

IN SITU HYBRIDIZATION PROTOCOL

PREHYBRIDIZATION AND HYBRIDIZATION USING DIG.LABELLED PROBES

MAKE UP 1X PBS in DEPC-treated H₂O. Turn on waterbath and ph meter

1. Bring slides to R.T.- Fix in 4 % Paraformaldehyde/PBS - **5 min at R.T.**
2. 0.3% Triton X-100 in PBS. **(0.9 ml/300 ml PBS/1.8 ml/600 ml)** - **10 min. R.T**
3. Wash briefly in PBS.
4. ProteinaseK solution(1 µg/ml in 20 mM Tris-Base and 1 mM EDTA, ph 7.2)
2 ml PK/600 ml.
for 15 min. at 37 ° C Waterbath.
5. Wash briefly in PBS.
6. Immerse in 4 % Paraformaldehyde / PBS - **5 min. at RT.**
7. Wash briefly in PBS.
8. Air dry for **at least 1 hour.** Standing slides up in fumehood in racks.

HYBRIDIZATION MIXTURE

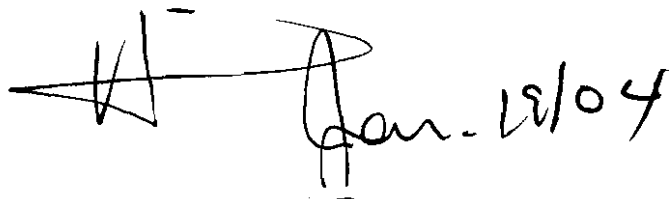
Hybridization buffer (is 90% of total volume)	250 µl
Herring Sperm DNA (denatured at 100°C)	12.5 µl
Dig-labelled Probe (1 µl/per section)	11µl
DEPC-treated H ₂ O (to make up total volume if needed)	1.5 µl
<u>This mixture is good for 10 slides.</u> (Calculation for 11 slides)	

9. Apply 25 µl per section, cover with coverslips and incubate overnight at 42°C in a humid chamber (moistened with 4x SSC)

NEXT DAY:

- Turn on waterbath at 37 C
- Make 1 x TBS

10. Wash sequentially in 4x SSC at 42° C for 10 min. **Twice.**
11. Incubate with 20µg /ml RNase A in 2x SSC (1 ml/600ml)
30 min at 37C Waterbath.
12. Wash in 2x SSC at 42° C for 10 min.
13. Wash in 0.1x SSC **10 min. at RT.**
14. Wash in TBS

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15. Incubate the sections with 3% of bovine serum albumin (BSA) /TBS **10 min. RT** (to reduce non-specific background) (9 gram / 300 ml/ 18 gram/600 ml)
16. Incubate with **1:1000** diluted <DIG-AP~conjugate (Sheep x digoxigenin-AP)
 i.e. 1 ml TBS
 1 μ l Triton X Mix and apply **40 μ l/slide** **Incubate 4 Hours at R.T.**
 1 μ l Dig-AP ab. Use the humid boxes. (moistened with TBS)
17. Wash the slides **3x of 15 min. at RT.** each in TBS.
18. Incubate with equalisation buffer (0.1M Tris-Base, 0.1 M NaCl, 50mM MgCl₂ ph 9.5) for **5 min. at RT.**
19. Colour development is performed by adding freshly prepared substrate solution:
 ~ X-phosphate-5-bromo-4-chloro-3-indolyl phosphate (**BCIP**) 0.350 mg/ml
 ~ Nitroblue tetrazolium salt (**NBT**) 0.740 mg /ml equalisation buffer.
 i.e. 120 ml equalisation buffer
 840 μ l BCIP
 888 μ l NBT
 Mix and divide over 3 cuvetts (40 ml each) And put in darkroom.
20. When the reaction is completed (usually **50 min. at RT**) wash slides in TBS followed by a wash in tap water, then counterstain with haematoxylin for **5- 20 seconds**, (depending on age) rinse in tapwater, then **5-10 sec.** in Lithium Carbonate to blue, and mount with crystal mount.
Note: To confirm the results, several controls should be performed, including:
 a) Running the ISH without ICC.
 b) ICC alone.
 c) Omitting the probe in ISH protocol or using a sense DIG-labelled riboprobe.
 d) Pre-treatment of slides with RNase A before ISH.
 e) Omitting the <DIG>-AP conjugate.

HYBRIDIZATION BUFFER - STOCK

- 50 % Formamide, deionized. 10 ml.
- 5x Denhards sol. 2 ml of a 50 x sol.
- 5x SSC 5 ml of a 20 x sol.
- Depc -H₂O 3ml
- **Dissolve and aliquot. Store at -20 C.**
- Right before use, add Salmon or Herring Sperm DNA **0.5 μ g/ μ l** final (Denatured at 94^o - 100^o C for 10 min.) **stock is 10 mg/ml**

Hybr. buffer volume x 0.5 μ g = Volume of herring sperm

PBS 10 X in Depec treated- H₂O

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1 Liter

NACL	80 Gr
KCL	2 Gr.
NA ₂ HPO ₄	6Gr.
KH ₂ PO ₄	2 Gr.

Adjust to ph 7.3 and autoclave

4 % Paraformaldehyde in 1 x PBS

<u>300 ml</u>	<u>600 ml</u>	<u>900 ml</u>	<u>1100 ml</u>
12 Gr.	24 Gr.	36 gr.	44 gr.

RNase A-stock solution: 12 mg /ml in dd H₂O.

Store at - 20 C.in 0.5 ml aliquots

Use for 300 ml : 0.5 ml	Final concentration is 20 µg /ml
450 ml : 0.75 ml	
600 ml : 1 ml	

20 x SSC solution in ddH₂O

NACL	175.3 Gram /Liter
Sodium Citrate	88.2 Gram /Liter

- Dissolve in 800 ml of H₂O. One salt at the time.
- Measure and adjust the ph to 7.0
- Adjust volume to 1 Liter.
- Autoclave for 20 min.

CHEMICALS FOR DIG-ISH

- DIG-UTP Boehringer Mannheim #1209256
- Herring Sperm DNA Promega # PR-D1811 @ 10 mg/ml
- BSA Fraction V Sigma # A-9647
- Dig-Ap conjugate Boehringer Mannheim #1093294
- BCIP " " " " #1383221
- NBR " " " " # 1383213
- MgCL₂-6H₂O Fisher # M33-500
- CaCL₂-2H₂O Fisher # C79-500
- HAE marker Gibco 100 bp ladder