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In situ hybridisation protocol

Probe synthesis

1. PCR and cloning

- Design primers to amplify approximately 800-1000 bp fragment of the gene of interest using a Taq polymerase that leaves TA overhangs.
- PCR reaction with GoTaq DNA Polymerase (Promega)

	Reagent	Final concentration
5 µl	5x GoTaq Reaction Buffer	1x
2 µl	$MgCl_2$ (25 mM)	2 mM
1 µl	Nucleotide mix (10 mM)	0.2 mM
1 µl	FW primer (10 µM)	0.4 µM
1 µl	RV primer (10 µM)	0.4 µM
x µl	DNA template	> 0.5 µg/20 µl
0.25 µl	GoTaq DNA Polymerase (5 u/µl)	1.25 u
x µl	MQ to final of 25 µl	

- PCR reaction (optimise according to amplicon and primers):

95°C	5 minutes
95°C	1 minute
x °C	1 minute
72°C	1 minute per kb
Repeat step 2-4 29x	
72°C	5 minutes

- Check 1 µl of PCR on gel and purify using Qiagen PCR Purification Kit.
- Clone the PCR product into pCR II TOPO Dual Promoter vector (Invitrogen), purify using Qiagen Plasmid DNA purification Kit (Midiprep) and check the insertion direction by restriction analysis.
- Linearise the vector with appropriate enzyme (4-5h or overnight) using approximately 5µg of plasmid.
- Purify linearised vector with Quiagen PCR Purification Kit, elute in 20µl, check linearisation on gel and measure the concentration.

2. Restriction

- Linearise the vector with appropriate enzyme (4-5h or overnight) using approximately 5µg of plasmid.
- Purify with Quiagen PCR Purification Kit, elute in 20µl and check 1µl on gel, 1µl in nanodrop (or Qubit).

2. In vitro transcription

- RNAse free eppendorf, mix on ice

8µl	5x Transcription buffer
4µl	DTT (10x)
4µl	DIG-nucteotide mix (or FLUO)
2µl	Rnase inhibitor (RNAsin)
2µl	Enzyme (SP6, T7, T3)
1-2µg	DNA template
X µl	MQ to final of 40µl

- Incubate at 37°C for 4h to O/N
- Take a 2µl aliquote as no DNase ctr and add 2µl of DNase to the rest. Incubate for 30min at RT (or 37°C, check the enzyme). Stop the reaction by adding 1.6µl 0.5M EDTA.

3. Probe purification

- Use RNAeasy Kit
- Elute in 50µl of nuclease free MQ
- Take 2µl for gel and 2µl, freeze at -20°C or dissolve and store in Hybridisation buffer (100-200ng/ml)

Embryo preparation

- Fix the embryos in 4% PFA at 4°C overnight (or at RT according to the age of embryos – 4h for 4dpf)
- Dissect the brains out if needed
- Wash in PBST 2x5min
- Transfer fixed embryos through graded methanol:PBST series (1:3, 1:1, 3:1 – 5min each) into methanol. Keep in methanol at -20°C for at least one hour (or overnight).

In situ hybridization

Day 1 – probe incubation

- Rehydrate embryos in graded methanol:PBST series (3:1, 1:1, 1:3 - 5 min each)
- Wash twice in PBST, 5min each
- Digest embryos with protein kinase at RT (dilute 1000x PK stock)

<2ss	no PK
2-10ss	in and out
10-15ss	1min
16-26ss	2min
24h	10-15min
30h	20min
36-48h	25min
>48h	30min
6 days brains	3min
9 days brains	7min

- Rinse with PBST
- Post-fix in 4% PFA for 20min
- Wash with PBST 4x5min
- Incubate in pre-Hyb for 1h at 70°C
- Hybridize the samples in probe/Hyb at 70°C overnight
  - ✓ For probes with concentrations <150ng/ul use 2ul probe in 400ul of Hyb mix
  - ✓ For probes with concentrations >150ng/ul use 1ul probe in 400ul of Hyb mix

Day 2 – washes, antibody incubation

- Washes at 70°C
  - ✓ 100% Hyb rinse
  - ✓ 75% Hyb/25% 2xSSC, 15min
  - ✓ 50% Hyb/50% 2xSSC, 15min
  - ✓ 25% Hyb/75% 2xSSC, 15min
  - ✓ 2xSSC, 15min
  - ✓ 0.2xSSC, 2x30min
- Washes at RT
  - ✓ 75% 0.2xSSC/25% PBST, 10min
  - ✓ 50% 0.2xSSC/50% PBST, 10min
  - ✓ 25% 0.2xSSC/75% PBST, 10min
  - ✓ 100% PBST, 10min
- Block in MaBl (2% sheep serum + 2mg/ml BSA in 150mM Maleic acid buffer) for >2h at RT
- Incubate in anti-DIG(or FLUO)-AP Fab fragments (Roche) at a 1:5000 dilution or in anti-DIG(or FLUO)-POD Fab fragments at a 1:1000 dilution in 1% block at 4°C O/N

Day 3 – washes, staining

For AP-conjugated antibodies:

1. BCIP/NBT in situ

- Wash with PBST 8x15min
- Wash in stain buffer 3x5min:

- Prepare 10ml of 1M Tris, pH 9.5 by mixing 9.44ml of 1M Tris Base and 0.56ml of 1M Trizma HCl
- Prepare the stain buffer (50ml)
    1. 5ml 1M Tris, pH 9.5
    2. 2.5ml 1M $MgCl_2$
    3. 1.25ml 4M NaCl
    4. 25ul 20% Tween-20

- Incubate embryos on stain solution (1ul NBT and 3,5ul BCIP in 1ml of stain buffer) for 30 minutes in dark, then keep checking if signal can be detected. Can take hours depending on the probe.

2. Fast red in situ (Roche)

- Wash with PBST 8x15min
- Wash with 0.1M Tris-HCl, pH8.2, 0.1% Tween20 for 10 min
- Dissolve Fast red tablets (Roche) in 0.1 M Tris-HCl, pH 8.2, 0.1% Tween20 (1 tablet in 2ml) and filter the solution through 0.22um filter unit.
- Develop the signals by incubating the samples in Fast Red solution (usually O/N at 4°C) in dark
- Wash with PBST 4x15 min (or more)

2. Fast red in situ (Sigma)

- Wash with PBST 8x15min
- Dissolve Fast red tablet and NAMP tablet in 1ml of mQ, filter and mix together
- Develop the signals by incubating the samples in Fast Red solution at RT or O/N at 4°C in dark
- Wash with PBST 4x15 min (or more)

3. Fast blue (Sigma)

- Wash with PBST 8x15min
- Prepare 10ml staining buffer (SB8.2)
    - 1ml Tris, pH 8.2
    - 250ul 4N NaCl
    - 500ul M1 $MgCl_2$
    - 50ul 20% Tween-20
    - 8.2ml mQ
- Prepare FastBlue solution 0.5mg/ml – from 100mg/ml stock (3ul in 597ul of SB8.2)
- Prepare NAMP buffer 0.5mg/ml – from 100mg/ml stock (3ul in 597ul of SB8.2)
- Add NAMP to FastBlue, stir while adding
- Develop the signals by incubating the samples in Fast Blue solution at RT or O/N at 4°C in dark. Signal is in far red but can check the colorimetric signal (embryos turn orange and blue crystals in the expression area)

For HRP-POD conjugated antibodies – TSA

- Wash with PBST 8x15min
- Spin down TSA Plus Cy3 (or TSA Plus Cy5 or TSA Plus Fluorescein) substrate and dilute 1:50 in Amplification Diluent buffer (from the kit).

  Tyramide reagent (Cy3, Cy5, fluorescein) stock solution – take a new tube of lyophilized tyramide reagent and add 60ul of DMSO. Make 10ul aliquotes and store at -20℃, protected from light.

- Incubate 45-60 minutes in TSA Plus Cy3 solution.
- Wash 3x10min in PBST

Double fluorescent *in situ* hybridisation with TSA

Notes:

- ✓ When doing a double *in situ*, FLUO-labelled probe should be used for mRNA that is expressed at higher levels (FLUO labelling is weaker than DIG).
- ✓ Cy5 should be used for mRNA that is expressed at higher levels (Cy5 detection is weaker than Cy3 or fluorescein and also has to be done as second staining).
- ✓ If mRNAs are expressed at similar levels, Cy5 staining should be used with DIG-labelled probe and Cy3 staining with fluorescein labelled probe.

1. Probe incubation

- Optional - for tyramide staining add 5% dextran sulphate to Hyb
- Incubate 5min in Hyb+ at 70°C (lower to 65°C if immuno follows)
- Change the Hyb+, incubate for 1.5h at 70°C (65°C)
- Incubate in probe solution, one probe DIG-labelled the other FLUO-labelled. For each probe:
  - ✓ If probe concentration is <150ng/ul use 2ul of probe per 400ul of Hyb+
  - ✓ If probe concentration is >150ng/ul use 1ul of probe per 400ul of Hyb+

2. Probe removal

- Remove probe – save to reuse next time
- Wash twice at 70°C in 50% formamide/2xSSCT for 30min
- Wash at 70°C in SSC + 0.1% Tween for 15min
- Wash twice at 70°C in 0.2xSSC for 30min (removing detergent from this washes from here onwards helps along with the staining but also makes embryos sticky)

3. Anti-fluo antibody incubation

- Block with MAB solution for 3hours at RT. MAB – maleic acid buffer (150mM maleic acid + 100mM NaCl, pH 7.5 + 2% Sheep serum +2mg/ml BSA) = Diz's MaBl stock
- Add anti-FLUO POD (Boehringer) at 1:500 into block and incubate at 4°C O/N
- Wash 6x20min in PBST at RT

4. Detection of fluorescein-labelled probe

- Keep the embryos on dark!
- Spin down TSA Plus Cy3 (or TSA Plus Fluorescein) substrate and dilute 1:50 in Amplification Diluent buffer (from the kit).

  Tyramide reagent (Cy3, Cy5, fluorescein) stock solution – take a new tube of lyophilized tyramide reagent and add 60ul of DMSO. Make 10ul aliquotes and store at -20°C, protected from light.

- Incubate 45-60 minutes in TSA Plus Cy3 solution.
- Incubate in 6% H_2O_2 in PBS for 20 min. to inactivate POD
- Wash 3x10min in PBS.

5. Anti-DIG antibody incubation

- Block with MAB solution for 3hours at RT
- Add anti-DIG POD (Boehringer) at 1:1000 into block and incubate at 4°C O/N
- Wash 6x20min in PBST at RT

6. Detection of DIG-labelled probe

- Spin down TSA Plus Cy5 (or TSA Plus Fluorescein) substrate and dilute 1:50 in Amplification Diluent buffer (from the kit).
- Incubate 45-60 minutes in TSA Plus Cy3 solution.
- Wash 3x10min in PBST (washes contain detergent again from here onwards)