In Situ Hybridization

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April 17, 2013

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Reagents

AP Buffer

Component	Location	Amount (per 50 ml)	Amount (per 250 ml)	Final
1M Tris pH 9.5	RT	5 ml	25 ml	100 mM
0.5 M MgCl2	RT	5 ml	25 ml	50 mM
5M NaCl	RT	1 ml	5 ml	100 mM
20% Tween-20	RT	250 µl	1.25 ml	0.1%

Developing Solution

Component	Location	Amount (per 1 ml)	Amount (per 5 ml)
AP Buffer	RT	1 ml	5 ml
100 mg/ml NBT	Freezer ISH components	4.5 µl	22.5 µl
50 mg/ml BCIP	Freezer ISH components	3.5 µl	17.5 µl

Hybridization buffer

To a 50 ml conical tube add:

Component	Location	Amount (per 50 ml)	Final
Formamide	4˚C	25 ml	50%
20X SSC	RT	12.5 ml	5X
Heparin (50 mg/ml)	Freezer ISH components	50 µl	50 µg/ml
Yeast tRNA (50 mg/ml)	Freezer ISH components	500 µl	500 µg/ml
20% Tween	RT	250 µl	0.1%
H_20	Sterile water	10.8 ml	
1M Citric acid, pH 6.0	RT	460 µl	

Label and date
Store up to 2 months at -20˚C

PBT

50 ml 1X PBS + 250 µl 20% Tween-20

PI Buffer (Blocking solution)

Component	Location	Amount (per 10 ml)	Amount (per 50 ml)
PBT	RT	9.6 ml	48 ml
BSA (100 mg/ml)	Freezer ISH components	200 µl	1 ml
Sheep serum	Freezer ISH components	200 µl	1 ml

SSCTween

50 ml 2X SSC + 250 µl 20% Tween-20 (prevents sticking)

Stop Solution

Component	Location	Amount (per 10 ml)	Amount (per 50 ml)
1x PBS (pH 5.5)	RT	9.9 ml	48 ml
EDTA (0.5 M)	RT	20 µl	100 µl
20% Tween20	RT	50 µl	250 µl

Riboprobe template preparation (from plasmid)

1. Set up digests of 10 µg of plasmid in 100 µl:

Component	Volume
DNA	Vol. to 10 µg
H_20	to final volume of 100 µl (including Buffer and enzyme)
Buffer	10 µl
Enzyme (10u/ µl)	2 µl

2. Incubate at 37˚C for 1-2 hours or O/N.
3. Run 1 µl on a gel to check for complete linearization.
4. Phenol:chloroform extract:
    a. Bring volume up to 200 µl w/H_20.
    b. Add 100 µl Tris saturated phenol.
    c. Add 100 µl chloroform.
    d. Vortex.
    e. Spin 2 minutes at maximum speed at RT.
    f. Carefully transfer top aqueous layer to a clean tube.
5. Chloroform extract:
    a. Add 200 µl chloroform.
    b. Vortex
    c. Spin 2 minutes at maximum speed at RT.
    d. Carefully transfer top aqueous layer to a clean tube.
6. Ethanol precipitate:
    a. Add 1/10 volume 3 M NaOAc (pH 5.2).
    b. Add 2 volumes 100% EtOH.
    c. Vortex.
    d. Spin 15 minutes at maximum speed at 4˚C.
    e. Carefully pour off supernatant.
7. Wash pellet by adding 1 ml 70% EtOH.
    a. Spin 5 minutes at maximum speed at 4˚C.
    b. Carefully pour off supernatant and air dry, inverted
8. Resuspend in 40 µl TE.
9. Proceed to Riboprobe synthesis or store at -20˚C.

Riboprobe template preparation (from genomic DNA)

1. PCR reaction to generate probe synthesis template

   Assemble the following in a PCR tube:
   a. Genomic DNA (~200 ng)
   b. ddH20 to 25 µl final volume
   c. 2.5 µl PCR buffer (10x stock)
   d. 0.5 µl dNTPs (10 mM)
   e. 1 µl 10 µM stock forward primer
   f. 1 µl 10 µM stock reverse primer
   g. 0.5 µl Taq Polymerase

   Perform a standard PCR reaction (adjust annealing temp if needed):
   95°C for 3 min with repetition of
   95°C for 30 sec,
   55°C for 30 sec,
   72°C for 30 sec for 30 cycles, then
   72°C for 10 min.

   Check an aliquot (1 µl) on an agarose gel to make sure the PCR product is the expected size.

Riboprobe synthesis

1. DIG-labeled probe synthesis (this is for T3, which uses a 5x buffer and requires a DTT supplement) – adjust accordingly if using a different polymerase with different reaction requirements (e.g., some brands include the DTT in a 10x buffer):

PCR reaction (not purified)	
or prepared template	2 µl
DEPC H20	5 µl
10x NTP mixture	2 µl
(10mM, ATP, CTP, GTP and 6.5 mM UTP)	
10x DIG-UTP (3.5 mM)	2 µl
5xTranscription Buffer	4 µl
DTT	2 µl
RNase Inhibitor (40 U/ µl)	1 µl
T3 Polymerase (17 U/ µl)	2 µl
	(total volume: 20 µl)

2. Incubate at 37˚C for 2 hours.
3. Remove 0.5 µl to confirm by gel electrophoresis (should see RNA *and* DNA template).

4. Add 2 µl RNase-free DNase (10 U/ µl) to reaction tube and incubate at 37˚C for 15 minutes.
5. During DNase incubation, prepare column (GE G-50 column).
6. Break off bottom.
7. Spin at 3K rpm for 1 min to remove liquid.
8. After the DNase incubation, transfer the probe mix over prepared column.
9. Spin for 2 min at 3K rpm **into a fresh tube**.
10. Remove 0.5 µl and run on a gel with the pre-DNAse treatment product (step 3)
11. Test probe and if it's good, make aliquots and store at -20˚C to avoid repeated freezing and thawing.

ISH Protocol

Day 1

Embryo preparation and fixation

1. Put up to 10 embryos each in microfuge tubes.
2. Under the hood, add 4% PFA to the tubes – WEAR GLOVES.
3. Incubate for 4h at RT or O/N @ 4˚C.
4. **If embryos have pigmentation bleach them before dehydration**.
    a. Incubate the embryos at room temperature in a 3% H_2O_2 /0.5% KOH medium until pigmentation has completely disappeared

       (Per ml: 100 ul of 30% solution of H_2O_2 [this is how it comes], 250 ul of 2% KOH, 500 ul H_20).

       This takes between 30 min and 1 h; progress can be checked by observing the embryos under a dissecting scope. There will be a lot of bubbles formed in the medium – DO NOT CAP TUBES DURING THIS PROCESS – THEY WILL POP. (HYDROGEN PEROXIDE SOLUTION SHOULD BE PREPARED FRESH IMMEDIATELY BEFORE USE).
    b. Wash the embryos 2x for 5 min each in 1x PBS to remove the H_2O_2 and stop the bleaching reaction.
    c. Progressively dehydrate the embryos by washing for 5 min in 33% MeOH, 66% MeOH and 100% MeOH.
5. **Store in MEOH at -20˚C for at least 2 hours before proceeding (or store long-term for later use).**

Hybridization

How you start, depends on whether you are using freshly fixed embryos or embryos that have been stored in MeOH.

- Freshly fixed embryos:
    a. Wash 5x, 5 min in PBT to remove PFA.
    b. Remove chorions if not already done:
        i. Transfer to Petri dishes and remove chorions.
        ii. Transfer embryos back to microfuge tube (~10/tube).
        iii. Wash 2x, 5 min PBT.
        iv. Remove chorions, if not already done.
- MeOH embryos (from storage) must be rehydrated:
    a. Wash 1X, 5 min in 66% MeOH:33% PBT.
    b. Wash 1X, 5 min in 33% MeOH:66% PBT.
    c. Wash 2X, 5 min each in PBT.

1. Treat with Proteinase K (ProtK) to permeabilize using the following incubation times at RT (or only if embryos are >20 somites).
    a. Treat embryos with 10 µg/ml (in PBT) of Proteinase K for the following time periods. DON'T OVER TREAT EMBRYOS!
        i. 3-5 somites 1 min
        ii. 8-10 somites 3 min
        iii. 16-20 somites 5 min
        iv. 24 hpf 10 min
        v. 30-36 hpf 15 min
    b. Fix embryos again in 4% PFA for 20 min at RT.
    c. Wash 5x, 5 min each with PBT.
2. Warm hybridization solution to 70˚C.
3. Add ~500 µl warm prehyb solution to each tube with embryos.
4. **Pre-hybridize embryos for 2-4 hours at 70˚C.**
5. Prepare hybridization solution with labeled probe of choice – start with a dilution of 1:200 probe:hybridization solution in a volume of 200 µl per tube. New probes may need to be titered.
6. **Hybridize O/N at 70˚C in water bath.**

Day 2

Washing out the probe

1. **Prewarm** the first 5 wash solutions – washes.
2. Remove tubes from water bath and
   SAVE THE PROBE IN THE HYBRIDIZATION SOLUTION AND STORE AT -20˚C– IT CAN BE RE-USED!!
3. 1x quick wash in prewarmed hyb solution
4. 1x, 5 min 66% hyb:33% 2X SSC at 65˚C.
5. 1x, 5 min 33% hyb:66% 2X SSC at 65˚C.
6. 1x, 5 min 2X SSC Tween at 65˚C.
7. 2x, 20 min 0.2X SSC Tween at 65˚C.
8. 1x, 5 min 66% 0.2X SSC:33% PBT at RT
9. 1x, 5 min 33% 0.2X SSC:66% PBT at RT
10. 1x, 5 min PBT at RT

Antibody (Ab) binding

1. Remove PBT and add 500 µl PI buffer. Incubate embryos for 2-4 hours in PI (blocking) buffer at RT to saturate non-specific binding sites for the antibody.
2. Prepare 1:5000 dilution of anti-dig Ab solution (1 µl anti-dig-AP Fab Ab to 5 ml of PI buffer). This dilution can be made using pre-bound Ab to further reduce non-specific staining.
    a. Pre-bind Ab by making a 1:100 dilution of the antibody in PI buffer. Incubate with fixed/dechorionated embryos that have been rocked for at least 2 hours at RT. Pre-bound Ab can be stored at 4 ˚C. To make a final dilution of 1:5000 of the antibody – dilute 4 ul of pre-bound Ab per 200 ul of PI buffer)

3. Remove PI buffer and add ~200 µl of 1:5000 dilution of anti-dig-AP Fab Ab.
4. Incubate **O/N at 4˚C** with tubes on their sides (rocking is not necessary but make sure embryos are covered with Ab-containing buffer).

Day 3

Develop

1. Wash 2x rapidly with PBT.
2. Wash 6x, 15 min with PBT on rocker. (At this point you can store O/N at 4˚C and wash the next day).
3. Wash 3x, 5 min in AP buffer. During washes, prepare developing solution (500 µl per tube). Do not leave embryos in AP buffer for extended periods of time.
4. Remove wash solution and add 500 µl of developing solution to each tube.
5. Transfer embryos to 9-well glass ISH dishes **USING A GLASS PASTEUR PIPET**, **(DO NOT USE A REGULAR PIPET TIP, THE EMBRYOS WILL STICK TO THE INSIDE WALL OF THE TIP!).**
6. Store in the dark (place the top of a cardboard freezer box over the ISH dishes).
7. Monitor samples for blue product every 5-15 minutes.
8. Once desired intensity is reached, transfer embryos to clean microfuge tube.
9. Wash several times in stop solution.
10. Store in stop solution (in brown tubes) 4˚C in the dark.

Imaging embryos

1. Dehydrate embryos through a graded series of methanol solutions (30%, 50%, 70% and 100%) for 5 minutes each.
2. To clear the embryos, transfer them to the well of a spot plate containing a 1:2 solution of benzyl alcohol:benzyl benzoate (300-400 µl per well). Allow embryos to sink in the well for 2-3 minutes.
3. Pick up the embryos with a glass hair (or the nylon loops) – NOT WITH A PIPET – try to reduce the volume of liquid transferred with the embryos! Place each embryo in a drop of Permount on a bridged coverslip*.
4. Gently lower another coverslip on top of the embryo. Roll the upper coverslip until the embryo is in the desired orientation.

**Bridged coverslips can be made by gluing (with crazy glue), three ~22x22 coverslips on either end of a microscope slide.*

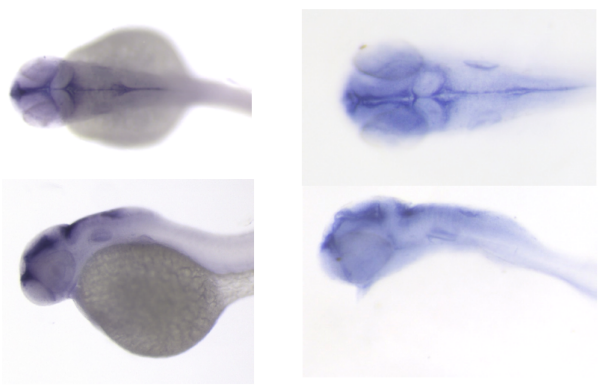

Figure 1. Embryos on left - without clearing. Embryos on right - with clearing.