Digoxigenin-labeled *in-situ* hybridization of urogenital sections

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This protocol has previously been described by Mendelsohn *et al* in Development 126, 1139-1148 (1999).

Embryo collection & cryosectioning

- Collect embryos in ice-cold 1X Phosphate Buffered Solutioin (PBS).
- Fix overnight in 4% Paraformaldehyde (PFA) / 0.1M PBS.
- Store in 100% methanol at -20ºC or process directly for embedding in OCT.
- Sections are cut to 14µm with a Leica CM3050 cryostat at -20ºC.
- Mount sections onto poly-lysine coated slides.
- Air-dry sections for 1-3 hours.
- Long term storage of cryosections is at -80°C. Transfer slides to -20°C the day before or early morning of the in situ.

Tissue preparation for hybridization

Prepare 250-300ml of each solution in glass dishes. Ensure RNase-free conditions and solutions at all times. Take the slides through the following washes.

- Wash in cold 4% PFA / 0.1M PBS for 10 minutes at room temperature.
- Wash three times with 1X PBS buffer for 3 minutes at room temperature.
- Treat with Proteinase K (1µg/ml) with buffer for 5 minutes at room temperature. Note: Conditions may have to be adjusted depending on the embryo stage.
- Wash in 4% PFA / 0.1M PBS for 5 minutes at room temperature.
- Wash three times in 1X PBS buffer for 3 minutes at room temperature.
- Treat with acetylate for 10 minutes at room temperature.
- Wash three times in 1X PBS buffer for 5 minutes at room temperature.

Hybridization buffer

Prepare 250-300ml of each solution in glass dishes.

- Dry the back of the slide with Kimwipes and carefully wipe around the sections to remove as much PBS as possible without allowing the sections to dry out.
- Add 1-1.5ml hybridization buffer to each slide.
- Incubate slides horizontally in a humidified chamber at room temperature for 2 hours to over-night.

Hybridization buffer with Probe

- Pour off hybridization buffer and carefully wipe the excess hybridization buffer from around the slide using Kimwipes without allowing the sections to dry out.
- Add 80μl of probe (in hybridization buffer, see below) and protect the section with a slide coverslip.
- Incubate the slides upright in a humidified chamber over-night at 68-72ºC.

Stingency washes & anti-digoxigenin immunological staining

Prepare 250-300ml of each solution in glass dishes. Stringency washes & immunological staining are carried out as follows.

- Wash slides in 5X SSC for 5 minutes at 72ºC. Coverslips should come off by themselves. Incubate longer if needed.
- Wash slides in 0.2X SSC for 1 hour at 72ºC.
- Wash slides in 0.2X SSC for 5 minutes at room temperature.
- Wash slides in 1X B1 for 5 minutes at room temperature.
- Incubate sections, 1ml/slide, in 1X B2 for 1 hour at room temperature. Keep slides horizontal in a humidified chamber.
- Incubate sections with anti-digoxigenin antibody, 1:5000 dilution, 0.5ml solution per slide, in a humidified chamber at 4ºC ovenight. Keep slides horizontal. A Pap Pen may be used to mark the sides of the slide to ensure the antibody solution does not leak while incubating overnight.
- Collect anitbody dilution after overnight incubation for use in another in situ. Reuse of anitbody solution will help reduce background in future experiments and can be re-used up to five times.

Alkaline Phosphatase activity detection

Alkaline phosphatase activity is detected by developing the slides in BCIP, NBT & 0.2mg/ml levamisole in a humidiefied chanber for 1-4 days in the dark.

- Wash slides 3X with 1X B1 for 5 minutes at room temperature.
- Incubate sections with 1X B3 for 2-3 minutes at room temperature.
- Carefully wipe the excess 1X B3 buffer from around the sections using a tissue without allowing the sections to dry out.
- Incubate sections with 70μl of 1X B4 and place a cover-slip over the sections. Maintain slides in a humidified and dark chamber from 6 hours to 3 days at room temperature.
- Can store overnight at 4°C to reduce the risk of overdeveloping.
- If B4 is to be left on for more than 48 hours, the solution should be replaced with fresh B4 to ensure the coverslips will come off.
- Wash slides in TE buffer (pH 8) for 5 minutes at room temperature to remove the cover-slip and stop the detection reaction.

Mounting of sections in Permount

Sections are dehydrated & mounted in Permount as follows.

- Rinse slides with water for 5 minutes at room temperature.
- Air dry overningt.
- Dehydrate in a 30%, 50%, 70%, 95% & 100% ethanol series for 45 seconds in each solution.
- Dip slides twice in Xylene (Fisher cat # x5-1) for 45 seconds.
- Coverslip with Permount (Fisher cat # SP15-100).

Mounting of bladder urothelium stained sections in glycergel mounting medium.

Sections staining for the bladder urothelium are dehydrated & mounted in glycergel mounting medium as follows. 100-150ml solutions are prepared in vertical glass dishes.

- Incubate slides in 4% PFA / 1X PBS for 15 minutes at room temperature. Add 1ml of solution to the section and incubate the slide in a horizontal poisition.
- Wash slides in TE (pH 8) for 5 minutes at room temperature.
- Wash slides in 1X PBS for 5 minutes at room temperature.
- Coverslip with Glycergel Mounting Medium (Dako cat # C0563)

Solutions

Note: All solutions (except for mounting steps) are made with either autoclaved water or RNASE free reagents

Tissue preparation solutions

4% Paraformaldehyde / 0.1M PBS (400ml)-make fresh
200ml H2O, heat to 70ºC.
16g paraformaldehyde
8 drops of 1N NaOH
Stir 10-15 minutes to dissolve.
Add 200ml 0.2M PBS (final concentration 0.1M)
Store on ice.

0.2M Phosphate Buffer (PBS, 4l)

165.3g Na2HPO4•7H2O (MW 268.07)
25.6g NaH2PO4•H2O (MW 137.99)
Add H2O to 4l.
Note: Make with RNASE free reagents.

2M Phosphate Buffer (500ml)

206.7g Na2HPO4•7H2O (109.5g anhydrous)
32g NaH2PO4•H2O
Make up to 500ml with H2O.
Note: Make with RNASE free reagents.

10X PBS (1l)

206.7gNa2HPO4x7H2O (109.5g anhydrous)
32g NaH2PO4
87.7g NaCl
Make up to 1l with H20.

Proteinase K with buffer (400ml)

Final concentration Prot K = 1µg/ml (Sigma cat #P6556)
5ml 0.5M EDTA (pH8.0)
20ml 1M Tris (pH7.5)
40µl (10mg/mL Prot K in 5mM EDTA, 50mM Tris)
Add H2O to 400ml

30% sucrose in 0.1M PBS

100ml 0.2M PBS
60g sucrose
Dissolve and make final volume up to 200 ml with H2O.
Filter to sterilise.

Acetylate (400 ml)

Add acetic anhydride before incubation with slides.
393ml H2O
5.3ml triethanolamine
0.7ml concentrated HCl

1ml acetic anhydride

Hybridization Buffers

Hybridization buffer (50 ml)

25ml formamide (Sigma cat #47671)
3.25ml 20X SSC
0.5ml 0.5M EDTA (pH8)
250µl 10% Tween
50µl baker's yeast RNA (50mg/ml stock, Sigma cat #R6750)
2.5ml 10% chaps
100µl Heparin (50mg/ml, Sigma cat #H3393)
17.6ml H20

Hybridization buffer with probe

4µl DIG-labelled in-situ probe (approx 150ng/ul depending on level of expression in the tissue and quality of probe)
250µl hybridization buffer
Boil for 5 minutes then immediately place on ice.
Add 80µl per slide.

Stingency washes & immunological staining

20X SSC (1l)

175.3g NaCl
88.2g $C_6H_5Na_3O_7 \cdot 2H_2O$ (sodium citrate)
Adjust pH to 7.0 with 10N NaOH.
Make up to 1l with H_2O & autoclave.

5X SSC (1l)

Make from 20X SSC stock solution.

0.2X SSC (1l)

Make from 20X SSC stock solution.

10X B1 (pH7.5)

121g Trizma base
68.7ml conc. HCL
87.7g NaCL
Make up to 1l with H_2O & autoclave.
B1 is 0.1M Tris (pH7.5) & 0.15M NaCl.

HIGS (Heat Inactivated Goat Serum)
Goat Serum (Invitrogen cat #16210-064)
Thaw bottle at 4°C overnight and incubate in a hot water bath at 56°C for 1 hour.
Aliquot out and store at -20°C.

B2 (10ml)
B1 (9ml B1)
10% HIGS (1ml HIGS)

Anti-Digoxigenin-AP, Fab fragments (5ml)
B1 (5ml B1)
0.01% Tween (5µl 10% Tween)
1% HIGS (50µl HIGS)
Anti-Digoxigenin-AP, 1:5000 dilution (1µl antibody, Roche cat #11 093 274 910)

B3 (1l)
0.1M Tris (pH 9.5) (50ml 2M Tris (pH 9.5)
0.1M NaCl (20ml 5M NaCl)
50mM MgCl2 (50ml 1M MgCl2)
H2O (830mL H2O)
Filter sterilise with a 0.45µm filter.

B4 (2.343ml)
8µl NBT (100mg/ml, NBT, Roche cat #11383213001, in 70% dimethylformamide)
8µl BCIP (50mg/ml, BCIP, Roche cat #11383221001, in 100% dimethylformamide)
4µl levamisole (500mM stock solution, Sigma cat #L9756)
23µl 10% Tween
2.3ml buffer B3

2M Tris pH 9.5 (1l)
242.2g Trizma Base
500mL H2O
Adjust pH to 9.5 with conc. HCl
H2O to 1l.
Autoclave.

TE pH 8.0 (1l)
10ml 1M Tris (pH 8.0)
2ml 0.5M EDTA
Sterile H2O to 1l.