

## Tissue In Situ Hybridization with a digoxigenin labeled probe (RON)

Grow up third in-star larvae in 18 bottles with ample yeast. Dissect out the eye discs in PBS. Dissect away all tissue except for the eye-antenna disc complexes. I dissect on silgard dishes. I transfer the dissected complexes into a drop of PBS in a small glass dissecting dish. Once I have all of the discs for a specific genotype dissected and waiting in the PBS, I add fix (4% formaldehyde in PBS) to the drop of PBS and allow the discs to fix for 15 minutes on ice. Then I transfer the discs to a basket (made from eppendorfs and wire mesh) which is sitting in fix (4% formaldehyde in PBS):

NOTE: When dissecting the larvae, after isolating each set of discs, shred the remaining tissue of the larva and put it into a basket immersed in the first fix solution. When the dissections are done, carry this basket through all of the fixation steps (1-8) along side your baskets of discs. This will give you tissue to preabsorb your antibody with on the second day of the protocol. Its a pain in the butt if you forget to do this.

### 1. fix in 4% formaldehyde for 15 min on ice

1 ml 37% formaldehyde  
800 10X PBS  
ddH<sub>2</sub>O to 9 ml

### 2. fix in 4% formaldehyde w/0.6% Triton X-100 for 15 min at RT

1 ml 37% formaldehyde  
900 10X PBS  
54 Triton X-100  
ddH<sub>2</sub>O to 9 ml

### 3. wash 3x in PBT for 5 min at RT

PBT: PBS with 0.1% Tween 20

### 4. digest with Proteinase K for 5 min at room temp

12.5µg/ml in PBT (store as a 1000x stock at -20° C)

NOTE: This step is critical, if the discs aren't digested enough you will get tons of background but no staining. If they are digested too much you will find that they disappear before the protocol is finished.

### 5. stop with glycine for 10 min at RT

2 mg/ml in PBT (store as 10x stock at -20° C)

6. wash 2x in PBT for 5 min at RT

7. refix in 4% formaldehyde & 0.2% glutaraldehyde for 15 min at RT

1 ml 37% formaldehyde  
72 25% glutaraldehyde  
900 10X PBS  
ddH<sub>2</sub>O to 9 ml

8. wash 5x in PBT for 5 min at RT

NOTE: Remember to now put your larval tissue (non-eye discs) into some PBT and keep it in the frig for tomorrow. Do not transfer it into any hybe solution.

9. wash with a 1:1 mixture of PBT and hybridization buffer for 10 min at RT

Hybridization buffer :	50 ml of soln	Stocks
50% formamide	25 ml (100% @ -20)	
5x SSC	12.5 ml	(20x stock)
100µg/ml salmon sperm DNA	500	(10mg/ml)
50µg/ml Heparin	50	(50mg/ml @ -20)
100µg/ml t-RNA (yeast)	100	(50mg/ml @ -20)
0.1% Tween 20 (make fresh)	50	(100%)

10. hybridization buffer for 10 min at RT

11. hybridization buffer for 60 min at 48

12. hybridize in hybe buffer with freshly denatured 50µg (labeled DNA in 100) overnight (at least 16 hours) at 48

NOTE: I wash the discs out of the baskets into glass dissecting wells with hybe soln, and then transfer them into pico-titer plates containing the different probes. This cuts down on the amount of probe that you need to dilute each time and it ensures that all of the discs will get exposed. The next morning, I transfer the discs out of the Nunc plates and back into baskets for the first wash.

13. wash in hybridization buffer for 20 min at 48

NOTE: Now is a good time to start pre-absorbing your antibody solution because you want to let it incubate with the non-specific tissue for a couple hours before using it on the discs. Dilute the antibody 1:2000 in PBT, then add the fixed larval tissue to it.

14. wash in hybe buffer : PBT (1:1) 20 min at 48
15. wash 5x in PBT for 20 min at 48
16. incubate in anti-digoxigenin antibody (1:2000; preabsorbed) 60 at RT or overnight at 4
17. wash 4x in PBT for 20 min at RT
18. incubate 2x in staining buffer for 20 min at RT

Staining buffer :	<u>50 ml of soln</u>	<u>Stocks</u>
100 mM NaCl	1 ml	5 M NaCl
50 mM MgCl <sub>2</sub>	2.5 ml	1 M MgCl <sub>2</sub>
100 mM Tris/HCl pH 9.5	2.5 ml	2 M Tris
1 mM Levamisol (Sigma) *	0.5 ml	100 mM Levamisol
0.1% Tween 20	50	100%

NOTE: The Levamisol is supposed to enhance the reaction. However, if it goes bad then it inhibits the color reaction completely. If this happens you can just make fresh staining soln with fresh Levamisol and transfer the discs into it. The color reaction will then proceed normally. I've found that the Levamisol lasts upto six months, but if you wanted to be on the safe side you could make it fresh each time.

19. incubate in color reaction for anywhere from 30 min to 5 hours.

Color Solution :	<u>10 ml of soln</u>
Staining buffer	10 ml
Tube 9 (NBT)	70
Tube 10 (X-phosphate)	35

20. stop by washing in PBT

To make the probe, follow the genus kit protocol. Label DNA in a 20 reaction volume just as the kit suggests. After EtOH precipitation, resuspend in 40TE buffer (or ddH<sub>2</sub>O). This will give you a final concentration of about 20 ng labeled DNA. As you want approximately 50 ng labeled DNA per 100 hybe buffer for your hybridization reaction you will only have to use 20 probe for each run.

To test the probe:

1. make a series of dilutions (1:30, 1:100, 1:300, 1:1000) both of the probe as well as a control whose concentration is already known.
2. spot 2 of each dilution onto a small piece of zeta probe paper

3. **bake in vaccum oven for 15 min**
4. **follow the Genius kit protocol for Immunological detection exactly as written, including the second step in which you incubate the filter in buffer 2. This is necessary to avoid background using zeta-probe.**