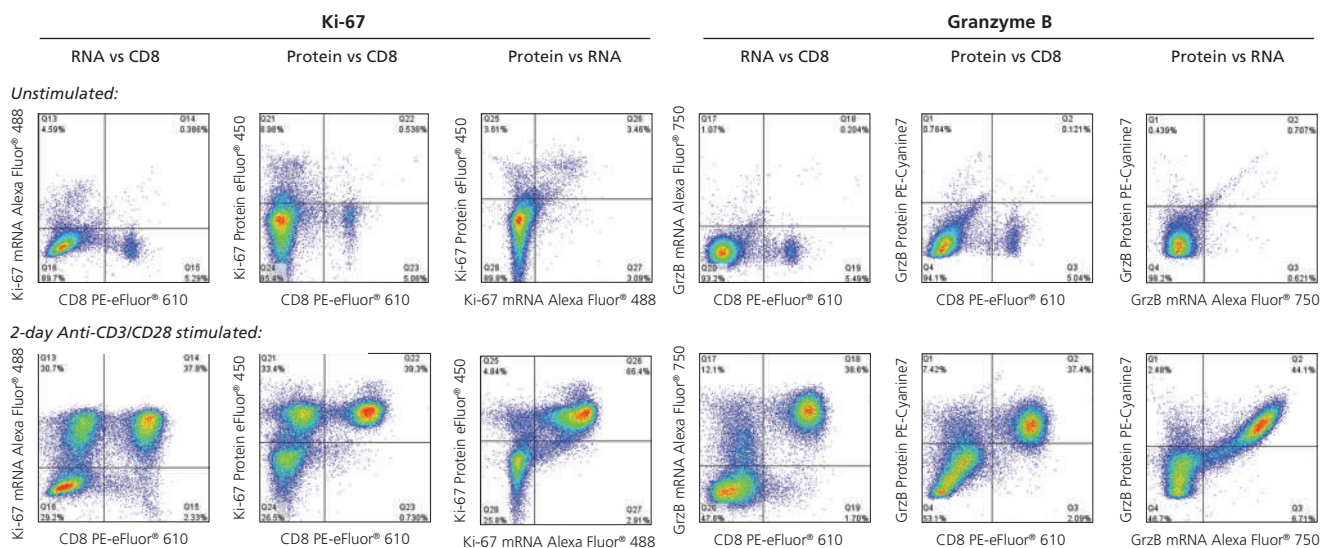# PrimeFlow™ RNA Assay
Technology Validation Paper

Introduction

PrimeFlow™ RNA Assay reveals the dynamics of RNA and protein expression within individual cells, facilitating unprecedented analysis of their correlation as the cells change over time or in response to stimulation. This novel assay uses fluorescent *in situ* hybridization (FISH) to enable simultaneous detection of as many as three RNA transcripts in a single cell using a standard flow cytometer. PrimeFlow RNA Assay is compatible with cell surface and intracellular staining, using common flow cytometry fluorochromes. The assay is based upon proven and well-published ViewRNA assays designed for microscopic analysis of RNA in cells and tissues that combine paired oligonucleotide probe design with branched DNA (bDNA) signal amplification to robustly detect gene expression at the single-cell level.

Coupling RNA expression with protein detection on a flow cytometer generates multiparametric data in heterogeneous cell populations and offers in-depth and high content details at the single-cell level. In contrast, microarrays and sequencing can provide comprehensive gene expression data in bulk sample preparations; however, the analysis of bulk samples can mask the individual effects of unique cellular subsets. Using PrimeFlow RNA Assay, specific cell populations may be analyzed for unique transcript expression levels, or cell subsets evaluated over time to determine transcriptional regulation and protein expression simultaneously. Such unique and valuable insights are highly applicable to answering previously unanswerable questions and have broad implications for advancing research across multiple fields of biology.

- Observe the heterogeneity of gene expression at a single-cell level
- Correlate RNA and protein kinetics within the same cell
- Detect non-coding RNA in cell subsets
- Evaluate viral RNA expression in infected cells
- Analyze mRNA expression levels when antibody is unavailable

Figure 1: Example data set
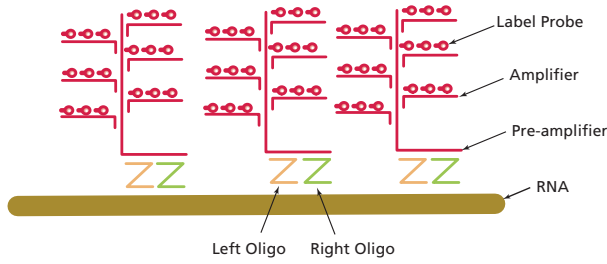

PrimeFlow™ RNA Assay in action
C57Bl/6 splenocytes were unstimulated (top row) or stimulated for 2 days with Anti-Mouse CD3 and CD28 Functional Grade Purified antibodies (cat. no. 16-0031 and cat. no. 16-0281) (bottom row) and in the presence of Protein Transport Inhibitor Cocktail (cat. no. 00-4980) for the last 3 hours of culture, followed by analysis using the PrimeFlow™ RNA Assay (cat. no. 88-18001). Cells were fixed and permeabilized using the PrimeFlow™ RNA Assay buffers and protocol, then intracellularly stained with Anti-Mouse CD8a PE-eFluor® 610 (cat. no. 61-0081), Anti-Mouse Ki-67 eFluor® 450 (cat. no. 48-5698), and Anti-Mouse Granzyme B PE-Cyanine7 (cat. no. 25-8898). Cells were then hybridized with Type 6 Mouse Granzyme B Alexa Fluor® 750 (cat. no. VB6-16522), Type 4 Mouse Ki-67 Alexa Fluor®488 (cat. no. VB4-16518), and Type 1 Mouse β-actin Alexa Fluor® 647 (cat. no. VB1-10350) target probes.

Assay technology

Fluorescent *in situ* hybridization (FISH) is a powerful technique that allows specific localization of ribonucleic acid targets in fixed cells. The basic premise of the application relies on detecting nucleic acids through sequential hybridization of nucleic acid probes that provides gene expression information at a single-cell level. Traditional FISH techniques are generally limited by high background and low sensitivity due to non-specific binding and inefficient signal amplification.

PrimeFlow™ RNA Assay incorporates a proprietary oligonucleotide probe set design and branched DNA (bDNA) signal amplification technology to analyze RNA transcripts by flow cytometry. bDNA technology provides a unique approach to RNA detection and signal amplification by amplifying the reporter signal rather than the target sequence (e.g., PCR) for consistent results, a common problem for PCR-based assays.

Figure 2: Branched DNA (bDNA) probe design principle

In the PrimeFlow™ RNA Assay, target-specific probe sets contain 20 to 40 oligonucleotide pairs that hybridize to the target RNA transcript. Signal amplification is achieved through specific hybridization of adjacent oligonucleotide pairs to bDNA structures, formed by Pre-amplifiers, Amplifiers, and fluorochrome-conjugated Label Probes, resulting in excellent specificity, low background, and high signal-to-noise ratio (Figure 2).

Figure 3: PrimeFlow™ RNA Assay workflow
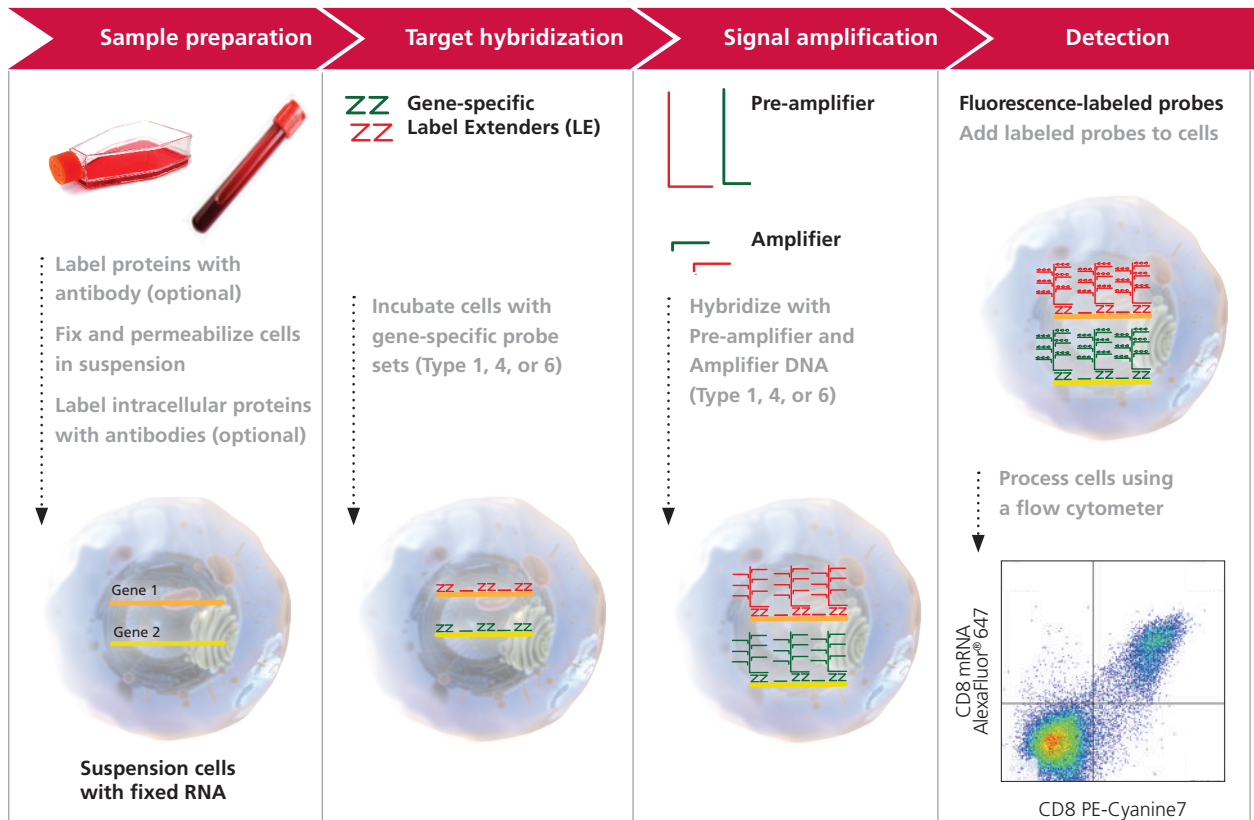
PrimeFlow™ RNA Assay principle

The assay workflow contains several steps: surface antibody staining; fixation and permeabilization; intracellular antibody staining, followed by target probe hybridization, with RNA-specific probe sets; signal amplification using bDNA constructs and detection by flow cytometry. For simplicity, detection of only two RNA targets are shown in orange and yellow (Figure 3, Page 2) with only three of the 20 to 40 oligonucleotide target probe pairs per target RNA.

Antibody staining, fixation, and permeabilization

Single-cell suspensions can be stained for cell surface markers and fixable viability dyes before the cells are fixed and permeabilized. Subsequently, the cells may be stained with antibody directed to intracellular targets, such as transcription factors and cytokines. After an additional fixation step, the cells are ready to proceed through the hybridization and signal amplification steps.

Target hybridization

A target-specific Probe Set contains 20 to 40 oligonucleotide pairs that hybridize to specific regions across the target RNA sequence. Subsequent signal amplification requires that each half of a given oligonucleotide pair binds to the target RNA in adjacent positions. Three types of Probe Sets are currently available to allow detection of RNA labeled with Alexa Fluor® 647 (Type 1 Probe Sets), Alexa Fluor® 488 (Type 4 Probe Sets), or Alexa Fluor® 750 (Type 6 Probe Sets). When detecting more than one RNA target in a single sample, each Probe Set must be a unique type to differentiate its signal from the others.
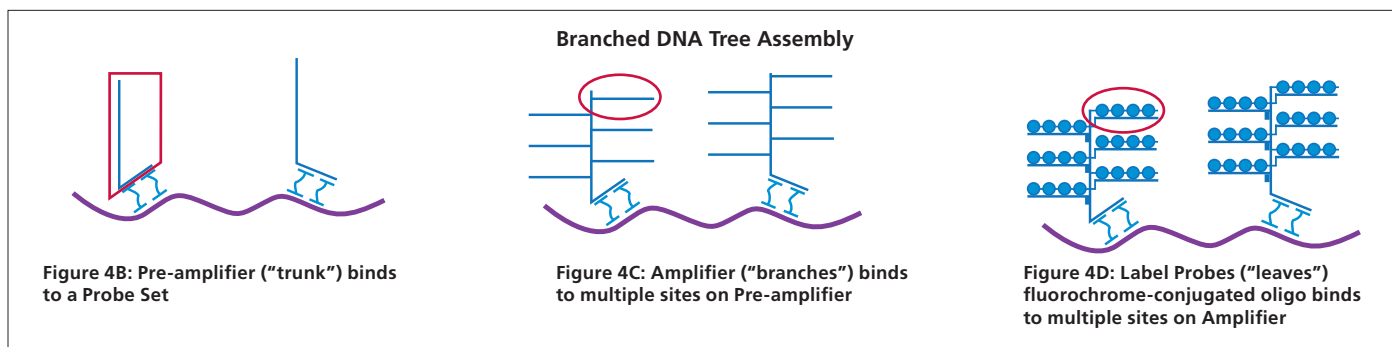
Figure 4A: Probe Set hybridization

Probe Set Type	Fluorochrome Label	Excitation Wavelength (max)	Emission Wavelength (max)	Laser Excitation Wavelength	Bandpass Filter Recommendation
Type 1	Alexa Fluor® 647	647 nm	668 nm	632-640 nm	660/20
Type 4	Alexa Fluor® 488	488 nm	519 nm	488 nm	530/30
Type 6	Alexa Fluor® 750	749 nm	775 nm	632-640 nm	780/60

Signal amplification

Signal amplification using bDNA technology is achieved through a series of sequential hybridization steps, that forms a tree-like structure. Pre-amplifier molecules hybridize to their respective pair of bound oligonucleotide probes to form the trunk of the tree. Multiple Amplifier molecules hybridize to their respective Pre-amplifier to create the branches. Finally, multiple Label Probes hybridize to the Amplifiers and form the "leaves" of the tree. A fully assembled signal amplification tree contains 400 Label Probe binding sites. If all target-specific oligonucleotides in a 20 oligonucleotide pair Probe Set bind to the target RNA transcript, an 8,000-fold amplification can be achieved.

Branched DNA Tree Assembly

Figure 4B: Pre-amplifier ("trunk") binds to a Probe Set

Figure 4C: Amplifier ("branches") binds to multiple sites on Pre-amplifier

Figure 4D: Label Probes ("leaves") fluorochrome-conjugated oligo binds to multiple sites on Amplifier

Fluorescence detection

Upon completion of the assay protocol, target RNA data is detected in cells by analyzing the sample on a standard flow cytometer equipped with 633-647 nm and 488 nm lasers and appropriate filter configurations, to capture the fluorescent signals (see table in Figure 4A).

Precision
Intra-assay variability

To assess intra-assay variability, samples from stimulated and unstimulated human peripheral blood mononuclear cells (PBMC) were divided into seven tubes and assessed for expression of Ribosomal Protein L13a (RPL13a), a positive control gene expressed in all PBMC, and interferon gamma (IFNγ), induced only upon stimulation in a subset of lymphocytes. As shown in Figures 5A and 5B, the assay shows robust intra-assay performance, with a CV% less than 10% for both RPL13a and IFNγ.
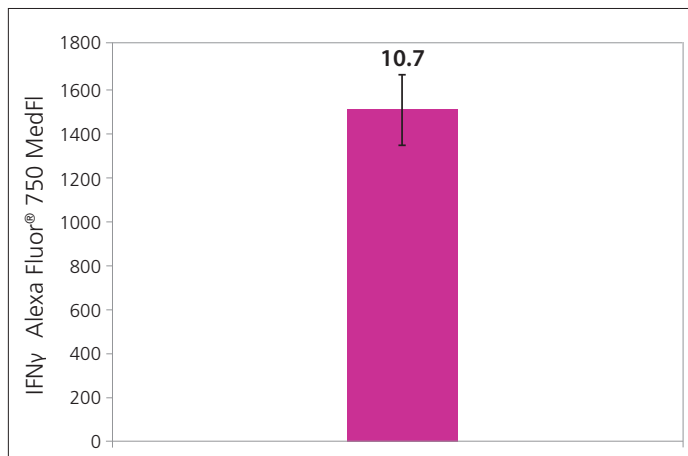
Figure 5A

Figure 5B
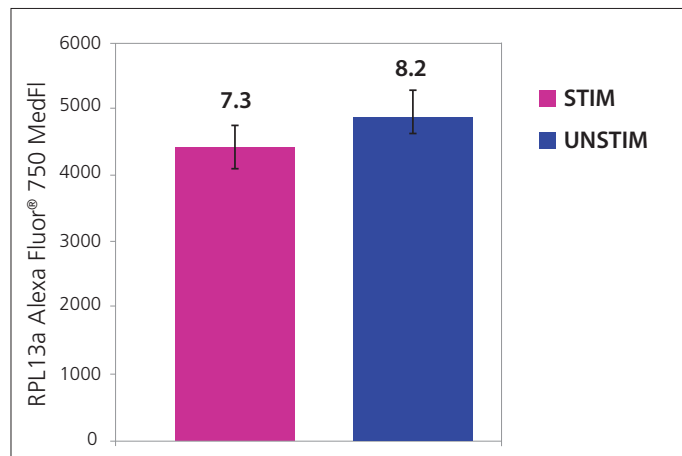
Assessing intra-assay variability
A single sample of stimulated or unstimulated human PBMC was divided into 7 tubes and assessed for expression of RPL13a mRNA in total lymphocytes (Figure 5B) or IFNγ mRNA in IFNγ + events (Figure 5A). Data shown are the average of the 7 replicates, and error bars represent standard deviation. Values shown above the bars represent the CV%.

To assess the effect of target probe handling on assay variability, a sample of mouse splenocytes was divided into three samples and assayed for expression of ß-actin, RPL13a, ß2-microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and Peptidyl-prolyl cis-trans isomerase B (PPIB). Target probes were diluted independently for each triplicate. As shown below, the CV% were typically less than 10%, while RPL13a was slightly higher, around 12%.
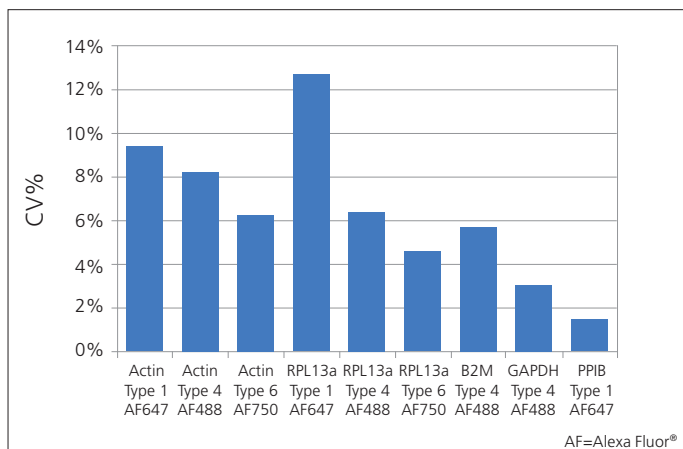

Figure 6: Contribution of target probe dilution to assay variability
Splenocytes from C57Bl/6 mice were assessed for expression of several positive control genes. Samples were prepared in triplicate with independent dilutions of target probes made for each sample. CV% of the median fluorescence intensity for each gene are shown.

Operator variability

To assess the contribution of technicians to assay variability, aliquots of U937 cells from the same cell culture were tested in triplicate for GAPDH, PPIB, and B2M gene expression by two different technicians. No Probe (NP) controls were used as negative controls, and samples were analyzed using the same cytometer settings. For each operator, the CV% of the mean fluorescence intensity (MFI) of triplicate samples was less than 6%, consistent with previous data for intra-assay variability. The variation between operators was approximately 5% for the MFI, and 20% for the signal-to-noise ratio, calculated as the ratio between the positive MFI and the No Probe control MFI.

Figure 7A
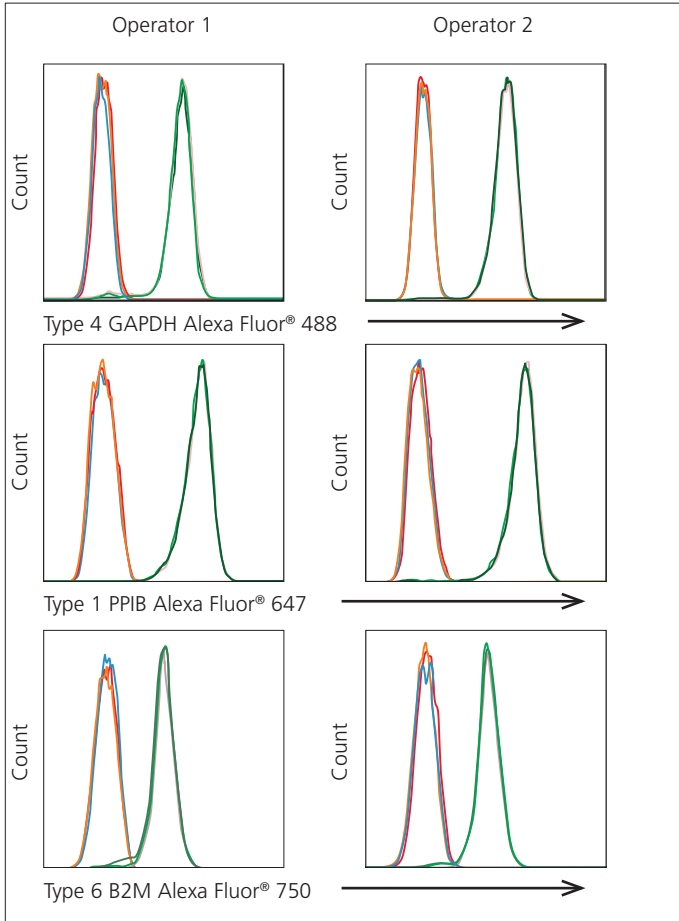
Figure 7B
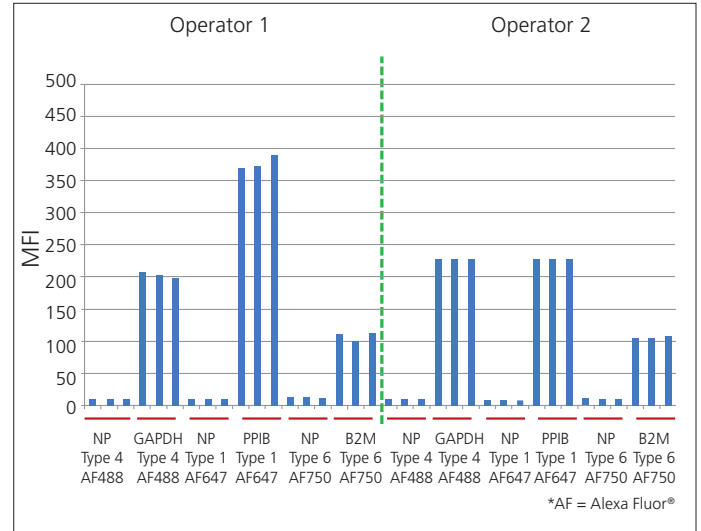
Contribution of operator variability
U937 cells were assessed for expression of several positive control genes. Samples were prepared in triplicate by two technicians. Histogram overlays of the triplicates for No Probe and target probes are shown on the left (Figure 7A). MFI for each sample are shown on the right (Figure 7B).

Additional studies to assess variation between operators were run with samples containing only a subpopulation of positive cells. C57Bl/6 splenocytes were stimulated for three days with anti-CD3 and anti-CD28 antibodies, and assessed for expression of Ki-67 and Granzyme B mRNA. The samples were divided among four technicians who independently performed the assay. As shown below, the mean percentage of positive events for Ki-67 or Granzyme B were 18.6% and 30.6%, respectively (Figure 8A). The MFI of the mRNA positive events are shown in the bar graph, and in this case the CV was 12-15% (Figure 8B), consistent with the results obtained with positive control genes in U937 cells (Figure 7A & 7B).

Figure 8A
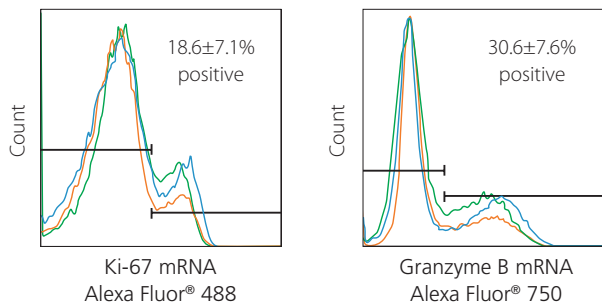
Contribution of operator variability in a bimodal expression model
Mouse splenocytes were stimulated with Anti-Mouse CD3 (cat. no. 16-0031) and Anti-Mouse CD28 (cat. no. 16-0281) Functional Grade Purified antibodies for 3 days, with the addition of brefeldin A and monensin in the last 2 hours. The cells were stained with Anti-Mouse CD8a PE-eFluor® 610 (cat. no. 61-0081), Anti-Mouse Ki-67 eFluor® 450 (cat. no. 48-5698), and Anti-Mouse Granzyme B PE-Cyanine7 (cat. no. 25-8898), followed by hybridization of Type 4 Mouse Ki-67 Alexa Fluor® 488 probe set (cat. no. VB4-16518) and Type 6 Mouse Granzyme B Alexa Fluor® 750 probe set (cat. no. VB6-16522). Cells in the lymphocyte gate or the mRNA+ events were used for analysis.

Figure 8B
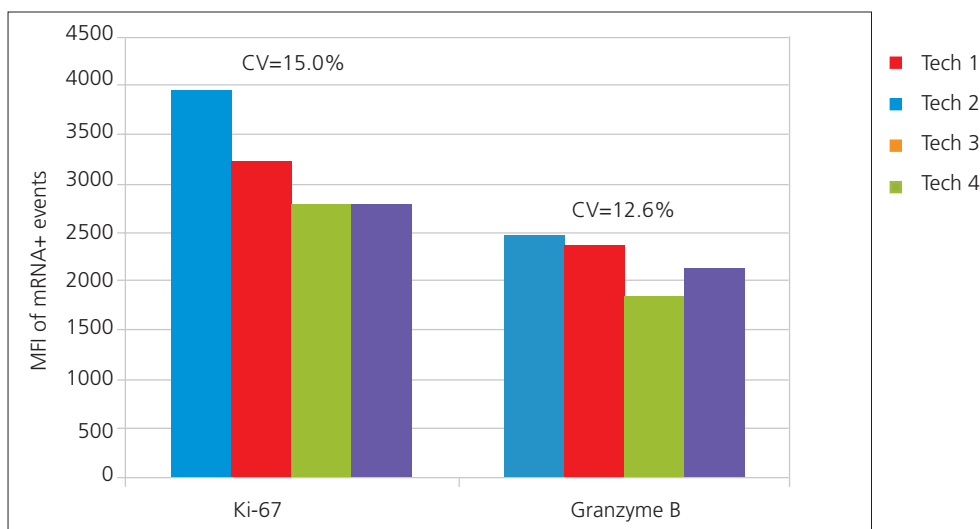
Day-to-day variability

To understand day-to-day variation, human PBMC stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitor cocktail) were analyzed fresh, or cryopreserved and analyzed one week later. As shown below, the percentage of positive events is virtually unchanged between the fresh and cryopreserved samples.

Day-to-day variation of PrimeFlow™ RNA Assay[a]	IFNγ mRNA[b]	IFNγ protein[b]
Experiment 1	58.0	90.3
Experiment 2	57.8	87.7
Average	57.9	89.0
Standard Deviation	0.14	1.84
CV (%)	0.24	2.07
[a]Normal human PBMC were stimulated and analyzed immediately (Experiment 1) or were cryopreserved and analyzed one week later (Experiment 2) for the expression of IFNγ mRNA and protein. Samples were surface stained with Anti-Human CD8a PE-eFluor® 610 (cat. no. 61-0088) and followed by hybridization of human IFNγ Alexa Fluor® 750 (cat. no. VA6-13121).		
[b]Data represent the percentage of IFNγ+ events, gated on viable CD8+ cells.		

Sensitivity and specificity

The central dogma of molecular biology states that DNA gives rise to RNA, which in turn gives rise to protein. However, studies have shown that the correlation between levels of RNA and protein products vary widely. Here we demonstrate how PrimeFlow™ RNA Assay can reveal the unique kinetics of mRNA and protein in the same cells to understand the correlation between the two over time, and in response to stimulation. Human PBMC were stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitors) for as long as five hours. Using PrimeFlow RNA Assay, the cells were assessed for IFNγ or TNFα mRNA expression and protein in CD8+ or CD8- lymphocytes at hourly intervals. As shown below, both CD8+ and CD8- lymphocytes respond to stimulation, but IFNγ and TNFα mRNA and protein each exhibit unique kinetics depending on the lymphocyte subset being analyzed. Of note, while IFNγ mRNA is rapidly upregulated after one hour of stimulation, IFNγ protein is not detected until the second hour. In contrast, TNFα mRNA and protein are both upregulated within the first hour, and although CD8+ cells maintain TNFα mRNA, the CD8- lymphocytes more rapidly downregulate TNFα mRNA, and show a subsequent slow decline in TNFα protein levels. Thus, PrimeFlow RNA Assay enables the study of gene expression at the single-cell level in heterogeneous samples without the need for sorting specific subsets and has the ability to elucidate the kinetics of mRNA and protein expression.
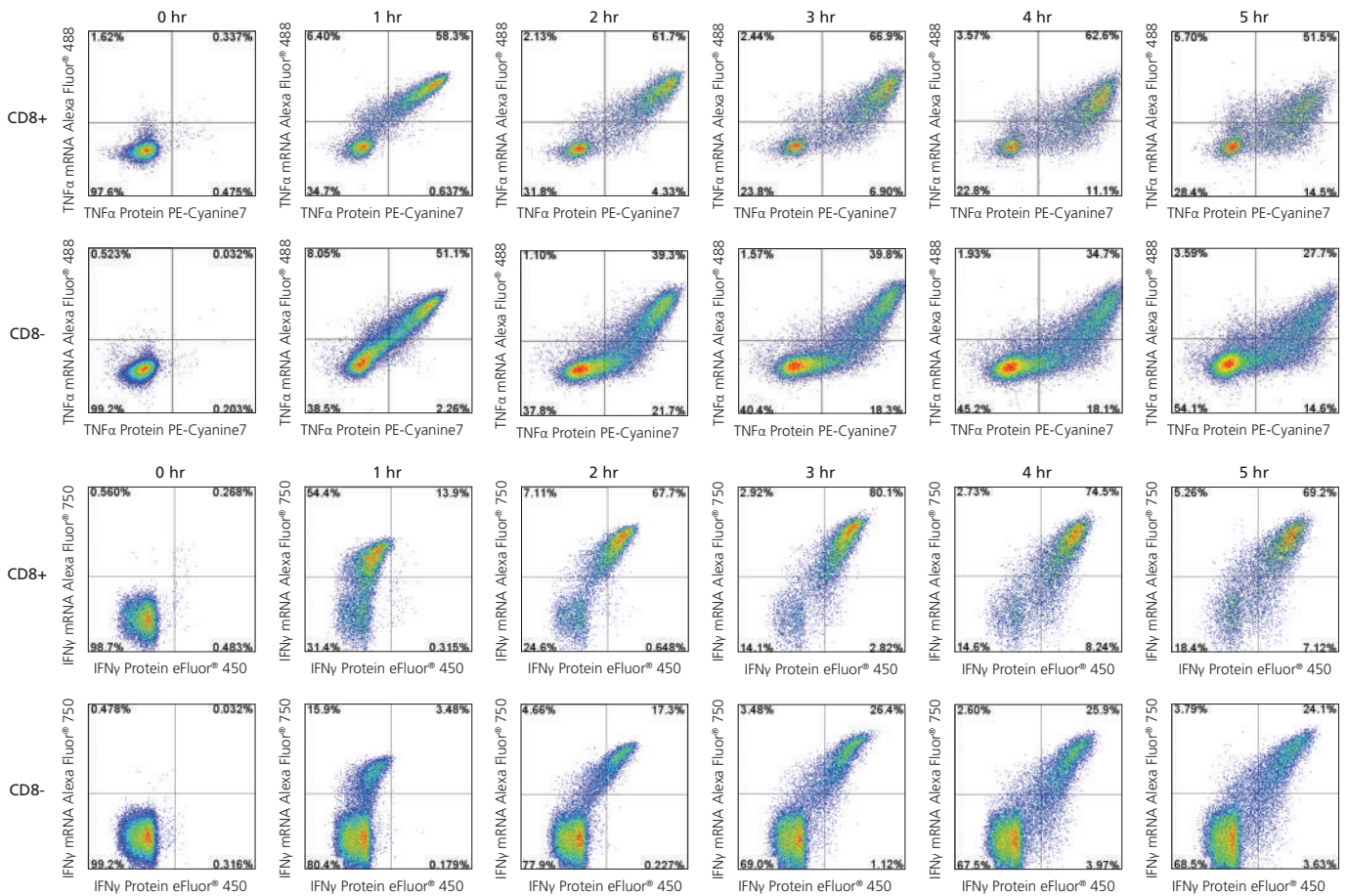
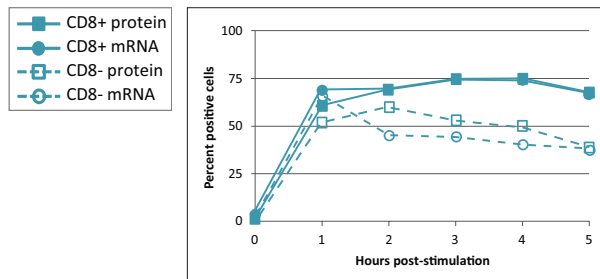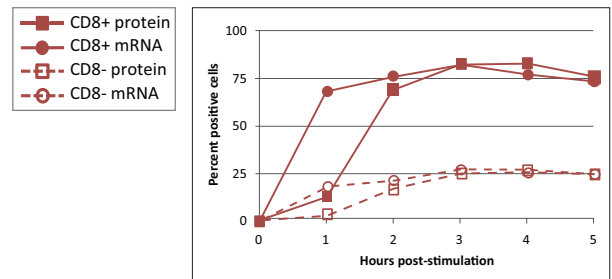**Figure 9A**

Figure 9B

Figure 9C

Correlation and kinetics of IFNγ and TNFα transcription and translation
Normal human PBMC were stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (cat. no. 00-4975) for 0-5 hrs, then subjected to PrimeFlow™ RNA Assay. Cells were intracellularly stained with Anti-Human CD8α PE-eFluor® 610 (cat. no. 61-0088), Anti-Human IFNγ eFluor® 450 (cat. no. 48-7319), and Anti-Human TNFα PE-Cyanine7 (cat. no. 25-7439), followed by hybridization with Human TNFα Alexa Fluor® 488 probe set (cat. no. VA4-10289) (Figure 9B) and Human IFNγ Alexa Fluor® 750 probe set (cat. no. VA6-13121) (Figure 9C). CD8+ or CD8- cells in the lymphocyte gate were used for analysis.

Orthogonal validation

PrimeFlow™ RNA Assay was assessed by examination of genes previously measured by QuantiGene® Plex Assay, a hybridization-based assay using the xMAP® Luminex® magnetic bead platform. As shown in Figure 10A, GAPDH showed the highest MFI in U937 cells, consistent with the highest relative signal captured in QuantiGene Plex Assay. HMBS had the lowest MFI, consistent with gene expression at low levels in U937 cells. Signal-to-noise ratios were calculated relative to the No Probe control sample and are shown in Figure 9B. Next, we assessed the expression of the same genes in normal human lymphocytes and monocytes (Figure 9C). Although U937 is a monocytic cell line, there were notable differences between primary monocytes and U937 cells. HMBS was completely undetectable in primary human monocytes, and instead of GAPDH, B2M was the most highly expressed gene in monocytes. Primary human lymphocytes diverged even further with very low levels of GAPDH and high levels of B2M. These data highlight the importance of understanding gene expression levels in the cells of interest, as expression can vary even in cell lines derived from the same primary cell type. Furthermore, based on QuantiGene Plex data, U937 cells are known to express 5-10 copies of HMBS mRNA, suggesting that in a fully optimized system it is possible to detect 5-10 RNA copies by PrimeFlow RNA Assay.
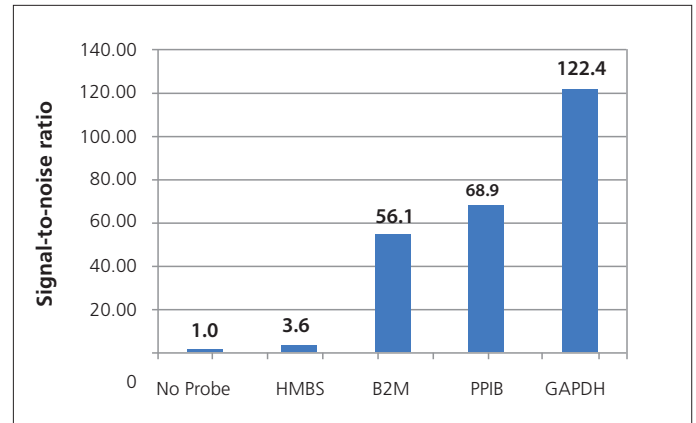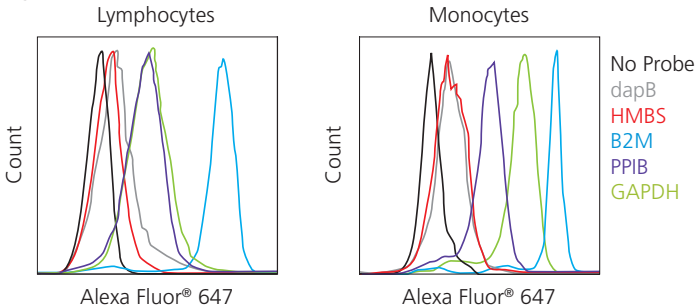
Figure 10A
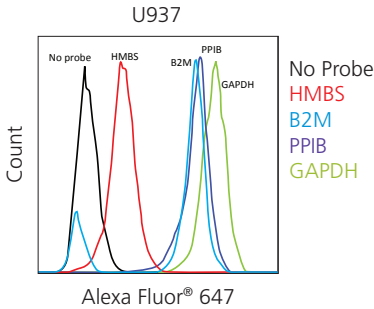
Figure 10B

Figure 10C

Orthogonal validation of QuantiGene® Plex and PrimeFlow™ RNA Assays
U937 (Figures 9A and 9B) or normal human PBMC (Figure 9C) were hybridized to a series of Type 1 Positive Control Alexa Fluor® 647 probe sets, following PrimeFlow™ RNA Assay protocol. The signal-to-noise ratio was calculated as the ratio between the MFI of the Positive Control gene and the MFI of the No Probe control.

Correlation of QuantiGene® Plex and TaqMan® data

To demonstrate the accuracy of QuantiGene® Plex assay, measurements of twenty transcripts from two reference RNA samples were made and compared to QuantiGene® and TaqMan® data. Reference RNA samples include human brain total RNA and universal human reference RNA as described in Canales et al. 2006. *Nature Biotechnology* 24(9):1115-1122. As shown below (Figure 11), excellent correlation is noted in RNA level fold changes in the reference samples, between QuantiGene® Plex and TaqMan® assays (R2 = 0.965). TaqMan® data are described in Canales, et al. 2006 publication. These studies show that TaqMan® based PCR, QuantiGene® Plex, and PrimeFlow™ RNA Assay provide consistent results.
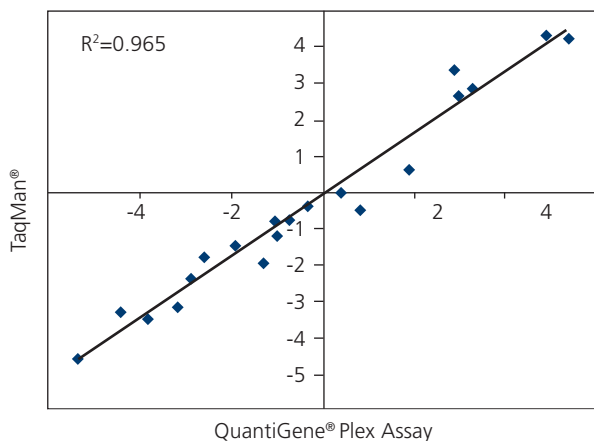

Figure 11: Comparison of QuantiGene® and TaqMan® RNA level fold changes in total and reference RNA.

Technology specificity

The bDNA technology achieves high target specificity with the use of oligonucleotide pairs—a design resulting in signal amplification only when two adjacent target probe oligonucleotides (left oligonucleotide and right oligonucleotide) bind to the specific target. To determine the assay specificity, probe sets for human GAPDH containing left oligonucleotides alone, or right oligonucleotides alone, were tested and compared to a complete probe set in human U937 cells. Fluorescent signal is detected only with the complete probe set, correlating to the abundant expression of GAPDH in U937 cells. In contrast, no signal is detected when either the left or right oligonucleotides are used individually, similar to the No Probe control. Additionally, a negative control probe set for DapB, a bacterial gene in *Bacilus subtillis*, yielded no specific signal.

Figure 12
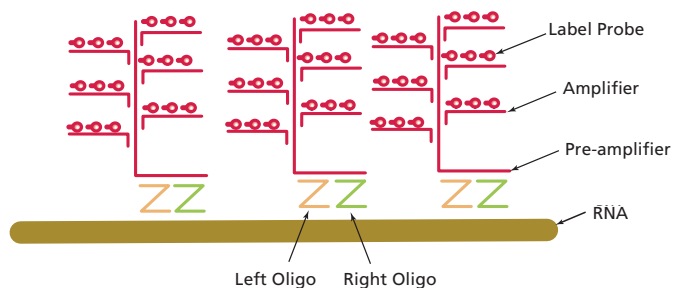
Figure 13
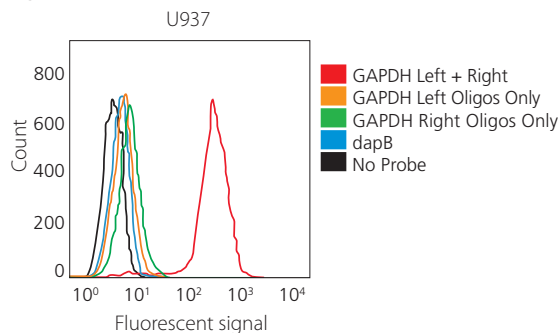
Figure 14
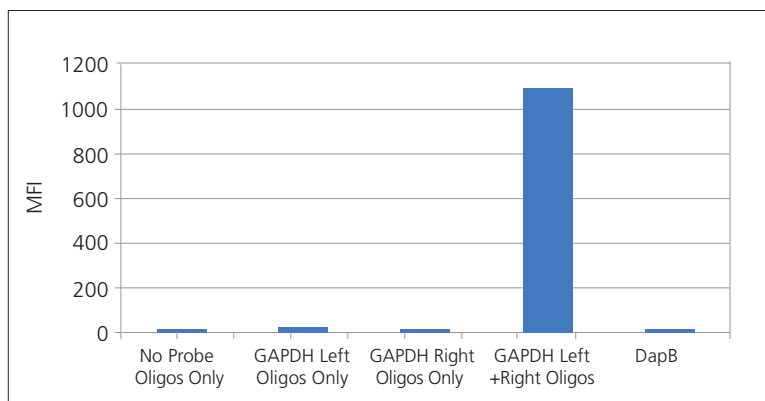
Demonstration of specificity of the PrimeFlow™ RNA Assay
Illustration of a Probe Set design containing both left and right oligonucleotide pairs, and signal amplifications reagents, Pre-amplifier, Amplifier, and Lable Probes (Figure 12). Flow cytometry histogram data of GAPDH RNA expression in U937 cells (Figure 13). Quantitative representation of GAPDH signal with different probe sets based on Mean Fluorescent Intensity (MFI) (Figure 14).

Assay channel sensitivity

PrimeFlow™ RNA Assay is capable of detecting three different RNA targets in a single cell through the use of three different fluorochromes. However, each fluorochrome provides different levels of sensitivity. To understand the relative sensitivity of the three RNA detection channels, we assessed the expression level of the same gene in each of the three probe set types using U937 samples or in mouse splenocytes. Human B2M probe sets in Type 1 (Alexa Fluor® 647), Type 4 (Alexa Fluor® 488), or Type 6 (Alexa Fluor® 750) were used to detect B2M RNA targets in human U937 cells (Figure 15A). The signal-to-noise ratio relative to the No Probe control was calculated and the relative sensitivity to Type 6 (Alexa Fluor 750) probe set was assessed. As shown in Figures 15A and 15B, Type 1 (Alexa Fluor 647) provides the greatest sensitivity. We observed similar sensitivity when assessing the expression of β-Actin in mouse splenocytes. For this reason, Type 1 probes for genes with low or unknown levels of expression. Type 4 (Alexa Fluor 488) and Type 6 (Alexa Fluor 750) probe sets have similar sensitivity and are recommended for genes with medium to high levels of expression. As with any multi color flow cytometry experiment, actual results depend on instrument configuration, detector sensitivity, instrument settings, and compensation, all of which require optimization to obtain the best results.

Figure 15A
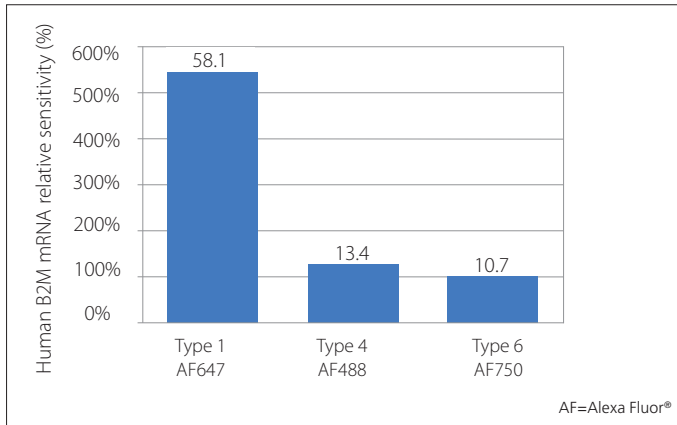
Assessing assay channel sensitivity
U937 cells were hybridized with B2M probe sets in each probe set type to assess the different sensitivity of each channel. Values above each bar represent the signal-to-noise ratio. Total viable cells were used for analysis (Figure 15A). Mouse splenocytes were hybridized with β-Actin (ACTB) probes, in triplicate. Total viable cells were used for analysis (Figure 15B).

Figure 15B
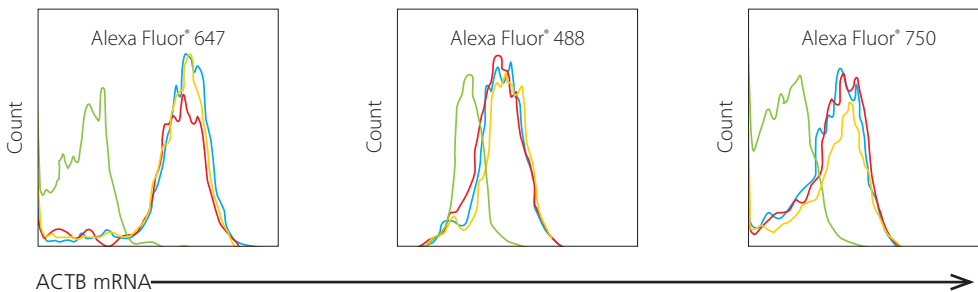
Sample types tested

PrimeFlow™ RNA Assay has been validated for use with suspension cells such as peripheral blood mononuclear cells (cryopreserved, stimulated cells and freshly isolated cells), mouse bone marrow cells, mouse tissue (fresh, stimulated and cryopreserved), cultured mammalian leukemic cell lines, and adherent cells.

Cell	Recommended Positive Control Gene
Human lymphocytes (PBMC) – fresh and cryopreserved	RPL13a, B2M
Human monocytes (PBMC) – fresh and cryopreserved	RPL13a, B2M
Mouse splenocytes (tissue) – fresh and cryopreserved	ACTB, RPL13a
Mouse thymocytes (tissue)	ACTB, RPL13a
Mouse bone marrow	ACTB
Human monocytic lymphoma, U937	RPL13a, B2M
Human T cell lymphoma, Jurkat	RPL13a, B2M
Human cervical carcinoma, HeLa*	RPL13a, GAPDH
Human lung carcinoma, PC9*	RPL13a, GAPDH

*adherent cells

Adherent cell data
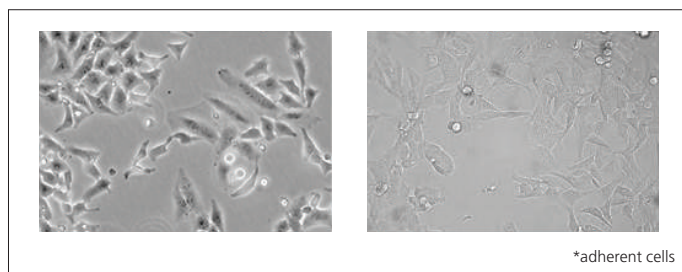
*adherent cells

Figure 16: Human cervical carcinoma cells, HeLa vs Human lung adenocarcinoma cells, PC9
Adherent cell lines, HeLa and PC9, were detached using either EDTA or typrsin and then subjected to PrimeFlow™ RNA Assay. HeLa and PC9 cells were hybridized with human KRT19, PPIB, and GAPDH probe sets.