Trichoplax *in-situ* hybridization protocol

DATE:_____ Hybe Temp:_____

Condition	Anti-sense probe	Sense probe	Other control

Fixation	**-**	Transfer animals from slides to gelatin coated dishes Allow animals to settle on the bottom of the dish (at least 1 hour)
	-	Fix animals by gently adding ice cold fix (4% PFA, 0.2% Glut, in high salt seawater 0.5g NaCl / 50ml) to the dish for 90 seconds.
	-	Gently remove solution, add ice cold 4% PFA (in high salt seawater), place at 4 C for 1 hour.
	-	Remove Fix, wash 3x Depc H2O. (ICE COLD)
	-	5 min 25% methanol-75% Depc H2O (ICE COLD)
	-	5 min 50% methanol-50% Depc H2O (ICE COLD)
	-	5 min 75% methanol-25% Depc H2O (ICE COLD)
	-	Store in 100% methanol (ICE COLD)
Pretreatment	**-**	Transfer animals (1 per well) to a 96 well plate(150µl / wash)
Rehydrate	-	5 min 75% methanol-25% Ptw 5 min 50% methanol-50% PTw
	-	5 min 25% methanol-75% PTw
	-	Wash 5x PTw
Proteinase	-	5 min Proteinase K (0.01 mg/ml) at RT *[5 µl per 10 ml]*
	-	2x 5 min PTw + 2 mg/ml glycine at RT *[100 mg/ml stock; 500 µl per 25 ml]*
	-	2x 5 min 0.1% triethanolamine in water *[332 µl per 25 ml]*
	-	Premix 0.1% triethanolamine with 3 µl/ml acetic anhydride and add to well for 5 min
	-	Repeat previous step but with 6 µl/ml acetic anhydride
	-	2x 5 min PTw
Refix	-	1 hour 4% paraformaldehyde in PTw at 4 C *[1 ml per 4 ml]*
	-	Wash 5x in PTw
Prehybe	**-**	2 times 10 min hybe buffer
	-	Hybe buffer 3 hrs to overnight at hybe temp ⊗ *[save this and previous used hybe buffer for washes]*
Hybe	**-**	Add probe (1 ng/µl) and hybridize overnight or the weekend ⊗
Washes	**-**	Remove probe
	-	10 min 100% hybe buffer *[used]* at hybe temp
	-	20 min 100% hybe buffer *[used]* at hybe temp
	-	20 min 75% hybe - 25% 2x SSC at hybe temp
	-	20 min 50% hybe - 50% 2x SSC at hybe temp

	-	20 min 25% hybe - 75% 2x SSC at hybe temp
	-	3x 20 min 2x SSC at hybe temp
	-	3x 10 min 0.05x SSC at hybe temp
	-	5 min 75% 0.05x SSC 25% PTw at room temp
	-	5 min 50% - 50% PTw
	-	5 min 25% - 75% PTw
	-	3x 10 min PTw
Block	-	1 hour (or longer) Blocking Buffer at RT
Antibody	-	Incubate with anti-Dig/AP (1:5000) at overnight at 4°C ⊗
Washes	-	10x 10 (to 30) minutes PTw or more 3-5x 5 min PBS only
Develop	-	2x 5 min AP Buffer WITHOUT $MgCl_2$ (USE FRESH TWEEN)
	-	2x 5 min AP Buffer (USE FRESH TWEEN) – Swirl and change quickly
	-	Stain in AP Buffer with NBT (3.3 µl/ml) and BCIP (3.3 µl/ml)
	-	To STOP, wash with PBS or PTw 3-5 times
	-	Mount in 70% glycerol in PBS (can go through 30% glycerol first)

Hybridization Buffer (Total: 40 ml)

Formamide	20 ml	50%
20x SSC pH4.5	10 ml	5x
Heparin 20 mg/ml	0.1 ml	50 µg/ml
*20% Tween-20	0.5 ml	0.5%
20% SDS	2.0 ml	1.0%
SS DNA 10mg/ml	0.2 ml	100 µg/ml
Distilled Water	7.5 ml	

PTw – PBS + 0.1% Tween-20 (5 ml 20% Tween-20 per 1 L of PBS)
PBT – PBS + 0.2% TritonX-100 + 0.1% BSA
PTr – PBS + 0.2% TritonX-100
AP Buffer – 100 mM NaCl + 50 mM $MgCl_2$ + 100 mM Tris pH9.5 + 0.5% Tween-20
AP minus Mg – 100 mM NaCl + 100 mM Tris pH 9.5 + 0.5% Tween-20

AP Stock Solution (Total: 10 ml)

Distilled Water	7.25 ml
1M NaCl	1.00 ml
1M Tris pH9.5	1.00 ml
20% Tween-20	0.25 ml
1M $MgCl_2$ (or water)	0.50 ml

MAKE UP FRESH 20% Tween-20!