Fluorescent in situ hybridization

(Based on the protocol from Molecular Cytogenetics lab, revised on 09/26/06)

Cell preparation

Solutions:

1. Hypotonic solution:
   0.075 M KCl

2. Fixative:
   3:1 ratio of methanol: acetic acid
   For 2 samples of 1 x 10^6 cells use 6 ml Methanol and 2 ml Acetic Acid
   *Prepare fresh before use

1. (For detection of metaphase chromosome) Add nocodazole (50 ng/ml, final) or colcemid (0.12 μg/ml, final) into medium and incubate for 30 min~16 h.
   - If not interested in synchronizing cells, do not add these reagents
2. Harvest the cell and transfer the cell suspension to the 15 ml tube.
   - Use more than 1 x10^6 cells for optimal analysis.
3. Centrifuge for 8 min at 800-1000 rpm.
4. Carefully aspirate the supernatant off and discard without disturbing the cell button. Gently break up the cell pellet by flicking the tube with your finger.
5. Resuspend the cell pellet in 4-6 ml (use 1 ml for less than 5 x10^5 cells) of Hypotonic Solution pre warmed to 37˚C. Incubate at 37˚C for 20 min.
6. Carefully add 8-10 drops (2 drops for less than 5 x 10^5 cells) of Working Fixative to the hypotonic solution, mix gently, and allow to stand at r.t. for 5 min. The cells are very delicate at this point and require very gentle fixation to prevent the cells from bursting.
7. Centrifuge for 8 min at 800-1000 rpm. Carefully aspirate the supernatant off and discard without disturbing the cell button. Gently break up the cell pellet by flicking the tube with your finger. Slowly add 5 ml (use 1 ml for less than 5 x10^5 cells) of fresh fixative down the side of the centrifuge tube and incubate for 30 min at r.t.
8. Repeat step 7 twice without the 30 min incubation period.

* For storage of cell pellet, spin the sample down and add about 1.5 ml (use 200~500 μl for less than 5 x 10^5 cells) of fresh fixative. Transfer the cell suspension to a 2.0 ml Cryogenic vials. The cell pellet can be stored for 1-2 month at 4˚C. For long storage, the cell pellet should be stored at –20˚C.

Sugden Lab.

Preparation of biotinylated probe by nick translation

Solutions:

1. 10 x NT buffer
   0.5 M Tris-HCl pH 8.0
   50 mM $MgCl_2$
   0.5 mg/ml BSA

2. 10 x nucleotide stock:
   0.5 mM dATP/dGTP/dCTP

3. 0.4 mM dTTP

4. DNase stock solution:
   0.15 M NaCl
   50% glycerol

   Dissolve DNase I (Roche:20 U/ml, final) into stock solution and store at –20˚C.
   1:1000 dilution in ice cold ddH_2O immediately before use

5. 0.1 M beta-Mecaptethanol

   Dilute 3.5 µl conc. beta-Mecaptethanol (14. 25 M) into 496.49 µl ddH_2O.

Nick translation:

10 x NT buffer	10 µl
0.1 M beta- Mecaptethanol	10 µl
10 x nucleotide stock	10 µl (0.05 mM, final)
E.coli polymerase I (NEB)	2 µl
DNase I dilution	5 µl
0.4 mM dTTP	2.5 µl (0.01 mM, final)
1 mM Biotinilated dUTP (Roche)	4.0 µl (0.04 mM, final)
Template DNA	1 µg

Adjust the final volume up to 100 µl by ddH_2O

1. Incubate 2 h at 15˚C, denature at 70˚C for 10 min using thermalcycler. Keep on ice after reaction.
2. Run 4 µl of the reaction on a 1% agarose gel.
3. Expect fragments in the 300-600 bp range. If fragments are larger than 1000 bp, reincubate reaction with 1.5~2.0 µl DNase I dilution for 15-30 min at r.t.
4. Precipitate labeled probe.

Updated 09/26/06

Probe	20 µl
Human Cot I DNA (GIBCO BRL)	4 µg
Salmon sperm DNA (Eppendorf)	6 µg
Adjust the final volume up to 60 ul by ddH_2O	

5. Add 6 µl 3 M NaOAC and 165 µl cold ethanol.
6. Centrifuge at 14,000 rpm for 20 min at r.t.
7. Remove supernatant and allow pellet to dry in air.
8. Resolubilize pellet in CEP hybridization buffer (Vysis Inc.) in 10~13 µl (for 3~4 samples).
9. Denature probe using thermalcycler as follow:
   70˚C for 10 min.
   4˚C for 5 min.
   Hold at 37˚C until ready to use (more than 30 min)

Hybridization of slides

Solutions:
1. Pre-hyb:
   2 x SSC+0.5% NP-40 pH 7.0 (adjust pH after addition of NP-40).

2. Denaturation Solution:
   28 ml formamide
   4 ml 20 x SSC (pH 5.3)
   8 ml ddH2O

3. Ethanols:
   70%, 80% and 95%

1. Turn on 72˚C water bath for hybridization.
2. Set up moist incubation chamber in 37˚C (Slide box with moist toweling).
3. Pour pre-hyb solution into Coplin jar and warm in 37˚C water bath.
4. Pre incubate an empty glass jar at 50˚C water bath.
5. Place denaturation solution in 72˚C water bath while it is warming.
6. Fill three Caplin jars with ethanols and hold them in the freezer just before step 8.
7. Place FISH slides into pre-hyb that has been warmed at 37˚C. (Check inside of jar for 37C reading.) Incubate slides for 30 min. (At this point the probe can be prepared.)
8. Remove ethanols from the freezer. Dehydrate each slid for 2 min in 70%, 80% and 95% ethanols. Reuse the ethanols for step 11. (The procedure can be stopped here, air-dried using pressurized air, and stored at -20˚C)
9. Preincubate the slide in the empty glass jar for 5 minutes in the 50˚C water bath.
10. Place slides into denaturation solution for 2 min.
11. Repeat step 8.
12. Dry slides and move them to the warming box until probe is prepared.

13. Apply mixed probe to slides (3 µl/sample).
14. Coverslip slides avoiding bubbles.
15. Seal coverslip with parafilm.
16. Place slide into moist incubation chamber and incubate at 37˚C overnight.

Detection

1. To remove unbound probe, wash the slides in 50% formamide, 2 x SSC for 30 min at 50˚C (remove the cover slips before washing).
2. Wash the slides in 2 x SSC for 30 min at 50˚C.
3. Add 25 µl (about 1 drop) ADR001 Texas Red-streptavidin solution (Rainbow Scientific inc.) and incubate in the dark for 20 min at 37˚C*.
4. Wash twice in 4 x SSC, 0.05% triton-X100 for 5 min at r.t.
5. Apply 1 drop of Vectashield with DAPI (Vector Laboratory Inc.), coverslip and incubate for 10 min in the dark.
6. Detect the signal by scope.
7. For short-term storage (~ 1 week), seal edges of the coverslip with nailpolish and store in a dark place. For long-term storage, there is no need to add nailpolish, just store at -20C.

* For FITC-streptavidin (green)

3. Block in 1% BSA, 4 x SSC for 30 min at r.t.
6. Apply 1% BSA, 4 x SSC containing 200 ug/ml FITC-straptavidin and incubate for 30 min at 37˚C.
7. Wash twice in 4 x SSC, 0.05% triton-X100 for 5 min at r.t.
8. Apply DAPI Vectashield with DAPI (Vector Laboratory Inc.), coverslip and incubate for 10 min in the dark.
9. Detect the signal by scope.