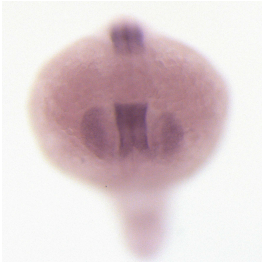
Jun 05, 2025 Version 2

Chromogenic in situ hybridisation V.2



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We use this protocol and it's working

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Abstract

The protocol for performing chromogenic *in situ* hybridisations in zebrafish embryos and larvae in the Wilson lab at UCL.

Image Attribution

Expression of *pax6a* in a 18-somite stage zebrafish embryo. In situ performed by the author.

Materials

Probe synthesis reagents

5x Transcription Optimised Buffer - Promega
DTT - Promega
10x DIG RNA labelling mix - Roche
T3/T7/SP6 polymerase - Promega

Buffers and solutions

Hybridization buffer (50ml):

Formamide	25 ml
20x SSC	12.5 ml
Torula RNA	500 µl
100mg/ml Heparin	25 µl
20% Tween-20	250 µl
1M citric acid	460 µl
MiliQ H_20	11.27 ml

Store at -20°C

Blocking Buffer:
PBST
2% sheep serum
0.2% BSA

20x SSC:
Final concentration
3M NaCl
0.3M $Na_3C_6H_5O_7$
Autoclave and store at room temperature

AP (alkaline phosphatase) buffer:
Final concentration
50mM $MgCl_2$
100 mM NaCl
100mM tris-HCL (pH 9.5)
0.1% Tween-20
Make fresh every time

Bleaching solution:
Final concentration
3% H_2O_2

0.5% KOH
Make fresh every time

Probe design

1 This *in situ* uses Digoxigenin-labelled antisense RNA probes. Probes are transcribed from DNA templates, which are themselves generally amplified by RT-PCR of mRNA extracted from zebrafish embryos, larvae or tissues. Antisense transcription is achieved by the addition of a promoter sequence to the reverse primer used in the RT-PCR. Add the following sequence to the beginning (5' end) of your reverse primer, depending on the polymerase being used:

T7 - GGATCCTAATACGACTCACTATAG
T3 - GGATCCATTAACCCTCACTAAAGG
SP6 - TATTTAGGTGACACTATAG

Generally speaking, T7 is the best of the three polymerases.

Alternatively, the PCR product can be cloned into a expression vector (e.g. a TOPO vector) such that one of these promoters is at the 3' end of the template. This allows for long term storage and re-use of the probe, as the plasmid can be amplified by bacterial transformation.

Probe length can vary from <200 to >1000 nucleotides. Somewhere in the middle of this range is usually considered ideal. The longer the probe, the more difficult it is for it to penetrate the sample. Shorter probes meanwhile are more prone to off-target binding, especially to similar mRNAs (e.g. paralogues).

Probe synthesis

2h 15m

2 If the template DNA is a plasmid, it must first be linearised by restriction enzyme digestion. If it is a PCR product, it can be used directly.

2h

Prepare probe synthesis reaction mix

Template DNA (PCR product or plasmid) - 0.5-1 µg
10x DIG RNA mix (Roche) - 2 µL
10x transcription buffer - 2 µL
RNA polymerase (T3/T7/SP6) - 2 µL
100mM DTT - 2 µL
RNase inhibitor - 0.5 µL
dH_20 to final volume 20 µL

Mix and incubate for 02:00:00 at 37-40 °C (temperature depending on the polymerase you are using-see polymerase sheet). Longer incubations, up to overnight, can be performed if yield is low with 2 hrs incubation.

3 OPTIONAL: add 2 µL of DNase1 to remove the template DNA; 00:15:00 at 37 °C. We tend to skip this step. If you are doing a precipitation step it's better if you have the DNA template because it helps the precipitation of your RNA. The DNA is not DIG labelled so it does not affect your in situ reaction. 15m

4 **Probe purification**

The best approach here is to perform a double purification of the probe, which removes almost all unicorporated nucleotides. This can allow you to use the probe at higher concentrations from the start without high background staining.

First, perform a purification with Sephadex G-50 columns, such as ProbeQuant columns. If unavailable, use another RNA/probe purification kit. Follow the manufacturers instructions.

Equipment

ProbeQuant G-50 micro columns	NAME
Cytiva	BRAND
28903408	SKU

5 Next, perform ethanol precipitation on the elute: 1h
Add 1/10 volume of 3M sodium acetate (pH 5) and 3 volumes of ice-cold 96-99% ethanol and mix well.
Incubate at -20 °C for at least 01:00:00 (longer incubations recover more RNA).
Centrifuge the mixture at 12000 x g, 4°C, 00:15:00. Dispose of the supernatant.
Wash the pellet by gently adding ice-cold 70% ethanol and centrifuge at 12000 rpm, 4°C, 00:10:00. Dispose of the supernatant.
Dry the pellet until the remaining ethanol has just evaporated. Drying for too long will impair subsequent resuspension.

Resuspend in nuclease free water.

6 We find it best practice to at this point, **check probe yield and integrity**. Measure concentration using a Nanodrop; there should be no less than 100ng/uL. Run on a 1% agarose gel, ideally there should be crisp bands (smearing on a gel indicates RNA degradation)

7 **Store probe**

Store the probe at -20 °C diluted at least 1:1 in hybridization buffer initially (the formamide protects the probe from degradation). The working concentration of a given probe is variable and requires optimization. Generally, 1:100, 1:250 or 1:500 are good starting points.

Sample collection

4h 15m

8 Perform in Eppendorf tubes or well plates, depending on number of samples. 4h 25m

Fix embryos/larvae at desired stage in 4% PFA in PBS for 04:00:00 at room temperature or Overnight at 4 °C.

Wash x4 with 1x PBS, 00:05:00 per wash.

Note

If you haven't used PTU and wish to bleach your larvae, do so at this point.
Incubate in bleaching solution (3% H_2O_2 and 0.5% KOH in water) for up to 1 hr.

Peroxide decomposition releases O_2 gas. DO NOT perform bleaching in a closed tube.

Observe the larvae every 5-10 mins and stop bleaching by washing with PBS x2 once the desired pigmentation reduction has been achieved.

9 Wash samples into methanol. Start with 50% for 00:10:00 at room temperature, then 100%. 20m

Store samples Overnight at -20°C in methanol.

Embryos or larvae can be stored in methanol at -20°C for several months.

In situ: Day 1 (Probe hybridisation)

4h 45m

10 Rehydrate samples: 20m

75% methanol/PBST 00:05:00
50% methanol/PBST 00:05:00
25% methanol/PBST 00:05:00
PBST 00:05:00 (x4)

> Note
>
> Bleaching can also be performed here.

11 Permeabilize samples with proteinase K digestion at room temperature. Dilute proteinase K stock (10mg/ml, 1000x) in PBST. Concentration and duration are stage dependent. 1h

3-9 somites - 1x for 30 s-1 min
10-18 somites - 1x for 2 mins
24 hrs - 1x for 10 mins
2-3 dpf - 2x for 20-30 mins
4-6 dpf - 3x for 45mins - 1 hr
Larval brains - 1x for 1 min (or not at all)
Juvenile brains - 2x for 20 mins

Quickly wash twice with PBST (no incubation) to stop digestion.

12 Post-fix with 4% PFA for 00:20:00 at room temperature. 25m

Wash x4 with PBST for 00:05:00 each.

Note

An alternative to proteinase K is incubation in ice-cold 80% acetone at -20°C for a duration dependent on stage.

1 dpf - 5 mins
2-3 dpf - 10 mins
>4 dpf - 20 mins

Wash back into PBST afterwards. No post-fixation necessary after acetone permeabilisation.

13 Pre-hybridize samples by incubation in hybridization buffer for 02:00:00 at 68 °C . 4h

Remove hybridization buffer and add probe diluted in the same buffer.

Incubate Overnight at 68 °C .

In situ: Day 2 (Antibody binding) 5h

14 Remove probe and save for reuse (working probe dilutions can be used many times). 1h

Wash once with 2x SSC for 00:30:00 (perform at 68 °C).

Wash twice with 0.2x SSC for 00:30:00 each (perform at 68 °C).

Note

SSC washes determine the stringency of probe binding. Low salt, high temperature conditions cause partial probe/mRNA duplexes to unravel, thereby reducing off-target staining of low complimentarity mRNAs.

15 Wash with PBST for 1 hr with several changes. 4h

Note

The times given for washes are the minimum times. In the experience of some lab members, elongating the PBST washes (especially those directly post-probe, above) can significantly reduce background staining. These washes can even be extended overnight, adding an extra day to the protocol.

Block in Blocking Buffer at room temperature for at least 02:00:00

Incubate samples in anti-DIG-AP Fab fragments (Roche) diluted 1:6000 in Blocking Buffer Overnight at 4 °C. Leave samples on a plate rocker if possible.

In situ: Day 3 (Development) 25m

16 Wash x3 with PBST. 25m

Wash multiple times in fresh AP buffer over 1-2 hrs.

Prepare NBT/BCIP (Roche) solution (1 µL NBT, 3.5 µL BCIP, 1 mL AP buffer) just prior to use.

Develop in situs in NBT/BCIP solution at room temperature. Protect samples from light at this point (e.g. cover with aluminium foil). Check development under a microscope every 5-10 minutes initially.
It is easier to do this if the samples are in a plate or dish. Depending on the probe/target mRNA, development can take minutes to hours (even overnight/several days).

Note

Concentration of probe, anti-DIG fragments and NBT/BCIP solution all affect the final staining intensity, development time and the amount of background staining. All can be freely varied, however you risk getting no staining or excessive background by doing so. Sometimes it is necessary to perform an *in situ* mutliple times to optimise these parameters, especially when using a new probe.

Stop reaction with two quick PBST washes.

Post-fix with 4% PFA for 00:20:00 at room temperature.

Wash x3 with PBS.

Transfer samples to glycerol through a graded series of glycerol/PBS washes (25%, 50% and 75%) for 00:05:00 each. Store samples in 100% glycerol at 4 °C, in the dark.

Image samples under a compound microscope or stereoscope, mounted in either glycerol or low-melting agarose in 80% glycerol.