

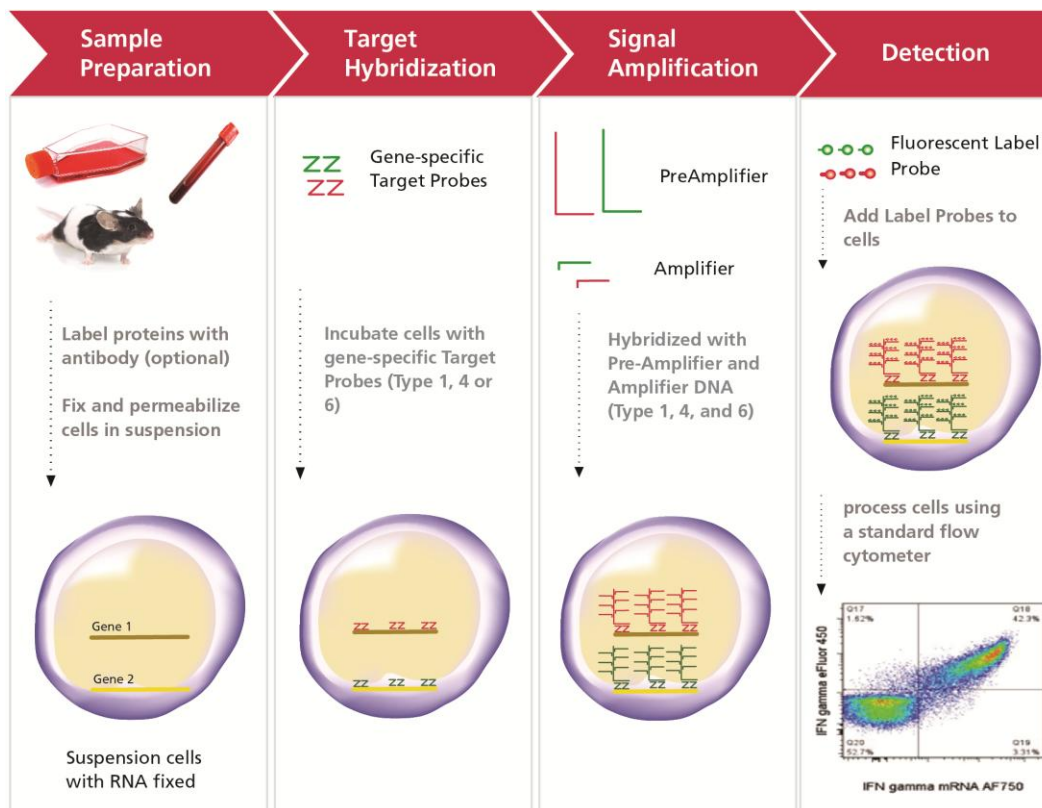
PrimeFlow™ RNA Assay User Manual and Protocol

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Introduction

The PrimeFlow™ RNA Assay is an *in situ* hybridization assay that combines the power of the Affymetrix ViewRNA™ branched DNA technology with the single-cell resolution of flow cytometry. This assay enables 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 a 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 sides of a respective probe pair have bound. Multiple Amplifier molecules then 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. eBioscience currently offers 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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General Notes

Assay Specifications	
Sample Type	Single cell suspensions, including human whole blood and PBMC, mouse dissociated tissues, and cell lines. See Appendix A5 for a complete list of validated cell types.
Species	Mammalian
Plex level	Up to 3 RNA targets simultaneously
Assay format	1.5-mL microcentrifuge tube
Instrumentation for RNA detection	Flow cytometer equipped with: <ul style="list-style-type: none"> • blue (488 nm) and red (633-640 nm) lasers • filter sets for FITC (bandpass 530/30), 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 $40 \pm 1^\circ\text{C}$. 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 (Affymetrix, cat. QV0523), following the instructions in Appendix A6 of this user manual.
- This assay allows detection of up to three (3) RNA targets in a single sample:
 - Type 1:** Alexa Fluor® 647; high sensitivity—best for low or unknown expression levels
 - Type 4:** Alexa Fluor® 488; intermediate to low sensitivity—best for medium to high expression levels
 - Type 6:** Alexa Fluor® 750; intermediate to low sensitivity—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)
- 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, but other channels (up to approximately 650 nm) may also be affected and depends on the cell type, laser line, and instrument settings. Please take this into consideration when setting voltages and when designing multicolor panels. Please contact eBioscience Technical Support (tech@ebioscience.com) for more information.
- Protect samples from light after they have been stained with fluorochrome-conjugated antibodies and labeled for RNA.
- We recommend performing the assay in two days, as follows:
 - Day 1
 - Antibody staining
 - Fixation & permeabilization
 - Target Probe hybridization
 - Day 2
 - Signal amplification
 - Flow cytometric analysis

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Fluorochrome compatibility:

1. Organic fluorochromes are compatible with this assay, such as FITC, eFluor® 450, 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 may 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 of eBioscience's antibodies used for intracellular staining. Some changes in performance are expected when compared to performance in the Intracellular Fixation & Permeabilization Buffer Set (cat. 88-8824) or Foxp3/Transcription Factor Staining Buffer Set (cat. 00-5523).
2. The fixation and permeabilization buffers in this kit are compatible with most of eBioscience's 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 β -actin (ACTB) for mouse tissues). See Appendix A5 for specific cell types and other recommended positive control genes.
2. For proper compensation, we recommend using the PrimeFlow™ Compensation Kit (cat. 88-17001-42). Please refer to the product data sheet on the product page for instructions for use. Alternatively, positive control probe sets may be used for single color compensation samples. Do not use fluorochrome-conjugated antibodies to set compensation for RNA signal. If using a combination of cells and beads as compensation controls, be sure to use the appropriate negative population for setting compensation (for beads, use the negative bead population; for cells, use unlabeled cells that have undergone the PrimeFlow™ RNA Assay). Please contact eBioscience Technical Support (tech@ebioscience.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 being used in the experiment. As with single-color controls, there should be an FMO control for every fluorochrome being 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 granulocytes from monocytes is recommended.
6. Please see Appendix A4 for examples of sample set-up and experimental design.

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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
- PrimeFlow™ RNA Fixation Buffer 1B
- PrimeFlow™ RNA Permeabilization Buffer (10X)
- PrimeFlow™ RNA Fixation Buffer 2 (8X)
- PrimeFlow™ RNA Wash Buffer
- PrimeFlow™ RNA Target Probe Diluent
- PrimeFlow™ RNA PreAmp Mix
- PrimeFlow™ RNA Amp Mix
- PrimeFlow™ RNA Label Probe Diluent
- PrimeFlow™ RNA Storage Buffer
- RNase Inhibitor 2 (100X)

Store at -20°C

- RNase Inhibitor 1 (1000X)
- Positive Control Target Probe Sets (20X) (one for each RNA detection channel)
- PrimeFlow™ RNA Label Probes (100X)

Materials required, but not included:

- Flow Cytometry Staining Buffer (cat. 00-4222)
- Fluorochrome-conjugated antibodies, as needed
- [Fixable Viability Dye](#), as needed
- 12x75 mm polystyrene tubes (e.g., BD Falcon, cat. 352008)
- PrimeFlow™ Compensation Kit (cat. 88-17001)
- IC Fixation Buffer (cat. 00-8222)

Instruments and equipment:

- Refer to Appendix A2 of this user manual.

Experiment duration:

Day 1 (~6-8 hours)

- Antibody staining
- Fixation & permeabilization
- Target Probe hybridization

Day 2 (~6 hours)

- Signal amplification
- Flow cytometric analysis

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Experimental Procedure

Day 1: Antibody staining, fixation, and permeabilization

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 & 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 1×10^7 cells/mL.

1. Pre-warm Wash Buffer to room temperature.
2. Aliquot $1-5 \times 10^6$ cells in Flow Cytometry Staining Buffer or 100 μ L 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 before beginning the assay. However, if lysis is desired, the use of [10X RBC Lysis Buffer \(cat. 00-4300\)](#) is recommended. Please see the product datasheet or [Best Protocols: Red Blood Cell Lysis Protocol, Protocol A](#) for detailed instructions for use.
3. 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.
4. Add 1 mL of Flow Cytometry Staining Buffer to each sample, invert to mix, and spin down at 600xg for 5 minutes, then discard supernatant and resuspend cells in the residual volume.
Note: When using whole blood, skip this step and proceed to Step 5.
5. Prepare Fixation Buffer 1 by mixing equal parts of Fixation Buffer 1A and 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.
6. Add 1 mL of prepared Fixation Buffer 1 to each sample, invert to mix, and then incubate for 30 minutes at 2-8°C.
7. Spin down at 800xg for 5 minutes, then discard supernatant and resuspend cells in the residual volume.
8. Prepare 1X Permeabilization Buffer with RNase Inhibitors by diluting Permeabilization Buffer (10X) to 1X with RNase-free water and then add RNase Inhibitor 1 at a 1/1000 dilution and RNase Inhibitor 2 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.
9. Add 1 mL of 1X Permeabilization Buffer with RNase Inhibitors to each sample, invert to mix, and spin down at 800xg for 5 minutes, then discard supernatant and resuspend cells in the residual volume.
10. Repeat wash with 1X Permeabilization Buffer with RNase Inhibitors.

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11. Intracellularly stain cells with fluorochrome-conjugated antibodies at their optimal concentration for 30 minutes at 2-8°C.
Note: *If intracellular staining is not desired skip this step and proceed to Step 12.*
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).*
12. Add 1 mL of 1X Permeabilization Buffer with RNase Inhibitors to each sample, invert to mix, and spin down at 800xg for 5 minutes, then discard supernatant and resuspend cells in the residual volume by vortexing gently.
13. Prepare 1X Fixation Buffer 2 by combining 125 µL of Fixation Buffer 2 (8X) with 875 µL of 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 Fixation Buffer 2 (8X) with 8.75 mL of Wash Buffer. Avoid vortexing or vigorously shaking this buffer. This buffer should be prepared fresh. Dispose of any unused buffer.*
14. Add 1 mL of 1X Fixation Buffer 2 to each sample, 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.*
15. [OPTIONAL] Cells may be stored in 1X Fixation Buffer 2 overnight in the dark at 2-8°C, instead of incubating for 60 minutes at room temperature (Step 14).
16. Spin down at 800xg 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.*
17. Add 1 mL of Wash Buffer to each sample, invert to mix, and spin down at 800xg for 5 minutes, then aspirate all but 100 µL of supernatant and resuspend cells in the residual volume by vortexing gently.
18. Repeat wash with Wash Buffer.
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.*
19. [OPTIONAL] Cells may be stored in Wash Buffer with RNase Inhibitor 1 overnight in the dark at 2-8°C. To do so, add RNase Inhibitor 1 to Wash Buffer at a 1/1000 dilution for the last wash (Step 18).
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.*

20. Thaw Target Probes, including Positive Control Probes, at room temperature.
21. Pre-warm Target Probe Diluent to 40°C.

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22. Dilute Target Probes 1/20 in 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 Target Probe Diluent accordingly so that the final volume remains 100 μ L per sample.
23. Add 100 μ L of diluted Target Probe(s) directly into the cell suspension for the appropriate samples, briefly vortex to mix, and then incubate for 2 hours at 40°C. Invert samples to mix after 1 hour.
24. Add 1 mL of Wash Buffer to each sample, invert to mix, and spin down at 800xg for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
25. Prepare Wash Buffer with RNase Inhibitor 1 by adding RNase Inhibitor 1 to Wash Buffer at a 1/1000 dilution. 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.
26. Add 1 mL of Wash Buffer with RNase Inhibitor 1 to each sample, invert to mix, and spin down at 800xg for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
27. 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 28 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: PreAmp Mix, 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.

28. Pre-warm samples and Wash Buffer to room temperature.
29. Pre-warm PreAmp Mix, Amp Mix, and Label Probe Diluent to 40°C.
30. Thaw Label Probes on ice in the dark.
Note: This can be done during the Amp Mix incubation (Step 34).
31. Add 100 μ L of PreAmp Mix directly into the cell suspension for each sample, briefly vortex to mix, and then incubate for 1.5 hours at 40°C.
32. Add 1 mL of Wash Buffer to each sample, invert to mix, and spin down at 800xg for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
33. Repeat wash with Wash Buffer two more times, for a total of three washes.
34. Add 100 μ L of Amp Mix directly into the cell suspension for each sample, briefly vortex to mix, and then incubate for 1.5 hours at 40°C.
35. Add 1 mL of Wash Buffer to each sample, invert to mix, and spin down at 800xg for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
36. Repeat wash with Wash Buffer.
37. Dilute Label Probes 1/100 in 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.
38. Add 100 μ L of diluted Label Probes directly into the cell suspension for each sample, briefly vortex to mix, and then incubate for 1 hour at 40°C.

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39. Add 1 mL of Wash Buffer to each sample, invert to mix, and spin down at 800xg for 5 minutes. Aspirate all but 100 μ L of supernatant and resuspend cells in the residual volume by vortexing gently.
40. Repeat wash with Wash Buffer.
41. Wash with 1 mL of Storage Buffer or Flow Cytometry Staining Buffer, then transfer samples to 12x75 mm polystyrene tubes 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 μ L of cells with 100 μ L of IC Fixation Buffer. Store samples in the dark at 2-8°C for up to 3 days.*

Quick Guide: PrimeFlow™ RNA Assay

Day 1:

1. Surface stain cells with antibody and/or fixable viability dye.
2. Wash once with Flow Cytometry Staining Buffer. Spin cells at 600xg for 5 minutes.
3. Fix cells in Fixation Buffer 1 for 30 minutes at 2-8°C.
4. Wash twice with 1X Permeabilization Buffer with RNase Inhibitors. Spin cells at 800xg for 5 minutes.
5. Intracellularly stain cells with antibody for 30 minutes at 2-8°C.
6. Wash once with 1X Permeabilization Buffer with RNase Inhibitors. Spin cells at 800xg for 5 minutes.
7. Fix cells in Fixation Buffer 2 for 60 minutes at room temperature.
8. Wash twice with Wash Buffer. Spin cells at 800xg for 5 minutes.
9. Target Probe hybridization for 2 hours at 40°C. Invert to mix after 1 hour.
10. Wash once with Wash Buffer. Spin cells at 800xg for 5 minutes.
11. Wash once with Wash Buffer with RNase Inhibitor 1. Spin cells at 800xg for 5 minutes.
12. Store samples overnight.

Day 2:

13. PreAmp hybridization for 1.5 hours at 40°C.
14. Wash three times with Wash Buffer. Spin cells at 800xg for 5 minutes.
15. Amp hybridization for 1.5 hours at 40°C.
16. Wash twice with Wash Buffer. Spin cells at 800xg for 5 minutes.
17. Label Probe hybridization for 1 hour at 40°C.
18. Wash twice with Wash Buffer. Spin cells at 800xg for 5 minutes.
19. Wash once with Storage Buffer. Spin cells at 800xg for 5 minutes.
20. Analyze samples on a flow cytometer.

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Appendix

A1: 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. 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 (xg), not RPM. 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- μ L pipette tip on the tip of the aspirator to help reduce aspiration rate. Follow the meniscus while aspirating down to the 100- μ L 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. 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. 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 \pm 1°C. 2. Use a metal heat block for hybridization. 3. Minimize traffic to incubator. Temperature tolerance is 1-2°C. 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. 5. Measure temperature using the ViewRNA™ Temperature Validation Kit (Affymetrix, cat. QV0523).
	5. Incorrect Wash Buffer	Do not substitute Wash Buffer with Storage Buffer or any other buffer.
	6. Incorrect diluents used	Ensure Target Probe Diluent is used with Target Probes, and Label Probe Diluent is used with Label Probes.

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	7. Incorrect dilution of Target Probes or Label Probes	<ol style="list-style-type: none"> 1. Ensure Target Probes are diluted 1/20 with Target Probe Diluent. 2. Ensure Label Probes are diluted 1/100 with Label Probe Diluent.
	8. Gene expressed at very low level	<ol style="list-style-type: none"> 1. Check biological model and confirm gene is expressed in sample by an alternative method such as QuantiGene® 2.0 Assay for lysate. 2. Keep in mind that protein expression might not correlate with RNA expression in some cases.
	9. Incorrect or insufficient RNase Inhibitor used	<ol style="list-style-type: none"> 3. Ensure that 1X Permeabilization Buffer contains both RNase inhibitor 1 at a final dilution of 1/1000 and RNase inhibitor 2 at a final dilution of 1/100. Ensure that the Wash buffer in Step 19 and/or Step 25 for overnight sample storage contains RNase inhibitor 1 at a final dilution of 1/1000.
C. High Background	1. Compensation not set up correctly	<ol style="list-style-type: none"> 1. See Appendix A3 for how to set compensation. 2. Use the PrimeFlow™ Compensation kit (sold separately) or samples labeled with one each of the included Positive Control Probe sets to set compensation. 3. Do not use fluorochrome-conjugated antibodies to set compensation for RNA detection.
	2. Incorrect incubator temperature	<ol style="list-style-type: none"> 1. Incubator must be able to hold temperature at 40±1°C. 2. Use a metal heat block for hybridization. 3. Minimize traffic to incubator. Temperature tolerance is 1-2°C. 4. Ensure incubator has been stable at 40°C for at least 12 hours before starting assay as temperature may drift during initial incubator setup. 5. Measure temperature using the ViewRNA™ Temperature Validation Kit (Affymetrix, cat. QV0523).
	3. Insufficient washing	<ol style="list-style-type: none"> 1. Ensure the Wash Buffer is used at room temperature. 2. Follow instructions for washing steps in the protocol. 3. Ensure uniform cell resuspension by gentle vortexing.
	4. Cytometer not set up properly	Reduce voltage settings of your cytometer. Note that this assay may require lower settings than typical antibody staining.
	5. Over-fixation	Follow the protocol for proper fixation time.
	6. Excessive Target Probe used	Dilute Target Probe at a 1/20 dilution.

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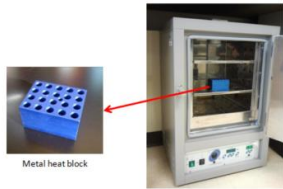




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A2: Instrument and Equipment Setup Guide

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 	1. Validated to maintain $40 \pm 1^\circ\text{C}$. 2. Metal heat block for 1.5-mL microcentrifuge tube. Example shown: Incubator (Affymetrix, cat. QS0704) Metal heat block (VWR, cat. 13259-002)
2. Refrigerated swinging bucket centrifuge 	Swinging bucket with adaptors for 15-mL conical tubes and 1.5-mL microcentrifuge tubes. (Optional: with refrigeration to 4°C .) Examples 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 	1. Two lasers - blue (488 nm) and red (633 nm or similar) 2. Detection optics optimized for FITC, APC, and APC-eFluor® 780 (APC-Cyanine7) Example shown: BD LSRFortessa
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). (Affymetrix, cat. QV0523).

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A3: Cytometer Setup

The PrimeFlow™ RNA Assay utilizes up to three 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

- Calibration beads
- Samples prepared using the PrimeFlow™ Compensation Kit (cat. 88-17001-42):
 - Unstained Ultracomp eBeads
 - Ultracomp eBeads labeled with PrimeFlow™ RNA Alexa Fluor® 647 Compensation Control
 - Ultracomp eBeads labeled with PrimeFlow™ RNA Alexa Fluor® 488 Compensation Control
 - Ultracomp eBeads labeled with PrimeFlow™ RNA Alexa Fluor® 750 Compensation Control
 - Single-color antibody-stained UltraComp eBeads (needed only if protein staining is performed)

Experimental Procedure

1. Ensure proper alignment following the instrument manufacturer's recommendations. Alternatively, calibration beads, such as Rainbow Calibration Particles (8-peak beads) (Spherotech, cat. RCP-30-5A) or Rainbow Fluorescent Particles (1-peak beads) (Spherotech, cat. RFP-30-5A), may be used to check the linearity of the PMT, PMT voltage range, and alignment of the instrument.
2. Setting PMT Voltages
 - a. Create an acquisition template/workspace that includes a forward scatter versus side scatter plot, a single-parameter histogram plot for every fluorescent channel being used, and a two-parameter plot for every pairwise combination of fluorescent channels being used in the experiment.
 - b. Place the unstained cell sample on the cytometer and begin to collect events.
 - i. 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.
 - ii. Looking at the histogram plots, ensure that the signals are on scale and adjust PMT voltages, if necessary.
 - iii. Stop collecting events and remove sample from the cytometer.
 - c. Place a single-color sample on the cytometer and begin to collect events.
 - i. Looking at the histogram plot for the fluorochrome being collected, ensure that events are on scale for the fluorescent channel being collected and adjust PMT voltages, if necessary.
 - ii. 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.
 - iii. Stop collecting events and remove sample from the cytometer.
 - iv. Repeat for each of the single-color samples.
 - d. Confirm that the voltage settings are appropriate for the experimental samples.
 - i. Place an experimental sample on the cytometer and begin collecting events.
 - ii. Looking at each of the histogram plots, confirm that all fluorescent signals are on scale and adjust PMT voltages, if necessary.
 - iii. Stop collecting events and remove sample from the cytometer.
3. Setting compensation

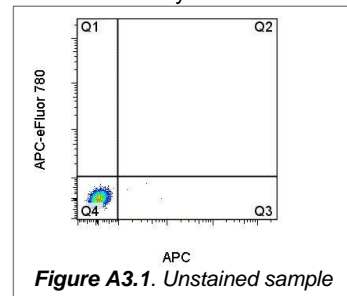
Note: Use the PrimeFlow™ Compensation Kit for single-color compensation controls. Please see the product data sheet for preparation of samples and instructions for use. Alternatively, cell samples labeled with individual Positive Control Probe Sets may be used for single-color

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compensation controls. Do not use fluorochrome-conjugated antibodies to compensate for RNA signal.

- a. If an autocompensation feature is available for the cytometer being used, follow the manufacturer's instructions for use.
- b. If an autocompensation feature is not available or to set compensation manually:
 - i. Place the unstained sample on the cytometer and begin to collect events.
 - ii. 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).
 - iii. 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.
 - iv. Stop collecting events and remove sample from the cytometer.

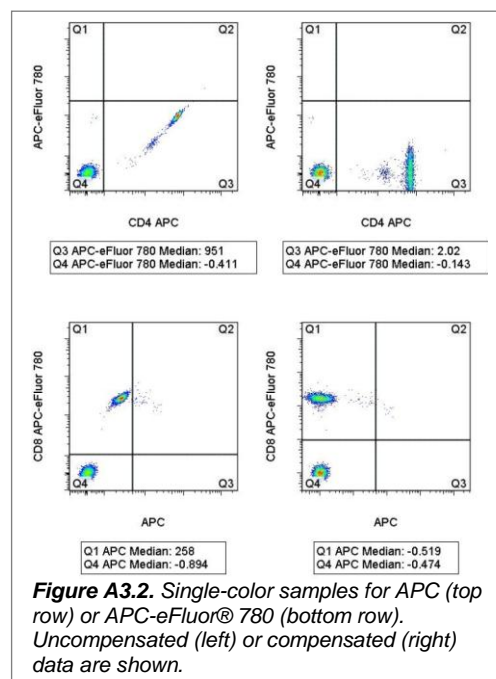


- c. Place a single-color sample on the cytometer and begin to collect events.

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.

- i. Looking at the two-parameter plots for the fluorochrome being 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).
- ii. 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).
- iii. Stop collecting events and remove sample from the cytometer.
- iv. Repeat for each of the single-color samples.

- d. Confirm that the compensation settings are appropriate for the experimental samples.
 - i. Place an experimental sample on the cytometer and begin collecting events.
 - ii. Looking at each of the two-parameter plots, confirm that the compensation is set correctly by looking at the medians.
 - iii. Stop collecting events and remove sample from the cytometer.



4. After setting compensation, you are ready to acquire your samples.
5. Use FMO controls after acquisition and during analysis to optimize compensation settings.

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A4: Examples of Expected Results

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

tube #	tube description	cell sample	488 Laser	PE-eFluor:610	633 Laser	405 Laser	eFluor 506 (510/20)
1	unstained/AF	stim	-	-	Alexa Fluor 750 780/60	eFluor 450 450/50	-
2	Alexa Fluor 488 single-color	stim	TNFa mRNA	-	-	-	-
3	PE-eFluor 610 single-color	stim	-	CD8a	-	-	-
4	PE-Cyanine7 single-color	stim	-	-	TNF alpha	-	-
5	Alexa Fluor 750 single-color	stim	-	-	IFNg mRNA	-	-
6	eFluor 450 single-color	stim	-	-	-	IFN gamma	-
7	eFluor 506 single-color	stim	-	-	-	-	FVD eF506
8	Alexa Fluor 488 FMO	stim	-	CD8a	TNF alpha	IFN gamma	FVD eF506
9	PE-eFluor 610 FMO	stim	TNFa mRNA	-	TNF alpha	IFN gamma	FVD eF506
10	PE-Cyanine7 FMO	stim	TNFa mRNA	CD8a	-	IFN gamma	FVD eF506
11	Alexa Fluor 750 FMO	stim	TNFa mRNA	CD8a	-	IFN gamma	FVD eF506
12	eFluor 450 FMO	stim	TNFa mRNA	CD8a	TNF alpha	-	FVD eF506
13	eFluor 506 FMO	stim	TNFa mRNA	CD8a	TNF alpha	IFN gamma	-
14	isotype/no probe control	stim	no probes	CD8a	mlgG1 isotype	mgG1 isotype	FVD eF506
15	6-color sample	stim	TNFa mRNA	CD8a	TNF alpha	IFN gamma	FVD eF506
16	Alexa Fluor 488 FMO	unstim	-	CD8a	TNF alpha	IFN gamma	FVD eF506
17	PE-eFluor 610 FMO	unstim	TNFa mRNA	-	TNF alpha	IFN gamma	FVD eF506
18	PE-Cyanine7 FMO	unstim	TNFa mRNA	CD8a	-	IFN gamma	FVD eF506
19	Alexa Fluor 750 FMO	unstim	TNFa mRNA	CD8a	TNF alpha	IFN gamma	FVD eF506
20	eFluor 450 FMO	unstim	TNFa mRNA	CD8a	TNF alpha	-	FVD eF506
21	eFluor 506 FMO	unstim	TNFa mRNA	CD8a	TNF alpha	IFN gamma	-
22	isotype/no probe control	unstim	no probes	CD8a	mlgG1 isotype	mgG1 isotype	FVD eF506
23	6-color sample	unstim	TNFa mRNA	CD8a	TNF alpha	IFN gamma	FVD eF506

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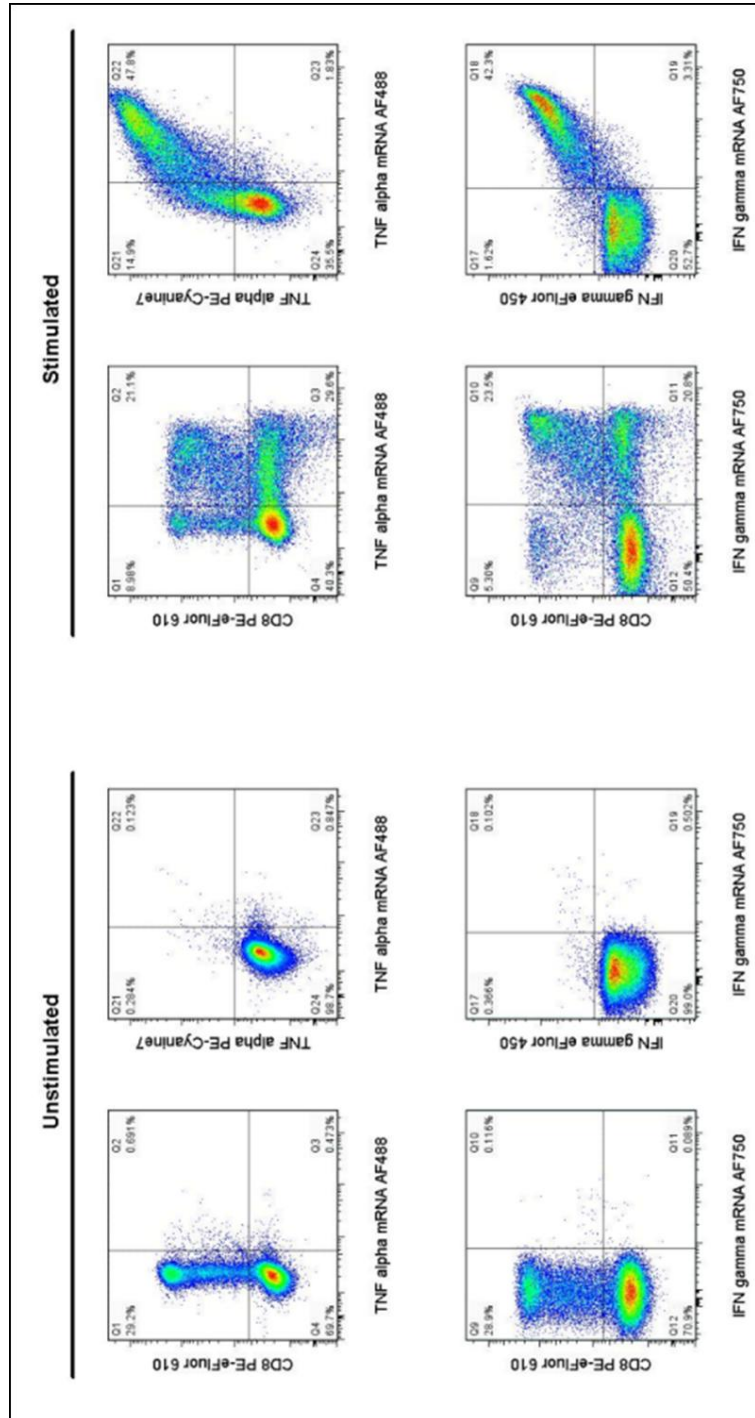


Figure A4.1. Normal human peripheral blood mononuclear cells were unstimulated (left) or stimulated with the Cell Stimulation Cocktail (plus protein transport inhibitors) (cat. 00-4975) for 3 hours (right) then analyzed using the PrimeFlow™ RNA Assay. Cells were intracellularly stained with Anti-Human CD8a PE-eFluor® 610 (cat. 61-0088), Anti-Human TNF alpha PE-Cyamine7 (cat. 25-7349), and Anti-Human IFN gamma eFluor® 450 (cat. 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 (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.

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Table A 4.2. Experimental set up for a 5-color panel to look at induction of Granzyme B and Ki-67 in stimulated mouse splenocytes.

tube #	tube 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/AF	stim	-	-	-	-	-	-	
2	Alexa Fluor 488 single-color	stim	Ki-67 mRNA	-	-	-	-	-	
3	PE-eFluor 610 single-color	stim	-	CD8a	-	-	-	-	
4	PE-Cyanine7 single-color	stim	-	-	Granzyme B	-	-	-	
5	Alexa Fluor 750 single-color	stim	-	-	-	Gzmb mRNA	-	-	
6	eFluor 450 single-color	stim	-	-	-	-	Ki-67	-	
7	Alexa Fluor 488 FMO	stim	-	CD8a	Granzyme B	Gzmb mRNA	Ki-67	-	
8	PE-eFluor 610 FMO	stim	Ki-67 mRNA	-	Granzyme B	Gzmb mRNA	Ki-67	-	
9	PE-Cyanine7 FMO	stim	Ki-67 mRNA	CD8a	-	Gzmb mRNA	Ki-67	-	
10	Alexa Fluor 750 FMO	stim	Ki-67 mRNA	CD8a	Granzyme B	-	Ki-67	-	
11	eFluor 450 FMO	stim	Ki-67 mRNA	CD8a	Granzyme B	Gzmb mRNA	-	-	
12	isotype/no probe control	stim	no probes	CD8a	rfgG2a isotype	no probes	rfgG2a isotype	-	
13	5-color sample	stim	Ki-67 mRNA	CD8a	Granzyme B	Gzmb mRNA	Ki-67	-	
14	Alexa Fluor 488 FMO	unstim	-	CD8a	Granzyme B	Gzmb mRNA	Ki-67	-	
15	PE-eFluor 610 FMO	unstim	Ki-67 mRNA	-	Granzyme B	Gzmb mRNA	Ki-67	-	
16	PE-Cyanine7 FMO	unstim	Ki-67 mRNA	CD8a	-	Gzmb mRNA	Ki-67	-	
17	Alexa Fluor 750 FMO	unstim	Ki-67 mRNA	CD8a	Granzyme B	-	Ki-67	-	
18	eFluor 450 FMO	unstim	Ki-67 mRNA	CD8a	Granzyme B	Gzmb mRNA	-	-	
19	isotype/no probe control	unstim	no probes	CD8a	rfgG2a isotype	no probes	rfgG2a isotype	-	
20	5-color sample	unstim	Ki-67 mRNA	CD8a	Granzyme B	Gzmb mRNA	Ki-67	-	

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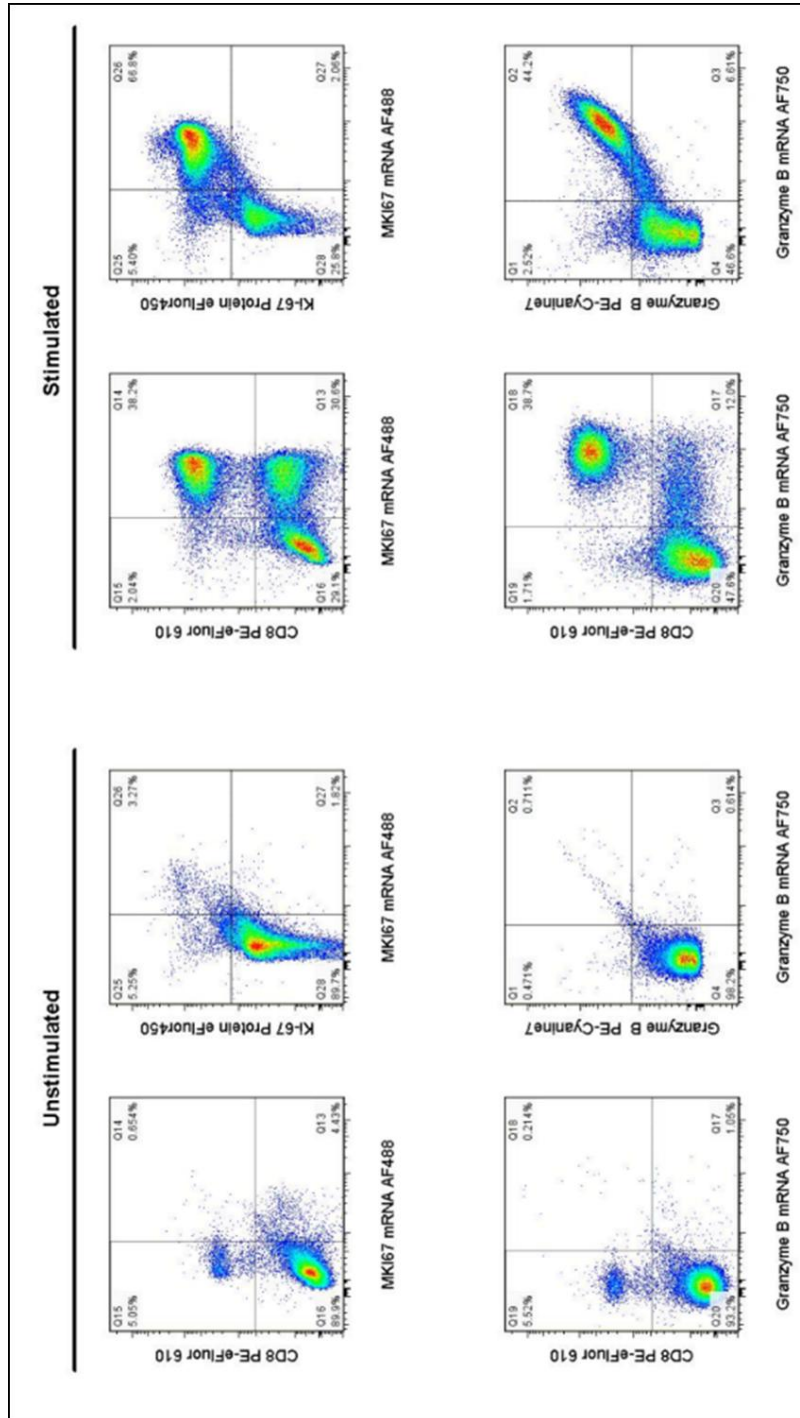


Figure A4.2. Mouse splenocytes were unstimulated (left) or stimulated with Anti-Mouse CD3 and CD28 Functional Grade Purifieds (cat. 16-0031 and 16-0281) for 2 days (right) then analyzed using the PrimeFlow™ RNA Assay. Cells were intracellularly stained with Anti-Mouse CD8a PE-eFluor® 610 (cat. 61-0081), Anti-Mouse Granzyme B PE-Cyanine7 (cat. 25-8898), and Anti-Mouse Ki-67 eFluor® 450 (cat. 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.

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A5: Validated Cells and Recommended Positive Control Genes

Cell	Recommended Positive Control Gene
Human peripheral blood (whole blood or PBMC) – fresh and cryopreserved	RPL13A, B2M
Mouse splenocytes (tissue) – fresh and cryopreserved	ACTB, RPL13A
Mouse thymocytes (tissue)	ACTB, RPL13A
Mouse bone marrow	ACTB
Human monocytic lymphoma, U937	RPL13A, B2M
Human T cell lymphoma, Jurkat	RPL13A, B2M
Human cervical carcinoma, HeLa*	RPL13A, GAPDH
Human lung carcinoma, PC9*	RPL13A, GAPDH

*adherent cells, limited testing

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A6: 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 and/or weak signal. The incubator should be validated before use following these instructions.

Materials required

- Incubator capable of maintaining temperature at $40\pm 1^\circ\text{C}$ (Affymetrix, cat. QS0704 or QS0712)
- ViewRNA™ Temperature Validation Kit (Affymetrix, cat. QV0523)
- 1.5-mL microcentrifuge tube
- Metal heat block for 1.5-mL microcentrifuge tube (VWR, cat. 13259-002)
- Parafilm

Experimental Procedure

1. Prepare the incubator.
 - a. Turn on the incubator.
 - b. Set the temperature to 40°C .
 - c. Place the metal heat block into the incubator near the center of the middle shelf of the incubator.
 - d. Allow the incubator and heat block to equilibrate overnight.
2. Assemble the temperature validation unit.
 - a. Insert the battery to activate the digital thermometer.
 - b. 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.
 - c. Add 0.2 mL of deionized water to the tube and then close the lid.
 - d. 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.
 - e. 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.
 - f. Turn on the digital thermometer.
3. Measure and adjust the temperature of the incubator.
 - a. Place the 1.5-mL tube containing the probe into the prewarmed heat block from Step 1.
 - b. Close the door, making sure there is sufficient slack in the wiring.
 - c. Wait 15 minutes for temperature to equilibrate.
 - d. 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.
 - e. Repeat the step above to adjust temperature until the incubator is $40\pm 1^\circ\text{C}$.

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

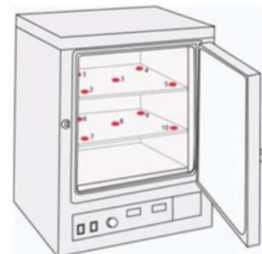


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4. Assess incubator temperature uniformity
 - a. Repeat Step 3 to measure the temperature at multiple positions in the incubator to determine temperature uniformity.

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



5. Assess temperature ramp-up time
 - a. Remove the 1.5-mL tube containing the probe from the prewarmed heat block from Step 3, and allow it to return to room temperature.
 - b. Open the incubator door for 1 minute then place the 1.5-mL tube containing the probe from Step 5a 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.
 - c. Repeat Steps 5a-b two more times.

Note: Do not use the incubator for the assay if it takes more than 5 minutes to return to 40°C or if it overshoots by more than 2°C during recovery.