Protocols for large scale *in situ* hybridization on *C. elegans* larvae*

Tomoko Motohashi, Hiroaki Tabara, Yuji Kohara§, Genome Biology Lab
Center for Genetic Resource Information, National Institute of Genetics,
Mishima 411–8540, Japan

Table of Contents

1. Preparation of staged worms 1
    1.1. Preparation of staged worms 1
    1.2. Cultivation for preparation of staged worms 2
2. Fixation 3
    2.1. Primary fixation of worms 3
    2.2. Fixation of worms onto slides 4
3. Hybridization and detection 5
    3.1. Hybridization 5
    3.2. Washing 6
    3.3. Staining by enzyme reaction 7
4. Reagents 7

1. Preparation of staged worms

1.1. Preparation of staged worms

1. Sieve a liquid culture containing a lot of gravid worms through nylon mesh (50 μml).
2. Clean up the collected worms thoroughly with DW on the nylon mesh.
3. Wash off the worms on the mesh with DW into a beaker.
4. Transfer the worms into a 50ml centrifuge tube.
5. Wash the worms by centrifugation (2000rpm for 1min at 4°C).

*Edited by Oliver Hobert. WormMethods editor, Victor Ambros. Last revised April 27, 2005. Published July 24, 2006. This chapter should be cited as: Motohashi, T. et al. Protocols for large scale *in situ* hybridization on *C. elegans* larvae (July 24, 2006). *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.103.1, http://www.wormbook.org.

§To whom correspondence should be addressed. Email: ykohara@lab.nig.ac or isugiura@lab.nig.ac.jp.

6. Aspirate the sup.

7. Measure the packed volume of the worms.

   - If it is 2–3 ml, add DW to 10 ml.
   - If it is 3–5 ml, add DW to 12.5 ml.
   - If it is larger than 5 ml, divide the worms into multiple tubes.

8. Add equal volume of 2X alkaline-bleach solution and mix gently.

   2X alkaline-bleach solution

NaClO	3 ml
5M KOH	2.5 ml
DW	19.5 ml

9. Lay the tube down, monitoring the breakage of the worms under a dissecting microscope.

10. When about 30% of the worms begin to break apart (usually 5–10 min later), load the suspension into a 50 ml disposable syringe.

11. Force it out through a needle (23G6) into a 50 ml Falcon tube.

12. Filtrate the suspension through a 50 mm nylon mesh, and wash the debris with M9 on the mesh to recover the trapped eggs.

13. Transfer the filtrate into 50 ml Falcon tubes.

14. Collect and wash eggs by centrifugation at 3000rpm for 1 min once and at 2000rpm for 1 min twice at 4°C.

15. Transfer the eggs into 15 ml Falcon tube and centrifuge at 2000rpm for 1min at 4°C.

16. Measure the packed volume of the eggs.

1.2. Cultivation for preparation of staged worms

To cover all larval stages, synchronization at L1 is not performed. We usually cultivate worms at 20°C.

1. Mix the eggs, S-basal and *E.coli* OP-50 suspension in a new 1L flask as follows:

	Eggs	S-basal	OP-50	Collect after	Expected vol of worms
For L1-L2	100 µl	200 ml	30 ml	20–24 hrs	150–200 µl
For L2-L3	100 µl	200 ml	80 ml	48 hrs	500 µl
For L3-L4	50 µl	200 ml	50 ml	60 hrs	500 µl
For L4-adult	50 µl	200 ml	90 ml	70–72 hrs	2.5 ml

2. After appropriate time, collect worms by: for L3 adults, sieving through 50µl nylon mesh and washing off with M9 into a 50ml Falcon tube. For L1-L2, centrifugation at 2000rpm and 4°C for 1 min.

3. Wash the worms with M9 by centrifugation (2000rpm, 4°C for 1 min.).

4. Transfer the worms into 2 ml eppendorf tubes at 200µl (packed volume) worms per tube.

5. Centrifuge the tubes at 3500rpm for 10sec at 4°C.

6. Let the tubes stand for 30 sec to settle the worms down to the bottom.
7. Remove the sup using aspirator (This procedure will be used for changing buffer in the subsequent steps.).

2. Fixation

2.1. Primary fixation of worms

1. Add 10mM DTT, 0.1% Tween-20 in 1X BO_3(pH9) equilibrated at 22°C.
2. Rotate the tubes for 20 min at 22°C.
3. Change the buffer to PBS (4°C), and rotate the tubes for 2 min at r.t.
4. Repeat step 3 once.
5. ProteinaseK digestion:
    a. Add PBT (at 22°C) to total 1ml.
    b. Add 5µl of ProteinaseK (20mg/ml).
    c. Rotate the tubes for 12 min at 22°C.
6. Change the buffer to Glycine in PBT (at 4°C) and rotate the tubes for 2 min at r.t.
7. Change the buffer to PBS and rotate for 2 min at r.t.
8. Repeat step 7 twice.
9. Fixation with Dent: Change the buffer to Dent (MeOH:DMSO = 8:2) pre-cooled at -20°C, and rotate for 5 min in cold room.
10. Rehydration: Change the buffer and rotate the tubes as follows:

MeOH	4°C	5 min
MeOH:0.2N HCl = 1:1	4°C	10 min
PBS	4°C	2 min
PBS	22°C	5 min
10mM DTT in 1X BO_3(pH9)	22°C	10 min
1X BO_3(pH9)	22°C	3 min, 2 min, 2 min (3 times)
0.6% H_2O_2 in 1X BO_3 (Add 1X BO_3 to total 1ml and then add 20µl of 30% H_2O_2)	22°C	10 min
PBS	22°C	2 min (3 times)
3.7% formaldehyde in hepes-PBS (Freshly prepared and stored in a refrigerator until use.)	22°C	2 hrs

11. Dehydration: Change the buffer and rotate the tubes at r.t. as follows:

EtOH:PBS = 3:7	5 min
EtOH:PBS = 1:1	5 min
EtOH:PBS = 7:3	5 min
EtOH	5 min (twice)

12. Store the fixed worms at –20°C in EtOH.

2.2. Fixation of worms onto slides

1. Resuspend the fixed worms (stored in EtOH at -20°C) and quickly transfer the following volume (variable depending on the sample worms) of the suspension into siliconized 2 ml eppendorf tubes:

L1-L2	ca. 200µl/tube
L2-L3	ca. 300µl/tube
L3-L4	ca. 900µl/tube
L4-adult	ca. 1100µl/tube

(The amounts of worms allows hybridization with 120 different probes.)

2. Rehydration: Change the buffer and rotate the tubes at r.t. as follows:

EtOH:PBS = 7:3	5 min
EtOH:PBS = 1:1	5 min
EtOH:PBS = 3:7	5 min

3. Wash with PBT for 5 min x 3 times and resuspend in about 700µl of PBT.

4. Check the density of the worms by counting worms in an aliquot of the suspension under a dissecting microscope.

5. Allow the worms to stick to slides as follows:

    1. Place poly-L-lysine coated 8 well test slides on the top of an aluminum block pre-cooled on ice.

    2. Dispense ice-cold PBS to individual wells at 30µl/well.

    3. Dispense the rehydrated worms to individual wells at 5µl/well as follows:

        - L1–L2 is in the wells #1 and 5
        - L2–L3 is in the wells #2 and 6
        - L3–L4 is in the wells #3 and 7
        - L4-adult is in the wells #4 and 8

    4. Let stand for 5 min to settle the worms to the bottom.

6. Fix the worms as follows:

    1. Soak the slides in MeOH pre-cooled at 4°C by arranging the slides in a stainless steel holder (15 slides/holder) that is placed in the MeOH.

    2. Let stand for 5 min.

3. Soak the holder with the slides in the following series of solution at 4°C in cold room:

MeOH:formaldehyde in hepes-PBS = 7:3	2 min
MeOH:formaldehyde in hepes-PBS = 1:1	2 min
MeOH:formaldehyde in hepes-PBS = 3:7	2 min
3.7% formaldehyde in hepes-PBS	60 min
PBT	5 min x 5 times at r.t.

7. ProteinaseK digestion:

   a. Add 60µl of 20mg/ml of ProteinaseK in 180ml of PBT pre-warmed at 37°C (final conc. µg/ml).

   b. Mix well by stirring.

   c. Transfer into a vat that fits the slide holder.

   d. Soak the holder containing the slides in the ProteinaseK solution.

   e. Incubate at 37°C for 30 min.

8. Transfer the holder in glycine in PBT pre-cooled at 4°C and let stand for 2 min to stop the digestion.

9. Acetylation

   a. Soak in 0.1% Triethanol amine for 2 min at r.t.

   b. Soak in 0.05% Acetic anhydride in Triethanol amine for 10 min.

10. Dehydrate the specimen by soaking the holder in the following series of solution at r.t.:

PBT	2 min
PBT	2 min
formaldehyde in hepes-PBS	20 min
EtOH:PBS = 3:7	5 min
EtOH:PBS = 1:1	5 min
EtOH:PBS = 7:3	5 min
EtOH	5 min twice

11. Store the slides in EtOH at –80°C.

3. Hybridization and detection

3.1. Hybridization

1. Take the fixed slides, arrange in a stainless holder and immersed in EtOH.

2. Rehydrate the specimen by soaking the holder in the following series of solutions:

EtOH:PBS = 7:3	5 min
EtOH:PBS = 1:1	5 min
EtOH:PBS = 3:7	5 min
PBT	5 min
50% formamide, 5XSSC, 100µ/ml heparin, 0.1% Tween:PBT = 1:1	10 min
50% formamide, 5XSSC, 100µ/ml heparin, 0.1% Tween	10 min

3. Prehybridization

    a. Take out the slides using forceps, wipe off the outside of the wells and draw a rectangle surrounding the 8 wells using a IMMUNO pen.

    b. Pour 250µl of hybridization solution (heat-denatured at 99°C for 10 min. and quickly chilled on ice-water for 5 min) inside the rectangle.

    c. Placed the slides in a moisture box.

    d. Place the moisture box in an oven at 48°C for 1hr.

4. Heat denature probes as follows:

    a. Dispense 9µl probe solution/well into 4 contiguous wells (e.g., A1-A4), since one probe is applied to 4 wells (for 4 different larval stages).

    b. Dispense 41µl of hybridization solution/well and mix by pipetting.

    c. Seal the plate using GeNunc Tape and centrifuge.

    d. Place the plate on a heated block at 99°C for 10 min and quickly chill on ice for 5 min.

5. Assembling of hybridization apparatus (S&S 96 well dot blotting apparatus):

    a. Place a silicon sheet (1 mm thick) on the top of the lower block.

    b. Clean up the surface of the silicon sheet with EtOH.

    c. Apply O-rings to the holes at the 4 corners and the holes used for hybridization of the upper 96-hole block.

    d. Take out the pre-hybridized slides, drain off the hybridization solution by tapping on the top of paper towel.

    e. Quickly arrange 4 slides at the fixed positions on the silicon sheet on the lower block.

    f. Cover the slides with the upper block and rock the complex.

6. Start of hybridization:

    a. Apply all of the heat denatured probes using a 4-channel pipette.

    b. Add 100µl of mineral oil per well.

    c. Seal the holes of the apparatus with microtiter plate sealing tape.

    d. Place the hybridization apparatus in a air-tight box.

    e. Incubate at 48°C overnight.

3.2. Washing

1. Pre-warm the following solutions:

    - solution-1: 50% formamide, 5XSSC, 100µg/ml heparin, 0.1% Tween : PBT = 1 : 1
    - solution-2: 0.8xPBS, 0,1% CHAPS

2. Dispense solution-1 into the hybridization holes (to dilute the probes).

3. Discard the solution in the holes by decantation.

4. Disassemble the apparatus, take the slides and arrange them in a holder soaked in solution-1.

5. Shake for 2 min in a 48°C incubator.

6. Transfer the holder containing the slides into a new vat containing solution-1 and shake for 10 min in the 48°C incubator. Repeat once.

7. Transfer the holder into a new vat containing solution-2 and shake for 20 min in the 48°C incubator. Repeat 3 times.

8. Transfer the holder into a new vat containing 1xPBT and shake for 5 min at r.t. Repeat once.

   You may store the slides in 1xPBT at 4°C overnight.

3.3. Staining by enzyme reaction

1. Transfer the holder containing the slides into a vat containing PBtr (PBS, 0.1% Triton-X100, 0.1% BSA, 0.01% NaN_3) and shake for 1.5 hr at r.t.

2. Take individual slides, remove the solution outside the wells and overwrite the rectangle using a PAP pen.

3. Apply 250µl of diluted anti-DIG antibody solution (diluted 1:2500 with PBtr) per slide.

4. Place the slides in a moist box.

5. Incubate for 2 hrs at r.t., or overnight at 4°C in the dark.

6. Transfer the slides into a vat containing PBtr (PBS, 0.1% Triton-X100, 0.1% BSA, 0.01% NaN_3) and shake for 10 min at r.t. Repeat 3 times.

7. Soak the slides in stain buffer (100mM NaCl, 5mM $MgCl_2$, 100mM TrisHCl pH9.5, 0.1% Tween, 1mM levamisol) and shake for 5 min at r.t. Repeat once.

8. Arrange the slides in glass vats (max. 8 slides per vat) containing the stain buffer.

9. Prepare the coloring solution by mixing 40ml of the stain buffer (at 22%C), 180µl of NBT and 140µl of BCIP.

10. Decant the stain buffer from the glass vat preventing the coming out of the slides, and add the coloring solution into the vat.

11. Incubate for 1hr 15 min in a 22°C incubator.

12. Wash the slides 3 times with PBS, 20mM EDTA to terminate the coloring reaction.

13. The slides can be stored in PBS, 20mM EDTA overnight at 4°C.

14. Mount the slides using glycerol solution.

15. Observe on a microscope equipped with Nomarski optics.

4. Reagents

M9

KH_2PO_4	3 g
Na_2HPO_4	6 g
1M $MgSO_4$	1 ml

Add DW to total 1 liter and autoclave

S-basal

NaCl	11.69 g
1M K-PO_4 (pH6)	100 ml
cholesterol (5 mg/ml in EtOH)	2 ml

Add DW to total 2 liter and autoclave

40X BO_3 (pH9)

H_3BO_3 1M Adjust pH to 9.0 using NaOH and autoclave

PBS

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	4.3 mM
KH_2PO_4	1.5 mM

Adjust pH to 7.2 and autoclave

PBT

PBS + 0.1% Tween 20

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 : 10X PBS = 8 : 1 : 1 hepes buffer

Hepes	100 mM
$MgSO_4$	2 mM
EGTA	0.04%

Add NaOH to pH6.9 and autoclave

Hybridization solution

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

CHAPS (349–04722, DOJINDO, Japan)

IMMUNO pen (Wako, Japan)