Validation of an Integrated MultiOmyx-RNAscope Assay for co-detection of RNA/protein

**Integrated MultiOmyx-RNAscope Assay Workflow and 2pkISH 17pxIF Validation Biomarker Panel**

**Figure 1. Integrated MultiOmyx-RNAscope Assay Workflow.** The integrated workflow combines RNAscope(RS) and MultiOmyx(CXCL10) ISH staining protocols. Slides were deparaffinized and rehydrated. Slides were incubated at 60°C for 20 minutes before proceeding to step 2. **A**) The slides were covered with 100μL of propylene glycol (PG) for 20 minutes, followed by rinsing with 100μL of PG for 5 minutes, before proceeding to step 3. **B**) Slides were blocked with 2%BSA for 10 minutes, followed by rinsing with PBS. **C**) Slides were covered with 100μL of 2% normal goat serum (NGS) for 10 minutes at room temperature followed by 3 rinses with PBS. **D**) Slides were covered with 100μL of MultiOmyx 17pxIF, 1:100 dilution, for 20 minutes at room temperature, with 3 rinses with PBS. **E**) Slides were blocked with 2%BSA for 10 minutes, followed by rinsing with PBS. **F**) Slides were covered with 100μL of RNAscope 2pkISH kits (R-19038/8) for 17pxIF, according to manufacturer's instructions. **G**) Slides were blocked with 2%BSA for 10 minutes, followed by rinsing with PBS. **H**) Slides were covered with 100μL of ISH detection solution for 30 minutes, followed by 3 rinses with PBS. **I**) Slides were washed with 100μL of RNase-free deionized water before proceeding to step 9. **J)** Slides were covered with 100μL of DAPI for 10 minutes. **K**) Slides were blocked with 2%BSA for 10 minutes, followed by rinsing with PBS. **L**) Slides were covered with 100μL of MultiOmyx 17pxIF, 1:100 dilution, for 20 minutes at room temperature, with 3 rinses with PBS. **M**) Slides were covered with 100μL of ISH detection solution for 30 minutes, followed by 3 rinses with PBS. **N**) Slides were washed with 100μL of RNase-free deionized water before proceeding to step 9. **O**) Slides were covered with 100μL of DAPI for 10 minutes. **P**) Slides were blocked with 2%BSA for 10 minutes, followed by rinsing with PBS. **Q**) Slides were covered with 100μL of ISH detection solution for 30 minutes, followed by 3 rinses with PBS. **R**) Slides were washed with 100μL of RNase-free deionized water before proceeding to step 9. **S**) Slides were covered with 100μL of DAPI for 10 minutes.

**Figure 2. Comparison of Integrated MultiOmyx-RNAscope Assay Version 1 and Version 2.** (A) Free-floating images show a single FFPE slide stained with MultiOmyx 17pxIF ISH (B) (BioTek) platform. MultiOmyx is a proprietary immunofluorescence (IF) platform for the visualization and characterization of up to 60 protein biomarkers in a single FFPE section. RNAscope Multiplex is a highly sensitive fluorescent in situ hybridization (ISH) assay that can detect up to 3 RNA markers in a single FFPE section. A unique feature of the Integrated RNAscope assay is the presence of a pretreatment step, which is required for RNAscope ISH staining but can damage proteins and consequently interfere with downstream antibody binding.

**Methods:** Optimization of both antigen retrieval and protein pretreatment steps was performed to improve protein biomarker accuracy. The optimized integrated Multioscope-RNAscope assay was then completed using a 2pkISH 17pxIF biomarker panel on FFPE human colonized cancer (CRC) samples. Expression of each ISH and protein biomarker was quantified using NeoTagSTM, the proprietary MultiOmyx Analytics pipeline and inter/intra run coefficient of variance (CV) were calculated for the precision assessment.

**Results:** The optimized integrated assay demonstrated improved protein biomarker staining/compatibility without compromising RNA ISH signal. Additionally, all markers evaluated showed highly reproducible results and passed successful criteria for precision evaluation. These results therefore demonstrate a highly robust assay with even improved performance observed for some markers previously evaluated.

**Conclusions:** Therefore, we successfully optimized the Integrated MultiOmyx-RNAscope assay for co-detection of protein/RNA in single FFPE specimen thereby improving assay development turnaround and protein biomarkers performance/compatibility.

**Summary**

- Integrated MultiOmyx-RNAscope assay V2 shows improved biomarker signal/noise and detection.
- Precision study of V2 assay in 3 CRC samples demonstrates robustness of biomarkers analyzed.
- Co-detection of RNA and protein shows correlation of cytokine RNA expression in different populations of the TME.
- The optimized Integrated MultiOmyx-RNAscope V2 assay is a robust and sensitive platform for co-detection of RNA/protein in a single FFPE sample.