RNAscope® Manual Reagents

Gene Expression Analysis by RNA *In Situ* Hybridization

Get quantitative molecular detection
with morphological context in a single assay

ACD
a bio-techne brand

RNAscope® Assay is the most advanced RNA *in situ* hybridization (ISH) assay based on ACD patented technology with signal amplification and simultaneous background noise suppression which advances RNA analysis in tissues and cells. Unique to this technology, RNAscope® 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 500 publications in the last 4 years, researchers have embraced RNAscope® technology in multitude of research areas, showing the universality of RNAscope® technology. With its unique features and high sensitivity, RNAscope® technology is an essential tool in any research and pre-clinical research laboratory.

- Cancer research
- Stem cell
- Neuroscience
- Cell biology
- Immunotherapy
- Xenograft models
- Infectious disease
- Preclinical safety assessment
- And more...

2011
4 publications

2012
11 publications

2013
47 publications

2014
94 publications

2015
>200 publications

Advanced Cell Diagnostics
Publication Count

500*+

*As of June 2016

Explore the RNAscope® world

Step 01. Permeabilize

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

Step 02. Hybridize

Double Z probe pools are hybridized to target RNA molecules.

Step 03. Amplify

Sequential hybridization of amplifiers and labeled probe.

Step 04. Visualize

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

Step 05. Quantify

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

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

This serial hybridization events—20 ZZ probe pairs, multiple amplifiers, multiple labeled probes—result in hybridization of thousands of labeled probes per RNA target.

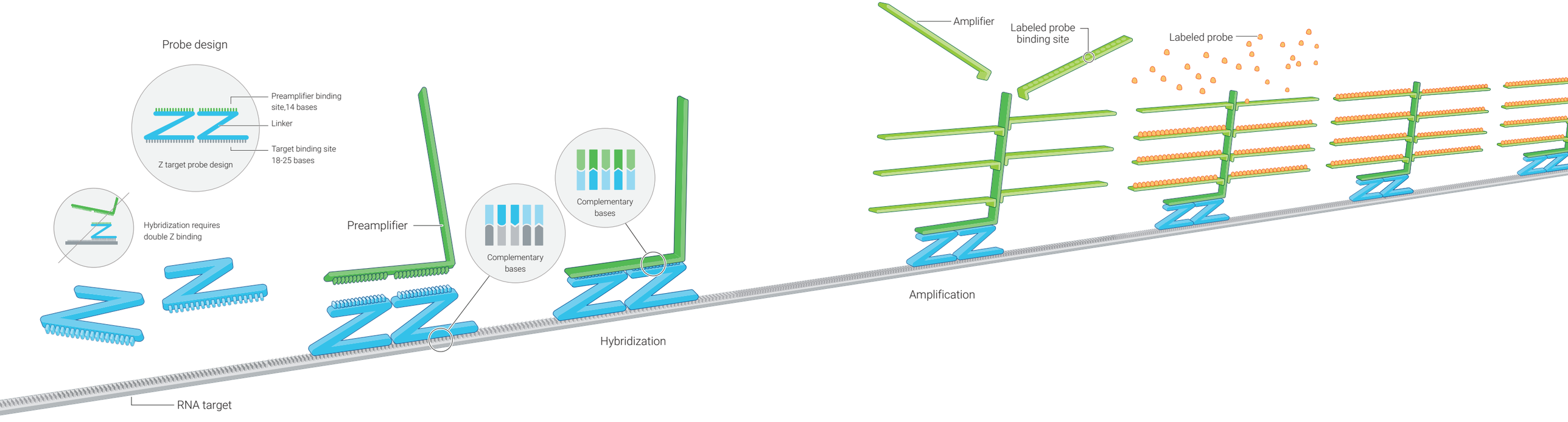
RNAscope® signal detection

Labeled probes contain chromogenic enzyme or 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 chromogenic signal by a brightfield microscope.

Benefits of RNAscope® technology

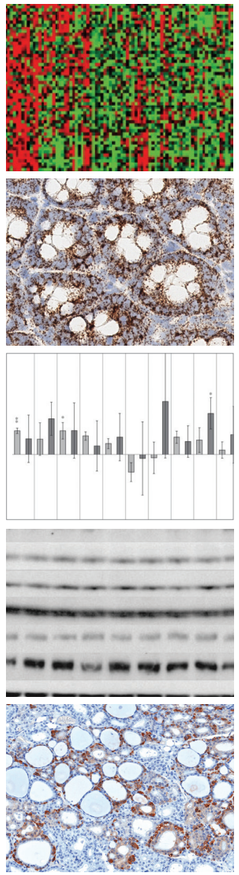
- **High sensitivity:** The serial signal amplification design increases sensitivity such that a single RNA molecule can be detected.
- **High specificity:** Proprietary probe design ensures target- specific binding while the double Z probe design prevents signal amplification of non-specific hybridization.
- **Morphological context:** Spatial resolution of gene expression in complex tissue environment.
- **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.



A solution for common research challenges

Rapid validation of biomarker discovery

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



Genomics/Proteomics
RNA/protein expression

RNAscope® Assay
RNA expression
Tissue localization

qRT-PCR
RNA expression

Western Blot
Protein expression
Protein modification

Immunohistochemistry
Protein expression
Tissue localization
Membrane localization

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

RNA expression analysis to complement or replace 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 (figure 2) 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 replace IHC.

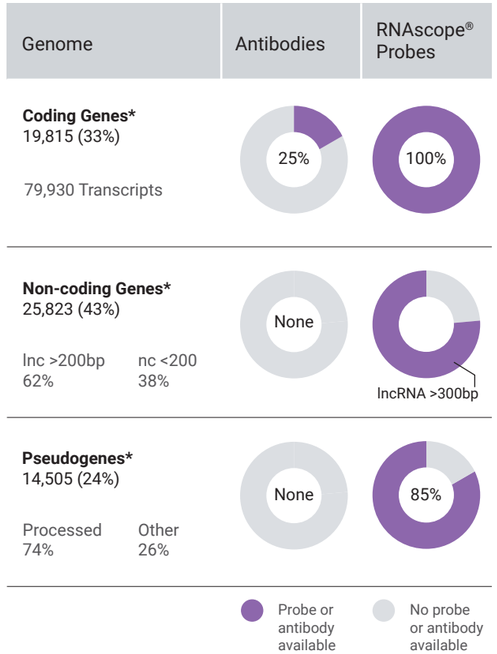


FIGURE 2. Availability of antibodies and RNAscope® probes for different gene categories.

*Human statistics based on Genecode v24, August 2015

Non-coding RNA expression

Long non-coding RNA (lncRNA) won one's spurs during the last decade and has shown some promises as biomarker for many areas of research. The absence of protein and corresponding antibody made RNA ISH the only method available to analyze the lncRNA expression in morphological context (figure 3).

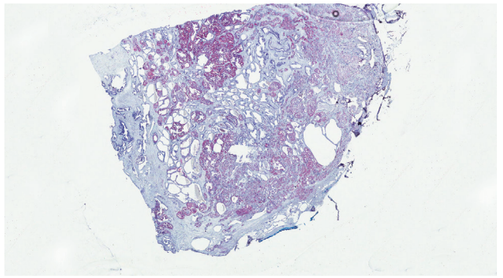
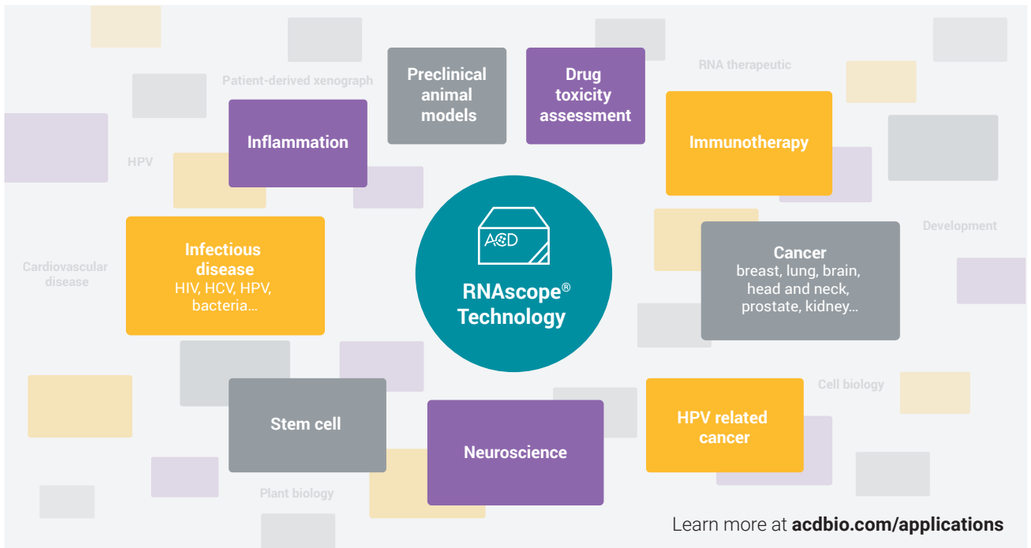


FIGURE 3. Non-coding *PCA3* transcript detection in whole prostate tumor whole tissue section using RNAscope® technology.

"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

Unique RNA ISH solution for all areas of research



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

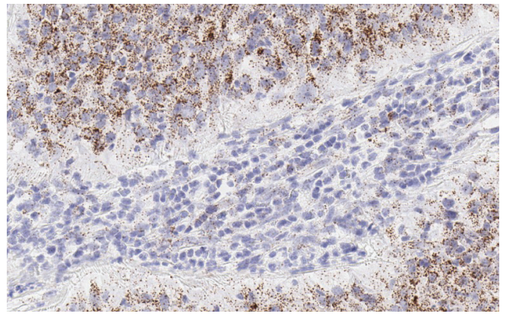


FIGURE 6. *PPIB* detection (brown punctate dots) in human cervix sample using RNAscope® HD 2.5 kit-BROWN.

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. 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 (H202)
- RNAscope® Target Retrieval
- RNAscope® Protease Plus
- RNAscope® Protease IV

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

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 9,000 *in situ* hybridization target probe pools for coding RNA and long non-coding 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, 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 quality 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). Two alternative configurations of this kit are available to enable fully automated walk-away ISH solutions:

- RNAscope® 2.5 VS Reagent Kit-BROWN for use on the DISCOVERY ULTRA and DISCOVERY XT automated tissue staining systems by Ventana Medical Systems, Inc.
- RNAscope® 2.5 LS Reagent Kit-BROWN for 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. Also for detection of target genes where a lower expression is anticipated, ACD recommends this assay as the red dots stand out more clearly against the hematoxylin staining and are more readily identifiable under a standard brightfield microscope. Two alternative configurations of this kit are available to enable fully automated walk-away ISH solutions

- RNAscope® 2.5 VS Reagent Kit-RED for use on the DISCOVERY ULTRA and DISCOVERY XT automated tissue staining systems by Ventana Medical Systems, Inc.
- RNAscope® 2.5 LS Reagent Kit-RED for use on the Leica Biosystems' BOND Rx System.

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

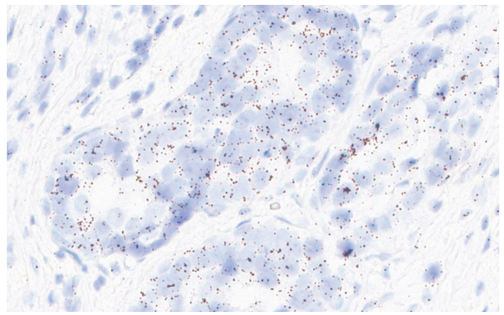


FIGURE 7. *POLR2A* detection (brown punctate dots) in colon cancer sample using RNAscope® 2.5 HD Reagent Kit-BROWN.

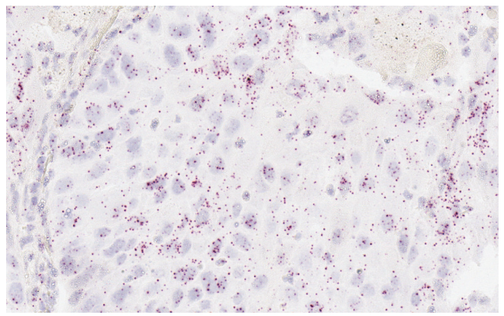


FIGURE 8. *PDL1* detection (red punctate dots) in lung cancer sample using RNAscope® 2.5 HD Reagent Kit-RED.

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 Channel1 (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.

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

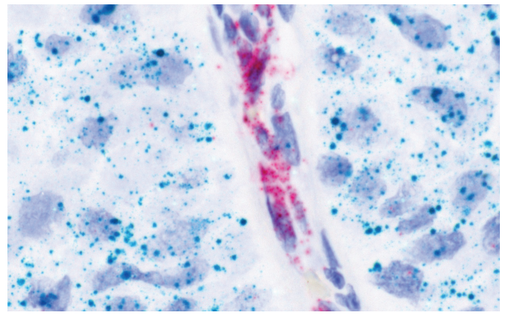


FIGURE 9. *EPCAM1* (red punctate dots) and *EGFR* (green punctate dots) expression in human breast cancer FFPE tissue using the RNAscope® 2-plex Chromogenic Kit.

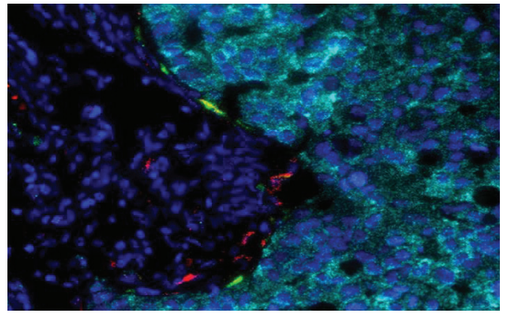


FIGURE 10. Expression analysis of *panCK* (aqua punctate dots), *uPA* (red punctate dots) and *Pai-1* (green punctate dots) in FFPE breast cancer tissue using RNAscope® Multiplex Fluorescent Assay.

RNAscope® Multiplex Fluorescent Assay

Ideal for co-localization studies of any expressed gene set in nearly any tissue type. The assay has exceptional sensitivity allowing simultaneous single-molecule detection of one, two or three different RNA targets. Each target probe pool is designed to a specific color detection channel referred to as C1, C2, and C3. With alternative color modules, users can mix-and-match the colors of C1, C2, and C3 probes according to their experimental design. This offers the highest flexibility to accommodate varying expression levels. ACD can customize 4 probe pools to enable simultaneous detection of four different RNA targets. This assay works for all sample types, but is the most ideal for fresh-frozen tissue sections. The stained slides can be visualized with a multispectral fluorescent, confoncal or standard fluorescent microscope. RNAscope® images are best visualized using the Nuance® multispectral imaging systems from Perkin Elmer.

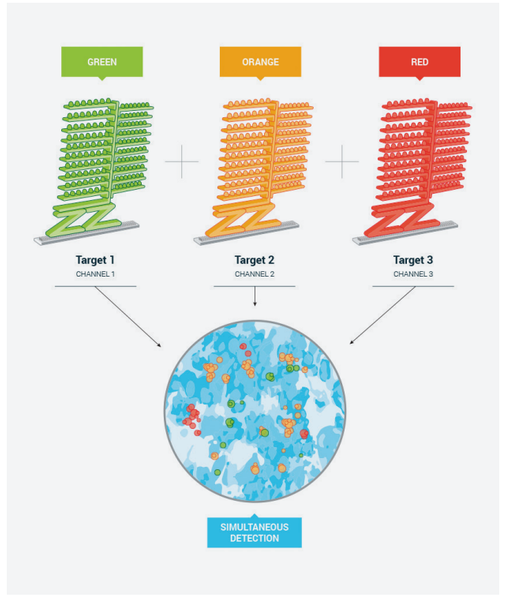


FIGURE 11. Multiplex RNA ISH analysis using RNAscope® Multiplex Fluorescent Assay.

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	RNAscope® Mulitplex Fluorescent Assay
Assay Type	Chromogenic	Chromogenic	Chromogenic	Fluorescent
Dye Used	Diaminobenzidine (DAB)	Fast Red	HRP-based Green and AP-based Fast Red	FITC, Cy3, Cy5, Cy7
RNAscope® Probes Channel Designation	Channel 1 (C1 Probes)	Channel 1 (C1 Probes)	Channel 1 & 2 (C1 & C2 Probes)	Channel 1-3 (C1, C2 & C3 Probes)
Multiplexing	Singleplex	Singleplex	Singleplex, Duplex	Single to Triplex
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	Utilizes up to 3 different spectral channels providing high flexibility
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	Co-expression studies of up to 3 genes simultaneously Experimental application requiring flexibility
Novel Gene or Unknown Expression	+++++	+++++	+++	+++
Archival Specimens	+++++	++	+++	++
Microscope Imaging System	Standard brightfield	Standard bright field Multispectral fluorescent imaging	Standard bright field	Multispectral fluorescent imaging
Sample Type	FFPE Tissues(TMAs) Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells PBMC	FFPE Tissues(TMAs) Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells PBMC	FFPE Tissues(TMAs) Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells PBMC	FFPE Tissues(TMAs) Fixed Frozen Tissues Fresh Frozen Tissues Cultured cells PBMC
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)	6.5 hours (with ~2 hours hands-on time)

RNAscope® accessories

Optimal temperature and humidity for optimal assay performance

HybEZ™ Hybridization System

HybEZ™ Hybridization System is recommended for the hybridization and incubation steps. The HybEZ™ Oven is a simple, easy-to-use, low-profile benchtop hybridization oven that provides stringent temperature conditions essential for RNA ISH. It is the only hybridization oven for which ACD guarantees RNAscope® performance. The HybEZ™ Oven provides a gasket-sealed, temperature-controlled humidifying chamber necessary for optimal RNAscope® assay performance.

This instrument system is capable of holding 20 slides at set temperature and high humidity for hybridization and other incubation steps specified in the manual RNAscope® FFPE Assay protocol.

The ACD HybEZ™ Hybridization System comprises (figure 12):

- HybEZ™ Oven (available for 110V and 220V)
- HybEZ™ Humidity Control Tray
- HybEZ™ Slide Rack

HybEZ™ Humidifying Paper is also required and can be purchased separately.

FIGURE 12. HybEZ™ Hybridization System with HybEZ™ Slide Rack.

RNAscope® EZ-Batch™ Slide Processing System

RNAscope® EZ-Batch™ Slide Processing System is designed for higher efficiency in running the manual assay protocol. The system comprises RNAscope® EZ-Batch™ Slide Holder and EZ-Batch™ Wash Tray. The RNAscope® EZ-Batch™ Slide Holder is fully compatible with the 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. Each RNAscope® EZ-Batch™ Slide Processing System can accommodate up to 20 slides.

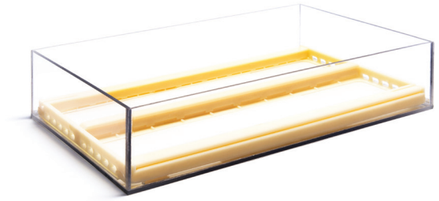


FIGURE 13. RNAscope® EZ-Batch™ Slide Processing System.

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 your microscope (figure 14).

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 DAB staining per 20X microscope field is acceptable.

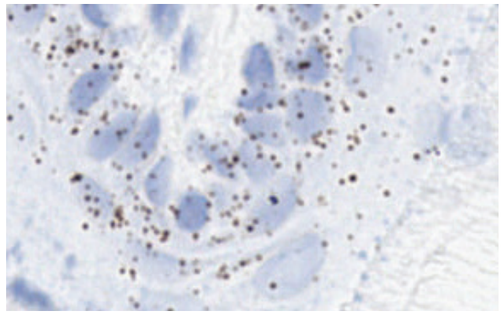


FIGURE 14. *PPIB* detection (brown punctate dots) in Rat Esophagus FFPE sample. Each brown punctate dot signal represents a single target RNA molecule and the size of the dot is proportional to the number of double Z probes hybridized on the target RNA molecule.

Step 05. Quantify

Software for quantitive analysis

Accurate quantification at your fingers

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

Advanced Cell Diagnostics have partnered with Delfiniens and Indica Lab to facilitate and improve quantitative scoring by providing software for automated analysis – SpotStudio™ and HALO Software. These advanced image analysis solutions bring objective and accurate quantification to RNA *in situ* hybridization. Gene expression can be measured quantitatively and interpreted by research pathologists within histopathological context. These softwares are designed for research pathologists with no prior training in image analysis software. They are intuitive automated solutions that generate standardized and objective results in minutes. SpotStudio™ software can be used to analyse data generated with chromogenic RNAscope® Assay. HALO Software can be used to analyze data generated with chromogenic, duplex chromogenic and fluorescent RNAscope® assay. HALO™ Software is commercialized by Indica Lab.

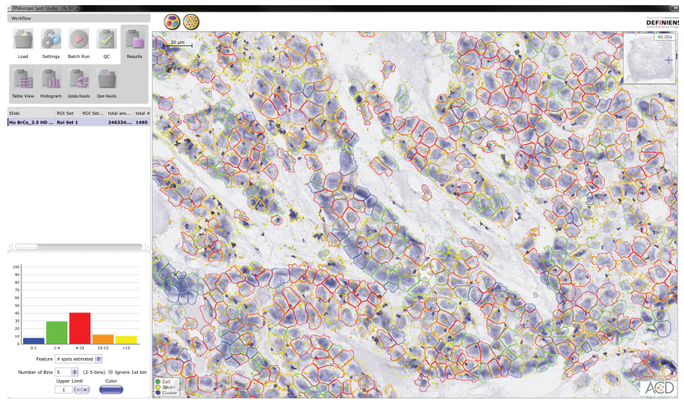


FIGURE 15. *PPIB* expression analysis in human breast carcinoma sample using SpotStudio™ software.

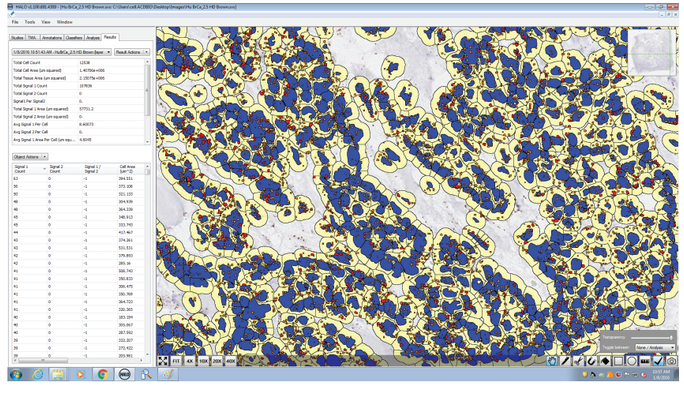


FIGURE 16. *PPIB* expression analysis in human breast carcinoma sample using HALO™ software.

ACD Support Scientists offer ISH expertise to ensure your success

Comprehensive worldwide support

Whether you need help selecting your target, deciding which RNA ISH 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 RNA ISH research analysis.

New user program

As you begin your first RNA ISH assay, we offer an exclusive new user program, to provide 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 webinar 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 or more members in your lab.

Contact us

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


Email Support: support@acdbio.com


Phone Contact: 1-877-576-3636

Europe ACD Support Scientists are available between 8:00 am to 6:00 pm Central European Time


Email Support: support@acdbio.com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