**Updated 7/21/22 Patricia Graham**

**Drosophila in situ with NBT/BCIP or INT/BCIP**

Use at least 10X the volume of sample for each rinse or wash (ie. For 50ul of sample use 500ul of liquid).

Note that the detergents in most of the washes help prevent the embryos from sticking to the tube.

Rinse = Add liquid. Allow sample to settle. Remove liquid.

Wash = Add liquid and either rock or let sit the indicated amount of time before removing the liquid.

**Day1 (about 2.5 hours)**

1. Rinse 2X with MeOH to remove any remaining heptane from embryos and tube.
	1. *If your embryos are sticking to the tube it sometimes helps to transfer them to a new tube.*
2. Rinse 1X with 1:1 MeOH:PBST.
3. Fix in 4% PFA for 25 minutes at RT.
	1. *For Drosophila the 4% PFA can be up to one month old, but this may not be great for other organisms. It may be best to make 1-2 ml aliquots of 12% and dilute only the amount you need that week.*
4. Wash at least 3 X 10 minutes with PBST or PBTT and make hybridization solutions.
	1. *For lower background you might find that PBTT works better. It also seems to help make the yolk less sticky/more permeable for those species where the embryos are surrounded by yolk.*
	2. *For Drosophila you only usually need to use hybridization buffer (HB) and follow the simple protocol below (2 ml HB/sample for 0.5 ml washes).*
	3. *If you have background issues, you may want to try the more complex protocol with wash 1 and wash 2 rather than the simple protocol. For that protocol, you need 2.5 ml of hybridization buffer (HB) and 1.5 ml each wash solution per sample for 0.5 ml washes.*
	4. *For Drosophila, HB can be made up to a 4 weeks ahead (ie. You can make a large batch and keep it at 4oC for about a month). If you work with another organism, you may find that making it fresh the day you plan to use it works better. You can make enough for the day you hybridize and for the washes the next day. Keeping the HB for the washes the next day on the bench ON seems to be ok for Tribolium.*
5. Remove most of the PBST and heat to 90oC for five minutes to denature proteins.
	1. *This is a replacement for a proteinase K treatment to remove proteins.*
6. Rinse 1X with 1:1 HB:PBST.
7. Incubate at least 2X 30 minutes in HB at 60oC. (Can go longer).
8. Heat probe 5 minutes at 95oC, then ice.
	1. *With Drosophila we usually use about 50ul of probe per sample. The concentration depends on the probe and must be determined by trying a range of concentrations. If you know the concentration starting with 10 and 1 ng/ul is often good. If you do not know the concentration, starting with 1/10, 1/50 and 1/100 seems to work well.*
9. Incubate embryos overnight with probe at 60oC.

**Day 2 (Simple protocol 4.5 hours plus staining time. More complex protocol 5.25 hours plus staining time.)**

1. Remove and save probe (Often a strong probe can be used several times).
2. Simplest protocol (for clean probes)
	1. Wash at least 1X 20 minutes at 60oC with HB.
	2. Wash at least 1X 20 minutes at 60oC with 1:1 HB:PBST
	3. Wash at least 4X 5 minutes at 60oC with PBST
3. More complex protocol to remove background
	1. Wash embryos at least 1X 30 minutes at 60oC in HB.
	2. Wash at least 3X 10 minutes at 60oC in wash 1
	3. Wash at least 3X 10 minutes at 60oC in wash 2
	4. Wash at least 3X 15 minutes at 60oC in PBST at RT.
4. Both protocols are the same from here on. Incubate 1-2 hours in appropriate antibody (eg. Anti-dig-frag AP conjugated, 1:2000 or Anti-FL-frag AP conjugated 1:2000 or Anti-biotin AP conjugated at 1:2000 in PBST) at RT.
	1. *Another option – incubate overnight at 4oC. May change the signal to noise ratio.*
5. Wash at least 4 X 10 minutes in PBST
6. Wash at least 1 X 5 minutes in hybridization staining buffer (SB)
7. A) Stain in
	1. 4.5ul NBT+3.5ul BCIP in 1 ml SB. Check using the microscope every 10-15 minutes. NBT/BCIP gives a nice dark purple/brown stain and is usually the best stain to use.
	2. 7.5ul INT/BCIP in 1ml SB. Check under the microscope every 10-15 minutes. This gives an orange red stain*. If you do double stain, do this one second as it washes out more readily than the NBT-BCIP.*
8. Stop reaction by rinsing 3X with PBST or PBTT when the signal is dark, and there is a bit of background stain in the rest of the embryo.
	1. *If you overstain – Wash at RT several times for 5-10 minutes with High Tween-salt buffer, then rinse 3X with PBST and wash 2X10 minutes with PBST. If the signal disappears too much, you can re-stain immediately or store ON at 4oC and re-stain the next day. You may want to watch during the first wash to see how quickly the background is disappearing vs. the pattern.*
9. Wash at least 3X 5 minutes with PBST.
10. Fix the stain in place
	1. **For NBT/BCIP**
		1. Rinse 1X with 1:1 PBST:MeOH
		2. Rinse 2X with MeOH
		3. Rinse 1X with EtOH
		4. Rinse 2X with MeOH
		5. Rinse 1X with 1:1 MeOH:PBST
		6. Rinse 3X with PBST
		7. Wash 3X 5 minutes with PBST
	2. **For (B) INT/BCIP**
		1. Fix 1X 30 minutes at RT with 4%PFA in PBST
		2. Rinse 3X with PBST
		3. Wash 3X 5 minutes in PBST
11. Mount in 70% glycerol, 0.1M Tris pH 8.0.
	1. *Put two small coverslips on the ends of the slide. They will prevent the main coverslip from crushing the embryos. Remove as much PBST as possible and add about 50-100 ul of glycerol solution. Cut the end off a blue tip to suck up the embryos. Place them in the middle of the slide and cover with a large coverslip that sits on the small coverslips on the ends of the slide. To keep for more than a few days seal the sides of the large coverslip with nail polish. Store at 4oC.*
	2. *Note that if the pH is off, the NBT/BCIP turns blue overnight.*

**Hybridization Buffer (HB)**

|  | **5 ml** | **10ml** | **15ml** | **20ml** | **50ml** |
| --- | --- | --- | --- | --- | --- |
| **dH2O** | 1ml | 2ml | 3ml | 4ml | 10ml |
| **Formamide** | 2.5ml | 5ml | 7.5ml | 10ml | 25ml |
| **20X SSC** | 1.25ml | 2.5ml | 3.75ml | 5ml | 12.5ml |
| **10% Tween** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |
| **Salmon Sperm DNA** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |
| **Heparin** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |
| **tRNA (20mg/ml)** | 25ul | 50ul | 75ul | 100ul | 250ul |

**Wash 1**

|  | **5 ml** | **10ml** | **15ml** | **20ml** | **50ml** |
| --- | --- | --- | --- | --- | --- |
| **dH2O** | 1.25ml | 2.5ml | 3.75ml | 5ml | 12.5ml |
| **Formamide** | 2.5ml | 5ml | 7.5ml | 10ml | 25ml |
| **20X SSC** | 1.25ml | 2.5ml | 3.75ml | 5ml | 12.5ml |
| **10% Tween** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |

**Wash 2**

|  | **5 ml** | **10ml** | **15ml** | **20ml** | **50ml** |
| --- | --- | --- | --- | --- | --- |
| **dH2O** | 2ml | 4ml | 6ml | 8ml | 20ml |
| **Formamide** | 2.5ml | 5ml | 7.5ml | 10ml | 25ml |
| **20X SSC** | 0.5ml | 1ml | 1.5ml | 2ml | 5ml |
| **10% Tween** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |

**High Tween-Salt PBST PBTT**

5%Tween 20 Phosphate buffered saline Phosphate buffered saline

650mM NaCl 0.05% Tween 20 0.05% Tween 20

200mM KCl 0.1% Triton X-100

125mM Tris pH 7.9

**4% PFA Hyb. Staining Buffer (SB) Hyb. Buffer (HB)**

4% paraformaldehyde in PBST 100mM NaCl 50% formamide

 50mM MgCl2 5X SSC

 100mM Tris pH 9.5 100ug/ml salmon sperm DNA

 0.1% Tween-20 50ug/ul heparin

 0.1% Tween-20

**Wash 1 Wash 2**

50% formamide 50% formamide

5X SSC 2X SSC

0.1% Tween-20 0.1% Tween-20

Just FYI, mostly from https://insitutech.wordpress.com/2016/04/20/hyb-buffer-what-does-it-do/

Formamide inactivates RNAses

SSC buffers the solution – higher pH = more stringent binding conditions. Also stabilizes the formation of the hybrid by decreasing the electrostatic repulsion between the strands.

Heparin – reduces background staining

tRNA and salmon sperm DNA– block non-specific hybridization

Tween-20 – prevents embryos from sticking to the tube

(dextran sulfate – found in some hyb buffers) – displaces water thereby effectively increasing the concentration of the probe)