Xenopus Whole Mount *In-Situ* Hybridization Protocol

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Day 1:

ON ICE — OFF ICE!! — ON ICE — 70°C

Fill a bucket with ice, place on bench with embryo rack (1 rack= 30 vials Fisherbrand cat# 03-339-25B, 15x45mm) sitting on top of ice.

Remove caps from vials, place in order on bench.

Turn on hot water bath.

Pre-treatment and Hybridization

Begin washing with either **Methanol** or **Ethanol** in **PBSw** (according to investigator's preference)*:

75% EtOH wait 5 mins
50% EtOH wait 5 mins
25% EtOH wait 5 mins

Fast wash with **1x PBSw**:

Wash #1 wait 5 mins
Wash #2 wait 5 mins
Wash #3 wait 5 mins

Remove embryos from ice before adding **Proteinase K**.

A. Prep **4% PFA** (retrieve from 4°C frig) simultaneously:
-USE GLOVES!-
-2 grams PFA per 50 mL 1xPBSw (50 mL per rack)
-Shake, heat in water bath to dissolve

B. Prep **4% PFA / 0.2% glutaraldehyde**
-100 µL glutaraldehyde per 50 mL 4% PFA (50 mL per rack)
-Filter thru 0.45 µM paper, use 60 mL syringe

C. Prep **Proteinase K** (Invitrogen cat# 25530-049):
-Want 20 µg per 50 mL 1x PBSw (50 mL per rack)

$C_1V_1 = C_2V_2$

[PK stock sol'n] V_1 = [desired] V_2

[0.020 µg/mL PK] x V_1 = [8.5 µg/mL PK] x 50 mL PBSw

V_1 = 21.25 µL per rack

D. Prep **Glycine**
-Want 100 mg glycine per 50 mL PBSw (50 mL per rack), or
0.1 grams glycine per 50 mL PBSw (per rack)

Add **Proteinase K**, swirl, wait 8 mins

Replace embryos to ice, fast wash with **glycine**, swirl.

Fast wash with **1x PBSw**:

Wash #1 wait 5 mins
Wash #2 wait 5 mins
Wash #3 wait 5 mins

Add **4% PFA / 0.2% glutaraldehyde** to refix embryos, wait 15 mins.

Fast wash with **1x PBSw**:

Wash #1 wait 5 mins
Wash #2 wait 5 mins

Wash with **50% PBSw/50 hyb sol'n**
Wait 3 mins.

E. **50% hybridization sol'n : 50% PBSw**
-hyb solution stored at -20°C
-Want total volume 50 mL per rack
-25 mL hybridization sol'n + 25 mL PBSw

Replace with 900 µL per vial of **100% hyb sol'n**.

Replace caps.

Set in water bath at 65-70°C for 3 hours. Check periodically that temp remains 65-70°C. Add probes after hybridizing for a minimum of 3 hours.

Day 2:

70 °C — RT — 70 °C — RT — 4°C

Remove both **goat serum** and **10x blocking solution** from -20°C frig, set on bench to thaw.

You will need approximately 100mL per rack of 10% goat serum + 10% 10x blocking solution + 80% PBSw.

Retrieve embryos from 70°C, unscrew caps.

Post-hybridization washes

Remove probe/hyb mix.

Use digital pipetter to add 800 µL **hyb sol'n** to each vial, replace to 70°C bath.
Wait 5 mins.

Use digital pipetter to add 400 µL **2x SSC** three times to each vial (in addition to hyb sol'n) in bath, without aspirating:

Add 400 µL **2x SSC** #1 wait 5 mins
Add 400 µL **2x SSC** #2 wait 5 mins
Add 400 µL **2x SSC** #3 wait 5 mins

Remove mix, wash embryos twice with **2x SSC / 0.1% CHAPS**, replace to 70°C bath with lid off to prevent condensation contamination.

Wash #1 wait 30 mins
Wash #2 wait 30 mins

Blocking and antibody staining

F. Prep **Ab Buffer**:
-Sol'n is 10% goat serum
10% 10x blocking sol'n
80% PBSw
-Want 100 mL per rack

Heat and filter the **Ab Buffer** through:
0.80 µM syringe filter
0.45 µM syringe filter

Incubate in 1 mL **Ab Buffer** (w/out Ab) per vial at 4°C, rocking, for 2 hours.
Swirl before placing on rocking tray.

Replace **Ab Buffer** in vials with pre-blocked **Ab Buffer + Ab**.

Incubate at 4°C, rocking, o/n.
Swirl before placing on rocking tray.

Wash embryos twice in **1x MAB** at RT:
Wash #1 wait 10 mins
Wash #2 wait 10 mins

Wash embryos twice in **1x MAB** at 70°C:
Wash #1 wait 30 mins
Wash #2 wait 30 mins

Wash embryos twice in **1x PBS** at RT:
Wash #1 wait 10 mins
Wash #2 wait 10 mins

G. Prep **Ab Buffer + Ab** (Anti-DIGoxygenin-AP is stored at 4°C; Roche cat# 11093274910):
-Dilute 10,000 : 1
-Calculate how much Ab Buffer is left after adding 1 mL to each vial
-Ex: 84 mL Ab Buffer + 8.4 µL Ab
-Add, then store with Ab Buffer + embryos at 4°C, rocking, for 2 hours.

RT

Day 3:

Post-antibody washes

Wash embryos five times in **0.1% BSA**, orbiting:
Fill caps with **0.1% BSA**
Place racks on orbital shaker, turn orbiting speed to 0, turn on, then turn speed to 4.

Wash #1	wait 1 hour
Wash #2	wait 1 hour
Wash #3	wait 1 hour
Wash #4	wait 1 hour
Wash #5	wait 1 hour

Wash embryos twice in **1xPBSw** at room temp:

Wash #1	wait 30 mins
Wash #2	wait 30 mins

Wash embryos twice in **AP 1 Buffer** for staining:

Wash #1	wait 10 mins
Wash #2	wait 10 mins

H. Prep **0.1% BSA** in **1xPBSw**
-Want 250 mL PBSw per rack
-BSA stored at 4°C
-Weigh out BSA:
0.001 = x grams BSA/250 mL 1xPBSw

Staining reaction

Remove **AP 1 Buffer**, replace with 1 mL per vial of **BM Purple Staining** (retrieve from 4°C; Roche/Sigma cat# 11 442 074 001).

Check embryos to make sure each is suspended in sol'n when laying down on aluminum foil.
Then wrap vials in aluminum foil and place on 4°C rocker o/n.
Wash 2x 10 minutes in **Stop solution**
Wash 3x over 24hours with 100% EtOH

Solutions

DEPC H_2O
Add 1 ml diethyl pirocarbonate to 1 liter nanopure water
Stir vigorously o/n and sterilize by autoclaving

10xPBS and **20xSSC** are DEPC treated (0.1% DEPC), autoclaved and filtered. All other solutions are made using **DEPC H_2O** and filtered

1xPBSw
100mL 10xPBS
900mL DEPC H_20
1ml Tween 20 (0.1% Tween 20 final)

Hybridization Solution: Prepare 1L, filter, aliquot and store at -20 °C
10g Blocking reagent (Roche/Sigma cat# 11 096 176 001)
500mL Formamide
250mL 20xSSC
Heat @ 65 C in waterbath until dissolved
120mL DEPC H_2O
100mL torula RNA (Roche/Sigma cat# 10109509001; 1g/100mL, filter .45µm)
2mL Heparin (50mg/mL in 1xSSC)
5mL 20% Tween ((20/80 Tween20/DEPC H_20)
10 ml 10% CHAPS
10mL 0.5M EDTA

Heat inactivated Goat Serum
Thaw at 37 °C or o/n at 4 °C
Incubate in a 56 °C waterbath for 1 hour
Mix during incubation
Filter using Nalgene microfilter and aliquot into 15mL Falcon tubes

10x Blocking solution: unfiltered, store at -20 °C
Roche cat# 11 096 176 001
Make 10% stock in 1x MAB

5xMAB (Maleic acid buffer)
To make 1L of 5x solution
600mL DEPC H_20
58g Maleic acid
43.65g NaCl
pH to 7.5 with NaOH
DEPC H_20 QSP 1L
Filter after diluting to 1xMAB

AP 1 buffer
0.1M NaCl
0.1M Tris-Hcl pH 9.5
50mM MgCl2

Stop solution
100mM Tris-HCl pH 7.4
1mM EDTA