# 191: Assessing differential expression of protein interactions and RNA by combining isPLA and HCR™ Pro RNA-FISH automated assays

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## 1. Introduction

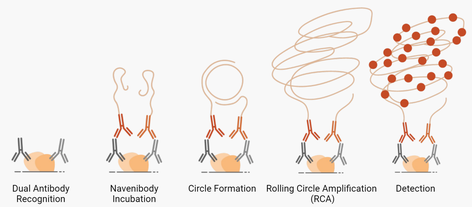
The advent of the spatial omics era has enabled researchers to acquire vast amounts of transcriptomics and proteomics data. The abundance of a transcript, however, does not necessarily display strong correlation with protein expression levels, so a holistic approach to sample analysis is pivotal. For the first time, we hereby combined two state-of-the art technologies for detecting RNA expression and protein interactions to better understand tissue phenotypes from a functional perspective. HCR™ Pro RNA-FISH (Molecular Instruments) enables high-resolution, multiplex detection of transcripts without requiring harsh protease-digestion steps. *In situ* proximity ligation assay (*is*PLA, Navinci) informs about protein functional states by sensitively detecting protein-protein interactions. Both technologies can be fully and robustly automated on the Leica BOND RX stainer. As a proof of principle, we focused on the PD1/PD-L1 inhibitory pathway, detecting the PD1 and PD-L1 transcripts, as well as the interaction between them on a protein level.

## 2. Methods and Technology Integration

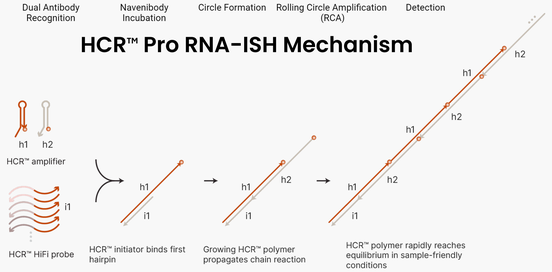
Human tonsil FFPE tissue sections, which are well known to be positive for PD1 and PD-L1 expression, were used for the combined isPLA/2-Plex HCR™ Pro RNA-FISH staining on the Leica BOND RX staining platform. In brief, after standard antigen retrieval, *is*PLA was performed using the Naveni® PD1/PD-L1 BOND RX HRP kit to detect the PD1/PD-L1 interaction. The *is*PLA signal was additionally amplified and detected by tyramide signal amplification (TSA, Atto647N). The tissue was then primed by adding the HCR™ Post-Process B reagent, followed by the HCR™ Pro RNA-FISH steps to detect PD1 (488) and PD-L1 (555) via TSA. Slides were imaged on the Olympus V200 automated slide scanner.

## 3. Mechanisms

### Naveni® *is*PLA Mechanism




### HCR™ Pro RNA-ISH Mechanism




## 4. Validation of RNA-ISH Performance for Clinical Grade Applications

Validating RNA-ISH is critical for ensuring high performance detection of RNA transcripts, especially for clinical-grade workflows where sensitivity and specificity are paramount. For this demonstration, we present a novel validation of PD-L1, a key clinical target in immuno-oncology known for its role in immune evasion and therapeutic response to checkpoint inhibitors. To achieve robust validation of the PD-L1 HCR™ HiFi Probe from Molecular Instruments, we applied a comprehensive approach that includes three key methods.

1. **Testing in different cell lines with varying PD-L1 RNA expression levels**: Demonstrates the assay's sensitivity, dynamic range, and reproducibility, ensuring reliable detection across biologically relevant and variable conditions
2. **Co-detection of PD-L1 RNA and Protein**: Confirms that RNA-ISH accurately identifies target transcripts by correlating RNA expression with protein expression via IHC, an established clinical method
3. **MI's Dual Channel™ Validation (DCV):** Builds confidence that the PD-L1 HCR™ HiFi Probe binds specifically to its target, effectively detecting genuine RNA transcripts for precise and reliable dot counting

**Conventional RNA Validation Methods**

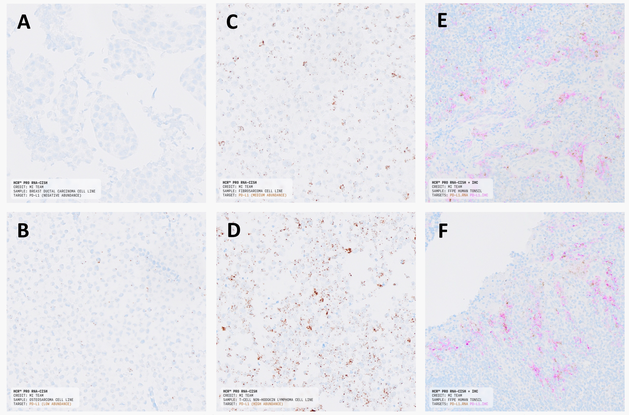



**Figure 1: HCR™ Pro RNA-CISH with PD-L1 and HCR™ Pro RNA-CISH + IHC with PD-L1.** (A) Negative PD-L1 in breast ductal carcinoma cell line. (B) Low PD-L1 abundance in osteosarcoma cell line. (C) Medium PD-L1 abundance in fibrosarcoma cell line. (D) High PD-L1 abundance in T-cell non-Hodgkin lymphoma cell line. (E+F) PD-L1 (RNA) and PD-L1 (Protein) in human tonsil FFPE tissue sections.

**Dual Channel™ Validation: A more robust tool for validation, particularly in cases where RNA and protein expression do not colocalize.**

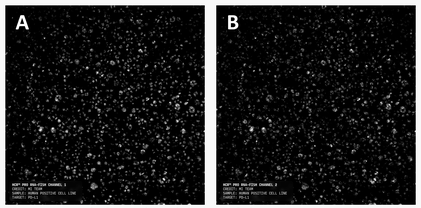



**Figure 2: Dual Channel™ Validation of the PD-L1 HCR™ HiFi Probe in Positive Cell Line.** Analysis reveals a colocalization factor of 93% between the two channels, with an intensity correlation coefficient of 0.88. (A) HCR™ Pro RNA-FISH targeting PD-L1 in the 546 channel. (B) HCR™ Pro RNA-FISH targeting PD-L1 in the 647 channel.

## 5. Combined Assay Results

Here, we present results from the combined HCR™ Pro RNA-ISH and *is*PLA assay, highlighting both 1-Plex RNA-ISH with PPIB detection (Fig. 3) and 2-Plex RNA-ISH with PD-1 and PD-L1 detection (Fig. 4) alongside *is*PLA. This integration of RNA and protein-level analyses enables precise co-detection of RNA transcripts and protein-protein interactions within the same tissue sample, maximizing the biological insights gained from each experiment. The ability to target multiple RNA molecules with HCR™ Pro RNA-ISH, while simultaneously performing *is*PLA, opens new opportunities for studying complex molecular interactions, supporting more advanced biological research, and conserving valuable resources, including time and precious tissue samples. The results demonstrate that this joint assay provides a scalable, high-performance solution ideal for biomarker studies, particularly in immuno-oncology, focusing on PD-1 and PD-L1 as critical immunotherapy targets.

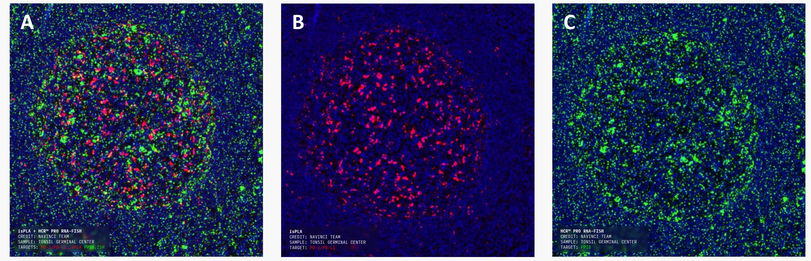



**Figure 3: *isPLA* with PD-1/PD-L1 + HCR™ Pro RNA-FISH with PPIB in a tonsil germinal center.** (A) Overlay image. (B). *is*PLA with PD-1/PD-L1. (C) HCR™ Pro RNA-FISH with PPIB.

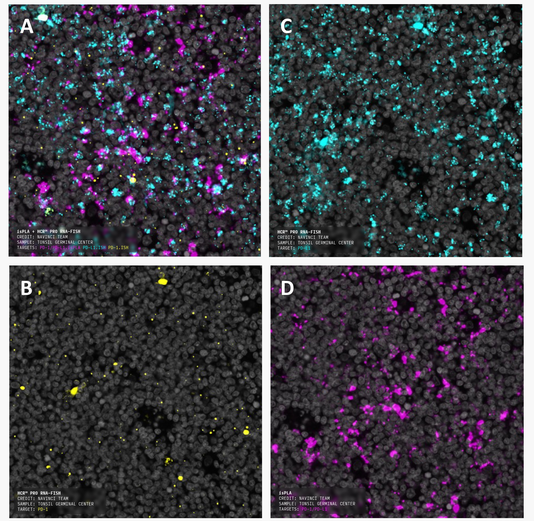



**Figure 4: *is*PLA with PD-1/PD-L1 + 2-Plex HCR™ Pro RNA-FISH with PD-1/PD-L1 in a tonsil germinal center.** (A) Overlay image. (B) HCR™ Pro RNA-FISH with PD-L1. (C) HCR™ Pro RNA-FISH with PD-1. (D) *is*PLA with PD-1/PD-L1.

This combined assay delivers powerful insights by connecting RNA expression with functional protein interactions, making it a comprehensive solution for understanding pathway regulation and immune interactions. It is particularly valuable for immuno-oncology research, paving the way for new biomarker discoveries and therapeutic advancements. For more information about this joint assay, reach out to MI or Navinci at info@molecularinstruments.com or contact@navinci.se.