PROTOCOL FOR REVERSE TRANSCRIPTION AND AMINO-ALLYL COUPLING (microarray probe Making)
updated by Pam Skinner and Hayet Abbassi 6-29-03


Take standard precautions for working with RNA during step 1
- use DEPC or eppendorf molecular biology grade water
- Wear gloves. Clean working area with RNase Zap.
- Use RNA only pipettes, filter tips and plastic ware. Bake glassware 150° C for 4 hours. Soak plastic stuff in 0.5M NaOH for 10 minutes, rinse in water, and autoclave. Can soak pipetteman barrels in RNAse Zap or NaOH, rinse with water.

1. RT Reaction*

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Volume (ul) for 1 Reaction</th>
<th>Volume for X Reactions</th>
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</thead>
<tbody>
<tr>
<td>Oligo dT</td>
<td>5ug/ul</td>
<td>2ul</td>
<td></td>
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<tr>
<td>pdN6</td>
<td>10ug/ul</td>
<td>1ul</td>
<td></td>
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<tr>
<td>Total RNA</td>
<td>12.5ug</td>
<td>Depends on the RNA concentration</td>
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<tr>
<td>RNase free water</td>
<td>To 18.5ul</td>
<td></td>
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<tr>
<td>Total volume</td>
<td>18.5 ul</td>
<td>X x 18.5ul</td>
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</table>

 Samples | RNA Concentration (ug/ul) | Ul for 12.5ug | Water added

1) For each sample, on ice, in 1.5 ml tube, add RNA and water to total 15.5 ul per reaction (31 ul for 2 Reactions)
2) On ice, in 1.5 ml tube make a primer cocktail of Oligo dT and pdN6 for the number of reactions (x reactions) planned for the RT.
3) To each RNA sample add 3ul of the primer cocktail for 1 reaction (or 6ul for 2 reactions).
4) Incubate RNA, oligo dT, and random hex. at 70° C for 10 min. (can do this in a thermocycler or a heater block)
5) Chill on ice for 10 min
6) Set up cDNA synthesis cocktail
<table>
<thead>
<tr>
<th>cDNA synthesis cocktail</th>
<th>UL per 1 reaction</th>
<th>uL per X reactions</th>
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</thead>
<tbody>
<tr>
<td>5X buffer (1st strand buffer)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>50X aa-dUTP/dNTP**</td>
<td>0.6</td>
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<tr>
<td>DTT (0.1M)</td>
<td>3</td>
<td></td>
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<tr>
<td>SuperScriptII (200U/ul)</td>
<td>1.9</td>
<td></td>
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<tr>
<td>Total</td>
<td>11.5</td>
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1) Mix the synthesis cocktail in a 1.5 uL eppendorf tube, on ice. Make 10% extra.
2) After RNA and primers have cooled, add 11.5 uL (or 23ul for 2 reactions) of the RT cocktail for a total volume of 30ul per reaction. Mix by flicking tube, and fling tube or pulse in centrifuge to pull reaction to base of tube.
3) Incubate reaction mixture at 42° C for 2 hours (For total RNA, the red book recommends incubating at 50degrees, as an increased temp will decrease mispriming on rRNA. See alternative protocol in Red book, 22.2.3)

*For polyA RNA omit the random hexamer (pdN6) from the priming and input at least 2ug of RNA (up to 50ug). TIGR protocol and red book protocols do not call for random hexamer, just oligo dT for priming total RNA. Kapur lab: For bacterial total RNA, we have had success using 10ug RNA and 30 ug of hexamers). If using more than 10ug of total RNA will want to double the reaction volume. Pam has had success using 12.5 ug total brain RNA with 10ug oligo dT and 10ug hexamers. May want to include control RNA such as Arabidopsis CAB, RCA or rbcl (Stratagene).

**

<table>
<thead>
<tr>
<th>1X dNTPs</th>
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<tr>
<td>500uM each dA, dC, dG</td>
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<tr>
<td>200uM aa-dUTP</td>
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<tr>
<td>300uM dTTP</td>
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<tr>
<th>50X recipe: For 2:3***</th>
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<tr>
<td>10uL each 100 mM dA, dG, dC</td>
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<tr>
<td>4uL 100 mM aa-dUTP</td>
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<tr>
<td>6uL 100 mM dTTP</td>
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***A ratio of 2 aa-dUTPs: 3 dTTP's was optimized for yeast chips. Altering the ratio to 3:2 or 4:1 may help increase signal in other systems. Optimizations are encouraged. May want to include an RNase inhibitor such as SUPERase•In™ from Ambion

2. Hydrolysis
For each reaction, add and Mix:
1) 10ul (20ul for two) 1 N NaOH. This step gets rid of the RNA.
2) 10ul (20ul for two) 0.5M EDTA
3) Incubate: 15 min. at 65 °C.
4) Neutralize with addition of 25ul (50 ul for two) 1M Tris-HCl pH 7.4, mixing well.
5) Samples may be stored at 4° C overnight at this point.

3. Cleanup
1) To continue with the amino-allyl dye coupling procedure all Tris must be removed from the reaction to prevent the monofunctional NHS-ester Cye-dyes from coupling to free amine groups in solution.
2) Label a set of concentrators and collection tubes with sample names.
3) Fill one Microcon 30 concentrator with 450 ul water (350 uL if 2 reactions). (don't need DEPC water anymore. DEPC inhibits some enzymes so only use when needed)
4) Add neutralized reaction.
5) Spin at 12K for 8 minutes.
6) Dump flow-thru.
7) Repeat process 2X, refilling original filter (with 500 uL of water each time. Spin for only 4-5 min the last time (be sure not to spin too long; we want at least 200 uL volume).
8) Elute.
   a) turn Microcon filter upside-down in clean tube and spin at 4000 rpm (1000XG) for 4 minutes
9) At this point a volume over 150ul should be concentrated again with a second Microcon filter :
   a) Transfer the cDNA in a new filter
   b) Add 300 to 350 uL of water
   c) Spin at 12K for 4-5 min
   d) Turn Microcon filter upside-down in clean tube and spin at 4000 rpm (1000XG) for 4 minutes
   e) Measure the volume of the elute
10) Run out 10-20% of sample onto a 1% agarose gel with DNA ladders in order to check the relative concentrations of cDNA made and see the size of the smear. It is a good idea to run the gel for 20 minute, take a photo (to be able to see easy the cDNA and have a visual estimation of the concentration) and run again for 25 to 30 min to be able to see the smears and compare them between the different samples).
11) Aliquot the remaining cDNA to 1 RT reaction per tube
12) Dry elute in speed vac.
13) Samples may be stored at -20°C indefinitely.

<table>
<thead>
<tr>
<th>Samples ID</th>
<th>Elute (ul)</th>
<th>10% (for gel)</th>
<th>Volume per reaction</th>
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4. Coupling
1) Resuspend cDNA pellet in 9 ul of 0.1M NaBicarbonate Buffer pH 9.0.
2) Let sit for 10-15 min at room temperature to ensure resuspension.
3) Transfer entire 9 ul volume to a dried aliquot of Cy3 or Cy5 dye (see below). Use Cy3 for one sample of the hybridization and Cy5 for the other. Note: Cy dyes are light sensitive.
   a) making cy dye aliquots
      i) Resuspend monofunctional NHS-ester in 42ul DMSO.
ii) Aliquot 5 ul x 8 tubes and dry in speed vac. (Hayet aliquots into 12 tubes). Decreasing the number of aliquots/dye tube may increase your signal. Optimizations are encouraged.

iii) dry aliquots in a speed vac

iv) store indefinitely at 4 °C

4) Mix dye and cDNA by pippeting up and down.

5) Incubate 1 hour at RT in dark.

5. Cleanup

1) To remove unincorporated Cy dyes proceed with Qia-Quick PCR Purification Kit (QIAGEN) and purify reactions separately at room temperature.

   a) Add 90 ul of molecular biology grade water to reaction.
   b) Add 500ul Buffer PB.
   c) Apply to Qia-quick column and spin at 13,000 rpm for 30-60 sec.
   d) discard flow-thru.
   e) Add 750ul Buffer PE and spin 30-60 sec.
   f) Aspirate (dump off) flow-thru and repeat with 500 uL of Buffer PE
   g) Aspirate flow-thru and spin for 1 min. at high speed to dry column.
   h) Transfer to fresh 1.5 ml (dark) tube
   i) Add 30ul Buffer EB to center of filter and let sit 1 min. at RT.
   j) Spin at 13,000 rpm for 1 min.
      i) Repeat elution step again with 30 uL of Buffer EB

2) Combine Cy3 (60 ul) and Cy5 (60 ul)

   a) Dry down Qia-quick eluate in speed vac. Don’t over dry or use heat as samples may be difficult to resuspend.
   b) The Samples are ready for the hybridization. At this point labeled cDNA may be stored for up to 48 hours before hybridization at 4°C DO NOT store at -20°C.
   c) See Hybridization protocol

* Alternative protocol for Cleanup : Quenching and Cleanup

1) Before combining Cy3 and Cy5 samples for hybridizations, the reactions must be quenched to prevent cross-coupling.
2) Add 4.5ul 4M hydroxylamine. Let reaction incubate 15 min. (no longer) at RT in dark.
3) To remove unincorporated/quenched Cy dyes proceed with Qia-Quick PCR Purification Kit (QIAGEN).
4) Combine Cy3 and Cy5 reactions
5) Add 70 ml of water and continue with the steps of the Qia-Quick PCR Purification Kit.

Note: another alternate is to use microcon 30 to purify.
MATERIAL AND REAGENT FOR REVERSE TRANSCRIPTION, AMINO-ALLYL COUPLING and HYBRIDISATION (By Hayet Abbassi and Pam Skinner 6-03)

RT: (Take standard precaution for working with RNA)
- Ice
- RnaseZap (Ambion: cat#9780, 9782, 9784) and or RnaseZap Wipes (Ambion cat#s 9786, 9788)
- Autoclaved 1.5 ml Rnase free microcentrifuge tubes handled only with gloves (dot scientific inc #509-FTG, U stores#?????)
- RNase free tips (10, 20, 200 and 1000ul)
- RNA: Stored at –80 C
- Oligo dT (ordered from AGAC, Rnase free box stored at – 20 C)
- PdN6 = random hexamers (Amersham Bioscience cat#27-2166-01, same box as oligo dT)
- 50X aa-duTP/dNTP (same box as oligo dT)
- DTT 0.1M (come with the SuperscriptII, stored in the same box with SSII)
- 5X Buffer (1st Strand Buffer, come with the superSriptII, stored in the same box with SSII)
- SuperScript™ II Rnase H⁻ Reverse transcriptase (10,000U (200U/ul) (Invitrogen cat# 18064-014)
- Water (not DEPC. Use molecular biology grade water; eppendorf; Brinkman instruments inc. cat# 955-15-503-3)
- Tubs for PCR (200ul flat top or others)
- Thermo-cycler or heater block set at 70 C
- Thermo-cycler or incubator or water bath set at 42 C

HYDROLYSIS AND CLEANUP
- 1N NaOH (stored at RT: room temperature)
- 0.5M EDTA pH 8 (stored at RT)
- Thermo-cycler or incubator set at 65 C
- 1M Tris-HCl, Ph 7.4
- MicroCon 30 (Millipore 42410; Amicon Bioseparation, Microcon ym-30 cat# 42210)
- Speed Vacuum
- λDNA/HindIII fragments(Invitrogen, cat# 15612-013; stored at –20C)
- 100 bp DNA ladder (Invitrogen cat# 15628-019; stored at –20C)
- Agarose
- Electrophoresis equipment
- Ethidium Bromide
- TAE 1X

COUPLING AND HYBRIDISATION
• 0.1M Na-Bicarbonate Buffer, PH 9.0 (RT)
• DMSO (Dimethyl sulphoxide; cat# D2650)
• Dried aliquots of Cy3 and Cy5 (Amersham Pharmacia Biotech, cat# PA23001 or PA23031 and PA25001 or PA25031; stored at 4 C)
• 1.5 ml dark Rnase free microcentrifuge tubes (dot scientific incorporated #509-FTGA, U stores #C55C7)
• cDNA (-20 C)
• 4 M fresh hydroxylamine (+4 C; optional)
• Qia-Quick PCR purification kit (50) (Qiagen cat# 28104, stored at RT)
• Ethanol 96-100% (for a new PCR purification kit)
• Molecular grade water (Eppendorf; Brinkman instruments inc. cat# 955-15-503-3)
• PolyA 10 mg/ml (?? Pams –20 C box #2)
• SSDNA 10mg/ml (?? Pams –20 C box #2)
• 5X and/or 10x SDS (stored at RT)
• 20x SSC (Stored at RT)
• Formamide (GibcoBRL cat# 15515-026; stored in hayet’s box + 4 C)
• Hybridization chambers (clean with water and dry after use)
• Lifter-slips (ERI Scientific Company; 25X60I-2-4789 portsmouth,NH,USA)
• Acetone (to clean the lifter-slips)
• Heater block adjusted at 95 C
• Water bath or incubator adjusted to 42 C

ARRAY WASHING (make 400 ml of each washing solution)

• 20X and /or 2X SSC
• 10% SDS
• Water
• Metallic slide racks (??…)
• Class staining dishes (????)
• Water bath at 37 or 42 C
• Centrifuge (HERMLE Z300 for plates to dry the slides???)