White Paper

QuantiGene® ViewRNA Assay: Simple, robust, and sensitive visualization of mRNA for *in situ* analysis

Abstract

The method of *in situ* hybridization is an invaluable technique that allows researchers to visualize their gene of interest in target cells and tissues. However, this technique is time and labor intensive and often limited by lack of sensitivity.

Here we discuss a new method, the QuantiGene® ViewRNA Assay, which can detect single copies of the target gene in cultured cells. The technique can also be applied to formalin-fixed, paraffin-embedded (FFPE) tissue sections. With a combination of greater sensitivity and shortened workflow, the QuantiGene® ViewRNA Assay markedly improves on currently available *in situ* hybridization methods.

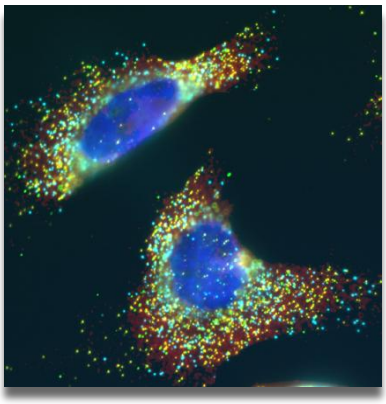
Introduction

Branched DNA (bDNA) detection is a clinically proven technology that quantitatively measures gene expression. The bDNA assay is a sandwich nucleic acid hybridization method that uses bDNA molecules to amplify signal from captured target RNA. Amplification of the signal to enhance assay sensitivity eliminates the need to amplify target RNA, as required in traditional PCR-based gene expression techniques.

Furthermore, bDNA assays measure RNA directly from the sample source, without RNA purification or enzymatic manipulation, thereby avoiding inefficiencies and variability introduced by errors inherent to these processes. bDNA assays have been available in the clinical market for more than a decade, forming the basis of the FDA-approved clinical diagnostic VERSANT® 3.0 assays for viral load in human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus infections (commercially offered by Siemens). This technique is also offered in the preclinical and life sciences markets in a single-plex and multiplex assay setup known as QuantiGene®2.0 and QuantiGene® Plex 2.0 Assays (offered by Affymetrix). Due to its system-wide accuracy, precision, and ease of use, bDNA is a superior method of analyzing gene expression in a variety of sample types[1].

The technique of *in situ* hybridization is used to visualize the localization of DNA or RNA within cells and tissues. However, this method has often been limited by low sensitivity and a complicated workflow as well as the inability to perform multiplex analysis. The QuantiGene ViewRNA Assay contains a novel mRNA *in situ* hybridization solution based on patent-pending probe set design and bDNA technology. The combination of probe design and signal amplification results in an assay that offers single-copy mRNA sensitivity in single cells in a multiplex assay format. Currently, up to four RNA targets can be visualized at a single transcript level within individual cells using this method (Figure 1).

Figure 1: Expression of four housekeeping genes in HeLa cells using the bDNA *in situ* hybridization method. RPLO (green), PPIB (yellow), HPRT (aqua) and β-actin (red). Nuclei were counterstained with DAPI.



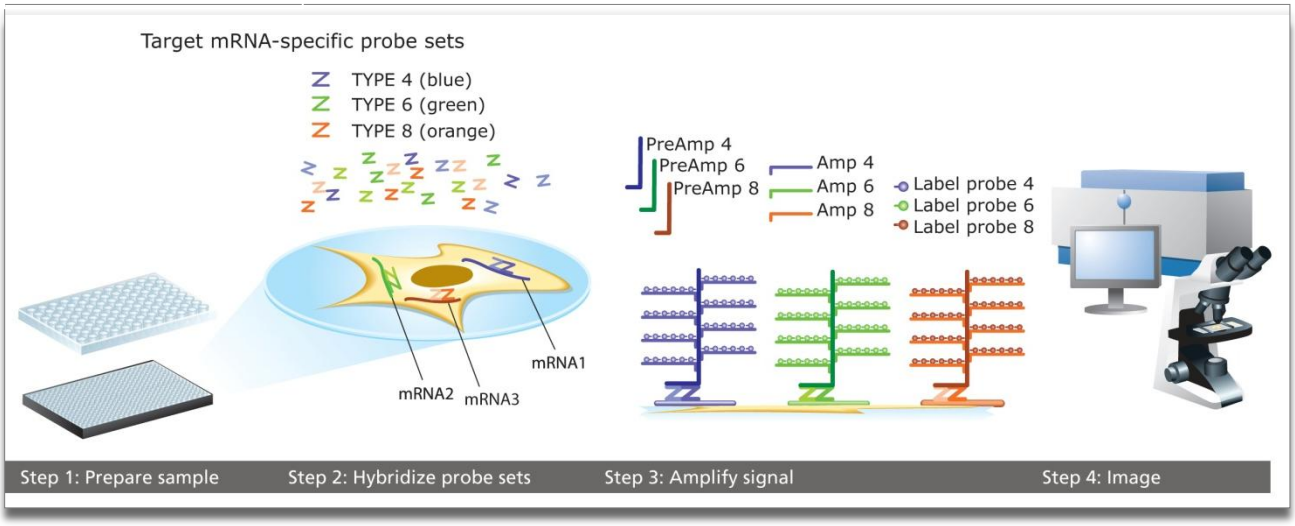
This technology enables transcriptional profiling of individual cells within a population and has broad applicability in research areas including biomarker validation as well as *in vitro* and *in vivo* quantitation of RNAi knockdown. The assay is amenable to automation and the simple assay workflow is suitable for high-throughput applications such as phenotypic or reporter gene screening. This method can also be used to detect individual RNA targets in FFPE tissue sections.

Methods

A schematic diagram of the workflow is represented in Figure 2A in cultured cells and Figure 2B in FFPE tissue sections. Unless otherwise noted, reagents for cultured cells, referred to in the workflow used, are provided in the QuantiGene® ViewRNA Plate-Based Assay and Signal Amplification Kits. For more information about the components provided in the assay, please refer to the *User Manual, QuantiGene® ViewRNA Plate-Based Assay*, available at www.panomics.com. Reagents for FFPE tissue sections, referred to in the workflow used, are provided in the QuantiGene ViewRNA FFPE Assay Kit and Chromogenic Signal Amplification Kit. QuantiGene® ViewRNA probe sets are ordered separately. For more information about the components provided in the assay, please refer to the *User Manual, QuantiGene® ViewRNA for FFPE Samples*, available at www.panomics.com.

Figure 2: Workflow of the *in situ* hybridization assay based on bDNA technology for (A) cultured cells and (B) FFPE tissue sections.

Figure 2A



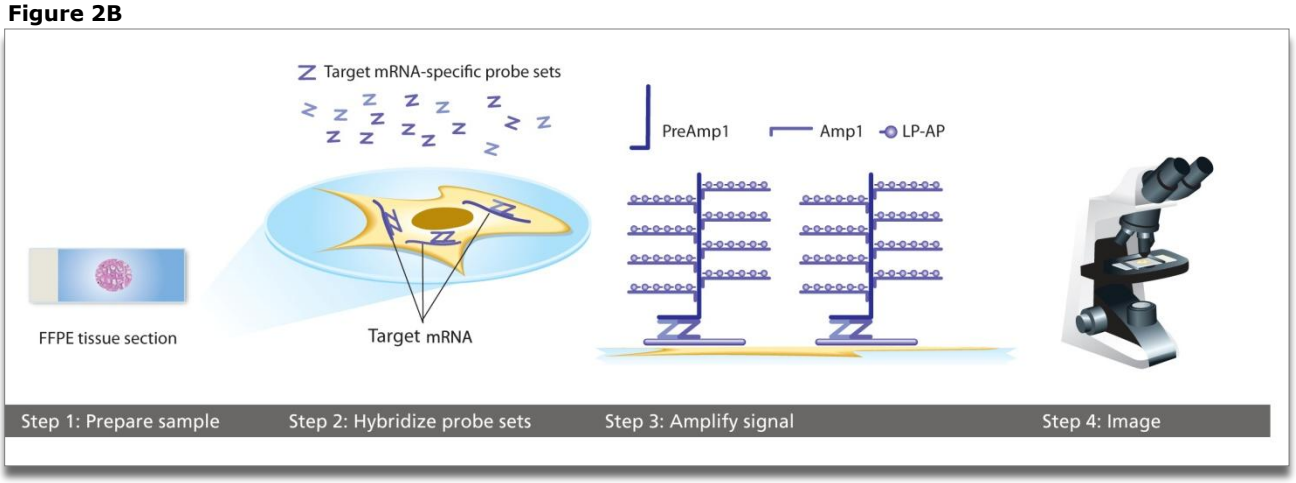
Step 1: Prepare sample
Adherent cells on a solid surface are fixed and permeabilized.

Step 2: Hybridize probe sets
Gene-specific probe sets hybridize to target mRNAs. For clarity, a three-plex assay with only single oligonucleotide pairs are shown; however, a typical probe set contains 20 or more oligonucleotide pairs. Each probe set type (e.g., type 4), interacts specifically with a corresponding signal amplification system (e.g., PreAmp 4, Amp 4, Label Probe 4), to generate signal for visualization.

Step 3: Amplify signal
Independent but compatible signal amplification systems enable simultaneous detection of multiple RNAs in a single assay. Distinct sets of Preamplifier (PreAmp), Amplifier (Amp), and Label Probe (LP) molecules are used to detect different target mRNAs. A PreAmp molecule hybridizes to each Probe Set oligonucleotide pair, then multiple Amp molecules hybridize to each PreAmp. Finally, multiple LP oligonucleotides conjugated to fluorescent dyes hybridize to each Amp.

Step 4: Image
Target mRNAs are visualized using a standard fluorescence microscope or automated imaging platform.

Figure 2B



Step 1: Prepare sample
FFPE tissue samples, fixed for 16 to 24 hours at room temperature (RT), are sectioned to 4-6 µm thickness and attached to a positively charged slide. FFPE tissue sections are treated with a pretreatment solution followed by protease digestion to allow target accessibility.

Step 2: Hybridize probe sets
A gene-specific probe set hybridizes to the target mRNA. For clarity, only single oligonucleotide pairs are shown. However, a typical probe set contains 10 or more oligonucleotide pairs.

Step 3: Amplify signal
A Preamplifier (PreAmp) molecule hybridizes to each pair of oligonucleotides, then multiple Amplifier (Amp) molecules hybridize to each PreAmp. Finally, multiple Label Probe oligonucleotides conjugated to alkaline phosphatase (LP-AP) hybridize to each Amp. Following the addition of the Fast Red Substrate, alkaline phosphatase breaks down the substrate to form a precipitate (punctated dots) that indicates the presence of the target RNA molecule.

Step 4: Image
Target mRNA is visualized using standard bright field microscopy and cell nuclei can be identified by the hematoxylin counterstain.

Sample preparation for cultured cells
Cells were plated in a 96-well plate at a density such that confluency was between 80 and 95 percent throughout the procedure. Dependent on the cell type used, wells were precoated to increase cell attachment to the surface. Cultured cells were washed two times in 150 µL of 1x phosphate buffered saline (PBS) to remove traces of culture medium. To fix cells, 60 µL of 4 percent formaldehyde was added to each well, and the plate was incubated for 30 minutes at room temperature. The formaldehyde solution was removed by gently washing cells with 150 µL of 1x PBS three times.

To permeabilize cells, 60 µL of working detergent solution was added to each well and the plate was incubated for 3 minutes at room temperature. Wells were washed once in 150 µL of 1x PBS before adding 60 µL of protease solution. After 10 minutes at room temperature, cells were gently washed three times with 150 µL of 1x PBS. To stop further permeabilization, 60 µL of protease stop buffer was added to each well and the plate was incubated for up to 30 minutes. All well contents were removed by expulsion before proceeding to probe set hybridization.

Sample preparation for FFPE tissue sections
FFPE samples, fixed in neutral buffer formalin for 16-24 hours at room temperature, were sectioned at 4 to 6 µm thickness and mounted onto positively charged Superfrost® slides (Fisher Scientific, USA). Tissue sections per slide were no larger than 22 mm x 22 mm.

Slides were incubated at 60°C for 30 minutes in a ThermoBrite® station (Abbott Molecular, USA) to increase tissue attachment to the slide. This was followed by submerging the slides in 10 percent formaldehyde for 1 hour at room temperature. Slides were washed briefly in 1x PBS twice before being air-dried. For deparaffinization, slides were incubated at 80°C for 3 minutes followed immediately by submersion in 200 mL of Histo-Clear® reagent (National Diagnostics, USA) for 10 minutes at room temperature with frequent agitation. Residual Histo-Clear reagent was removed by

agitating slides in 95 percent ethanol twice. Slides were allowed to dry at room temperature for 5 minutes before a hydrophobic barrier was drawn around the tissue section using an ImmEdge™ Hydrophobic Pen (Vector Laboratories, USA). Once the barrier had dried, slides were placed into boiling pretreatment solution and incubated for between 5 and 20 minutes, depending on tissue type. After pretreatment, slides were submerged in distilled water twice.

Protease solution was prepared by diluting protease 1:100 in 1x PBS and warmed to 40°C. Slides were also prewarmed to 40°C with 200 µL of 1x PBS covering the tissue section to avoid dehydration. Once warmed, the 1x PBS solution was decanted from the section and 200 µL of the prewarmed protease solution was added. The slides were incubated at 40°C for 10 to 40 minutes, depending on tissue type. Following this step, the protease solution was decanted and the slides were washed by submersion and agitation in 1x PBS. After three washes, the slides were transferred into 4 percent formaldehyde for 5 minutes followed by rinsing once in 1x PBS.

Hybridization of probe sets for cultured cells
Probe sets were diluted 1:100 to form a working probe set solution. To hybridize the probe set, 60 µL of diluted probe set was added to each well and the plate was incubated for 3 hours at 40°C. Then, wells were washed three times with 150 µL of 1x PBS buffer and the contents completely expelled. For signal amplification, 60 µL of prewarmed Preamplifier reagent (PreAmp) was added to the wells and the plate was incubated at 40°C for 60 minutes. Wells were subsequently washed with 150 µL of 1x PBS three times before adding 60 µL of Amplifier reagent (Amp). The plate was incubated at 40°C for 60 minutes before the unbound reagent was removed by three washes with 150 µL of 1x PBS. To visualize, 60 µL of Label Probe reagent (LP) was added to each well and the plate was incubated at 40°C for 60 minutes. Following three washes with 150 µL of 1x PBS, nuclei were counterstained with DAPI (10 mg/mL) at a 1:10,000 ratio. After 1 minute incubation at room temperature, the DAPI was removed by washing once with 1x PBS. To visualize, 150 µL of fresh 1x PBS was added to each well and the plate was examined under a fluorescent microscope using the following filter sets: FITC (Ex 501 nm/Em 523 nm), Cy3 (Ex 554 nm/Em 576 nm), Cy5 (Ex 644 nm/Em 669 nm), Cy7 (Ex 740 nm/Em 764 nm), and DAPI (Ex 358 nm/Em 461 nm).

Hybridization of probe sets for FFPE tissue sections
Probe sets were diluted 1:50 in prewarmed Hyb A buffer and 200 µL was added to each tissue section, which was incubated for 3 hours at 40°C. Following this step, slides were washed by submersion and agitation for 2 minutes in wash buffer twice. Slides were removed from the wash buffer and 200 µL of PreAmp 1 solution was added to the tissue section and incubated at 40°C for 25 minutes. Following three washes with wash buffer, 200 µL of Amp 1 reagent was added to the tissue section and the slides were incubated at 40°C for 15 minutes. Again, slides were washed three times in wash buffer and then incubated with Label Probe-AP reagent for 40° C for 15 minutes. After three washes with wash buffer, 200 µL of the AP-enhancer solution was added to the section and incubated at room temperature for 5 to 10 minutes. Fast Red solution was prepared by dissolving one tablet in 5 mL of naphthol buffer and 200 µL was added to the tissue section, which was incubated at 40° C for 30 minutes in the dark. Slides were then submerged in 1x PBS in order to rinse off the Fast Red substrate. To fix, slides were incubated in 4 percent formaldehyde for 5 minutes at room temperature before being washed in 1x PBS to eliminate traces of formaldehyde. Slides were counterstained with Gill's hematoxylin (American Master Tech Scientific, USA) before being mounted using Dako® Ultramount medium (Dako, USA). Results were viewed under a bright-field microscope.

Results and discussion

In the past, *in situ* hybridization has been limited by sensitivity and a complicated workflow. To enhance sensitivity, probes were often radiolabeled and exposed to film for several weeks before results were visualized. Despite the time-intensive procedure, detection of mRNA was still limited to approximately 20 copies per cell.

We report a novel method for visualizing localization of mRNA in cultured cells and FFPE sections. Specificity of the assay was achieved by hybridizing multiple "double Z" structured oligonucleotide pairs along a target region (Figure 2A and 2B). By using this method of probe design, sequences with more than 90 percent homology can still be readily distinguished. Furthermore, the double Z structure incorporated into the oligonucleotide pair design is critical to the assay as each oligonucleotide pair

forms the base of the signal amplification. By subsequent addition of the preamplifier, amplifier, and label probe, the signal is amplified 400-fold per each oligonucleotide pair.

To verify that the assay is sensitive enough to detect single-copy transcript in a cell, probe sets were designed against *HER2* DNA so that copy number could be quantified. *HER2* is a gene that is overexpressed in certain types of aggressive breast cancers[2,3]. In HeLa cells, normal diploid copies of the *HER2* gene were expressed as well as diploid copies of the housekeeper gene for IL-8. However, in the SKBR3 breast cancer cell line, multiple copies of *HER2* were apparent while copies of the IL-8 gene remained at normal levels (Figure 3A). This was also observed at the mRNA level (Figure 3B).

Figure 3: DNA (3A) and mRNA (3B) expression of *HER2* in HeLa and SKBR3 cells. Her2C, a probe directed to the unprocessed intron region of *HER2*, served as a negative control. For DNA detection, cells were first treated with RNAse, then DNA was denatured prior to *in situ* hybridization. Nuclei were counterstained with DAPI.

Figure 3A

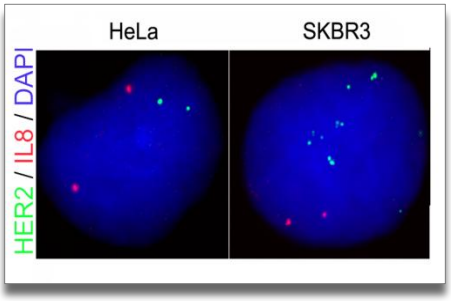
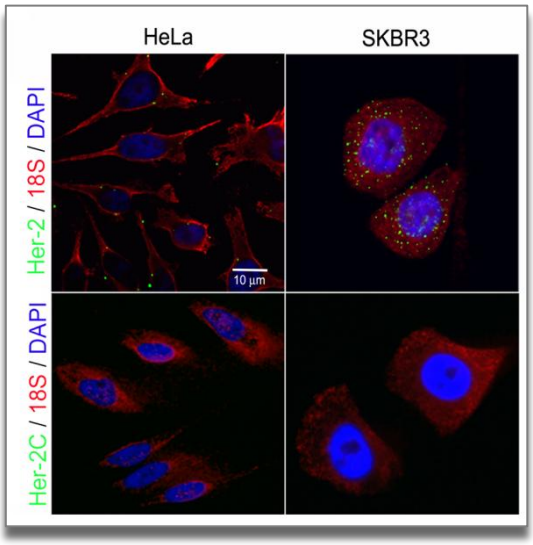


Figure 3B



This assay has also been used in FFPE sections, which are notoriously difficult samples due to the fixation processes involved in preserving them. We examined the expression of the *HPRT* gene in FFPE rat kidney tissue sections using bDNA technology. Results demonstrated that individual copies of *HPRT* mRNA were visible in rat kidney tissue sections (Figure 4). To compare sensitivity between the use of radiolabeled probes and our reported method, IGFBP-3 mRNA expression was visualized in human liver sections using either ^{35}S-UTP probe or probes designed for use with the bDNA method. IGFBP-3 mRNA expression was apparent in liver Kupffer cells using both methods. However, radiolabeled probes required exposure to X-ray film for three weeks before results could be visualized. This was in contrast to the bDNA method, where IGFBP-3 expression was apparent after a 30-minute chromogenic substrate reaction (Figure 5, page 6).

Figure 4: Expression of *HPRT* in FFPE ray kidney tissue. *HPRT* mRNA (red); nuclei were counterstained using hemotoxylin-1 (blue).

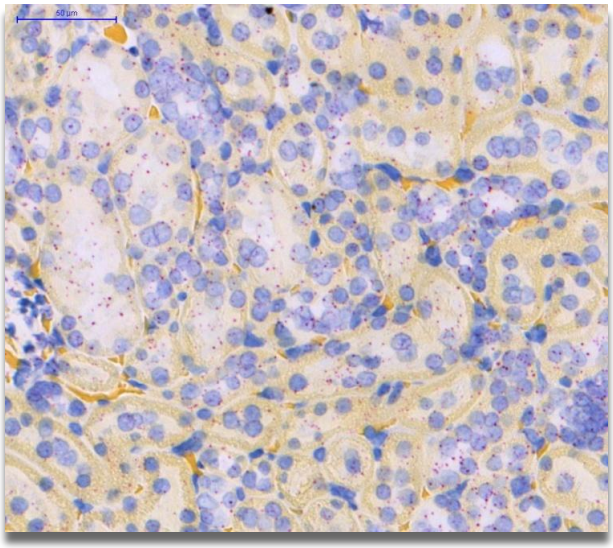
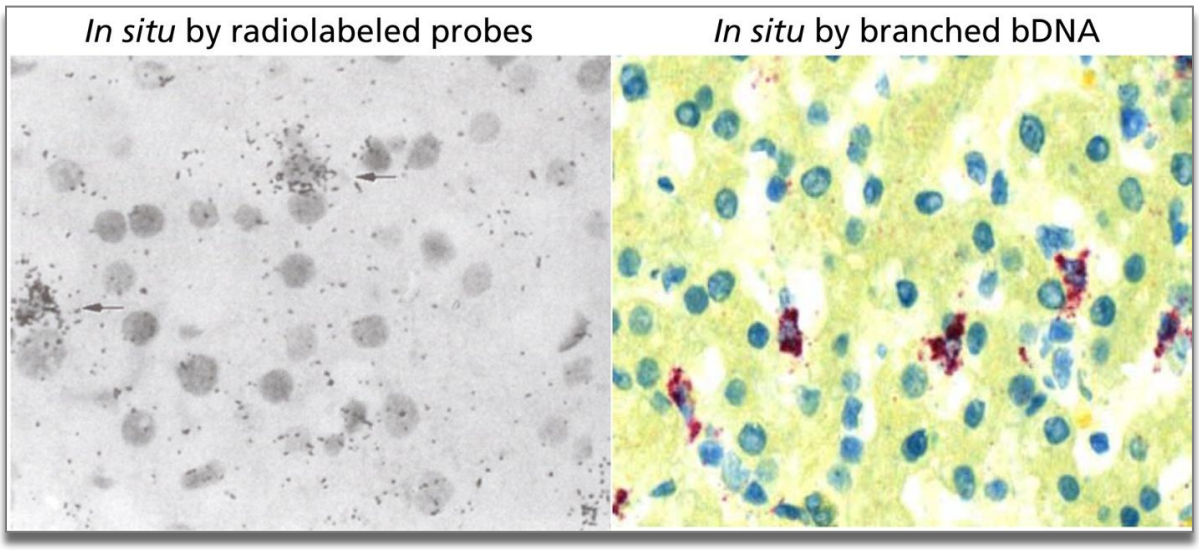


Figure 5: IGFBP-3 mRNA expression in FFPE human liver tissue samples detected using radioactivity and QuantiGene® ViewRNA Assay.



Taken together, these results highlight the specificity and sensitivity of the bDNA method for *in situ* hybridization. Furthermore, this method has considerably improved the workflow, such that the time to obtain results has been significantly reduced compared to traditional methods.

Please visit www.panomics.com to learn more about the QuantiGene® ViewRNA Assay.

References

1. Canales R. D., *et al.* Evaluation of DNA microarray results with quantitative gene expression platforms. *Nature Biotechnology* **24**(9):1115-1122 (2006).

2. Zhao J., *et al.* Secreted antibody/granzyme B fusion protein stimulates selective killing of HER2-overexpressing tumor cells. *Journal of Biological Chemistry* **279**(20):21343–21348 (2004).

3. Yu C. J., *et al.* Selective proapoptotic activity of a secreted recombinant antibody/AIF fusion protein in carcinomas overexpressing HER2. *Gene Therapy* **13**(4):313–320 (2006).

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