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M. tuberculosis Protocols for Operon Oligo Microarrays

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I Post Processing

Before you start

a) Heat up heat block to 90°C and invert the insert
b) Place slide-staining chambers in front of the heating block.
c) Fill slide staining chamber with water.
d) Get a dust-free board needed for the cross-linking step.
e) Fill one of the slide dishes with 95% ethanol
f) Place in the chem. hood:
   - slide washing tray with 350ml dH_2O
   - slide washing tray with 350ml 95% ethanol
   - stirring plate for preparation of blocking solution
   - shaker
g) For the preparation of the blocking solution, have a clean 500 ml beaker ready.
h) Check if the following chemicals are available:
   - succinic anhydride (6.0 g)
   - 1-Methyl-2-pyrrolidinone (350 ml)
   - 15 ml 1 M Na-Borate, pH 8.0 (filtered)

i) Make a template slide which marks the boundaries of you array. The array will be invisible after post processing.

1. Rehydrate the slides
   a) Rehydrate slides by inverting (array side down toward water) them over water in a slide-staining chamber. Let the spots become glistening and juicy.
   Be careful not to allow the water to touch the array.

   b) Immediately flip them (array side up!) onto a heating block (inverted about 90°C). Watch the steam evaporate. When the array spots dry in a rapid wave-like pattern, remove them from the heating block. This takes about 5 seconds. Do 1 slide at a time.

2. UV cross link

   a) Place the slides, array side up, on a flat, dust-free board that fits into the UV cross-linker.

   Do not put them on a seran wrap surface since the slides stick to it.

   b) Irradiate with 600 □Joules UV light. (press *power*, press *energy*, set *600*, press *start*)

3. **Wash slides with 2x SSC / 0.15%SDS for 30 plunges up and down <u>@ 50°C</u>**.

3. Wash slides with dH2O for 30 plunges up and down.
4. Wash slides with dH2O for 30 plunges up and down.
5. Wash slides with 95% ethanol for 30 plunges up and down.
6. Dry slides in centrifuge at 600rpm 1-2 min.

7. Block free lysines (wear a lab coat when working with methyl pyrrolidinone)

   a) Add 6.0 g of succinic anhydride into 350 ml 1-Methyl-2-pyrrolidinone while stirring.

   b) As soon as the solids dissolve, quickly add 15 ml of the 1M Na-Borate pH 8, and pour the mixed solution into a slide washing tray (make sure the tray is small enough that 350ml of solution will completely cover the slides when they are placed inside).

   c) Quickly place the slides into the succinic anhydride solution and vigorously plunge for 60 seconds. Shake without exposing the slides to air for15 min.

   d) Remove the slide rack from the organic reaction mixture and plunge it immediately into the slide washing tray with 350ml dH_2O for 90 seconds.

   e) Transfer the slide rack to the slide washing tray with 350ml 95% ethanol and plunge 20X.

   f) Spin dry the slides by centrifugation at 600 rpm for 2 min. Use a counter balance with the same number of slides in a rack.

   g) Carefully transfer the slides to a dry slide box for storage in a dessicator.

NOTE: The slides should be rapidly plunged into the organic succinic anhydride solution to quickly wash the unattached DNA away from the slides.
Do not pour the solution over the slides.

Supplies

Gloves
Hood
Slide Dishes
Slide Racks -- washed and DRY 4 liter beaker
500 ml beaker and stir bar, dry and clean
Hot plate in the hood
Rotating shaker in the hood
Slide staining chamber
Heating block
95% Ethanol
Glass-distilled, deionized water
1M Sodium Borate, pH 8 (Prepare with Boric acid, adjust pH to 8 with sodium hydroxide.) Filtered.
1-Methyl-2-pyrrolidinone --Aldrich 32,863-4 ($51.35/liter). Anhydrous, 99.5%, FW=99.13, irritant, hygroscopic. Undergoes condensation reactions with arylthioacetamides and methyl salicylates. Versatile solvenet for water miscible applications.
Succinic Anhydride -- Aldrich 23,969-0 ($13.56 /50g) 99+%, FW=100.07. Moisture sensitive, irritant. Used for post-processing slides to block free poly-L-lysine groups.

II RNA isolation and Purification.

M. tb. RNA isolation.

1. Centrifuge 20-30 ml of early- to mid-log culture (O.D. 0.15-0.2) 5m at 3700 rpm, at RT-37C. Pipette off supernatant and immediately freeze on dry ice and store at –80C.
2. Add 1 ml Trizol (Gibco-BRL) to each of 4-8 pellets, suspend first pellet by vortexing, while other pellets are still frozen. Add suspension to 0.5 ml glass beads in a 2 ml screw cap tube.
3. Shake 30s at maximum speed in bead beater. Suspend next pellet and add to glass beads. Apply next sample to bead beater. Repeat bead beating two more times with each sample. Continue to periodically invert samples for at least 5m in Trizol.
4. Centrifuge samples 45s at max speed, remove Trizol solution to a 2 ml screw cap tube containing half of the Heavy Phase Lock Gel I, supplied by 5 Prime 3 Prime, Inc. in 1.5 ml tubes and transferred with sterile stick to 2 ml tubes, and 300µl Chloroform. Invert rapidly for 15s, and continue inverting periodically for 2 m.[a]
5. Centrifuge 5-10m, remove aqueous layer (540µl) and add to a 1.5 ml tube containing 270µl isopropanol then add 265µl high salt solution (0.8M Na Citrate, 1.2M NaCl). Invert several times and spray with Staphene and remove from the P3. Typically ppted at 4C O/N.
6. Centrifuge 10m at 4C and remove isopropanol. Add 1 ml 75% EtOH[b], invert several times and centrifuge 5min.
7. Remove EtOH by drying under vacuum 2m [Do not over dry].
8. Suspend RNA in 90 ul RNase free water (don't suspend in DNaseI buffer), may need to heat 10m at 55-60C to dissolve RNA. (Optional: obtain RNA concentration)
9. Add 10µl 10X DNaseI buffer to RNA (use no more than 80 µg RNA) and add 2µl DNaseI. Incubate 30m at 37C.

Qiagen RNeasy purification.

1. Add 350µl RLT buffer (add 10µl BME to 1 ml RLT before using), vortex, and add 265µl 95% EtOH to each sample, vortex.
2. Add to RNeasy spin column, centrifuge 15s, transfer column to a new 2 ml collection tube. Add 500µl RPE, centrifuge 15s, discard flow-through, add 500µl additional RPE and centrifuge 2m. If column still wet on sides, remove wash solution from tube and spin 1m to dry.
3. Transfer to a 1.5 ml collection tube, elute with 40µl RNase free water, centrifuge 1m.
4. Determine RNA concentration with A_{260}/A_{280} readings. Dilute 1µl in 199µl TE. [200(dilution factor)x 40 µg/A_{260}x A_{260} = µg/µl].
5. Run 1µl RNA on 2% Agarose TAE gel. Run gel 45m at 100 volts.

[a]If Phase Lock Gel is not used, decrease Chloroform:isoamyl alcohol to 200µl. The yield will be less. Be careful not to remove any of the interface layer.

[b]Using 100% EtOH should be avoided in all steps of preparation of RNA intended for array analysis. Instead use 95% EtOH in preparation of solutions. Benzene contamination may fluoresce.
[c]Instead of using the RNeasy purification, the DNaseI can be inactivated at 65-70C for 15m and then EtOH precipitated. The RNeasy purification may not be necessary in all applications. We find the purification gives more consistent results and is less time consuming, compared to precipitation. In addition, other purifications can be used. Note: The RNeasy column will remove much of the small tRNA and degraded RNA.

III Synthesis and Labeling of cDNA.

1. Bring 0.5-5µg RNA (Typically 3 µg) to 11µl and add 2.2µl (2 mg/ml) N6 primers
2. Heat 2m at 98C, snap cool on ice.

3. Add 11.1µl (keep on ice)
   2.5µl 10X Stratoscript Buffer
   5.0µl Rnase free H_20
   2.3µl dNTP (5mM A,G,C and 0.2mM T)
   1.5µl Cy3 or Cy5 (Cy5 for exp. sample)

   Then add 2.0µl Stratoscript RTase.

4. Incubate 10m at 25C followed by 90m at 42C in PCR machine. Can freeze or leave at 4C o/n
5. Add both rxns to a microcon10 (Amicon) with 400µl TE. Centrifuge at max speed until ~25µl remaining (~20m). Add 200µl more TE and spin until almost dry (~12m)
6. (< 5µl).
7. Recover cDNA by inverting microcon into new collection 1.5 ml tube and centrifuge at half max speed 1m.
8. Bring sample to 5µl with TE and transfer to a 0.5 ml tube.

VI Prehybridization

1. Prepare Prehybridization Solution (Fresh Each Time)
   32.0ml dH2O
   15.0ml 20xSSC
   12.0ml 5% BSA
   0.6ml 10%SDS
2. Add Prehybridization solution to a glass slide-staining jar and place post-processed slides into solution. Make sure there are no bubbles on the surfaces of the slides.
3. Place the staining jar containing the prehybridization solution and slides into a waterbath and hybridize for 1 hour underwater at 42^{o}C.
4. Take the prehybridized slides out of the prehybridization solution and place into a slide holder submerged in a slide washing tray containing MilliQ water. Wash slides for 2 minutes with constant shaking up and down.

5. Transfer slide rack to a slide washing tray containing Isopropanol and wash for 2 minutes with constant shaking up and down.
6. Take the slides in the Isopropanol bath to the centrifuge to keep the slide wet until you can dry it rapidly. Spin at 600 RPM for 3 minutes to dry. *Compleate this step within one hour of beginning the hybridization.

V Hybridization

Add the following, **in order**, to each sample.
Mix well when adding each reagent (very important!)!

0.5µl tRNA 10 mg/ml
1.0µl 20X SSC
2.5ul Formamide
1.0µl 1% SDS

1. Heat to 98C for 2m. Spin in centrifuge for 4 minutes at 12,000x g.
2. Apply 1 microdot of rubber cement with 25G needle/syringe; remove point with wire cutters, to each corner of the array. Apply 10µl hybe solution to microarray. Do no apply very hot hybe solution to array. Apply 22 mm coverslip with bent precision forceps. If bubbles under coverslip try to free the bubbles by tapping on the top of coverslip with the forceps. COMPLETELY seal edges of coverslip with a bead of rubber cement from the (practice this). It is not critical to move quickly at this step. The rubber cement can be allowed to dry for a few minutes before sealing the chambers to avoid sticking to the top of the chamber. Hybe in standard hybe chambers with ~100µl water for 6 hr to O/N in 50°C water bath.
3. Remove coverslip with a razorblade at corner of coverslip (pull up) while array is submerged in first wash solution, transfer to submerged microscope slide staining racks and rinse for 1-2m in 1X SSC+0.05% SDS.
4. Rinse in 0.06X SSC. Transfer to another submerged staining rack and wash 2m in fresh 0.06X SSC.
5. Centrifuge 1m at 500 rpm to dry and scan.