

# Ciona in-situ protocol using Mobicols

## PROTOCOL FOR:

### Whole-mount in situ hybridization of small invertebrate embryos using laboratory mini-columns

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*BioTechniques 43:XXX-XXX (December 2007)*

## LEGEND

 **ATTENTION**

 **HINT**

 **REST**

## REAGENTS

RNAse-Away (Molecular BioProducts, San Diego, CA, USA)

Digoxygenin-UTP (Roche, Indianapolis, IN, USA)

2% Blocking Reagent (Roche)

Anti-digoxygenin antibody conjugated to alkaline phosphatase (Roche)

NBT/BCIP (Roche)

## PROCEDURE

 USE ALL RNAse-FREE SOLUTIONS UP TO POST-HYB WASH STEPS

### REHYDRATION

1. Begin with fixed demembrated embryos stored in ethanol
2. Wash once with ethanol
3. Wash once each with 75% ethanol
4. 50% ethanol
5. 30% ethanol
6. Wash three times with PTw

### PERMEABILIZATION: Proteinase K treatment

7. Reduce volume to 0.5 ml
8. Add 0.5 ml of 4 µg/ml Prot K

9. Incubate at 37°C for the following times:  
Eggs to early tailbud—5 min  
Mid to late tails—9 min



FOR LARVAE:

- Make 20 µg/ml ProtK
  - Add 0.5 ml to 0.5 ml of larvae in PTw
  - Incubate for 4 min at 37°C
  - Remove fluid to 0.5 ml
  - Add 1 ml of PTw
  - Incubate another 8 min at 37°C
  -
10. Wash 2× with PTw + glycine
  11. Reduce volume to 0.5 ml
  12. Post-fix by adding 0.5 ml of 8% paraformaldehyde in PTw for 30 min at RT

ACETYLATION

13. Take off as much liquid as feasible
14. Add 1% triethanolamine in PTw
15. Repeat steps 13-14
16. Remove most liquid
17. Add 1.3 µl of acetic anhydride to 0.5 ml of 1% triethanolamine/PTw in another tube
18. Vortex and immediately add 400 µl to the tube with embryos and gently mix
19. After 5 min, repeat with a fresh tube of acetic anhydride in triethanolamine



Acetic anhydride has a short half-life in aqueous solution, so work quickly to add to embryos

20. Wash 3× with PTw



Embryos can remain at 4°C overnight

Optional: Embryos may be dehydrated through the graded ethanol series and stored at -70°C

PRE-HYBRIDIZATION—HYBE TEMP 60°C

21. Transfer embryos to prepared Mobicols



Mobicols hold 0.5 ml of solution up to the bottom of the screw cap

- Make sure there is fluid over the upper filter when transferring embryos to the column
- Hybridization is done in the hybe oven
- Take care not to let the solutions cool below hybe temp
- Do transfers quickly without leaving the door to the oven open or the columns out of the oven any more than necessary to change solutions
- For incubations longer than a few minutes close the snap cap on the column(s)



CHANGING SOLUTIONS IN THE MOBICOLS:

- *Gently* push the solution through the column using the syringe attached to tubing, applying only enough pressure to cause the solution to pass through drop by drop.
- When the solution approaches the top filter, back off the plunger slightly so that embryos do not become dry.
- Always leave a couple of millimeters of solution covering the embryos.

#### Adding new solutions

- Add 400–500  $\mu$ l of liquid through the luer opening in the cap.
- Keep the pipette tip against the side of the opening and leave a slight air space next to the pipette tip so that you don't create bubbles.

(Modified Corbohyb: see below)—Add denatured HS DNA just before hybe and keep above 60°C.

22. Wash for 5 min in 1:1 Hybridization solution/PTw mix
23. Reduce vol to 1/2 level and add 250  $\mu$ l of Hybridization solution
24. Remove solution and wash for 5 min in 0.5 ml Hybe
25. Wash once more in Hybe
26. Close cap and invert quickly to mix
27. Transfer to oven at hyb temp for a 1+ h prehyb period

#### HYBRIDIZATION

28. Use 30–300 ng of probe per ml of hybe solution. (For *Dl1A* and *Dl1B* 300 ng/ml worked well. For *Pax6* try 30 ng per ml.) Since the column only holds 0.5 ml you'd be using 1/2 this much RNA.
29. Add probe to 250  $\mu$ l of hyb in a 1.5 ml tube
30. Heat at 80°–90°C for 8 min
31. Add directly to 250  $\mu$ l of hyb + embryos in the appropriate Mobicol
32. Mix gently but quickly by closing the lid and inverting once
33. Place back in hybe oven

Total amt. of probe used:  $\mu$ l \_\_\_\_\_ ng \_\_\_\_\_

#### WASH

34. Wash 2 $\times$  for 5 min. with hyb + DNA (no probe) at hybe temp
35. Wash 1 $\times$  for 30 min. with hyb + DNA (no probe) at hybe temp
36. Denature HS DNA and add to 4 ml of hybe per tube



Solutions need not be RNase-free from this point on. Don't need gloves or baked pipettes.

At hybe temp—20 min each:

37. 75% hybe/25% 2 $\times$  SSC/0.1% Tw
38. 50% hybe/50% 2 $\times$  SSC/0.1% Tw
39. 25% hybe/75% 2 $\times$  SSC/0.1% Tw
40. 100% 2 $\times$  SSC/0.1% Tw
41. 3 $\times$  in 0.1 $\times$  SSC/0.1% Tw

At RT—10 min each:

42. 75% 0.1 $\times$  SSC/0.1% Tw/25% PTw
43. 50% 0.1 $\times$  SSC/0.1% Tw/50% PTw
44. 25% 0.1 $\times$  SSC/0.1% Tw/75% PTw
45. 2 $\times$  in 100% PTw

#### DETECTION

46. Wash 2 $\times$  10 min with 2% B-M Block in PTw.
47. Wash in 2% B-M Block in PTw w/rocking for at least 1 h
48. Reduce vol to .25 ml
49. Add .25 ml of 2 $\times$  anti-Dig antibody
50. Incubate at RT for 90 min with rocking



May also be incubated overnight at 4°C without rocking.

2× anti-Dig: For each 2.5 ml of block add 1 µl of anti-Dig Fab fragments, i.e. 1:5000 final dilution after adding to column.

51. Wash out antibody 3× 5 min. with PT w/2% block

52. Wash 6× 15 min with PT w/0.1% BSA.

53. Remove embryos from Mobicols

54. Transfer to a 12-well plate or glass staining dish

55. Wash 2× 5 min with AP Detection Buffer

56. Reduce vol. to 750 µl (about 2 mm level in 12-well plate)

\* If buffer is cloudy, filter through a syringe filter.

57. Add 750 µl of 2× AP Substrate Solution and watch for color reaction. The reaction could take anywhere from a few minutes to a few days.

58. When fully developed but without excessive background staining, wash 4× 5 min with PTw.

\* If clearing is necessary, try 70% glycerol with 0.01% Tween-20.

Probe/stage	Treatment	Comments/Results

Embryos:

Stage(s)	No. reqd.	Tube(s)	Stage(s)	No. reqd.	Tube(s)

## RECIPES

### Modified Corbohyb

Component	Volume (ml)	[final]
Formamide	15	50%
20× SSC, pH 4.5	7.5	5×
15% Tween-20	0.2	0.1%
50 mg/ml heparin	30 $\mu$ l	50 $\mu$ g/ml
10 mg/ml yeast RNA	0.15	50 $\mu$ g/ml
50× Denhardt's	1.2	2×
10 mg/ml herring sperm DNA *	0.15	50 $\mu$ g/ml
Water	5.77	
Volume	30	

\* Add herring sperm DNA just before prehyb after boiling 10 min to denature. Keep hyb at 60°C+ after adding DNA. Add just enough HS DNA for the amount you need for hyb and prehyb. You will need about 6 ml of hybe with DNA per tube.

### AP Detection Buffer (25 ml) *make fresh for each use—only lasts a few hours*

Component	Volume (ml)	[final]
5 M NaCl	0.5	100 mM
1 M MgCl	0.625	25 mM
1 M Tris, pH 9.5	2.5	100 mM
15% Tween-20	0.17	0.1%
dH2O	21.2	
TOTAL	25	

### 2× AP Substrate Solution

To AP Detection Buffer add 6.6  $\mu$ l/ml NBT [stock: 100 mg/ml in DMF (Roche, Indianapolis, IN, USA)] and then 6.6  $\mu$ l/ml BCIP [stock: 50 mg/ml in DMF (Roche)].

### 4 $\mu$ g/ml Prot K

1  $\mu$ l of 20 mg/ml Prot K

5 ml of PTw

this gives a final conc of 2  $\mu$ g/ml

### 20 $\mu$ g/ml ProtK

1  $\mu$ l per 1 ml of PTw

### PTw + glycine

2 mg/ml glycine in PTw

### PTw

1x PBS, pH 7.5

BioTechniques Protocol

0.1% Tween-20

## **TROUBLESHOOTING**

### **NO SIGNAL**

Reduce fixation time

Increase probe concentration

Increase detection time

### **EQUIPMENT**

Autoblot Microhybridization Oven (Bellco Glass Inc., Vineland, NJ, USA)

Mobicol mini-columns (MoBiTec GmbH, Gottingen, Germany; U. S. distributor, Boca Scientific, Boca Raton, FL)