# Modified Eberwine ("ANTISENSE") RNA Amplification Protocol

**Chris Barry, MD, PhD, Pat Brown Lab, 4/22/99**
**barry_c@cmgm.stanford.edu**

**The optimal range for amplifying from mRNA is 20-100 ng. The optimal range for total RNA is 1-3 µg.**

## First Strand Synthesis:

**(First and second strand synthesis reactions are performed in 0.2 mL RNase free PCR tubes.)**

1. **Mix mRNA, trehalose (Sigma #T-5251, make 1.7M stock in DEPC water)/DEPC water to 9 µL (final concentration of 0.6M trehalose/20 µL) and "Eberwine" oligo-dT/T7 primer (5'AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T15-3')1 uL (1 µg/µL). May dry down RNA in Speedvac to concentrate. Trehalose is viscous; mix well by pipeting.**
2. **Heat 65°C for 10 minutes then put on ice.**
3. **Add 4µL 5X First Strand Buffer (GibcoBRL, comes with Superscript II enzyme) 2µL 0.1M DTT (GibcoBRL, comes with Superscript II enzyme) 1 µL RNAsin (GibcoBRL #15518-012) 1µL 10 mM dNTP mix (Pharmacia #27-2035-01, resuspend in DEPC water) 1 µL linear acrylamide (0.1 µg/µL, Ambion #9520 or homemade--see recipe below) 1 µL Superscript II (reverse transcriptase, GibcoBRL #18064-014)**
4. **Thermocycle: 37°C 5 min, 45°C 5 min, then 10 cycles alternating between 60°C 2 min and 55°C 2min.**
5. **Place on ice and keep cool while adding second strand components.**

## Second Strand Synthesis:

1. **Add to 1st strand reaction: 106 µL DEPC water, 15 µL 10X Second Strand Buffer (recipe below) 3 µL 10 mM dNTP mix (dilute in DEPC water, Pharmacia #27-2035-01), 1 µL E. coli DNA Ligase (10 U/µL, NEB #205L), 4 µL E. coli DNA Polymerase I--holoenzyme (10 U/µL, NEB #209L), 1 µL RNase H (2 U/µL, GibcoBRL #18021-071)**
2. **Incubate 16°C for 2 hours. If amplifying from mRNA, stop reaction with 10 µL 0.5M EDTA and incubating at 65°C for 10 minutes. If amplifying from total RNA, stop with 7.5 µL 1M NaOH/2mM EDTA and incubating at 65°C for 10 minutes (latter degrades ribosomal and tRNA that can interfere with subsequent in vitro transcription reaction).**

## Sample Extraction and Precipitation:

1. **Phenol:chloroform:isoamyl (25:24:1) extract once (use 0.5 ml "phase lock gel" tubes from 5'-3' Inc, #p1-257178). Add organics (150 µL) directly to PCR tubes, being careful not to spill. Mix by pipeting 5-10X, then transfer slurry to Phase lock gel tubes. Spin 5 minutes maximum speed (15k x g) at room temperature. Transfer aqueous phase to RNase free 1.5 ml eppendorf tube.**
2. **Add 70 µL 7.5M ammonium acetate (in DEPC water, 0.2 micron filtered) to aqueous phase, then 1 mL absolute ethanol (-20°C). Vortex, centrifuge immediately for 20 minutes, maximum speed, at room temperature. It is important to spin right away so as not to precipitate residual proteins or low molecular weight nucleotides (i.e., free nucs or primer).**

3. Wash with 100 µL absolute ethanol once, spin 5 minutes. At this point, a large (salt) pellet should be visible; if no pellet is seen at all, suspect RNase contamintion of reagents or degraded starting RNA. Remove all excess ethanol, dry briefly at room temperature (not completely or resuspension will be difficult).
4. Resuspend in 10 µL DEPC water. (May stop here indefinitely at -20°C.)
5. Prepack Sephadex G75 spin columns (Pharmacia #17-0050-01, make slurry by adding 3g of powder to 50 mL DEPC TE, use 1 cc syringes and glass wool plug, prespin twice for 5 minutes, 700xG, room temperature, adding resin between first and second spin for final packed resin volume=0.8-1.0 mL), then pass cDNA (10 µL) over column for 5 minutes at 700xG. When loading column, be careful to put the sample in the center of the matirx in order to prevent wicking of sample onto side. Lyophilize flow through (20-50 µL) to 16 µL or less in Speedvac.

## In Vitro Transcription:

1. Ambion T7 Megascript Kit (#1334), double the standard 20 µL reaction (total volume=40 µL), 37°C for 4 hours. (Follow manufacturer's instructions verbatim.)
2. Phenol:chloroform:isoamyl extract once (in 5'-3' phase lock gel tubes).
3. Pass over ChromaSpin TE+30 column (Clontech #K1321-2). Prepare columns while performing the organic extraction: remove top cap then snap off bottom; prespin 5 minutes at 700 x g, room temperature. When loading column, be careful to put the sample in the center of the matirx in order to prevent wicking of sample onto side.
4. Transfer flow through to Rnase free 1.5 mL Eppendorf tube. Lyophilize to 13 µL or less.
5. Adjust volume to 13 µL DEPC water, if necessary.
6. Quantitate approximate yield by 1% agarose electrophoresis (1uL/lane) and/or spectrophotometry (1:50 or 1:100 dilution). OD is less accurate due to contaminating primer/free nucleotides. Gel shows whether there is product or not. Do not procede with labeling if no product seen. Ready for Cy3/5 dUTP labeling with random hexamer priming. Label at least 5 µg of amplified RNA per reaction. Use 6-8 µg of random hexamers per labeling reaction.

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10X Second Strand Buffer:

200 mM Tris pH 6.9
900 mM KCl
46 mM $MgCl_2$
1.5 mM Nicotine Adenine Dinucleotide (Calbiochem #481915)
100 mM $(NH_4)_2SO_4$

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Linear Acrylamide Recipe
(from Joe Derisi, Brown lab)

for 375 µL of 10mg/ml stock:

75 µL 5% acrylamide:
3.75 mg acrylamide
1.5 µL 2M Tris pH 8

**0.5 µL 3M NaAc**
**0.1 µL 0.5M EDTA**
**73 µL water**

**add 1 µL 10% ammonium persulfate and 0.1 µL TEMED**
**polymerize 30 minutes**
**add 2.5 volumes absolute etoh**
**spin 5 minutes top speed**
**aspirate supernatant**
**redissolve in 375 µL (DEPC) water for final concentration of 10 µg/µL**

**dilute 1:100 in DEPC water for use in Eberwine antisense RNA amplification protocol**

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**References:**

**Luo, et al., Nature Medicine 5(1):117, 1999.**
**Carninci, et al., PNAS 95:520, 1998.**
**Eberwine, et al., PNAS 89:3010, 1992.**
**Van Gelder, et al., PNAS 87:1663, 1990.**