JAN 10, 2024

OPEN ACCESS

Protocol Citation: Andrew J. Flores 2024. v2 RNAscope in situ hybridization . **protocols.io** https://protocols.io/view/v2-rnascope-in-situ-hybridization-c66kzhcw

Protocol status: Working
We use this protocol and it's working

Created: Jan 08, 2024

Last Modified: Jan 10, 2024

PROTOCOL integer ID: 93100

v2 RNAscope in situ hybridization

Andrew J. Flores[1]

[1]University of California, San Diego

Andrew J. Flores
University of California, San Diego

ABSTRACT

This is a protocol for performing RNAscope® in situ hybridization analysis on fixed-frozen mouse brain tissue using the RNAscope® Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics; ACD). This RNAscope® protocol similar to the v2 protocol provided by ACD; places where it deviates from ACD's protocol have been indicated. This protocol also includes steps for sample preparation (including cryosectioning) and imaging of slides using a slide scanner.

MATERIALS

RNAscope® H2O2 and Protease Reagents (REF 322381)
RNAscope® Target Retrieval Reagents (REF 322000)
RNAscope® Protease III (REF 322337)
RNAscope® Wash Buffer Reagents (REF 310091)

RNAscope® Multiplex Fluorescent Detection Reagents (REF 323110)

- RNAscope® Multiplex FL v2 AMP 1 (REF 323101)
- RNAscope® Multiplex FL v2 AMP 2 (REF 323102)
- RNAscope® Multiplex FL v2 AMP 3 (REF 323103)
- RNAscope® Multiplex FL v2 HRP C1 (REF 323104)
- RNAscope® Multiplex FL v2 HRP C2 (REF 323105)
- RNAscope® Multiplex FL v2 HRP C3 (REF 323106)
- RNAscope® Multiplex FL v2 HRP Blocker (REF 323107)
- RNAscope® Multiplex FL v2 DAPI (REF 323108)

RNAscope® Probes

- RNAscope® Probe tdTomato (REF 317041)
- RNAscope® Probe Mm-Drd1-C2 (REF 461901-C2)
- RNAscope® Probe Mm-Drd2-C3 (REF 406501-C3)
- RNAscope® Probe Mm-Drd3-C3 (REF 447721-C3)
- RNAscope® Probe Mm-Pdyn-C3 (REF 318771-C3)

Keywords: in situ hybridization, RNAscope, multiplex

Cryosectioning, sample preparation, and storage

1 Collect sections spaced 20 µm apart between Bregma = +1.0–0.0 in RNase-free PBS.

2 Immediately mount sections onto Superfrost® plus microscopy slides. Mount sections spaced 120 microns apart 4 per slide (Note that this is different from the ACD protocol, which recommends mounting only 1 section per slide). This amounts to a total of 8 sections between Bregma = +1.0–0.0 mounted onto 2 slides.

3 Allow slides to dry for 60 minutes at -20°C, then, immediately store at −80°C in a slide box placed inside of a Ziploc bag with drierite desiccant until the day of the RNAscope procedure.

Pretreatment of fixed-frozen tissue samples

4 **Baking.** Bake slides for 60 min at 60°C in the HybEZ™ oven. Note that this is longer than the time recommended in the ACD v2 protocol.

5 **Post-fixation.** Post-fix the slides by immersing them in 4% PFA in 1X PBS for 30 min at 4°C. Note that this post-fixation time is longer than the time recommended in the ACD v2 protocol.

6 **Serial dehydration.** Slides are then rinsed once in ddH_2O and then serially dehydrated using an ethanol series in the following order: 50%, 70%, two times 100% ethanol (5 min each).

7 **Hydrogen peroxide treatment.** Take the dehydrated slides and add ~5–8 drops of RNAscope Hydrogen Peroxide to cover the sections on each slide. Treat sections with hydrogen peroxide (CAT no) for 10 minutes at RT.

7.1 Rinse slides with ddH_2O. Repeat with fresh ddH_2O water.

8 **Manual target retrieval.** Perform manual target retrieval using Target Retrieval Reagents () as described in Appendix B of the ACD v2 protocol.

8.1 Prepare 700 mL of fresh RNAscope 1X Target Retrieval Reagents by adding 630 mL ddH2O to 1 bottle (70 mL) 10X Target Retrieval Reagents in a clean 1 L beaker. Mix well with stir bar on stir plate.

8.2 Place the beaker containing RNAscope 1X Target Retrieval Reagents on the hot plate. Cover the beaker with foil, and turn the hot plate on high until 1X Target Retrieval Reagents reaches **98–102°C.**

8.3 With a pair of forceps, slowly submerge a Tissue-Tek Slide Rack containing the slides into RNAscope 1X Target Retrieval Reagents solution. Cover the beaker with foil and boil the slides for **5 min.**

8.4 Wash slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water. Then Transfer the slides to 100% ethanol for 3 min.

9 Slides are then air dried for 5 min and a hydrophobic barrier is drawn around sections using an ImmEdge™ Pen (H-4000; Vector Laboratories, Inc.).

10 Incubate slides with Protease III (ACD) reagent for 30 min at 40°C in the HybEZ™ oven.

10.1 Wash slides 2x with ddH_2O.

Probe Hybridization

11 **Prepare probe mixture.** C1 probes are 1X; C2 and C3 probes come as 50X solutions and therefore must be diluted 1:50 with the C1 probe.

12 **Add probe mixture to slides.** Refer to Appendix C. Reagent Volume Guidelines to determine the volume of probe mixture to add to each slide.

13 Insert into HybEZ™ oven for **2 hrs** at **40°C.**

14 (Optional stopping point). You can store the slides in 5X SSC **overnight** at **RT.**

Amplification

15 **Hybridize AMP 1.** Add RNAscope Multiplex FL v2 Amp1 to each slide.

15.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

15.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

16 **Hybridize AMP 2.** Add RNAscope Multiplex FL v2 Amp2 to each slide.

16.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

16.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

17 **Hybridize AMP 3.** Add RNAscope Multiplex FL v2 Amp3 to each slide Incubate in the HybEZ™ oven for **15 min** at **40°C**.

17.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

17.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

Develop HRP Signals

18 **Develop HRP-C1 signal.** Add RNAscope Multiplex FL v2 HRP-C1 to slides.

18.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

18.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

19 Add 150-200 µL of fluorophore to label the C1 probe.

19.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

19.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

20 Add RNAscope Multiplex FL v2 HRP blocker to slides.

20.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

20.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

21 **Develop HRP-C2 signal.** Add RNAscope Multiplex FL v2 HRP-C2 to slides.

21.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

21.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

22 Add 150-200 µL of fluorophore to label the C2 probe.

22.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

22.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

23 Add RNAscope Multiplex FL v2 HRP blocker to slides.

23.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

23.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

24 **Develop HRP-C3 signal.** Add RNAscope Multiplex FL v2 HRP-C3 to slides.

24.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

24.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

25 Add 150-200 µL of fluorophore to label the C3 probe.

25.1 Incubate in the HybEZ™ oven for **30 min** at **40°C.**

25.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

26 Add RNAscope Multiplex FL v2 HRP blocker to slides.

26.1 Incubate in the HybEZ™ oven for **15 min** at **40°C.**

26.2 Wash slides **2x** for **2 min** at **RT** with 1X Wash Buffer.

27 **Counterstain and mount slides.** Add enough DAPI to cover each section and incubate for **30 sec** at **RT.**

27.1 Remove DAPI and place 1-2 drops of ProLong Gold Antifade Mountant on each slide

27.2 Carefully place a 24 mm x 50 mm glass coverslip over the tissue section.

27.3 Dry slides 30 min to overnight in the dark and store slides in the dark at **2-8°C.**