

Protocol for mRNA Amplification and Target Preparation

Adaptation of Wang et. al., *Nature Biotechnology*, April, 2000

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RNA amplification is of interest to a broad range of researchers in genomics. Often, primary tissue samples or other sources of RNA yield only small amounts of material. Therefore, several protocols have been developed for “linear” amplification, in an attempt to retain the relative levels of each transcript in the starting material. All of these protocols use a RNA polymerase after first synthesizing first and second strands of cDNA.

Materials

Isolate Total RNA using Qiagen mini kit (Cat#75142) (see manufacturer's protocol) or by Trizol (Gibco BRL Cat# 15596-026) extraction (see manufacturer's protocol). Resuspend total RNA in DEPC water at 1 µg/µl concentration.

Eppendorf Phase Lock Tubes

RNeasy Kit

Phenol/Chloroform

RNaseIN (Promega Cat# N2111)

SuperScript II (Gibco BRL Cat# 18064-071) or PowerScript, Clontech

RNase H (2U/µl Gibco BRL Cat# 18021-071)

Advantage Polymerase (Clontech Cat# 8417-1)

Linear Acrylamide (Ambion Cat# 9520)

Bio-6 Chromatograph column (Bio-Rad Cat# 732-6222)

Standard Stocks of NaOH and EDTA

T7 Transcription Kit (Ambion; T7 Megascript Kit #1334)

Primer sequences:

oligo dT(15)-T7 primer:

5' AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T(15) [57-mer]

TS (template switch) oligo primer:

5' AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG [30-mer]

All incubations are done in a thermal cycler. For starting amounts of less than or equal to 1ug of total RNA a second round of amplification may be required to generate enough aRNA for a microarray hybridization (2.5-3µg aRNA/hybridization.)

Protocol

TUBE PREPARATION

Prepare (and label) 8 sets of tubes:

4 sets of 1.5 ml tubes:

- 1 set for accepting the aqueous phase from the phase lock tubes following cDNA synthesis
- 1 set for receiving the flow-through following the Bio-6 column purification
- 1 set for pre-aliquoting the RNeasy reagent and receiving of the in vitro transcription products
- 1 set for the final aRNA product eluted off RNeasy columns

1 set of 0.5 ml tubes for pre-aliquoting the phenol:chloroform:isoamyl alcohol mix

2 sets (plus 2 optional) of PCR tubes:

- 1 set for the cDNA synthesis
- 1 set for the in vitro transcription

1 set of 0.5 ml eppendorf phase lock tubes. **Be sure to spin the gels down first!**

1. FIRST STRAND CDNA SYNTHESIS

In PCR reaction tube, mix:

Ammt	Reagent
0.5-3 µg	total RNA
9 µl	DEPC H ₂ O
1 µl	0.5 µg/µl oligo dT(15)-T7 primer

70 °C for 3-4 min., snap cool on ice.

Make 1st Strand Master Mix:

Ammt/rxn	Reagent
4 µl	5X First strand buffer
1 µl	1 µg/µl TS (template switch) primer
2 µl	0.1M DTT
1 µl	RNaseIN (Promega Cat# N2111)
2 µl	10mM dNTP (Pharmacia Cat# 27-2035-02)
2 µl	Superscript II (Gibco BRL Cat# 18064-071)

Add total of 12µl to each tube.

42 °C for 90 min. in thermal cycler.

(Note: buffer and 0.1M DTT come with SS II)

2. SECOND STRAND SYNTHESIS

Make 2nd Strand Master Mix:

Ammt/rxn	Reagent
106 μ l	DEPC H ₂ O
15 μ l	Advantage PCR buffer
3 μ l	10 mM dNTP mix
1 μ l	RNase H (2U/ μ l Gibco BRL Cat# 18021-071)
3 μ l	Advantage Polymerase (Clontech Cat# 8417-1)

Add total of 128 μ l to each tube.

37 °C for 5 min. to digest mRNA, 94 °C for 2 min. to denature, 65 °C for 1 min. for specific priming and 75 °C for 30 min. for extension.

Stop reaction with 7.5 μ l 1M NaOH solution containing 2mM EDTA.

Incubate at 65 °C for 10 min. to inactivate enzyme.

3. DS cDNA CLEANUP

Add to PCR Tube

Ammt	Reagent
1 μ l	0.1 μ g/ μ l or 1 μ g/ μ l Linear Acrylamide (Ambion Cat# 9520; comes at 5 μ g/ μ l)
150 μ l	Phenol: Chloroform: Isoamyl alcohol 25:24:1 (Boehringer Mannheim Cat #101001)

Mix well by pipeting (be careful not to spill or contaminate).

Transfer the slurry solution to Phase Lock gel tube (5'-3' Inc. Cat# p1-257178)

Spin at 14,000 rpm for 5 min. at room temperature.

Transfer the aqueous phase to RNase/DNase-free tube (**stopping point, -80 °C**)

Add 70 μ l of 7.5M ammonium acetate (Sigma Cat# A2706) and gently mix.

Add 1 ml 95% room temperature ethanol.

Centrifuge at 14,000 rpm for 20 min. at room temperature.

Prepare Bio-6 Chromatograph column (Bio-Rad Cat# 732-6222)

Shake well before draining to get rid of air bubbles - otherwise it drains very slowly! When opening column, sometimes you will observe gel in the underside of the cap. This should be aspirated off to prevent contamination.

Wash column one time with 700 μ l DEPC H₂O and spin at 700xg for 2 min. at room temperature.

Remove flow-through. Make sure all liquid is drained out of column. Spin again at 700xg for 2 min. to dry column completely.

Wash pellet with 500 μ l 95% EtOH and spin pellet down at maximum speed for 6 min.

Air dry pellet and resuspend ds cDNA in 60 μ l DEPC H₂O.

Load 60 μ l sample to the center of the Bio-6 column and spin at 700xg for 4 min. **(stopping point, -80 °C)**

Transfer samples to new PCR tubes.

Completely dry samples by speed vac. **(stopping point, -80 °C)**

4. IN VITRO TRANSCRIPTION

(Ambion; T7 Megascript Kit#1334)

Make up Master Mix:

Amount	Reagent
8 μ l	of 75mM NTP Mix (A, G, C and UTP) (if new kit, combine NTPs into one tube)
2 μ l	Reaction buffer
2 μ l	Enzyme mix (RNase inhibitor and T7 phage polymerase)
8 μ l	H ₂ O

Add 20 μ l of mix to each PCR tube.

Incubate at 37 °C for 6 hrs.

5. a-RNA PURIFICATION USING Qiagen RNeasy COLUMNS

Make up RLT w/ Beta-ME and H₂O Master Mix:

Per sample:

3.5 μ l β -ME

80 μ l H₂O

350 μ l RLT

Pre-alias 430 μ l RLT w/ β -ME and H₂O to 1.5ml Rnase/Dnase-free tubes.

Transfer contents of in vitro transcription mix to the tube.

Mix well. **(stopping point -80 °C overnight)**

Add 250 μ l ethanol (95%) and mix well by pipetting. (Do not centrifuge!)

Apply sample (700 μ l) to RNeasy mini spin column sitting in a collection tube.

Centrifuge 15 sec. at \geq 8000 x g. Discard flow through.

Transfer RNeasy column to a new 2-ml collection tube (supplied). Add 500 μ l Buffer RPE (which must contain ethanol) and centrifuge 15 sec. at \geq 8000 x g. Discard flow-through but re-use tube.

Pipet 500 μ l Buffer RPE onto RNeasy column and centrifuge for 2 min. at maximum speed.

Remove flow through and pipet another 500 μ l Buffer RPE onto column. Centrifuge for 2 min. at maximum speed.

Place RNeasy spin column into a new 1.5-ml or 2-ml collection tube (not supplied) and spin at full speed for 1 min. to completely dry column.

Transfer RNeasy column into a new 1.5-ml collection tube (supplied) and add 30 μ l RNase-free water directly onto membrane. Centrifuge for 1 min. at $\geq 8000 \times g$ to elute. Repeat if expected yield is $\geq 30 \mu$ g.

Check RNA concentration and quality by measuring OD₂₆₀ and OD_{260/280}.

6. Second round amplification

You only need to perform a second round of amplification if the starting input was too low to result in adequate yield of aRNA for labeling (2.5-3 μ g).

Mix aRNA(0.5-1 μ g) in 9 μ l DEPC H₂O with 1 μ l (2 μ g/ μ l) random hexamer (i.e. dN6) and heat to 70 °C for 3 min., cool to room temperature. Let sit for 10 minutes. Then add the following reagents:

- 4 μ l 5 X First strand buffer
- 1 μ l (0.5 μ g/ μ l) oligo dT-T7 primer
- 2 μ l 0.1 M DTT
- 1 μ l RNAsin (Promega Cat# N2111)
- 2 μ l 10mM dNTP (Pharmacia Cat# 27-2035-02)
- 2 μ l Superscript II (SS II) (Gibco BRL Cat# 18064-071)

42 °C for 90 minutes

From here, follow the procedure of first round amplification for ds cDNA synthesis and cleanup.

Follow standard microarray labeling techniques, using between 3 - 6 μ g of aRNA as input. Remember to use dN6 primer instead of oligo-dT. Use between 5 - 10 μ g of primer.