TriDix Bio
INNOVATING DRUG DISCOVERY

RNA FISH Protocol for Adherent Cells

I . Product Description

RNA FISH Probes consist of 25-48 fluorescently labeled oligonucleotides specifically designed to bind to RNA or DNA targets. These probes enable the detection of single RNA molecules as diffraction-limited spots using conventional or confocal fluorescence microscopy.

II . Content of the Kit

Name	Cat#	Storage
FISH probe		-80°C
10x Hybridization buffer	TDP-HB-25	-80°C
5x Washing buffer	TDP-WB-25	4°C
20x SSC	TDP-SSC-25	Room Temperature
Positive control		-80°C

III . Materials without in the kit

Name	Vendor	Cat.#
16% paraformaldehyde solution	Electron Microscopy Sciences	15710
10X Phosphate Buffered Saline (PBS)	Fisher Scientific	SH3002803
Ethanol for molecular biology	Fisher Scientific	04-355-450
Nuclease-free water	Thermo Fisher Scientific	10-977-015
Deionized formamide	Millipore	4650-500ML
4',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific	D3571
20x SSC buffer	Invitrogen	AM9770
Mounting Medium	Thermo Fisher Scientific	P10144
12 mm round coverslips	California Bio-supply	633029

IV . Other Reagents and Consumables:

1. 12- or 24-well culture plates
2. RNase-free consumables (e.g., pipette tips)
3. Humidified chamber (or equivalent): 150 mm tissue culture plate or plastic box with a flat bottom, lined with water-saturated paper towels and a single layer of Parafilm® placed on top
4. Super-frost Plus Microscope slides
5. 37°C laboratory oven

V . Buffer Preparations

1. **Fixation Buffer**:
   The final composition should be 4% (vol./vol.) formaldehyde in 1X PBS.
   For a final volume of 10 mL, mix:
   - 2.5 mL 16% formaldehyde solution
   - 7.5 mL 1X PBS

2. **Hybridization Buffer:**
   The final composition should be 15% (vol./vol.) formamide in Hybridization Buffer. Prepare fresh Hybridization Buffer for each experiment. To account for solution viscosity, it is recommended to prepare an excess of 5% in volume.
   For a final volume of 1 mL, mix:
   - 850 µL RNA FISH Hybridization Buffer (9 mL)
   - 150 µL deionized formamide
     Mix thoroughly by vertexing and pipetting up and down.

3. **1x Wash Buffer:**
   Prepare the 1x Wash Buffer freshly for each experiment. For a final volume of 10 mL, mix:
   - 2 mL 5x TriDixBio RNA FISH Wash Buffer
   - 6.5 mL nuclease-free water

- 1.5 mL deionized formamide
  Mix thoroughly by gentle vertexing.

4. **DAPI Nuclear Stain Solution:**
   - 4',6-diamidino-2-phenylindole (DAPI) dissolved in 1x Wash Buffer at 5 ug/mL. This solution is used for DAPI staining for use after hybridization.

5. **Mounting Media:**
   - Prolong mounting media: Use 10 µL for each coverslip.

VI . Protocol for Adherent Cell Fixation

1. Fixation of Adherent Cell Lines

1) Grow cells on 12 mm round coverslips in a 12-well or 24-well cell culture plate.
2) Aspirate the growth medium and wash cells once with 0.5 mL (for 24-well) or 1 mL (for 12-well) of 1x PBS.
3) dd 0.5 mL (for 24-well) or 1 mL (for 12-well) of fixation buffer (4% PFA in 1x PBS).
4) Incubate at room temperature for 10 minutes.
5) Wash twice with 0.5 mL (for 24-well) or 1 mL (for 12-well) of 1X PBS.
6) To permeabilize, immerse cells in 1 mL of 70% (vol./vol.) ethanol for at least 2 hours at 4°C.
7) Cells can be stored at 4°C in 70% ethanol for up to one week before hybridization.

2. Hybridization, Washing, and Sealing Coverslips in Adherent Cells

1) If frozen before use, warm the reconstituted probe solution to room temperature. Mix well by vortexing and briefly centrifuge.
2) To prepare the Hybridization Buffer containing the probe, add 1 µL of probe stock solution to 50 µL of Hybridization Buffer with 15% formamide. Vortex and centrifuge. This creates a working probe solution of 1 ng/µL, enough for one coverslip, which will be used in steps 6 and 7.
3) Aspirate the 70% ethanol from the coverslip containing adherent cells within the 12-well or 24-well plate.

4) Add 1 mL of 2x SSC buffer with 15% formamide and incubate at room temperature for 5 minutes.
5) Prepare the humidified chamber by placing water-saturated paper on the bottom, and insert a flat rack wrapped with Parafilm into the chamber.
6) Within the humidified chamber, dispense 50 µL of the Hybridization Buffer containing the probe onto the Parafilm on top of the rack.
7) Gently transfer and flip over the coverslip, with cells side down, onto the 50 µL drop of Hybridization Buffer containing the probe.
8) Cover the humidified chamber with the lid and wrap it with foil to protect from light.
9) Incubate the chamber in the dark at 37°C for at least 4 hours (incubation can be extended up to 16 hours).
10) Gently transfer the coverslip, with cells side up, to a fresh 12-well plate containing 1 mL of 1x Wash Buffer.
11) Incubate in the dark at 37°C for 30 minutes.
12) Aspirate the 1x Wash Buffer, then add DAPI (final concentration of 5 ng/mL) within 1 mL fresh 1x Wash Buffer for nuclear staining and second washing.
13) Incubate in the dark at 37°C for another 30 minutes.
14) Aspirate the DAPI staining Wash Buffer, then add 1 mL of 2x SSC buffer. Incubate at room temperature for 5 minutes.
15) Add a small drop (~10 µL) of Prolong mounting media to a glass slide. Use Kim-wipes paper to soak up 2x SSC buffer from the edge of coverslip. Mount the coverslip onto the slide with cells side down.
16) Gently wick away any excess mounting media from the perimeter of the coverslip (optional).
17) Place the sealed slide into a drawer at room temperature to avoid light for at least 3 hours or overnight.
18) The slides with sealed coverslips are ready for imaging.

3. Imaging

1) Microscope Requirements:

- Wide-field fluorescence microscope (e.g., Leica TIRF & D-STORM/THUNDER) or Confocal (e.g., Confocal SP8 Light-Sheet)
- High numerical aperture (>1.3) and 60-100x oil-immersion objective
- Strong light source (e.g., mercury or metal-halide lamp; newer LED-based light sources may also suffice)
- Appropriate filter sets for fluorophores

- Standard cooled CCD or sCMOS camera (ideally optimized for low-light-level imaging with a pixel size of 13 µm or less)