Digoxigenin whole mount in situs

Dianne M. Duncan

(protocol originally from Michael Levine's lab)

Digoxigenin-UTP labeling of RNA probes

10X transcription buffer	1 μl
10X dig U NTP mix	1 μl
50 mM DTT	1 μl
RNase inhibitor (50 u/μl)	1 μl
linearized DNA (1μg)*	5 μl
T7 or T3 RNA polymerase	1 μl (10 to 20 u)

--incubate at 37 C for 2 hr
--add 40 μl H_20, run 5 μl on gel to check
--add 50 μl stop solution
--add 10 μl 4 M LiCl
--add 5 μl 20 mg/ml tRNA or 20 mg/ml glycogen as carrier
--add 300 μl ethanol
--mix and freeze (-20 C) at least 15 min
--spin top speed in microfuge 20 min in cold
--wash pellet in 70% ethanol
--dry, dissolve in 150 μl dH_20, store at -70 C
--use 1-3 μl probe per 100 μl hybridization buffer in reaction

10X transcription buffer

0.4 M Tris pH 7.5
0.06 M $MgCl_2$
0.1 M NaCl
0.02 M Spermidine-HCl

10X dig U NTP mix

10 mM ATP
10 mM GTP
10 mM CTP
6 mM UTP
4 mM dig UTP (Boehringer)

stop solution

0.2 M NaOAc
pH to 6.0 with acetic acid

*DNA must first be cut with an emzyme that lies upstream of the 5' end but does not cut within the insert. After digestion, phenol/chloroform extract and ethanol precipitate, then resuspend in 5 μl of DEPC-treated sterile distilled water.

Protocol for whole-mount in situs using RNA probes

A. Embryo fixation

1. collect and age embryos
2. wash in PBST (PBS + 0.1% Triton X 100)
3. dechorionate in 50% bleach/water
4. wash with copious amounts of dH_2O
5. wash with PBST to disaggregate
6. transfer embryos to 10 ml scintillation vial, add 3.2 ml PMG buffer + 0.8 ml formaldehyde + 4 ml heptane
7. shake vigorously for 30 min
8. remove bottom (aqueous) phase and replace with 4 ml methanol, shake vigorously 30 seconds to 1 minute
9. allow embryos to sink to bottom and then remove all solution and wash 3X with methanol
10. wash 3X with absolute ethanol and store at -20 C if desired
11. transfer embryos to a microfuge tube (no more than 100 μl packed volume per tube)
12. rinse 5X in DEPBT
13. incubate 2-3 minutes in 4 μg/ml non-digested Proteinase K in DEPBT with rocking
14. rinse 2X quickly in DEPBT, then 4X in DEPBT for 2 min each
15. postfix in 5% formaldehyde in DEPBT for 25 min.
16. rinse 5X 5 min each in DEPBT
17. incubate 5 min in 50/50 hyb soln/DEPBT with rocking
18. incubate 5 min in hyb at room temp with rocking
19. prehyb 45 min to several hours in hyb soln + sonicated salmon sperm DNA at 55 C*

*The hybridization temperature must be determined empirically for each probe.

B. Imaginal disc fixation

1. wash well fed 3rd instar larvae in PBS to remove junk
2. pull larvae apart under PBS with sharp forceps such that the point of transection lies about 1/3 of the way back from the anterior end
3. invert the head section so that the discs are exposed but still attached to the body wall. Collect as many as desired in 30 minutes or less and transfer to microfuge tube.
4. fix 20 min in 4% formaldehyde/DEPBT (1ml/sample, all subsequent washes in 1 ml)
5. wash 3X in DEPBT
6. digest 8-10 min in 10μg/ml Proteinase K
7. wash 2X 1 min in DEPBT
8. postfix 20 min in 4% formaldehyde/DEPBT
9. wash 2X 5 min in DEPBT
10. wash 5 min in 50/50 hyb/DEPBT
11. wash 5 min in hyb at room temp
12. incubate 45 min to several hours in hyb + salmon sperm DNA at 55 C*

--we use normal 37% formaldehyde solution for the first embryo fixation (step 6 in embryo protocol), but we buy special formaldehyde solution for the disc fixation steps and embryo post-fixation:

10% ultrapure EM Grade Formaldehyde (methanol free)
catalogue number 04018
Polysciences, Inc.
Warrington, PA 18976
(800) 523-2575

*See note for embryo fixation.

Hybridization and Detection

1. Heat probe to 70 C for 5 min and then put on ice
2. Dump prehyb and add 500 μl fresh hyb+salmon sperm DNA, add 5 - 10 μl of heat treated probe.
3. Hybridize overnight (10 hours or more) at 55 C* with rocking
4. The next day, dump probe and rinse 1X in 1 ml hyb (- salmon sperm DNA), all subsequent washes in 1 ml
5. Wash 5X 30 min each in hyb at 55* C
6. Wash 20 min in 8/2 hyb/DEPBT at room temp (all subsequent steps at room temp with rocking)
7. Wash 20 min in 6/4 hyb/DEPBT
8. Wash 20 min in 4/6 hyb/DEPBT
9. Wash 20 min in 2/8 hyb/DEPBT
10. Wash 3X10 min in DEPBT
11. Incubate 1 hr. at room temp with rocking in 300 to 500 μl DEPBT with Alkaline Phosphatase anti DIG antibody at 1:2000 final dilution.
    The antibody should be prepared ahead of time by diluting 1:200 in DEPBT and preabsorbing 1 to 2 hours against 100 μl packed volume of fixed tissue (i.e. fixed embryos if embryos are being stained or fixed inverted larvae if larvae are being stained). Preabsorbed antibody appears to be stable for several weeks at 4 C. Dilute the preabsorbed antibody an additional 10 fold for the incubation.
12. Wash 4X 20 min each in DEPBT (1 ml)
13. Rinse in staining buffer
14. Wash 2X 5 min each in staining buffer
15. Put sample in 24 well plate and add 1 ml staining solution. Incubate in dark for 1 to 2 hours, monitoring periodically under dissecting scope until the pattern is clear.
16. Wash 5X 5 min in DEPBT and mount in 50/50 PBS/glycerol (discs) or 80% permount/20%methyl salicylate (embryos).

* See note for embryo fixation.

Solutions

PBS

140 mM NaCl
7 mM Na_2HPO_4
3 mM KH_2PO_4

DEPBT

Treat PBS with 0.1% DEPC, autoclave, and add 0.1% Tween 20

Hybridization Buffer

5X SSC or SSPE
50 μg/ml heparin
0.1% Tween 20
(100 μg/ml denatured Salmon Sperm DNA)
50% formamide (deionized)

Staining Buffer

100 mM NaCl
50 mM $MgCl_2$
100 mM Tris pH 9.5
0.1% Tween 20
1 mg/ml levamisole

Staining solution

4.5 μl NBT and 3.5 μl x-phosphate (both from Boehringer kit) per ml of staining buffer

PMG

0.1 M PIPES
2 mM EGTA
1 mM $MgSO_4$
adjust pH to 6.9 by NaOH addition (PIPES will not dissolve until pH is increased)