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Protocol for Fluorescent *in situ* hybridization (FISH) on cultured cells

Day 1

Cell seeding

↓ Seed the proper number of cells on the dish (Nunc Lab-Tek II Chamber Slide, code no. 154534; Thermo Scientific) and incubate it at 37°C overnight.

Day 2

Pretreatment

↓ Wash wells with PBS twice.
↓ Fix in 4% paraformaldehyde at room temperature for 20 min.
↓ Wash wells with PBS twice.
↓ Incubate cells in 0.2 N HCl at room temperature for 20 min.
↓ Wash wells with Nuclease free water.
↓ Incubate cells in 37°C prewarmed Proteinase K solution (0.6 µg/mL) at 37°C for 7 min. (Optimizing the Proteinase K concentration and the reaction time is recommended.)
↓ To stop the protease reaction, incubate cells in 0.2% Glycine/ PBS at room temperature for 10 min.
↓ Wash wells with PBS twice.
↓ Wash wells with Nuclease free water.
↓ Fix in 4% paraformaldehyde at room temperature for 20 min.
↓ Wash wells with PBS.
↓ Wash wells with Nuclease free water.
↓ Incubate cells in Acetylation solution (1.5% Triethanol amine, 0.03 N HCl, 0.25% Acetic anhydride) at room temperature for 15 min.
↓ Wash wells with PBS.
↓ Incubate cells in 55°C prewarmed Prehybridization solution (50% formamide, 1×Denhardt's solution, 2×SSC, 10 mM EDTA (pH8.0), 100 µg/mL yeast tRNA, 0.01% Tween-20) at 55°C for 2 hr.

Hybridization

↓ Denature 50 µg/sample of Probe solution (1 µg/mL DIG-labeled RNA probe, 400 µg/mL yeast tRNA diluted with formamide) at 80°C for 10 min., then quench at 4°C for 5 min.
↓ Mix 50 µL of 55°C prewarmed Hybridization Solution (2×Denhardt's solution, 4×SSC, 20 mM EDTA (pH8.0), 0.02% Tween-20, 10% Dextran sulfate) and 50 µL

of Probe Solution, then vortex well.

↓ Add the mixed solution and transfer the dish in humidity chamber to avoid dry. Incubate it at 55°C for at least 16 hr. (The condition of the hybridization reaction depends on the probe being used.)

Day 3

Wash step after hybridization

↓ Wash wells twice with 55°C prewarmed Wash Buffer A (50% formamide, 2×SSC, 0.01% Tween 20) at 55°C for 30 min.

↓ Wash wells with NTET Buffer (10 mM Tris-HCl (pH8.0), 1 mM EDTA, 0.01% Tween 20, 500 mM NaCl).

↓ Incubate cells in 37°C prewarmed NTET Buffer with RNase A (10 μg/mL) at 37°C for 1 hr.

↓ Wash wells with NTET Buffer, twice.

↓ Wash wells with 55°C prewarmed Wash Buffer B (2×SSC, 0.01% Tween 20) at 55°C for 30 min.

↓ Wash wells twice with 55°C prewarmed Wash Buffer C (0.1×SSC, 0.01% Tween 20) at 55°C for 30 min.

↓ Wash wells with PBS.

Detection

↓ Add Blocking Buffer (1× Blocking Reagent (code no. 1158576200; Roche), 1×PBS, 0.01% Tween 20), and incubate cells at room temperature for 1 hr.

↓ Add Anti-Digoxigenin (DIG) mAb (MBL; code no. M227-3) diluted with Blocking Buffer at room temperature for 1 hr.

↓ Wash wells three times with 0.2% Tween 20/ PBS at room temperature for 5 min. each.

↓ Wash wells with PBS.

↓ Add Alexa fluor 594 conjugated anti-mouse IgG (code no. A11032; Invitrogen) diluted with Blocking Buffer at room temperature for 1 hr.

↓ Wash wells three times with 0.2% Tween 20/ PBS at room temperature for 5 min. each.

↓ Wash wells with PBS.

↓ Mount the slide with Mount medium with DAPI.