

PrimeFlow[™] RNA Assay Kit

USER GUIDE

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For descriptions of symbols on product labels or product documents, go to [thermofisher.com/symbols-definition](https://www.thermofisher.com/symbols-definition).

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Revision	Date	Description
B.0	22 February 2022	• The storage temperature was updated to 2-8°C for the PrimeFlow Compensation Kit (see “Contents and storage” on page 6). • The dilution ratios were corrected, and RNase Inhibitor 2 was deleted throughout the document. • The document template was updated including associated updates to trademarks, logos, licensing, and warranty. • This was the initial release with the Revision history table.

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Product information



WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from [thermofisher.com/support](https://www.thermofisher.com/support).

Product description

The PrimeFlow™ RNA Assay Kit 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 four 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 Kit, 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 four different RNA targets for multicolor flow cytometric analysis. Once the cells have been processed by the PrimeFlow™ RNA Assay Kit, the data can be collected and analyzed on any standard flow cytometer. The workflow illustrates the detection of two unique targets.

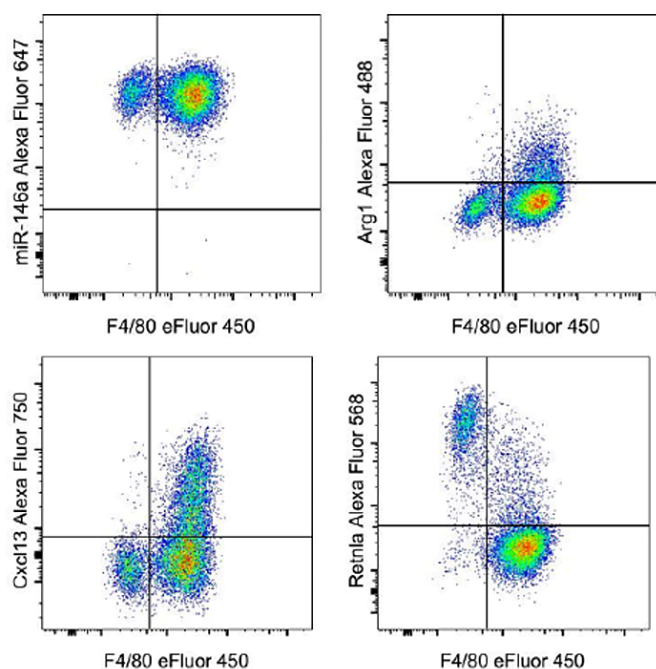


Figure 1 C57Bl/6 mouse peritoneal exudate cells analyzed using the PrimeFlow™ RNA Assay

CD16/CD32 Monoclonal Antibody (93) (Cat. No. 14-0161) and Normal Mouse Serum (Cat. No. 24-5544) to block Fc receptors, then surface-stained with anti-mouse F4/80 Monoclonal Antibody (BM8), eFluor™ eFluor™ 450 (Cat. No. 48-4801) and anti-mouse CD11b Monoclonal Antibody (M1/70), PE-Cyanine7 (Cat. No. 25-0112), before analysis with the PrimeFlow™ RNA Assay. 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

Unless otherwise indicated, all materials are available through [thermofisher.com](https://www.thermofisher.com). Catalog numbers that appear as links open the web pages for those products.

Contents	Cat. No.	Storage
PrimeFlow™ RNA Tubes	19197	Store at room temperature
PrimeFlow™ RNA Fixation Buffer 1A	00-18100-52, 00-18100-54	Store at 2–8°C
PrimeFlow™ RNA Fixation Buffer 1B	00-18200-52, 00-18200-54	
PrimeFlow™ RNA Permeabilization Buffer (10x)	00-18300-12, 00-18300-43	
PrimeFlow™ RNA Fixation Buffer 2	00-18400-104, 00-18400-53	

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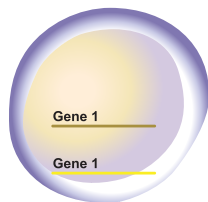
Contents	Cat. No.	Storage
PrimeFlow™ RNA Wash Buffer	00-19180-26, 00-19180-58, 0019180-56	Store at 2–8°C
PrimeFlow™ RNA Target Probe Diluent	00-19185-19	
PrimeFlow™ RNA PreAmp Mix	00-16000-104, VIEW-01-00-16000	
PrimeFlow™ RNA Amp Mix	00-16001-104, VIEW-01-00-16001	
PrimeFlow™ RNA Label Probe Diluent	00-19183-19, VIEW-01-00-19183	
PrimeFlow™ RNA Storage Buffer	00-19178-56, 00-19178-140	
PrimeFlow™ RNase Inhibitors (100X)	00-16002-19, VIEW-01-00-16002V	
PrimeFlow™ Compensation Kit ^[1]	88-17009-42	
PrimeFlow™ RNA Label Probes	00-16003-32, 00-16003-92	
eBioscience™ IC Fixation Buffer	00-8222-49	

^[1] PrimeFlow™ Compensation Kit includes UltraComp eBeads™ Compensation Beads (Cat. No. [01-2222-41](#), [01-2222-42](#)).

PrimeFlow™ RNA Assay Kit workflow

PrimeFlow™ RNA Assay Kit workflow

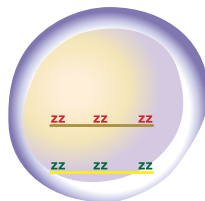
Sample Preparation



Suspension cells with RNA fixed

1. Label proteins with antibody (*optional*).
2. Fix and permeabilize cells in suspension.

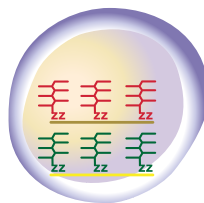
Target Hybridization



ZZ ZZ Gene-specific Target Probes

1. Incubate cells with gene-specific Target Probes (Type 1, 4, 6 or 10).

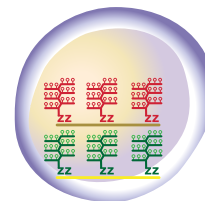
Signal Amplification



L L PreAmplifier
L L Amplifier

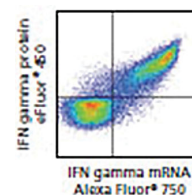
1. Hybridize with Pre-Amplifier and Amplifier DNA (Type 1, 4, 6 or 10)

Detection



o o Fluorescent Label Probe

1. Add Label Probes to cells.
2. Process cells using a standard flow cytometer.



General notes

Table 1 Assay specifications

Sample type	Single-cell suspensions, including human whole blood and peripheral blood mononuclear cells (PBMC), mouse dissociated tissues, and cell lines. See “Validated cells and recommended positive control genes” on page 38 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 D, “Protocol using 96-well plates” for the protocol to use 96-well plates.
Instrumentation for RNA detection	Flow cytometer equipped with: • Blue (488 nm), yellow-green (561 nm), and red (633–640 nm) lasers • 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

- 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.
- This assay is highly temperature dependent. Please ensure that the incubator holds temperature at $40\pm1^{\circ}\text{C}$. 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 (Cat. No. [QV0523](#)), following the instructions in “Temperature validation procedure for incubator” on page 31.

- 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.
- 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 Scientific Technical Support (techsupport@thermofisher.com) for more information.
- Protect samples from light after they have been stained with fluorochrome-conjugated antibodies and labeled for RNA.
- PrimeFlow™ RNA Assay Kit may be used to detect microRNA. For optimal detection of microRNA, the PrimeFlow™ microRNA Pretreatment Buffer (Cat. No. [88-18006](#)) 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.

We recommend performing the assay in two days

Day 1

- Antibody staining
- Fixation and permeabilization
- Target probe hybridization

Day 2

- Signal amplification
- Flow cytometric analysis

Fluorochrome compatibility

- 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.
- 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.
- PerCP, PerCP-Cyanine5.5, and PerCP-eFluor™ 710 should not be used. We recommend using PE-Cyanine5 or PE-Cyanine5.5 instead.
- Qdot™ nanocrystal- and eVolve™-conjugated antibodies are not compatible with this assay.

Intracellular antibody staining compatibility

- The fixation and permeabilization buffers in this kit are compatible with most Thermo Fisher Scientific antibodies used for intracellular staining. Some changes in performance are expected when compared to performance in the eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (Cat. No. [88-8824-00](#)) or eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Cat. No. [00-5523-00](#)). Antibody performance should be determined empirically using the PrimeFlow™ Fixation/Permeabilization Buffer Set (Cat. No. [88-17000-210](#)).
- The fixation and permeabilization buffers in this kit are compatible with most Thermo Fisher Scientific 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.
- 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

- 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 Kit (RPL13A for human leukocytes and beta-actin [ACTB] for mouse tissues). See “Validated cells and recommended positive control genes” on page 38 for specific cell types and other recommended positive control genes.
- For proper compensation, use the compensation kit that is included with the PrimeFlow™ RNA Assay Kit. See “Cytometer setup and compensation” on page 25 for instructions for use and for other methods to set compensation. Please contact Thermo Fisher Scientific Technical Support (techsupport@thermofisher.com) for more information.
- 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.
- 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.
- 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.
- See “Examples of expected results” on page 34 for examples of sample set-up and experimental design.
- This protocol is intended for use with 1.5-mL microcentrifuge tubes. See Appendix D, “Protocol using 96-well plates” for the 96-well plate protocol.

Materials Included

Product	Cat. No.	Storage
PrimeFlow™ RNA tubes (1.5-mL microcentrifuge tubes)	N/A	Store at room temperature
PrimeFlow™ RNA Fixation Buffer 1A	00-18100-52, 00-18100-54	Store at 2–8°C
PrimeFlow™ RNA Fixation Buffer 1B	00-18200-52, 00-18200-54	
PrimeFlow™ RNA Permeabilization Buffer (10X)	00-18300-12, 00-18300-43	

(continued)

Product	Cat. No.	Storage
PrimeFlow™ RNA Fixation Buffer 2	00-18400-104, 00-18400-53	Store at 2–8°C
PrimeFlow™ RNA Wash Buffer	00-19180-26, 00-19180-58, 0019180-56	
PrimeFlow™ RNA Target Probe Diluent	00-19185-19	
PrimeFlow™ RNA PreAmp Mix	00-16000-104, VIEW-01-00-16000	
PrimeFlow™ RNA Amp Mix	00-16001-104, VIEW-01-00-16001	
PrimeFlow™ RNA Label Probe Diluent	00-19183-19, VIEW-01-00-19183	
PrimeFlow™ RNA Storage Buffer	00-19178-56, 00-19178-140	
PrimeFlow™ RNase Inhibitor (100X)	00-16002-19, VIEW-01-00-16002V	
PrimeFlow™ Compensation Kit ^[1]	88-17009-42	
PrimeFlow™ RNA Label Probes	00-16003-32, 00-16003-92	
eBioscience™ IC Fixation Buffer	00-8222-49	

^[1] PrimeFlow™ Compensation Kit includes UltraComp eBeads™ Compensation Beads (Cat. No. 01-2222-41, 01-2222-42).

Required materials not included

"MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Product	Cat. No.
Flow Cytometry Staining Buffer	00-4222
12 × 75 mm polystyrene tubes (e.g., Corning™)	352008
(OPTIONAL) 96-well, v-bottom polystyrene plate ^[1]	44-17005
Fixable Viability Dye, as needed	MLS
Target Probe sets, as needed	
Fluorochrome-conjugated antibodies, as needed	

^[1] See Appendix D, "Protocol using 96-well plates" for modified protocol.

Instruments and equipment

- Refer to “Instrument and equipment setup guide” on page 24 of this document.

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 (step 1 to step 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 1×10^7 cells/mL. To perform the assay in 96-well plates, refer to Appendix D, “Protocol using 96-well plates” 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 on page 15.
2. Aliquot $1\text{--}5 \times 10^6$ 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. 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 “Cytometer setup and compensation” on page 25 for detailed instructions. Store compensation control samples in the dark at 2–8°C in eBioscience™ IC Fixation Buffer by resuspending the stained UltraComp eBeads™ Compensation Beads in 100 μL of Flow Cytometry Staining Buffer. Then add 100 μL of eBioscience™ 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 on page 15.

5. Add 1 mL of Flow Cytometry Staining Buffer to each sample, invert to mix, and centrifuge at $500 \times g$ 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 on page 15.

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. Centrifuge at $800 \times g$ 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 (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 $800 \times g$ for 5 minutes, then discard supernatant and resuspend cells in the residual volume.

11. Repeat step 10 on page 15.

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 on page 16.

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 $800 \times g$ 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 μ L of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 875 μ L 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 on page 16).
17. Spin down at $800 \times g$ for 5 minutes, then aspirate all but 100 μ L 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 $800 \times g$ for 5 minutes, then aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
19. Repeat step 18 on page 16.

Note: It is critical that the residual volume is as close to 100 μ L 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 (100X) to PrimeFlow™ RNA Wash Buffer at a 1/100 dilution and use in step 19 on page 16.

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.

1. Thaw Target Probes, including Positive Control Target Probe Sets (20X), at room temperature.
2. Pre-warm PrimeFlow™ RNA Target Probe Diluent to 40°C.
3. 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.

4. 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.
5. 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.
6. Prepare PrimeFlow™ RNA Wash Buffer with RNase Inhibitor 1 by adding RNase Inhibitor 1 (100X) at a 1/100 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.

7. Add 1 mL of PrimeFlow™ RNA Wash Buffer with RNase Inhibitor 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.
8. 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 7 on page 17 may be skipped. If skipping this step, proceed to step 1 on page 18 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 μ L 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 μ L of residual volume and samples should be mixed well before incubating. Do not pipette these solutions onto the walls of the tubes.

1. 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 μ L of diluted Label Probes for each sample. Prepare diluted Label Probes in bulk to accommodate all samples.

2. Pre-warm PrimeFlow™ RNA PreAmp Mix, PrimeFlow™ RNA Amp Mix, and PrimeFlow™ RNA Label Probe Diluent to 40°C.
 3. Add 100 μ L 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.
 4. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at $800 \times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
 5. Repeat step 4 on page 18 two times, for a total of three washes.
 6. Add 100 μ L 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.
 7. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at $800 \times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
 8. Repeat step 7 on page 18.
 9. Dilute PrimeFlow™ RNA Label Probes (100X) 1/100 in PrimeFlow™ RNA Label Probe Diluent.
-

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

10. Add 100 μ L 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.
11. Add 1 mL of PrimeFlow™ RNA Wash Buffer to each sample, invert to mix, and spin down at $800 \times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.

12. Repeat step 11 on page 18.
13. Add 1 mL of PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer to each sample, invert to mix, and spin down at $800 \times g$ for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
14. Transfer samples to 12 $\times$ 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 eBioscience™ IC Fixation Buffer by mixing 100 μ L of cells with 100 μ L of eBioscience™ 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 $500 \times g$ 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. Centrifuge cells at $800 \times g$ 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. Centrifuge cells at $800 \times g$ 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 $800 \times g$ 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. Centrifuge cells at $800 \times g$ for 5 minutes at room temperature.
11. Wash once with PrimeFlow™ RNA Wash Buffer with RNase Inhibitors. Centrifuge cells at $800 \times g$ for 5 minutes at room temperature.
12. Store samples overnight.

Day 2

1. Perform PreAmp hybridization for 1.5 hours at 40°C.
2. Wash three times with PrimeFlow™ RNA Wash Buffer. Centrifuge cells at $800 \times g$ for 5 minutes at room temperature.
3. Perform Amp hybridization for 1.5 hours at 40°C.
4. Wash twice with PrimeFlow™ RNA Wash Buffer. Centrifuge cells at $800 \times g$ for 5 minutes at room temperature.
5. Perform Label Probe hybridization for 1 hour at 40°C.
6. Wash twice with PrimeFlow™ RNA Wash Buffer. Centrifuge at $800 \times g$ for 5 minutes at room temperature.
7. Wash once with PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining. Centrifuge cells at $800 \times g$ for 5 minutes at room temperature.
8. Analyze samples on a flow cytometer.



Troubleshooting

Observation	Possible cause	Recommended action
Poor cell recovery or low cell count on cytometer	Cell lysis due to cells in poor physiological condition during processing	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.
		Check cell viability using trypan blue or other viability dye before beginning the assay.
	Improper centrifuge settings	Confirm settings are for RCF ($\times g$), not RPM.
		Set centrifuge to the speed specified in the protocol.
	Improper tubes used	Only use the 1.5-mL tubes provided with the kit.
		Tubes from other vendors may result in significant cell loss.
	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- μ L pipette tip on the tip of the aspirator to help reduce aspiration rate. While aspirating, follow the meniscus down to the 100- μ L mark on the side of the tube.
Weak or no signal	Rough handling	Avoid excessive vortexing to prevent cell damage. Vortex in short pulses.
	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. 580).
	Cytometer not set up properly	Check that your instrument is properly set up according to the manufacturer's recommendations.
		Use samples labeled with one each of the included Positive Control Probe Sets to set compensation.
		Do not use fluorochrome-conjugated antibodies to set compensation for RNA detection.
	Incorrect preparation of signal amplification reagents	Make sure PreAmp Mix, Amp Mix, and Label Probes are used in the specified order.

Observation	Possible cause	Recommended action
Weak or no signal (continued)	Incorrect incubator temperature	Incubator must be able to hold temperature at 40±1°C.
		Use a metal heat block for hybridization.
		Minimize traffic to incubator. Temperature tolerance is 1–2°C.
		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.
		Measure temperature using the ViewRNA™ Temperature Validation Kit (Cat. No. QV0523).
	Incorrect Wash Buffer	Do not substitute PrimeFlow™ RNA Wash Buffer with Storage Buffer or any other buffer.
	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).
	Incorrect dilution of Target Probes or Label Probes	Ensure Target Probes are diluted 1/20 with PrimeFlow™ RNA Target Probe Diluent.
		Ensure PrimeFlow™ RNA Label Probes (100X) are diluted 1/100 with PrimeFlow™ RNA Label Probe Diluent.
	Gene expressed at very low level	Check biological model and confirm gene is expressed in sample by an alternative method such as QuantiGene™ 2.0 Assay for lysate.
		Keep in mind that protein expression might not correlate with RNA expression in some cases.
	Incorrect or insufficient RNase Inhibitor used	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.
High background	Compensation not set up correctly	See “Cytometer setup and compensation” on page 25 for how to set compensation.
		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.
		Do not use fluorochrome-conjugated antibodies to set compensation for RNA detection.

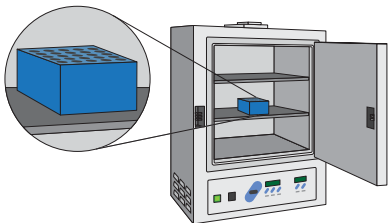
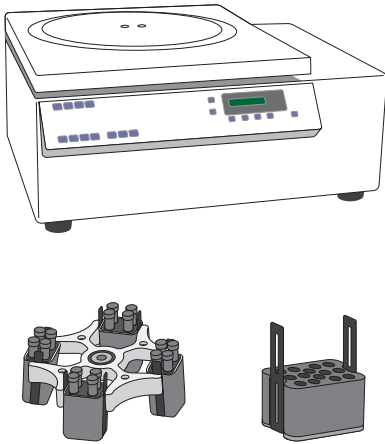
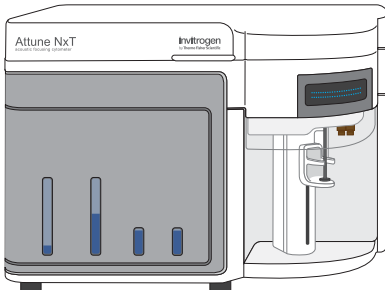
Observation	Possible cause	Recommended action
High background (continued)	Incorrect incubator temperature	See “Temperature validation procedure for incubator” on page 31 on how to validate an incubator.
		Incubator must be able to hold temperature at $40 \pm 1^\circ\text{C}$.
		Use a metal heat block for hybridization.
		Minimize traffic to incubator. Temperature tolerance is $1\text{--}2^\circ\text{C}$.
		Ensure incubator has been stable at 40°C for at least 12 hours before starting assay as temperature may drift during initial incubator setup.
		Measure temperature using the ViewRNA™ Temperature Validation Kit (Cat. No. QV0523).
	Insufficient washing	Ensure the PrimeFlow™ RNA Wash Buffer is used at room temperature.
		Follow instructions for washing steps in the protocol.
		Ensure uniform cell resuspension by gentle vortexing.
	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.
	Over-fixation	Follow the protocol for proper fixation time.
	Excessive Target Probe used	Dilute Target Probes at a 1:20 dilution.



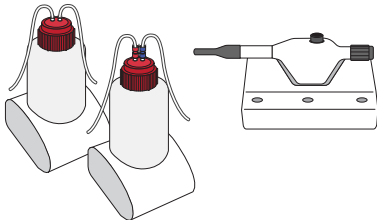
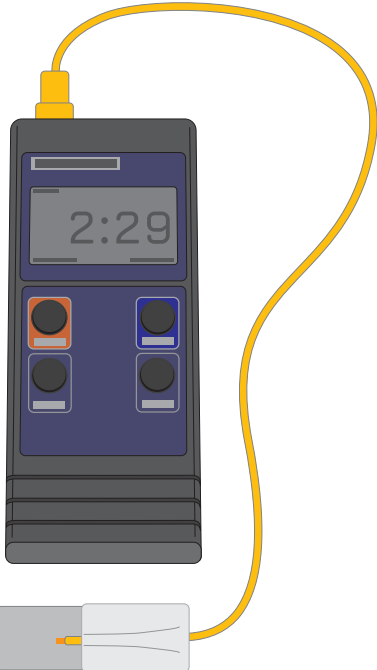
Instruments and equipment guides

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 	- Validated to maintain $40 \pm 1^\circ\text{C}$ - Metal heat block for 1.5-mL microcentrifuge tube Example shown: Metal heat block (Cat. No. 13-687-210)
2. Refrigerated swinging bucket centrifuge 	Swinging bucket centrifuge with adaptors for 15-mL conical tubes and 1.5-mL microcentrifuge tubes. (Optional: with refrigeration to 4°C) Example shown: Centrifuge (Eppendorf™, model 5810R) Rotor (Eppendorf™, model A-4-44) Adaptor for 15-mL conical tube (Eppendorf™, model 5804 755.006) Adaptor for 1.5-mL microcentrifuge tube (Eppendorf™, model 5804 750.004)
3. Flow cytometer 	- Three lasers: blue (488 nm), yellow-green (561 nm), and red (633 nm or similar) - Detection optics optimized for FITC, PE-eFluor™ 610 (PE-Texas Red™), APC, and APC-eFluor™ 780 (APC-Cyanine7) Example shown: Invitrogen™ Attune™ NxT Flow Cytometer

(continued)

Equipment	Specifications
4. Aspiration system for washing 	Aspiration rate adjusted to 0.5 mL/sec. Can use in-house vacuum line or vacuum pump Example shown: Vacuum™ bottle (Argos Technologies™, model EV432) Aspirator (Argos Technologies™, model EV514)
5. ViewRNA™ Temperature Validation Kit 	NIST™ traceable thermometer, with temperature probe in 1.5-mL microcentrifuge tube (shown on left). (Cat. No. QV0523)

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

Catalog numbers that appear as links open the web pages for those products.

Product	Contents	Cat. No.
PrimeFlow™ Compensation Kit		88-17009
	UltraComp eBeads™ Compensation Beads	01-2222-41 , 01-2222-42
	PrimeFlow™ Compensation Control Alexa Fluor™ 647	PF51-17003-41
	PrimeFlow™ Compensation Control Alexa Fluor™ 488	PF53-17002-41
	PrimeFlow™ Compensation Control Alexa Fluor™ 568	PF54-17006-41
	PrimeFlow™ Compensation Control Alexa Fluor™ 750	PF55-17004-41
eBioscience™ IC Fixation Buffer		00-8222-49

Materials required not provided

Product	Cat. No.
Calibration beads	MLS
Experimental, fluorochrome-conjugated antibodies, as needed	
Unstained cells that have undergone PrimeFlow™ assay	N/A
Fully stained cells that have undergone PrimeFlow™ assay	

Experimental procedure

Step I: Stain UltraComp eBeads™ Compensation Beads.

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 D, “Protocol using 96-well plates”. 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™ Compensation Beads by vigorously inverting at least 10 times or pulse-vortexing.
3. Add 1 drop of UltraComp eBeads™ Compensation Beads 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 to 30 minutes at 2 to 8°C for up to 5 days.
8. Add 2 mL of Flow Cytometry Staining Buffer to each tube and centrifuge 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 eBioscience™ IC Fixation Buffer to each tube and briefly vortex to mix.
10. Store in the dark at 2 to 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 centrifuge 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 200 to 400 µL of Flow Cytometry Staining Buffer to each tube and briefly vortex to mix each tube immediately before analysis in “Step IV: Assess incubator temperature uniformity.” on page 33.

Step II: Ensure proper instrument alignment.

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

Note: UltraComp eBeads™ Compensation Beads 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 on page 28.

- 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 IV: Set compensation.

1. Return forward- and side-scatter voltages to those noted in step III3 on page 29 in order to visualize the UltraComp eBeads™ Compensation Beads. 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 2).

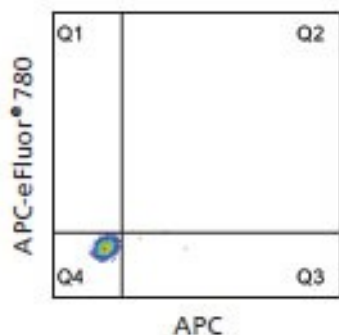


Figure 2 Unstained sample.

- 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 3).

- 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 3).

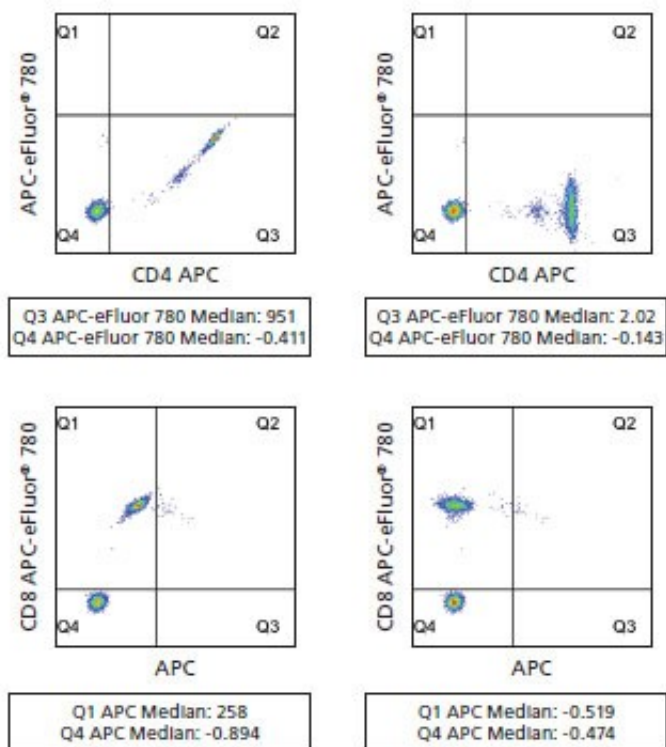


Figure 3 Single-color samples for APC (top row) or APC-eFluor™ 780 (bottom row). Uncompensated (left) or compensated (right) data are shown.

- 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 on page 28. 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.

Temperature validation procedure for incubator

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 required

Product	Cat. No.
ViewRNA™ Temperature Validation Kit	QV0523
Metal heat block for 1.5-mL microcentrifuge tube	VWR™ 13-687-210
1.5-mL microcentrifuge tube ^[1]	MLS
Incubator capable of maintaining temperature at 40±1°C	
Parafilm™ wrap	

^[1] Included in PrimeFlow™ RNA Assay Kit

Calibration procedure

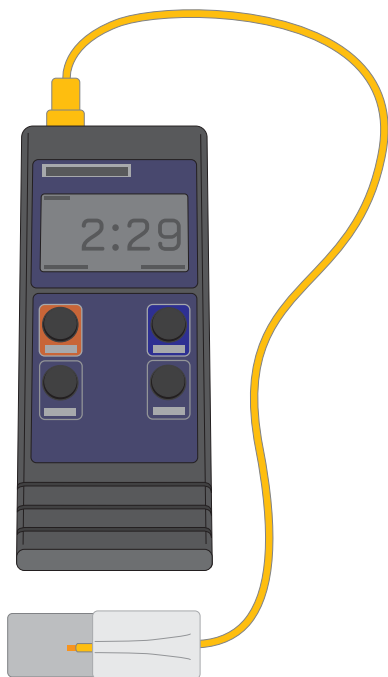
Step I: Prepare the incubator.

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.

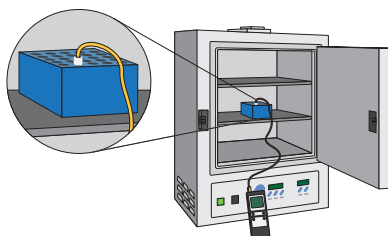
5. Wrap Parafilm™ around the top of the 1.5-mL tube and probe to form a seal. Avoid an excessive amount of Parafilm™ around the sides of the tube; otherwise it may not fit properly into the heat block.
6. Turn on the digital thermometer.



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

1. Place the 1.5-mL tube containing the probe from “Step II: Assemble the temperature validation unit.” on page 31 into the prewarmed heat block from “Step I: Prepare the incubator.” on page 31.
2. Close the door, making sure there is sufficient slack in the wiring.
3. Wait 15 minutes for temperature to equilibrate.
4. 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.
5. 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.

Repeat “Step III: Measure and adjust the temperature of the incubator.” on page 32 to measure the temperature at multiple positions in the incubator to determine temperature uniformity.

Note: The temperature for all positions should be $40 \pm 1^\circ\text{C}$.

Step V: Assess temperature ramp-up time.

1. Remove the 1.5-mL tube containing the probe from the prewarmed heat block from “Step III: Measure and adjust the temperature of the incubator.” on page 32, and allow it to return to room temperature.
2. 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.
3. Repeat steps V1–2 on page 33 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.





Expected results, validated cells, and recommended control genes

Examples of expected results

Table 2 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).

Tube no. and description	Cell sample	488 Laser			633 Laser	405 Laser	
		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	TNFα mRNA	-	-	-	-	-
3—PE-eFluor™ 610 single-color	stimulated	-	CD8a	-	-	-	-
4—PE-Cyanine7 single-color	stimulated	-	-	TNF alpha	-	-	-
5—Alexa Fluor™ 750 single-color	stimulated	-	-	-	IFNγ 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	IFNγ mRNA	IFN gamma	FVD eF506
9—PE-eFluor™ 610 FMO	stimulated	TNFα mRNA	-	TNF alpha	IFNγ mRNA	IFN gamma	FVD eF506
10—PE-Cyanine7 FMO	stimulated	TNFα mRNA	CD8a	-	IFNγ mRNA	IFN gamma	FVD eF506
11—Alexa Fluor™ 750 FMO	stimulated	TNFα mRNA	CD8a	TNF alpha	-	IFN gamma	FVD eF506



Table 2 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). *(continued)*

Tube no. and description	Cell sample	488 Laser			633 Laser	405 Laser	
		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
12—eFluor™ 450 FMO	stimulated	TNFα mRNA	CD8a	TNF alpha	IFNγ mRNA	-	FVD eF506
13—eFluor™ 506 FMO	stimulated	TNFα mRNA	CD8a	TNF alpha	IFNγ mRNA	IFN gamma	-
14—Isotype/no probe control	stimulated	-	CD8a	mIgG1 isotype	-	mIgG1 isotype	FVD eF506
15—6-color sample	stimulated	TNFα mRNA	CD8a	TNF alpha	IFNγ mRNA	IFN gamma	FVD eF506
16—Alexa Fluor™ 488 FMO	unstimulated	-	CD8a	TNF alpha	IFNγ mRNA	IFN gamma	FVD eF506
17—PE-eFluor™ 610 FMO	unstimulated	TNFα mRNA	-	TNF alpha	IFNγ mRNA	IFN gamma	FVD eF506
18—PE-Cyanine7 FMO	unstimulated	TNFα mRNA	CD8a	-	IFNγ mRNA	IFN gamma	FVD eF506
19—Alexa Fluor™ 750 FMO	unstimulated	TNFα mRNA	CD8a	TNF alpha	-	IFN gamma	FVD eF506
20—eFluor™ 450 FMO	unstimulated	TNFα mRNA	CD8a	TNF alpha	IFNγ mRNA	-	FVD eF506
21—eFluor™ 506 FMO	unstimulated	TNFα mRNA	CD8a	TNF alpha	IFNγ mRNA	IFN gamma	-
22—Isotype/no probe control	unstimulated	-	CD8a	mIgG1 isotype	-	mIgG1 isotype	FVD eF506
23—6-color sample	unstimulated	TNFα mRNA	CD8a	TNF alpha	IFNγ mRNA	IFN gamma	FVD eF506

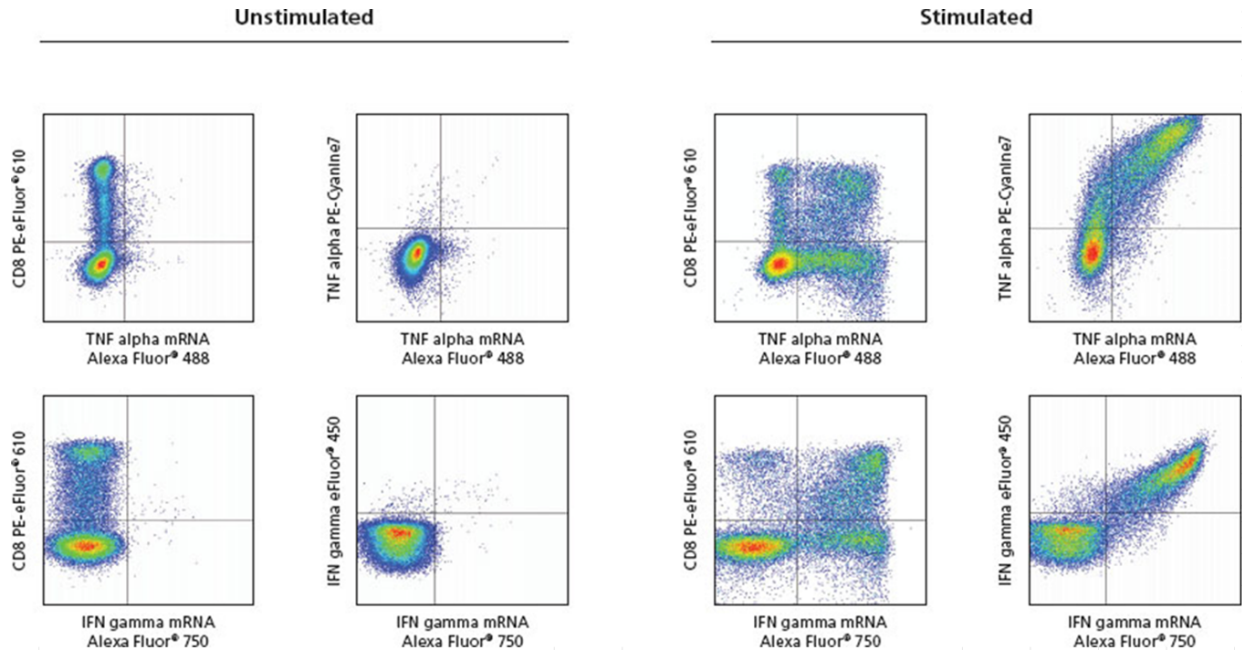


Figure 4 Normal human peripheral blood mononuclear cells were unstimulated (left) or stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitors) (Cat. No. No. 00-4975) for 3 hours (right) and then analyzed using the PrimeFlow™ RNA Assay.

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

Tube No. and description	Cell sample	488 Laser			633 Laser	405 Laser
		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	-



Table 3 Experimental set up for a 5-color panel to detect induction of Granzyme B and Ki-67 in stimulated mouse splenocytes. *(continued)*

Tube No. and description	Cell sample	488 Laser			633 Laser	405 Laser
		Alexa Fluor™ 488 530/30	PE-eFluor™ 610 610/20	PE-Cyanine7 780/60	Alexa Fluor™ 750 780/60	eFluor™ 450 450/50
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	-	CD8a	rlgG2a isotype	-	rlgG2a 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	-	CD8a	rlgG2a isotype	-	rlgG2a isotype
20—5-color sample	unstimulated	Ki-67 mRNA	CD8a	Granzyme B	Gzmb mRNA	Ki-67

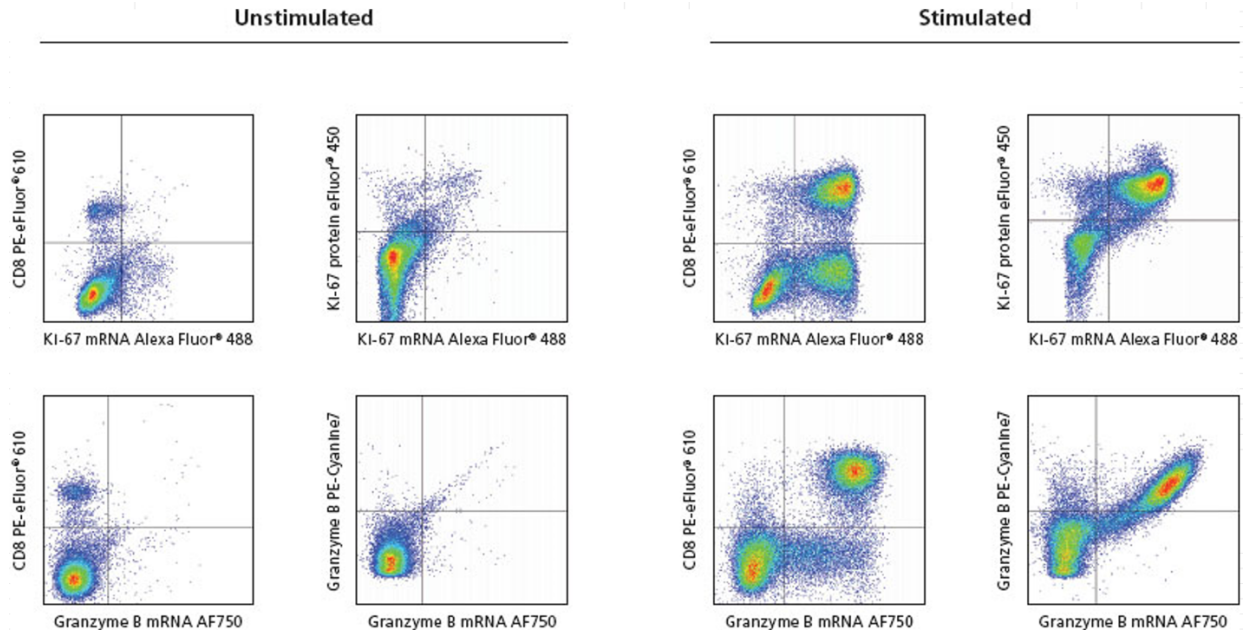


Figure 5 Mouse splenocytes were unstimulated (left) or stimulated with Anti-Mouse CD3 and CD28 Functional Grade Purifieds (Cat. No. 16-0031 and 16-0281) for 2 days (right) then analyzed using the PrimeFlow™ RNA Assay.

Cells were intracellularly stained with Anti-Mouse CD8a PE-eFluor™ 610 (Cat. No. 61-0081), Anti-Mouse Granzyme B PE-Cyanine7 (Cat. No. 25-8898), and Anti-Mouse Ki-67 eFluor™ 450 (Cat. 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.

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



(continued)

Cell	Recommended Positive Control Gene	Type 1	Type 2	Type 6	Type 10
PC9 ^[2] , 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



Protocol using 96-well plates

This protocol is validated based on the use of a polystyrene 96-well v-bottom plate (Cat. 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 μ L.

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 on page 42.
2. Aliquot $1-5 \times 10^6$ cells in 100 μ L 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. 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 to 12 in bulk (refer to the 1.5-mL tube protocol, steps 2 to 41, to maintain the optimal sample to reagent ratio).

3. In 12 $\times$ 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 “Cytometer setup and compensation” on page 25 for detailed instructions. Store compensation control samples in the dark at 2–8°C in eBioscience™ IC Fixation Buffer by resuspending the stained UltraComp eBeads™ Compensation Beads in 100 μ L of Flow Cytometry Staining Buffer, then add 100 μ L of eBioscience™ 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 on page 41.

5. Centrifuge at $500 \times g$ for 4 minutes, then discard supernatant.
6. Add 200 μL of Flow Cytometry Staining Buffer to each sample, pipet to mix, and centrifuge at $500 \times g$ 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 $2-8^{\circ}\text{C}$.
9. Centrifuge at $1,000 \times g$ 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 (100X) at a 1/100 dilution. Mix gently by inverting. Keep at $2-8^{\circ}\text{C}$.

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 centrifuge at $1,000 \times g$ 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 $2-8^{\circ}\text{C}$.

Note: If intracellular staining is not desired, skip this step and proceed to step 15 on page 42.

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 μ L of 1X PrimeFlow™ RNA Permeabilization Buffer with RNase Inhibitors to each sample, pipet to mix, and centrifuge at $1,000 \times g$ 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 μ L of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 175 μ L of PrimeFlow™ RNA Wash Buffer per well. Mix gently by inverting.
-

Note: You will need 200 μ L of this buffer per well. Prepare this buffer in bulk to accommodate all samples. For example, for 10 samples, combine 250 μ L of PrimeFlow™ RNA Fixation Buffer 2 (8X) with 1,750 μ L 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 μ L 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 16).
 18. Centrifuge at $1,000 \times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 19. Add 200 μ L of PrimeFlow™ RNA Wash Buffer to each well, pipet to mix, and centrifuge at $1,000 \times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 20. Add 200 μ L of PrimeFlow™ RNA Wash Buffer to each well, pipet to mix, and centrifuge at $1,000 \times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 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 (100X) to PrimeFlow™ RNA Wash Buffer at a 1/100 dilution and use in step 19.
-

Note: You will need 100 μ L 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 μ L.

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 μ L per well.

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

Note: You will need 100 μ L 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 μ L per sample.

4. Add 100 μ L of PrimeFlow™ RNA Wash Buffer to each well. Then add 100 μ L 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.

5. Centrifuge at 1,000 $\times g$ for 4 minutes. Discard supernatant and resuspend cells in the residual volume by vortexing.
 6. Add 200 μ L of PrimeFlow™ RNA Wash Buffer, and centrifuge at 1,000 $\times g$ for 4 minutes. Discard supernatant and resuspend cells in the residual volume by vortexing.
 7. Repeat step 6.
 8. 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 μ L 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.

9. Add 100 μ L of PrimeFlow™ RNA Wash Buffer with RNase Inhibitors, and pipet to mix.
 10. 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 10 may be skipped. If skipping this step, proceed to step 1 on page 44 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 μ L.

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 μ L per well. During hybridization, keep lid on the plate. Place plates directly on the incubator shelf. Do not stack plates.

1. 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.

2. Pre-warm PrimeFlow™ RNA PreAmp Mix, PrimeFlow™ RNA Amp Mix, and PrimeFlow™ RNA Label Probe Diluent to 40°C.
 3. Add 100 μ L 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.
 4. Centrifuge at 1,000 $\times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 5. Add 200 μ L of PrimeFlow™ RNA Wash Buffer, and centrifuge at 1,000 $\times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 6. Repeat step 5 on page 44.
 7. Add 100 μ L of PrimeFlow™ RNA Wash Buffer to each well. Then add 100 μ L 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.
 8. Centrifuge at 1,000 $\times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 9. Add 200 μ L of PrimeFlow™ RNA Wash Buffer, and spin down at 1,000 $\times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
 10. Repeat step 9 on page 44.
 11. Dilute PrimeFlow™ RNA Label Probes (100X) 1:100 in PrimeFlow™ RNA Label Probe Diluent.
-

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

12. Add 100 μ L of PrimeFlow™ RNA Wash Buffer to each well. Then add 100 μ L 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.



13. Spin down at $1,000 \times g$ for 4 minutes. Discard supernatant and resuspend cells in the residual volume by vortexing.
14. Add 200 μL of PrimeFlow™ RNA Wash Buffer, and centrifuge at $1,000 \times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
15. Repeat step 14 on page 45.
16. Add 200 μL of PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer, and centrifuge at $1,000 \times g$ for 4 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing.
17. 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 $\times$ 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 eBioscience™ IC Fixation Buffer by mixing 100 μL of cells with 100 μL of eBioscience™ IC Fixation Buffer.

Quick guide: PrimeFlow™ RNA Assay Kit 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. Centrifuge cells at $500 \times g$ 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. Centrifuge cells at $1,000 \times g$ 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. Centrifuge cells at $1,000 \times g$ 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. Centrifuge cells at $1,000 \times g$ 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. Centrifuge cells at $1,000 \times g$ for 4 minutes at room temperature.



11. Resuspend cells in PrimeFlow™ RNA Wash Buffer with RNase Inhibitors.
12. Store samples overnight.

Day 2

1. Perform PreAmp hybridization for 1.5 hours at 40°C.
2. Wash twice with PrimeFlow™ RNA Wash Buffer. Centrifuge cells at 1,000 × *g* for 4 minutes at room temperature.
3. Perform Amp hybridization for 1.5 hours at 40°C.
4. Wash twice with PrimeFlow™ RNA Wash Buffer. Centrifuge cells at 1,000 × *g* for 4 minutes at room temperature.
5. Perform Label Probe hybridization for 1 hour at 40°C.
6. Wash twice with PrimeFlow™ RNA Wash Buffer. Centrifuge cells at 1,000 × *g* for 4 minutes at room temperature.
7. Wash once with PrimeFlow™ RNA Storage Buffer or Flow Cytometry Staining Buffer. Centrifuge cells at 1,000 × *g* for 4 minutes at room temperature.
8. Analyze samples on a flow cytometer.



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