PrimeFlow RNA Assay technology verification

Introduction

The Invitrogen™ 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 assay uses fluorescence *in situ* hybridization (FISH) to simultaneously detect as many as four RNA transcripts in a single cell using a standard flow cytometer. The PrimeFlow RNA Assay can detect mRNA, long noncoding RNA, and microRNA, as well as viral RNA and telomere DNA. The PrimeFlow RNA Assay is compatible with cell surface and intracellular staining using common flow cytometry fluorophores.

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 (Figure 1). 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 the PrimeFlow RNA Assay, specific cell populations may be analyzed for unique transcript levels, or cell subsets may be evaluated over time to determine transcriptional regulation and protein expression simultaneously. Such valuable insights can help answer previously intractable questions and have broad implications across multiple fields of biology.

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

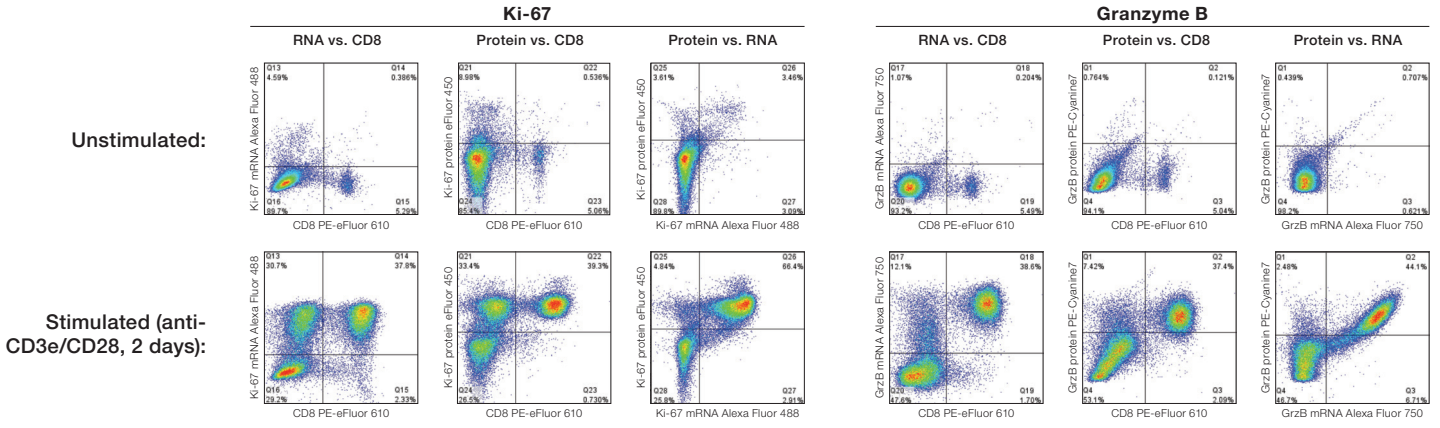

Figure 1. Example data set for the PrimeFlow RNA Assay in action. C57Bl/6 splenocytes were left unstimulated, or stimulated for 2 days with Invitrogen™ eBioscience™ Anti–Mouse CD3e and CD28 Functional Grade Monoclonal Antibodies, and in the presence of Invitrogen™ eBioscience™ Protein Transport Inhibitor Cocktail for the last 3 hours of culture, followed by analysis using the PrimeFlow RNA Assay. Cells were fixed and permeabilized using the PrimeFlow RNA Assay buffers and protocol, then intracellularly stained with Invitrogen™ eBioscience™ Anti–Mouse CD8α PE-eFluor™ 610, Anti–Mouse Ki-67 eFluor™ 450, and Anti–Mouse Granzyme B PE-Cyanine7 antibodies. Cells were then hybridized with Invitrogen™ Type 6 Mouse Granzyme B Alexa Fluor™ 750, Type 4 Mouse Ki-67 Alexa Fluor™ 488, and Type 1 Mouse β-actin Alexa Fluor™ 647 probe sets.

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Assay technology

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

The 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., as in PCR) to enable consistent results, a common concern for PCR-based assays.

In the PrimeFlow RNA Assay, target-specific probe sets contain 20–40 oligonucleotide pairs that hybridize to the target RNA transcript. For microRNA (miRNA), a single pair of oligonucleotides hybridizes to the target RNA transcript. Signal amplification is achieved through specific hybridization between adjacent oligonucleotide pairs and bDNA structures, formed by preamplifiers, amplifiers, and fluorophore-conjugated label probes, resulting in excellent specificity, low background, and high signal-to-noise ratios (Figure 2).

PrimeFlow RNA Assay principle

The assay workflow contains several steps: surface antibody staining, fixation and permeabilization, intracellular antibody staining, target probe hybridization with RNA-specific probe sets, signal amplification using bDNA constructs, and detection by flow cytometry. For simplicity, Figure 3 illustrates an example with only two RNA targets and with only three of the 20–40 oligonucleotide probe pairs per target RNA.

Antibody staining, fixation, and permeabilization

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

Target hybridization

A target-specific probe set contains 20–40 oligonucleotide pairs that hybridize to specific regions across the target RNA sequence. Subsequent signal amplification requires that the two oligonucleotides of each pair bind to the target RNA in adjacent positions. Four types of probe sets are currently available for detection of RNA: type 1 (Alexa Fluor™ 647 label), type 4 (Alexa Fluor™ 488 label), type 6 (Alexa Fluor™ 750 label), and type 10 (Alexa Fluor™ 568 label). When detecting more than one RNA target in a single sample, each probe set must be of a unique type to differentiate its signal from the others (Table 1).

Signal amplification

Signal amplification by bDNA technology is achieved through a series of sequential hybridizations that form a tree-like structure. A preamplifier molecule hybridizes specifically to a pair of bound oligonucleotide probes to form the trunk of the tree. Multiple amplifier molecules hybridize to their respective preamplifier 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 probe set containing 20 oligonucleotide pairs bind to the target RNA transcript, 8,000-fold amplification can be achieved.

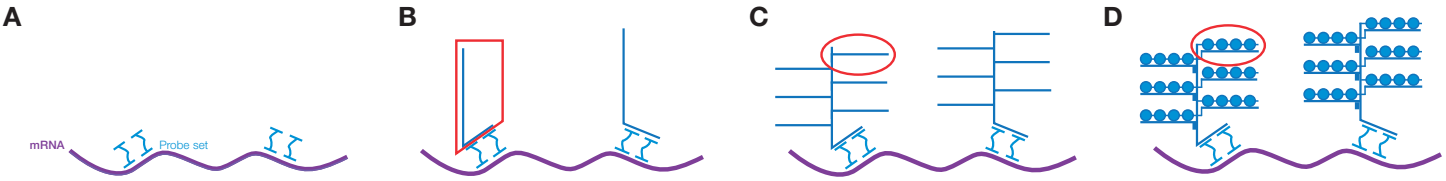


Figure 2. Signal amplification by sequential hybridization of oligonucleotides. (A) Gene-specific probe sets are hybridized to target RNA transcripts. **(B)** Preamplifier (“trunk”) binds to a probe set. **(C)** Amplifiers (“branches”) bind to multiple sites on the preamplifier. **(D)** Fluorophore-conjugated label probes (“leaves”) bind to multiple sites on the amplifiers.

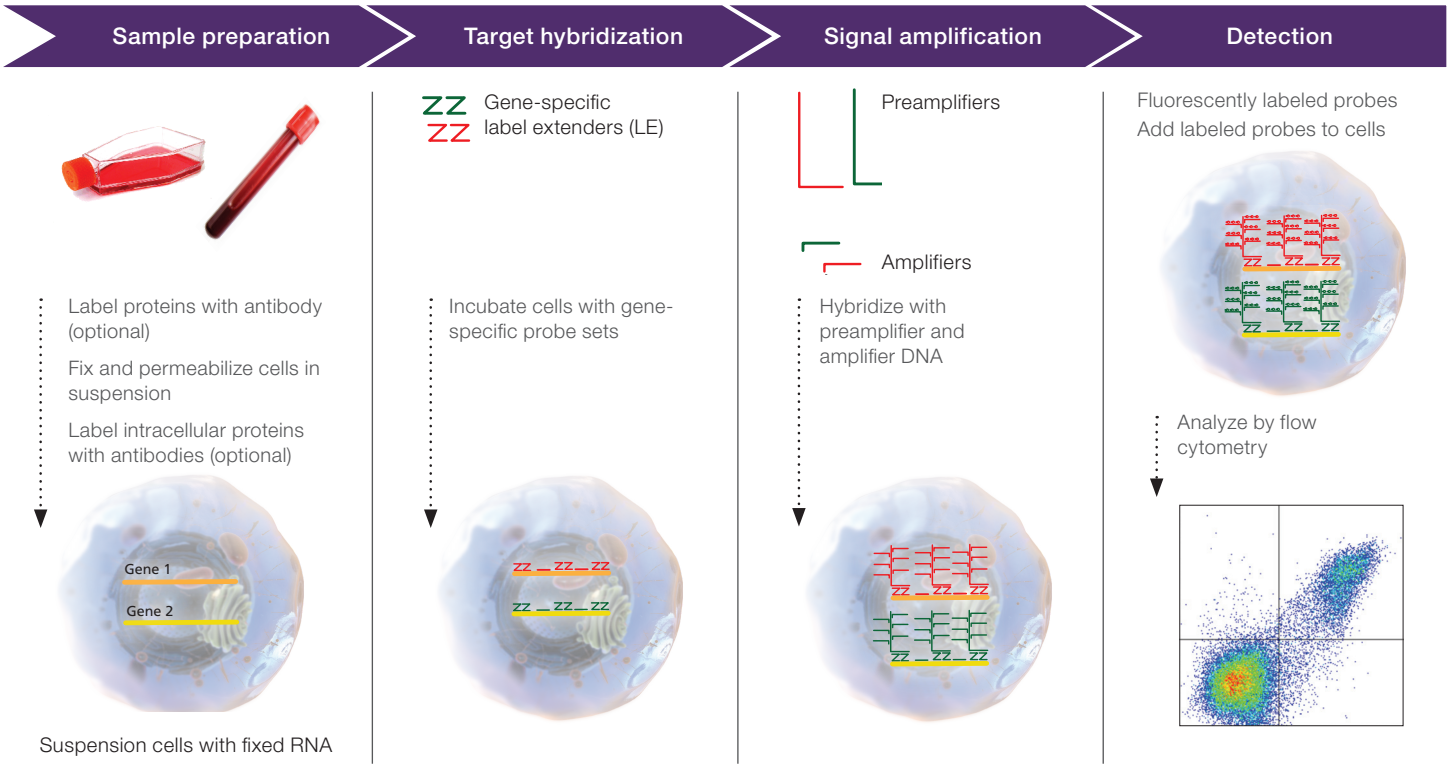


Figure 3. PrimeFlow RNA Assay workflow.

Fluorescence detection

Upon completion of the assay, target RNA is detected by analyzing the cells on a standard flow cytometer equipped with 633–647 nm, 561 nm, and 488 nm lasers and appropriate filter configurations to capture the fluorescence signals (Table 1).

Table 1. Probe set hybridization.

Probe set type	Fluorophore label	Excitation wavelength (max)	Emission wavelength (max)	Laser excitation wavelength	Bandpass filter recommendation
Type 1	Alexa Fluor 647	647 nm	668 nm	633–647 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	633–647 nm	780/60
Type 10	Alexa Fluor 568	561 nm	610 nm	561 nm	610/20

Precision

Intra-assay variability

To assess intra-assay variability, samples from stimulated and unstimulated human peripheral blood mononuclear cells (PBMCs) were divided into seven replicates and assessed for expression of ribosomal protein L13a (RPL13a), a positive control expressed in all PBMCs, and interferon gamma (IFNγ), induced only upon stimulation in a subset of lymphocytes. As shown in Figure 4, the assay shows robust intra-assay performance, with a coefficient of variation (CV) less than 11% for both RPL13a and IFNγ.

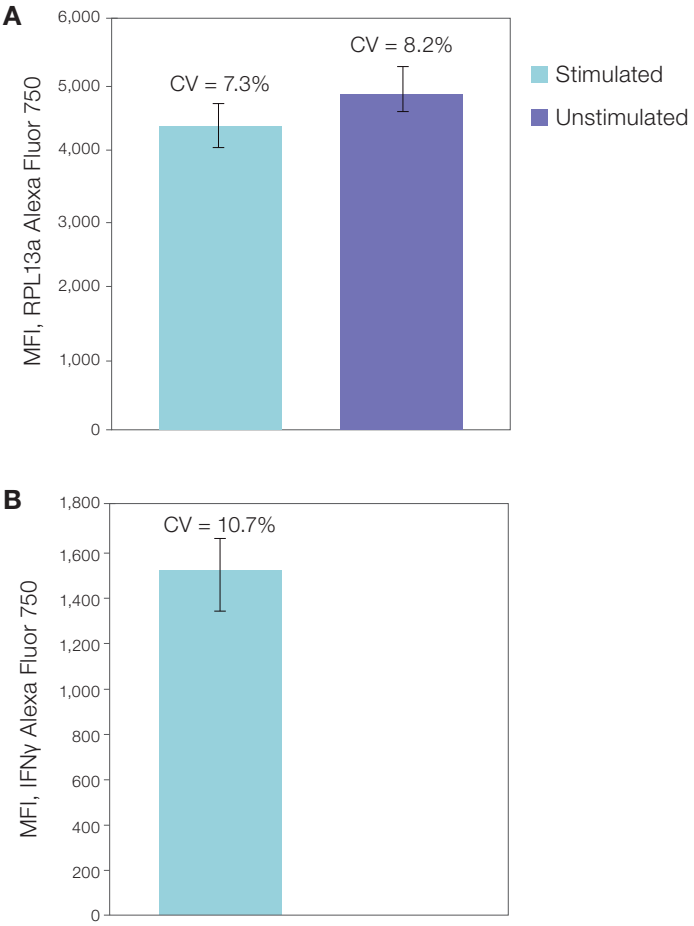


Figure 4. Intra-assay variability. A single sample of stimulated or unstimulated human PBMCs was divided into 7 replicates and assessed for expression of **(A)** RPL13a mRNA in total lymphocytes or **(B)** IFNγ mRNA in IFNγ-positive events. Median fluorescence intensity (MFI) data shown are the average of the 7 replicates, and error bars represent standard deviation.

To assess the effect of target probe handling on assay variability, a sample of mouse splenocytes was divided into three replicates and assayed for expression of β-actin, RPL13a, β2-microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and peptidyl-prolyl cis-trans isomerase B (PPIB). Target probes were diluted independently for each replicate. The CVs were typically less than 10%; one assay for RPL13a had a CV of ~12% (Figure 5).

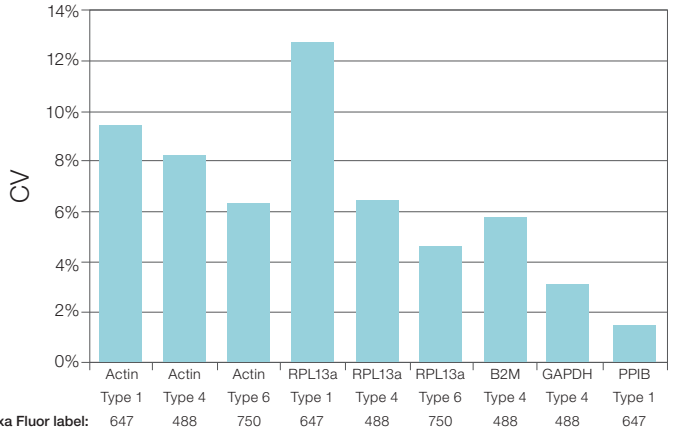


Figure 5. Contribution of target probe dilution to assay variability. Splenocytes from C57Bl/6 mice were assessed for expression of several positive control genes. Samples were prepared in triplicate, with independent dilutions of target probes made for each sample. The CV of the MFI for each assay is shown.

Operator variability

To assess the contribution of operator variability, aliquots of a single culture of U937 cells were tested in triplicate for GAPDH, PPIB, and B2M expression by two different technicians. No-probe controls were used as negative controls, and samples were analyzed using the same flow cytometer settings. For each operator, the CV of the MFI of triplicate samples was less than 6%, consistent with previous data for intra-assay variability (Figure 4). 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 6).

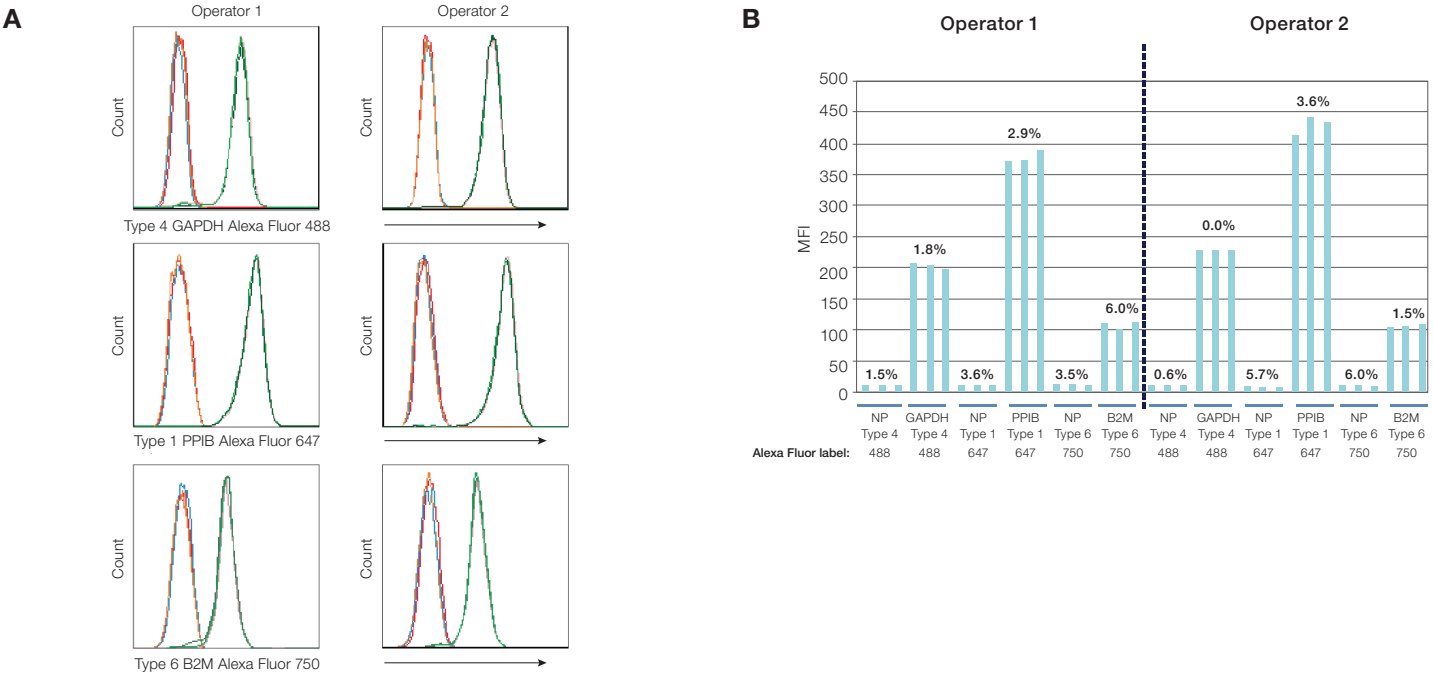


Figure 6. Contribution of operator variability. U937 cells were assessed for expression of several positive-control genes. Samples were prepared in triplicate by two technicians. **(A)** Histogram overlays of the triplicates for no-probe controls and target probes are shown. **(B)** The MFI is shown for each sample. Values above the bars represent CV. NP = no probe.

Additional studies to assess variation between operators were run with samples containing only a subpopulation of positive cells. C57Bl/6 splenocytes were stimulated for 3 days with anti-CD3e and anti-CD28 antibodies, and assayed for expression of Ki-67 and granzyme B mRNA. The samples were divided among four technicians who independently performed the assay. The mean percentages of positive events for Ki-67 or granzyme B were 18.6% and 30.6%, respectively Figure 7, upper graphs. The MFIs of the mRNA-positive events are shown in the bar graph, and in this case the CVs were 12–15% Figure 7, lower graph, in a range similar to the results obtained with positive-control genes in U937 cells (Figure 6B).

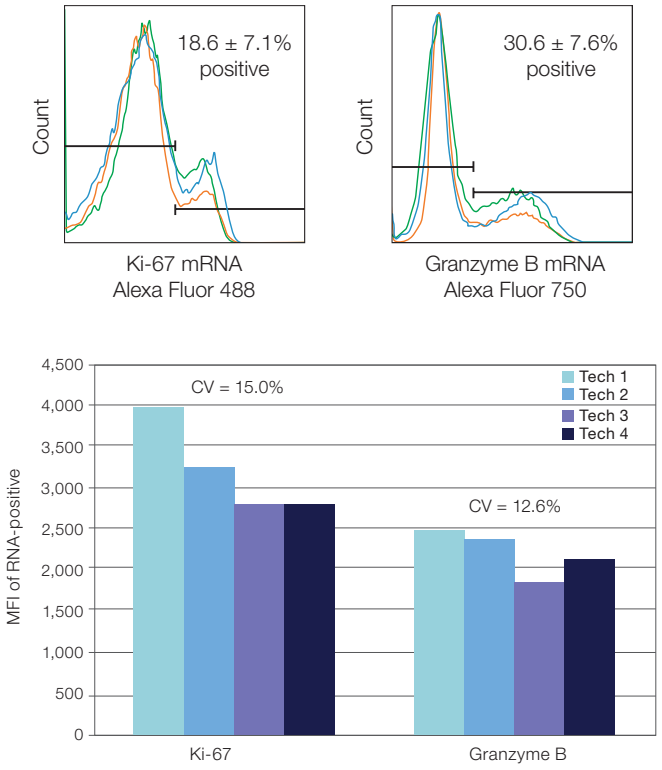


Figure 7. Contribution of operator variability in a bimodal expression model. Mouse splenocytes were stimulated with eBioscience Anti–Mouse CD3e and CD28 Functional Grade Monoclonal Antibodies for 3 days, with the addition of brefeldin A and monensin in the last 2 hours. The cells were stained with eBioscience Anti–Mouse CD8α PE-eFluor 610, Anti–Mouse Ki-67 eFluor 450, and Anti–Mouse Granzyme B PE-Cyanine7, followed by hybridization of Invitrogen™ Type 4 Mouse Ki-67 Alexa Fluor 488 and Type 6 Mouse Granzyme B Alexa Fluor 750 probe sets. Cells in the lymphocyte gate or the mRNA-positive events were used for analysis.

Day-to-day variability

To understand day-to-day variation, human PBMCs stimulated with Invitrogen™ eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitor cocktail) were analyzed fresh, or cryopreserved and analyzed 1 week later. The percentages of positive events were virtually unchanged between the fresh and cryopreserved samples.

Table 2. Day-to-day variation of the PrimeFlow RNA Assay.*

	IFNγ mRNA**	IFNγ protein**
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

* Normal human PBMCs were stimulated and analyzed immediately (experiment 1) or were cryopreserved and analyzed 1 week later (experiment 2) for the expression of IFNγ mRNA and protein. Samples were surface stained with eBioscience Anti–Human CD8α PE-eFluor 610 antibody followed by hybridization of Invitrogen™ Human IFNγ Alexa Fluor™ 750 probe set.

** Data represent the percentage of IFNγ$^+$ events, gated on viable CD8$^+$ cells.

Sensitivity and specificity

The central dogma of molecular biology states that genetic information flows from DNA to RNA to protein. However, studies have shown that the correlation between levels of RNA and protein products vary widely. Here we demonstrate how the 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 PBMCs were stimulated with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) for as long as 5 hours. Using the PrimeFlow RNA Assay, the cells were assayed for IFNγ or TNFα mRNA and protein expression in CD8$^+$ or CD8$^-$ lymphocytes at hourly intervals. As shown in Figure 8, both CD8$^+$ and CD8$^-$ lymphocytes responded to stimulation, but IFNγ and TNFα mRNA and protein each exhibited unique kinetics depending on the lymphocyte subset being analyzed. Of note, while IFNγ mRNA was rapidly upregulated after 1 hour of stimulation, IFNγ protein was not detected until the second hour. In contrast, TNFα mRNA and protein were both upregulated within the first hour, and although CD8$^+$ cells maintained TNFα mRNA, the CD8$^-$ lymphocytes downregulated TNFα mRNA, and showed a subsequent slow decline in TNFα protein levels. Thus, the 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 can be used to elucidate the kinetics of mRNA and protein expression.

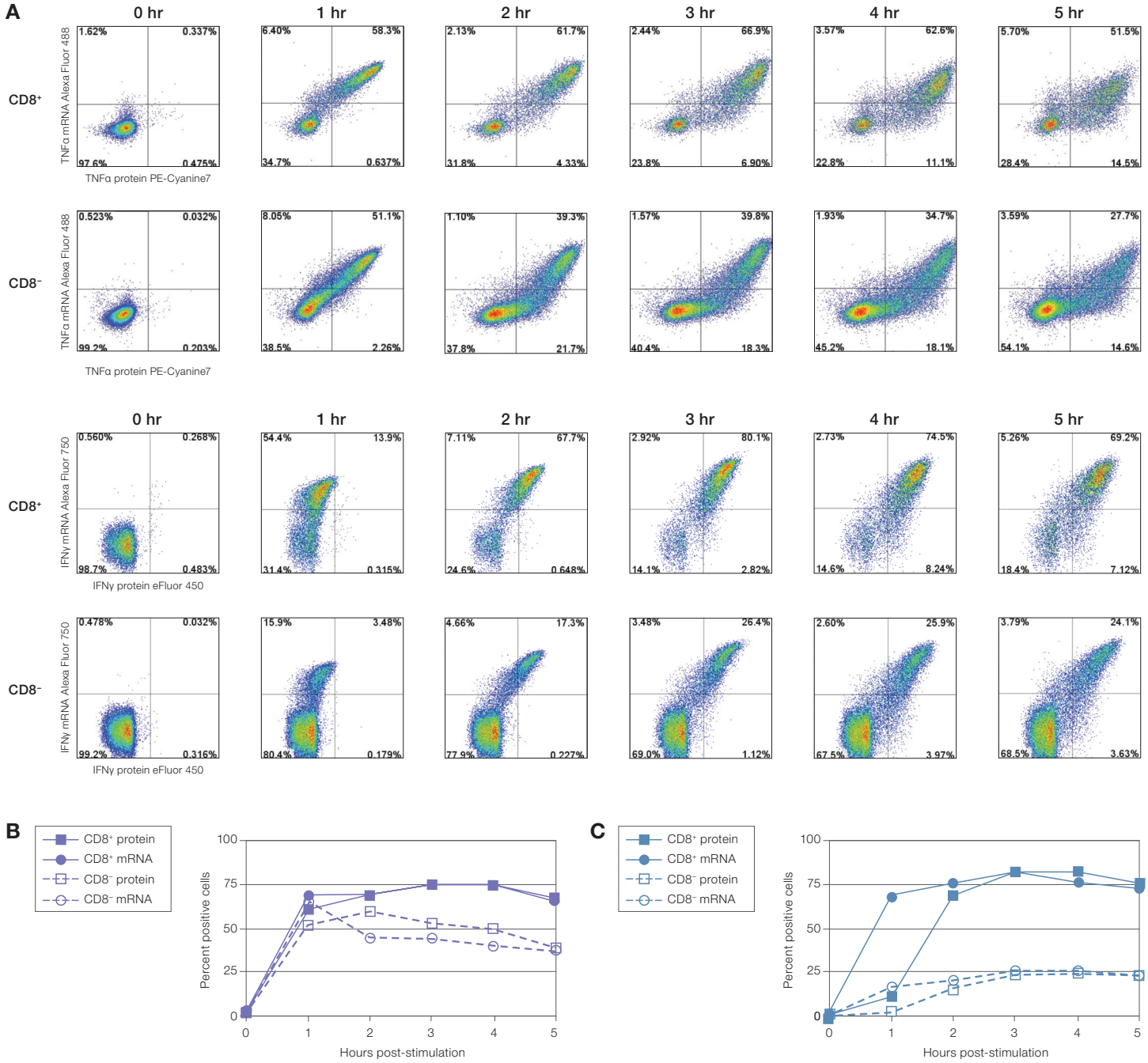
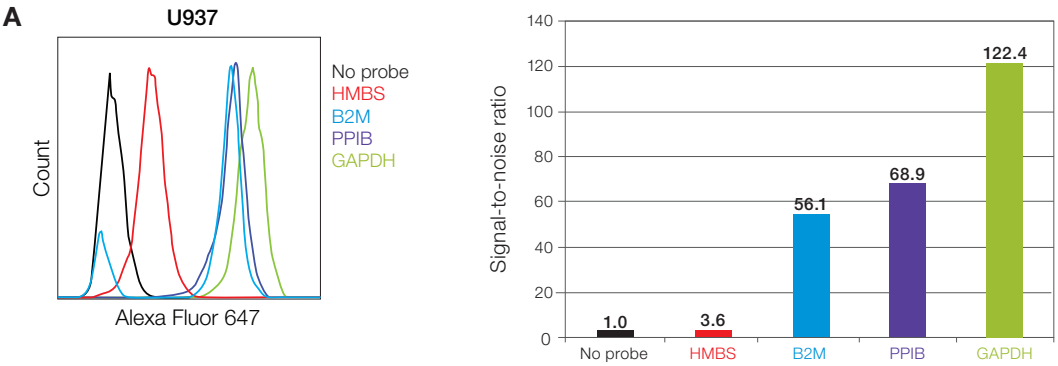


Figure 8. Correlation and kinetics of IFNγ and TNFα transcription and translation. Normal human PBMCs were stimulated with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) for 0–5 hr, then subjected to the PrimeFlow RNA Assay. **(A)** Cells were intracellularly stained with eBioscience Anti–Human CD8α PE-eFluor 610, Anti–Human IFNγ eFluor 450, and Anti–Human TNFα PE-Cyanine7 antibodies, followed by hybridization with Invitrogen™ Human TNFα Alexa Fluor 488 and Human IFNγ Alexa Fluor 750 probe sets. $CD8^+$ or $CD8^-$ cells in the lymphocyte gate were used for analysis. Results are plotted for **(B)** TNFα and **(C)** IFNγ expression.

Orthogonal verification

The PrimeFlow RNA Assay was evaluated by examination of targets previously measured by the Invitrogen™ QuantiGene™ Plex Assay, a hybridization-based assay using the Luminex® xMAP® magnetic bead platform. With the PrimeFlow assay on U937 cells, GAPDH had the highest MFI and HMBS had the lowest MFI (Figure 9A). These results are consistent with the expression levels of these targets determined using the QuantiGene Plex Assay (data not shown). Signal-to-noise ratios were calculated relative to the no-probe control sample and are shown in Figure 9A. Next, we examined the same targets in normal human monocytes and lymphocytes (Figure 9B). 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 mRNA in monocytes. Primary human lymphocytes diverged even further, with 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 data from the QuantiGene Plex Assay, 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 per cell by the PrimeFlow RNA Assay.



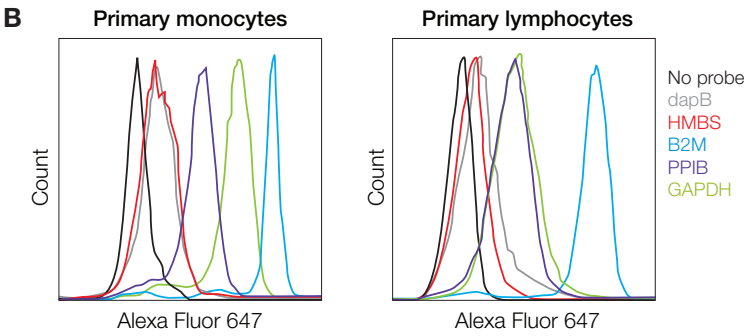


Figure 9. Orthogonal verification of PrimeFlow RNA Assays. (A) U937 or **(B)** normal human PBMCs were hybridized to a series of Invitrogen™ Type 1 Positive Control Alexa Fluor 647 probe sets, following the PrimeFlow RNA Assay protocol. The signal-to-noise ratios were calculated for the positive control targets relative to the no-probe control.

Correlation of data from QuantiGene Plex and TaqMan Assays

To demonstrate the accuracy of the QuantiGene Plex Assay, measurements of twenty transcripts from two reference RNA samples were made and compared to data from Applied Biosystems™ TaqMan® Assays. Reference RNA samples included human brain total RNA and universal human reference RNA [1]. As shown in Figure 10, there is excellent correlation between the QuantiGene Plex and TaqMan Assays. Together, these studies show consistency among the TaqMan, QuantiGene Plex, and PrimeFlow RNA Assays.

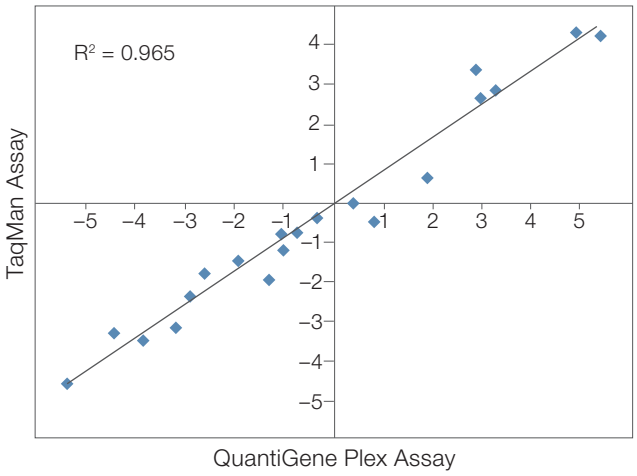


Figure 10. Comparison of fold changes in RNA levels (human brain total RNA vs. universal human reference DNA) as measured by QuantiGene Plex and TaqMan Assays.

Specificity of bDNA technology

The bDNA technology achieves high target specificity with the use of oligonucleotide pairs—a design resulting in signal amplification only when two adjacent (“left” and “right”) target probe oligonucleotides 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 (Figure 11). The fluorescence 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 a bacterial gene yielded no specific signal.

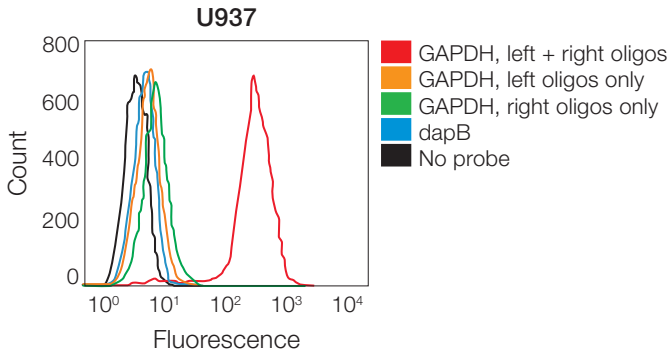


Figure 11. Specificity of the PrimeFlow RNA Assay. Flow cytometry histogram of GAPDH RNA detection in U937 cells.

Assay channel sensitivity

The PrimeFlow RNA Assay is capable of detecting four different RNA targets in a single cell through the use of four different fluorophores. However, each RNA deetection channel provides different levels of sensitivity. To understand the relative sensitivity of the four RNA detection channels, we assessed the expression level of HMBS mRNA in human U937 cells using each of the four probe set types: type 1 (Alexa Fluor 647), type 4 (Alexa Fluor 488), type 6 (Alexa Fluor 750), and type 10 (Alexa Fluor 568) (Figure 12A). The signal-to-noise ratio relative to the no-probe control was calculated, and a positive signal was defined as a signal-to-noise ratio of ≥2. As shown, HMBS expression was detectable using type 1 and type 10 target probes but not type 4 and type 6 target probes.

Fluorescence imaging can detect RNA at the subcellular level with higher resolution and sensitivity. Imaging results of samples measured with the PrimeFlow RNA Assay indicate that HMBS mRNA is expressed at ~10 copies per cell in U937 cells. While not able to robustly detect HMBS expression, type 4 and type 6 target probes were able to discern signal for POLR2A with signal-to-noise ratios of 4.6 (type 4) and 4.5 (type 6) (Figure 12B). POLR2A mRNA is found to be expressed at ~30 copies per cell by fluorescence imaging analysis. Taken together, we recommend type 1 (Alexa Fluor 647) or type 10 (Alexa Fluor 568) probe sets for genes with low or unknown levels of expression, and type 4 (Alexa Fluor 488) or type 6 (Alexa Fluor 750) probe sets for genes with medium to high levels of expression. As with any multicolor 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.

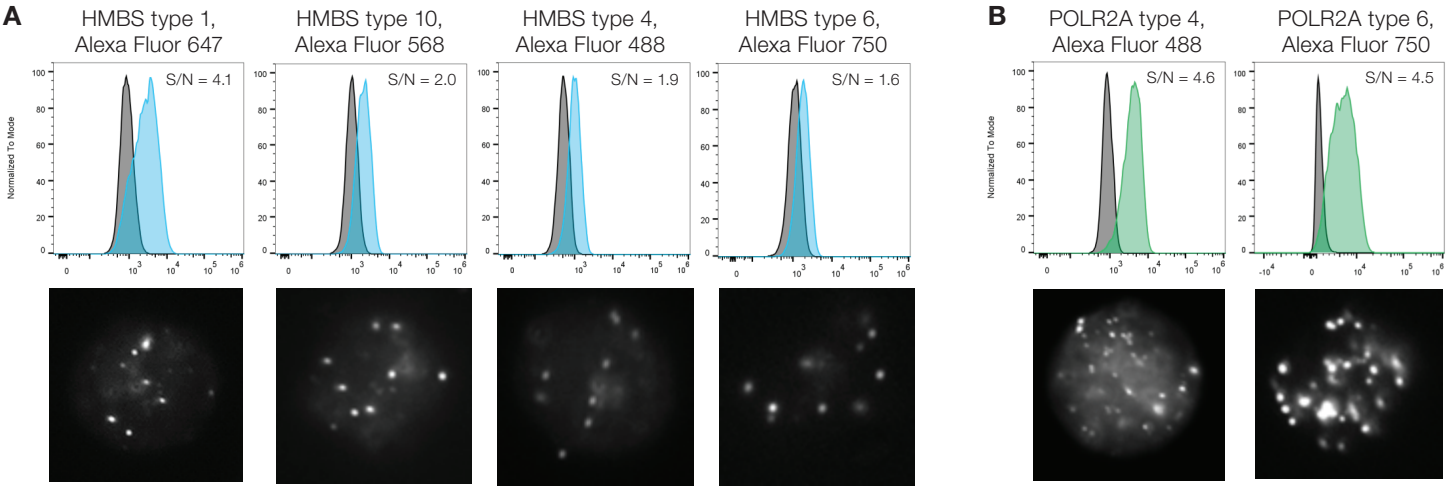


Figure 12. Sensitivity of RNA detection channels. (A) HMBS and **(B)** POLR2A expression levels were assessed using the indicated probe sets. Flow cytometry and fluorescence imaging results are shown for each sample. S/N = signal-to-noise ratio

Sample types tested

The PrimeFlow RNA Assay has been verified for use with suspension cells such as PBMCs (cryopreserved, stimulated, and freshly isolated), mouse bone marrow cells, mouse tissue (cryopreserved, stimulated, and freshly isolated), cultured mammalian leukemic cell lines, and adherent cells (Table 3 and Figure 13).

Table 3. Sample types tested with PrimeFlow RNA Assays.

Cell type	Recommended positive control
Human lymphocytes (PBMCs), fresh and cryopreserved	RPL13a, B2M
Human monocytes (PBMCs), 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.

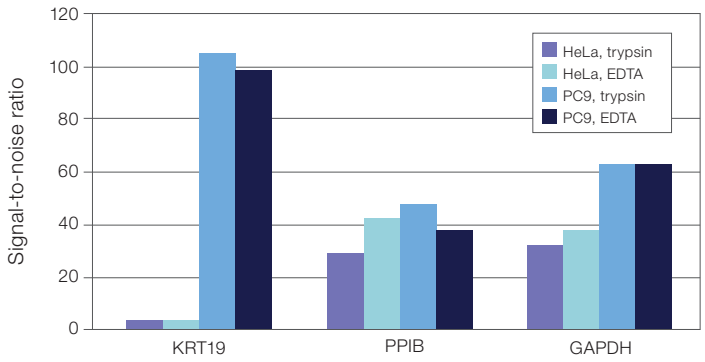


Figure 13. Compatibility of the PrimeFlow RNA Assay with HeLa human cervical carcinoma cells vs. PC9 human lung adenocarcinoma cells. HeLa and PC9 adherent cell lines were detached using either EDTA or trypsin and then subjected to the PrimeFlow RNA Assay. HeLa and PC9 cells were hybridized with human KRT19, PPIB, and GAPDH probe sets.

Conclusion

The PrimeFlow RNA Assay is capable of simultaneous detection of RNA and protein within millions of cells at single-cell resolution. With the PrimeFlow RNA Assay, researchers can now incorporate the simultaneous analysis of RNA transcripts and proteins into a better understanding of single-cell dynamics, by mechanistically and phenotypically characterizing the coexpression of RNAs and functional proteins at the single-cell level. With its high sensitivity and specificity, the PrimeFlow RNA Assay is an invaluable tool for studying complex biological systems in a high-throughput manner by flow cytometry.

Reference

1. Canales RD, Luo Y, Willey JC et al. (2006) Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat Biotechnol* 24:1115-1122.

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