WHOLE MOUNT mRNA *IN SITU* HYBRIDIZATION

by Pengfei Lu

- ***In situ* hybridization**
  - Pre-hybridization & hybridization (Day1):
    1. Rehydrate embryos in a MeOH:PBT series (90%→75%→50%→25% MeOH:PBT). Each wash is 10mins.
    2. Wash embryos two times in PBT.
    3. Bleach embryos in 6% H_2O_2/PBT (10ml 30% H_2O_2 to 40ml PBT) for 1hr at RT. Shake gently.
    4. Wash 3 x 10mins in PBT (examine conditions of embryos; make sure the mesh is not blocked).
    5. Pre-hybridize embryos/tissues in 1ml pre-hyb buffer (pre-warmed, in 10ml tubes) for 1hr at 70 ºC.

	16.846ml	33.712ml
Formamide	8ml	16ml
20X SSC, **pH4.5**	4ml	8ml
Yeast tRNA (20mg/ml)	30λ	80λ
20%SDS	800λ	1.6ml
Heparin (50mg/ml)	16λ	32λ
DEPC-H_2O	4ml	8ml

    6. Replace pre-hyb with 0.5ml hyb containing probes (dilute probe stock 1:200). Store pre-hyb at -20ºC; it can be re-used many times.
    7. Incubate O/N at 70 ºC.

  - Post-hybridization & antibody incubation (Day2):
    8. Remove baskets from individual tubes containing probes and wash embryos in Solution I (pre-warmed, 80ml each time) 2 X 30min at 70 ºC. Store probes at -20ºC; it can be re-used many times.

Solution I:	200ml
Formamide	100ml
20X SSC, **pH4.5**	40ml
20%SDS	10ml
ddH_2O	50ml

    9. Wash in 1:1 mixture (pre-warmed, 40ml:40ml) of Solution I and Solution II for 10mins at 70 ºC.

Solution II:	480ml
5M NaCl	48ml
1M Tris (pH7.6)	4.8ml
10% Tween20	4.8ml
ddH_2O	423ml

    10. Wash in Solution II (80ml each time) 3 X 5mins at RT.
    11. Treat embryos in 20ug/ml RNase (75λ 10mg/ml RNase to 50ml Solution II) at RT for 1hr.
    12. Wash in Solution II for 5mins at RT.
    13. Wash in Solution III (50ml) for 5mins at RT.

Solution III:	200ml
Formamide	100ml

20X SSC, **pH4.5**	20ml
ddH_2O	80ml

14. Wash in Solution III (pre-warmed, 75ml each time) for 2 X 30mins at 65 ºC.

❖ **Antibody staining and color reaction**

➢ Antibody blocking and staining:

1. Wash embryos in MABT buffer (0.1% Tween20 in MAB) 3 X 10mins at RT.
2. Incubate in 100ml Blocking Solution (MABT + 2% BMB reagent + 10% inactivated sheep serum + sodium azide) for 2hrs at RT. Shake gently. Blocking Solution is stored at 4 ºC until reuse.
3. Incubate in 100ml Antibody Solution (100ml Blocking Solution + 1:500 anti-Dig-Fab (200ul)) O/N at 4 ºC. Shake gently. Antibody Solution is stored at 4 ºC until reuse.

➢ Post-antibody staining washes and color reaction:

1. Wash embryos 5hrs-O/N at RT (1X5mins, 1X15mins, 1X30mins, 4X1hr or longer).
2. Wash in NTMT for 3 X 10mins.

NTMT:	500ml	3L
5M NaCl	10ml	60ml
1M Tris (pH9.5)	50ml	150ml
10% Tween20	5ml	30ml (or 3ml Tween20)
ddH_2O	to 500ml	to 3L

3. Transfer embryos a 24/48-well plate; remove excess buffer.
4. Incubate embryos in BM purple in the dark at RT until desirable signal intensity has been reached. This could range from several hours to several days.
5. To stop color reaction, fix embryos in 4% PFA for 1hr at RT and store in 100% MeOH at 4 ºC. Cover plate with Parafilm to reduce MeOH evaporation during storage.
6. Photograph embryos on a 1% agar plate (Φ=60mm).

- **Reagents/Solutions/Equipments:**

10ml tubes
30% H_2O_2 (Sigma)
Formamide (Sigma, F9037, SIGZR198)
SDS (Sigma, L6926-50G)
anti-Dig-Fab (Roche, 1093274, ROCZR223)
BM purple (Roche, 1442074)
BM Blocking Reagent (Roche, 1096176, ROCZR241)
Yeast tRNA (20mg/ml) (Sigma R8508)
Heparin (50mg/ml, 10,000 units in 1.11ml DEPC-H_2O) (Sigma H3393)
Sheep Serum
RNase

1N NaOH
5M NaCl
1M Tris pH7.6
1M Tris (pH9.5)

20X SSC, **pH4.5**
Adjust pH to 4.5 with HCl

pre-hyb buffer
Blocking Solution (MABT + 2% BMB reagent + 10% inactivated sheep serum + sodium azide)

10xMAB (pH7.5)

	1L	3L
Maleic acid	116 g	348 g
NaCl	174.9 g	525 g

NaOH	70 g	210 g
ddH_2O	to 1L	to 3L