# A Novel Protease-Free Method for the Co-detection of RNA and Protein Biomarkers using the RNAscope™ Technology

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## Background

RNAscope™ *in situ* hybridization (ISH) technology, capable of highly sensitive single-molecule RNA detection, can be combined with immunohistochemistry (IHC) or immunofluorescence (IF) for the co-detection of clinically relevant biomarkers on the same slide with morphological context. This application is especially important in immuno-oncology research to profile immune cell populations using protein markers and characterize their activation states by detecting cytokine and chemokine expression with RNA. Proteases are normally required for the RNAscope technology, but they can impact some epitopes when combined with certain antibodies. A novel RNA-protein co-detection workflow has been developed which eliminates the need for proteases, resulting in high detection sensitivities for both protein and RNA markers.

## Methods

To maintain the same RNA detection sensitivity without the use of proteases, we formulated a new protease-free pretreatment buffer to replace the existing protease step within the current RNAscope ISH-IHC co-detection workflow which allows adequate accessibility of RNAscope probes to the target RNAs. Following this protease-free pretreatment, tissue specimens were assayed with various RNAscope assays to detect RNA in tissue, followed by standard IHC or IF staining to co-detect protein biomarkers.

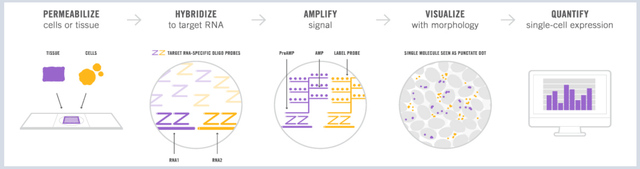


**Figure 1: Overview of the RNAscope workflow. Protease-free step occurs during permeabilization or pretreatment step.** This workflow is manual or automated on Leica BOND RX and Ventana BenchMark Ultra.

## Results

**Some antibodies are sensitive to protease treatment.** CD8 and FOXP3 antibodies show negative impact due to proteases, whereas CD68 and CD3 antibodies show minimal to no negative impact due to proteases. IHC with protease-free shows similar staining patterns as IHC, suggesting negligible impact to epitopes from the workflow for both protease-sensitive and protease-resistant antibodies.

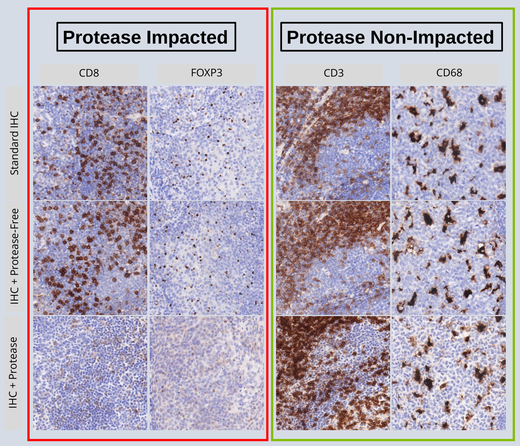


**Figure 2**: **Comparison of antibody staining on human tonsil tissue using standard IHC, IHC with protease-free reagent and IHC with protease. 50% of 12 tested antibodies show impact from Protease.**

**Protease sensitive neuronal marker NeuN antibody works with protease-free reagent on mouse brain in sequential co-detection workflow on Leica BOND RX platform**

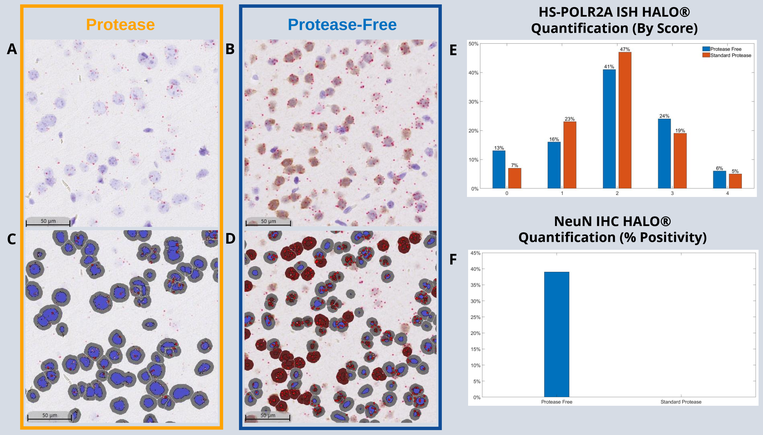


**Figure 3: Comparison of NeuN antibody signal (brown) with *Polr2a* mRNA probe (red) expression in mouse brain using RNAscope LS Red ISH-sequential IHC** with standard protease (A) and protease-free reagent (B). Quantitative analyses performed by Indica Labs HALO® software, indicating improvement for NeuN protein detection (F) while maintaining comparable *Polr2a* RNA dot counts (E). HALO® overlays shown for standard protease (C) and protease-free (D).

**Compatibility of protease-free reagent with RNAscope Multiplex Fluorescent assay in sequential co-detection workflow on Leica BOND RX**

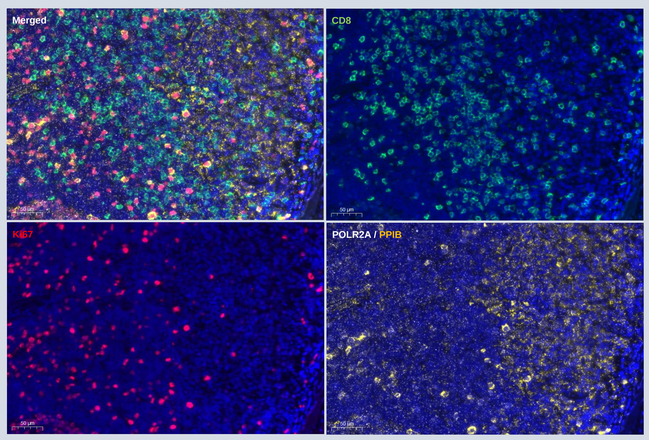


**Figure 4: Protease-sensitive CD8 (green) and Ki67 (red) antibody signal with human *POLR2A* (white) and *PPIB* (yellow) mRNA in normal human tonsil tissue** using RNAscope LS ISH-sequential IF with protease-free reagent.

**Protease sensitive CD45 antibody works with protease-free reagent on Human bladder cancer in sequential co-detection workflow on Ventana BenchMark Ultra**

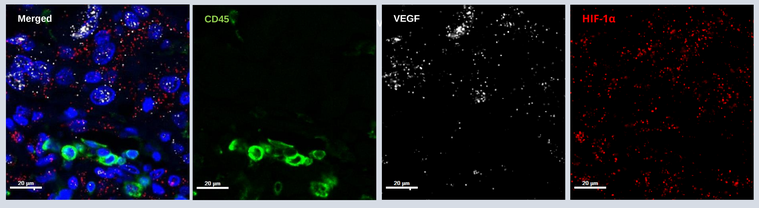


**Figure 5: CD45 antibody signal (green) with *VEGF* (white) and *HIF-1α* (red) mRNA probe expression in human bladder cancer** using RNAscope VS ISH-sequential IF with protease-free reagent.

***CCL5* and *CXCL9* (induced by *IFN-γ*) are over-expressed in CD8+ T-cell infiltration sites in solid tumors.** T-cell infiltration requires tumor cell-derived CCL5 and is amplified by IFN-γ-inducible, myeloid cell-secreted CXCL9. The interaction between tumor-derived CCL5 and IFN-γ-inducible CXCR3 ligands produced by myeloid cells is key for coordinating T-cell recruitment to immunoreactive tumors.

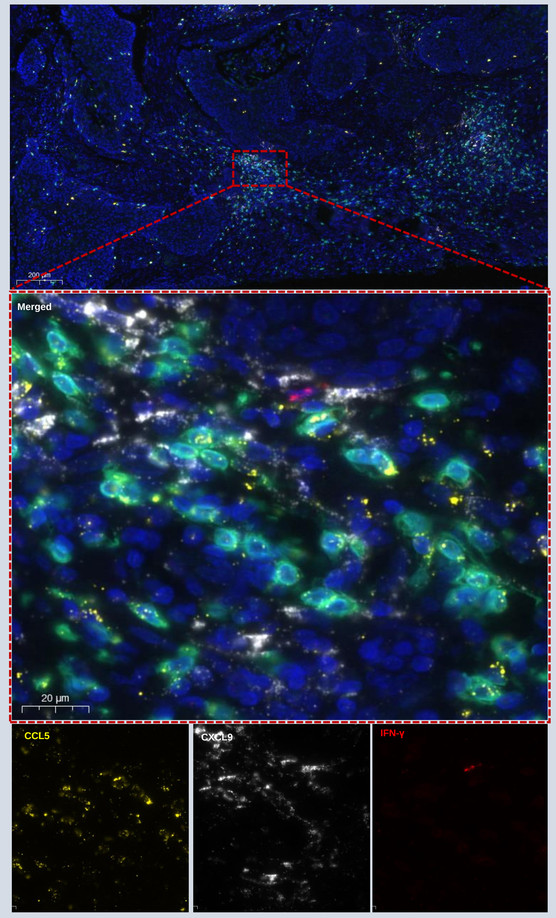


**Figure 6: Protease-sensitive CD8 antibody signal (green) with *CCL5* (yellow), *CXCL9* (white) and *IFN-γ* (red) mRNA probe expression in human cervical cancer** using RNAscope LS ISH-sequential IF with protease-free reagent.

## Summary

- Protease-free workflow was developed to allow co-detection of RNAscope and IHC for protease-sensitive antibodies.
- We present results using protease-free reagent in a variety of different IHC and ISH-IHC co-detection assays on Leica and Ventana auto-staining platforms, suggesting compatibility with different RNAscope offerings and resulting in complete protein restoration with negligible impact to RNA while maintaining tissue integrity.
- The protease-free RNAscope co-detection workflow will serve as a powerful multi-omics staining technique for a wider range of antibodies by enabling visualization of RNA-protein co-detection for the comprehensive profiling of tissue microenvironments, facilitating faster breakthroughs in the discovery of therapeutics.

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