Whole-mount RNA in-situ hybridization on butterfly wings (with Acetone)

BUFFERS (make fresh)

PBST (Phosphate Buffered Saline with Tween® 20)

50 µl (0.1%) of TWEEN in 50 ml of 1X PBS (pH:7)

Note: Add PBS before adding TWEEN

or

PTwx

PBS 0.1% Tween-20 and 0.1% Triton X-100

Fixative (5% Formaldehyde in PBST)

[Prepare 5 ml of fixative before dissection in 15 ml tube (0.5 ml of 10X PBS, 0.5 ml of 0.5M EGTA, 1.2 ml of 37% Formaldehyde, rest DEPC-water) and keep on ice]

[The fixative without EGTA also works equally well!]

E.g. For 15 samples: 1 ml Formaldehyde (37%) + 7ml PBST = 8 ml (Need 500 µl for 1 sample)

Pre-hybridization buffer (5X saline sodium citrate pH 4.5, 50% formamide, 0.01% Tween20, final pH 5-6 at 22°C)

20 ml Formamide

10 ml 20X SSC

10 ml of DEPC treated water

40 µl of TWEEN (0.1%)

H**ybridization buffer**

Hybridization buffer (Pre-hyb supplemented with 1g/L glycine, 100µg/mL denatured salmon sperm DNA and 30 ng/ml riboprobe)

10 µl of salmon sperm DNA (10 mg/ml in water_D7656-1ML) + 10 µl of glycine + 1 µl of Riboprobe in 100 µl of Pre-hyb

Block buffer

[PBST supplemented with 1% w/v bovine serum albumin]

100 ml of Block Buffer + 1 g of BSA (bovine serum albumin)

Alkaline phosphatase buffer

(100 mM Tris- HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween20)

1 ml Tris (1M) + 400 µl NaCl (2.5M)+ 250 µl of MgCl2 (200mM) + 10 µl of TWEEN + water till 10 ml

DAY 1 (all the steps from 1 to 12 are on ice)

1. Dissect wings in PBS at room temperature. (Use standard pyrex glass well plates for dissection).
2. Wipe the wells with chloroform or RNAse Away. Transfer dissected wings to wells containing PBST (30-40 seconds) with a spatula and then replace the solution with fixative **30mins** (on ice for larval wings/RT for pupal wings). For pupal wings, keep fix at room temp. Make sure the wings are flat after transferring into PBST. Then when all pupal wings are fixed, move the plate to ice.
3. Remove the peripodial membrane at this point if working on larval wings (not necessary).
4. Wash in abs. EtOH - 2×5 mins.
5. Incubate the wings in 1:1 (v/v) Xylene:EtOH mixture for 60 mins. **The solution will freeze within minutes of incubation. This will not affect the efficacy of the whole process. To remove the solution after incubation, move the wells to room temperature. Place them back on ice for the next step.
6. Wash in abs EtOH - 2×5 mins.
7. Rehydrate the wings by immersing in graded MeOH (80%, 50%, 25% in water) and finally in H_2O.
8. Incubate in 80% Acetone (in water) for 10 mins at -20°C.
9. Wash in PTwx/PBST - 2×5 mins.
10. Fix the wings again using 4% Formaldehyde (4.4 ml of 37% Formaldehyde in 36 ml of PBST) for 30 mins.
11. Wash in PTwx/PBST - 3×5 mins. (Ref: Nagaso, H., Murata, T., Day, N. & Yokoyama, K. K. Simultaneous Detection of RNA and Protein by In Situ Hybridization and Immunological Staining. **49,** 1177–1182 (2001)).
12. Gradual transfer to a standard Pre-hybridization buffer (5X saline sodium citrate pH 4.5, 50% formamide, 0.1% Tween20, final pH 5-6 at 22°C) 200 µl for each well.

- 3:1 PBST:Pre-Hyb buffer – 5 min
- 1:1 PBST:Pre-Hyb buffer – 5 min
- 1:3 PBST:Pre-Hyb buffer – 5 min
- Bring to the room temperature
- Pre-Hyb buffer only (tissue can be stored in the Pre-hyb at 4°C)

Note: You can keep the plate inside a covered container at 4 ℃ overnight.

13. Pre-incubation in Pre-hybridization buffer at 60°-65°C – **1 hr**. You don't need to wait till the incubator reaching to 60 ℃. Just place the plate into the incubator. But keep them minimum at 60 ℃ for 1 hour. Take out salmon sperm from freezer for the next step as it takes a while to thaw out.

14. Incubation in Hybridization buffer (Pre-hyb supplemented with 1g/L glycine, 100µg/mL denatured salmon sperm DNA and 30 ng/ml riboprobe) at 62°C for **12-16 hr.** This step is on the rotator shaker. Add 500 µl of Hybridization buffer for each well. Be fast because wings might dry out. The speed of the rotator shaker is 2.

(Place the dissection tray inside a water-tight container containing moist tissue pad; cover the tray with a parafilm to prevent condensed water droplets from dropping into the wells!).

To make 5mls (5000ul) Hyb:

Salmon sperm: (10,000ug/ml)(x) = (100ug/ml)(5mls) = 0.05ml (50ul)

Probe: 50-100 ng/ml

Glycine Stock = 100mg/ml, you need 1mg/ml: (100mg/ml)(x)=(1mg/ml)(5mls) = 0.05ml (50ul)

DAY 2

15. Wash in Pre-hybridization buffer at 60°C – 2 X 5 min.

16. Wash in Pre-hybridization buffer (50% Formamide, 2X SSC at 60°C – 5 X 10 min.

For 10 ml:
5 ml Formamide

1 ml 20X SSC
4 ml water
10 µl TWEEN

Note: This step is on the rotator shaker. After this step, switch off the machine.

17. Bring to room temperature. Gradually step back to PBST.
    - 3:1 Pre-Hyb buffer:PBST – 5-10 min, Room temperature.
    - 1:1 Pre-Hyb buffer:PBST – 5-10 min, Room temperature.
    - 1:3 Pre-Hyb buffer:PBST – 5-10 min, Room temperature.
    - PBST only

18. Wash in PBST – 2 X 5 min.

19. Incubate in Block buffer [PBST supplemented with 1% w/v bovine serum albumin] – 30-60 min at room temperature.

    100 ml of Block Buffer + 1 g of BSA (bovine serum albumin) or 30ml of BB + 0.3g BSA

20. Remove Block buffer. Incubate with 1:3000 dilution of anti-digoxigenin alkaline phosphatase Fab fragments in Block buffer – 30-60 min at room temperature.

    Eg: 1 µl Anti-Dig in 3ml of Block Buffer (3mls is enough for 9 wells)

    If you need to use less buffer than 3mls e.g. 2mls then: 2000/3000 = 0.67ul in 2ml

    Note: This step is on the rotator shaker. Don't keep the antibody tube outside too long. No heat.

21. Wash in Block buffer – 5 X 5 min on a rotatory shaker. Add 500 µl to each well.

22. Incubate in an alkaline phosphatase buffer (100 mM Tris- HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween20) – 2 X 5 min. Add 600 µl for each well.

    1 ml Tris (1M)

    400 µl NaCl (2.5M)

250 µl of MgCl2 (200mM)

10 µl of TWEEN

water till 10 ml

23. Remove alkaline phosphatase buffer. Incubate in NBT/BCIP mix added to alkaline phosphatase buffer at room temperature – till color develops (4-8 hrs). Develops in the dark.

    33 µl of NBT+ **16 µl** BCIP mix in **5 ml** alkaline phosphatase buffer

24. Wash in PBST supplemented with 2mM ethylene diamine tetra acetic acid (EDTA).

25. Mount in PBS containing 60% glycerol or Crystal Mount Aqueous Mounting Medium.