In-situ Hybridization on Vibrating Microtome-cut Mouse Tissue Sections

Vezina Group, GUDMAP Consortium

Prior to starting

Prepare stock solutions, make DIG-labeled riboprobes, section mouse tissues with a vibrating microtome, make sample baskets and embryo powder as described below.

Make sample baskets

- **1.** Use heated razor blade to cut off bottom of microcentrifuge tube (at or just below 100mL mark).
- **2.** Cut polyester mesh into squares that are large enough to cover the cut part of the microfuge tube.
- **3.** Heat cut edge of tube in flame until softened.
- **4.** Press melted surface of microfuge tube firmly onto center of mesh square & hold for a few seconds to seal the mesh along entire circumference of tube.
- **5.** Trim away excess mesh with scissors (may also heat near flame to melt down excess).

Make embryo powder

- **1.** Collect mouse embryo tissue & store at –80°C until ready to make powder.
- **2.** Place small amounts of frozen tissue into mortar, add liquid nitrogen & use pestle to grind tissue into powder (add more liquid nitrogen as needed).
- **3.** Combine embryo powder with 4 volumes of acetone and homogenize with several strokes of dounce homogenizer until tissue is ground to a fine powder.
- **4.** Transfer homogenate to a 15mL glass screw-top vial. Mix well and extract overnight at 4°C.
- **5.** Pellet the embryo powder by centrifugation at 5000rpm for 10 min at 4°C.
- **6.** Remove and discard supernatant.
- **7.** Resupspend pellet in 4 volumes of fresh acetone and shake 2 hr at 4°C.
- **8.** Pellet the embryo powder by centrifugation at 5000rpm for 10 min at 4°C.
- **9.** Remove and discard supernatant.
- **10.** Spread pellet onto #2 Whatman filter paper and air dry in fume hood.

- **11.** Use mortar and pestle to grind dried pellet into fine powder.
- **12.** Store powder in tightly sealed glass vial at 4°C.

DAY 1 – All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

- **1.** Prep baskets
    - **A.** Close basket cap, affix sticker to cap and label with probe name.
    - **B.** Use heated 18 gauge needle to puncture two holes per basket cap (facilitates air movement).
- **2.** Prepare supplies and preheat solutions.
    - **A.** Transfer prehyb stock solution into sealed conical tube and preheat to 60.5°C.
    - **B.** Fill a small plastic storage container (used as a hybridization chamber) with about ½ inch of tap water, cover container and preheat to 60.5°C.
- **3.** Prep samples.
    - **A.** Use spring scissors to remove most of the agarose from the vibrating microtome-cut tissue sections.
    - **B.** Remove any debris if stuck to tissue sections.
    - **C.** Add 2mL PBSTw and one basket to each well of a 24-well culture plate.
    - **D.** Transfer sections into labeled baskets.
- **4.** Incubate tissues for 30 min at 25°C in 2mL/well of 6% H_2O_2.
- **5.** Wash tissues 4 x 5 min at 25°C with 2mL/well PBSTw.
- **6.** Incubate tissues 12 min at 25°C in 2mL/well proteinase K solution.
- **7.** Wash tissues 1 x 5 min at 25°C with 2mL/well PBSTw.
- **8.** Incubate tissues 20 min at 25°C in 2mL/well post-fix solution.
- **9.** Wash tissues 2 x 5 min at 25°C with 2mL/well PBSTw.
- **10.** Prehybridization step: Incubate tissues inside hybridization chamber for at least 1 hr at 60.5°C in 2mL/well prehyb buffer.
- **11.** Hybridization step: Add 0.65mg probe/well and incubate sections overnight in hybridization chamber at 60.5°C in probe+prehyb buffer.
- **12.** Prepare and preheat solutions for Day 2: Add SDS to solution 1 (to a final concentration of 1%) and preheat overnight at 60.5°C.

DAY 2 - All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

- **1.** Tissue sections are to remain in the baskets. Remove hyb buffer from each well and wash tissues 3 X 30 min at 60.5°C with 2mL/well Solution 1.
- **2.** Prepare and preheat solutions.

    - **A.** Prepare 50/50% mix of Solution1/Solution 2 and preheat to 60.5°C.
    - **B.** Add RNase to Solution 2 and preheat to 37°C.
    - **C.** Preheat one wash volume of Solution 3 to 25°C and two wash volumes to 60.5°C.
- **3.** Wash tissues 1 X 10 min at 60.5°C with 2mL/well of 50/50% mixture of Solution 1/ Solution 2.
- **4.** Wash tissues 4 X 10 min at 25°C with 2mL/well Solution 2.
- **5.** Incubate tissues 15 min at 37°C in 2mL/well RNase solution.
- **6.** Wash tissues 1 X 10 min at 25°C with 2mL/well Solution 2.
- **7.** Wash tissues 1 X 10 min at 25°C with 2mL/well Solution 3.
- **8.** Wash tissues 2 X 1 hr at 60.5°C with 2mL/well Solution 3.
- **9.** Preheat Tissue Blocking (TB), Antibody Dilution (AD), and Antibody Absorption buffers to 25°C.
- **10.** Wash tissues 3 X 10 min at 25°C with 2ml/well TBSTw.
- **11.** Blocking step
    - **A.** Incubate tissues at least 2 hr at 25°C in 2mL/well TB buffer.
    - **B.** Add 3.3mL anti-DIG antibody per 600µL AA buffer & incubate at least 2 hr at 4°C.
- **12.** Antibody step
    - **A.** Centrifuge AA buffer containing antibody at 10,000 rpm for 1 min.
    - **B.** Remove supernatant and add entire supernatant volume to 6mL AD buffer.
    - **C.** In a humidified chamber, incubate tissues overnight at 4°C in 2mL AD buffer + antibody.

Day 3 - All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

- **1.** Remove antibody solution and save.
    - **A.** Add sodium azide to a final concentration of 0.2mM to prevent microbial growth.
    - **B.** Store antibody solution at 4°C and reuse up to two additional times.
- **2.** Wash tissues 8 X 10 min at 25°C with 2mL/well TBSTw containing 2 mM levamisole.
- **3.** Remove tissues from baskets, use forceps to separate sections & remove visible debris, transfer tissues into clean microcentrifuge tubes.
- **4.** Wash tissues 1 X 10 min at 25°C with 1mL NTMT containing 2 mM levamisole.
- **5.** Detection step
    - **A.** Protect tissues from light and incubate at 25°C in 1mL/tube of a 60/40% mixture of NTMT containing 2mM levamisole/BM Purple.

    - **B.** Color development time ranges from several hours to several days. Change NTMT/BM Purple solution as needed (substrate will precipitate over time).
    - Once color is fully developed, wash tissues 2 X 5 min at 25°C with 1mL/tube NTMT containing 2mM levamisole.
- **6.** Bleaching step
    - **A.** Post-fix tissues overnight at 4°C in 1mL/tube 4% PFA.
    - **B.** Remove PFA and incubate tissues for 30 min at 25°C in 1mL/tube PBSTw containing 3% H2O2.
    - **C.** Wash tissues 1 X 10 min at 25°C in 1mL/tube PBSTw.
    - **D.** Store tissues at 4°C in 1mL/tube 4% PFA.

Solutions

Day 1
PBSTw: 1X PBS containing 0.1% Tween-20 and 0.2mM sodium azide. Pass through 0.2µm filter to remove insoluble material/contaminants.
6% H_2O_2: 1mL 30% H_2O_2 per 4mL PBS
Proteinase K: 0.25µL 20mg/mL proteinase K per 1mL PBSTw
Post-fix: 8µL 25% glutaraldehyde per 1mL 4% PFA

Prehybridization solution

stock solution	**final conc**	**for 1000mL**
100% formamide	50%	500mL
20X SSC	5X	250mL
Blocking reagent	1%	10g
10mg/mL yeast tRNA	10µg/mL	1mL
10mg/mL heparin	10µg/mL	1mL
dH_2O to vol.	--	to 1000mL

Aliquot 50mL volumes into conical tubes & store at –20°C.

Solution 1

stock solution	**final conc**	**for 500mL**
100% formamide	50%	250mL
20X SSC	5X	125mL
dH_2O	--	75mL
10% SDS	1%	50mL

Mix formamide, SSC & dH_2O as shown above & store at –20°C.
DO NOT add SDS to freezer stock solution because SDS will precipitate in the cold.
Prior to using Solution 1 for ISH, add 1mL of 10% SDS per 9mL Solution 1 stock.

Day 2

Solution 2

stock solution	**final conc**	**for 500mL**
1M Tris-HCl, pH 7.5	10mM	5mL
5M NaCl	0.5M	50mL
100% Tween-20	0.1%	0.5mL
0.2M sodium azide	0.2mM	0.5mL
dH_2O	--	444mL

Pass through 0.2µm filter to remove insolubles/contaminants, store at 25°C.
RNase: 5µL RNase (50µg/mL) per 1mL Solution 2

Solution 3

stock solution	final conc	for 500mL
20X SSC	2X	50mL
100% formamide	50%	250mL
dH_2O	--	200mL

Store at –20°C

Tissue blocking (TB) buffer

stock solution	final conc	for 500mL
10X TBS	1X	50mL
100% sheep serum	10%	50mL
10% blocking reagent	1%	50mL
BSA	1%	0.5g
dH_2O to vol.	--	to 500mL
100% Tween-20	0.1%	0.5mL

Mix TBS, serum, blocking reagent, BSA & dH_2O as shown above.
Filter through #2 Whatman filters.
Add Tween-20.
Aliquot 6mL volumes into conical tubes & store at –20°C.

Antibody dilution (AD) buffer

stock solution	final conc	for 500mL
10X TBS	1X	50mL
100% sheep serum	5%	25mL
10% blocking reagent	1%	50mL
BSA	1%	0.5g
dH_2O to vol.	--	to 500mL
100% Tween-20	0.1%	0.5mL

Mix TBS, serum, blocking reagent, BSA & dH2O as shown above.
Filter through #2 Whatman filters.
Add Tween-20.
Aliquot 6mL volumes into conical tubes & store at –20°C.
TBSTw: 1X TBS containing 0.1% Tween-20 and 0.2mM sodium azide. Pass through 0.2µm filter to remove insoluble material/contaminants.

Antibody absorption (AA) buffer

stock solution	final conc	for 20mL
1X TBSTw	--	17mL
100% sheep serum	5%	1mL
10% blocking reagent	1%	2mL
BSA	1%	0.2g
embryo powder	--	0.12g

Shake at 4°C for 30 min to rehydrate embryo powder.
Aliquot 600µL volumes & store at –20°C.
Sheep serum (must be heat-inactivated before use).
To heat inactivate:

- thaw new bottle of serum
- incubate at 70°C for 30 min
- aliquot & store at –20°C

10% Blocking reagent

stock solution	final conc	for 100mL
maleic acid	100mM	1.2g
5M NaCl	150mM	3mL
dH_2O to vol.	--	to 100mL
Blocking reagent	10%	10g

Mix maleic acid, NaCl & dH_2O according to above, pH to 7.5 (note: strong buffer so difficult to pH, try using solid NaOH pellets to raise pH initially).
Add blocking reagent, microwave briefly to aid solubility (avoid boiling over, solution will be cloudy & viscous so watch carefully to ensure blocking reagent is completely dissolved in solution).
Aliquot 10 mL volumes into conical tubes & store at –20°C.

Day 3
2M Levamisole: Dissolve 4.82g levamisole in ~7mL double-distilled H_2O (total volume should equal 10mL), aliquot 200µL volumes & store stocks at –20°C.
TBSTw + levamisole: 1X TBSTw containing 2mM levamisole.

NTMT + levamisole (inhibits endogenous alkaline phosphatases):

stock solution	**final conc**	**for 500mL**

1M Tris-HCl, pH 9.5	100mM	50 mL
5M NaCl	100mM	10mL
1M $MgCl_2$	50mM	25mL
dH_2O	--	415mL
100% Tween-20	0.1%	0.5 mL
2M Levamisole	2mM	0.5mL

Mix Tris, NaCl, $MgCl_2$ & dH_2O as shown above.
Pass through 0.2µm filter to remove insoluble material/contaminants. Store at 25°C.
DO NOT add Tween or levamisole to stock solution.
Prior to using NTMT for ISH, add 1µL of 100% Tween-20 and 1µL of 2M levamisole per 1mL NTMT stock.
3% H_2O_2: 1mL 30% H_2O_2 per 9mL PBSTw.

Abbreviations

- BSA = bovine serum albumin
- DIG = digoxigenin
- H_2O_2 = hydrogen peroxide
- PFA = paraformaldehyde
- PBS = phosphate-buffered saline
- PBSTw = 1X PBS + 0.1% Tween-20
- SDS = sodium dodecyl sulfate (aka lauryl sulfate)
- SSC = saline sodium citrate
- TBS = Tris-buffered saline
- TBSTw = 1X TBS + 0.1% Tween-20

Reagents and Supplies

- Anti-DIG antibody, Fab fragments, cat # 11214667001, Roche
- Blocking reagent, cat # 11096176001, Roche
- BM Purple AP substrate, precipitating, cat # 11442074001, Roche
- BSA, cat # BP1600-100, Fisher
- Formamide, cat # F5786-1L, Sigma

- Glutaraldehyde, 25% solution in H_2O, cat # G6257-100ML, Sigma
- Heparin, sodium salt, cat # H3393, Sigma
- Hydrogen peroxide, 30% solution in H_2O, cat # BP2633-500, Fisher
- Levamisole, cat # L9756, Sigma
- Magnesium chloride, cat # M33-500, Fisher
- Maleic acid, cat # M0375-500G, Sigma
- Paraformaldehyde, cat # 101176-014, VWR
- PBS, w/out Ca & Mg, MP Biomedicals powdered media, cat # ICN1760420, Fisher
- Proteinase K solution, 20mg/mL, biotechnology grade, cat # E195-5ML, Amresco
- RNase, cat # R6513, Sigma
- SDS, cat # S529-500, Fisher
- Sheep serum, cat # S2263-500mL, Sigma
- Sodium azide, granular, cat # S227I-100, Fisher
- Sodium chloride, cat # BP358-212, Fisher
- SSC, 20X solution, cat # S24022-4000.0, Research Products International
- Tris-HCl, cat # BP153-1, Fisher
- Tween-20, cat # BP337-100, Fisher
- Yeast tRNA, cat # 109495, Roche
- Polyester mesh, 33 micron, 12” x 24”, cat # CMY-0033-D, Small Parts Inc.