Updated 7/25/22 by Patricia Graham

**Tribolium in situ**

**Day1 (4 hours)**

1. Rinse 2X with MeOH.
2. Incubate in 1:1 MeOH:Xylenes for 1 hour at room temperature (RT). Vortex for 35 seconds every 10 minutes (0 min, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min). This removes the vitelline membrane from early embryos and some of the yolk from older embryos.
	1. Make Hyb buffer and Wash buffers. You need 2 ml of hyb buffer and 1.5 ml of each wash per sample for 0.5 ml washes. *Making the buffers fresh each time seems to be important for reliable staining*.
3. Rinse 2X with MeOH.
4. Rinse 1X with 1:1 MeOH:PBST.
5. Rinse 3X with PBST
6. Wash 3X 10 minutes in PBTT at RT.
7. Rinse 1X with PBST
8. Fix in 4% PFA for 15 minutes at RT.
9. Wash 3X 10 minutes with PBTT.
10. Rinse 1X with PBST
11. Remove PBST and heat to 90oC for five minutes.
12. Rinse 1X with 1:1 hybridization buffer (HB):PBST.
13. Incubate 2x 30 minutes in HB at 60oC. (Can go longer).
14. Heat probe 5 minutes at 95oC, then ice.
15. Incubate embryos overnight with a probe at 60oC.

**Day 2 (About 5.5 hours plus staining time)**

1. Remove and save probe.
2. Wash embryos 1X 30 minutes at 60oC in HB. Invert every 10 minutes.
3. Wash 3X 10 minutes in wash 1 at 60oC
4. Wash 3X 10 minutes in wash 2 at 60oC
5. Wash 3X 15 minutes in PBTT at RT.
6. Rinse 1X with PBST
7. Incubate 1-2 hours in appropriate antibody (eg. Anti-dig-frag AP conjugated, 1:2000) at RT.
8. Wash 3-5 X 10 minutes in PBTT
9. Rinse 1X with PBST
10. Wash 1X 10 minutes in SB (make fresh SB to avoid precipitation of salts).
11. A) Stain in 4.5 ul NBT + 3.5 ul BCIP in 1 ml SB. Check under a microscope every 10-15 minutes.

B) Stain in 7.5 ul INT/BCIP solution in 1 ml SB. Check every 10-15 minutes as above.

1. Stop reaction with PBST or PBTT.
	1. Optional – Wash up to 3X 10 minutes at RT with High Tween-salt buffer, then rinse 3X with PBST and wash 2X10 minutes with PBST. 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 stain is coming out of the background vs. the pattern.

**For (A) NBT/BCIP follow 13-20**

1. Rinse 1X and wash 2X 10 minutes with PBST.
2. Rinse 1X with 1:1 PBST:MeOH
3. Rinse 2X with MeOH
4. Rinse 1X with EtOH
5. Rinse 2X with MeOH
6. Rinse 1X with 1:1 MeOH:PBST
7. Rinse 3X with PBST
8. Mount and view.

**For (B) INT/BCIP follow 21-23**

1. Fix 1X 30 minutes at RT with 4%PFA in PBST
2. Rinse 3X with PBST
3. Mount

**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**

4% paraformaldehyde in PBST

**SB, staining buffer pH 9.5 for 30 mL**

24.6 mL H2O

600 uL 5M NaCl

1.5 mL 1M MgCl2

300 uL 10% Tween 20

3 mL 1M Tris HCl pH 9.5