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# *In situ* hybridization on whole mount embryos of *C. elegans*\*

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## 1. Preparation of DIG-labeled DNA probes for *in situ* hybridization

### 1.1. DIG-labeling by linear PCR

1. Make the following reaction mix (total 10  $\mu$ l):

distilled water	4.4 $\mu$ l
anchored oligo dT primers*	3.0 $\mu$ l
10 $\times$ Taq buffer	1 $\mu$ l
Taq polymerase	0.1 $\mu$ l
10 $\times$ DIG-dUTP/dNTP mix**	1.5 $\mu$ l
cDNA insert (> 3.5 ng/ $\mu$ l) amplified from yk clone using T7/T3 primers	0.6 $\mu$ l
<p>* anchored oligo dT primers: 5.3 <math>\mu</math>M (dT)<sub>17</sub>dG/5.3 <math>\mu</math>M (dT)<sub>17</sub>dC/21.3 <math>\mu</math>M (dT)<sub>17</sub>dA (This is to avoid the effect of poly-A stretch. Other vector primers may be used.)</p> <p>** 10 <math>\times</math> DIG-dUTP/dNTP mix: 0.35 mM DIG-dUTP/0.65 mM dTTP/1mM each d(A, G, C)TP</p>	

2. Subject to thermal cycling:

hot start at 95  $^{\circ}$ C for 45 sec

95  $^{\circ}$ C  $\times$  15 sec

45  $^{\circ}$ C  $\times$  1 min

72  $^{\circ}$ C  $\times$  1 min

50 cycles

3. Add 10  $\mu$ l of 10 mM EDTA
4. Pass through G-50 spin column chromatography (ca. 250  $\mu$ l bed volume)
5. Add 5  $\mu$ l of TSE

### 1.2. Chopping the probes by DNaseI digestion

6. Make the following reaction mix (total 25  $\mu$ l) on ice:

The G-50 elutate	20 $\mu$ l
10 mg/ml Salmon sperm DNA	1 $\mu$ l
distilled water	0.5 $\mu$ l
10 $\times$ DNase buffer*	2.5 $\mu$ l
DNaseI (16 $\mu$ g/ml)**	1 $\mu$ l
<p>* 10 <math>\times</math> DNase buffer: 0.5 M TrisHCl pH 7.5, 0.1 M MgCl<sub>2</sub></p>	

\*\* Dilute stock solution (1 mg/ml) with 0.1 M NaCl.

(Note : Best size of probes is about 100 bases. Longer probes may cause high background. The concentrations of the enzyme should be optimized by pilot experiments.)

7. Incubate at 37 °C for 30 min
8. Transfer on ice
9. Add 5 µl of 0.1 M EDTA
10. Heat at 75 °C for 5 min
11. Check the size by alkaline agarose gel electrophoresis and DIG detection, if necessary
12. Store frozen

## **2. Fixation of embryos from a small number of worms**

1. Take siliconized 1.5 ml eppendorf tubes
2. Place about 100 µl of distilled water on the (inside) top of the lids
3. Pick and transfer 40-50 gravid worms into the water. If you need very late stage embryos:
  - a) Add 50 µl of suspension of *E. coli* OP50 in S-basal
  - b) Cover the lid with the body of the tube
  - c) Let stand at 20 °C overnight
4. Spin down the worms.
5. Add equal volume of 2 × alkaline-bleach solution and mix well
6. Leave at r.t. for 10 min to dissolve the adult bodies
7. Add 1 ml of M9 buffer at 4 °C
8. Centrifuge at 2500 rpm for 30 sec at 4 °C in a swing rotor
9. Remove the sup carefully, leaving about 100 µl of the sup to avoid removing the embryos
10. Repeat steps 7-9 three more times
11. Add an equal volume of 3 mg/ml chitinase
12. Mix and incubate at r.t. for 3 min
13. Spin at 2500 rpm for 30 sec at 4 °C
14. Reduce the volume to about 50 µl
15. Transfer the embryos to a poly-L-lysine coated 3-well slide using a siliconized pipette tip
16. Add a half volume of 4% gelatin, 2% BSA, and mix gently by pipeting

17. Let stand for several minutes to allow the embryos settled down to the bottom
18. Cover with a cover slip (24 × 40 mm)
19. Place it on the top of a dry ice block
20. Freeze for 7 min at -70 °C
21. Peel off the cover slip quickly
22. Soak the slide in methanol cooled at -20 °C for 5 min
23. Rehydrate by soaking the slide in the series of the following solutions pre-cooled at 4 °C:

methanol	for 5 min
methanol:formaldehyde-Hepes-PBS* = 35:15	for 2 min
methanol:formaldehyde-Hepes-PBS* = 25:25	for 2 min
methanol:formaldehyde-Hepes-PBS* = 15:35	for 2 min
formaldehyde-Hepes-PBS*	for 20 min

*formaldehyde-Hepes-PBS	
Hepes	200ml
10xPBS	25ml
formaldehyde	25ml

24. Dehydrate by soaking the slide in the series of the following solutions at r.t.:

ethanol:PBS=15:35	for 5min
ethanol:PBS=25:25	for 5min
ethanol:PBS=35:15	for 5 min
ethanol	for 5 min × 2 times

25. Store in ethanol at -20 °C.

### 3. Large scale fixation of embryos

#### 3.1. Harvesting of embryos

1. Get a plenty of worms from a mixed stage population
2. Wash the worms 2 times with M9 buffer
3. Collect L1-L3 by sieving through 50 µm Nylon mesh
4. Allow the collected worms to grow to young adults in liquid culture
5. Take 1 ml packed worms from the culture, which will give 8-15 slides for *in situ*
6. Resuspend the worms in 4 ml water in a 15 ml Falcon tube (clear type)

7. Add 5 ml of 2 × alkali-bleach solution, mix well and let stand for 10 min
8. Force the worms out through a 23-gauge needle onto nylon mesh
9. Collect embryos by spinning the filtrate at 800 × g using a swing rotor
10. Wash the embryos 4 times with M9 and transferred into a siliconized eppendorf tube

### 3.2. Fixation

1. Take 100 µl (packed volume) of the embryos and adjust the volume to 200 µl with M9
2. Add 200 µl of yatalase (15 mg/ml in 0.3 M mannitol) and vigorously shake for 75 sec
3. Wash the embryos 3 times with EH buffer (Embryo Handling buffer)
4. Wash the embryos with Basal EH buffer
5. Resuspend the embryos in 1 ml of Basal EH buffer. (Note: For success, it is desired that 20-30% of embryos are devitellinized at this step)
6. Place 30 µl/well of Basal EH buffer onto each well of poly-L-lysine coated 8-well slides
7. Dispense 5 µl/well of the embryo suspension into the buffer at each well
8. Let stand for 10 min at 4 °C to settle the embryos to the bottom
9. Remove the buffer, and immediately immerse in methanol at –20 °C for 5 min
10. Rehydrate the embryos by immersing the slides in the following series at 4 °C. The solutions must be pre-cooled at 4 °C.

methanol	5 min
methanol:3.7% formaldehyde in hepes-PBS = 7:3	2 min
methanol:3.7% formaldehyde in hepes-PBS = 1:1	2 min
methanol:3.7% formaldehyde in hepes-PBS = 3:7	2 min
3.7% formaldehyde in hepes-PBS	75 min at 22 °C

11. Dehydrate the embryos by immersing the slides in the following series at r.t.

ethanol:PBS = 3:7	5 min
ethanol:PBS = 1:1	5 min
ethanol:PBS = 7:3	5 min
ethanol	5 min × 2 times

12. Store in ethanol at –20 °C.

## 4. Hybridization and signal detection

### 4.1. Proteinase K treatment

1. Rehydrate the embryos by immersing the following ethanol series:

0.03% H <sub>2</sub> O <sub>2</sub> in ethanol:PBS = 7:3	2 min
ethanol:PBS = 1:1	5 min
ethanol:PBS = 3:7	5 min

2. Wash the slides once by immersing in PBT for 5 min. \*For late stage embryos, additional HCl treatment is effective, which can cut glycosid bonds of the proteoglycan that appear on late stage embryos.
  - a) Immerse the slides in 0.2 N HCl for 20 min at r.t.
  - b) Wash the slides 2 times in PBT for 5 min
3. Immerse the slides in proteinase K (10 µg/ml in PBT) and incubate at r.t. for 11 min
4. Stop the digestion by immersing the slides in 2 mg/ml glycine in PBT for 2 min
5. Wash the slides twice by immersing them in PBT for 2 min each
6. Refix by immersing the slides in 3.7% formaldehyde in hepes-PBS at r.t. for 50 min
7. Wash the slides twice in PBT for 5 min each
8. Immerse the slides in 2 mg/ml glycine in PBT at r.t. for 5 min
9. Wash the slides in PBT for 5 min

### 4.2. Pre-Hybridization

1. Immerse the slides in the following series of mixtures;

50% formamide, 5 × SSC, heparin, 0.1% Tween:PBT = 1:1	10 min
50% formamide, 5 × SSC, heparin, 0.1% Tween	10 min

2. Wipe off the slides
3. Draw a rectangle surrounding the sample wells using a IMMUNO pen to make a ridge
4. Add 250 µl of heat denatured (at 99 °C for 10 min and quickly chilled for 5 min) hybridization solution for each 8-well slide
5. Place the slide in a moist chamber containing a paper towel wetted with 50% formamide, 5 × SSC. (No need to use coverslips)
6. Incubate at 48 °C for 1 hr

### 4.3. Hybridization

1. Add 50 µl of heat-denatured DNA probes for each slide. (The final concentrations of probes is about 0.06 µg/ml)
2. Cover the slide with a parafilm coverslip to reduce evaporation
3. Incubate the slides at 48 °C overnight in the moist chamber

### 4.4. Washing

1. Wash the slides in the following series of washing solutions at 48 °C with slight agitation

50% formamide, 5 × SSC, heparin, 0.1% Tween:PBT = 1:1 (First washing is performed in separate containers for every 2 min slides)	
50% formamide, 5 × SSC, heparin, 0.1% Tween:PBT = 1:1	10 min × 2 times
0.8 × PBS, 0.1% CHAPS	20 min × 4 times

2. Wash the slides twice in PBT for 5 min at r.t. to remove CHAPS.

### 4.5. Probe detection

1. Incubate the slides in PBtr (PBS, 0.1% Triton-X100, 0.1% BSA, 0.01% NaN<sub>3</sub>) for 1.5 hr at r.t.
2. Cover the embryos with 250 µl of anti-DIG conjugate (dilute 1:2500)/8-well slide
3. Incubate for 2 hrs at r.t. in a moist chamber. (no need to use coverslips)
4. Wash the slides with PBtr 4 times with slight agitation
5. Wash the slides with the staining buffer (see reagents) twice for 5 min each at r.t.
6. Color development
  - a) Mix 180 µl of NBT and 140 µl of BCIP in 40 ml of staining buffer.
  - b) Immerse the slides in the mixture for 1 hr at 22 °C in the dark, monitoring the extent of the staining.
7. Wash the slides three times with PBS, 20 mM EDTA to stop the reaction.
8. If necessary, incubate the slides in 1 µg/ml DAPI in Tris buffer at 4 °C for 30 min.

### 4.6. Mounting

#### 4.6.1. Permanent mount 1

1. Add about 90 µl of “MOUNT-QUICK AQUEOUS” onto the embryos on the slide
2. Cover with a coverslip
3. Let stand one day to dry up
4. Seal up the edge of the coverslip using nail varnish

#### 4.6.2. Permanent mount 2

1. Dehydrate with the following ethanol series:

ethanol:PBS = 3:7	5 min
ethanol:PBS = 1:1	5 min
ethanol:PBS = 7:3	5 min
ethanol	5 min × 2 times

2. Wash once with ethanol:Histo-Clear (National Diagnostics) = 1:1
3. Wash once with Histo-Clear
4. Add drops of Mount-Quick onto the embryos and cover with a coverslip
5. Leave the slide at 40 °C for several hours

(Note : Hybridization signals by this method tend to be weaker than those by other methods and to diffuse, but preservation of morphology is better than other methods)

#### 4.6.3. Glycerol mount

1. Add drops of 90% glycerol, 10 mM Tris, 1% n-propylgallate onto the embryos
2. Cover with a coverslip which are dotted with vaselin:solid paraffin = 9:1 at the 4 corners as spacer

## 5. Reagents

### M9

$\text{KH}_2\text{PO}_4$	3 g
$\text{Na}_2\text{HPO}_4$	6 g
1M $\text{MgSO}_4$	1 ml
Add DW to total 1 liter and autoclave	

### S-basal

NaCl	69 g
1M $\text{K-PO}_4$ (pH6)	100 ml
cholesterol (5 mg/ml in EtOH)	2 ml
Add DW to total 2 liter and autoclave	

### 2 × alkali-bleach solution

NaClO	3.0 ml
5M KOH	2.5 ml
DW	19.5 ml



**PBS**

NaCl	13.7 mM
KCl	2.7 mM
Na <sub>2</sub> HPO <sub>4</sub>	4.3 mM
KH <sub>2</sub> PO <sub>4</sub>	1.5 mM
Adjust pH to 7.2 and autoclave	

**PBT**

PBS + 0.1% Tween 20

**EH buffer (Embryo Handling buffer)**

mannitol	0.3 M
Hepes pH 7.2	50 mM
NaCl	10 mM
MgCl <sub>2</sub>	10 mM
EGTA	0.04%
NH <sub>4</sub> NO <sub>3</sub>	2 mM
gelatin	0.1%
DTT	2 mM

**Basal EH buffer**

(= EH buffer without EGTA, NH<sub>4</sub>NO<sub>3</sub>, gelatin and DTT)

**Glycine in PBT**

Glycine	2 mg/ml in PBS
	autoclave, then add 0.1% Tween 20

**3.7% Formaldehyde in hepes-PBS**

hepes buffer\*:formalin:10 × PBS = 8:1:1

**\*hepes buffer**

Hepes	100 mM
MgSO <sub>4</sub>	2 mM
EGTA	0.04%
Add NaOH to pH6.9 and autoclave	

### Hybridization solution

deionized formamide	50%
SSC (pH7, autoclaved)	5×
sonicated salmon testis DNA	100 µg/ml
yeast tRNA	100 µg/ml
heparin	100 µg/ml
Tween 20	0.1%

**yatalase** (15 mg/ml) and **chitinase** (1 mg protein/ml = 5 mg crude/ml)

1. Dissolve powder of yatalase (TAKARA No.T017) or chitinase (SIGMA No. C-6137) in 0.3 M mannitol, 50 mM Hepes pH 7.2, 10 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM DTT
2. Filtrate through a 0.45 µm syringe filter.
3. store at -20 °C.

### Digoxigenin-11-dUTP (Roche 1570013)

#### PBT

0.1% Tween-20 in PBS (0.01% DEPC treated)

#### PBtr

0.1% BSA (Fraction V), 0.1% Triton X-100 in PBS proteinase K stock solution

20 mg Proteinase K (Roche 30U/mg)/ml water

### Staining buffer (Alkaline phosphatase reaction buffer)

NaCl	100 mM
MgCl <sub>2</sub>	5 mM
TrisHCl pH 9.5	100 mM
Levamisol	1 mM
Tween-20	0.1%

### poly-L-lysine coated slides

1. Immerse glass slides in solution of kitchen detergent for 20 min
2. Wash in tap water for 1 hr
3. Wash in ion-exchanged water
4. Autoclave and dry at 80 °C
5. Drop poly-L-lysine solution (SIGMA P-8920) onto individual wells of the slides
6. Leave for 25 min

7. Aspirate off the excess solution (only when 3-well slides are used)
8. Dry at 65 °C for 1 hr

**Parafilm coverslips**

1. Dribble beads of rubber cement along the edge of square pieces of parafilm
2. Dry briefly at 35-40 °C

