Application Note

QuantiGene® Assays for cancer and disease research

QuantiGene® Assays are based on the clinically proven branched DNA (bDNA) signal amplification technology used in the FDA-approved HIV and HCV viral load assays sold by Siemens under the trade name VERSANT®. More than 450 studies have been published using QuantiGene Assays.

QuantiGene Assays are versatile and enable a comprehensive approach to systems biology and translational sciences for validation and quantitation of biomarkers identified by next generation sequencing, microarray studies or from published literature. QuantiGene Assays include singleplex and multiplex assay formats for cell lysate/tissue homogenate analysis as well as cell and tissue formats for RNA *in situ* hybridization (ISH) analysis.

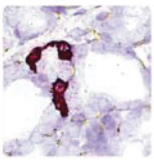
QuantiGene® Lysate/homogenate Assays enable accurate and precise quantitation of RNA, miRNA, and DNA directly from the sample. These direct hybridization assays avoid biases associated with nucleic acid purification, cDNA synthesis and PCR amplification. QuantiGene® ViewRNA ISH Assays enable RNA target localization and quantitation with single-copy sensitivity at single-cell resolution.

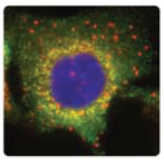
FFPE tissues and blood are clinically important sample types that can be technically challenging for PCR-based methods. RNA degradation and chemical modifications of bases pose a challenge when working with FFPE tissue samples. Additional processing steps, fractionation steps, and/or globin RNA reduction, are required when working with blood samples. QuantiGene Assays are not affected by the technical challenges associated with FFPE tissues and blood samples.

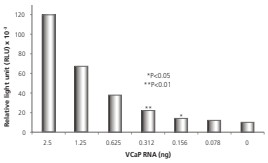
QuantiGene Assay highlights

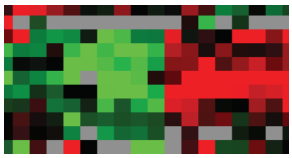
- Proven successful across diverse and difficult samples: FFPE (including H&E stained) and fresh frozen tissues, blood, urine, cultured cells, isolated nucleic acids, and more
- Direct, spatial visualization of RNA in complex tissue architectures while retaining tissue morphology
- Insensitive to chemical modifications of bases and RNA degradation associated with formalin fixation
- Eliminates unnecessary steps and biases associated with miRNA, RNA, or DNA purification, cDNA synthesis, and PCR amplification

QuantiGene Assays deliver unparalleled flexibility in three formats:









In situ RNA assays

QuantiGene® ViewRNA

- Measure 1-4 miRNA or RNA targets per assay in cells
- Measure 1-2 RNA targets per assay in FFPE and fresh frozen tissue sections

Key application areas

- Biomarker validation
- Study of non-coding RNAs
- Cell differentiation
- Toxicology and safety assessment

Detection

- Chromogenic signal visualized using a brightfield microscope (tissues)
- Fluorescent signal detected using a fluorescence microscope or imaging system (tissues and cells)

Cell lysate/tissue homogenate assays

QuantiGene® 2.0

Measure 1 miRNA, RNA, or DNA target per well

Key application areas

- Biomarker validation
- Quantitate siRNA knockdowns
- DNA copy number analysis
- Quantitate miRNAs
- Detect translocations and fusion genes

Detection

Glow chemiluminescent signal detected using a standard 96-well plate laboratory luminometer

QuantiGene® Plex

- Measure 3–80 mRNA targets per well
- Measure 3–33 DNA targets per well

Key applications areas

- Biomarker signature validation
- Pathway analysis
- DNA copy number analysis
- Validation of microarray and sequencing results

Detection

Fluorescent signal detected using a Luminex® Instrument (FLEXMAP 3D®, Luminex 100/200™ and MAGPIX®)

QuantiGene® ViewRNA

QuantiGene® ViewRNA Assays have the sensitivity and robustness to detect single RNA molecules *in situ* at single-cell resolution and quantitate expression heterogeneity. This level of sensitivity and resolution is critical in understanding the important biological role played by lower expressed genes. As noted by Zhang L., *et al. Science* **276**(5316):1268-72 (1997), 80 percent of mRNAs are present at fewer than five copies per cell.

Benefits

- Direct, spatial visualization of RNA in complex tissue architectures while retaining tissue morphology
- Single-copy sensitivity at single-cell resolution
- Simple hybridization assay to any RNA target, no radioactivity and no antibodies
- Flexible probe design to target any gene or sequence within a gene; new assays developed in one week

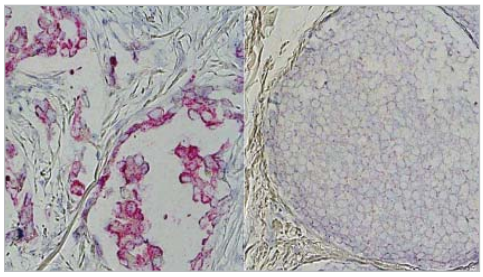
QuantiGene ViewRNA application areas

QuantiGene ViewRNA Assays have broad applicability in cancer and disease research, stem cell biology and regenerative medicine, mRNA knockdown measurements, neurobiology, biomarker validation, and more. These assays are a great alternative/companion to *in situ* immunohistochemistry (IHC) and immunofluorescence (IF) assays when antibodies are not available or inadequate.

Biomarker quantitation in FFPE tissue samples

Visualize and quantitate gene expression *in situ* in FFPE and fresh frozen tissue samples with excellent specificity and without interference from formalin fixation, RNA degradation, or H&E staining, in less than two days. In this application (Figure 1), Her2 expression was measured in a disease progression series of FFPE breast cancer tissues using the QuantiGene ViewRNA ISH Tissue Assay (Figure 1A), and the results were confirmed and quantitated using the QuantiGene 2.0 Lysate Assay (Figure 1B).

1A High expression Low expression



1B

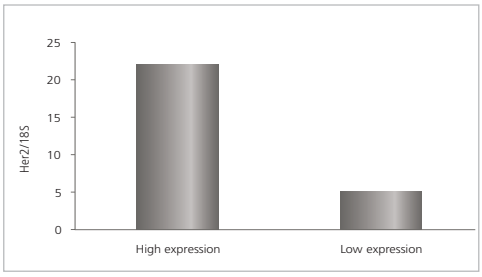
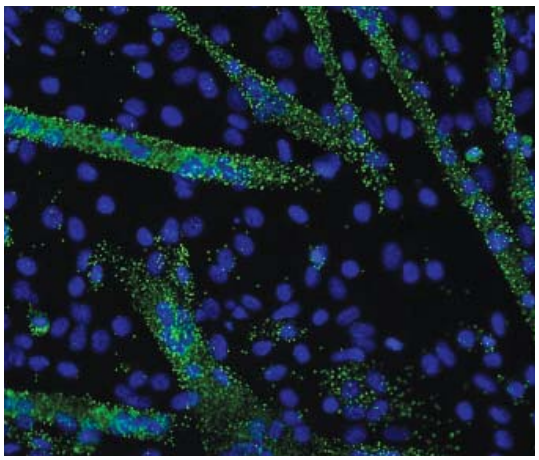


Figure 1: Her2 gene expression analysis in breast cancer FFPE tissue sections using QuantiGene Assays.
A: QuantiGene ViewRNA ISH Tissue Assay for Her2 (red staining) as seen using a brightfield microscope.
B: Quantitative measurement of Her2 expression normalized to 18S control using the QuantiGene 2.0 Lysate Assay and measured by a chemiluminescence microplate reader.

miRNA and mRNA multiplex *in situ* analysis

With the QuantiGene ViewRNA Cell Assay, for the first time, you can simultaneously detect miRNA and mRNA *in situ (coming soon)*. In this application, miR-133 miRNA and myogenin mRNA are co-detected in differentiated myoblast C2C12 cells (Figure 2).

2A: miR-133



2B: miR-133 and myogenin

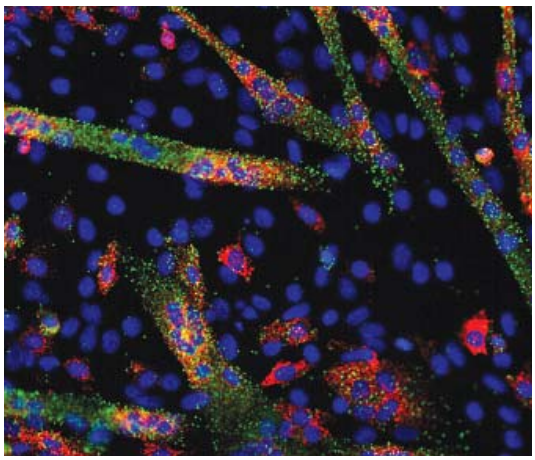


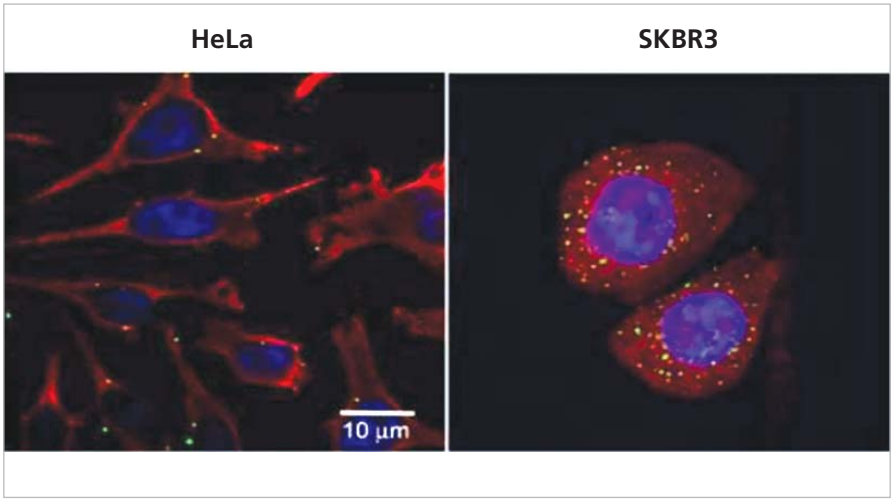
Figure 2: QuantiGene ViewRNA Cell Assay detects *in situ* miRNAs and mRNAs. C2C12 cells were grown in 96-well plates and induced to myoblast differentiation. After five days, myotubes were detected with fluorescent probes for miRNA and mRNA using fluorescence microscopy as follows:
A: miR-133 miRNA (green).
B: miR-133 miRNA (green) and myogenin RNA (red) – merged image.

Quantitation of expression heterogeneity in cancer samples

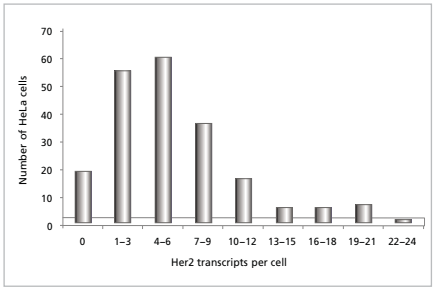
The quantitation of Her2 in HeLa cells compared to SKBR3 cells clearly demonstrates the QuantiGene® ViewRNA *in situ* assay's ability to visualize and quantitate gene expression heterogeneity in cancer specimens. Lysate-based and qPCR assays result in an average signal for a given clinical specimen and can mask potentially important information relating to expression heterogeneity in tissue samples consisting of multiple cell types or within a population of cells.

In this example (Figure 3A), the QuantiGene ViewRNA ISH Cell Assay accurately visualized Her2 expression levels in HeLa cells known to express an average of four to six copies per cell and in SKBR3 breast cancer cells known to have higher expression levels of Her2. In Figure 3B, Her2 spots were counted in 200 HeLa cells and plotted as a histogram. The histogram of Her2 expression clearly indicates a high degree of expression heterogeneity from cell to cell with a range of expression from 0 to 21 transcripts per cell and an average of 5.4 transcripts per cell. The QuantiGene 2.0 Lysate Assay was then used to confirm these measurements, and the resulting 6.4 transcripts per cell correlated nicely with the results of the QuantiGene ViewRNA *in situ* Assay (Figure 3C).

3A



3B



3C

Assay	Ave. number of transcripts/cell
QuantiGene ViewRNA	5.4
QuantiGene 2.0	6.4±2.3

Figure 3: Visualization and quantitation of transcript heterogeneity in cell lines.
A: QuantiGene ViewRNA multiplex analysis of Her2 mRNA (green) and control 18S rRNA (red) in HeLa cells (left image) and SKBR3 cells (right image). Nuclei were stained with DAPI (blue) and visualized by fluorescence microscopy.
B: Histogram of Her2 expression per HeLa cell based on counting dots per cell for 200 cells.
C: Comparison of Her2 expression results using the QuantiGene ViewRNA *in situ* assay vs. the QuantiGene 2.0 Lysate Assay.

Infectious diseases

The QuantiGene ViewRNA ISH Cell Assay is also well suited to investigate the presence, location, and trafficking of viral genes within individual cells and the host response to virus and other infectious disease agents (Figure 4).

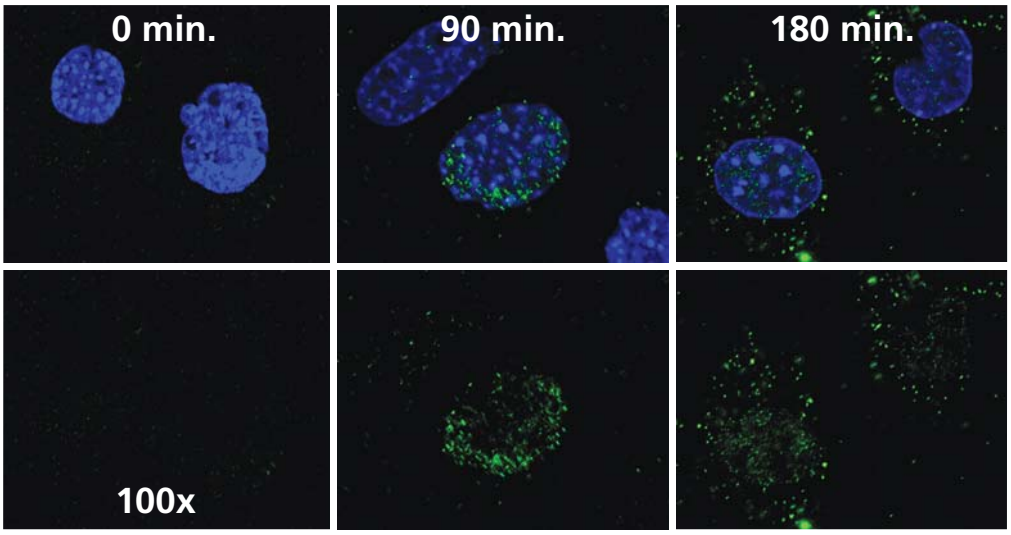


Figure 4: Detection of H1N1 Influenza A RNA migration in MEF cells. Confocal image. Nuclear translocation of viral RNA is necessary for replication of the H1N1 virus. Murine embryonic fibroblasts (MEFs) were incubated on ice with influenza A virus (H1N1, PR8 strain). At time 0, virus was removed and 37° C media was added. At the indicated time points after warming, the cells were fixed and processed with the QuantiGene ViewRNA Kit and a probe set against the nucleoprotein (NP) viral genomic segment (green). DNA is stained blue with DAPI. Time-dependent nuclear translocation of the NP genome is seen at 90 minutes post infection, and by 180 minutes the nuclear export of newly synthesized viral genomes to the cytosol is also observed.

Data courtesy of Dr. Abraham L. Brass, MD, PhD, Ragon Institute of MGH, MIT and Harvard

QuantiGene® 2.0 Assay

The QuantiGene® 2.0 Assay enables sensitive and direct quantitation of any miRNA, RNA, or DNA copy number in any sample. This simple hybridization-based assay with an ELISA-like workflow results in a glow chemiluminescent signal that can be detected using a standard 96-well plate luminometer.

Benefits

- Accurate and precise results (MAQC study, *Nature Biotechnology*, September 2006)
- Direct quantitation (lyse and go) across diverse and difficult sample types: FFPE (including H&E stained) and fresh frozen tissues, blood, urine, cells, isolated miRNAs, RNAs or DNAs, and more
- Eliminates unnecessary steps and biases associated with miRNA, RNA, or DNA purification methods, cDNA synthesis, and PCR amplification
- Insensitive to chemical modification of bases and RNA degradation associated with formalin fixation
- Does not require blood fractionation or globin RNA reduction steps and typically requires less than 10 µL of blood per assay
- Ultimate specificity by using multiple probes per target and based on cooperative hybridization events to generate signals

QuantiGene 2.0 application areas

QuantiGene 2.0 Assays are widely used in quantitative gene expression of signal transduction pathways, biomarker validation in retrospective and prospective FFPE and blood studies, validation of microarray and sequencing results, predictive toxicology, quantitation of RNAi knockdowns, quantitation of DNA copy number variation, and more.

Prostate cancer biomarker validation in archived 9- to 13-year-old H&E-stained FFPE tissue samples
As shown in Figure 5, the QuantiGene 2.0 Assay was used to analyze RNA in 9- to 13-year-old H&E-stained prostate cancer FFPE tissue samples. The authors conclude that in macro-dissected tissues from 9- to 13-year-old blocks with poor RNA quality, the QuantiGene 2.0 Assay correctly identified the over-expression of known cancer genes (arrows) and that the QuantiGene Assay appears to be well suited for clinical analysis of FFPE tissues with diagnostic or prognostic gene expression biomarker panels (Knudsen, *et al.*, *Journal of Molecular Diagnostics* **10**:169-176 (2008).

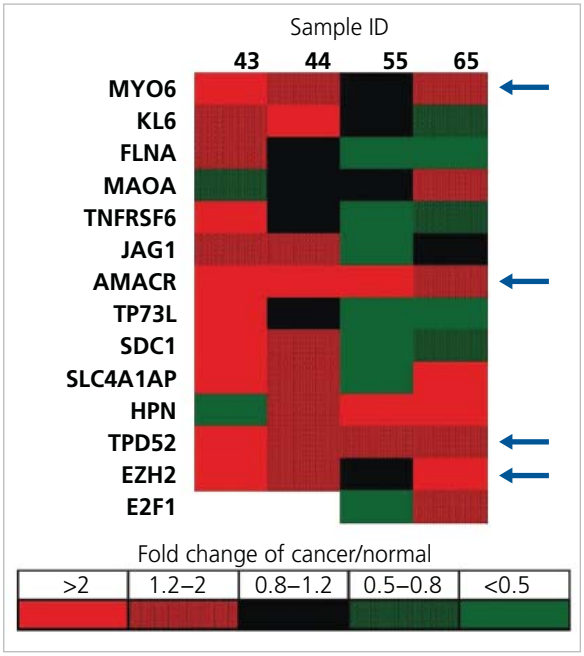


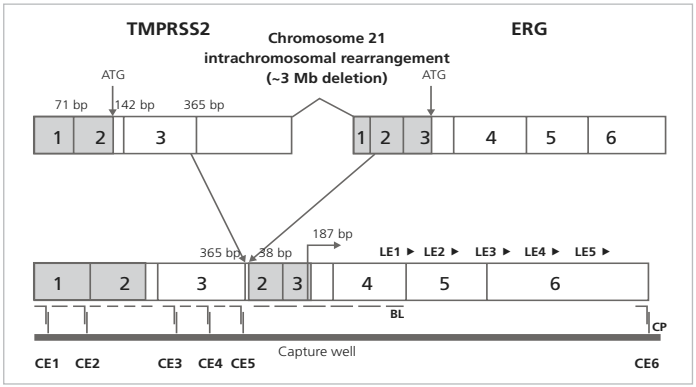
Figure 5: Quantitation of fold change in prostate cancer markers. Cancer and normal tissues from the same FFPE blocks were macro-dissected and dissolved in homogenizing solution using the QuantiGene 2.0 Assay. A panel of 14 prostate cancer genes was measured in cancer and adjacent normal tissues. Values for each gene were normalized to a housekeeping gene (RPL19). The ratio between cancerous and normal tissues is calculated and shown in a five-tiered categorical scale.

Detection of TMPRSS2-ERG fusion gene expression in prostate cancer specimens

In an evaluation of clinical prostatectomy specimens, the QuantiGene® 2.0 Assay detected 8 known TMPRSS2-ERG gene fusion subtypes from less than 200 pg of prostate cancer RNA (Figure 6). Fusion gene detection with one-step RT-PCR required more than 600 pg of RNA.

The QuantiGene 2.0 Assay showed a concordant detectable fusion signal in all nine clinical samples that had fusion detected by nested RT-PCR or FISH. Moreover, bDNA detected gene fusion in 2 of 16 prostate cancer tissue specimens that were not detected by FISH or nested RT-PCR. These findings demonstrate a bDNA assay that is effective for detection of TMPRSS2-ERG gene fusion in prostate cancer clinical specimens, thus providing an alternative method to ascertain TMPRSS2-ERG gene fusion in human prostate cancer tissue (Lu, *et al. Journal of Urology*, 2009).

6A



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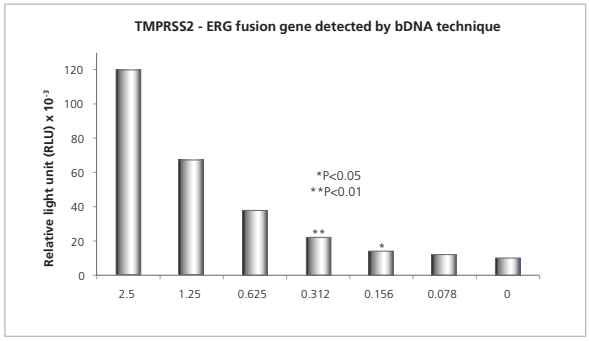

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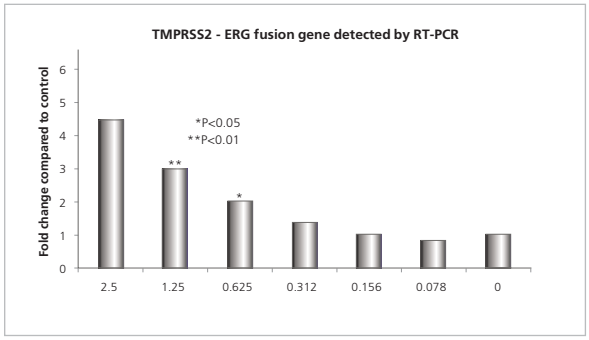


Figure 6: Detection of TMPRSS2-ERG fusion gene expression in total RNA from VCaP cells spiked with total RNA from normal human prostate tissue.
A: Schematic illustration of the bDNA probe set for TMPRSS2-ERG fusion. Probe sets for the fusion gene are designed to capture the 5′ portion of the TMPRSS2 gene and exons 5-6 of the ERG gene and to quantify the expression of the 8 known TMPRSS2-ERG subtypes. The capture extender (CE) probes are within exons 1-3 (1-365 bp) of the TMPRSS2 gene, and the label extender probes are within exons 5 and 6 (453-652 bp) of the ERG gene.
B: Quantitation data for the TMPRSS2-ERG fusion gene from the QuantiGene 2.0 Assay, which detected TMPRSS2-ERG gene fusion from less than 200 pg of prostate cancer RNA.
C: Quantitation data for the TMPRSS2-ERG fusion gene from nested RT-PCR assay based on input of 600 pg of prostate cancer RNA.

Accurate determination of miRNA copies in cancer tissue

QuantiGene 2.0 miRNA Assay provides accurate determination of miRNA copy number directly from FFPE or fresh frozen tissues. Equivalent amounts of breast tissues (normal and tumor) were used to analyze expression levels of miR-155. Using a linear standard curve, the absolute copy number of miR-155 in the two tissues samples was determined.

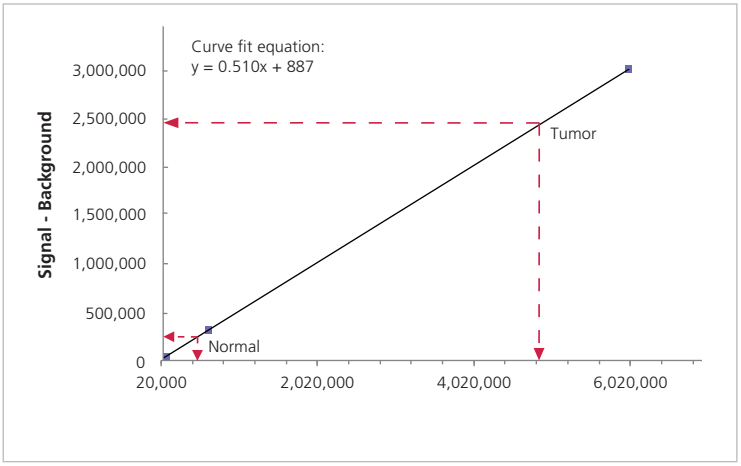


Figure 7: Absolute miRNA copy number determination.
Equivalent amounts of normal and tumor breast tissues were lysed and used to measure miR-155 expression. A synthetic miR-155 control was used to generate the standard curve, and the miR-155 expression was calculated based on a linear curve fit. The miR-155 expression in normal tissues was 488,476 copies and in the cancer tissue was 4,900,241 copies.

QuantiGene® Plex Assay

Identifying and assessing gene expression signatures allows researchers to identify targets and optimize lead compounds by tracking multiple genes associated with specificity, potency and toxicity. Gene expression profiling can also provide important information for tracking disease type and progression in biomarker validation studies.

QuantiGene® Plex Assays are a cost-effective way to generate high-confidence, high-quality, reproducible results for multiple targets across large numbers of samples. Affymetrix also provides complementary multiplex protein assays, Procarta® Immunoassays, with more than 250 validated assays in 6 species. Fluorescence signals generated in both QuantiGene Plex and Procarta Immunoassays are detected on Luminex® based systems (FLEXMAP 3D®, Luminex 100/200™ and MAGPIX®).

Benefits

- Save time and money with true same well multiplexing of up to 80 targets (RNA) or 33 targets (DNA)
- Eliminate unnecessary steps and biases associated with RNA and DNA purification methods, cDNA synthesis and PCR amplification
- Insensitive to chemical modification of bases and RNA degradation associated with formalin fixation
- Does not require blood fractionation or globin RNA reduction steps and typically requires less than 10 µL of blood per assay
- Ultimate specificity by using multiple probes per target and based on cooperative hybridization events to generate signals

QuantiGene Plex application areas

QuantiGene® Plex 2.0 RNA and QuantiGene® Plex DNA Assays are widely used to profile and quantitate gene signatures, biomarker signature validation in retrospective and prospective studies using FFPE tissues and blood samples, validation of microarray and sequencing results, predictive toxicology, DNA copy number analysis, and more.

Multiplex quantitative gene expression testing in blood samples

The QuantiGene Plex Assay can be used to rapidly quantify and profile gene expression signatures from microliters of whole blood, PAXgene™ blood tubes, isolated blood cells, or purified RNA or DNA. In this application (Figure 8), a QuantiGene Plex Assay was used to quantify the relative abundance of 17 transcripts per well from isolated intact platelet fractions, bypassing the need for RNA purification. Simultaneous quantitation of 17 platelet transcripts was assayed using intact platelet-rich plasma (PRP) or gel-filtered platelets (GFP) lysed *in vitro*.

Accurate and reproducible profiles could be obtained from as few as 5 × 10^7 platelets (~100 µL of blood), even for the low-abundance platelet transcripts. Correlation coefficients of this 17-member gene set to Affymetrix® microarrays were excellent, with no correlation to in kind-derived leukocyte profiles, highlighting the cell specificity of the platform. Despite the broad range of relative expression, all transcripts were detected, and the correlation coefficients comparing each of the starting materials were excellent (Gnatenko D., *et al*. *Blood* (ASH Annual Meeting Abstracts) **110**:3645, 2007).

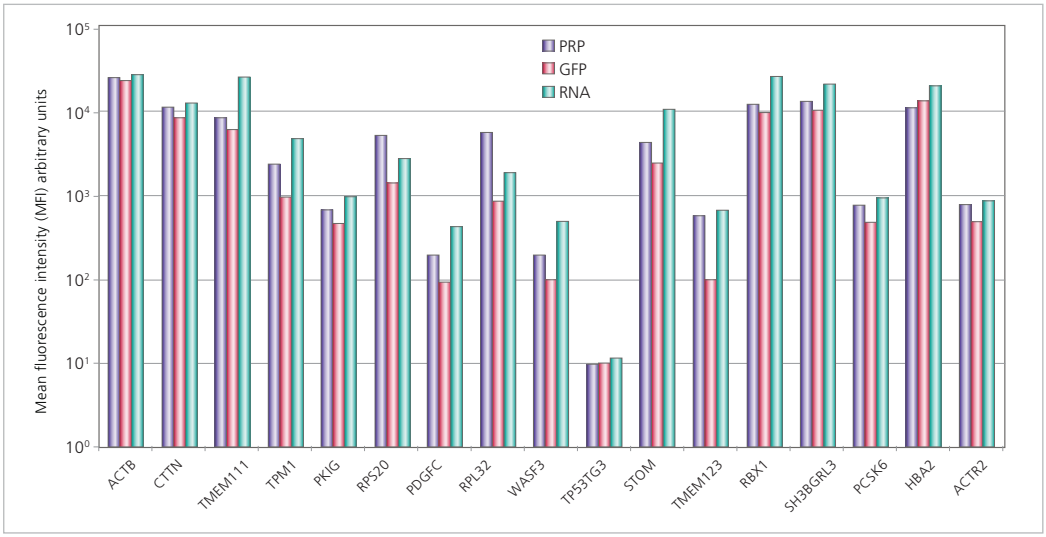


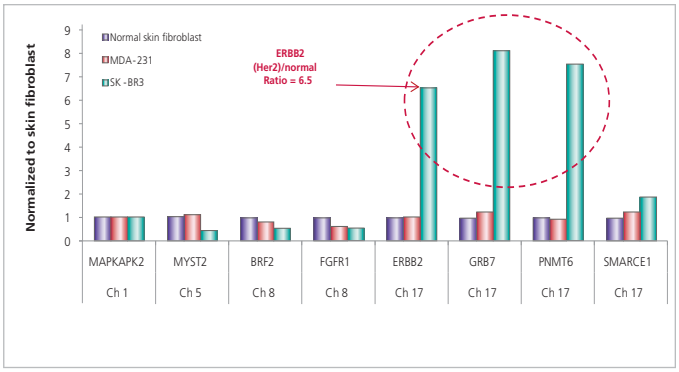
Figure 8: QuantiGene 17-plex RNA assay on purified RNA and intact platelet fractions.

Multiplex DNA copy number analysis

With the QuantiGene® Plex DNA Assay, you can quantify single-copy differences in DNA copy number and multiplex up to 34 targets per well. This enables you to process more samples per plate with fewer reagents, less hands-on time, and increased accuracy.

In Figure 9A, a QuantiGene Plex DNA Assay for copy number analysis was used to detect Her2 and adjacent genes on chromosome 17 as well as control genes on chromosomes 1, 5, and 8 in SKBR3 breast cancer cells and control cell lines (normal skin fibroblasts and MDA-231 cells). As expected, 7-fold amplification (6.5 normalized ratio) of Her2 were quantified. The QuantiGene Plex result was concordant with that of a bDNA FISH assay (note: the DNA assay is not currently commercially available, Figure 9B), in which 14 copies can be counted in SKBR3 cells vs. 2 copies in the HeLa cells as expected, again showing a 7-fold amplification. Amplification of PNMT6 and GRB7, 2 genes adjacent to Her2, were also detected at 7- to 8-fold, whereas control genes were detected at the expected single copy number.

9A: QuantiGene Plex DNA Assay



9B: QuantiGene DNA FISH Assay

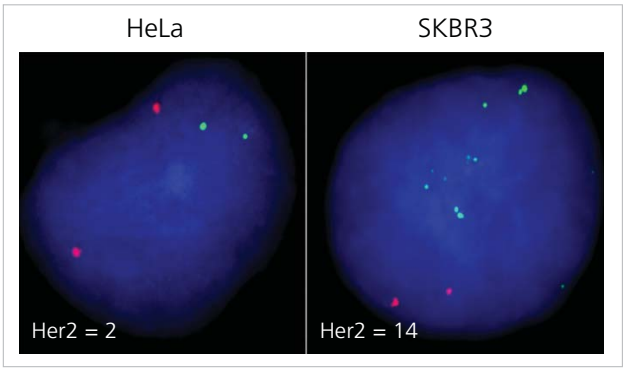


Figure 9: DNA copy number analysis.
A: QuantiGene Plex DNA copy number assay, for 8 targets, in SKBR3, normal skin fibroblast, and MDA-231 cells. Ratio of SKBR3/control = ~7.
B: DNA *in situ* hybridization of Her2 (green) and IL8 (red) in HeLa and SKBR3 cell lines. Her2 was amplified in SKBR3 cells vs. HeLa cells. Ratio of SKBR3/HeLa = 7. IL8 (a control gene) showed no amplification in HeLa or SKBR3 cells. Nuclei (blue) were stained with DAPI.

Classification of breast cancer

The QuantiGene Plex Assay was used to classify a library of breast cancer cell lines that represents recurrent genetic abnormalities as well as biological variability in primary breast cancer tumors (Figure 10). The data demonstrate strong correlation of the cell lines into basal or luminal subtypes and thus can be utilized to predict response to new drugs for these subtypes of breast cancer.

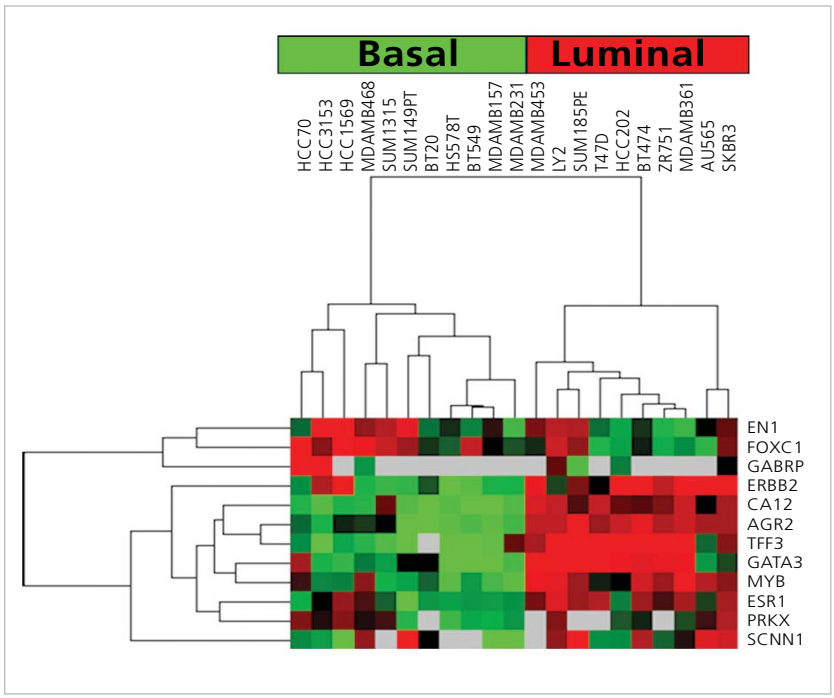


Figure 10: Classification of breast cancer cell lines. A 12-plex RNA measurement from the cell lines was derived using the QuantiGene Plex 2.0 Assay, and their normalized gene expression levels were plotted using a heat map.

Data Courtesy of Nicholas Wang, Joe Gray, Lawrence Berkeley National Laboratories, Department of Cancer & DNA Damage Responses

Partial list of available QuantiGene® Probe Sets

Visit our website or contact your local sales representative for a complete list of available targets. Currently, we have over 1,300 probes that are compatible with the QuantiGene® ViewRNA Assays, over 3,200 probes that are compatible with the QuantiGene® 2.0 Assays, and more than 5,000 probes that are compatible with the QuantiGene® Plex Assays. New probe sets can be developed with a fast turnaround time.

Cancer targets (human): ALK, AMACR, AR, BCL2, BCL6, BIRC5, BRAF, BTG2, CCR2, CLEC3B, CXCL12, EGFR, ERBB2 (Her2), ERG, EZH2, GLI1, KLK3, KRT17, KRT19, KRT5, KRT7, MACC1, MAGEA2, MCM6, MKI67, MMP1, MMP2, MMP9, MYC, NKX3-1, OR51E2, PCA3, PCGEM1, PCNA, PDZK1, PTEN, SPARC, SPP1, TEMPRSS2-ERG fusion, TP53, VEGF

Cytokine, chemokines and growth factors (human): CCL2, CSF2, FGF21, FGFR3, HGF, IL10, IL13RA2, IL17, IL17F, IL17RB, IL1RL1, IL23A, IL25, IL6, IL8, PDGFBR, SERPINE1; TGFA, TNF, TNFRSF12A, VEGF

Partial list of QuantiGene publications

- Yu M., et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. Nature 487 (7408):510-3 (2012).
- Ting T., *et al*. Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. *Science* **331**(6017):593-6 (2011).
- Kang Y. G., *et al.* Prognostic significance of S100A4 mRNA and protein expression in colorectal cancer. *Journal of Surgical Oncology* doi: 10.1002/jso.22070 (2011).
- Hall J. S., *et al*. Exon-array profiling unlocks clinically and biologically relevant gene signatures from formalin-fixed paraffin-embedded tumor samples. *British Journal of Cancer* **104**(6):971-81 (2011).
- Gan J., *et al. In vitro* assessment of cytochrome P450 inhibition and induction potential of tanespimycn and its major metabolite, 17-amino-17-demethoxygeldanamycin. *Cancer Chemotherapy and Pharmacology* doi 10.1007/s00280-011-1672-2 (2011).
- Chae B. J., *et al.* Measurement of ER and PR status in breast cancer using the QuantiGene2.0 assay. *Pathology* **43**(3):248-53 (2011).
- Babij C., *et al.* STK33 Kinase Activity is Non-Essential in KRAS-Dependent Cancer Cells. *Cancer Research* **71**(17):5818-26 (2011).
- Yim H. W., *et al.* Branched-chain assay for ER, PR, and HER2 RNA levels is a useful adjunct in the evaluation of ER, PR, and HER2 in Breast cancer. *Journal of Breast Cancer* **13**(3):267-74 (2010).
- Talbot D. C., *et al*. Tumor survivin is downregulated by the antisense oligonucleotide LY2181308: a proof-of-concept, first-in-human dose study. *Clinical Cancer Research* **16**(24):6150-8 (2010).
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