

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)

Sigma-Aldrich, Cat. 78460

Bovine Serum Albumin (BSA)

Roche Diagnostics, Cat. 100350

DAPI

Sigma-Aldrich, Cat. 18860

Dextran Sulfate (50%)

Millipore Cat. S 4031

Dimethyl sulfoxide (DMSO)

Sigma-Aldrich, Cat. D2650

Ethylene glycol bis(succinimidyl succinate)

Sigma-Aldrich, Cat. E3257

Formamide

Sigma-Aldrich, Cat. 47670

Formamide, deionized

Ambion, Cat. 9342

Goat anti-mouse-FITC (FISH 2° Ab)

Sigma-Aldrich, Cat. F0257

Goat anti-rabbit-TRITC (ICC 2° Ab)

Sigma-Aldrich, Cat. T-5268

Normal Goat Serum

Sigma-Aldrich, Cat. G6767

HCl, 1N

Human Cot-1 DNA

Life Technologies, Cat. 15279-011

Methanol

Mouse anti-biotin-FITC (FISH 1° Ab)

Sigma-Aldrich, Cat. F4024

Cot-I DNA (Mouse)

Life Technologies, Cat. 18440-016

Para-Formaldehyde

Sigma-Aldrich, Cat. P6148

Phosphate Buffered Saline, pH 7.4

Life Technologies, Cat. 10010-023

Rabbit polyclonal antibodies (ICC 1° Ab)

Specific for desired protein

RNase A

Roche Diagnostics, Cat. 10109169001

Salmon testes DNA

Sigma-Aldrich, Cat. D-7656

NaOH, 0.1 M

20X SSC

Tween 20

Sigma-Aldrich, 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 µl 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	f.c. [20%]
20X SSC, pH 7.0	20 ml	f.c. [4x SSC]
Sterile dH ₂ O	40 ml	
Total	100 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	20 ml
dH ₂ O	80 ml
Formamide	100 ml
Total	200 ml

*Adjust pH to 7-7.5 with 1M HCl

Pre-warm to 45°C

0.1X SSC

20X SSC	2.5 ml
dH ₂ O	498 ml
Total	500 ml

Pre-warm to 60°C

4X SSC/Tween 20

20X SSC	200 ml
dH ₂ O	799 ml
Tween 20	1 ml
Total	1000 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 µl
4X SSC/Tween 20	666 µl

DAPI (stock solution)

DAPI	2 mg	f.c. [0.2 ng/ml]
dH ₂ O	10 ml	

Aliquot and store at -80°C

DAPI (staining solution)

DAPI stock solution	40 µl	f.c. [80 ng/ml]
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 µl 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 µl 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 to coverslip (as per standard FISH Protocol, probe is deatured at 80°C, 5 min, in 50% Deionized Formamide/Master Mix and pre-annealed if necessary at 37°C in the presence of Cot I DNA for 60-90 min).
 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 µl 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.