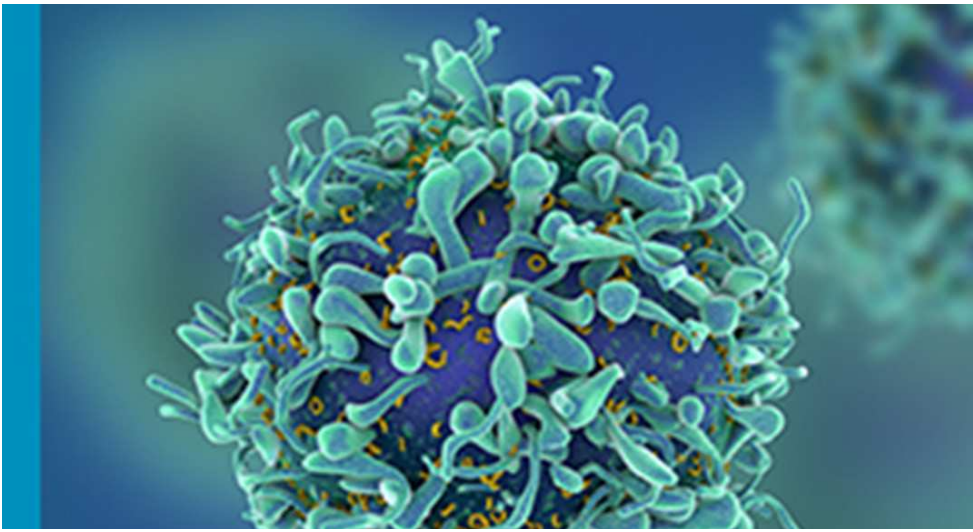
sartorius

Immune Cell Phenotype and Function

Mouse T Cell and Cytokine Profiling Kit

Cat No. 97042 for 1 x 96-well format
Cat No. 97043 for 5 x 96-well format
Cat No. 97044 for 1 x 384-well format
Cat No. 97045 for 5 x 384-well format



Open immediately upon arrival and store reagents at temperatures stated on labels. For research use only.

Publication No. 17094 Rev A.

Table of Contents

Section 1. Quick guides

The quick guides summarize the protocol. Detailed instructions are provided in Section 10 (Assay protocol for all formats). Workflow and volumes added are identical for all formats.

1.1 Reagent preparation (96- and 384-well formats)

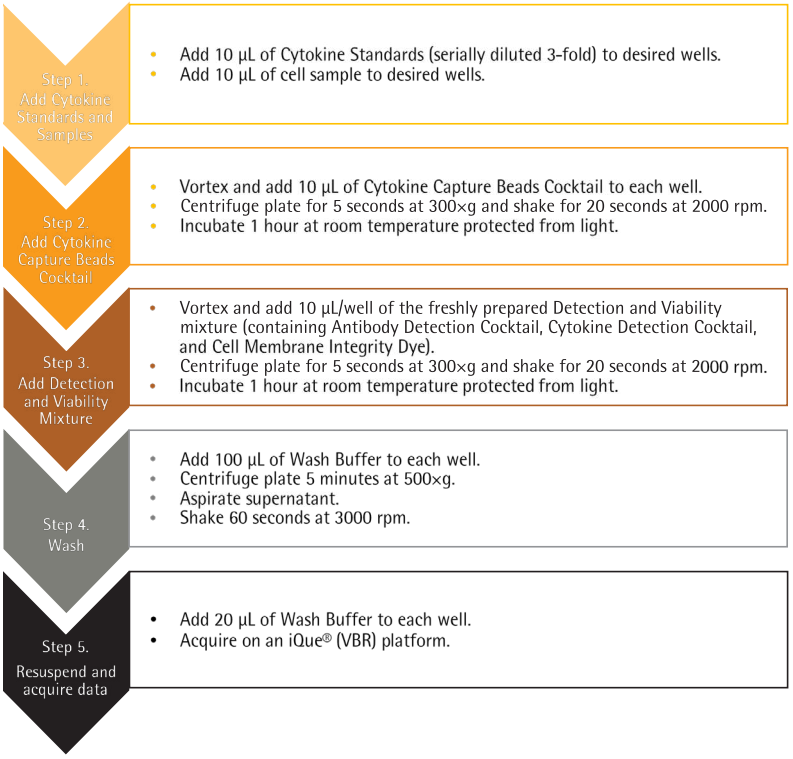
a. In advance of the assay, stain the cells of interest with the Proliferation and Encoding Dye by following the protocol in **Appendix A**. Prepare cells at a concentration of 1-4x10^6/mL.

b. On the day of the assay, prepare Cytokine Standards and prepare the standard curve. For details see Reagent and cell preparation (**Section 9**).

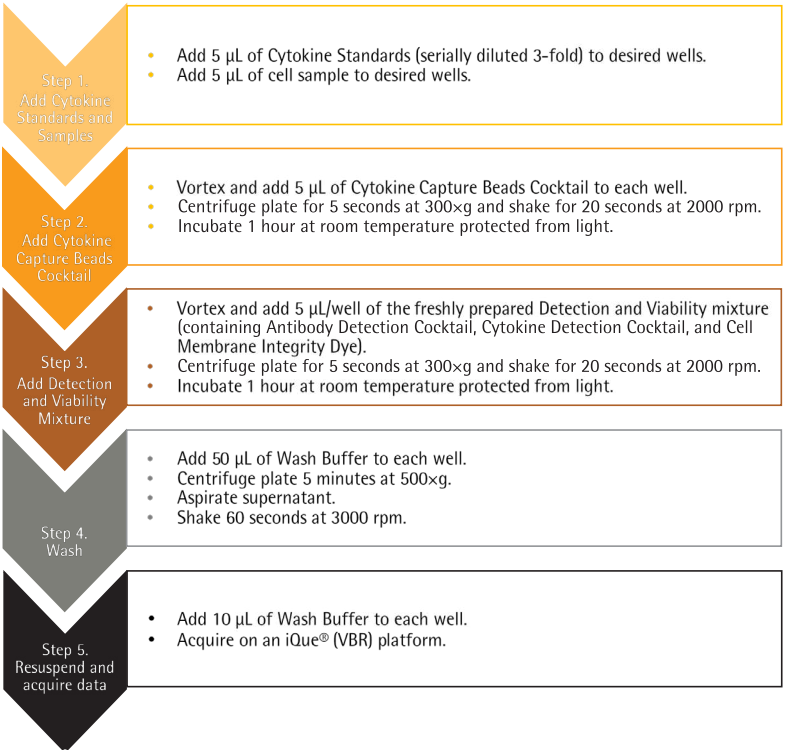
c. Prepare the Detection and Viability mixture according to the kit specific volumes described in **Table 2**.

NOTE: For first time assay users, refer to **Section 10**. Assay protocol for all formats, for detailed step-by-step procedures. The quick guide is a tool you can utilize once you are familiar with the protocol.

1.2 Quick guide for 96-well format



1.3 Quick guide (384-well Format)



Section 2. Introduction

In the Immune Cell Phenotype and Function series of reagents, the Mouse T Cell and Cytokine Profiling Kit is intended for use with mouse specimens to comprehensively assess mouse T cell phenotype and function. This kit is designed to run on the Intellicyt® iQue® VBR platforms. In a single experiment this kit enables high resolution of multi-color stained cells with multiplex cytokine-detecting QBeads® in the same sample well. High content functional readouts allow for the simultaneous evaluation of the cell membrane integrity as an indicator of cell viability (live/dead), the interrogation of helper (CD4+) and cytotoxic (CD8+) T cells for markers of activation (CD69 and CD44) and exhaustion (PD-1), and the identification of key memory subsets (naive, effector memory, and central memory T cells) in addition to the quantification of secreted cytokines (IFNγ and IL-2).

An optional protocol to incorporate the supplied Cell Proliferation and Encoding Dye into the workflow is also provided with this kit. During cell culture preparation, cells can be stained to identify and measure the proliferating cell population or to encode cells for easy identification based on a signature fluorescence intensity.

Data analysis is made easy with the aid of pre-configured templates (ForeCyt® versions 6.0, 7.0, and 8.0) provided with the kit. The templates include a compensation matrix and population gating strategy that expedites generating actionable results. In contrast, acquisition of comparable data points performed by traditional methods such as FACS or ELISA platforms are labor intensive and have limited throughput. These methods also require large amounts of reagents, cell or tissue specimens, and considerable user training. The ability to quickly profile T cell phenotype and function within the same sample vastly reduces the actual time and resources needed for investigative target biology research and expedites the development of therapeutic drug candidates.

Section 3. Assay principles

3.1 Multiplex assay in a single well

The Mouse T Cell and Cytokine Profiling Kit enables simultaneous measurement of the following in a single well (**Figure 1**):

- Cell count and viability
- T cell phenotypes
- Markers of activation and exhaustion
- Secreted cytokines
- Cell encoding and proliferation (optional)

In each assay well, cell viability is evaluated with Cell Membrane Integrity Dye, a fluorescent DNA intercalating dye that enters through the compromised membranes of dead or dying cells, thus distinguishing them from live cells. Specific T cells are identified based on their CD3, CD4, and CD8 profile by using antibody-fluorochrome conjugates. The memory T cell subsets: specifically naïve (Tn), central memory (Tcm), and effector memory, (Tem) are determined by their CD62L/CD44 expression signatures. T cells are also interrogated for markers commonly associated with activation and/or exhaustion: CD69 (early activation), CD44 (previous/ongoing activation), and PD-1 (exhaustion and transiently in activation). Secreted IFNγ and IL-2 cytokines are captured in a sandwich immunoassay format using QBeads® included in the same assay well (**Figure 1**). For cells stained prior to culture with the optional Proliferation and Encoding Dye (**Appendix A**), simultaneous T cell proliferation (measured via dye dilution) or identification of encoded target cells is possible.

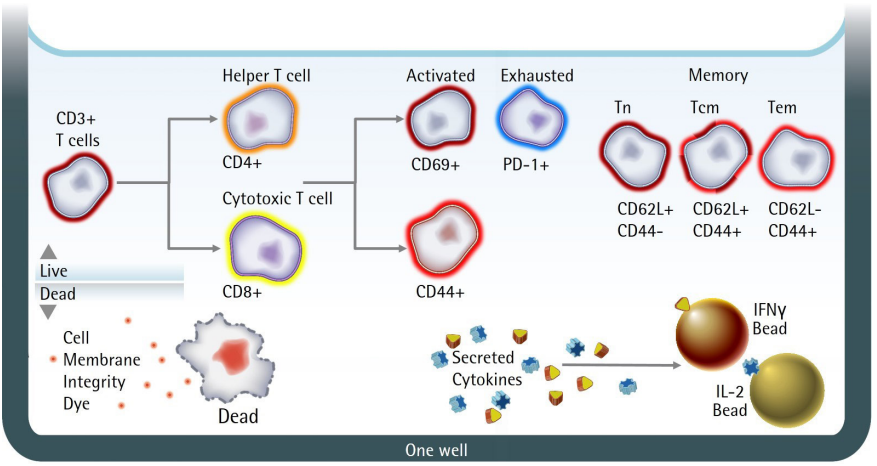


Figure 1. Simultaneous endpoint measurement in a single well.

3.2 Workflow overview

Enriched T cells or isolated immune cells such as splenocytes are treated in the culture plate. Samples or prepared Cytokine Standards are transferred to their respective wells in an assay plate along with the Cytokine Capture Beads cocktail (QBeads® capture beads and Fc-blocking antibody). After 1 hour of incubation, a freshly prepared mixture combining Antibody Detection Cocktail (phenotyping antibodies), Cytokine Detection Cocktail (QBeads® detection reagent), and Cell Membrane Integrity Dye, is added. Following a second, 1 hour incubation, the assay plate is washed once prior to acquisition on an iQue® (VBR) platform.

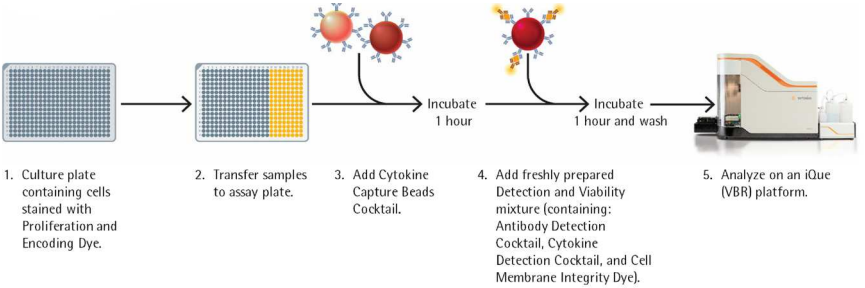


Figure 2. Assay workflow.

Section 4. Reagents provided

Table 2. Mouse T Cell and Cytokine Profiling Kit contents

Reagent	Catalog No. 97042 1 x 96-well	Catalog No. 97043 5 x 96-well	Catalog No. 97044 1 x 384-well	Catalog No. 97045 5 x 384-well
Mouse IFNγ Standard	lyophilized 1 vial	lyophilized 5 vials	lyophilized 1 vial	lyophilized 5 vials
Mouse IL-2 Standard	lyophilized 1 vial	lyophilized 5 vials	lyophilized 1 vial	lyophilized 5 vials
Cell Proliferation and Encoding Dye (B/Green)	25 µL 1 vial	25 µL 5 vials	25 µL 1 vial	25 µL 5 vials
Cytokine Capture Beads Cocktail	2 mL 1 bottle	2 mL 5 bottles	5.4 mL 1 bottle	5.4 mL 5 bottles
Antibody Detection Cocktail	2 mL 1 bottle	2 mL 5 bottles	5.4 mL 1 bottle	5.4 mL 5 bottles
Cytokine Detection Cocktail	125 µL 1 vial	125 µL 5 vials	325 µL 1 vial	325 µL 5 vials
Cell Membrane Integrity Dye (B/Red)	100 µL 1 vial	100 µL 5 vials	250 µL 1 vial	250 µL 5 vials
Wash Buffer	25 mL 1 bottle	125 mL 1 bottle	50 mL 1 bottle	250 mL 1 bottle

Section 5. Storage and stability

With the exception of the lyophilized Cytokine Standards and the Wash Buffer, all other reagents are light sensitive and should be stored in dark. Store lyophilized Cytokine Standards, Cytokine Capture Beads Cocktail, Cytokine Detection Cocktail, Antibody Detection Cocktail, and Cell Membrane Integrity Dye (B/Red) at 2-8°C. The Cell Proliferation and Encoding Dye (B/Green) should be stored at -20°C. Avoid repeated freezing and thawing. Do not use after the expiration date stated on the kit.

Section 6. iQue®3 (VBR) detector channels

This kit is compatible with the iQue®3 (VBR) and iQue® Screener PLUS (VBR) platforms.

Detector	Spectrum	Violet Laser (405 nm)		Blue Laser (488 nm)		Red Laser (640 nm)	
445/45 nm		VL1	CD279 (PD-1) (V/Blue)				
530/30 nm		VL2		BL1	Cell Proliferation and Encoding Dye (B/Green)		
572/28 nm		VL3	CD8 (V/Yellow)	BL2	QBeads (B/Yellow)		
615/24 nm		VL4	CD4 (V/Orange)	BL3			
675/30 nm		VL5		BL4	Cell Membrane Integrity Dye (B/Red)	RL1	CD44 (R/Red)
780/60 nm		VL6	CD3 (V/Crimson)	BL5	CD69 (B/Crimson)	RL2	CD62L (R/Crimson)

Figure 3. iQue®3 (VBR) lasers, detector channels and markers panel.

Section 7. Materials required but not provided

- Intellicyt® iQue®3 VBR platform (iQue®3 or iQue® Screener PLUS)
- Cell population of interest and appropriate complete cell culture medium
- Centrifuge (up to 500×g capability for use with microplates and microfuge tubes)
- Vortex mixer
- 96- or 384-well V-bottom assay plate (e.g., Greiner #651101 or Greiner #781280)
- 96- or 384-well V-bottom assay plate (e.g., Greiner #651101 or Greiner #781280)
- Microfuge tubes and/or 15 mL conical tubes
- Reagent reservoirs
- Universal opaque lid or foil to limit light exposure and evaporation
- Single-channel or multi-channel pipette (See **Appendix C**)
- Plate washer (e.g., BioTek model ELx405 Select)

Section 8. Recommended materials

We strongly recommend running positive and negative controls with this assay.

- Positive control: Dynabeads™ Mouse T Activator CD3/CD28 (ThermoFisher Scientific #11452D).
- Negative control: Culture medium without stimulating agents may be used as a negative control.

Section 9. Reagent and cell preparation

9.1 Software

- The assay plate design can be found in the Design tab of ForeCyt® and in the template provided (USB flash drive in kit package).
- This assay uses serially diluted Cytokine Standards to generate two standard curves for quantitation of IFNγ and IL-2 in the sample.

9.2 Setting up standards in ForeCyt®

A template with the standards plate design is provided in the kit (**Figure 4**). The Standards sub-section can be located within the Design section of ForeCyt®. The Standard Set is preconfigured with the lowest value set to 0 in the template provided. It is recommended to load standards in duplicate from low to high concentration in the direction of the plate read (96-well format: left to right; 384-well format: top to bottom). For ForeCyt® version 7.1 and later this is the default setting, however for earlier versions, this format requires the "Reverse Series" box to be checked (**Figure 5**). If necessary, the template configuration can be altered in the Design section of the experiment: Design → Standards → Edit Standard Set. Representative standard curves are shown in **Figure 6**.

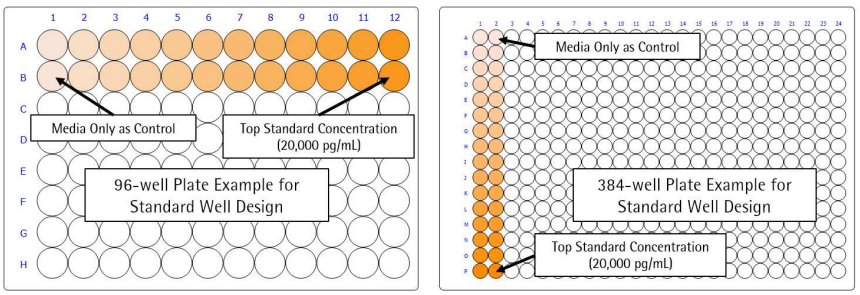


Figure 4. Configuration of the Standard Set.

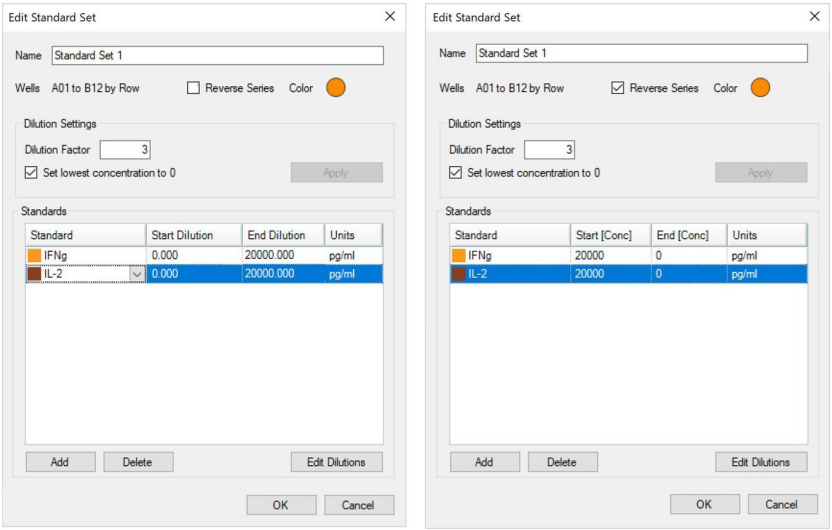


Figure 5. Editing the Standard Set. (Left) The provided assay template is preset to have Cytokine Standards in the low to high configuration with the lowest concentration set to zero. If a different orientation or lowest concentration is used, the Standard Set may be edited as necessary. (Right) In versions of ForeCyt® (prior to 7.1), to achieve a left to right (from low concentration to high concentration) in 96-well plate, the "Reverse Series" checkbox must be selected. Check the "Set lowest concentration to 0" checkbox in all ForeCyt® versions.

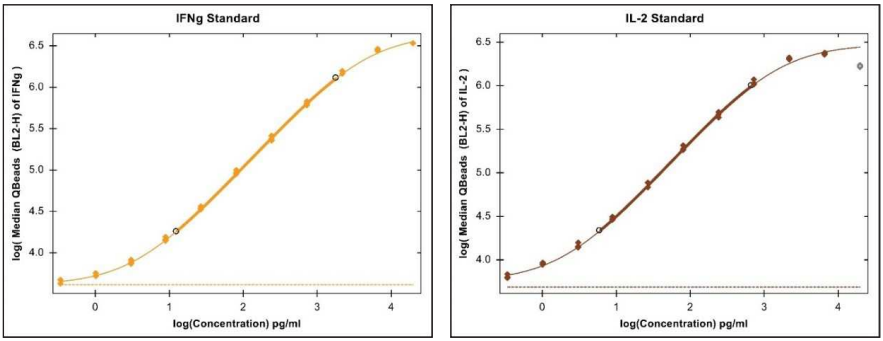


Figure 6. Representative standard curves (IFNγ and IL-2) with 1:3 serial dilutions. The linear ranges (bolded) for IFNγ and IL-2 are approximately 10-1,800 pg/mL, and 10-700 pg/mL, respectively. The dashed line represents the fluorescent background when the standard concentration is zero.

9.3 Reagents

- Briefly centrifuge all vials before use to prevent reagent loss.
- Vortex the Cytokine Capture Beads Cocktail and the freshly prepared Detection and Viability mixture (**Section 9.5**) prior to use to ensure homogenous solution and consistent concentration in the assay. These reagents contain QBeads® and/or antibodies that tend to settle and aggregate over time.

9.4 Cytokine Standard preparation

Materials required: Cytokine Standards (IFNγ and IL-2)

Cytokine standard curve range is 0.0 pg/mL – 20,000 pg/mL

a. From the glass vials provided in the kit, combine the lyophilized Cytokine Standard spheres (IFNγ and IL-2) into a 1.5 mL microfuge tube or 15 mL conical tube.

b. Add 500 µL fresh culture medium to the tube with the Cytokine Standard spheres. DO NOT MIX. Mixing at this step causes the reagent to foam.

c. Allow the spheres to dissolve for 15 minutes at room temperature.

d. Once dissolved, pipette up and down to mix Cytokine Standards.

e. Perform 3-fold serial dilutions of Cytokine Standards (i.e., 100 µL of top standard into 200 µL of culture medium serially). For the 96-well format perform a 12-point curve, including a blank medium control (**Figure 7**, top). For the 384-well format, perform a 16-point curve, including a blank medium control (**Figure 7**, bottom).

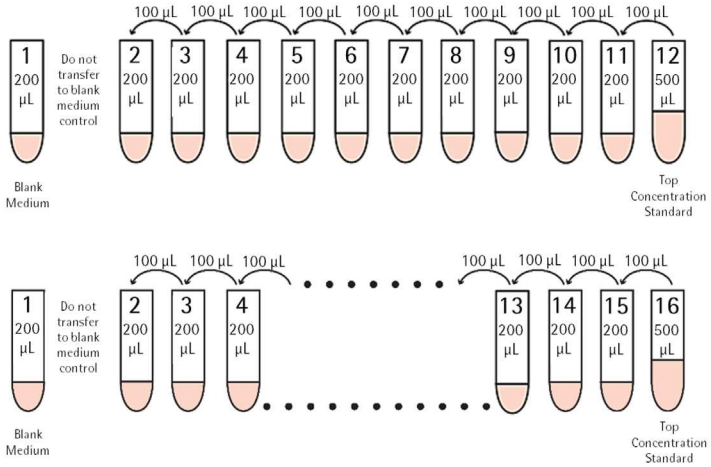


Figure 7. Serial dilution of Cytokine Standards.

9.5 Detection and Viability mixture preparation

Label a 15 mL conical tube, "Detection and Viability mixture" and add together the appropriate volumes of Antibody Detection Cocktail, Cytokine Detection Cocktail, and Cell Membrane Integrity Dye (**Table 2**). This mixture should be prepared fresh and used on the day of assay.

Table 2. Volumes needed for Detection and Viability Mixture according to kit format.

Format	Antibody Detection Cocktail	Cytokine Detection Cocktail	Cell Membrane Integrity Dye (B/Red)
1 × 96-wells	1.9 mL	82 µL	62 µL
1 × 384-wells	5.1 mL	219 µL	166 µL

Section 10. Assay protocol for all formats

This Protocol describes **96-well** (and 384-well) plate formats.
Total time: 3 hours
Hands-on time: Approximately 30 minutes

Assay protocol

a. Prepare Cytokine Standards.
b. Add **10 µL** (5 µL for 384-well format) of cytokine standards or cell sample.
c. Vortex the Cytokine Capture Beads Cocktail and add **10 µL** (5 µL for 384-well format) to each well.
d. Centrifuge the assay plate (300×g, 5 seconds) and follow with a brief shake (2000 rpm, 20 seconds).
e. Incubate for 1 hour at room temperature, protected from light.
f. Vortex the Detection and Viability Mixture and add **10 µL** (5 µL for 384-well format) to each well.
g. Centrifuge the assay plate (300×g, 5 seconds) and follow with a brief shake (2000 rpm, 20 seconds).
h. Incubate for 1 hour at room temperature, protected from light.
i. Add **100 µL** (50 µL for 384-well format) of Wash Buffer to each well.
j. Centrifuge the assay plate (500×g, 5 minutes).
k. Aspirate supernatant.
l. Agitate residual liquid (3000 rpm, 60 seconds).
m. Add **20 µL** (10 µL for 384-well format) Wash Buffer to each well.
n. Acquire data on iQue®3 (VBR) or iQue® Screener PLUS (VBR).

Section 11. Plate acquisition and data analysis

11.1 Acquire plate

a. Launch ForeCyt® software.

b. Import the template "Mouse T Cell and Cytokine Template". Use the most appropriate template for the kit type and version of ForeCyt® being used.

c. Create a New Experiment using the template.

d. In the Design section:

   1. Well Type sub-section: Assign positive and negative control wells in addition to sample wells. (Positive and negative controls are essential for fine-tuning activated/exhausted populations during data analysis.)

   2. Series/Standards sub-section: Assign wells for compound series or cytokine standards.

e. In the Protocol section: Adjust sip times as needed to achieve statistical significance for the cell population of interest.

f. Click "Run" on the Controller to acquire the plate.

NOTE: Remove the plate lid prior to clicking "Run" on the Controller.

11.2 Data analysis and gating hierarchy

The Mouse T Cell and Cytokine Profiling Kit template includes pre-defined population gates. Each gate can be adjusted to improve fit on populations of interest (**Figures 8–10**). An optimized compensation spillover matrix has also been included in the kit template (**Figure 11**).

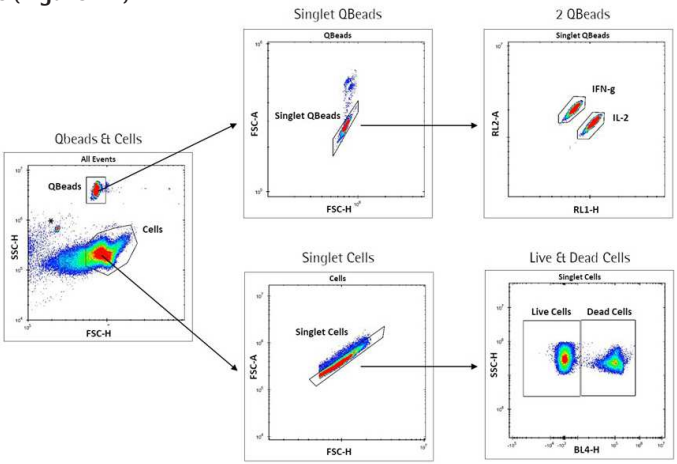


Figure 8. Gating QBeads® and cell populations. From All Events identify the QBeads® and cell populations followed by identification of singlet populations and furthermore individual IFNγ/IL-2 QBeads® populations and live/dead cell populations.

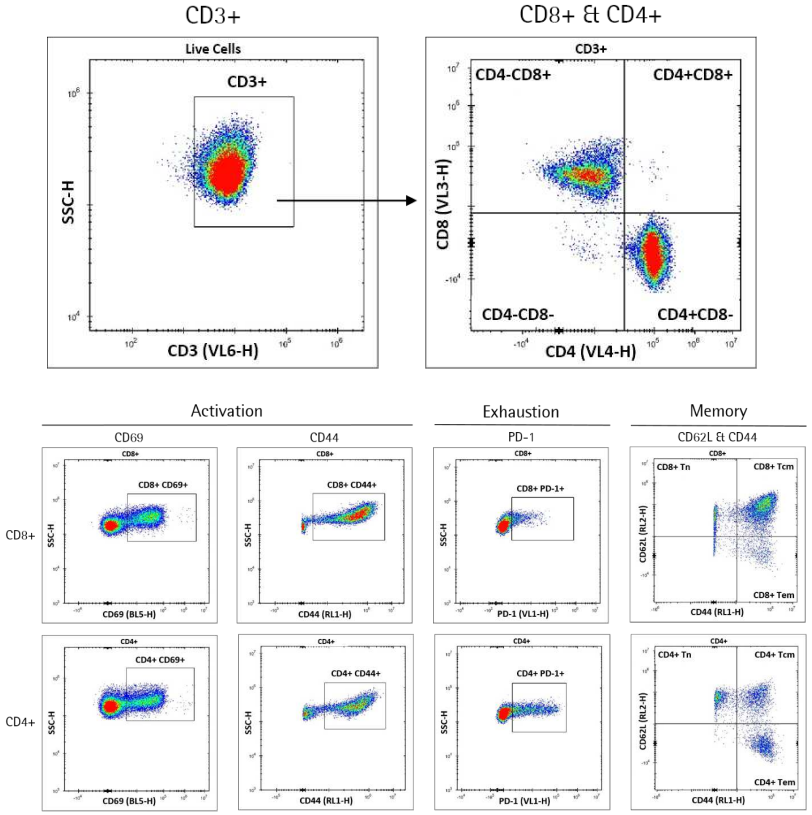


Figure 9. Gating different cell phenotypes from Live Cells. From Live Cells, identify CD3+ T cells. Next, identify CD4+ or CD8+ T cells followed by activated, exhausted, or memory subset populations. To improve the separation of different populations, manually adjust the linear range of the dot plot bi-exponential scale.

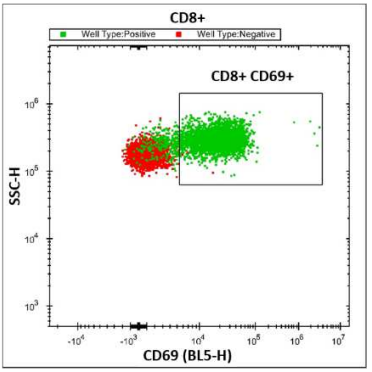


Figure 10. Use positive and negative control wells to fine tune gates. Once positive and negative wells have been designated in the Design section, use an overlay plot to fine tune the gates of activated/exhausted cell populations.

11.3 Compensation spillover matrix

Primary Channel \ Spillover Channel	Proliferation (BL1-H)	QBeads (BL2-H)	Viability (BL4-H)	CD69 (BL5-H)	CD44 (RL1-H)	CD62L (RL2-H)	PD-1 (CD279) (VL1-H)	CD8 (VL3-H)	CD4 (VL4-H)	CD3 (VL6-H)
Proliferation (BL1-H)		0.00	0.83	0.02	0.00	0.00	0.00	2.50	0.82	0.00
QBeads (BL2-H)	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Viability (BL4-H)	1.75	0.00		12.81	6.35	0.78	0.24	3.81	14.83	2.68
CD69 (BL5-H)	0.47	0.00	0.28		0.02	22.69	0.01	0.28	0.20	13.43
CD44 (RL1-H)	0.00	0.00	0.77	0.04		6.15	0.00	0.01	0.09	0.32
CD62L (RL2-H)	0.01	0.00	0.03	0.67	5.76		0.01	0.03	0.03	4.96
PD-1 (CD279) (VL1-H)	0.01	0.00	0.00	0.00	0.00	0.00		4.98	2.07	0.03
CD8 (VL3-H)	0.48	0.00	3.00	0.12	0.01	0.00	4.53		100.00	1.36
CD4 (VL4-H)	0.01	0.00	2.53	0.16	0.10	0.01	0.94	10.00		2.99
CD3 (VL6-H)	0.03	0.00	0.02	3.48	0.17	19.22	3.24	0.40	0.42	

Figure 11. This compensation matrix is included in the ForeCyt® template and should be applied with or without use of the optional Cell Proliferation and Encoding Dye.

Section 12. Best practices and tips

12.1 Plate-type recommendations and automated wash protocols

The assay protocols described in this manual are designed for both 96-well and 384-well plate formats. The Mouse T Cell and Cytokine Profiling Kit has been extensively tested with both 96-well, and 384-well V-bottom plates (Griener #651101 and #781280, respectively) with the BioTek ELx405 Select aspiration settings (**Table 3**). If a different brand/model of plate washer/aspirator is used, these settings can be used to approximate the aspiration settings on a different system. It is recommended that an automatic plate washer be used for aspiration steps as manual aspiration or plate-inversion techniques can result in severe sample loss.

Table 3. Aspiration settings using the BioTek ELx405 Select.

Plate Type	Height Setting	Height Offset (mm)	Rate Setting	Aspiration Rate (mm/s)
Greiner 96-well, V bottom (#651101)	#40	5.08	#6	15
Greiner 384-well, V bottom (#781280)	#31	3.937	#6	15

12.2 Manual pipetting recommendation

This protocol requires pipetting volumes between 5 µL and 100 µL depending on the plate formats. When manual pipetting it is important to change tips between wells to avoid cross-contamination. When pipetting small volumes (e.g., 5 µL), it is a best practice to either touch the bottom of a well (in an empty plate) or the side-wall of a well (when occupied with sample/reagent) to ensure release of the liquid into the assay well. Touching the wall of the well prevents the liquid droplet from hanging on the pipette tip instead of releasing into the assay well. A quick spin will force the newly dispensed liquid to the well bottom to mix with the existing reagent/sample already in the well. For liquid handler recommendations, see **Appendix C**.

12.3 Mixing plate contents using a shaker

Use of a plate shaker to mix plate contents is required when performing this assay. If a separate plate shaker is not available, the shaker on the iQue® (VBR) platform can be used without exceeding the volume and speed limitations (**Table 4**).

Table 4. Volume and speed limitations when using the iQue®3 (VBR) or iQue® Screener PLUS (VBR) shaker.

Plate Type	Well Volume (µL)	Maximum Speed (rpm)
96-well	20–40	2600
96-well	40–60	2200
96-well	60+	*
384-well	10–30	3000
384-well	30–50	2800
384-well	50+	*
* To determine ideal shake speeds for high volume assays, it is recommended to begin at a lower rpm value and gradually increase to a higher rpm value.		

The steps to use the iQue® (VBR) platform shaker are described below. (See also **Figure 12**).

a. Click on Device in the menu bar.

b. Scroll down to Manual Control Mode.

c. In the Manual Control Mode window, set the desired shake speed (rpm).

d. Once the checkbox "On" is clicked, the shaker will begin to shake and continue to shake until disabled by clicking the checkbox a second time.

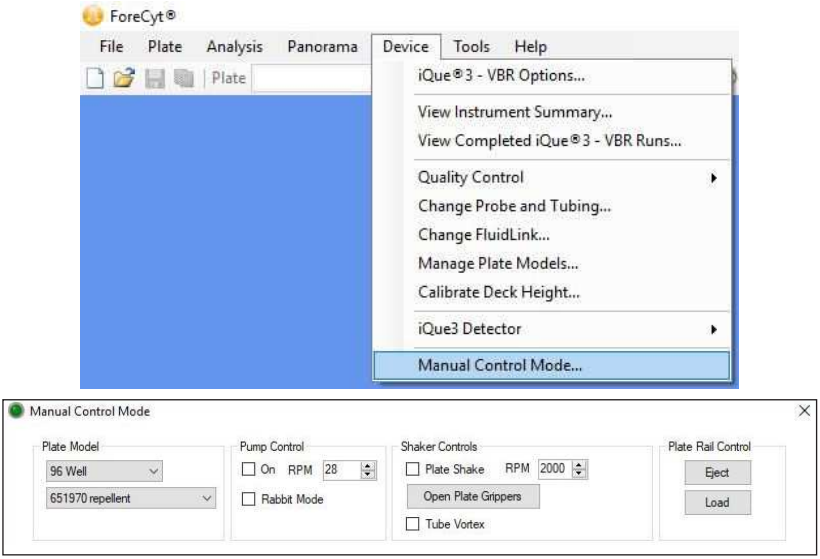


Figure 12. Steps for using the shaker on an iQue® (VBR) platform. Set the shaker speed to either 2000 rpm or 3000 rpm depending on the assay requirements. It is important to note that shaking at 3000 rpm is reserved only for the step post-aspiration. Shaking the plate at 3000 rpm with liquid in the wells will result in cross-contamination.

12.4 Dilute protein standards with fresh culture medium

It is critical to use fresh culture medium when reconstituting the Cytokine Standards to ensure data reproducibility and reliability. Culture medium should not differ from that used in the cell culture sample. A specific diluent for protein standards dilution is not provided with this kit. It has been tested and found that use of recombinant human IL-2 in culture medium is compatible with mouse QBeads® in this kit and does not influence the cytokine standard curves or IL-2 measurements.

12.5 Making sure sample cytokines are within the linear range of standard curves

The ForeCyt® template defaults to 4 Parameter Logistic with $1/Y^2$ weighting for fitting the standard curves. At the log scale, ForeCyt® can provide the linear range for each standard curve. If adjustments are necessary for concentration, dilution factor, or Standard plate layout modification, refer to the ForeCyt® Reference Guide and make the adjustment in the Design section. Use of a different culture medium for the standard dilution may have a slight impact on the standard curve and the linear range.

12.6 Adjust the sip time to acquire sufficient cell events

The number of cell events acquired from each well is determined by the sip time. The kit template default sip time is 10 seconds per well for 96-well plates and 8 seconds per well for 384-well plates. Adjust sip time as necessary to ensure that enough cell events from the population of interest are acquired to reach statistical significance during data analysis. Sip volume per second is approximately 1.5 µL per second. When adjustments to sip time are necessary, refer to **Tables 5** and **6**. Refer to **Appendix B** for additional measures to improve cell event acquisition in addition to increasing acquisition sip time.

Table 5. Data acquisition adjustments for 96-well format.

Sip Time Per Well	Culture Plate Cell Density	Sample Transfer Volume	Final Volume (following resuspension)	Estimated Cell Density in Assay Plate	Estimated Acquired Volume*
4 s	$1x10^6$ cells/mL (minimum seeding density)	10 µL (from culture plate to assay plate)	25 µL (20 µL + residual volume)	$0.3x10^6$ cells /mL (assuming 20% loss during wash)	6 µL
6 s					9 µL
8 s					12 µL
10 seconds (default)					15 µL
12 seconds					18 µL

* Assuming a 1.5 µL/s sip per well.

Sip Time Per Well	Estimated Cell Events Acquired/Well	Inter-well Shake Frequency	Inter-well Shake Duration	Acquisition Time/Plate
4 s	1800	Every 4 wells	4 s	~17 min
6 s	2700	Every 4 wells	4 s	~20 min
8 s	3600	Every 4 wells	4 s	~24 min
10 s default)	4500	Every 4 wells	4 s	~27 min
12 s	5400	Every 4 wells	4 s	~30 min

Table 6. Data acquisition adjustments for 384-well format.

Sip Time Per Well	Culture Plate Cell Density	Sample Transfer Volume	Final Volume (following resuspension)	Estimated Cell Density in Assay Plate	Estimated Acquired Volume*
4 seconds	$1x10^6$ cells per mL (minimum seeding density)	5 µL (from culture plate to assay plate)	15 µL (10 µL + residual volume)	$0.3x10^6$ cells per mL (assuming 20% loss during wash)	6 µL
6 seconds					9 µL
8 seconds (default)					12 µL

* Assuming a 1.5 µL/s sip per well.

Sip Time Per Well	Estimated Cell Events Acquired/Well	Inter-well Shake Frequency	Inter-well Shake Duration	Acquisition Time/Plate
4 seconds	1800	Every 4 wells	4 s	~54 min
6 seconds	2700	Every 4 wells	4 s	~68 min
8 seconds (default)	3600	Every 4 wells	4 s	~72 min

Section 13. Appendices

13.1 Appendix A: Optional Proliferation and Encoding Dye Protocol (B/Green) for target cells

a. Completely thaw the vial of Proliferation and Encoding Dye before use.

b. Dilute Proliferation and Encoding Dye at 1:1250 with either HBSS or PBS.

c. Collect the sample to stain (i.e., T cells or target cells) in a 50 mL conical tube.

   1. Centrifuge cells (500×g, 5 minutes).
   2. Remove supernatant.
   3. Resuspend cells in protein-free HBSS or PBS to 1-4x10^6 cells/mL.

d. Combine equal volume of the prepared cells and the dilute dye. The final dye concentration will be 1:2500.

e. Mix gently and incubate for 15 minutes at room temperature.

f. Wash sample by adding 2X volume of complete culture medium to the staining sample. Centrifuge (500×g, 5 minutes). Remove supernatant and resuspend the sample in residual liquid.

g. Repeat Step f. two more times.

h. After the final wash, resuspend cells to the desired cell density for the culture/assay.

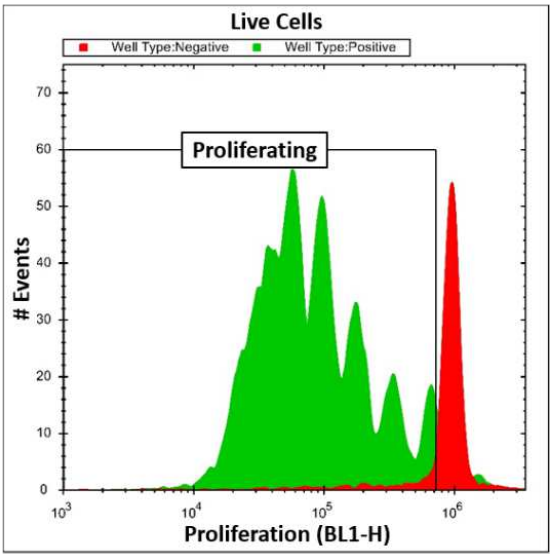


Figure 13. Example of proliferating mouse T cells. Mouse T cells stained with Proliferation and Encoding Dye following a 3-day culture with CD3/CD28 activating beads.

13.2 Appendix B: Improving cell event acquisition

Option 1: Adjust acquisition sip time (**Section 12.6**).

Option 2: Concentrate cell samples in the original culture plate prior to next assay run.

a. Spin down cells (300×g, 5 minutes) in the original culture plate.

b. Remove up to half the supernatant to double the cell density in the culture well.

c. Re-suspend cells in the culture plate by pipetting the sample up and down (5-6 times) in the remaining supernatant.

d. Transfer the concentrated cell samples to the assay plate before running the assay.

Option 3: Use cell-repellent or ultra-low binding plates to prevent cell attachment.

Some user-defined biological conditions may cause partial attachment of cells to the assay well surface resulting in inconsistent cell count. To achieve a more precise cell count use cell-repellent plates (e.g., Greiner #651970 or Greiner #781970) or ultra-low attachment plates (e.g., Corning #7007 or Corning #4516). To add new plate models into ForeCyt®, click on Device → Manage Plate Models → Add.

Option 4: Run daily volumetric calibration to get more precise cell density data.

Running a daily volumetric calibration on the iQue® (VBR) Platform using SPHERO™ AccuCount beads (Spherotech #ACBP-50-10) is recommended if very precise cell density information is required. This bead has an absolute count per volume unit.

a. Follow the Spherotech protocol to mix and transfer the beads to a testing plate.

b. Mimic the run protocol in the Mouse T Cell Biology Cell and Cytokine Profiling Kit by using the same plate type, sample volume, and sip time.

c. Measure the sip volume by running at least three wells of AccuCount beads.

d. Use this volume measurement to calculate the cell density.

e. Adjust the final calculation by considering the sip time (in ForeCyt® Protocol) and the sample dilution in the final assay reaction volume.

13.3 Appendix C: Pipette recommendations

a. Multi-Channel Pipettes

- Manual 12-channel pipette, Tacta®, 5-120 µL (Sartorius)
- Manual 12-channel pipette, Tacta®, 30-300 µL (Sartorius)
- Electronic 12-channel pipette, Picus®, 5-120 µL (Sartorius)
- Electronic 12-channel pipette, Picus®, 10-300 µL (Sartorius)

b. Single-Channel Pipettes

- Manual single-channel pipette, Tacta® (Sartorius)
- Electronic single-channel pipette, Picus® (Sartorius)

13.4 Appendix D: FAQ

Q1. Can I apply the standard curves acquired from one day to another day's experiment for cytokine quantitation?

A1. Standard curves should be run on each day of assay and applied only to experiments run on the same day. This eliminates potential day-to-day variation that may affect cytokine quantitation. Standards can be included in-plate or run as a stand-alone plate. For in-plate standards, cytokine quantitation is automatically included in the ForeCyt® template. However, cytokine quantification can be achieved from a stand-alone plate by sharing the standard-curve fit to other assay plates. Once the curve fit has been shared, cytokine quantitation can be performed using the Derived Concentration advanced metric. More information on the Share Fit feature and calculating a derived concentration from a shared cure can be found in the ForeCyt® Reference Guide.

Q2. Can I fix my samples in the plate with fixatives?

A2. Samples may be fixed with certain fixatives (i.e., 1% PFA) however it is important to understand how fixation may affect biological outcomes. Use of methanol for fixation is highly discouraged as it affects bead-based cytokine detection. Fixation and further wash steps may cause cell loss and affect the final event acquisition and therefore warrant additional optimization. If significant cell loss is observed, perform the fixation in a cell-repellent plate (i.e., Greiner #651970 or Greiner #781970) which may reduce cell loss due to fixation or fixation-related cell cross-linking to the well bottom.

Q3. Do I need to dilute my samples for the assay if my samples have high cytokine levels?

A3. Diluting samples is appropriate when cytokine levels are beyond the linear range. When diluting samples, consider adjusting the sip-time to assure enough cellular events are collected for analyses.

Q4. The cell count is too low. How do I acquire more cells?

A4. Increase the sip time in the Protocol in ForeCyt® to acquire more cell events **(Tables 5** and **6).** If the sip time cell events remain low, consider concentrating samples in a future assay (**Appendix B**).

Q5. Can I use 1 x 384-well kit to run a 96-well plate assay? How many 96-well plates can I run?

A5. Yes. A 1 x 384-well kit can be used for 2 assay plates in a 96-well format. Both 1 x 96-well kits and 1 x 384-well kits provide 1 vial of each Cytokine Standard. Additional standards are also available for purchase. For all kits, both a 96- and 384-well ForeCyt® template is provided.

Q6. Can I multiplex this assay with other cellular or cytokine endpoints?

A6. Outside the use of the optional Proliferation and Encoding Dye (T cell proliferation or target cell measurement), additional multiplexing is not recommended. The ForeCyt® template includes a compensation matrix that accounts for these measurements without a need for additional adjustments.

Q7. Why do I get very few beads and/or cells from the sample in data acquisition?

A7. In most cases, more than 50 QBeads® per cytokine population will be counted in each well.

If there are significantly less QBeads®, it may be due to:

a. Insufficient mixing of the Cytokine Capture Beads Cocktail vial (containing QBeads®).

b. Settling of QBeads® due to improper agitation of residual liquid following aspiration.

c. Loss of QBeads® due to improper aspiration.

If significantly fewer cells are observed, it may be due to:

a. The effect of biological processes on growth/viability.

b. Insufficient mixing of cells in the culture plate prior to transferring to the assay plate.

c. Settling of cells due to improper agitation of residual liquid following aspiration.

d. Loss of cells due to improper aspiration.

Q8. Certain markers in the dot plot show as smears rather than discreet populations. Can I adjust the plot and the gating?

A8. The assay template includes several phenotype 2D plots on a biexponential scale. Manual adjustment of the biexponential scale linear range (by increasing or decreasing 2-10 times of the linear range value) may improve visualization and signal separation. Gating can then be adjusted to include the corresponding cell population of interest. It is useful to note that certain proteins can present as a continuous expression pattern (i.e., smear). Visualization and signal separation of continually expressed proteins can also be improved by the adjustments mentioned above.

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