

Modified 9/12/07 from the Rebeiz lab

Tissue Preparation (Abdomen)

It is very important that the tissue used for hybridization is treated gently. Don't bend or stretch samples more than needed.

- 1.) Heat pupae for 1 min @ 100 degrees C in PBS. This relaxes the muscles, helping to maintain the morphology during fixation. Don't heat longer than a minute, as the fat bodies and other tissues start to melt, it will be harder to clean out the abdomen.
- 2.) Dissect fly open, with a cut down the ventral midline.
- 3.) Immerse in cold PBS and clean the specimen: Gently wash away fat bodies by blowing with a P-200. Remove reproductive organs, trachea, etc using forceps and scissors.
- 4.) Add to a tube of PBT + 4% paraformaldehyde fix on ice
- 5.) When batch of pupae has been dissected, fix at room temperature 30 minutes
- 6.) Wash 2X in MeOH
- 7.) Wash 2X in EtOH
- 8.) Store in EtOH at -20C indefinitely

in situ day 1

Sometimes abdomens collect bubbles, and float to the top of the tube. The bubbles can be teased out with forceps in a staining dish.

Wash = 5 min for organic solution, 10 min for aqueous solution rinse is a rinse

- 1.) take carcasses from -20C freezer (all should be in 100% EtOH). If in scintillation vials, transfer to eppendorf tube with cut blue pipet tip
- 2.) wash 3X in 100% EtOH ○○○
- 3.) incubate in 50:50 xylenes:EtOH for 30 min in hood ○
- 4.) wash 5X in 100% EtOH ○○○○○
- 5.) wash 2X in MeOH ○○
- 6.) Wash 3X in PBT ○○○
- 7.) fix 30 min in PBT + 4% paraformaldehyde ○
- 8.) wash 5X in PBT ○○○○○
- 9.) incubate 8-10 in PBT + Proteinase K ○
Final [] 1:25,000 – 10mg/ml Stock -> 1:100 -> 6 ul in 1.5 ml PBT
- 10.) Rinse 2X in PBT; store all on ice until all have been rinsed, then wash 2X for 10 min in PBT ○○○○
- 11.) Post-fix 30 min in PBT + 4% paraformaldehyde ○
- 12.) Wash 5X in PBT ○○○○○
- 13.) Wash in 50:50 PBT/hyb for 10 min ○

- 14.) Wash 3X in Hyb at Room temp ○○○
- 15.) Prehybridize for 1 hr in hyb solution @ 65C ○
- 16.) While hybridizing, prepare probes; heat @ 80C for 3-5 min then ice till use ○
- 17.) Setup 6-10 carcasses (the more the merrier) in each tube for each probe. remove all old hyb with p200 pipetperson ○
- 18.) Add probe to each tube. flick tubes to mix. incubate at least 18 hrs @ 65C. mix a couple times during incubation ○

in situ day 2

- 1.) Rinse once in hyb solution pre-warmed to 65 C. ○
- 2.) Incubate in hyb solution (pre-warmed) 1 hr at 65 C. ○
- 3.) Incubate 3X for 30 min in hyb solution (pre-warmed) at 65 C. ○○○
- 4.) Wash 2X for 15 min in 50/50 PBT/hyb solution. ○○
- 5.) Wash 5X for 10 min each in PBT. ○○○○○
- 6.) Remove all solution, add 0.3 mL antibody. Incubate overnight at 4 C. ○
Antibody: Roche anti-DIG AP Fab fragments. 1:6000 in PBT

in situ day 3

- 1.) Wash 5X for 10 min in PBT. ○○○○○
- 2.) Wash 3X for 10 min in Staining Buffer. ○○○
- 3.) Remove carcasses from tubes into glass viewing dishes. ○
- 4.) Remove liquid, add 0.4 mL Staining Solution. ○
- 5.) Stain in the dark, watching the pattern periodically develop (20 min to 2+ hrs, depending on probe). ○
- 6.) To stop staining, wash 2X for 10 min in Staining Buffer. ○○
- 7.) Wash 2X in PBT. ○○
- 8.) Add 0.5 mL Glycerol Mountant. ○
- 9.) Incubate overnight at 4 C. ○
- 10.) For Abomens, I mount samples on slides that are supported by 2-3 layers of double sticky tape. Cut a hole in the stack of tape, and place the tape on a new slide. After adding mount to the center and placing samples, add a cover slip. This method keeps the abdomen from getting crushed by the coverslip, and allows the sample to be preserved indefinitely.

PBT

1X PBS
0.1% Triton-X

PBT + 4% Paraformaldehyde

4 mL 10X PBS
10 mL 16% paraformaldehyde
400 uL 10% Triton-X

PBT + Proteinase K (for 15 mL)

1.5 mL 10X PBS
6 uL 10 mg/mL stock Proteinase K
150 uL 10% Triton-X

Glycerol Mountant

0.1 M Tris Hcl pH 8.0
80% glycerol

<u>Hybridization (hyb) Solution</u>	STOCK	Amt/200mL
50% Formamide		100mL
5X SSC	20X	50mL
pH to 5.5 (for 65C Hyb)		
Filter Sterilize		
100 ug/mL SSp DNA	10mg/mL	2mL
100 ug/mL Heparin		.02g
0.1% Tween		200ul

<u>Staining Buffer</u>	STOCK	Amt/50mL
100 mM NaCl	5M	1mL
50 mM MgCl ₂	1M	2.5mL
100 mM Tris HCl pH 9.5	2M	2.5mL
0.1% Tween-20		50ul

Staining Solution (per 1.5 mL)

1.5 mL Staining Buffer
10.2 µL NBT (promega)
5.25 µL BCIP (promega)

Probes

I find that raw PCR products make the best template. If I have a pGem or Topo clone, I determine the directionality, and amplify my template using vector-specific primers, so as to include the right promoter (T7, T3, SP6). If you get a single, robust band, the raw product will work great for *in vitro* transcription to make a dig labeled probe. I use the 10X Dig mix from Roche, and the polymerases from promega.

When the *in vitro* transcription is finished, I ethanol precipitate (using linear acrylamide as a carrier), and resuspend the probe in hyb solution.