Whole Mount Drosophila Embryo In Situ Hybridization with RNA probes
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DAY ONE

- All incubations are done at room temperature unless otherwise noted.
- All solutions and all containers used to make solutions and all tips should be RNASE FREE!!!!!
- For all washes, fill tubes nearly to the top with wash solution and place embryos on a nutator during the washing period.

1. Prepare fixed embryos according to attached protocol until 70% ethanol step.
2. Remove an aliquot of embryos sufficient for the number of probes you will use. Typically, use a volume of approximately 200 ul of packed embryos per probe. There is a fair amount of attrition at each step and starting with this large number of animals will ensure that a sufficient number are available for analysis at the end of the protocol.
3. Rehydrate by incubating with 50% ethanol/dH2O. Allow embryos to incubate for 5 minutes.
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5. Wash 3 X 5 minutes with PTw (1XPBS, 0.1% Tween). Place embryos on a nutator.
6. Treat embryos for 8 minutes with 50 ug/ml Proteinase K dissolved in PTw. Stock solution of Proteinase K is at –20^{o}C and is a 10 mg/ml stock. Thaw one small aliquot and discard after taking what you need. Do not refreeze or reuse this aliquot. Embryos should be nutated to ensure even digestion with Proteinase K.
7. Stop Proteinase K by washing for 2 minutes on a nutator with 2 mg/ml glycine dissolved in PTw.
8. Wash 2 X 5 minutes with PTw on a nutator.
9. Refix in 4% Paraformaldehyde/1XPBS for 20 minutes while shaking or nutating tube gently.
10. Wash 5 X 5 minutes with PTw on a nutator.
11. Equilibrate in Hybridization buffer by incubating in 1:1 PTw: Hybridization buffer, 10 minutes. Do not nutate—allow embryos to settle.
12. Remove 1:1 and replace with Hybridization buffer; incubate 10 minutes (or until embryos sink in hyb buffer.
13. Remove Hybridization buffer, replace with fresh Hybridization buffer and prehybridize for one hour at 60^{o}C in a water bath. Ensure that the tubes are firmly inserted in the floating rack so that the embryos are submerged in the water.
14. Make up sufficient probe to cover the embryos, using 1-2 ul probe per 100ul Hybridization buffer.
15. Denature probe by incubating at 80^{o}C for 5 minutes, then chill on wet ice.
16. Remove Hybridization buffer from embryos and replace with denatured probe in Hybridization buffer. Use enough probe to ensure that embryos are covered with a thin layer of probe.
17. Hybridize overnight at 60^{o}C in a water bath, ensuring that the tubes are firmly inserted in the rack and that the water level is above that of the embryos. Prewarm enough Hybridization buffer in the same water bath for use in the washes on Day Two.

DAY TWO

1. Dilute the embryos and probe with 500 ul of prewarmed Hybridization buffer. Return the embryos to the 60°C water bath and wait for them to sink. Remove the Hybridization buffer, and replace with 500 ul of prewarmed Hybridization buffer. Allow to wash for 30 minutes at 60°C.
2. Wash for 30 minutes at 60°C in 500 ul of 1:1 Hybridization buffer:1XTBST.
3. Wash 3 X 5 minutes in 1XTBST at room temperature. Fill the tubes nearly to the top with this wash buffer. Lay the tubes sideways and allow to rotate on a nutator.
4. Wash 1 X 5 minutes in 1XTAE (gel running buffer). Again, fill tubes nearly to the top and wash on a nutator.
5. Cast a 1% agarose gel in 1XTAE (no ethidium bromide), using 2 ml microcentrifuge tubes as a "comb." When gel has set, situate in a gel box and add 1XTAE, using enough gel buffer to just cover the gel.
6. Gently pipet embryos into wells, keeping track of which genotype is in which well, and make sure that embryos do not float out of wells. Use either a wide bore pipet tip or a transfer pipet with a trimmed end to move the embryos out of the tube into the well.
7. "Electrowash" the embryos by electrophoresing for 30 minutes at 100V.
8. Gently remove embryos from well and return to tubes filled with 1XTBST.
9. Wash 1 X 5 minutes with 1XTBST on a nutator.
10. Block embryos by nutating 1 hour in Block (1XTBST, 5% HINGS, 1 mg/ml BSA).
11. While embryos are blocking, dilute anti-DIG-AP antibody 1:2500 in the above Block solution. Preabsorb this antibody by incubating approximately 100 ul of packed, fixed embryos in the diluted antibody on a nutator.
12. At the conclusion of the blocking period, allow the embryos to settle. Also allow the embryos in the antibody preabsorption step to settle. Transfer the preabsorbed antibody to a clean tube, being careful not to transfer any embryos while doing so. Remove the block from the experimental embryos, leaving only a small volume to just cover the embryos and prevent them from drying out. Add the preabsorbed antibody to the experimental embryos.
13. Incubate overnight in the refrigerator.

DAY THREE

1. Remove the antibody solution and rinse three times with 1XTBST, allowing the embryos to settle on your bench after each wash.
2. Wash at least 3 X 30 minutes with 1XTBST. Place the embryos on a nutator during the washes. Extended washing periods will decrease background, and the washes can be done during the course of a day or even overnight, with no ill effect upon the tissue.
3. Wash 2X10 minutes with NTMT to equilibrate embryos to alkaline pH. Nutate.
4. Make up 1.5 ml staining solution for each sample as follows: 4.5 ul NBT and 3.5 ul BCIP per 1 ml of NTMT.
5. Incubate the embryos with staining solution by placing on a nutator. Cover samples with foil to protect from light exposure.
6. Depending on the probe you use, staining will be complete in 2 to 24 hours. Check progress of the developing reaction occasionally under a dissecting microscope.
7. When the desired level of signal has developed, rinse embryos twice with 1XTBST.

8. Remove last 1XTBST wash and replace with 50% glycerol/1XPBS. Allow embryos to sink in this solution for several hours.
9. Once embryos have settled in 50% glycerol/1XPBS, replace solution with 70% glycerol/1XPBS.
10. Mount embryos on slides with bridge coverslips and view by DIC optics.

SOLUTIONS

PTw	Stock solutions	For 50 ml	For 500 ml
1X PBS	10X	5 ml	50 ml
0.1% Tween 20	10%	0.5 ml	5 ml
ultrapure water		to 50 ml	to 500 ml

Proteinase K	Stock solutions	For 5 ml	For 10 ml
50 ug/ml Proteinase K	10 mg/ml	25 ul	50 ul
PTw		to 5 ml	to 10 ml

Glycine/PTw		For 10 ml	For 50 ml
2 mg/ml Glycine		20 mg	100 mg
PTw		to 10 ml	to 50 ml

Hybridization buffer

50% Formamide
5X SSC
100 ug/ml yeast tRNA
50 ug/ml heparin
100 ug/ml herring sperm DNA
0.1% Tween 20

10XTBST	Stock solutions	For 250 ml
1.37 M NaCl		20 g
27 mM KCl		0.5 g
25 mM Tris-HCl pH 7.5	1M	62.5 ml
11% Tween 20		27.5 g

Tare a 400 ml glass beaker on the balance. Weigh out 27.5 g of Tween 20 by pouring into glass beaker. Add ~150 ml ultrapure water and mix gently on a magnetic stir plate until detergent is dissolved. Add salts and Tris solution. Mix until all components are dissolved. Bring volume to 250 ml with ultrapure water.

1XTBST
137 mM NaCl
2.7 mM KCl
2.5 mM Tris-HCl pH7.5
1.1% Tween 20

	Stock solutions	For 50 ml	For 500 ml
	10XTBST	5 ml	50 ml
	ultrapure water	45 ml	450 ml

Block solution	Stock solutions	For 10 ml	For 50 ml
1X TBST	10X TBST	1 ml	5 ml
5% HINGS	100%	0.5 ml	2.5 ml
1 mg/ml BSA		10 mg	50 mg
ultrapure water		to 10 ml	to 50 ml

NTMT	Stock solutions	For 10 ml	For 50 ml
100 mM NaCl	5 M	0.2 ml	1 ml
50 mM MgCl2	1 M	0.5 ml	2.5 ml
100 mM Tris, pH 9.5	1 M	1 ml	5 ml
0.1% Tween 20	10%	0.1 ml	0.5 ml
1 mM Levamisole	100 mM (in water)	0.1 ml	0.5 ml
ultrapure water		to 10 ml	to 50 ml

Staining Solution	For 5 ml	For 10 ml
NBT	22.5 ul	45 ul
BCIP	17.5 ul	35 ul
NTMT	to 5 ml	to 10 ml