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QuantiGene® FlowRNA Assay

A Novel RNA ISH Multicolor Application for Flow Cytometry

Introduction

QuantiGene FlowRNA is a novel *in situ* hybridization assay enabling simultaneous detection of up to three RNA transcripts in a single cell using a standard flow cytometer. The assay combines a proprietary probe design using oligonucleotide (oligo) pairs with branched DNA (bDNA) signal amplification to robustly detect gene expression at the single cell level. Additionally, the assay is compatible with standard antibody staining methods for simultaneous detection of cell surface markers within the same cells.

Coupling RNA expression with protein detection on a flow cytometer allows simultaneous analysis of both levels within complex cell populations. Microarrays and sequencing provide only broad comprehensive gene expression data. Using the QuantiGene FlowRNA Assay, specific cell populations may be analyzed for unique transcript expression levels or kinetic evaluation of cell subsets to determine transcriptional upregulation versus protein expression. Such unique and valuable insights are highly applicable to answering previously unanswerable questions in cellular immune response or cancer therapies.

At a glance

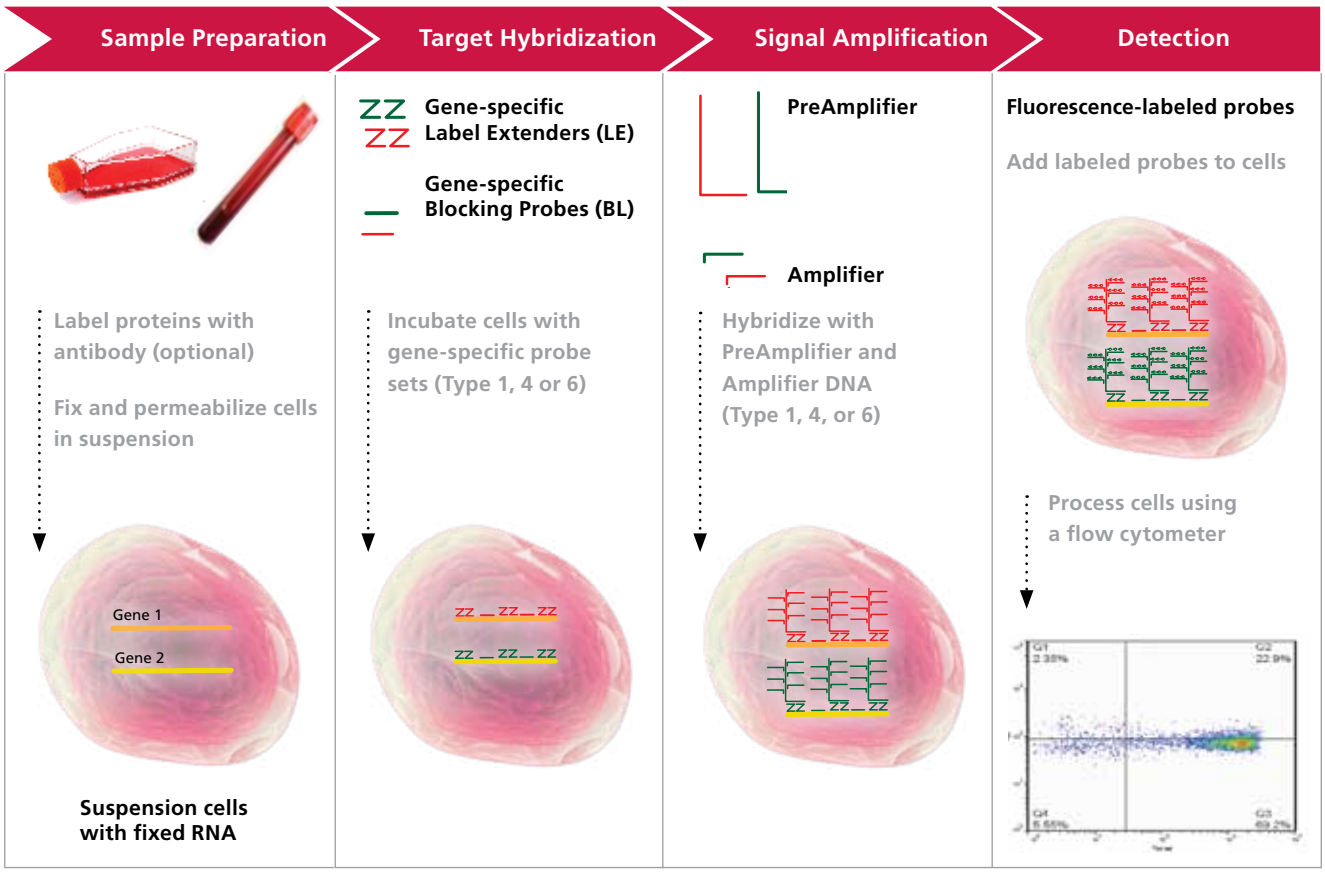
- Multiplexed gene expression in a single cell
- Molecule-level RNA sensitivity
- Simultaneous detection of RNA and protein expression
- Genomic answers in heterogeneous samples

Assay principle

The QuantiGene FlowRNA Assay is based on a proprietary oligo probe design and bDNA signal amplification technology. A typical target-specific probe set containing 20 oligo pairs hybridizes to the target RNA. Signal amplification is detected through specific hybridization of an adjacent probe set of oligo pairs to a target RNA. The result is low background, excellent specificity and high signal-to-noise ratio. Independent but compatible signal amplification systems enable simultaneous detection of up to three RNA targets in a single cell.

The assay workflow (Figure 1) contains four steps: sample preparation, target hybridization with probes, signal amplification using bDNA technology and detection. For simplicity, two RNA targets are shown (orange and yellow) and only three of the 20 oligo pairs per target RNA.

Figure 1: The QuantiGene FlowRNA Assay Workflow



Assay features

- Simultaneous detection of three RNA transcripts in a single cell
- Data analysis using a standard flow cytometer
- Over 3000 existing QuantiGene RNA ISH probes
- Compatible with antibody labeling of cell surface proteins
- Probe design and shipment in five days or less for custom probe sets – from sequence submission to probe delivery

Focus areas

- Cancer research
- Infectious diseases
- Immunology
- Stem cell research
- Toxicology
- Biomarker validation
- Analysis of non-coding RNA and RNA viruses

Specificity validation

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 oligos (left oligo and right oligo) bind to the specific target (Figure 3A). To determine the assay specificity, probe sets for human GAPDH containing left oligo alone or right oligo alone are tested and compared to a complete probe set in human U937 cells (Figures 3B & 3C). Strong signal is detected with the complete probe set, correlating to the abundant expression of GAPDH in U937 cells. In contrast, no significant signal is detected when either left oligos or right oligos are used individually as compared to the "no probe" control. A negative control with a complete probe set (DapB gene in Gram-positive bacterium, *Bacillus subtillis*) yielded no specific signal.

Figure 3A

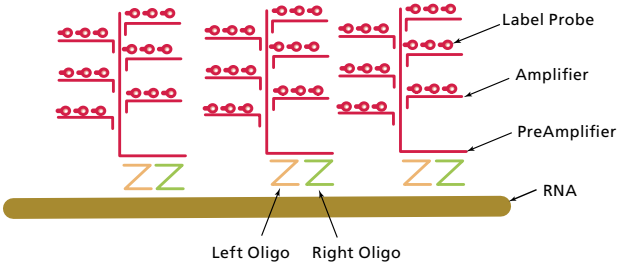


Figure 3B

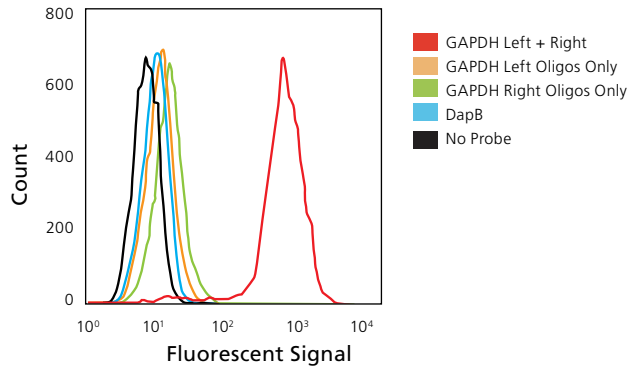


Figure 3C

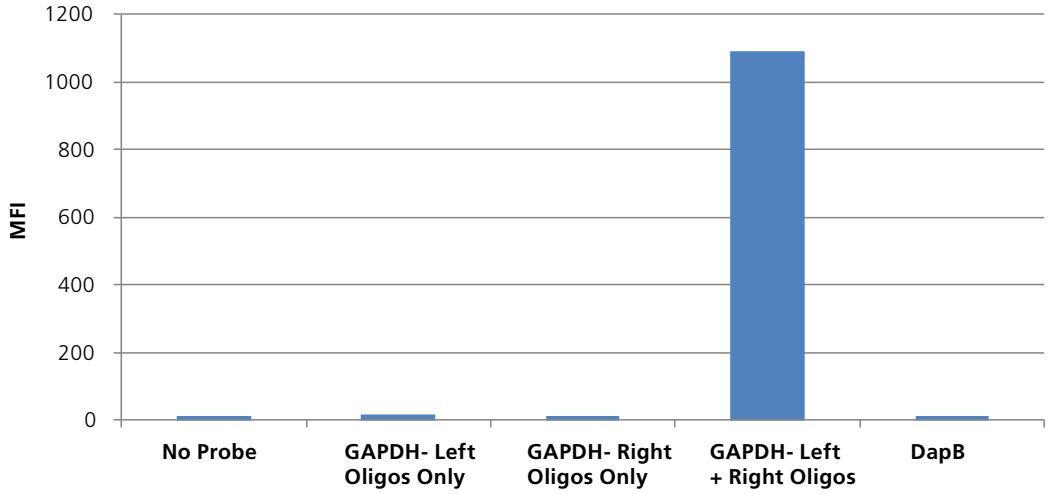


Figure 3: Demonstration of the specificity of the Quantigene FlowRNA Assay. Illustration of a probe set design containing both left and right oligonucleotide pairs, and amplification reagents pre-amplifier, amplifier and labeled probe (Figure 3A). Flow cytometry histogram data of GAPDH RNA expression in U937 cells (Figure 3B) . Quantitative representation of GAPDH signal with different probe sets based on Mean Fluorescent Intensity (MFI) (Figure 3C).

Detect RNA and protein levels simultaneously

The QuantiGene FlowRNA Assay may be used with antibody labeling of cell surface proteins to isolate cell populations or simultaneously measure protein and RNA transcript levels. To demonstrate, human peripheral blood mononuclear cells (PBMCs) are stained for a monocyte-specific marker with Anti-CD14, conjugated to FITC. The antibody-stained cells are hybridized and amplified to detect CD14 RNA expression per protocol instructions. After data acquisition on a flow cytometer, cells are gated using forward scatter (FSC) and side scatter (SSC) parameters to isolate single cell monocyte and lymphocyte populations (Figure 2A). The monocyte population stains positively for both CD14 RNA and CD14 protein levels (Figure 2B). Gated lymphocytes do not express CD14 RNA or protein (not shown).

Alternatively, an antibody/fluorochrome-labeled secondary antibody system may be used. Human PBMCs are stained with an unconjugated antibody to beta 2-microglubulin (B2M), a housekeeping gene expressed in all blood cells. Antibody-labeled cells are next probed for B2M RNA per protocol instructions, and subsequently stained with a PE-conjugated secondary antibody. Using a similar gating strategy indicated above, data analysis indicates co-expression of both B2M protein and RNA levels in lymphocytes as well as monocytes (Figure 2C, 2D). Together, the data presented show CD14 RNA signal correlates well with CD14 protein expression in the specific target cells. Furthermore, protein detection is possible within the protocol conditions for this RNA ISH assay with cell surface markers.

Figure 2A

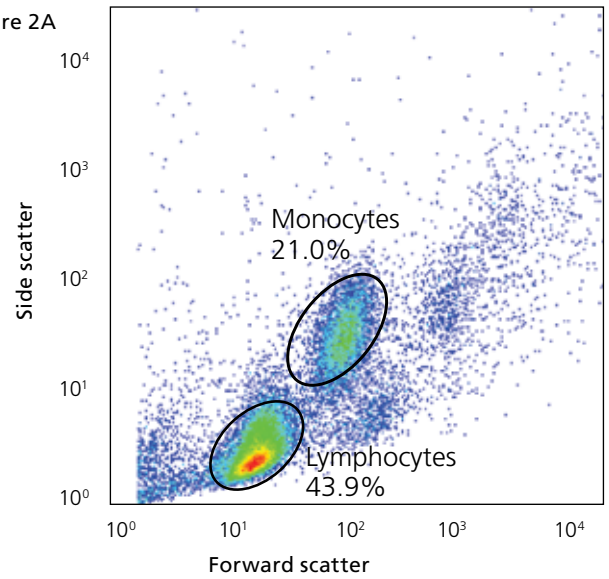


Figure 2B

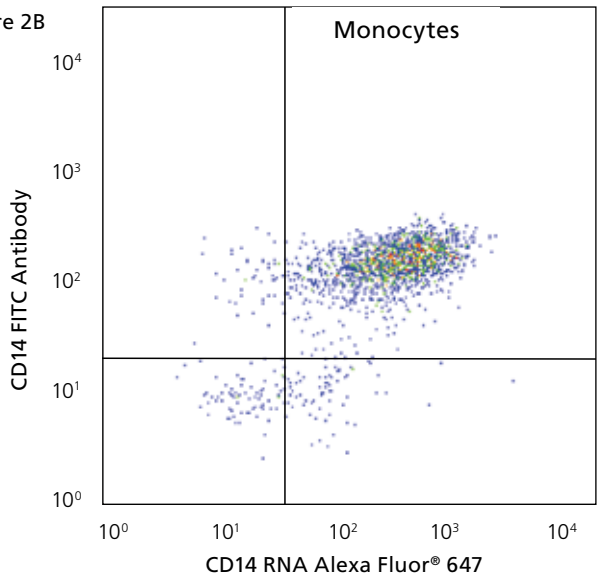


Figure 2C

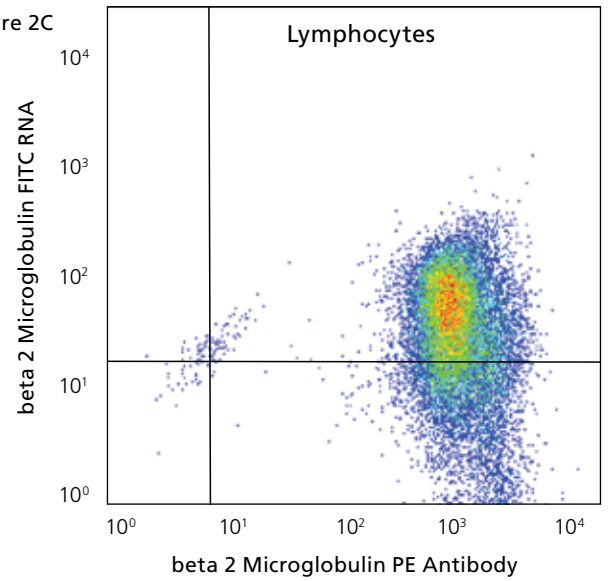


Figure 2D

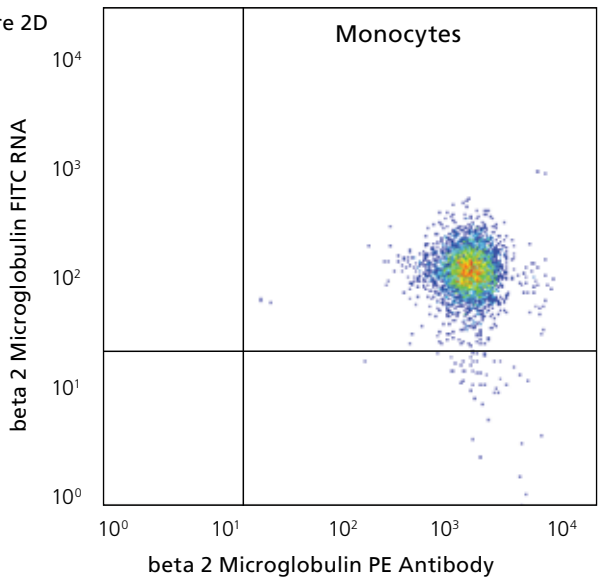


Figure 2: Human PBMCs are stained with a fluorochrome-conjugated monocyte-specific surface marker, Anti-Human CD14 FITC. Antibody-labeled PMBCs are subjected to the QuantiGene FlowRNA Assay, incorporating an RNA probe set with an antibody for CD14. Gating strategy is applied to PBMCs (Figure 2A). CD14 protein and RNA levels displayed for a monocyte population (Figure 2B). PBMCs are stained with a B2M antibody/FITC conjugated-secondary antibody system followed by B2M RNA probe set. Both lymphocytes and monocytes populations showed positive for B2M protein and RNA (Figures 2C & 2D).

Cytokine RNA profiling of a PBMC subset

In inflammation, blood cells respond to stimuli such as lipopolysaccharides (LPS) by the activation of proinflammatory cytokines such as IL-1 beta, IL-6 and TNF alpha. The expression of these cytokine proteins is routinely measured by flow cytometry using specific antibodies. To investigate the feasibility of detecting a subset of cells expressing cytokine RNA transcripts, PBMCs are treated with LPS and R848 for 4 hours. Cells are then stained with FITC-conjugated Anti-CD14 antibody and processed by the QuantiGene FlowRNA Assay. For analysis, a monocyte subset is selected based upon a FSC/SSC gating strategy (Figure 4A). In monocytes that stain positively for CD14, the expression of cytokine genes including IL-1 beta, IL-6, IL-8 and TNF alpha is strongly up-regulated after 4 hours of stimulation (Figure 4B).

Figure 4A

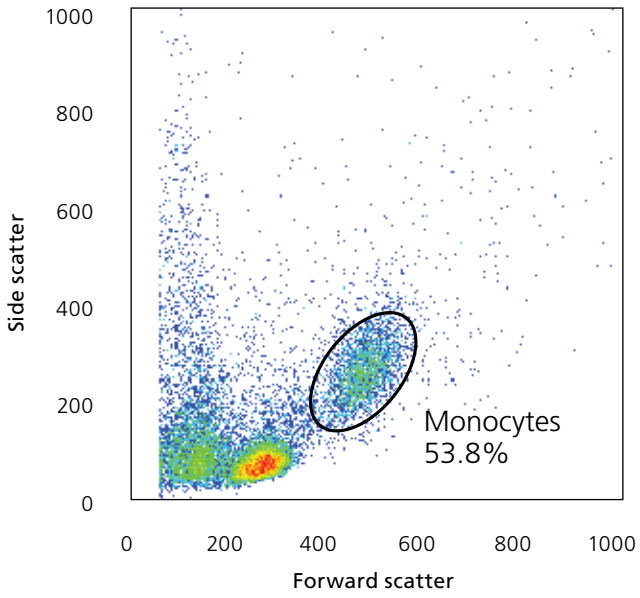
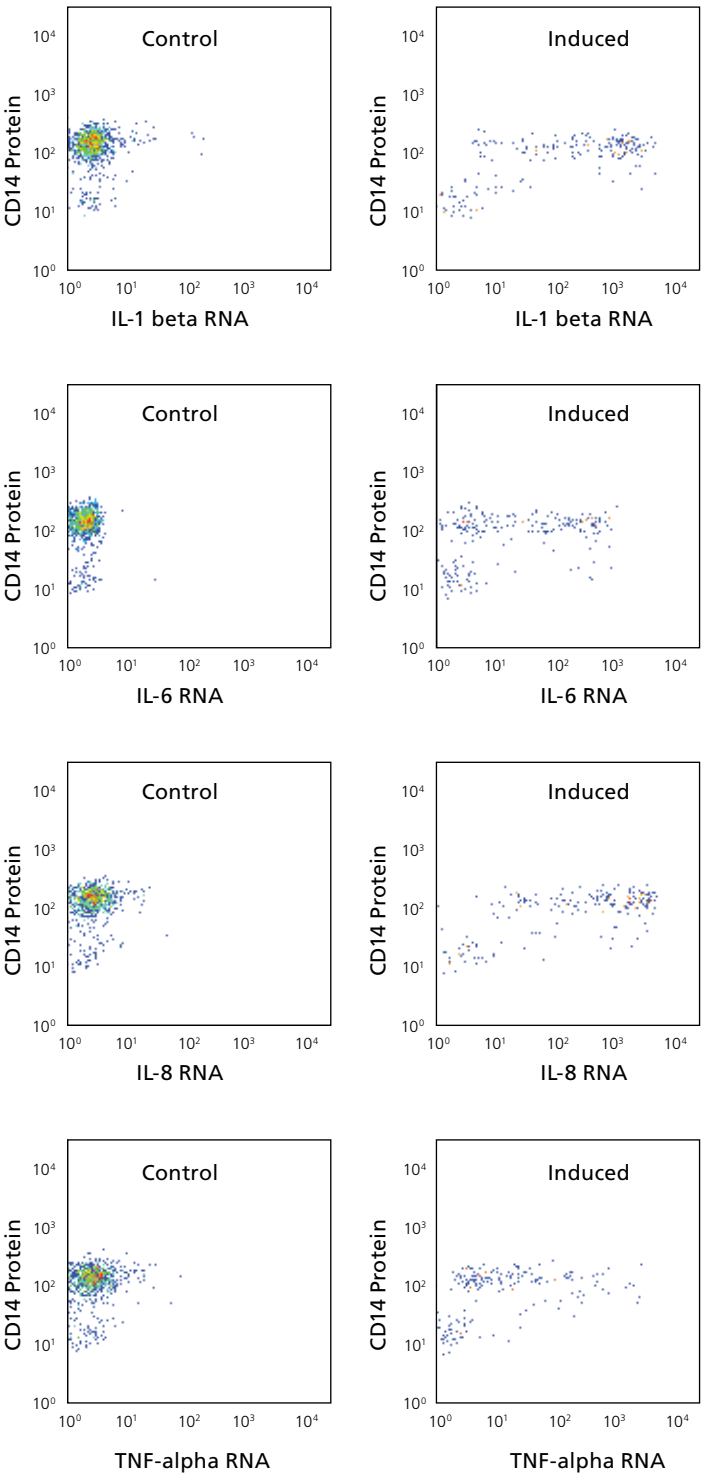


Figure 4: Human PBMCs treated with 1 µg/mL LPS and 2.5 µg/mL R-848 for 4 hours, and processed using the QuantiGene FlowRNA Assay. Gating strategy displayed for a monocyte cell subset (Figure 4A). Expression of IL-1 beta, IL- 6, IL-8 and TNF alpha in monocytes (Figure 4B).

Figure 4B



Concordance with QuantiGene® Plex

Human PBMCs are treated under the same conditions as in Figure 4 and cell lysates are prepared for analysis with the QuantiGene Plex Assay to quantitate RNA expression levels (Figure 5). The results show cytokine transcripts IL-1 beta, IL-6, TNF alpha and IL-2 are up-regulated after LPS stimulation, consistent with the QuantiGene FlowRNA data in Figure 4B. This study highlights the use of both antibody and RNA probe sets in a gene expression study. It is particularly useful in cases when the antibodies are unavailable or inadequate. This has also been demonstrated recently in the characterization of the transcription factor profile in bone marrow cells based on a derivative of this assay.

Figure 5

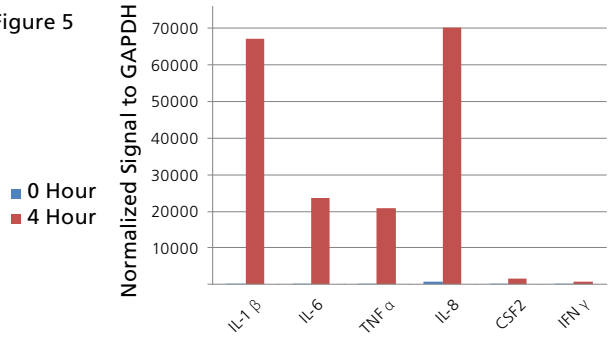


Figure 5: Human PBMCs are treated with 1 µg/mL LPS and 2.5 µg/mL R-848 for 4 hours. Sample is processed and analyzed following protocol conditions in the QuantiGene Plex Assay. Results show correlation of RNA levels to that of the QuantiGene FlowRNA assay (Figure 4B).

Identify cell population subsets

Due to the highly specific probe set design within the QuantiGene FlowRNA Assay, small subsets of cell populations are easily isolated when no antibody is available. To demonstrate the assay sensitivity, human U937 cells are spiked into a population of murine M1 cells. A mixed cell population of 3 million cells is assayed with a human specific probe set for beta2-microglobulin (B2M). The B2M probe set does not cross react with murine M1 cells (data not shown). As little as 1% of human U937 cells are easily identified in the mixed cell populations (Figure 6). When used in combination with established antibodies for specific cell markers, this assay can provide a more comprehensive profile of the cell population.

Figure 6

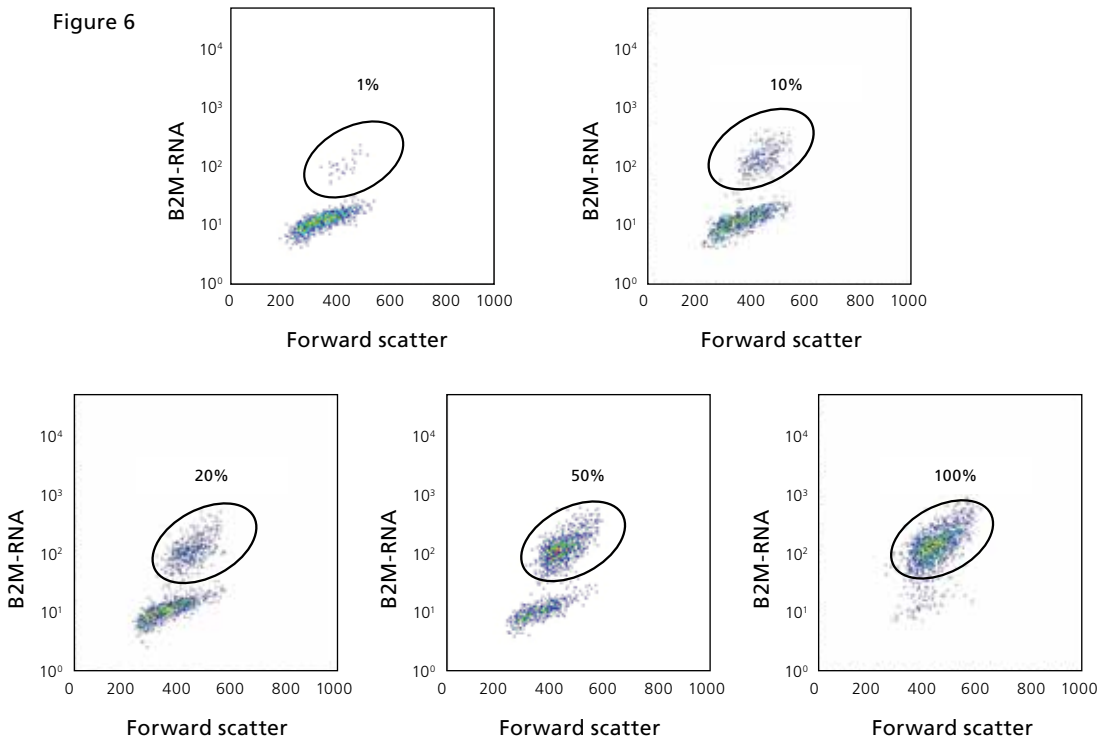


Figure 6: Cultured mouse M1 cells are spiked with varying numbers of human U937 cells. The percentages of U937 cells in the mixed cell populations are shown within each graph. A total of 3 million cells from each mixed population sample are probed with human B2M probe set. The cells encircled within each black region represent the B2M+ subset of positive cells. U937 cells at a level as low as 1% are detected.

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