Updated 7-26-22 by Patricia Graham

***Drosophila* TSA Double Stain as successful with biotin and dig probes (Modified from Jie Xiang’s protocol by Tricia 10/17)**

**Day 1 (2 hours)**

1. Rinse 2X with MeOH
2. Wash 2X 5 minutes RT with MeOH
3. Wash 1X 5 minutes RT with 1:1 MeOH:PBST
	1. Start the heat block and set to 95oC.
	2. Make hybridization buffer (2.5 ml/sample for 500ul washes)
4. Fix 25 minutes RT with 4% PFA in PBST
5. Heat to 95oC 5 minutes
6. Wash 1X 10 minutes 60oC with 1:1 HB:PBST
7. Wash 2X 30 minutes 60oC with HB
	1. Heat probes to 95oC 5 minutes and ice
8. Incubate ON 60oC with probes
	1. *Probe concentrations must be determined empirically. I have had different probes work at concentrations from 0.1 ng/ul to 25 ng/ul. Jie said that all of her probes worked at the same concentration in TSA as in colorometric, but this was not true for mine, and there seemed to be no consistent pattern as to whether you would need more or less concentrated probe for TSA.*

**Day 2 (1.5 hours)**

1. Wash 2X 30 minutes 60oC with HB
2. Wash 1X 10 minutes 60oC with 1:1 HB:PBST
3. Wash 4X 5 minutes 60oC with PBST
4. Incubate ON 4oC with one of the primary antibodies (Note that the antibody to biotin is conjugated to HRP and so acts as both a primary and a secondary for our purposes. The antibody to dig is not conjugated to HRP so you need to apply a secondary antibody before you can stain that probe.
	1. *In general, it worked best to stain my weaker probe first and use TSA 488 for that one.*
	2. *I used pre-absorbed G anti-biotin HRP conjugated at 1/1000 and pre-absorbed Mouse anti-dig at 1/250 in PBTH (250ul/sample for either).*
	3. *Biotin is probably not your best choice for a label as there is a lot of endogenous biotin around that gives a high background. You can mitigate this somewhat by staining for a shorter time (see below), but it’s probably better to use dig, fluorescein or DNP.*

**Day 3 (6 hours)**

1. Wash 4X 15 minutes RT with PBST
2. If needed, incubate for two hours at room temperature with the secondary antibody, then wash again 4X 15 minutes RT with PBST.
3. Stain 1X 15 minutes with tyramide 488 (used 100ul)
	1. **Tyr** in amplification buffer working solution (add 1 ul of 30% H2O2 from the kit into 200 ul of amplification buffer from the kit to get 0.15% H2O2, then add 1:100 this intermediate dilution to amplification buffer to make 0.0015% H2O2), rock at RT for 15 min. (Wrap the tube with foil to block light).

*Note: the amplification buffer working solution has to be fresh before each use* *and H2O2 has a limited shelf life (probably something around 6 months). If your reactions stop working, try getting new H2O2).*

*Also note that ThermoFisher said that a solution of 50mM Tris pH 7.4 is a good amplification buffer.*

* 1. *The time you stain with the TSA reagent can be varied. Jie tried a lot of different times and found that 15 minutes usually worked well for Drosophila embryos. I found that for my biotin probes staining for 5-6 minutes worked better (gave a better signal to noise ratio).*
1. Rinse 1X with PBST
2. Wash 2X 5 minutes with PBST
	1. Stop here and mount if you are doing a single stain.
3. Inactivate HRP with 1% H2O2 in PBST (4ul of 30% H2O2 with 116 ul of PBST) 15 minutes RT
	1. *Again this solution must be made fresh just before use.*
4. Rinse 3X with PBST
5. Wash 3X 10 minutes with PBST
6. Leave ON 4oC in the second primary Ab.

**Day 4 (1 hour)**

1. Wash 4X 15 minutes RT with PBST
2. If needed, incubate for two hours at room temperature with the secondary antibody, then wash again 4X 15 minutes RT with PBST.
3. Stain 1X 15 minutes with tyramide 555 (see above).
4. Rinse 1X with PBST
5. Wash 2X 5 minutes RT with PBST
6. Mount in VectaShield.

**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 |