


Aug 22, 2025

v2 RNAscope

 Forked from [v2 RNAscope](#)

 In 1 collection

DOI

dx.doi.org/10.17504/protocols.io.yxmvmmdw5v3p/v1

Thomas Hnasko¹, María Sanchiz Calvo²

¹University of California San Diego; ²KU Leuven



María Sanchiz Calvo

KU Leuven

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.yxmvmmdw5v3p/v1>

Protocol Citation: Thomas Hnasko, María Sanchiz Calvo 2025. v2 RNAscope. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.yxmvmmdw5v3p/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



Protocol status: Working

We use this protocol and it's working

Created: June 24, 2025

Last Modified: August 22, 2025

Protocol Integer ID: 220884

Keywords: frozen mouse brain tissue, v2 protocol, similar to the v2 protocol, advanced cell diagnostic, mouse brain, sample preparation, steps for sample preparation

Funders Acknowledgements:

ASAP

Grant ID: ASAP-000458

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). It is similar to the v2 protocol provided by ACD; places where it deviates from ACD's protocol have been indicated. This protocol provides steps for staining sections through dorsal striatum of mouse brain, but can also be applied to other brain areas. It also has steps for sample preparation, including cryosectioning.

Materials

RNAscope® H2O2 and Protease Reagents (REF 322381)

- RNAscope® Hydrogen Peroxide (REF 322335)
- RNAscope® Protease III (REF 322337)

RNAscope® Target Retrieval Reagents (REF 322000)

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)





ProLong™ Gold Antifade Mountant (REF P36930)

Troubleshooting

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 ddH₂O 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 RNAscope® Hydrogen Peroxide for **10 min** at **RT**.
- 7.1 Rinse slides with ddH₂O. Repeat with fresh ddH₂O water.
- 8 **Manual target retrieval.** Perform manual target retrieval using RNAscope® 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 RNAscope® Protease III reagent for **30 min** at **40°C** in the HybEZ™ oven.
- 10.1 Wash slides 2x with ddH₂O.

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 AMP 1 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 AMP 2 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 AMP 3 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 diluted 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 diluted 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 diluted 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.

Counterstaining and mounting

- 27 Add enough DAPI to cover each section and incubate for **30 sec** at **RT**.
- 28 Remove DAPI and place 1-2 drops of ProLong™ Gold Antifade Mountant on each slide
- 29 Carefully place a 24 mm x 50 mm glass coverslip over the tissue section.
- 30 Dry slides 30 min to overnight in the dark and store slides in the dark at **2-8°C**.