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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LEGEND

ATTENTION
HINT
REST

REAGENTS

RNAse-Away (Molecular BioProducts, San Diego, CA, USA)
Digoxigenin-UTP (Roche, Indianapolis, IN, USA)
2% Blocking Reagent (Roche)
Anti-digoxigenin 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 demembranated 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 μl 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 μl 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 *DlIA* and *DlIB* 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 μl of hyb in a 1.5 ml tube
30. Heat at 80°–90°C for 8 min
31. Add directly to 250 μl 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: μl _________  ng __________

WASH
34. Wash 2× for 5 min. with hyb + DNA (no probe) at hybe temp
35. Wash 1× 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× SSC/0.1% Tw
38. 50% hybe/50% 2× SSC/0.1% Tw
39. 25% hybe/75% 2× SSC/0.1% Tw
40. 100% 2× SSC/0.1% Tw
41. 3× in 0.1× SSC/0.1%Tw

At RT—10 min each:
42. 75% 0.1× SSC/0.1%Tw/25% PTw
43. 50% 0.1× SSC/0.1%Tw/50% PTw
44. 25% 0.1× SSC/0.1%Tw/75% PTw
45. 2× in 100% PTw

DETECTION
46. Wash 2× 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× 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.

<table>
<thead>
<tr>
<th>Probe/stage</th>
<th>Treatment</th>
<th>Comments/Results</th>
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<tbody>
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Embryos:

<table>
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<tr>
<th>Stage(s)</th>
<th>No. reqd.</th>
<th>Tube(s)</th>
<th>Stage(s)</th>
<th>No. reqd.</th>
<th>Tube(s)</th>
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RECIPES

Modified Corbohyb

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
<th>[final]</th>
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<tbody>
<tr>
<td>Formamide</td>
<td>15</td>
<td>50%</td>
</tr>
<tr>
<td>20× SSC, pH 4.5</td>
<td>7.5</td>
<td>5×</td>
</tr>
<tr>
<td>15% Tween-20</td>
<td>0.2</td>
<td>0.1%</td>
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<tr>
<td>50 mg/ml heparin</td>
<td>30 µl</td>
<td>50 µg/ml</td>
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<tr>
<td>10 mg/ml yeast RNA</td>
<td>0.15</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>50× Denhardt’s</td>
<td>1.2</td>
<td>2×</td>
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<tr>
<td>10 mg/ml herring sperm DNA</td>
<td>0.15</td>
<td>50 µg/ml</td>
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<tr>
<td>Water</td>
<td>5.77</td>
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<tr>
<td>Volume</td>
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* 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*

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<th>Volume (ml)</th>
<th>[final]</th>
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<tr>
<td>5 M NaCl</td>
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<tr>
<td>1 M MgCl</td>
<td>0.625</td>
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<tr>
<td>1 M Tris, pH 9.5</td>
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<td>100 mM</td>
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<tr>
<td>15% Tween-20</td>
<td>0.17</td>
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<tr>
<td>dH2O</td>
<td>21.2</td>
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<td>TOTAL</td>
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2× AP Substrate Solution
To AP Detection Buffer add 6.6 µl/ml NBT [stock:100 mg/ml in DMF (Roche, Indianapolis, IN, USA)] and then 6.6 µl/ml BCIP [stock: 50 mg/ml in DMF (Roche)].

4 µg/ml Prot K
1 µl of 20 mg/ml Prot K
5 ml of PTw
this gives a final conc of 2 µg/ml

20 µg/ml ProtK
1 µl 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)