

Anti-sense strand amplification of RNA using RT-PCR and Ambion MEGA script T7 and direct labelling for cDNA microarrays

Outline

Amplification is sometimes used to produce sufficient RNA for labelling when the amount of biological material is limiting. The method we use is based on a protocol from Gertrud Woerfel (<http://www.gen.cam.ac.uk/Research/russell.htm>). The samples or samples and controls to be compared are each then labelled with a different fluorescent dye and subjected to paired competitive hybridisations.

Equipment and reagents

- (dT)-T7 primer: GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA-(T)_{n=21}-[A or G or C]
- T4 Gene 32 protein (Amersham; Cat. No. E70029Z)
- Second strand buffer (Invitrogen; Cat. No. 10812-014)
- DNA Polymerase I (Invitrogen; Cat. No. 1801-025)
- RNaseH (New England Biolabs; Cat. No. M0297S)
- *E. coli* DNA ligase (Amersham; Cat. No. E70020Z)
- T4 DNA polymerase (Roche Diagnostics; Cat. No. 1004786)
- Microspin columns with Bio-Gel P6 in Tris (Bio-Rad; Cat. No. 732-6221)
- Phenol:Chloroform:Isoamylalcohol (Fisher Scientific; Cat. No. BPE 1754 I-100)
- Sodium Chloride (VWR; Cat. No. 102414P)
- Gene Elute LPA (Sigma; Cat. No. 56575)
- 100% Ethanol
- MEGA-Script T7 kit (Ambion; Cat. No. 1334)
- Sodium Acetate
- Phenol, saturated pH 4.3 (Fisher Scientific; Cat. No. BPE 1751 I-100)
- 70% Ethanol
- RQ1 RNase-free DNase (Promega; Cat. No. M6101)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Random primer (Promega; Cat. No. C1181)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- DEPC-treated MilliQ water
- Cy3 dCTP (Amersham; Cat. No. PA 53021)
- Cy5 dCTP (Amersham; Cat. No. PA 55021)
- RNasin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- EDTA (BDH; Cat. No. 100935V)
- NaOH, (BDH; Cat. No. 102525P)
- Tris-HCl solid (BDH; Cat. No. 443864E)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- AutoSeq G-50 column (Amersham, Cat. No. 27-5340-01)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

RNA amplification

Making the dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
 - ◆ 100 µl 100mM dNTA
 - ◆ 100 µl 100mM dNTT
 - ◆ 100 µl 100mM dNTG
 - ◆ 100 µl 100mM dNTC
2. Then make up to 1 ml using DEPC-water
3. Aliquot the dNTP mix and then store at -20 °C

Reverse Transcription reaction:

The following steps are performed in 200 µl PCR tubes and the PCR block

1. Take up to 4.5 µl of the extracted RNA (small scale RNA extraction protocol), add 0.5 µl of Primer T7-dT (200 ng/µl) and then make up to a total volume of 5 µl with DEPC- water
2. Incubate at 65 °C for 10 min
3. Then snap freeze on ice
4. Make up a premix for the RT reaction:
 - ◆ 2 µl Superscript Buffer (from superscript kit)
 - ◆ 1 µl 0.1M DTT (from superscript kit)
 - ◆ 1 µl 10 mM dNTP mix
 - ◆ 0.8 µl T4 Gene 32 protein
 - ◆ 0.5 µl RNasin
 - ◆ 1 µl Superscript III
5. Add 6.3 µl to each sample
6. Incubate at 42 °C for 2 hours
7. Incubate at 65 °C for 15 minutes
8. Then snap freeze on ice

Second strand synthesis:

The following steps are performed in 200 µl PCR tubes and the PCR block

9. Make up a premix for the second strand synthesis:
 - ◆ For 1 reaction:
 - ◆ 45 µl DEPC-water
 - ◆ 15 µl Second strand buffer
 - ◆ 1.5 µl 10 mM dNTP mix
 - ◆ 4 µl DNA Polymerase I
 - ◆ 0.2 µl RNaseH
 - ◆ 1 µl *E. coli* Ligase
10. Add 66.7 µl to each sample

11. Incubate at 16 °C for 2 hours
12. Add 2.0 µl T4 DNA polymerase
13. Incubate at 15 °C for 15 minutes, then 70 °C for 10 minutes
14. Prepare Microspin columns with Bio-Gel P6 in Tris:
 - ◆ Invert columns sharply 2-3 times
 - ◆ Snap off the bottom and remove the lid
 - ◆ Let the column drain by gravity flow
 - ◆ Shortly before use spin for 2 minutes at 2500 rpm
15. Phenolisation: add 75 µl PCI (Phenol/Chloroform/Isoamylalcohol) to the sample and vortex
16. Centrifuge for 10 minutes at 13000 rpm
17. Transfer upper phase to the Microspin columns with Bio-Gel P6 in Tris columns and centrifuge for 4 minutes at 2500 rpm (1000 x g)

Precipitate cDNA:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

18. Transfer the second strand synthesis product to 1.5 ml microfuge tubes and then add
 - ◆ 3.5 µl 5M Sodium Chloride
 - ◆ 0.5 µl LPA
 - ◆ 220 µl 100% Ethanol
19. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
20. Centrifuge for 30 minutes at 13000 rpm
21. Remove supernatant and wash with 70% Ethanol, centrifuge for 2-3 minutes
22. Dry pellet and dissolve in 8 µl DEPC-H₂O

In vitro transcription using Ambion Megascript T7 Kit:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

23. Make a premix (per sample) using the Ambion Megascript T7 Kit
 - ◆ 2 µl Txn buffer
 - ◆ 2 µl 75 mM ATP
 - ◆ 2 µl 75 mM CTP
 - ◆ 2 µl 75 mM GTP
 - ◆ 2 µl 75 mM UTP
 - ◆ 2 µl Enzyme Mix
24. Add 12 µl of the premix to each sample
25. Incubate at 37 °C for 9-16 hours
26. Add 1 µl RQ1 RNase-free DNase
27. Incubate 1 hour at 37 °C
28. Take 1 µl and analyse on a 1% agarose gel (RNA quality control protocol)

Purification of the now amplified RNA:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

29. To the Ambion Megascript T7 processed material add:
 - ◆ 20 µl 3M Sodium Acetate
 - ◆ 16 µl DEPC-water
 - ◆ 200 µl Phenol (pH4.5)
30. Vortex and centrifuge for 10 minutes at 13000 rpm
31. Transfer upper phase to a fresh 1.5 ml microfuge tube
32. Add 600 µl 100% EtOH
33. Incubate at -20 °C for at least 30 minutes (or 10 minutes at -80 °C)

Second strand synthesis:

34. Centrifuge for 30 minutes at 13000 rpm
35. Wash pellet with 500 ml 70% Ethanol
36. Dry pellet and dissolve in 10 µl DEPC-water
37. Measure concentration on NanoDrop (RNA quality control protocol)

Reverse transcription and direct labelling reaction:

38. Prepare a concentrated stock of low-C dNTP mix:
 - ◆ 25 µl of 100 mM dATP
 - ◆ 25 µl of 100 mM dGTP
 - ◆ 25 µl of 100 mM dTTP
 - ◆ 10 µl of 100 mM dCTP
 - ◆ Make to 500 µl with DEPC-treated MilliQ water
 - ◆ Store in small aliquots at -20 °C
39. Mix together up to 10 µg amplified RNA, DEPC MilliQ water and spike mix to a total volume of 28 µl in an RNase-free 1.5 ml tube. Add 1 µl of 500 µg/ml random primer.
40. Incubate at 65 °C for 10 minutes in a hot-block to denature RNA tertiary structure, then place on ice.
41. Mix together the following to make a master mix:
 - ◆ 8 µl of 5x first strand buffer
 - ◆ 2 µl of conc. low-C dNTP mix
 - ◆ 2 µl of 1 mM Cy3 or Cy5 dCTP
 - ◆ 2 µl of 0.1 M DTT
 - ◆ 0.5 µl of RNAsin
 - ◆ 2 µl of Superscript III reverse transcriptase
42. Add 16.5 µl master mix to each tube of RNA/MilliQ water mixing carefully to avoid bubbles. Do not expose samples to light any more than necessary, ie. wrap in foil when possible.
43. Incubate at 42 °C for 1-2 hours.

Hydrolysis and neutralisation:

44. Hydrolyse the remaining RNA by mixing equal volumes of 0.5 M EDTA and 1 M NaOH. Then add 20 µl of this mix to the reaction and incubate at 65 °C for 15 minutes.
45. Bring samples to room temperature and add 25 µl of 1 M Tris-HCl (pH 7.5) to neutralise. If required, the labelled probe can be stored at -20 °C in the dark at this point.

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

46. Reduce volume of probe to approximately 25 µl, by placing in a speed vac with medium heat. With our machine, this takes about 30 mins. Then combine the Cy3- and Cy5-labelled probe (sample and control) into one 1.5 ml microfuge tube.
47. Resuspend the resin in the G-50 column by vortexing gently.
48. Loosen the cap a quarter turn and snap off the bottom closure.
49. Place the column in a 1.5 ml tube.
50. Pre-spin column at 5,000 rpm for 1 minute to remove the buffer. Blot the tip of the column dry using a clean paper towel.
51. Remove the top cap and place column in a new 1.5 ml tube. Pipette half of the sample onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.

52. Spin for 1 minute at 5,000 rpm. The unincorporated dye and nucleotides should be retained by the column and the purified labelled probe should pass through into the support tube. Discard the column.
53. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
54. Reduce volume of probe to between 2 to 5 μ l by placing in a speed vac with medium heat
55. Add 2 μ l of sonicated salmon sperm DNA

The samples have now been amplified and combined together for hybridisation to a microarray with the blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (07-06-2004).