Application Note

Affymetrix®
Revolutionize life

QuantiGene® Assays for cancer and disease research in molecular pathology labs

QuantiGene® Assays enable direct quantification of miRNA, mRNA, or DNA targets directly from any sample. More than 400 studies have been published using QuantiGene Assays, which are based on the clinically proven branched DNA (bDNA) signal amplification technology used in Siemens' FDA-approved VERSANT™ assays for HIV and Hepatitis C viral load testing.

Each QuantiGene Assay kit contains everything you need to successfully detect and quantify or view your genes or transcripts of interest. Simply provide the accession number or sequence information for those genes or transcripts and Affymetrix will generate the necessary probe sets to be utilized in each assay kit.

QuantiGene Assays:

- Have proven successful across diverse and difficult samples: FFPE (including H&E stained), fresh frozen or cryogenic tissues, blood, urine, saliva, cultured cells, isolated RNAs or DNAs, and more
- Offer ultimate specificity by using multiple probes per target and cooperative hybridization
- Eliminate the need for miRNA, RNA, or DNA purification, cDNA synthesis via reverse transcription, and PCR amplification

QuantiGene Assays deliver unparalleled flexibility in three formats:

QuantiGene® ViewRNA

- Measure 1-4 mRNAs per well
- Measure 1 miRNA plus 1-3 mRNAs per well
- Measure 1 mRNA target/assay in FFPE tissue sections
- Available in CISH and FISH formats
- Requires a bright field and/or fluorescent microscope or high content imager

Key applications

- Expression heterogeneity
- Infectious diseases
- Stem cells and differentiation
- miRNA analysis
- Cancer tissue analysis

Benefits

- Contextual cell biology in a one-day assay
- Ultimate sensitivity of single-copy RNA at single-cell resolution

QuantiGene® 2.0

- Measure 1 miRNA, RNA, or DNA target per well
- Requires a standard laboratory microplate luminometer

Key applications

- Validate microarray results
- Quantify siRNA knockdowns
- Quantify DNA copy number
- Quantify miRNAs
- Detect translocations and fusion genes

Benefits

- Sensitivity comparable to RT-PCR
- Eliminate sample loss and variances associated with miRNA, RNA, or DNA isolation
- Avoid bias and false positive/negative results associated with cDNA synthesis or PCR amplification
- Obtain high precision and accuracy with tight correlation coefficients even at low expression levels
- Get excellent results in difficult clinical research samples, including H&E-stained FFPE, blood, and skin

QuantiGene® Plex

- Measure 3-36 mRNAs per well
- Measure 3-34 DNAs per well
- Requires a Luminex® instrument

Key applications

- Gene signatures
- Pathway analysis
- DNA copy number analysis
- Biomarker detection in clinical samples

Benefits

- True, same-well multiplexing of up to 36 targets per well
- Quantify directly from sample and eliminate RNA and DNA isolation step
- Avoid bias and false positive/negative results associated with cDNA synthesis or PCR amplification
- Obtain high precision and accuracy with tight CVs, even at low expression levels
- Get excellent results in difficult clinical research samples, including H&E-stained FFPE, blood, and skin

QuantiGene® ViewRNA

QuantiGene® ViewRNA technology has the sensitivity and robustness to detect single RNA molecules *in situ* at single-cell resolution and quantify expression heterogeneity. This level of sensitivity and resolution is critical in understanding the important biological role played by lowly expressed genes. As noted by Zhang (Zhang L., *Science*, 276(5316):1268-1272, 1997), 80 percent of mRNAs are present at fewer than five copies per cell.

Benefits of QuantiGene ViewRNA Assays

- Detection of single transcripts at the individual cell level in a one-day assay protocol
- In tissue assay, maintain tissue architecture and cell morphology
- Faster, more robust alternative to immunohistochemistry (IHC) and immunofluorescence (IF) when antibodies are not available or inadequate; also RNA alternative to DNA FISH
- Flexible probe design to target any gene or sequence within a gene; new assays developed in one week

QuantiGene ViewRNA applications

QuantiGene ViewRNA Assays have broad applicability in disease research, stem cell biology and regenerative medicine, mRNA knockdown measurements, neurobiology, biomarker validation testing, and more.

Biomarker quantification in FFPE tissue samples

In a single day, you can visualize and quantify gene expression *in situ* in FFPE samples with excellent specificity and without interference from formalin fixation, RNA degradation, or H&E staining. In this application (Figure 1), Her2 expression was measured in a disease progression series of FFPE breast cancer tissues using the QuantiGene ViewRNA tissue assay (Figure 1A) and the results were confirmed and quantified using the QuantiGene 2.0 lysate assay (Figure 1B).

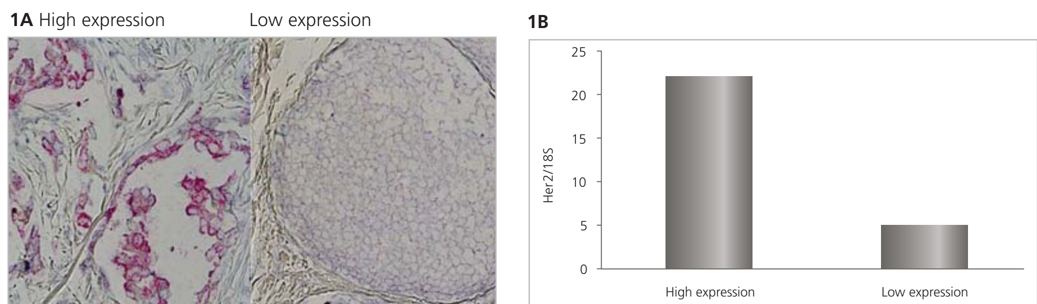


Figure 1: Her2 gene expression analysis in breast cancer FFPE tissue sections using QuantiGene Assays. **A:** QuantiGene ViewRNA CISH assay of Her2 (red staining) as seen using a bright field microscope. **B:** Quantitative measurement of Her2 expression normalized to 18S control using the QuantiGene 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*.
In this application, miR-133 miRNA and myogenin mRNA are co-detected in differentiated myoblast C2C12 cells (Figure 2).

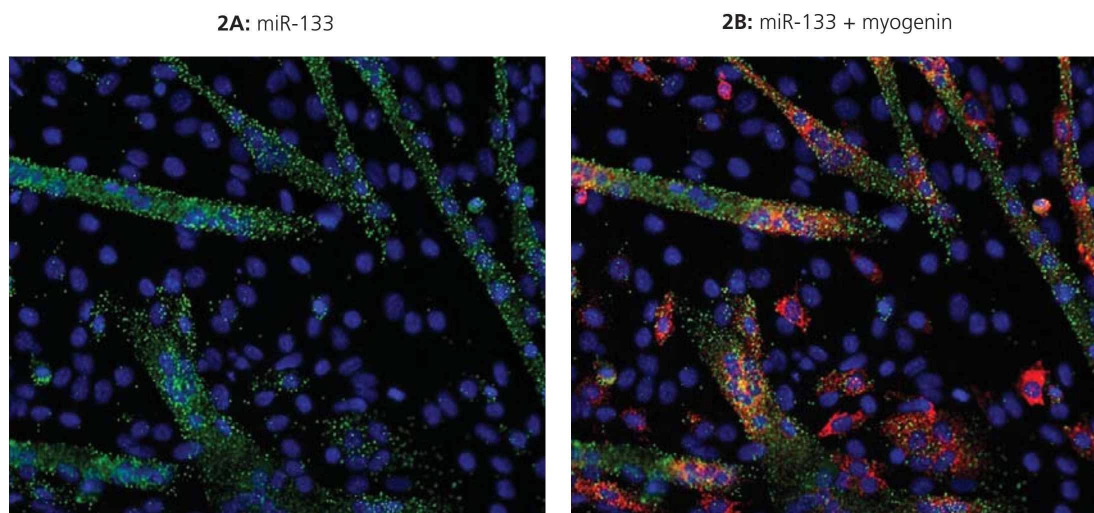
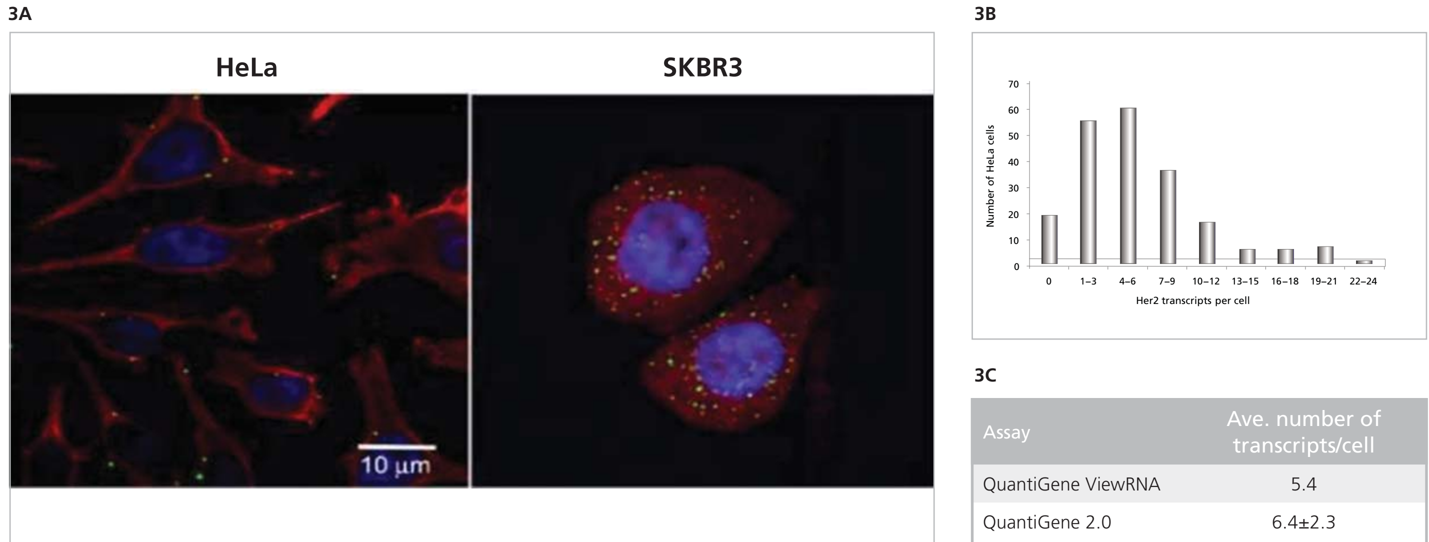


Figure 2: QuantiGene ViewRNA Assay detects *in situ* miRNAs and mRNAs. C2C12 cells were grown in 96-well plates and induced to myoblast differentiation. After five days, myotubes were detected with fluorescent probes for miRNA and mRNA using fluorescent microscopy as follows: **A:** miR-133 miRNA (green).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

Quantification of expression heterogeneity in cancer samples

The quantification of Her2 in HeLa cells compared to SKBR3 cells clearly demonstrates the QuantiGene ViewRNA *in situ* assay's ability to visualize and quantify gene expression heterogeneity in cancer specimens. Lysate and qPCR assays of clinical specimens average quantities of expressed genes of all cells within the population of cells comprising the tissue and can generate misleading results.

In this example (Figure 3A), the QuantiGene ViewRNA 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 with highly amplified Her2. But visualization of expression in the microscope field of view clearly indicates a high degree of expression heterogeneity from cell to cell. In Figure 3B, Her2 spots were counted in 200 HeLa cells and plotted as a histogram, revealing a range of expression from 0-21 transcripts per cell and with 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).



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

Figure 3: Visualization and quantification 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 was stained with DAPI (blue) and visualized by fluorescent microscopy.
B: Histogram of Her2 expression per HeLa cell, showing transcription heterogeneity within the cell population, based on counting 200 cells and dots per cell under the microscope.
C: Comparison of Her2 expression results using the QuantiGene ViewRNA Assay vs. the QuantiGene 2.0 lysate assay.

Infectious diseases

The QuantiGene ViewRNA 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).

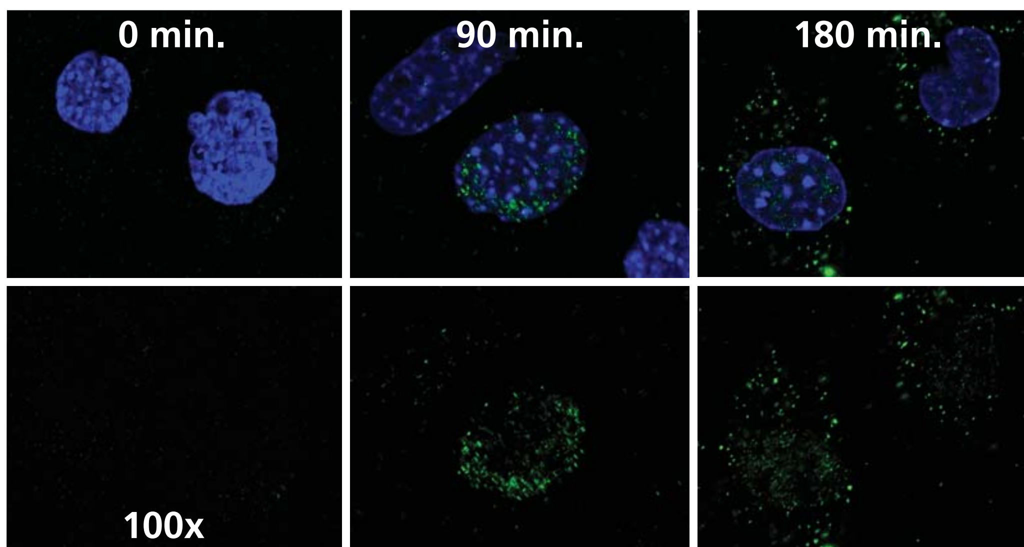


Figure 4: Detection of H1N1 Influenza A RNA migration in MEF cells. Confocal image. Nuclear translocation of viral RNA is necessary for replication of the H1N1 virus. Murine embryonic fibroblasts (MEFs) were incubated on ice with influenza A virus (H1N1, PR8 strain). At time 0, virus was removed and 37° C media was added. At the indicated time points after warming, the cells were fixed and processed with the QuantiGene ViewRNA slide-based kit reagents 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

The QuantiGene 2.0 Assay enables you to measure **any** miRNA, RNA, or DNA copy number in **any** sample.

Benefits

- Get accurate and precise results (MAQC study, *Nature Biotechnology*, September 2006)
  - Detect subtle changes – less than 10 percent change in gene expression vs. fold changes detected by RT-PCR
  - Measure single-copy differences between zero, one, two, three, or four DNA copies per cell
  - Specificity to two-base differences in miRNAs
- Quantify directly from your sample
  - Lyse and go: no RNA or DNA extraction
  - Works well with FFPE samples – no interference from H&E stain or formalin and can detect degraded RNA
  - Works well with blood samples or PAXgene™ tubes – requires no globin reduction and typically less than 10 µL blood
- Avoid bias and false negative and positive results due to RNA/DNA extraction, reverse transcriptase, and DNA polymerase

QuantiGene 2.0 applications

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

Prostate cancer biomarker validation in archived 9- to 13-year-old H&E-stained FFPE samples
As shown in Figure 5, the QuantiGene 2.0 Assay was used to analyze RNA in prostate cancer in 9- to 13-year-old H&E-stained FFPE 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).

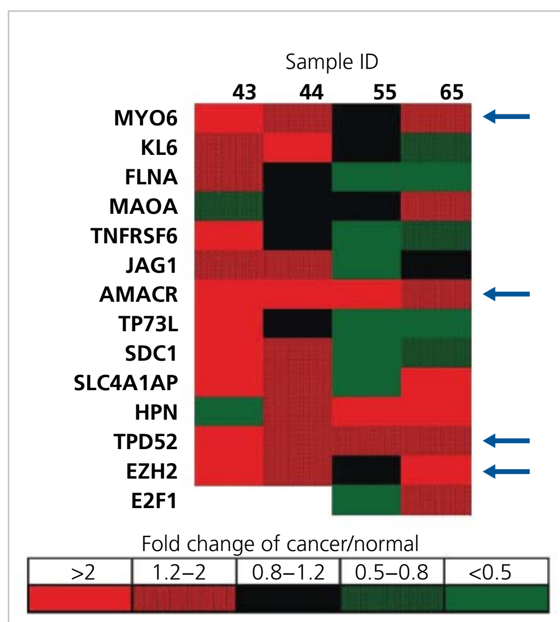


Figure 5: Quantification of fold change in prostate cancer markers. Cancer and normal tissues from the same FFPE blocks were macro-dissected and dissolved in homogenizing solution using the QuantiGene 2.0 Assay. A panel of 14 prostate cancer genes was measured in cancer and adjacent normal tissues. Values 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.

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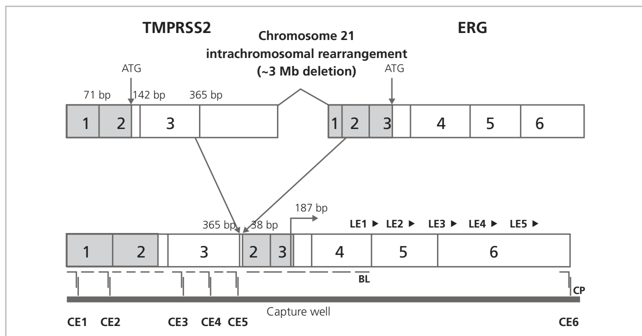
Detection of TMPRSS2-ERG fusion gene expression in prostate cancer specimens

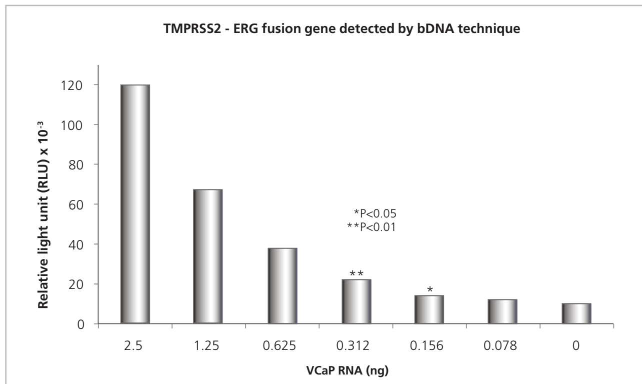
In an evaluation of clinical prostatectomy specimens, the QuantiGene 2.0 Assay detected 8 known TMPRSS2-ERG gene fusion subtypes from less than 200 pg of prostate cancer RNA (Figure 6). Fusion gene detection with one-step RT-PCR required more than 600 pg of RNA.

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 was 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



6B



6C

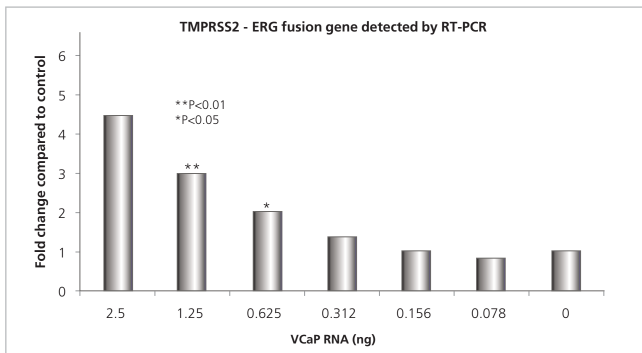


Figure 6: Detection of TMPRSS2-ERG fusion gene expression in total RNA from VCaP cells spiked with total RNA from normal human prostate tissue.
A: Schematic illustration of the bDNA probe set for TMPRSS2-ERG fusion. Probe sets for the fusion gene are designed to capture the 5′ portion of the TMPRSS2 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: Quantification 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: Quantification data for the TMPRSS2-ERG fusion gene from nested RT-PCR assay based on input of 600 pg of prostate cancer RNA.

QuantiGene® Plex

QuantiGene Plex is a novel quantitative assay with true same-well multiplexing of up to 36 targets per well with proven performance in difficult archived clinical samples such as FFPE and blood. The QuantiGene Plex Assay is a fluorescent assay that is run on commonly used Luminex instruments and has many of the same benefits as the QuantiGene 2.0 single-plex and QuantiGene ViewRNA *in situ* assays. Affymetrix also provides multiplex protein and cytokine assays with more than 250 validated assays in 6 species. For more information, please contact your local Affymetrix representative.

Benefits

- Plex up to 34 DNA copy number targets and up to 36 RNA transcripts per well from a single sample
- No sample extraction required; can detect purified RNAs or DNAs as well as poor-quality, degraded RNA
- No false negative, false positive, or biased results due to reverse transcriptase or DNA polymerase
- Highly accurate and precise measurements from plate to plate, day to day, and operator to operator
- No interferences from formalin, H&E stain, enzyme inhibitors such as melanin, globin or other highly expressed genes

QuantiGene Plex applications

QuantiGene Plex RNA and QuantiGene Plex DNA Assays are widely used to profile and quantify gene signatures in applications such as signal transduction pathways, biomarker validation in retrospective and prospective FFPE and blood studies, validation of microarray results, predictive toxicology, quantification of RNAi knockdowns, quantification of DNA copy number variation, 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, PAX tube blood, isolated blood cells, or purified RNA or DNA. In this application (Figure 7), a 17-plex QuantiGene Plex Assay was used to quantify the relative abundance of 17 transcripts per well from isolated intact, bypassing the need for RNA purification. Simultaneous quantification 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).

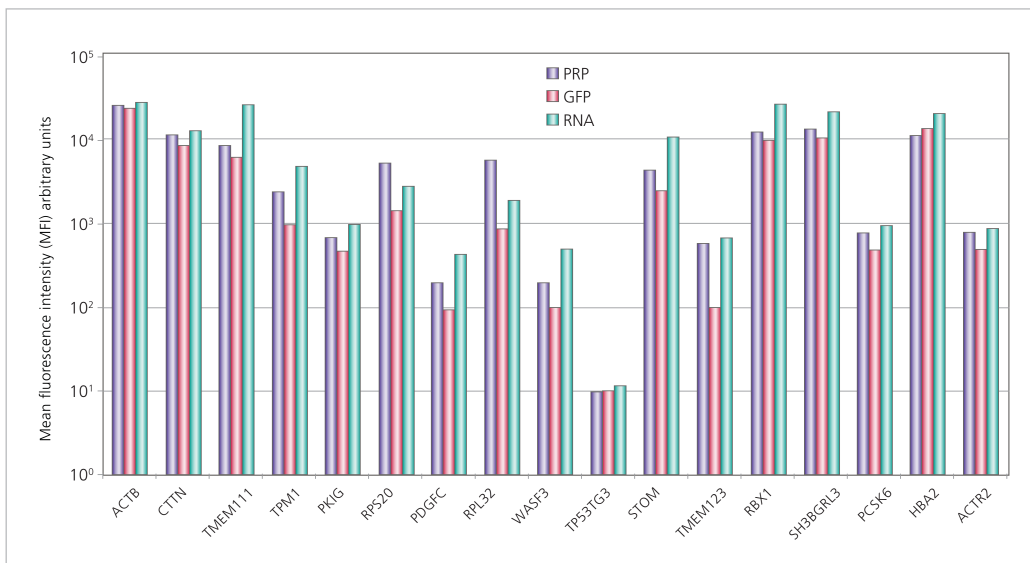


Figure 7: QuantiGene 17-plex RNA assay on purified RNA and intact platelet fractions.

Multiplex DNA copy number analysis

With the QuantiGene Plex DNA Assay, you can quantify single-copy differences between zero, one, two, three, or four DNA copies per cell and multiplex up to 34 targets. This enables you to process more samples per plate with fewer reagents, less hands-on time, and increased accuracy.

In Figure 8, a QuantiGene 8-plex DNA copy number assay 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 (Figure 9), in which ~13 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.

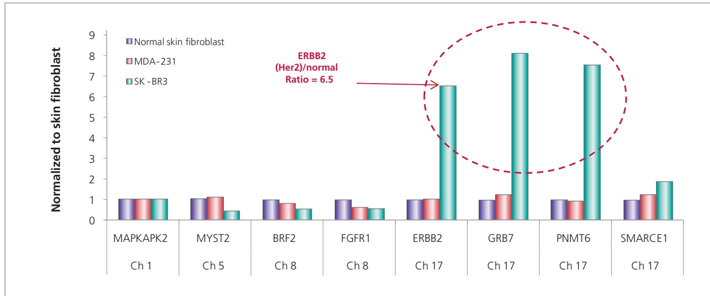


Figure 8: QuantiGene 8-plex DNA copy number assay in SKBR3, normal skin fibroblast, and MDA-231 cells. Ratio of SKBR3/control = ~7.

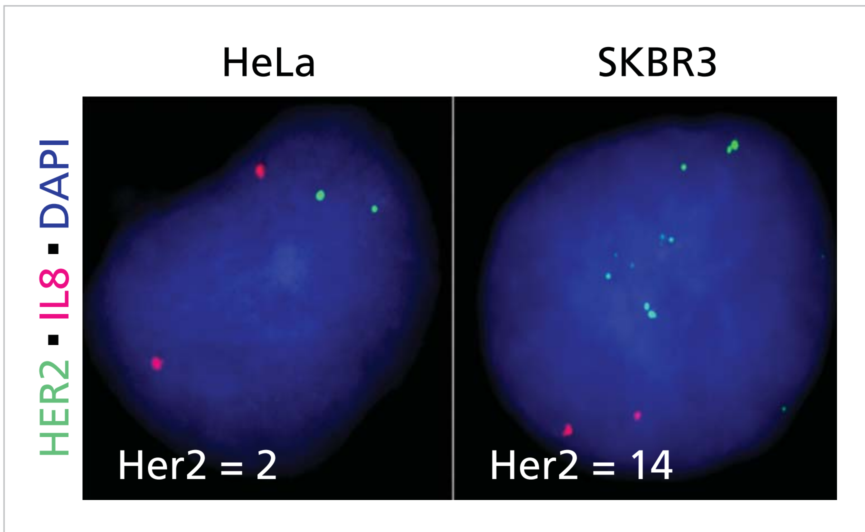
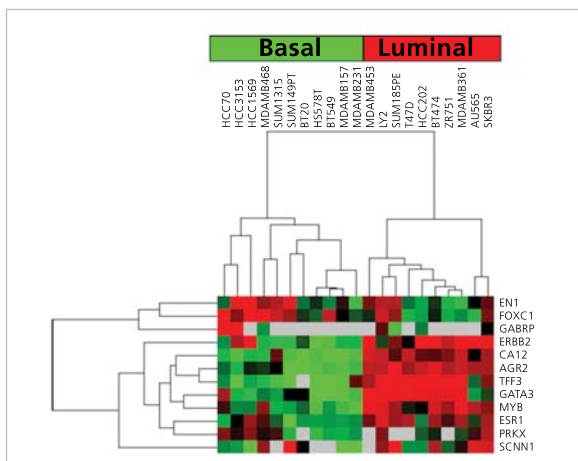


Figure 9: FISH assay: Her2 amplified in SKBR3 cells vs. HeLa cells. Ratio of SKBR3/HeLa = ~7.



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: A 12-plex RNA measurement from the cell lines was derived using the QuantiGene 2.0 Plex 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

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P/N AN101 Rev. 1
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