

## Preparation of total RNA, two rounds of amplification, and labeling of aRNA

### Materials:

MessageAmp aRNA Kit

Ambion (www.ambion.com) Cat# 1750. Good for one round of amplification for 20 samples, or two rounds for 10 samples.

BioArray HighYield RNA Transcript Labeling Kit (T7) Enzo (www.enzo.com)  
No. 42655-10 (10 samples) or 42655-20 (20 samples)

RNeasy RNA purification spin column kit  
Qiagen 74104

DEPC water

Glycoblue

100%, 75% nuclease-free ethanol

RNase inhibitor (Promega)

2M Na OAC (sodium acetate)

TE

A	sort and RNA extraction with Trizol	
B	1st strand cDNA synthesis	
C	2nd strand cDNA synthesis	
D	cDNA purification	
E	1st round aRNA synthesis	<b>8h</b>
F	aRNA purification and quantification	<b>3h</b>
G	2nd round 1st strand cDNA synthesis	messageAmp kit
H	2nd round 2nd strand cDNA synthesis	
I	cDNA purification	
J	2nd round aRNA synthesis with biotin labeling	Enzo kit
K	Purification of labeled aRNA	RNAeasy <b>13h</b>

Note: large quantities of RNA only require ONE round of amplification. Smaller quantities (e.g. less than a million cells) require 2 rounds.

### **A Trizol extraction**

- 1 Sort cells into 1.5ml Eppendorf tubes. Has been successfully done with 30,000 cells (RO).
- 2 Spin 3' @ 4000rpm, 4°C.
- 3 Aspirate sup, leaving 50µl behind.
- 4 Remove remaining liquid with a P200, leaving 10µl behind is OK.
- 5 Add 500µl Trizol; pipet 8x up and down
- 6 Incubate 5' @ RT.
- 7 Add 100µl of Chloroform.
- 8 Shake vigorously for 15".
- 9 Spin 15' @ 12000g, 4°C.

- 10 Transfer 2 x 125µl sup to new tubes. Don't transfer interface.
- 11 Add 1µl glycogen, vortex briefly.
- 12 Add 300µl isopropanol, vortex right away.
- 13 Vortex again.
- 14 Store at -20°C

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In general:

- flick or pipet tubes with enzyme solution to mix, don't vortex.
- PCR machines for incubations, set lid to tracking 5°C, so that lid does not heat up to 95°C.

### **B 1st strand cDNA synthesis**

- 1 Spin RNA 15' @ 13000.
  - 2 Remove sup.
  - 3 Wash with 75% ethanol.
  - 4 Remove sup. Dry at RT, ~5' so that no liquid is left.
  - 5 Resuspend in 20µl DEPC water, 11µl if you have very little RNA. [Store @ -80°C.]
  - 6 Turn on the PCR block @ 70°C.
  - 7 Transfer 11µl into thin wall PCR tubes.
  - 8 Add 1µl of T7-oligo-dT primer (Ambion kit).
  - 9 Incubate for 10' @ 70°C.
  - 10 Transfer to 4°C. Spin briefly.
  - 11 Add 8µl of RT MasterMix consisting of
    - 2µl 10x 1st strand buffer
    - 1µl RNase Inh
    - 4µl dNTP mix
    - 1µl RT
- Mix by gentle pipetting. Spin briefly.
- 12 Incubate for 2h @ 42°C.
  - 13 Spin briefly, Transfer to 4°C.

### **C 2nd strand cDNA synthesis**

- 1 Prepare master mix:
    - 63µl nuclease-free water
    - 10µl 10x 2nd strand buffer
    - 4µl dNTP mix
    - 2µl DNA polymerase
    - 1µl RNase H
- Mix by gentle pipetting. Spin briefly.
- 2 Add 80µl of master mix to cDNA sample.
  - 3 Incubate for 2h @ 16°C.

4 [Freeze at -20°C. Stopping here is possible.]

#### **D cDNA purification**

Ambion kit, small columns

- First time use of message AmpKit: add 11.2ml ethanol to cDNA Wash Buffer and 20ml ethanol to aRNA Wash Buffer.
- 1 Preheat Nuclease-free water to 50°C.
  - 2 Place cDNA Filter Cartridge into 2ml Wash Tube and pipet 50µl cDNA Binding Buffer onto the filter. Incubate for 5' @ RT. Do NOT spin through.
  - 3 Add 250µl cDNA Binding Buffer (make sure there is no precipitate in the buffer) to each cDNA sample and mix by gentle pipetting.
  - 4 Pipet it on the equilibrated cDNA Filter Cartridge in the 2ml Wash Tube.
  - 5 Spin for 1' @ 10,000 rpm.
  - 6 Discard flow-through, wash with 500µl cDNA Wash Buffer.
  - 7 Spin again to remove traces of ethanol.
  - 8 Transfer the cDNA Filter Cartridge to a cDNA Elution Column.
  - 9 Elute with preheated Nuclease-free Water:  
Pipet 8µl onto the filter, incubate 2' @ RT, then elute with 10,000rpm.
  - 10 Repeat with 11µl.
  - 11 Continue with 15µl to the next step. Do not stop here. (If you have a lot of RNA, you can elute in a greater volume, use 15ul for the next step, and freeze the rest for later Q-RT-PCR)

#### **E 1st round aRNA synthesis: 1st IVT**

- 1 Prepare In Vitro Transcription (IVT) Master Mix:
  - 4µl T7 ATP solution
  - 4µl T7 CTP solution
  - 4µl T7 GTP solution
  - 4µl T7 UTP solution
  - 4µl T7 10x Reaction Buffer
  - 4µl T7 Enzyme Mix
  - 1µl RNaseIn (Promega; Tetsuya)

Mix by gentle pipetting.

- 2 Add 25ul Master Mix to each sample.
- 3 Incubate for 6-14h @ 37°C (we do 14h).

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- 3 Add 2µl DNaseI and mix by pipetting.
- 4 Incubate for 30' @ 37°C.
- 5 Add aRNA Elution Solution to bring the final volume to 100µl. Mix by pipetting.

#### **F aRNA purification, precipitation, and quantification**

- 1 Preheat nuclease-free water to 50°C.
- 2 Add 350µl of aRNA Binding Buffer to each sample, mix by pipetting.
- 3 Add 250µl ethanol to each sample, mix by pipetting.
- 4 Place aRNA Filter Cartridge In an aRNA Collection Tube and pipet each sample onto the filters.
- 5 Spin for 1' @ 10,000rpm.
- 6 Discard flow-through and wash with 650µl aRNA Wash Buffer.
- 7 Spin again to remove traces of ethanol.
- 8 Transfer Filter Cartridge to fresh aRNA Collection Tube.
- 9 Add 50µl of preheated Nuclease-free water to filter. Incubate for 2'.
- 10 Spin 2' @ 10,000rpm.
- 11 Repeat with another 100µl.
- 12 Precipitate aRNA by adding:
  - 22.5µl 2M NaOAc
  - 0.5µl Glycoblue
  - 450µl cold 100% ethanolMix well by pipetting. Incubate for 30' @ -20°C.
- 13 Spin 15' @ 13,000rpm.
- 14 Wash with 500µl cold 70% ethanol. Air dry. Resuspend in 15µl nuclease-free water.
- 15 Dilute 1ml in 100µl TE and measure OD.
- 16  $\mu\text{g}/\text{m} = \text{OD}_{260} \cdot \text{dil} \cdot 40 \mu\text{g}/\text{ml}$ .  $\text{OD}_{260}:\text{OD}_{280} \approx 1.5$
- 17 [Freeze 1st round aRNA @ -80°C. Stopping here is possible.]

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### **G 2nd round 1st strand cDNA synthesis**

- 1 Turn on the PCR block @ 70°C.
- 2 Place 2µg aRNA, add 2µl of 2nd round primer (Ambion kit) and bring total volume with nuclease-free water to 12µl.
- 3 Incubate for 10' @ 70°C.
- 4 Spin briefly, place on ice.
- 5 Prepare Master Mix:
  - 2µl 10x 1st strand buffer
  - 1µl RNaseIn
  - 4µl dNTP Mix
  - 1µl RT
- 6 Add 8µl Master Mix, Incubate for 2h @ 42°C.
- 7 Add 1µl RNase H and incubate for 30' @ 37°C.

### **H 2nd round 2nd strand cDNA synthesis**

- 1 Add 5µl T7 Oligo(dT) Primer to each reaction. Mix and spin.
- 2 Incubate for 10' @ 70°C.
- 3 Place on ice. To each 26ml sample, add
  - 58µl nuclease-free water
  - 10µl 10x 2nd strand buffer

- 4µl dNTP Mix
  - 2µl DNA Polymerase
- Mix by pipetting.

4 Incubate for 2h @ 16°C.

### **I cDNA purification**

Ambion kit, small columns

- 1 Preheat Nuclease-free water to 50°C.
- 2 Place cDNA Filter Cartridge into 2ml Wash Tube and pipet 50µl cDNA Binding Buffer onto the filter. Incubate for 5' @ RT.  
Do NOT spin through.
- 3 Add 250µl cDNA Binding Buffer to each cDNA sample and mix by gentle pipetting.
- 4 Pipet it on the equilibrated cDNA Filter Cartridge in the 2ml Wash Tube.
- 5 Spin for 1' @ 10,000 rpm.
- 6 Discard flow-through, wash with 500µl cDNA Wash Buffer.
- 7 Spin again to remove traces of ethanol.
- 8 Transfer the cDNA Filter Cartridge to a cDNA Elution Column.
- 9 Elute with preheated Nuclease-free Water:  
Pipet 10µl onto the filter, incubate 2' @ RT, then elute with 10,000rpm.
- 10 Repeat with 16µl.
- 11 Continue with 22µl. Do not stop here.

### **J 2nd round aRNA synthesis with biotin labeling**

Enzo kit

- 1 Continue with 22µl of the above.
- 2 Prepare Master Mix:
  - 4µl 10x Reaction Buffer (vial 1)
  - 4µl Biotin-labeled nucleotides (vial 2)
  - 4µl DTT (vial 3)
  - 4µl RNase Inh (vial 4)
  - 2µl 20x T7 RNA Polymerase (vial 5)
 Mix and spin.
- 3 Incubate for 4-5h @ 37°C with mixing and spinning every 45'.

### **K Purification of labeled aRNA**

Qiagen RNeasy

First time use of RNeasy Kit:

- RPE buffer: Add 4 volumes of ethanol.
- RLT buffer: 350µl/sample needed. Add 10µl β-ME/1ml RLT.

- 1 Add 60µl nuclease-free water to each sample: total is now 100µl. Transfer to Eppi.
- 2 Add 350µl RLT and mix.
- 3 Add 250µl ethanol to each sample, mix by pipetting and apply to RNeasy column. Spin for 15" @ 10,000rpm. Discard collection tube and flow-through.
- 4 Transfer cartridge into new collection tube. Add 500µl RPE and spin for 2' @ 10,000rpm. Discard the flowthrough.
- 5 Wash again with 500µl RPE, discard flowthrough, spin again and make sure no ethanol is being transferred.

- 6 Transfer cartridge to new collection tube and add 50 $\mu$ l nuclease-free water. Incubate for 2'. Spin for 1' @ 10,000.
- 7 Quantify 1 $\mu$ l.