

IN SITU HYBRIDIZATION PROTOCOL
BASIC SOLUTIONS TO BE USED FOR *IN SITU* HYBRIDIZATION

NOTE: On the day before usage, make the following solutions and set the incubator at 37C.

I. 4% Paraformaldehyde in DEPC-treated PBS.

	<u>100 ml</u>	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ Paraformaldehyde	4 gram	12 gram	20 gram	24 gram	36 grams

1. Heat PBS to 59-60C on hot plate/stirrer and then add paraformaldehyde in the fumehood.
2. Maintain the temperature of the solution at 59-60C until paraformaldehyde is dissolved.
3. **Store overnight in fridge.**

II. 0.1M Glycine in DEPC-treated PBS:

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ Glycine	2.25 gram	3.75 gram	4.5 gram	6.75 gram

1. Add glycine to PBS and stir until glycine is dissolved.
2. **Store at room temperature.**

III. 0.3% Triton in DEPC treated PBS:

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ Triton X-100	0.9 ml	1.5 ml	1.8 ml	2.7 ml

1. Add Triton to PBS and stir solution in the fumehood.
2. **Store at room temperature.**

IV. ProteinaseK solution in DEPC-treated water:

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ DEPC H ₂ O	240 ml	398.33	480 ml	720 ml
◆ 0.1M Tris	30 ml(of a 10X)	50 ml	60 ml	90 ml
◆ 0.05M EDTA	30 ml (of a 10X)	50 ml	60 ml	90 ml
◆ Proteinase K	1 ml	1.67 ml	2 ml	3 ml

1. **Put water in flask and keep it at 37C.** Next day: Add chemicals and mix.
2. Just before use, add the Proteinase K (1 μ g/ml). (The stock solution of Proteinase K in DEPC-treated water is in the freezer at 300 μ g/ml in 1 ml aliquots.

V. Autoradiography solution (1) in DEPC-treated water:

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ DEPC water	296 ml	492 ml	594 ml	888 ml
◆ Triethanolamide (10 mM)	4 ml	6.67 ml	8 ml	12 ml
◆ Acetic Anhydride(0.25)	0.75 ml	1.25 ml	1.5 ml	2.25 ml

1. Place DEPC water in conical flask and leave overnight at 37C.
2. Before use, add triethanolamide and acetic anhydride and stir until dissolved.
3. **Keep at 37C until needed.**

VI. Autoradiography solution (2) in DEPC-treated water:

VII. (To prevent non-specific binding of the Probe)

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ DEPC water	300 ml	500 ml	600 ml	900 ml
◆ N-ethylmalamide	0.375 gram	0.625 gram	0.75 gram	1.125 gram
◆ Iodoacetamide (0.01 M)	0.555 gram	0.925 gram	1.11 gram	1.665 gram

1. Place DEPC water in conical flask and keep at 37C overnight.
2. Before use, add N-ethylmalamide and iodoacetamide and stir in fumehood.
3. **Keep at 37C until needed.**

VII. 50% Formamide in 2X SSC:

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ <u>4X SSC:</u>	30 ml-20xSSC--	50 ml-20xSSC--	60 ml-20xSSC----	90 ml-20xSSC
	120 ml H2O	200 ml H2O	240 ml H2O	360 ml H2O
◆ <u>Formamide:</u>	150 ml	250 ml	300 ml	450 ml

1. Make 4 x SSC from 20x SSC in DEPC-H₂O , put in marked bottle and leave overnight at 37C.
2. Before use, add formamide and stir in the fumehood.
3. **Keep at 37C until needed.**

FOR POSTHYBRIDIZATION

VIII. RNase solution:

(to be made the day before post-hybridization)

Method I: (Usually for double staining and DIG-ISH)

	<u>300 ml</u>	<u>500 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ 2X SSC	300 ml	500 ml	600 ml	900 ml
◆ RNase (final conc. 20g/ml)	0.5 ml	0.83 ml	1.0 ml	1.5 ml

Method II:(For regular ISH)

	<u>300 ml</u>	<u>600 ml</u>	<u>900 ml</u>
◆ NaCl (4 M)	37.5 ml	75 ml	112.5 ml
◆ Tris (1 M)	3 ml	6 ml	9 ml
◆ EDTA (0.5 M)	0.6 ml	1.2 ml	1.8 ml
◆ dd Water	258.2 ml	516.6 ml	775 ml
◆ RNase (final conc. 20g/ml)	0.5 ml	1.0 ml	1.5 ml

1. Place 2X SSC (Method I) or dd Water(Method II) in labelled bottle and leave overnight at 42C.
2. Before use, add salts to solution (Method II) and stir on hot plate to dissolve.
3. RNase (12 mg/ml) stock solution in freezer made up in 1 ml aliquots in sterile water.
Add to prewarmed solution, stir and keep warm until needed.
Rnase from Sigma # R-4875 500 mg.

Basic solutions to be used for In Situ Hybridization

The solutions used for ISH must be RNase-free (except the RNase solution used in the post-hybridization stage) this is to ensure that the mRNA is not broken down before the probe is hybridized to the signal mRNA in the target tissue.

0.1 M Phosphate buffered saline (PBS)

	<u>1000 ml</u>
NaCl	8.7 g
KH_2PO_4	0.272 g
Na_2HPO_4 (anhydrous)	1.14 g

Make up to 1 litre using double distilled water.

For RNase-free PBS (to be used for ISH), add 1 ml of DEPC to 1000 ml of PBS. Let the solution stir at room temperature in the fumehood for a minimum of 2 hours. Autoclave for 25 mins to destroy the DEPC.

DEPC-treated water (DEPC water)

Add 1 ml of DEPC to every 1000 ml of double distilled water and let the solution stir for a minimum of 2 hours in the fumehood. Autoclave for 25 mins to destroy the DEPC.

Paraformaldehyde (4 %)

Heat RNase-free PBS on the magnetic stirrer in the fumehood until it reaches ~~59~~ 60 °C. Add preweighed paraformaldehyde powder (weigh it out using a mask !! and use 4 g per 100 ml) in the fumehood and stir continuously until dissolved (1.5 - ~~2 hours~~). The solution should not exceed 60 °C or else it will be useless for ISH tissue fixation. Cool to room temperature (RT) and use or store overnight in fridge and allow to come to RT before use. The solution should be used within 24 hours. For small batches, cool and aliquot in 10 - 50 ml portions and freeze at - 80 °C until needed.

15% Sucrose PBS

15 gram Sucrose | per 100 ml PBS DEPC treated.

Stir until dissolved.

Keep at R.T.