RNAscope on cryosections using chromogenic substrate

(adapted from *RNAscope® 2.5 HD Reagent Kit -RED User Manual*)

1. Prepare the Slides

a. Air dry slides in oven for 30 min -1 hour.

b. Prepare target retrieval solution.

   i. Add 45 mL 10X Target Retrieval stock solution to 405 mL DI water for 450 mL 1X target retrieval working solution.

   ii. Pour the target retrieval solution into a glass dish (e.g PYREX™ Crystallizing Dish)

c. Heat target retrieval solution in the glass dish over flame until boiling.

d. Transfer the glass dish with target retrieval solution into the steamer and add slides to dish.

e. Steam for 10 minutes.

f. After steaming, transfer the slides to DI water.

g. Rinse the slides in 100% EtOH (200 proof) inside a slide staining jar for 2 min twice

h. Dry the slides on a slide rack.

i. Create a hydrophobic barrier around the sections using an ImmEdge™ Hydrophobic Barrier Pen.

j. Air dry the slides thoroughly in the fume hood for 30 minutes or overnight.

Note: The slides can be dried at room temperature overnight for use the following day (must use within 24 hours) or proceed directly to the next section.

2. Applying RNAscope Protease Plus

a. Moisten humidity of tray by adding DI water.

b. Add ~5 drops of RNAscope Protease Plus to each slide.

c. Cover the sections with paraffin to help spread solution.

d. Close lid and let sit for 10 min at RT.

e. Wash slides in DI water.

3. Hybridize Probe

a. Remove excess liquid from slides.
b. Add ~4 drops of appropriate probe to each slide. (different probe for each gene)
c. Cover the slides with paraffin to help spread solution.
d. Close lid and incubate in the oven for 2 hours at 40 °C.
e. Wash slides in 1X Wash Buffer for 2 min at RT.
f. Repeat wash step.

Note: Slides can be kept overnight at RT in 5X SSC (25 mL 20X SSC + 100 mL DI H_2O) or proceed directly to the next section.

4. Hybridize AMP 1

a. If removing slides from 5X SSC, wash in 1X Wash Buffer 1-2X.
b. Add ~ 4 drops of AMP 1 to entirely cover each slide.
c. Close tray and incubate in the oven for 30 min at 40 °C.
d. Wash in 1X Wash Buffer for 2 min at RT.
e. Repeat wash step.

5. Hybridize AMP 2

a. Remove excess liquid from slides.
b. Add ~4 drops of AMP 2 to entirely cover each slide.
c. Close tray and incubate in the oven for 15 min at 40 °C.
d. Wash in 1X Wash Buffer for 2 min at RT.
e. Repeat wash step.

6. Hybridize AMP 3

a. Remove excess liquid from slides.
b. Add ~4 drops of AMP 3 to entirely cover each slide.
c. Close tray and incubate in the oven for 30 min at 40 °C.

d. Wash in 1X Wash Buffer for 2 min at RT.
e. Repeat wash step.

7. Hybridize AMP 4

a. Remove excess liquid from slides.
b. Add ~4 drops of AMP 4 to entirely cover each slide.
c. Close tray and incubate in the oven for 15 min at 40 °C.
d. Wash in 1X Wash Buffer for 2 min at RT.

8. Hybridize AMP 5

a. Remove excess liquid from slides.
b. Add ~4 drops of AMP 5 to entirely cover each slide.
c. Close tray and incubate for 30min at RT.
d. Wash in 1X Wash Buffer for 2 min at RT.
e. Repeat wash step.

Note: Staining intensity can be modified by adjusting the AMP 5 incubation time. We are able to get stronger signals by incubating in AMP5 for more than 3 hours at RT without increasing the background.

9. Hybridize AMP 6

a. Remove excess liquid from slides.
b. Add ~4 drops of AMP 6 to entirely cover each slide.
c. Close tray and incubate for 15 min at RT.
d. Wash in 1X Wash Buffer for 2 min at RT.

10. Detect the Red Signal.

a. Use a 1:60 ratio of Red B: Red A. (For example, add 2.5 µl of Red B to 150 µl of Red A). Mix well.

   Note: Use the freshly prepared RED solution within 5 min. Protect from light.

b. Remove excess liquid from slides.
c. Pipette ~150 µl RED solution onto each slice. Observe red signaling developing under the microscope. Stop the reaction by immersing the slices in DI water.
   Note: We recommend only working with 2 slides at a time (e.g. one mutant and one control).

11. Counterstain the slides.

a. Dilute hematoxylin 1:10 with DI water.
b. Stain the slides with hematoxylin staining solution for ~10 s at RT.
c. Immediately replace the hematoxylin with DI water. Transfer the slides to tap water and wait for 20-30 s while agitating the slides.
d. Rinse the slides with DI water.
e. Mount slides with VectaMount Aqueous Mounting Medium and leave on bench to dry.