Automation of RNAscope™ assays in immuno-oncology research on LabSat® Research

Why RNA in situ hybridization (ISH)?

Most cancer types display a very high intra-tumoral and microenvironmental heterogeneity, as well as an elevated phenotypic diversity. The study of such complex systems directly correlates with the surge of “personalized medicine” and, more specifically, with the growing needs in biomarker discovery.

New transcriptomic profiling technologies, such as microarrays, Real-Time PCR (qPCR), or Next-Generation Sequencing (NGS) start to reveal the intricacies of long non-coding RNAs (lncRNA) as well as microRNAs (miRNA), transcripts’ splice variants, and the existence of numerous clinically relevant RNA biomarkers. However, these methods destroy tissue in the process and deliver no spatial information essential to understand the tumor microenvironment. Hence, their results do not accurately reflect the great cell-to-cell variation of RNA expression.

Protein quantification through fluorescent immunohistochemistry (IHC), also referred to as immunofluorescence (IF), has often been used as a source of spatial information. Nevertheless, coding mRNAs represent only a fraction of the human genome, leaving researchers with no protein targets to analyze in many cases and, in some others, with only a few quality antibodies for the coding portion. Additionally, inaccuracies or information losses take place for a number of reasons: when protein are secreted they are not observed in their original location, stable genomes are subject to processes that regulate their RNA patterns of expression without DNA alterations, etc. When studying RNA as a biomarker, observation of targets in its cellular origin, provides a more robust spatial characterization.

How does RNAscope work?

Derived from RNA ISH (in situ hybridization), RNAscope (Fig. 1) uses engineered double Z probes to target and hybridize with specific mRNA or lncRNA molecules. A signal amplification strategy via sequential hybridization of probes and amplifiers provides high sensitivity and specificity to the test and allows the visualization under the microscope of a single molecule of RNA as one signal dot. The two independent Z probes must hybridize to the target sequence in tandem in order for signal amplification to occur, which is behind the high specificity of the test. The Z probe technology also amplifies the signal x400 times as compared to conventional ISH. Moreover, standard RNA ISH methods often display issues surrounding the potential degradation of RNA material. RNAscope’s Z probes which target short regions are specifically designed to overcome these issues as they will successfully hybridize even with partially degraded RNA.

RNAscope offers overall an innovative probe and amplification design to perform ISH, in order to acquire quantifiable spatial and morphological RNA information over single-cell gene expression with high signal-to-noise ratios in a large range of tissues.

1 Wang et al. 2012

RNAscope in short

Advanced Cell Diagnostics (ACD), a Bio-Techne brand, has developed the RNAscope technology, an advanced platform for in situ RNA detection. This ISH technique enables the detection of almost any RNA biomarker with single-molecule sensitivity and high specificity in tissues.
**Application Note**

**RNAscope on LabSat® Research**

LabSat® Research is an automated tissue stainer device, allowing high-quality fast multiplexing up to 6 markers within a few hours. Lunaphore’s core technology, the Fast Fluidic Exchange (FFeX), utilizes a microfluidic Staining Chip that delivers reagents sequentially onto a tissue sample. The staining chamber is filled almost instantaneously, preventing different areas of the tissue from being incubated unevenly hence providing a great degree of signal uniformity in an ultra-controlled environment, allowing more robust and reliable results. The system is pressurized and provides temperature control in order to fine-tune and optimize staining conditions. This is key for image processing purposes with digital pathology tools such as signal quantification.

**USE CASE: Single-plex chromogenic ISH (CISH) RNAscope on LabSat® Research**

**Robust and reliable automation**

As with other hybridization methods, performing the RNAscope assays requires incubation and amplification cycles. These are performed under temperature controlled conditions. The LabSat® Research platform allows for automation of these important steps (Fig. 2) resulting in time savings and walk-away opportunities for scientists. During the single plex proof-of-concept test on the platform, results were achieved in 6 hours and 30 minutes following a fully automated protocol, from pre-treatment to detection and counterstaining. The same experiment, when performed manually, required up to 8 hours.

**High quality data**

During the test, all CISH protocol steps (except slide baking, deparaffinization, dehydration, bluing), were transferred onto LabSat® Research, resulting in specific and reproducible detection of TBP (TATA-box-binding protein) positive control probe on Hela cells, followed by negative control with a DapB probe (Dihydodipicolinate reductase mRNA transcript) (Figure 3).

**FIGURE 3** - Automated RNAscope CISH on LabSat® Research with the positive (top) and negative control (bottom) probes on Hela Cell Block. LabSat® Research results were compared with ACD reference results.

Following the standard protocol, a shorter one was developed to show the potential for time savings without compromising the quality of results. Results of the three protocols performed on a mouse multi-tissue block are shown in Figure 4.

**FIGURE 4** - Automated standard and short RNAscope CISH on LabSat® Research with PPIB positive control probe on FFPE mouse multi-tissue block. Deparaffinization and bluing/mounting not included in the time calculation.
Workflow: RNAscope on LabSat® Research

Both the standard and the shortened automated CISH protocols were fully run on LabSat® Research, with probes for six relevant targets on human tissue. Results showed adequate detection comparable to the manual reference (Figure 5).

**Turnaround and hands-on time reduction**

The standard and shortened automated CISH protocols as run on LabSat® Research allowed a significant time reduction and hands-off time compared to the manual protocol (Fig. 6). The shortest timeframe reached for a qualitative result was 4.5 hours for a chromogenic single-plex. The use of alternative reagents and concentrated probe may allow for further time reduction down to 3.5 hours.

**Easy step-by-step guidance**

Lunaphore provides LabSat® Research customers with protocol templates in a user-friendly software interface to automate these steps. Additionally, Lunaphore offers substantial field technical support and training to users getting started with RNAscope assays on LabSat® Research to ensure the understanding and confidence in the test.
**Conclusion**

RNA biomarker research has the potential to unravel the relationship between the tumor and its immune microenvironment and assess the complex heterogeneity held by tumors. RNAscope allows spatial visualization of RNA targets and protein expression that may help researchers to harvest key data without compromising tissue integrity.

Lunaphore is dedicated to bringing omics-like approaches to tissue analysis tests. With LabSat® Research, we provide a simple and affordable automation solution to handle sophisticated tests like RNAscope, rendering it into a faster, easier, and highly reliable method.

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**The potential of RNAscope & IHC in one single platform**

The possibilities opened by performing IHC, ISH (in situ hybridization) as well as a combination of both staining methods on the same tissue slide are numerous. Some of the counted benefits of this approach could be:

1. The possibility of identifying the location of a secreted protein as well as its cellular origin.
2. In an heterogenous samples, it can be challenging to segment cell population. In these cases, it is possible to combine, on the same slide, protein staining IHC to identify the cell population, and RNA ISH quantification to characterize it in depth.
3. Identify gene regulation mechanisms where variations in protein presence are observed along with stable levels in the RNAs of origin.
4. Detect non-coding RNA such as IncRNA and mRNA for targets where no antibodies are available or offer poor results.
5. In case of uncertainty or to increase data robustness for publication purposes, validate high-throughput transcriptomic analyses and/or confirm results obtained through qPCR by providing specific cellular localization and the cell type of origin of a given protein in the tumor microenvironment.

**Multiplex RNAscope ISH**

There are several different RNAscope kits available for the field of immuno-oncology:

ACD custom-made panels for new target probe

Chromogenic 2-marker panel:
- ISH: PD1/PD-L1

Fluorescent 4-marker panels:
- ISH: Breast cancer (KRT19/CD8A/CD4/CD68)
- ISH: Lung cancer (FoxP3orKRT19/CD8A/CD4/CD68 or TNFA)