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.

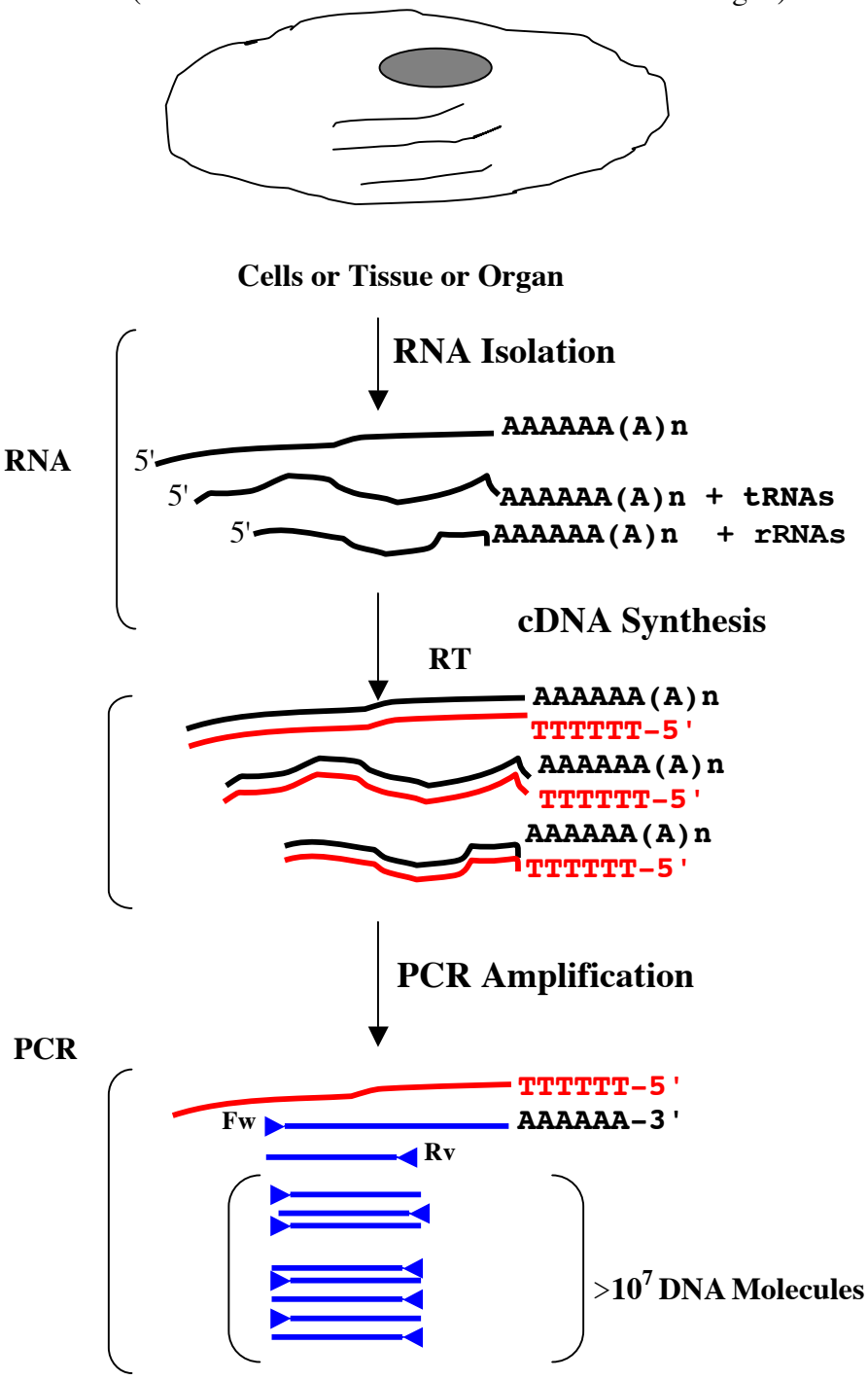
OVERVIEW OF RT-PCR STRATEGY

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?