In situ protocol for embryos and juveniles of Convolutriloba longifissura

CURRENT STATUS: POSTED

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DOI:

10.1038/nprot.2008.201

SUBJECT AREAS

Cell biology Developmental biology

KEYWORDS

fixation, in situ hybridization, acoel, embryos

Introduction

This protocol is based on a in situ hybridization protocol used for embryos of the sea anemone

Nematostella vectensis (Martindale et al. 2004).

Reagents

Hybe Buffer (40 mL)

See figure in Figures section.

 $10x PBS = 18.6 \text{ mM NaH}_2PO_4$ (2.56 g NaH₂PO₄-H₂O per liter dH₂O)

84.1 mM Na2HPO4 (11.94 g Na₂HPO₄-H₂O per liter dH₂O)

1,750 mM NaCl (102.2 g NaCl per liter dH_2O)

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

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

20x SSC = 0.3 M Na citrate + 3 M NaCl, pH 7

Alkaline Phosphatase buffer (50mL)

See figure in Figures section.

AP Substrate Solution

To AP buffer, add 3.3 $\mu l/ml$ NBT (stock: 50 mg/ml in 70% dimethyl formamide:30% water) and then

3.3 μ I/ml BCIP (stock: 50 mg/ml in dimethyl formamide). Keep this solution dark.

Equipment

Rocker table at RT and at 4 degrees

Procedure

Fixation

Fixative: 3.7% formaldehyde, in Sea water (make up fresh)

- 1. Starve Adults for two days. Fix hatchlings on the same day they hatched.
- 2. Relax adults and juveniles in 7.14% MgCl₂ up to 10 min
- 3. Fix animals in 3.7% formaldehyde only for 4 hours at 4°C.
- 4. Wash 5x in PTw, 1x in dH_2O , and transfer to fresh 100% MeOH
- 5. Replace MeOH 2x, store at -20°C in a screw cap tube

In situ hybridization

Use RNAse-free equipment and solutions through hybridization step. All washes are 5 min. at RT on rocker table unless otherwise stated.

DAY 1

Pretreatment

- 1. Transfer embryos to a 24 well dish and use 500µl for each wash.
- 2. Rehydrate through: 60% MeOH/40% PTw, 30% MeOH/70%, PTw 4 x PTw washes
- Digest with Proteinase-K (0.01 mg/ml in PTw make fresh) for 2-3 minutes (no shaker). (Use 4 μl of a 20 mg/ml stock in 8 ml)
- 4. Stop digestion with 2 (PTw + 2 mg/ml glycine) washes.
- 5. Wash with 1% triethanolamine in PTw
- 6. Add 1.5 μ l acetic anhydride to 500 μ l 1% triethanolamine, Vortex it an put on the probe for 5 minutes.
- 7. Take the solution, add 1.5 μl more acetic anhydride, vortex again and put it again on the probe.
- 8. Wash briefly in Ptw, then wash 2 x 5 min in PTw
- 9. Refix in 3.7% formaldehyde in PTw for 1 hour at RT.
- 10. Wash 5 x in PTw
- 11. Heat animals for 10 min at 80°C to destroy endogenous phosphatases

Prehybridization

- Remove as much liquid as possible without letting the embryos falling dry, and add 500 μl hybe
 buffer B incubate for 10 minutes at RT.
- 13. Remove liquid add 500 µl hybe buffer. Place at hybe temp overnight
- 14. Wash embryos once with prewarmed Hybe to get rid of traces of the dissolved egg cluster jelly

Hybridisation

15. Dilute probe to a final concentration of 3-0.05 ng/ μ l (usually 2.0 ng/ μ l) in hybe solution (diglabeled probe should be stored as a 50 ng/ μ l stock in hybe buffer at -20 degrees). Denature probe at 80-90°C max for 10 minutes.

16. Remove prehybe and add probe to each well. Hybridize overnight or the weekend.

DAY 2

- 17. Remove Probe (can be reused 4-5 times)
- 18. Wash 1 x for 10 minutes and 1 x for 40 minutes with hybe buffer at hybe temp. (Do not forget

to prewarm hybe buffer)

19. Wash usung the following steps:

30 min in 75% hybe + 25% 2X SSC at hybe temp 30 min in 50% hybe + 50% 2X SSC at hybe temp 30 min in 25% hybe + 75% 2X SSC at hybe temp 30 min in 100% 2X SSC at hybe temp 3 x 20 min in 0.2X SSC at hybe temp 10 min in 75% 0.2X SSC + 25% PTw at RT 10 min in 50% 0.2X SSC + 50% PTw at RT 10 min in 25% 0.2X SSC + 75% PTw at RT

20. 10 min in 100% PTw at RT

Visualization of Probe

- 21. Wash 3 x with PBT at RT
- 22. Block in Boehringer-Mannheim Blocking buffer (diluted to 1x with maleic acid buffer)

1 hr at RT on rocker.

Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer to 1:5000) at 4°C overnight on rocker.

DAY3

- 24. Wash 10x (or more) for 20-30 minutes in PBT.
- 25. Wash 3 x for 10 minutes in AP buffer (embryos tend to stick a lot).
- 26. Develop in AP substrate solution (make fresh) at RT in dark. Monitor color development. (Can also develop slower at 4 degrees)
- 27. Stop reaction by washing 5 x with PTw.

Timing

4 - 5 days

References

Martindale, M. Q., Pang, K., & Finnerty, J. R. Investigating the origins of triploblasty: 'mesodermal'

gene expression in a diploblastic animal, the sea anemone Nematostella vectensis (phylum, Cnidaria;

class, Anthozoa). Development 131, 2463-2474 (2004).

Acknowledgements

Many thanks go to Kevin Pang for optimizing the in situ protocol in Nematostella and for the help

adjusting it to acoel flatworm embryos.

Figures

	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	0.5 mL	0.1%
20% SDS	2.0 mL	1.0%
10 mg/mL SS DNA	0.2 mL	100 µg/mL
dH ₂ O	7.5 mL	
10 mg/mL tRNA	0.2 mL	100µg/ml

Figure 1

		ADD	[FINAL]
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		1.25 ml	0.5%
dH ₂ O		36.25 ml	

Figure 2

Acoel development indicates the independent evolution of the bilaterian mouth and anus

by Andreas Hejnol & Mark Q. Martindale

Nature (30 July, 2008)