EXPERIMENT 3 – GENE EXPRESSION STUDY IN *ARABIDOPSIS THALIANA* (GENE ONE)

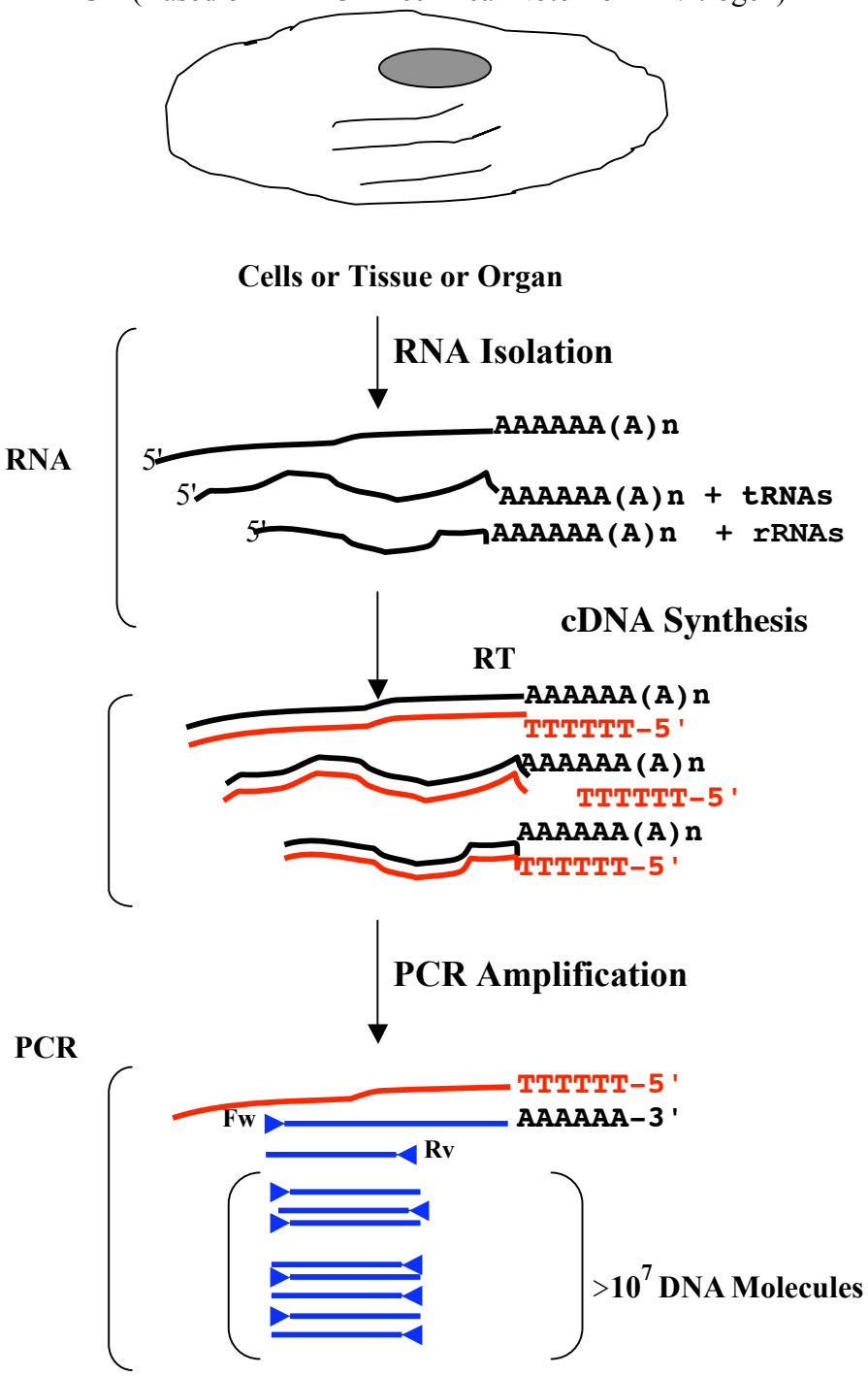
Purpose: To determine mRNA accumulation patterns of genes encoding transcription factors in Arabidopsis leaves and seeds.

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 tissues/organs for gene expression study.

Reference:

- Qiagen's RNeasy Plant Mini Kit Protocol (accompanied the kit).
- Sambrook et al. (1989). Molecular Cloning Manual Volume 1.
- Ambion's DNase-Free Technical Bulletin

FREQUENT ASKED QUESTIONS

PROCEDURE

A. RNA ISOLATION

B. REMOVING CONTAMINATED GENOMIC DNA FROM TOTAL RNA SOLUTION USING RNase-FREE DNase I

C. DETERMINING QUALITY OF ISOLATED TOTAL RNA BEFORE AND AFTER DNase I-TREATMENT VIA GEL ELECTROPHORESIS

Materials and Reagents Needed:

- Plant organs: leaves and seeds from Arabidopsis.
- Qiagen RNeasy Plant Mini Kit: (Cat. # 74903 for 20 extractions or 74904 for 50 extractions) containing extraction buffer, PE buffer, RNase-free water.
- DiEthyl PyroCarbonate (DEPC). *Note: DEPC is suspected to be carcinogen and corrosive. Therefore, it is handled with care! DEPC inhibits RNase*.
- One to Three Gel apparatus (gel boxes, 20-tooth combs, gel trays and casts) treated with DEPC water and air-dried on a drying rack overnight.
- **500 mL** of freshly prepared 0.05% **DEPC**-treated water (**non-autoclaved**) for cleaning up pipetman, microcentrifuge rotor and chamber, racks for microcentrifuge tubes, SpeedVac rotor and chamber, vortex mixer.
- β-mercaptoethanol. *Caution: work in the fume hood because this chemical has very bad odor*.
- RNA ladder (Invitrogen)
- Formamide (Ultrapure grade, Invitrogen)
- 37% formaldehyde (Fisher)
- Ambion DNase I kit (stored at -20^{o}C)
- Sevag (24 chloroform :1 isoamyl alcohol, v/v)
- Buffer-saturated phenol (Invitrogen)
- 3M NaOAc, pH 5.2 or 6.0
- Ice-cold 100% and 80% ethanol solutions dedicated for RNA work
- Loading dye for RNA samples
- 10X MOPS buffer
- **Autoclaved** DEPC-treated (**DEPC'd**) water
- Agarose dedicated for RNA work
- 5 mg/mL ethidium bromide. *Caution: this chemical is suspected carcinogen*.
- Liquid Nitrogen. *Caution: It is very cold (at least -100˚C). Avoid getting frost-bite.*
- Black ultra-fine sharpie
- RNase-free spatulas
- RNase-free 14-mL disposable centrifuge tubes
- RNase-free 1.5 mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- RNase-free filtered PCR tips for P-20, P-200, P-1000
- White Revco storage boxes
- Gloves (small, medium, large, or extra-large)
- Microcentrifuges
- Razor blades
- Kimwipes
- Plastic wrap
- SpeedVac
- Spectrophotometer (Nanodrop)
- Vortex
- Ice bucket or plastic container
- 55 ^{o}C Water bath
- 37 ^{o}C Heat block

FREQUENT ASKED QUESTIONS
(Taken from Qiagen RNeasy Plant Mini Handbook June 2001)

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 **RLT** (Guanidine Isothiocyanate) is used for all tissues except endosperm and tissues containing endosperms (e.g., seeds).
- Buffer **RLC** (Guanidine Hydrochloride) is used for seeds with endosperm

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 I-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.

<u>Cautions:</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 less than 20˚C.
- **Keep** all reagents, glassware, plasticware, and equipment **RNase-free.**
- **Use aerosol-barrier pipet tips throughout the procedure.**
- **Change GLOVES frequently!**

PROCEDURE

Attention: *Before isolating RNA, use Kimwipes wetted with freshly prepared non-autoclaved DEPC treated water to clean all equipments (pipetman sets, pipetman stand, microcentrifuge-tube racks, microcentrifuges and its rotors, speedvac, test-tube racks, pens and sharpies) to be used in isolating RNA*.

A. RNA ISOLATION

1. Label on the WHITE area on the side of TWO RNase-free **14-mL disposable centrifuge tubes "Leaf" or "Seed" and your initial**. Chill them on either **crushed dry ice** or a styrofoam floater in a styrofoam box containing **liquid nitrogen** (filling up to one-third of the styrofoam).
2. Chill **RNase-free** spatulas in a Dewar flask containing liquid nitrogen.
3. Remove bottles/tubes containing **frozen ground organs** from a **–80 °C freezer** and set them on **crushed dry-ice** in a styrofoam container or in **liquid nitrogen**.
4. (Option) Add **small amount of liquid nitrogen** to the bottles containing **ground organs** (leaves, seeds) to ensure that the frozen powder is not partially thawed out.
5. Use a **chilled spatula** to transfer small amount **(~100 mg)** of **frozen ground material** from the bottle to a chilled **14-mL centrifuge tube**. **Keep** the tube on dry ice or in liquid nitrogen. Proceed with other tubes for all organs.

Organs	Type of Lysis Buffer used
➢	RLT or RLC (circle one)
➢	RLT or RLC (circle one)
➢	RLT or RLC (circle one)
➢	RLT or RLC (circle one)
➢	RLT or RLC (circle one)
➢	RLT or RLC (circle one)

6. Aliquot an **appropriate volume** (**= # of organs x 500 µL**) of **lysis buffer** to a **14-mL disposable centrifuge tube** standing on a test-tube rack. (TA will prepare lysis buffer and β-mercaptoethanol).

7. Add **10 μL** of **β-mercaptoethanol** for every **1 mL** of **lysis buffer** in a fume hood. Mix well 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 LS 2828.*

Volume of lysis buffer (**mL**)	Volume of β-mercaptoethanol **(μL)**
_____ RLT	_____
_____ RLC	_____

8. Remove the chilled tube containing **~100mg** of ground organ powder from the styrofoam container and set on the rack at room temperature. Briefly, tap the tube on the bench to loosen frozen powder.
9. **Immediately**, pipet **450 μL of lysis buffer containing β-mercaptoethanol** into the 14-mL tube containing **~100 mg** of starting organ. Cap the tube. **Immediately**, vortex the tube **vigorously** for at least **1-2 minutes**. Then set the tube back on a tube rack. *The **lysate** should appear clear with no lumps of ground organ powder.*
   (Optional) A short incubation time (1-3 minutes) at 56^{o}C may help to disrupt the tissue. But NOT appropriate for an organ, such as seeds or old leaves, rich in starch.
10. **Repeat steps 8-9** for all organs.
11. Label **QIAshredder** (**purple**) spin columns placed in **2-mL collection tubes**.
12. Pipet the lysate directly onto a QIAshredder spin column.
13. Centrifuge at **FULL speed** (13,200 rpm) for **2 minutes**.
14. Meanwhile, label on the lids of TWO 1.5-mL RNase-free microcentrifuge tubes "**Leaf**" or "**Seed**" and **your initial**. Set the labeled tubes on a microcentrifuge-tube rack at room temperature.
15. Transfer ~ **450 μL** of the **supernatant** (**= volume of sample**) of the **flow-through solution** to a **NEW RNase-free 1.5-mL microcentrifuge tube** without disturbing the cell-debris pellet.
16. Add **0.5 volume** (or **225 μL**) of **room temperature 96-100% ethanol** to the **clear lysate**. **Immediately**, mix the mixture by pipetting **up and down for 10 times**.
17. Apply **~675 μL** of the **mixture** (including any precipitate) to an **RNeasy** (**pink**) **mini column** placed in a **2-mL collection tube**. Close the lid of the tube **gently**.

18. Centrifuge for **15 seconds** at **10,000 rpm** (or FULL speed).
19. Remove the **spin column** from the collection tube with one hand and hold it while **pouring** off the **flow-through solution** in the **collection tube**. Put the column back on the collection tubes.

    Note: if the sample volume is >700 μL, load aliquots successively onto the RNeasy column and centrifuge as before.
20. Add **700 μL** of **buffer RW1** to the RNeasy column. Close the lid of the tube.
21. Centrifuge for **15 seconds** at **10,000 rpm** to wash the column.
22. Transfer the column(s) to **NEW 2-mL collection tube**(s).
23. Discard the flow-through solution and collection tubes.
24. Pipet **500 μL** of **buffer RPE** onto each RNeasy column.
25. Centrifuge for **15 seconds** at **10,000 rpm** to wash the column.
26. Discard the flow-through solution as in step 18.
27. Add another **500 μL** of **buffer RPE** to the column.
28. Centrifuge for **1 minute** at **10,000 rpm** to wash the RNeasy silica-gel membrane again.
29. Discard the flow-through solution as in step 18.
30. Spin the column **again** for **1 minute** to ensure that ethanol is removed completely from the membrane. *Caution: This step is **crucial** because if residual ethanol is still on the membrane, it will be eluted with RNA in steps 32-35. If this is the case, RNA solution will float up when it is loaded on an agarose gel.*
31. While spinning at step 29, label on the **lid** and **side** of RNase-free 1.5-mL microcentrifuge tubes "**Name of Sample RNA**", "**your initial**", and "**date**".
32. Transfer the **spin columns** to these **NEW labeled tubes**.
33. Pipet 30 μL of **RNase-free water** (supplied with the kit) or DEPC-treated water directly onto the center of the silica-gel membrane of the RNeasy columns.
34. Wait for **1 minute** to allow water to evenly absorbed in the membrane.
35. Centrifuge for **1 minute** at **10,000 rpm** to elute RNA.
36. Repeat steps 32-34 with **another 20 μL** of **RNase-free water**.
37. Mix the content in the tubes by gently flicking. Put tubes **on ice**. *Note: From this step on, **KEEP** RNA solution **ON ICE** to prevent **RNA degradation**.*

38. Determine the **total volume** of **RNA solution** using a P-200 pipetman. The volume should be **~48 μL**.

39. Determine **RNA concentration** and **total amount** using a **spectrophotometer**.

*Note: (a) If **Nanodrop** spectrophotometer is used, there is **NO** need to make a dilution of RNA solution, and the concentration is directly given in **ng/μL**;(b) however, if **Beckman** or **other brand** spectrophotometer is used, dilute a small volume of RNA solution to 1/50 dilution (i.e., 4 μL RNA solution in 200 μL total volume), and the calculation is determined as shown below:*

Calculations:

[RNA] = (OD_{A260} reading) (Dilution factor) (40 μg/mL.OD) = **X μg/mL** or

= **X μg/μL**

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

Records of organs and their RNA concentration and total amount

Organs	**[RNA] (μg/μL)**	**Estimated Total Amount (μg)**
Leaves		
Seeds		

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

B. REMOVING CONTAMINATING GENOMIC DNA FROM TOTAL RNA SOLUTION USING RNase-FREE DNase I

Reference: Modification from the Ambion protocol accompanied the DNase-Free components (Cat # 1906).

Important Note: *This protocol is suitable for* ***removing up to 1 μg of DNA*** *from RNA in a* ***25-100 μL*** *reaction volume.*

PROCEDURE

1. Add **0.1 volume** of **10X DNase I buffer** and **1 μL** of **2 Units/μL DNase I** (Ambion) to the RNA solution. **One unit** of **DNase I** is defined as the amount of enzyme that degrades **1 μg of DNA** in **10 minutes** at **37°C** (Ambion).

	RNA solution			
DEPC'd water	**1.0** μL			
RNA sample	**25.0** μL			
10X DNase I buffer	**3.0** μL			
DNase I (2 Units/μL)	**1.0** μL			
Total volume	**30.0** μL			

2. Mix the solutions gently by flicking the tubes. Spin briefly (**10 seconds**).
3. Incubate at **37 °C** in a **heat block** for **20-30 minutes**. Spin tubes for **10 seconds** in a microcentrifuge.
4. To inactivate DNase I, add **0.1 volume** (or **3.0 μL**) of the **DNase inactivation reagent** (**slurry)** to the sample. Mix well by **flicking the tube**.
   Note: Make sure the slurry is WHITE. If the DNase inactivation reagent is CLEAR, revortex the mixture for a few seconds.
5. Incubate the tube at **room temperature** for **2 minutes**. Flick the tube **once more during the incubation** to re-disperse the **DNase inactivation reagent**.
6. At the meantime, label on the **lids** and **sides** of **NEW RNase-free microcentrifuge tubes** "**Purified Leaf RNA**" or "**Purified Seed RNA**", "**your initial**", and "**date**".

7. Spin the tube at ~**10,000 rpm** for **1 minute** to pellet the **DNase inactivation reagent**.
8. **Carefully**, Pipet ~**30 µL** of the **RNA solution** (*AVOID pipetting the PELLET!*) and transfer it into NEW labeled tubes. *Note: It is **okay** if **tiny amount** of the pellet **is carried over** in the RNA solution*.
9. Keep RNA tubes **on ice**.
10. Determine RNA concentration using a Nanodrop or Beckman spectrophotometer

    Calculations (if Using Beckman spectrophotometer):

    [RNA] = (OD_{A260} reading) (Dilution factor) (40 µg/mL.OD) = X µg/mL

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

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

Organs	**[RNA] (µg/µL)**	**Estimate Total Amount (µg)**
Purified Leaf RNA		
Purified Seed RNA		

11. Store the RNA solution at **-20°C for up to 1 week** or **-70°C for up to 6 months**. **Alternatively**, precipitate RNA by adding **0.1 volume** (or **3.0 µL**) of **3 M NaOAc, pH 5.2** and 3 volumes (or **90 µL**) of **ice-cold 100% ethanol** (dedicated for RNA work); mix well by **inverting 10 times** and keep in the **-70°C freezer** until use.

C. DETERMINING QUALITY OF ISOLATED TOTAL RNA BEFORE AND AFTER DNase I-TREATMENT VIA GEL ELECTROPHORESIS

1. Prepare a **1.3% A**garose **F**ormaldehyde (**AF**) midi gel (10 cm x 17 cm)

	100 mL
Agarose	**1.3 g**
DEPC'd water	**80.0 mL**
10X MOPS buffer	**10.0 mL**

2. Microwave for **2-5 minutes**. Swirl to ensure that all agarose granules are melted.
3. Cool the agarose mixture in a **55^{o}C water bath** for **at least 30 minutes**.
4. In a fume hood, add **9.8 mL** of **37% formaldehyde solution** to the warm agarose mixture using a sterile (RNase-free) disposable 10-mL pipet. ***Immediately***, swirl **gently** to mix the mixture. *Caution: Avoid creating many bubbles*.
5. Cast the gel and allow agarose to solidify for ~30 minutes in a **fume hood** (room LS 2828).
6. Prepare **700 mL** of **1X MOPS running buffer** (**70** mL of **10X MOPS buffer** + **630 mL** of **DEPC'd water**) in a **1-L Erlenmyer flask**.

   Note: 10X MOPS buffer is prepared as follows:

10X MOPS buffer	**1 Liter**	**Final Concentration**
MOPS	46.2 g	0.2 M or 200 mM
Sodium Acetate (trihydrates)	10.9 g	0.08 M or 80 mM
0.5 M EDTA, pH 8.0	20 mL	0.01 M or 10 mM
DEPC'd water	800 mL	

   - Adjust **pH to 7.0** with a **NaOH solution**.
   - Bring the **final volume** to **1 Liter** with DEPC'd water.
   - Filter the buffer using a **Nalgene filtering unit**.
   - Autoclave for **15 minutes**. The buffer turns **yellow**.
   - Store on a **reagent shelf dedicated for RNA** work at room temperature.

7. Prepare RNA samples for loading on the gel

How many RNA samples will be analyzed on the gel? **5**

2 RNA (leaves and seeds) samples **before** DNase I treatment

2 RNA (leaves and seeds) samples **after** DNase I treatment

1 RNA ladder

a. Label on the lids of 5 RNase-free microcentrifuge tubes **numbers "1-5"** and **your initial**. Set tubes **on ice**.

b. Label **one** RNase-free microcentrifuge tube as **Mmix** and **your initial**. Set the tube on ice.

- Prepare a **master mix (Mmix)** (for **6** = **5** Samples + **1** extra; see table below). Mix the contents well.

	Mmix for **1** RNA sample	**Mmix** for **6** RNA samples
10X MOPS buffer	1.5 µL	9.0 µL
37% formaldehyde	2.6 µL	15.6 µL
Formamide (Ultrapure)	7.5 µL	45.0 µL
5 mg/mL Ethidium Bromide	1.0 µL	6.0 µL
Total Volume	**12.6 µL**	**75.6 µL**

- Aliquot **12.6 µL** of the **Mmix** into each of the labeled RNase-free microcentrifuge tubes in **step (a)** and add **3.4 µL** of **RNA** to each tube as shown on the table below

	Tube #1	Tube #2	Tube #3	Tube #4	Tube #5
Mmix	**12.6 µL**	**12.6 µL**	**12.6 µL**	**12.6 µL**	**12.6 µL**
Leaf RNA **before** DNase I	**3.4 µL**	-------	-------	-------	-------
Leaf RNA **after** DNase I	-------	**3.4 µL**	-------	-------	-------
Seed RNA **before** DNase I	-------	-------	**3.4 µL**	-------	-------
Seed RNA **after** DNase I	-------	-------	-------	**3.4 µL**	-------
RNA Ladder	-------	-------	-------	-------	**3.4 µL**
Total Volume	**16.0 µL**	**16.0 µL**	**16.0 µL**	**16.0 µL**	**16.0 µL**

- Mix the contents by flicking the tubes several times or pipetting gently up and down for 5 times.
- Heat samples to **70 °C** for **10 minutes to denature secondary structure of RNA**. Quench tubes **on ice** for **2 minutes**.
- Add **2 µL** of **10X loading dye** for **RNA samples** to each tube. Centrifuge tubes briefly to bring water condensation from the lids down.
    - Preparation of the 10X loading buffer is shown below:

10X Loading Dye	**Volume**	**Stock**
50% Glycerol	250 µL	100%
1 mM EDTA	1 µL	0.5 M EDTA, pH8.0
Bromophenol blue	~10 mg (a pinch to get blue color)	
DEPC'd H_2O	249 µL	

8. Load samples on the gel. **Record** a pattern of the loaded samples **below**.

—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

9. Run the gel at ~**70 volts** for **1-2 hours** in the **fume hood**.

   Starting time:

   Ending time:

   *Note: To reduce the brightness of the ethidium bromide band migrated in the gel, the top of the gel is excised after the ethidium bromide band ran into it (usually 15 minutes after turning on the power supply). However, it is **okay** if you forget to excise the top of the gel after the ethidium bromide band ran into it.*

10. Take a picture of the gel. Paste the picture below.

    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 before and after DNase I 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:

- Instruction Manual for iScript cDNA Synthesis Kit (Bio-Rad, Cat.#170-8890).
  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 <1kb 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**. For examples, 40 μL reaction for 2 μg, 100 μL reaction for 5 μg to ensure optimum synthesis efficiency*.

Note:

- *For **every** 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.*

Materials and Reagents Needed:

- 5x iScript Reaction mix (came with the iScript RT, Bio-Rad, stored in a **-20** ^{o}C **RNA** Freezer, room 2918)
- Nuclease-free water (came with the iScript RT, Bio-Rad, stored in a **-20** ^{o}C **RNA Freezer**, room 2918)
- iScript Reverse transcriptase (iScript RT, Bio-Rad, stored in a **-20** ^{o}C **RNA Freezer**, room 2918)
- Total RNA samples (stored in a **-20** ^{o}C **RNA Freezer**, room 2918)
- DEPC'd water
- 42 ^{o}C and 85 ^{o}C dry baths (or heating blocks)
- RNase-free 1.5 mL microcentrifuge tubes
- Aerosol-barrier PCR pipet tips
- Pipetman sets
- Microcentrifuge-tube rack

PROCEDURE

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

   *Note: 1 μg = 1,000 ng. Therefore, the concentration determined by using the Nanodrop spectrophotometer as **ng/μL** needs to be converted into **μg/μL**.*

	Leaves	**Seeds**
RNA concentration	_____ μg/μL	_____ μg/μL

2. Determine a **volume** for **1 μg** of Total RNA to be added to **RT reactions**.

   Volume of **1 μg RNA** = (**Amount** of **RNA**) / (**concentration** of **RNA**).

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

	Leaves	**Seeds**
Volume	_____ μL	_____ μL

3. **Use the following table as the guide** to fill in volumes for total RNA and DEPC'd water.
   - **The volume of DEPC'd water** is the **difference** between the **Total Reaction Volume** and the **volumes** of **other components**.

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

4. Get a bucket full of **ice** from an icemaker in **room 2911** or **3906**.
5. Label on the lids of RNase-free 1.5 mL microcentrifuge tubes as "**Name of the plant organ**" and either "**+RT**" or "**-RT**". *For example, **Leaf +RT** and **Leaf -RT**.* Keep tubes **on ice**.

6. Thaw out the following tubes of **5x iScript Reaction Mix** and **Nuclease-free Water** at room temperature. Once the solutions are **thawed out**, keep the tubes **on ice**.
7. Prepare **two** tubes of **Master mixes** (**+RT Mix** and **-RT Mix**) as follows:
   - Determine a number of RT reactions to be set up.
     Note: ***# RT reactions*** = ***# of RNA samples*** + *1 Extra*
     Example: ***# RT reactions*** = ***3*** = *Leaves + Seeds + 1 Extra*
   - Write on the **lid** of each of RNase-free microcentrifuge tubes as **"+RT mix**" and **"-RT mix**". Keep tubes **on ice**.
   - Remove a tube of iScript Reverse transcriptase from a **-20 °C RNA freezer** (dedicated for RNA Work, in room LS 2918). Keep the **tube on ice** at all time to prevent degradation of enzymes such as RNase-inhibitor and reverse transcriptase in this tube.
   - Pipet the **following components** into appropriate tubes as shown below.
     Master Mixes:

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	---- µL	---- µL	1.0 µL	3.0 µL
5x iScript Reaction mix	4.0 µL	12.0 µL	4.0 µL	12.0 µL
iScript Reverse transcriptase	1.0 µL	3.0 µL	---- µL	---- µL
Total volume	**5.0 µL**	**15.0 µL**	**5.0 µL**	**15.0 µL**

   - Mix the contents by pipetting up and down **five times** or flicking the tube several times. Repeat for all reaction tubes.
   - Spin the tubes in a microcentrifuge for **10 seconds**. Put tubes on ice.
8. Using the "**+RT & -RT" chart** written up earlier in **step 3**, pipet into **+RT** and **-RT** tubes (labeled in **step 5**) the following components:
   - DEPC'd or Nuclease-free water
   - Total RNA
   - **+RT Mix** into **+RT** tubes

- **-RT Mix** into **-RT** tubes
- Mix the contents in each tube by pipetting **gently** up and down **five times**. Keep tubes on ice until the last component is added into the last RT tube.

9. Transfer all +RT and -RT tubes from the ice bucket to a rack for microcentrifuge tubes on the bench.
10. Incubate reaction tubes at **25 °C** (or room temperature) for **5 minutes**. *This step is to allow oligo(dT) and random primers annealing to messenger RNA in the reactions*.
11. Incubate reaction tubes at **42 °C** for **30 minutes** on a **dry bath** (or heating block). *This step is to synthesize first strand cDNAs*.
12. **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 mixture at **85 °C** for **5 minutes**.
13. Chill the tubes **on ice** for at least **2 minutes**.
14. Centrifuge the tubes at room temperature for **1 minute** to bring down water condensation on the lids of the tubes. <u>*Note:*</u> *The RT reactions are ready for PCR amplification step*.
15. Store RT reactions in a **-20°C freezer** if they are not used for 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 DNA template corresponding to mRNA that is either absent or present at different levels in different plant organs throughout plant development.

Materials and Reagents Needed:

- Reverse transcription (+RT & -RT) reactions
- 10X Ex-Taq buffer (Takara Mirus Bio)
- dNTP mix (Takara Mirus Bio)
- **12 μM** Gene-specific **RT-PCR Fw** primers
- **12 μM** Gene-specific **RT-PCR Rv** primers
- **12 μM Tubulin Fw** primers
- **12 μM Tubulin Rv** primers
- Sterile water
- Ex-Taq DNA polymerase (Takara Mirus Bio, 5 U/ μL)
- Agarose
- 1X TAE buffer
- 1 KB DNA ladder (Invitrogen)
- 6X Loading buffer
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube racks
- PCR Machine (Bio-Rad MyCycler or PE 9700)
- Gel apparatus
- Power supplies

PROCEDURE

1. Get ice from the icemaker in room 2911 or 3906.
2. Determine **how many RT reactions**, including **+RT's** and **-RT's**, will be amplified.
3. Make a **Table** with information sush as **tube #**, **plant organ(s)**, and **+RT's/-RT's** (see the **example** Table below)

Tube #	**1**	**2**	**3**	**4**	**5 (Positive)**	**6 (Negative)**
Organ & RT	Leaf +RT	Leaf -RT	Seed +RT	Seed -RT	Genomic DNA	Sterile Water

4. Label on the **lids** and **sides of FIVE 0.2 mL PCR tubes** with **Number** and **your initial**.
5. Put the labeled tubes on a PCR rack sitting **on ice**.
6. Prepare a **master mix** in a **1.5-mL microcentrifuge tube** for the **number** of **PCR solutions** being carried out **plus** 1 **extra solution volume** as followings: **(How many reactions are carried out?)**

	Mmix for 1 Reaction	**Mmix** for **7** Reactions
10X Ex-Taq buffer	5.0 μL	35.0 μL
dNTP mix	4.0 μL	28.0 μL
12 μM RT-PCR Gene-specific **Fw** primer	1.0 μL	7.0 μL
12 μM RT-PCR Gene-specific **Rv** primer	1.0 μL	7.0 μL
***12 μM Control** (**Tubulin**) **Fw** primer	1.0 μL	7.0 μL
***12 μM Control** (**Tubulin**) **Rv** primer	1.0 μL	7.0 μL
Sterile water	34.5 μL	241.5 μL
Ex-Taq DNA Polymerase (5 U/μL)	0.5 μL	3.5 μL
Total Volume	**48.0** μL	**336.0 μL**

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

7. Pipet **48 μL** of the **master mix** to the labeled tubes and **2 μL** of **appropriate RT** to each of the tubes shown on the table below. Mix the contents by pipetting **gently** up and down for five times.

Tube #	1	2	3	4	5 (Positive)	6 (Negative)
Mmix	**48 µL**	**48 µL**	**48 µL**	**48 µL**	**48 µL**	**48 µL**
Leaf +RT	**2 µL**	---- µL	---- µL	---- µL	---- µL	---- µL
Leaf -RT	---- µL	**2 µL**	---- µL	---- µL	---- µL	---- µL
Seed +RT	---- µL	---- µL	**2 µL**	---- µL	---- µL	---- µL
Seed -RT	---- µL	---- µL	---- µL	**2 µL**	---- µL	---- µL
0.2 ng/µL Genomic DNA	---- µL	---- µL	---- µL	---- µL	**2 µL**	---- µL
Water	---- µL	---- µL	---- µL	---- µL	---- µL	**2 µL**
Total Volume	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**

8. Carry out PCR reactions with the **RT-PCR program** containing the following profile: 1 cycle of 96 °C, 3 min. → 40 cycles of 94 °C, 10 sec./60 °C, 30 sec./72 °C, 45 sec. → 1 cycle of 72 °C, 4 min. → 4 °C, ∞.
9. Prepare **100 mL** of **1.5% agarose** gel in **1X TAE** buffer as usual (Use a **20-tooth** comb).
   *Note: The **percentage** of **agarose gels** depends on the **difference** in **size** of **two PCR products**. If there is **at least 100-bp difference** between two PCR products, then use a **1% agarose gel**. However, if there is **50-100 bp difference** between two PCR products, then use **1.5-2% agarose gel**. For example, the size of PCR products is 0.6 kb and 0.55 kb for the control and gene A, respectively. The **2.0%** agarose gel resolves these two PCR products as **two** discreet DNA bands whereas the **1.0%** agarose gel shows these two PCR products as a **single** DNA band.*
10. Label 1.5 mL microcentrifuge tubes according to the PCR solutions being performed.
11. Add to the labeled 1.5 mL microcentrifuge tubes **10 µL** of **PCR solution** and **2 µL** of **6X loading dye**.
12. Load samples on a 1.5% agarose gel along with **10 µL** of **50 ng/µL 1-Kb ladder** solution. Record RNA loading pattern.

—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

13. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is about two-thirds of the gel.

    Starting time:

    Ending time:

14. Take a picture of the gel.
15. Analyze the data.

    How many DNA fragments do you see on the gel?

    Is there different brightness between the fragments from one organ to the other?

    What are the sizes of DNA fragments?

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

    What is a conclusion on gene expression of the gene-of-interest for the tested plant organs, leaves and seeds?

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

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	4 µL
iScript Reverse Transcriptase	1 µL
Nuclease-free water	x µL
RNA template (100fg to 1µg Total RNA)*	x µL
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

Pipette tips, aerosol barrier tips
Nuclease-free tubes
RNA purification kit

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. 40 µL reaction for 2µg, 100 µL reaction for 5 µg to ensure optimum synthesis efficiency.

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