

Reduced Volume RNA Amplification Protocol

The following mRNA amplification protocol is a modified version of that published by Baugh et al., (2001). This is an alternative to the standard amplification protocol, which minimizes the amount of reagents by reducing the overall reaction volume during the cDNA synthesis step. In addition to reduced cost, this approach has been optimized to improve the quality of transcripts generated by utilizing T4gp32 single stranded binding protein (USB) and by minimizing secondary reactions. Please refer to the author's notes in the original protocol found at <http://www.mcb.harvard.edu/hunter/Protocols/aRNAprotocol.pdf>.

Starting material and rounds of amplification:

- ≥ 1.0 ug totRNA → 1 Round amplification
- ≥ 1.0 ng totRNA → 2 Rounds amplification
- ≥ 1.0 pg totRNA → 3 Rounds amplification

Starting material and RT reaction volumes:

- ≥ 100ng total RNA → 10 ul reverse transcription (RT) reaction
- < 100ng total RNA → Reduce volume by 1ul per 10 ng < 100 (10 ng total RNA per ul) down to 2 ul
- *Note- 1ug of totRNA has been successfully amplified using a 2ul 1st strand cDNA synthesis reaction for the first round of amplification.

To maintain a good ratio of RNA to primer, the concentration of primer used should be constant as reaction volumes are scaled down. The use of premixes in setting up the reactions is essential as they are scaled down.

Round 1 Amplification

starting material ≥ 100 ng totRNA (10 ul RT reaction)

1st Strand cDNA Synthesis

1. Combine 1 ul (dT)-T7 primer (100ng/ul) with your total RNA in a 1.5 ml tube.
2. Speed-vac, no heat, the primer + RNA down to 5.0 ul if needed WITHOUT DRYING COMPLETELY.
Have as a visual gauge 5 ul of water in a 1.5 ml tube, avoid measuring the volume by pipetting to prevent the loss of material.
3. Denature RNA + primer at 70°C for 4 minutes in a thermal cycler with a heated lid. Snap cool on ice, and keep on ice.
The volume may drop after denaturation. You should determine with controls beforehand how much it drops in your hands so as to add the appropriate amount of extra dH2O to the premix to make up for it.

4. Prepare RT premix/5ul:

5x First Strand Buffer	2 ul
100 mM DTT	1 ul
10 mM dNTP	0.5 ul
T4gp32 (8.0 mg/ml)	0.5 ul
Rnase Inhibitor (~20 U)	0.5 ul
SuperScript II (100 U)	0.5 ul
Water (after denaturation to restore volume)	?

5. Add 5 ul ice cold RT premix for a RT rxn volume of 10 ul and mix by pipetting.
6. Incubate for 1 hr at 42°C in either a thermal cycler with a heated lid or an air incubator, but not in a water bath.
7. Heat inactivate the rxn at 65°C for 15 min and immediately chill on ice.

2nd Strand cDNA Synthesis & Clean-up

1. Prepare second strand synthesis (SSS) premix:

5x Second Strand Buffer	15 ul
10 mM dNTP	1.5 ul
20 U DNA Polymerase I (10U/ul)	2 ul
1 U <i>E. coli</i> Rnase H (2U/ul)	0.5 ul
5 U <i>E. coli</i> DNA Ligase (10U/ul)	0.5 ul
Water → 65ul final volume	45.5 ul

2. Add 65 ul ice-cold SSS premix for a 75 ul SSS rxn volume, and mix by pipetting.
3. Be sure the premix is cold when you add it.
4. Incubate at 14-16°C for 2 hours.
5. On ice, add 2 ul T4 DNA Polymerase (5U/ul) and mix by flicking and gentle vortexing.
6. Incubate at 14-16°C for 15 min.
7. Immediately heat inactivate the reaction by heating it to 70°C for 10 min.
Go directly from 15C to 70°C to avoid undesirable enzyme activities.
8. Add 75 ul phenol:chloroform (1:1), and mix by pipetting vigorously.
9. Transfer to prespun PLG Heavy (Phase Lock Gel-Eppendorf), and spin 5 min. at 13K rpm.
10. Carefully transfer aqueous layer to a new 1.5 ml eppendorf tube.
12. Ethanol precipitation

7.5M NH ₄ Oac	38 ul
Cold 100% EtOH	300 ul
5 µg/µl Glycogen (can inhibit subsequent RT rxns)	4 ul

13. Incubate for 10 min. at – 80°C to precipitate pellet (or overnight)
14. Spin at 14,000 rpm at 4°C for 25 min.
15. Carefully remove the EtOH and wash pellet with 500ul cold 80% EtOH.
16. Centrifuge for 10 min. at 4°C at 14,000 rpm.
17. Carefully remove EtOH and air-dry pellet.
18. Immediately resuspend the pellet in 8ul DEPC H₂O.

Round 1 IVT: Epicentre T7 Ampliscribe protocol

1. Add the following to each 8ul reaction:

10X T7 Reaction Buffer	2 ul
ATP	1.5 ul
CTP	1.5 ul
GTP	1.5 ul
TTP	1.5 ul
DTT	2 ul
T7 Enzyme Solution	2 ul

*Hold at room temperature (minus T7 enzyme solution) to avoid precipitation.

2. Mix by gently flicking and gently vortexing.
3. Incubate at 42°C for at least 3 hrs.
4. Degrade the cDNA by adding 1ul Rnase free Dnase I (Ambion) for at least 15 min. at 42°C.
5. Freeze or proceed to clean-up.

IVT Clean-up (Qiagen RNeasy) I:

1. Add Rnase free DEPC-treated H₂O to 100 ul.
2. Add 350 ul Buffer RLT, mix.
3. Add 250 ul 100% EtOH, mix.
4. Apply mix to RNeasy column. Wait 3 min.
5. Spin at 10,000 rpm for 1 min.
8. Transfer column to a new 2 ml collection tube.
9. Add 500 ul Buffer RPE.
10. Spin at 10,000 rpm for 1 min.
11. Discard flow-through. Add 500 ul Buffer RPE. Spin at 10,000 rpm for 2 min.
12. Discard flow-through and spin column at 10,000 rpm for 3 min.
14. Add 50 μ l Rnase free DEPC-treated H₂O. Soak for 3 min. Spin at full speed for 2.5 min. Quantify amount of IVT product on Nanodrop.

Round 2 Amplification***1st Strand cDNA Synthesis II***

1. Add 1 ul random primers (0.5 ug/ul) (mostly hexamers, Invitrogen) to aRNA from round 1.
2. Speed-vac, no heat aRNA + hexamers to 5.0 ul. (25-35 min.)
3. Heat to 70°C for 5 min. in a thermal cycler with a heated lid.
4. Snap cool on ice.
5. Allow to sit at room temperature for 5 min.
6. Prepare RT premix/5ul:

5x 1 st Strand Buffer	2 ul
100 mM DTT	1 ul
10 mM dNTP	0.5 ul
T4gp32 (8.0 mg/ml)	0.5 ul
Rnase Inhibitor (~20 U)	0.5 ul
SuperScript II (100 U)	0.5 ul
Water (after denaturation to restore volume)	?

7. Add 5 ul room temperature RT premix, and mix by pipetting.
8. Incubate for 1 hr at 42°C in either a thermal cycler with a heated lid or an air incubator, but not in a water bath.
9. Add 0.5 ul (2U/ul) Rnase H, and mix by vortexing gently.
10. Incubate for 30 min at 37°C and then heat to 95°C for 2 min.
11. Chill on ice and then spin briefly to collect condensation and return to ice.

2nd Strand cDNA Synthesis & Clean-up II

1. Add 1 ul (100ng/ul) (dT)-T7 while on ice.
2. Incubate at 42°C for 10 min. to anneal the primer.
3. Snap-cool samples on ice.
4. Prepare SSS premix (minus ligase).

5x Second-Strand Buffer	15 ul
10 mM dNTP	1.5 ul
20 U DNA Polymerase I (10U/ul)	2 ul
1 U <i>E. coli</i> Rnase H (2U/ul)	0.5 ul
Water → 65ul final volume	46.0 ul

5. Add 65 ul ice-cold SSS premix for a 75 ul SSS rxn volume. Make sure both are cold before adding premix.
6. Incubate at 14-16°C for 2 hrs.
7. Add 2ul T4 DNA Polymerase(5U/ul) and mix by gentle flicking and vortexing.
8. Incubate at 14-16°C for 15 min.
9. Immediately heat inactivate the reaction by heating it to 70°C for 10 min.
10. Add 75 ul phenol:chloroform (1:1), and mix by pipetting vigorously.
11. Transfer to prespun PLG Heavy, 0.5 ml tubes (Phase Lock Gel-Eppendorf), and spin 5 min. at 13,000 rpm and transfer aqueous layer to a new 1.5ml eppendorf tube.
12. Ethanol precipitation

7.5M NH ₄ OAc	38 ul
Cold 100% EtOH	300 ul
5 µg/ul Glycogen (can inhibit subsequent RT rxns)	4 ul

13. Incubate for 10 min. at – 80°C to precipitate pellet (or overnight).
14. Spin at 14,000 rpm at 4°C for 25 min.
15. Carefully remove the EtOH and wash pellet with 500 ul cold 80% EtOH.
16. Centrifuge for 10 min. at 4°C at 14,000 rpm.
17. Carefully remove EtOH and air-dry pellet.
18. Immediately resuspend the pellet in 8 ul DEPC H₂O (or 22 ul if proceeding to ENZO's IVT)

Round II IVT: Epicentre T7 Ampliscribe Protocol

1. Add the following to each 8ul reaction:

10X T7 Reaction Buffer	2 ul
ATP	1.5 ul
CTP	1.5 ul
GTP	1.5 ul
TTP	1.5 ul
DTT	2 ul
T7 Enzyme Solution	2 ul

*Hold at room temperature (minus T7 enzyme solution) to avoid precipitation

2. Mix by gently flicking and gently vortexing.
3. Incubate at 42°C for at least 3 hrs.
4. Degrade the cDNA by adding 1ul Rnase free Dnase I (Ambion) for at least 15 minutes at 42°C.
5. Freeze or proceed to clean-up.

IVT Clean-up (Qiagen RNeasy) II:

1. Add Rnase free DEPC-treated H₂O to 100 ul
2. Add 350 ul Buffer RLT, mix.
3. Add 250 ul 100% EtOH, mix.
4. Apply mix to RNeasy column. Wait 3 min.
5. Spin at 10,000 rpm for 1 min.
8. Transfer column to a new 2 ml collection tube.
9. Add 500 ul Buffer RPE.
10. Spin at 10,000 rpm for 1 min.
11. Discard flow-through. Add 500 ul Buffer RPE. Spin at 10,000 rpm for 2 min.
12. Discard flow-through and spin column at 10,000 rpm for 3 min.
14. Add 50-100 ul Rnase free DEPC-treated H₂O. Soak for 3 min. Spin at full speed for 2.5 min. Quantify amount of IVT product on Nanodrop.