High-Performance *In Situ* Imaging of Short RNA Targets with Next-Generation HCR™ HiFi Probes

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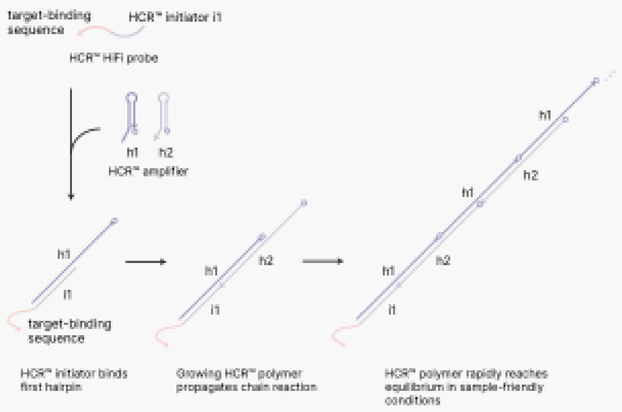
1. Introduction

Short RNA targets such as microRNAs (miRNAs) and small interfering RNAs (siRNAs) are essential regulators of gene expression and increasingly serve as RNA-based therapeutics. Their compact length (~16–22 nt) poses challenges for *in situ* detection, including limited hybridization sequence, higher off-target risk, and susceptibility to non-specific signal from unbound or partially bound probes. Traditional detection methods often rely on high-stringency conditions to suppress background, which can compromise sensitivity and limit assay robustness. Here, we present a next-generation HCR™ HiFi Probe architecture that achieves high-performance, background-suppressed detection of short RNA targets in challenging FFPE tissue.

2. Approach

As the most challenging class of short RNAs, we focused on miRNAs, ≈22-nucleotide non-coding RNAs that regulate a significant fraction of vertebrate mRNAs. Due to their short length, miRNAs must be detected using a single probe carrying a full HCR™ initiator, placing a significant premium on signal amplification. To suppress background, we developed a new probe architecture that minimizes non-specific binding while preserving robust HCR™ signal amplification. Validation was conducted in FFPE human tissue samples using HCR™ Pro signal amplification platform. The assay is compatible with both manual and automated workflows including the BOND RX, DISCOVERY ULTRA, and ONCORE Pro X platforms.

3. HCR™ Pro RNA-ISH Platform



4. Validation of Next-Generation HCR™ HiFi Probes

For regular length RNA transcripts, HCR™ HiFi Probes have been demonstrated to achieve excellent performance with high signal-to-background ratio with the convenience of using unoptimized probe sets for new targets and organisms. The next-generation HCR™ HiFi Probes for short RNA targets were engineered to achieve the same high-performance with background suppression. In this experiment, we compared the performance of our state-of-the-art, commercially available HCR™ HiFi Probes, legacy HCR™ Probes for short RNAs, and next-generation HCR™ HiFi Probes with background suppression for short RNAs.

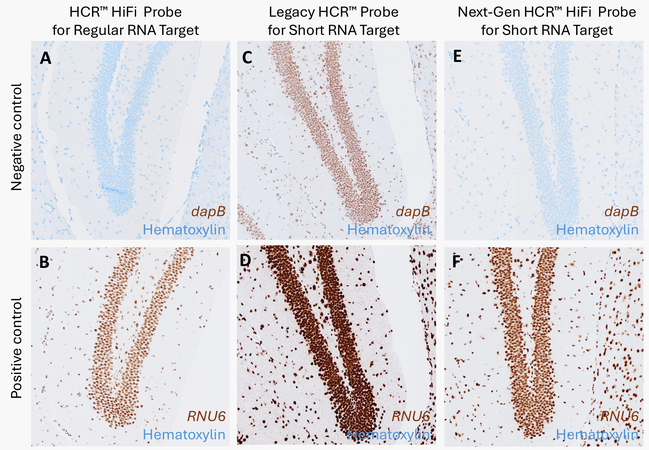


Figure 1: Background suppression using next-generation HCR™ HiFi Probes for a short RNA target. HCR™ HiFi Probes for regular length RNA targets achieve excellent signal-to-background ratio (panels A and B). Negative control probe (dapB) generates high background with legacy HCR™ Probe (panel C) and no visible background with next-generation HCR™ HiFi Probe with background suppression capability (panel E). Next-generation HCR™ HiFi Probe yields high contrast for short RNA target RNU6 (panel F). Brightfield images in 5 µm FFPE mouse brain sections. Automated HCR™ Pro RNA-CISH using Roche DISCOVERY ULTRA. Slide scanner objective: 20×.

This novel probe architecture for short target detection enables the same level of specificity and sensitivity as our current HCR™ HiFi Probe for regular RNA targets. We further validated this new probe technology on the most challenging class of short RNAs, miRNAs.

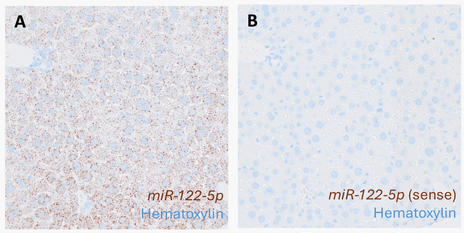


Figure 2: miRNA imaging with next-generation HCR™ HiFi Probe. (A) High-contrast staining for miR-122. (B) Negative control using a sense probe has minimal background. Brightfield images in 5 µm FFPE mouse liver sections. Automated HCR™ Pro RNA-CISH using Roche DISCOVERY ULTRA. Slide scanner objective: 20×.

5. Combined Assay Results

The next-generation HCR™ HiFi Probes for short RNA targets are compatible with our current HCR™ HiFi Probes for regular RNA targets and traditional IHC assays for protein imaging. In this experiment, we demonstrated co-detection of a long RNA (*Polr2a*), a short RNA (*RNU6*), and a membrane protein with our protease-free automated workflow in FFPE tissue sections, preserving tissue morphology and maintaining protein target integrity.

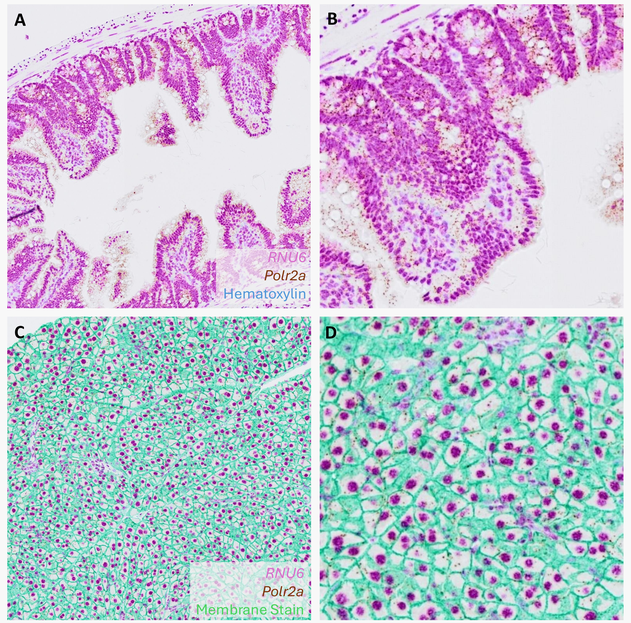


Figure 3: Protease-free HCR™ Pro RNA-CISH with traditional IHC. (A) Co-detection of a long RNA (*Polr2a*) and a short RNA (*RNU6*). (B) Zoom of panel A. Brightfield images in 5 µm FFPE mouse intestine tissue. (C) Simultaneous imaging of a long RNA (*Polr2a*) and a short RNA (*RNU6*) with HCR™ Pro RNA-CISH and a membrane protein with traditional IHC. (D) Zoom of panel C. Brightfield images in 5 µm FFPE mouse pancreas tissue. Automated slide stainer: Roche DISCOVERY ULTRA. Slide scanner objective: 20×.

6. Conclusions

This next-generation HCR™ HiFi Probe architecture enables background-suppressed *in situ* detection of short RNA targets by integrating full-length HCR™ initiators with a novel background suppression strategy. The platform supports sensitive and specific spatial analysis of small RNA biomarkers and therapeutics in FFPE tissue sections and is deployable in both manual and automated formats, including the BOND RX, DISCOVERY ULTRA, and ONCORE Pro X systems.

For more information about this next-generation HCR™ HiFi Probe technology, please reach out to MI at info@molecularinstruments.com.