Vezina Lab RNA *in situ* hybridization on vibratome sections

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1 *Works for me* This protocol is published without a DOI.

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PROTOCOL CITATION

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Prior to starting

1 Prep stock solutions, make baskets, make probes, section samples.

Day 1

2 Prep baskets:
a. close cap of tube, affix sticker to cap and label with probe name

b. to facilitate air movement, heat large gauge needle in flame & push through plastic cap to puncture two holes in each basket

c. partially submerge baskets in PBSTw in 24-well culture dish

3 Prep samples:
a.sort through tissue section pictures if applicable, choose which sections to use & mark with name of probe to be applied

b.remove samples from 4°C, use spring scissors to remove majority of agarose from those sections that will be used in experiment

c.carefully remove any visible debris that may be stuck to section

d.transfer each section into basket that corresponds with probe to be used

4 Prepare supplies and preheat solutions
a.remove needed volume of prehybridization solution from –20°C stock & transfer into conical tube, place in hybridization oven

b.prep humidified chamber by adding enough tap water to small plastic storage container to cover bottom, cover container & place in hybridization oven

c.turn hybridization oven on at 60.5°C so everything at desired temperature when reach prehybridization step

5 Add 2 mL of 6% H2O2 per well of 24-well culture dish, place baskets in wells, incubate 30 min at room temperature with rocking.

6 Wash 4 x 5 min with PBSTw at RT with rocking.

7 Add 2 mL proteinase K soln to wells, incubate 12 min at RT with rocking.

8 Rinse 1 x 5 min with PBSTw at RT with rocking.

9 Add 2 mL post-fix to wells, incubate 20 min at RT with rocking.

10 Wash 2 x 5 min with PBSTw at RT with rocking.

11 Prehybridization step:
a. add 1 mL prewarmed prehybridization to wells

b. place culture dish in humidified chamber & cover

c. incubate at least 1 hr in 60.5°C hybridization oven with rocking

12 Hybridization step
a.for each probe, calculate vol needed to yield 0.325 mg

b.add appropriate vol of each probe to prehybridization soln in corresponding well

c.replace culture dish in humidified chamber

d.incubate overnight in 60.5°C hybridization oven with rocking

13 Prepare and preheat solution for Day 2
a. remove needed vol of Solution 1 from –20°C stock

b. add appropriate vol of 10% SDS (may want to wait until Solution 1 warms to RT)

c. heat in hybridization oven overnight so ready for Day 2 wash steps

Day 2

14 Wash 3 X 30 min with Soln 1 in 60.5°C hybridiation oven with rocking

15 Prepare and preheat solutions:
a.once samples in first Soln 1 wash, remove enough Soln 2 from RT stock to make Soln 1 / Soln 2 mix, warm in 60.5°C hyb oven

b.remove enough Soln 2 from RT stock for one wash, add RNase, warm at 37°C

c.remove needed vol of Soln 3 from –20°C stock, set aside enough for one wash at RT, start warming rest of Soln 3 in 60.5°C hyb oven

16 Wash 1 X 10 min with 50% Soln 1 / 50% Soln 2 in 60.5°C hyb oven with rocking

17 Wash 4 X 10 min with Soln 2 at RT with rocking

18 Add 2 mL RNase soln to wells, incubate 15 min at 37°C

19 Wash 1 X 10 min with Soln 2 at RT with rocking

20 Wash 1 X 10 min with Soln 3 at RT with rocking

21 Wash 2 X 1 hr with Soln 3 in 60.5°C hyb oven with rocking

22 Begin thawing aliquots of Tissue Blocking (TB) & Antibody Dilution (AD) buffers

** you do not need to thaw AD buffer for every run because antibody soln can be reused several times—check fridge for antibody soln before thawing AD buffer**

23 Wash 3 X 10 min with TBSTw at RT with rocking

24 Begin thawing aliquot of Antibody Absorption (AA) buffer

you do not need to thaw AA buffer for every run because antibody soln can be reused several times—check fridge for antibody soln before thawing AA buffer

25 Blocking step:
a.add 2 mL TB buffer to wells

b.incubate at least 2 hr at RT with rocking

c.if fresh antibody soln is needed, add 3.3 mL anti-DIG antibody per 600 mL aliquot

of AA buffer & incubate at least 2 hr at 4°C with rocking

26 Antibody step using **fresh** antibody soln [to reuse antibody soln, see step 27]
a.spin AA buffer + antibody at 10,000 rpm for 1 min to collect embryo powder

b.carefully draw off supernatant, add entire volume to AD buffer & mix

c.add 2 mL AD buffer + antibody to wells

d.place culture dish in humidified chamber

e.incubate overnight at 4°C with rocking

27 Antibody step **reusing** antibody soln
a.remove antibody soln from 4°C, add 1 mL soln to wells

b.place culture dish in humidified chamber

c.incubate overnight at 4°C with rocking

Day 3

28 Remove antibody soln
a. if soln is to be reused, collect total vol in conical tube & store at 4°C

b. following initial use only, add 1 µL of 0.2 M sodium azide soln per 1 mL antibody soln to prevent contamination

c. clearly mark tube with number of times soln used, antibody soln should be discarded after three applications

29 Wash 8 X 10 min with TBSTw + levamisole at RT

30 Remove sections from baskets, use forceps to separate sections & remove any visible debris, transfer into clean microcentrifuge tubes according to probe

31 Wash 1 X 10 min with NTMT + levamisole at RT with rocking

32 Detection step
a. prep 40% BM Purple substrate in NTMT + levamisole, add 1 mL per tube

b. wrap tubes in foil, incubate at RT with rocking while color develops (color development time ranges from several hours to several days)

c. change BM Purple + levamisole soln as needed (substrate will precipitate over time).

d. Switch over to 100% BM purple (Containing a final concentration of 2mM levamisol and 0.1% Tween-20) if staining is not apparent after about 20 hours.

e. Once color fully developed, wash 2 X with NTMT + levamisole at RT

33 Bleaching step
a. post-fix in 1 mL 4% PFA at least overnight, rinse with 1 mL PBSTw

b. make 3% H_2O_2 soln, add 1 mL per tube, rock at RT for at least 30 min

c. wash 1 X 10 min with 1 mL PBSTw at RT with rocking

d. post-fix in 4% PFA, image samples & store at 4°C

Solution Recipes

34 PBSTw:
1X PBS + 0.1% Tween 20, add 1 mL of 0.2 M sodium azide per 1 mL PBSTw

to prevent contaminating growth, sterile filter to remove insolubles/contaminants

35 0.2 M sodium azide: Dissolve 1.3 g sodium azide in 100 mL double-distilled H_20, pH to 7.6 (note: quite sensitive to pH change so need very little NaOH to adjust)

36 6% H_2O_2: 1 mL 30% H_2O_2per 4 mL PBSTw

37 Proteinase K: 0.25 mL 20 mg/mL prot K per 1 mL PBSTw

38 Post-fix: 8 mL 25% glutaraldehyde per 1 mL 4% PFA

39 **Prehybridization soln**

stock solution	final conc.	for 1000 mL
100% formamide	50%	500 mL
20X SSC	5X	250 mL
Blocking reagent	1%	100 mL of a 10% solution in Maleic acid buffer, pH 7.5
10 mg/mL yeast tRNA	10 mg/mL	1 mL
10 mg/mL heparin	10 mg/mL	1 mL
dH2O to vol.	--	to 1000 mL

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

40 Solution 1:

stock solution	final conc	for 500 mL
100% formamide	50%	250 mL
20X SSC	5X	125 mL
dH2O	--	75 mL
10% SDS	1%	50 mL

Mix formamide, SSC & dH_2O according to above & store at –20°C

DO NOT add SDS to freezer stock soln because SDS will precipitate in the cold
Prior to using Soln 1 for ISH, add 1 mL of 10% SDS per 9 mL Soln 1 stock

41 Solution 2

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

dH2O	--	444 mL

Sterile filter to remove insolubles/contaminants, store at RT

42 RNase:5 mL RNase (50 mg/mL) per 1 mL Soln 2

43 Solution 3

stock soln	final conc	for 500 mL
20X SSC	2X	50 mL
100% formamide	50%	250 mL
dH2O	--	200 mL

Store at –20°C

44 Tissue blocking (TB) buffer

stock soln	final conc	for 500 mL
10X TBS	1X	50 mL
100% sheep serum	10%	50 mL
10% blocking reagent	1%	50 mL
BSA	1%	0.5 g
dH2O to vol.	--	to 500 mL
100% Tween 20	0.1%	0.5 mL

Mix TBS, serum, blocking reagent, BSA & dH_2O according to above

Filter through #2 Whatman filters

Add Tween 20, aliquot 6 mL volumes into conical tubes & store at –20°C

45 Antibody dilution (AD) buffer

stock solution	final conc	for 500 mL
10X TBS	1X	50 mL
100% sheep serum	5%	25 mL
10% blocking reagent	1%	50 mL
BSA	1%	0.5 g
dH2O to vol.	--	to 500 mL
100% Tween 20	0.1%	0.5 mL

Mix TBS, serum, blocking reagent, BSA & dH_2O according to above

Filter through #2 Whatman filters

Add Tween 20, aliquot 6 mL volumes into conical tubes & store at –20°C

46 TBSTw:1X TBS + 0.1% Tween 20, add 1 mL of 2 M sodium azide per 1 mL TBSTw to prevent contaminating growth, sterile filter to remove insolubles/contaminants

47 Antibody absorption (AA) buffer

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

Shake at 4°C for 30 min to rehydrate embryo powder
Aliquot 600 mL volumes & store at –20°C

Sheep serum (must be heat-inactivated before use)
To heat inactivate: thaw new bottle of serum
incubate 70°C for 30 min
aliquot & store at –20°C

48 10% Blocking reagent

stock soln	final conc	for 100 mL
maleic acid	100 mM	1.2 g
5 M NaCl	150 mM	3 mL
dH2O to vol.	--	to 100 mL
Blocking reagent	10%	10 g

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, soln will be cloudy & viscous so watch carefully to ensure blocking reagent is completely in soln)
Aliquot 10 mL volumes into conical tubes & store at –20°C

49 2 M Levamisole: Dissolve 4.82 g levamisole in 7 mL double-distilled H_2O (total vol should equal 10 mL), aliquot 200 mL volumes & store stocks at –20°C

50 TBSTw + levamisole: 1 mL of 2 M levamisole per 1 mL 1X TBSTw

51 NTMT + levamisole (inhibits endogenous alkaline phosphatases):

stock soln	final conc	for 500 mL
1 M Tris-HCl, pH 9.5	100 mM	50 mL
5 M NaCl	100 mM	10 mL
1 M MgCl2	50 mM	25 mL
0.2 M sodium azide	0.2 mM	0.5 mL
dH2O	--	414.5 mL

100% Tween 20	0.1%	0.5 mL
2 M Levamisole	2 mM	0.5 mL

Mix Tris, NaCl, $MgCl_2$, sodium azide & dH_2O according to above

Sterile filter to remove insolubles/contaminants, store at RT

DO NOT add Tween or levamisole to stock soln

Prior to using NTMT for ISH, add 1 mL of100% Tween 20 and 1 mL of 2 M levamisole per 1 mL NTMT stock

52 3% H_2O_2:1 mL 30% H_2O_2per 9 mL PBSTw

53 Embryo powder:

a. Collect embryo tissue & store at –80°C until ready to make powder

b. Place small amounts of frozen tissue into mortar, add liquid nitrogen & use pestle to grind tissue into powder (add more liquid nitrogen as needed)

c. Use dounce homogenizer to grind tissue further: combine embryo powder with 4 volumes of acetone in homogenizer, homogenize with several strokes of dounce until ground to fine powder

d. Transfer acetone + powder to 15 mL glass screw-top vials, make sure powder is in 4 volumes of acetone & shake overnight at 4°C

e. Wrap vials in 2 folded paper towels, place wrapped vials in tabletop centrifuge adaptors for 50 mL conical tubes & spin at 5000 rpm for 10 min at RT

f .Remove & discard supernatant

g. Add 4 volumes fresh acetone to powder in vials & shake 2 hr at 4°C

h. Spin according to above

i. Remove & discard supernatant

j. Spread pellet onto #2 Whatman filter paper & allow to air dry in hood

k. Once dry, use mortar and pestle to grind pellet into fine powder

l. Store powder in tightly sealed glass vial at 4°C

m. The approximate yield is 50 mg powder per 1 g embryo wet weight.