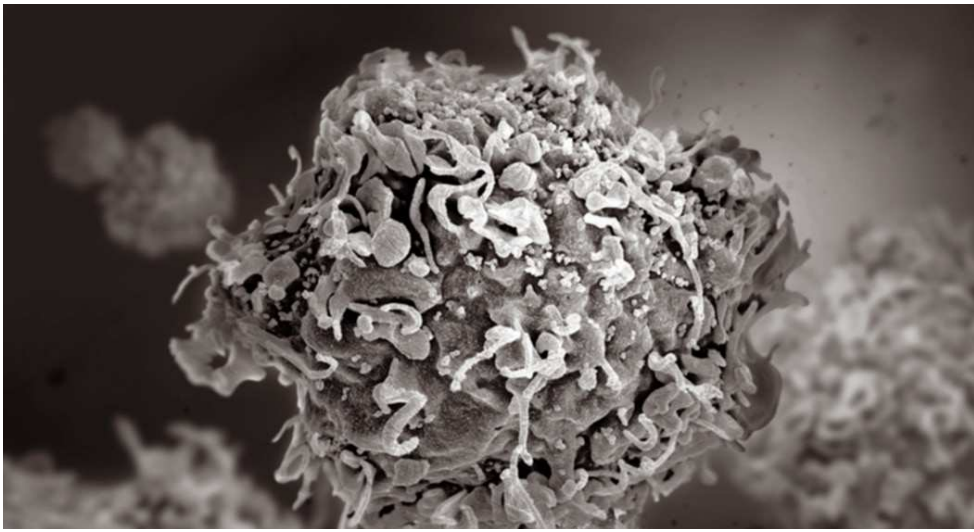
SARTORIUS

Immune Cell Phenotype and Function

Human T Cell Exhaustion Cell and Cytokine Profiling Kit

Cat. No. 97069 for 1 x 96-well format
Cat. No. 97070 for 5 x 96-well format
Cat. No. 97071 for 1 x 384-well format
Cat. No. 97072 for 5 x 384-well format



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

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Table of Contents

Section 1. Quick guides

The quick guides summarize the protocol. Detailed instructions are provided in **Section 10** (Assay protocols for all formats) and **Appendix A** (Optional proliferation staining of cells).

NOTE: Note: For first time assay users, refer to **Section 10** for detailed step-by-step procedures. The Quick guides serve as aids once familiar with the protocol.

1.1 Quick guide for optional proliferation staining of T cells

Wash cells with protein free buffer.
Re-suspend in protein free buffer at **1-4 million/mL**. ☐

↓

Dilute Cell Proliferation and Encoding Dye in protein free buffer at **1:1250**. ☐

↓

Combine cells and the diluted encoding dye solution **1:1**. | Incubate RT, 15 minutes, Dark ☐

Start time _____ ↓ *Stop time* _____

Wash cells by adding 2-fold volume of fresh culture medium. Spin **500 xg, 5 minutes**. Remove supernatant. Repeat wash 2 more times. ☐

↓

Re-suspend cells at the density needed for assay. ☐

Notes

1.2 Quick guide for 96-well assay format

1. Reagent preparation

Combine two different lyophilized Cytokine Standards into the same tube.
IFNγ ☐ **TNF** ☐
Add 200 µL fresh culture medium to solubilize.
Incubate RT, 15 minutes ☐

Start time _____ *Stop time* _____

Prepare **1:3 serial dilution** of Cytokine Standards with fresh culture medium. ☐

Dilute Cytokine Capture Beads Cocktail with **9-fold** volume of fresh culture medium. ☐

Add Membrane Integrity Dye to Antibody Detection Cocktail (**1:250 dilution**). ☐

2. Assay protocol

Add **10 µL/well** samples and standards to the assay plate. ☐

Add **100 µL/well** diluted Cytokine Capture Beads Cocktail.
DO NOT SHAKE
Incubate RT, 60 minutes, Dark ☐

Start time _____ *Stop time* _____

Long spin **(300 xg, 5 minutes).** Aspirate supernatant.
Agitate in residual liquid with Strong shake* ☐

Add **10 µL/well** Cytokine Detection Cocktail.
Quick spin | Brief shake*
Incubate RT, 60 minutes, Dark ☐

Start time _____ *Stop time* _____

Add **10 µL/well** combined Antibody Detection Cocktail with Cell Membrane Integrity Dye.
Quick spin | Brief shake*
Incubate RT, 60 minutes, Dark ☐

Start time _____ *Stop time* _____

Add **100 µL/well** Wash Buffer.
Long spin **(300 xg, 5 minutes)**. Aspirate supernatant.
Agitate in residual liquid with Strong shake* ☐

Add **20 µL/well** Wash Buffer. Acquire data. ☐

Notes

* Quick spin: 300 xg, 5 seconds | Brief shake: 2000 RPM, 20 seconds | Strong shake: 3000 RPM, 60 seconds

1.3 Quick Guide for 384-well assay format

1. Reagent preparation

Combine two different lyophilized Cytokine Standards into the same tube.
IFNγ ☐ **TNF** ☐
Add 200 µL fresh culture medium to solubilize.
Incubate RT, 15 minutes ☐

Start time _____ *Stop time* _____

Prepare **1:3 serial dilution** of Cytokine Standards with fresh culture medium. ☐

Dilute Cytokine Capture Beads Cocktail with **9-fold** volume of fresh culture medium. ☐

Add Membrane Integrity Dye to Antibody Detection Cocktail (**1:250 dilution**). ☐

2. Assay protocol

Add **10 µL/well** samples and standards to the assay plate. ☐

Add **100 µL/well** diluted Cytokine Capture Beads Cocktail.
DO NOT SHAKE
Incubate RT, 60 minutes, Dark ☐

Start time _____ *Stop time* _____

Long spin **(300 xg, 5 minutes).** Aspirate supernatant.
Agitate in residual liquid with Strong shake* ☐

Add **10 µL/well** Cytokine Detection Cocktail.
Quick spin | Brief shake*
Incubate RT, 60 minutes, Dark ☐

Start time _____ *Stop time* _____

Add **10 µL/well** combined Antibody Detection Cocktail with Cell Membrane Integrity Dye.
Quick spin | Brief shake*
Incubate RT, 60 minutes, Dark ☐

Start time _____ *Stop time* _____

Add **50 µL/well** Wash Buffer.
Long spin **(300 xg, 5 minutes)**. Aspirate supernatant.
Agitate in residual liquid with Strong shake* ☐

Add **10 µL/well** Wash Buffer. Acquire data. ☐

Notes

* Quick spin: 300 xg, 5 seconds | Brief shake: 2000 RPM, 20 seconds | Strong shake: 3000 RPM, 60 seconds

Section 2. Introduction

T cells can become exhausted following persistent antigen exposure during chronic infections and cancer. Exhausted T cells exhibit decreased effector functions and show expression of inhibitory receptors (IRs) on the cell surface. The exhausted phenotype may be protective in the context of chronic infection, however, it allows tumors to avoid immune surveillance and immune cell mediated killing. Recent therapies to block the IRs to reinvigorate exhausted T cells have shown promise in the treatment of cancers.

This Human T Cell Exhaustion Cell and Cytokine Profiling Kit was designed for ease of use in multiplexing markers of T cell exhaustion, phenotyping T helper and cytotoxic T cells, and bead-based measurement of secreted cytokines, all in the same assay. This optimized assay offers these unique advantages:

- Simultaneous measurement of cells and secreted cytokines in a mixed cells and beads assay.
- An improvement over common immunology research workflows that require multiple assays. This kit offers a single multiplexed assay optimized for use on the Intellicyt® platform (iQue®3 and iQue® Screener PLUS) equipped with Violet, Blue, and Red lasers (VBR, this assay is not compatible with BR configurations).
- Integration of a single platform and data analysis package provides rapid data acquisition, analysis workflow, and solves data synchronization issues.
- Measurement and analysis of T cell exhaustion phenotypic and functional markers in one single, high-content assay allows screening for molecules that reverse T cell exhaustion, screening of modified cells such as CAR-Ts or to select clones that exhibit low levels of IRs and show a robust response to stimulation.
- Simplified 'plug-and-play' assay workflow with no additional color compensation, and pre-mixed reagents for CD antibody staining and for secreted protein detection. Total assay time is approximately 3 hours, with a hands-on time of about 30 minutes. An included template with pre-set compensation enables data acquisition of the multiplexed, phenotyping assay without the need for single stain color compensation.

Section 3. Assay principles

3.1 Multiplexed assay in a single well

The Human T Cell Exhaustion Cell and Cytokine Profiling Kit is a multiplexed assay that simultaneously measures these endpoints in a cells and beads assay:

- T cell phenotype markers: CD3, CD4, and CD8
- T cell exhaustion markers (IRs): PD-1, Lag-3, and Tim-3
- Secreted cytokines Interferon gamma (IFNγ) and Tumor Necrosis Factor (TNF)
- Cell count, viability, and optional proliferation

In each assay well, live T cells are distinguished from the dead cells by staining with Intellicyt® Cell Membrane Integrity Dye which enters only dead cells or those with a compromised membrane, staining the nuclear DNA by intercalation. Live cells are immunophenotyped by staining with a fluorescent antibody panel to distinguish $CD3^+$ T cells, $CD3^-$ non-T cells, $CD3^+CD4^+$ helper T cells, and $CD3^+CD8^+$ cytotoxic T cells. The panel also includes three markers of T cell exhaustion (IRs): PD-1, Lag-3, and Tim-3. The capacity of the sample to proliferate can also be determined with an optional proliferation dye included with the kit. Cytokines secreted by activated T cells (IFNγ and TNF) are measured in a sandwich immunoassay by using two different QBeads® included in the same well.

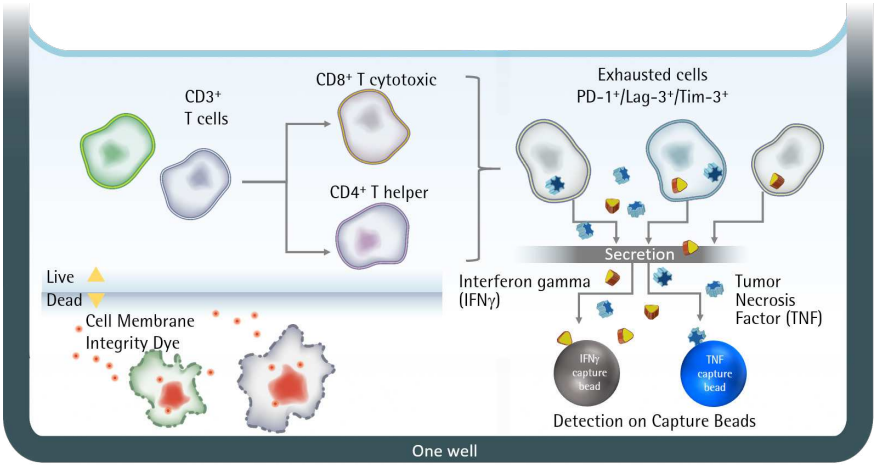

Figure 1. Simultaneous endpoint measurement in a single well.

3.2 Workflow overview

T cells are stimulated in culture plates to test for hallmark markers of exhaustion (expression of IRs and decreased secretion of IFNγ and TNF upon stimulation). Sample aliquots of the cells/supernatant mixture from each well are transferred to 96-well or 384-well assay plates along with diluted IFNγ/TNF Capture Beads Cocktail. After 60 minutes of incubation, the plate is centrifuged, supernatant aspirated, and the cells/beads are resuspended in Cytokine Detection Cocktail. After a second 60 minutes of incubation, a fluorescent Antibody Detection Cocktail with Cell Membrane Integrity Dye is added to the assay plate. After a final 60 minutes of incubation, the assay plate is washed once before acquisition on the iQue®3 (VBR) or iQue® Screener PLUS (VBR). The cells may be stained with optional proliferation dye before culture (**Appendix A**).

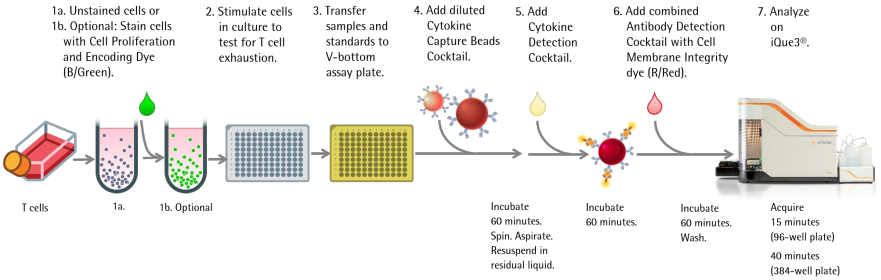

Figure 2. Assay workflow.

Table 1. Assay result readouts

T cell type	T cell ID			IRs			Secreted effector cytokines		Cell count	Cell viability	Cell proliferation (Optional)
	CD3	CD4	CD8	PD-1	Lag-3	Tim-3	IFNγ	TNF			
Cytotoxic T cells	+	-	+	+/-	+/-	+/-	+/-	+/-	#	0 – 100%	+
Helper T cells	+	+	-	+/-	+/-	+/-	+/-	+/-	#	0 – 100%	+

Identification of basic T cell subtypes at different stages of activation and exhaustion. Secreted IFNγ and TNF are included in the final readouts. In **Table 1**, "+" means highly expressed/secreted, "-" means low or no expression/secretion, "+/-" means partially expressed/secreted, "#" means a certain number of cells, and cell viability ranges between 0 -100%.

Section 4. Reagents provided

Table 2. Human T Cell Exhaustion Cell and Cytokine Profiling Kit contents

Intellicyt® reagents	Catalog No. 97069 1 x 96-well	Catalog No. 97070 5 x 96-well	Catalog No. 97071 1 x 384-well	Catalog No. 97072 5 x 384-well
Human IFNγ Lyophilized Cytokine Standard	1 vial	5 vials	1 vial	5 vials
Human TNF Lyophilized Cytokine Standard	1 vial	5 vials	1 vial	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
Cell Membrane Integrity Dye (R/Red)	25 µL 1 vial	25 µL 5 vials	25 µL 1 vial	25 µL 5 vials
Cytokine Capture Beads Cocktail	2.0 mL 1 vial	2.0 mL 5 vials	5.4 mL 1 vial	5.4 mL 5 vials
Cytokine Detection Cocktail	2.0 mL 1 vial	2.0 mL 5 vials	5.4 mL 1 vial	5.4 mL 5 vials
Antibody Detection Cocktail	2.0 mL 1 vial	2.0 mL 5 vials	5.4 mL 1 vial	5.4 mL 5 vials
Wash Buffer	25 mL	125 mL	50 mL	250 mL

One printed manual, a flash drive with templates, and a printable manual is also included in the package with the reagents kit.

Section 5. Storage and stability

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

Section 6. iQue®3 (VBR) and iQue® Screener PLUS (VBR) detector channels

Detector	Spectrum	Violet Laser (405 nm)		Blue Laser (488 nm)		Red Laser (640 nm)	
445/45 nm		VL1	CD3				
530/30 nm		VL2		BL1	Proliferation/ Encoding dye (B/Green)		
572/28 nm		VL3		BL2	QBeads Detection		
615/24 nm		VL4		BL3			
675/30 nm		VL5	Tim-3	BL4	Lag-3	RL1	Cell Membrane Integrity Dye (R/Red)
780/60 nm		VL6	CD8	BL5	PD-1	RL2	CD4

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

Section 7. Materials required but not provided

- iQue®3 (VBR) or iQue® Screener PLUS (VBR)
- Cell population of interest and appropriate complete cell culture medium
- Centrifuge (up to 500 xg capability for use with microplates and microfuge tubes)
- Vortex mixer
- 96- or 384-well V-bottom assay plates (e.g., Intellicyt® Cat. No. 90151 or Greiner Cat. No. 781280)
- Microfuge tubes and/or 15 mL conical tubes
- Reagent reservoirs
- Universal black lid (e.g., Corning Cat. No. 3935) or foil to protect from light or evaporative losses
- Multi-channel pipettes (See **Appendix C** for recommendations)
- Plate washer (e.g., BioTek model ELx405)

Section 8. Recommended materials

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

- Positive control option: Dynabeads™ Human T-Activator CD3/CD28 (ThermoFisher Cat. No. 11131D)
- Negative control option: Culture medium without stimulating agents may be used as a negative control (blank)

Section 9. Cells and reagents preparation

9.1 Samples

a. This assay is designed to detect markers of T cell exhaustion in cultures. Prior to preparing cultures, sample cells may be optionally stained with Intellicyt® Proliferation and Encoding Dye (B/Green), included with this kit (**Appendix A**). Before running the assay, prepare cultures in appropriate culture medium and conditions, including initial input cell density. If the assay cell density is too low, it may be difficult to achieve statistical significance in the cell population of interest (See **Table 7** below and **Appendix B** for recommendations).

b. This assay is validated in cell culture with RPMI 1640 medium with 10% fetal bovine serum. Other similar culture medium may also work in this assay.

c. If necessary, include recombinant human cytokines such as IL-2 or other cytokine cocktails with biological activity in the culture medium to help maintain or promote the health and growth of T cells.

9.2 Assay plate design

a. The recommended assay plate design can be found in the Design section of ForeCyt®, and in the template provided (USB flashdrive in kit package). For latest electronic revisions of this manual and ForeCyt® templates, contact askascientist@sartorius.com.

b. This assay uses serially diluted cytokine standards to generate a standard curve for quantitation of IFNγ and TNF in the sample.

9.3 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 zero 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 may be altered in the Design section of the experiment: Design → Standards → Edit Standard Set. Representative standard curves are shown in **Figure 6**.

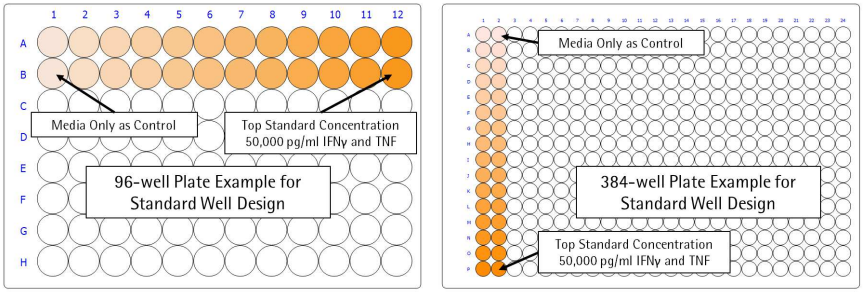

Figure 4. Configuration of the Standard Set. The Standard Set provided in the kit template is arranged from left to right for 96-well formats, and from top to bottom for 384-well formats.

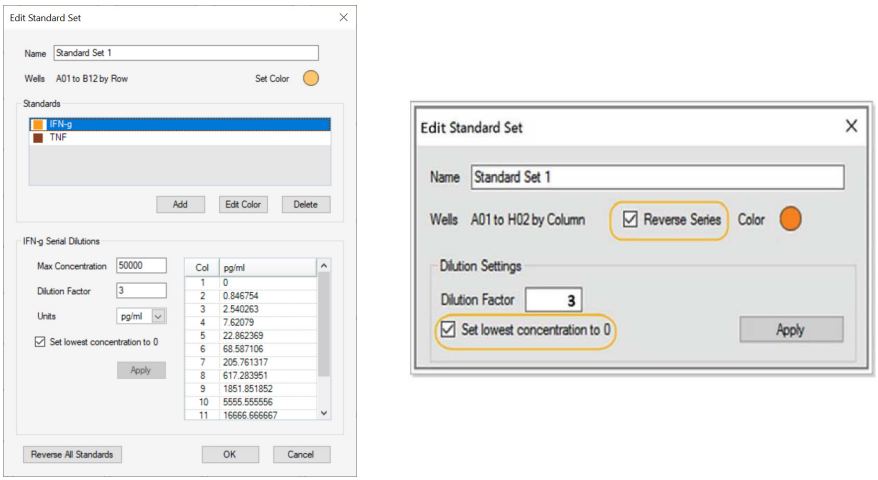

Figure 5. Editing the Standard Set. (Left) The provided assay template is preset to have cytokine standards in the low to high configuration with the lowest concentration set to zero. If a different orientation or lowest concentration is used, the Standard Set may be edited as necessary. (Right) In versions 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.

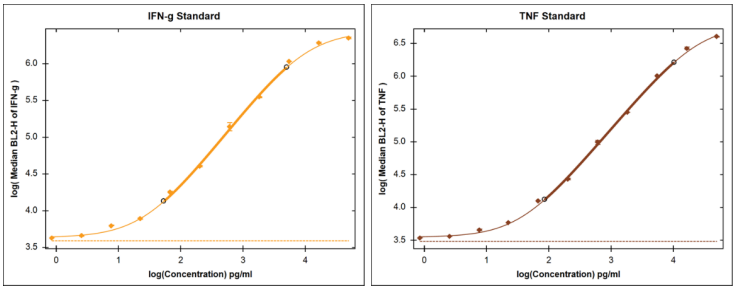

Figure 6. Representative standard curves (IFNγ and TNF) with 1:3 serial dilutions. The bold lines indicate the linear range in each graph, with the detection range wider than the linear range. The linear ranges for IFNγ and TNF are 55-5,000 pg/mL, and 85-10,000 pg/mL, respectively. The dashed line represents the fluorescent background when the standard concentration is zero.

9.4 Reagents

a. Briefly centrifuge all vials before use to prevent reagent loss.

b. Vortex the Cytokine Capture Beads Cocktail, Cytokine Detection Cocktail and the Antibody Detection Cocktail 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.

NOTE: Do not use expired QBeads® or antibodies.

9.5 Cytokine Standard preparation

a. Cytokine Standard curve ranges are 0.0 pg/mL – 50,000 pg/mL for IFNγ and for TNF.

b. From the provided glass vials, combine the two lyophilized Cytokine Standard spheres (IFNγ and for TNF) into a 1.5 mL microfuge tube or 15 mL conical tube. Use only 1 glass vial of each cytokine for the standard preparation on each assay day.

c. Slowly add 200 µL fresh culture medium to the tube with the Cytokine Standard spheres. DO NOT MIX. Mixing at this step causes the reagent to foam.

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

e. Once dissolved, pipette up and down gently to mix the Cytokine Standards.

f. Perform 1:3 serial dilutions of Cytokine Standards (i.e. 50 µL of top standard into 100 µL of culture medium serially). For 96-well format perform a 12-point curve, including blank medium control (**Figure 7**, top). For 384-well format, perform a 16-point curve, including blank medium control (**Figure 7**, bottom).

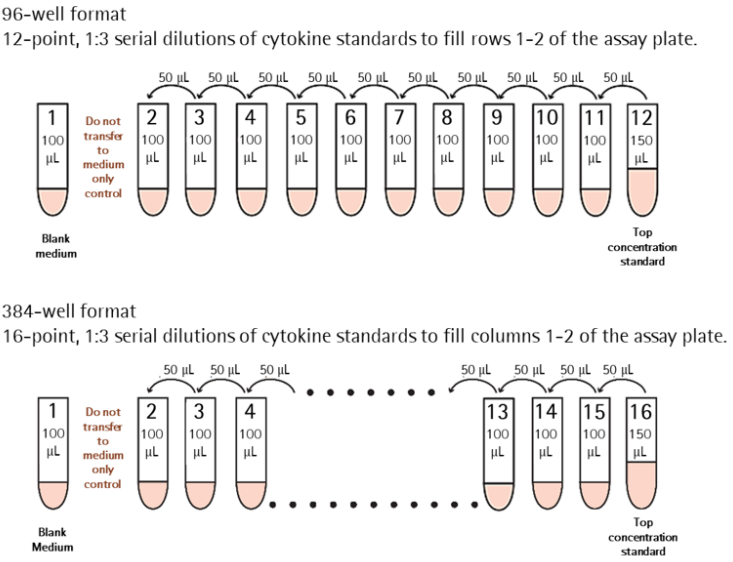

Figure 7. Serial dilution of Cytokine Standards. For both 96- and 384-well formats, Cytokine Standards are to be serially diluted 1:3 by adding 50 µL from the top concentration standard into 100 µL of culture medium serially. A blank medium tube should be included as a control.

9.6 Dilute the Cytokine Capture Beads Cocktail

A vial of pre-mixed Cytokine Capture Beads Cocktail is provided in this kit, which requires dilution with fresh culture medium prior to assay.

a. Label a 50 mL conical tube, or larger container such as bottle or reservoir, "Diluted Cytokine Capture Beads Cocktail."

b. Dilute the provided Capture Beads Cocktail with a 9-fold volume of fresh culture medium (final 1:10 dilution) by transferring the volumes to an appropriate container (See **Table 3** below).

c. Vigorously vortex the container for 10 seconds. If the dilution is prepared in a reservoir, mixing may be performed by manual pipetting the solution

Table 3. Volumes for dilution of Cytokine Capture Beads Cocktail

Kit Format	Cytokine Capture Beads Cocktail IFNγ/TNF (Volume per plate)	Fresh culture medium Final 1:10 dilution (Volume per plate)
1 × 96-wells 5 × 96-wells	2.0 mL	18.0 mL
1 × 384-wells 5 × 384-wells	5.4 mL	48.6 mL

9.7 Preparation of Cell Membrane Integrity Dye and Antibody Detection Cocktail

An appropriate amount of Cell Membrane Integrity Dye (R/Red) should be added to the Antibody Detection Cocktail fresh on the day of the assay according to the kit format.

Table 4. Volumes for Cell Membrane Integrity Dye addition to Antibody Detection Cocktail

Kit Format	Antibody Detection Cocktail (Volume per plate)	Cell Membrane Integrity Dye (R/Red) Final 1:250 dilution (Volume per plate)
1 × 96-wells 5 × 96-wells	2.0 mL	8 µL
1 × 384-wells 5 × 384-wells	5.4 mL	21.6 µL

Section 10. Assay protocol for all formats

NOTE: The following protocol includes brief shaking steps (2,000 RPM for 20 seconds) and strong shaking steps (3,000 RPM for 60 seconds).

WARNING: Make sure that the RPM for these shakes are correct to avoid well cross-contamination.

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

10.1 Add cell/supernatant mixture samples and Cytokine Standards

a. Ensure the cell/supernatant mixture in the original culture plate is in suspension by manually pipetting up and down 6-8 times, then transfer **10 µL** of sample to each well of the assay plate (96- or 384-well format) designated as Sample during the plate set up on the ForeCyt® Design section.

b. Transfer **10 µL** of cytokine standards prepared earlier (**Section 9.5**) to each well of the assay plate (96- or 384-well format) designated as Standards in the ForeCyt® Design section.

10.2 Add the diluted Cytokine Capture Beads Cocktail to the assay plate

a. Vigorously vortex diluted capture beads prepared earlier (**Section 9.6**) and transfer to a reservoir.

b. Transfer **100 µL** of diluted capture beads to each assay well (96- or 384-well format). Agitate the reagent in the reservoir occasionally to prevent bead settling.

c. Cover the plate to prevent evaporation and protect from light. Incubate the plate in the dark at room temperature for 60 minutes. Do NOT shake.

NOTE: During this liquid transfer, change pipette tips to avoid cross-well contamination.

10.3 Spin/aspiration/resuspension

a. After incubation, spin the assay plate (300 xg, 5 minutes).

b. Aspirate the supernatant.

c. Resuspend samples in the residual liquid with a strong shake on the iQue®3 (VBR) or iQue® Screener PLUS (VBR) plate shaker (3000 RPM, 60 seconds).

NOTE: It is recommended that the aspiration step be carried out using a plate washer following the manufacturer's recommendations (see **Section 12, Best practices and tips**). The specific plate washer must first be optimized to avoid sample loss during the aspiration. If a plate washer is not available, manual aspiration using a multichannel pipette or plate flicking may be employed.

10.4 Add the Cytokine Detection Cocktail

a. Transfer the Cytokine Detection Cocktail to a reservoir. Add **10 µL** per well to the assay plate (96- or 384-well format).

b. Quick spin (300 xg, 5 seconds) and brief shake (2,000 RPM, 20 seconds) using the iQue®3 (VBR) or iQue® Screener PLUS (VBR) plate shaker to ensure thorough mixing.

c. Cover and incubate in the dark at room temperature for 60 minutes.

10.5 Add the combined Antibody Detection Cocktail with Cell Membrane Integrity Dye

a. Transfer the combined Antibody Detection Cocktail with Cell Membrane Integrity Dye prepared earlier (**Section 9.7**) to a reservoir. Add **10 µL** per well to the assay plate (96- or 384-well format).

b. Quick spin (300 xg, 5 seconds) and brief shake (2,000 RPM, 20 seconds) using the iQue®3 (VBR) or iQue® Screener PLUS (VBR) plate shaker to ensure thorough mixing.

c. Cover and incubate the plate in the dark at room temperature for 60 minutes.

10.6 Wash and resuspension

a. Add **100 µL** (50 µL for 384-well format) per well of Wash Buffer to the assay plate.

b. Centrifuge the plate (300 xg, 5 minutes).

c. Aspirate the supernatant.

d. Resuspend samples in residual liquid with a strong shake on the iQue®3 (VBR) or iQue® Screener PLUS (VBR) plate shaker (3000 RPM, 60 seconds).

e. Add **20 µL** (10 µL for 384-well format) per well Wash Buffer to the assay plate. An additional quick spin (300 xg, 5 seconds) and brief shake (2000 RPM, 20 seconds) should be performed to ensure that all samples are at the well bottom.

f. Secure the assay plate onto the plate loader of the iQue®3 (VBR) or iQue® Screener PLUS (VBR) system. The samples are now ready for acquisition.

Section 11. Plate acquisition and data analysis

11.1 Acquire plate

a. Launch ForeCyt® software.

b. Import the provided experiment template (included on USB key in the kit package). Create a New Experiment using the provided template.

c. In the Design section:

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

   ii. Series sub-section: Assign wells for compound series (dose-responses).

   iii. Standards sub-section: Edit standard set if necessary – only when different plate location, orientation or lowest concentration has been employed to ensure proper plate layout.

d. In the Protocol section: Adjust sip times and inter-well shaking as needed to achieve statistical significance for your cell population of interest (Refer to the tables in **Section 12**, **Best practices and tips**).

e. 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 template gates are preset for different populations. If preferred, below are the gating details to manually draw the gates or fine-tune the existing gates in the template: 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**).

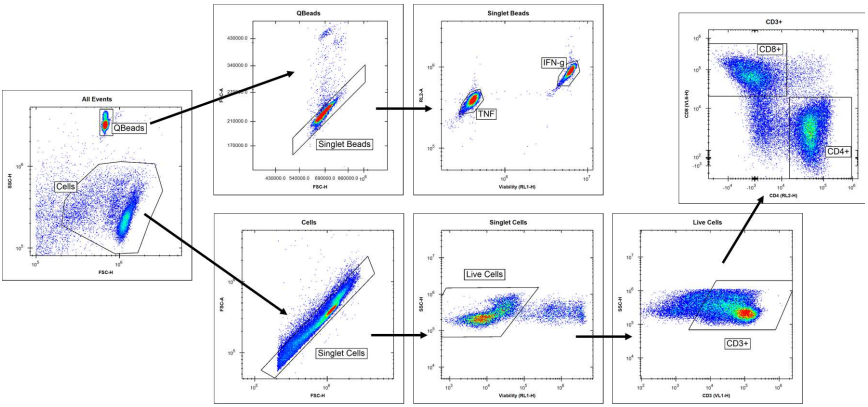

Figure 8. Gating QBeads® and cell populations. From All Events identify the QBeads® and cell populations followed by identification of singlet populations. Furthermore, individual IFNγ and TNF QBead® populations and live/dead cell populations are identified.

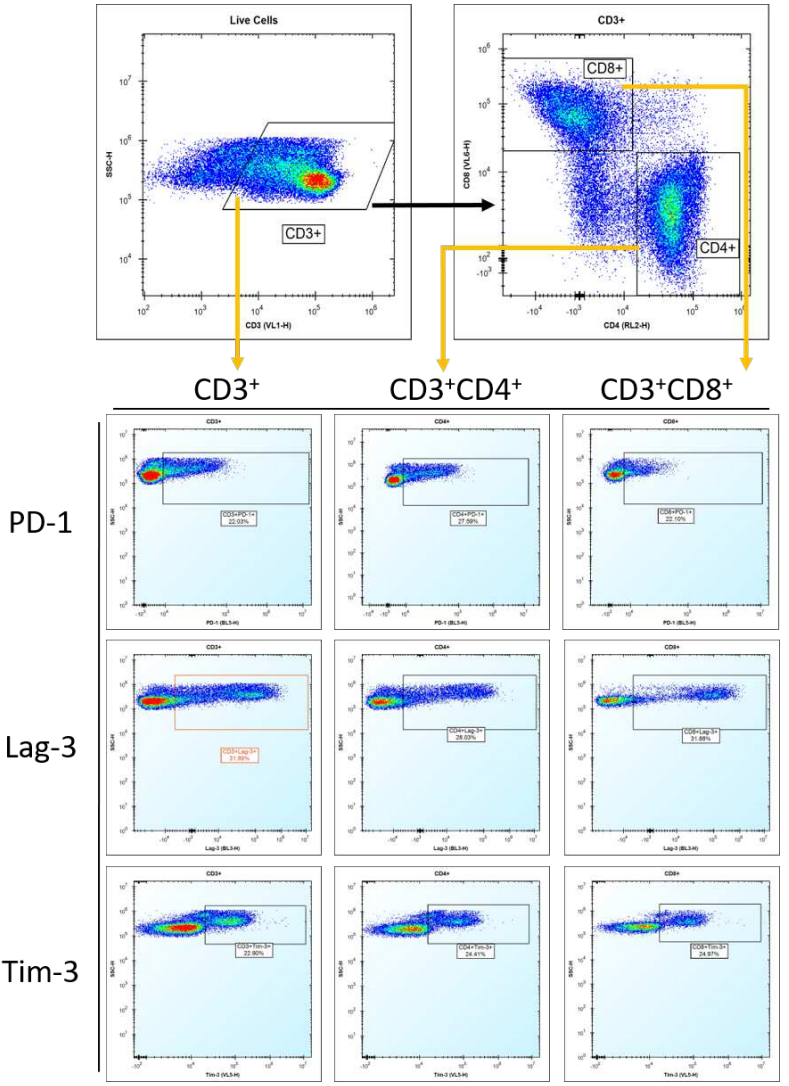

Figure 9. Gating different cell phenotypes from Live cells. From $CD3^+$ cells, identify $CD4^+$ and $CD8^+$ cells followed by Inhibitory Receptor subset populations. To improve the separation of different populations, manually adjust the linear range of the dot plot bi-exponential scale.

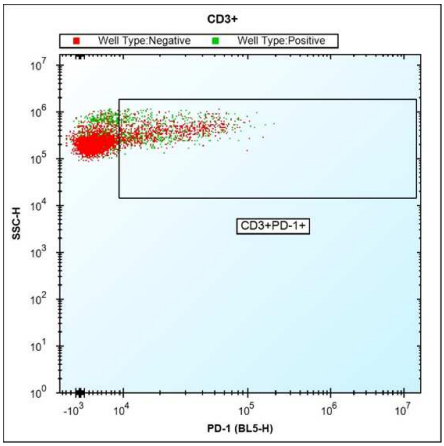

Figure 10. Use positive and negative control wells to fine tune gates. Once positive and negative wells have been designated in the Design section, use the overlay plots in the template to fine tune the gates of exhausted cell populations.

11.3 Compensation spillover matrix

Primary Channel \ Spillover Channel	Proliferation (BL1-H)	Lag-3 (BL3-H)	PD-1 (BL5-H)	Viability (RL1-H)	CD4 (RL2-H)	CD3 (VL1-H)	Tim-3 (VL5-H)	CD8 (VL6-H)
Proliferation (BL1-H)		7.19	0.03	0.00	0.00	0.00	0.13	0.00
Lag-3 (BL3-H)	0.19		1.70	0.09	0.01	0.00	3.20	0.10
PD-1 (BL5-H)	0.37	1.04		0.02	21.17	0.00	0.01	10.43
Viability (RL1-H)	0.70	0.54	0.00		8.42	0.08	0.47	0.11
CD4 (RL2-H)	0.00	0.00	0.73	6.45		0.00	0.20	4.03
CD3 (VL1-H)	0.00	0.00	0.01	0.01	0.00		0.16	0.02
Tim-3 (VL5-H)	0.00	0.16	0.05	35.29	2.19	1.46		6.15
CD8 (VL6-H)	0.00	0.00	3.35	0.15	19.44	3.62	0.39	

Figure 11. Compensation spillover matrix. This compensation matrix is included in the ForeCyt® template and is applied when experiment templates are employed for data acquisition. There is no need to adjust any compensation settings.

Section 12. Best practices and tips

12.1 Dilute the protein standards with fresh culture medium

It is critical to use fresh culture medium when reconstituting the Cytokine Standards to avoid possible matrix effect and to ensure data reproducibility and reliability. Medium should not differ from that used in the cell culture sample. A specific diluent for protein standards is not provided with this kit.

12.2 Plate type

The assay protocols in this manual are designed for both 96-well and 384-well plate formats. The Human T Cell Exhaustion Cell and Cytokine Profiling Kit has been tested with both 96-well, and 384-well V-bottom plates (Intellicyt® Cat. No. 90151 and Greiner Cat. No. 781280, respectively). This assay kit provides templates for both 96-well and 384-well formats.

12.3 Manual pipetting recommendation

This protocol requires pipetting volumes between 10 µL and 100 µL depending on the plate formats. Care should be taken during liquid transfers so that volumes are fully dispensed with appropriate pipettes. Avoid well cross-contamination by changing pipette tips between wells. When pipetting small volumes, it is best practice to touch the bottom of the 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 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 reagent to the well bottom to mix with the existing reagent/sample already in the well. For single and multi-channel pipette recommendations, see **Appendix C**.

12.4 Mixing plate contents using a shaker

The 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®3 (VBR) or iQue® Screener PLUS (VBR) may be used without exceeding the volume and speed limitations (**Table 5**). From the ForeCyt® menu bar, select Device → Manual Control. In the Manual Control window, set the desired shake speed.

Table 5. 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+	*
* Larger volumes will require additional optimization. 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. Care should be taken to avoid well cross-contamination.		

From the ForeCyt® menu bar, select Device → Manual Control. In the Manual Control window, set the desired shake speed (**Figure 12**). As soon as the "On/Plate Shake" Shaker Controls checkbox is selected, the shaker will begin to shake and continue to shake until disabled by deselecting the checkbox.

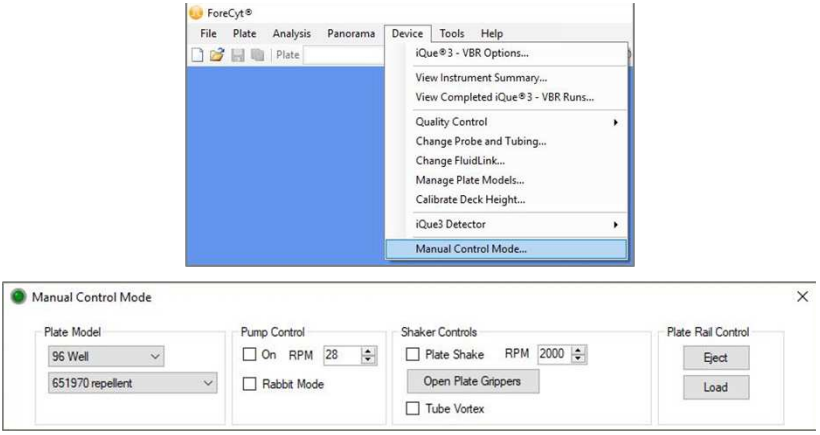

Figure 12. Steps for using the shaker on iQue®3 (VBR) or iQue® Screener PLUS (VBR). Set the shaker speed to either 2000 RPM or 3000 RPM depending on the assay requirements. It is important to note that shaking at 3000 RPM is reserved only for the step post-aspiration. Shaking the plate at 3000 RPM with liquid in the wells will result in cross-contamination.

12.5 Use a plate washer for aspiration

For assay aspiration steps it is best to use an automated plate washer. If a plate washer is not available, manual aspiration using a multichannel pipette or plate flicking may be employed; however these techniques may result is severe sample loss. An automated washer is the preferred method. Aspiration programs for this assay have been tested on a BioTek ELx405 Select (**Table 6**). For a different plate washer brand or model, it is best practice to optimize specific plate washer settings so that sample loss is avoided.

Table 6. Aspiration settings using the BioTek ELx405 Select

Plate Type	Height setting	Height offset (mm)	Rate setting	Aspiration rate (mm/s)
Intellicyt® 96-well, V-bottom (#90151)	#40	5.08	#6	15
Greiner 384-well, V-bottom (#781280)	#31	3.937	#6	15

12.6 Adjust the sip time to acquire enough cell events

Sip time determines how many cell events are acquired from each well. The template in the kit has a default sip time of 4 seconds per well. 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 may vary slightly from machine to machine and day to day. Sip volume is approximately 1.5 µL per second. Adjustment of sip time from the 4 second default may be made in the ForeCyt® Protocol section. Inter-well shaking may need to be adjusted as well, as settling will occur over time. If necessary, **Tables 7** and **8** below, may help with sip time and shaking adjustments, assuming the lowest cell density in the culture plate is 1 million/mL. Refer to **Appendix B** for additional measures to improve cell event acquisition in addition to increasing sip time.

Table 7. 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 seconds (default)	1 x 10^6 cells/mL (minimum seeding density)	10 µL (from culture plate to assay plate)	25 µL (20 µL + residual volume)	0.3 x 10^6 cells /mL (assuming 20% loss during wash)	6 µL
6 seconds					9 µL
8 seconds					12 µL
10 seconds					15 µL
12 seconds					18 µL

* Assuming a 1.5 µL/s sip per well.

Sip time per well	Estimated cell events acquired per well	Inter-well shake frequency	Inter-well shake duration	Acquisition time per plate
4 seconds (default)	1800	Every 4 wells	4 s	~16 min
6 seconds	2700	Every 4 wells	4 s	~20 min
8 seconds	3600	Every 4 wells	4 s	~23 min
10 seconds	4500	Every 3 wells	4 s	~27 min
12 seconds	5400	Every 3 wells	4 s	~30 min

Table 8. 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 (default)	1 x 10^6 cells/mL (minimum seeding density)	5 µL (from culture plate to assay plate)	15 µL (10 µL + residual volume)	0.3 x 10^6 cells /mL (assuming 20% loss during wash)	6 µL
6 seconds					9 µL
8 seconds					12 µL

* Assuming a 1.5 µL/s sip per well.

Sip time per well	Estimated cell events acquired per well	Inter-well shake frequency	Inter-well shake duration	Acquisition time per plate
4 seconds (default)	1800	Every 6 wells	4 s	~45 min
6 seconds	2700	Every 6 wells	4 s	~57 min
8 seconds	3600	Every 4 wells	4 s	~75 min

12.7 How to ensure sample cytokines are within the linear range of the standard curves

The ForeCyt® template defaults to 4 parameter logistic (4PL) with $1/Y^2$ weighting for the standard curves. ForeCyt® can provide the linear range for each standard curve. Use a 1:3 serial titration with the top concentrations at 50,000 pg/mL for IFNγ and TNF. If adjustments for concentration, dilution factor, or plate layout for the standard are necessary, refer to the ForeCyt® Reference Guide and make the adjustment in the Design section. The use of a different culture medium may have a slight impact on the standard curve linear range.

Section 13. Appendices

13.1 Appendix A: Proliferation and Encoding Dye protocol for target cells

The following protocol uses Intellicyt® Cell Proliferation and Encoding Dye (B/Green) to label the cells before culture. Cell proliferation is detected and quantified based on the halving or "dilution" of the loaded dye after each round of cell division. Each of the two daughter cells retains half the original intensity of the dye, and all subsequent rounds of division further divide out the original dye signal. The assay template provided in the USB drive in the kit includes the compensation matrix for the dye detection channel, BL1 in iQue®3 (VBR) and iQue® Screener PLUS (VBR). Below are instructions for loading the cells using the Cell Proliferation and Encoding Dye (B/Green):

a. Before beginning, ensure that the dye is completely thawed. If necessary, place the dye vial in a 37°C water bath for 5-10 minutes before use.

b. Prepare the working dye stock by diluting the Proliferation and Encoding Dye (B/Green) into Hank's Balanced Salt Solution (HBSS) buffer or Phophate Buffered Saline (PBS) buffer (dilution factor 1:1250). The HBSS or PBS buffer must be protein-free. Select one buffer and use it consistently across the protocol when it is required.

c. Collect cells in a 50 mL conical tube. Spin cells down (500 xg, 5 minutes) and remove the original culture medium.

d. Resuspend cells in 20 mL protein-free HBSS or PBS. Spin cells down (500 xg, 5 minutes). Remove the supernatant. Resuspend cells in protein-free HBSS or PBS at 1-4 million/mL.

e. Combine an equal volume of the prepared cells and the prepared working dye stock. The final dye concentration in the staining tube will be 1:2500 diluted. Thoroughly mix, and incubate the cells at room temperature for 15 minutes, covered with a lid, and protected from light.

f. After staining, wash by adding at least 2x volume of complete culture medium (with 10% serum) to the staining sample. Spin (500 xg, 5 minutes). Remove the supernatant. Resuspend cells manually in the residual liquid.

g. Repeat wash (described in step f.) two more times.

h. After the final wash, carefully resuspend cells at the desired cell density for assay. **Figure 13** illustrates gating to proliferation of a sample acquired by iQue®3 (VBR) or iQue® Screener PLUS (VBR) after 3 days of culture.

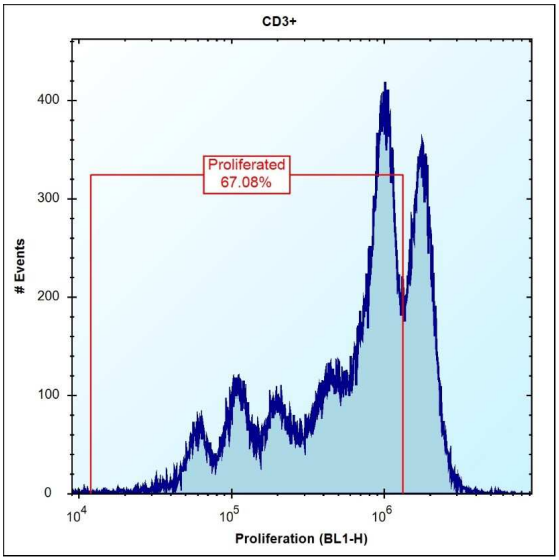

Figure 13. Gating example to determine proliferation of cells. Cells were loaded with Proliferation and Encoding Dye (B/Green), followed by a 3 day culture with activators.

13.2 Appendix B: Options for improving cell event acquisition

Option 1: Adjust acquisition sip time (See **Section 12, Best practices and tips**).

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

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

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

c. Resuspend 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: 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®3 (VBR) or iQue® Screener PLUS (VBR) using SPHERO™ AccuCount beads (Spherotech Cat. No. #ACBP-50-10) is recommended if precise cell density information is required. This product 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 Human T Cell Mediated Killing Kit by using the same plate type, sample volume, and sip time

c. Measure the sip volume by sampling 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

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)

Single-channel pipettes

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

13.4 Appendix D: Modified workflow for samples with low levels of IFNγ and TNF

If samples are expected to have very low levels of both IFNγ (<55 pg/mL) and TNF (<85 pg/mL), adjustments to the workflow may be made to extend the linear range of the standards at the low end of the standard curve. The recommendation is below:

a. Eliminate reagent preparation step in Section 9.6: The original assay protocol requires diluting the Cytokine Capture Beads Cocktail with a 9-fold volume of fresh culture medium. This step should be bypassed. Do NOT dilute the Cytokine Capture Beads Cocktail provided in the kit.

b. Modify plate assay protocol steps in Sections 10-1 through 10-3 as described below:

   i. Add 10 µL/well Samples and Cytokine Standards

   ii. Add 10 µL/well Cytokine Capture Beads Cocktail to the assay plate. Quick spin (300 xg, 5 seconds). Brief Shake (2000 RPM, 20 seconds). Incubate 60 minutes RT, Dark.

   iii. Add 100 µL/well fresh culture medium to each well. Spin the plate (300 xg, 5 minutes). Aspirate supernatant. Resuspend cells/beads in the residual volume in the assay plate by a strong shake (3000 RPM, 60 seconds).

c. Continue the assay by following the assay protocol steps 10-4 through 10-6 as described.

13.5 Appendix E: 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 the assay, and applied only to experiment plates 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 curve can be found in the ForeCyt® Reference Guide.

Q2: Can I use fixatives in my samples?

A2: Samples may be fixed with certain fixatives (e.g., 1% PFA) however it is important to understand how fixation may affect biological outcomes. The 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 (e.g., Greiner #651970 or Greiner #781970), which may reduce cell loss due to fixation or fixation-related cell cross-linking to the well bottom.

Q3: Can I use a 1 x 384-well kit to run 96-well plate assay? How many 96-well plates can I run?

A3: Yes. A 1 x 384-well kit can be used for 4 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 96- and 384-well ForeCyt® templates are provided.

Q4: Can I multiplex this assay with other cellular or cytokine endpoints?

A4: We recommend not multiplexing this assay with other cellular endpoints. The ForeCyt® template includes a compensation matrix that accounts for these measurements without a need for additional adjustments.

Q5: Why do I get very few capture beads and/or cells from the sample in data acquisition?

A5: If capture beads and cell numbers are low following sample acquisition, increase the sip time and re-read the plate. Each well should yield greater than 50 capture beads for each bead-based population. A number of situations could be responsible:

- Capture beads have not been agitated adequately in their original vial.
- Capture beads were not mixed in the reservoir during transfer to the assay plate.
- The sample was not agitated in the residual buffer liquid after the final centrifugation and aspiration step.
- Capture beads were washed away or lost during the aspiration steps.

For low cell counts, consider the following possibilities:

- Cell proliferation/viability was affected during sample preparation.
- Cells were not mixed before transferring cell/supernatant sample from the culture plate to assay plate.
- The sample was not agitated in the residual buffer after the final centrifugation and aspiration step.
- Cells were washed away or lost during aspiration steps.

Q6: I may have some well cross-contamination. What could be the causes?

A6: There are several assay steps that may have caused well cross-contamination:

- Pipette tips touched samples in the well and were used for reagent transfer for other wells. Be sure to change pipette tips at each reagent addition.
- Use of the strong shake (3,000 RPM) for brief shake (2,000 RPM). Ensure that shake speeds are as described in the assay protocol.

Q7: Do I need to dilute my samples for the assay if my samples have high cytokine levels?

A7: This assay is designed to measure moderately high levels of IFNγ (as high as 5,000 pg/mL) and TNF (as high as 10,000 pg/mL) without sample dilution. Diluting samples is appropriate when cytokine levels are beyond the linear range of the standard curve. When diluting samples, consider adjusting the sip-time to assure enough cellular events are collected for analyses.

Additionally, if sample cytokine levels are expected to be lower than the linear range of the standard curve, a modified assay workflow may be utilized to extend the lower end of the standard curve for your samples. See **Appendix D**.

Q8: What if I do not have access to an automated plate washer for liquid aspiration step?

A8: If you do not have access to the plate washer, you may carefully and slowly aspirate the liquid in the assay well with a manual multi-channel pipette. The pipette tips should be at a 45 degree angle against the wall of assay well. Touching the cell/bead pellet at the well bottom should be avoided. The tips should be changed after each liquid aspiration step in order to avoid cross-contamination across the wells.

Another option to aspirate the liquid in the assay well is to quickly flick the assay plate into a sink. This is a one-time flick, with force. DO NOT flick the plate repeatedly. After plate flicking, wipe the liquid on the top of the plate with a tissue paper. Make sure to bleach your waste liquid in the sink, if necessary.

All above techniques may need some practice and testing, and are not guaranteed to be successful. The data in your assay may be skewed due to sample loss.

Q9: Why do I sometimes get cell membrane integrity/live/viable cell readings from wells which only contain capture beads (e.g. wells designated for cytokine standards)?

A9: These cell membrane integrity readings are typically caused by stray events. You may use the plate view option of live cells to verify that the observed cell numbers are low. These stray events can be considered background noise, and we suggest you exclude the wells designated as standards when viewing heat maps containing live cell data to eliminate any confusion.

Q10: Can I use this assay to measure secreted proteins in human sera?

A10: This assay is only optimized for cell culture samples, and is not optimized to measure secreted proteins in human sera. If you need to measure the same proteins from human sera samples, you may purchase QBeads® kits from Intellicyt® which include a special diluent for human sera samples. The QBeads® kits for human sera samples may NOT be multiplexed with Human T Cell Exhaustion Cell and Cytokine Profiling Kit.

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