Completing the Expression Catalog of the Arabidopsis Transcriptome by Quantitative Real-Time RT-PCR

QPCR SOP

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SECTION 1: PLANT RNA EXTRACTION

NOTES BEFORE BEGINNING:

--For most tissues/treatments, 2g of tissue will produce plenty of RNA for our needs.

--ALWAYS use blocked tips, clean pipettes, and gloved hands when working with RNA. If you wear a lab coat, DO NOT use the same one used for activities like seed harvesting or bacterial mini-preps.

--Day 1 steps using phenol and chloroform should be carried out in the fume hood.

Reagents and Supplies

- RNase free tubes
- Mortar and pestle
- liquid Nitrogen
- 5:1 Acid Phenol:Chloroform (Ambion p/n 9722)
- Chloroform:IAA
- DEPC-treated H2O (Ambion p/n 9920)
- 8M LiCl (Sigma p/n L-7026)
    - 2M LiCl, 4C
- 80% EtOH, -20C
- **Extraction Buffer**, made fresh each time (Per 10mL needed)
    - 50mM Tris pH 7.5 (0.5mL 1M)
    - 100mM LiCl (0.125mL 8M)
    - 5mM EDTA, pH8 (0.1mL 0.5M)
    - 1% SDS (1mL 10%)
    - 1% (beta)-Mercaptoethanol (0.1mL)
    - DEPC-treated H_2O (8.2mL)

- **Day 1**
    - Grind 1g of tissue in mortar and pestle under liquid nitrogen, adding sand if necessary, to fine powder. Transfer to tube in liquid nitrogen.
        - While the protocol may be scaled down, scaling up above 1g per tube is not recommended. Instead, make multiple preps when more RNA is needed.
        - *Tissue preparation may be done in advance, storing the tissue at -80C and keeping under liquid nitrogen until added to buffer*
    - Dump tissue into 10mL Extraction buffer. Cap and vortex briefly to break clumps.
    - Quickly add 10mL 5:1 Acid Phenol:Chloroform. Vortex 1min.
    - Centrifuge 4000rpm, 10min at RT in table top centrifuge.

- Recover aqueous phase, avoiding any floating particles and the interface.
- Add 10mL Chloroform:IAA (using glass pipette) and vortex or shake vigorously.
- Centrifuge 4000rpm, 10min at RT.
- Recover ~9mL aqueous phase.
- Add 1/3 volume 8M LiCl (3mL) and vortex.
- Incubate at least overnight at -20C.

- **Day 2**
  - Remove tubes from -20C and let thaw briefly.
  - Centrifuge 4000rpm, 10min at 4C.
  - Remove supernatant and SAVE pellet.
  - Resuspend pellet in 3mL DEPC-treated H_2O.
    - *If there are duplicate tubes, they may be combined at this point*
  - Add 1/3 vol 8M LiCl and vortex.
  - Incubate at least overnight at -20C.

- **Day 3**
  - Remove tubes from -20C and let thaw briefly.
  - Centrifuge 4000rpm, 10min at 4C.
  - Decant supernatant.
  - Wash pellet with 4C 2M LiCl.
    - Add 4mL and vortex.
    - Centrifuge 4000rpm, 5min at 4C.
    - Decant supernatant.
  - Repeat wash once.
  - Wash pellet with -20C 80% EtOH.
    - Add 4mL and vortex.
    - Centrifuge 4000rpm, 5min at 4C.
    - Decant supernatant.
  - Repeat wash once.
  - Invert tube and air dry pellet no more than 15min. Over dried RNA is hard to dissolve.
  - Resuspend pellet in 200ul DEPC-treated H_2O per 1g starting material.
  - Check yield.
  - Store at -80C, aqueous.

SECTION 2: RNA PROCESSING

NOTES BEFORE BEGINNING:

--ALWAYS use blocked tips, clean pipettes, and gloved hands when working with RNA. If you wear a lab coat, DO NOT use the same one used for activities like seed harvesting or bacterial mini-preps.

Supplies

- RNase free tubes
- TURBO DNA-free™ (Ambion p/n AM1907)
- RNeasy Mini Kit (TIGR stock room # 5802)
- Mini-Gel box and accessories
- Gel Reagents
    - 1M $NaPO_4$ pH 6.5
    - Agarose
    - Formaldehyde
    - Formamide
    - 0.5M EDTA pH 8.0
    - Ethidium Bromide
    - 6x loading dye
    - RNA Millenium Markers (Ambion p/n AM7150)

- **DNase Treatment**
    - Aliquot 300ug Total RNA
    - Add DEPC-treated H_2O to 267ul, 30ul 10x DNase buffer, and 3ul DNAse I; flick to mix.
    - Incubate 30min at 37C.
    - Move directly to filtration.

- **RNeasy Filtration**
    - *Apply about 50ug RNA to each column for best results*
    - Add 300ul DEPC-treated H_2O, aliquot 100ul per tube.
    - Continue with the QIAGEN protocol for RNA cleanup as stated in the book, with the following notes in mind:
        - Pass the sample over the column twice.
        - Include optional step 6a.
        - Elute in 50ul DEPC-treated H_2O, allowing water to sit on column 10 min prior to centrifuging.

- Combine like samples.
- Check yield.

- **RNA Denaturing Gel Analysis**
  - *Make gel and buffer fresh each time, excess sample buffer may be kept up to 2 months at 4C*
  - Gel Preparation (appropriate amount for mini-gel)
    - 44mL dH_2O
    - 1mL 1M $NaPO_4$ pH 6.5
    - 0.5g Agarose
    - Microwave to melt, cool to hand-hot.
    - Add 4mL Formaldehyde, mix and pour.
  - Running Buffer
    - 20mL Formaldehyde
    - 2.5mL 1M $NaPO_4$ pH 6.5
    - dH2O to 250mL
  - 2x Sample Buffer
    - 50ul Formamide
    - 27.5ul DEPC-treated H_2O
    - 16.5ul Formaldehyde
    - 2ul 0.5M EDTA
    - 2ul Ethidium Bromide
  - Sample preparation
    - Mix RNA (usually 1ul is appropriate) with DEPC-treated H_2O to 5ul.
    - Add 5ul 2x Sample Buffer
    - Incubate at 65C for 15min
    - Quick cool.
    - Add 2ul loading dye.
  - Load and Run gel
    - 80V for 45-60min.
    - *Running gel longer than 60min can lead to poor results.*
    - Results should show 2 or 3 clean bands, depending on tissue type.

SECTION 3: cDNA SYNTHESIS FOR RT-qPCR

>>Batch synthesis with SuperScript III, modified from Invitrogen protocol<<

NOTES BEFORE BEGINNING:

--ALWAYS use blocked tips, clean pipettes, and gloved hands when working with RNA. If you wear a lab coat, DO NOT use the same one used for activities like seed harvesting or bacterial mini-preps.

Reagents and Supplies

- 25ug Total RNA, DNase treated and filtered.
- 1:100 dilution of Spike Master Mix.
    - Pseudotranscript (pmol/ul), in Master Mix
      LYS (0.00001)
      PHE (0.0001)
      THR (0.001)
      TRP (0.01)
      DAP (0.1)
    - Pseudotranscript (fmol), **target usage *per 1ug Total RNA***
      LYS (0.00002)
      PHE (0.0002)
      THR (0.002)
      TRP (0.02)
      DAP (0.2)
- SuperScriptIII (Invitrogen p/n 18080-051)
    - Includes Oligo dT(20), dNTP's, buffers, etc.
- Water bath set at 65C.
- Air incubator or water bath at 50C.
- Water baths or heat blocks set at 85C and 37C.
- DEPC-treated H2O.
- RNase free tubes

- **cDNA Synthesis**
    - Aliquot 25ug Total RNA.
    - Add:
        - DEPC-treated H_2O to 35ul
        - 5ul 1:100 spike mix dilution
        - 5ul 50uM Oligo(dT)20
        - 5ul 10mM dNTP mix
    - Incubate 5min at 65C.

- Quick cool, 1 min.
- Add:
  - 10ul 10x RT Buffer
  - 20ul 25mM $MgCl_2$
  - 10ul 0.1M DTT
  - 5ul RNaseOUT
  - 5ul SuperScript III Reverse Transcriptase
- Incubate 50min at 50C.
- Incubate 15min at 85C. Chill.
- Add 5ul RNase H.
- Incubate 20min at 37C.
- Add DEPC-treated H_2O to 3mL (2.895mL).

SECTION 4: cDNA QC TESTING

NOTES BEFORE BEGINNING:

--ALWAYS wear gloves when touching the optical plates.

--NEVER write on the optical plates before a reaction.

--Try not to touch the optical seals in the center.

--Pay extra attention to seating the seal well, especially around the edges, with the sealing tool. Neglecting this can result in leakage during cycling.

Supplies and Reagents

- Finished and diluted cDNA populations from above.
- 2x SYBR Green Master mix (ABI p/n 4364346)
- 0.8uM Primer Mixes, including:
    - GAPDH 5' End
    - GAPDH 3' End
    - LYS
    - TRP
    - THR
- Optically clear 384-well plates, barcoded (ABI p/n 4309849)
- Optical seals (ABI p/n 4311971)

** GAPDH primer sequences used came from Michael Udvardi lab.*

- **Reaction Setup**
    - Run one reaction with each primer pair for each cDNA population to be checked.
    - 5ul 2x SYBR Green Master Mix, 2.5ul cDNA, 2.5ul 0.8uM primer mix per well.
    - Seal plate and centrifuge 4000rpm, 5min at 4C.

- **Cycling Parameters and document setup**
    - Open a new 384-well, 'Absolute quantification' document in SDS. Enter the barcode.
    - Click 'Add Detector', bottom middle.
        - Select 'SYBR Green' and 'Copy to Plate Document'
    - Select wells in use and click the 'use' box in the setup pane.
    - Navigate to the 'Instrument' pane.

- Click the end of the cycling line, and then click the 'Add Dissociation Stage' button.
- Alter the volume to 10.
- Save the document.
- Click 'Open/Close', insert the plate in the carrier with well A1 in the corner indicated, and click 'Start'.

- **Data Recovery and Analysis**
  - When the protocol is complete, click the 'Analysis Settings' button (green arrow with a blue bar on top)
    - Select the 'Automatic Baseline' radial, and 'Apply'.
  - Click 'Analyze' (green arrow).
  - Switch to the 'Dissociation Curve' pane, and check for abnormalities. Make notes as necessary.
  - Switch to the 'Results' pane, and again check for abnormalities.
  - Using the 'Export' function under 'File', export the Results Table.
  - Using EXCEL, or similar:
    - Calculate 2^(Ct(GAPDH 5') - Ct(GAPDH 3')) for each cDNA.
      - This value should ideally be ~1.
      - Values above 2 may indicate poor transcription or degradation of transcripts.

SECTION 5: qPCR REACTION SETUP

NOTES BEFORE BEGINNING:

--ALWAYS wear gloves when touching the optical plates.

--NEVER write on the optical plates before a reaction.

--Try not to touch the optical seals in the center.

--Pay extra attention to seating the seal well, especially around the edges, with the sealing tool. Neglecting this can result in leakage during cycling.

Supplies and Reagents

- Finished and diluted cDNA populations.
- 2x SYBR Green Master mix (ABI p/n 4364346)
- 0.8uM Primer Mixes, in 384-well plates.
- Optically clear 384-well plates, barcoded (ABI p/n 4309849)
- Optical seals (ABI p/n 4311971)

- **Reaction Setup**
    - Run one reaction with each primer pair for each cDNA population to be checked.
    - PER REACTION: 5ul 2x SYBR Green Master Mix, 2.5ul cDNA, 2.5ul 0.8uM primer mix per well.
        - Master mix: 1mL cDNA, 2mL 2x SYBR Green Master Mix.
            - Dispense 7.5ul per well.
        - Add 2.5ul 0.8uM primer mix per well, aligning primer plate positions to reaction plate positions.
    - Seal plate and centrifuge 4000rpm, 5min at 4C.

- **Cycling Parameters and document setup**
    - Open a new 384-well, 'Absolute quantification' document in SDS. Enter the barcode.
    - Click 'Add Detector', bottom middle.
        - Select 'SYBR Green' and 'Copy to Plate Document'
    - Select wells in use and click the 'use' box in the setup pane.
    - Navigate to the 'Instrument' pane.
    - Click the end of the cycling line, and then click the 'Add Dissociation Stage' button.

- Alter the cycle number to 45, and the volume to 10.
- Save the document.
- Click 'Open/Close', insert the plate in the carrier with well A1 in the corner indicated, and click 'Start'.

- **Data Recovery**
  - When the protocol is complete, click the 'Analysis Settings' button (green arrow with a blue bar on top)
    - Select the 'Automatic Baseline' radial, and 'Apply'.
  - Click 'Analyze' (green arrow).
  - Switch to the 'Dissociation Curve' pane, and check for. Make notes as necessary.
  - Switch to the 'Results' pane, and again check for abnormalities.
  - Using the 'Export' function under 'File':
    - Export the Results Table.
    - Export the Clipped data.

- **Fluorescence Data and Efficiency Calculations**
  - You'll need: Excel and LinRegPCR, clipped files.
    - Reference for LinRegPCR:

      Ramakers C, Ruijter JM, Deprez RH, Moorman AF. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. **Neurosci Lett**, **339**(1): 62-66.

  - Open the clipped file in Excel.
    - Delete the first range of data. Use the second (Delta Rn).
    - Delete *contents* of the column with the header 'Reporter'. Leave the column there.
    - Save as Excel format. You may put several clipped files in here.
  - Open LinRegPCR.
    - A dialog box will offer to pull data from the *active* Excel sheet. Please make a note of which one you are working with.
    - Indicate the locations of your data (columns A-AU, rows 2-386).
    - Select the 'Applied Biosystems' radial button and click 'OK'.
    - If you wish to change the fit options, select the 'Fit Options' tab.
      - 'Number of points between 4 and 6 and best correlation coefficient' is default and most popular.
      - 'Correlation Coefficient of at least 0.999 and highest number of points' is also acceptable.
    - Click 'Fit All'.

        - Review each sample; look for anomalies and correct them manually. Examples:
            - Correct: Points along the exponential portion of the curve should be selected. You are looking at a log plot, so this portion of the curve will be linear.
            - Wrong: Points along the leveled-off portion (linear) of the curve are selected.
            - Wrong: Points along the transition area between exponential and linear.
            - Questionable: A point at the bottom of the exponential phase, just near the 'disorganized' points has been selected. This may be a skewed point due to proximity to the lower limit of detection or background correction range. The author likes to test how the values will change if this point is not used.
        - Select 'File > Save to Excel'.
            - In the dialog box enter 'A' and '1' and click 'ok'.
    - Back in Excel, change the name of the tab to the plate barcode and your initials. Save the file.

>>SEE DATA ANALYSIS DOCUMENTATION FOR MORE INFORMATION<<