RNA Extraction and cDNA Synthesis

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Materials:
Retsch Mill, M300
Microfuge tube holder for Retsch Mill (Qiagen, 24 tube holder)
Stainless steel balls, autoclaved
2 Styrofoam boxes, one with ice, other for LN_2
2 ml Microfuge tubes, autoclaved (Sarstadt work best)
LN_2
Qiagen RNeasy Plant Mini Kit (74904) On bench
2-Mercaptoethanol, 14.3 M (Sigma M6250) in fume hood cabinet
Qiagen DNase Set (79254) 4°C
DNaseI -80°C I1
1.5 ml microfuge tubes, autoclaved
100% Ethanol
RNase free water On bench
SuperScript II reverse trascriptase (Invitrogen, 18064) -20°C
Oligo(dT) primer (Invitrogen, 18418) -20°C
10 mM (ea) dNTP mix (Invitrogen, 18427) -20°C
PCR tubes
Thermocycler, programs are in small Perkin Elmer machine

Procedure:

**** When working with RNA cleanliness is extremely important. Make sure you are always wearing clean gloves, change gloves frequently and avoid sample contact with potential sources of RNase, i.e. dust falling into tubes, water from ice, dirty microfuge tube racks etc.****

Tissue Grinding
1. Label and weigh 2 ml microfuge tubes, be sure to use sterile tubes and used gloved hands to avoid RNase contamination
2. Harvest leaf material, you want no more than 100 mg FW of tissue, this is approximately 1 fully expanded mature leaf, and immediately freeze in LN_2
**** Make sure there is not too much material, < 100 mg FW maximum, this is approximately 1 fully expanded mature leaf, or RNA yield will suffer****
3. After freezing, weigh again, to determine fresh weight
4. Store in -80º C until ready to continue with assay
6. Place Retch mill microfuge holder in Styrofoam box and cool with LN_2
7. Get plant samples from step 4 and place an autoclaved stainless ball in each sample tube
8. Place samples in cooled microfuge tube holder
9. Place and secure holder containing vials into Retsch Mill, you will need cryo gloves
** **Make sure each holder has a microfuge tube holder, however the second microfuge tube holder does not have to be filled with tubes****
10. Gind samples for 20 sec at a frequency of 30 (maximum speed)

If using RNeasy plant mini kit for the first time
1. In the fume hood add 450 µl 2 Mercaptoethanol to the buffer RLT bottle (10 µl β-ME per 1 ml Buffer RLT)
2. Date bottle of RLT, once β-ME is added RLT will be good at room temp for 1 month
3. Add 95% - 100% EtOH to Buffer RPE bottle according to directions on bottle, check box on lid of

bottle
4. Prepare DNase I stock solution by injecting 550 µl RNase free water into vial sing an RNase-free needle and syringe
**** Do not vortex DNase I, DNase I is sensitive to physical denaturation. Mix by gently inverting the tube ****
5. Aliquot DNaseI solution into 3, 150 µl aliquots and 1, 100 µl aliquot, label tubes and store at -20 or -80°C for up to 9 months, Thawed aliquots can be stored at 4°C for 6 weeks, **Do not refreeze**

RNA extraction
**** Perform all steps at room temperature, and centrifuge at room temperature. Make sure centrifuge does not cool below 20°C, work quickly ****
1. Buffer RLT may form a precipitate. If necessary, redissolve by warming and then place at room temp.
2. Add 450 µl buffer RLT to tissue powder, vortex vigorously, A short 1-3 min incubation at 56°C may help to disrupt tissue. Do not incubate samples with high starch at elevated temperatures or the sample will swell.
3. Transfer sample to the lilac colored QIAshredder column and place in a provided 2 ml collection tube. You may need to cut the tip off the pipette tip in order to transfer the sample to the QIAshredder column
4. Centrifuge for 2 minutes at full speed
5. Transfer the supernatant of the flow-through to a new autoclaved 1.5 ml microfuge tube
6. Add 225 µl 96 – 100% ethanol to supernatant of the flow-through, precipitates may be visible after addition of ethanol, this is not a problem
7. Transfer the sample including any precipitate that may have formed to a pink RNeasy spin column placed in a 2 ml collection tube, close lid, and centrifuge for 15 seconds at 8,000 g, save collection tube
8. Discard flow through
9. Add 350 µl buffer RW1 to the pink RNeasy spin column
10. Centrifuge for 15 seconds at 8000 g, discard flow-through, save collection tube
11. In an autoclaved 1.5 ml microfuge tube Prepare a DNase I according to formula below
    + 10 µl DNase I stock solution * # of samples
    + 70 µl RDD buffer * # of samples
    **** Mix by gently inverting tube, Do not vortex, DNase I is sensitive to physical denaturation ****
    Centrifuge briefly to collect residual liquid from sides of tube
12. Add 80 µl of DNase I mix prepared above directly to the RNeasy spin column membrane, allow to incubate on bench at room temperature for 15 minutes
    Be sure to add DNase I mix directly to membrane and is not sticking to walls or the O-ring of the spin column
13. Add 350 µl buffer RW1 to the RNeasy spin column.
14. Centrifuge for 15 seconds at 8000 g, discard flow-through, save collection tube
15. Add 500 µl buffer RPE to RNeasy spin column
16. Centrifuge at 8000 g for 15 seconds, discard flow-through, save collection tube
17. Add 500 µl buffer RPE to RNeasy spin column
18. Centrifuge for 2 min at 8000 g to dry column, discard flow-through, discard collection tube
    In this step residual ethanol from buffer RPE must be eliminated, gently remove EtOH around inside rim that surrounds column membrane with a 20 µl pipette
19. Place RNeasy spin column on a fresh 1.5 ml microfuge tube with cap add 30 – 50 µl of RNase-free water directly to the spin column membrane
20. Centrifuge at 8000 g for 1 minute to elute RNA
21. If the expected RNA yiled is > 30 µg, repeat step 20 using another 30-50 µl RNase free water
22. Measure RNA amount using spectrophotometer at λ = 260 nm and 280 nm, using Nanodrop select RNA on the right of the screen
If the ratio of 260/280 is below 1.75 this is not good

cDNA Synthesis
1. In a PCR tube add the following reagents
    + 1 µl Oligo (dT) primer 0.5 µg/µl
    + 1 ng – 5 µg RNA (usually use 1 µg)

+ 1 μl 10 mM (ea) dNTP mix
+ H_2O, RNase free up to 12 μl

2. Heat to 65°C for 5 minutes using thermocycler and chill on ice 1 – 2 minutes, Program RT1
3. After sample has chilled on ice centrifuge briefly to collect residual liquid from sides of tube
4. Add the following reagents to the PCR tube
    + 4 μl First-Strand buffer
    + 2 μl 0.1 M DTT
    + 1 μl H_2O, RNase free
5. Mix contents of tube gently by up and down pipetting
6. Incubate sample (19 μl) in thermocycler at 42°C for 2 minutes, Program RT2
7. Add 1 μl SuperScript II RT and mix by gently pipetting up and down
8. Incubate sample (20 μl) in thermocycler at 42°C for 50 minutes, Program RT3
9. Inactivate reverse transcriptase by heating at 70°C for 15 minutes, Program RT3