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# PrimeFlow™ RNA Assay User Manual and Protocol

Catalog No. 88-18005
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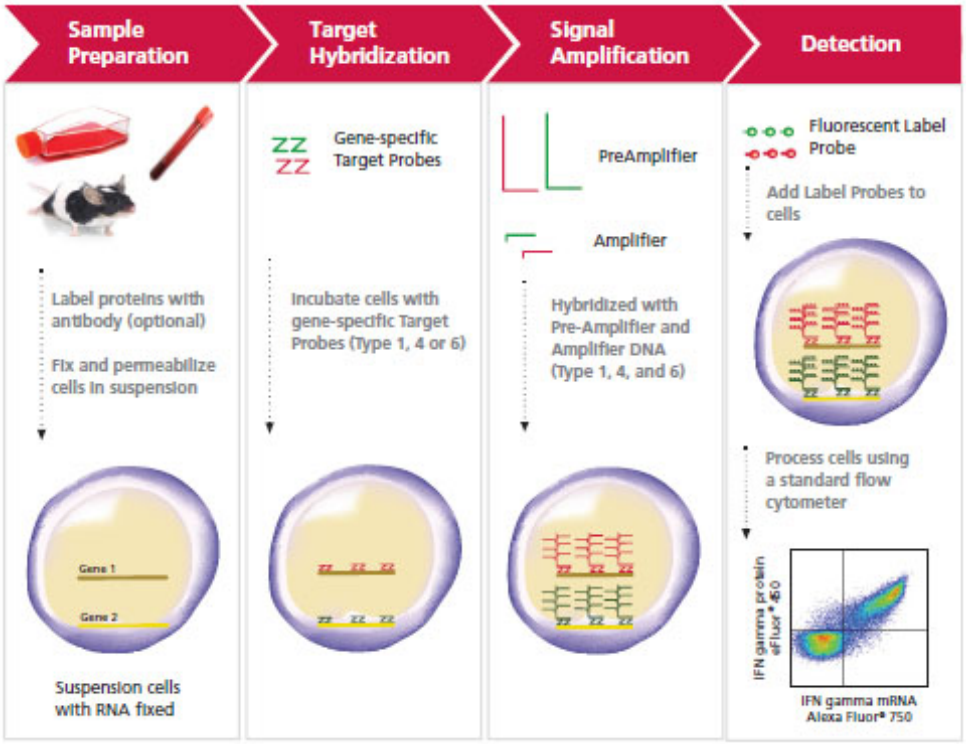
## Introduction

The PrimeFlow™ RNA Assay is an in-situ hybridization assay that combines the power of branched-DNA technology with the single-cell resolution of flow cytometry. This assay enables the simultaneous detection of up to three RNA targets in combination with immunophenotyping for cell surface and intracellular proteins using fluorochrome-conjugated antibodies to allow further discrimination of specific cell subpopulations.

In the PrimeFlow™ RNA Assay, branched-DNA technology is used to amplify the detection of an RNA transcript, rather than the target RNA itself. In the first hybridization step of the assay, a gene-specific oligonucleotide Target Probe set that contains 20–40 probe pairs binds to the target RNA sequence. An individual probe pair is designed to bind adjacent to each other in order for signal amplification to take place. Signal amplification is then achieved through a series of sequential hybridization steps. The PreAmplifier molecules confer an additional level of specificity because they will hybridize to the Target Probes only when both halves of a respective probe pair have bound to their target sequence. Multiple Amplifier molecules subsequently hybridize to their respective PreAmplifier. Finally, Label Probe oligonucleotides conjugated to a fluorescent dye hybridize to their corresponding Amplifier molecules. A fully assembled signal amplification "tree" has 400 Label Probe binding sites. When all target-specific oligonucleotide probes in the Target Probe set bind to the target RNA transcript, 8,000–16,000-fold amplification is achieved. We currently offer three different amplification structures that allow simultaneous measurement of up to three different RNA targets for multicolor flow cytometric analysis. Once the cells have been processed by the PrimeFlow™ RNA Assay, the data can be collected and analyzed on any standard flow cytometer. The schematic below illustrates the detection of two unique targets.

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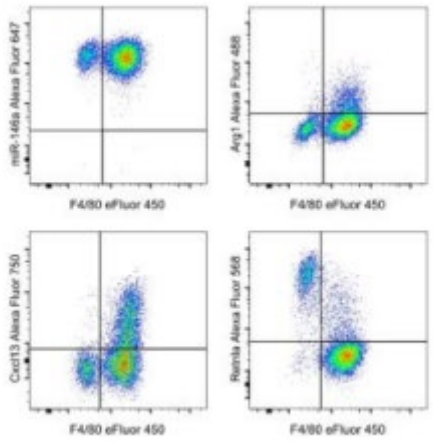


**Figure 1.** C57Bl/6 mouse peritoneal exudate cells were incubated with Anti-Mouse CD16/CD32 Purified (Cat. No. 14-0161) and Normal Mouse Serum (Cat. No. 24-5544) to block Fc receptors, and then surface stained with Anti-Mouse F4/80 Antigen eFluor™ 450 (Cat. No. 48-4801) and AntiMouse CD11b PE-Cyanine7 (Cat. No. 25-0112). Subsequently, the cells were fixed with the PrimeFlow™ microRNA Pretreatment Buffer (Cat. No. 88-18006). Then, using the PrimeFlow™ RNA Assay (Cat. No. 88-18005), the cells were hybridized to the following target probe sets: Type 1 Mouse miR-146a Alexa Fluor™ 647 Target Probe Set (Cat. No. VM1-10253), Type 4 Mouse Arginase-1 (Arg1) Alexa Fluor™ 488 Target Probe Set (Cat. No. VB4- 15827), Type 6 Mouse Cxcl13 Alexa Fluor™ 750 Target Probe Set (Cat. No. VB6-20633), and Type 10 Mouse RELM alpha (Retnla) Alexa Fluor™ 568 Target Probe Set (Cat. No. VB10-20634). CD11b positive cells were used for analysis.

## Contents and storage

| Contents | Catalog No. | Storage |
|---|---|---|
| PrimeFlow™ RNA Tubes | 19197 | Store at room temperature. |
| PrimeFlow™ RNA Fixation Buffer 1A | 00-18100 | Store at 2-8°C. |
| PrimeFlow™ RNA Fixation Buffer 1B | 00-18200 | |
| PrimeFlow™ RNA Permeabilization Buffer (10x) | 00-18300 | |
| PrimeFlow™ RNA Fixation Buffer 2 | 00-18400 | |
| PrimeFlow™ RNA Wash Buffer | 00-19180 | |
| PrimeFlow™ RNA Target Probe Diluent | 00-19185 | |
| PrimeFlow™ RNA PreAmp Mix | 00-16000 | |
| PrimeFlow™ RNA Amp Mix | 00-16001 | |
| PrimeFlow™ RNA Label Probe Diluent | 00-19183 | |
| PrimeFlow™ RNA Storage Buffer | 00-19178 | |
| PrimeFlow™ RNase Inhibitors (100X) | 00-16002 | |
| PrimeFlow™ Compensation Kit | 88-17009 | |
| IC Fixation Buffer | 00-8222 | |
| PrimeFlow™ RNA Label Probes | 00-16003 | |

## General notes

| Assay specifications | |
|---|---|
| Sample type | Single-cell suspensions, including human whole blood and peripheral blood mononuclear cells (PBMC), mouse dissociated tissues, and cell lines. See Appendix 5 for a complete list of validated cell types. |
| Species | Mammalian |
| Plex level | Up to four RNA targets simultaneously |
| Assay format | 1.5-mL microcentrifuge tube or 96-well v-bottom plate. See Appendix 7 for the protocol to use 96-well plates. |
| Instrumentation for RNA detection | Flow cytometer equipped with:<br>■ Blue (488 nm), yellow-green (561 nm), and red (633-640 nm) lasers<br>■ Filter sets for FITC (bandpass 530/30), PE-eFluor™ 610 (PE-Texas Red™) (bandpass 610/20), APC (bandpass 660/20), and APC-eFluor™ 780 (APC-Cyanine7) (bandpass 780/60) |

### Assay guidelines

1. Best results are obtained when starting with healthy cells. Always begin with cells that are in good physiological condition. Cells should be in active growth phase to preserve RNA integrity and minimize cell lysis during processing. If using sorted primary cells, make sure the cells are healthy after purification. Addition of a Fixable Viability Dye is recommended to ensure analysis is restricted to those cells that were alive at the start of the protocol.
2. This assay is highly temperature dependent. Please ensure that the incubator holds temperature at 40±1°C. A significant reduction in signal will result from temperature deviations greater than 1°C. The incubator must be validated using the ViewRNA™ Temperature Validation Kit (Thermo Fisher, Cat. No. QV0523), following the instructions in Appendix 6 of this user manual.
3. This assay allows detection of up to four (4) RNA targets in a single sample:

   **Type 1**: Alexa Fluor™ 647; high sensitivity that is best for low or unknown expression levels

   **Type 4**: Alexa Fluor™ 488; intermediate-to-low sensitivity that is best for medium to high expression levels

   **Type 6**: Alexa Fluor™ 750; intermediate-to-low sensitivity that is best for medium to high expression levels (data for Type 6, Alexa Fluor™ 750 probe sets should be collected in the APC-eFluor™ 780 channel with a 780/60 bandpass filter, or equivalent).

   **Type 10**: Alexa Fluor™ 568; high sensitivity that is best for low or unknown expression of levels (data for Type 10, Alexa Fluor™ 568 probe sets should be collected in the PE-eFluor™ 610 channel off of the yellow-green [561 nm] laser with a 610/20 bandpass filter, or equivalent. The blue [488 nm] laser should not be used.
4. The autofluorescence of cells is increased following this protocol when compared to live cells or fixed cells. This primarily impacts the FITC, PE, eFluor™ 450, and eFluor™ 506 channels. Other channels (up to approximately 650 nm) may also be affected, depending on the cell type, laser line, and instrument settings. Please take this into consideration when setting voltages and when designing multicolor panels. Please contact Thermo Fisher Technical Support (**techsupport@thermofisher.com**) for more information.
5. Protect samples from light after they have been stained with fluorochrome-conjugated antibodies and labeled for RNA.
6. PrimeFlow™ RNA Assay may be used to detect microRNA. For optimal detection of microRNA, the PrimeFlow™ microRNA Pretreatment Buffer (Cat. No. 88-18006-210) and protocol should be used in conjunction with this assay kit. Please refer to the product datasheet for additional details specific for microRNA detection and instructions for use.
7. We recommend performing the assay in two days, as follows:

#### Day 1

- Antibody staining
- Fixation and permeabilization
- Target Probe hybridization

#### Day 2

- Signal amplification
- Flow cytometric analysis

### Fluorochrome compatibility

1. Organic fluorochromes are compatible with this assay, such as FITC, eFluor™ 450, eFluor™ 506, eFluor™ 660, and Alexa Fluor™ 700. BV dyes are also reported to work.
2. Most protein-based fluorochromes are compatible with this assay, including PE, PE-eFluor™ 610, PE-Cyanine5, PE-Cyanine5.5, PE-Cyanine7, APC, and APC-eFluor™ 780.
3. PerCP, PerCP-Cyanine5.5, and PerCP-eFluor™ 710 should not be used. We recommend using PE-Cyanine5 or PE-Cyanine5.5 instead.
4. Qdot™ nanocrystal- and eVolve™-conjugated antibodies are not compatible with this assay.

### Intracellular antibody staining compatibility

1. The fixation and permeabilization buffers in this kit are compatible with most Thermo Fisher™ antibodies used for intracellular staining. Some changes in performance are expected when compared to performance in the Intracellular Fixation and Permeabilization Buffer Set (Cat. No. 88-8824) or Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523). Antibody performance should be determined empirically using the PrimeFlow™ Fixation/Permeabilization Buffer Set (Cat. No. 88-17000).
2. The fixation and permeabilization buffers in this kit are compatible with most Thermo Fisher phospho-specific antibodies; however, phospho-specific antibodies that will work only in the IC Fixation/Methanol protocol are not compatible. Please see the Phospho Flow Cytometry Antibody Clone Buffer Selection Guide or the datasheet for the individual antibody for more information.
3. Staining for some surface markers may be done after fixation and permeabilization. Please see the Antibody Clone Performance Following Fixation/Permeabilization table and refer to the column for "After IC Fixation and Perm Wash" to determine if the antibody clone will recognize a fixed epitope.

### Experimental design guidelines

1. To ensure proper assay performance, use Positive Control Probe Sets in every experiment. For your convenience, these probe sets are included with the PrimeFlow™ RNA Assay (RPL13A for human leukocytes and beta-actin [ACTB] for mouse tissues). See Appendix A5 for specific cell types and other recommended positive control genes.
2. For proper compensation, use the PrimeFlow™ Compensation Kit that is included with the PrimeFlow™ RNA Assay. Please refer to Appendix 3 for instructions for use and for other methods to set compensation. Please contact Thermo Fisher Technical Support (techsupport@thermofisher.com) for more information.
3. Fluorescence-Minus-One (FMO) controls are highly recommended. The FMO control is a sample that contains all but one of the fluorochromes used in the experiment. As with single-color controls, there should be an FMO control for every fluorochrome used in the experiment. FMO controls facilitate assessment of background on gated events and allow fine-tuning of compensation for optimal performance.
4. Negative controls, such as samples with the target probe omitted or samples labeled with a target probe not expressed in the cells of interest (e.g., DapB, a bacterial gene), are highly recommended. Negative control samples comprised of or containing cells known to be negative for the gene of interest (e.g., unstimulated) are also recommended to confirm specificity of the target probes.
5. If using whole blood, there is no need to pre-lyse red blood cells; however, due to changes in forward-scatter and side-scatter properties of granulocytes, the use of antibodies to distinguish leukocyte subpopulations is recommended.
6. Please see Appendix 4 for examples of sample set-up and experimental design.

## This protocol is intended for use with 1.5-mL microcentrifuge tubes.

(See Appendix 7 for the 96-well plate protocol.)

### Materials included

#### Store at room temperature

- PrimeFlow™ RNA tubes (1.5-mL microcentrifuge tubes)

#### Store at 2–8°C

- PrimeFlow™ RNA Fixation Buffer 1A (Cat. No. 00-18100)[1]
- PrimeFlow™ RNA Fixation Buffer 1B (Cat. No. 00-18200)
- PrimeFlow™ RNA Permeabilization Buffer (10X) (Cat. No. 00-18300)
- PrimeFlow™ RNA Fixation Buffer 2 (8X) (Cat. No. 00-18400)
- PrimeFlow™ RNA Wash Buffer (Cat. No. 0019180)
- PrimeFlow™ RNA Target Probe Diluent (Cat. No. 00-18185)[2]
- PrimeFlow™ RNA PreAmp Mix (Cat. No. 00-16000)[2]
- PrimeFlow™ RNA Amp Mix (Cat. No. 00-16001)
- PrimeFlow™ RNA Label Probe Diluent (Cat. No. 00-19183)
- PrimeFlow™ RNA Storage Buffer (Cat. No. 00-19178)
- PrimeFlow™ RNase Inhibitor (100X) (Cat. No. 00-16002)
- PrimeFlow™ Compensation Kit (Cat. No. 88-17009)
- IC Fixation Buffer (Cat. No. 00-8222)[2]
- PrimeFlow™ RNA Label Probes (100X) (Cat. No. 00-16003)

*[1] DANGER: Contains formaldehyde classified as a poison and irritant. See the Safety Data Sheet for more information.*

*[2] DANGER: Contains formamide classified as a teratogen, irritant, and possible carcinogen. See the Safety Data Sheet for more information.*

## Materials required, but not included

- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- Fluorochrome-conjugated antibodies, as needed
- Fixable Viability Dye, as needed
- Target Probe sets, as needed
- 12 × 75 mm polystyrene tubes (e.g., Corning, Cat. No. 352008)
- PrimeFlow™ Compensation Kit (Cat. No. 88-17001)
- *(OPTIONAL)* 96-well, v-bottom polystyrene plate (Cat. No. 44-17005; see Appendix 7 for modified protocol)

## Instruments and equipment

- Refer to Appendix 2 of this user manual

## Experiment duration

### Day 1 (~6–8 hours)

- Antibody staining
- Fixation and permeabilization
- Target probe hybridization

### Day 2 (~6 hours)

- Signal amplification
- Flow cytometric analysis

## Experimental procedure

***Note:*** *This procedure is written based on the use of the 1.5-mL tubes provided in the kit throughout the assay. The use of these tubes is important during the hybridization and signal amplification steps to control residual volumes. However, staining with antibodies and/or fixable viability dyes as well as fixation and permeabilization (Steps 1–16) may be done in bulk and in any tube desired. If these procedures are done in bulk, use volumes such that cells do not exceed* $1 \times 10^7$ *cells/mL. To perform the assay in 96-well plates, refer to Appendix A7 of this user manual.*

### Day 1: Antibody staining, fixation, and permeabilization

1. Pre-warm PrimeFlow™ RNA Wash Buffer to room temperature. This buffer will first be used in Step 13.
2. Aliquot 1–5 × $10^6$ cells in Flow Cytometry Staining Buffer or 100 µL of whole blood into the 1.5-mL tubes provided in the kit.

   ***Note*****:** *When using whole blood, it is not necessary to lyse the red blood cells before beginning the assay. However, if pre-lysis is desired, the use of 10X RBC Lysis Buffer (Cat. No. No. 00-4300) is recommended. Please see the product datasheet or Best Protocols: Red Blood Cell Lysis Protocol, Protocol A for detailed instructions for use.*
3. Prepare single-color compensation control samples using the PrimeFlow Compensation Kit and any experimental antibodies used. Refer to Appendix 3 for detailed instructions. Store compensation control samples in the dark at 2–8°C in IC Fixation Buffer by resuspending the stained UltraComp eBeads™ microspheres in 100 µL of Flow Cytometry Staining Buffer. Then add 100 µL of IC Fixation Buffer and mix gently.
4. Surface stain cells with fluorochrome-conjugated antibodies at their optimal concentration for 30 minutes at 2–8°C.

   ***Note*****:** *If needed, please see Best Protocols: Staining Cell Surface Antigens for Flow Cytometry: Protocol A: Cell Suspensions for detailed instructions for staining.*

   ***Note*****:** *Use antibodies that are conjugated to approved fluorochromes only (see general notes, fluorochrome compatibility section above).*

   ***Note*****:** *Cells may be stained with a Fixable Viability Dye before or after surface staining (see Best Protocols: Viability Staining Protocol, Protocol C for detailed instructions).*

   ***Note*****:** *Staining for some surface markers may be done after fixation and permeabilization. Please see the Antibody Clone Performance Following Fixation/Permeabilization table on our website and refer to the column for "After IC Fixation and Perm Wash" to determine if the antibody clone will recognize a fixed epitope. If you prefer to stain after fixation, skip this step and proceed to Step 6.*
5. Add 1 mL of Flow Cytometry Staining Buffer to each sample, invert to mix, and spin down at 500 × *g* for 5 minutes. Discard supernatant and resuspend cells in the residual volume.

   ***Note*****:** *When using whole blood, skip this step and proceed to Step 6.*
6. Prepare Fixation Buffer 1 by mixing equal parts of PrimeFlow™ RNA Fixation Buffer 1A and PrimeFlow™ RNA Fixation Buffer 1B. Mix gently by inverting.

   ***Note*****:** *You will need 1 mL of this buffer per sample. Prepare this buffer in bulk to accommodate all samples. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh. Dispose of any unused buffer.*
7. Add 1 mL of prepared Fixation Buffer 1 to each sample and invert to mix. Incubate for 30 minutes at 2–8°C.
8. Spin down at 800 × *g* for 5 minutes. Discard supernatant and resuspend cells in the residual volume.

9. Prepare 1X PrimeFlow™™ RNA Permeabilization Buffer with RNase Inhibitors by diluting PrimeFlow™™ RNA Permeabilization Buffer (10X) to 1X with RNase-free water. Then add RNase Inhibitor 1 (1,000X) at a 1/1,000 dilution and RNase Inhibitor 2 (100X) at a 1/100 dilution. Mix gently by inverting. Keep at 2–8°C.

   ***Note:*** *You will need 3 mL of this buffer per sample. Prepare this buffer in bulk to accommodate all samples. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh. Dispose of any unused buffer.*

10. Add 1 mL of 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes, then discard supernatant and resuspend cells in the residual volume.
11. Repeat Step 10.
12. Intracellularly stain cells with fluorochrome-conjugated antibodies at their optimal concentration in the 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors for 30 minutes at 2–8°C.

    ***Note:*** *If intracellular staining is not desired, skip this step and proceed to Step 14.*

    ***Note:*** *Staining for some surface markers may be done after fixation and permeabilization. Please see the Antibody Clone Performance Following Fixation/Permeabilization table on our website and refer to the column for "After IC Fixation and Perm Wash" to determine if the antibody clone will recognize a fixed epitope.*

    ***Note:*** *Use antibodies that are conjugated to approved fluorochromes only (see General notes, Fluorochrome compatibility section above).*

13. Add 1 mL of 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing gently.
14. Prepare 1X PrimeFlow™ RNA Fixation Buffer 2 by combining 125 µL of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 875 µL of PrimeFlow™ RNA Wash Buffer per sample. Mix gently by inverting.

    ***Note:*** *You will need 1 mL of this buffer per sample. Prepare this buffer in bulk to accommodate all samples. For example, for 10 samples, combine 1.25 mL of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 8.75 mL of PrimeFlow™ Wash Buffer. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh. Dispose of any unused buffer.*

15. Add 1 mL of 1X PrimeFlow™ RNA Fixation Buffer 2 to each sample and invert to mix, and then incubate for 60 minutes in the dark at room temperature.

    ***Note:*** *It is important to fix the samples at room temperature. Do not perform this step on ice.*

16. *(OPTIONAL)* Cells may be stored in 1X PrimeFlow™ RNA Fixation Buffer 2 overnight in the dark at 2–8°C, instead of incubating for 60 minutes at room temperature (i.e., skip Step 14).
17. Spin down at 800 × *g* for 5 minutes, then aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.

    ***Note:*** *If staining, fixation, and permeabilization were performed in bulk, the cells should be transferred into the 1.5-mL tubes provided in the kit during the following wash steps and before storage overnight.*

18. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at 800 ***x g*** for 5 minutes, then aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
19. Repeat Step 18.

    ***Note:*** *It is critical that the residual volume is as close to 100 µL as possible. Use the markings on the 1.5-mL tubes provided in the kit to assist.*

20. *(OPTIONAL)* Cells may be stored in PrimeFlow™ Wash Buffer with RNase Inhibitor 1 overnight in the dark at 2–8°C. To do so, add RNase Inhibitor 1 (1,000X) to PrimeFlow™ RNA Wash Buffer at a 1/1,000 dilution and use in Step 19.

    ***Note:*** *You will need 1 mL of this buffer per sample. Prepare this buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose of any unused buffer.*

### Day 1: Target probe hybridization

***Note:*** *It is critical that the residual volume after all washes be as close to 100 µL as possible. Use the markings on the 1.5-mL tubes provided in the kit to assist.*

***Note:*** *Diluted Target Probes should be pipetted directly into the 100 µL of residual volume and samples should be mixed well before incubating. Do not pipette solutions onto the walls of the tubes.*

21. Thaw Target Probes, including Positive Control Target Probe Sets (20X), at room temperature.
22. Pre-warm PrimeFlow™ RNA Target Probe Diluent to 40°C.
23. Dilute Positive Control Target Probe Sets (20X) 1/20 in PrimeFlow™ RNA Target Probe Diluent. Mix thoroughly by pipetting up and down.

    ***Note:*** *You will need 100 µL of diluted Target Probes for each sample. If you are adding more than one Target Probe per sample, adjust the volume of the PrimeFlow™ RNA Target Probe Diluent accordingly so that the final volume remains 100 µL per sample.*

24. Add 100 µL of diluted Target Probe(s) directly into the cell suspension for the appropriate samples and briefly vortex to mix, and then incubate for 2 hours at 40°C. Invert samples to mix after 1 hour.
25. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.

26. Prepare PrimeFlow™ RNA Wash Buffer with RNase Inhibitor 1 by adding RNase Inhibitor 1 (1,000X) at a 1/1,000 dilution to the PrimeFlow™ RNA Wash Buffer. Mix gently by inverting.

    ***Note***: *You will need 1 mL of this buffer per sample. Prepare this buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose of any unused buffer.*

27. Add 1 mL of PrimeFlow™ RNA Wash Buffer with RNase Inhibitor 1 to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
28. Store samples overnight in the dark at 2–8°C.

    ***Note***: *We recommend this stopping point for ease-of-use and a more manageable workflow. However, if desired, Step 27 may be skipped. If skipping this step, proceed to Step 29 and continue through to the end of the protocol.*

### Day 2: Signal amplification

***Note***: *It is critical that the residual volume after all washes be as close to 100 µL as possible. Use the markings on the 1.5-mL tubes provided in the kit to assist.*

***Note***: *PrimeFlow™ RNA PreAmp Mix, PrimeFlow™ RNA Amp Mix, and diluted Label Probes should be pipetted directly into the 100 µL of residual volume and samples should be mixed well before incubating. Do not pipette these solutions onto the walls of the tube*s.

29. Pre-warm samples and PrimeFlow™ RNA Wash Buffer to room temperature.

    ***Note***: *Some precipitation in the Wash Buffer may occur. After warming to room temperature, an aliquot for the volume needed for the day may be prepared and allowed to settle or briefly centrifuged to remove precipitates. Do not repeatedly centrifuge the Wash Buffer to remove precipitates. will need 100 µL of diluted Label Probes for each sample. Prepare diluted Label Probes in bulk to accommodate all samples.*

30. Pre-warm PrimeFlow™ RNA PreAmp Mix, PrimeFlow™ RNA Amp Mix, and PrimeFlow™ RNA Label Probe Diluent to 40°C.
31. Add 100 µL of PrimeFlow™ RNA PreAmp Mix directly into the cell suspension for each sample and briefly vortex to mix. Incubate for 1.5 hours at 40°C.
32. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
33. Repeat Step 32 two times, for a total of three washes.
34. Add 100 µL of PrimeFlow™ RNA Amp Mix directly into the cell suspension for each sample and briefly vortex to mix. Incubate for 1.5 hours at 40°C.
35. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
36. Repeat Step 35.
37. Dilute PrimeFlow™ RNA Label Probes (100X) 1/100 in PrimeFlow™ RNA Label Probe Diluent.

    ***Note***: *You will need 100 µL of diluted Label Probes for each sample. Prepare diluted Label Probes in bulk to accommodate all samples.*

38. Add 100 µL of diluted Label Probes directly into the cell suspension for each sample and briefly vortex to mix, and then incubate for 1 hour at 40°C.
39. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
40. Repeat Step 39.
41. Add 1 mL of PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer to each sample, invert to mix, and spin down at 800 × *g* for 5 minutes. Aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
42. Transfer samples to 12 x 75 mm polystyrene tubes, resuspend in an appropriate volume of PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer, and analyze samples on a flow cytometer.

    ***Note***: *Samples may be stored before analysis. If samples have been stained with antibodies conjugated to tandem dyes, we recommend storing the samples in IC Fixation Buffer by mixing 100 µL of cells with 100 µL of IC Fixation Buffer. Store samples in the dark at 2–8°C for up to three days.*

## Quick guide: PrimeFlow™™ RNA Assay in 1.5-mL tubes

### Day 1:

1. Surface stain cells with antibody and/or fixable viability dye and prepare compensation controls.
2. Wash once with Flow Cytometry Staining Buffer. Spin cells at 500 × *g* for 5 minutes at 2-8°C.
3. Fix cells in PrimeFlow™ RNA Fixation Buffer 1 for 30 minutes at 2–8°C.
4. Wash twice with 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors. Spin cells at 800 × *g* for 5 minutes at 2-8°C.
5. Intracellularly stain cells with antibody for 30 minutes at 2–8°C.
6. Wash once with 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors. Spin cells at 800 × *g* for 5 minutes at 2-8°C.
7. Fix cells in 1X PrimeFlow™ RNA Fixation Buffer 2 for 60 minutes at room temperature.
8. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 800 × *g* for 5 minutes at room temperature.
9. Perform Target Probe hybridization for 2 hours at 40°C. Invert to mix after 1 hour.
10. Wash once with PrimeFlow™ RNA Wash Buffer. Spin cells at 800 × *g* for 5 minutes at room temperature.
11. Wash once with PrimeFlow™ RNA Wash Buffer with RNase Inhibitors. Spin cells at 800 × *g* for 5 minutes at room temperature.
12. Store samples overnight.

### Day 2:

13. Perform PreAmp hybridization for 1.5 hours at 40°C.
14. Wash three times with PrimeFlow™ RNA Wash Buffer. Spin cells at 800 × *g* for 5 minutes at room temperature.
15. Perform Amp hybridization for 1.5 hours at 40°C.
16. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 800 × *g* for 5 minutes at room temperature.
17. Perform Label Probe hybridization for 1 hour at 40°C.
18. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 800 × *g* for 5 minutes at room temperature.
19. Wash once with PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining. Spin cells at 800 × *g* for 5 minutes at room temperature.
20. Analyze samples on a flow cytometer.

## Appendix 1: Troubleshooting

| Problem | Possible Reasons | Solution |
|---|---|---|
| A. Poor Cell Recovery / Low Cell Count on Cytometer | 1. Cell lysis due to cells in poor physiological condition during processing | 1. If using frozen cells, thaw cells carefully following proper cell culture procedures, and rest cells for 30 minutes at 37°C before starting the assay. Make sure cells are cryopreserved while still in log-phase growth.<br>2. Check cell viability using trypan blue or other viability dye before beginning the assay. |
| | 2. Improper centrifuge settings | 1. Confirm settings are for RCF (× *g*), not RPM.<br>2. Set centrifuge to the speed specified in the protocol. |
| | 3. Improper tubes used | Only use the 1.5-mL tubes provided with the kit. Tubes from other vendors may result in significant cell loss. |
| | 4. High aspiration rate by pipetting or vacuum aspirator when removing supernatant | Ensure vacuum setting for aspirator is low. Aspirate no faster than 0.5 mL/sec. Place a 10-µL pipette tip on the tip of the aspirator to help reduce aspiration rate. While aspirating, follow the meniscus down to the 100-µL mark on the side of the tube. |
| | 5. Rough handling | Avoid excessive vortexing to prevent cell damage. Vortex in short pulses. |
| | 6. Incorrect cell count by flow cytometer | Verify the flow rate of cytometer using beads of known concentration such as the Flow Cytometry Absolute Count Standard (Bangs Laboratories, Cat. No. No. 580). |
| B. Weak / No Signal | 1. Cytometer not set up properly | Check that your instrument is properly set up according to the manufacturer's recommendations. |
| | 2. Incorrect samples used for setting compensation | Use samples labeled with one each of the included Positive Control Probe Sets to set compensation.<br>Do not use fluorochrome-conjugated antibodies to set compensation for RNA detection. |
| | 3. Incorrect preparation of signal amplification reagents | Make sure PreAmp Mix, Amp Mix, and Label Probes are used in the specified order. |
| | 4. Incorrect incubator temperature | 1. Incubator must be able to hold temperature at 40±1°C.<br>2. Use a metal heat block for hybridization.<br>3. Minimize traffic to incubator. Temperature tolerance is 1–2°C.<br>4. Ensure incubator has been stable at 40°C for at least 12 hours before starting the assay as temperature may drift during initial incubator setup.<br>5. Measure temperature using the ViewRNA™ Temperature Validation Kit (Thermo Fisher, Cat. No. No. QV0523). |
| | 5. Incorrect Wash Buffer | Do not substitute PrimeFlow™ RNA Wash Buffer with Storage Buffer or any other buffer. |
| | 6. Incorrect diluents used | Ensure PrimeFlow™ RNA Target Probe Diluent is used with Positive Control Target Probe Sets (20X), and PrimeFlow™ RNA Label Probe Diluent is used with PrimeFlow™ RNA Label Probes (100X). |

| Problem | Possible Reasons | Solution |
|---|---|---|
| B. Weak / No Signal | 7. Incorrect dilution of Target Probes or Label Probes | 1. Ensure Target Probes are diluted 1/20 with PrimeFlow™ RNA Target Probe Diluent.<br>2. Ensure PrimeFlow™ RNA Label Probes (100X) are diluted 1/100 with PrimeFlow™ RNA Label Probe Diluent. |
| | 8. Gene expressed at very low level | 1. Check biological model and confirm gene is expressed in sample by an alternative method such as QuantiGene™ 2.0 Assay for lysate.<br>2. Keep in mind that protein expression might not correlate with RNA expression in some cases. |
| | 9. Incorrect or insufficient RNase Inhibitor used | 3. Ensure that 1X PrimeFlow™ RNA Permeabilization Buffer contains RNase Inhibitors (100X) at a final dilution of 1/100. Ensure that the PrimeFlow™ RNA Wash Buffer for overnight sample storage contains RNase Inhibitors at a final dilution of 1/100. |
| C. High Background | 1. Compensation not set up correctly | 1. See Appendix A3 for how to set compensation.<br>2. Use the PrimeFlow™ Compensation Kit (sold separately) or samples labeled with one each of the included Positive Control Target Probe Sets (20X) to set compensation.<br>3. Do not use fluorochrome-conjugated antibodies to set compensation for RNA detection. |
| | 2. Incorrect incubator temperature | 1. See Appendix A6 on how to validate an incubator.<br>2. Incubator must be able to hold temperature at 40±1°C.<br>3. Use a metal heat block for hybridization.<br>4. Minimize traffic to incubator. Temperature tolerance is 1–2°C.<br>5. Ensure incubator has been stable at 40°C for at least 12 hours before starting assay as temperature may drift during initial incubator setup.<br>6. Measure temperature using the ViewRNA™ Temperature Validation Kit (Thermo Fisher, Cat. No. No. QV0523). |
| | 3. Insufficient washing | 1. Ensure the PrimeFlow™ RNA Wash Buffer is used at room temperature.<br>2. Follow instructions for washing steps in the protocol.<br>3. Ensure uniform cell resuspension by gentle vortexing. |
| | 4. Cytometer not set up properly | Reduce voltage settings on your cytometer. Note that this assay may require lower settings than typical antibody staining, but do not set voltages outside the linear range for the PMT. |
| | 5. Over-fixation | Follow the protocol for proper fixation time. |
| | 6. Excessive Target Probe used | Dilute Target Probes at a 1/20 dilution. |

## Appendix 2: Instrument and equipment setup guide

This guide illustrates the setup of typical equipment and their specifications for the PrimeFlow™ RNA Assay. Consult your equipment manufacturers to make sure the equipment meets the specifications. The major equipment include: incubator, swinging bucket centrifuge, flow cytometer, aspiration system for washing, and temperature validation kit.

| Equipment | Specifications |
|---|---|
| 1. Incubator with heat block inside<br>  | 1. Validated to maintain 40±1°C<br>2. Metal heat block for 1.5-mL microcentrifuge tube<br><br>**Example shown:**<br>Incubator (Cat. No. nos. QS0704 or QS0712)<br>Metal heat block (Cat. No. 13-687-210) |
| 2. Refrigerated swinging bucket centrifuge<br>  | Swinging bucket centrifuge with adaptors for 15-mL conical tubes and 1.5-mL microcentrifuge tubes. (Optional: with refrigeration to 4°C)<br><br>**Example shown:**<br>Centrifuge (Eppendorf, model 5810R)<br>Rotor (Eppendorf, model A-4-44)<br>Adaptor for 15-mL conical tube (Eppendorf™, model 5804 755.006)<br>Adaptor for 1.5-mL microcentrifuge tube (Eppendorf™, model 5804 750.004) |
| 3. Flow cytometer<br>  | 1. Three lasers: blue (488 nm), yellow-green (561 nm), and red (633 nm or similar)<br>2. Detection optics optimized for FITC, PE-eFluor™ 610 (PE-Texas Red™), APC, and APC-eFluor™ 780 (APC-Cyanine7)<br><br>**Example shown:**<br>Invitrogen™ Attune™ NxT Flow Cytometer |
| 4. Aspiration system for washing<br>  | Aspiration rate adjusted to 0.5 mL/sec. Can use in-house vacuum line or vacuum pump<br><br>**Example shown:**<br>Vacuum bottle (Argos Technologies™, model EV432)<br>Aspirator (Argos Technologies™, model EV514) |
| 5. ViewRNA™ Temperature Validation Kit<br>  | NIST traceable thermometer, with temperature probe in 1.5-mL microcentrifuge tube (shown on left). (Cat. No. No. QV0523) |

## Appendix 3: Cytometer setup and compensation

The PrimeFlow™ RNA Assay utilizes up to four fluorescent channels for detection of RNA on a flow cytometer. To ensure optimal detection of RNA, it is important that the cytometer is set up properly. In a multicolor assay, signal from a given fluorochrome often spills over into the other detection channels due to overlapping emission spectra. The process of subtracting the spillover signal is called compensation. The procedures below describe how to set proper PMT voltage and compensation for multicolor PrimeFlow™ RNA analysis.

### Materials included

- PrimeFlow™ Compensation Kit (Cat. No. 88-17009)
  - UltraComp™ eBeads™ microspheres (Cat. No. 01-2222)
  - PrimeFlow™ Compensation Control Alexa Fluor™ 647 (Cat. No. PF51-17002)
  - PrimeFlow™ Compensation Control Alexa Fluor™ 647 (Cat. No. PF51-17002)
  - PrimeFlow™ Compensation Control Alexa Fluor™ 647 (Cat. No. PF51-17002)
  - PrimeFlow™ Compensation Control Alexa Fluor™ 647 (Cat. No. PF51-17002)
- IC Fixation Buffer (Cat. No. 00-8222)[1]

### Materials required

- Calibration beads
- Experimental, fluorochrome-conjugated antibodies, as needed
- Unstained cells that have undergone PrimeFlow™ assay
- Fully stained cells that have undergone PrimeFlow™ assay

### Experimental procedure

#### Step I: Stain UltraComp eBeads™ microspheres

***Note***: *When using the PrimeFlow™ Compensation Kit, the compensation samples should be prepared on day 1 of the assay, as noted in the procedure above or in Appendix 7. Alternatively, single-color stained cells may be used, and single-color compensation controls for the RNA channels should be prepared using Target Probes for highly expressed RNA targets, according to the PrimeFlow™ RNA Assay protocol. Do not use fluorochrome-conjugated antibodies to set compensation for the RNA detection channels.*

1. Label a tube for each fluorochrome that will be used in the experiment for both antibodies and RNA Target Probes, plus one additional tube for unstained control.
2. Mix UltraComp eBeads™ microspheres by vigorously inverting at least 10 times or pulse-vortexing.
3. Add 1 drop of UltraComp eBeads™ microspheres to each tube.
4. For each RNA detection channel used, add 5 µL of the appropriate Compensation Control to the appropriate tube.
5. For each experimental antibody used, add 1 test or less of conjugated antibody to the appropriate tube.
6. Mix briefly by flicking or pulse-vortexing.
7. Incubate for 15-30 minutes at 2-8°C for up to 5 days.
8. Add 2 mL of Flow Cytometry Staining Buffer to each tube and spin down at 400-600 × *g* for 3-5 minutes at room temperature. Decant the supernatant and resuspend beads in the residual volume by vortexing gently. Approximately 100 µL of residual volume should remain.
9. Add 100 µL of IC Fixation Buffer to each tube and briefly vortex to mix.
10. Store in the dark at 2-8°C for up to 5 days.
11. Before acquiring on a cytometer, add 2 mL of Flow Cytometry Staining Buffer to each tube and spin down at 400-600 × *g* for 3-5 minutes at room temperature. Decant the supernatant and resuspend beads in the residual volume by vortexing gently.
12. Add 0.2-0.4 mL of Flow Cytometry Staining Buffer to each tube and briefly vortex to mix each tube immediately before analysis in Step IV below.

#### Step II: Ensure proper instrument alignment.

1. Follow the instrument manufacturer's recommendations for checking instrument alignment. Alternatively, calibration beads, such as Rainbow Calibration Particles (8-peak beads) (Spherotech, Cat. No. RCP-30-5A) or Rainbow Fluorescent Particles (1-peak beads) (Spherotech, Cat. No. RFP-30-5A), may be used to check the linearity of the PMT, PMT voltage range, and alignment of the instrument.

#### Step III: Set PMT voltages.

1. Create an acquisition template/workspace that includes a forward-scatter versus side-scatter plot, a single-parameter histogram plot for every fluorescent channel used, and a two-parameter plot for every pairwise combination of fluorescent channels used in the experiment.
2. Place the unstained cell sample on the cytometer and begin to collect events.
   a. Adjust voltages for forward scatter and side scatter so that cells/events of interest are on scale. Set a threshold on forward scatter to eliminate debris from acquisition. Make a note of these settings.

b. Looking at the histogram plots, ensure that the signals are on scale and adjust PMT voltages, if necessary.

***Note:*** *Unstained cells that have undergone the PrimeFlow™ assay may appear higher along the axis than live or traditionally fixed cells due to an increase in autofluorescence. Do not adjust voltages outside of the linear range of the PMT to ensure optimal resolution of fluorescence signal.*

c. Stop collecting events and remove sample from the cytometer.

[1] *DANGER: Contains formaldehyde classified as a poison and irritant. See the Safety Data Sheet for more information.*

3. Place a single-color sample on the cytometer and begin to collect events.

***Note:*** *UltraComp eBeads may require different forward- and side-scatter voltage settings to be visualized. Adjust these settings as needed and make a note of these settings.*

a. Looking at the histogram plot for the fluorochrome collected, ensure that events are on scale for the fluorescent channel collected and adjust PMT voltages, if necessary, keeping them within the linear range of the PMT.

b. Ensure that the signal is at least 0.5 log brighter in the channel of interest than in all other channels. Adjust other PMT voltages as necessary.

c. Stop collecting events and remove sample from the cytometer.

d. Repeat for each of the single-color samples.

4. Confirm that the voltage settings are appropriate for the experimental samples.

a. Place a fully stained sample on the cytometer and begin collecting events.

***Note:*** *If forward- and side-scatter voltages were changed between Steps III2a and III3, adjust voltages back to those noted in Step III2a.*

b. Looking at each of the histogram plots, confirm that all fluorescent signals are on scale and adjust PMT voltages, if necessary, keeping them within the linear range of the PMT.

**c.** Stop collecting events and remove sample from the cytometer.

## Step III: Set compensation.

1. Return forward- and side-scatter voltages to those noted in Step III3 in order to visualize the UltraComp eBeads™ microspheres. Do not change voltages for any of the fluorescent PMT channels.
2. If an autocompensation feature is available for the cytometer used, follow the manufacturer's instructions for use.
3. If an autocompensation feature is not available or to set compensation manually:
   a. Place the unstained sample on the cytometer and begin to collect events.
   b. Looking at the two-parameter plots, create quadrant regions on each plot such that the cells fall within the lower left quadrant (Figure A3.1).
   c. For each two-parameter plot, create statistics windows according to your instrument's acquisition software and include the x- and y-median for all parameters.
   d. Stop collecting events and remove sample from the cytometer.
4. Place a single-color sample on the cytometer and begin to collect events.

***Note:*** *If using a single-color cell sample that does not contain any negative events, a small amount of unstained cells may be added to the single-color samples just before loading the sample onto the cytometer. Alternatively, an unstained cell sample that has been processed through the PrimeFlow™ assay must be used and the median fluorescence intensity of that sample should be noted for all parameters.*

a. Looking at the two-parameter plots for the fluorochrome collected, adjust the quadrants so that the positive events are fully contained within either the lower right or the upper left quadrant (Figure A3.2, left).

b. Looking at the statistics, note the y- or x-median of the two populations. Adjust the compensation for the fluorochrome being collected (subtract its fluorescence out of the other channels being used in the experiment) until the medians of the two populations are the same (Figure A3.2, right).

c. Stop collecting events and remove sample from the cytometer.

d. Repeat for each of the single-color samples.

5. Confirm that the compensation settings are appropriate for the experimental samples.
   a. Adjust forward- and side-scatter voltages back to those noted in Step III2a. Do not change voltages for any of the fluorescent PMT channels.
   b. Place a fully stained sample on the cytometer and begin collecting events.
   c. Looking at each of the two-parameter plots, confirm that the compensation is set correctly by looking at the medians.
   d. Stop collecting events and remove sample from the cytometer.
6. After setting compensation, you are ready to acquire your samples.
7. Use FMO controls after acquisition and during analysis to optimize compensation settings.

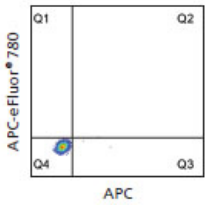


**Figure A3.1.** Unstained sample.

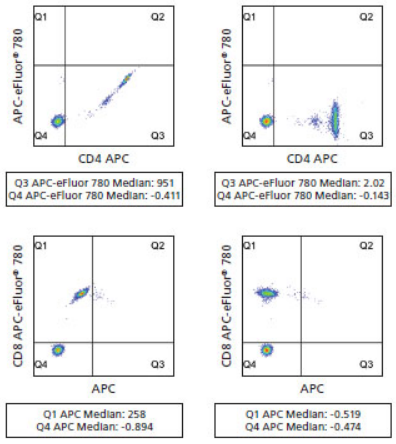


**Figure A3.2.** Single-color samples for APC (top row) or APC-eFluor™ 780 (bottom row). Uncompensated (left) or compensated (right) data are shown.

## Appendix 4: Examples of expected results

**Table A4.1.** Experimental set up for a 6-color panel to detect induction of IFN gamma and TNF alpha in stimulated normal human peripheral blood mononuclear cells (PBMC).

| | | | 488 Laser | | | 633 Laser | 405 Laser | |
|---|---|---|---|---|---|---|---|---|
| **tube #** | **Tube description** | **Cell sample** | **Alexa Fluor™ 488 530/30** | **PE-eFluor™ 610 610/20** | **PE-Cyanine7 780/60** | **Alexa Fluor™ 750 780/60** | **eFluor™ 450 450/50** | **eFluor™ 506 (510/20)** |
| 1 | Unstained/ autofluorescence | stimulated | - | - | - | - | - | - |
| 2 | Alexa Fluor™ 488 single-color | stimulated | TNFa mRNA | - | - | - | - | - |
| 3 | PE-eFluor™ 610 single-color | stimulated | - | CD8a | - | - | - | - |
| 4 | PE-Cyanine7 single-color | stimulated | - | - | TNF alpha | - | - | - |
| 5 | Alexa Fluor™ 750 single-color | stimulated | - | - | - | IFNg mRNA | - | - |
| 6 | eFluor™ 450 single-color | stimulated | - | - | - | - | IFN gamma | - |
| 7 | eFluor™ 506 single-color | stimulated | - | - | - | - | - | FVD eF506 |
| 8 | Alexa Fluor™ 488 FMO | stimulated | - | CD8a | TNF alpha | IFNg mRNA | IFN gamma | FVD eF506 |
| 9 | PE-eFluor™ 610 FMO | stimulated | TNFa mRNA | - | TNF alpha | IFNg mRNA | IFN gamma | FVD eF506 |
| 10 | PE-Cyanine7 FMO | stimulated | TNFa mRNA | CD8a | - | IFNg mRNA | IFN gamma | FVD eF506 |
| 11 | Alexa Fluor™ 750 FMO | stimulated | TNFa mRNA | CD8a | TNF alpha | - | IFN gamma | FVD eF506 |
| 12 | eFluor™ 450 FMO | stimulated | TNFa mRNA | CD8a | TNF alpha | IFNg mRNA | - | FVD eF506 |
| 13 | eFluor™ 506 FMO | stimulated | TNFa mRNA | CD8a | TNF alpha | IFNg mRNA | IFN gamma | - |
| 14 | Isotype/ no probe control | stimulated | no probe | CD8a | mIgG1 isotype | no probe | mIgG1 isotype | FVD eF506 |
| 15 | 6-color sample | stimulated | TNFa mRNA | CD8a | TNF alpha | IFNg mRNA | IFN gamma | FVD eF506 |
| 16 | Alexa Fluor™ 488 FMO | unstimulated | - | CD8a | TNF alpha | IFNg mRNA | IFN gamma | FVD eF506 |
| 17 | PE-eFluor™ 610 FMO | unstimulated | TNFa mRNA | - | TNF alpha | IFNg mRNA | IFN gamma | FVD eF506 |
| 18 | PE-Cyanine7 FMO | unstimulated | TNFa mRNA | CD8a | - | IFNg mRNA | IFN gamma | FVD eF506 |
| 19 | Alexa Fluor™ 750 FMO | unstimulated | TNFa mRNA | CD8a | TNF alpha | - | IFN gamma | FVD eF506 |
| 20 | eFluor™ 450 FMO | unstimulated | TNFa mRNA | CD8a | TNF alpha | IFNg mRNA | - | FVD eF506 |
| 21 | eFluor™ 506 FMO | unstimulated | TNFa mRNA | CD8a | TNF alpha | IFNg mRNA | IFN gamma | - |
| 22 | Isotype/ no probe control | unstimulated | no probe | CD8a | mIgG1 isotype | no probe | mIgG1 isotype | FVD eF506 |
| 23 | 6-color sample | unstimulated | TNFa mRNA | CD8a | TNF alpha | IFNg mRNA | IFN gamma | FVD eF506 |

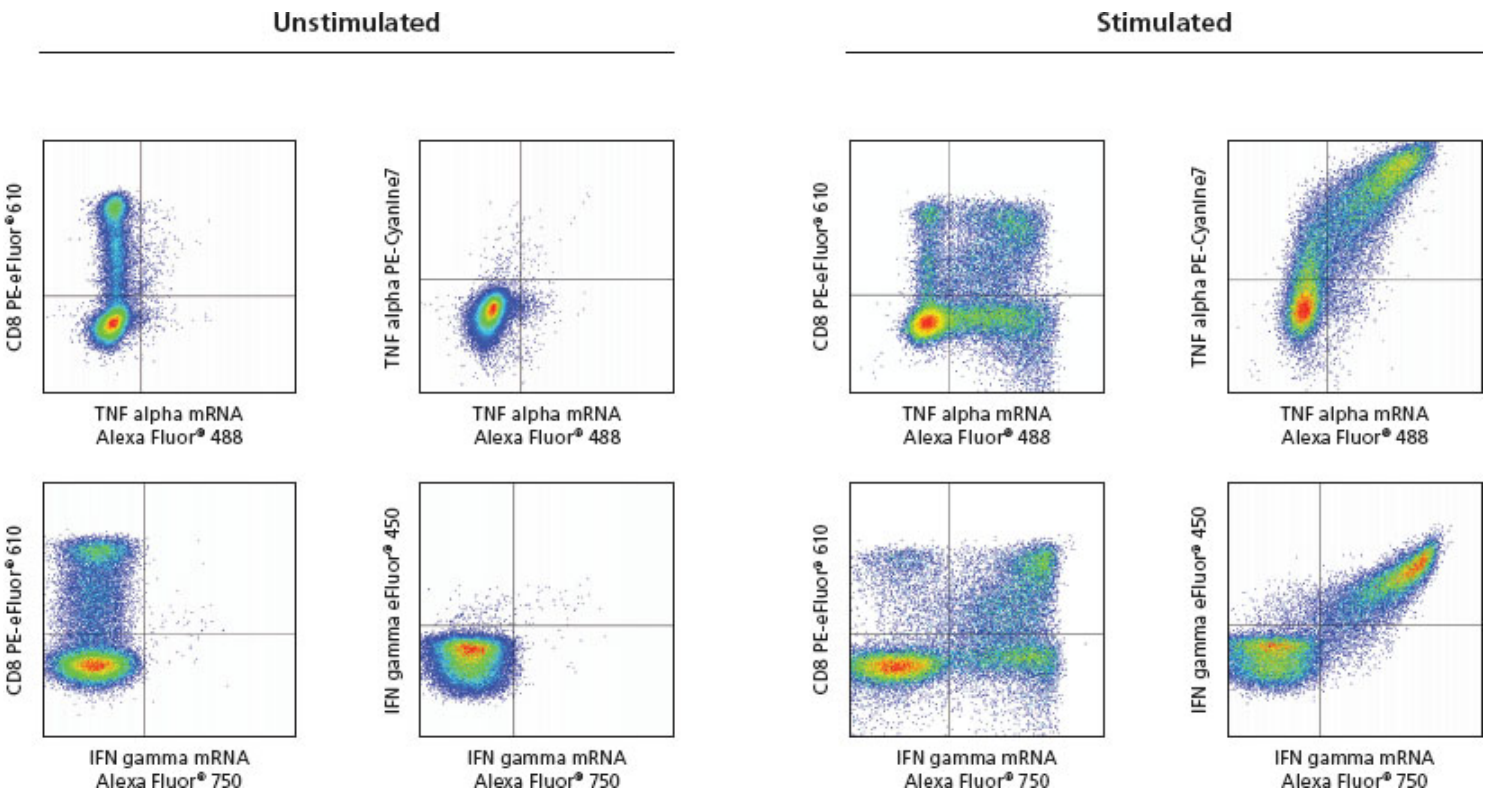


**Figure A4.1.** Normal human peripheral blood mononuclear cells were unstimulated (left) or stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitors) (Cat. No. No. 00-4975) for 3 hours (right) and then analyzed using the PrimeFlow™™ RNA Assay. Cells were intracellularly stained with Anti-Human CD8a PE-eFluor™ 610 (Cat. No. No. 61-0088), Anti- Human TNF alpha PE-Cyanine7 (Cat. No. No. 25-7349), and Anti-Human IFN gamma eFluor™ 450 (Cat. No. No. 48-7319). Cells were then labeled with Type 4 Human TNF alpha Alexa Fluor™ 488 or Type 6 Human IFN gamma Alexa Fluor™ 750 Target Probes (top or bottom row, respectively) for the measurement of mRNA. Cells in the lymphocyte gate were used for analysis. Detection of RNA are shown on the x-axes, while the y-axes show antibody staining of either CD8 or the corresponding cytokine as indicated.

**Table A4.2.** Experimental set up for a 5-color panel to detect induction of Granzyme B and Ki-67 in stimulated mouse splenocytes.

| | | | 488 Laser | | | 633 Laser | 405 Laser |
|---|---|---|---|---|---|---|---|
| **tube #** | **Tube description** | **Cell sample** | **Alexa Fluor™ 488 530/30** | **PE-eFluor™ 610 610/20** | **PE-Cyanine7 780/60** | **Alexa Fluor™ 750 780/60** | **eFluor™ 450 450/50** |
| 1 | Unstained/ autofluorescence | stimulated | - | - | - | - | - |
| 2 | Alexa Fluor™ 488 single-color | stimulated | Ki-67 mRNA | - | - | - | - |
| 3 | PE-eFluor™ 610 single-color | stimulated | - | CD8a | - | - | - |
| 4 | PE-Cyanine7 single-color | stimulated | - | - | Granzyme B | - | - |
| 5 | Alexa Fluor™ 750 single-color | stimulated | - | - | - | Gzmb mRNA | - |
| 6 | eFluor™ 450 single-color | stimulated | - | - | - | - | Ki-67 |
| 7 | Alexa Fluor™ 488 FMO | stimulated | - | CD8a | Granzyme B | Gzmb mRNA | Ki-67 |
| 8 | PE-eFluor™ 610 FMO | stimulated | Ki-67 mRNA | - | Granzyme B | Gzmb mRNA | Ki-67 |
| 9 | PE-Cyanine7 FMO | stimulated | Ki-67 mRNA | CD8a | - | Gzmb mRNA | Ki-67 |
| 10 | Alexa Fluor™ 750 FMO | stimulated | Ki-67 mRNA | CD8a | Granzyme B | - | Ki-67 |
| 11 | eFluor™ 450 FMO | stimulated | Ki-67 mRNA | CD8a | Granzyme B | Gzmb mRNA | - |
| 12 | Isotype/ no probe control | stimulated | no probe | CD8a | rIgG2a isotype | no probe | rIgG2a isotype |
| 13 | 5-color sample | stimulated | Ki-67 mRNA | CD8a | Granzyme B | Gzmb mRNA | Ki-67 |
| 14 | Alexa Fluor™ 488 FMO | unstimulated | - | CD8a | Granzyme B | Gzmb mRNA | Ki-67 |
| 15 | PE-eFluor™ 610 FMO | unstimulated | Ki-67 mRNA | - | Granzyme B | Gzmb mRNA | Ki-67 |
| 16 | PE-Cyanine7 FMO | unstimulated | Ki-67 mRNA | CD8a | - | Gzmb mRNA | Ki-67 |
| 17 | Alexa Fluor™ 750 FMO | unstimulated | Ki-67 mRNA | CD8a | Granzyme B | - | Ki-67 |
| 18 | eFluor™ 450 FMO | unstimulated | Ki-67 mRNA | CD8a | Granzyme B | Gzmb mRNA | - |
| 19 | Isotype/ no probe control | unstimulated | no probe | CD8a | rIgG2a isotype | no probe | rIgG2a isotype |
| 20 | 5-color sample | unstimulated | Ki-67 mRNA | CD8a | Granzyme B | Gzmb mRNA | Ki-67 |

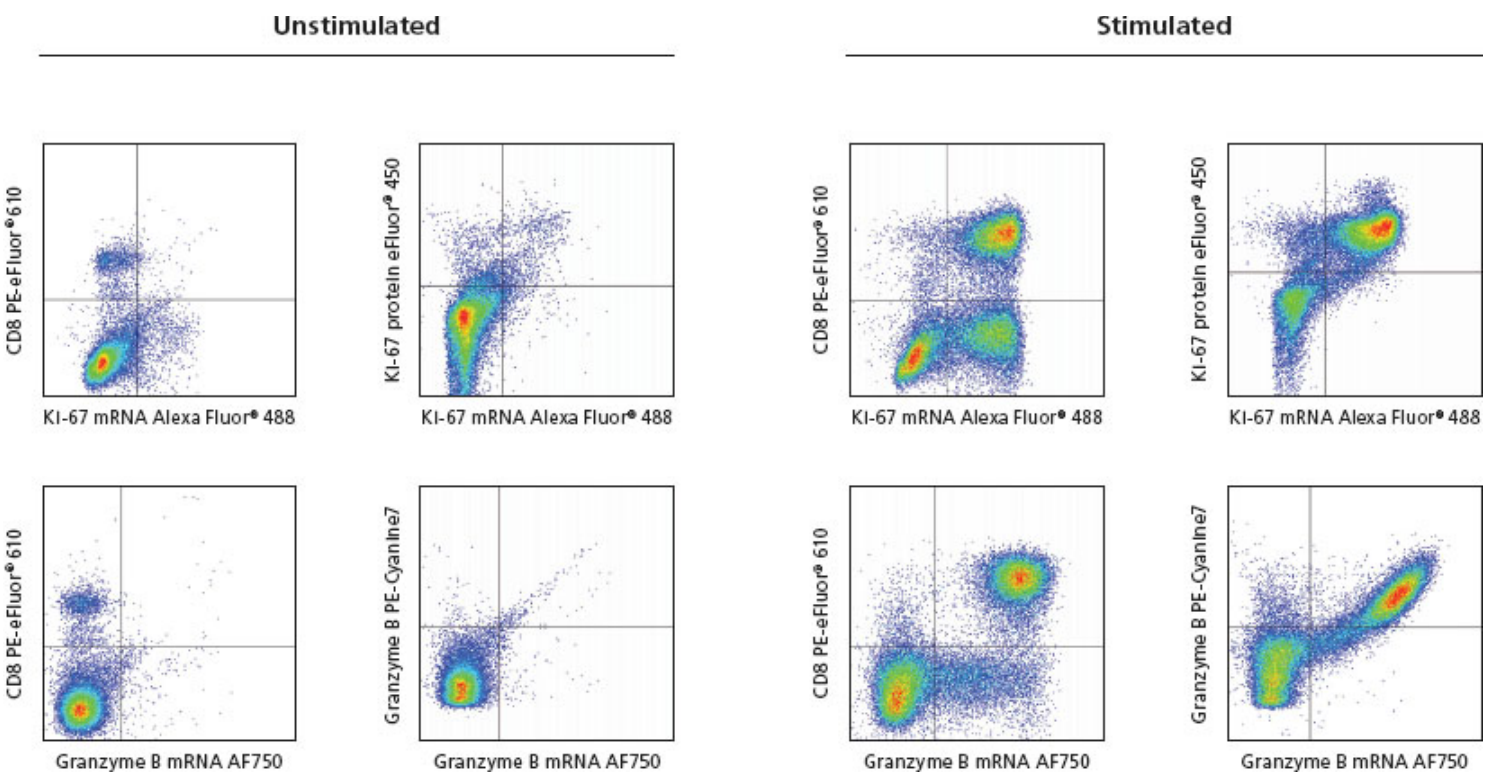


**Figure A4.2.** Mouse splenocytes were unstimulated (left) or stimulated with Anti-Mouse CD3 and CD28 Functional Grade Purifieds (Cat. No. 16-0031 and 16-0281) for 2 days (right) then analyzed using the PrimeFlow™TM RNA Assay. Cells were intracellularly stained with Anti-Mouse CD8a PE-eFluor™ 610 (Cat. No. 61-0081), Anti-Mouse Granzyme B PE-Cyanine 7 (Cat. No. 25-8898), and Anti-Mouse Ki-67 eFluor™ 450 (Cat. No. No. 48-5698). Cells were then labeled with Type 4 Mouse Ki-67 Alexa Fluor™ 488 or Type 6 Mouse Granzyme B Alexa Fluor™ 750 Target Probes (top or bottom row, respectively) for the measurement of mRNA. Detection of RNA are shown on the x-axes, while the y-axes show antibody staining of either CD8 or the corresponding protein, as indicated.

## Appendix 5: Validated cells and recommended positive control genes

| Cell | Recommended Positive Control Gene | Type 1 | Type 2 | Type 6 | Type 10 |
|---|---|---|---|---|---|
| Human peripheral blood cells[1,2] | RPL13A | VA1-13100 | VA4-13187 | VA6-13186 | VA10-19016 |
| | B2M | VA1-10611 | VA4-13460 | VA6-11782 | VA10-10297 |
| U937, human monocytic lymphoma | RPL13A | VA1-13100 | VA4-13187 | VA6-13186 | VA10-19016 |
| | B2M | VA1-10611 | VA4-13460 | VA6-11782 | VA10-10297 |
| Jurkat, human T cell lymphoma | RPL13A | VA1-13100 | VA4-13187 | VA6-13186 | VA10-19016 |
| | B2M | VA1-10611 | VA4-13460 | VA6-11782 | VA10-10297 |
| HeLa[3], human cervical carcinoma | RPL13A | VA1-13100 | VA4-13187 | VA6-13186 | VA10-19016 |
| | GADPH | VA1-10119 | VA4-10641 | VA6-10337 | VA10-10458 |
| PC9[3], human lung carcinoma | RPL13A | VA1-13100 | VA4-13187 | VA6-13186 | VA10-19016 |
| | GADPH | VA1-10119 | VA4-10641 | VA6-10337 | VA10-10458 |
| Mouse splenocytes[1] | ACTB | VB1-10350 | VB4-10432 | VB6-12823 | VB10-20653 |
| | RPL13A | VB1-16196 | VB4-16154 | VB6-15315 | VB10-20652 |
| Mouse thymocytes | ACTB | VB1-10350 | VB4-10432 | VB6-12823 | VB10-20653 |
| | RPL13A | VB1-16196 | VB4-16154 | VB6-15315 | VB10-20652 |
| Mouse bone marrow cells | ACTB | VB1-10350 | VB4-10432 | VB6-12823 | VB10-20653 |

[1] Tested on fresh and cryopreserved samples
[2] BMC and whole blood, for granulocytes in whole blood samples, B2M is recommended over RPL12a
[3] Adherent cells, limited testing

## Appendix 6: Temperature validation procedure for incubator

Temperature control is critical for the success of the PrimeFlow™™ RNA Assay. Improper hybridization temperature will result in high background or weak signal, or both. The incubator should be validated before use following these instructions. This temperature validation procedure is appropriate for both tube- and plate-based protocols in this user manual.

### Materials required

- Incubator capable of maintaining temperature at 40±1°C (Thermo Fisher, Cat. No. QS0704 or QS0712)
- ViewRNA™ Temperature Validation Kit (Thermo Fisher, Cat. No. QV0523)
- 1.5-mL microcentrifuge tube (included in PrimeFlow™ RNA Assay kit)
- Metal heat block for 1.5-mL microcentrifuge tube (VWR, Cat. No. 13-687-210)
- Parafilm™ wrap

### Calibration procedure

#### Step I: Prepare the incubator.

1. Turn on the incubator.
2. Set the temperature to 40°C.
3. Place the metal heat block into the incubator near the center of the middle shelf of the incubator.
4. Allow the incubator and heat block to equilibrate overnight.

#### Step II: Assemble the temperature validation unit.

1. Insert the battery to activate the digital thermometer.
2. Use a pointed object (e.g., a ballpoint pen) to drill a hole into the center of the lid of a 1.5-mL microcentrifuge tube.
3. Add 0.2 mL of deionized water to the tube and then close the lid.
4. Insert the Type-K beaded probe into the digital thermometer, and place the other end of the probe through the pre-drilled hole of the 1.5-mL tube and into the water.
5. Wrap Parafilm around the top of the 1.5-mL tube and probe to form a seal. Avoid an excessive amount of Parafilm around the sides of the tube; otherwise it may not fit properly into the heat block.
6. Turn on the digital thermometer.

#### Step III: Measure and adjust the temperature of the incubator.

7. Place the 1.5-mL tube containing the probe from Step II into the prewarmed heat block from Step I.
8. Close the door, making sure there is sufficient slack in the wiring.
9. Wait 15 minutes for temperature to equilibrate.
10. Record the temperature. If necessary, adjust the temperature settings so that the digital thermometer reads 40°C. After adjustment, allow the incubator and heat block to equilibrate. Then recheck the temperature.
11. Repeat the step above to adjust temperature until the incubator is 40±1°C.

***Note***: *We recommend calibrating the incubator at least once a month to ensure accuracy.*

#### Step IV: Assess incubator temperature uniformity.

12. Repeat Step III to measure the temperature at multiple positions in the incubator to determine temperature uniformity.

***Note***: *The temperature for all positions should be 40±1°C.*

#### Step V: Assess temperature ramp-up time.

13. Remove the 1.5-mL tube containing the probe from the prewarmed heat block from Step III, and allow it to return to room temperature.
14. Open the incubator door for 1 minute, then place the 1.5-mL tube containing the probe from Step V1 into the heat block and close the door. Measure the time needed for the temperature to return to 40°C and monitor the temperature profile during recovery.
15. Repeat Steps V1–2 two more times.

***Note***: *Do not use the incubator for the assay if it takes more than 10 minutes to return to 40°C or if it overshoots by more than 2°C during recovery.*

### Appendix 7: Protocol using 96-well plates

This protocol is validated based on the use of a polystyrene 96-well v-bottom plate (Cat. No. No. 44-17005); polystyrene u-bottom plates may also be used. To discard supernatant from the wells, the plate may be inverted, using a single motion with adequate force, and then gently blotted on a paper towel. Alternatively, aspiration may be used, being careful to not disrupt the pellet. The residual volume inside each well should not exceed 10 µL.

***Note:*** *Flat-bottom plates are not recommended for use with this protocol.*

## Experimental procedure

### Day 1: Antibody staining, fixation, and permeabilization

1. Pre-warm PrimeFlow™ RNA Wash Buffer to room temperature. This buffer will first be used in Step 15.
2. Aliquot 1–5 x $10^6$ cells in 100 µL of Flow Cytometry Staining Buffer per well.

   ***Note***: *To use whole blood, it is necessary to pre-lyse the red blood cells before beginning the assay using the 10x RBC Lysis Buffer (Cat. No. No. 00-4300). Please see the product datasheet or Best Protocols: Red Blood Cell Lysis Protocol, Protocol A for instructions for use. Alternatively, perform Steps 2–12 in bulk (refer to the 1.5-mL tube protocol above, Steps 2–11, to maintain the optimal sample to reagent ratio).*
3. In 12 × 75 mm polystyrene tubes or in a separate 96-well plate, prepare single-color compensation control samples using the PrimeFlow™ Compensation Kit and any experimental antibodies used. Refer to Appendix 3 for detailed instructions. Store compensation control samples in the dark at 2–8°C in IC Fixation Buffer by resuspending the stained UltraComp eBeads™ microspheres in 100 µL of Flow Cytometry Staining Buffer, then add 100 µL of IC Fixation Buffer and mix gently.
4. Surface stain cells with fluorochrome-conjugated antibodies at their optimal concentration for 30 minutes at 2–8°C.

   ***Note***: *If needed, please see Best Protocols: Staining Cell Surface Antigens for Flow Cytometry: Protocol A: Cell Suspensions for detailed instructions for staining.*

   ***Note***: *Use antibodies that are conjugated to approved fluorochromes only (see General notes, Fluorochrome compatibility section above).*

   ***Note***: *Cells may be stained with a Fixable Viability Dye before or after surface staining (see Best Protocols: Viability Staining Protocol, Protocol C for detailed instructions).*

   ***Note***: *Staining for some surface markers may be done after fixation and permeabilization. Please see the Antibody Clone Performance Following Fixation/Permeabilization table on our website and refer to the column for "After IC Fixation and Perm Wash" to determine if the antibody clone will recognize a fixed epitope. If you prefer to stain after fixation, skip this step and proceed to Step 5.*
5. Spin down at 500 × *g* for 4 minutes, then discard supernatant.
6. Add 200 µL of Flow Cytometry Staining Buffer to each sample, pipet to mix, and spin down at 500 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
7. Prepare Fixation Buffer 1 by mixing equal parts of PrimeFlow™ RNA Fixation Buffer 1A and PrimeFlow™ RNA Fixation Buffer 1B. Mix gently by inverting.

   ***Note***: *You will need 200 µL of this buffer per well. Prepare this buffer in bulk to accommodate all samples. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh. Dispose of any unused buffer.*
8. Add 200 µL of prepared PrimeFlow™ RNA Fixation Buffer 1 to each sample and pipet to mix. Incubate for 30 minutes at 2–8°C.
9. Spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
10. Prepare 1x PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors by diluting PrimeFlow™ RNA Permeabilization Buffer (10x) to 1x with RNase-free water. Then add RNase Inhibitor 1 (1,000x) at a 1/1,000 dilution and RNase Inhibitor 2 (100x) at a 1/100 dilution. Mix gently by inverting. Keep at 2–8°C.

    ***Note***: *You will need 700 µL of this buffer per sample. Prepare this buffer in bulk to accommodate all samples. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh.*
11. Add 200 µL of 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors to each sample, pipet to mix, and spin down at 1,000 ***x g*** for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
12. Repeat Step 11.

    ***Note***: *Whole blood samples being prepared in bulk should be transferred into a 96-well plate before proceeding to Step 12.*
13. Intracellularly stain cells with fluorochrome-conjugated antibodies at their optimal concentration in the 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors for 30 minutes at 2–8°C.

    ***Note***: *If intracellular staining is not desired, skip this step and proceed to Step 14.*

    ***Note***: *Staining for some surface markers may be done after fixation and permeabilization. Please see the Antibody Clone Performance Following Fixation/Permeabilization table on our website and refer to the column for "After IC Fixation and Perm Wash" to determine if the antibody clone will recognize a fixed epitope.*

    ***Note***: *Use antibodies that are conjugated to approved fluorochromes only (see general notes, fluorochrome compatibility section above).*
14. Add 200 µL of 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors to each sample, pipet to mix, and spin down at 1,000 ***x g*** for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
15. Prepare 1X PrimeFlow™ RNA Fixation Buffer 2 by combining 25 µL of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 175 µL of PrimeFlow™ RNA Wash Buffer per well. Mix gently by inverting.

    ***Note***: *You will need 200 µL of this buffer per well. Prepare this buffer in bulk to accommodate all samples. For example, for 10 samples, combine 250 µL of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 1,750 µL of PrimeFlow™ RNA Wash Buffer. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh. Dispose of any unused buffer.*

16. Add 200 µL of 1X PrimeFlow™ RNA Fixation Buffer 2 to each well and pipet to mix. Incubate for 60 minutes in the dark at room temperature.

    ***Note***: *It is important to fix the samples at room temperature. Do not perform this step on ice.*

17. *(OPTIONAL)* Cells may be stored in 1X PrimeFlow™ RNA Fixation Buffer 2 overnight in the dark at 2–8°C, instead of incubating for 60 minutes at room temperature (i.e., skip Step 15).
18. Spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
19. Add 200 µL of PrimeFlow™ RNA Wash Buffer to each well, pipet to mix, and spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
20. Repeat Step 18.
21. *(OPTIONAL)* Cells may be stored in PrimeFlow™ RNA Wash Buffer with RNase Inhibitor 1 overnight in the dark at 2–8°C with lid on. To do so, add RNase Inhibitor 1 (1,000X) to PrimeFlow™ RNA Wash Buffer at a 1/1,000 dilution and use in Step 19.

    ***Note***: *You will need 100 µL of this buffer per well. Prepare this buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose of any unused buffer.*

### Day 1: Target probe hybridization

***Note***: *It is critical that the residual volume after all washes does not exceed 10 µL.*

***Note***: *Diluted Target Probes should be pipetted directly into the cell suspension, and samples should be mixed well before incubating. The total volume for Target Probe hybridization should be 200 µL per well.*

22. Thaw Target Probe Sets (20X) at room temperature.
23. Pre-warm PrimeFlow™ RNA Target Probe Diluent to 40°C.
24. Dilute Target Probes 1/20 in PrimeFlow™ RNA Target Probe Diluent. Mix thoroughly by pipetting up and down.

    ***Note***: *You will need 100 µL of diluted Target Probes for each sample. If you are adding more than one Target Probe per sample, adjust the volume of the PrimeFlow™ RNA Target Probe Diluent accordingly so that the final volume remains 100 µL per sample.*

25. Add 100 µL of PrimeFlow™ RNA Wash Buffer to each well. Then add 100 µL of diluted Target Probe(s) directly into the cell suspension for the appropriate samples and pipet to mix. Incubate plate with the lid on for 2 hours at 40°C.

    ***Note***: *Plate sealing is not necessary for hybridization. Plates should be placed directly onto the incubator shelf. Do not stack plates.*

26. Spin down at 1,000 × *g* for 4 minutes. Discard supernatant and resuspend cells in the residual volume by vortexing.
27. Add 200 µL of PrimeFlow™ RNA Wash Buffer, and spin down at 1,000 × *g* for 4 minutes. Discard supernatant and resuspend cells in the residual volume by vortexing.
28. Repeat Step 27.
29. Prepare PrimeFlow™ RNA Wash Buffer with RNase Inhibitors by adding RNase Inhibitors (100X) to PrimeFlow™ RNA Wash Buffer at a 1/100 dilution. Mix gently by inverting.

    ***Note***: *You will need 100 µL of this buffer per well. Prepare this buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose of any unused buffer.*

30. Add 100 µL of PrimeFlow™ RNA Wash Buffer with RNase Inhibitors, and pipet to mix.
31. Store samples overnight in the dark at 2–8°C with lid on.

    ***Note***: *We recommend this stopping point for ease-of-use and a more manageable workflow. However, if desired, Step 31 may be skipped. If skipping this step, proceed to Step 32 and continue through to the end of the protocol.*

### Day 2: Signal amplification

***Note***: *It is critical that the residual volume after all washes does not exceed 10 µL.*

***Note***: *PrimeFlow™ RNA PreAmp Mix, PrimeFlow™ RNA Amp Mix, and diluted Label Probes should be pipetted directly into the samples and mixed well before incubating. The total volume for each hybridization is 200 µL per well. During hybridization, keep lid on the plate. Place plates directly on the incubator shelf. Do not stack plates.*

32. Pre-warm samples and PrimeFlow™ RNA Wash Buffer to room temperature.

    ***Note***: *Some precipitation in the Wash Buffer may occur. After warming to room temperature, an aliquot for the volume needed for the day may be prepared and allowed to settle or briefly centrifuged to remove precipitation. Do not repeatedly centrifuge the Wash Buffer to remove precipitation.*

33. Pre-warm PrimeFlow™ RNA PreAmp Mix, PrimeFlow™ RNA Amp Mix, and PrimeFlow™ RNA Label Probe Diluent to 40°C.
34. Add 100 µL of PrimeFlow™ RNA PreAmp Mix directly into the cell suspension for each sample, and pipet to mix, and then incubate plate with the lid on for 1.5 hours at 40°C.
35. Spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
36. Add 200 µL of PrimeFlow™ RNA Wash Buffer, and spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
37. Repeat Step 36.
38. Add 100 µL of PrimeFlow™ RNA Wash Buffer to each well. Then add 100 µL of PrimeFlow™ RNA Amp Mix directly into the cell suspension, and pipet to mix. Incubate plate with the lid on for 1.5 hours at 40°C.
39. Spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
40. Add 200 µL of PrimeFlow™ RNA Wash Buffer, and spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.

41. Repeat Step 40.
42. Dilute PrimeFlow™ RNA Label Probes (100X) 1/100 in PrimeFlow™ RNA Label Probe Diluent.

    ***Note***: *You will need 100 µL of diluted Label Probes for each sample. Prepare diluted Label Probes in bulk to accommodate all samples.*
43. Add 100 µL of PrimeFlow™ RNA Wash Buffer to each well. Then add 100 µL of diluted PrimeFlow™ RNA Label Probes directly into the cell suspension, and pipet to mix. Incubate plate with the lid on for 1 hour at 40°C.
44. Spin down at 1,000 × *g* for 4 minutes. Discard supernatant and resuspend cells in the residual volume by vortexing.
45. Add 200 µL of PrimeFlow™ RNA Wash Buffer, and spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
46. Repeat Step 45.
47. Add 200 µL of PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer, and spin down at 1,000 × *g* for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
48. If a plate adapter is used for sample acquisition, resuspend cells in an appropriate volume of PrimeFlow™ RNA Fixation Buffer or Flow Cytometry Staining Buffer, and analyze on a flow cytometer. Otherwise, transfer samples to 12 × 75 mm polystyrene tubes, resuspend cells in an appropriate volume of Storage Buffer or Flow Cytometry Staining Buffer, and analyze on a flow cytometer.

    ***Note***: *Samples may be stored in the dark at 2-8°C for up to 3 days before analysis. We recommend storing the samples in IC Fixation Buffer by mixing 100 µL of cells with 100 µL of IC Fixation Buffer.*

## Quick guide: PrimeFlow™ RNA Assay in 96-well plates

### Day 1:

1. Surface stain cells with antibody and/or fixable viability dye and prepare compensation controls.
2. Wash once with Flow Cytometry Staining Buffer. Spin cells at 500 × *g* for 4 minutes at 2–8°C.
3. Fix cells in PrimeFlow™ RNA Fixation Buffer 1 for 30 minutes at 2–8°C.
4. Wash twice with 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors. Spin cells at 1,000 × *g* for 4 minutes at 2–8°C.
5. Intracellularly stain cells with antibody for 30 minutes at 2–8°C.
6. Wash once with 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors. Spin cells at 1,000 × *g* for 4 minutes at 2–8°C.
7. Fix cells in 1X PrimeFlow™ RNA Fixation Buffer 2 for 60 minutes at room temperature.
8. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 1,000 × *g* for 4 minutes at room temperature.
9. Perform Target Probe hybridization for 2 hours at 40°C.
10. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 1,000 × *g* for 4 minutes at room temperature.
11. Resuspend cells in PrimeFlow™ RNA Wash Buffer with RNase Inhibitors.
12. Store samples overnight.

### Day 2:

13. Perform PreAmp hybridization for 1.5 hours at 40°C.
14. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 1,000 × *g* for 4 minutes at room temperature.
15. Perform Amp hybridization for 1.5 hours at 40°C.
16. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 1,000 × *g* for 4 minutes at room temperature.
17. Perform Label Probe hybridization for 1 hour at 40°C.
18. Wash twice with PrimeFlow™ RNA Wash Buffer. Spin cells at 1,000 × *g* for 4 minutes at room temperature.
19. Wash once with PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer. Spin cells at 1,000 × *g* for 4 minutes at room temperature.
20. Analyze samples on a flow cytometer.

Notes

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