IN SITU HYBRIDIZATIONS

NOTES

1. This protocol is based (with modifications) on a protocol by Judice *et al*. (2001), published in Brain Research Protocols.
2. All probes have been labeled according to package instructions with DIG Oliginucleotide 3'-End Labeling Kit, 2nd Generation (Roche Applied Science catalog # 03 353 575 910).
3. Decant all solutions with a Pasteur pipette in order to visibly see that you have not removed any specimens/parts of specimens.
4. Specimens will stick to 24-well plate when transferred there, unless you have a plate for suspension cultures. Be sure to have it loosened from the plastic to ensure even detection.
5. Make all solutions with DEPC-treated water. I make all my working solutions fresh each week.
6. Sterile filter all solutions.
7. Recipes for solutions are available at the end of the protocol. Adjust the volumes according to the number of samples you will be doing.

DAY 1:

1. Wash area with RNase Zap (Ambion catalog # 9780). Always work with a clean, neat work area.

2. Place each specimen in a 2 mL microcentifuge tube. For each tissue type, have a blank control tube, your desired probe(s), and if possible, a probe that will not be detected in your particular tissue type to diagnose non-specific binding.

3. Label the tubes with ethanol resistant marker.

4. Dehydrate through a graded ethanol series, dilute ethanol with DEPC water and sterile filter:
   - 50% ethanol x 5 minutes @ 4°C, rotate
   - 70% ethanol x 5 minutes @ 4°C, rotate
   - 100% ethanol x 15 minutes @ 4°C, rotate

5. Rehydrate through graded ethanol series:
   - 70% ethanol x 5 minutes @ 4°C, rotate
   - 50% ethanol x 5 minutes @ 4°C, rotate
   - 25% ethanol x 5 minutes @ 4°C, rotate

6. Wash in PBS_w,:
   - PBS_w x 5 minutes @ 4°C, rotate
   - PBS_w x 5 minutes @ 4°C, rotate
   - PBS_w x 5 minutes @ 4°C, rotate

7. Digestion: Stock concentration of Proteinase K (Sigma catalog # P4850) is 20 mg/mL. . Make a solution of 1 part Proteinase K: 7 parts PBS_w. This will give you a 2.5 mg/ml working solution. Add 4 µL of the Proteinase K: PBS_w solution to 2 mL of PBS_w and rotate according to desired time. Below is a guide for timing of mouse embryo samples. Always dissect out desired parts of a mouse embryo for embryos older that E10.5. These times have been altered for tissue from other species.

Embryo age	Duration of Incubation
E 6.5	4 minutes
E 7.5	4-5 minutes
E 8.5	6 minutes
E 9.5	10 minutes
E 10.5	15 minutes
Other	18 minutes

8. Stop digestion with glycine/tween-20.
   - Glycine/Tween 20 x 5 minutes @ 4°C, rotate

9. Refix specimens in 4% PFA in PBS.
   - 4% PFA x 15 minutes @ 4°C, rotate

10. Wash in PBS_w,:
    - PBS_w, x 1 minute @ 4°C, rotate
    - PBS_w, x 5 minutes @ 4°C, rotate

11. Prehybridization: Denature 150 µL h. sperm DNA (Roche Applied Science catalog # 223 646) concentration 10mg/mL, by placing in a 98°C heatblock for 10 minutes. Add the h. sperm DNA to 1850 µL prehybridization mix.
    - Prehybridize x 1 hour @ 52°C, rotate

12. Hybridization: Add 6 µL DIG-labeled probe (@ 2 pmol/µL stock concentration) to 24 µL prehybridization mix. Denature by placing in a 68°C heatblock for 10 minutes. Remove 30 µL prehybridization mix from your samples and add the 30 µL probe:prehyb mix.
    - Hybridize overnight @ 52°C, rotate

DAY 2:

1. Replace hybridization mix with 2X SSC. Dilute concentrated 20X SSC with DEPC-treated water and sterile filter.
   - 2X SSC x 1 minute @ RT, rotate
   - 2X SSC x 30 minutes @ 50°C, rotate
   - 2X SSC x 30 minutes @ 50°C, rotate
   - 0.5X SSC x 30 minutes @ 50°C, rotate

2. Replace SSC with RNase A Buffer.
    - RNase A Buffer x 5 minutes @ RT, rotate. (RT = Room Temperature)

3. Treat with RNase A (10 mg/mL stock concentration). Make a solution of 2 µL RNase A in 2 mL RNase A buffer.
    - RNase A Buffer x 60 minutes @ 37°C , rotate

4. Kill RNase A enzyme. Dilute concentrated SSC and SDS solution with DEPC-treated water and sterile filter.
    - 0.5X SSC with 0.1% SDS x 10 minutes @ RT, rotate

5. Wash.
    - 1X Wash solution x 10 minutes @ RT, rotate
    - 1X Wash solution x 30 minutes @ RT, rotate

6. Block
    - 1X Block Buffer x 60+ minutes @ 4°C, rotate

7. Antibody Treatment: Add 1 µL anti-DIG AP (Roche Applied Science catalog # 11 093 274 910), a 1:2,000 dilution, to blocking buffer.
    - Antibody solution overnight @ 4°C, rotate

DAY 3:

1. Levamisole washes. (0.1 g Tetramisole / 100 mL 1X Wash buffer)
    - 1X Levamisole Wash solution x 15 minutes @ 4°C, rotate
    - 1X Levamisole Wash solution x 30 minutes @ 4°C, rotate
    - 1X Levamisole Wash solution x 60 minutes @ 4°C, rotate
    - 1X Levamisole Wash solution x 120 minutes @ 4°C, rotate
    - 1X Levamisole Wash solution x 120 minutes @ 4°C, rotate
    - 1X Levamisole Wash solution x 120 minutes @ 4°C, rotate

2. Detection Solution Wash
    - 1X Detection Solution overnight @ 4°C, rotate

DAY 4:

1. Detection. BM Purple solution (light sensitive)(Roche Applied Science catalog # 11 422 074 001) at RT for at least 10 minutes.
    - Transfer specimens to 24-well plate. Decant off the detection buffer. Add 1 mL BM Purple solution, do not dilute. Due to light sensitivity, samples must be rotated in the dark at RT. Check periodically for cell staining.
2. Storage: Once detection is finished, stop the reaction in 1X PBS pH 5.5. Store the samples at 4°C. For long-term storage, place the samples in 4% PFA in PBS at 4°C.

SOLUTIONS FOR IN SITU HYBRIDIZATIONS

1X Block Buffer 100 mL (Roche Applied Science catalog # 11 585 762 001)

10 mL	10X Maleic Acid Buffer from Roche Kit
10 mL	10X Block Solution from Roche Kit
80 mL	DEPC Water

- Always apply Maleic Acid Buffer to DEPC water before adding Solution.
- Sterile filter.

DEPC Water (1 Liter)

1 mL	DEPC (Sigma catalog # D5758)
1 L	Sterile ultrapure water

- Autoclave.

Detection Buffer (100 mL) (Roche Applied Science catalog # 11 585 762 001)

10 mL	10X Detection Buffer from Roche Kit
90 mL	DEPC Water

- Sterile filter.

Glycine/.Tween 20 (50 mL)

- Premix a solution with 50 µL Tween 20 (Sigma catalog # P9416) and 450 µL DEPC water. In another tube mix:

100 mg	Glycine (Sigma catalog # G8898)
50 mL	DEPC water
500 µL	Tween 20/DEPC water solution

- Sterile filter.

Levamisole Wash Buffer (100 mL) (Roche Applied Science catalog # 11 585 762 001)

0.1 g	Tetramisole hydrochloride (Levamisole) (Sigma catalog # L9756)
90 mL	DEPC Water
10 mL	10X Wash buffer from Roche kit

- Sterile filter.

4% Paraformaldehyde in PBS

8 g	NaCl (Fisher catalog # PB368-212)
0.2 g	KCl (Fisher catalog # BP366-1)
1.44 g	Na_2HPO_4 (Sigma catalog # S7907)
0.23 g	KH_2PO_4 (Sigma catalog # 450200)
40 g	PFA (Fisher catalog # 04042-500)
1 mL	4 M NaOH (Fisher catalog # 8318-1)

- **ADD PFA UNDER HOOD!**
- Adjust volume with DEPC Water to 1000 mL.
- Adjust pH to 7.4 with NaOH or HCl. Filter sterilize.

1X PBS

8 g	NaCl (Fisher catalog # PB368-212)
0.2 g	KCl (Fisher catalog # BP366-1)
1.44 g	Na_2HPO_4 (Sigma catalog # S7907)
0.23 g	KH_2PO_4 (Sigma catalog # 450200)
800 mL	DEPC Water

- Adjust the pH to 7.4 with HCl.
- Adjust volume with DEPC water to 1000 mL. Autoclave.

PBS_w (100 mL)

100 µL	Tween 20 (Sigma catalog # P9416)
100 mL	1X PBS

- Sterile filter.

Prehybridization Mix (100 mL)

50 mL	Formamide (Sigma catalog # F7508)
10 mL	20X SSC
100 µL	Tween 20 (Sigma catalog # P9416)

- Adjust volume to 100 mL with DEPC water and filter sterilize.
- Formamide has a 6 month shelf life unless frozen at -20°C.

Proteinase K Solution (40 µL)

5 µL	Proteinase K (Sigma catalog # P4850)
35 µL	PBS_w

- Add 4 µL of the working 2.5 mg/mL solution to 2 mL PBS_w.

RNase A Buffer (100 mL)

2.922 g	NaCl (Fisher catalog # BP358-212)
1.2114 g	Tris (Fisher catalog # BP152-5)
100 µL	Tween 20 (Sigma catalog # P9416)

- Adjust pH to 7.5 with HCl or NaOH.
- Adjust volume to 100 mL with DEPC water and filter sterilize.

20% SDS (100 mL)

20 g	SDS (Fisher catalog # BP166-500)
70mL	DEPC water

- Adjust volume to 100 mL with DEPC water.

20X SSC (1 Liter)

175.3 g	NaCl (Fisher catalog # BP358-212)
88.2 g	Sodium Citrate (Fisher catalog #S279-500)
700 mL	DEPC water

- Adjust pH to 7.0 with HCl or NaOH. (pH adjustment not crucial if pH already between 6.8 and 7.2)

- Adjust volume to 1 L with DEPC water. Autoclave.

1X Wash Buffer (100 mL) (Roche Applied Science catalog # 11 585 762 001)

10 mL 10X Wash Buffer from Roche Kit
90 mL DEPC water

- Sterile filter.