DIG In Situ Hybridization Protocol (with detergent)

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DAY ONE

*Prepare fresh frozen tissue in OCT and section at 15 µm. Collect sections on Superfrost PLUS slides. Let slides dry 20 minutes to 3 hours.

*Fix in 4% Paraformaldehyde/1XPBS 10 minutes

To make fixative:
Add 20g paraformaldehyde to 450 ml boiling dH2O + 50 µl 10N NaOH. Stir on magnetic stirrer in hood until paraformaldehyde is well dissolved. Add 50 ml 10XPBS. Sterile filter and chill on ice. Use within 3 days of preparation.

*Wash 3X3 minutes in 1XPBS.

*Acetylate for 10 minutes.

To make acetylation solution:
Combine 9.25 g triethanolamine HCl Sigma Cat.#T1502 and 1.12 ml 10N NaOH in 500 ml dH2O. Mix well. Immediately before adding solution to sections, add 1.25 ml acetic anhydride and shake solution vigorously.

*Wash 3X5 minutes in 1XPBS.

*Prehybridize by placing slides vertically in slide mailers filled with hybridization solution at 65^{o}C for 1 hour. You can reuse hybridization solution for 1-2 months if you store it at -20^{o}C.

Hybridization solution:
50% formamide
5X SSC
5X Denhardts
250 µg/ml yeast tRNA
500 µg/ml herring sperm DNA
50 µg/ml Heparin
2.5 mM EDTA
0.1% Tween-20
0.25% CHAPS

*For each slide, prepare 100 µl hybridization buffer + 1-2 µl of DIG probe
(see section below for probe synthesis).

*Denature probes by heating to 80 degrees C for 5 minutes. Chill on ice. Pipet carefully onto

tissue and coverslip with Hybrislip plastic cover slips.

*Place slides horizontally in slide mailers. Do not mix slides containing different probes in the same slide mailer, because cross-contamination may occur.

*Hybridize slides by placing horizontally in slide mailers in a humidified box at 65^{o}C for 16 to 24 hours.

DAY TWO

*Remove coverslips by soaking slides in 5X SSC prewarmed to 65^{o}C

*Wash 3X20 minutes in 0.2X SSC at 65^{o}C

*Wash 1X10 minutes in 1XPBS, 0.1% Triton X-100

*Block 1 hour in B2 GOAT in vertical slide mailers. You can reuse blocking solutions for 4-6 weeks or until they turn cloudy, if you store them at 4^{o}C.

B2 Goat:
1XPBS
0.1% Triton X-100
10% heat inactivated normal goat serum

*Incubate with anti-DIG-Alkaline Phosphatase antibody (BMB Cat. #1 093 274) at 4^{o}C overnight. Dilute antibody 1:1000 in B2 GOAT.

DAY THREE
*Wash 3X30 minutes in 1XPBS, 0.1% Triton X-100.

*Equilibrate slides in B3 for 10 minutes.

B3 Buffer:
0.1M Tris pH 9.5
0.1M NaCl
50 mM MgCl2,
0.1% Tween-20

*Develop inverted on parafilm in 300 µl drops of B4. Develop at room temperature in the dark for 10 minutes to 3 days, depending on abundance of transcript.

B4 Solution:
45 µl NBT
35 µl BCIP
100 µl 100mM Levamisole
10 ml B3

*Stop reaction by washing for 1X5 minutes in 1XPBS, 0.1% Triton X-100. Rinse with distilled water, air dry, and mount in 160 ul melted Glycergel (DAKO) with a 24x60 mm cover glass.

VARIATIONS FOR FLUORESCENT VISUALIZATION

DIG and FITC probes work at very good sensitivity, while BIOTIN is considerably less sensitive. Therefore I recommend that you use BIOTIN probes only for very abundant transcripts. Substitution of FITC-labeled secondary antibodies is not recommended. Only Alexa488 has worked in my hands. This method works well for high and medium abundance transcripts. If your transcript is not detectable by these methods, you may want to try tyramide signal amplification as described in: Paratore, Suter, and Sommer. Embryonic gene expression at the cellular level by fluorescence in situ hybridization. Histochem. Cell Biol. (1999) 111:435-443.

DAY ONE

Add DIG, FITC, or BIOTIN probes, singly, or in double or triple combinations to tissue. Do not exceed 6 µl of riboprobe per 100µl applied probe mixture.

DAY TWO

*Block sections in B2 HORSE Block:

B2 HORSE Block:
1XPBS
0.1% Triton X-100
10% Heat Inactivated Normal Horse Serum)
***** Important!!!! Do not use Goat Serum by mistake!!!!*****

*Use these primary antibodies diluted in B2 HORSE:

DIG	Sheep anti-DIG (BMB Cat. #1 333 089) 1:500
BIOTIN	Rabbit anti-BIOTIN (Chemicon Cat. #AB1708) 1:500
FITC	Mouse IgG anti-FITC (BMB Cat. #1 426 320) 1:500

DAY THREE

*After doing 3X30 minute washes: Block sections in B2 HORSE, 30 minutes 25^{o}C.

*Incubate with secondary antibodies against DIG, FITC, and BIOTIN labeled RNA for 2 hours 25^{o}C in the dark. Dilute all in B2 HORSE 1:500:

Donkey anti-Sheep-Alexa Fluor 488	(Molecular Probes Cat.#A-11015)
Donkey anti-MouseIgG-CY3	(Jackson Cat. #715-165-151)
Donkey anti-Rabbit CY5	(Jackson Cat. #711-175-152)

*Wash 3X10 minutes in 1XPBS, 0.1% Triton X-100.

*Mount in Vectashield, store in the dark, view immediately in confocal. Stable for 5-30 days.

DIG RNA RIBOPROBES

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In my experience, the purity of the template DNA is the major determinant of successful RNA synthesis. This protocol therefore takes special care in post-digestion purification. It is recommended that these steps are followed to ensure consistently good RNA probes.

Make Template

*Digest the equivalent of 1 µg of insert DNA in 50 µl with 3 µl of enzyme (to generate blunt ends or 5' overhang; no 3' overhangs) for 3 hours. [Calculate amount of DNA to yield 1 µg of insert. e.g. 0.5 kb insert in pBluescript--digest 7 µg of plasmid DNA]

*Check 5 µl of digest in a gel to make sure DNA is linearized.

*If linear, dilute remaining 45 ul of digest with 50 ul RNase free water and purify DNA by extracting once with 80 ul of 1:1 phenol:chloroform and once with 80 ul of chloroform. Vortex each extraction well and spin for 3 minutes at maximum speed at room temperature.

*Pass aqueous phase over a G50 ProbeQuant column (Pharmacia) to remove residual organic solvents.

*Precipitate eluate with 10 ul sodium acetate (3M, pH5) and 250 ul ethanol. Spin out DNA, 30 minutes 4 degrees. Wash pellet with 70% ethanol. Dry and resuspend in 10.5 µl of RNAse free water

Synthesize Riboprobes

*To 10.5 µl of template in dH2O
add in order for a total volume of 20 ul:
4 µl Promega 5X Transcription Buffer
2 µl BMB 10X DIG RNA labeling mix (BMB Cat. #1 277 073)
1 µl 0.1M DTT
0.5 µl Promega rRNAsin
2 µl RNA polymerase (SP6, T3, or T7)

*Mix gently, briefly spin down tube at low speed, and incubate at 37 degrees for 2 hours.

*Pass probes over G50 ProbeQuant columns, bring volume of eluate to a final volume of 100 µl with hybridization buffer. Use 10µl-20µl/ml for in situ hybridization.

FITC RNA RIBOPROBES

Substitute 10X Fluorescein RNA labeling mix (BMB Cat. #1 685 619) for 10X DIG RNA labeling mix in protocol above. Do all incubations in the dark. Eluted probe will be yellow if synthesis has succeeded.

BIOTIN RNA RIBOPROBES

Substitute 10X BIOTIN RNA labeling mix (BMB Cat. #1 685 597) for 10X DIG RNA labeling mix in protocol above.