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Plant Genomics

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T7 RNA POLYMERASE-BASED RNA AMPLIFICATION

(Last Revised: April, 2007)

This protocol is used by the Schnable Laboratory (Iowa State University; edited by Dr. David Skibbe, Dr. Kazuhiro Ohtsu, and Marianne B. Smith). Please contact Dr. Patrick Schnable (schnable@iastate.edu) regarding questions or corrections.

The amount of RNA collected in a standard microdissection is often insufficient for global gene expression analysis. However, the amount of RNA can be increased via linear amplification. The procedure described here is a modified Eberwine procedure (Eberwine et al., 1992), in which an oligo(dT)-T7 chimeric primer is used to preferentially select polyadenylated RNA species (e.g., mRNA) through two rounds of sequential reverse transcription and RNA transcription. Amplification of RNA isolated from maize via this procedure has been shown to be reproducible (Nakazono et al., 2003). The procedure described below was optimized using RNA isolated from laser microdissected tapetal cells from maize anthers. It can be completed in two to four days and typically results in an amplification of 50,000 to 500,000 fold (e.g., assuming 1% poly(A) mRNA in 10 ng of total RNA starting material, yields of 5 to 50 µg of amplified RNA are routinely obtained). A list of the materials and suppliers used for optimizing this protocol is in Appendix A.

Eberwine J, H Yeh, K Miyashiro, Y Cao, S Nair, R Finnell, M Zettel, Coleman P (1992) Analysis of gene expression in single live neurons. Proceedings National Academy Sciences, 89:3010-3014.

Nakazono M, F Qiu, LA Borsuk, PS Schnable (2003) Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. Plant Cell, 15:583-596.

NOTE: All centrifugation steps are performed in a standard benchtop microcentrifuge at room temperature.

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1. First-round RNA amplification

(1) First strand synthesis

- In a 1.5 ml tube (or PCR tube) **mix**:
  1 μl 0.5 μg/μl T7dT primer
  5-100 ng total RNA extracted from LCM sample
  dH_2O (DEPC-treated) to make 11.0 μl
- **Incubate** the samples at 65 ^oC for 10 min, and then quickly chill on ice.
- **Keep** sample on ice for 5-10 min.
- **Spin** down the sample, and then **equilibrate** at 42 ^oC for 5 min.
- **Add** 8.0 μl premix containing:
  1 μl 10 mM dNTPs mix
  4 μl 5x first strand buffer (Invitrogen)
  2 μl 0.1 M DTT (Invitrogen)
  0.5 μl 40 U/μl RNase inhibitor (RNaseOUT, Invitrogen)
  0.5 μl 5 μg/μl T4 gene 32 protein (USB)
- **Mix** gently, and **add:**
  1 μl 200 U/μl Superscript II (Invitrogen)
- **Incubate** at 42 ^oC for 1 hr (stopping point: -20^oC)

(2) Second strand synthesis

- To each 20 μl reaction, **add** 130 μl premix containing:
  15 μl 10x NEBuffer 2 (NEB)
  3 μl 10 mM dNTPs mix
  15 μl 260 μM β-NAD^+
  4 μl 10 U/μl *E. coli* DNA polymerase I (NEB)
  1 μl 2 U/μl RNase H (Invitrogen)
  1 μl 10 U/μl *E. coli* DNA ligase (NEB)
  91 μl dH_2O
- **Mix** gently, and **incubate** at 16 ^oC for 2 hr.
- Then **add**:
  2 μl 3 U/μl T4 DNA polymerase (NEB), and incubate at 16 ^oC for 10 min.

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(3) Clean-up of cDNA

- **Extract** with an equal amount (150 µl) of phenol (pH 6.6)/chloroform (1:1), and then **extract** with an equal amount (150 µl) of chloroform.
- **Purify** the cDNA using a QiaQuick PCR Purification Kit (QIAGEN)
  a. **Add** 35 µl 100 mM sodium acetate pH5.2 to each tube.
  b. **Add** 500 µl Buffer PB to each tube.
  c. **Proceed** as per manufacturer's instructions until elution.
  d. **Elute** two times in 15 µl dH_2O (in total, 30 µl).
- **Concentrate** sample to 8 µl with a concentrator/evaporator (50 ^oC, approximately 10 minutes).

(4) T7 *in vitro* transcription

Prepare the reagents from MEGAscript T7 Kit (Ambion)

- **Thaw** the rNTP solutions, **mix** by vortexing, **spin** down, and **put** them on ice.
- **Thaw** 10x reaction buffer, **mix** until the precipitate has dissolved, and keep **<u>at room temperature</u>** **<u>(not on ice)</u>**.
- **Assemble** the reaction in this order:
  To 8 µl cDNA **add**:
  8 µl rNTP mix (2 µl each of ATP, CTP, GTP and UTP)
  2 µl 10x reaction buffer
  2 µl T7 RNA polymerase enzyme mix
- **Incubate** the reaction mix at 37 ^oC for 5 hr.
- **Add:**
  1 µl 2 U/µl RNase-free DNase I, and **incubate** at 37 ^oC for 15 min.

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(5) Clean-up of aRNA

- **Add** 80 μl nuclease-free dH_2O to sample (total vol: 100 μl)
- **Extract** with an equal amount (100 μl) of phenol (pH4.3)/chloroform (1:1), and then **extract** with an equal amount (100 μl) of chloroform.
- **Concentrate** sample in RNeasy mini column (QIAGEN)
- **Add** 350 μl Buffer RLT (with 3.5 μl β-mercaptoethanol), and **mix** thoroughly.
- **Add** 250 μl ethanol, and **mix** thoroughly by pipetting. **Do not centrifuge**.
- **Apply** the sample (700 μl) to an RNeasy mini column placed in a 2 ml collection tube.
- **Spin** 15 sec at 10,000 rpm, and **discard** the flow-through.
- **Transfer** the RNeasy column into a new 2 ml collection tube.
- **Pipette** 500 μl Buffer RPE onto the RNeasy column.
- **Spin** 15 sec at 10,000 rpm, and **discard** the flow-through.
- **Add** another 500 μl Buffer RPE to the RNeasy column.
- **Spin** 2 min at 10,000 rpm to dry the RNeasy silica-gel membrane.
- To elute**, transfer** the RNeasy column to a new 1.5 ml collection tube.
- **Pipette** 15 μl dH_2O onto the RNeasy column, and **spin** 1 min at 10,000 rpm.
- **Pipette** another 15 μl dH_2O onto the RNeasy column, and **spin** 1 min at 10,000 rpm.
- **Concentrate** the sample to 10 μl with a concentrator/evaporator (50 ^{o}C, approximately 10 minutes).

***** At this stage it is optional to remove a 1 μl aliquot for RNA quantification. This is only feasible if enough RNA starting material was used for amplification. For this option the RNA sample should be concentrated to 11 μl rather than 10 μl.

2. Second-round RNA amplification

(1) First strand synthesis

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- **Assemble** the solution:
  1 µl 1 µg/µl random hexamer (Roche) plus
  10 µl aRNA
- **Incubate** at 70 °C for 10 min, and then quickly **chill** on ice.
- **Keep** the sample on ice for 5-10 min.
- **Spin** down the sample, and then **equilibrate** tube at room temperature for 10 min.
- **Add** 8 µl premix containing:
  1 µl 10 mM dNTPs mix
  4 µl 5x first strand buffer (Invitrogen)
  2 µl 0.1 M DTT (Invitrogen)
  0.5 µl 40 U/µl RNase inhibitor (RNaseOut, Invitrogen)
  0.5 µl 5 µg/µl T4 gene 32 protein (USB)
- **Mix** gently, and **add**:
  1 µl 200 U/µl Superscript II (Invitrogen)
- **Incubate** at 37°C for 1 hr.
- **Add:**
  1 µl 2 U/µl RNase H (Invitrogen), and **incubate** at 37°C for 30 min.

(2) Second strand synthesis

- **Heat** at 95 °C for 2 min, and then quickly **chill** on ice.
- **Keep** sample on ice for 5 min.
- **Add** 1 µl 0.5 µg/µl T7dT primer, and **incubate** at 70 °C for 5 min.
- **Incubate** at 42 °C for 10 min, and **place** sample on ice for 5 min.
- **Add** 128 µl premix containing:
  15 µl 10x NEBuffer 2 (NEB)
  3 µl 10 mM dNTPs mix
  15 µl 260 µM β-NAD^+
  4 µl 10 U/µl *E. coli* DNA polymerase I (NEB)
  1 µl 2 U/µl RNase H (Invitrogen)
  90µl dH_2O
- **Mix** gently, and incubate at 16 °C for 2 hr,
- **Add:**
  2 µl 3 U/µl T4 DNA polymerase (NEB), and **incubate** at 16 °C for 10 min.

(3) Clean-up of cDNA

- **Extract** with an equal amount (150 µl) of phenol (pH 6.6)/chloroform (1:1),

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and then **extract** with an equal amount (150 µl) of chloroform.

- **Purify** the cDNA using a QiaQuick PCR Purification Kit (QIAGEN)
  a. **Add** 35 µl 100 mM sodium acetate pH5.2 to each tube.
  b. **Add** 500 µl Buffer PB to each tube.
  c. **Proceed** as per manufacturer's instructions until elution
  d. **Elute** two times in 15 µl dH_2O (in total, 30 µl).
- **Concentrate** sample to 8 µl with a concentrator/evaporator (50 ^oC, approximately 10 minutes).

(4) T7 *in vitro* transcription

Prepare the reagents from MEGAscript T7 Kit (Ambion)

- **Thaw** the rNTP solutions, **mix** by vortexing, **spin** down, and **put** them on ice.
- **Thaw** 10x reaction buffer, **mix** until the precipitate has dissolved, and keep **at room temperature (not on ice)**.
- **Assemble** the reaction in this order:
  To 8 µl cDNA **add**:
  8 µl rNTP mix (2 µl each of ATP, CTP, GTP and UTP)
  2 µl 10x reaction buffer
  2 µl T7 RNA polymerase enzyme mix
- **Incubate** the reaction mix at 37^oC for 5 hr.
- **Add:**
  1 µl 2 U/µl RNase-free DNase I, and **incubate** at 37 ^oC for 15 min.

(5) Clean-up of aRNA

- **Add** 80 µl nuclease-free dH_2O to sample (total volume: 100 µl)

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- **Extract** with an equal amount (100 µl) of phenol (pH4.3)/chloroform (1:1), and then extract with an equal amount (100 µl) of chloroform.
- **Concentrate** sample in RNeasy mini column (QIAGEN)
- **Add** 350 µl Buffer RLT (with 3.5 µl β-mercaptoethanol), and **mix** thoroughly.
- **Add** 250 µl ethanol, and **mix** thoroughly by pipetting. **Do not centrifuge**.
- **Apply** the sample (700 µl) to an RNeasy mini column placed in a 2 ml collection tube.
- **Spin** 15 sec at 10,000 rpm, and **discard** the flow-through.
- **Transfer** the RNeasy column into a new 2 ml collection tube.
- **Pipette** 500 µl Buffer RPE onto the RNeasy column.
- **Spin** 15 sec at 10,000 rpm, and **discard** the flow-through.
- **Add** another 500 µl Buffer RPE to the RNeasy column.
- **Spin** 2 min at 10,000 rpm to dry the RNeasy silica-gel membrane.
- To elute, **transfer** the RNeasy column to a new 1.5 ml collection tube.
- **Pipette** 30 µl dH_2O onto the RNeasy column, and spin 1 min at 10,000 rpm.
- **Pipette** another 30 µl dH_2O onto the RNeasy column, and spin 1 min at 10,000 rpm.
- **Quantify** RNA concentration (e.g. with RiboGreen assay).

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APPENDIX A—MATERIALS AND SUPPLIERS

Although specific suppliers are included, this does not indicate that materials/equipment from other suppliers would not be suitable; we simply have not tested them.

Materials	Supplier	Catalog number
T7-oligo(dT) primer (5'-TCTAGTCGACGGCCA GTGAATTGTAATACGACTCACTATAGGGCGTTT TTTTTTTTTTTTTTTTTT-3'), HPLC Purified	Integrated DNA Technologies (Coralville, IA)	N/A
Diethylpyrocarbonate, 50 ml	Sigma (St. Louis, MO)	D5758
100 mM dNTP Set: dATP, dCTP, dGTP, dTTP; 4 x 25 μmole	ISC BioExpress (Kaysville, UT)	C-5012-4X25
SuperScript™ II Reverse Transcriptase; 10,000 units, 200 units/μl, supplied with 5x first strand buffer and 0.1 M DTT	Invitrogen (Carlsbad, CA)	18064-014
RNaseOUT™ Recombinant Ribonuclease Inhibitor, 5000 units, 40 units/μl	Invitrogen (Carlsbad, CA)	10777-019
T4 gene 32 protein, 500 μg, 5 μg /μl	USB Corporation (Cleveland, OH)	70029Z 500 UG
E. coli DNA Polymerase I, 2500 units, 10 units/μl, supplied with 1X NEBuffer 2	New England Biolabs (Ipswich, MA)	M0209L
β-nicotinamide adenine dinucleotide hydrate (β-NAD+), min. 98 % from yeast, 250 mg	Sigma (St. Louis, MO)	N7004
Ribonuclease H (RNase H), 30 units, 2 units/μl	Invitrogen (Carlsbad, CA)	18021-014
E. coli DNA ligase, 1000 units, 10 units/μl	New England Biolabs (Ipswich, MA)	M0205L
T4 DNA polymerase, 750 units, 3 units/μl	New England Biolabs (Ipswich, MA)	M0203L
Saturated phenol pH 6.6, 400 ml	Fisher Scientific (Pittsburgh, PA)	BP1750I-400
Chloroform, approx. 0.75% ethanol as preservative, Technical grade, 4L	Fisher Scientific (Pittsburgh, PA)	C295-4
Sodium acetate, anhydrous, fused crystals, certified ACS, 500 g	Fisher Scientific (Pittsburgh, PA)	S210-500
Concentrator/evaporator, Labconco CentriVap DNA system	Fisher Scientific (Pittsburgh, PA)	16-315-47
QIAquick PCR Purification Kit, 250 columns, includes Buffer PB, Buffer PE, and Bufffer EB	Qiagen (Valencia, CA)	28106
MEGAscript® T7 Kit, 40 reactions, includes rNTP solutions, 10x reaction buffer, T7 RNA polymerase enzyme mix, and RNase-free DNase I	Ambion (Austin, TX)	1334
Saturated phenol, pH 4.3, 400 ml	Fisher Scientific (Pittsburgh, PA)	BP1751I-400
RNeasy Mini Kit, 50 columns, includes 1.5 and 2.0 ml collection tubes and RNase-free reagents and buffers	Qiagen (Valencia, CA)	74104
Random hexamer primer, 50 A_{260} units (2 mg)	Roche Diagnostics Co. (Indianapolis, IN)	11034731001
Ethyl alcohol USP, Absolute—200 proof, 1 pint plastic bottle	Aaper Alcohol (Shelbyville, KY)	N/A