Protocol for Probe Production Using Ulysis Alexafluor and Hybridization of ISB Yeast Oligo Chips

Materials Needed:

Isolated and quantitated total RNA samples
Reverse Transcriptase (eg. Superscript II), 5X buffer
Microarray slides
Contents of Ulysis Alexafluor kit ("Components" below refer to kit)
PCR Purification kit (eg. Qiagen's QIAquick, cat. 28104)
RNase-free water
Coverslips, 22 x 30mm size, Corning
oligo dT (15) primers, 1 ug/ul
DIG Easy hyb buffer
Small volume of following (<1 ml):
- 0.5 M NaOH / 50 mM EDTA
- 1 M Tris-HCl, pH 7.5
- 10 mM Tris-HCl, pH 8.0 / 1 mM EDTA
- 100% Ethanol, 70% Ethanol
- 3M Sodium Acetate (NaOAc) pH 5.2
- 0.1 M DTT

100 ml each of following:
- 1X SSC / 0.1% SDS / 0.1% BSA
- 1X SSC/0.2% SDS
- 1X SSC
- 0.1 X SSC
- 0.2 % SDS

Get Ready Beforehand:

Dry incubator set to hybridization temp of 37 C
Preheat 50 ml of each of the following to 37 C in dish or Coplin jar:
- 1X SSC / 0.1% SDS / 1% BSA
- 1X SSC/0.2% SDS
- 50 ml of 1X SSC
- 50 ml of 0.1X SSC

Water baths or thermal cycler set to 42 C, 70 C, 80 C, 95 C

Scheduling – Estimated times are indicated for each part. The best stopping points throughout are when precipitations are in the freezer and when pellets are drying.

Reverse Transcription (1 hour set-up and 2 hour incubation)

Set up a reverse transcription reaction for RNA isolated from each yeast treatment. Since the bulk of the total RNA is rRNA, oligo dT primers are used to prepare first-strand cDNA from only polyA+ mRNA. The RNA is then hydrolyzed before the primers are removed.

1. Precipitate 25 ug total yeast RNA using 1/10 volume 3M NaOAc and 2 volumes 100% EtOH, -70 C 30 minutes, spin >12000 xg 15 minutes. Wash with 100 ul 70% EtOH, allow pellet to air dry.

2. Resuspend pellet in 20 ul RNAse free H2O then add:

   2 ul (1 ug/ ul) oligo dT (15)

3. Heat to 70 C for 10 minutes, then 42 C for 5 minutes, then slowly cool to room temp.

4. Add the following:

   8 ul 5X RT buffer
   2 ul dNTP mix (10 mM each)
   2 ul RNasin (40 units)
   4 ul 0.1 M DTT
   2 ul RT enzyme (200 units/ul)

5. Incubate at 42 C for 2 hours

6. Hydrolyze RNA with 7 ul 0.5 M NaOH/50 mM EDTA, heat at 70 C for 10 minutes.

7. Neutralize with 10 ul 1 M Tris 7.5

8. Precipitate with 6 ul 3M NaOAc and 130 ul 100% EtOH, -70 C 30 minutes, spin >12000 x g 15 minutes.

9. Resupend pellet in 40 ul RNAse free H2O, heat at 37 C for 10 minutes, vortex.

Probe Purification

There are lots of ways to purify the probe away from the oligo dT primers. One could use the SCL columns from the 3DNA procedure, or a PCR purification kit such as QIAquick. For QIAquick, do 2 washes with buffer PE instead of one. Elute with 30 ul H20 warmed to 37 C.

Alexafluor Labeling (~2 hours)

1. Precipitate probe using 1/10 volume 3M NaOAc and 2 volumes 100% EtOH, -70 C for 30 minutes, spin >12000 x g 15 minutes. Wash with 100 ul 70% EtOH, allow pellet to air dry.

2. Resupend pellet in 20 ul labeling buffer (component C), heat at 95 C for 5 minutes, cool on ice

3. Add 5 ul of labeling stock solution (Alexafluor 546 or 647) heat at 80 C for 15 minutes, cool on ice

4. Precipitate with 2.5 ul 3 M NaOAc (pH 5.2) and 55 ul 100% EtOH, -70 C for 30 minutes, spin >12000 x g 15 minutes

5. Air dry pellet for use in hybridization

Optional: Resuspend probe in H2O and measure absorbance at 260, 555, and 647 nm. (Hint: In order to get reliable readings, measure the entire probe volume, eg. In a 50 ul cuvette. Rinse the cuvette with 0.1 N HCl to prevent cross contamination.) In Ulysis directions they give formulae for determining the amount of labeling incorporation in bases/dye.

Hybridizations – The ISB yeast chips have two arrays on them. A separate experiment can be done on each using a 22 x 30 mm coverslip and 40 ul of probe.

Pre-Hybridization (~45 minutes)

1. Hold microarray over boiling water for 10 seconds

2. Snap dry in oven at 95 C until dry (about 30 seconds)

3. Place microarray slide in Coplin jar with 50 ml of 2X SSC / 0.1% SDS / 0.1% BSA, preheated to hybridization temp of 37 C, incubate for 30 minutes

4. Transfer slide to dish or Coplin jar with 50 ml of 2X SSC for 5 minutes at 37 C

5. Transfer slide to dish or Coplin jar with 50 ml of 0.2X SSC for 5 minutes at 37 C

6. Dry slide by placing in 50 ml tube and spinning 2-3 minutes at 500 rpm in clinical centrifuge

7. Prepare coverslip by dipping into 0.2 % SDS, then water. Blot and let dry.

Hybridization (15 to 24 hours)

1. Resuspend probe pellets in 9 ul DIG Easy hyb each. Combine the two probes and add 2 ul of 0.1 ug/ul yeast tRNA for a total of 20 ul per microarray.

2. Heat the probe at 95 C for 3 minutes and place on ice.

3. Add the probe to the microarray slide on one end of the array.

4. Place coverslip on same end and let fall slowly. Probe should spread out with no bubbles

5. Transfer slide to 50 ml tube. Keep the slide and tube horizontal and add 300 ul water

6. Seal tube with cap and parafilm and place in dry incubator at hybridization temp of 37 C for 15 to 24 hours.

Post-Hybridization (~40 minutes)

1. Place microarray slide in a Coplin jar at room temp with 1X SSC/0.2% SDS for 15 seconds. When removing the slide the cover slip should just slide off

2. Transfer the slide to dish or Coplin jar with 1X SSC/0.2% SDS at 37 C for 10 minutes

3. Transfer to dish or Coplin jar with 0.1X SSC/0.2% SDS for 10 minutes at room temp

4. Transfer to dish or Coplin jar with 0.1X SSC for 10 minutes at room temp

5. Transfer to 50 ml tube and spin at 500 rpm in clinical centrifuge for 5minutes to dry. Wipe the back (be sure it's the back) of the slide.

6. Add microarray slide to package, tape shut, send off for scanning