**Insitu Protocol** *(modified 8/25/16 by Robert G)*

Reagents:

*AP Buffer (50 mL):*

5mL of 1M Tris HCl pH 9.5

2.5 mL of 1M MgCl2

1.25 mL of 4M NaCl

250 **uL** of 20% Tween 20

*1X PBS (1L):*

100 mL 10X PBS (Duelbecco’s Phosphate Buffered Saline)

 900 mL MilliQ H2O

*2x SSC (1L):*

100 mL 20X SSC

 900 mL MilliQ H2O

*0.2X SSC (1L):*

10 mL 20X SSC

 990 mL MilliQ H2O

*1 M Citric Acid (250 mL):*

64.52 g Citric Acid

 250 mL MilliQ H2O

*75% Methanol in PBS (500 mL):*

375 mL Methanol

 125 mL 1X PBS

*50% Methanol in PBS (500 ml):* 250 mL Methanol

 250 mL 1X PBS

*25% Methanol in PBS (500 ml):* 125 mL Methanol

 375 mL 1X PBS

*Tris HCl 9.5 1M (1L):*

121.1 g Tris HCl

 900 mL H20

**Titrate with HCl until pH~ 9.5**

*0.1% Tween 20 in 1X PBS (1X PBT) (1 L)):*

1 mL 100% Tween 20

 999 mL 1X PBS

*Hyb Buffer (50% Formamide)*

100 mL Formamide

 50 mL 20X SSC

 1 mL 20% Tween 20

 10 mg Heparin Sodium Salt (50 ug/mL)

 100 mg tRNA

*Alpha Blocking Buffer*

50 mL 1X PBT

 1 mL 100% Sheep Serum

 100 mg Bovine Serum Albumin

*4% PFA (5 mL):*

541 uL 37% Formaldehyde

 4460 uL 1X PBS

*Bleaching Solution (5 mL):*

4.425 mL RNase Free H2O

 250 uL Formamide

 125 uL 20X SSC

 200 uL 30% H2O2

**Take care in handling H2O2, it is very caustic do not add directly to formamide, add everything to H20.**

*Proteinase K (10 g/mL)*

1 uL Proteinase K 10 mg/mL

 999 uL 1X PBS

In situ Hybridization Protocol:

Day 1:

Fixation of embryos/ larval fish

* Collect fish in Eppendorf tubes, if > 5dpf, add Tricaine and let sit for ~ 15 minutes (otherwise, the fish may become contorted), be sure to lay the tube flat so that the fish do not become contorted in the bottom of the vial
* Fix tissue in 4% PFA over night @4degrees

Dehydrate to 100% Methanol (MeOH)

* Replace PFA solution with 25% Methanol ~5 min
* Replace with 50% Methanol ~5 min
* Replace with 75 % Methanol ~5 min
* Replace with 100% Methanol ~ 5 min
* Replace with fresh 100% Methanol ~5 min

**Leave at -20 Degree Freezer O/N**

Day 2:

Rehydrate fish

* Replace with 75% MeOH ~ 5 min 
* Replace with 50% MeOH ~ 5 min 
* Replace with 25% MeOH ~ 5min 
* Replace with 1X PBT 4 washes ~ 5 min each 

Proteinase K treatment

* Replace PBT with 10 g/mL Proteinase K
	+ <5 dpf, no proteinase K
	+ 5-7 dpf, 15 minutes proteinase K
	+ 7 dpf or more, 30 minutes proteinase K
	+ Stop proteinase K with 2 quick PBT washes then 2 EDTA washes [0.5M] 5mins each then fix with 4% PFA ~ 20 - 60 min
	+ Wash with PBT 4 washes ~ 5 mins each 

Prehybridization treatment and probe addition

* + Replace with Hyb Buffer (50% Formamide) at 70 degrees for ~2-5 hours
	+ Replace with antisense DIG labeled RNA Probe (150 ng) O/N at 70 degrees

**Make sure the water bath has sufficient water to make it through the night with evaporation**

Day 3:

* *Collect antisense DIG labeled RNA probe for later use, be sure to label the collection for number of uses.*

Stringency washes for removing excess probe (all solutions and washes must be at 70 degrees)

* Add Hyb Buffer (50% Formamide) 10 min at 70 degrees 
* Replace with 75% Hyb Buffer 10 min at 70 degrees 
* Replace with 50% Hyb Buffer 10 min at 70 degrees 
* Replace with 25% Hyb Buffer 10 min at 70 degrees 
* Replace with 2X SSC 10 min at 70 degrees 
* 2 washes with 0.2X SSC 30 min at 70 degrees 

Rehydration in PBT (perform all washes at room temperature)

* Replace with 75% 0.2X SSC ~ 10 min at RT
* Replace with 50% 0.2X SSC ~ 10 min at RT
* Replace with 25% 0.2X SSC ~ 10 min at RT
* 4 washes with PBT ~ 10 min at RT 

Antibody block/incubation

* Replace PBT with alpha antibody blocking buffer for ~ 2-3 hours at RT
* Replace with 1:10,000 anti-DIG antibody (in alpha antibody blocking buffer) O/N at 4 degrees, gentle rocking.

**Make sure to be gentle when changing solutions, do not use a micropipette, use a transfer pipette (thin tip), and squeeze solution onto the wall of the tube.**

Day 4:

* Discard anti-DIG antibody
* Replace with PBT ~ 10 min
* Transfer fish from Eppendorf tube to a glass vial or plastic wells **gently** with a glass pipette
* Replace with fresh PBT ~ 10 min
* Replace with fresh PBT ~ 10 min
* Replace with fresh PBT ~ 10 min
* Replace with fresh PBT ~ 10 min
* Replace with fresh PBT ~10 min
* Replace with AP Buffer ~ 10 min
* Replace with fresh AP Buffer ~ 10 min
* Replace with fresh AP Buffer ~ 10 min
* Discard AP Buffer, add ~ 700 uL BM Purple (shake BM Purple vigorously prior to adding to fish), allow **gentle** agitation
* Cover the fish in saran wrap and aluminum foil, ensure no entry of light
* Check fish staining (a purple stain within the fish at various places) about every 30 minutes

**Before you leave, ensure the fish are well covered and place in fridge at 4 degrees O/N, in the morning, replace BM Purple solution**

Day 5:

* Once stained, stop the staining with stop solution(?) and remove most of the BM Purple
* Replace with 25% Glycerol (cut with 1X PBS), invert vial that contains fish 2 times **gently**
* After ~ 30 min of sitting, replace with 50% Glycerol
* For imaging, use 80% glycerol
* Save in 100% glycerol indefinitely at 4 degrees

**The glycerol with obliterate the fish if placed on a rocker, do not allow ANY agitation of the fish. Place on desk.**