RNAscope® Manual Reagents
Gene Expression Analysis by RNA *In Situ* Hybridization

Get quantitative molecular detection
with morphological context in a single assay

Advanced Cell Diagnostics

The RNAscope® assay is the most advanced RNA *in situ* hybridization (ISH) methodology based on ACD's patented technology, with signal amplification and simultaneous background noise suppression that advances RNA analysis in tissues and cells. Unique to this technology, the RNAscope® assay delivers quantitative, sensitive and specific molecular detection of RNA species on a cell-by-cell basis with morphological context in a single assay. This enables researchers to visualize which genes are expressed, localize where they are expressed, and quantify the level of expression.

Research Areas

With more than 1,800 publications since 2011, researchers have embraced the RNAscope® technology in a multitude of research areas, showing the universality of the RNAscope® technology. With its unique features and unequal performances, the RNAscope® technology is an essential tool in any research and pre-clinical research laboratory.

- Cancer research
- Stem cell
- Neuroscience
- Immunotherapy
- Xenograft models
- Infectious disease
- Preclinical safety assessment
- Gene and cell therapy
- And more...

2011
4 publications

2012
11 publications

2013
47 publications

2014
95 publications

2015
>200 publications

2016
>300 publications

2017
>450 publications

2018
>650 publications

Advanced Cell Diagnostics
Publication Count

1,800+*

*In November 2018

Explore the RNAscope® World

Principle and features of the RNAscope® Technology 4

A solution for common research challenges 6

RNAscope® *in situ* Manual Assay Workflow 8

Step 01. Permeabilize

Tissue sections or cells are fixed onto slides and pretreated with RNAscope® Pretreatment Kit to unmask target RNA and permeabilize cells.

Pretreatment Reagents / pg. 8

Step 02. Hybridize

Double Z probe pools are hybridized to target RNA molecules.

RNAscope® Target Probes / pg. 9
RNAScope® Control Probes / pg. 9

Step 03. Amplify

Sequential hybridization of amplifiers and labeled probe(s).

RNAscope® Detection Reagents / pg. 10
Accessories / pg. 13

Step 04. Visualize

Each punctate dot signal represents a single target RNA molecule and can be visualized with a standard microscopes.

pg. 14

Step 05. Quantify

Single molecule signals are quantified on a cell-by-cell basis by manual counting or automated image analysis with RNAscope® and HALO™ and other software.

RNAscope® & HALO™ software/ pg. 14

Principle and features of RNAscope® Technology

Innovative solution for single RNA molecule detection and quantification in single cells

RNAscope® probe design

A standard target probe consists of a pool of 20 double Z probes targeting a region of 1,000 bases. Each Z target probe contains three elements: The lower region is complementary to the target RNA and is selected for target specific hybridization and uniform hybridization properties. A spacer sequence links the lower region to an upper region. The two adjacent upper regions from a double Z target probe forms a 28 base binding site for the pre-amplifier.

Two independent Z probes, designed as probe pairs, need to hybridize to the target sequence in tandem in order to enable binding of the pre-amplifier.

A single Z probe hybridization onto a non-specific RNA target can happen, but the resulting hybridization of the pre-amplifier onto the upper region of a single Z will be unstable and therefore will be removed during the wash steps. This design ensures a low background noise level.

RNAscope® probe hybridization and amplification occurs as a cascade of events:

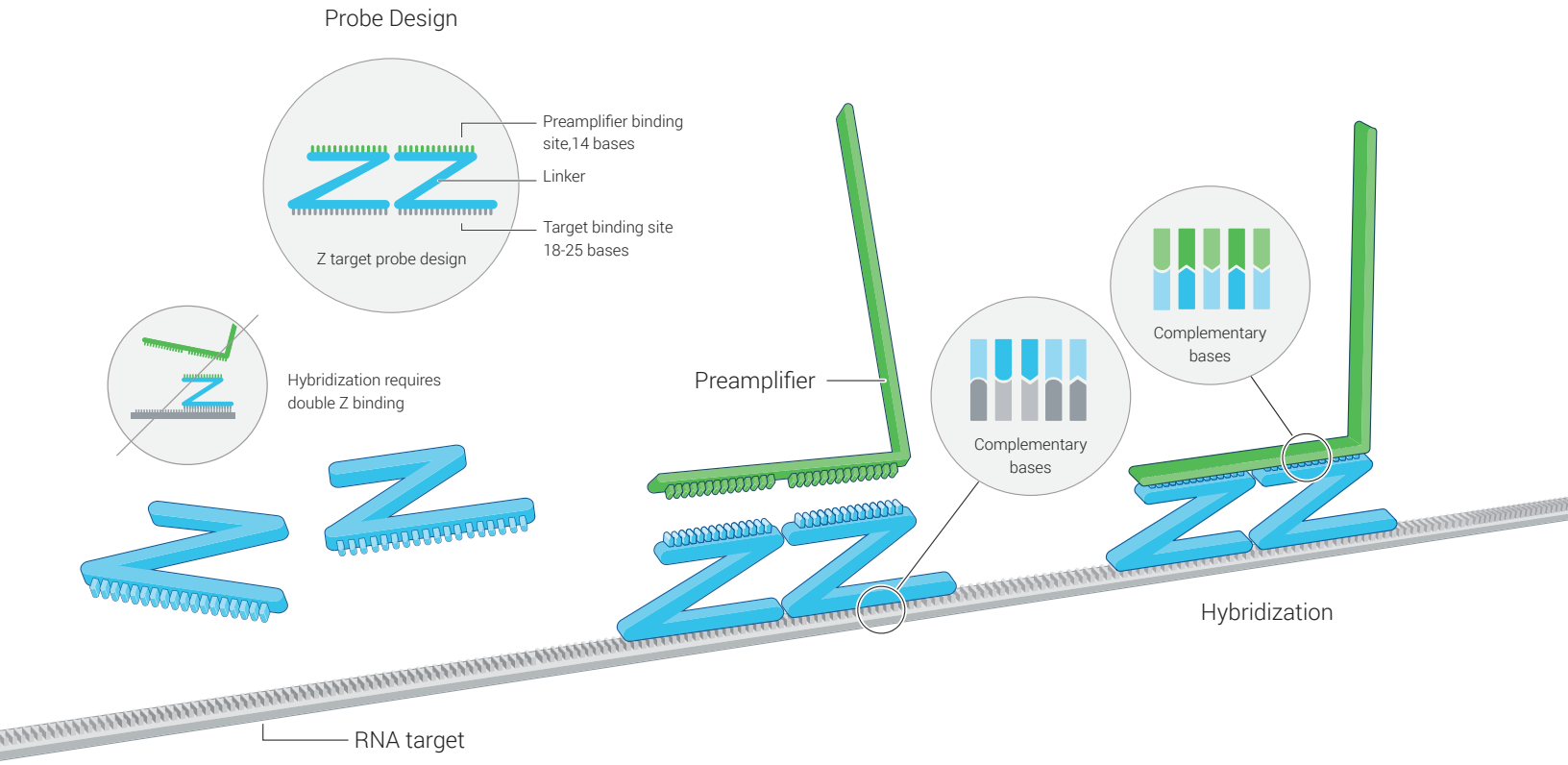
Step 1: Hybridization of 20 ZZ probe pairs to the RNA target

Step 2: Hybridization of the pre-amplifier to the upper regions of the Z probe pairs

Step 3: Hybridization of multiple amplifiers per pre-amplifier

Step 4: Hybridization of multiple labeled probes per amplifier

Serial hybridization events - 20 ZZ probe pairs, multiple amplifiers, multiple labeled probes - result in hybridization of thousands of labeled probes per RNA target.



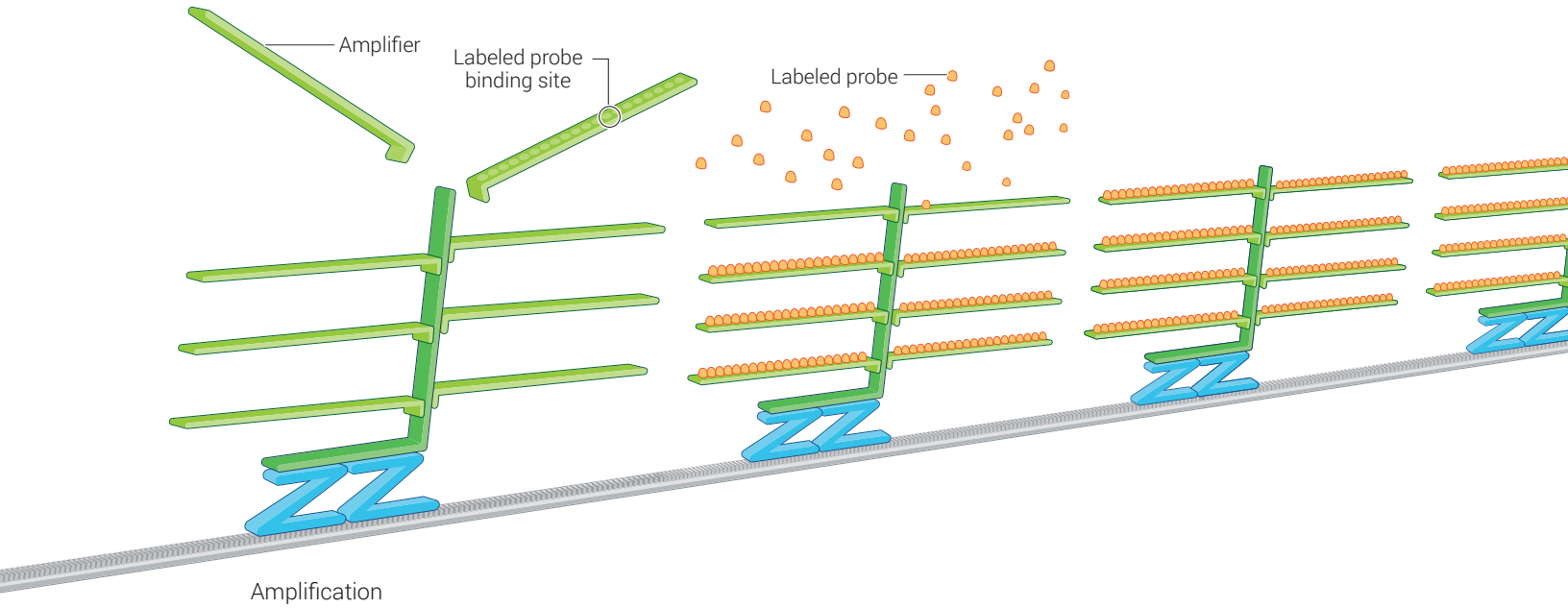
RNAscope® signal detection

Labeled probes contain either a chromogenic enzyme or a fluorophore signal generating one punctate dot per RNA target. (See page 14 - Visualize Step).

Hybridization of only three Z probe pairs is sufficient to obtain a detectable signal by a standard microscope.

Benefits of the RNAscope® technology

- **High sensitivity:** The serial signal amplification design increases sensitivity such that a single RNA molecule can be detected.
- **High specificity:** Proprietary probe design ensures target- specific binding while the double Z probe design prevents signal amplification of non-specific hybridization.
- **Morphological context:** Spatial resolution of gene expression in the complex tissue environmet creates a spatial map
- **Per-cell quantitation:** High sensitivity combined with morphological context results in single-molecule detection at single-cell resolution.
- **Universal:** Works for virtually ANY gene from ANY species in ANY tissue.



Amplification

A solution for common research challenges

Rapid validation of biomarker discovery

Whether you are characterizing biomarkers discovered by single-cell RNA sequencing, NGS, microarray or high throughput qPCR, the RNAscope® technology is a quick and easy tool to use across the different stages of the biomarker validation. With the RNAscope® technology you have access to unique gene expression information with morphological context: digital RNA expression at single cell level in complex tissue structure Theses.

RNA expression analysis to complement or combine with IHC-based protein analysis

Examination of protein as a biomarker with immunohistochemistry (IHC) technique is a widely used and accepted approach for diagnosis, prognosis, and therapy development for clinical diseases. However, the number of high quality and reliable antibodies is limited and IHC is not without issues, and the use of sometimes poorly characterized antibodies and insufficient overall standardization often leads to questionable results. At the contrary, RNAscope® technology is based on probe designed to be highly specific to the target and reproducibly manufactured. With a unique and reproducible protocol, RNAscope® assay is an ideal solution to validate, supplement or combine with IHC.

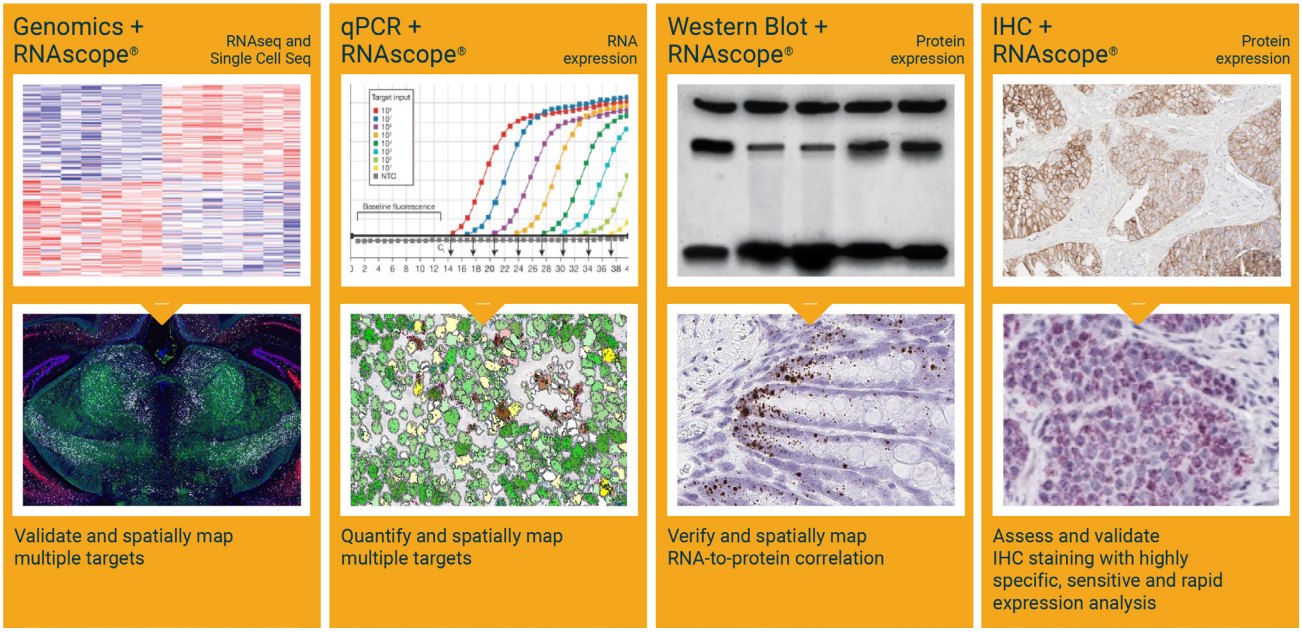


FIGURE 1. RNAscope® assay, a simple, first filter in target validation.

Non-coding RNA expression

In the most recent statistics from the GENCODE project (v29, October 2018), the human genome contains 23,643 non-coding RNA (ncRNA) genes, surpassing the number of protein-coding genes (19,940). Of the non-coding RNA species, some 30% (7,577) are less than 200 bases long, termed as small non-coding RNA. They comprise of transfer RNA (tRNA) and ribosomal RNA (rRNA), as well as RNAs such as snoRNAs, microRNAs, siRNAs, snRNAs, exRNAs and piRNA. About 60% (16,066) of the non-coding RNAs are longer than 200 bases and are operationally designated as long non-coding RNAs (lncRNAs).The functions of lncRNAs are still characterized; their abundance and diversity add to the challenge. Some lncRNAs have been shown to regulate gene expression through a diversity of mechanisms and play important roles in chromatin modification (HOTAIR), transcriptional and post-transcriptional regulation (ZEB2). Dysregulation of lncRNA is being found to have relevance not only in tumorigenesis, but also to neurological, cardiovascular, developmental and other diseases.

The discovery of a previously unknown universe of lncRNAs has created an unprecedented demand for effective RNA ***in situ*** hybridization tools. Unlike protein coding genes, for which immunohistochemistry (IHC) and RNA ***in situ*** hybridization are complementary for mapping gene expression to specific cells ***in situ***, lncRNA expression can only be investigated by RNA ***in situ*** hybridization. The generally lower expression levels of lncRNAs than their protein coding counterparts demand the highest sensitivity from RNA ***in situ*** hybridization methods. The single-molecule sensitivity and rapid assay development time (<2 weeks) of ACD's RNAscope® technology make RNAscope® ideally suited for localizing lncRNAs expression to specific cell types and sub-cellular structures. RNAscope® *in situ* assays will undoubtedly accelerate lncRNA research and become an indispensable tool for lncRNA-based molecular diagnostics.

"This technology allows us to directly visualize gene expression in the target tissue of interest – for example, within the same sample we can tell whether gene overexpression occurs in benign prostate glands, high grade prostatic intraepithelial neoplasia (HGPIN – a pre-cancerous state) or prostate cancer."

Dr. Mehra, Clinical Assistant Professor of Pathology
at Michigan Center for Translational Pathology

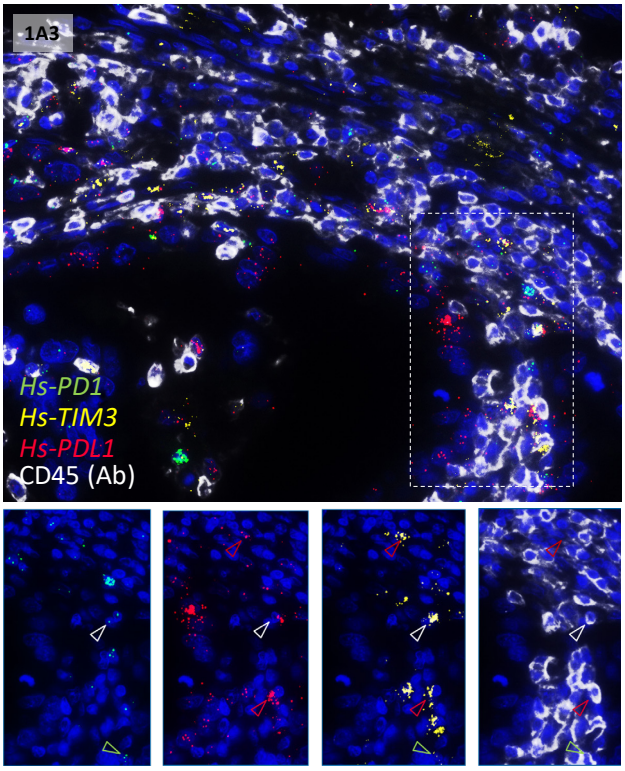


FIGURE 2. Single-cell co-expression profiles of checkpoint molecules in selected non-small cell lung cancer (NSCLC) cores.

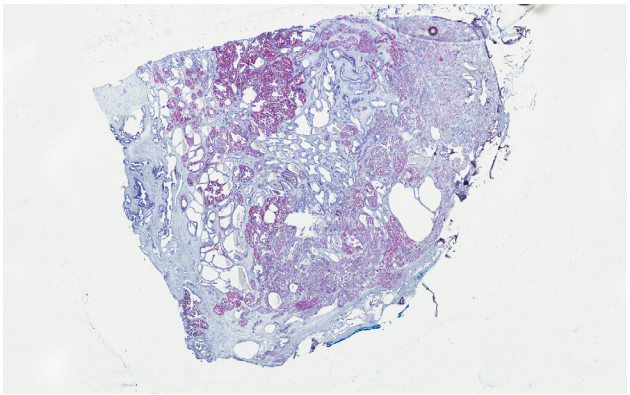


FIGURE 3. Non-coding PCA3 transcript detection in whole prostate tumor whole tissue section using RNAscope® technology.

RNAscope® *in situ* Manual Assay

Workflow and associated products

Step 01. Permeabilize

RNAscope® Pretreatment Reagents

Optimized permeabilization for optimal target accessibility.

In order to perform the RNAscope® assay, start with properly prepared and pretreated samples.

Sample preparation and pretreatment include the following steps:

- Fixation of cells or tissues if needed (fresh-frozen, cultured cells, etc.)
- Deparaffinization if needed (FFPE)
- Applying pretreatment reagents included in the RNAscope® Reagent Kit

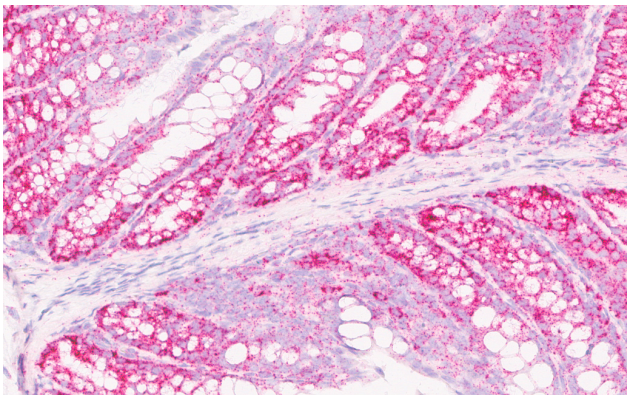


FIGURE 4. *Ppib* detection (red punctate dots) in mouse intestine using RNAscope® 2.5 HD Assay-RED.

RNAscope® 2.5 Pretreatment Reagents provide improved accessibility to target RNA reducing the time and effort in assay optimization. These reagents include hydrogen peroxide to block endogenous peroxidase activity. Additional pretreatment reagents such as target retrieval and protease pretreatment reagents allow the RNAscope® probes to better access the RNA, breaking cross links that could occur with the tissue during fixation and permeabilizing cells to allow target accessibility. Pretreatment reagents are available and suitable for multiple tissue types including: formalin-fixed, paraffin-embedded (FFPE) tissue including archival tissue, fresh frozen (FF) tissue, fixed frozen tissue, tissue microarray (TMA), and cell preparations.

RNAscope® 2.5 Universal Pretreatment Reagents is recommended when working or switching between different tissue types such as fresh-frozen or FFPE or cultured cells. It contains all the pretreatment reagents in one kit:

- RNAscope® Hydrogen Peroxide (H_20_2)
- RNAscope® Target Retrieval
- RNAscope® Protease Plus
- RNAscope® Protease III

For further information on our pretreatment reagents please visit **acdbio.com/pretreatment**

For more information request or download the RNAscope® Reference Guide containing guidelines and protocols for obtaining optimal RNAscope® *in situ* hybridization results with any tissue type from any species.

acdbio.com/referenceguide

Step 02. Hybridize

RNAscope® Target Probes

Unique probe design provides highly specific hybridization to the target molecule.

RNAscope® Catalog Target Probes

Using the proprietary ACD RNAscope® Probe Design algorithm, we design double-Z oligo probe pools that hybridize to your specific RNA target of interest. We can design probe pools for virtually ANY gene in ANY genome for interrogation in ANY tissue. The probe pools consist of proprietary oligonucleotides designed for detecting specific targets (page 4-5). Every target probe pool also contains a tag that enables the associated target to be visualized in a specific "color channel" under the microscope (page 12).

Select from our growing catalog of over 20,000 *in situ* hybridization target probe pools for mRNA and long noncoding RNA (lncRNA). Our RNA ISH probe pools span a variety of species including human, mouse, rat, dog, cow, zebrafish, rabbit, pig, chicken, monkeys, HPV, HIV, HCV, and many others.

Search for an assay targeting your gene of interest at **acdbio.com/probesearch**

RNAscope® Made-to-Order Target Probes

If ACD catalog probes are not available for your gene of interest, we can create new probes within two weeks using public or proprietary sequences. ACD probe design algorithm can also accommodate non-standard designs such as probe pools for detection of fusion genes, species-specific detection of biomarkers in xenografts, or any other non-standard application in any species. Standard and non-standard RNA ISH probe pools can be designed for use with any of our RNAscope® Reagent Kits, including singleplex, duplex, multiplex, manual, or automated assay configurations.

Interested in custom probes? Tell us your gene of interest and let's get started: **acdbio.com/target-probes-made-order**

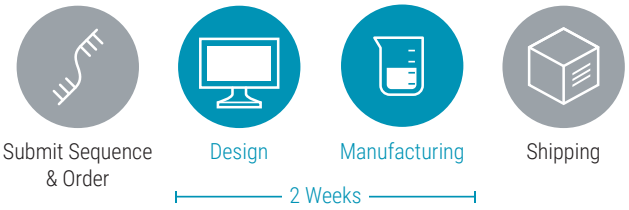


FIGURE 5. An easy and quick design and manufacturing process for highly specific and reproducible RNAscope® probes.

Ensure your success with good qualilty controls

RNAscope® Control Probes

In addition to target probes, we also provide species-specific housekeeping gene positive control probes and DapB negative control probes, designed to work with RNAscope® Reagent Kits. The positive control probes span from high to very low levels of expression, providing appropriate experimental controls for RNA *in situ* hybridization and ensuring high confidence when working with varying or unknown levels of gene expression. See our list of species-specific control probes at **acdbio.com/controlprobes**

RNAscope® Control Slides

The RNAscope® control slides are essential to verify assay conditions. The first run would serve as a technique quality control check and should be run with the assay, using control probes prior to using your samples and target probes. Two types of control slides are offered: Human control slides contains FFPE cultured cell pellets of human HeLa cells and mouse control slides contains FFPE cultured cell pellets from mouse NIH 3T3 cells.

RNAscope® *in situ* Manual Assay

Workflow and associated products

Step 03. Amplify

RNAscope® Reagents

Multiplex your possibilities from single to 4-plex analysis.

RNAscope® 2.5 HD Reagent Kit-BROWN

The ideal starter kit for first-time users and is universal in applications. This very robust assay gives high-definition staining results, which can be archived permanently due to the permanent staining. The chromogen diaminobenzidine (DAB) used in the assay is the standard in molecular pathology and suitable for a wide range of sample types as well as readily visible under a standard brightfield microscope.

The RNAscope® 2.5 HD Reagent Kit-BROWN is ideal for detection of target genes with anticipated low expression levels (1–20 copies per cell). Three alternative configurations of this kit are available to enable fully automated walk-away ISH solutions:

- RNAscope® VS Universal HRP Reagent Kit-BROWN for use on the DISCOVERY ULTRA automated tissue staining systems by Roche Tissue Diagnostics.
- RNAscope® LSx Reagent Kit- Prefilled BROWN kits for the seamless use on the use on the Leica Biosystems' BOND Rx System.
- RNAscope® LS Reagent Kit-BROWN kits for the seamless use on the use on the Leica Biosystems' BOND Rx System.

For further information on our automated solutions please visit **acdbio.com/automated-assays**

The RNAscope® 2.5 HD Reagent Kit-RED

A Fast Red dye which offers a higher contrast and is the first choice for in situ hybridization applications where chromogenic staining with DAB is less desirable, such as staining of highly pigmented lung, liver, retina and skin tissue specimens. ACD also recommends this assay for detection of target genes where a lower expression is anticipated, as the red dots stand out more clearly against the hematoxylin staining and are more readily identifiable under a standard brightfield microscope. Three alternative configurations of this kit are available to enable fully automated walk-away ISH solutions

- RNAscope® VS Universal AP Reagent Kit-RED for use on the DISCOVERY ULTRA automated tissue staining systems by Roche Tissue Diagnostics.
- RNAscope® LSx Reagent Kit- Prefilled RED kits for the seamless use on the use on the Leica Biosystems' BOND Rx System.
- RNAscope® LS Reagent Kit-RED kits for the seamless use on the use on the Leica Biosystems' BOND Rx System

For further information on our automated solutions please visit **acdbio.com/automated-assays**

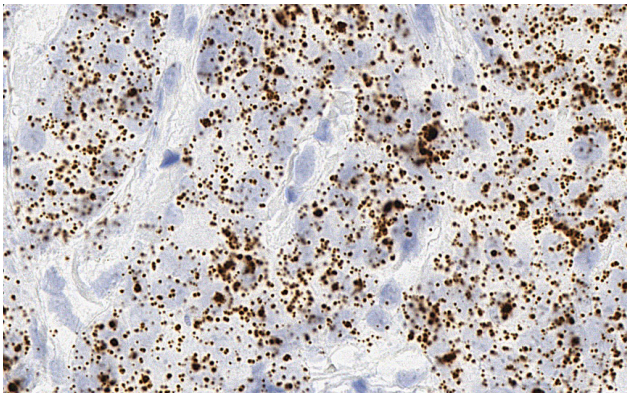


FIGURE 6. *ERB2* detection (brown punctate dots) in uman breast cancer sample using RNAscope® 2.5 HD Reagent Kit-BROWN.

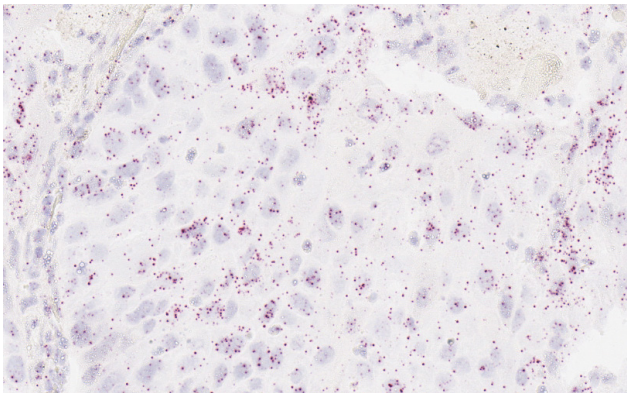


FIGURE 7. *PDL1* detection (red punctate dots) in lung cancer sample using RNAscope® 2.5 HD Reagent Kit-RED.

RNAscope® 2.5 HD Duplex Assay

Designed for simultaneous ***in situ*** detection of two RNA species. Common applications include co-localization studies to map co-expression of two targets within the same cellular context (e.g. secreted ligand and its receptor) or to profile gene expression in a specific cell type expressing a known marker (e.g. a specific stem cell marker). To distinguish between the two chromogenic colors, ACD has employed the naming convention of Channel 1 (C1) to refer to green and Channel 2 (C2) to Fast Red, hence RNAscope® probe pool names often include C1 or C2. The stained slides are visualized with bright-field microscopes.

Two alternative configurations of this kit are available to enable fully automated walk-away ISH solutions:

- RNAscope® VS Duplex Assay for use on the DISCOVERY ULTRA automated tissue staining systems by Roche Tissue Diagnostics.
- RNAscope® 2.5 LS Duplex Assay for use on the Leica Biosystems' BOND Rx System.

For further information on our Leica automated solutions please visit **acdbio.com/automated-assays**

RNAscope® Multiplex Fluorescent Assay

The RNAscope® Multiplex Fluorescent assays provide the same exceptional sensitivity as our singleplex assays, allowing single-molecule detection of up to four RNA targets simultaneously. The RNAscope® Multiplex Fluorescent assays are ideal for co-localization studies of any genes in nearly any tissue-type using fluorescent labels. ACD offers two types of multiplex fluorescent assays. The RNAscope® Fluorescent Assay, our first generation assay, is an all in one kit, ideal for fresh frozen tissue samples and cultured cells, also compatible with fixed frozen samples. The RNAscope® Multiplex Fluorescent Assay v2, ideal for FFPE and fixed frozen samples and compatible with all sample preparation methods, is a TSA-based assay ideal for FFPE tissues and requires Perkin Elmer's proprietary Tyramide Signal Amplification technology (TSA™) or Opal™ Dyes for visualization.

For a deatiled comparison of those two assays please have a look at page 15.

For further information on our Leica automated solutions please visit
acdbio.com/rnascope®-ls-multiplex-fluorescent-assay

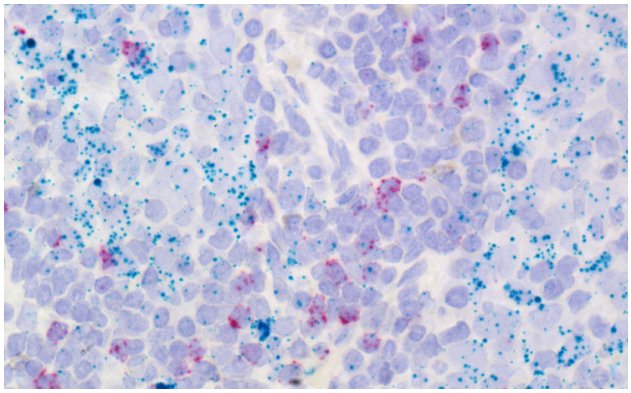


FIGURE 8. Expression analysis of *PD-L1* (green) and *CD8a* (red) in human lung cancer tissue using RNAscope® 2.5 HD Duplex Assay.

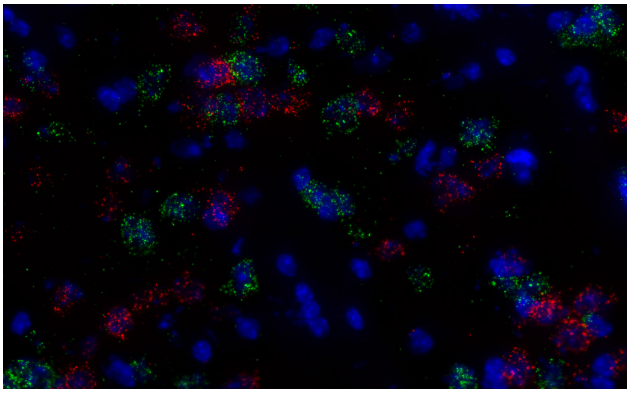


FIGURE 9. Expression analysis of *Drd1* (red) and *Drd2* (green) in mouse brain striatum tissue tissue using RNAscope® Multiplex Fluorescent Assay v2

RNAscope® *in situ* Manual Assay

Workflow and associated products

Step 03. Amplify

RNAscope® Reagents

One assay for short sequence and exon junction analysis

The BaseScope™ Assay

The BaseScope™ Assay is a unique product from ACD, based on the same platform of proven and established RNAscope® technology. This powerful assay provides new data dimensions and unique insight into biological mechanisms. The BaseScope™ Assay enables applications such as exon junctions for the analysis of splice variants, detection of short RNA targets (50-300 bases), highly homologous sequences, RNA mutations and, circular RNA, or gene fusions.

With ACD's continued commitment to provide researchers advanced capabilities of performing RNA ISH, the development of the BaseScope™ product provides a key benefit to detect biological events *in situ* using a single Z pair (i.e. RNA sequence spanning 50nt). The BaseScope™ assay was developed with advances in probe design, leveraging core concepts of the ZZ patented technology, and applies a novel amplification system that generates increased signal with simultaneous background noise suppression.

BaseScope™ Reagent Kit v2-RED

The BaseScope™ Reagent Kit v2-RED, with its high contrast Fast Red dye, uses alcaline phosphatase mediated detection with the FastRed substrate.

Two alternative configurations of this kit are available to enable fully automated walk-away ISH solutions

- BaseScope™ VS Reagent Kit for use on the DISCOVERY ULTRA automated tissue staining systems by Roche Tissue Diagnostics.
- BaseScope™ LS Reagent Kit for use on the Leica Biosystems' BOND Rx System.

For further information on our automated solutions please visit **acdbio.com/automated-assays**

BaseScope™ Duplex Assay

The BaseScope™ Duplex Assay can be used for simultaneous visualization of two RNA targets while maintaining single cell resolution.

Applications include:

- Discerning bi-allelic vs. mono-allelic CRISPR-mediated mutations
- Co-detection of circRNAs and linear RNAs
- Simultaneous visualization of 2 splice variants or short targets
- Profiling expression of a splice variant, short target, or gene edit in a cell-specific manner targets

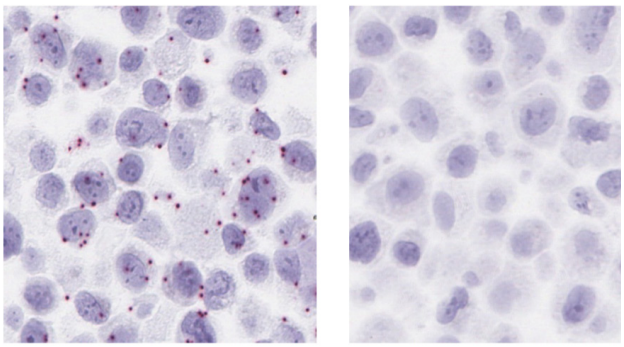


FIGURE 10. Detection of exon 14 skipped variant of MET mRNA (MET Δ14) in lung cancer cell line H596 using the BaseScope™ Reagent Kit-RED

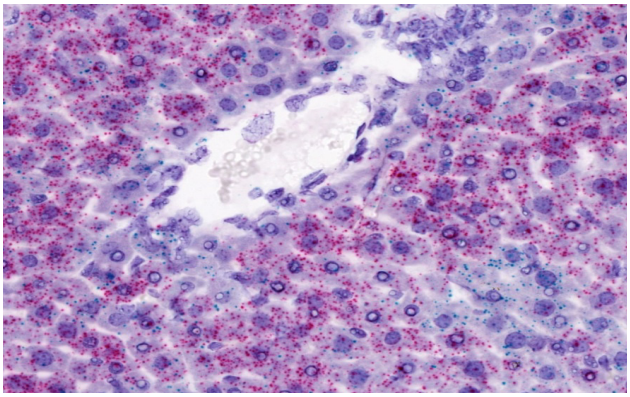


FIGURE 11. Visualization of CRISPR-mediated gene editing in the mouse liver using the BaseScope™ Duplex Assay. Wild-type sequence in green, edited sequence in red.

RNAscope® *in situ* Manual Assay

Featured Applications

Dual ISH-IHC

RNA *in situ* hybridization (ISH) and immunohistochemistry (IHC) are well-established methods providing unique RNA and protein expression with morphological context. Often considered complementary technologies, they bridge the gap between RNA and protein analysis. By targeting different molecules, one the precursor of the other, performing ISH and IHC together can provide complementary information to:

- Identify the origin of secreted proteins – ISH identifies cells that produce of the protein, IHC cells that take up the secreted protein
- Identify complex tissue structure – in complex structures with multiple cell types, IHC identifies the cell type and ISH detects RNA expression inside these cells
- Identify regulation of gene expression - translational regulation controls can shut down protein synthesis, or protein instability can render IHC ineffective. Analysis of both RNA and protein expression in the same tissue allows differentiation between inhibition of transcription and protein instability
- Assess gene therapy – a combination of ISH and IHC can provide a useful comparison between levels of transduction and levels of protein. Successful transduction may not always translate to successful expression due to regulation or protein instability.

RNAscope for Spatial Validation of Single-Cell Transcriptomes

The RNAscope® technology is a robust, highly specific and sensitive RNA ISH methodology with multiplexing capabilities to validate and provide spatial information for high-throughput single cell transcriptomic results. This technology allows for cell type-specific expression profiles to be mapped back to the complex tissue context of organs.

Benefits of the RNAscope® technology include:

- Visual confirmation of individual gene and gene signature expression
- Single cell resolution of gene expression and co-localization
- Spatial localization of cell types and states in tissue environment
- Identifying proper single cells for quality transcriptome analysis

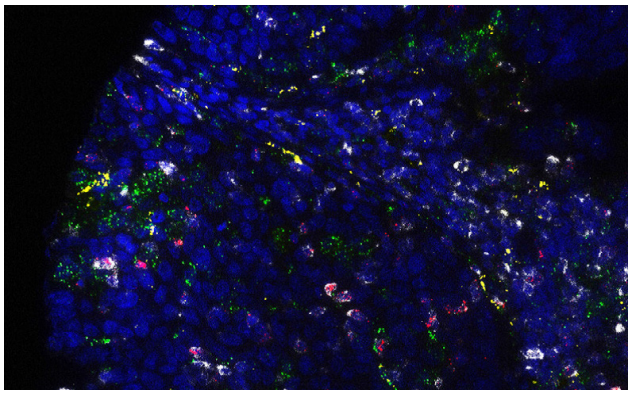


FIGURE 12. Detection of ***CD4*** (green), ***FOXP3*** (yelow), *IFNg* (red) combined with an CD8 antibody (white) in lung cancer tissue using the RNAscope® LS Multiplex Fluorescent Assay

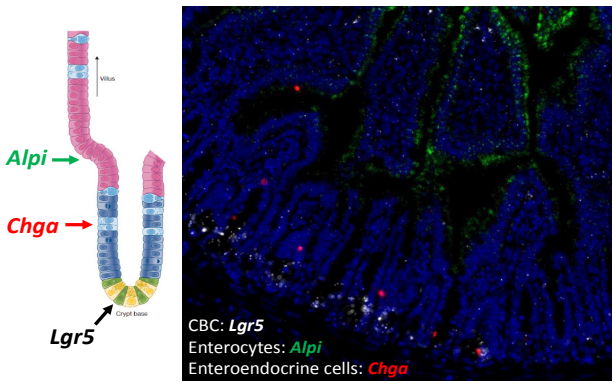


FIGURE 13. Single Cell Analysis - Simultaneous detection of 3 intestinal cell populations. Here, ***Lgr5*** (white), ***Alpi*** (green) and ***Chga*** (red) in mouse intestine.

RNAscope® detection kit selection guide

A RNA ISH solution for every need

	RNAscope® 2.5 HD Assay-BROWN	RNAscope® 2.5 HD Assay-RED	RNAscope® 2.5 HD Duplex Assay
Target	mRNA >300 bases lncRNA > 300 bases	mRNA >300 bases lncRNA > 300 bases	mRNA >300 bases lncRNA > 300 bases
Assay Type	Chromogenic	Chromogenic	Chromogenic
Dye Used	Diaminobenzidine (DAB)	Fast Red	HRP-based Green and AP-based Fast Red
Probes Channel Designation	Channel 1 (C1 Probes)	Channel 1 (C1 Probes)	Channel 1 & 2 (C1 & C2 Probes)
Multiplexing	Singleplex	Singleplex	Singleplex, Duplex
Key Benefit	Robust, sensitive, permanent stain. Most widely used RNAscope assay	Provides bright color stains with high contrast to background	Utilizes 2 different staining enzymes therefore avoiding any cross talk between the two stains
Ideal For	First time user Routine applications	Studies of tissues with endogenous color background such as melanin in skin, liver, or lungs from smokers	Studies interrogating two RNA biomarkers simultaneously
Novel Gene or Unknown Expression	+++++	+++++	+++
Archival Specimens	+++++	++	+++
Microscope Imaging System	Standard brightfield	Standard bright field Multispectral fluorescent imaging	Standard bright field
Sample Type	FFPE Tissues Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells	FFPE Tissues Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells	FFPE Tissues Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells
Assay protocol length (steps 1-3)	8 hours (with ~2 hours hands-on time)	8 hours (with ~2 hours hands-on time)	13 hours (with ~3 hours hands-on time)

	RNAscope® Mulitplex Fluorescent Assay	RNAscope® Multiplex Fluorescent v2	BaseScope™ Reagent Kit v2-RED	BaseScope™ Duplex Assay
Target	mRNA >300 bases lncRNA > 300 bases	mRNA >300 bases lncRNA > 300 bases	RNA 50 to 300 bases Exon junction	RNA 50 to 300 bases Exon junction
Assay Type	Fluorescent	Fluorescent	Chromogenic	Chromogenic
Dye Used	FITC, Cy3, Cy5, Cy7	Perkin Elmer: - PN NEL741001KT - PN NEL744001KT - NEL745001KT Opal™ Dyes - Part No. FP1487001KT - Part No. FP1488001KT - Part No. FP1495001KT - Part No. FP1497001KTP	Fast Red	HRP-based Green and AP-based Fast Red
Probes Channel Designation	Channel 1-3 (C1, C2 & C3 Probes)	Channel 1-4 (C1, C2, C3 & C4 Probes)	Channel 1 (C1 Probes)	Channel 1 & 2 (C1 & C2 Probes)
Multiplexing	Single to Triplex	Single to 4-plex	Singleplex	Singleplex, Duplex
Key Benefit	Utilizes up to 3 different spectral channels providing high flexibility	TSA-based Fluorescent RNAscope® detection assay for up to 4-plex capability using TSA fluorophores (sold separately). Ideal for FFPE tissues	Provides bright color stains with high contrast to background	Utilizes 2 different staining enzymes therefore avoiding any cross talk between the two stains
Ideal For	Co-expression studies of up to 3 genes simultaneously Experimental application requiring flexibility	Co-expression studies of up to 4 genes simultaneously Experimental application requiring flexibility, FFPE tissue	circRNA, splice variants, short targets, gene editing	circRNA, splice variants, short targets, gene editing, Studies interrogating two RNA biomarkers simultaneously
Novel Gene or Unknown Expression	+++	++++	+++++	+++
Archival Specimens	++	++++	++	+++
Microscope Imaging System	Multispectral fluorescent imaging	Multispectral fluorescent imaging	Standard bright field Multispectral fluorescent imaging	Standard bright field
Sample Type	Fresh Frozen Tissues Cultured cells Fixed Frozen Tissues	FFPE Tissues Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells	FFPE cells & tissues Fresh frozen tissues Cultured cells	FFPE cells & tissues Fresh frozen tissues Cultured cells
Assay protocol length (steps 1-3)	6.5 hours (with ~2 hours hands-on time)	14 hrs (with ~5 hours hands-on time, can split over 2 days)	10 hours (with ~2 hours hands-on time , can split over 2 days)	12 hours (with ~3 hours hands-on time , can split over 2 days)

RNAscope® accessories

Optimal temperature and humidity for optimal assay performance

HybEZ™ II Hybridization System

Successful implementation of RNAscope® Assay is directly linked to hybridization environment. The ACD HybEZ™ II Hybridization System and its ability to accurately keep the temperature stable is essential to the success of RNAscope® and BaseScope™ workflows.

The HybEZ™ II Oven is a simple, easy-to-use, low-profile benchtop hybridization oven that provides superior conditions for RNA-ISH, and is the only hybridization oven that guarantees optial performances of the RNAscope® and BaseScope™ assays. The HybEZ™ II Oven provides a gasket-sealed, temperature-controlled humidifying chamber necessary for optimized RNAscope® assay performance.

This instrument system is capable of holding 20 slides at a set temperature and high humidity for hybridization and other incubation steps as specified in the manual RNAscope® and BaseScope™ protocols.

The system comprises: (figure 14): HybEZ oven (PN 321710/321720), a humidity control tray (PN 310012), and HybEZ Humidifying Paper (2 sheets PN 310025), EZ-Batch Wash Tray (PN 310019), EZ-Batch Slide Holder (PN 310017)

Processing System

The ACD EZ-Batch™ Slide Processing System facilitates processing multiple sample slides simultaneously. We designed this product for higher efficiency in running the manual RNAscope® assay protocol. The system comprises RNAscope® EZ-Batch™ Slide Holder and EZBatch™ Wash Tray.

The ACD EZ-Batch™ Slide Holder is fully compatible with the ACD HybEZ™ Humidity Control Tray and is designed with an easy locking mechanism to keep slides intact during washing steps. This design eliminates the time-consuming transfer of slides between the slide rack and Tissue-Tek washing tray during wash steps and ensure optimal washing efficiency. Each ACD EZ-Batch™ Slide Processing System can accommodate 20 slides.

Scientists and lab technicians routinely employ this efficient tool to RNAscope® Assay and also to other protocols where handling multiple slides is required. Why handle one slide at a time when you can process all 20 at once?

RNAscope® EZ-Batch™ Slide

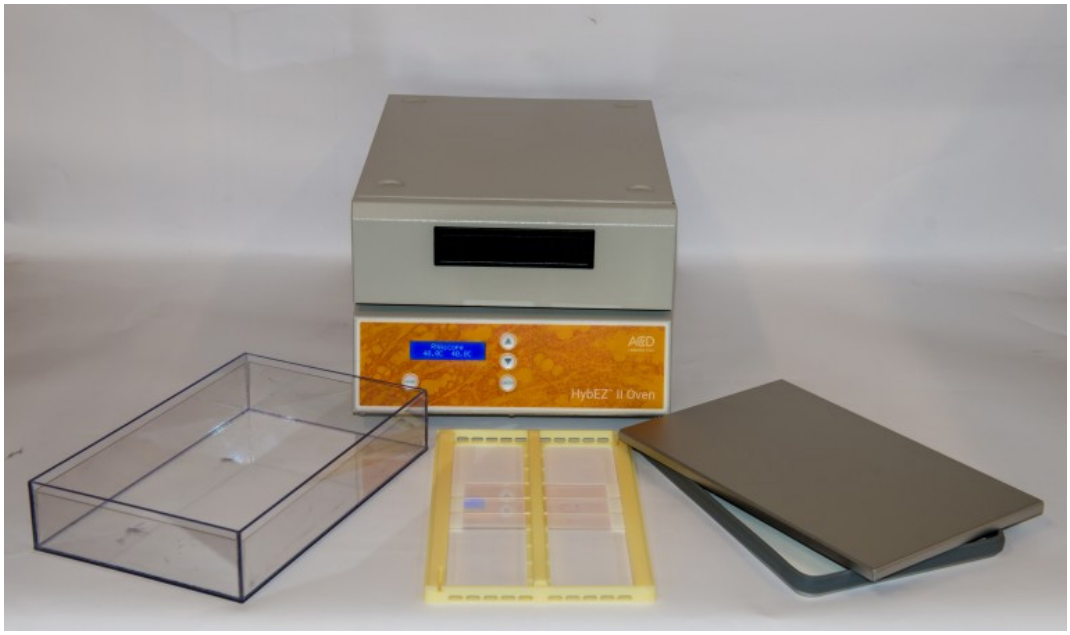


FIGURE 14. HybEZ™ II Hybridization System

RNAscope® *in situ* Manual Assay

Workflow and associated products (continued)

Step 04. Visualize

View your results

Each punctate dot signal represents a single target RNA molecule and can be visualized with a standard microscope (figure 15).

Examine tissue sections under a standard brightfield microscope or standard fluorescent microscope at 20–40X magnification or with a multispectral fluorescent imaging microscope to:

- Assess tissue and cell morphology and quality.
- Assess positive control signal strength. Positive control signal should be visible as punctuate dots at 20–40X magnification.
- Assess negative control background. One dot to every < 10 cells displaying background *DapB* staining per 20X microscope field is acceptable.
- Assess differences between single dots versus dot clusters. This is important for scoring for quantification.

Singleplex semi-quantitative scoring

Visual scoring is performed at 40x magnification to assign a single score to a sample based on the predominant staining pattern seen throughout the entire sample. Intensity of the stain does not have an impact on scoring. Dots correlate to the number of individual RNA molecules, whereas dot intensity reflects the number of probe pairs bound to each molecule. Percentage of cells positive is scored visually based on number of cells with ≥1 dot/cell and binned into categories.

Visual H-scoring

To evaluate heterogeneity in marker expression, H-score calculation is performed and a single score is assigned to a region of interest (ROI). Cells are grouped into 5 bins based on the number of dots per cell. Clusters are divided by the typical probe signal area to calculate a dot number for the cluster. Each sample is evaluated for the percentage of cells in each bin. The H-score is calculated by totaling the percentage of cells in each bin according to the weighted formula shown. H-scores are typically provided on a scale of 0–400.

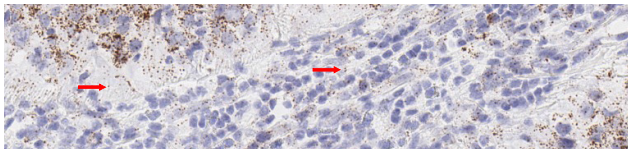
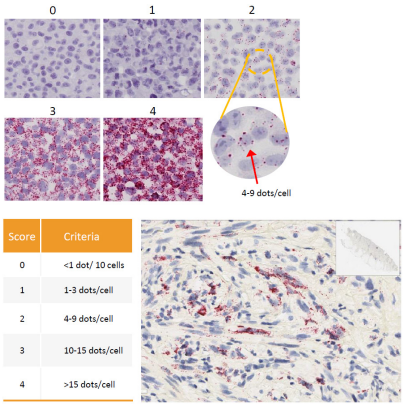
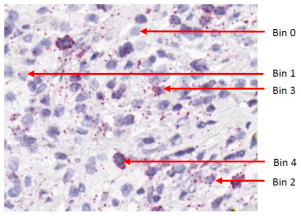


FIGURE 15. ***PPIB*** detection (brown punctate dots) in human cervix using RNAscope® HD 2.5 kit-BROWN. Each brown punctate dot signal represents a single target RNA molecule and the size of the dot is proportional to the number of double Z probes hybridized on the target RNA molecule.



Score	Criteria
0	<1 dot/ 10 cells
1	1-3 dots/cell
2	4-9 dots/cell
3	10-15 dots/cell
4	>15 dots/cell

FIGURE 16. Singleplex semi-quantitative scoring



	% of Cells	Weighted Formula
Bin 0 (0 dots/cell)	8	0 * 8
Bin 1 (1-3 dots/cell)	46	+ 1 * 46
Bin 2 (4-9 dots/cell)	39	+ 2 * 39
Bin 3 (10-15 dots/cell)	5	+ 3 * 5
Bin 4 (>15 dots/cell)	2	+ 4 * 2
H-Score		147

H-Score = 0*(% of cells in bin 0) + 1*(% of cells in bin 1) + 2*(% of cells in bin 2) + 3*(% of cells in bin 3) + 4*(% of cells in bin 4)

FIGURE 17. Visual H-scoring

For more information request or download the RNAscope® Data Analysis Guide containing data analysis guidelines for several types of RNAscope® staining results. **acdbio.com/dataanalysisguide**

RNAscope® *in situ* Manual Assay

Workflow and associated products (continued)

Step 05. Quantify

Software for quantitive analysis

Accurate quantification at your fingertips.

The single-molecule sensitivity and visualization of the RNAscope® technology makes quantitative RNA ***in situ*** hybridization analysis a reality.

ACD as partnered with Indica Labs to facilitate and improve quantitative scoring by providing software for automated analysis – HALO™ Software. This advanced image analysis solution brings objective and accurate quantification to RNA ***in situ*** hybridization. Gene expression can be measured quantitatively and interpreted by research pathologists within histopathological context. This softwares is designed for research pathologists with no prior training in image analysis software. It is an intuitive automated solution that generates standardized and objective results in minutes. HALO™ Software can be used to analyze data generated with chromogenic, duplex chromogenic and fluorescent RNAscope® Assays. HALO™ Software is commercialized by Indica Labs.

An additional collaboration with Leica Biosystems enables reasearchers using the Leica Biosystems BOND RX to use the Aperio RNA ISH Algorithm for accurate identification and standardized quantification of individual signals and clusters on single- or dual-plex stained slides by using the Aperio image analysis software.

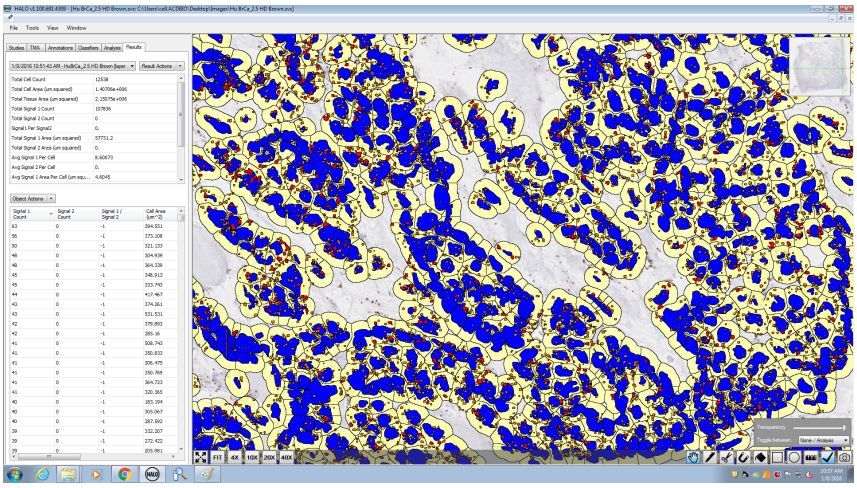
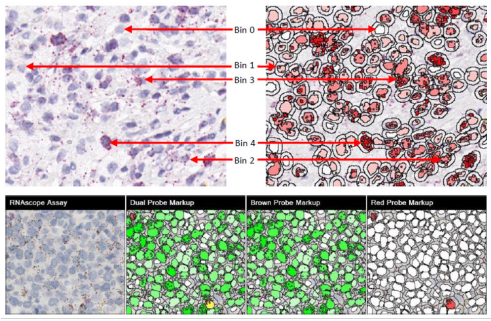


FIGURE 18. PPIB expression analysis in human breast carcinoma sample using HALO™ software (Indica Labs).



HALO Input Parameters	HALO Output Parameters
Minimum nuclear and probe optical density	Total cell and probe area and count
Nuclear and probe contrast thresholds	Average probe copies and area per cell
Cell detection and cytoplasm radius	% of cells in each bin
Nuclear and probe segmentation aggressiveness	Area and number of probe copies per cell
Minimum and typical probe spot size	Probe H-Score
Minimum probe counts for bins (if different from standard criteria)	% Dual positive and negative (if duplex)

FIGURE 19.Software-based image image analysis is performed using HALO™ software (Indica Labs).

ACD Support Scientists offer ISH expertise to ensure your success

Comprehensive worldwide support

Whether you need help selecting your target or deciding which RNAscope® assay format best suits your needs, our Global Technical and Field Support Scientists are here to assist you through the process of selecting your assay, designing your experiments, and guiding you through the interpretation for a successful RNAscope® research analysis.

New user program

As you begin your first RNAscope® assay, we offer an exclusive new user program, providing guidance for every step from experimental design and set up, to interpretation of results. With our support scientists' assistance, we are confident you can obtain publication quality results from your first assay. We also offer monthly technical support webinars for new or existing users with details on our manual chromogenic, fluorescent and/or automated assay procedures. You can always watch support videos and recorded webinars available on our website at **acdbio.com/learn-more**

Field support and onsite trainings

We pride ourselves in advancing our client's research by offering the best scientific support or guidance either via phone, email or onsite visits. If you would like our support scientists to visit your lab and provide training on site, please contact your sales or account executive. A Field Application Scientist will visit you shortly and provide an onsite training to you and members of your lab.

Contact us

North America ACD Support Scientists are available between 7:30 am to 6:00 pm Pacific Standard Time



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Product overview

		Chromogenic Singleplex Brown	Chromogenic Singleplex Red	Duplex	Fluorescent Multiplex
RNAscope® Assay	Manual assays	■	■	■	■
	Automated on Leica BOND RX System	■	■	■	■
	Automated on DISCOVERY ULTRA automated tissue staining systems by Roche Tissue Diagnostics	■	■	■	
BaseScope™ Assay	Manual assays		■	■	
	Automated on Leica BOND RX System		■		
	Automated on DISCOVERY ULTRA and DISCOVERY XT* automated tissue staining systems by Roche Tissue Diagnostics		■		
Pharma Assay Services	Manual assays	■	■	■	■
	Automated on Leica BOND RX System	■	■	■	■
	Automated on DISCOVERY ULTRA automated tissue staining systems by Roche Tissue Diagnostics	■	■	■	

Learn more about the RNAscope® assay at acdbio.com

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