Updated 7/25/22 by Patricia Graham

***Tribolium* colorimetric double 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 30 seconds every 10 minutes. 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.
	1. *Note that I have found that it works better to use whichever will be the “second” probe (developed second) at a higher concentration than I would if I were using it alone. The one time I explicitly tried it, double the “normal” concentration worked well.*
15. Incubate embryos overnight with 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 60oC.
6. Rinse 1X with PBST
7. Incubate 1-2 hours in appropriate 1st 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 5 minutes in SB.
11. Stain in 4.5ul NBT+3.5ulBCIP in 1 ml SB. Check under a microscope every 10-15 minutes.
12. Stop reaction with PBST or PBTT.
13. Rinse 1X and wash 2X 10 minutes with PBST.
14. Rinse 1X with 1:1 PBST:MeOH
15. Rinse 2X with MeOH
16. Rinse 1X with EtOH
17. Rinse 2X with MeOH
18. Rinse 1X with 1:1 MeOH:PBST
19. Wash 3X 10 minutes with PBTT
20. Rinse 1X with PBST (Can stop here or after step 23)
21. Inactivate AP antibody with inactivation buffer (IB) for 15 minutes at 60oC.
22. Wash 3x 10 minutes with PBTT at RT
23. Store ON 4oC in PBST

**Day 3 (4.25 hours plus staining time)**

1. Rinse 1X in PBST at RT
2. Incubate 2 hours RT in second Ab (anti-FL Fab AP conjugated, at **1/200** in PBST)
3. Wash 4X 10 minutes at RT with PBTT
4. Rinse 1X with HSB
5. Wash 1X 5 minutes at RT with HSB
6. Stain with 7.5ul INT/BCIP in HSB
7. Rinse 3X with PBST
8. Wash 3X 5 minutes with PBST
9. Fix with 4% PFA for 30 minutes at RT
10. Wash 3X 10 minutes with PBST
11. Mount (70% glycerol in 0.1M Tris pH8.0)

**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 Inactivation Buffer**

4% paraformaldehyde in PBST 480ul Formamide

 240ul water

 240ul 10X SSC

 30ul 10% SDS

 10ul 10% Tween 20