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Capitella teleta in situ hybridization protocol

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*Use RNase-free equipment and solutions through hybridization step. Can use RNase-away or concentrated NaOH to wipe down work area. **All washes are 500 µl for 5 min. at RT on rocker table unless otherwise stated.**

-DAY 1-

Pretreatment

- Transfer embryos (20 – 40/well) to a 24 well dish (in 100 % MeOH) and use 500µl for each wash.
  Rehydrate through (5 min ea. wash): 60% MeOH/40% PTw
  30% MeOH/70% PTw
  4 x PTw washes
- Digest with Proteinase-K (0.01 mg/mL in PTw – dilute just prior to use) for 2-5 min (no rocker). (Use 4 µl of a 20 mg/mL stock in 8 mL)
- Stop digestion by carefully pipetting 2 x PTw + 2 mg/mL glycine washes. (store glycine stock at 4°C – 40mg/mL (20x) glycine stock). Note: until re-fixation (below), the animals can be fragile, so take care not to pipet liquid directly onto them.
- Wash 1 x in 1% triethanolamine in PTw with 1.5 µl acetic anhydride added per 500µl. Make sure the acetic anhydride is in solution and work quickly since its efficacy goes down with time. Wash 1 x in 1% triethanolamine in PTw with 3.0 µl acetic anhydride per 500 µl added.
- Wash 2 x in PTw.
- Refix in 3.7% formaldehyde in PTw for 30-60 min at RT.
- Wash 5 x in PTw.
  Heat embryos (still in PTw) at 80°C for 10 min to kill endogenous alkaline phosphatase activity.

Prehybridization

- Remove as much liquid as possible, wash in 500 µl hybe buffer (store at –20°C, heat to dissolve) - for 10 min at RT.
- Remove liquid - add 500 µl pre-heated hybe buffer. Place at hybe temp (**hybe temp=65°C**) for 4 hr (or overnight).

Hybridization

- Denature probe at 80-90°C max for 10 min. Dilute probe to a final concentration of 0.05-3.0 ng/µl (usually 1.0 ng/µl) in hybe solution (dig-labeled probe should be stored as a 50 ng/µl stock in hybe buffer at -20°C). Remove prehybe and add probe to each well. Parafilm 24 well plate. Place in tupperware container with diH_2O dampened towels with lid on but cracked at corner. Gentle rocking. Hybridize ≥48 hours (usually 72 hrs).

-DAY 2-

- Remove probe (Some probes can be reused 4-5 times. Store at –20°C.)
- Wash 1 x for 5 min and 1 x for 20 min with hybe buffer at hybe temp. (Do not forget to pre-warm hybe buffer, 2XSSC solutions and the 0.05x SSC – do not warm the 0.05x SSC + PTw solutions)

- Wash:
  - 10 min in 75% hybe + 25% 2X SSC at hybe temp (SSC pH 7)
  - 10 min in 50% hybe + 50% 2X SSC at hybe temp
  - 10 min in 25% hybe + 75% 2X SSC at hybe temp
  - 10 min in 100% 2X SSC at hybe temp

- gloves no longer required -

  - 2x 30 min in 0.05X SSC at hybe temp (pre-warm 0.05X SSC-hybe temp)
  - 5 min in 75% 0.05X SSC + 25% PTw at RT
  - 5 min in 50% 0.05X SSC + 50% PTw at RT
  - 5 min in 25% 0.05X SSC + 75% PTw at RT
  - 5 min in 100% PTw at RT

Visualization of Probe

- Wash 5 x with PBT (Store at 4°C.) (NOTE: this PBT is "special" for in situ – see the recipe on final page).
- Block in Boehringer-Mannheim Blocking buffer for 1 hour at RT – make fresh! (dilute autoclaved 10X block buffer to 1X with filter sterilized or autoclaved maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, pH 7.5).
- Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer to 1:5000) at 4°C overnight on rocker.

-DAY 3-

NBT/BCIP detection

- Wash 7-8 x for 10 min in PBT
- Rinse 1 x quickly in 1mL AP buffer and then 2 x in 500ul for 5 min each (The initial wash is to prevent precipitation. Make AP buffer for approximately 5 washes/well).
- Develop in AP substrate solution (make fresh - add 4.4 µl of 75 mg/mL NBT and then 3.3 µl of 50 mg/mL BCIP per mL of AP buffer) at RT in dark. Monitor color development, change AP substrate solution when it turns from yellow to pink. (Can also develop slower at 4°C or faster at 37°C).
- Stop color reaction by washing 5 x with PTw. Keep in PTw (at RT or 4°C) for ≥ 1 day. This can help clear non-specific background.

Alternative for removing high background: transfer to AP stop buffer without washing in PTw. Change buffer every 24 hours (3x). Embryos clear up after 4-6 days (faster at RT).

- Final fix: 20-30 min incubation in 3.7% Formaldehyde+PTw solution; after incubation wash 2-3 x in PTw.
- Store in either PTw or 70-80% glycerol in PBS in the dark at 4°C.
- Use 70-80% glycerol in 1x PBS with 0.25 µl/mL of Hoechst (this amount of Hoechst may need to be adjusted depending on animal) as mounting medium. It may be necessary to clear in glycerol for a few hours or ideally overnight before imaging.

-DAY 3- fast red detection

- When using DIG probes, the whole mount in situ protocol is the same as detailed above
- Wash 8 x for 10 min in PBT
- Rinse 1 x quickly and then 2 x 5min in 0,1M Tris HCl pH 8,25; 0.1%Tween-20
- Develop at RT in dark: Dissolve (vortexing) 1 Tris buffer tablet (gold foil wrapping) in 1 mL dH_2O. Drop 1 fast red tablet (silver foil wrapping) into that solution and dissolve by vortexing. The manufacturer recommends to sterile filter this solution since there always is precipitation. Monitor color development, entire embryo will become yellowish. Sometimes the red color is a little hard to see, but the detection time is around the same time as for NBT/BCIP detection. The fluorescence will nevertheless be bright – visible with Texas Red or rhodamine filter sets (excitation optimum: 543nm)
- Stop color reaction by washing 5 x with PTw. Keep in PTw (at RT or 4°C) for ≥ 1 day. This can help whiten non-stained tissue.

Solutions

Hybe Buffer (40 mL)	ADD	[FINAL]
Formamide	20 mL	50%
20x SSC (pH 4.5)	10 mL	5x
20 mg/mL heparin	0.1 mL	50 µg/mL
20% Tween-20	0.2 mL	0.1%
10% SDS	4.0 mL	1.0%
10 mg/mL SS DNA (*boil!)	0.2 mL	50 µg/mL
dH_2O (DEPC)	5.5 mL	

*must heat at 100°C for ~5-10 min, then can ice-shock for ~2 min before adding to hybe → store at –20°C

10x PBS = 18.6 mM NaH_2PO_4 (2.23 g NaH_2PO_4 per liter dH_2O)
84.1 mM Na_2HPO_4 (11.94 g Na_2HPO_4 per liter dH_2O)
1,750 mM NaCl (102.2 g NaCl per liter dH_2O)

Mix phosphates in about 800 mL of dH_2O for a 1.0 L volume. Check pH. It should be 7.4 ± 0.4. If more than 0.4 off, start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl and rest of dH_2O.

PTw = 1x PBS + 0.1% Tween-20 detergent - filter sterilize

PBT = 1x PBS + 0.2% Triton X-100 + 0.1% BSA - filter sterilize (store at 4°C)

20x SSC = 0.3 M Na citrate + 3 M NaCl (We have a lab supply of 20x SSC pH7, Rnase-free)
(for 1 L, add 175.3 g NaCl + 88.2 g Na citrate, pH to 7.0 with HCl, and autoclave)

Alkaline Phosphatase buffer (50mL)		ADD	[FINAL]
	dH_2O	36.25 mL	
1 M	NaCl	5.0 mL	100 mM
1 M	$MgCl_2$	2.5 mL	50 mM
1 M	Tris, pH 9.5	5.0 mL	100 mM
20%	Tween-20 (make fresh)	1.25 mL	0.5%

(Prepare AP buffer just prior to use. The solution will become cloudy after a few hours and will no longer work for the enzymatic reaction.)

AP Substrate Solution

To AP buffer, add 4.4 μl/ml NBT (stock: 75 mg/mL in 70% dimethyl formamide: 30% water) and then 3.3 μl/mL BCIP (stock: 50 mg/mL in dimethyl formamide). Keep this solution in the dark.

Maleic Acid Buffer (500 mL)

Bring 5.804g maleic acid ($C_4H_4O_4$) and 4.383g NaCl up to 500mL (100mM $C_4H_4O_4$, 150mM NaCl). Add concentrated NaOH until pH 7.5. Filter sterilize and store at 4°C.

Product Information

Product	Vendor	Catalog Number
anti-DIG/AP	Roche	11093274910/NC0391726
BCIP	US Biological	B0800
Blocking Buffer	Boehringer-Mannheim	1 096 176
NBT	Thermo	FERR0842
Proteinase-K	GibcoBRL (Invitrogen)	25530-049
Salmon Sperm DNA	Ambion/Life Tech	AM9680