**Manual non-radioactive**

**Fluorescent in situ hybridization (FISH)**

**with amplification**

**Materials:**

1. 3% H2O2 in methanol1
2. 1x PBS2
3. 0.2 HCL3
4. PK- buffer4
5. proteinase K (Roche 3115828)
6. 4% PFA5
7. Hyb-Buffer6
8. DTT (1,4-Dithiothreitol) (Merch 11583786001)
9. Parafilm/Coverslide
10. SSC7
11. PBST8
12. 10% Sheep-Serum- inactivated (Equitech-bio SSA62)9
13. TNT buffer10
14. Anti-DIG (POD) antibody (Merck 11093274910)
15. Glass slides and coverslips and mounting media (water based)
16. RNA probe
17. TNB11
18. DMSO
19. TSA Signal Amplification (TSA) Systems – PerkinElmer or equivalent

**Method:**

Manual non-radioactive RNA *in situ* hybridization

Tissue: fresh frozen tissue that are fixed in PFA, acetylated, and dehydrated 12

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Day 1** |  | | | |
| *Incubate* |  | 3% H2O2 in Methanol(prepare freshly) | 5 min | RT |
| *Wash* |  | PBS | 2 x 5min | RT |
| *Denature* | 0.2 N | HCl | 10 min | RT |
| *Wash* |  | PBS | 2 x 5 min | RT |
| *Deproteinate* |  | PK-buffer + 30-40 µg/ml proteinase K13 | 2 x 10 min | RT |
| *Wash* |  | PBS | 2 x 5 min | RT |
| *Fix* | 4 % | PFA in PBS | 15 min | RT |
| *Wash* |  | PBS | 2 x 5 min | RT |
| *Pre-hybridize*  *Hybridize* |  | Hyb-Buffer (add 1mg/ml DTT)  Hyb-Buffer + 0.5-2µg/ml probe (parafilmed/coversliped) | 30 min  o/n | 60 oC  60 oC |
| **Day 2** |  |  |  |  |
| *Wash* | 2x | SSC | 15 min | 60 oC |
| *Wash* | 2x | SSC | 5 min | RT |
| *Wash* | 0.2x | SSC | 2 x 30 min | 60 oC |
| *Wash* | 0.2x | SSC | 2 min | RT |
| *Wash* |  | PBST (0.1% Tween 20 in PBS) | 2 x 20 min | RT |
| *Block* | 10 % | inactivated sheep serum (SS) in TNB | 30 min | RT |
| *Antibody* | 10 % | SS in TNB+ anti-DIG Peroxidase (POD) antibody (1:500) | 30 min | RT |
| *Wash* |  | TNT (Tris-NaCl-Tween buffer) | 3 x 5 min | RT |
| *Incubate* |  | Tyramide working solution14, 15 | 3-5 min | RT |
| *Wash* |  | TNT buffer | 3 x 5 min | RT |
|  |  | *Continue* |  |  |
|  |  |  |  |  |
|  |  |  |  |  |

Immunohistochemistry

DAPI

Double/Triple FISH16

Notes:

1. 3% H2O2 in methanol

[Hydrogen peroxide (3% in methanol) (cshlp.org)](http://cshprotocols.cshlp.org/content/2010/12/pdb.rec12371.full)

1. 1x PBS solution preparation-

[Phosphate-buffered saline (PBS) (cshlp.org)](http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8247)

1. 0.2 HCL solution preparation- To make a 0.2M solution, slowly add 16.42mL of your stock 37% solution of HCLE to 250mL deionized H2O. Adjust the volume of the solution to 1000mL with Deionized water.

N.B. Always add the hydrochloric acid to the water, not the water to the hydrochloric acid!

For other calculation you can use this calculator-

[Molarity Calculator & Normality Calculator for Acids & Bases | Sigma-Aldrich](https://www.sigmaaldrich.com/chemistry/stockroom-reagents/learning-center/technical-library/molarity-calculator.html)

1. PK (proteinase K) buffer-

[Proteinase K buffer (10X) (cshlp.org)](http://cshprotocols.cshlp.org/content/2009/10/pdb.rec11994.full). After you prepare 10X dilute the solution to 1X.

1. 4% PFA solution preparation-

[Paraformaldehyde (PFA; 4%) (cshlp.org)](http://cshprotocols.cshlp.org/content/2009/12/pdb.rec12044.full?sid=0aa5e5c0-f1c0-49e1-87c2-1c51f93c834d)

1. Hyb-buffer alternative to the commercially ready-made solutions:

[Hybridization buffer (A) (cshlp.org)](http://cshprotocols.cshlp.org/content/2009/9/pdb.rec11932.full) + [Denhardt’s solution (100X) (cshlp.org)](http://cshprotocols.cshlp.org/content/2008/12/pdb.rec11538.full?sid=f63bfddf-cbbf-4030-9c00-035857290a90)

1. SSC solution preparation- [SSC (cshlp.org)](http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8297.full?sid=cc2310c8-ccdd-4e64-af53-851ce9b42895).
2. PBST solution preparation- [PBST (cshlp.org)](http://cshprotocols.cshlp.org/content/2007/1/pdb.rec10851.full?sid=eda590f6-409b-41e0-8255-2a4ad85c2d1e)
3. To inactivate the sheep serum heating to 56°C for 30 minutes, then store -20°C
4. TNT buffer solution preparation protocol- [TNT (Tris-NaCl-Tween buffer) (cshlp.org)](http://cshprotocols.cshlp.org/content/2009/12/pdb.rec12060.full)
5. TNB - [TNB Blocking Buffer (cshlp.org)](http://cshprotocols.cshlp.org/content/2012/8/pdb.rec070235.full)
6. Check the Protocol for fixation, acetylation, and dehydration.
7. The required Proteinase K concentration depends largely on the tissue type. By default, we use 5µl/100ml of proteinase K for E14.5 mouse embryo and 35-40 µl/100ml for adult mouse, monkey or human brain.
8. Fluorophore Tyramide Stock Solution

Fluorophore Tyramide Reagent is supplied as a solid. The company supplies vials which should be reconstituted with DMSO, or water as indicated in the brochure to make Fluorophore Tyramide Stock Solution.

1. Fluorophore Tyramide Working Solution

To make Tyramide Working Solution before each staining, dilute Fluorophore Tyramide Stock Solution 1:50 in 1X Amplification Diluent to make Fluorophore Tyramide Working Solution. Add enough of the working solution ~ 100-300 µL of Fluorophore Tyramide Working Solution per slide.

1. If you want to do double or triple FISH staining you should use differently labelled anti-sense probes using DIG (digoxigenin), Fluorescein and Biotin as hapten-labels (labelling agent). Each of those transcripts is detected with appropriate anti-body directed towards DIG, Fluorescein and biotin, respectively so they can be differentially recognised. Between each detection, the anti-hapten antibody should be eliminated before using the next one. The elimination is achieved with 0.2 M HCL for 15 min. The specimen should be rinsed 3X with TNT buffer, blocked again for 30 min with TNB and SS. The next antibody should be again applied. Here we apply a step-by-step workflow to achieve double or triple FISH label.

Do do double or triple staining continue after step 8

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Wash* |  | 0.2M HCL | 15 min | RT |
| *Wash* |  | TNT wash | 3 x 5 | RT |
| *Block* |  | *TNB with SS* | 15 min | RT |
| *Incubate* |  | *Anti-FITC- POD* | 30 min | RT |
| *Wash* |  | *TNT buffer* | 3 x 5 min | RT |
| *Incubate* |  | *Tyramide FITC* | 7-10 min | RT |
| *Wash* |  | *TNT buffer* | 3 x 5 min | RT |
|  |  | *Continue with another probe, antibody or DAPI* |  |  |