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THE UNIVERSITY OF TEXAS MD Anderson Cancer Center
Making Cancer History®

RNAscope *In Situ* Hybridization Assay SOP

Version 2.0

1. **Analyte(s):** *CTLA4, CD274* (PD-L1), *IL10, IFNG*, *TNFRSF8* (CD30)

2. **Technical Platform(s):**

Sample Preparation for RNAScope assay - This protocol is intended to be applied in formalin-fixed paraffin embedded (FFPE) tissue, including whole tissue sections or tissue microarrays. Tissue should be properly fixed according to ACD guidelines, namely *"FFPE samples should be fixed in FRESH 10% NBF (neutral buffered formalin) for 16 – 32 hrs at RT (1)*. FFPE tissues should be sectioned at 5 +/-1 µm in Superfrost® Plus Slides. When studying tumor tissue, a minimum of 100 viable tumor cells are required.

The RNAscope Assay use *in situ* hybridization (ISH) to visualize single RNA molecules per cell in formalin-fixed, paraffin-embedded (FFPE) tissue mounted on slides. The tissue section is pretreated with heat and enzyme, then hybridized with RNA-specific probes to target RNAs. The signal is then amplified using multiple steps, followed by hybridization to horseradish peroxidase (HRP)-labeled probes and detected using the 3,3'-diaminobenzidine (DAB) chromogenic substrate. Each single RNA transcript will appear as a distinct dot of chromogen precipitate that is visible using a common brightfield microscope.(1, 2)

3. **Reagents, controls, and calibrators**
   a) RNAscope probes:
      ✓ Target probes: *CTLA4, CD274* (PD-L1), *IL10, IFNG*, *TNFRSF8* (CD30)

- RNAscope® LS 2.5 Probe- Hs-CTLA4: Cat# 554348
- RNAscope® LS 2.5 Probe- Hs-CD274 (PD-L1): Cat# 600868
- RNAscope® LS 2.5 Probe- Hs-IL-10: Cat# 602058
- RNAscope® LS 2.5 Probe- Hs-IFNG: Cat# 310508
- RNAscope® LS 2.5 Probe- Hs-TNFRSF8 (CD30): Cat # 593458

      ✓ Control probes: *PPIB, DapB.*

- RNAscope® 2.5 LS Positive Control Probe – Hs-PPIB (Cat# 313908)
- RNAscope® 2.5 LS Negative Control Probe – DapB (Cat# 312038)

   b) Reagents, controls, and calibrators

      Reagents:

- RNAscope 2.5 LSx Reagent Kit – Brown (Cat# 322700)
  - RNAscope 2.5 LSx Rinse
  - RNAscope 2.5 LSx Hematoxylin
  - RNAscope 2.5 LSx DAB
  - RNAscope 2.5 LSx Bluing
  - RNAscope 2.5 LSx AMP 1 DAB
  - RNAscope 2.5 LSx AMP 2 DAB

- RNAscope 2.5 LSx AMP 3 DAB
- RNAscope 2.5 LSx AMP 4 DAB
- RNAscope 2.5 LSx AMP 5 DAB
- RNAscope 2.5 LSx AMP 6 DAB
- RNAscope 2.5 LSx H_2O_2
- RNAscope 2.5 LSx Protease

- Leica BOND Reagents
  - BOND Epitope Retrieval Solution 2-1L (RTU) (Cat# AR9640)
  - BOND Dewax Solution – 1L (RTU) (Cat# AR9222)
  - BOND Wash Solution 10X Concentrate – 1L (Cat# AR9590)

Equipment:

- BOND RX Fully Automated Research Stainer from Leica Biosystems

Control slides:

- RNAscope® Control Slides - Human HeLa Cell Pellet (Cat# 310045)

4. Automated Steps (Deparaffination, epitope retrieval, hybridization and counterstaining):

Pretreatment

1) Bake at 60℃ for 30 minutes.
2) BOND Dewax Solution (AR9222) at 72℃ with 3 changes.
3) 100% Alcohol with 3 changes
4) Epitope Retrieval solution (ER2, pH9, AR9640) at 95℃ for 15 minutes *
5) RNAscope 2.5 LSx Protease at 40 ℃ for 15 minutes **
6) RNAscope 2.5 LSx H_2O_2 for 10 minutes

Hybridization

7) RNAScope probe hybridization at 42℃ for 120 minutes

Pre-Amplification

8) RNAscope 2.5 LSx AMP 1 DAB for 30 minutes
9) RNAscope 2.5 LSx Rinse for 10 minutes

Amplification

10) RNAscope 2.5 LSx AMP 2 DAB for 15 minutes
11) RNAscope 2.5 LSx Rinse for 10 minutes
12) RNAscope 2.5 LSx AMP 3 DAB for 30 minutes
13) RNAscope 2.5 LSx Rinse for 10 minutes
14) RNAscope 2.5 LSx AMP 4 DAB for 15 minutes
15) RNAscope 2.5 LSx Rinse for 10 minutes
16) RNAscope 2.5 LSx AMP 5 DAB for 30 minutes
17) RNAscope 2.5 LSx Rinse for 10 minutes
18) RNAscope 2.5 LSx AMP 6 DAB for 15 minutes
19) RNAscope 2.5 LSx Rinse for 10 minutes

Signal Detection

20) RNAscope 2.5 LSx DAB for 20 minutes

Counter Staining

21) RNAscope 2.5 LSx Hematoxylin for 5 minutes
22) RNAscope 2.5 LSx Bluing for 2 minutes.

Notes:

* Epitope retrieval condition in step 4 can be modified according to tissue type. See Appendix B in the reference (3).
** Protease pretreatment in step 5 can be modified according to tissue type. See Appendix C in the reference (3).

5. Manual Completion Steps:

1) Unload the slides from the instrument and place them into Deionized (DI) water.
2) Dehydrate and clear sections through 2 changes of 95% alcohol, 2 changes of 100% alcohol and 2 changes of xylenes (10 dips each for all solutions).
3) Mount the section with cover glass using xylene based mounting medium, such as Cytoseal.

6. Scoring

The workflow for histological evaluation and scoring includes the following steps:

1.*Evaluation of tissue and cell morphology* in H&E-stained slides from FFPE sample. Using a standard bright-field microscope at 20-40x magnification a pathologist ensures presence and quality of tissue of interest in the sample. For neoplastic tissue a minimum of 100 viable tumor cells are required.

2.*Evaluation of signal strength in the positive control probe* (*PPIB*, Homo Sapiens peptidylprolyl isomerase B) RNAScope stained slide. The positive control signal should be visible as ≥4 punctuate dots within the cell using a standard bright-field microscope at 20-40X magnification. Ensure that tissue of interest is present in stained section.

3.*Evaluation of negative control probe* (*dapB*, Bacillus subtilis dihydrodipicolinate reductase gene). No dots visible or 1 dot to every <10 cells at 20x magnification with standard bright-field microscope, is considered negative. Ensure that tissue of interest is present in stained section.

4. *Evaluation of target probe* can be performed manually or digitally using the semi-quantitative histological scoring methodology based on ACD scoring criteria to visualize dot signals (representing single target RNA molecules) (**Table 1**). This scoring guideline uses the estimated number of punctuate dots present within each cell boundary. A score of 0 has no staining or less than 1 dot for every 10 cells, while a score of 4+ has greater than 15 dots per cell. The manual evaluation is performed using standard bright-field microscope at 20-40x magnification.

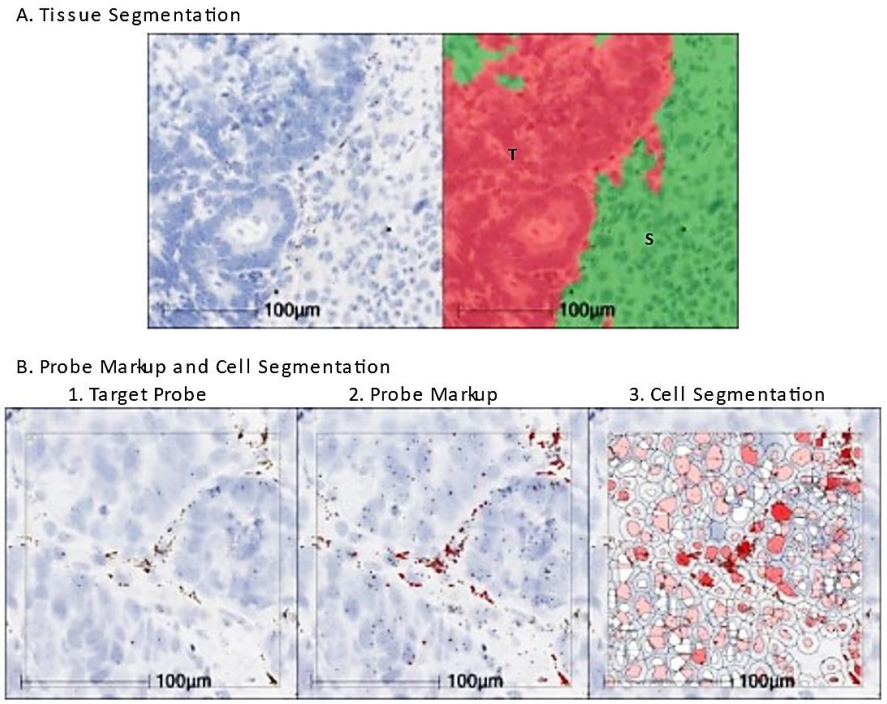

Figure 1. Microphotograph of FFPE colorectal carcinoma showing the algorithm in Halo software to quantify the number of target probes signals **A**. Tissue segmentation showing Tumor (T) in red, and stroma (S) compartments. **B.** Probe mark up and cell segmentation **1.** target probes identified as brown dots **2.** probe mark-up identifying target probes (shown as red dots), and **3.** cell segmentation with target probe mark up. The different color of the cell segmentation illustrates the staining score based on Advanced Cell diagnostic Scoring guidelines.

The digital image evaluation includes the scanning of slides with Aperio AT2 scanner (Leica Biosystem) at 40x and the creation of an algorithm using ISH v.4.1.3 settings in HALO Software to quantify the number of target probe signals in tumor cells. This algorithm starts with the creation of annotation layers to perform tissue (tumor/stroma) and cell segmentation when needed with subsequent target probe markup using ACD scoring criteria. These settings are analyzed obtaining graphed plots for the target probe cell bins and target probe copies per cell. The results are reported as copies per cell, H-score [(range 0-400, calculated as H-score = Σ (ACD score or bin number x percentage of cells per bin)] and percentage of tumor cells positive for staining score 0, 1+, 2+, 3+ and 4+ (Advanced Cell Diagnostics Scoring Guidelines) **Figure 1**.

For the validation, both methods, manual and digital analysis were used subsequently in the same samples.

Table 1. Advanced Cell Diagnostics Scoring Guidelines

Score	RNA ISH score (ACDbio Scoring Criteria)
0	No staining or <1 dot/ 10 cells
1+	1-3 dots/cell
2+	4-9 dots/cell. None or very few dot clusters
3+	10-15 dots/cell and <10% dots are in clusters
4+	>15 dots/cell and >10% dots are in clusters

7. References

1. ACDbio. Technical support 2023 [Available from: https://acdbio.com/technical-support/solutions/faq.
2. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2012;14(1):22-9.

3. ACDbio. RNAscope® 2.5 LSx Reagent Kit – BROWN User Manual for BDZ 15/BXD 15 2018 [Available from: https://acdbio.com/sites/default/files/322700-USM%20RNAscope%20LSx%20BROWN%2005102018.pdf.

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Signature (Luisa M. Solis Soto) **Date: 12/15/2023**