

## Anti-sense strand amplification of RNA and indirect labelling of amino-allyl RNA for oligo microarrays (2 rounds)

### Outline

Amplification can be used to produce sufficient RNA for labelling when the amount of biological material is limiting. The method we use is based on the Eberwine method. The 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)<sub>n=21</sub>-[A or G or C]
- T4 Gene 32 protein (GE Healthcare Bio-Sciences AB; 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 (GE Healthcare Bio-Sciences AB; 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 (Sigma; Cat. No. P2069)
- Sodium Chloride (VWR; Cat. No. 102414P)
- Gene Elute LPA (Sigma; Cat. No. 56575)
- 100% Ethanol
- MEGA-Script T7 kit (Ambion; Cat. No. 1334)
- 70% Ethanol
- RQ1 RNase-free DNase (Promega; Cat. No. M6101)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- DEPC - Diethyl pyrocarbonate (Sigma; Cat. No. D 5758)
- DEPC-treated MilliQ water
- RNAsin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB; Cat. No. 27-5340-01)
- Phase Lock Gel (Helena Bioscience; Cat. No. 0032 007.953)
- Aminoallyl-UTP-sodium salt (Sigma; Cat. No. A-5660)
- RNeasy Mini Kit (Qiagen; Cat. No. 74104)
- Cy Dye Post-Labelling Reactive Dye Pack (GE Healthcare Bio-Sciences AB; Cat. No. RPN 5661)
- Sodium Bicarbonate (Sigma; Cat. No. S7277)
- Hydroxylamine hydrochloride (Sigma; Cat. No. H9876)
- Ammonium Acetate (VWR; Cat. No. 1.01116.0500)
- Random Primer (Promega; Cat.No. C1181)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- 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

### 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

### Making the amino-allyl UTP stock:

1. For 75mM amino-allyl UTP stock, resuspend 1mg Aminoallyl-UTP-sodium salt in 24.7 µl DEPC-water

## RNA amplification - first round

### 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 5 µg/µl T4 Gene 32 protein
  - ◆ 0.5 µl 40 U/µl RNasin
  - ◆ 1 µl 200 U/µl Superscript III
5. Add 6.3 µl to each sample
6. Incubate at 46 °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 10 U/µl DNA Polymerase I
  - ◆ 0.2 µl 5 U/µl RNaseH
  - ◆ 1 µl 10 U/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 1 U/µl T4 DNA polymerase
  13. Incubate at 15 °C for 15 minutes, then 70 °C for 10 minutes

**Purification of double stranded DNA:**

The following steps are performed in 1.5 ml microfuge tubes

14. Make the sample up to 100 µl (add 20 µl DEPC-water)
15. Prepare Microspin columns with Bio-Gel P6 in Tris:
  - ◆ Invert columns sharply 2-3 times and remove any air bubbles
  - ◆ 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 (1000 x g)
16. Immediately before use pellet Phase Lock Gel (PLG) tube at 13,000 rpm for 30 seconds
17. Add the cDNA and an equal volume (100 µl) of (Phenol/Chloroform/Isoamylalcohol pH 8.0) to the PLG tube and shake for 15 seconds (do not vortex)
18. Centrifuge for 5 minutes at 13000 rpm
19. 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)
18. Precipitate the dsDNA:
  - ◆ 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-H<sub>2</sub>O

**In vitro transcription using Ambion Megascript T7 Kit:**

The following steps are performed in 1.5 ml microfuge tubes and in a 37°C Incubator

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 1 U/µl RQ1 RNase-free DNase
27. Incubate 1 hour at 37 °C

**Purification of the amplified RNA using Qiagen RNeasy Mini Kit RNA cleanup protocol followed by precipitation:**

The following steps are performed in 1.5 ml microfuge tubes

28. To the Ambion Megascript T7 processed material add:
  - ◆ 80 µl DEPC-water

Second strand synthesis:

- ◆ 350 µl RLT buffer (add 10 µl beta-mercaptoethanol to 1ml RLT buffer)
- 29. Mix thoroughly
- 30. Add 250 µl ethanol (96-100%) and mix by pipetting
- 31. Apply sample (700 µl) to an RNeasy mini column placed in a 2ml collection tube
- 32. Centrifuge at 13,000 rpm 15 seconds
- 33. Transfer RNeasy column into a new 2 ml tube and add 500 µl Buffer RPE onto the column
- 34. Centrifuge at 13,000 rpm 15 seconds. Discard the flow-through
- 35. Add another 500 ul Buffer RPE to the column
- 36. Centrifuge at 13,000 rpm for 2 minutes
- 37. Transfer RNeasy column into a new 2 ml tube and centrifuge at 13,000 rpm for 1 minute to dry the membrane
- 38. Transfer RNeasy column into a new 1.5 ml tube
- 39. Pipette 50 µl DEPC-water directly onto membrane, incubate for 1 minute
- 40. Centrifuge at 13000 rpm for 1 minute to elute
- 41. Repeat step 39 and 40
- 42. Take 2.0 µl aliquot and analyse on a 1% agarose gel (ethidium bromide)
- 43. Precipitate with:
  - ◆ 0.5 µl LPA (25 µg/µl)
  - ◆ 50 µl 7.5M Ammonium acetate
  - ◆ 250 µl 100% Ethanol
- 44. Mix and centrifuge immediately at 13,000 rpm for 30 minutes
- 45. Wash pellet with 70% Ethanol
- 46. Dry and resuspend in 5.0 µl DEPC-water
- 47. Take 0.5 µl aliquot and measure concentration on NanoDrop

## RNA amplification - second round

### Reverse Transcription reaction:

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

- 48. Take up to 1 µg of the amplified RNA from the first round, add 0.5 µl of 0.5 µg/µl Random Primer and then make up to a total volume of 5 µl with DEPC- water
- 49. Incubate at 65 °C for 10 min
- 50. Then snap freeze on ice
- 51. 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 5 µg/µl T4 Gene 32 protein
  - ◆ 0.5 µl 40 U/µl RNasin
  - ◆ 1 µl 200 U/µl Superscript III
- 52. Add 6.3 µl to each sample
- 53. Incubate at 46 °C for 1 hour
- 54. Incubate at 65 °C for 15 minutes
- 55. Hold temperature at 37 °C and add 0.2 µl 5 U/µl RNase H
- 56. Incubate at 37 °C for 30 minutes
- 57. Incubate at 95 °C for 2 minutes
- 58. Then snap freeze on ice
- 59. Add 0.5 µl Primer T7-dT (200 ng/µl)
- 60. Incubate at 42 °C for 10 minutes

Purification of the amplified RNA using Qiagen RNeasy Mini Kit RNA cleanup protocol followed by precipitation

### **Second strand synthesis:**

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

61. 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 10 U/µl DNA Polymerase I
  - ◆ 0.2 µl 5 U/µl RNaseH
62. Add 65.7 µl to each sample
63. Incubate at 16 °C for 2 hours
64. Add 2.0 µl 1 U/µl T4 DNA polymerase
65. Incubate at 15 °C for 15 minutes, then 70 °C for 10 minutes

### **Purification of ds DNA:**

The following steps are performed in 1.5 ml microfuge tubes

66. Make the sample up to 100 µl (add 20 µl DEPC-water)
67. Prepare Microspin columns with Bio-Gel P6 in Tris:
  - ◆ Invert columns sharply 2-3 times and remove any air bubbles
  - ◆ 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 (1000 x g)
68. Immediately before use pellet Phase Lock Gel (PLG) tube at 13,000 rpm for 30 seconds
69. Add the cDNA and an equal volume (100 µl) of (Phenol/Chloroform/Isoamylalcohol pH 8.0) to the PLG tube and shake for 15 seconds (do not vortex)
70. Centrifuge for 5 minutes at 13000 rpm
71. 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)
72. Precipitate the dsDNA:
  - ◆ 3.5 µl 5M Sodium Chloride
  - ◆ 0.5 µl LPA
  - ◆ 220 µl 100% Ethanol
73. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
74. Centrifuge for 30 minutes at 13000 rpm
75. Remove supernatant and wash with 70% Ethanol, centrifuge for 2-3 minutes
76. Dry pellet and dissolve in 8 µl DEPC-H<sub>2</sub>O

### **In vitro transcription using Ambion Megascript T7 Kit:**

The following steps are performed in 1.5 ml microfuge tubes and in a 37°C Incubator

77. 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
  - ◆ 1.5 µl 75 mM UTP
  - ◆ 0.5 µl 75 mM amino-allyl UTP
  - ◆ 2 µl Enzyme Mix
78. Add 12 µl of the premix to each sample
79. Incubate at 37 °C for 9-16 hours

80. Add 1  $\mu$ l 1 U/ $\mu$ l RQ1 RNase-free DNase
81. Incubate 1 hour at 37 °C

**Purification of the amplified RNA using Qiagen RNeasy Mini Kit RNA cleanup protocol followed by precipitation:**

The following steps are performed in 1.5 ml microfuge tubes

82. To the Ambion Megascript T7 processed material add:
  - ◆ 80  $\mu$ l DEPC-water
  - ◆ 350  $\mu$ l RLT buffer (add 10  $\mu$ l beta-mercaptoethanol to 1ml RLT buffer)
83. Mix thoroughly
84. Add 250  $\mu$ l ethanol (96-100%) and mix by pipetting
85. Apply sample (700  $\mu$ l) to an RNeasy mini column placed in a 2ml collection tube
86. Centrifuge at 13,000 rpm 15 seconds
87. Transfer RNeasy column into a new 2 ml tube and add 500  $\mu$ l Buffer RPE onto the column
88. Centrifuge at 13,000 rpm 15 seconds. Discard the flow-through
89. Add another 500  $\mu$ l Buffer RPE to the column
90. Centrifuge at 13,000 rpm for 2 minutes
91. Transfer RNeasy column into a new 2 ml tube and centrifuge at 13,000 rpm for 1 minute to dry the membrane
92. Transfer RNeasy column into a new 1.5 ml tube
93. Pipette 50  $\mu$ l DEPC-water directly onto membrane, incubate for 1 minute
94. Centrifuge at 13000 rpm for 1 minute to elute
95. Repeat step 93 and 94
96. Take 2.0  $\mu$ l aliquot and analyse on a 1% agarose gel (ethidium bromide)
97. Precipitate with:
  - ◆ 0.5  $\mu$ l LPA (25  $\mu$ g/ $\mu$ l)
  - ◆ 50  $\mu$ l 7.5M Ammonium acetate
  - ◆ 250  $\mu$ l 100% Ethanol
98. Mix and centrifuge immediately at 13,000 rpm for 30 minutes
99. Wash pellet with 70% Ethanol
100. Dry and resuspend in 5.0  $\mu$ l DEPC-water
101. Take 0.5  $\mu$ l aliquot and measure concentration on NanoDrop

**Dye coupling:**

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

102. Resuspend 1 aliquot of monofunctional dye in 4.5  $\mu$ l 0.1M sodium bicarbonate (pH 8.5)
103. Mix with 4.5  $\mu$ l amplified amino-allyl RNA
104. Incubate at 23 °C for 1 hour in heating block
105. Quench reaction by adding 4.5  $\mu$ l 4M Hydroxylamine
106. Incubate at 23 °C for 15 minutes in heating block
107. Combine Cy3 and Cy5 sample pairs

**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:

108. Resuspend the resin in the G-50 column by vortexing gently.
109. Loosen the cap a quarter turn and snap off the bottom closure.

In vitro transcription using Ambion Megascript T7 Kit:

110. Place the column in a 1.5 ml tube.
111. 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.
112. 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.
113. 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.
114. 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.
115. Reduce volume of probe to between 2 to 5  $\mu$ l by placing in a speed vac with medium heat
116. Add 2  $\mu$ 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 (12-06-2006).