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

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# 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  $4^{\circ}$ C).

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- 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 μ1
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 μ1
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<sub>3</sub>(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 <sub>3</sub> (pH9)	22°C	10 min
1X BO <sub>3</sub> (pH9)	22°C	3 min, 2 min, 2 min (3 times)
0.6% H <sub>2</sub> O <sub>2</sub> in 1X BO <sub>3</sub>	22°C	10 min
(Add 1X BO $_3$ to total 1ml and then add 20 $\mu$ l of 30% $H_2O_2$ )		
PBS	22°C	2 min (3 times)
3.7% formaldehyde in hepes-PBS	22°C	2 hrs
(Freshly prepared and stored in a refrigerator until use.)		

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  $-20^{\circ}$ 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:

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EtOH:PBS = 7:3 5 min 

EtOH:PBS = 1:1 5 min 

EtOH:PBS = 3:7 5 min 

PBT 5 min 

50% formamide, 5XSSC, 100\mu/ml heparin, 0.1% 10 min 

Tween:PBT = 1:1 50% formamide, 5XSSC, 100\mu/ml heparin, 0.1% 10 min 

Tween
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#### 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<sub>2</sub>) 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<sub>3</sub>) and shake for 10 min at r.t. Repeat 3 times.
- Soak the slides in stain buffer (100mM NaCl, 5mM MgCl<sub>2</sub>, 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 <sub>2</sub> PO <sub>4</sub>	3 g
Na HPO	6 g
1M MgSO	1 ml
Add DW to total 1 liter and autoclave	

#### S-basal

NaCl 11.69 g 1M K-PO<sub>4</sub> (pH6) 100 ml cholesterol (5 mg/ml in EtOH)  $2 \, ml$ Add DW to total 2 liter and autoclave

#### 40X BO<sub>2</sub> (pH9)

H<sub>2</sub>BO<sub>2</sub> 1M Adjust pH to 9.0 using NaOH and autoclave

#### **PBS**

NaCl 137 mM KC1 2.7 mM Na HPO 4.3 mM KH, PO. 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<sub>4</sub> 2 mMEGTA<sup>†</sup> 0.04%

Add NaOH to pH6.9 and autoclave

#### **Hybridization solution**

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

## **CHAPS (349–04722, DOJINDO, Japan)**

IMMUNO pen (Wako, Japan)

