

## **Immunocytochemistry Followed by FISH (Version 2)**

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**\*We present multiple versions of the Immunocytochemistry / FISH protocols because the conditions under which each antibody and DNA probe work can be different and must be determined empirically.**

### **Reagents**

**Antifade (1,4-phenylene-diamine)**

**Bovine Serum Albumin (BSA)**

Boehringer Mannheim Biochemicals (BMB), Cat. 100 350

**DAPI**

BMB, Cat. 236 276

**Dextran Sulfate (50%)**

Intergen, S4030

**Dimethyl sulfoxide (DMSO)**

**Ethylene glycol bis(succinimidyl succinate)**

Sigma, Cat. E3257

**Formamide**

FLUKA BioChemica, Cat. 47670

**Formamide, deionized**

Ambion, Cat. 9342

**Goat anti-mouse-FITC (FISH 2° Ab)**

BMB, Cat. 605 240

**Goat anti-rabbit-TRITC (ICC 2° Ab)**

Sigma, Cat. T-5268

**Normal Goat Serum**

Sigma, Cat. G6767

**HCl, 1N**

**Human Cot-1 DNA**

Gibco BRL, Cat. 15279-01

**Methanol**

**Mouse anti-biotin-FITC (FISH 1° Ab)**

Sigma, Cat. F4024

**Cot-I DNA (Mouse)**

Gibco BRL, Cat. 18440-016

**Para-Formaldehyde**

Sigma, Cat. P6148  
**Phosphate Buffered Saline, pH 7.4**  
Gibco/BRL, Cat. 10010-023  
**Rabbit polyclonal antibodies (ICC 1° Ab)**  
Specific for desired protein  
**RNase A**  
BMB, Cat. 109 169  
**Salmon testes DNA**  
Sigma, Cat. D-7657  
**NaOH, 0.1 M**  
**20X SSC**  
**Tween 20**  
Sigma, Cat. P1379

## Preparation

### **Methanol**

Pre-chill to -20°C

### **Blocking Solution I (5% NGS/1% BSA/1X PBS)**

NGS                      500 µl  
1%BSA/1X PBS        10 ml

Store at 4°C

### **Antibody Solution I (1% NGS/1% BSA/1X PBS)**

NGS                      10 µl  
1%BSA/1X PBS        1 ml

### **Ethylene glycol bis(succinimidyl succinate) (EGS) Solution**

Weigh volume of EGS powder [i.e., 100 µl powder] in eppendorf tube

Add equal volume of DMSO [i.e. 100 µl DMSO]

Incubate at 37°C until dissolved and re-determine volume

Calculate concentration based on weight of EGS used, molecular weight of EGS, and final volume of solution (should be ~ 500-650 mM)

Store at RT <1 month

Dilute stock into 1X PBS immediately prior to use for final conc. 50 mM, discard unused portion

### **1% p-formaldehyde**

p-formaldehyde        1 g  
1X PBS                    100 ml

0.1N NaOH,            500  $\mu$ l                    f.c. [0.5 mM]  
pH 7.4 w/ HCl  
Store <1 month at 4°C

**RNase A (DNase-free)**

20 mg/ml in sterile water  
Boil 15 min, cool to RT, aliquot and store at -20°C

**Master Mix**

Dextran sulfate, 50%            40 ml  
20X SSC, pH 7.0                10 ml  
Sterile dH<sub>2</sub>O                    50 ml

Vortex solution and place tube on a shaking platform overnight to insure proper mixing.

Aliquot, and store at -20°C.

**50% FA/SSC**

20X SSC                        30 ml  
dH<sub>2</sub>O                            120 ml  
Formamide                    150 ml  
Adjust pH to 7-7.5 with 1M HCl

**Pre-warm to 45°C**

**0.1X SSC**

20X SSC                        2.5 ml  
dH<sub>2</sub>O                            498 ml

**Pre-warm to 60°C**

**4X SSC/Tween 20**

20X SSC                        200 ml  
dH<sub>2</sub>O                            799 ml  
Tween 20                        1 ml

**Pre-warm to 45°C**

**Blocking Solution II ( 3% BSA/4X SSC/Tween20)**

BSA                                0.3 g  
4X SSC/Tween 20    10 ml  
Store at 4°C

**Antibody Solution II ( 1% BSA/4X SSC/Tween20)**

Blocking Solution II    333  $\mu$ l  
4X SSC/Tween 20    666  $\mu$ l

**DAPI (stock solution)**

DAPI 2 mg

dH<sub>2</sub>O 10 ml

Aliquot and store at -80°C

**DAPI (staining solution)**

DAPI stock solution 40 µl

2X SSC 100 ml

**Antifade (1,4-phenylene-diamine)**

See Antifade preparation procedure in CGH Protocols

**Procedure**

1. Grow adherent cells in chamber slides or cytopsin suspension cells onto poly-L-lysine coated slides.
2. Fix cells in methanol (pre-chilled to -20°C) for 10 min at RT.
3. Wash 3 x 5 min 1X PBS at RT.
4. Block coverslips with 25 µl blocking solution I in hybridization chamber 30 min at 37°C.
5. Incubate with rabbit polyclonal (ICC 1° Ab) in 25 µl antibody solution I in hybridization chamber at 37°C for 60 min.
6. Wash 3 x 5 min with 1X PBS at RT.
7. Incubate with ICC 2° Ab [goat anti-rabbit-TRITC; 1:200 in 25 µl antibody solution I] in hybridization chamber at 37°C for 60 min.
8. Wash 3 x 5 min with 1X PBS at RT.
- 9a. Incubate with 25 µl EGS solution [dilute stock to 50mM in 1X PBS prior to use and mix well (will be turbid)] in hybridization chamber at 37°C for 30 min to allow postfixation cross-linking of the Ab to the target protein.
- 10a. Wash 3 x 5 min with 1X PBS at RT.

**OR**

9b. Incubate with 25  $\mu$ l 1% p-formaldehyde [1g p-formaldehyde, 100 ml 1X PBS, 0.5 mM NaOH, adjust to pH 7.4 with HCl (store <1 month at 4°C)] at RT for 5 min.

10b. Wash 3 x 5 min with 1X PBS at RT.

**Note:**

**Can counterstain with DAPI at this point and mount slides with antifade to have a look at them and determine if Ab detection worked.**

**Wash coverslips 3 x 5 min in 2X SSC before continuing with procedure.**

11. Incubate with RNaseA (1:200 in 1xPBS) in hybridization chamber 60 min at 37°C

12. Wash 3 x 5 min with 1X PBS.

13. Denature chromosomal DNA by inverting coverslips (18 mm x 18 mm) onto 25  $\mu$ l drop of NaOH (pH 13.0 - ~0.1M) for exactly 2 min.

14. Rinse immediately in cold 1X PBS.

15. Hybridize denatured/pre-annealed biotin-labeled probe (as per standard FISH Protocol in 50% Deionized Formamide/Master Mix at 80°C 8 min and pre-annealed if necessary at 37°C in the presence of Cot I DNA for 60-90 min) to coverslip.

16. Seal with rubber cement and incubate in hybridization chamber at 37°C overnight.

17. Remove rubber cement.

18. Wash coverslips 3 x 5 min in FA/SSC (pre-warmed to 45°C), shaking.

19. Wash coverslips 3 x 5 min in 0.1X SSC (pre-warmed to 60°C), shaking.

20. Dip slides in 4X SSC/Tween 20 (pre-warmed to 45°C); do not let dry.

21. Block with 25  $\mu$ l blocking solution II in hybridization chamber 30 min at 37°C.

22. Dip slides in 4X SSC/Tween20; do not let dry.

Note: Centrifuge all fluorescent-conjugated Ab for 3 min at 13,000 rpm.

23. Incubate with FISH 1° Ab [mouse anti-biotin-FITC, 1:200 in 25 µl antibody solution II] in hybridization chamber 45 min at 37°C.
24. Wash coverslips 3 x 5 min in 4X SSC/Tween20 (pre-warmed to 45°C), shaking.
25. Incubate with FISH 2° Ab [goat anti-mouse-FITC, 1:200 in 25 µl antibody solution II] in hybridization chamber 45 min at 37°C.
26. Wash coverslips 3 x 5 min in 4X SSC/Tween 20 (pre-warmed to 45°C), shaking.
27. Stain for 2 min with DAPI.
28. Wash in 1X PBS for 10 min, shaking.
29. Mount coverslip with antifade on microscope slide.