Optimized Vibratome Section in situ Hybridization For P1 Mouse Kidney Sections

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I. Preparation of tissues for vibratome section

1. Dissect newborn kidneys in cold PBS.

2. Fix kidneys in 4% paraformaldehyde for 1hr.

3. Blot dry kidneys on paper towel briefly (Important! Otherwise kidneys are easy to pop out of the section during sectioning).

4. Embed kidneys in 15%gelatin (300 Bloom)/PBS in cryomolds and set at 4C for 1 hour.

   Note: Gelatin of 175 and 300 Bloom were tested. 300 Bloom gelatin makes blocks of higher hardness which is important for sectioning of consistent thickness.

5. Take gelatin blocks out of cryomolds, fix blocks with 4%PFA for 18 hrs at 4C.

   Note: Fixations of 6h and 18h were tested with probes of cortex and medulla, stroma and epithelium (BF2 and Wnt7b probes). 18h fixation allows more sensitive detection of signals.

6. Wash with PBS 5min, 3 times.

7. Trim blocks with a razor blade, blot dry blocks on Kimwipe paper, glue blocks to the specimen disc with superglue, tissue facing up. Keep blocks to cut on ice.

8. Filled the buffer tray with ice-cold PBS to the rim of the block, but not cover the block. Fill the cooling chamber with ice. Keeping all solutions and blocks cold help cutting sections of consistent thickness.

9. Cut with low speed (4.5) and high vibration frequency (10, maximum) at 75um.

   Note: Different thickness of 50um, 75, and 150um were tested to find the thickest thickness that allows sensitive detection of expression. 150um thickness reduces probe penetration, while 75um allow sensitive detection of both cortical and medullary signals when tested with BF2 and Wnt7b probes.

10. Collect the sections in PBS in 24-well plates.

11. Dehydrate the sections as with tissues for wholemount in situ hybridization and store in 100% Methanol.

    Note: microwave gelatin to dissolve it in PBS, be careful not to boil over. Store the stock at 4C. When making many blocks, keep the melted gelatin warm in 55C water bath.

II. Pretreatment and hybridization of vibratome sections

1. Rehydrate samples 75%, 50%, 25% Methanol in 0.85%NaCl, rinse 2x in PBT 5' at RT. Upon completion of this step, put vibratome sections in transwell filters, using 12-well plates, 5-6 sections/well. The sections will stay in the transwell filters for the remainder of pretreatment.
2. Bleach with 6% Hydrogen Peroxide in PBT 1 hr at RT (3 ml 30% + 12 ml PBT) wash with PBT 3x for 5' at RT.
3. Treat with 10 µg/ml proteinase K in PBT(Boehringer 161 519) for 25min. Timing is important!

   Note: 1. Tissues are very fragile after prot K treatment before second fixation.
   2. Proteinase K (10ug/ml) treatment of 15min, 30min and 1h were tested. 15min and 30min gave comparable level of signal, while 1h digestion reduced signal intensity. Therefore 25 min digestion is used.

4. Wash with fresh 2 mg/ml glycine in PBT. (filtered)
   Wash 2x with PBT for 5' at RT.
5. Refix with 0.2% glutaraldehyde/4% paraformaldehyde in PBT 20' at RT.
   (80µl 25% glutaraldehyde per 10 ml para/PBT)
   Wash 2x with PBT for 5' at RT (transfer into o-ring screw top vials).
6. Add 3ml Prehyb solution 1 hr at 70°C in Hybaid oven.
   (can store in Prehyb at -20C - before or after heating)
7. Thaw frozen probe at 65C, dilute probe with Prehyb solution to make Hyb. solution, heat at 80C for 3 min.
8. Hybridize sections in 48-well plates in humidifying chambers with 50%formamide, 5xSSC. Remove Prehyb solution and replace with 200ul of Hyb solution, o/n at 70°C in Hybaid oven, do not need to rotate.

Solutions

4% paraformaldehyde: dilute 1 part 16% paraformaldehyde stock (EMS, cat# 15710) into 3 parts PBS.

Prehyb and Hyb solution: 50% formamide(Fisher BP 227 100), 5x SSC, pH 4.5.(use citric acid to pH),50 ug/ml yeast tRNA(Gibco 15401-011), 1% SDS, 50 µg/ml Heparin(Sigma H8514).

25% glutaraldehyde (Sigma G5882).should be stored as aliquots at -20°C.

III. Post hybridization wash and Ab hybridization

All washes until BM purple color reaction are performed in BioLane HTI (Hulle &Huttner AG Information Technology)

1. Add 90ml/plate Soltion I to soak the 48-well plates for BioLane HTI. Transfer UGS to the plates.
2. Wash 4x with 75ml/plate solution I for 15' at 70°C (the volume for all washes in BioLane HTI is 75ml/plate)

   pre-warm solution I; 30 ml formamide
   12 ml 20 x SSC pH 4.5
   6 ml 10% SDS
   60 ml

3. Wash with 1:1 solution I: solution II for10' at 70°C.

   pre-warm mix.; solution II; 12 ml 5M NaCl
   1.2 ml 1M Tris pH 7.5

120.0 µl tween -20
120.0 ml

4. Wash 3x with solution II for 5' at RT.

5. Wash 1x with 100 µg/ml RNase A (Sigma R 6513) in solution II at 37°C for 1 hr.

6. Wash with solution II then solution III 5' at RT.
   solution III; 30 ml formamide
   6 ml 20x SSC 4.5
   1.2ml 10% SDS (optional, decreases sticking in younger embryos)
   60 ml

7. Wash 4x with solution III for 15' at 65°C. Make up MBST buffer during this incubation.
8. Wash 3x for 5', with MBST. MBST is better made as 10xMBST stock (without Tween-20). For each hyb, it can be freshly diluted in water, with .1% Tween -20 added.

   MBST is: 100mM Maleic acid(Sigma M 0375), 150mM NaCl, pH 7.5, .1% tween -20.
   + 2mM levamisole (use NaOH to bring to pH 7.5, start with 7g NaOH pellets/l)

9A. Preblock embryos with 3 ml 10% sheep serum in MBST plus 2% Boehringer Mannheim Blocking Reagent (Roche 1 096 176) Heat briefly to dissolve BMB, cool to RT, before adding sheep serum, will remain cloudy) 3.0-4.0 hr RT.
Sheep serum (Sigma S2263) should be heat inactivated, to kill endogeneous alkaline phosphatase activity by heating serum at 70°C for 30'; can then be stored at -20°C in aliquots, be sure to open cap slightly and swirl serum, occasionally, during inactivation, we have found certain lots coagulate.

9B. For each plate, weigh out 1 mg embryo powder into a 50ml Falcon tube.
add 15 ml MBST plus 2% BMB 70°C for 30' rotating in Hybaid oven, vortex for 10' RT
cool on ice, add 18.75 µl anti-dig AP (Roche 1 093 274))
shake at 4°C gently for 1 hr on Nutator.
spin at 3500rpm for 10' at 4°C
Transfer the supernatant to a new Falcon tube and add 150 µl sheep serum. This is further diluted in the BioLane HTI with 1% sheep serum in MBST plus 2%BMB to make 1:4000 dilution of anti-Dig AP.

Note: 1:2000, 1:4000, 1:5000, 1:6000, 1:7500, 1:8000 and 1:10,000 dilution of anti-Dig AP were tested. No difference of signal intensity and background was seen.
Note: Preabsorption is not necessary for anti-Dig AP.

10. Incubate with anti-Dig Ab at 4 C, overnight.

Preparation of embryo powder

1. Homogenize ~12.5-14.5 d mouse embryos in a minimum volume of ice-cold PBS.
2. Add 4 volumes of ice-cold acetone, mix and incubate on ice for 30 min.
3. Centrifuge at 10 000 g for 10', remove the supernatant, and then wash the pellet with ice-cold acetone and spin again.
4. Spread the pellet out, let dry o/n at RT, covered.
5. Grind it into a fine powder using a mortar and pestle. Store dessicated at 4C.

It is important to use embryo powder prepared from the species that you are studying, e.g. for the analysis of chick embryos, prepare chick embryo powder by this method.

IV. Post Ab hybridisation washes and detection

1. Wash 3x with MBST for 5' at RT (make up 1000 mls + 2 mM Levimasole).

2. Wash 10x with MBST for 1 hr each at RT.

3. Wash 8x with MBST for 2hr each at 4C.

4. Wash 3x with NTMT for 5' at RT (make up 60 ml + 2 mM Levimasole).

   2.0 ml 5 M NaCl
   5.0 ml 2 M Tris 9.5
   5.0 ml of 1 M $MgCl_2$
   0.1 ml tween -20
   100 ml

5. Transfer sections to 48-well tissue culture plates and then replace NTMT with 0.5ml BM Purple (Roche 1 442 074). Wrap in foil, monitor signal every few hrs, usually takes from 6hrs to 48hrs.

   Note: Shh, a weakest probe, produces signals after 48hrs of color reactions with acceptable level of background. So 48hrs was chosen as the longest time of color development.

6. When reaction is complete; wash 3x in PBT, *pH 4.5,(pH PBS with HCl), for 5 RT, keep in dark.

7. Fix in 4% paraformaldehye plus 0.1% glutaraldehyde, for 1 hr-o/n.

8. Wash in 50% then 80% glcerol/PBS. Mount slides on VWR precleaned superfrost glass slides. Remove excess glycerol with Wattman paper. Mount coverslip using Glycergel.
   (*Need to drop pH of PBT to 4.5 to stop reaction completely, also whitens up sections).

IV. Archiving and Photography

1. We photograph samples with a Nikon DXM1200 digital camera attached to an Edge scope.

2. We take 6 photos per gene: 1 global view at 100X and 5 regional (outter cortex, juxtamedullary cortex, outter medulla, inner medulla, renal pelvis), each at 4000X.

   White balance should be adjuted so that the color of the digital image matches the color of the real image. The center of the bladder generally provides a good white reference point. Non-expressing regions can also provide a white reference.

Naming protocol is “YYMMDDi[type][initial]_###”. For example, the eighth photograph of a whole mount *in situ* hybridization sample taken by John Harvard on May 15, 2001 is called “010515iWMjh_008.”

Also, as each sample is photographed, a separate Excel document is maintained to record filename, gene name, MTF, zoom, and BM Purple development time