Jeffrey Lab RNA Amplification Protocol

First strand cDNA synthesis

Mix the following contents in 0.2 ml PCR tube and spin briefly:

Total RNA	Eberwine primer*	RNase-free H_2O
N μl (3 μg)	1 μl (1 μg/μl)	8 – N μl

*Eberwine primer = oligo-dT(15)-T7 (5'-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC-3'). Protocol performs equally well with primer input of 0.5 – 1 μg.

Incubate in PCR machine at 70 C for 3 min, cool on ice for 2 min.
Spin briefly.
Add the following contents:

5X first strand buffer	0.1M DTT	RNasin	10mM dNTP mix	Superscript™II*
4 μl	2 μl	1 μl	2 μl	2 μl

*Invitrogen

Mix contents, spin briefly, and incubate at 42 C for 1.5 hours.

Second strand cDNA synthesis

Add the following contents to the first strand synthesis reaction:

RNase-free H_2O	106 μl
10X Advantage™ PCR buffer*	15 μl
10mM dNTP mix	3 μl
RNase H (2U/μl)	1 μl
Advantage™ Polymerase Mix*	3 μl

*Clontech

Incubate in PCR machine at 37 C for 5 min, 94 C for 2 min, 65 C for 1 min, 75 C for 30 min. Stop reaction by adding 7.5 μl of 1M NaOH with 2mM EDTA and incubate in PCR machine at 65 C for 10 min.

ds cDNA cleanup

If necessary, transfer to a larger tube. Add 150 μl phenol:chloroform:isoamyl alcohol 25:24:1 and mix by pipetting. Transfer to phase lock gel tube and spin at ~16,000g for 5min.
Transfer aqueous layer to new tube. Add 1 μl linear acrylamide (0.1 μg/μl), 70 μl 7.5M NH_4Ac, 1 ml 100% EtOH. Spin at ~16,000g for 20 min at room temperature (not at 4 C).
Wash pellet with 500 μl 75% EtOH, spin at ~16,000g for 5 min.
Air dry, and suspend the pellet in 16 μl RNase-free H_2O.

In vitro transcription (IVT)

Mix at room temperature (the spermidine in the 10X transcription buffer can coprecipitate the template DNA if the reaction is assembled on ice):

cDNA	10X reaction buffer (room temperature)	75mM NTP mix (on ice)	T7 enzyme mix*
16 μl	4 μl	16 μl	4 μl

*Ambion T7 MEGAscript™ kit

Incubate at 37 C for 5 hours (we have tried incubating for 2-6 hours and achieved the highest correlation between amplified and unamplified samples at 5 hours incubation).

aRNA cleanup (using Qiagen RNeasy® mini kit)

Add 10 μl β-mercaptoethanol to 1ml buffer RLT before use (stable for 1 month).

Transfer IVT reaction into a 1.5 ml new tube, add 60 μl RNase-free H_2O, add 350 μl buffer RLT, mix by pipetting. Add 250 μl 100% EtOH, mix by pipetting.
Apply sample to RNeasy® column, spin at ~12,000g for 15 sec at room temperature. Transfer column to new collection tube.
Add 500 μl buffer RPE, spin at ~12,000g for 15 sec. Discard flow through, add 500 μl buffer RPE, spin at ~12,000g for 2 min.
Transfer column to new collection tube, add 30 μl RNase-free H_2O to the membrane, spin at ~12,000g for 1 min to elute

Measure aRNA yield at A_{260}