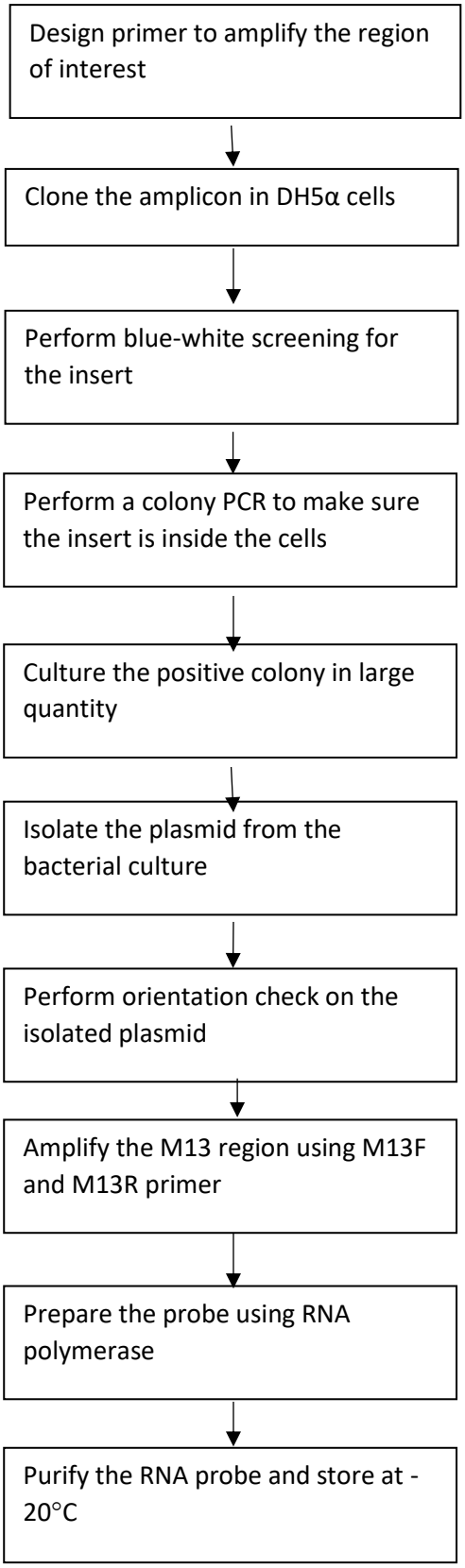
Whole-mount RNA in-situ hybridization on butterfly wings (v5.0)

Probe Preparation for *in-situ* Hybridization



Design of primers for amplification of DNA fragment of interest.

1. Copy the sequence of interest and paste it into the box on the webpage: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi.
2. Under the 'General Setting' tab change the values:
   Primer Tm: - Min: 55; Max: 65
   Primer GC%: - Min: 45; Opt: 60; Max: 60
   Max Tm Difference: - 3
   Under 'Advanced Settings' tab
   GC Clamp: 2
3. Click on the 'Pick Primers' tab in the top right corner.
4. Select the best set from the list of primers.

Amplification of DNA fragment of interest

5. Resuspend the lyophilized primers using molecular grade water to make a stock solution of 100 ng/µl. Prepare a working solution of 10 mM as described above.
6. Add the following reagents in a 200 µl PCR tube:

Table. Reaction mixture for amplification of DNA of interest.

Reagents	Volume (µl)
2X PCRBIO Taq Mix Red	12.5
Gene F	1
Gene R	1
Template (gDNA/plasmid/cDNA)	1
Molecular grade water	9.5

⚠ **CRITICAL STEP:** Prepare at least 5 tubes in order to identity the most optimal annealing temperature in a gradient PCR reaction.

7. Setup the gradient PCR reaction with following conditions:

Table. PCR condition for amplifying DNA of interest.

Temperature (°C)	Time (secs)	Number of cycles
95	60	1
95	15	40
Gradient (55-65)	15	
72	15	
4	∞	1

8. Run the reaction mixture in 1% agarose gel for 30 mins.
   ⏸ **PAUSE STEP:** The PCR reaction mixture can be stored at 4°C overnight.

Cloning of amplified DNA fragments (using pGEM®-T Vector System)

1. Add the following reagents in a 1.5 ml microcentrifuge tube (ligation mixture):

Table. Reaction mixture for ligating DNA of interest to plasmid.

Reagents	Volume (µl)

2X Rapid ligation buffer	5
pGEM®-T vector	0.5
Amplified DNA (200ng/µl)	0.5
T4 DNA ligase	1
Molecular grade water	3

2. Incubate the reaction mixture at 4°C for 16 hrs.
3. Take out 1 vial of competent cells and keep the tube on ice for 15 mins.
4. Transfer 5 µl of ligation mixture into the competent cell tube and tap gently to mix the solution.
5. Leave the mixture on ice for 30 mins.
6. Heat shock the cells by transferring the tube into a water bath at 42°C for 45 secs.
   ⚠**CRITICAL STEP:** Be careful not to exceed the heat shock step above 45 secs.
7. Transfer the tube into ice and leave it for 2 mins.
8. Add 500 µl of autoclaved LB broth and incubate the cells in bacterial incubation chamber at 37°C with shaking speed of 225 rpm for 2 hrs.
9. Centrifuge the tube at 3000 rpm for 4 mins.
10. Inside a biological safety cabinet add the following reagents to an LB agar plate:

Table. Reagents for the screening of positive bacterial colonies.

Reagents	Volume (µl)
IPTG	25
X-GAL	25
Ampicillin	25

11. Spread the reagents on the plate using glass beads and let the plate dry inside the hood.
12. Add 50 µl of supernatant from step 9 and spread across the plate using the glass beads.
13. Once dried, seal the plate using parafilm and incubate the plate inside a bacterial incubator at 37°C for 14 hrs.

Colony PCR on transformed clones

1. In a 1.5 ml microcentrifuge tube add 10 µl molecular grade water. Pick the transformed white colonies and transfer them into the tube. Vortex gently to homogenize the colony.
   ⚠**CRITICAL STEP:** Prepare at least 10 clones for testing.
2. Add the following reagents in 200 µl PCR tubes:

Table 21. Reaction mixture to verify DNA inserted into plasmid.

Reagents	Volume (µl)
2X PCRBIO Taq Mix Red	12.5
M13F primer	1
M13R primer	1
Homogenized clone	1
Molecular grade water	9.5

3. Setup the PCR reaction with the following conditions:

Table. PCR settings to amplify inserted DNA along with the M13 region of the plasmid.

Temperature (°C)	Time (secs)	Number of cycles
95	60	1
95	15	30
57	15	
72	15	

4	∞	1

4. Run the reaction mixture in a 1% agarose gel for 30 mins and note down the colonies with a single band of the expected size, e.g., those that don't have an empty plasmid.
5. Inside a laminar hood add 5 µl of Ampicillin stock solution into a test tube with 5 ml LB broth. Transfer 5 µls of homogenized cells from step 1. Do this step for every positive colony.
6. Incubate the tubes in a bacterial incubation chamber at 37°C and 225 rpm for 14-16 hrs.

Isolation of plasmids from transformed clones (using GeneJET Plasmid Miniprep Kit)

1. Harvest the cells in a 1.5 ml centrifuge tube at 3000 rpm for 5 mins (pellet can be stored in 40% glycerol at -80°C for future use).
2. Discard the supernatant and resuspend the pellet in 250 µl of resuspension buffer.
3. Add 250 µl of lysis buffer and mix by inverting the tube 6-10 times.
4. Add 350 µl of neutralization buffer and mix by inverting the tube 6-10 times.
5. Centrifuge at 14000 rpm for 5 mins and transfer the supernatant to GeneJET spin column.
6. Centrifuge the column at 14000 rpm for 30 secs.
7. Add 500 µl of wash buffer and centrifuge at 14000 rpm for 30 secs. Discard the flow through and repeat this step one more time.
8. Centrifuge the empty column at 14000 rpm for 1 min.
9. Transfer the column to a 1.5 ml microcentrifuge tube and add 20 µl of elution buffer or molecular grade water. Incubate the mixture at room temperature for 3 mins.
10. Centrifuge the column at 14000 rpm for 1 min and measure the concentration of plasmid using Nanodrop.
    - **PAUSE STEP:** Prepare a working concentration of 100ng/µl. The purified plasmid can be stored at 4°C for over one month. For long-term storage use a -20°C freezer.

Perform orientation check on the isolated plasmid

1. Perform PCR with following set of primers to see which one is giving band.

1	2	3	4
M13F	M13F	M13R	M13R
Gene F	Gene R	Gene F	Gene R

Orientation 1

You will observe band with Primer set 2 and 3. Use SP6 polymerase for AS probe.

Orientation 2

You will observe band with Primer set 1 and 4. Use T7 polymerase for AS probe.

M13 amplicon preparation for probe

1. Add the following reagents in 200 µl PCR tubes:

Table. Reaction mixture to verify DNA inserted into plasmid.

Reagents	Volume (µl)
2X PCRBIO Taq Mix Red	12.5
M13F primer	1
M13R primer	1
plasmid	1
Molecular grade water	9.5

2. Setup the PCR reaction with the following conditions:

Table. PCR settings to amplify inserted DNA along with the M13 region of the plasmid.

Temperature (°C)	Time (secs)	Number of cycles
95	60	1
95	15	30
57	15	
72	15	
4	∞	1

Run the reaction mixture in a 1% agarose gel for 30 mins.

RNA probe preparation

1. Add the following reagents in 1.ml tube:

Table. Reaction mixture for probe synthesis

Reagents	Volume (µl)
10x RNA polymerase buffer	2.5
Dig Mix	2.5
M13 amplified DNA	3
T7/SP6 polymerase	2
Ribolock	0.5
Molecular grade water	14.5

2. Incubate the tube at 37°C for 2 hrs.

Purification of RNA probe (via ethanol precipitation)

3. Add 80 µl of molecular grade water to the reaction tube from the previous step to raise the volume to 100 µl.
4. Add 10 µl of 3M NaOAc, 10µl Linear acrylamide, and 200 µl of 100% ethanol.
5. Vortex the mixture for 10 secs and store at -20°C for 15-20 mins.
6. Centrifuge the mixture at 4°C, 14000 rpm for 15 mins.
7. Carefully remove the supernatant.
   ⚠**CRITICAL STEP:** Be very careful not to disturb the pellet.
8. Dry the sample in a vacuum concentrator and add 20 µl of molecular grade water.
9. Prepare a stock concentration of 600 ng/µl by adding additional water (after a Nanodrop reading) and store aliquots at -20°C.
   ⏸**PAUSE STEP:** RNA can be stored at -20°C for over 1 year.

Cheers,

Tirtha

BUFFERS (make fresh)

1x PBST (Phosphate Buffered Saline with Tween® 20)

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

Note: Add 1x PBS before adding TWEEN

Fixative (<u>4% Formaldehyde</u> in 1x PBST)

55 µl of 37% formaldehyde in 500 µl of 1x PBST.

20x SSC

800ml dH_2O (RNase free if required)

175.3g NaCl (3M)

88.2g trisodium citrate

adjust the pH to 7.0 with a few drops of 1M HCl

adjust the volume to 1L with dH_2O

sterilize by autoclaving

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%)

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

1 ml Tris (1M; pH9.5)

400 µl NaCl (2.5M)

250 µl of $MgCl_2$ (200mM)

10 µl of TWEEN

water till 10 ml

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

1. Wipe the wells with RNAse zap.
2. Dissect wings in 1x PBS at room temperature. (Use standard silicone plates for dissection).
3. Transfer dissected wings to wells containing 1X PBST with a spatula.
4. Fix the wings using 4% Formaldehyde (0.055 ml of 37% Formaldehyde in 0.5 ml of PBST) for 30 mins.
5. After fixing transfer the plate to ice.

Note: For larval wings you can transfer the wings in 1x PBS on ice before fixation. For pupal wings transfer the wings with mixture of 1x PBST and 4% formaldehyde at room temperature. Fix the wings for 30 mins and then move on ice.

6. Wash in cold 1x PBST – 3x5 mins.

7. Add 1.25 µl of protenase K diluted in 1 ml of 1x PBST in the wells for 5 mins. Remove the solution.
8. Add 100 ul of glycine (100 mg/ml) diluted in 5 ml of 1x PBST in the wells for 5 mins.
9. Wash in cold 1x PBST - 3×5 mins.
10. Remove the peripodial membrane at this point if working on larval wings.

    Two alternative methods:

    1) Physical removal (requires fine tweezers)

    2) Chemical removal: Either via (a) glycolic acid or (b) acetone

    (a) glycolic acid

    For larval wings only: add 500 µl of glycolic acid solution (400 µl of 7% glycolic acid in every 1 ml of 1X PBST) and leave it for 5 mins.

    (b) acetone

    Wash in cold 70% EtOH for 2 mins

    Wash in cold 100% EtOH - 2×2 mins.

    Incubate the wings in cold 1:1 (v/v) Xylene: EtOH mixture for 60 mins.

    Note: The solution might 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.

    Wash in cold abs EtOH - 2×5 mins.

    Rehydrate the wings by immersing in graded MeOH (80%, 50%, 25% in cold 1x PBS) and finally in 1x PBST.

    Incubate in 80% Acetone (in cold 1X PBS) for 10 mins at -20°C.

    Wash in cold 1xPBST - 2×5 mins.

    Fix the wings again using 4% Formaldehyde (4.4 ml of 37% Formaldehyde in 36 ml of 1x PBS) for 30 mins on ice.

    Wash in cold 1x PBS - 3×5 mins.

    (Ref (for acetone method): 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)).

11. Gradual transfer to a standard Pre-hybridization buffer 500 µl for each well.
    - 1:1 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: a) Add 40 ul Salmon sperm DNA and 40 ul (100mg/ml) glycine in 4 ml prehyb buffer before incubation of the wings.

b) You can keep the plate inside a covered container at 4 ℃ overnight/over the weekend.

12. Pre-incubation in Pre-hybridization buffer at 60° °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.

13. Incubation in Hybridization buffer (Pre-hyb supplemented with 100 mg/ml glycine, 100µg/mL denatured salmon sperm DNA and 50-100 ng/ml riboprobe) at 60°C for **12-16 hr.** This step is on the rocker/orbital shaker. Add 500 µl of Hybridization buffer for each well. Be fast because wings might dry out.

    (Place the dissection tray inside a water-tight container containing).

To make 5mls (5000ul) Hyb:

Pre-hyb buffer: 4.9 ml

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

Probe: 50-100 ng/ml

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

DAY 2

14. Wash in Pre-hybridization buffer at 60°C- 5x10 mins.

Note: After this step, switch off the machine.

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

16. Wash in RT 1x PBST – 2 X 5 min.

17. Incubate in Block buffer [1x 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).

18. Remove Block buffer. Incubate with 1:3000 dilution of anti-digoxigenin alkaline phosphatase Fab fragments in Block buffer – 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 rocker/orbital shaker. Don't keep the antibody tube outside too long. No heat is required.

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

20. Incubate in an alkaline phosphatase buffer– 2 X 5 min. Add 500 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

21. 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

    26.4 µl of NBT+ **12.8 µl** BCIP mix in **4 ml** alkaline phosphatase buffer

    19.8 µl of NBT+ **9.6 µl** BCIP mix in **3 ml** alkaline phosphatase buffer

    13.2 µl of NBT+ **6.4 µl** BCIP mix in **2 ml** alkaline phosphatase buffer

22. Wash in 1x PBST supplemented with 2mM ethylene diamine tetra acetic acid (EDTA): 2x5mins (this step is not necessary if you are imaging immediately).

23. Mount in 1x PBS containing 60% glycerol or fluorescent Mounting Medium.