RNAscope Staining Protocol

Skop Lab, updated 2025 by Sungjin Park

Overview

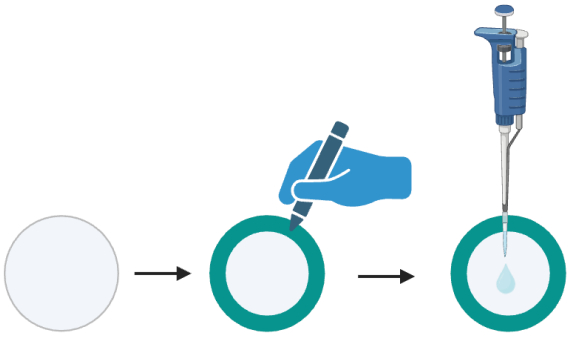
RNA in situ hybridization was performed using the RNAscope® Multiplex Fluorescent kit v2 (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions.

1. Cover Glass Preparation

1. Sonicate #1.5 18mm circular cover glass (Cat# 72222-01, Electron Microscopy Sciences) in 1M KOH for 30 minutes.
2. Rinse with distilled water 10 times.
3. Store in 100% ethanol until use.
4. Before use, dry the coverslip using a vacuum-connected Pasteur pipette.
5. (Optional for mitotic cell attachment): Incubate coverslips with Poly-L-lysine (Cat# P4832, Sigma) at room temperature overnight or 37℃ for 1 hour.
6. Wash with distilled water 10 times.
7. Dry completely. (Coated coverslips can be stored at -20℃.)

2. Cell Fixation & Pre-treatment

8. Seed cells on prepared 18mm #1.5 circular cover glass.
9. Fix with 4% paraformaldehyde in 0.1M PBS (Cat# 15735-85) for 30 minutes at room temperature.
10. Hydrate in cold 70% ethanol (prepared with DEPC-treated water) for at least 1 hour at 4℃, or store up to 48 hours.
11. Dehydrate with 1× SSC buffer for 1 minute, then 1× SSC buffer for 10 minutes.
12. Aspirate excess liquid from the boundary using a vacuum-connected Pasteur pipette.
13. Within 1 minute, draw a hydrophobic barrier using Advanced PAP Pen.
14. Immediately add 1× SSC buffer inside the barrier to prevent drying.



3. RNAscope Hybridization

15. Pre-warm the RNAscope probe at 37℃ for 10 minutes.

16. Apply Hydrogen Peroxide buffer, incubate 10 minutes at room temperature (drop along the barrier line to avoid cell detachment).
17. Wash with 1× SSC buffer.
18. Apply Protease III solution (1 drop per 150 µL 1× SSC), incubate 30 minutes at room temperature.
19. Wash with 1× SSC buffer.
20. Apply pre-warmed RNAscope probe, incubate 2 hours at 37℃.

21. Wash with RNAscope wash buffer.
22. Apply AMP 1, incubate 30 minutes at 37℃.
23. Wash with RNAscope wash buffer (100 µL).
24. Apply AMP 2, incubate 30 minutes at 37℃.
25. Wash with RNAscope wash buffer (100 µL).
26. Apply AMP 3, incubate 15 minutes at 37℃.
27. Wash with RNAscope wash buffer (100 µL).
28. Apply C1-HRP, incubate 15 minutes at 37℃.
29. Wash with RNAscope wash buffer (100 µL).
30. Apply TSA Vivid™ Fluorophore 650 (1:750 dilution in TSA buffer), incubate 30 minutes at 37℃ in the dark.
31. Wash with RNAscope wash buffer (100 µL).
32. Apply HRP blocker, incubate 15 minutes at 37℃.
33. Wash with RNAscope wash buffer (100 µL).

4. Immunofluorescence (Optional)

34. Apply blocking buffer (PBS + 3% BSA + 0.1% Saponin), incubate 30 minutes at room temperature.
35. Incubate with primary antibodies (e.g., α-tubulin DM1A, MKLP1) in blocking buffer overnight at 4℃.
36. Wash with RNAscope wash buffer (100 µL).
37. Incubate with secondary antibodies in blocking buffer for 30 minutes at room temperature. (Optional: include DAPI)
38. Wash 3 times with RNAscope wash buffer (100 µL each).

5. Mounting & Imaging

39. Mount coverslips on microscope slides using Fluoro-Gel mounting medium (Cat# 17985-30, Electron Microscopy Sciences).

40. Acquire 3–5 z-stack images (0.5 µm intervals) to minimize photobleaching and project signals.

Reagents Checklist

- Circular Coverglass, 18mm, #1.5 (Cat# 72222-01, Electron Microscopy Sciences)
- 1M KOH
- 100% EtOH (Cat# BP2818-500, Fisher)
- Cold 70% EtOH (DEPC-treated)
- DEPC-treated water (Cat# BP561-1, Fisher)
- 20xSSC (Cat# J60561, Alfa Aesar)
- Poly-L-lysine (Cat# P4832, Sigma)
- 4% PFA in 0.1M PBS (Cat# 15735-85, Electron Microscopy Sciences)
- Advanced PAP Pen (Cat# Z672548, Mililipore Sigma)
- RNAscope® Multiplex Fluorescent Reagent Kit v2 (Cat# 323100, ACD)
- TSA Vivid™ Fluorophore 650 (Cat# 323273, ACD)
- Blocking buffer (PBS + 3% BSA + 0.1% Saponin)
- BSA (Cat# AM2616, Thermo Fisher Scientific)
- Saponin (Cat# A18820, Alfa Aesar)