Enhanced Two-Color Fluorescent *In Situ* Hybridization for Low-Abundance Genes

In situ **hybridization two-color fluorescent detection using TSA-FITC/ TSA-CY5**

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DAY ONE

*Prepare fresh frozen tissue in OCT and section at 14 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 65oC for 1 hour. You can reuse hybridization solution for 1-2 months if you store it at -20oC.

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 and/or FITC probe (see section below for probe synthesis; can adjust amount of probe used based on strength, eg between 1:2500 and 1:10).

*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 65oC for 16 to 24 hours.

DAY TWO

Necessary Reagents:
anti-FLUORESCEIN-POD, Fab fragments (Roche 1 426 346)
anti-DIGOXIGENIN-POD, Fab fragments (Roche 11 207 733 910)
TSA™ Plus Fluorescein Fluorescence System (Perkin Elmer NEL741)
TSA™ Plus Cy5 Fluorescence System (Perkin Elmer NEL745)

Roche
Anti-digoxigenin-POD, Fab fragments
Catalog 11 207 733 910

Roche
Anti-Fluorescein-POD, Fab fragments
1 426 346

Buffers:

TN
100 mM Tris-HCl (pH 7.5)
150 mM NaCl

TNB
0.5% Blocking Reagent (Perkin Elmer) in TN buffer

TNT
0.05% Tween20® in TN buffer

*Remove coverslips by soaking slides in 5X SSC prewarmed to 65oC

*Wash 3X20 minutes in 0.2X SSC at 65oC

*Equilibrate in TN for 5 min.

*Block in TNB for 30 min in a vertical slide mailer @ RT.

*Dilute anti-FITC-POD 1:500 in TNB.
Apply 100 µl/ slide, coverslip, and incubate for 30 min @ RT.

*Wash slides with TNT - 3 x 5 min.

*Dilute FITC-Tyramide 1:50 in Amplification Reagent (TSA™ kit).
Apply 100 µl of working solution per slide, coverslip, incubate for 10 min @ RT.

*Wash slides with TNT - 1 x 5 min.

*To quench peroxidase activity, incubate in 3% H_2O_2 for 1hr.

*Wash slides with TNT - 3 x 5 min.

*Dilute anti-DIG-POD 1:500 in TNB.
Apply 100 µl/ slide, coverslip, and incubate for 30 min @ RT.

*Wash slides with TNT - 3 x 5 min.

*Dilute Cy5-Tyramide 1:50 in Amplification Reagent (TSA™ kit).
Apply 100 µl of working solution per slide, coverslip, incubate for 10 min @ RT.

*Wash slides with TNT - 3 x 5 min.

*Mount Slides in 60ul VectaShield®.

NOTE: If doing double in situ, do weaker probe first. Eg, if DIG probe is weaker than FITC probe, incubate with anti-DIG-POD first and anti-FITC-POD second. If using this protocol for a single in situ, ignore quenching.

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; **also note** .5ug of insert DNA can be used with success if DNA is limited]

*Check 1 µl of digest in a gel to make sure DNA is linearized.

*If linear, dilute remaining 49 ul of digest with 151 ul RNase free water and purify DNA by extracting once with 200 ul of 1:1 phenol:chloroform and once with 200 ul of chloroform. Vortex each extraction for 3 minutes and spin for 4 minutes at maximum speed at room temperature.

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

*Measure volume and 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 11 µl of RNAse free water

*Check .5 ul of DNA on gel to make sure template is still present.

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 (Amersham/Pharmacia Cat.#)

*Check 2 ul of probe on a gel (can use normal agarose gel) to ensure transcription reaction was successful.

*Bring volume of eluate to a final volume of 100 µl with hybridization buffer (i.e., add ~82 ul). Use 1µl-2µl/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.