

Optimized binding buffers for every DNA cleanup task

All QIAquick Spin Kits contain identical QIAquick spin columns but different binding buffers optimized for each specific application:

- Buffer PB in the QIAquick PCR Purification Kit allows the efficient binding of single- or double-stranded PCR products as small as 100 bp and the quantitative (99.5%) removal of primers up to 40 nucleotides. This kit can therefore be used to remove oligo-dT primers after cDNA synthesis or to remove unwanted linkers in cloning experiments.
- Buffer PN in the QIAquick Nucleotide Removal Kit promotes the adsorption of both oligonucleotides ≥17 bases and DNA fragments up to 10 kb to the membrane.
- Buffer QG in the QIAquick Gel Extraction Kit solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane.

All of these buffers are available separately (see ordering information, page 37).

pH indicator

Binding buffer PB and binding and solubilization buffer QG are specially optimized for use with the QIAquick silica membrane. Buffer QG contains an integrated pH indicator, while an optional pH indicator can be added to Buffer PB allowing easy determination of the optimal pH for DNA binding. DNA adsorption requires a pH ≤7.5, and the pH indicator in the buffers will appear yellow in this range. If the pH is >7.5, which can occur if during agarose gel electrophoresis, the electrophoresis buffer had been used repeatedly or incorrectly prepared, or if the buffer used in an enzymatic reaction is strongly basic and has a high buffering capacity, the binding mixture turns orange or violet (Figure 2). This means that the pH of the sample exceeds the buffering capacity of Buffer PB or QG and DNA adsorption will be inefficient. In these cases, the pH of the binding mixture can easily be corrected by addition of a small volume of 3 M sodium acetate*, pH 5.0, before proceeding with the protocol. In addition, in the QIAquick Gel

Figure 3. Indicator enables easy checking of the optimal pH. Indicator dye in solubilization and binding Buffers QG and PB identifies optimal pH for DNA binding.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs) available from the product supplier.

Extraction Kit procedure, the color of the binding mixture allows easy visualization of any unsolubilized agarose, ensuring complete solubilization and maximum yields. The indicator dye does not interfere with DNA binding and is completely removed during the cleanup procedure. Buffers PB and QG do not contain sodium iodide (NaI). Residual NaI may be difficult to remove from DNA samples, and reduces the efficiency of subsequent enzymatic reactions such as blunt-end ligation.

Washing

During the DNA adsorption step, unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, and detergents (e.g., DMSO, Tween® 20) do not bind to the silica membrane but flow through the column. Salts are quantitatively washed away by the ethanol-containing Buffer PE. Any residual Buffer PE, which may interfere with subsequent enzymatic reactions, is removed by an additional centrifugation step.

Elution in low-salt solutions

Elution efficiency is strongly dependent on the salt concentration and pH of the elution buffer. Contrary to adsorption, elution is most efficient under basic conditions and low salt concentrations. DNA is eluted with 50 or 30 µl of the provided Buffer EB (10 mM Tris·Cl, pH 8.5), or water. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water to elute, make sure that the pH is within this range. In addition, DNA must be stored at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent. Elution with TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) is possible, but not recommended because EDTA may inhibit subsequent enzymatic reactions.

DNA yield and concentration

DNA yield depends on the following three factors: the volume of elution buffer, how the buffer is applied to the column, and the incubation time of the buffer on the column. 100–200 µl of elution buffer completely covers the QIAquick membrane, ensuring maximum yield, even when not applied directly to the center of the membrane. Elution with ≤50 µl requires the buffer to be added directly to the center of the membrane, and if elution is done with the minimum recommended volume of 30 µl, an additional 1 minute incubation is required for optimal yield. DNA will be up to 1.7 times more concentrated if the QIAquick column is incubated for 1 minute with 30 µl of elution buffer, than if it is eluted in 50 µl without incubation (Figure 4, page 14).

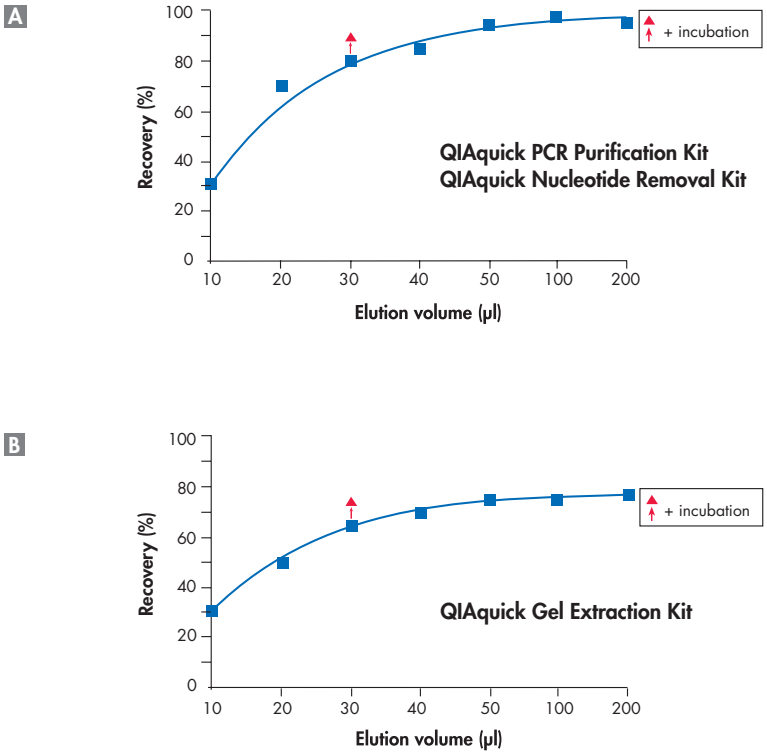


Figure 4. Highly concentrated DNA. Effect of elution buffer volume on DNA yield for A the QIAquick PCR Purification and QIAquick Nucleotide Removal Kit; B the QIAquick Gel Extraction Kit. 5 µg of a 2.9 kb DNA fragment were purified and eluted with the indicated volumes of Buffer EB. 30 µl plus 1 minute incubation on the QIAquick column gives DNA yields similar to 50 µl without incubation, but at a concentration 1.7 times greater.

Loading dye

Loading dye is provided for analysis of purified DNA samples using electrophoresis. It contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type. Loading dye is supplied as a 5x concentrate; thus 1 volume of loading dye should be added to 5 volumes of purified DNA.

Table 2. Migration Distance of Gel Tracking Dyes

%TAE (TBE) agarose gel	Xylene cyanol (light blue)	Bromophenol blue (dark blue)	Orange G (orange)
0.8	5000 bp (3000 bp)	800 bp (400 bp)	150 bp (<100 bp)
1.0	3000 bp (2000 bp)	400 bp (250 bp)	<100 bp (<100 bp)
1.5	1800 bp (1100 bp)	250 bp (100 bp)	<100 bp (<100 bp)
2.0	1000 bp (600 bp)	200 bp (<100 bp)	<100 bp (<100 bp)
2.5	700 bp (400 bp)	100 bp (<50 bp)	<50 bp (<50 bp)

Agarose gel analysis of yield

Yields of DNA following cleanup can be determined by agarose gel analysis. Table 3 shows the total yield obtained following extraction of 1 µg or 0.5 µg starting DNA from an agarose gel with a recovery of 80% or 60% using the QIAquick Gel Extraction Kit. The corresponding amount of DNA in a 1 µl aliquot from 50 µl eluate is indicated. Quantities of DNA fragment corresponding to these 1 µl aliquots are shown on the agarose gel in Figure 4.

Table 3. Amount of DNA in 1 µl aliquots of a 50 µl eluate following QIAquick purification

Starting DNA	Recovery	Total yield (50 µl eluate)	Amount of DNA in 1 µl
1 µg	80%	0.8 µg	16 ng
	60%	0.6 µg	12 ng
0.5 µg	80%	0.4 µg	8 ng
	60%	0.3 µg	6 ng

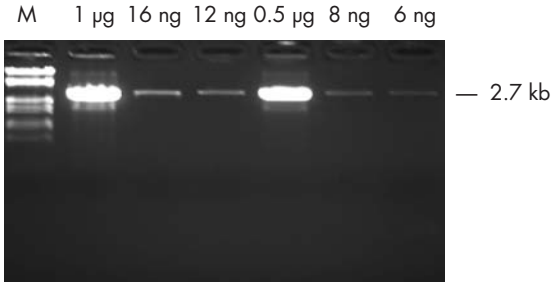


Figure 5. High DNA recovery. Quantities of purified 2.7 kb DNA fragment corresponding to 1/50 of the DNA obtained following purification from 1 µg or 0.5 µg starting DNA with a recovery of 80% or 60% (see Table 1). Samples were run on a 1% TAE agarose gel. **M**: lambda–EcoRI-HindIII markers.

Quantification of DNA fragments

DNA fragments can be quantified by running a sample alongside standards containing known quantities of the same-sized DNA fragment. The amount of sample DNA loaded can be estimated by visual comparison of the band intensity with that of the standards (Figure 5).

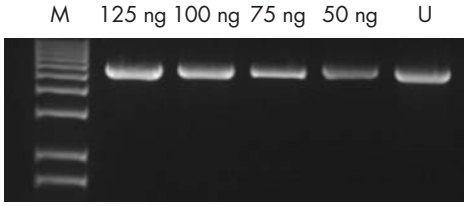
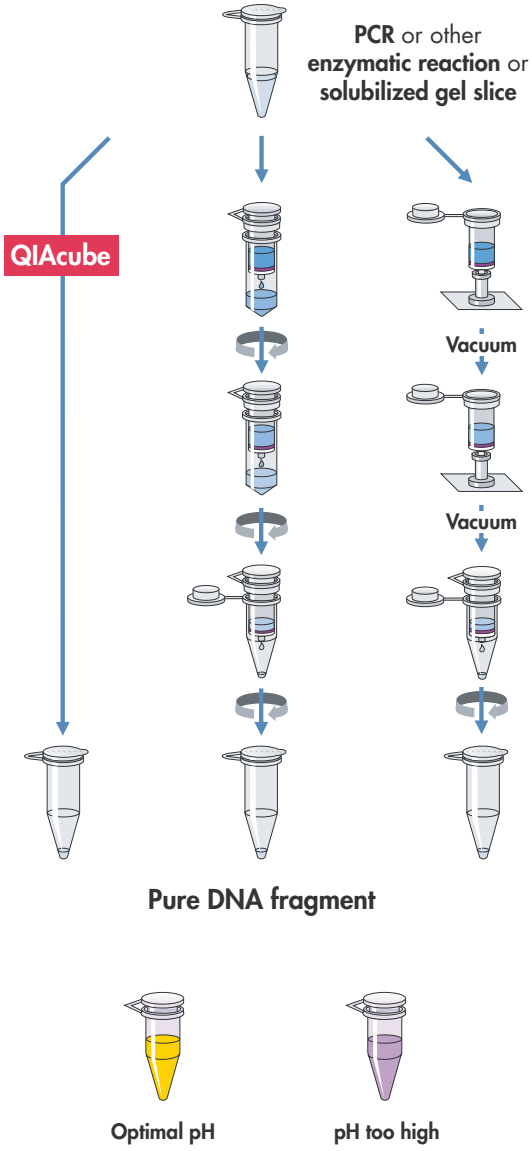


Figure 6. Agarose gel analysis. An unknown amount of a 5.5 kb DNA fragment (**U**) was run alongside known quantities (as indicated in ng) of the same DNA fragment. The unknown sample contained 75–100 ng DNA, as estimated by visual comparison with the standards. **M**: 1 kb DNA ladder.

Applications using QIAquick purified DNA

DNA purified with QIAquick is suitable for any subsequent application, such as restriction, labeling, hybridization, PCR, ligation and transformation, radioactive and fluorescent sequencing, in vitro transcription, or microinjection.

The QIAquick Procedure



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs) available from the product supplier.

For all protocols

- Ethanol (96–100%)*
- Microcentrifuge
- 1.5 or 2 ml microcentrifuge tubes
- 3 M sodium acetate, pH 5.0, may be necessary for PCR purification and gel extraction protocols.
- Optional: Distilled water or TE buffer (10 mM Tris·Cl. 1 mM EDTA, pH 8) for elution of DNA.

Vacuum protocols

- Vacuum manifold (e.g., QIAvac 24 Plus or QIAvac 6S)
- Vacuum pump (e.g., QIAGEN Vacuum Pump, see ordering information).

Gel extraction protocols

- Isopropanol (100%)
- Heating block or water bath set at 50°C

* Do not use denaturated alcohol, which contains other substances such as methanol or methylethylketone.

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx . The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and Suggestions
Low or no recovery		
a)	Buffer PE did not contain ethanol	Ethanol must be added to Buffer PE (concentrate) before use. Repeat procedure with correctly prepared Buffer PE.
b)	Inappropriate elution buffer	DNA will only be eluted efficiently in the presence of low-salt buffer (e.g., Buffer EB: 10 mM Tris·Cl, pH 8.5) or water. See "Elution in low-salt solutions", page 13.
c)	Elution buffer incorrectly dispensed	Add elution buffer to the center of the QIAquick membrane to ensure that the buffer completely covers the membrane. This is particularly important when using small elution volumes (30 µl).
Gel		
d)	Gel slice incompletely solubilized	After addition of Buffer QG to the gel slice, mix by vortexing the tube every 2–3 min during the 50°C incubation. DNA will remain in any undissolved agarose.
e)	pH of electrophoresis buffer too high (binding mixture turns orange or violet)	The electrophoresis buffer has been repeatedly used or incorrectly prepared, resulting in a sample pH that exceeds the buffering capacity of Buffer QG and leads to inefficient DNA binding. Add 10 µl of 3 M sodium acetate, pH 5.0, to the sample and mix. The color of the mixture will turn yellow indicating the correct pH for DNA binding. Even for binding mixtures with only small color changes (slight orange color), add the 10 µl sodium acetate.
f)	Gel slice was too large (>400 mg)	70–80% recovery can only be obtained from ≤400 mg gel slice per QIAquick column. For gel slices >400 mg, use multiple QIAquick columns.

Gel: refers to QIAquick Gel Extraction Kits only.

PCR: refers to QIAquick PCR Purification Kits only.

Other notes refer to all kits.

		Comments and Suggestions
PCR		
g)	Insufficient/no PCR product	Estimate DNA recovery by running 10% of PCR product before and after purification on an agarose gel.
PCR/Gel		
h)	Cloudy and gelatinous appearance of sample mixture after addition of isopropanol	This may be due to salt precipitation, and will disappear upon mixing the sample. Alternatively, the gel slice may not be completely solubilized. In this case, apply the mixture to the QIAquick column, centrifuge, and then add 0.5 ml Buffer QG to the column. Let stand for 1 min at room temperature, and then centrifuge and continue with the procedure. This additional wash will solubilize remaining agarose.
i)	Binding mixture turns orange or violet	The pH in the sample exceeds the buffer capacity of Buffer QG or PB respectively. Add 20 µl of 3 M sodium acetate, pH 5.0, to the sample and mix. The color of the mixture will turn yellow indicating the correct pH for DNA binding. Even for samples with slight color changes (orange color), add 10 µl sodium acetate.
DNA does not perform well (e.g., in ligation reactions)		
a)	Salt concentration in eluate too high	Modify the wash step by incubating the column for 5 min at room temperature after adding 750 µl of Buffer PE, then centrifuge.
b)	Eluate contains residual ethanol	Ensure that the wash flow-through is drained from the collection tube and that the QIAquick column is then centrifuged at 17,900 x *g* (13,000 rpm) for an additional 1 min.
Gel		
c)	Eluate contaminated with agarose	The gel slice is incompletely solubilized or weighs >400 mg. Repeat procedure, including the optional Buffer QG column-wash step.
PCR		
d)	Eluate contains primer-dimers	Primer-dimers formed are >20 bp and are not completely removed. After the binding step, wash the QIAquick column with 750 µl of a 35% guanidine hydrochloride aqueous solution (35 g in 100 ml). Continue with the Buffer PE wash step and the elution step as in the protocol.

		Comments and Suggestions
e)	Eluate contains denatured ssDNA, which appears as smaller smeared band on an analytical gel	Use the eluted DNA to prepare the subsequent enzymatic reaction but omit the enzyme. To reanneal the ssDNA, incubate the reaction mixture at 95°C for 2 min, and allow the tube to cool slowly to room temperature. Add the enzyme and proceed as usual. Alternatively, the DNA can be eluted in 10 mM Tris buffer containing 10 mM NaCl. The salt and buffering agent promote the renaturation of DNA strands. However the salt concentration of the eluate must then be considered for subsequent applications.

References

1. Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA ***76***, 615.
2. Hamaguchi, K. and Geiduschek, E.P. (1962) The effect of electrolytes on the stability of deoxyribonucleate helix. J. Am. Chem. Soc. ***84***, 1329.

Appendix 1G
Qiagen RNeasy Plant Mini Kit Handbook

Fourth Edition September 2010

RNeasy® Mini Handbook

RNeasy Mini Kit

For purification of total RNA from animal cells, animal tissues, bacteria, and yeast, and for RNA cleanup

RNeasy Protect Mini Kit

For immediate stabilization of RNA in harvested animal tissues and subsequent total RNA purification

RNeasy Plant Mini Kit

For purification of total RNA from plants and filamentous fungi

WWW.QIAGEN.COM

RNeasy Plant Mini Kit	(20)	(50)
Catalog no.	74903	74904
Number of preps	20	50
RNeasy Mini Spin Columns (pink)	20	50
QIAshredder Spin Columns (lilac)	20	50
Collection Tubes (1.5 ml)	20	50
Collection Tubes (2 ml)*	20	50
Buffer RLT*†	18 ml	45 ml
Buffer RLC†	18 ml	45 ml
Buffer RW1†	18 ml	45 ml
Buffer RPE‡ (concentrate)	5 ml	11 ml
RNase-Free Water	10 ml	10 ml
Handbook	1	1

* Also available separately. See page 74 for ordering information.

† Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 8 for safety information.

‡ Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Storage

The RNeasy Mini Kit, RNeasy Protect Mini Kit (including RNA*later* RNA Stabilization Reagent), and RNeasy Plant Mini Kit should be stored dry at room temperature (15–25°C) and are stable for at least 9 months under these conditions.

Storage of RNA*later* Reagent at lower temperatures may cause precipitation. Before use, redissolve the precipitate by heating to 37°C with agitation.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RNeasy Mini Kit, RNeasy Protect Mini Kit, and RNeasy Plant Mini Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

The RNeasy Mini Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer RLT contains guanidine thiocyanate, Buffer RLC contains guanidine hydrochloride, and Buffer RW1 contains a small amount of guanidine thiocyanate. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is split, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the RNeasy Mini Kit, RNeasy Protect Mini Kit, and/or RNeasy Plant Mini Kit.

Buffer RLT

Contains guanidine thiocyanate: harmful. Risk and safety phrases:* R20/21/22-32, S13-26-36-46

Buffer RLC

Contains guanidine hydrochloride: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-26-36-46

Buffer RW1

Contains ethanol: flammable. Risk phrase:* R10

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R22: Harmful if swallowed; R32: Contact with acids liberates very toxic gas; R36/38: Irritating to eyes and skin; S13: Keep away from food, drink and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S46: If swallowed, seek medical advice immediately and show the container or label.

Introduction

The *RNeasy Mini Handbook* provides protocols for use with the following kits:

- **RNeasy Mini Kit** — for purification of total RNA from animal cells, animal tissues, and yeast, and for cleanup of RNA from crude RNA preps and enzymatic reactions (e.g., DNase digestion, proteinase digestion, RNA ligation, and labeling reaction)
- **RNeasy Protect Mini Kit** — for immediate stabilization of RNA in harvested animal tissues and subsequent purification of total RNA
- **RNeasy Plant Mini Kit** — for purification of total RNA from plant cells and tissues and filamentous fungi

The RNeasy Mini Kit can also be used to purify total RNA from bacteria. In this case, we strongly recommend using the kit in combination with RNAprotect® Bacteria Reagent (available separately), which provides in vivo stabilization of RNA in bacteria to ensure reliable gene expression analysis. Various protocols for stabilizing and purifying RNA from different bacteria species are included in the *RNAprotect Bacteria Reagent Handbook*. The RNeasy Mini Kit and RNAprotect Bacteria Reagent can also be purchased together as the RNeasy Protect Bacteria Mini Kit. For ordering information, see pages 75–76. It is also possible to use the RNeasy Mini Kit to purify cytoplasmic RNA from animal cells. The protocol can be downloaded at www.qiagen.com/literature/protocols/RNeasyMini.aspx .

The RNeasy Kits are designed to purify RNA from small amounts of starting material. They provide a fast and simple method for preparing up to 100 µg total RNA per sample. The purified RNA is ready for use in downstream applications such as:

- RT-PCR and real-time RT-PCR
- Differential display
- cDNA synthesis
- Northern, dot, and slot blot analyses
- Primer extension
- Poly A^+ RNA selection
- RNase/S1 nuclease protection
- Microarrays

The RNeasy Kits allow the parallel processing of multiple samples in less than 30 minutes. Time-consuming and tedious methods, such as CsCl step-gradient ultracentrifugation and alcohol precipitation, or methods involving the use of toxic substances, such as phenol and/or chloroform, are replaced by the RNeasy procedure.

Principle and procedure

RNA purification using RNeasy technology

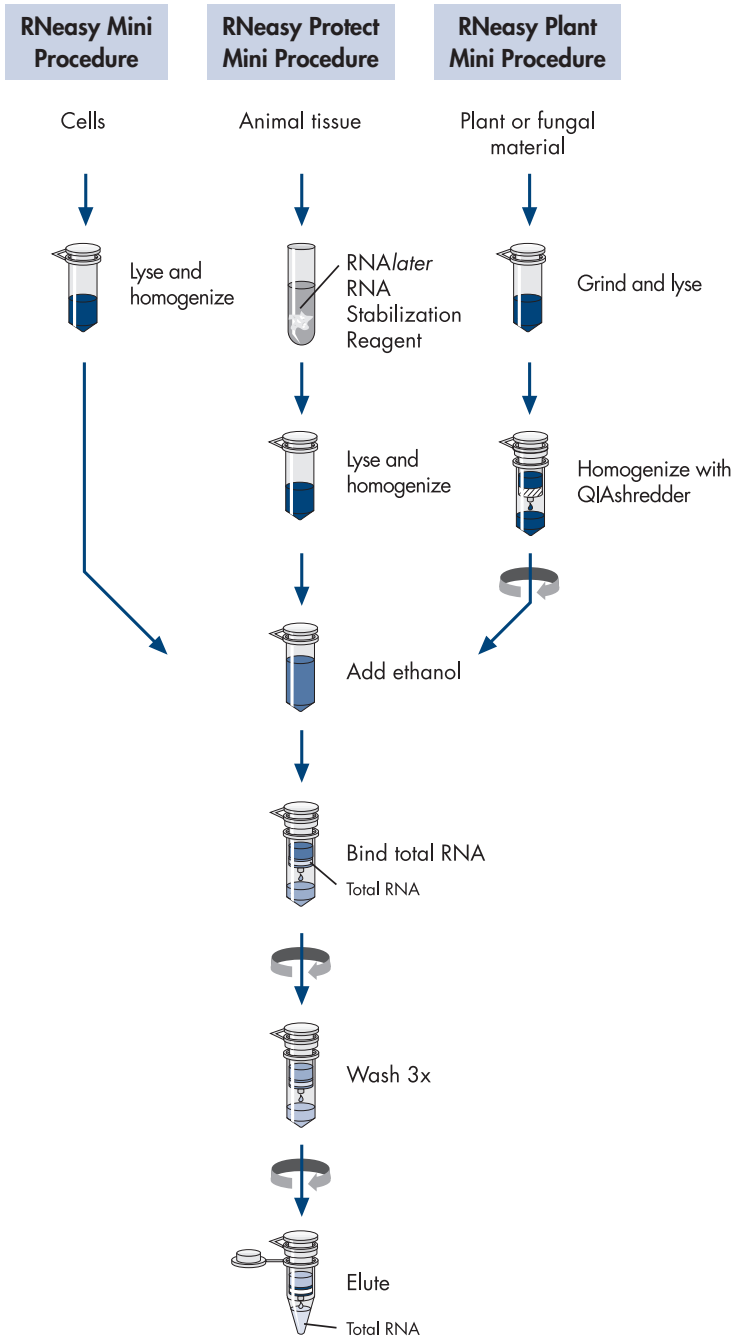
The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl water.

With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently. Protocols for purification of small RNA using RNeasy Kits are available at www.qiagen.com/goto/microRNAprotocols .

In this handbook, different protocols are provided for different starting materials. The protocols differ primarily in the lysis and homogenization of the sample and in the adjustment of the conditions for binding RNA to the RNeasy membrane. Once the sample is bound to the membrane, the protocols are similar (see flowchart, next page).

RNA stabilization using RNA*later* technology

RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analyses, such as microarray analyses, quantitative RT-PCR, such as TaqMan® and LightCycler® technology, and other nucleic acid-based technologies.


RNeasy Mini Procedure
RNeasy Protect Mini Procedure
RNeasy Plant Mini Procedure
Cells
Animal tissue
Plant or fungal material
Lyse and homogenize
RNA*later* RNA Stabilization Reagent
Grind and lyse
Lyse and homogenize
Homogenize with QIAshredder
Add ethanol
Bind total RNA
Total RNA
Wash 3x
Elute
Total RNA

The RNeasy Protect Mini Kit is supplied with RNA*later* RNA Stabilization Reagent, which represents a novel technology for the immediate preservation of the gene expression pattern in animal tissues, enabling reliable gene expression analysis. After harvesting, tissues are immediately submerged in RNA*later* RNA Stabilization Reagent, which rapidly permeates the tissues to stabilize and protect cellular RNA in situ. The reagent preserves RNA for up to 1 day at 37°C, 7 days at 15–25°C, or 4 weeks at 2–8°C, allowing transportation, storage, and shipping of samples without ice or dry ice. Alternatively, the samples can be archived at –20°C or –80°C. During storage or transport in RNA*later* RNA Stabilization Reagent, even at elevated temperatures (e.g., room temperature or 37°C), the cellular RNA remains intact and undegraded. RNA*later* technology allows large numbers of samples to be easily processed and replaces inconvenient, dangerous, and equipment-intensive methods, such as snap-freezing of samples in liquid nitrogen, storage at –80°C, cutting and weighing on dry ice, or immediate processing of harvested samples.

Note: RNA*later* RNA Stabilization Reagent is not for stabilization of RNA in animal cells, whole blood, plasma, or serum.

Description of protocols

Purification of Total RNA from Animal Cells Using Spin Technology

Up to 1 x 10^7 cells, depending on the cell line, are disrupted in Buffer RLT and homogenized. An overview of disruption and homogenization methods is given on pages 20–23. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. All bind, wash, and elution steps are performed by centrifugation in a microcentrifuge.

Purification of Total RNA from Animal Cells Using Vacuum/Spin Technology

Up to 1 x 10^6 cells, depending on the cell line, are disrupted in Buffer RLT and homogenized. An overview of disruption and homogenization methods is given on pages 20–23. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. The bind and wash steps are performed on a QIAvac 24, QIAvac 24 Plus, or QIAvac 6S vacuum manifold, and the final elution step is performed by centrifugation in a microcentrifuge.

Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi

Up to 100 mg of sample is first ground in liquid nitrogen and then lysed under highly denaturing conditions. The RNeasy Plant Mini Kit provides a choice of lysis buffers: Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively. The higher cell disruption and denaturing properties of Buffer RLT frequently make it the buffer of choice. However, some tissues, such as milky endosperm of maize or mycelia of filamentous fungi, solidify in Buffer RLT, making the extraction of RNA impossible. In these cases, Buffer RLC should be used instead. After lysis with either buffer, samples are centrifuged through a QIAshredder homogenizer. This simultaneously removes insoluble material and reduces the viscosity of the lysates by disrupting gelatinous material often formed in plant and fungal lysates. Ethanol is added to the cleared lysate, creating conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

RNA Cleanup

This protocol can be used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labeling) or to desalt RNA samples (up to 100 µg RNA). Buffer RLT and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For all protocols

- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- 96–100% ethanol*
- Disposable gloves
- Equipment for sample disruption and homogenization (see pages 20–23). Depending on the method chosen, one or more of the following are required:
  - Trypsin and PBS
  - QIAshredder homogenizer (see ordering information, page 76)
  - Blunt needle and syringe
  - Mortar and pestle
  - TissueLyser (see ordering information, page 76)
  - Rotor–stator homogenizer

For RNA purification from animal cells

- 70% ethanol*

For RNA purification from animal cells using vacuum technology

- QIAvac 24 (no longer available); QIAvac 24 Plus (cat. no. 19413); QIAvac 6S (cat. no. 19503) with the QIAvac Luer Adapter Set (cat. no. 19541); or other vacuum manifold with luer connectors and capable of dealing with vacuum pressures of –800 to –900 mbar
- QIAGEN Vacuum Pump (see page 75 for ordering information); or other vacuum pump capable of generating a vacuum pressure of –800 to –900 mbar and with a capacity of 18–20 liter/min

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Note: Use of insufficient vacuum pressure may reduce RNA yield and purity. The RNeasy procedure requires higher vacuum pressures compared with other QIAGEN procedures. Most water pumps or house vacuums do not provide sufficient vacuum pressure.

- Optional: Vacuum Regulator (cat. no. 19530) to measure the pressure difference between the inside and outside of a vacuum system

  A vacuum pressure of –800 to –900 mbar should develop when RNeasy Mini spin columns are used on the vacuum manifold. Vacuum pressures exceeding –900 mbar should be avoided. The vacuum pressure is the pressure difference between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 mbar or 760 mm Hg) and can be regulated and measured using a pressure gauge or vacuum regulator. Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere.

- Optional: VacConnectors (cat. no. 19407)

  These disposable connectors fit between the RNeasy Mini spin columns and the luer extensions on the QIAvac 24 or QIAvac 24 Plus or the QIAvac Luer Adapters on the QIAvac 6S. They prevent direct contact between the RNeasy Mini spin columns and luer connectors during RNA purification, avoiding any cross-contamination between samples. VacConnectors are discarded after single use.

For RNA purification from animal tissues

- 70% ethanol*
- Optional: Dithiothreitol (DTT)

For RNA purification from yeast using enzymatic lysis

- 70% ethanol*
- Buffer for enzymatic lysis

  In most cases, Buffer Y1 (containing sorbitol, EDTA, β-ME, and lyticase or zymolase) can be used. See the protocol on page 45 for details on preparing Buffer Y1.

For RNA purification from yeast using mechanical disruption

- 70% ethanol*
- Glass beads, 0.45–0.55 mm diameter

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

- Concentrated nitric acid, deionized water, and baking oven
- TissueLyser or other bead-mill homogenizer

For RNA purification from plants and fungi

- Liquid nitrogen
- Mortar and pestle (alternatively, TissueLyser or other bead-mill homogenizer)

Suppliers of equipment for disruption and homogenization*

Rotor–stator homogenizers can be purchased from:

- BioSpec Products, Inc. (www.biospec.com): Tissue-Tearor™ homogenizer
- Charles Ross & Son Company (www.mixers.com)
- IKA (www.ika.de): ULTRA-TURRAX® dispersers
- KINEMATICA AG (www.kinematica.ch) or Brinkmann Instruments, Inc. (www.brinkmann.com): POLYTRON® laboratory dispersing devices
- Omni International, Inc. (www.omni-inc.com)
- Silverson (www.silverson.com)
- VirTis (www.virtis.com)

Bead-mill homogenizers and stainless steel and tungsten carbide beads can be purchased from:

- QIAGEN (TissueLyser system, see page 76 for ordering information)

Glass, stainless steel, and tungsten carbide beads can be purchased from:

- Retsch (www.retsch.de)

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

Important Notes

Determining the amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The maximum amount that can be used is determined by:

- The type of sample and its RNA content
- The volume of Buffer RLT required for efficient lysis
- The RNA binding capacity of the RNeasy spin column

When processing samples containing high amounts of RNA, less than the maximum amount of starting material shown in Table 1 should be used, so that the RNA binding capacity of the RNeasy spin column is not exceeded.

When processing samples containing average or low amounts of RNA, the maximum amount of starting material shown in Table 1 can be used. However, even though the RNA binding capacity of the RNeasy spin column is not reached, the maximum amount of starting material must not be exceeded. Otherwise, lysis will be incomplete and cellular debris may interfere with the binding of RNA to the RNeasy spin column membrane, resulting in lower RNA yield and purity.

More information on using the correct amount of starting material is given in each protocol. Table 2 shows expected RNA yields from various sources.

Table 1. RNeasy Mini Spin Column Specifications

Maximum binding capacity	100 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >200 nucleotides
Minimum elution volume	30 µl
Maximum amount of starting material	
■ Animal cells	1×10^7*
■ Animal tissues	30 mg*
■ Yeast	5×10^7*
■ Plant tissues	100 mg
■ Filamentous fungi	100 mg

* For larger sample sizes, RNeasy Kits and RNeasy Protect Kits are available in midi and maxi formats. For details, visit www.qiagen.com/RNA .

Note: If the binding capacity of the RNeasy spin column is exceeded, RNA yields will not be consistent and may be reduced. If lysis of the starting material is incomplete, RNA yields will be lower than expected, even if the binding capacity of the RNeasy spin column is not exceeded.

Table 2. Typical Yields of Total RNA with RNeasy Mini Spin Columns

Source	Yield of total RNA* (µg)
Cell cultures (1 x 10^6 cells)	
NIH/3T3	10
HeLa	15
COS-7	35
LMH	12
Huh	15
Mouse/rat tissues (10 mg)	
Embryo (13 day)	25
Kidney	20–30
Liver	40–60
Spleen	30–40
Thymus	40–50
Lung	10–20
Yeast (1 x 10^7 cells)	
S. cerevisiae	25
Plants (100 mg leaves)	
Arabidopsis	35
Maize	25
Tomato	65
Tobacco	60

* Amounts can vary due to factors such as species, developmental stage, and growth conditions. Since the RNeasy procedure enriches for mRNA and other RNA species >200 nucleotides, the total RNA yield does not include 5S rRNA, tRNA, and other low-molecular-weight RNAs, which make up 15–20% of total cellular RNA.

Handling and storing starting material

RNA in animal and plant tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent (animal tissues only), flash-frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that tissue samples are immediately frozen in liquid nitrogen and stored at –70°C, or immediately immersed in RNA*later* RNA Stabilization Reagent.

The procedures for tissue harvesting and RNA protection should be carried out as quickly as possible. Frozen tissue samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT (lysis buffer), samples can be stored at –70°C for months.

Animal and yeast cells can be pelleted and then stored at –70°C until required for RNA purification. However, if performing RNA purification from yeast cells with enzymatic lysis, only freshly harvested samples can be used.

Disrupting and homogenizing starting material

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA purification procedures. Disruption and homogenization are 2 distinct steps:

- **Disruption**: Complete disruption of cell walls and plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced RNA yields.
- **Homogenization**: Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy spin column membrane and therefore significantly reduced RNA yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 3 (page 21) gives an overview of different disruption and homogenization methods, and is followed by a detailed description of each method. This information can be used as a guide to choose the appropriate methods for your starting material.

Note: After storage in RNA*later* RNA Stabilization Reagent, tissues become slightly harder than fresh or thawed tissues. Disruption and homogenization of these tissues, however, is usually not a problem.

Table 3. Disruption and Homogenization Methods

Sample	Disruption method	Homogenization method	Comments
Animal cells	Addition of lysis buffer	Rotor–stator homogenizer or QIAshredder homogenizer* or syringe and needle	If processing ≤1 x 10^5 cells, lysate can be homogenized by vortexing
Animal tissues	TissueLyser	TissueLyser	The TissueLyser gives results comparable to using a rotor–stator homogenizer
	Rotor–stator homogenizer	Rotor–stator homogenizer	Simultaneously disrupts and homogenizes
	Mortar and pestle	QIAshredder homogenizer* or syringe and needle	Rotor–stator homogenizer usually gives higher yields than mortar and pestle
Yeast	Enzymatic digestion of cell wall followed by lysis of spheroplasts	Vortexing	
	TissueLyser with glass beads	TissueLyser with glass beads	TissueLyser simultaneously disrupts and homogenizes; cannot be replaced by vortexing
Plants and filamentous fungi	Mortar and pestle	QIAshredder homogenizer*	Mortar and pestle cannot be replaced by rotor–stator homogenizer

* QIAshredder homogenizers are supplied in the RNeasy Plant Mini Kit and can be purchased separately for use with the RNeasy Mini Kit or RNeasy Protect Mini Kit. See page 76 for ordering information.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the animal or plant tissue immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen–cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the homogenization according to one of the 2 methods below.

Note: Grinding the sample using a mortar and pestle will disrupt the sample, but will not homogenize it. Homogenization must be performed afterwards.

Homogenization using QIAshredder homogenizers

Using QIAshredder homogenizers is a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples. Up to 700 µl of lysate is loaded onto a QIAshredder spin column placed in a 2 ml collection tube, and spun for 2 minutes at maximum speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column. QIAshredder spin columns are supplied in the RNeasy Plant Mini Kit and can be purchased separately for use with the RNeasy Mini Kit and RNeasy Protect Mini Kit. See page 76 for ordering information.

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. Lysate is passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize loss.

Eliminating genomic DNA contamination

Generally, DNase digestion is not required with RNeasy Kits since RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). In these cases, residual DNA can be removed by optional on-column DNase digestion using the RNase-Free DNase Set (see Appendix D, page 69). The DNase is efficiently removed in subsequent wash steps. Alternatively, residual DNA can be removed by a DNase digestion after RNA purification (see Appendix E, page 71). The DNase digestion can then be cleaned up, if desired, using "Protocol: RNA Cleanup" (page 56).

The RNeasy Plus Mini Kit, which is designed for RNA purification from animal cells and tissues, integrates unique gDNA Eliminator spin columns with RNeasy technology. Genomic DNA is effectively removed in a single, rapid centrifugation step, avoiding the need for DNase digestion. See page 76 for ordering information.

If the purified RNA will be used in real-time, two-step RT-PCR, we recommend using the QuantiTect® Reverse Transcription Kit. The kit provides a fast and convenient procedure, enabling cDNA synthesis and genomic DNA removal in only 20 minutes. For ordering information, see page 78.

Protocol: Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi

This protocol requires the RNeasy Plant Mini Kit.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 100 mg plant material or 1 x 10^7 cells can generally be processed. For most plant materials, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Average RNA yields from various plant materials are given in Table 2 (page 19).

If there is no information about the nature of your starting material, we recommend starting with no more than 50 mg plant material or 3–4 x 10^6 cells. Depending on RNA yield and purity, it may be possible to use up to 100 mg plant material or up to 1 x 10^7 cells in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

Counting cells or weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5 cm diameter leaf disc weighs 25–75 mg.

Important points before starting

- If using the RNeasy Plant Mini Kit for the first time, read "Important Notes" (page 18).
- If working with RNA for the first time, read Appendix A (page 63).
- Fresh or frozen tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.
- The RNeasy Plant Mini Kit provides a choice of lysis buffers: Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively. In most cases, Buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of guanidine thiocyanate. However, depending on the amount and type of secondary metabolites in some tissues (such as milky endosperm of maize or mycelia of filamentous fungi), guanidine thiocyanate can cause solidification of the sample, making extraction of RNA impossible. In these cases, Buffer RLC should be used.

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT, Buffer RLC, and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 8 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT or Buffer RLC before use. Add 10 µl β-ME per 1 ml Buffer RLT or Buffer RLC. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT or Buffer RLC containing β-ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69).

Procedure

1. **Determine the amount of plant material. Do not use more than 100 mg.**

   Weighing tissue is the most accurate way to determine the amount.

2. **Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 3.**

   RNA in plant tissues is not protected until the tissues are flash-frozen in liquid nitrogen. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

3. **Add 450 µl Buffer RLT or Buffer RLC (see "Important points before starting") to a maximum of 100 mg tissue powder. Vortex vigorously.**

   A short 1–3 min incubation at 56°C may help to disrupt the tissue. However, do not incubate samples with a high starch content at elevated temperatures, otherwise swelling of the sample will occur.

Note: Ensure that β-ME is added to Buffer RLT or Buffer RLC before use (see "Things to do before starting").

4. **Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.**

   It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.

5. **Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.**

   Note: The volume of lysate may be less than 450 µl due to loss during homogenization.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. **Transfer the sample (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   Reuse the collection tube in step 7.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.*

   Optional: If performing optional on-column DNase digestion (see "Eliminating genomic DNA contamination", page 23), follow steps D1–D4 (page 69) after performing this step.

7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.***

   Reuse the collection tube in step 8.

* Flow-through contains Buffer RLT, Buffer RLC, or Buffer RW1 and is therefore not compatible with bleach. See page 8 for safety information.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

Skip this step if performing optional on-column DNase digestion (page 69).

8. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Reuse the collection tube in step 9.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.**

   The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

    If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back cover for contact information).

		Comments and suggestions
Clogged RNeasy spin column		
a)	Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (pages 20–23) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see protocols) and/or increase the volume of lysis buffer and the homogenization time. If working with tissues rich in proteins, we recommend using the RNeasy Fibrous Tissue Mini Kit (see page 76 for ordering information).
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material. It is essential to use the correct amount of starting material (see protocols).
c)	Centrifugation before adding ethanol not performed (protocols for tissues and mechanical disruption of yeast)	Centrifuge the lysate before adding ethanol, and use only this supernatant in subsequent steps (see protocols). Pellets contain cell debris that can clog the RNeasy spin column.
d)	Centrifugation temperature too low	The centrifugation temperature should be 20–25°C. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause formation of precipitates that can clog the RNeasy spin column. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring it to the RNeasy spin column.

		Comments and suggestions
Low RNA yield		
a)	Insufficient disruption and homogenization	See "Disrupting and homogenizing starting material" (pages 20–23) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see protocols) and/or increase the volume of lysis buffer and the homogenization time. If working with tissues rich in proteins, we recommend using the RNeasy Fibrous Tissue Mini Kit (see page 76 for ordering information).
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material. It is essential to use the correct amount of starting material (see protocols).
c)	RNA still bound to RNeasy spin column membrane	Repeat RNA elution, but incubate the RNeasy spin column on the benchtop for 10 min with RNase-free water before centrifuging.
d)	Ethanol carryover	During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C to dry the RNeasy spin column membrane. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. To eliminate any chance of possible ethanol carryover, place the RNeasy spin column in a new 2 ml collection tube and perform the optional 1-min centrifugation step as described in the protocols.
e)	Incomplete removal of cell-culture medium (cell samples)	When processing cultured cells, ensure complete removal of the cell-culture medium after harvesting cells (see protocols).

	Comments and suggestions
Low A_{260}/A_{280} value	
Water used to dilute RNA for A_{260}/A_{280} measurement	Use 10 mM Tris·Cl, pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 65).
RNA degraded	
a) Harvested animal tissue not immediately stabilized	Submerge the tissue in the appropriate volume of RNA*later* RNA Stabilization Reagent immediately after harvesting.
b) Too much animal tissue for proper stabilization	Reduce the amount of tissue or increase the amount of RNA*later* RNA Stabilization Reagent used for stabilization (see protocol on page 36).
c) Animal tissue too thick for stabilization	Cut large samples into slices less than 0.5 cm thick for stabilization in RNA*later* RNA Stabilization Reagent.
d) Frozen animal tissue used for stabilization	Use only fresh, unfrozen tissue for stabilization in RNA*later* RNA Stabilization Reagent.
e) Storage duration in RNA*later* RNA Stabilization Reagent exceeded	RNA*later* stabilized tissue can be stored for up to 1 day at 37°C, up to 7 days at 15–25°C, or up to 4 weeks at 2–8°C, and can be archived at –20°C or –80°C.
f) Inappropriate handling of starting material	Ensure that tissue samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent. For frozen cell pellets or frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the RNeasy procedure quickly, especially the first few steps. See Appendix A (page 63), "Handling and storing starting material" (page 20), and the RNA*later* protocol (page 36).

		Comments and suggestions
g)	RNase contamination	Although all RNeasy buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the RNeasy procedure or later handling. See Appendix A (page 63) for general remarks on handling RNA. Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
DNA contamination in downstream experiments		
a)	Optimal procedure not used (cell samples)	For animal cells, we recommend purifying cytoplasmic RNA for applications where the absence of DNA contamination is critical, since intact nuclei are removed at the start of the procedure. The protocol can be downloaded at www.qiagen.com/literature/protocols/RNeasyMini.aspx .
b)	No incubation with Buffer RW1	In subsequent preparations, incubate the RNeasy spin column for 5 min at room temperature (15–25°C) after addition of Buffer RW1 and before centrifuging.
c)	No DNase treatment	Perform optional on-column DNase digestion using the RNase-Free DNase Set (see Appendix D, page 69) at the point indicated in the individual protocols. Alternatively, after the RNeasy procedure, DNase digest the RNA eluate. After inactivating the DNase by heat treatment, the RNA can be either used directly in the downstream application without further treatment, or repurified using the RNA cleanup protocol (page 56).

		Comments and suggestions
RNA does not perform well in downstream experiments		
a)	Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C. When reusing collection tubes between washing steps, remove residual flow-through from the rim by blotting on clean paper towels.
b)	Ethanol carryover	During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C to dry the RNeasy spin column membrane. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. To eliminate any chance of possible ethanol carryover, place the RNeasy spin column in a new 2 ml collection tube and perform the optional 1-min centrifugation step as described in the protocols.

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 64). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS),* thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

† Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.

Appendix B: Storage, Quantification, and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260}=1 → 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 66), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 64). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)

A_{260} = 0.2

Concentration of RNA sample = 44 µg/ml x A_{260} x dilution factor
= 44 µg/ml x 0.2 x 50
= 440 µg/ml

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Total amount	= concentration x volume in milliliters
	= 440 µg/ml x 0.1 ml
	= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA", page 65).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While RNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample.

For analysis of very low abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in real-time RT-PCR applications, such as with ABI PRISM® and LightCycler instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Assays from QIAGEN are designed for real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible. For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination (see ordering information, page 78).

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

For other sensitive applications, DNase digestion of the purified RNA with RNase-free DNase is recommended. A protocol for optional on-column DNase digestion using the RNase-Free DNase Set is provided in Appendix D (page 69). The DNase is efficiently washed away in subsequent wash steps. Alternatively, after the RNeasy procedure, the RNA eluate can be treated with DNase. The RNA can then be repurified according to the RNA cleanup protocol (page 56), or after heat inactivation of the DNase, the RNA can be used directly in downstream applications.

The protocol for purification of cytoplasmic RNA from animal cells (available at www.qiagen.com/literature/protocols/RNeasyMini.aspx) is particularly advantageous in applications where the absence of DNA contamination is critical, since intact nuclei are removed. Using this protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and RNeasy technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, even further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). Using the cytoplasmic RNA protocol with optional DNase digestion results in undetectable levels of DNA, even in sensitive quantitative RT-PCR analyses.

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using an Agilent 2100 bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S RNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

RNeasy® Plant Mini Kit

The RNeasy Plant Mini Kit (cat. nos. 74903 and 74904) can be stored at room temperature (15–25°C) for at least 9 months.

For more information, additional and more detailed protocols, and safety information, please refer to the *RNeasy Mini Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- The RNeasy Plant Mini Kit provides a choice of lysis buffers. Buffer RLT is the lysis buffer of choice but Buffer RLT can cause solidification of some samples, depending on the amount and type of secondary metabolites in the tissue. In these cases, Buffer RLC should be used.
- Add either 10 µl β-mercaptoethanol (β-ME), or 20 µl 2 M dithiothreitol (DTT)*, to 1 ml Buffer RLT or Buffer RLC before use. Buffers with DTT or β-ME can be stored at room temperature for up to 1 month.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

* This option not included for plant tissue in handbook; handbook to be updated.

1. Disrupt a maximum of 100 mg plant material according to step 1a or 1b.

1a. Disruption with mortar and pestle
Immediately place tissue in liquid nitrogen. Grind thoroughly. Decant tissue powder and liquid nitrogen into RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 2.

1b. Disruption using the TissueLyser II, TissueLyser LT, or TissueRuptor®
For detailed information on disruption of plant tissues for purification of RNA, see *TissueLyser Handbook*, *TissueLyser LT Handbook,* or *TissueRuptor Handbook*. (The *RNeasy Mini Handbook* will be updated with this option.)

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2. Add 450 µl Buffer RLT or Buffer RLC to a maximum of 100 mg tissue powder. Vortex vigorously.
3. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at full speed. Transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet.
4. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 5.
5. Transfer the sample (usually 650 µl), with any precipitate, to an RNeasy Mini spin column (pink) in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.
6. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*.

   Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to dry the membrane.
9. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x *g* to elute the RNA.
10. If the expected RNA yield is >30 µg, repeat step 9 using another 30–50 µl of RNase-free water. Alternatively, use the eluate from step 9 (if high RNA concentration is required). Reuse the collection tube from step 9.

Appendix 1H
Turbo DNA-free kit

TURBO DNA-*free*™ Kit

REVISED 4/2009
Inactivation time extended in step 4 on page 7.

TURBO DNase Treatment and Removal Reagents
Part Number AM1907

Applied Biosystems

A. Product Description

Ambion® TURBO DNA-*free*™ DNase Treatment and Removal Reagents are designed to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the sample. The included TURBO DNase (patent pending) is an engineered version of wild type DNase I with 350% greater catalytic efficiency. TURBO DNase has a markedly higher affinity for DNA than conventional DNase I, and is thus more effective in removing trace quantities of DNA contamination. In addition, TURBO DNase maintains up to 50X greater activity than DNase I in solutions containing physiological salt concentrations. The TURBO DNase provided in the kit is overexpressed in an animal-free system, and is then extensively purified in a bovine-free process and tested. It is guaranteed to lack any contaminating RNase activity. The kit also includes an optimized DNase reaction buffer that contains a small molecule enhancer that extends the activity of the TURBO DNase enzyme by 100-fold or more. Using TURBO DNA-*free*, contaminating DNA is digested to levels below the limit of detection by routine PCR (Figure 1). The DNase is then removed rapidly and easily using a novel method which does not require phenol/chloroform extraction, alcohol precipitation, heating, or the addition of EDTA (see Table 1). TURBO DNA-*free* treated RNA is suitable for endpoint or real-time RT-PCR, microarray analysis, RPAs, Northerns, and all other RNA analysis methods.

Ambion®

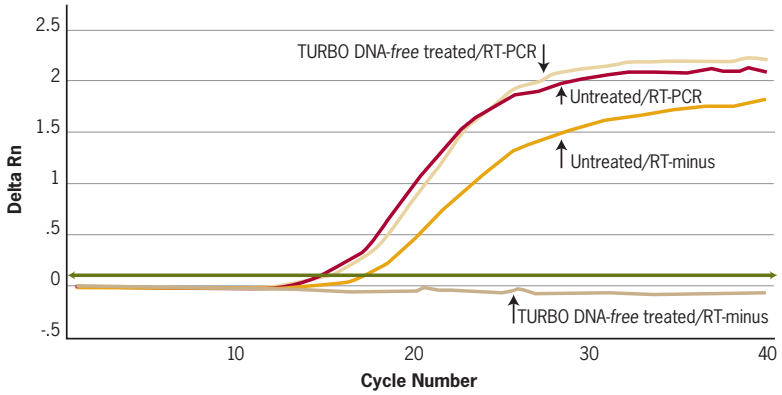


Figure 1. TURBO DNA-*free*™ Reduces Genomic DNA Contamination by Greater than 5 Million Fold.

Equal amounts of mouse spleen total RNA (purified using Ambion's RNAqueous® Kit) were either treated with 7.8 U of TURBO DNase in a 130 µL reaction for 20 min at 37°C, or were left untreated. The digestions were stopped by adding 22 µL DNase Inactivation Reagent. 5 µL (1 µg RNA) was amplified in a one step 25 µL RT-PCR using a TaqMan® primer probe set for mouse GAPDH. Treated and untreated samples were reverse transcribed with Ambion's MessageSensor™ RT Kit. RT-minus samples were subjected to PCR to control for DNA contamination. Results are shown using a linear scale so that the amplification plot for the TURBO DNase-treated, RT-minus sample is visible.

The fold-removal (5.4×10^6 fold) of genomic DNA was calculated as follows: The C_t value from the untreated RNA in the RT-minus reaction is the level of gDNA contamination. The fold-removal was determined by subtracting the RT-minus reaction C_t value for the treated RNA sample, 39.5 (the other duplicate's signal was undetectable) from the C_t value of the untreated sample, 17.13, and raising the 17.13 as the exponent with a base of 2.

Table 1. Treatment of RNA with TURBO DNA-*free*™ Maintains Target Sensitivity in Real-time RT-PCR

	Ct for β-actin (duplicates)	
RNA treatment	100 pg RNA	1 pg RNA
none	24.78 / 24.67	31.83 / 31.53
TURBO DNA-*free* treated	24.50 / 24.62	30.89 /30.88

	Ct for CDC-2 (duplicates)	
none	28.88 / 28.24	34.41 / 35.50
TURBO DNA-*free* treated	27.71 / 28.10	34.04 / 33.99

Total RNA from HeLa S3 cells was treated with the TURBO DNA-*free*™ Kit following the standard protocol. 5 µL of the treated RNA was then reverse transcribed using Ambion's MessageSensor RT Kit, and the resulting cDNA was amplified by real-time RT-PCR using primer and probe sets for either human β-actin or CDC-2 with TaqMan® detection.

In addition to removing the DNase enzyme, DNase Inactivation Reagent also removes divalent cations, such as magnesium and calcium, which can catalyze RNA degradation when RNA is heated with the sample (Figure 2).

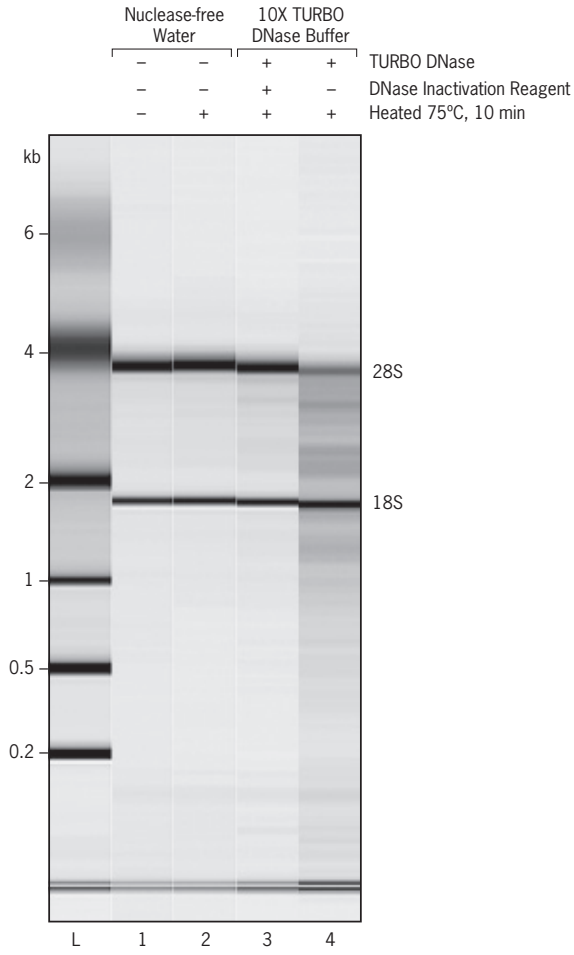


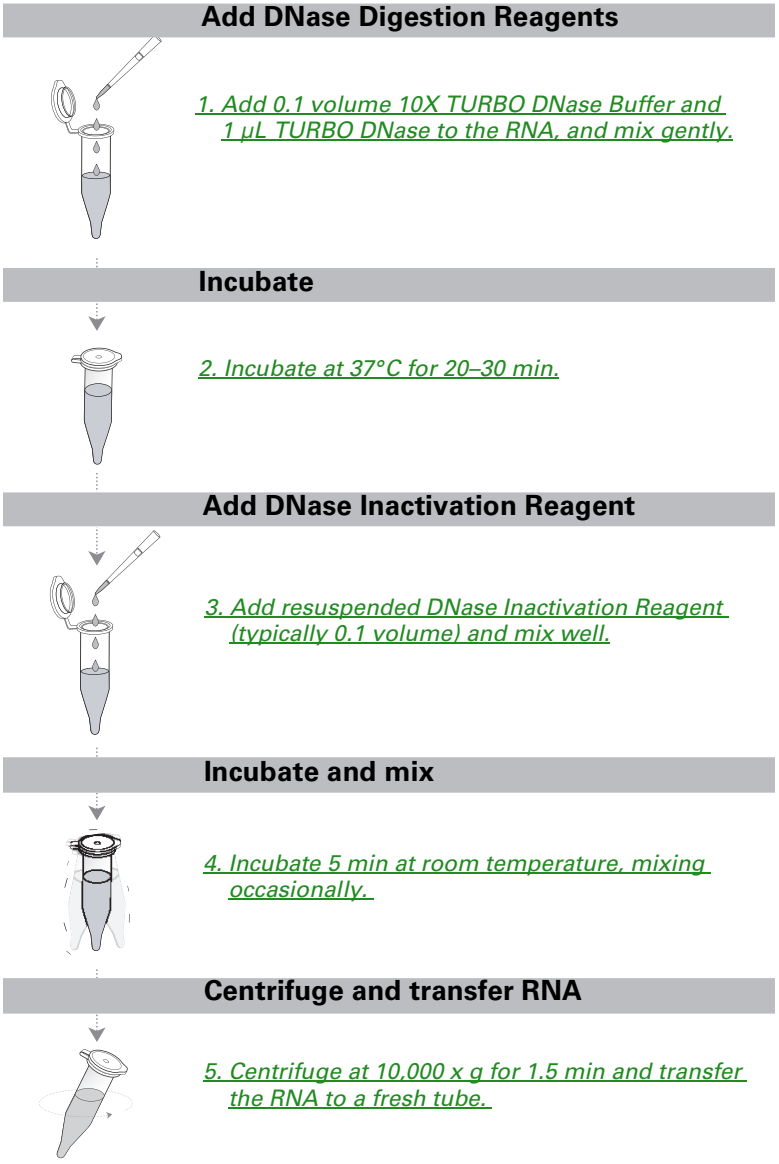
Figure 2. Removal of divalent cations by DNase Inactivation Reagent

HeLa-S3 total RNA (100 ng), in 50 µL 1X TURBO DNase Buffer or in nuclease-free water, was treated with components from the TURBO DNA-*free*™ kit as indicated. Samples were heated for 10 min at 75°C (Lanes 2, 3, & 5), or 3 min at 90°C (Lane 4), to determine if divalent cations from the TURBO DNase Buffer remained in solution, and degraded the RNA. 1 µL of each sample was analyzed on an RNA LabChip® using the Agilent 2100 bioanalyzer. Note that RNA was degraded in the sample that contained TURBO DNase Buffer, but was not treated with the DNase Inactivation Reagent (Lane 5); this degradation is due to the presence of divalent ions that induce heat-mediated RNA cleavage.

B. Procedure Overview

For the detailed procedure, see section E on page 5.

Figure 3. TURBO DNA-*free*™ Procedure Overview



C. How Much RNA Can Be Treated with TURBO DNA-*free*™?

This protocol is designed to remove trace to moderate amounts of contaminating DNA (up to 50 µg DNA/mL RNA) from purified RNA to a level that is mathematically insignificant by RT-PCR. No RNA isolation method can extract RNA that is completely free from DNA contamination; in fact, RNA isolated from some tissues, such as spleen, kidney, or thymus, often contain relatively high levels of DNA. Other potential sources of DNA contamination include carryover of the interface during organic extractions, and overloaded glass-fiber filters during RNA purification.

D. TURBO DNA-*free* Components and Storage

Reagents are provided for 50 TURBO DNA-*free* treatments (up to 100 µL each).

Amount	Component	Storage
120 µL	TURBO DNase (2 Units/µL)	–20°C
600 µL	10X TURBO DNase Buffer	–20°C
600 µL	DNase Inactivation Reagent	–20°C
1.75 mL	Nuclease-free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C or room temp

Store the TURBO DNA-*free* Kit at –20°C in a non-frost-free freezer for long-term storage. For convenience, the 10X TURBO DNase Buffer and the DNase Inactivation Reagent can be stored at 4°C for up to 1 week.

E. TURBO DNA-*free* Procedure

Procedure Notes

- We recommend conducting reactions in 0.5 mL tubes to facilitate removal of the supernatant after treatment with the DNase Inactivation Reagent.

- TURBO DNA-*free* reactions can be conducted in 96-well plates. We recommend using V-bottom plates because their shape makes it easier to remove the RNA from the pelleted DNase Inactivation Reagent at the end of the procedure.
- The recommended reaction size is 10–100 µL. A typical reaction is 50 µL.

1. Add 0.1 volume 10X TURBO DNase Buffer and 1 µL TURBO DNase to the RNA, and mix gently.

There are separate DNase digestion conditions depending on the amount of contaminating DNA and the nucleic acid concentration of the sample.

- ***Routine DNase treatment:*** ≤200 µg nucleic acid per mL
- ***Rigorous DNase treatment:*** >200 µg nucleic acid per mL or RNA that is severely contaminated with DNA (i.e. >2 µg DNA/50 µL)

Routine DNase treatment: Use 1 µL TURBO DNase (2 U) for up to 10 µg of RNA in a 50 µL reaction. These reaction conditions will remove up to 2 µg of genomic DNA from total RNA in a 50 µL reaction volume.

Rigorous DNase treatment: If the RNA contains more than 200 µg of nucleic acid per mL, dilute the sample to 10 µg nucleic acid/50 µL before adding the TURBO DNase Buffer and TURBO DNase. It is also helpful to add only half of the TURBO DNase to the reaction initially, incubate for 30 min, then add the remainder of the enzyme and incubate for another 30 min.

If the sample cannot be diluted, simply increase the amount of TURBO DNase to 2–3 µL (4–6 U). It may be possible to successfully remove contaminating DNA from samples containing up to 500 µg/mL nucleic acid in a 10–100 µL TURBO DNA-*free* reaction. However, the efficacy of treating highly concentrated nucleic acid samples depends on the absolute level of DNA contamination, and residual DNA may or may not be detectable by PCR after 35–40 cycles.

2. Incubate at 37°C for 20–30 min.

If only half of the TURBO DNase was added in step 1, incubate for 30 min, then add the rest of the TURBO DNase and incubate for 30 min more.

3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume) and mix well.

Always resuspend the DNase Inactivation Reagent by flicking or vortexing the tube before dispensing it.

- For ***routine DNase treatment*** use 2 µL or 0.1 volume DNase Inactivation Reagent, whichever is greater. For example, if the RNA volume is 50 µL, and 1 µL of TURBO DNase was used in step 1, add 5 µL of DNase Inactivation Reagent.
- For ***rigorous DNase treatments***, where 2–3 µL of TURBO DNase was used, add 0.2 volumes of DNase Inactivation Reagent.

 **IMPORTANT**

Always use at least 2 µL of DNase Inactivation Reagent, even if it is more than 0.1 volume.

 **NOTE**

The DNase Inactivation Reagent may become difficult to pipette after multiple uses due to depletion of fluid from the interstitial spaces. If this happens, add a volume of Nuclease-free Water (supplied with the kit) equal to approximately 20–25% of the bed volume of the remaining DNase Inactivation Reagent, and vortex thoroughly to recreate a pipettable slurry.

4. Incubate 5 min at room temperature, mixing occasionally.

Flick the tube 2–3 times during the incubation period to redisperse the DNase Inactivation Reagent.

 **NOTE**

If room temperature cools below 22–26 °C, move the tubes to a heat block or oven to control the temperature. Cold environments can reduce the inactivation of the TURBO DNase, leaving residual DNase in the RNA sample.

5. Centrifuge at 10,000 x g for 1.5 min and transfer the RNA to a fresh tube.

- For 96-well plates, centrifuge at 2000 x g for 5 min.

This centrifugation step pellets the DNase Inactivation Reagent. After centrifuging, carefully transfer the supernatant, which contains the RNA, into a fresh tube. Avoid introducing the DNase Inactivation Reagent into solutions that may be used for downstream enzymatic reactions, because it can sequester divalent cations and change the buffer conditions.

F. Troubleshooting

1. No RT-PCR product is detectable from RNA treated with TURBO DNA-*free*

DNase Inactivation Reagent could inhibit RT-PCR.
In step .E.5 on page 8, remove the RNA solution from the pelleted DNase Inactivation Reagent carefully to avoid transferring it to the tube of RNA. You may have to leave a small amount of RNA behind to accomplish this. If you accidentally touch the pellet while removing the RNA, recentrifuge to pack the DNase Inactivation Reagent.

TURBO DNA-*free* treated RNA should comprise only ~20% of an RT-PCR reaction volume.
For RT-PCR, we recommend that TURBO DNA-*free* treated RNA makes up ~20%, and no more than 40%, of the final RT-PCR volume. Otherwise, components from the TURBO DNase Buffer and the DNase Inactivation Reagent could interfere with the reaction. If necessary, RT-PCR volumes can be increased to 50 µL or more to accommodate your RNA without exceeding the 20–40% limit.

RNA used in RT-PCR should be treated only once with TURBO DNA-*free*.
The salt in TURBO DNA-*free* reactions is carefully balanced for optimal TURBO DNase activity. Subjecting RNA to a second TURBO DNA-*free* treatment will introduce additional salts that could interfere with downstream enzymatic reactions. If a second DNase treatment is required, please refer to the "TURBO DNA-*free* 2nd Digest Protocol" available online at:

www.ambion.com/techlib/append/supp/digest.html

2. RNA is degraded upon heating to >60°C

RNA samples that contain divalent cations, such as magnesium or calcium, will degrade when heated to temperatures above 60°C. To ensure that divalent cations are removed during step .E.4 on page 7, redisperse the DNase Inactivation Reagent by mixing the reaction 2–3 times during the incubation period.

3. The RNA absorbance spectrum has an unusual profile after treatment with TURBO DNA-*free*.

If the concentration of RNA in the sample is less than about 50 ng/µL, you may notice significant absorbance at ~230 nm. A_{260}/A_{280} ratios may also be slightly lower than normal when the RNA concentration is ≤25 ng/µL. These differences in the absorbance profile are caused by the enhancer in the TURBO DNase Buffer. Exhaustive comparisons at Ambion with both treated and untreated RNA samples indicate that the enhancer has no affect on accurate RNA quantification unless the RNA concentration is below 10 ng/µL. For more information, please see:

www.ambion.com/catalog/supp/absorbance.html

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.

Appendix 1I
iScript cDNA Synthesis

BIO-RAD

iScript™cDNA Synthesis Kit

25 x 20 µl reactions	170-8890
100 x 20 µl reactions	170-8891

For Research purposes only
Store at -20 °C (not frost-free)

iScript cDNA Synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. This kit includes just three tubes - comprehensive of the reagents required for successful RT-PCR.

The iScript reverse transcriptase is RNAse H+, resulting in greater sensitivity than RNAse H- enzymes. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided pre-blended with RNAse inhibitor. The unique blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length.

iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

Storage and Stability

Store the iScript™ cDNA Synthesis Kit at -20 °C in a constant temperature freezer. When stored under these conditions the kit components are stable for a minimum of one year after ship date. Nuclease-free water can be stored at room temperature.

Kit Contents

Reagent	Volume
25 reaction kit	
5x iScript Reaction Mix	100µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	25µl
100 reaction kit	
5x iScript Reaction Mix	400µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	100µl

Reaction Set Up

Component	Volume per reaction
5x iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	xµl
RNA template (100fg to 1µg Total RNA)*	xµl
Total Volume	20 µl

Reaction Protocol

Incubate complete reaction mix:
- 5 minutes at 25ºC
- 30 minutes at 42ºC
- 5 minutes at 85ºC
- Hold at 4ºC (optional)

Reagents and Materials Not Supplied

Reagents for PCR or real-time PCR
- Such as:
- iTaq™ DNA polymerase, 170-8870
- iQ™ Supermix, 170-8860 or
- iQ™ SYBR® Green Supermix, 170-8880

Pipette tips, aerosol barrier tips
- Such as:
- the Xcluda® Style B, 211-2006

Nuclease-free tubes
- Such as:
- 0.2ml Thin-Wall Tubes, 223-9473 or
- plates, 223-9441

RNA purification kit
- Such as the:
- Aurum™ Total RNA Mini Kit, 732-6820 or
- Aurum Total RNA Kit, 2 x 96 well, 732-6800

Recommendations for optimal results using the iScript cDNA Synthesis Kit:

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2µl.

*When using larger amounts of input RNA (>1µg) the reaction should be scaled up e.g. 40µl reaction for 2µg, 100µl reaction for 5µg to ensure optimum synthesis efficiency.

Appendix 1J
iProof High-Fidelity DNA Polymerase

BIO-RAD

iProof™ High-Fidelity PCR Kit

2 units/µl, 25 µl	50U	172-5330
2 units/µl, 100 µl	200U	172-5331

For research purposes only
Store at -20°C

iProof is a high-fidelity DNA polymerase that offers extreme performance for all PCR applications. Incorporating an exciting new and patented technology, iProof DNA polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity enhancing domain. This allows for the generation of long templates with an accuracy and speed previously unattainable with a single enzyme. The extreme fidelity of iProof makes it a superior choice for cloning. The error rate of iProof polymerase is determined to be 4.4×10^{-7} in iProof HF buffer, which is approximately 50-fold lower than that of *Thermus aquaticus*, and 6-fold lower than that of *Pyrococcus furiosus*.

The iProof™ High Fidelity PCR Kit includes lambda DNA control template and primers for 1.3 kb and 10 kb positive control amplicons. Sufficient template is included for performing 20 x 50 µl or 50 x 20 µl reactions.

Storage and Stability

Store the iProof™ High-Fidelity PCR Kit at -20°C in a constant temperature freezer. When stored under these conditions, the polymerase is stable for one year after the ship date.

Kit Contents

Reagent	50U	200U	Description
iProof Polymerase	25 µl	100 µl	iProof™ High Fidelity DNA Polymerase, 2 units/µl
iProof HF Buffer	1.5 ml	3 x 1.5 ml	5X HF Buffer, 7.5 mM $MgCl_2$
iProof GC Buffer	1.5 ml	3 x 1.5 ml	5X GC Buffer, 7.5 mM $MgCl_2$
dNTP mix	100 µl	100 µl	dNTP solution, 10 mM each
$MgCl_2$	1.5 ml	1.5 ml	50 mM $MgCl_2$ solution
Control 1template	40 µl	40 µl	Control 1template, 0.5 ng/µl
1.3 kb primers	50 µl	50 µl	4 µM each
10 kb primers	50 µl	50 µl	4 µM each
DNA Standard	200 µl	400 µl	DNA size standard
DMSO	500 µl	500 µl	100% DMSO solution

iProof DNA polymerase is unlike other enzymes. Please read the *QuickGuide* to modify your protocol for optimal results.

QuickGuide (See *Notes About Cycling Conditions* for details)

- Use 98°C for denaturation.
- Anneal at T_m +3°C (>20nt oligo).
- Use 15–30 sec/kb for extension times. Do not exceed 1 min/kb.
- Use iProof at 0.5–1.0 U per 50 µl reaction. Do not exceed 2 U/50 µl.
- Use 200 µM dNTPs. Do not use dUTP.
- iProof produces blunt end DNA products.

Notes About Cycling Conditions

1. **Denaturation**
   Template denaturation should be performed at 98°C. Due to the high thermostability of iProof, denaturation temperatures greater than 98°C can be used. A 30 s initial denaturation time is recommended, but this can be extended to 3 min for difficult DNA templates. Subsequent denaturation should be performed for 5–10 s at 98°C.
2. **Annealing**
   When using iProof, a general rule is to anneal primers (>20 nt) for 10–30 s at +3°C above the primer with the lowest T_m. Primer T_m should be calculated using the nearest-neighbor method as results can vary significantly depending on the method used. For primers $\leq$ 20 nt, use an annealing temperature equal to the primer with the lowest T_m.
3. **Extension**
   Template extension should be performed at 72°C and extension time depends on amplicon length and complexity. For low complexity DNA (e.g. plasmid, lambda, or BAC DNA) use 15 s per kb. For high complexity DNA (e.g. genomic DNA) use 30 s per kb. **Do not exceed 1 min per kb for amplicons that are >5 kb.**

Related Amplification Products From Bio-Rad Laboratories

Reagents for PCR or Real-Time PCR

iProof™ High-Fidelity DNA Polymerase	172-5301
iProof HF Master Mix	172-5310
iProof GC Master Mix	172-5320
iTaq™ DNA Polymerase	170-8870
iTaq Supermix With ROX	170-8854
iTaq SYBR Green Supermix With ROX	170-8850
iQ™ Supermix	170-8860
iQ SYBR Green Supermix	170-8880
iScript™ cDNA Synthesis Kit	170-8890
iScript Select cDNA Synthesis Kit	170-8896
iScript One-Step RT-PCR Kit with SYBR Green	170-8892
iScript One-Step RT-PCR Kit for Probes	170-8894

For ordering information on larger pack sizes, or to learn more about Bio-Rad amplification reagents and instruments, visit www.bio-rad.com/amplification/

Bio-Rad Laboratories
2000 Alfred Nobel Drive, Hercules, CA 94547
510-741-1000

10002300 Rev B

Reaction Setup

Important Note – Please Read Before Starting

Spin all tubes before opening to improve recovery. Reactions should be set up on ice. Pipet all components in the order given below. Always add iProof DNA Polymerase last to the reaction as primer degradation may occur in the absence of dNTPs. It is recommended that you prepare a master mix for the appropriate number of samples to be amplified.

Typical Reaction Setup

Component	Volume for 50 µl reaction	Volume for 20 µl reaction	Final Conc.
5X iProof HF Buffer*	10 µl	4 µl	1X
dNTP mix	1 µl	0.4 µl	200 µM each
Primer 1**	x µl	x µl	0.5 µM
Primer 2**	x µl	x µl	0.5 µM
DNA template	x µl	x µl	
Sterile H_20	x µl	x µl	
iProof DNA Polymerase	0.5 µl	0.2 µl***	0.02 U/µl
Total Volume	50 µl	20 µl	

* For difficult or GC-rich templates, 5X iProof GC Buffer can be used.
** Recommended final primer concentration is 0.5 µM; can range between 0.2–1.0 µM.
*** Enzyme should be diluted to avoid pipeting errors.

Control Template Reaction Setup

Component	Volume for 50 µl reaction	Volume for 20 µl reaction	Final Conc.
5X iProof HF Buffer	10 µl	4 µl	1X
dNTP mix	1 µl	0.4 µl	200 µM each
Primers *	2.5 µl	1 µl	0.2 µM
Control DNA Template	2 µl	0.8 µl	
Sterile H_20	34 µl	13.6 µl	
iProof DNA Polymerase	0.5 µl	0.2 µl**	0.02 U/µl
Total Volume	50 µl	20 µl	

* Either 1.3 kb or 10 kb primers
** Enzyme should be diluted to avoid pipetting errors.

Notes About Reaction Components

1. **iProof DNA Polymerase**
   The optimal amount of enzyme depends on the amount of template and the length of the PCR product. Usually 1 unit of iProof DNA polymerase per 50 µl reaction will give good results, but optimal amounts could range from 0.5–2 units per 50 µl reaction depending on amplicon length and difficulty. **Do not exceed 2 U/50 µl (0.04 U/µl), especially for amplicons that are > 5kb**.
2. **Buffers**
   Two buffers are provided: 5x iProof HF buffer and 5x iProof GC buffer. The error rate of iProof polymerase in HF buffer (4.4×10^{-7}) is lower than that in GC buffer (9.5×10^{-7}). Therefore, the HF buffer should be used as the default buffer for high fidelity amplification. However, the GC buffer can improve iProof performance on certain difficult or long templates, i.e. GC rich templates or those with complex secondary structures. Only use GC buffer when amplification with HF buffer does not provide satisfactory results.

3. **Mg^{2+} and dNTP**
   Mg^{2+} concentration is critical since iProof is a Mg^{2+}-dependent enzyme. Excessive Mg^{2+} stabilizes dsDNA, preventing complete denaturation, and can also promote inaccurate priming. Conversely, insufficient amounts of Mg^{2+} can lead to low product yield. The optimal Mg^{2+} concentration also depends on dNTP concentration, the specific DNA template and the sample buffer composition. The optimal Mg^{2+} concentration is 0.5 to 1 mM over the total dNTP concentration for standard PCR. For optimization, increase or decrease Mg^{2+} concentration in 0.2 mM increments.

   Only high quality dNTPs should be used. Use of dUTP or other dUTP-derivatives or analogs is not recommended. Due to the increased processivity of iProof, there is no advantage to increasing dNTP amounts. For optimal results, use 200 mM dNTPs.

4. **DNA Template**
   General guidelines are 1 pg–10 ng of DNA template in a 50 µl reaction for low complexity DNA (e.g. plasmid, lambda, or BAC DNA). For high complexity DNA (e.g. genomic DNA), 50–500 ng of template DNA should be used in a 50 µl reaction.

5. **PCR Additives**
   The recommended reaction conditions for GC-rich templates include the addition of 3% DMSO which aids in template denaturation. Further optimization of DMSO should be made in 2% increments. In some cases, DMSO may be used to help relax supercoiled plasmid DNA. High DMSO concentrations (10%) will require lowering the annealing temperature by 5.5–6.0°C. Other PCR additives such as formamide, glycerol, and betaine are also compatible with iProof.

Cycling Conditions

Important Note – Please Read

Due to the novel nature of iProof DNA polymerase, optimal reaction conditions may differ from standard PCR protocols. iProof works better at elevated denaturation and annealing temperatures due to higher salt concentration in the reaction buffer.

Typical Thermal Cycling Protocol

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	5–10 s	
Annealing	45–72°C	10–30 s	25–35
Extension	72°C	15–30 s / kb	
Final Extension	72°C	5–10 min	1

Control Template (1.3 kb) Cycling Protocol (2-step)

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	1 min	1
Denaturation	98°C	5 s	
Annealing/Extension	72°C	20 s	25–35
Final Extension	72°C	10 min	1

Control Template (10 kb) Cycling Protocol (3-step)*

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	1 min	1
Denaturation	98°C	5 s	
Annealing	60°C	15 s	25–35
Extension	72°C	2 min 30 sec	
Final Extension	72°C	10 min	1

* Both control template reactions can be run using the 10 kb cycling protocol.

Appendix 1K
pENTR/D-TOPO Cloning Instruction Manual

pENTR™ Directional TOPO® Cloning Kits

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an entry vector for the Gateway® System

Catalog nos. K2400-20, K2420-20, K2525-20, K2535-20, K2435-20, and K2635-20

Version G
6 April 2006
25-0434

User Manual

Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Design PCR Primers</td><td>• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer.
• Design the primers such that your gene of interest will be optimally expressed and fused in frame with the TEV recognition site (in pENTR™/TEV/D-TOPO® only) or any N- or C-terminal tags, if desired (after recombination with the Gateway® destination vector).</td></tr>
<tr><td>Amplify Your Gene of Interest</td><td>1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product.
2. Use agarose gel electrophoresis to check the integrity and determine the yield of your PCR product.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td>1. Set up the following TOPO® Cloning reaction. For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
<table>
<tr><th>Reagent</th><th>Chemical Transformation</th><th>Electroporation</th></tr>
<tr><td>Fresh PCR product</td><td>0.5 to 4 µl</td><td>0.5 to 4 µl</td></tr>
<tr><td>Salt solution</td><td>1 µl</td><td>--</td></tr>
<tr><td>Dilute salt solution (1:4)</td><td>--</td><td>1 µl</td></tr>
<tr><td>Water</td><td>to a final volume of 5 µl</td><td>to a final volume of 5 µl</td></tr>
<tr><td>TOPO® vector</td><td>1 µl</td><td>1 µl</td></tr>
<tr><td>Total volume</td><td>6 µl</td><td>6 µl</td></tr>
</table>
2. Mix gently and incubate for 5 minutes at room temperature.
3. Place on ice and proceed to transform One Shot® chemically competent E. coli, below.</td></tr>
<tr><td>Transform One Shot® Chemically Competent E. coli</td><td>1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® chemically competent E. coli cells and mix gently.
2. Incubate on ice for 5 to 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.
4. Add 250 µl of room temperature S.O.C. Medium.
5. Incubate at 37°C for 1 hour with shaking.
6. Spread 50-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.</td></tr>
</table>

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 23-25 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Size	Catalog no.
pENTR™/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2400-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2435-20
pENTR™/SD/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2420-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2635-20
pENTR™/TEV/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2525-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2535-20

Shipping/Storage

Each pENTR™ Directional TOPO® Cloning Kit is shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pENTR™ TOPO® Reagents	-20°C
2	One Shot® Chemically Competent *E. coli*	-80°C

continued on next page

Kit Contents and Storage, continued

pENTR™ TOPO® Reagents

The following reagents are supplied with each pENTR™ TOPO® vector (Box 1). **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pENTR™ TOPO® vector, TOPO®-adapted (pENTR™/D-TOPO® **or** pENTR™/SD/D-TOPO® **or** pENTR™/TEV/D-TOPO®)	15-20 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Water	---	1 ml
M13 Forward (-20) Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
M13 Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl

Sequences of the Primers

The table below provides the sequences of the M13 Forward (-20) and M13 Reverse sequencing primers.

Primer	Sequence	pMoles Supplied
M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´	407
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385

continued on next page

Kit Contents and Storage, continued

One Shot® Reagents

The following reagents are included with the One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store Box 2 at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 or Mach1™-T1^R cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of *E. coli* Strains

TOP10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Mach1™-T1^R: F$^-$ Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(r_k^-, m_k^+) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

Information for Non-U.S. Customers Using Mach1™-T1^R Cells

The parental strain of Mach1™-T1^R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S.A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Accessory Products

Introduction

The products listed in this section may be used with the pENTR™ Directional TOPO® Cloning Kits. For more information, refer to www.invitrogen.com or call Technical Service (see page 35).

Additional Products

Many of the reagents supplied in the pENTR™ Directional TOPO® Cloning Kits and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® TOP10 **Chemically Competent** *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 **Electrocompetent** *E. coli*	10 reactions	C4040-50
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03
M13 Forward (-20) Primer	2 µg (407 pmoles)	N520-02
M13 Reverse Primer	2 µg (385 pmoles)	N530-02
Kanamycin Sulfate	1 g	11815-016
LB Broth	500 ml	10855-021
LB Agar	500 g	22700-025
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
Gateway® LR Clonase™ Plus Enzyme Mix	20 reactions	12538-013
MultiSite Gateway® Three-Fragment Vector Construction Kit	1 kit	12537-023
AcTEV™ Protease	1,000 units	12575-015
	10,000 units	12575-023

x

Introduction

Overview

Introduction

The pENTR™ Directional TOPO® Cloning Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for entry into the Gateway® System or the MultiSite Gateway® System available from Invitrogen. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

A choice of pENTR™ Directional TOPO® vectors is available for optimal expression of your PCR product after recombination with the Gateway® destination vector of interest (see table below).

Vector	Benefit
pENTR™/D-TOPO®	For efficient expression of your gene of interest after recombination with a Gateway® destination vector
pENTR™/SD/D-TOPO®	Contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) for optimal expression of native protein after recombination with a prokaryotic Gateway® destination vector **Note:** Also suitable for efficient expression of your gene of interest in other hosts after recombination with a Gateway® destination vector (*e.g.* mammalian, insect, yeast)
pENTR™/TEV/D-TOPO®	Contains a Tobacco Etch Virus (TEV) recognition site for efficient TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein after recombination and expression from a Gateway® destination vector

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway® Technology, simply:

1. TOPO® Clone your blunt-end PCR product into one of the pENTR™ TOPO® vectors to generate an entry clone.
2. Generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway® destination vector of choice.
3. Introduce your expression construct into the appropriate host (*e.g.* bacterial, mammalian, yeast, insect) and express your recombinant protein.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 35).

continued on next page

Overview, continued

MultiSite Gateway® Technology

The MultiSite Gateway® Technology uses modifications of the site-specific recombination reactions of the Gateway® Technology (see the previous page) to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation. The MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023) facilitates simultaneous cloning of DNA fragments in three entry vectors to create your own expression clone. For more information about the MultiSite Gateway® Technology and the MultiSite Gateway® Three-Fragment Vector Construction Kit, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual which is available for downloading from our Web site or by contacting Technical Service.

Features of the pENTR™ TOPO® Vectors

The pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for entry into the Gateway® System. Features of the vectors include:

- *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway® destination vector
- T7 gene 10 translation enhancer and ribosome binding site for efficient translation of the PCR product in prokaryotes **(pENTR™/SD/D-TOPO® only)**
- TEV recognition site for TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein **(pENTR™/TEV/D-TOPO® only)**
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

How Directional TOPO® Cloning Works

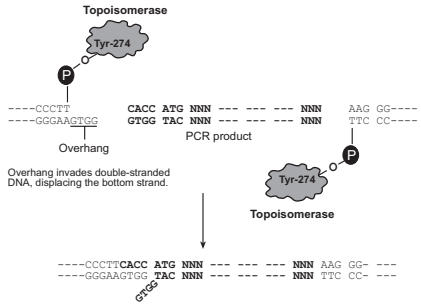
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT; see **Note** below) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



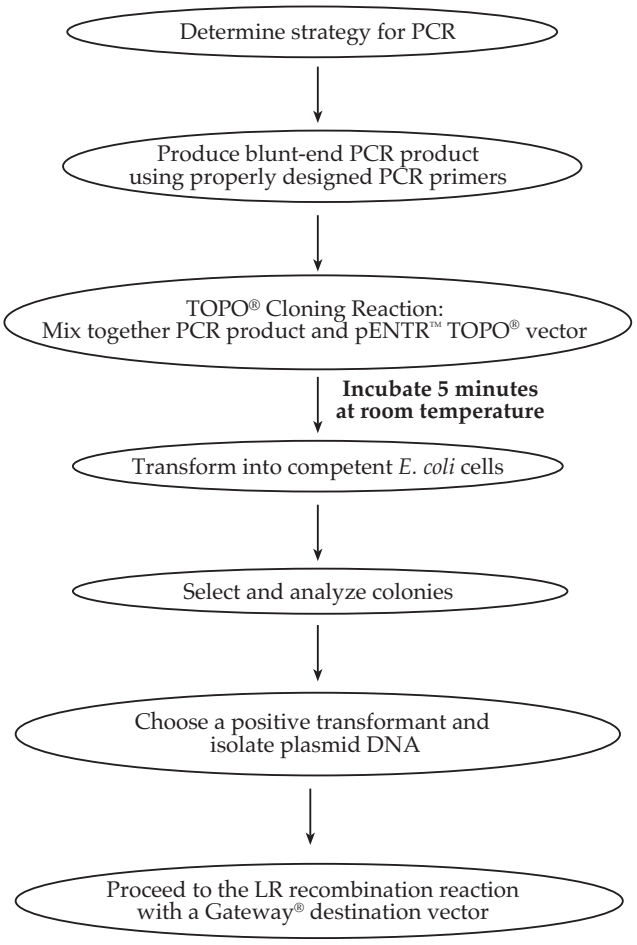
Note

The 5′ TOPO® recognition site in pENTR™/TEV/D-TOPO® is encoded by the sequence TCCTT rather than CCCTT. This is because the 5′ TOPO® recognition site directly follows the TEV recognition site, and studies have shown that TEV protease does not cleave efficiently if the first amino acid following the TEV recognition sequence is proline (Kapust *et al.*, 2002) as would be the case if the 5′ TOPO® recognition site was encoded by **CCC**TT. By changing the sequence of the 5′ TOPO® recognition site to **TCC**TT, the first amino acid following the TEV recognition site is now serine. **This change does not affect TOPO® Cloning efficiency and allows efficient TEV cleavage.**

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and clone your blunt-end PCR product.



4

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pENTR™ TOPO® vector you are using, consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal tag after recombination of your entry clone with a Gateway® destination vector

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pENTR™ TOPO® vector.
- If you plan to express your PCR product in mammalian cells as a native or C-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest should include a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)**NNATG**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** on the next page).
- If you plan to express your PCR product in mammalian cells as an N-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest does not need to contain a Kozak translation initiation sequence. A Kozak sequence is provided by the appropriate destination vector. **Note:** In this case, internal initiation may occur if your PCR product contains an endogenous Kozak sequence.
- If you plan to express your PCR product in **prokaryotic** cells without an N-terminal fusion tag (following recombination of the entry clone with a Gateway® destination vector), you should TOPO® Clone your PCR product into pENTR™/SD/D-TOPO®. pENTR™/SD/D-TOPO® contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) to enable efficient translation of the PCR product in *E. coli*. **To ensure optimal spacing for proper translation, design your forward PCR primer so that the ATG initiation codon of your PCR product directly follows the CACC necessary for directional cloning** (see **Example** on the next page).

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Designing PCR Primers, continued

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then:

- The ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.
- The ATG initiation codon is properly spaced from the RBS (in pENTR™/SD/D-TOPO® only), allowing proper translation of the PCR product in prokaryotic cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 on the next page).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you **do not** wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

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Designing PCR Primers, continued

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector). The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

…**GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′**

- To fuse the ORF in frame with a C-terminal tag (supplied by the destination vector after recombination), remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.
- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that the pENTR™ TOPO® vectors accept blunt-end PCR products.
- Do not add 5´ phosphates to your primers for PCR. This will prevent ligation into the pENTR™ TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

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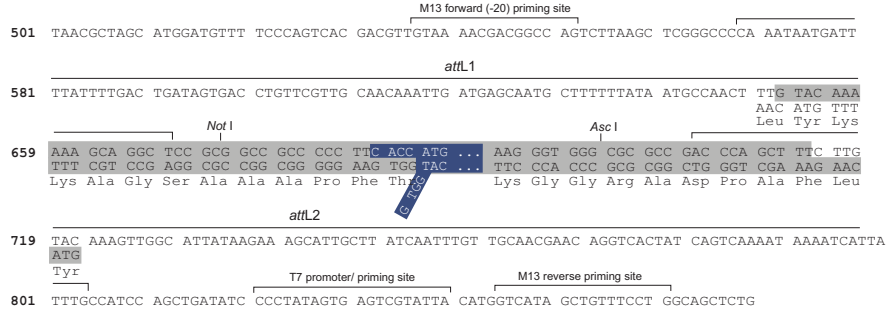
Designing PCR Primers, continued

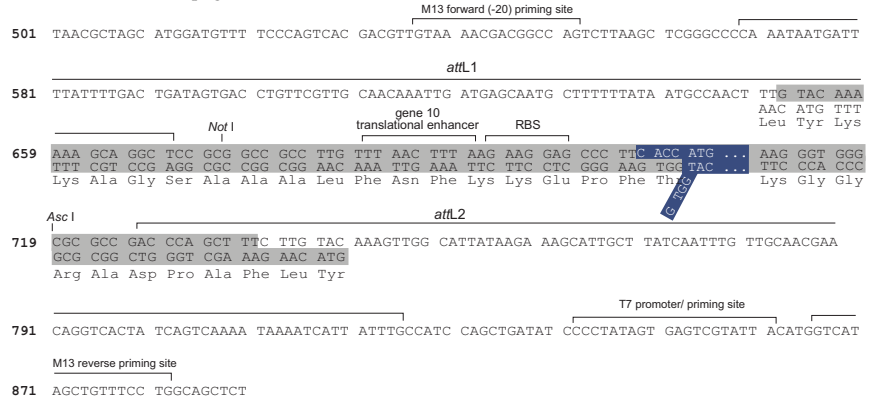
TOPO® Cloning Site for pENTR™/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/D-TOPO®, see pages 28-29.



TOPO® Cloning Site for pENTR™/SD/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/SD/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/SD/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/SD/D-TOPO®, see pages 30-31.



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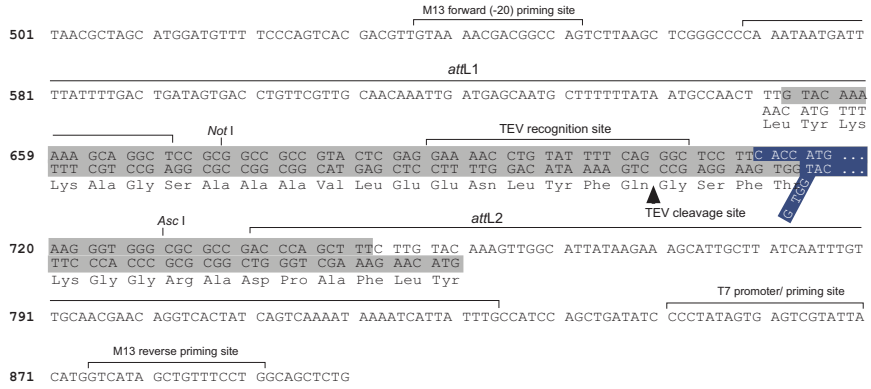
Designing PCR Primers, continued

TOPO® Cloning Site for pENTR™/TEV/ D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/TEV/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/TEV/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/TEV/D-TOPO®, see pages 32-33.

Note: The sequence of the 5′ TOPO® recognition site has been changed from **C**CCTT to **T**CCTT, resulting in an amino acid substitution of serine for proline**. This amino acid change increases the efficiency of TEV protease cleavage (Kapust *et al.*, 2002), but does not affect the efficiency of TOPO® Cloning.**



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

You will need the following reagents and equipment for PCR. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers to produce the PCR product

Producing Blunt-End PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at –20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 26-27).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Setting Up the TOPO® Cloning Reaction

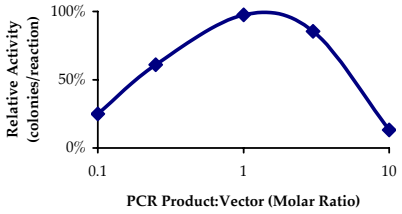
Introduction

Once you have produced the desired blunt-end PCR product, you are ready to TOPO® Clone it into the pENTR™ TOPO® vector and transform the recombinant vector into One Shot® competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent *E. coli*** (pages 13-14) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 23-25 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For pENTR™ TOPO® vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Setting Up the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page x for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Competent *E. coli***, next page.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pENTR™ TOPO® construct into competent *E. coli*. One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Needed

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment:

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* (supplied with the kit, Box 2)
- S. O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (to verify transformation efficiency, if desired, Box 2)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 50 µg/ml kanamycin (two for each transformation)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of One Shot® competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw **on ice** one vial of One Shot® cells from Box 2 for each transformation.

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Transforming One Shot® Competent *E. coli*, continued

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1^R chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature S.O.C. Medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the kanamycin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

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Transforming One Shot® Competent *E. coli*, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5-10 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml kanamycin.

   Note: If you transformed One Shot® Mach1™-T1^R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for only 4 hours in pre-warmed LB medium containing 50 µg/ml kanamycin before isolating plasmid DNA. For optimal results, inoculate as much of a single colony as possible.

2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

3. Analyze the plasmids by restriction analysis or PCR (see below) to confirm the presence and correct orientation of the insert.

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a combination of the M13 Forward (-20) primer or the M13 Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template.

Materials Needed:

- PCR Super Mix High Fidelity (Invitrogen, Catalog no. 10790-020)
- Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5-10 colonies and resuspend them individually in 50 µl of the PCR SuperMix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation. Use the M13 Forward (-20) and M13 Reverse included to help you sequence your insert (see the diagrams on pages 8-9 for the location of the priming sites in each pENTR™ TOPO® vector). For the complete sequence of each pENTR™ TOPO® vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 35).

Note: The M13 Forward (-20) and M13 Reverse primers are available separately from Invitrogen (see page x for ordering information).

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Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 23-25 or refer to the **Troubleshooting** section, page 21 for tips to help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Guidelines to Perform the LR Recombination Reaction

Introduction

Once you have obtained your entry clone, you may:

- Perform an LR recombination reaction using Gateway® LR Clonase™ II enzyme mix (see page x for ordering information) to transfer your gene of interest from the entry construct into any Gateway® destination vector of choice to generate an expression clone.
- Perform a MultiSite Gateway® LR recombination reaction with 5′ and 3′ entry clones, the appropriate MultiSite Gateway® destination vector, and LR Clonase™ Plus enzyme mix (see page x for ordering information) to generate an expression clone.

General guidelines are provided below.

Important

For most applications, we recommend performing the LR recombination reaction or the MultiSite Gateway® LR recombination reaction using a:

- Supercoiled entry clone(s) **and**
- Supercoiled destination vector

To catalyze the LR recombination reaction, we recommend using Gateway® LR Clonase™ II Enzyme Mix (see page x for ordering information). The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied by Invitrogen as separate components in LR Clonase™ enzyme mix (Catalog no. 11791-019) into an optimized single tube format to allow easier set-up of the LR recombination reaction. Follow the instructions included with the product to perform the LR recombination reaction.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired.

Destination Vectors

A large selection of Gateway® destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available, see our Web site (www.invitrogen.com) or call Technical Service (see page 35). Manuals supporting all of the destination vectors are available for downloading from our Web site or by contacting Technical Service.

***E. coli* Host**

Once you have performed the LR recombination reaction or the MultiSite Gateway® LR recombination reaction, you will transform the reaction mixture into competent *E. coli* and select for expression clones. You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α™, or equivalent for transformation. Do not transform the Gateway® or MultiSite Gateway® LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

continued on next page

Guidelines to Perform the LR Recombination Reaction

Performing the LR Recombination Reaction

To perform the Gateway® LR recombination reaction, you will need:

- Purified plasmid DNA of the entry clone containing your gene of interest
- A destination vector of choice
- LR Clonase™ II enzyme mix (see page x for ordering information)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate chemically competent *E. coli* host and growth media for expression
- Appropriate selective plates

For instructions to perform the LR recombination reaction, refer to the Gateway® Technology with Clonase™ II manual or to the manual for the destination vector you are using.

Performing the MultiSite Gateway® LR Recombination Reaction

Before you can perform the MultiSite Gateway® LR recombination reaction, you will first need to generate 5′ and 3′ entry clones using Invitrogen's MultiSite Gateway® Three-Fragment Vector Construction Kit (Catalog no. 12537-023). Once you have generated the 5′ and 3′ entry clones, you will use the 5′ and 3′ entry clones, the entry clone containing your gene of interest, and the other reagents supplied in the MultiSite Gateway® Three-Fragment Vector Construction Kit (including LR Clonase™ Plus enzyme mix and the pDEST™R4-R3 destination vector) in a MultiSite Gateway® LR recombination reaction to generate an expression clone.

For instructions to generate 5′ and 3′ entry clones and to perform the MultiSite Gateway® LR recombination reaction, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 23-25) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	• Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector. • Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify your PCR product.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies, continued	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and smaller PCR products. • Optimize your PCR. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	Make sure that the forward and reverse PCR primers are designed correctly.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the proofreading polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Component	Amount
Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile water	41.5 µl
Proofreading polymerase (1-2.5 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. Make sure that you see a single, discrete 750 bp band.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pENTR™ TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pENTR™/D-TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® competent cells using the protocol on page 14.
4. Spread 50-200 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

continued on next page

Performing the Control Reactions, continued

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pENTR™/D-TOPO®	*Not* I	Correct orientation: 127, 3203 Reverse orientation: 646, 2684 Empty vector: 2580
pENTR™/SD/D-TOPO®	*Not* I	Correct orientation: 148, 3203 Reverse orientation: 667, 2684 Empty vector: 2601
pENTR™/TEV/D-TOPO®	*Eco*R V/*Pst* I	Correct orientation: 757, 2602 Reverse orientation: 250, 3109 Empty vector: 2610

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation.

Relatively few colonies should be produced in the vector-only reaction.

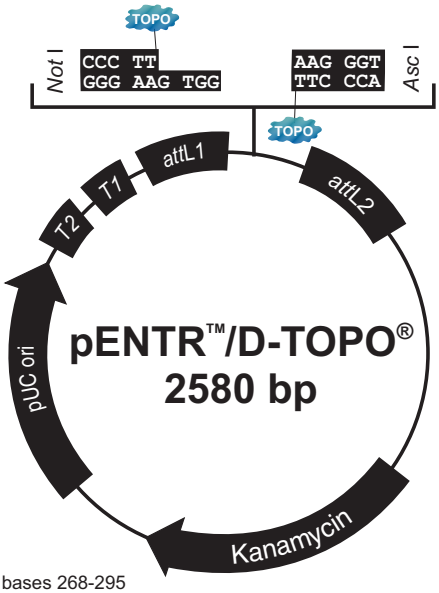
Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® competent cells with 10 pg of pUC19 using the protocol on page 14. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^9 cfu/µg DNA.

Map and Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® Map

The figure below shows the features of pENTR™/D-TOPO® vector. **The complete sequence of pENTR™/D-TOPO® is available for downloading from www.invitrogen.com)or by contacting Technical Service (see page 35).**



Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

continued on next page

Map and Features of pENTR™/D-TOPO®, continued

Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® (2580 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (-20) priming site	Allows sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Appendix 1L
QIAprep Spin Miniprep Handbook

Second Edition December 2006

QIAprep® Miniprep Handbook

For purification of molecular biology grade DNA

Plasmid
Large plasmids (>10 kb)
Low-copy plasmids and cosmids
Plasmid DNA prepared by other methods

QIAGEN

WWW.QIAGEN.COM

Kit Contents

QIAprep Spin Miniprep Kit	(50)	(250)
Catalog no.	**27104**	**27106**
QIAprep Spin Columns	50	250
Buffer P1	20 ml	73 ml
Buffer P2	20 ml	73 ml
Buffer N3*	30 ml	140 ml
Buffer PB*	30 ml	150 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
LyseBlue	20 µl	73 µl
RNase A†	200 µl	730 µl
Collection Tubes (2 ml)	50	250
Handbook	1	1

* Buffers N3 and PB contain chaotropic salts which are irritants and not compatible with disinfecting agents containing bleach. Take appropriate laboratory safety measures and wear gloves when handling. See page 9 for further information.

† Provided as a 10 mg/ml solution.

Storage

QIAprep Miniprep Kits should be stored dry at room temperature (15–25°C). Kits can be stored for up to 12 months without showing any reduction in performance and quality. For longer storage these kits can be kept at 2–8°C. If any precipitate forms in the buffers after storage at 2–8°C it should be redissolved by warming the buffers to 37°C before use.

After addition of RNase A and optional LyseBlue reagent, Buffer P1 is stable for 6 months when stored at 2–8°C. RNase A stock solution can be stored for two years at room temperature.

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of QIAprep Miniprep Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

QIAprep Miniprep Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

The QIAcube, BioRobot 3000, BioRobot 8000 and BioRobot Universal System workstations are intended for research applications. No claim or representation is intended for their use to provide information for the diagnosis, prevention, or treatment of a disease.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffers N3 and PB contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to QIAprep Miniprep Kits.

Buffer N3

Contains guanidine hydrochloride, acetic acid: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-23-26-36/37/39-46.

Buffer P2

Contains sodium hydroxide: irritant. Risk and safety phrases:* R36/38, S13-26-36-46.

Buffer PB

Contains guanidine hydrochloride, isopropanol: harmful, flammable, irritant. Risk and safety phrases:* R10-22-36/38, S13-23-26-36/37/39-46

RNase A

Contains ribonuclease: sensitizer. Risk and safety phrases:* R42/43, S23-24-26-36/37.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R22: Harmful if swallowed; R36/38: Irritating to eyes and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink, and animal feedingstuffs; S23; Do not breathe spray; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protecting clothing and gloves; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S46: If swallowed seek medical advice immediately and show the container or label.

Introduction

The QIAprep Miniprep system provides a fast, simple, and cost-effective plasmid miniprep method for routine molecular biology laboratory applications. QIAprep Miniprep Kits use silica membrane technology to eliminate the cumbersome steps associated with loose resins or slurries. Plasmid DNA purified with QIAprep Miniprep Kits is immediately ready for use. Phenol extraction and ethanol precipitation are not required, and high-quality plasmid DNA is eluted in a small volume of Tris buffer (included in each kit) or water. The QIAprep system consists of four products with different handling options to suit every throughput need.

Low throughput

The **QIAprep Spin Miniprep Kit** is designed for quick and convenient processing of 1–24 samples simultaneously in less than 30 minutes. QIAprep spin columns can be used in a microcentrifuge or on any vacuum manifold with luer connectors (e.g., QIAvac 24 Plus, or QIAvac 6S with QIAvac Luer Adapters).

The **QIAprep Spin Miniprep Kit** can be fully automated on the **QIAcube**. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute) enabling you to continue using the QIAprep Spin Miniprep Kit for purification of high-quality plasmid DNA.

The QIAcube is preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/MyQIAcube .

Medium throughput

For medium throughput requirements the **QIAprep 8 Miniprep Kit and QIAprep 8 Turbo Miniprep Kit** utilize 8-well strips on QIAvac 6S allowing up to 48 minipreps to be performed simultaneously in approximately 40 and 30 minutes respectively. In addition, the **QIAprep 8 Turbo BioRobot® Kit** enables automated purification of up to 48 minipreps in 50 minutes on BioRobot systems.

High throughput

The **QIAprep 96 Turbo Miniprep Kit** enables up to 96 minipreps to be performed simultaneously in less than 45 minutes on the QIAvac 96. For automated high-throughput plasmid purification the **QIAprep 96 Turbo BioRobot Kit** enables up to 96 minipreps to be processed in 70 minutes.

Applications using QIAprep purified DNA

Plasmid DNA prepared using the QIAprep system is suitable for a variety of routine applications including:

- Restriction enzyme digestion
- Library screening
- In vitro translation
- Sequencing
- Ligation and transformation
- Transfection of robust cells

Principle

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (1). The unique silica membrane used in QIAprep Miniprep Kits completely replaces glass or silica slurries for plasmid minipreps.

The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrane
- Washing and elution of plasmid DNA

All steps are performed without the use of phenol, chloroform, CsCl, ethidium bromide, and without alcohol precipitation.

Preparation and clearing of bacterial lysate

The QIAprep miniprep procedure uses the modified alkaline lysis method of Birnboim and Doly (2). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. After lysate clearing, the sample is ready for purification on the QIAprep silica membrane. For more details on growth of bacterial cultures and alkaline lysis, please refer to Appendix A on pages 39–42. In the QIAprep Spin and QIAprep 8 miniprep procedures, lysates are cleared by centrifugation, while the QIAprep 8 and 96 Turbo Miniprep kits provide TurboFilter strips or plates for lysate clearing by filtration.

LyseBlue reagent*

Use of LyseBlue is optional and is not required to successfully perform plasmid preparations. See “Using LyseBlue reagent” on page 14 for more information.

* LyseBlue reagent is only supplied with QIAprep Spin Miniprep Kits since multiwell or automated formats do not allow visual control of individual samples.

LyseBlue is a color indicator which provides visual identification of optimum buffer mixing. This prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. This makes LyseBlue ideal for use by researchers who have not had much experience with plasmid preparations as well as experienced scientists who want to be assured of maximum product yield.

DNA adsorption to the QIAprep membrane

QIAprep columns, strips, and plates use a silica membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. The optimized buffers in the lysis procedure, combined with the unique silica membrane, ensure that only DNA will be adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane but are found in the flow-through.

Washing and elution of plasmid DNA

Endonucleases are efficiently removed by a brief wash step with Buffer PB. This step is essential when working with *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, to ensure that plasmid DNA is not degraded. The Buffer PB wash step is also necessary when purifying low-copy plasmids, where large culture volumes are used.

Salts are efficiently removed by a brief wash step with Buffer PE. High-quality plasmid DNA is then eluted from the QIAprep column with 50–100 µl of Buffer EB or water. The purified DNA is ready for immediate use in a range of applications — no need to precipitate, concentrate, or desalt.

Note: Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range. Store DNA at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent.

DNA yield

Plasmid yield with the QIAprep miniprep system varies depending on plasmid copy number per cell (see page 39), the individual insert in a plasmid, factors that affect growth of the bacterial culture (see pages 39–42), the elution volume (Figure 1), and the elution incubation time (Figure 2). A 1.5 ml overnight culture can yield from 5 to 15 µg of plasmid DNA (Table 1, page 14). To obtain the optimum combination of DNA quality, yield, and concentration, we recommend using Luria-Bertani (LB) medium for growth of cultures (for composition see page 41), eluting plasmid DNA in a volume of 50 µl, and performing a short incubation after addition of the elution buffer.

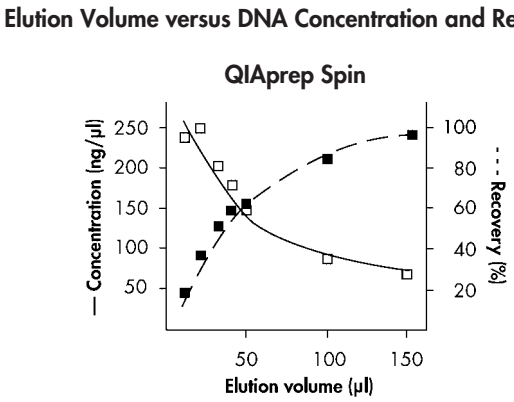


Figure 1 10 µg pUC18 DNA was purified using the QIAprep Spin protocol and eluted with the indicated volumes of Buffer EB. The standard protocol uses 50 µl Buffer EB for elution, since this combines high yield with high concentration. However the yield can be increased by increasing the elution volume.

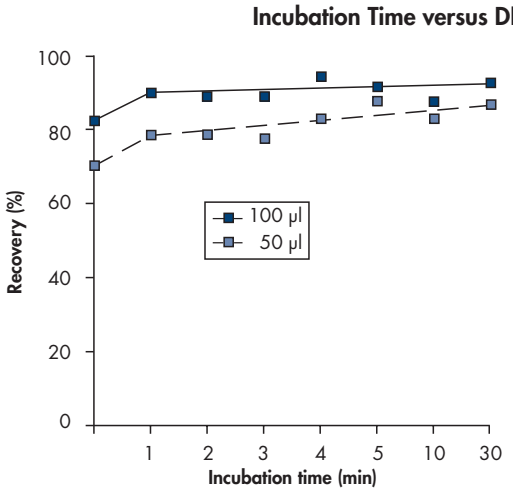


Figure 2 10 µg pBluescript DNA was purified using the QIAprep Spin Miniprep protocol and eluted after the indicated incubation times with either 50 µl or 100 µl Buffer EB. The graph shows that an incubation time of 1 minute and doubling the elution buffer volume increases yield.

Table 1. Effect of Different Compositions of Growth Medium LB on DNA Yield

Culture media	Yield
LB (containing 10 g/liter NaCl)	11.5 µg
LB (containing 5 g/liter NaCl)	9.5 µg

QIAprep Spin Miniprep Kit was used to purify DNA from 1.5 ml LB overnight cultures of XL1-Blue containing pBluescript®. Elution was performed according to the standard protocol (50 µl Buffer EB and 1 min incubation). Use of the recommended LB composition (with 10 g/liter NaCl, also see Appendix A, p. 43) provides optimal plasmid yield.

Using LyseBlue reagent

Using a simple visual identification system, LyseBlue reagent prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, cell debris, and genomic DNA.

LyseBlue can be added to the resuspension buffer (Buffer P1) bottle before use. Alternatively, smaller amounts of LyseBlue can be added to aliquots of Buffer P1, enabling single plasmid preparations incorporating visual lysis control to be performed.

LyseBlue reagent should be added to Buffer P1 at a ratio of 1:1000 to achieve the required working concentration (e.g., 10 µl LyseBlue into 10 ml Buffer P1). Make sufficient LyseBlue/Buffer P1 working solution for the number of plasmid preps being performed.

LyseBlue precipitates after addition into Buffer P1. This precipitate will completely dissolve after addition of Buffer P2. Shake Buffer P1 before use to resuspend LyseBlue particles.

The plasmid preparation procedure is performed as usual. After addition of Buffer P2 to Buffer P1, the color of the suspension changes to blue. Mixing should result in a homogeneously colored suspension. If the suspension contains localized regions of colorless solution or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

Upon addition of neutralization buffer (Buffer N3), LyseBlue turns colorless. The presence of a homogeneous solution with no traces of blue indicates that SDS from the lysis buffer has been effectively precipitated.

Important Notes

Please read the following notes before starting any of the QIAprep procedures.

Growth of bacterial cultures in tubes or flasks

1. **Pick a single colony from a freshly streaked selective plate and inoculate a culture of 1–5 ml LB medium containing the appropriate selective antibiotic. Incubate for 12–16 h at 37°C with vigorous shaking.**

   Growth for more than 16 h is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 x *g*) in a conventional, table-top microcentrifuge for 3 min at room temperature (15–25°C).**

   The bacterial cells can also be harvested in 15 ml centrifuge tubes at 5400 x *g* for 10 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

Buffer notes

- Add the provided RNase A solution to Buffer P1, mix, and store at 2–8°C.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Check Buffers P2 and N3 before use for salt precipitation. Redissolve any precipitate by warming to 37°C. Do not shake Buffer P2 vigorously.
- Close the bottle containing Buffer P2 immediately after use to avoid acidification of Buffer P2 from CO_2 in the air.
- Buffers P2, N3, and PB contain irritants. Wear gloves when handling these buffers.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (spin down briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

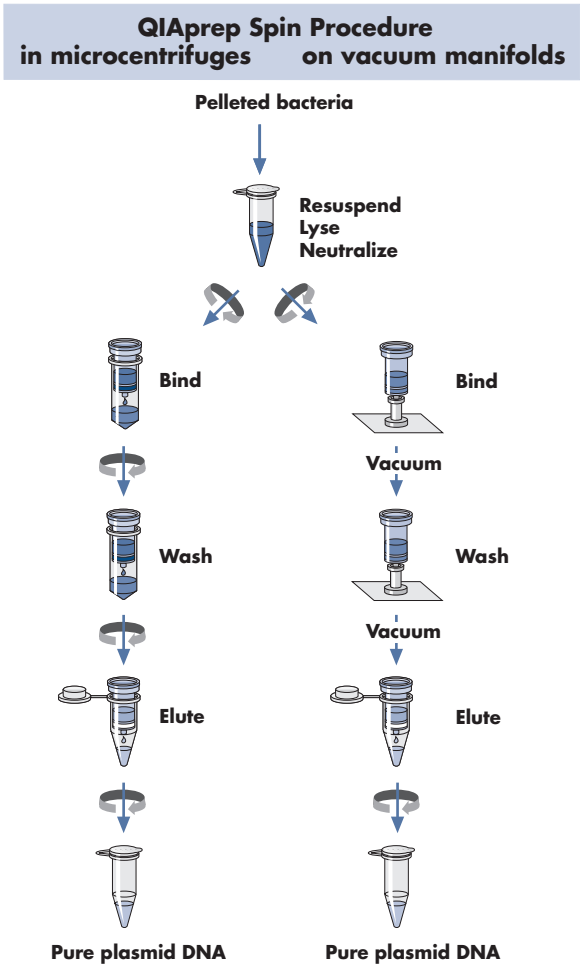
Centrifugation notes

- All centrifugation steps are carried out at 13,000 rpm (~17,900 x *g*) in a conventional, table-top microcentrifuge.

Elution notes

- Ensure that the elution buffer is dispensed directly onto the center of the QIAprep membrane for optimal elution of DNA. Average eluate volume is 48 µl from an elution-buffer volume of 50 µl (QIAprep spin procedures), and 60 µl from an elution-buffer volume of 100 µl (QIAprep multiwell procedures).
- For increased DNA yield, use a higher elution-buffer volume. For increased DNA concentration, use a lower elution-buffer volume (see "DNA yield", pages 13–14).
- If water is used for elution, make sure that its pH is between 7.0 and 8.5. Elution efficiency is dependent on pH and the maximum elution efficiency is achieved within this range. A pH <7.0 can decrease yield.

  Note: Store DNA at –20°C when eluted with water, as DNA may degrade in the absence of a buffering agent.
- DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.


QIAprep Spin Procedure
in microcentrifuges
on vacuum manifolds
Pelleted bacteria
Resuspend
Lyse
Neutralize
Bind
Bind
Vacuum
Wash
Wash
Vacuum
Elute
Elute
Pure plasmid DNA
Pure plasmid DNA

Protocol: Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 44.

Please read "Important Notes" on pages 15–21 before starting.

Note: All protocol steps should be carried out at room temperature.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.
8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
10. **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol(s) in this handbook or molecular biology applications (see back cover for contact information).

		Comments and suggestions
Low or no yield		
General		Low yields may be caused by a number of factors. To find the source of the problem, analyze fractions saved from each step in the procedure on an agarose gel (e.g., Figure 6, page 43). A small amount of the cleared lysate and the entire flow-through can be precipitated by adding 0.7 volumes isopropanol and centrifuging at maximum speed (13,000 rpm or ~17,000 x *g*) for 30 minutes. The entire wash flow-through can be precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.0, and 0.7 volumes of isopropanol.
No DNA in the cleared lysate before loading		
a)	Plasmid did not propagate	Read "Growth of bacterial cultures" (pages 39–41) and check that the conditions for optimal growth were met.
b)	Lysate prepared incorrectly	Check storage conditions and age of buffers.
c)	Buffer P2 precipitated	Redissolve by warming to 37°C.
d)	Cell resuspension incomplete	Pelleted cells should be completely resuspended in Buffer P1. Do not add Buffer P2 until an even suspension is obtained.
DNA is found in the flow-through of cleared lysate		
a)	QIAprep membrane overloaded	If rich culture media, such as TB or 2x YT are used, culture volumes must be reduced. It may be necessary to adjust LB culture volume if the plasmid and host strain show extremely high copy number or growth rates. See "Culture media" on page 41.

		Comments and suggestions
b)	RNase A digestion omitted	Ensure that RNase A is added to Buffer P1 before use.
c)	RNase A digestion insufficient	Reduce culture volume if necessary. If Buffer P1 containing RNase A is more than 6 months old, add additional RNase A.

DNA is found in the wash flow-through

Ethanol omitted from wash buffer		Repeat procedure with correctly prepared wash buffer (Buffer PE).

Little or no DNA in eluate

a)	Elution buffer incorrect	DNA is eluted only in the presence of low-salt buffer (e.g., Buffer EB [10 mM Tris·Cl, pH 8.5] or water). Elution efficiency is dependent on pH. The maximum efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range.
b)	Elution buffer incorrectly dispensed onto membrane	Add elution buffer to the center of the QIAprep membrane to ensure that the buffer completely covers the surface of the membrane for maximum elution efficiency.

Low DNA quality

DNA does not perform well in downstream applications

a)	Eluate salt concentration too high	For the QIAprep spin column, modify the wash step by incubating the column for 5 minutes at room temperature after adding 0.75 ml of Buffer PE and then centrifuging. For QIAprep 8 preparations and QIAprep 8 and 96 Turbo preparations, ensure that two wash steps are carried out prior to elution.
b)	Nuclease contamination	When using *endA*$^+$ host strains such as HB101 and its derivatives, the JM series, or any wild-type strain, ensure that the wash step with Buffer PB is performed.
c)	Eluate contains residual ethanol	Ensure that step 9 in the QIAprep Spin Miniprep protocol and steps 9 and 10 in the QIAprep 8 Miniprep, QIAprep 8 Turbo Miniprep, or QIAprep 96 Turbo Miniprep protocols are performed.

		Comments and suggestions
RNA in the eluate		
a)	RNase A digestion omitted	Ensure that RNase A is added to Buffer P1 before use.
b)	RNase A digestion insufficient	Reduce culture volume if necessary. If Buffer P1 containing RNase A is more than 6 months old, add additional RNase A.
Genomic DNA in the eluate		
a)	Buffer P2 added incorrectly	The lysate must be handled gently after addition of Buffer P2 to prevent shearing. Reduce culture volume if lysate is too viscous for gentle mixing.
b)	Buffer N3 added incorrectly	Upon addition of Buffer N3 in step 3, mix immediately but gently.
c)	Lysis too long	Lysis in step 2 must not exceed 5 minutes.
d)	Culture overgrown	Overgrown cultures contain lysed cells and degraded DNA. Do not grow cultures for longer than 12–16 hours.

Appendix A: Background Information

Growth of bacterial cultures

Plasmids are generally prepared from bacterial cultures grown in the presence of a selective agent such as an antibiotic (3,4). The yield and quality of plasmid DNA may depend on factors such as plasmid copy number, host strain, inoculation, antibiotic, and type of culture medium.

Plasmid copy number

Plasmids vary widely in their copy number per cell (Table 5), depending on their origin of replication (e.g., pMB1, ColE1, or pSC101) which determines whether they are under relaxed or stringent control; and depending on the size of the plasmid and its associated insert. Some plasmids, such as the pUC series and derivatives, have mutations which allow them to reach very high copy numbers within the bacterial cell. Plasmids based on pBR322 and cosmids are generally present in lower copy numbers. Very large plasmids and cosmids are often maintained at very low copy numbers per cell.

Table 5. Origins of replication and copy numbers of various plasmids (3).

DNA construct	Origin of replication	Copy number	Classification
Plasmids			
pUC vectors	pMB1*	500–700	high copy
pBluescript vectors	ColE1	300–500	high copy
pGEM® vectors	pMB1*	300–400	high copy
pTZ vectors	pMB1*	>1000	high copy
pBR322 and derivatives	pMB1*	15–20	low copy
pACYC and derivatives	p15A	10–12	low copy
pSC101 and derivatives	pSC101	~5	very low copy
Cosmids			
SuperCos	ColE1	10–20	low copy
pWE15	ColE1	10–20	low copy

* The pMB1 origin of replication is closely related to that of ColE1 and falls in the same incompatibility group. The high-copy-number plasmids listed here contain mutated versions of this origin.

Host strains

Most *E. coli* strains can be used successfully to isolate plasmid DNA, although the strain used to propagate a plasmid has an effect on the quality of the purified DNA. Host strains such as DH1, DH5α, and C600 give high-quality DNA. The slower growing strain XL1-Blue also yields DNA of very high-quality which works extremely well for sequencing. Strain HB101 and its derivatives, such as TG1 and the JM series, produce large amounts of carbohydrates, which are released during lysis and can inhibit enzyme activities if not completely removed (4). In addition, these strains have high levels of endonuclease activity which can reduce DNA quality. The methylation and growth characteristics of the strain should also be taken into account when selecting a host strain. XL1-Blue and DH5α are highly recommended for reproducible and reliable results.

Inoculation

Bacterial cultures for plasmid preparation should always be grown from a single colony picked from a freshly streaked selective plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures may lead to uneven plasmid yield or loss of the plasmid. Inoculation from plates that have been stored for a long time may also lead to loss or mutation of the plasmid.

The desired clone should be streaked from a glycerol stock onto a freshly prepared agar plate containing the appropriate selective agent so that single colonies can be isolated. This procedure should then be repeated to ensure that a single colony of an antibiotic-resistant clone can be picked. A single colony should be inoculated into 1–5 ml of media containing the appropriate selective agent, and grown with vigorous shaking for 12–16 hours. Growth for more than 16 hours is not recommended since cells begin to lyse and plasmid yields may be reduced.

Antibiotics

Antibiotic selection should be applied at all stages of growth. Many plasmids in use today do not contain the *par* locus which ensures that the plasmids segregate equally during cell division. Daughter cells that do not receive plasmids will replicate much faster than plasmid-containing cells in the absence of selective pressure, and can quickly take over the culture.

The stability of the selective agent should also be taken into account. Resistance to ampicillin, for example, is mediated by β-lactamase which is encoded by the plasmid-linked *bla* gene and which hydrolyzes ampicillin. Levels of ampicillin in the culture medium are thus continually depleted. This phenomenon is clearly demonstrated on ampicillin plates, where "satellite colonies" appear as the ampicillin is hydrolyzed in the vicinity of a growing colony. Ampicillin is also very sensitive to temperature, and when in solution should be stored frozen in single-use aliquots. The recommendations given in Table 6 are based on these considerations.

Table 6. Concentrations of Commonly Used Antibiotics

Antibiotic	Stock solutions Concentration	Storage	Working concentration (dilution)
Ampicillin (sodium salt)	50 mg/ml in water	–20°C	100 µg/ml (1/500)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml (1/200)
Kanamycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Streptomycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Tetracycline HCl	5 mg/ml in ethanol	–20°C	50 µg/ml (1/100)

Culture media

Luria-Bertani (LB) broth is the recommended culture medium for use with QIAprep Kits, since richer broths such as TB (Terrific Broth) or 2x YT lead to extremely high cell densities, which can overload the purification system. It should be noted that cultures grown in TB may yield 2–5 times the number of cells compared to cultures grown in LB broth. If these media are used, recommended culture volumes must be reduced to match the capacity of the QIAprep membrane. If excess culture volume is used, alkaline lysis will be inefficient, the QIAprep membrane will be overloaded, and the performance of the system will be unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing, which may result in shearing of bacterial genomic DNA and contamination of the plasmid DNA. Care must also be taken if strains are used which grow unusually fast or to very high cell densities. In such cases, doubling the volumes of Buffers P1, P2, and N3 may be beneficial. It is best to calculate culture cell density and adjust the volume accordingly.

Please note that a number of slightly different LB culture broths, containing different concentrations of NaCl, are in common use. Although different LB broths produce similar cell densities after overnight culture, plasmid yields can vary significantly.

Table 7. Recommended composition of Luria Bertani medium

Contents	Per liter
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Preparation of cell lysates

Bacteria are lysed under alkaline conditions. After harvesting and resuspension, the bacterial cells are lysed in NaOH/SDS (Buffer P2) in the presence of RNase A (2, 5). SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents while the alkaline conditions denature the chromosomal and plasmid DNAs, as well as proteins. The optimized lysis time allows maximum release of plasmid DNA without release of chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline conditions may cause the plasmid to become irreversibly denatured (2). This denatured form of the plasmid runs faster on agarose gels and is resistant to restriction enzyme digestion.

The lysate is neutralized and adjusted to high-salt binding conditions in one step by the addition of Buffer N3. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate, while the smaller plasmid DNA renatures correctly and stays in solution. It is important that the solution is thoroughly and gently mixed to ensure complete precipitation.

To prevent contamination of plasmid DNA with chromosomal DNA, vigorous stirring and vortexing must be avoided during lysis. Separation of plasmid from chromosomal DNA is based on coprecipitation of the cell wall-bound chromosomal DNA with insoluble complexes containing salt, detergent, and protein. Plasmid DNA remains in the clear supernatant. Vigorous treatment during the lysis procedure will shear the bacterial chromosome, leaving free chromosomal DNA fragments in the supernatant. Since chromosomal fragments are chemically indistinguishable from plasmid DNA under the conditions used, the two species will not be separated on QIAprep membrane and will elute under the same low-salt conditions. Mixing during the lysis procedure must therefore be carried out by slow, gentle inversion of the tube.

Appendix B: Agarose Gel Analysis of Plasmid DNA

The QIAprep Miniprep procedure can be analyzed using agarose gel electrophoresis as shown in Figure 6. Samples can be taken from the cleared lysate and its flow-through, precipitated with isopropanol and resuspended in a minimal volume of TE buffer. In Figure 6 the cleared lysate shows closed circular plasmid DNA and degraded RNase A-resistant RNA. The flow-through contains only degraded RNA and no plasmid DNA is present. The eluted pure plasmid DNA shows no contamination with other nucleic acids.

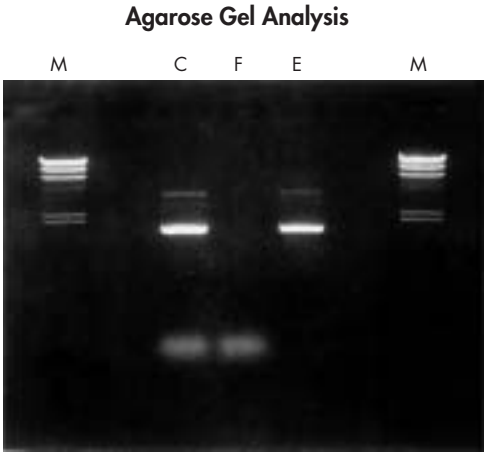


Figure 6 Agarose gel analysis of the QIAprep Miniprep procedure. **C**: cleared lysate; **F**: flow-through; **E**: eluted plasmid; **M**: markers.

Appendix C: Special Applications

Purification of low-copy plasmids and cosmids

All QIAprep miniprep protocols in this handbook can be used for preparation of low-copy-number plasmid or cosmids from 1–10 ml overnight *E. coli* cultures grown in LB medium.

Only two slight modifications to the protocols are required:

- The wash step with Buffer PB is required for all strains.
- When plasmid or cosmids are >10 kb, pre-heat Buffer EB (or water) to 70°C prior to eluting DNA from the QIAprep membrane. A 10 ml overnight LB culture typically yields 5–10 µg DNA.

Note: When using 10 ml culture volume, it is recommended to double the volumes of Buffers P1, P2, and N3 used.

Purification of very large plasmids (>50 kb)

Plasmids >50 kb elute less efficiently from silica than smaller plasmids, but do elute efficiently from QIAGEN anion-exchange resin. QIAGEN provides the anion-exchange-based QIAGEN Large-Construct Kit for efficient large-scale purification of ultrapure genomic DNA-free BAC, PAC, P1, or cosmid DNA. For high-throughput, small-scale purification of BACs, PACs, and P1s, an optimized alkaline lysis protocol in R.E.A.L.® Prep 96 Kits yields DNA suitable for sequencing and screening. Call QIAGEN Technical Services or your local distributor for more information on these kits, or see ordering information on page 47.

Purification of plasmid DNA prepared by other methods

Plasmid DNA isolated by other methods can be further purified using QIAprep modules and any of the QIAprep protocols in this handbook.

1. Add 5 volumes of Buffer PB to 1 volume of the DNA solution and mix (e.g., add 500 µl Buffer PB to 100 µl of DNA sample).
2. Apply the samples to QIAprep spin columns or to the wells of a QIAprep 8 strip or 96-well plate. Draw the samples through the QIAprep membrane by centrifugation or vacuum, and continue the appropriate protocol at the Buffer PE wash step. The optional wash step with Buffer PB is not necessary.

References

1. Vogelstein, B., and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**, 615–619.
2. Birnboim, H.C., and Doly, J. (1979) A rapid alkaline lysis procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**, 1513–1522.
3. Sambrook, J. et al., eds. (1989) Molecular cloning: a laboratory manual. 2nd ed., Cold Spring Harbor Laboratory Press.
4. Ausubel, F.M. et al., eds. (1991) Current protocols in molecular biology. Wiley Interscience, New York.
5. Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100**, 243–255.

Bench Protocol: QIAprep Spin Miniprep Kit Using a Microcentrifuge

This protocol is designed for the purification of up to 20 µg high-copy plasmid DNA from 1–5 ml overnight *E. coli* culture in LB medium. New users and users wanting to purify low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods should refer to the detailed protocols provided in the *QIAprep Miniprep Handbook*, 2nd ed.

Things to do before starting

- Add RNase A solution to Buffer P1.
- Optional: Add LyseBlue reagent to Buffer P1.
- Add ethanol (96–100%) to Buffer PE.
- Check Buffers P2 and N3 for salt precipitation and redissolve at 37°C if necessary.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**
2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   If using LyseBlue reagent, solution turns blue.
3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   If using LyseBlue reagent, solution turns colorless.
4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**
5. **Apply the supernatant (from step 4) to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is only required when using *endA*$^+$ or other bacteria strains with high nuclease activity or carbohydrate content (see *QIAprep Miniprep Handbook* for more details)
8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**
10. **To elute DNA, place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 50 µl Buffer EB or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

APPENDIX 2 - BIOINFORMATICS (PARTS I AND II)

HC70AL Spring 2004

An Introduction to Bioinformatics -- Part I

By

Brandon Le

April 6, 2004

What are the Characteristics of a Gene?

- An ordered sequence of nucleotides
- A unique position/location in the genome
- Polarity (5' to 3')
- Exons and Introns

What are the Anatomical Features of Genes?

- Discrete beginning and discrete end
- Two strands of DNA
- Double helical
- Strand one (5' to 3')
- Strand two (3' to 5')
- Sense strand (5' to 3')
  - specifies the trait
- Nonsense strand (3' to 5')
  - template for transcription

Sense Strand

```
5' - ACGTCAGTCGATGCATGCTAGCTAGC - 3'
3' - TGCAGTCAGCTACGTACGATCGATCG - 5'
```

Nonsense Strand

Genes Have a Unique Position in the Genome!

Task: Where is your gene located in the genome?

Tools: The Arabidopsis Information Resources (TAIR)
(http://www.arabidopsis.org)

Procedure:

1. Select Seqviewer
2. Enter gene number (ex. AT1G18260)
3. Submit

Results/Question:

1. What chromosome is your gene in?
2. What other genes/markers are next to your gene?
3. What is the exact position of your gene in the genome?

01	AT2G22800
02	AT2G23290
03	AT2G37120
04	AT3G09735
05	AT3G12840
06	AT3G50060
07	AT3G53370
08	AT4G37260
09	AT4G37790
10	AT5G03220
11	AT5G03500
12	AT5G19490
13	AT5G67300

Genes Have a Unique Order of Nucleotides!

Task: What is the order of nucleotides for your gene?

Tools: The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org)

Procedure: (Continue from previous slide)

1. Click on Location

Results/Question:

1. What are your neighbor genes?
2. What is the orientation of your gene?
3. How big is your gene?

Genes Have Exons and Introns!

Task: How many exons and introns does your gene have?

Tools: The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org)

Procedure: (Continue from previous slide)

1. Click on gene information on the right

Results/Question:

1. How many exons/introns in your gene?
2. What are exons?
3. What are introns?

Gene Encodes a Protein

Task: Determine the protein encoded by gene?

Tools: The Arabidopsis Information Resources (TAIR)
(http://www.arabidopsis.org)

Results/Question:

1. **How large is your protein?**
2. **What are the anatomy of a protein?**

N-terminal  **C-terminal**

What is the identity of your gene?

Task: What does your gene code for?

Tools: NCBI BLAST Tools
(http://www.ncbi.nlm.nih.gov/BLAST)

What is BLAST?

Basic Local Alignment Search Tool (BLAST)

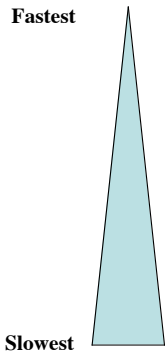
What does BLAST do?

A family of programs that allows you to input a query sequence and compare it to DNA or protein sequences in db.

What are the steps to performing BLAST search?

Paste sequence of interest into BLAST input box
Select BLAST program
Select db
Select Optional Parameters

What are the different BLAST Programs?



blastp - protein query vs protein db

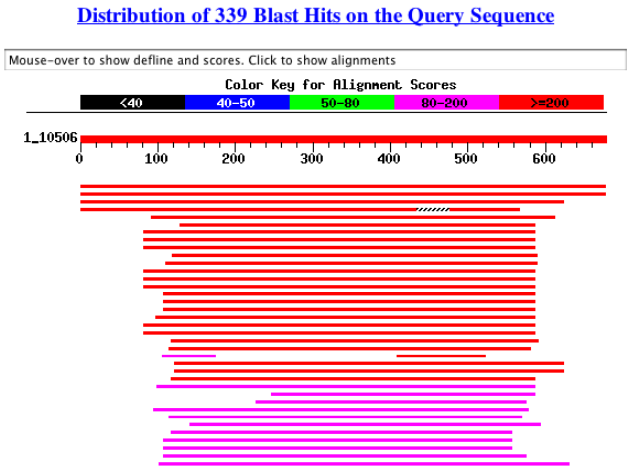
blastn - DNA query vs DNA db

blastx - translated DNA query vs protein db

tblastx - protein query vs translated DNA db

tblastn - translated DNA query vs translated DNA db

Anatomy of a BLAST Result -- Part I



Anatomy of a BLAST Result -- Part II

Sequences producing significant alignments:	Score (bits)	E Value
gi|14532716|gb|AAK64159.1| unknown protein [Arabidopsis tha...	1206	0.0
gi|18394588|ref|NP_564049.1| suppressor of lin-12-like prot...	1209	0.0
gi|15219499|ref|NP_177498.1| suppressor of lin-12-like prot...	877	0.0
gi|11120786|gb|AAG30966.1| hypothetical protein, 3' partial...	426	e-118
gi|41151276|ref|XP_046437.5| chromosome 20 open reading fra...	291	3e-77
gi|13559241|emb|CAB65792.2| dJ842G6.2 (novel protein imilar...	282	2e-74
gi|19923669|ref|NP_005056.3| sel-1 suppressor of lin-12-lik...	268	4e-70
gi|6851089|gb|AAF29413.1| SEL1L [Homo sapiens] >gi|17646138...	268	4e-70
gi|9967440|dbj|BAB12403.1| SEL1L [Mesocricetus auratus]	264	4e-69
gi|31203035|ref|XP_310466.1| ENSANGP00000019196 [Anopheles ...	263	1e-68
gi|21355295|ref|NP_651179.1| CG10221-PA [Drosophila melanog...	263	1e-68
gi|20857527|ref|XP_127076.1| Sel1 (suppressor of lin-12) 1 ...	261	4e-68
gi|4159995|gb|AAD05210.1| SEL1L [Mus musculus] >gi|20073079...	259	1e-67
gi|29336095|ref|NP_808794.1| Sel1 (suppressor of lin-12) 1 ...	259	2e-67
gi|29612522|gb|AAH49959.1| Sel1h protein [Mus musculus]	258	4e-67
gi|17563256|ref|NP_506144.1| Suppressor/Enhancer of Lin-12 ...	247	9e-64
gi|1255199|gb|AAC47112.1| sel-1 gene product	247	9e-64

Anatomy of a BLAST Result -- Part III

```
>gi|14532716|gb|AAK64159.1|   unknown protein [Arabidopsis thaliana]
          Length = 678

 Score = 1206 bits (3120), Expect = 0.0
 Identities = 614/678 (90%), Positives = 614/678 (90%)

Query: 1   MRILSYGIVILSLLVFSFIEFGVHARPVVLVXXXXXXXXXXXXXXXVXXXXXXXXXXXXXX 60
           MRILSYGIVILSLLVFSFIEFGVHARPVVLV              V
Sbjct: 1   MRILSYGIVILSLLVFSFIEFGVHARPVVLVLSNDDLNSGGDDNGVGESSDFDEFGESEP 60

Query: 61  XXXXXLDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120
                LDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS
Sbjct: 61  KSEEELDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120

Query: 121 AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV 180
           AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV
Sbjct: 121 AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV 180

Query: 181 QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG 240
           QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG
Sbjct: 181 QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG 240

Query: 241 NANAMYKIGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
           NANAMYK GLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT
Sbjct: 241 NANAMYKNGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
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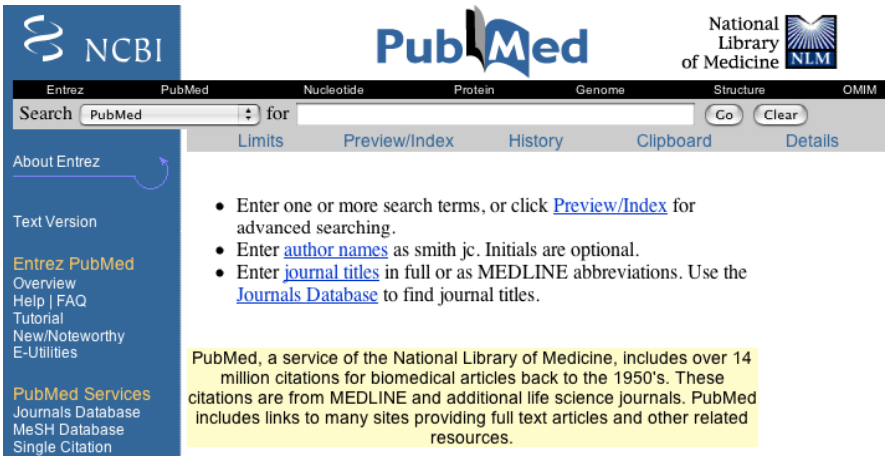
PubMed - Endless Resources



HC70AL Spring 2004

An Introduction to Bioinformatics -- Part II

By

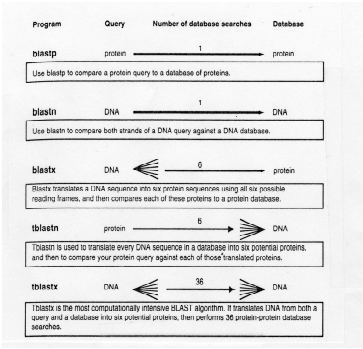
Brandon Le

April 8, 2004

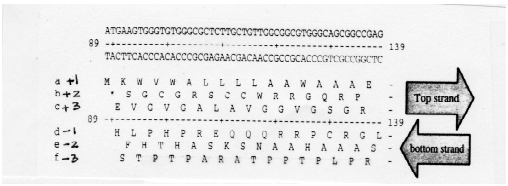
Review of BLAST Search

1. What is the purpose of running BLAST Search?
2. What are the steps to performing BLAST search?
3. What does the e-value from a blast result tell you?
4. How may BLAST program can you perform?
5. What BLAST program(s) takes the least computational time?
6. What BLAST program(s) takes the most computational time? Why?

What are the Five BLAST Search Programs?



•How many proteins can a short DNA sequence potentially encode?



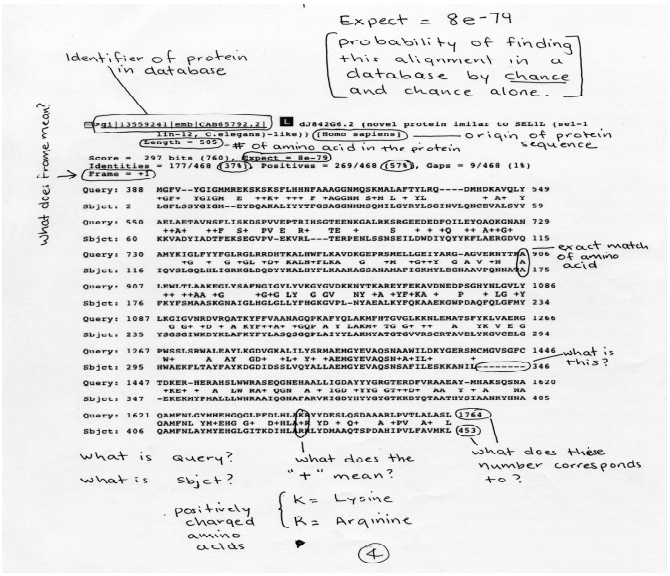
Question:

You have DNA Sequence. You want to know which protein in the main protein database is most similar to some protein encoded by your DNA.

Which BLAST program should you use?

Suppose you have a protein sequence.
Which BLAST program should you use?

HOW to interpret BLAST results?



Review of gene transcription

1. What product is made after transcription?
2. How is the product similar/different from the gene?
3. What is cDNA?
4. What important information does a cDNA tell you about a gene?
5. What are ESTs?
6. What important information does ESTs tell you about a gene?

Annotation of your gene

1. What chromosome is your gene in?
2. How "big" is your gene?
3. How many exons and introns in your gene?
4. What orientation is your gene in the genome?
5. What is the specific position of your gene in the genome?
6. What gene is "upstream" of your gene?
7. What gene is "downstream" of your gene?
8. How far are the other genes (6 & 7) from your gene?
9. What is the "structure" of your gene?
10. What is the size of the protein in your gene encodes?
11. What protein does your gene encode
12. Is your gene structure predicted by a program?

Webbook - A Virtual Lab Notebook

Webbook is a web lab notebook

Purpose/goal: To have access to experiments carried out b
Lab members, etc… from anywhere
Also serves as a repository for protocols, stocks/reagents

Created by: Harry Hahn
Brandon Le
Bob Goldberg

http//estdb.biology.ucla.edu/webbook

Using the Webboook

1. **Username:** email username
   Password: 9 digit student id
2. **Check message board for important news/updates**
3. **An overview of the different sections**

Projects - **list of experiments**

Stocks - **catalog of stocks/reagent in the lab**

Protocols - **procedures carried out in the lab (pdf format)**

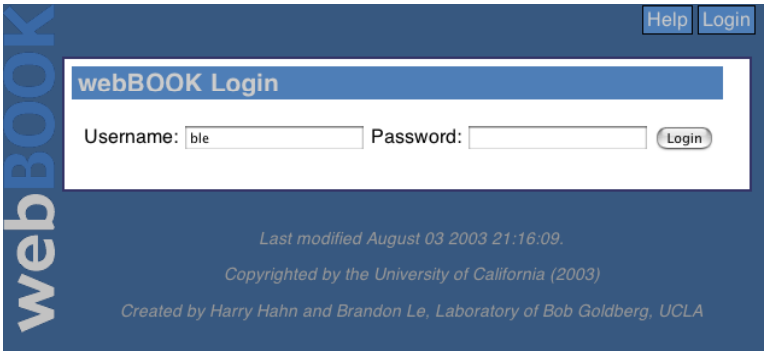
Calendar - **calendar to plant your experiments**

Browse - **search and look at other members experiments**

Contact - **email for help**

Logout - **will logout if idle for 30 min**

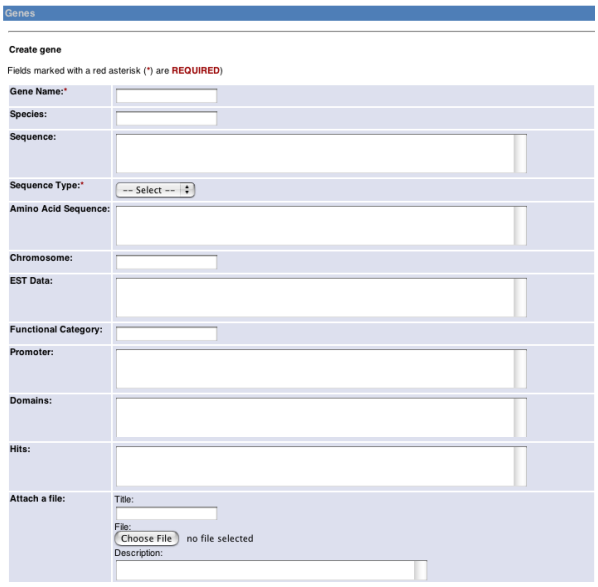
Webbook Login Page



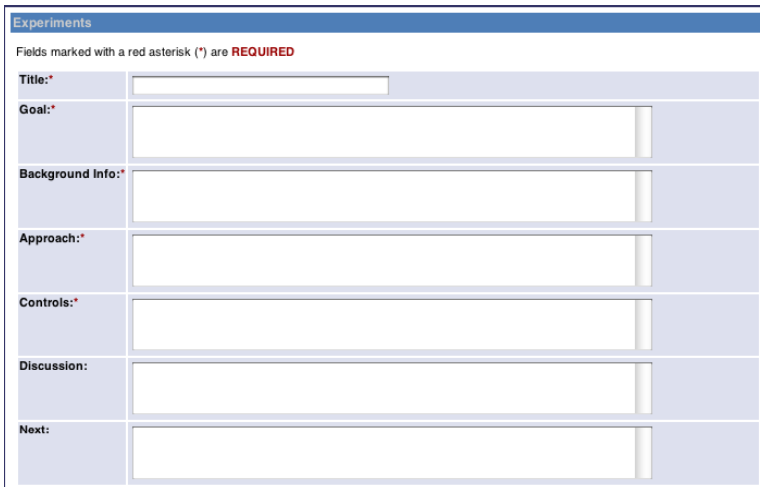
Creating Projects / Experiments

1. Title of project
2. Questions/Purpose of project
3. Summary of project (ideas)

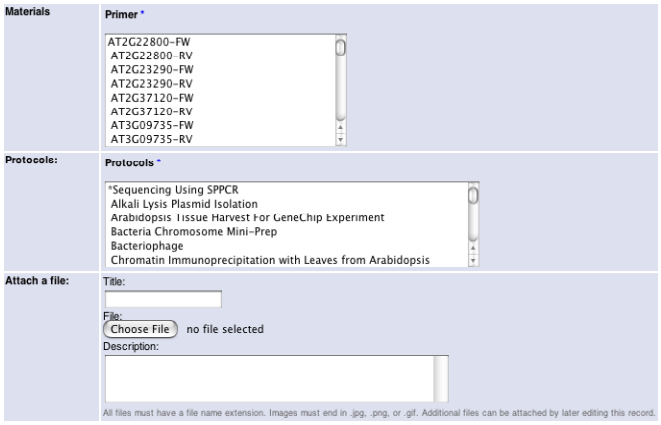
Entering Gene Information



Entering Experiments Information
Part 1



Entering Experiment Information
Part II



Entering References Relating to your Gene