

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 ₂ O
N μ l (3 μ g)	1 μ l (0.5 μ g/ μ l)	9 μ l N μ l

* Eberwine primer = T7-oligo-dT(15) (5'-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC TTT TTT TTT TTT TTT-3')

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 μ l	2 μ l	1 μ l	2 μ l	2 μ l

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 ₂ O	106 μ l
10X Advantage [®] PCR buffer*	15 μ l
10mM dNTP mix	3 μ l
RNase H (2U/ μ l)	1 μ l
Advantage [®] Polymerase Mix*	3 μ l

*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 μ l 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 μ l 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 μ l acrylamide (0.1 μ g/ μ l), 70 μ l 7.5M NH₄Ac, 1 ml 100% EtOH. Spin at ~16,000g for 20 min at room temperature (not at 4°C).

Wash pellet with 500 μ l 75% EtOH, spin at ~16,000g for 5 min.

Air dry, and suspend the pellet in 16 μ l RNase-free H₂O.

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 μ l	4 μ l	16 μ l	4 μ l

*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 μ l β -mercaptoethanol to 1ml buffer RLT before use (stable for 1 month).

Transfer IVT reaction into a 1.5 ml new tube, add 60 μ l RNase-free H₂O, add 350 μ l buffer RLT, mix by pipetting. Add 250 μ l 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 μ l buffer RPE, spin at ~12,000g for 15 sec. Discard flow through, add 500 μ l buffer RPE, spin at ~12,000g for 2 min.

Transfer column to new collection tube, add 30 μ l RNase-free H₂O to the membrane, spin at ~12,000g for 1 min to elute

Measure aRNA yield at A₂₆₀