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| Genome Innovation Hub  Standard Operating Procedure (SOP) |

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# A Purpose and Application

This SOP describes the implementation of ACD RNAscope® HiPlex12 v2 assay on formalin fixed paraffin embedded (FFPE) tissue. The RNAscope® HiPlex12 v2 Assay is the most advanced RNA in situ hybridization (ISH) assay based on ACD patented technology with signal amplification and simultaneous background noise suppression which advances RNA analysis in tissues and cells. And now it is available for archival FFPE tissue as well as fresh frozen tissue. The assay as described in this SOP allows simultaneous detection of up to 12 targets per slide. It enables the user to investigate expression as well as positional relationships between multiple genes at a single cell level. The assay may utilise an automated slide scanner to create high-quality virtual slides and capture fluorescence images with high throughput.

This SOP does not include any procedures on preparing the tissue sample (i.e. fixation) or complementary analyses such as H&E staining.

# B Brief Summary of Method

Properly prepared tissue slides are deparaffinized and are treated with protease, and then RNA-specific probes (off-the-shelf or customized) designed for different detection tails are hybridized to multiple RNAs (up to 12 targets). After a series of highly effective and specific signal amplification steps, single RNA transcripts for up to four targets genes at a time can be visualised as punctate dots in four distinct fluorescence channels using the cleavable versions of the fluorophores AF488, Atto550, Atto647, and AF750. These dots are visible with an epifluorescence microscope and the appropriate filters. After imaging, the fluorophores from the first four targets are cleaved off and the next four targets are labelled and imaged. Images from each round can be merged to produce a single image containing spatially resolved gene expression data for multiple genes at single cell resolution.

# C Definitions and Abbreviations

ACD – Advanced Cell Diagnostics

H&E – hematoxylin and eosin

FFPE – Formalin Fixed Paraffine Embedded

# D Occupational Health and Safety

Users must read, understand, and sign on to Risk Assessment ID #2905 “RNAscope Hiplex Assay on Formalin Fixed paraffin embedded (FFPE) tissue” in the IMB Risk Management database.

If you are using a microtome in the SBMS Histology facility, you must get trained beforehand by facility staff and read, understand, and sign on to Risk Assessment ID #3320 in the UQ Risk Assessment database.

If you are using a microtome in IMB level 4, you must get trained beforehand by facility staff in IMB microscopy facility and read, understand, and sign on to Risk Assessment ID #1643 in the IMB Risk Management Database.

After fixation in formalin or paraformaldehyde, the biological risk associated with High-Risk Biological materials is negated in most cases. However, all staff must still be vaccinated for hepatitis B and show 10IU minimum titre before beginning work.

# E Cautions

* Use SuperFrost Plus Adhesion Slides for all tissue types to avoid tissue detachment.
* It is recommended to always perform initial positive and negative control tests with your particular tissue, to assess sample RNA quality and optimal set up for imaging.
* Flick or tap the slides to remove residual reagents, but do not let the slides dry out at any time otherwise stated.
* We have noted significant autofluorescence detection in FFPE tissue. This background fluorescence makes it difficult to determine true signal for these probes. To reduce auto-fluorescence, use RNAscope HiPlex FFPE reagent at each round of image and perform an extra imaging round for blank image.
* RNA in tissue sections is easily degraded, which can result in poor detection of transcripts. Ensure your workplace and all materials used are clean and free of RNases. It is recommended that all measuring cylinders, bottles, forceps etc that are used throughout the procedure are baked before use to destroy any RNases.
* It is highly recommended to read the entire user manual before performing the assay.

# F Personnel Qualifications, Training and Responsibilities

This SOP is maintained and updated as required by the GIH Operations Manager.

Training Requirements for users:

X

X

Read and understand documents Training required

# G Equipment and Materials

#### Equipment

1. HybEZ™ II Oven (ACD Cat. No. 321720)
2. ZEISS Axio Scan.Z1 slide scanner microscopy or ZEISS LMS900 microscopy (SBMS imaging facility)
3. Waterbath (Benchmark)
4. Microtome (IMB imaging facility or SBMS histology facility)
5. Heatblock

#### Materials

1. RNAscope HiPlex Probe Diluent (ACD Cat. No. 324301)
2. RNAscope HiPlex 12 Positive Control Probe-Hs (ACD Cat. No. 324311)
3. RNAscope HiPlex 12 Negative Control Probe (ACD Cat. No. 324341)
4. RNAscope HiPlex Protease III (ACD Cat. No. 322340)
5. RNAscope HiPlex 12 Detection reagents Kit (ACD Cat. No. 324409)
6. RNAscope Wash Buffer Reagents (50X) (ACD Cat. No. 310091)
7. RNAscope HiPlex Cleaving stock solution v2 (ACD Cat. No. 324399)
8. HybEZ™ Humidity Control Tray (with lid) (ACD Cat. No. 310012)
9. ACD EZ-Batch™ Wash Tray (ACD Cat. No. 321717)
10. ACD EZ-Batch™ Slide Holder (ACD Cat. No. 321716)
11. HybEZ™ Humidifying paper (ACD Cat. No. 310015)
12. SuperFrost Plus Adhesion Microscope slides (Bio-Strategy Pty Limited Cat. No. EPBRSF41296SP)
13. ImmEdge™ Hydrophobic Barrier Pen (ACD Cat. No. 310018)
14. Xylene (Thermo Fisher, Cat. No. FSBX/0250/17)
15. 100% alcohol, molecular grade
16. 10X PBS (Fisher Scientific Cat. No. BP3991)
17. 10% Tween-20 (Fisher Scientific Cat. No. PI85115)
18. 20X SSC (Fisher Scientific Cat. No. BP1325)
19. Thermometer
20. ProLong Gold Antifade Mountant (Fisher Scientific Cat. No. P36930)
21. Cover glass (Zeiss, Cat. No. 474030-9000-000)
22. Ultra pure water, Dnase/Rnase free
23. Coplin jars (baked or RNAase free)
24. Staining jars (baked or RNAase free)
25. Tubes (50ml, 15ml, 1.5ml, various sizes)
26. RNase Zap
27. Paper towel

**Reagent setup**

Prepare 1X Wash Buffer

1. Take out 10 ml from 500ml of Ultra-pure water bottle and add 10 ml of 50X Wash Buffer.

2. Mix thoroughly by inverting the container at least ten times.

3. Make up at least 3 L of Wash buffer. Scale up and down as needed.

Note: If precipitation occurs in the 50X Wash Buffer, warm it up at 40 °C for 10 – 20 min before making the 1X Wash.

Prepare 4X SSC

1. To prepare 4X SSC, dilute 20X SSC with distilled water by pipetting one volume of 20X SSC with four volumes of Ultra-pure water. (i.e., 10 ml of 20 X SSC + 40 ml of water)

2. Mix thoroughly by inverting the container at least ten times.

Prepare PBST (0.5 % Tween)

1. To make 500 ml PBST (0.5 % Tween), add 50 ml 10X PBS, 425 ml distilled water, and 25 ml of 10 % Tween in a container.

2. Mix thoroughly by inverting the container at least ten times.

3. Make up at least 3 L of Wash buffer. Scale up and down as needed.

# H Procedure

# **Workflow outline:**

|  |  |  |  |
| --- | --- | --- | --- |
| Steps | Procedure | Duration | Note |
| FFPE sample preparation | FFPE sample sections | ~ 1 hr | Optional stopping point (1) |
|  | Bake slides | 2 hr | Optional stopping point (2) |
|  | Deparaffinization | ~ 30 min |  |
|  | Perform target retrieval | 30 min |  |
|  | Create a barrier | 10 min | Optional stopping point (3) |
|  | Apply RNAscope Protease III | 30 min |  |
| RNAscope assay for Round 1 | Hybridize probes | 2 hr | Optional stopping point (4) |
|  | Hybridize RNAscope HiPlex Amp 1 | 40 min |  |
|  | Hybridize RNAscope HiPlex Amp 2 | 40 min |  |
|  | Hybridize RNAscope HiPlex Amp 3 | 40 min |  |
|  | Incubate with RNAscope HiPlex FFPE reagent | 40 min |  |
|  | Hybridize RNAscope Fluoro T1-T4 v2 | 25 min |  |
|  | Counterstain the slides | 2 min |  |
|  | Image the samples for Round 1 |  |  |
| RNAscope assay for Round 2 | Remove the coverslips |  | Optional stopping point (5) |
|  | Cleave the fluorophores and wash (1) | 25 min |  |
|  | Cleave the fluorophores and wash (2) | 25 min |  |
|  | Incubate with RNAscope HiPlex FFPE reagent | 40 min |  |
|  | Hybridize RNAscope Fluoro T5-T8 v2 | 25 min |  |
|  | Image the samples for Round 2 |  |  |
| RNAscope assay for Round 3 | Remove the coverslips |  | Optional stopping point (6) |
|  | Cleave the fluorophores and wash (1) | 25 min |  |
|  | Cleave the fluorophores and wash (2) | 25 min |  |
|  | Incubate with RNAscope HiPlex FFPE reagent | 40 min |  |
|  | Hybridize RNAscope Fluoro T9-T12 v2 | 25 min |  |
|  | Image the samples for Round 3 |  |  |
| RNAscope assay for Round 4 | Remove the coverslips |  | Optional stopping point (7) |
|  | Cleave the fluorophores and wash (1) | 25 min |  |
|  | Cleave the fluorophores and wash (2) | 25 min |  |
|  | Image the samples for Round 4 |  | For blank image |
| Image analysis | Transfer and analyse images |  |  |

# Day 1

## Step 1 Sectioning FFPE tissue (~ 4hr)

1. Soak the FFPE block in iced cold water for minimum 1 hr before sectioning. Clean the microtome with the RNase Zap.

2. Section the block with 5 µm thickness and float the section onto 42°C water bath filled with RNase free water. Place the section onto a super frost slide.

3. Dry the slide on the heat block set up 42°C for at 3 hours.

4. Store the slide with desiccants for <1 week. Prolonged storage many degrade sample RNA.

*Optional stopping point (1)*

# Day 2

## Step 2 Bake slides (~ 2hr)

1. Bake slides on a heat block for < 2 hour at 60°C.

2. Prepare materials for the next step if you continue with the procedure.

*OPTIONAL STOPPING POINT (2)*. Use immediately, or store at RT with desiccants for ≤ 1 week. Prolonged storage may degrade sample RNA.

## Step 3 Deparaffinize FFPE sections (~ 30min)

Reagents may be prepared ahead of time. Ensure that all containers remain covered.

1. In a fume hood, fill two coplin jars with ~50 mL fresh xylene. Fill two staining jars ~200 mL fresh 100% ethanol.

2. Place slides in a coplin jar and submerge in the first xylene-containing dish in the fume hood.

3. Incubate the slides in xylene for 5 MIN at RT. Agitate the slides by occasionally lifting the slide rack up and down in the dish.

4. Remove the slide rack from the first xylene-containing dish and immediately place in the second xylene-containing jar in the fume hood.

5. Incubate the slides in xylene for 5 MIN at RT with agitation.

6. Remove the slide rack from the second xylene-containing jar and immediately place in a jar containing 100% ethanol.

7. Incubate the slides in 100% ethanol for 2 MIN at RT with agitation.

8. Remove the slide rack from the first ethanol-containing jar and immediately place in the second ethanol-containing dish.

9. Incubate the slides in 100% ethanol for 2 MIN at RT with agitation.

10.Remove the slides from the rack and place on absorbent paper with the section face-up. Dry slides in a drying oven for 5 MIN at 60°C (or until completely dry).

## Step 4 Prepare pretreatment materials (~ 30min)

1. Turn on the HybEZ Oven and set temperature to 40°C.

2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.

3. Insert covered tray into the oven and close the oven door. Warm the tray for 30 MIN at 40°C before use. Keep the tray in the oven when not in use.

4. Prepare 400 mL of fresh RNAscope 1X Target Retrieval Reagents by adding 360 mL distilled water to 40 mL 10X Target Retrieval Reagents in the beaker. Mix well.

5. Place the beaker containing RNAscope 1X Target Retrieval Reagents in the water bath. Cover the beaker with foil, and turn the water bath on high for 10–15 MIN.

6. Once the 1X RNAscope Target Retrieval Reagents reach a mild boil (< 98°C), turn the hot plate to a lower setting to maintain the correct temperature. Check the temperature with a thermometer. Do not boil the 1X RNAscope Target Retrieval Reagents more than 15 min before use.

## Step 5 Perform target retrieval using a water bath (30min)

1. With a pair of forceps very slowly submerge the slide rack containing the slides into the mildly boiling RNAscope 1X Target Retrieval Reagents solution. Cover the beaker with foil and boil the slides for 15 min.

2. Use the forceps to immediately transfer the hot slide rack from the RNAscope 1X Target Retrieval Reagents to the 50ml tubes containing distilled water. Do not let the slides cool in the Target Retrieval Reagents solution.

3. Wash slides 3–5 times by moving the slides up and down in the distilled water (for 15 sec).

4. Wash slides in fresh 100% alcohol (use 50ml tubes) for 3 min, and allow the slides to dry completely at 60°C for 5 MIN.

## Step 6 Create a barrier (10min)

1. Use the following template to draw a barrier 2–4 times around each section with the ImmEdge hydrophobic barrier pen.

2. Let the barrier dry completely ~10 min or OVERNIGHT at RT.

*OPTIONAL STOPPING POINT (3).* Dry slides overnight at room temperature for use the following day or proceed directly to the next section.

## Step 7 Apply RNAscope Protease III (30min)

1. Load the dry slides into the ACD EZ-Batch Slide Holder by opening the swing clamp.

2. Add ~3 drops of RNAscope Protease III to entirely cover each section.

3. Place the ACD EZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray. Close the lid, seal, and insert the tray back into the oven.

4. Incubate at 40°C for 30 min.

Note: If needed, prepare probes as described in the ‘Prepare probes’ section below.

5. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.

6. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.

7. Place the ACD EZ-Batch Slide Holder into the clear plastic wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation.

8. Repeat the wash step with fresh distilled water.

## Step 8 Hybridize probe (2hr 10min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into HybEZ Humidity Control Tray.

2. Add enough of the appropriate probe to entirely cover each section. For example, add 2 ~3 drops (~50ul) to cover a 1.5 cm x 1.5 cm barrier.

3. Close the tray and insert into the oven for 2 hrs at 40°C.

4. Bring RNAscope HiPlex FFPE Reagent to RT.

5. Briefly spin down the contents of the FFPE reagent tube to ensure content is at the bottom of the tube before opening the cap.

6. Cover with a foil and leave at RT.

7. After 2 hr, pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

8. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.

9. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.

10. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

*OPTIONAL STOPPING POINT (4)*. You can store the slides in 5X SSC (not provided in the kit) overnight at RT. Before continuing with the assay, wash the slides twice with 1X Wash Buffer for 2 MIN at RT.

# Day 3

## Step 9 Hybridize RNAscope HiPlex Amp 1 (40min)

1. Prepare 5% FFPE reagent to cover each section by using a 1:20 ratio of FFPE reagent to 4X SSC (for example 5 ul of FFP reagent in 95 ul of 4X SSC buffer). Mix well and cover with foil. Store at RT.

2. Place RNAscope HiPlex Amp 1–3 and RNAscope HiPlex Fluoro T1–T4 v2 reagents at RT. Place the humidity tray inside of the HybEZ oven and prewarm the oven at 40°C.

3. Wash the slides with twice with 1X wash buffer for 2 min at RT if the assay was paused after hybridization step

4. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

5. Add enough RNAscope HiPlex Amp 1 to entirely cover each section.

6. Close the tray and insert into the HybEZ Oven for 30 MIN at 40°C.

7. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.

8. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

9. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 MIN at RT.

10. Repeat the wash step with fresh 1X Wash Buffer for 2 MIN at RT.

## Step 10 Hybridize RNAscope HiPlex Amp 2 (40min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough RNAscope HiPlex Amp 2 to entirely cover each section.

3. Close the tray and insert into the HybEZ Oven for 30 MIN at 40°C.

4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.

5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 MIN at RT.

7. Repeat the wash step with fresh 1X Wash Buffer for 2 MIN at RT.

## Step 11 Hybridize RNAscope HiPlex Amp 3 (40min)

1. Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough RNAscope HiPlex Amp 3 to entirely cover each section.

3. Close the tray and insert into the HybEZ Oven for 30 MIN at 40°C.

4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven or keep it at RT if you are using FFPE Reagent.

5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 MIN at RT.

7. Repeat the wash step with fresh 1X Wash Buffer for 2 MIN at RT.

## Step 12 Apply RNAscope HiPlex FFPE Reagent to reduce autofluorescence (40min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough freshly prepared 5% FFPE reagent to entirely cover each section.

3. Close the tray and incubate for 30 min at RT.

4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

5. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.

6. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

## Step 13 Hybridize RNAscope HiPlex Fluoro T1–T4 v2 (25min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough RNAscope HiPlex Fluoro T1–T4 to entirely cover each section.

3. Close the tray and insert into the HybEZ Oven for 15 MIN at 40°C.

4. Remove the tray from the oven and remove the slide holder.

5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-BatchWash Tray.

6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 MIN at RT.

7. Repeat the wash step with fresh 1X Wash Buffer for 2 MIN at RT.

## Step 14 Counterstain and mount the slides (2min)

1. Remove excess liquid from the slides and add ~4 drops of DAPI to each section.

2. Incubate for 30 SEC at RT.

3. Remove DAPI from slides and immediately place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.

4. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store slides in the dark at 2–8°C.

Note: Store slides in the dark at 2–8°C before and after imaging. To the extent possible, it is ideal to keep slides cool while imaging to slow mountant curing process. As mountant cures, autofluorescence may increase and subsequent removal of coverslips will take longer. Preventing the slides from completely curing can shorten the assay running time and help to preserve tissue morphology throughout different rounds. When possible, imaging slides within 30 MIN of mounting is ideal.

## Step 15 Image the slides for Round 1 (the duration varies depending on the microscope setup)

Note : Zeiss LSM900 microscope was used for the assay.

1. Image the slides under Zeiss LMS900 microscope.

2. Turn on the key on the box at the left side of the microscope.

3. Switch on the two buttons above the key box.

4. Switch on 3 main switches on the workstation (under the monitor desk).

5. Log into the PC use UQ credentials.

6. Double click the Zen 3.1 (blue edition) software icon on the desktop.

7. Select ‘Zen System’ to initiate the confocal component.

8. Go to ‘Acquisition’ and load a predefined configuration (ie “RNAscope SY Melanoma WF 4 colours’)

9. Change the object to 63X oil and put a drop of oil onto the lens.

10. Place the slide upside down, fix and close the cover.

11. Go to ‘Tiles’ -> ‘Show Viewer’ -> Tiles -> Click ‘Set up new tile regions from predefined size’

12. Click ‘Live’ to see you have cells, otherwise move slide until you see the tissue area.

13. Change to ‘2 containers’ mode to have ‘Tile view’ and “Live’ side by side.

14 .Click ‘New tile regions by drawing contour’ -> Click ‘Draw a tile region with a rectangle contour’ -> Generate a preview -> Leave ‘DAPI’ only -> Start

15. Move the tile region to the area you want to capture.

16. Once the area of interest is covered, ‘Draw a tile region with a polygon contour’.

17. Click ‘Z-stack’ and drag it out.

18. Set up the ‘Range (thickness of the tissue)’, ‘Slices (number of z-slice)’, ‘Interval’.

19. Click the area you want to set up a ‘Centre’ and go live and focus.

20. Click ‘Centre’ to designate the area as a centre.

21. Go to ‘Tiles’ and click your current tile region from the table.

22. Click ‘Set current Z’

23. Move around to focus manually.

24. If it is out of the current z-stack range, change the Z-setting by increasing the ‘Range’.

25. Go to ‘Channels’.

26. Check each fluorescence signal and change exposure time if necessary.

27. Snap to preview.

28. Go to ‘Autosave’ and designate the folder and name for image (Save in D drive -> SBMS users -> GIH)

29. Start Experiment.

30. Copy the files into SBMS voltage drive.

31. Turn off the Zen blue software and log out from the PC.

32. Take out the slide and wipe lens with the provided cleaner.

33. Return the lens to 10X.

34. Switch off the 3 buttons under the monitor desk.

35. Turn off the Key first and switch off the 2 buttons above.

36. As there will be three rounds of detection and imaging, we recommend including the initials R1 (round 1), R2 (round 2), R3 (round 3) and the target names when saving image files. Implementing a naming convention will help you to identify each group of images during the image registration process.

37. Store the slides in 4X SSC buffer and leave at RT.

# Day 4

## Step 16 Cleave the fluorophores (50min)

1. Place RNAscope HiPlex Fluoro T5–T8 v2 reagents at RT. Place the humidity tray inside of the HybEZ oven and prewarm the oven at 40C.

2. Gently remove each coverslip. Once the coverslips have been removed, briefly wash the slides once in 4X SSC.

3. Break open a FRESH glass ampoule of provided Cleaving Stock Solution v2.

4. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.

Note: Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

5. Load the slides in the ACD EZ-Batch Slide Holder.

6. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

7. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.

8. Close the tray and incubate for 15 MIN at RT.

9. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.

10. Remove the slide holder from the tray.

11. Place the slide holder into the wash tray and wash the slides for 2 MIN at RT.

12. Repeat the wash step one more time with fresh PBST (0.5% Tween) for 2 MIN at RT.

13. Repeat a second cleaving reaction starting with the following step.

14. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

15. Apply enough 10% cleaving solution v2 to entirely cover each section.

16. Close the tray and incubate for 15 MIN at RT.

17. Remove the slide holder from the tray and place the tray into the HybEZ Oven to warm for the next step.

18. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.

19. Place the slide holder into the wash tray and wash the slides for 2 MIN at RT.

20. Repeat the wash step one more time with fresh PBST (0.5% Tween) for 2 MIN at RT.

## Step 17 Apply RNAscope HiPlex FFPE Reagent to reduce autofluorescence (40min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough freshly prepared 5% FFPE reagent to entirely cover each section.

3. Close the tray and incubate for 30 min at RT.

4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

5. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.

6. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

## Step 18 Hybridize RNAscope HiPlex Fluoro T5–T8 v2 (25min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough RNAscope HiPlex Fluoro T1–T4 to entirely cover each section.

3. Close the tray and insert into the HybEZ Oven for 15 MIN at 40°C.

4. Remove the tray from the oven and remove the slide holder.

5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-BatchWash Tray.

6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 MIN at RT.

7. Repeat the wash step with fresh 1X Wash Buffer for 2 MIN at RT.

## Step 19 Counterstain and mount the slides (2min)

1. Remove excess liquid from the slides and add ~4 drops of DAPI to each section.

2. Incubate for 30 SEC at RT.

3. Remove DAPI from slides and immediately place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.

4. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store slides in the dark at 2–8°C.

Note: Store slides in the dark at 2–8°C before and after imaging. To the extent possible, it is ideal to keep slides cool while imaging to slow mountant curing process. As mountant cures, autofluorescence may increase and subsequent removal of coverslips will take longer. Preventing the slides from completely curing can shorten the assay running time and help to preserve tissue morphology throughout different rounds. When possible, imaging slides within 30 MIN of mounting is ideal.

## Step 20 Image the slides for Round 2 (the duration varies depending on the microscope setup)

1. Image the slides with the same method described at step 15 Image the slides for Round 2.

2. Store the slides in 4X SSC buffer and leave at RT.

# Day 5

## Step 21 Cleave the fluorophores (50min)

1. Place RNAscope HiPlex Fluoro T9–T12 v2 reagents at RT. Place the humidity tray inside of the HybEZ oven and prewarm the oven at 40C.

2. Gently remove each coverslip. Once the coverslips have been removed, briefly wash the slides once in 4X SSC.

3. Break open a FRESH glass ampoule of provided Cleaving Stock Solution v2.

4. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.

Note: Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

5. Load the slides in the ACD EZ-Batch Slide Holder.

6. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

7. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.

8. Close the tray and incubate for 15 MIN at RT.

9. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.

10. Remove the slide holder from the tray.

11. Place the slide holder into the wash tray and wash the slides for 2 MIN at RT.

12. Repeat the wash step one more time with fresh PBST (0.5% Tween) for 2 MIN at RT.

13. Repeat a second cleaving reaction starting with the following step.

14. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

15. Apply enough 10% cleaving solution v2 to entirely cover each section.

16. Close the tray and incubate for 15 MIN at RT.

17. Remove the slide holder from the tray and place the tray into the HybEZ Oven to warm for the next step.

18. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.

19. Place the slide holder into the wash tray and wash the slides for 2 MIN at RT.

20. Repeat the wash step one more time with fresh PBST (0.5% Tween) for 2 MIN at RT.

## Step 22 Apply RNAscope HiPlex FFPE Reagent to reduce autofluorescence (40min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough freshly prepared 5% FFPE reagent to entirely cover each section.

3. Close the tray and incubate for 30 min at RT.

4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.

5. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.

6. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

## Step 23 Hybridize RNAscope HiPlex Fluoro T9–T12 v2 (25min)

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

2. Add enough RNAscope HiPlex Fluoro T9–T12 to entirely cover each section.

3. Close the tray and insert into the HybEZ Oven for 15 MIN at 40°C.

4. Remove the tray from the oven and remove the slide holder.

5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-BatchWash Tray.

6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 MIN at RT.

7. Repeat the wash step with fresh 1X Wash Buffer for 2 MIN at RT.

## Step 24 Counterstain and mount the slides (2min)

1. Remove excess liquid from the slides and add ~4 drops of DAPI to each section.

2. Incubate for 30 SEC at RT.

3. Remove DAPI from slides and immediately place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.

4. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store slides in the dark at 2–8°C.

Note: Store slides in the dark at 2–8°C before and after imaging. To the extent possible, it is ideal to keep slides cool while imaging to slow mountant curing process. As mountant cures, autofluorescence may increase and subsequent removal of coverslips will take longer. Preventing the slides from completely curing can shorten the assay running time and help to preserve tissue morphology throughout different rounds. When possible, imaging slides within 30 MIN of mounting is ideal.

## Step 25 Image the slides for Round 3 (the duration varies depending on the microscope setup)

1. Image the slides with the same method described at step 15 Image the slides for Round 3.

2. Store the slides in 4X SSC buffer and leave at RT.

# Day 6 (optional for high auto-fluorescent tissue)

Note: This round of blank slide imaging is recommended if the tissue specimen exhibited high autofluorescence background that interfered with the detection of positive fluorescent signals.

## Step 26 Cleave the fluorophores (50min)

1. Gently remove each coverslip. Once the coverslips have been removed, briefly wash the slides once in 4X SSC.

2. Break open a FRESH glass ampoule of provided Cleaving Stock Solution v2.

3. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.

Note: Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

4. Load the slides in the ACD EZ-Batch Slide Holder.

5. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

6. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.

7. Close the tray and incubate for 15 MIN at RT.

8. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.

9. Remove the slide holder from the tray.

10. Place the slide holder into the wash tray and wash the slides for 2 MIN at RT.

11. Repeat the wash step one more time with fresh PBST (0.5% Tween) for 2 MIN at RT.

12. Repeat a second cleaving reaction starting with the following step.

13. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.

14. Apply enough 10% cleaving solution v2 to entirely cover each section.

15. Close the tray and incubate for 15 MIN at RT.

16. Remove the slide holder from the tray and place the tray into the HybEZ Oven to warm for the next step.

17. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.

18. Place the slide holder into the wash tray and wash the slides for 2 MIN at RT.

19. Repeat the wash step one more time with fresh PBST (0.5% Tween) for 2 MIN at RT.

## Step 27 Counterstain and mount the slides (2min)

1. Remove excess liquid from the slides and add ~4 drops of DAPI to each section.

2. Incubate for 30 SEC at RT.

3. Remove DAPI from slides and immediately place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.

4. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store slides in the dark at 2–8°C.

Note: Store slides in the dark at 2–8°C before and after imaging. To the extent possible, it is ideal to keep slides cool while imaging to slow mountant curing process. As mountant cures, autofluorescence may increase and subsequent removal of coverslips will take longer. Preventing the slides from completely curing can shorten the assay running time and help to preserve tissue morphology throughout different rounds. When possible, imaging slides within 30 MIN of mounting is ideal.

## Step 28 Image the slides to use with background subtraction function in HiPlex Registration Software v2.0.1.

1. Image the slides with the same method described at step 15 Image the slides for blank image.

## Step 29 Image registration using RNAscope HiPlex Registration Software v2.0.1

1. Image registration using RNAscope HiPlex Registration Software v2.0.1

NOTE: Contact ACD Technical Support team (support.acd@bio-techne.com) or local distributor (Aus/NZ : InVitro Technologies) to download software.

2. Register the DAPI, AF488, Dylight550, Dylight650, and AF750 fluorescent images generated from all rounds.

3. To ensure accuracy, make sure that the DAPI channel images are similarly exposed.

4. Refer to the RNAscope HiPlex Registration Software User Manual (Doc. No. 300065-USM). A step-by-step guide for how to use the software is also available in the installer package of the software.

# I Worked Example

Two melanoma FFPE slides were provided by Dr Mitchell Stark group in UQ Fraser Instititue and were used for the assay according to the SOP. For publication purpose only 8 probes (two of image rounds) were selected for the assay to avoid excessive staining and washing steps that may lead tissue detachment. Total 3 rounds of image were performed including blank image round and the single images at each round were composited using RNAscope HiPlex image registration software according to the software user manual. Each punctate dot represents single mRNA molecules. The expression level of the target molecules varies depending on the histopathological information of the tissue. The 8 of different target mRNA molecules are expressed in the two-melanoma tissue (A and B). The negative control probe is targeting a bacterial gene (dapB), with each 12 tail respectively (C, left). The positive control probes are an RTU mixture of 12 probes targeting 12 housekeeping genes with each 12 tails, respectively (C, right).



# J SOP Validation Details

This SOP has been developed according to the manufacturer's protocol. Gene expression data has been assessed by Dr. Quan Nguyen and PhD student Quang Anh Tuan Vo based on Visium spatial transcriptomic data from the same FFPE blocks and has been considered to show a good co-relationship with the Visium data.

# K Troubleshooting

|  |  |  |
| --- | --- | --- |
| Step | Issue | Recommendations |
| All step | Tissue detachment | * Use SuperFrost Plus Adhesion Microscope slides * Bake slide more than 1hr at step 2 * Wash gently |
| Imaging step | Weak/no signal | * May be due to under digestion: increase the incubation time with Protease III * May be due to RNA degradation: use sectioned tissue within 1 week from sectioning |
| High background | * May be due to dried tissue: use desiccant to store slides. * Run extra imaging round to have a blank image |
| Step 29 | Failing to register and overlay images | * This may be cause of the location of the same region are not matched or the resolution and pixel by pixel dimensions between two image rounds. This can be resolved by using a microscope with a precise stage recorder. |

# L Waste Management and Disposal

Solid and low-volume liquid waste generated through performing this protocol are to be disposed of into clinical waste bins according to IMB waste management protocol. Sharps including slides and coverslips are to be disposed of into puncture-resistant clinical sharps bins. There are no special waste disposal requirements associated with this SOP.

# M Data Records Management

All generated image files (.czi and .tiff) and are saved in GIHEX21PRO-UQRDM.

# N Reference Documents

ACD RNAscope HiPlex12 Reagent Kit (488,550,650,750) v2 Standard Assay (document No. 324409-UM)

ACD RNAscope HiPlex Image registration software v2.0.1 user manual (document No. 300065-UM)

Risk Assessments associated with this SOP are available in the IMB Risk Assessment Database in WebDB:

* Risk Assessment ID #2905 “RNAscope Hiplex Assay on Formalin Fixed paraffin embedded (FFPE) tissue”

Other references Risk Assessments are available in the IMB Risk Assessment Database or UQSafe-Risk system.

* IMB Risk Assessment ID #1643 “Use of a manual rotary microtome”
* UQ Risk Assessment ID #2916 “SBMS Histology – Disposal of Waste”
* UQ Risk Assessment ID #1365 “SBMS Histology – Use of Sharps/Blades”
* UQ Risk Assessment ID #3320 “SBMS Histology – Use of the Sliding Microtome”

# O Quality Control (QC) & Quality Assurance (QA) Section

* Recommend running both positive and negative control. If not practical, run negative control with your test slide at least.
* Perform H&E staining on a consecutive tissue section to check the morphology of the tissue.
* Before starting imaging, confirm that nuclei are stained with DAPI properly.

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1. **Collaborator name (optional)**
2. **Collaborator title (optional)**

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