

Whole Mount In Situ Hybridization of *Drosophila* Embryo

Modify from BDGP 96-well plate in situ hybridization protocol

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Day 1

1. Put 50 μ l of fixed embryos into an eppendorf tube.
2. Rehydrate in 3:1 methanol:4% formaldehyde in 1X PBS for 2 min.
 - * PBS: Dissolve 8 g of NaCl, 0.2 g KCl, 0.24 g KH₂PO₄ and 2.72 g Na₂HPO₄·7H₂O in 800 ml of DEPC treated H₂O, adjust pH to 7.4 with HCl, adjust volume to 1L, sterilize by autoclaving
 - * Prepare freshly just before starting the hybridization steps.
3. Rehydrate in 1:3 methanol:4% formaldehyde in 1X PBS for 5 min.
 - * Prepare fresh just before starting the hybridization steps.
4. Post-fix in 4% formaldehyde in 1X PBS for 10 min.
 - * Prepare freshly just before starting the hybridization steps.
5. Rinse 4x in PBT.
 - * PBT: 0.1% Tween-20 in 1X PBS
6. Add 700 μ l of hybridization buffer without dextran sulfate to embryos.
 - * Hybridization buffer: 50% formamide, 4X SSC, and 0.01% Tween-20. Prepare freshly just before starting the hybridization.
7. Incubate with rocking or rotating for at least 1 hr at room temperature to pre-hybridize embryos.
8. During pre-hybridization make fresh hybridization buffer with 5% dextran sulfate.
 - * 50% Dextran Sulfate stock: dissolve 25g of dextran sulfate in DEPC-H₂O, adjust volume to 50ml
9. Carefully remove pre-hybridization buffer from embryos. Put 700 μ l fresh hybridization buffer with 5% dextran sulfate and 1:300 diluted probe.
10. Incubate embryos at 55 °C with shaking overnight.

Day 2

11. Add 700 μ l of room temperature wash buffer.
 - * Wash buffer: 50% formamide, 2X SSC and 0.01% Tween 20; prepare fresh before use
12. Let embryos settle down. Remove the hybridization buffer without touching the embryos to prevent embryos from

getting flattened, crushed.

13. Rinse 1x with wash buffer.

14. Incubate in wash buffer at 55 ° C with shaking 5 times, 30 min each. Incubate another wash at 55 °C with shaking overnight.

Day 3

15. Rinse 2x in PBT.

16. Incubate in PBT, 1% blocking reagent (Roche) at RT with shaking for 30 min.

17. Incubate in PBT, 1% blocking reagent, Anti-DIG-AP (Roche) at RT for 2 hrs.

** If a **POD-conjugated antibody and TSA amplification kit** will be used, incubation in PBT, 1% blocking reagent, 1:100 dilution Anti-DIG-POD (Roche) O/N in cold room, then go directly to step 29.*

18. Rinse 1x with PBT.

19. Wash in PBT at RT 4~5 x with shaking, 10 min each.

20. Rinse 1x with 1ml AP buffer.

* AP Buffer: 0.1M NaCl, 0.05M MgCl₂, 0.1M Tris pH 9.5, 0.1% Tween 20; prepare fresh before use.

21. Wash in AP buffer at RT for 5 min; remove AP buffer.

22. Add developing solution. (Roche, add 200 ul of NBT/BCIP stock solution per 10 ml of AP Buffer; add NBT/BCIP just before use)

23. Incubate with shaking and check the staining every 15min until desired color development is achieved (1~2hour at RT or O/N at 4C).

24. Rinse 3x in PBT to stop the color reaction.

25. Rinse 3x in ethanol.

26. Rinse 1x in PBT.

27. Add 70% glycerol to the tube. Store at 4 ° C.

28. Check individual samples under a low power magnification microscope. Embryos are ready to be photographed.

* Check individual samples to make sure that the embryos as a whole are of high quality, that the controls worked properly, that the hybridization signal quality is high (no or minimal background) and that the embryo morphology is good (e.g. embryos are not broken, flattened nor squashed).

Day 4

29. Rinse 1x with PBT.

30. Wash in PBT at RT 4~5 x with shaking, 10 min each.

31. Rinse twice in PBS.
32. Add 100ul of 1:100 diluted Tyramide (Invitrogen TSA kit), and incubate at RT for 15 min. Tap the tube with finger several times during incubation.
35. Wash 4x 15 min in PBT. Now embryos are ready to be photographed.

RNA probe labeling by in vitro transcription (Roche)

The DNA to be transcribed should be cloned into the polylinker site of a transcription vector, which contains a promoter for SP6, T7, or T3 RNA Polymerase

1. Linearize the template plasmid DNA using a restriction enzyme that creates a 5'-overhang end.
2. Purify linearized template DNA with phenol/chloroform extraction and ethanol precipitation. Resuspend the pellet in 10 mM Tris-HCl/DEPC H₂O, pH 8.0.

3. Set up the transcription reaction:

1 µg of purified linearized plasmid DNA.

2 µl of either 10× concentrated DIG or Biotin or Fluorescein RNA Labeling Mix.

2 µl of 10× concentrated Transcription Buffer.

2 µl of RNA Polymerase (SP6, T7, or T3).

Add water supplied by RNA Polymerase kit to final volume of 20 µl.

4. Mix the components and centrifuge briefly.
5. Incubate for 2 h at 37°C.
6. If you want to remove the template DNA, add 2 U DNase I, RNase-free to the tube and incubate for 15 min at 37°C.
7. Add 2 µl of 0.2 M EDTA (pH 8.0) to the tube to stop the polymerase reaction.
8. Precipitate the labeled RNA transcript by performing the following steps:

To the reaction tube, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.

Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.

Centrifuge the tube (at 13,000 × g) for 15 min at 2–8°C. Discard the supernatant.

Wash the pellet with 50 µl of ice-cold 70% (v/v) ethanol.

Centrifuge the tube (at 13,000 × g) for 5 min at 2–8°C. Discard the supernatant.

Dry the pellet in the air.

Dissolve the RNA pellet for 30 min at 37°C in 100µl of DEPC-treated water.

9. Run an aliquot of the transcript on an agarose gel to estimate the size and yield of the transcript.
10. If you are not going to use the labeled probe immediately, store the probe solution at -70°C. Avoid repeated freezing and thawing of the probe.

Regulation of RNA Probe Length by Alkaline Hydrolysis (Roche)

When performing in situ hybridizations, probes must be short enough to allow diffusion into and out of the tissue. Alkaline hydrolysis allows you to regulate the size of RNA probes.

1. Hydrolyze 1 µg RNA by adding an equal volume of DEPC treated H₂O and two volumes of carbonate buffer. Incubate for 10–60 min at 60°C.

* Carbonate buffer: 60 mM Na₂CO₃; 40 mM NaHCO₃; pH 10.2

* Calculate the incubation time according to the formula below,

$$t = \frac{L_o - L_f}{k \times L_o \times L_f}$$

L_o = initial length of transcript (in kb)

L_f = desired probe length (in kb)

K = constant = 0.11 kb/min

2. Add an equal volume of hydrolysis-neutralization buffer to stop the hydrolysis.

* Hydrolysis-neutralization buffer: 3 M sodium acetate; 1% (v/v) acetic acid; pH 6.0

3. Add 3 volumes of prechilled absolute ethanol to precipitate the RNA.

4. Centrifuge at 13,000 × g for 15 min at 4°C. Decant the ethanol.

5. Wash the pellet with 100 µl of cold 70% ethanol. Centrifuge at 13,000 × g for 5 min at 4°C. Remove the 70% ethanol.

6. Dry the pellet and resuspend in 100 µl DEPC treated H₂O. If not use immediately, store the probe at -70°C.

7. Check the resulting probe length by electrophoresis of 10 µl hydrolyzed RNA on agarose gel.

RNA Probe Quantification

1. Pipette spot 1 µl of RNA probe onto a positively charged nylon membrane.
2. Pipette spot 1 µl of a series of diluted controls DIG labeled RNA onto the same nylon membrane.

3. Crosslink in UV Stratelinker.
4. Wash 2X with blocking solution for 5 minutes.
5. Incubate in blocking solution at room temperature for 30 minutes.
6. Incubate in 1:2000 dilution of Anti-Digoxigenin-AP Fab Fragment in blocking solution at RT for 30 minutes.
7. Wash 4X with blocking solution, 15 min each.
8. Wash 2X with AP buffer, 5 min each.
9. Develop color in the dark with developing solution at RT for 20 min.
10. Wash 3X in blocking solution to stop the color reaction, 3 min each.
11. Compare the RNA probes with controls and determine the success rate.
12. RNA probe is now ready to be used in hybridization.

Mass Embryo Fixation

Keep embryos of different time periods separate during fixation

1. Collect embryos from food tray by rinsing the tray with deionized water removing yeast paste completely. Dry embryos with paper towel.
 2. Dechorionate embryos with 50% bleach, shaking for 3 min.
 3. Wash embryos well with distilled water.
 4. Move embryos to 20ml glass vial. Fix embryos by gently shaking in 50-50 mix of heptane and 4% formaldehyde/PBS fixative for 15~20 min.
 5. Remove lower aqueous phase and replace with equal volume of methanol.
 6. Shake for 1 min then allow embryos to settle.
 7. Remove upper phase containing the vitelline membranes and embryos remaining at interphase.
 8. Remove remaining methanol.
 9. Wash 3x in methanol.
 10. Add 10 ml of methanol into embryos.
 11. Store embryos at -20 °C.
- * Good embryos, unbroken embryos devoid of chorion and vitelline membranes, will sink to the bottom. Embryos remaining at interphase are damaged and should not be used in hybridization.
12. Embryos are now ready to be hybridized.

