WHITE PAPER

THE RNA REVOLUTION
A GUIDE TO RNA AS A BIOMARKER AND ITS DETECTION

EXPLORING BIOMARKERS WITH THE NEW CENTRAL DOGMA

WHEN IS IT BEST TO MEASURE DNA, RNA OR PROTEIN?

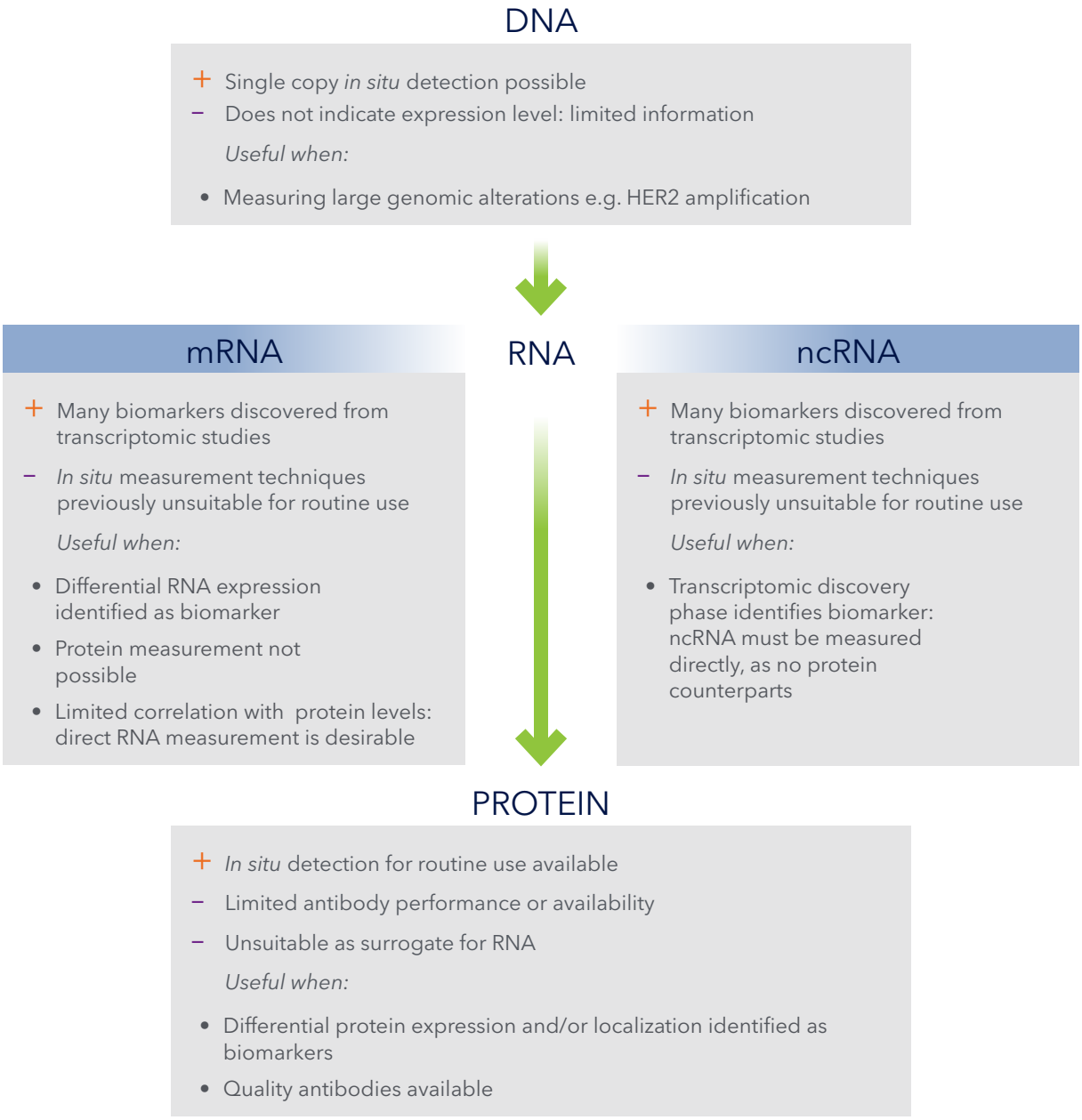

FIGURE 1. Exploring biomarkers with the new central dogma. It can be a daunting decision for scientists to know when to focus on DNA, messenger RNA (mRNA), non-coding RNA (ncRNA) or protein biomarkers for routine clinical use, with each exhibiting its own strengths and limitations.

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INTRODUCTION

One of the biggest surprises of the human genome project was that the number of protein coding genes found in our genome was unexpectedly small: a mere 20,000 versus the anticipated 100,000 or more. This presented a real conundrum for the research community – how does our incredible level of phenotypic complexity and diversity arise from such a modest set of genes? Discovering the world of non-coding RNAs revealed the answer, thanks to new transcriptomic technologies such as microarrays and next generation sequencing (NGS) enabling new avenues in RNA research. Today, new classes of RNAs are being discovered on a regular basis that do not code for proteins, but instead have a hand in genetic regulatory control and a wide range of cellular activities. Amazingly diverse and changeable, these transcripts have the potential to produce any number of splice variants, and in the latest catalog compiled by the ENCODE project,[1] we now know that there are approximately 60,000 genes and ~200,000 RNA species. This RNA revolution has created a fundamental shift in how researchers now view "the central dogma", challenging the traditional idea that DNA is the master and RNA merely the messenger. The revelation of RNA's vast number and diverse role in gene regulation has brought to light the many possibilities for using RNA as an indicator of biological states: the biomarker.

Indeed, the widespread use of transcriptomic profiling in cancer research over recent years has proven that, like protein, RNA is a rich source of clinically valuable biomarkers for diagnosis, prognosis and predicting therapeutic response.[2, 3] Although such transcriptomic profiling may identify many potential biomarkers, translating these discoveries into the clinic for routine RNA biomarker measurement presents challenges in terms of established analytical technologies. While it is commonplace to detect and visualize DNA and proteins in their native context within single cells, until now the best routine measurement tools for RNA analysis have been those that detect and quantify RNA in solution. However, these methods only provide an "average" measurement in a cell population, masking the incredible level of cell-to-cell variation in RNA expression. With its central role in cell physiology, protein has been the more popular biomarker traditionally, providing functional insights into disease states while also lending itself to well-established detection techniques. The lack of effective RNA *in situ* detection methods has often resulted in the use of DNA and protein as surrogates for those RNA biomarkers initially discovered, however this can be problematic. Changes in RNA expression may not result from DNA alterations and may not correlate with protein levels – or there may be no protein counterparts at all in the case of non-coding RNAs. The bottom line is, when utilizing RNA biomarkers initially discovered during microarray or RNA-seq programs, the best approach is direct RNA measurement *in situ*, since the use of a DNA or protein surrogate, or solution-based RNA analysis, inevitably leads to information loss, compromising the full diagnostic utility of the RNA biomarker.

The discovery of the "new world" of RNA has sparked an unprecedented drive towards better tools to characterize the complexity of RNA – in terms of quantity, function and spatial distribution. Presenting a vital piece of the puzzle in elucidating the role played by RNA in disease states, pinpointing the localization of specific RNAs within cells and tissue architecture is an important factor in realizing its true potential as a biomarker. Here we examine the utility of RNA as a biomarker, and how this is profoundly linked to the developing technologies now available for its detection, localization and validation.

1. WHAT IS A BIOMARKER?

The term biomarker is currently defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention'.[4] Biomarkers have long been used for diagnostic testing and the identification of potential therapeutic targets, and are growing exponentially in their importance in the era of personalized medicine. A valuable biomarker is capable of uncovering a specific biological trait or measurable change directly associated with a physiological condition or disease status[5], and they therefore tend to be one of the three main functional components of the cell: DNA, RNA or protein. As we will explain in more depth, while DNA biomarkers provide a static view of the cells and protein biomarkers suffer from variable detection success (albeit through well-established techniques), RNA biomarkers are both functionally significant and now benefit from new robust tools for detection (**FIGURE 1**).

Of course, a biomarker is only as good as its routine analysis methodology – which demands the capabilities highlighted in Box 1.

BOX 1: THE OPTIMAL BIOMARKER METHOD

DETECTION

Accurate and precise biomarker analysis requires sensitivity to detect even the most scarce molecules, and specificity to differentiate between multiple related molecular species.

QUANTIFICATION

While measuring biomarker levels at a continuous scale provides more accurate information than detection alone, levels can vary significantly even between individual cells. An average measurement over a population of cells may therefore obscure significant events, while single-cell analysis can reveal important intercellular heterogeneity, yielding more insightful data.

LOCALIZATION

Since differential expression across cell populations forms the basis for multicellular physiology, analyzing a biomarker in its morphological context presents an especially powerful tool. Visualizing such expression *in situ*, within intact tissue morphology also opens many doors for researchers, particularly in the area of cell-to-cell communication. For example, examining autocrine and paracrine networks, and communication within tumors or between tumor and stroma.

2. THE VALUE OF RNA BIOMARKERS

Our understanding of RNA is constantly evolving, with discoveries over the last few decades including alternative splicing and the catalytic activity of some RNAs, along with the identification of many small non-coding and long non-coding RNAs (lncRNAs).[6] It has become increasingly clear that RNA is a key player in a myriad of both normal and disease-related cellular activities, and genetic regulatory control. For this reason, RNA has become a molecule of great interest and value across the research, diagnostic and pharmaceutical arenas, and since RNA expression reflects the state of a biological system, it presents an ideal choice of biomarker. RNA expression levels are highly dynamic and integrate both genetic and epigenetic mechanisms of gene regulation, serving as an excellent molecular phenotypic readout of the functional state of the cell. In order to utilize the full potential of these biomarkers, there is an unprecedented demand for the next generation of more effective tools for routine RNA biomarker analysis. This demand is particularly acute when it comes to *in situ* techniques, in order to map important pathways and networks within the morphological context (FIGURE 2).

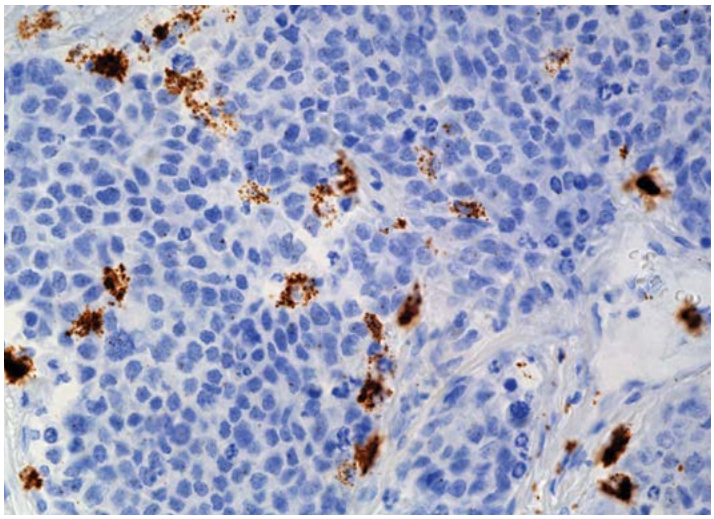


FIGURE 2. Revealing the RNA expression signature of individual cells within the tissue architecture. Human breast cancer FFPE tissue is probed for MMP9 mRNA expression using RNAscope® 2.0 HD Reagent Kit-BROWN. Overexpression of this matrix metalloprotease facilitates tumor growth and invasion; and although signal (brown) is abundant in scattered stromal cells of the tumor microenvironment, it is barely detectable within tumor cells.

3. THE CONCEPT OF ALTERNATIVE BIOMARKERS

As previously discussed, the majority of biomarkers discovered today are RNA derived from transcriptomic studies, but the lack of standardized and robust technologies for measuring RNA biomarkers *in situ* within clinical samples has been a significant bottleneck in advancing these discoveries to the clinic. The inability to accurately detect RNA *in situ* has therefore prompted the continued use of alternative biomarkers as a surrogate for RNA or bulk tissue, and in solution analysis techniques such as RT-PCR. While researchers may seek to bypass RNA detection by looking towards the molecule's counterparts (i.e. measuring upstream DNA or downstream protein products, where present), in many cases such approaches lead to significant information loss. There is no substitute for directly measuring RNA itself *in situ*, leading to the most accurate biomarker validation and assay development program.

3.1 PROTEIN-BASED BIOMARKER DETECTION

The number one limitation of analyzing a protein in place of the RNA biomarker initially discovered is that, due to both transcriptional and post-transcriptional mechanisms of gene regulation, protein and RNA levels rarely exhibit a linear correlation. This means that although during RNA biomarker discovery a particular RNA molecule may follow an expression pattern indicative of a biological state, its protein counterpart may exhibit a very different expression signature altogether. Measuring such a protein is therefore unlikely to capture the biological state indicated by the mRNA, while in the case of non-coding RNA molecules, which have no protein counterparts, measuring RNA is the only option.

Nearly every protein detection technique fundamentally relies upon the use of antibodies, with the main method of protein biomarker detection *in situ* being immunohistochemistry (IHC). However, antibody quality or availability can be limiting.

The development and validation of a new antibody requires considerable resources[7], and can take six months to more than a year with no guarantee of success – while it is not always possible to raise an antibody against a poorly immunogenic or toxic protein. Moreover, antibody quality can vary significantly between batches, and many often have uncertain specificity. The determination of antibody specificity is not as straightforward as one might imagine[8], as antibodies can often cross react with similar proteins from the same family or even have more unexpected cross reactions. Added to this is the problem that only 25% of the proteins within the human proteome have validated antibodies[9], and reliable and sensitive antibodies are lacking for many important proteins such as receptors, GPCRs, transcriptional factors and secreted proteins. Research applications can also be hampered where antibody availability is limited for certain key model organisms such as drosophila and zebrafish.

Assuming high-quality antibodies are available, the long-standing semi-quantitative technique of IHC detects and locates antigens (proteins) in specific cellular compartments in tissue sections. It has been in routine use in diagnostic and research laboratories since 1942 when the first IHC study was reported. [10] The technique is advantageous as it conserves tissue morphology and permits the visual localization of the biomarkers *in situ*, and the widespread availability of automated immunostainers today makes the method commonplace.[7] However since the method was introduced, apart from improvements in protein conjugation, tissue fixation methods, detection labels and microscopy, the core technique itself has remained much the same with little scope for advances in the methodology, and is not without challenges.

Although this affordable technique lends itself to widespread use, lack of absolute specificity in IHC can lead to problems with background staining in histological sections, obscuring the ability to accurately interpret the results. This can be somewhat corrected by the use of dilutions, but it is time and reagent-consuming to set-up, and is a major source of variability. Diffusion of secreted proteins into the intercellular space of the tissue can also be problematic, resulting in dilution of antigens to a level below the sensitivity of the antibody, while diffusion of secreted proteins from sources other than the cells of interest may contribute to background staining, making it difficult to identify the cell of origin of the secreted proteins. In contrast, as RNA is only found within cells, it enables the precise identification of the cell of origin – and these may, for example, be important signaling molecules such as growth factors and cytokines.

In summary, depending on the antibody or antigen, detecting protein as a biomarker has important limitations as indicated in Box 2. Even when high quality antibody is available and the methods do work well, *in situ* detection of RNA can provide additional information complementary to protein detection methods.[11]

3.2 DNA-BASED BIOMARKERS

Like RNA and protein biomarkers, DNA biomarkers are also important in many research and clinical applications. A common technique for the detection and validation of DNA biomarkers is DNA *in situ* hybridization (DNA ISH).

BOX 2: PROTEIN AS AN ALTERNATIVE BIOMARKER

BENEFITS

- Measurement of the final functional product of a gene
- Well-established methodologies are broadly available
- Options for automation (e.g. IHC)
- Cost effective when antibodies are available
- Rapid and easy to perform

HOWEVER…

- Limited antibody availability
- Antibodies can lack specificity or have unknown specificity
- Low sensitivity of antibody based methods makes it challenging to interrogate secreted and scarce proteins
- Antibody development & validation can be time consuming, challenging and cost-prohibitive
- Antibody batches can exhibit high batch-to-batch variation
- RNA and protein levels may not correlate linearly
- Limited to protein-coding genes
- Lack of standardization in both assay and data interpretation (IHC)

It is also important to note that structural rearrangements such as DNA amplification or translocation do not always result in changes in transcriptional activity or levels of expression. Thus, an increased DNA copy number does not necessarily translate to an increased amount of RNA or protein.[12] A single gene can have multiple transcriptional start sites and alternative splicing sites that produce multiple RNA transcripts or isoforms, which may be subject to differential regulation, making one form more informative as a biomarker than another in a given disease. Such transcripts and isoforms are not identifiable from *in situ* detection of DNA. Furthermore, at the DNA level, gene fusion can involve two nearby genes, rendering the transcript undetectable by break-apart fluorescent *in situ* hybridization (FISH) probes. However, at the RNA level, fusion transcripts can be detected regardless of the distance between the two partner genes.

In addition to the inherent issues with DNA biomarkers, current DNA ISH technologies have limited sensitivity for short, low-copy DNA sequences due to limited probe size. In contrast, a single-copy of a gene may produce high copies of RNA levels, enabling more sensitive detection at the RNA level. Confirmation of RNA expression from altered DNA provides key functional evidence relevant to disease processes.

The field of DNA biomarkers has also benefited greatly from the rapidly changing landscape of DNA sequencing technologies (such as NGS) and the wealth of data produced. Although DNA sequencing can detect and measure alterations such as single nucleotide variation, copy number variation, fusion genes and structural anomalies, this approach is not without its limitations – primarily being the lack of data on spatial and cellular distribution within the tissue context. In such cases, RNA analysis can confirm and complement DNA analysis in many applications and research areas.

BOX 3: DNA AS AN ALTERNATIVE BIOMARKER

BENEFITS

- Methods can detect structural alterations such as translocations, deletions and amplifications
  - FISH: exhibits single copy sensitivity - specificity is easily verified through visual evidence
  - NGS: ever-evolving NGS technology presents many benefits e.g. targeted gene panels through to genome wide, de novo discovery; high throughput

HOWEVER…

- DNA alterations do not always lead to changes in RNA and protein expression
- Not single-gene resolution - since typical probes are hundreds of kb long, spanning multiple genes
  - FISH: not feasible to detect small DNA alterations e.g. micro-deletions & micro-amplifications, or gene rearrangements between nearby genes
  - NGS: no information on morphological context, bioinformatics often complex and requires specialized skills

4. SELECTING THE RIGHT RNA BIOMARKER DETECTION METHOD

Many methods exist for the analysis of RNA, but they can be loosely categorized into in-solution and *in situ*. The ability of *in situ* techniques to visualize the location of RNA directly at single-cell resolution within the morphological context of its surroundings provides valuable insight, unattainable by mere in-solution detection and quantification alone.

4.1 IN-SOLUTION METHODS

In-solution based methods are the so-called 'grind-and-bind' methods that analyze RNA within cell populations and provide quantitative analysis, but destroy all morphological context and spatial resolution in the process. Researchers are therefore limited to comparing quantitative information between heterogeneous cell populations. Such methods include real-time polymerase chain reaction (PCR), nuclease protection assays and molecular bar-coding.

4.1.1 REAL-TIME PCR

Real-time PCR (qPCR) is perhaps the best known and most widespread of the in-solution methods available for RNA quantification. The method, which remains the gold standard for RNA measurements today, records data at the same time as the PCR reaction occurs, combining amplification and detection into one step. The method is known for its large dynamic range, high sensitivity and specificity, and is also amenable to increasing throughput, which is ideal for every stage of a biomarker discovery and validation program. In theory, PCR itself is robust and predicable, but in reality, however, it is quite susceptible to variations, especially in the efficiency of the RNA extraction and reverse transcription steps. The numerous stages of the protocol also increase the potential for error and introduction of contaminants, and the method requires expensive reagents.[13]

4.1.2 NUCLEASE PROTECTION ASSAYS

Nuclease protection assays (NPAs) are used to identify individual RNA molecules in a heterogeneous sample of extracted RNA. The technique can identify one or more RNA molecules of known sequence at low concentrations. The extracted RNA is mixed with RNA or DNA probes that are complementary to the sequence or sequences of interest, which hybridize to form double-stranded RNA (or a DNA-RNA hybrid). The mixture is then exposed to nucleases that specifically cleave single-stranded RNA and have no activity against double-stranded RNA or DNA-RNA hybrids. Unhybridized RNA regions are therefore degraded, leaving only the RNA fragments of interest. The technique has historically been low throughput, and complex in comparison to *in situ* hybridization methods. Although advances have been made, such as those by High Throughput Genomics (HTG Molecular Diagnostics Inc., Tucson, AZ), to multiplex andsimplify the assay, this method still does not provide morphological context.

4.1.3 NANOSTRING® nCOUNTER® TECHNOLOGY

Single molecule imaging assays with molecular bar coding, such as NanoString's technology (NanoString Technologies Inc, Seattle, WA), employ two probes per mRNA that hybridize in solution. A 'reporter probe' carries the signal while a 'capture probe' allows the complex to be immobilized for data collection. After hybridization, excess probes are removed and the probe-target complexes are aligned and immobilized in a cartridge. The cartridge is then digitally analyzed for image acquisition and the data processed, with hundreds of thousands of color-codes denoting RNA targets of interest. Gene expression levels are measured by counting the number of times the color-coded barcode for that gene is detected.[14]

While the NanoString approach offers certain benefits, such as the ability to assay many targets directly from tissue extracts and whole blood lysates, the hybridization step of the method is slow (typically 20 hours or more with pre-processing steps included). In addition, the analysis system comprises specialist instruments, requiring significant capital investment and specialized training, making the technology less accessible to all but the largest laboratories.

4.1.4 SUMMARY

In-solution methods provide useful molecular profiles of diseases and accurate quantitative data, however clinically relevant information regarding cellular and tissue context, as well as spatial variation of the expression patterns, is lost in the process. This information is vital in order to clinically validate biomarkers, and *in situ* methods must therefore fulfill this requirement.

4.2 IN SITU METHODS

In situ hybridization (ISH) was first introduced in 1969, and first applied to RNA in 1981.[15,16] However, until recently methods have lacked the sensitivity, specificity and ease of use required to make the method commonplace in both research and diagnostics labs. The morphological context and spatial resolution provided by ISH enables valuable benefits, e.g. providing precise localization of target RNA in single cells, and therefore much work has been done to advance the technique.

In situ methods allow localization of RNA expression in specific cell types e.g. stromal verses tumor expression (FIGURE 2). Although laser micro-dissection for in-solution methods can provide similar information to a limited extent, it lacks single-cell resolution. *In situ* methods also enable the detection of expression in rare cell populations such as cancer stem cells and circulating tumor cells (CTCs) that is not possible with other methods. In the case of circulating tumor cells, RNA ISH can detect both expression and count the number of cells that are expressing (FIGURE 3), while in-solution methods can yield expression but cannot enumerate cells expressing.

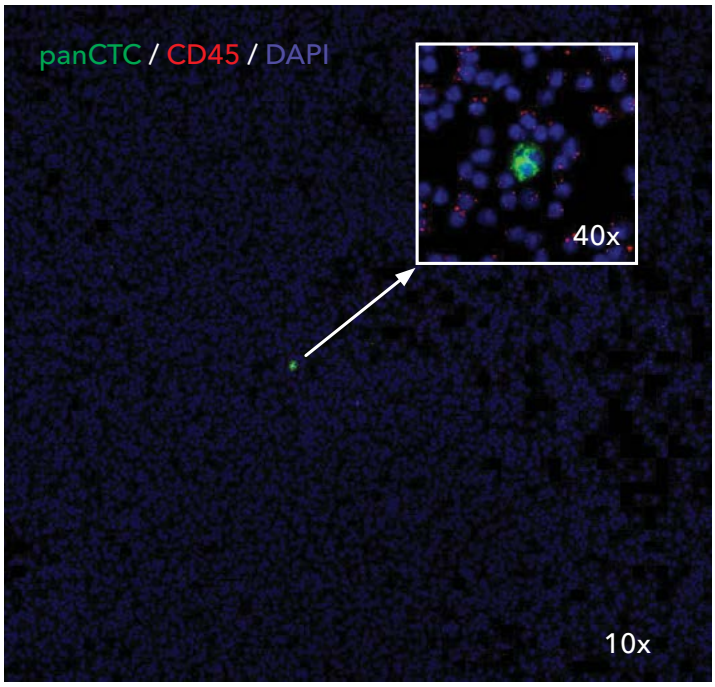


FIGURE 3. Highly specific cell identification using RNA ISH. A circulating tumor cell within metastatic breast cancer blood sample is here identified by its positive staining with a CTC-specific probe cocktail (panCTC), and absence of staining for the leukocyte marker CD45. Picking out this single cell against a large background is here achieved with RNAscope – showing 10x and 40x magnification.[20]

4.2.1 CONVENTIONAL RNA ISH METHODS

Isotopic ISH The first examples of RNA ISH as a technology involved the use of radioactively labeled RNA probes. Isotopic ISH can be highly sensitive but requires long exposure times (days to weeks) and only allows limited morphological correlation. It is also hampered by non-specific signals due to the use of long probes and long exposure times, leading to higher background noise. These shortcomings and the use of radioactivity prohibit the technology from routine use.[17]

Non-isotopic ISH The advent of fluorescently labeled or biotinylated oligonucleotide probes advanced the use of RNA ISH greatly, improving both turnaround time and sensitivity without the use of radioactivity.[18] Such probes can be visualized with fluorescence or chromogenic enzymatic reactions after hybridization. As an alternative to DNA probes, RNA hybridization probes are also available – termed riboprobes, which are produced by *in vitro* transcription and are typically hundreds of bases long. Compared to DNA oligonucleotide probes, riboprobes form more stable hybrids with target RNA allowing more stringent hybridization for improved specificity. However, due to the short nature of RNA targets allowing only a limited amount of labels to be incorporated into the probes, both DNA oligo probes and riboprobes lack sufficient sensitivity for the majority of expressed genes. Signal-to-noise ratio is also limited with these methods due to a high probability of non-specific binding and cross hybridization in a highly complex tissue section. Although researchers have continued to develop RNA ISH, only recently have we approached a level of specificity and sensitivity that enables its widespread use.

4.2.2 DIRECT DETECTION RNA ISH

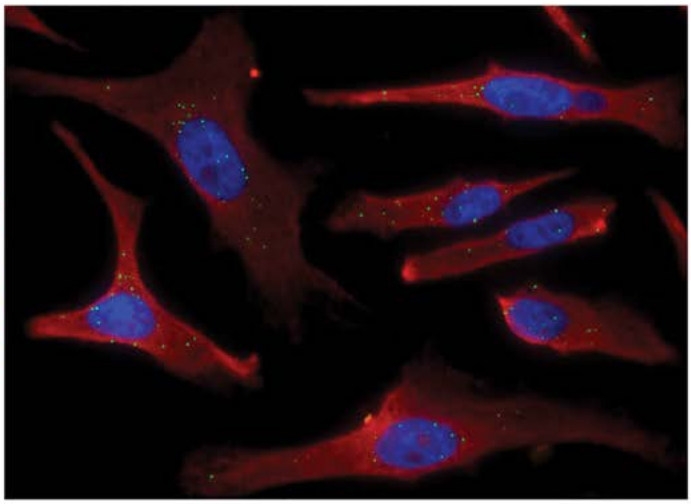
Direct detection RNA ISH, such as the method marketed by Biosearch (Stellaris™, Biosearch Technologies Petaluma, CA) involves small single stranded oligonucleotide probes directly labeled with fluorophores. Multiple probes are created for each target RNA, each complementary to a short stretch, which combined span the length of the sequence. Use of multiple probes in this way ensures enhanced sensitivity and that the fluorescent signal is strong enough to be visible above the background of one or two probes hybridizing non-specifically, which cannot be avoided completely. The technique is simple and fast to perform owing to the direct detection of the labeled probes. However, the sensitivity and signal-to-noise ratio of this method remains limited, requiring specialized background subtraction algorithms for data interpretation.[19]

4.2.3 RNAscope® ISH

RNAscope is a new multiplex nucleic acid *in situ* hybridization technology, based on ACD's (Advanced Cell Diagnostics Inc., Hayward, CA) unique probe design and signal amplification methodology. The RNAscope approach is an alternative to conventional ISH/FISH *in situ* RNA detection, and the method provides the opportunity to profile single-cell gene expression *in situ* with single-molecule detection sensitivity, unlocking the full potential of RNA biomarkers (FIGURE 4).

To date, this method is the only platform that has the sensitivity to detect most genes in the human transcriptome *in situ*, as well as simultaneously quantifying multiple RNA transcripts at a single-cell resolution. In order to substantially improve the signal-to-noise ratio of RNA ISH, RNAscope employs a probe design strategy very similar to fluorescence resonance energy transfer (FRET), in which two independent probes (doubleZprobes)musthybridizetothetargetsequenceintandemin order for signal amplification to occur (FIGURE 5). Since it is highly unlikely that two independent probes will hybridize to a nonspecific target right next to each other, this design concept ensures selective amplification of target-specific signals.[21]

A



HER2 mRNA dots/cell: 14.4 ± 1.75

B

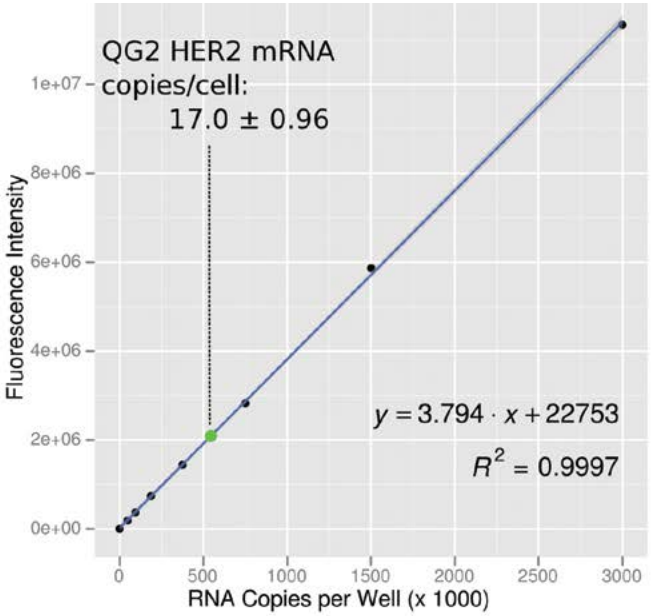


FIGURE 4. Quantitative RNA ISH profiles *in situ* at single-cell resolution. (A) HER2 mRNA was visualized with RNAscope Multiplex Fluorescent Kit and quantified by counting signal dots in individual HeLa cells. Nuclei were counterstained with DAPI (blue) and a probe set to 18S rRNA was used as internal control for RNA detection. (B) HER2 mRNA in the same HeLa cell culture was quantified using a "grind-and-bind" method (QuantiGene2®) to estimate the absolute copy number of HER2 mRNA in single cells. These two estimates were in close agreement, demonstrating the quantitative capacity of RNAscope. [21]

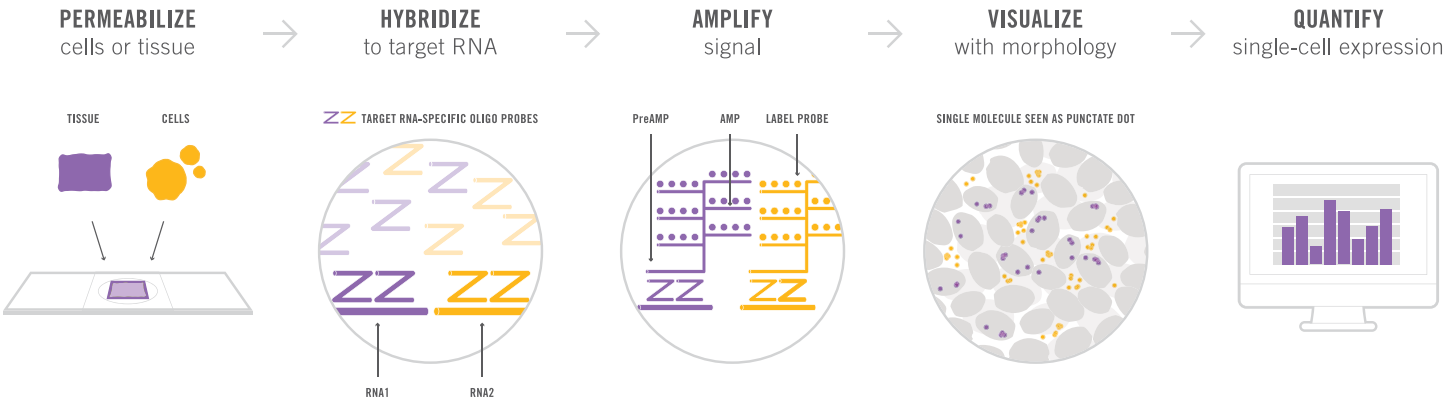


FIGURE 5. The RNAscope assay procedure is completed within a single day. Once the sample is prepared, oligo probes complementary to the RNA target of interest are added and hybridization takes place. Specific signal is subsequently amplified with the double Z probe system, and immediately detected and quantified via standard brightfield microscope or multi-spectral fluorescent imaging system.

For each target RNA species, a pool consisting of about 20 double Z target probe pairs are designed to specifically hybridize to the target molecule, and signal amplification is achieved through a cascade of hybridization events involving pre-amplifiers, amplifiers and label probes containing fluorescent molecules or chromogenic enzymes.

The current RNAscope probe design method of 20 double Z probe pairs requires 1KB of unique sequence. It can be applied to targets as few as 300 bases long but but this sequence length requirement does exclude the ability to interrogate small coding or non-coding RNAs such as snoRNAs, microRNAs, siRNAs, snRNAs, exRNAs, piRNAs - which can be as small as 18 bases. Classified as 200 bases or more, long non-coding RNAs (lncRNAs) are suitable targets for RNAscope technology, which is by far the most sensitive *in situ* method available for this gene class.

This breakthrough method confers numerous advantages including increased sensitivity (**FIGURE 6**), specificity, single molecule visualization and quantification – in addition to compatibility with partially degraded RNA samples (common in FFPE tissue sections), as detailed in Box 3.

The method also enables multiplex staining of RNA transcripts (**FIGURE 7**), which had previously been extremely difficult to achieve due to the complications of finding compatible assay optimization and probe hybridization conditions for multiple target probe sequences. RNAscope can be used to visualize virtually any gene combination of up to four RNA transcripts simultaneously. Furthermore, multiple RNA species can be interrogated in a pooled signal strategy, for example when investigating the human papilloma virus (HPV), where 18 strains are considered high risk and must all be detected if present.

Biomarker validation and routine diagnostic pathology can be time consuming and require the analysis of hundreds of samples.[21] For labs that require this kind of high throughput in their RNA ISH analysis, ACD has developed RNAscope for use on fully automated stainers. These walk-away solutions are available on Leica Biosystems' BOND RX System (Leica Biosystems Nussloch GmbH) and the DISCOVERY ULTRA and DISCOVERY XT systems from Ventana (Ventana Medical Systems, Inc., Tucson AZ). In addition, new target probes can be developed for any public or proprietary target sequence within just two weeks, enabling the rapid development of new assays for biomarker validation.

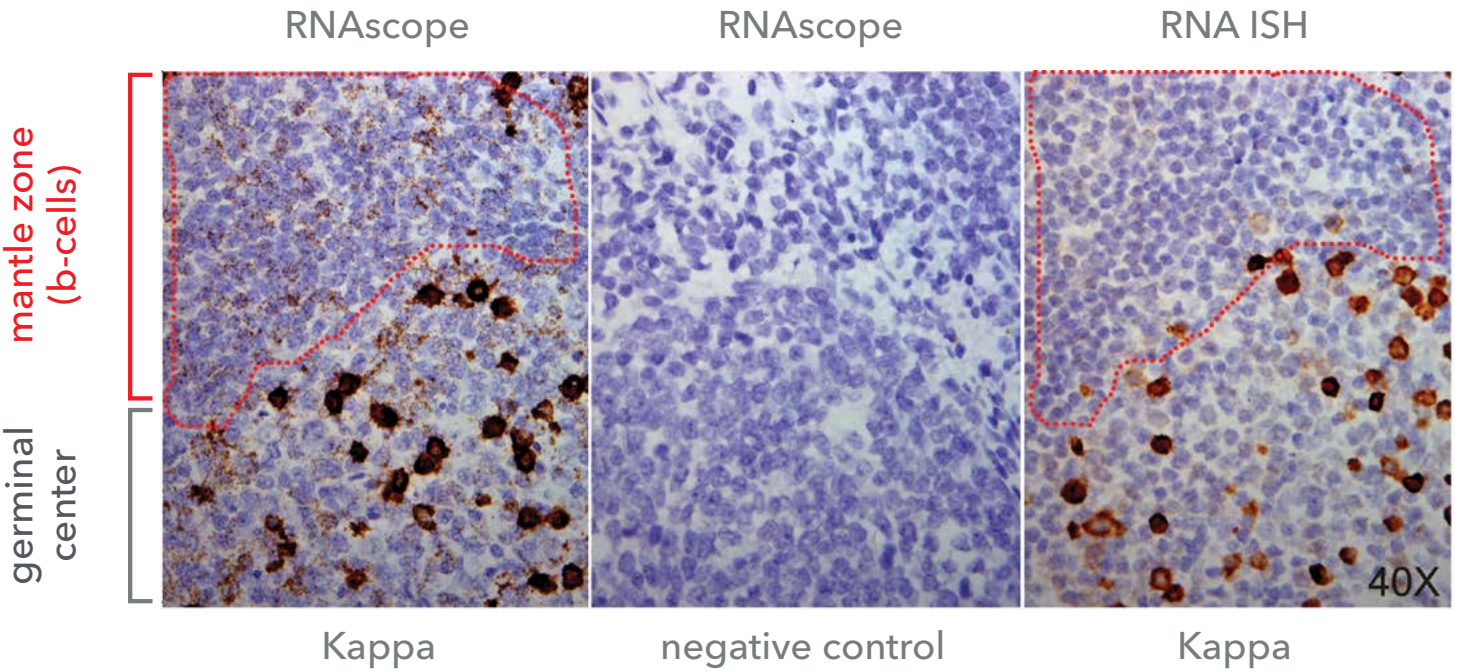


FIGURE 6. Highly sensitive RNA detection compared to conventional ISH. Sensitivity of Ig κ chain mRNA detection in the B lymphocytes of FFPE human tonsil tissue is vastly improved with RNAscope, when compared to conventional non-isotopic ISH. κ light chain mRNA transcripts were stained using RNAscope or a commercial non-radioisotopic RNA ISH (RISH) kit, with a negative control (bacterial gene dapB) included for RNAscope.

BOX 4: ADVANTAGES OF RNAscope STRATEGY

Sensitivity: Detection of each single RNA molecule requires only three double Z probe pairs to bind to target RNA. The 20 double Z probe pairs provide robustness against partial target RNA accessibility or degradation.

Specificity: The double Z probe design prevents background noise. Single Z probes binding to nonspecific sites will not produce a full binding site for the pre-amplifier, thus preventing amplification of non-specific signals and enhancing specificity.

Single molecule visualization and single-cell quantitation: Hybridization of three or more double Z probe pairs is visualized as a punctate signal dot under a standard microscope. Analysis software quantifies RNA expression levels for each single-cell.

Compatible with degraded RNA: The double Z probe design, with its relatively short target region (36-50 bases of the lower region of the double Z), allows for successful hybridization of partially degraded RNA.

Widespread application: As long as at least a 300-base unique sequence is available, RNAscope can be applied to virtually any gene, species or tissue.

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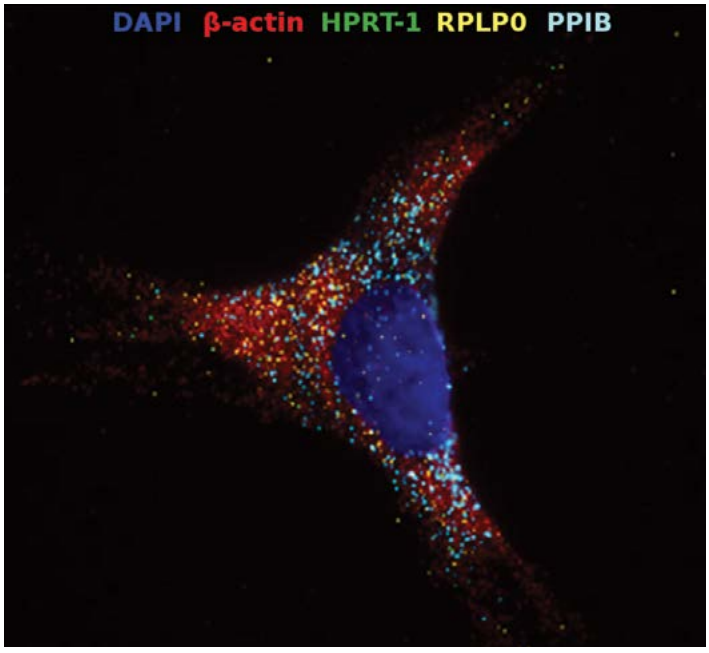

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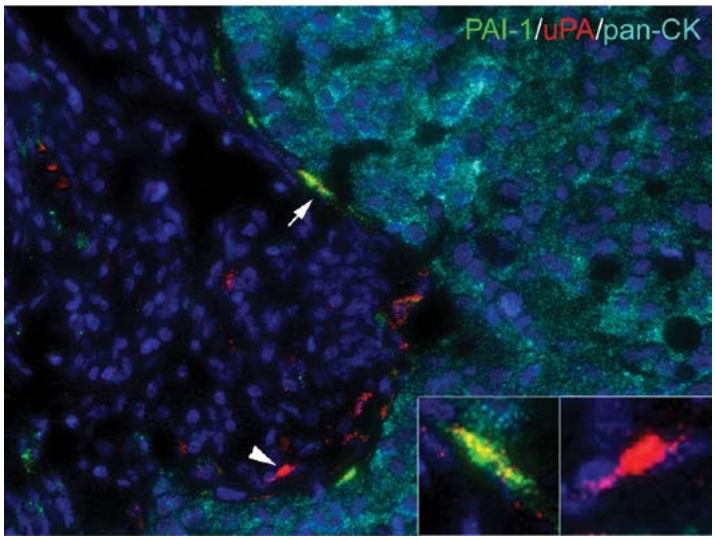

FIGURE 7. Multiplexing with RNAscope provides powerful morphological information both within and between cells. (A) HeLa cells were hybridized with probes to ß-actin, RPLP0 (60S acidic ribosomal protein P0), PPIB (peptidylprolylisomerase (**B**), and HPRT-1 (hypoxanthine phosphoribosyltransferase 1) in multiplex fluorescence format. Nuclei were counterstained with DAPI. B) Multiplex fluorescence detection of uPA and PAI mRNAs in breast cancer. Merged pseudo-colored image of a metastatic breast cancer tissue section stained with probes specific to cytokeratins [PanCK (CK-8, CK-18, and CK-19), labeled with Alexa Fluor 647], uPA (labeled with Alexa Fluor 546), and PAI-1 (labeled with Alexa Fluor 488). Both uPA expression (arrowhead and right inset) and coexpression with PAI-1 (arrow and left inset) were detected.[21]

5. SUMMARY

Whether searching for gene expression information or studying the function of RNA species themselves, it is evident that there is no better marker of RNA than RNA itself. DNA and protein surrogates do not always correlate with their RNA expression patterns due to pre- and post-translational modifications, but until now these biomarkers have been predominantly analyzed following the discovery phase, since routine RNA analysis just wasn't good enough. A method was required that was both high-throughput and would fit simply into the current pathology workflow for validation and translation into diagnosis. ACD's RNAscope assay technology fills that gap by overcoming the pitfalls of other methods, while providing a direct path from discovery to clinical assays by maintaining biomarkers at the RNA level.

Next generation sequencing approaches will continue to fuel RNA biomarker discoveries and the need for RNA biomarker validation within tissue morphology will continue to increase in order to fully understand disease relevance and the complex biology of the marker. RNAscope is an ideal platform that can be used downstream of NGS and microarrays for translating new scientific advances into clinical research applications due to both the speed of assay development and its superior sensitivity and specificity. There are already numerous published papers utilizing RNAscope across many fields of research, with more emerging every month. Topics include cancer,[22, 23, 24] where researchers have studied the heterogeneity of tumor micro-environments, circulating tumor cells and the detection of fusion genes. Valuable insights into stem cell signaling have also been gained,[25] as well as single-cell gene expression profiling[26] for researching predictive gene expression signatures. Virology and neurology studies have also been published,[27, 28] and with thousands of RNAscope probes currently available, many more studies are on the horizon.

The RNA revolution is here and with it, finally, the ability to easily and effectively visualize RNA *in situ*. The only question is - what discoveries will this new-found ability lead us to?

To find out more about how RNAscope can transform your research, please contact Advanced Cell Diagnostics at info@acdbio.com or call + 1 (510) 576-8800.

	RNA DETECTION METHOD					
	IN-SOLUTION			*IN SITU*		
BENEFIT	RT-PCR	NPA	MOLECULAR BAR CODING	CONVENTIONAL ISH	DIRECT DETECTION ISH	RNAscope
SENSITIVITY	✓	✓	✓			✓
CONSERVED MORPHOLOGY				✓	✓	✓
SINGLE-CELL RESOLUTION			✓		✓	✓
QUANTATIVE	✓	✓	✓		✓	✓
FFPE COMPATIBLE	✓	✓	✓	✓	✓	✓
WALK AWAY AUTOMATION				✓		✓
MULITPLEXING	✓	✓	✓	✓	✓	✓
BACKGROUND SUPPRESION	✓					✓

TABLE 1. Summary of the key benefits of various RNA detection methods.

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