Liu Lab protocol
Bruno Fang/Jacy Villa
Updated by Amir Jazaeri
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Jazaeria@mail.nih.gov

Protocol for Amplification of RNA –Modified from Eberwine

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Reagents

- Superscript II RT, Gibco Life technologies # 18064-014
- *E. coli* DNA Polymerase I, Gibco Life technologies # 18010-025
- *E. coli* DNA Ligase, Gibco Life technologies # 18052-019
- *E. coli* RNAse H, Gibco Life technologies # 18021-014
- T4 DNA Polymerase, Gibco Life technologies # 18005-025
- 5X second strand buffer, Gibco Life technologies # 10812-014
- dNTP Set, 10mM Gibco Life
- T7-(dT)24 Primer, GENSET Corp), HPLC purified DNA-5'-GGC-CAG-TGA-ATT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-AGG-CGG-(dT)24-3'
- DEPC treated water, Ambion #9902 or Water, Molecular Biology Grade, BioWhittaker, #16-001Y
- RNAsin, Promega #N2515
- EDTA 0.5 M
- NaOH 1 M
- Phase Lock gel, 5 prime to 3 prime, Inc #pl-188233 for 200 or #pl-175850 for 50
- Phenol/Chloroform/isoamyl alcohol, Ambion #9732
- 7.5M Ammonium Acetate, Sigma #A2706
- 95% Ethanol
- 80% Ethanol
- Linear acrylamide, Ambion #9520
- T7 Megascript Kit, Ambion #1334
- Random hexamers, Gibco Life technologies #48190-011
- Qiagen Rneasy Mini Kit (50 samples) #74104

Check Before Starting

- Heating Block at 70ºC
- 95% and 80% ethanol in -20ºC
- Cold bath at 16ºC

First Strand Synthesis

1. Primer hybridization:
   - 1-3 µg   Total RNA
   - (Variable) Nuclease free H_2O (to complete 1st strand final reaction volume of 20 µl)
   - 1 µl T7-$(dT)_{24}$ primer (2µg/µl)

2. Incubate at 70ºC for 10 minutes
3. Quick spin and put on ice
4. Add:
   - 4 µl   5X First strand cDNA buffer
   - 2 µl   0.1M DTT
   - 2µl   10mM dNTP mix
   - 1µl   RNAsin
5. Add 2 µl Superscript II
6. Mix well and incubate at 42ºC for 1 hour
7. Centrifuge briefly, place on ice

Second Strand Synthesis

8. Add:
   - 91 µl   DEPC treated H_2O
   - 30 µl   Second strand buffer
   - 3 µl   10 mM dNTP mix
   - 4 µl   DNA Polymerase I (10U/ µl)
   - 1 µl   DNA Ligase (10U/µl)
   - 1 µl   RNAse H (2U/µl)

   Final Volume: 150 µl

9. Gently tap tube to mix, briefly centrifuge
10. Incubate at 16ºC for 2 hours
11. Add 2 µl (10U) T4 DNA Polymerase
12. Cool for 5 minutes at 16ºC
13. Stop reaction with 10 µl of 0.5 M EDTA, then add 10 µl of 1M NaOH
14. Incubate at 65ºC for 10 minutes, then neutralize solution with 25 µl Tris-HCl (pH=7.5)

Clean Up of Double Stranded cDNA

15. Pellet the Phase Lock Gel in a microcentrifuge at maximum speed for 30 seconds
16. Add 198 µl (equal volume) of (25:24:1) Phenol : chloroform : isoamyl alcohol (saturated with 10 mM Tris-HCL pH 8.0/1mM EDTA) to the final DNA synthesis preparation (198µl) to a final volume of 396 µl. Mix well by pipetting up & down vigorously.

17. Transfer the entire cDNA-phenol/chloroform mixture to the PLG tube
18. Do not vortex. Microcentrifuge at maximum speed for 2 minutes
19. Transfer the aqueous supernatant to a new 1.5 ml tube
20. Add 1 µl linear acrylamide
21. Add 0.5 volumes of 7.5M Ammonium Acetate + 2.5 volumes (include the added Ammonium Acetate) of 95% ethanol stored at –20 to the sample and vortex. (may stop here, keep sample at -20ºC and continue next day)
22. Centrifuge at maximum speed in a microcentrifuge at room temperature for 20 minutes
23. Remove supernatant. Wash pellet (may be invisible) with 0.5 ml of 80% ethanol
24. Centrifuge at maximum speed for 5 minutes at room temperature
25. Carefully pour-off the 80% ethanol.
26. Repeat the 80% ethanol wash once again
27. Air dry the pellet (~ 15 min.)
28. Resuspend the pellet in 16 µl of Nuclease-free water

In Vitro Transcription

29. Ambion T7 Megascript kit- Follow manufacturer's instructions for a total 40 µl reaction (double the 20 µl standard reaction, 37ºC for 4-5 hours)
    SET UP REACTION AT ROOM TEMPERATURE
    If done on ice, spermidine in the buffer can lead to template precipitation

- 16µl of template DS DNA (from step 28).
- 4µl of 10x Reaction Buffer
- 4µl of ATP solution (75mM T7)
- 4µl of CTP solution (75mM T7)
- 4µl of GTP solution (75mM T7)
- 4µl of UTP solution (75mM T7)
- 4µl of Enzyme Mix
- Incubate at 37ºC for 5-6 hours.

30. Add 60 µl Nuclease free water to bring total volume up to 100µl.
31. Follow "RNA clean-up" protocol in the Qiagen RNeasy mini handbook
32. Elute with 30µl of Nuclease free water.
33. Check O.D. and ratio

    Expected yield: ~10 X starting amount of total RNA

34. Proceed using the Liu lab protocol for generating probe for microarrays using total RNA. Use 3 to 5 µg amplified RNA and **random hexamer primers (2 µl of 3 µg/µl instead of oligo-dT primer)**

For 2nd Round Amplifications:

- Ressuspend 0.5-1.0µg of amplified RNA in 11 µl ultrapure water

First Strand Synthesis

1. Add 1 µl Random hexamer (1 mg/ml)
2. Incubate 70ºC for 10 minutes, then chill on ice
3. Equilibrate at room temperature for 10 minutes
4. Add:
    - 4 µl 5X First strand cDNA buffer
    - 2 µl 0.1M DTT
    - 2µl 10mM dNTP mix
    - 1µl RNAsin
5. Mix and incubate at 42ºC for 2 minutes
6. Add 2 µl Superscript II
7. Mix well and incubate at 42ºC for 1 hour
8. Add 1 µl RNAse H and incubate at 37ºC for 20 minutes
9. Heat to 95ºC for 2 minutes and chill on ice

Second Strand Synthesis

10. Add 1 µl T7-oligodT primer (0.5 mg/ml), incubate 70ºC for 5 minutes and at 42ºC for 10 minutes
11. Then add:
    - 91 µl DEPC treated H_2O
    - 30 µl Second strand buffer
    - 3 µl 10 mM dNTP mix
    - 4 µl DNA Polymerase I (10U/ µl)
    - 1 µl DNA Ligase (10U/µl)
    - 1 µl RNAse H (2U/µl)

    Final Volume: 150 µl

12. Gently tap tube to mix, briefly centrifuge
13. Incubate at 16ºC for 2 hours
14. Add 2 µl (10U) T4 DNA Polymerase
15. Cool for 5 minutes at 16ºC
16. Stop reaction with 10 µl of 0.5 M EDTA, then add 10 µl of 1M NaOH
17. Incubate at 65ºC for 10 minutes, then neutralize solution with 25 µl Tris-HCl (pH=7.5)

Clean Up of Double Stranded cDNA and In Vitro Transcription: ***Proceed as above***