

Day 1 Re-fixing and equilibration (~5 hours)

- Start early and take lunch during re-fixing stage)

Reagent	Volume required/ week total (x n)	Storage	Function
95% Methanol	1 ml	Room	Serial Rehydration
75% Methanol/PBSAT	1 ml	Room	Serial Rehydration
50 % Methanol/PBSAT	1 ml	Room	Serial Rehydration
25% Methanol/PBSAT	1 ml	Room	Serial Rehydration
PBSAT (1xPBS + 0.1% Tween-20)	10.02 ml/ 12.02 ml	Room	Phosphate Buffered Saline helps to maintain a constant pH. + Tween-20 prevents tissues sticking together
Proteinase K 25 mg/ml (50-100 µl)	2 µl	-20°C	Removal of outer proteins allows better probe penetration for deeper tissues
TEA 0.1 M, pH 7-8 (~100-200 ml)	2 ml	Room	Triethanolamine - Buffer
Acetic Anhydride (125 - 250 µl)	5 µl	Fume hood	Enables probe to bind specifically to mRNA rather than nonspecifically due to electrostatic interactions
4% Paraformaldehyde in PBS pH 7.5	2 ml/3ml	-20°C	Fixative
Hybridization Buffer (-)	1.25 ml	-20°C	Used to equilibrate the specimens
Hybridization Buffer (+)	1ml/4.1 ml	-20°C	+ Yeast RNA to provide competition in non-specific binding

Rehydration:

- Wash 1 x 95% Methanol, 5 minutes on roller
- Wash 1 x 75% Methanol/PBSAT, 10 minutes on roller
- Wash 1 x 50% Methanol/PBSAT, 10 minutes on roller (Worms can be separated and sectioned at this stage in a petri dish)
- Wash 1 x 25% Methanol/PBSAT, 10 minutes on roller
- Wash 3 x PBSAT, 5 minutes on roller

Proteinase K treatment and fixation:

- Remove PFA from -20°C and thaw on ice
- Turn on oven @ 60°C for warming hybridisation buffer (+) later
- Thaw aliquot of Proteinase K on ice to make mix, (for 1 ml reactions mix 2 µl stock in 20µl PBSAT)
- Add 4 µl Proteinase K mix/ml PBSAT
- Incubate at room temperature for 10 minutes. Gently invert, but do not agitate
- Rinse 2 x in 1 ml TEA for 5 minutes on roller
 - On 2nd wash, add 2.5 µl acetic anhydride to TEA under fume hood. Swirl often and keep under hood
 - After 5 minutes add another 2.5 µl acetic anhydride for 5 minutes
- Wash 2 x with PBSAT for 5 minutes on roller
- Re-fix for 20 minutes - 1 ½ hours in paraformaldehyde in PBSAT on roller
 - Turn on heating block @ 60°C
- Wash 3 x for 5 minutes, then 2 x for 10 minutes in PBSAT, to wash off excess paraformaldehyde

- During PBSAT washes, aliquot (1.25 ml x n) hybridisation buffer (-), prepare (4.1 ml x n) hybridisation buffer (+) and incubate @ 60°C

Equilibrium of specimens/tissues in hybridisation buffer:

- Add 250 µl hybridisation buffer (-) to 1 ml PBSAT, invert, and allow specimens to settle (~ 1 minute)
- Remove the buffer and replace with 1 ml hybridisation buffer (-)
- Incubate @ 60°C for 10 minutes in heating block (@ 300 rpm). Invert before removing
 - Note: Remove Yeast RNA from -20°C during wait

Pre-hybridisation

- Keep samples @ 60°C throughout
- Make enough hybridisation buffer (+) for n x 4.1 ml. For 10 ml mix:

100µl Yeast RNA
10 ml hybridisation Buffer
- Note: Make sure to label (+)
- Replace the hybridisation buffer (-) with fresh hybridisation buffer (+) @ 60°C
- Seal tubes with parafilm
- Pre-hybridise overnight in rocking heater @ 60 °C (rocker @ 15 rpm, heating block @ 300 rpm)
- Store hybridisation buffer (+) @ -20°C for day 2 use

Day 2 - Hybridisation of probes (~ 45 minutes)

- Can be started anytime

Reagent	Volume required/ week total (x n)	Storage	Function
Hybridization Buffer (+)	1.1 ml/4.1 ml	-20°C	+ Yeast RNA to provide competition in non-specific binding
1 µl/ml digoxigenin-labelled riboprobe	~ 2 µl	-20°C	Probe

Hybridisation

- Warm buffer (+) (n x 1.1 ml) @ 60°C
- Replace hybridisation buffer with fresh hybridisation buffer (+) @ 60 °C
 - Note: Skip this step if re-using probe
 - Spin down unused probes before denaturing
- Denature probe ([~2 - 4 µg/ml] in 100 µl of 60°C hybridisation buffer [+]) for 3 minutes @ 80°C in PCR machine (setting 'probe denaturation')
 - Note: If re-using probe this can be done in heating block @ 80°C for ~ 4 minutes
- Add PCR tube contents to sample (1 ml buffer @ 60°C + 100 µl buffer [+ ~ 1 µg/ml probe])
- Hybridise overnight @ 60°C with the same motion settings as day 1 pre-hybridisation

Day 3 - Antibody Hybridisation (~ 7 hours)

- Start as soon as possible to take lunch break during one of the 30 minute 0.2 SSC washes

Reagent	Volume required/ week total (x n)	Storage	Function
Hybridization Buffer (+) @ 60°C	2 ml/4.1 ml	-20°C	+ Yeast RNA to provide competition in non-specific binding
2 x SSC + 0.1% Tween 20 @ 60°C	3 ml/3 ml	Room	Saline sodium citrate - controls stringency for washing steps
0.2 x SSC + 0.1% Tween 20 @ 60°C	3 ml/3 ml	Room	Saline sodium citrate - controls stringency for washing steps
MAB (100mM Maleic Acid, 150mM NaCL, pH 7.8, 0.1% Tween-20)	2 ml/8 ml	Room	Dilutes blocking solution
MAB + 2% (w/v) Blocking reagent (BMB) + 20% (v/v) Lamb serum	2 ml/2 ml	-20°C	Blocking of unspecific binding sites
Sheep Anti-DIG-AP FAB fragments (i.e. alkaline-phosphatase-coupled antibody)	0.5 µl	4°C	Enables detection of DIG labeled compounds

Probe removal and washing

- Turn on oven @ 60°C and incubate hybridisation buffer (+) and SSC x 0.2/x 2
 - Note: while changing solutions, keep samples @ 60°C at all times
- Remove probe solution from samples. Store probe in 1.5 ml tubes @ -20°C. Probes can be reused several times - often showing improved performance in successive runs (reduced background staining)
- Rinse x 2 in 1 ml of pre-warmed hybridisation (+) buffer, wash @ 60°C on rocking heater for 10 minutes
- Wash 3 x in 2 x SSC (warmed to 60°C) for 20 minutes on rocking heater
- Wash 3 x in 0.2 x SSC (warmed to 60°C) for 30 minutes on rocking heater
 - Remove Lamb Serum and BMB from -20°C
- Wash 2 x in MAB for 15 minutes @ room temperature for 15 minutes

Antibody incubation and washing

- Pre-incubate in 1 ml MAB + 2% BMB + 20% heat treated lamb serum and roll for 2 hours @ room temperature
 - Note: Make enough for 2 ml x n
- e.g. for 10 ml solution use:

1 ml BMB
2 ml Lamb serum
7 ml MAB

 - Spin down sheep DIG for 5 minutes before use + pipette from top surface of liquid
- Remove solution and replace with fresh solution containing 1/2000 dilution affinity purified sheep anti digoxigenin antibody coupled to AP (1 µl in 2 ml of previous solution)
- Place tubes in 50 ml Falcon tubes and secure on rocker (very gentle setting) overnight @ 4°C (put rocker in fridge)

Days 4 & 5 - Colour development and clearing

- Start in morning, day 4 can be finished in ~ 6 hours if the staining is complete within 2 hours.

Reagent	Volume required/ week total (x n)	Storage	Function
MAB	6 ml/8 ml	Room	Maleic Acid washing buffer
Alkaline Phosphatase Buffer	2 ml	Room	Inhibits endogenous phosphatases (make fresh on day)
NBT/BCIP	5 µl	4°C	Staining mix
PBSAT	2 ml/12.02 ml	Room	Buffer solution
4% Paraformaldehyde in PBS pH 7.5	1 ml/3 ml	-20°C	Increases rigidity of samples and further stabilises reaction
50% EtOH/PBS	1 ml	Room	Serial dehydration
75% EtOH/PBS	1 ml	Room	Serial dehydration
90% EtOH/PBS	1 ml	Room	Serial dehydration
100% EtOH	1 ml	Room	Serial dehydration
1:1 Benzyl Alcohol/Benzyl Benzoate	1.5 ml	Room	Clearing agent

Washing

- Remove antibody and keep at 5°C for future use (optional)
- Wash 3 x for 5 minutes with MAB, on roller
- Wash 3 x for 1 hour with MAB, on roller placed on rocking platform
 - Note: use largest volume possible (preferably, should be washed in larger vials...)
- Wash 1 x for 3 minutes in Alkaline Phosphatase Buffer @ room temperature
- Wash 1 x for 10 minutes in Alkaline Phosphatase Buffer @ room temperature

Chromogenic colour reaction

- Add 5µl/ml NBT/BCIP staining mix and place in the dark (cover in foil) until colour develops (varies from 1 - 24 hours, check every 30 minutes). If leaving overnight, specimens must be wrapped in foil and stored at 4°C
 - Note: Larvae stain much more quickly than adults (check after 15 minutes...)
- After staining wash 2 x in PBSAT for 15 minutes to stop the reaction
- Post fix specimens in 4% paraformaldehyde in PBS for 1 hour at room temperature, or overnight at 4°C. This increases the rigidity of the specimens and further stabilises the colour reaction

Clearing

- Gradually dehydrate on roller @ RT using ethanol dilution series:
 - 50% EtOH/PBS for 5 minutes
 - 70% EtOH/PBS for 5 minutes
 - 90% EtOH/PBS for 5 minutes
 - 100% EtOH/PBS for 5 minutes
- Transfer samples into pre-cleaned (100% EtOH) embryo dishes
- Clear dehydrated specimens in a 1:1 solution of Benzyl Alcohol:Benzoate by removing 500µl 100% EtOH and add 500µl BA:BB very slowly, so as not to introduce bubbles

Whole Mount In situ Hybridisation Protocol

- Allow to stand for 10 minutes, before removing EtOH/BA:BB and replacing with 1 ml BA:BB
- Specimens can now be imaged. Store specimens @ 4°C in BA:BB