Jul 25, 2025

Expanded RNAscope™ In Situ Hybridization Assay

Applications in Plant Sciences

DOI
https://dx.doi.org/10.17504/protocols.io.14egn6pp6l5d/v1

Lena Hileman[1], Brooklyn Anaya[1], Austin Nguyen[1], Kelly Matsunaga[1]

[1]University of Kansas

Brooklyn Anaya
Academic

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DOI: https://dx.doi.org/10.17504/protocols.io.14egn6pp6l5d/v1

External link: https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/aps3.70020

Protocol Citation: Lena Hileman, Brooklyn Anaya, Austin Nguyen, Kelly Matsunaga 2025. Expanded RNAscope™ In Situ Hybridization Assay . **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn6pp6l5d/v1

Manuscript citation:
Anaya, B. M., A. T. Nguyen, K. K. S. Matsunaga, and L. C. Hileman. 2025. Expanded application to plant reproductive tissues of a branched DNA probe-based in situ hybridization method. *Applications in Plant Sciences* e70020. https://doi.org/10.1002/aps3.70020

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

Created: October 22, 2024

Last Modified: July 25, 2025

Protocol Integer ID: 110575

Keywords: branched DNA probes, CYCLOIDEA, Histone H4, in situ hybridization, inflorescence, reproductive cone, RNAscope, Z-probe, situ hybridization assay, histone h4 expression in reproductive tissue, rnascope assay, detection of histone h4 expression, expanded rnascope, reproductive tissues of mimulus lewisii, rnascope ish, cycloidea transcription factor, expression of cycloidea transcription factor, quality ish results in reproductive tissue, histone h4 expression, challenging approaches to gene expression, breadth of plant species, situ hybridization, gene expression, plant development, gymnosperm species, assay, mrna, model angiosperm, specific patterns of gene expression, plant species, reproductive tissue, mimulus lewisii

Funders Acknowledgements:
NSF
Grant ID: 2338756
NSF
Grant ID: 2421327
NSF-GRFP
Grant ID: 1940699
NSF-RaMP
Grant ID: 2319820
K-INBRE
Grant ID: #P20 GM103418
NIGMS
Grant ID: P30GM145499
NSF Major Research Instrumentation Award
Grant ID: 2117449

Abstract

Detecting clear tissue- and organ-specific patterns of gene expression is key to understanding the genetic programs that control plant development. In situ hybridization (ISH) of mRNA is one of the clearest, yet most challenging approaches to gene expression assays. Detection of Histone H4 expression in reproductive tissues of *Mimulus lewisii*, a model angiosperm, was optimized using the RNAscope ISH assay. The optimized protocol was used to detect Histone H4 expression in reproductive tissues of two gymnosperm species, *Taxodium distichum* and *Juniperus virginiana*, without further need for species-specific optimization. Additionally, the optimized protocol was used to detect expression of CYCLOIDEA transcription factors in *M. lewisii* reproductive tissues without further optimization and with results similar to those previously reported. The RNAscope assay can quickly and sensitively generate high-quality ISH results in reproductive tissues across a breadth of plant species.

Materials

Instruments/Software:

- Fisher Scientific Isotemp Vacuum Oven (**Model 280A[HL1]**)
- Inlet DryFast Vacuum (Model 2022B-01), or similar (e.g., Welch DryFast Vacuum Pump **Model 2014**)
- Barnstead/Thermolyne Vari-Mix™ Platform Mixer (**Model M48725**)
- Boekel HybEZ II Oven ACD (**Cat. No 240200ACD[HL5]**)
- Cimarec+™ Stirring Hotplates Series (**SP88854100**)
- Epredia HistoStar Embedding Workstation (**A81000002**)
- Slide Warmer (**Model 77**, Cat. 12-594)
- AO Rotary Microtome (**Model 820**)
- Nikon Eclipse LV100ND Microscope (**205555**)
- Nikon DS-Fi3 Camera (**122519**)
- **NIS Elements D 5.20.02 64-bit**

Materials:

- Fisherbrand™ Polypropylene Centrifuge Tubes (**05-539-9**)
- Glass Scintillation vials [20 mL] (**03-341-73**)
- Stainless Steel General-purpose Forceps
- DABPRESS 6X7 Rosin Cold Plate (**dp-cp67-us**)
- Fisherbrand™ Tissue Path™ MACROSETTE™ Processing/Embedding Cassettes (**15-182-706**)
- Bell Jar: Thermo Scientific™ Nalgene™ Transparent Polycarbonate Classic Design Desiccator (**08-642-7**)
- Vollrath Stainless Steel Beakers [50 mL, 100 mL]
- Chemglass Life Sciences Beaker, Low Form, Griffin, Pyrex [400 mL]
- DWK Life Sciences Wheaton™ 20-Slide Glass Staining Dish (**08-812-1A**)
- Reynolds Wrap Aluminum Foil
- AccuForge Single Edge Blade Clamshell (**AGBL-7033-0000**)
- Epredia™ Ultra Disposable Microtome Blades (**30-538-35**), Gold Microtome Blades (**D554G50**)
- FisherBrand Glass Dropper [2.5 mL]
- Peel-a-Way Embedding Molds - Truncated (**50-465-348**)
- Fisherbrand™ Superfrost™ Plus Microscope Slides (**22-037-246**), Fisherbrand™ ProbeOn Plus Microscope Slides (**22-230-900**)
- Epredia™ Richard-Allan Scientific™ Cover Glass [22 × 50 mm 1.5] (**22-050-238**)
- Electron Microscopy Sciences Easy Dip™ Kit [Slide Staining Jars and Racks] (**NC0263028**)

Protocol materials

- Citrisolv (d-Limonene) **Fisher Scientific Catalog #**22-143-975
- Paraplast Plus Tissue Embedding Medium **Grayine Medical Catalog #**39502004
- Epredia Signature Series Paraffin **Fisher Scientific Catalog #**22-050-128
- Epredia Tissue Section Adhesive **Fisher Scientific Catalog #**86-014
- PARA/Gard Paraffin Repellent **Fisher Scientific Catalog #**15-183-15
- Silica Gel Desiccant **Fisher Scientific Catalog #**S684-212
- Xylenes (Histological) **Fisher Scientific Catalog #**X3P-1GAL
- RNAscope® Target Retrieval Reagents **Advanced Cell Diagnostics Catalog #**322000
- RNAscope Hydrogen Peroxide (H2O2) **Advanced Cell Diagnostics Catalog #**322330
- ImmEdge Hydrophobic Barrier Pen **Advanced Cell Diagnostics Catalog #**310018
- RNAscope Protease Plus Reagents **Advanced Cell Diagnostics Catalog #**322330
- RNAscope® Wash Buffer Reagents **Advanced Cell Diagnostics Catalog #**310091
- RNAscope 2.5 HD AMP 1 **Advanced Cell Diagnostics Catalog #**322311
- RNAscope 2.5 HD AMP 2 **Advanced Cell Diagnostics Catalog #**322312
- RNAscope 2.5 HD AMP 3 **Advanced Cell Diagnostics Catalog #**322313
- RNAscope 2.5 HD AMP 4 **Advanced Cell Diagnostics Catalog #**322314
- RNAscope 2.5 HD AMP 5 RED **Advanced Cell Diagnostics Catalog #**322361
- RNAscope 2.5 HD AMP 6 RED **Advanced Cell Diagnostics Catalog #**322362
- Hematoxylin Solution, Gill No. 1 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**GHS116-500ML
- RNAscope Fast Red B **Advanced Cell Diagnostics Catalog #**320459
- RNAscope Fast Red A **Advanced Cell Diagnostics Catalog #**320458
- Ecomount **Biocare Medical Catalog #**EM897L
- Vectamount **Advanced Cell Diagnostics Catalog #**321584
- EUKITT Neo Special Mounting Medium **Electron Microscopy Sciences Catalog #**15320-25
- 10X Hank's Balanced Salt Solution (HBSS) **Thermo Fisher Scientific Catalog #**14185-052
- Molecular Biology Grade Water, Corning **Fisher Scientific Catalog #**MT46000CM
- Pierce 16% Formaldehyde (w/v), Methanol-free **Thermo Fisher Scientific Catalog #**28908
- Silwet-L77 [VAC-N-STUFF] **Phytotech Labs Catalog #**S7777

Tissue Fixation & Embedding

1 Preparation

1.1 Make appropriate volume (40 mL , more if needed) of 4% Formaldehyde Solution (form.) by adding

10X Hank's Balanced Salt Solution (HBSS) **Thermo Fisher Scientific Catalog #**14185-052

, 26 mL

Molecular Biology Grade Water, Corning **Fisher Scientific Catalog #**MT46000CM

(MG Water), 10 mL

Pierce 16% Formaldehyde (w/v), Methanol-free **Thermo Fisher Scientific Catalog #**28908

, and 4 µL Silwet-L77 [VAC-N-STUFF] **Phytotech Labs Catalog #**S7777 in a 50 mL falcon tube, distribute into the appropriate amount of scintillation (scint.) vials.

Note

Make 4% form. in the safety hood. Keep on ice for entire procedure.

Molecular Biology Grade water (not DEPC-treated water) is used throughout the protocol to reduce the possibility of RNase contamination.

1.2 Fill scint. Vials approximately half full with 4% form.

1.3 Dissect the plant material and submerge tissue immediately into 4% form. in scint. vials.

Note

Tissue should be less than approximately 5 mm in any dimension.

1.4 Place scint. vials uncapped and packed into ice in the bottom of the bell jar.

2 Vacuum Infiltration

2.1 Using the vacuum pump attached to the bell jar, pull a full vacuum to -27 inHg , hold for 00:01:00 , quickly release vacuum. 1m

2.2 go to step #2.1 until tissue no longer floats.

2.3 Cap scint. vials and place on the rocker at 1 °C to 4 °C for 12:00:00 to 24:00:00 .

Note

Fixation time is critical, optimize to between 12-24 hours. While standard ISH protocols for plant tissues often recommend 12 hr fixation, we found that 24 hr fixation, coupled with 10 min. Protease Plus treatment (see below) worked well.

2.4 *Prep EtOH solutions for dehydration steps and place in* 4 °C *for the following day.*

3 Dehydration

Ethanol Series (Formaldehyde solution to EtOH)

3.1 Dehydrate the samples through the following EtOH series **on the rocker at** 1 °C **to** 4 °C , keeping samples in scint. vials:

Note

All EtOH solutions should be pre-chilled at 1-4°C and *made from fresh 200 proof EtOH with MG water.*

3.2 1. 50% EtOH for 01:30:00 — 7h 30m
2. 70% EtOH for 01:15:00
3. 85% EtOH for 02:15:00
4. 95% EtOH for 01:00:00
5. 100% EtOH for 01:30:00
6. 100% EtOH for Overnight at 4 °C .

4 Wax Infiltration

Citrisolv Series (EtOH to Wax)

4.1 Ensure the vacuum oven is set to 60 °C

4.2 Move the rocker to Room temperature

4.3 Put samples though the following Citrisolv (d-Limonene) **Fisher Scientific Catalog** #22-143-975 series **on the rocker at** Room temperature **,** keeping samples in scint. vials**:**

4.4 1. 100% EtOH for 01:00:00 — 5h
2. 50:50 EtOH:Citrisolv for 02:00:00

Note

Equal parts 100% EtOH and 100% Citrisolv

3. 100% Citrisolv for 02:00:00

Note

Remove as much 50:50 EtOH:Citrisolv as possible with pipette before adding 100% Citrisolv.

4. 50:50 Citrisolv:Wax Chips 60 °C Overnight (*oven instructions below*)

Note

Fill glass staining dish ½ with Citrisolv
Place tissue cassette(s) open and submerged in Citrisolv.

Move tissue into cassette(s):

Note

Discard most of Citrisolv from scint. vials, but keeping tissue submerged. Quickly dump tissue with remaining Citrisov from scint. vial into open, submerged cassettes in the glass staining dish. Use forceps to take hold of the cassette(s) and shake while submerged in Citrisolv to release bubbles.

Note

Multiple tissues can be co-processed in single cassettes, but do not over-crowd tissue.

4.5 Bring the glass staining dishes that are half-full with Citrisolv to completely full with Paraplast Plus Tissue Embedding Medium **Grayine Medical Catalog #**39502004 .

Safety information

Paraplast Plus contains DMSO for higher efficiency infiltration. Handle with caution.

4.6 Allow the samples to infiltrate with Citrisolv:wax at 60 °C , Overnight , under -20 inHg vacuum.

Note

Once the vacuum reaches target pressure, close the oven vacuum valve and turn off vacuum pump.

Note

Put two additional glass staining dishes filled with molten wax (or wax chips) in the oven at this time so these dishes are ready to use the following morning for wax changes.

4.7 Make 3 changes into fresh molten wax per day for the next 2 days. All changes at 60 °C, under vacuum, as described above.

Note

Approximately 9:00 am, 1:00 pm, 4:30 pm.

Note

With the forceps, quickly transfer the cassette(s) from the current glass dish to the fresh dish. Shake to release bubbles.
Do not reuse the Citrisolv:wax glass dish for the 100% wax steps.

5 **Embedding**

For embedding, use DMSO-free

Epredia Signature Series Paraffin **Fisher Scientific Catalog #**22-050-128 .

5.1 Move the glass staining dish of molten wax containing cassette(s) from the vacuum oven to the heating compartment of the Epredia HistoStar embedding station.

5.2 Embed tissue in the molds. Alternating between heat and cold to orient and solidify the tissue in the bottom of molds as desired.

5.3 Once opaque, move the mold to the cold block to set for 02:00:00 .

2h

Note

Once fully solidified, either store molds at 4C or proceed to Tissue Sectioning.

Tissue Sectioning & Slide Mounting

6 **Sectioning**

6.1 ~30 minutes prior to sectioning:
Pre-heat the warming plate to 40 °C and coat “Superfrost Plus” microscope slides with a drop of 100%
Epredia Tissue Section Adhesive **Fisher Scientific Catalog #**86-014 . Spread over the slide surface and remove excess with a gloved finger (see video), place slides on the warmer to dry.

Note

Do not begin mounting until the adhesive has dried. Approximately ten minutes on the warming plate until slides are dry.

6.2 Using a razor blade, trim the wax molds into a trapezoidal shape down to the bevel or depth of embedded tissue. Mount the wax block to a microtome mounting block and set the microtome to section at 7 µm .

6.3 With a kimwipe, wipe the blade and blade holder with
PARA/Gard Paraffin Repellent **Fisher Scientific Catalog #**15-183-15 , wait a few seconds and then wipe the blade and blade holder with water.

Note

The Paraguard will melt away any wax and prevent adherence of wax slices onto the blade holder, allowing the ribbon to form.
The water will prevent static, helping to keep the ribbons from wrinkling. Blade holder should ideally be damp to allow ribbon to glide over smoothly.

Note

Recommended: section entire volume of embedded tissue laying out ribbons onto static-free, clean surface; then spot check sections for "good morphology"; only mount sections with target morphology onto coated slides for ISH.

7 Wet-Mounting Sections to Slides

7.1 With coated slides and a beaker of MG water on the warmer at 40 °C , use a disposable glass dropper to place 4 drops of MG water evenly spaced from top to bottom of the slide.

Note

~1 cm of space between each drop, or your sections will be too crowded and make later assay steps difficult (i.e., creating hydrophobic barriers).

7.2 Leave the slide with water on the warming plate for ~ 00:00:10 before adding the sections. 10s

7.3 Move single sections onto single drops of water on the slide by gently laying the slice onto the pool of water.

Note

Leave section on the water bubble for ~ 00:02:00 *. This allows the water to "stretch" out the wax section and remove wrinkles.*
Ideally, your wax sections should be large enough that the corners adhere to the glass slide, leaving the middle on top of the bubble (see image).

7.4 With the tissue sections bubbled on top of the water droplets, slowly tilt and tap the droplets out from under the sections onto a paper towel, leaving the sections behind on the slide surface. Flick to remove excess water from under sections. Replace slide back onto the warmer

7.5 Leave slides on warmer for ~ 00:02:00 , flick again to remove excess water. 2m

7.6 Once all desired sections are mounted to slides, close the warmer and leave slides Overnight .

7.7 The following day, move slides from the warmer into a slide box with a layer of Silica Gel Desiccant **Fisher Scientific Catalog** #S684-212 beads inside unless moving directly to ISH protocol.

Note

*Optional stopping point (1). The slides can be preserved in desiccant for **3 months maximum** at* Room temperature *before moving to RNAscope ISH assay.*

RNAscope 2.5 HD Detection Kit (RED) ISH Assay

8 **Part 1: Prepare and Pretreat Samples** 3h 30m

8.1 Prep:
Turn on oven to 60 °C .

Move hot plate into fume hood.
Open **new** bottle of 200 proof EtOH for specific use during ISH protocol.

Note

Note: each EasyDip slide container used throughout ISH protocol will hold ~100 mL of liquid. Each EasyDip slide rack holds 12 slides.
EasyDip container and slide rack hereafter referred to as "jars" and "racks"

8.2 Bake Slides: Place slides on a flat, clean surface (e.g. tray) and bake in a dry oven for 01:00:00 at 60 °C .
After, reduce oven to 40 °C .

1h

Note

*Optional stopping point (2). Use sectioned tissue within **1 week**. Store sections with desiccants at* Room temperature .

8.3 *During "Bake Slides" step, prepare HybEZ oven:* Set HybEZ oven to 40 °C and warm humidity control tray containing paper towel (wetted with DI water) for 00:30:00 before use. (*Keep tray warm during assay*).

30m

8.4 Deparaffinize FFPE sections (in fume hood):
1. Load slides into rack and place into a jar with Xylenes (Histological) **Fisher Scientific Catalog #**X3P-1GAL 2 x 00:05:00 , *agitate gently*.
2. Wash slides in 100% EtOH 2 x 00:01:00 .
3. Remove slides from rack. Air dry slides for 00:10:00 at Room temperature (*or until completely dry*).

16m

8.5 *At the start of the 10 minute drying period:* Prepare 300 mL fresh 1X Target Retrieval Solution in a 400 mL beaker. Do this by adding 30 mL RNAscope® Target Retrieval Reagents **Advanced Cell Diagnostics Catalog #**322000 to 270 mL MG water.

Cover with foil, bring to a mild boil (see video), and maintain.

Note

Do not boil more than 00:30:00 *before use.*

Note

Optional stopping point (3). Air dry overnight at Room temperature *(**must use within*** 24:00:00 *) or proceed directly to the next step.*

8.6 Apply hydrogen peroxide: 10m

Note

Recommended to start the hydrogen peroxide incubation 10-15 minutes after placing Target Retrieval Solution on hot plate.

1. Place slides back on flat, clean, dark-colored surface (e.g., tray lined with foil so you can see tissue on slides) and add 2-3 drops (or full coverage) of
   RNAscope Hydrogen Peroxide (H2O2) **Advanced Cell Diagnostics Catalog #**322330
   to each deparaffinized section for 00:10:00 at Room temperature .
2. Remove hydrogen peroxide from the slide by tapping the side onto absorbent paper (i.e., small stack of paper towels).
3. Place slides into rack, rinse in MG water; dunk 3-5x.
4. Repeat wash with fresh MG water.

8.7 Perform RNAscope Target Retrieval: 18m

1. *Very slowly,* submerge the slide rack into the simmering 1X Target Retrieval Solution for 00:05:00 .
2. *Immediately* transfer hot slide rack to a jar containing MG water.

3. Wash slides in MG water; dunk 3-5 times.
4. Repeat wash with fresh MG water.
5. Wash slides in 100% EtOH 1x for 00:03:00, *agitate gently*.
6. Remove slides from rack and air dry 00:10:00 at Room temperature.

8.8 Create Hydrophobic Barrier: Place slides back on a flat, clean, dark-colored surface draw around tissue 2-4x using 10m

ImmEdge Hydrophobic Barrier Pen **Advanced Cell Diagnostics Catalog #**310018

(see image). Let dry completely (~ 00:10:00) at Room temperature .

Note

Shake hydrophobic barrier pen well before use, press tip to a glass surface (e.g., spare slide) to dispense fluid prior to use on sample slides.

8.9 Apply Protease Plus: 10m

1. Place slides into HybEZ Slide rack and lock; add 1-2 drops

   RNAscope Protease Plus Reagents **Advanced Cell Diagnostics Catalog #**322330

   to each section resulting in full coverage.
2. Place HybEZ slide rack in the prewarmed HybEZ Humidity Control Tray with lid and insert back into HybEZ oven. Incubate for 00:10:00 at 40 °C.
3. Remove slides and gently tap on absorbent paper to remove excess liquid.
4. Load slides into rack.
5. Wash slides in MG water; dunk 3-5x.
6. Repeat wash with fresh MG water.

Note

When first putting slides in to incubate, move probes to 40 °C *oven for ~* 00:10:00 *, then let probes come to* Room temperature .

9 Part 2: RNAscope 2.5 Assay

5h

9.1

2h 2m

Hybridize Probe:

1. Remove excess liquid from slides by flicking aggressively (see video), place in the HybEZ Slide rack and lock, add ~4 drops of probe to each section (fully cover).

Note

If, at any time, probe solution spills onto another section, dunk slide into Wash Buffer immediately to prevent any unwanted reactions

1. Insert the covered tray containing slide rack back into the HybEZ oven for 02:00:00 at 40 °C .

Note

Prep materials during this time:
1st hour:
1. Put the bottle of RNAscope 50X
RNAscope® Wash Buffer Reagents **Advanced Cell Diagnostics Catalog #**310091
in the 40 °C oven for 01:00:00 .
2. Make 0.02% Ammonia water (bluing reagent). *If making from scratch, begin preparation now; prepare in fume hood.* Make this by combining 1.43 mL 1N Ammonium Hydroxide and 250 mL DI water.
2nd hour:
Remove the 50X Wash Buffer from the oven, reset the oven to 60 °C
1. Prepare 1.5 L of 1X Wash Buffer by adding 30 mL 50X Wash Buffer to 1.47 L DI water.
2. Move Amps 1-6 to the bench, let come to Room temperature (if light sensitive, leave in insulated box).

1. Remove slides from the HybEZ tray and rack, tap off excess liquid from slides onto absorbent paper.
2. Wash slides in 1X Wash Buffer for 00:02:00 at Room temperature , *agitate gently*.
3. Repeat wash with fresh 1X Wash Buffer.

9.2 Amp 1 Hybridization: 32m

1. Remove excess liquid from slides, place in HybEZ slide rack and lock, add ~2 drops of RNAscope 2.5 HD AMP 1 **Advanced Cell Diagnostics Catalog #**322311 to each section (full coverage).
2. Insert the closed tray containing slide rack back into the HybEZ oven for 00:30:00 at 40 °C.
3. Remove slides from rack and tap off excess liquid.
4. Wash slides in 1X Wash Buffer for 00:02:00 at Room temperature, *agitate gently*.
5. Repeat wash with fresh 1X Wash Buffer.

9.3 Amp 2 Hybridization: 17m

1. Remove excess liquid from slides, place in HybEZ slide rack and lock, add ~4 drops of RNAscope 2.5 HD AMP 2 **Advanced Cell Diagnostics Catalog #**322312 to each section.
2. Insert the covered tray containing slide rack back into the HybEZ oven for 00:15:00 at 40 °C.
3. Remove slides from rack and tap off excess liquid.
4. Wash slides in 1X Wash Buffer for 00:02:00 at Room temperature, *agitate gently*.
5. Repeat wash with fresh 1X Wash Buffer.

9.4 Amp 3 Hybridization: 32m

1. Remove excess liquid from slides, place in HybEZ slide rack and lock, add ~4 drops of RNAscope 2.5 HD AMP 3 **Advanced Cell Diagnostics Catalog #**322313 to each section.
2. Insert the covered tray containing slide rack back into the HybEZ oven for 00:30:00 at 40 °C.
3. Remove slides from rack and tap off excess liquid.
4. Wash slides in 1X Wash Buffer for 00:02:00 at Room temperature, *agitate gently*.
5. Repeat wash with fresh 1X Wash Buffer.

9.5 Amp 4 Hybridization: 17m

1. Remove excess liquid from slides, place in HybEZ slide rack and lock, add ~4 drops of RNAscope 2.5 HD AMP 4 **Advanced Cell Diagnostics Catalog #**322314 to each section.

2. Insert the covered tray containing slide rack back into the HybEZ oven for 00:15:00 at 40 °C.
3. Remove slides from rack and tap off excess liquid.
4. Wash slides in 1X Wash Buffer for 00:02:00 ta@t Room temperature , *agitate gently*.
5. Repeat wash with fresh 1X Wash Buffer.

Note

*Turn **OFF** HybEZ oven at this time. **Following incubations are at** Room temperature .*

9.6 Amp 5 Hybridization: 1h 2m

1. Remove excess liquid from slides, place in HybEZ slide rack and lock, add ~4 drops of RNAscope 2.5 HD AMP 5 RED **Advanced Cell Diagnostics Catalog #**322361 to each section.
2. Incubate the covered tray containing the HybEZ slide rack for 01:00:00 at Room temperature .
3. Remove slides from rack and tap off excess liquid.
4. Wash slides in 1X Wash Buffer for 00:02:00 at Room temperature , *agitate gently*.
5. Repeat wash with fresh 1X Wash Buffer.

9.7 Amp 6 Hybridization: 17m

1. Remove excess liquid from slides, place in HybEZ slide rack and lock, add ~4 drops of RNAscope 2.5 HD AMP 6 RED **Advanced Cell Diagnostics Catalog #**322362 to each section.
2. Incubate the covered tray containing the HybEZ slide rack for 00:15:00 at Room temperature .
3. Remove slides from rack and tap off excess liquid.
4. Wash slides in 1X Wash Buffer for 00:02:00 at Room temperature , *agitate gently*.
5. Repeat wash with fresh 1X Wash Buffer.

9.8 Detect Signal: 10m

Briefly spin down

RNAscope Fast Red B **Advanced Cell Diagnostics Catalog #**320459 and pipette mix 1 volume of RED-B to 60 volumes of

RNAscope Fast Red A **Advanced Cell Diagnostics Catalog #**320458 .

Note

Do NOT prep ahead of time: *must use within 3-5 minutes.*

Note

Be aware of the light sensitivity of the reagents.

RED-A is stored in a dropper tube. Drop solution into an epi tube to approximate amount needed.
(150 µL total is enough to cover 12 sections).

1. Remove excess liquid from slides, place in the HybEZ slide rack and lock, pipette RED solution onto tissue section to cover.
2. Incubate covered tray containing HybEZ slide rack for 00:10:00 at Room temperature .
3. Remove slides and tap off excess liquid onto absorbent paper.
4. Wash slides in **TAP** water, dunk 3-5x

Note

Prepare 50% Hematoxylin counterstain at this time. Add 50 mL Hematoxylin Solution, Gill No. 1 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**GHS116-500ML to 50 mL DI water.

9.9 *At this time the expression of the probes can be visualized under the microscope. Observe the slides before deciding whether or not to continue to the counterstain.*

9.10 Counterstain: 2m 10s

1. Place slides in 50% Hematoxylin I for 00:02:00 at Room temperature . wash in **TAP** water, dunk 3-5x, repeat with fresh tap water.
2. Wash slides for 00:00:10 in 0.02% Ammonia water, followed by dunking 3-5x in **TAP** water.

Expected result

Hematoxylin should render slides purple. Ammonia water should render slides blue.

9.11 Mount Slides: 25m

1. Place slides on a flat, clean surface and dry slides in 60 °C dry oven for 00:15:00 .
2. Dip slides into fresh pure (not recycled) xylene, dunk 5-6x, to remove the hydrophobic barrier.
3. Immediately apply mounting media (either Ecomount **Biocare Medical Catalog #**EM897L , Vectamount **Advanced Cell Diagnostics Catalog #**321584 , or EUKITT Neo Special Mounting Medium **Electron Microscopy Sciences Catalog #**15320-25) directly onto the sections and slide (~4 drops total) *before* the xylene dries. Place a coverslip over the section.
4. Air dry until cover slip is set (00:05:00 or Overnight).

Note

RED substrate is alcohol sensitive. Do not dehydrate in alcohol!

Imaging

10 Image on a compound microscope without filters (white light) equipped with digital camera and image capture software.

Note

For example, Nikon Eclipse scope with DS-Fi3 camera and NIS Elements D software.

Protocol references

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