**YILDIZ Lab Protocols**

## AMINOALLYL LABELING OF BACTERIAL RNA FOR MICROARRAYS

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Sources: -Hasseman, J., TIGR Aminoallyl Labeling of RNA for Microarrays & TIGR Microarray Labeled Probe Hybridization

-Gilbert et al. TIGR Microbial RNA Aminoallyl Labeling for Microarrays & Hybridization of probed labels

-Hedge et al., A concise guide to cDNA Microarray analysis-II

- **Reverse Transcriptase Reaction**

*Turn heating block to 70-80°C and 42°C. Water baths can also be used in that step. If you are using heating blocks, put some water so that the heat is uniform.*

1) Priming reaction
   - Take 3 µg of RNA
   - Adjust volume to 13 µl with RNase free water
   - Add 2.5 µl of random hexamer primers (2mg/mL)

*You can make a table like shown below:*

| Sample Id and concentration | ul RNA | ul water |
|---|---|---|
| | | |
| | | |
| | | |
| | | |

2) Mix well and incubate at 70-80°C for 8 minutes. Then place on ice for 5 min.

3) Prepare RT cocktail (14.5 µl/ rxn):

| **Component** | **Volume** | **Adjusted volume for master mix*** |
|---|---|---|
| 5X First Strand buffer | 6 ul | |
| 0.1 M DTT | 3 ul | |
| 25X aminoallyl-dNTP mix | 1.2 ul | |
| SuperScript II RT (200U/uL) | 2.3 ul | |
| Water | 2.0 µl | |

***** For **n** reactions, prepare **n+0.5** aliquots of cocktail to assure that you will not run out.

4) Add 14.5 μl to cooled reactions.

5) Mix (do not vortex) and incubate at 42°C for 3-4 hours

6) Samples can be stored at –20°C if necessary.

- **Hydrolyze RNA**

1) To each sample, add 3 uL of 1 N NaOH and 0.6 uL of 0.5 M EDTA. (Final conc.s = 100mM NaOH, 10mM EDTA)

2) Mix and incubate at 65°C for 10 minutes.

3) Neutralize by adding 33.6 μl 1M HEPES, pH=7.0 (final conc.= 500mM HEPES)

4) Samples can be stored at –20°C if necessary.

- **Reaction Purification : Removal of unincorporated aa-dUTP and free amines**

**Note:** This purification protocol is modified from the Qiagen QIAquick PCR purification kit protocol. The phosphate wash and elution buffers are substituted for the Qiagen supplied buffers because the Qiagen buffers contain free amines that compete with the Cy dye coupling reaction. The columns from the mini-prep kit can also be used.

1) Mix cDNA reaction with 300 uL (or 5X reaction volume = 336 μl) buffer PB (Qiagen supplied) and transfer to QIAquick column.

2) Place the column in a 2 ml collection tube (Qiagen supplied) and centrifuge at ~13,000 rpm for 1 minute. Empty collection tube.

3) To wash, add 750 uL phosphate wash buffer (not Qiagen's) to the column and centrifuge at ~13,000 rpm for 1 minute.

5) Empty the collection tube and repeat the 750 uL wash and centrifugation step.

5) Empty the collection tube and centrifuge the column an additional 1 minute at maximum speed.

6) Transfer column to a new 1.5 mL microfuge tube and carefully add 30 uL phosphate elution buffer. (see solution preparation section)

7) Incubate for 1 minute at room temperature.

8) Elute by centrifugation at ~13,000 rpm for 1 minute.

9) Elute a second time (with another 30 uL of phosphate elution buffer) into the same tube by repeating steps 6-8. The final elution volume should be ~60 uL.

7) The amount of cDNA can be quantified at this stage:

- ng cDNA = (dilution factor)($OD_{260}$)(37)(total volume eluted from column)

8) Dry sample in a speed vac at 45°C.

9) Samples can be stored at –20°C if necessary.

- **Coupling aa-cDNA to Cy Dye Ester**

1) Resuspend aminoallyl-labeled cDNA with 10 μl 50 mM Na-bicarbonate, pH=9.0 if necessary. (This buffer should be made fresh every two weeks)

2) Working in a dark place, add the probe to a tube of the appropriate Cy dye. Pipet up and down several times to resuspend the dye.

3) Incubate the reaction for 1 hour in the dark at room temperature. (Incubation can go up to two hours)

- **Reaction Purification II: Removal of uncoupled dye**

1) To the reaction add 35 uL 100 mM NaOAc pH 5.2. Mix thoroughly.

2) Add 250 uL ( or 5X reaction volume = 225 μl) Buffer PB (Qiagen supplied) to each sample and mix by pipetting up and down.

3) Place a QIAquick spin column in a 2 mL collection tube (Qiagen supplied), apply the sample to the column, and centrifuge at ~13,000 for 1 minute. Empty collection tube.

4) To wash, add 0.75 mL Buffer PE (Qiagen supplied) to the column and centrifuge at ~13,000 for 1 minute.

5) Repeat step 4.

6) Empty collection tube and centrifuge column for an additional 1minute at maximum speed.

7) Place column in a clean 1.5 mL microfuge tube and carefully add 30 uL Buffer EB (Qiagen supplied) to the center of the column membrane.

8) Incubate for 1 minute at room temperature.

9) Elute by centrifugation at ~13,000 rpm for 1 minute.

10) Elute a second time into the same tube by repeating steps 7-9. The final eluted volume should be ~60 uL.

**Analysis of Labeling Reaction**

The amount of cDNA and label can be quantified at this stage:

- Measure OD 260, 550 and 650 (Blank is water.) Use nanodrop microarray program.

- Calculate the pmol of dyes incorporation and pmol DNA and nuc/dye ratio with given equations below:

- pmol nucleotides = $\frac{[OD260 * volume (\mu L) * 37 ng/\mu L * 1000 pg/ng]}{324.5 pg/pmol}$

**Note:** 1 OD260 = 37 ng/μL for cDNA; 324.5 pg/pmol average molecular weight of a dNTP)

pmol Cy3 = $\frac{OD550 * volume (\mu L)}{0.15}$

pmol Cy5 = $\frac{OD650 * volume (\mu L)}{0.25}$

nucleotides/dye ratio = $\frac{pmol\ cDNA}{pmol\ Cy\ dye}$

*If you are using nanodrop, it already gives pmol/μl for each dye. So, you only need to multiply this number by volume to get total pmol dye incorporation.*

**Note:** >150-200 pmol of dye incorporation per sample and a ratio of less than 20 nucleotides/dye molecules is optimal for hybridizations.

- Samples can be stored at –20°C at dark.

- Combine probes to be hybridized together and dry down in speedvac at 42°C.

- Samples can be stored at -20°C until ready to hyb.

- **Pre-hyb slides**

1) Place slide face down over RT 100 mL water in a clean red slide holder for 3-5 minutes
2) Snap dry the slide by placing face up on a 100°C heating block for a few seconds – watch for the condensation to vanish
3) UV cross-link the slide at 250 mJoules ( Enter 2500 to the UV crosslinker)
4) Hydrate slides by dipping in pre-warmed 42°C water. Spin 5 min at 700 RPM at RT.
5) Incubate slides in 50ml Coplin jar containing pre-warmed pre-hyb (5XSSC, 1% BSA, 0.1% SDS) for at least 45 minutes @ 42°C, and incubation can go longer if necessary.

   50 mL pre-hyb buffer:
   12.5 mL 20x SSC
   10 ml 5% BSA
   0.5 ml 10 % SDS
   27 mL water

6) Dip slides in pre-warmed water (42°C) and agitate a couple of minutes to remove pre-hyb buffer.
7) Rinse in isopropanol and spin 5 min at 700 rpm, room temp, to dry.

- **Preparing samples for hybridization**

1) Resuspend probe in water (volumes are given below)

| For total vol. = 30µl | For total vol. = 35µl | For total vol.= 40µl |
|---|---|---|
| 19.8 µl water | 23.55 µl water | 27.2 µl water |

Add:

| | For total vol. = 30µl | For total vol. = 35µl | For total vol. = 40µl |
|---|---|---|---|
| 10 mg/ml salmon sperm DNA (Invitrogen) | 1.5 µl | 1.5 µl | 1.5 µl |
| 12.5 mg/ml tRNA | 1.2 µl | 1.2 µl | 1.2µl |
| 20XSSC (3X final) | 4.5 µl | 5.25 µl | 6 µl |
| **1** % SDS | 3 µl | 3.5 µl | 4 µl |

**NOTE:** The order of addition of the reagents are important. Do not prepare master mix.

2) Boil 5 minutes and then spin 5 minute at 14,000 rpm. Cool 2 min at room temp.

- **Hybridization**

1) Add 10-12 uL of 3X SSC to the wells of the hybridization chamber on each edge and in the middle.

2) Add a clean lifter slip to the appropriate area of the slide and gently load in hybridization solution (30 -40 µl). There should be extra sample on either edge of the cover slip.

3) Place the slide in the chamber. Tighten the screws and place in a 65°C water bath overnight. Do not place the chamber directly on the bottom of the water bath. Place a block on top of the uppermost chamber to hold everything in place. Keep the lid on the water bath to protect the light sensitive samples.

- **Hyb Washes**

1) Prepare the following buffers:

- 1X SSC, 0.05 % SDS
- 0.06 X SSC

2) After the incubation unseal hybridization chamber. Remove the slide from the chamber, taking care not to disturb the coverslip.

3) To remove coverslips, submerge slides in a dish containing warm 1X SSC, 0.05 % SDS wash buffer coverslip facing the bottom of the dish. The coverslip will fall off by moving the slide side to side.

4) After the coverslip is removed place the slide in a rack a fresh bath with warm 1XSSCm 0.05% SDS. Agitate for a couple of minutes

5) Transfer slides plus rack to a bath of 0.06 X SSC.

6) Transfer slides to another bath of 0.06 X SSC containing a fresh rack, blotting the slide on a paper towel to remove as much SDS-containing buffer as possible before placing in the fresh bath.

7) Agitate slides for a couple of minutes.

8) Spin immediately room temperature for 5 min @ 700 rpm, and slides are ready to scan. Store in the dark until scanned.

## MATERIALS

- ✓ 5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate (AA-dUTP) (Sigma; Cat# A0410)
- ✓ 100 mM dNTP Set PCR grade (Amersham; Cat # 27-2035-01)
- ✓ Random Hexamer primers (3mg/mL) (Amersham; Cat # 27-2166-01)
- ✓ SuperScript III RT (200U/μL) (Invitrogen; Cat # 180800444)
- ✓ CyDye™ Post-Labelling Reactive Dye Pack (Amersham; Cat# RPN5661)
- ✓ QIAquick PCR Purification kit (QIAGEN Cat# 28104)

## REAGENT PREPARATION

### ❖ Phosphate Buffers

1) Prepare 2 solutions: 1M $K_2HPO_4$ and 1M $KH_2PO_4$

2) To make a 1M Phosphate buffer ($KPO_4$, pH 8.5-8.7) combine:

1M $K_2HPO_4$……..9.5 mL

1M $KH_2PO_4$……..0.5 mL

3) For 100 mL Phosphate wash buffer (5 mM $KPO_4$, pH 8.0, 80% EtOH) mix:

1 M KPO4 pH 8.5…. 0.5 mL
MilliQ water…….. 15.25 mL
95% ethanol…….. 84.25 mL

**Note:** Wash buffer will be slightly cloudy.
** **IMPORTANT:** Phosphate wash buffer should be prepared daily.

4) Phosphate elution buffer is made by diluting 1 M $KPO_4$, pH 8.5 to 4 mM with sterile water.

❖ **Aminoallyl dUTP (aa-dUTP)**

NOTE: *Make two vials of aminoallyl dUTP (SIGMA) at a time; add 34 uL of RNase free water and 1.4 uL NaOH to one vial, mix well, and transfer entire contents to second vial. Knowing the concentration is extremely important.*

For a final concentration of 100mM:

- 1 mg aa-dUTP
- add 17 uL of Rnase free water
- add 0.7 uL 1N NaOH
- pH should be 7
- store at –20 C

You can measure the concentration of the aa-dUTP solution by diluting an aliquot 1:5000 in 0.1 M $KPO_4$ (pH 7.5) and measuring the $OD_{289}$.
The concentration of your stock solution in mM = $OD_{289}$ x 704

**NOTE:** aa-dUTP (50 mM) can be ordered from Ambion (Cat # 10297-018). However, we haven't got consistent results with Ambion.

❖ **dNTP Labeling Mix (25X) with 3:2 aa-dUTP: dTTP ratio**

Mix the following reagents:

| | | Final concentration |
|---|---|---|
| dATP (100 mM) | 10µL | (12.5 mM) |
| dCTP (100 mM) | 10µL | (12.5 mM) |
| dGTP (100 mM) | 10µL | (12.5 mM) |
| dTTP (100 mM) | 4µL | (5 mM) |
| aa-dUTP (50 mM) | 12µL | (7.5 mM) |
| Water | 34µl | |
| | **80 ul (Prepare 20 ul aliquots)** | |

The ratio of aa-dUTP to dTTP can be modified to optimize labeling.
Other ratios I have seen are 2:3 for yeast chips and 4:1.

Store unused solution at –20°C.

- **Sodium Carbonate Buffer ($Na_2CO_3$): 0.5M, pH 9.0**

- Dissolve 4.2 g $NaHCO_3$ in 80 mL of sterile water and adjust pH to 9.0 with 10 N NaOH; bring volume up to 100 mL with sterile water.

- To make a 50 mM solution for the dye coupling reaction dilute 1:10 with water.

**Note:** Carbonate buffer changes composition over time; make it fresh every couple of weeks to a month.