Whole Mount In Situ Hybridization of *Drosophila* Embryo

Modify from BDGP 96-well plate in situ hybridization protocol

Lili Qin edited on April, 2011

Day 1

1. Put 50 µl 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 Na2HPO4·7H20 in 800 ml of DEPC treated H20, 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.

* Preparely fresh just before starting the hybridization steps.

**** If a POD-conjugated antibody and TSA amplification kit will be used, quench embryos with 1~3% H_2O_2 in 0.1% PBT for 30 min, and wash 3x5 min each with 0.1% PBT, and then go to step4.***

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 µl 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-H_20, adjust volume to 50ml

9. Carefully remove pre-hybridization buffer from embryos. Put 700 µl fresh hybridization buffer with 5% dextran sulfate and 1:200 diluted probe.

10. Incubate embryos at 55 °C with shaking overnight.

Day 2

11. Add 700 µl 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 H_2O, 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. Mix 2ul of labeled RNA probe with 8ul of Gel Loading Buffer provided by MEGAscript Kit and boil the sample 2min to denature RNA. Keep the tube on ice for 5min, and then run sample on a 1% agarose gel.

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 H_2O 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,

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

Lo = initial lenght of transcript (in kb)

Lf = desired probe lenght (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.