

# PrimeFlow™ RNA Assay

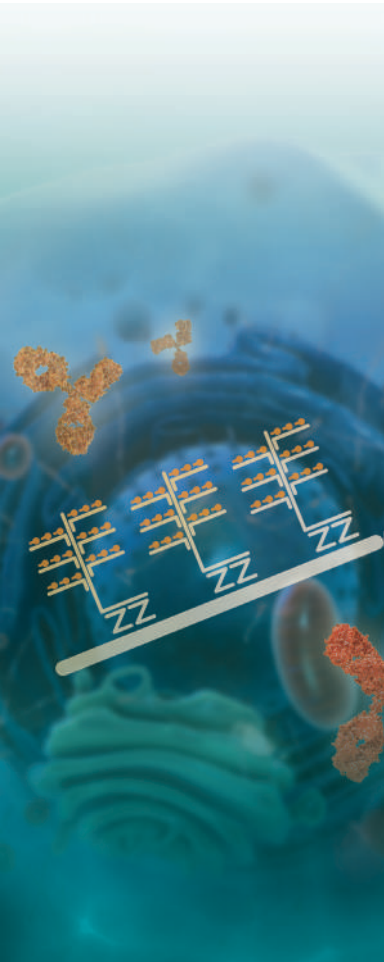
## Enter a new dimension of single-cell analysis

Detect RNA and protein simultaneously by flow cytometry

- See gene expression heterogeneity at the single-cell level
- Compare RNA and protein kinetics in the same cell
- Detect non-coding RNA in cellular subsets
- Evaluate viral RNA in infected cells
- Analyze mRNA expression levels when antibody selection is limited

PrimeFlow™ RNA Assay reveals the dynamics of RNA and protein expression within individual cells, facilitating unprecedented analysis of their correlation as cells change over time or in response to stimulus. This novel assay employs a proprietary fluorescent *in situ* hybridization (FISH) and branched DNA (bDNA) signal amplification technique, enabling simultaneous detection of as many as three RNA transcripts in a single cell using a standard flow cytometer. RNA detection may be teamed together with intracellular and cell-surface antibody staining to elevate the understanding of single-cell dynamics to a new dimension in cellular analysis.

Coupling RNA and protein expression on a flow cytometer generates multi-parametric data in heterogeneous cell populations and offers in-depth details at the single cell level. In contrast, microarrays and sequencing provide comprehensive gene expression data in bulk sample preparations. However, such bulk sample preparation can mask the individual effects of different cellular subsets. Analysis of expression levels in individual cells provide a clearer picture of transcriptional dynamics within any definable population of cells. 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.



# Compare RNA and protein kinetics in the same cell

In response to external stimuli, differential regulation of RNA transcription leads to changes in protein level. However, the levels of RNA and protein products of specific genes may vary at any given time point. Using current methods such as qPCR or microarrays, researchers must choose between measuring mRNA or protein because they cannot be measured simultaneously due to the limitations of prevailing techniques. PrimeFlow™ technology can reveal the unique kinetics of mRNA and protein in different cell subsets, and identify expression differences as they change over time in response to the stimulus.

## Correlation and kinetics of IFN $\gamma$ and TNF $\alpha$ transcription and translation

### Aims

Intracellular staining and flow cytometric analysis of lymphocytes is commonly used to assess cytokine production at the single-cell level in heterogeneous samples. Here, PrimeFlow™ RNA Assay is used in combination with intracellular antibody staining to study the kinetics of the transcription and translation of IFN $\gamma$  and TNF $\alpha$  in lymphocyte subsets.

### Results

IFN $\gamma$  mRNA was upregulated in CD8+ and CD8- lymphocytes within 1 hour after stimulation, while protein levels were not detected until 2 hours. Both IFN $\gamma$  mRNA and protein were maintained for the next 3–4 hours (Figure 1A and 1C). In contrast, TNF $\alpha$  mRNA and protein were both upregulated within 1 hour after stimulation

and expression was maintained in CD8+ cells, while expression in CD8- cells peaked between 1–2 hours and then decreased over the next 4 hours, with the decrease in mRNA preceding the decrease in protein (Figure 1B and 1D).

### Conclusions

Using PrimeFlow RNA Assay, we find that induction of IFN $\gamma$  and TNF $\alpha$  mRNA and protein exhibit unique kinetics, and that TNF $\alpha$  protein and mRNA are differentially regulated in CD8+ and CD8- lymphocytes. This assay enables the study of gene expression at the single-cell level in heterogeneous samples without the need for sorting specific subsets, as well as the ability to compare and contrast the kinetics of mRNA and protein induction.

Figure 1A

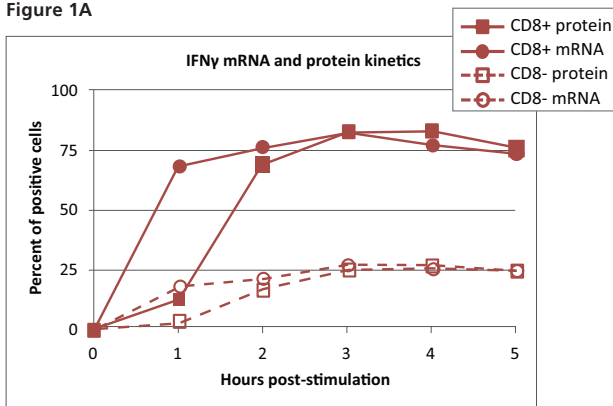
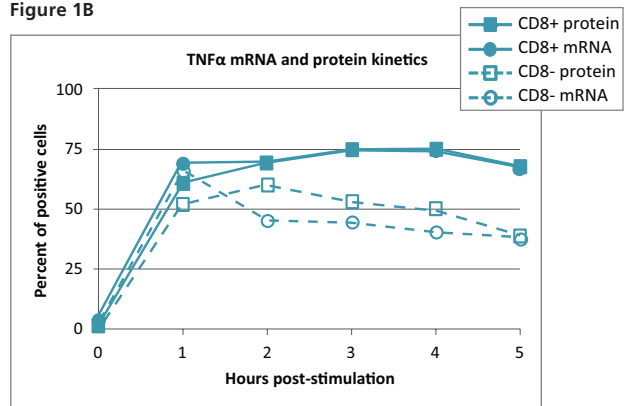


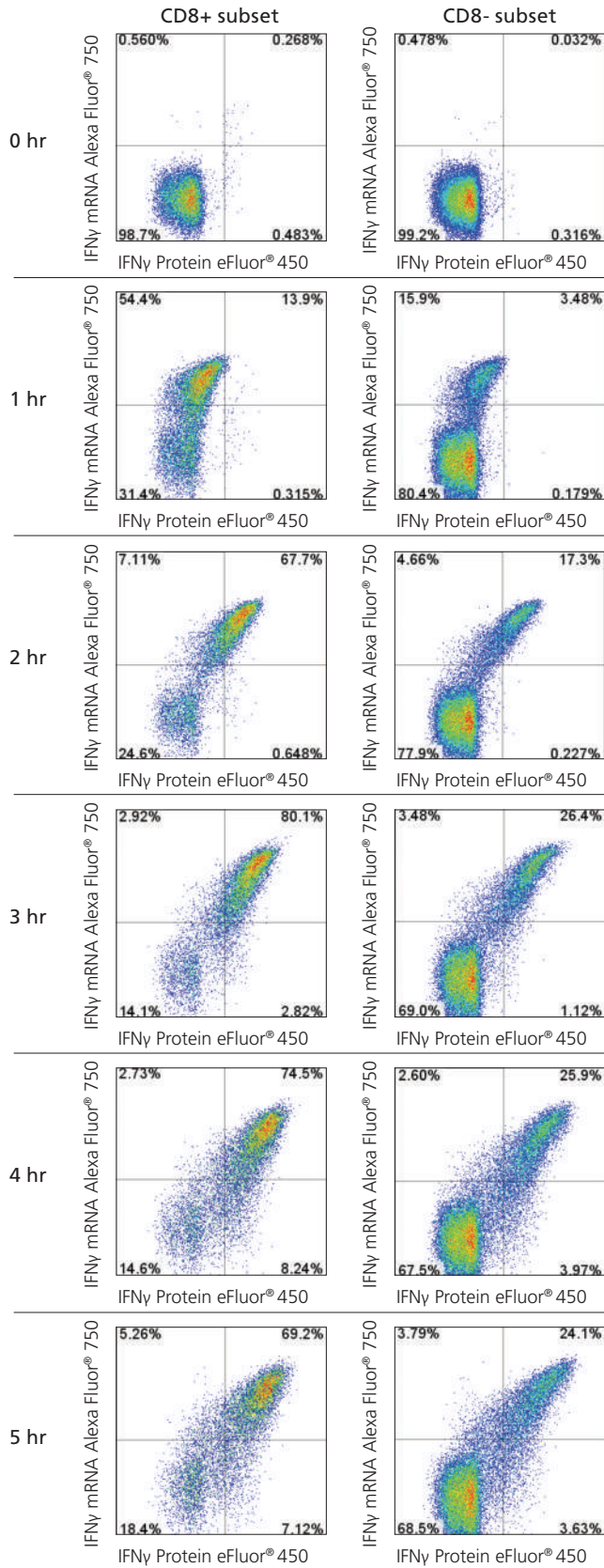
Figure 1B



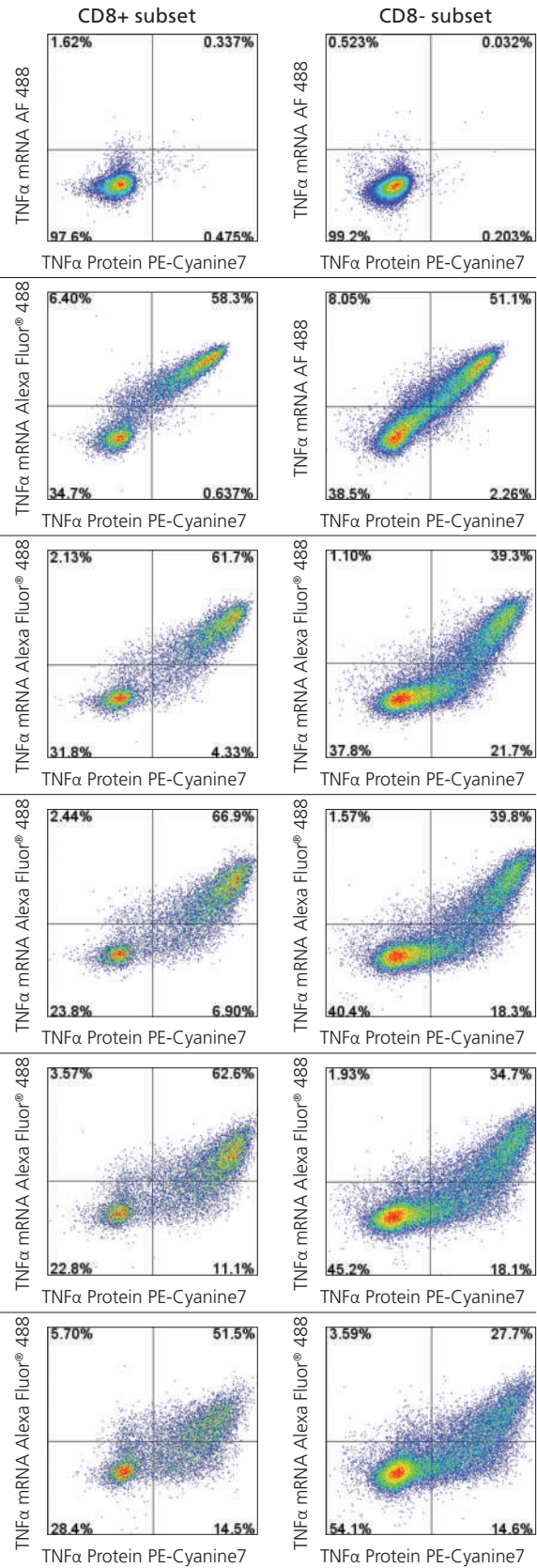
### Kinetics of IFN $\gamma$ and TNF $\alpha$ transcription and translation measured by PrimeFlow™ RNA Assay

Normal human peripheral blood mononuclear cells were stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitors) (cat. no. 00-4975) for 0-5 hours. Using PrimeFlow™ RNA Assay, cells were fixed, permeabilized, and intracellularly stained with antibodies for CD8, IFN $\gamma$ , and TNF $\alpha$ . Next, cells underwent a series of hybridization steps to label mRNA for IFN $\gamma$  and TNF $\alpha$ . Viable CD8+ and CD8- cells in the lymphocyte gate were used for analysis. Figures 1A and 1C reveal the kinetics of IFN $\gamma$ , and Figures 1B and 1D illuminate the kinetics of TNF $\alpha$ .

**Figure 1C: Human IFN $\gamma$  induction and correlation between mRNA and protein**



**Figure 1D: Human TNF $\alpha$  induction and correlation between mRNA and protein**



# Observe gene expression heterogeneity at the single-cell level

Cellular heterogeneity is present in any biological sample, from low- to high-protein levels in cell subsets or gene expression differences from cell to cell. Paradoxically, most of our understanding of gene expression is based upon bulk population averages. This analysis, although informative, often leads to conclusions that assume ensemble averages reflect the dominant biological mechanism operating within an entire population. Using such measurements and assumptions can mask the presence of rare or small subpopulations of cells or bimodal cellular behaviors, and ignores essential cell-to-cell differences. To fully understand if cellular heterogeneity contributes to biological function or contains relevant information, a single-cell approach must be applied. The PrimeFlow™ RNA Assay reveals differential responses following stimulation and, thus, the hidden story that is otherwise masked by using qRT-PCR to analyze the entire population.

## Revealing a hidden story beneath bulk masking

### Aims

mRNA is commonly assessed by quantitative RT-PCR (qRT-PCR) where cells are isolated after stimulation so that total RNA can be extracted and then undergo RT-PCR reactions. This results in amplified bulk measurements that mask the differences in gene expression that occur at the single-cell level. In this example, PrimeFlow RNA data are compared to qRT-PCR.

### Results

The same cell populations in Figure 1 (pages 2 and 3) were analyzed by qRT-PCR. Using qRT-PCR, it appears that IFN $\gamma$  and TNF $\alpha$  are initially induced at similar levels and IFN $\gamma$  plateaus, while TNF $\alpha$  declines slowly. However, with the PrimeFlow RNA assay, it is observed that TNF $\alpha$  mRNA is rapidly and highly induced at 1 hour, dramatically declines between 1–2 hours, and continues to decline more slowly 3–5 hours after stimulation. In contrast, IFN $\gamma$  is maintained throughout the time course. As shown in Figure 1, PrimeFlow RNA data can be further analyzed into CD8+ or CD8- cell subsets, which reveal additional sample heterogeneity that qRT-PCR cannot achieve from a single sample. PrimeFlow RNA Assay shows the percentage of cells expressing IFN $\gamma$  is actually less than the percentage of cells expressing TNF $\alpha$ .

### Conclusions

Examination of stimulated cells over time using current qRT-PCR technology shows similar kinetics as with PrimeFlow RNA technology. However, PrimeFlow RNA Assay uncovers the finer details of the kinetics at a single-cell level and allows users to study multiple parameters within the same sample, thereby eliminating the bulk averages previously masking cellular heterogeneity.

Figure 2A: Cytokine mRNA expression measured by qRT-PCR

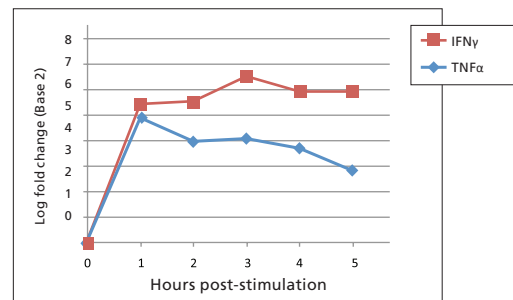
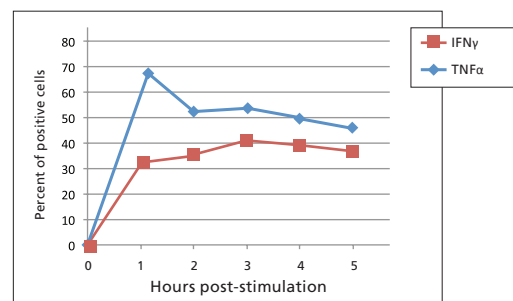


Figure 2B: Percentage of cells expressing cytokine mRNA measured by PrimeFlow™ RNA Assay



### IFN $\gamma$ and TNF $\alpha$ mRNA measured by qRT-PCR and PrimeFlow™ RNA assays

Normal human peripheral blood mononuclear cells were stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitors) (cat. no. 00-4975) for 0–5 hours (same sample from Figure 1). RNA was isolated and was analyzed by qRT-PCR (Figure 2A). Using PrimeFlow™ RNA Assay, the cells were fixed, permeabilized, and intracellularly stained with antibodies for CD8, IFN $\gamma$ , and TNF $\alpha$ . Next, cells underwent a series of hybridization steps to label mRNA for IFN $\gamma$  and TNF $\alpha$ . Viable cells in the lymphocyte gate were analyzed for percentage of cytokine-producing cells (Figure 2B).

# Analyze mRNA expression levels when antibody is limited

Flow cytometry, with its ability to look at millions of cells and multiplexing capabilities, and its straightforward workflow to detect both cell surface and intracellular proteins with single-cell resolution is the gold standard for the study of heterogeneous cell populations. However, flow cytometry historically has been constrained by the availability and adequacy of antibodies. Non-coding RNA, viral transcripts, unique model organisms/targets, or markers for which antibody development is troublesome have not benefitted from the power of flow cytometry and have required numerous disconnected experiments to analyze their impact on cell subsets. PrimeFlow™ RNA Assay can detect target-specific RNA for which available flow cytometry antibodies are nonexistent.

## IL-23R mRNA expression by flow cytometry

### Aims

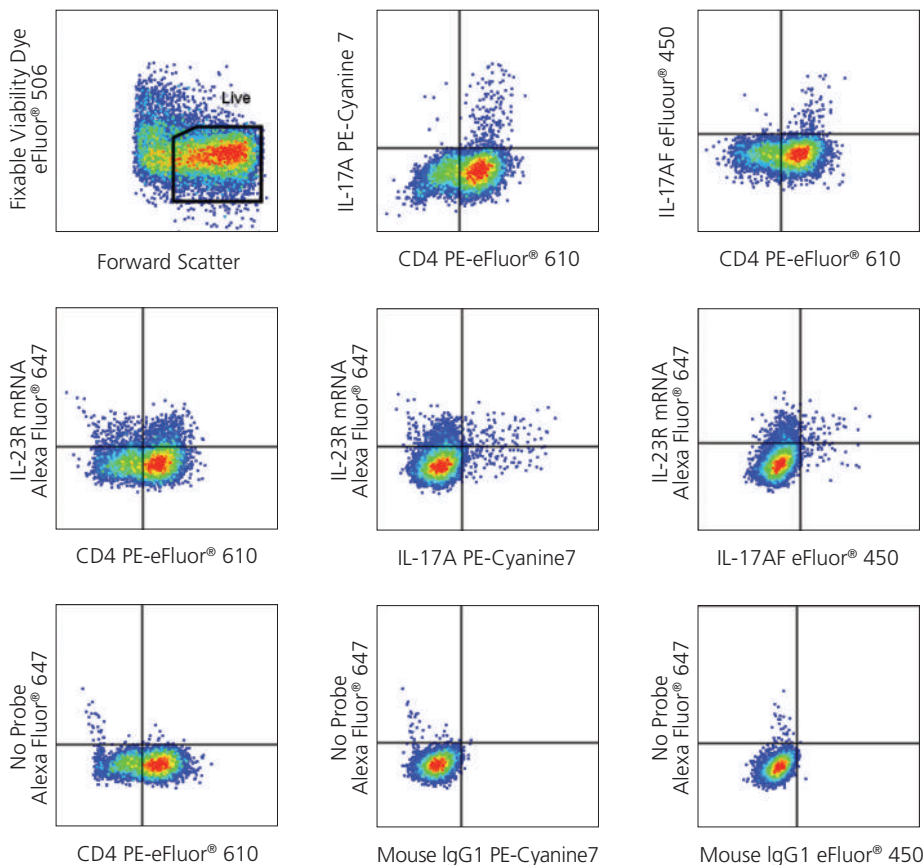
IL-23R is expressed by Th17 cells, a subset of activated CD4+ T cells that play a key role in defense of mucosal barriers against extracellular bacteria and fungi. While Th17 cell differentiation is controlled by the TGFβ, IL-6, and IL-1, IL-23 is crucial for their survival and function and has been implicated in many autoimmune diseases. The study of IL-23R is impaired by the lack of antibodies with appropriate sensitivity; most studies involving IL-23R in Th17 cells examine gene expression at the population level. The data obtained are unquestionably informative; however, it masks the actual expression heterogeneity. Here, PrimeFlow RNA Assay is used in combination with antibody staining to interrogate the heterogeneity of IL-23R mRNA expression in polarized Th17 cell subsets.

### Results

Normal human peripheral blood cells were cultured under Th17-polarizing conditions for 3 days and then restimulated. Under these conditions, only a subset of IL-17A+ or IL-17AF+ cells expressed low levels of IL-23R mRNA.

### Conclusions

A strength of the PrimeFlow RNA Assay is its ability to detect most mRNAs in individual cells without being limited by antibody availability. This applies to targets for which antibody development is difficult (e.g., IL-23R, GPCRs), unique model organisms (e.g., canine, fish), or esoteric markers for which no commercial antibodies are available. The PrimeFlow RNA Assay can also be used to detect non-protein coding RNA targets (lnc-RNA, viral transcripts) against which antibodies cannot be made.



**Figure 3: IL-23R mRNA expression in Th17 cell subsets by PrimeFlow™ RNA Assay**

Normal human peripheral blood cells were cultured under Th17-polarizing conditions for 3 days, then restimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (cat. no. 00-4975) for 5 hours. Cells were labeled with Fixable Viability Dye eFluor®506 (cat. no. 65-0866), fixed and permeabilized using the PrimeFlow™ RNA Assay buffers, then intracellularly stained with Anti-CD4 PE-eFluor®610, Anti-IL-17A PE-Cyanine7, and Anti-IL-17AF eFluor®450. Next, cells underwent a series of hybridization steps to label mRNA for IL-23R.



# Evaluate viral RNA in infected cells

Coinfection by multiple virus particles can lead to increased pathology and morbidity as is the case of Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) coinfections seen in older AIDS patients or super-infection with Hepatitis B and HCV. Understanding the lack of virus interference in these cases, on a per-cell basis, may have implications in the future design and delivery of vaccines. To date, there are no robust methods to track and study individual coinfecting cells. PrimeFlow™ RNA Assay can be used for the direct detection of multiple viral transcripts within a single cell by flow cytometry, thus facilitating the detailed study of coinfecting populations of cells.

## Detection of Hepatitis C viral RNA in coinfecting human hepatocytes

### Method

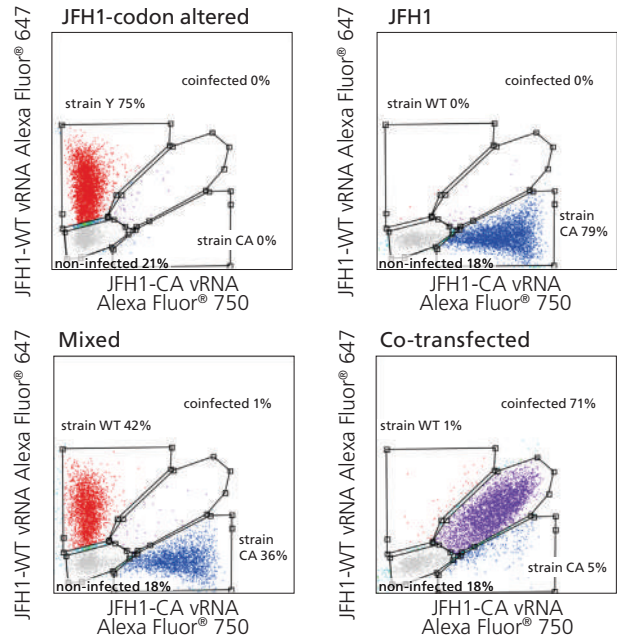
Hepatocytes were coinfecting with two strains of HCV, and JFH1-wild type (WT), and JFH1-codon altered (CA). Probes were designed to differentiate the two strains of HCV to study dominance relationships in coinfecting cells. These probes were then applied using FlowRNA and ViewRNA™ assays.

### Results

FlowRNA, clearly defined JFH1-wild type single positive, JFH1-codon altered single positive and double positive populations were observed. The orthogonal ViewRNA assay was used to verify that the probe design was highly specific for the indicated strains of Hepatitis C.

### Conclusions

The FlowRNA assay is the first commercial assay that can study individual cells that have been coinfecting.

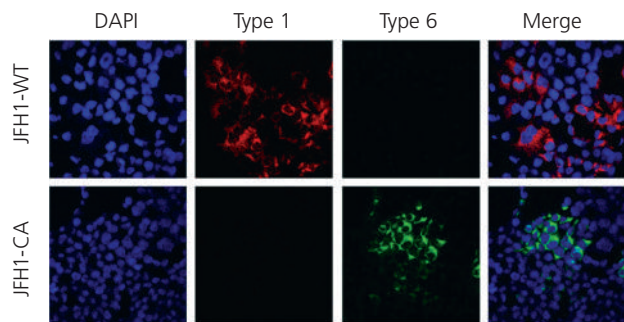


**Figure 4: Coinfecting viral RNA proof of principle\***

Hepatocytes were infected with JFH1-WT strain (top left), JFH1-CA (top right). Hepatocytes from top left and top right were mixed post infection (bottom left) or hepatocytes were coinfecting with JFH1-WT and JFH1-CA (bottom right). Using QuantiGene® FlowRNA Assay, hepatocytes were analyzed for strain-specific viral RNA.

**Figure 5: Visualization of specificity\***

Hepatocytes infected with JFH1-WT (top row) or JFH1-CA (bottom row) were analyzed using the ViewRNA Assay and the same Target Probe Sets used in the FlowRNA Assay.



\* Data courtesy of Nicholas J. van Buuren, Ph.D. Microbiology and Immunology, Karla Kirkegaard (PI) Stanford University School of Medicine. Data obtained using version 1, QuantiGene® FlowRNA. Both QuantiGene FlowRNA Assay and PrimeFlow™ RNA Assay have the same RNA hybridization and branched DNA amplification protocol.

# Create new discoveries and challenge current conventions

Most diseases, such as cancer, are complex and highly heterogeneous. With its ability to look at millions of single cells in a high-throughput manner, flow cytometry is one of the most prevalent technologies to study complex disease. However, there is still a great need to improve therapeutic targeting and efficacy through improved understanding of disease states. RNA detection, teamed together with intracellular and cell-surface antibody staining, elevates the understanding of single-cell dynamics to a new dimension, thereby allowing researchers to further unmask these complex diseases with a unique tool.

## Cell cycle regulation

### Aims

Proliferation and cell cycle progression are tightly controlled biological processes mediated by cyclins and cyclin inhibitors. Each phase of the cell cycle can be characterized by the expression of specific cyclins; however, cells must be synced *in vitro* in order to assay the kinetics of cyclin expression, using bulk sample methods currently available. Here, PrimeFlow™ RNA Assay is applied for the detection of cyclins in unsynchronized U937 cells, untreated or treated with nocodazole, allowing for analysis of cells in different stages of the cell cycle.

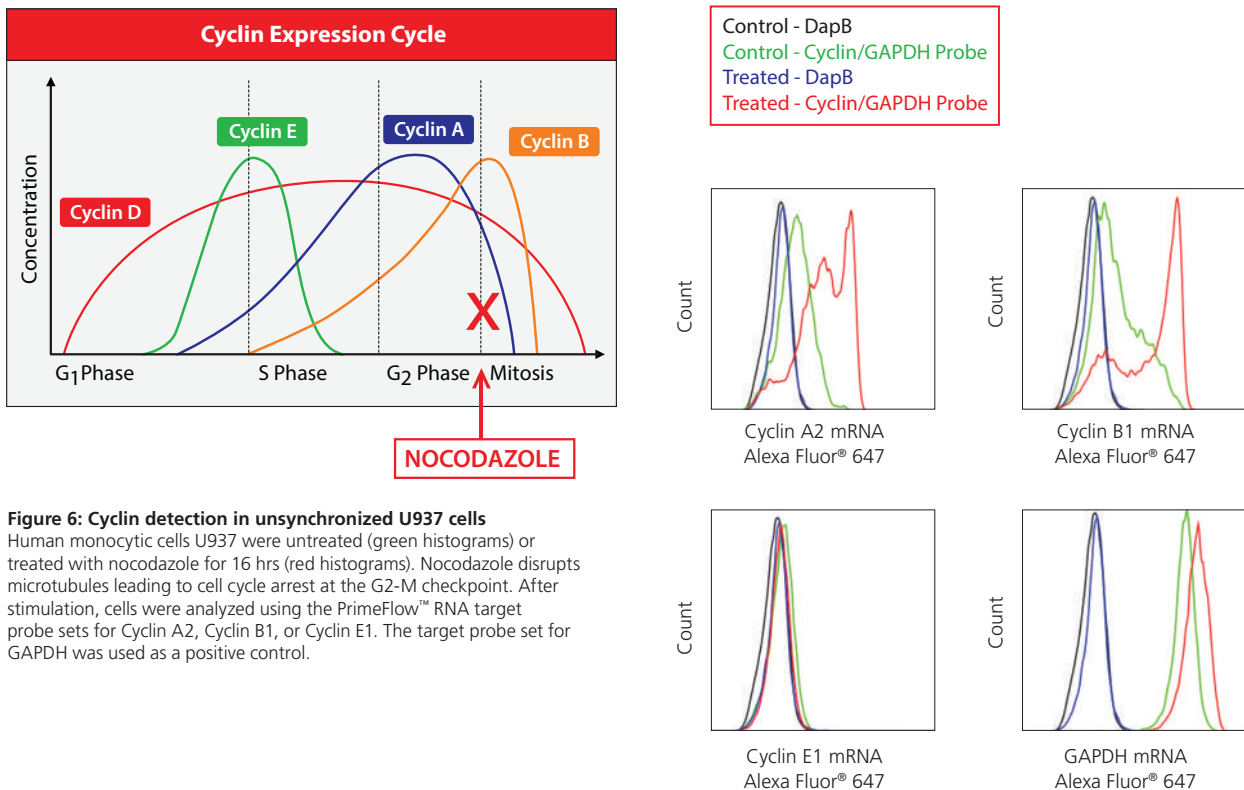
### Results

U937 cells showed upregulation of Cyclins A and B mRNA, but no Cyclin E mRNA in the treated cells in comparison to the untreated cells. Positive control gene (GAPDH) expression

was minimally affected by nocodazole treatment. Therefore, the mechanics of Nocodazole were confirmed with PrimeFlow RNA Assay (Figure 6).

### Conclusions

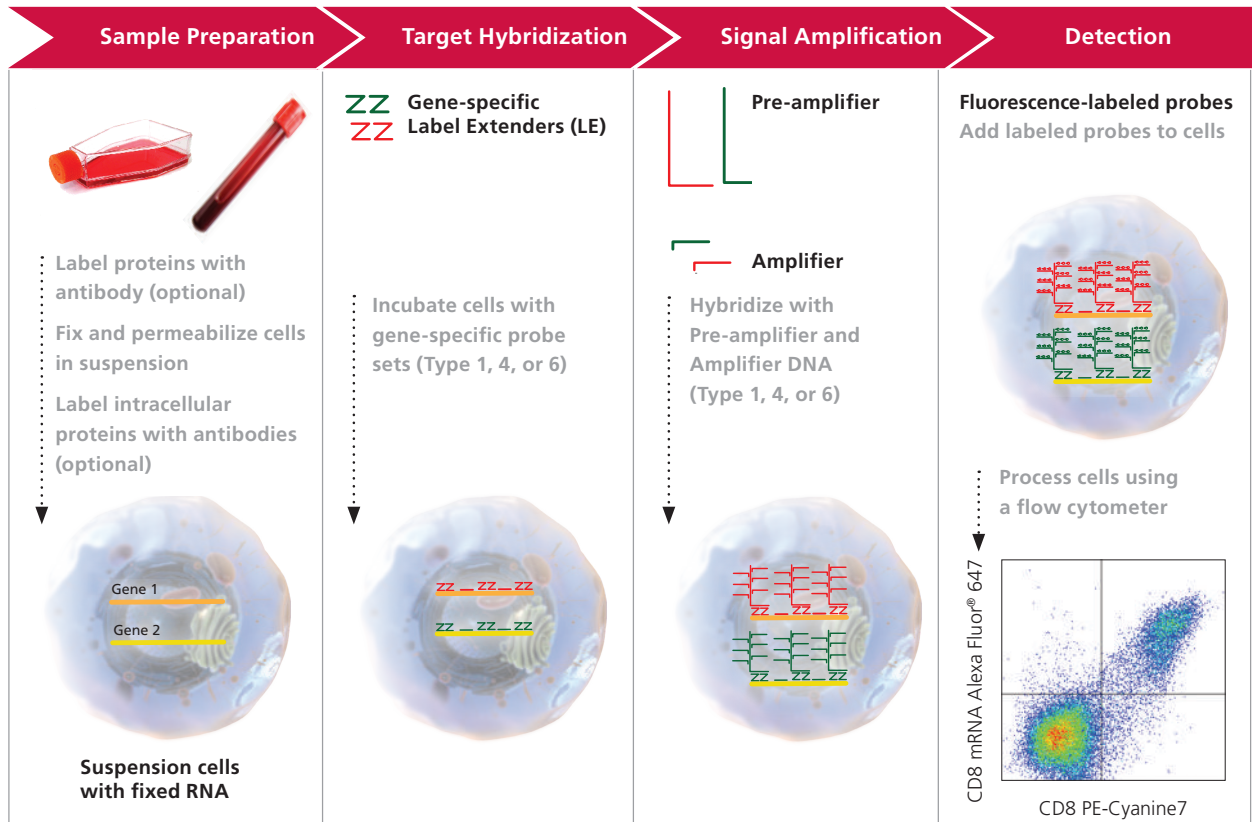
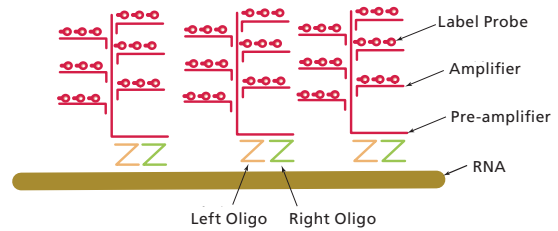
PrimeFlow RNA Assay answers previously unanswerable questions in numerous fields, from immunology to cancer research to cell biology. Life happens at a single-cell level. However more than just a single cell, life is a collection of millions of cells working together in complex environments. The assay is the first and only tool that combines single-cell resolution with the acquisition of millions of cells to study both RNA and protein expression, allowing researchers to create new discoveries and challenge current conventions.



**Figure 6: Cyclin detection in unsynchronized U937 cells**  
Human monocytic cells U937 were untreated (green histograms) or treated with nocodazole for 16 hrs (red histograms). Nocodazole disrupts microtubules leading to cell cycle arrest at the G<sub>2</sub>-M checkpoint. After stimulation, cells were analyzed using the PrimeFlow™ RNA target probe sets for Cyclin A2, Cyclin B1, or Cyclin E1. The target probe set for GAPDH was used as a positive control.

# Principle of the PrimeFlow™ RNA Assay

Development of the PrimeFlow™ RNA Assay is based upon proven and well-published ViewRNA™ technology designed for microscopic analysis of RNA in cells and tissues. The assay combines paired oligonucleotide probe design with branched DNA (bDNA) signal amplification to robustly detect up to three RNA transcripts at the single-cell level using a standard flow cytometer.



**Figure 7: The PrimeFlow™ RNA Assay workflow**

The assay workflow contains several steps: antibody staining; fixation and permeabilization, including intracellular staining, if desired; followed by target hybridization with a target-specific probe set containing 20 to 40 oligonucleotide pairs. Next, branched DNA signal amplification is achieved through a series of sequential hybridization steps consisting of Pre-amplifiers, Amplifiers, and Label Probe, followed by detection by flow cytometric analysis. This results in excellent specificity, low background and high signal-to-noise ratio. For simplicity, two RNA targets are shown in the schematic above (red and green), and only three of the 20 to 40 oligonucleotide target probe pairs per target RNA.

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