Macroarray hybridization

1. Plasmid DNA Denaturation
   - Take the frozen 96 well plates containing DNA and let them at room temperature until the DNA is melt.
   - Transfer plates to 65°C for 5 min
   - Transfer 50 µL NaOH 0.32M into the wells of a new plate. Transfer 30 µL of each DNA sample (containing ~130 ng DNA/ml) to the wells with NaOH (final concentration will be 0.2 N; final volume will be 80 µL and ~50 ng/ml DNA). Avoid bubbles in the wells. Let the plates at 37°C for 15 min.
   - Transfer 1 mL of each DNA sample to Hybond-N membranes using the V&P Scientific 96 pins device (delivers ~0.1 µl/sample). Repeat the spotting once more to deliver ~10 ng in each spot. Before changing to another DNA plate, wash the pins: H2O: 20 s; H2O and in a water bath sonicator: 10 s; ethanol abs: 10 s; ethanol abs again: 10 s, let it dry at room temperature for 1 min.
   - Fix the DNA at 80°C for 2h and store them at room temperature between filter paper sheets.

2. Hybridization with plasmid probe

2.1 Pre-hybridization
   - If this is first time membranes are used, pour boiling 0.1% SDS solution in the membranes to de
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2.2 Probe labeling
   - For two membranes:
     1 mL denatured primer mix (10 pmol/ml each; 80°C 5 min, spin, 37°C 10 min, then ice)
     0.5 mL BSA (2mg/mL)
     2.0 mL OLB solution
     0.5 mL 33P dCTP (5 mCi)
     1 mL Klenow (2 U/mL)
     5 mL water
   - Let it at room temperature (~25 °C) for 1h.
   - Count the total cpm in a scintillation counter.
   - Adjust the volume to 50mL with STE and purify with G50 columns (Pharmacia). Count the total cpm scintillation counter.

Primer sequences (Amp gene)
OSG208: 5’ GTG GTC CTG CAA CTT TAT CCG C 3’
OGA243: 5’ TAG ACT GGA TGG AGG CGG ATA A 3’

OLB solution:
Sol A : 10 mL; Sol B:  25 mL; Sol C: 15 mL

Sol A:  66.6 mL O solution (1.25 M Tris-HCl pH 8.0 and 125 mM MgCl₂; store at -20°C), 1.2 ml β-mercaptoethanol and 1.0 ml mix dATG (100 mM).
Sol B:  2M HEPES-NaOH (pH 6.6).
Preparation: dissolve 2.60 g HEPES in 5 mL water and adjust the pH to 6.6 with HCl pure. Store at -20°C.
Sol C:  3 mM Tris-HCl (pH 7.4); 0.2 mM EDTA. Store at -20°C.

2.3. Hybridization
- Denature the probe at 94°C for 3 min, spin briefly and transfer to ice.
- Take 10 mL from the pre-hyb solution in use with the membranes and mix with the denatured probe in Falcon tube.
- Transfer the solution back to the hybridization cylinder. Hybridize for 16-18 h.

2.4. Washes and exposition
- Discard the hyb solution and add 100 ml 2 x SSC , 0.1% SDS in the hybridization cylinder (we pre-heat the washing solutions). Wash at 58°C for 30 min.
- Transfer the membranes to plastic trays and wash with:
  1,5 x SSC, 0,1% SDS a 58°C for 30 min (once)
  0,5 x SSC, 0,1% SDS a 58°C for 30 min (once).
- Seal the membranes with a plastic sheet and expose them in an imaging plate for 96 h. We use FLA3000 FUJI PhosphorImager).

3. Hybridization with cDNA probe

3.1. Pre-hybridization
- Pour boiling 0.1% SDS solution in the membranes to decrease background (let them in the SDS solution for 5 min with gentle shaking). In the next time membranes are hybridized, jump this step.

- Pre-hybridize two membranes in a 30x3.5 cm cylinder with 20 mL solution for 4 h at 42°C the first time (2 hours the other times). Use the regular hybridization cylinders or group the membranes in a tray Pre-hyb solution: 5x SSC, 10x Denhardt’s, 20mM Tris-HCl pH 7.5, 1% SDS, 50% formamide and 100 µg/ml denatured salmon sperm DNA.

3.2. cDNA probe synthesis (Schummer et al, 1999, Gene 238: 375-385, with modifications)
- Mix in a 1.5 ml eppendorf tube: 6 ml total RNA total (30 mg) and 1.5mL Oligo dT₁₈V (100mM stock). Heat at 75°C/ 10 min. Spin briefly and transfer to ice.
- Add:
  5 mL First Strand Buffer (5x, BRL)
  2.5 mL DTT (100 mM)
  2.0 mL RNAguard (Pharmacia)
  2.5 mL dATG (10 mM each)
  5 mL ^3^P de dCTP (50 mCi)
- Heat at 42\(^\circ\)C for 5 min and add 1.25 mL Superscript II (200 U/mL). Mix gently and spin quickly. Incubate at 42\(^\circ\)C for 20 min, spin, and add 125 mL dCTP (10 mM). Incubate at 42\(^\circ\)C for 60 min.
- Spin quickly and add 1 mL Poly-A (2 mg/mL) and denature at 94\(^\circ\)C for 5 min.
- Spin and add 1.4 mL NaOH (5 N). Incubate at 37\(^\circ\)C for 15 min.
- Spin and add 1.4 mL HCl (4 N) and 7.0 mL Tris-HCl 1M pH 7.5.
- Add 19 ml STE and quantify all the reaction in the eppendorf tube a scintillation counter. Usually the counts are 600,000 cpm.
- Purify the reaction with G50 columns (Pharmacia) and count all the purified probe in the eppendorf. Counts will be ~300,000-450,000 with an incorporation of ~50-75%. Some authors did not purify the cDNA probe.

3.3 Hybridization
- Prepare the hybridization solution (7.5 mL/membrane). Keep it at 65 \(^\circ\)C in Falcon tubes.
  Hyb-solution: 5x SSC, 2x Denhart’s, 20mM Tris-HCl pH 7.5, 1% SDS, 50% formamide, 5% Dextran sulfate and 100µg/ml denatured salmon sperm DNA.
- Denature the probes at 94\(^\circ\)C for 3 min and transfer to ice. Spin.
- Transfer the probes to the Falcon tubes containing the pre-heated hyb solution and mix.
- Discard the remaining pre-hyb solution and add the probe. Hybridize for 18 h at 42\(^\circ\)C. Do not overtime.

3.4 Washes and exposition
- Discard the probes.
- Wash the membranes with 200 mL of pre-heated:
  - 2x SSC, 0.1% SDS 15 min/ 65\(^\circ\)C
  - 2x SSC, 0.1% SDS 15 min/ 65\(^\circ\)C
  - 1x SSC, 0.1% SDS 15 min/ 65\(^\circ\)C
  - 0.1x SSC, 0.1% SDS 15 min/ 65\(^\circ\)C
  - 0.1x SSC, 0.1% SDS 15 min/ 65\(^\circ\)C
- Never let the membranes dry! Pack the membranes with plastic sheets.
- Expose to imaging plates for 96 h.

3.5 Probe stripping
- Pour boiling 0.1% SDS and let the membranes for 5 min with gentle shaking. Repeat this step two more times.
- Pack with plastic and expose to imaging plates for 96 h. If no signal is detected, store the membranes at -20 \(^\circ\)C. Otherwise, strip again, 1-3 times, according to the signal detected.