



sartorius

Human T Cell Activation Cell and Cytokine Profiling Kit



Protocol Guide

Open immediately upon arrival and store reagents at temperatures stated on labels. For Research Use Only.

Notice to Purchaser

The Human T Cell Activation Cell and Cytokine Profiling Kit is a member of the Intellicyt product line that has been tested extensively for live cell analysis applications. These screening kits are validated as complete screening assays and are optimized for use in high content screening applications. Intellicyt's building blocks and reagents are designed for flexibility in multiplexing and incorporation into screening assays. Intellicyt reagent kits are specifically formatted for optimal performance on Intellicyt Screening platforms.

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These products are offered under a limited warranty. The products are guaranteed to meet appropriate specifications described in the product insert at the time of shipment. Intellicyt Corporation will provide product replacement for valid claims. All claims should be made within five (5) days of receipt of order.

Trademarks and Patents

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List of Catalog Numbers

Description	Cat. No
Human T Cell Activation Cell and Cytokine Profiling Kit 1x96 wells	90560
Human T Cell Activation Cell and Cytokine Profiling Kit 5x96 wells	90561
Human T Cell Activation Cell and Cytokine Profiling Kit 1x384 wells	90562
Human T Cell Activation Cell and Cytokine Profiling Kit 5x384 wells	90563

NOTE: The 1x 384-well kit has enough reagents to run 2x 96-well plates, NOT 4x 96-well plates.







Kit Contents

Component	Quantity Provided
Human IFN γ and TNF α Capture Beads (Pre-mixed)	1 vial
IFN γ and TNF α 2 Separate Standards	1 vial for each cytokine
Cytokine Detection Cocktail	1 bottle
Antibody Panel Detection Cocktail (Pre-mixed: 6 FL antibodies)	1 bottle
Cell Membrane Integrity Dye (R/Red)	1 vial
Wash Buffer	1 bottle
Cell Proliferation and Encoder Dye (B/Green)	1 vial

NOTE: A kit manual and a USB key with assay templates are also included the kit Package.

Detection Channels

iQue Screener PLUS Detector Channels

Detector	Spectrum	Violet Laser (405 nm)		Blue Laser (488 nm)		Red Laser (640 nm)	
445/45 nm		VL1	CD25 (V/Blue)				
530/30 nm		VL2		BL1-H	Prolif. (B/Green)		
572/28 nm		VL3	CD8 (V/Yellow)	BL2-H	QBeads Det.		
615/24 nm		VL4	CD4 (V/Orange)	BL3-H	HLA-DR (B/Orange)		
675/30 nm		VL5		BL4-H		RL1	Cell Viability (R/Red)
780/60 nm		VL6	CD3 (V/Crimson)	BL5_H	CD69 (B/Crimson)	RL2	

NOTE: This assay is only compatible with the iQue® Screener PLUS with VBR option. Other iQue platforms including iQue Screener, iQue Screener PLUS (VYB lasers), iQue Screener PLUS (BR lasers) will NOT work with this assay due to the detection channel limitation.

BL1 channel: Optional proliferation measurement with Cell Proliferation and Encoder Dye (B/Green) included with kit.

RL1 and RL2 channel: Classification of 2 QBeads (IFN γ and TNF α). The RL1 channel is also used for cell viability detection.

Materials Needed but Not Provided

- Intellicyt iQue Screener PLUS platform with VBR Lasers
- Centrifuge capable of spinning microcentrifuge tubes and/or 15 mL conical tubes at up to 500g
- Centrifuge capable of spinning microplates
- Vortex mixer
- Fresh complete cell culture media (Same media used to grow your sample cell culture)
- 96 or 384 well assay plate (Example Source: Costar, Cat#3897 or Greiner Cat#781280)
- Microcentrifuge tubes and/or 15 mL conical tubes
- Reagent reservoirs (Example source: VWR, Cat#89094-680)
- Universal black lid (Example source: Corning, Cat#3935) or foil to protect from light/evaporation)
- 12-channel pipette reservoir (Example source: VWR, Cat#80092-466) (optional for preparing serial titrations)
- Appropriate liquid handler or multi-channel pipette (See [Appendix F](#))
- Plate washer (such as BioTek model ELx405)
- T cell activation reagents such as CD3/CD28 DynaBeads or Phytohemagglutinin (PHA) for use as positive control

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Introduction

The Human T Cell Activation Cell and Cytokine Profiling Kit was designed for ease of use in multiplexing cellular measurements and bead-based quantitation of secreted cytokines in the same assay sample. This optimized assay offers these unique advantages:

- **Simultaneous measurement of cells and secreted cytokines.** This assay is optimized to run on the iQue Screener PLUS (VBR configuration) which has a wide dynamic range without PMT adjustment, and enables high resolution of multi-color stained cells and multiplexed cytokine-detecting beads from the cell/beads mixture in each assay well.
- **Simultaneous measurement of T cell phenotypes, early and late cell activation markers in each phenotype, cell viability, cell count, and secreted effector cytokines.** High content functional readouts profile T cell activation from multiple endpoints at the same time.
- **Optimized workflow to enable measurement of high-level IFN γ /TNF α in the same assay well.** The optimized workflow enables the measurement of high levels of IFN γ /TNF α generally observed in T cell activation cultures without sample dilution, while maintaining sufficient cell counts for phenotyping measurement.
- **One-wash assay.** As a multiplexed assay with multiple staining dyes, the one-wash assay workflow achieves excellent signal resolution with minimal hands-on time.
- **The ability to multiplex T cell proliferation measurements along with the immunotyping and cytokine analysis.** The kit provides the Cell Proliferation and Encoding dye (B/Green) to identify and measure the proliferative cell population.

Assay Principles

The Human T Cell Activation Cell and Cytokine Profiling Kit is a cell/bead multiplex assay that simultaneously measures these endpoints:

- Immune cell phenotypes
- Early and late markers of T cell activation for each phenotype
- Secreted effector cytokines
- Cell count and cell viability

In each assay well, live immune cells are distinguished from dead cells by staining with a fluorescent membrane integrity dye which enters only dead cells or those with a compromised membrane, staining the nucleic DNA by intercalation. Both late apoptotic cells and necrotic cells with compromised membranes will stain marking them for exclusion in further analyses. Live cells are immunophenotyped by staining with a fluorescent antibody panel to separate CD3+ T cells, CD3- non-T cells, CD3+CD4+ T helper cells, CD3+CD8+ T cytotoxic cells. The panel also includes 3 different T cell activation markers: CD69 (early), CD25 (late), and HLA-DR (even later, with strong stimulation). Effector cytokines secreted by activated T cells, including Th1 cytokine IFN γ , and multifunctional cytokine TNF α (secreted mostly by CD8+ T cytotoxic cells and also by Th1 cells) are measured in a sandwich immunoassay format by 2 different QBeads[®] included in the same well.

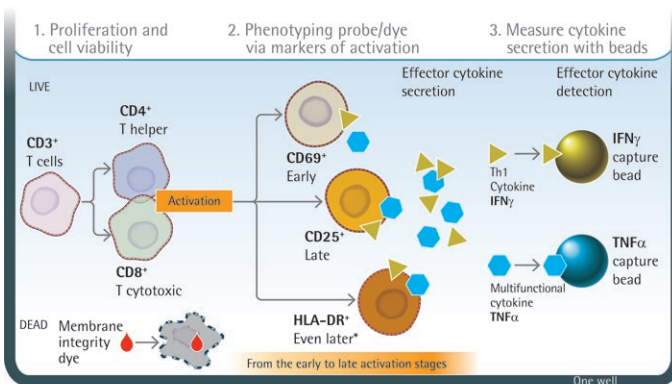


Figure 1. Illustration of Human T Cell Activation Cell and Cytokine Profiling Kit assay principles. Different T cell phenotypes are profiled for the expression of 3 early/late activation markers: CD69 (early), CD25 (late), and HLA-DR (even later). *(May require strong stimulation such as TCR and CD28 co-stimulation). The 2 effector cytokines (IFN γ and TNF α) are also quantified in a 2-plex QBeads in a sandwich immunoassay format in the same assay well. Simultaneous measurement of T cell proliferation or encoded target cells is possible, but is not included in this illustration.

Activation Panel Assay Workflow Overview

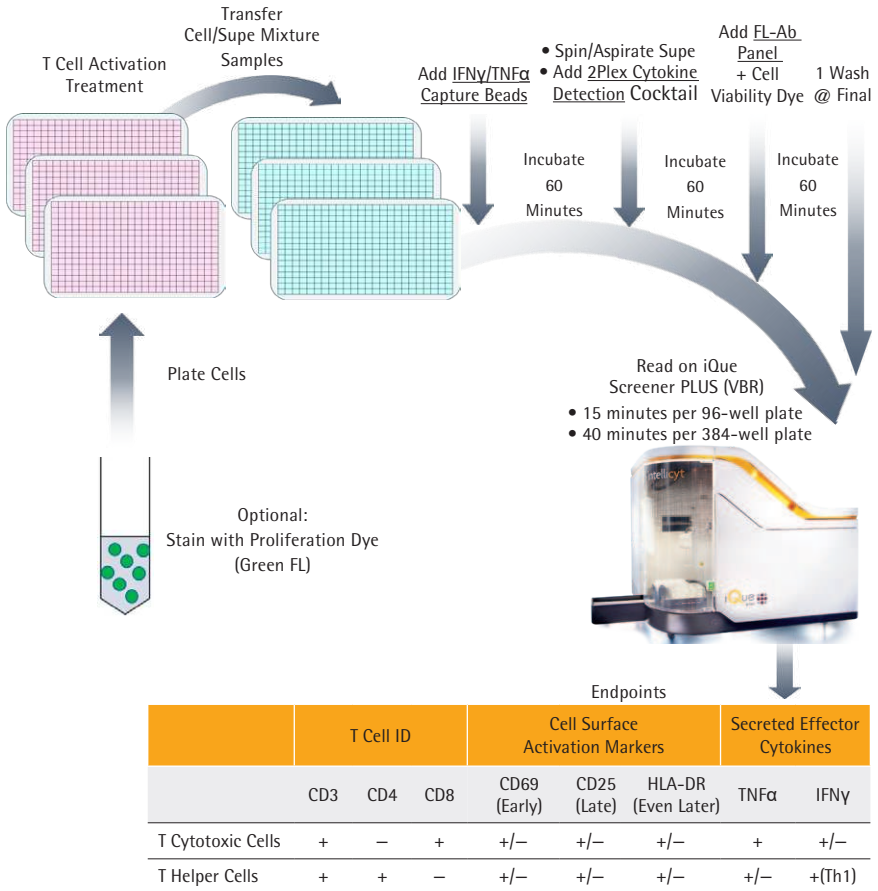


Figure 2. Assay Workflow. T cells or immune cells such as PBMCs are activated in the culture plates. An aliquot of the cells/supernatant mixture from each well is transferred into assay plates along with IFN γ /TNF α capture beads. After incubating 60 minutes, the plate is centrifuged, the supernatant aspirated, and the cell/beads are resuspended in cytokine detection cocktail (anti-IFN γ , and anti-TNF α). After 60 minutes incubation, a fluorescent antibody panel cocktail with cell viability dye is added to the assay plate, including antibodies against CD3, CD4, CD8, CD69, CD25 and HLA-DR, as well as a cell viability dye. After a 60 minute incubation, the assay plates are washed once before sample acquisition on the Intellicyt iQue Screener PLUS using the violet, blue and red (VBR) laser configuration. T cell and T cell subtypes, 3 cell surface activation markers expressed at different stages, and 2 secreted effector cytokines will be measured as the final readouts. In the table, "+" means: highly expressed/secreted. "+/-" means: partially expressed/secreted. "-" means: low or no expression/secretion.

Please Note: If you want to measure T cell proliferation simultaneously, please stain the cells first in the test tube with the optional proliferation dye provided in the kit before plating cells in the culture plate with appropriate T cell activation treatment.

Best Practices and Tips

These best practices and tips will help ensure the success and accuracy of your assay.

Plate Type

The assay protocol described in this manual is designed for both a 96-well and 384-well plate format. You can use one 384-well kit to run two 96-well plate assays. Intellicyt recommends the use of 96-well V-bottom plates (Costar, Cat#3897) for 96-well plate assay, and 384-well V-bottom plates (Greiner, Cat#781280) for a 384-well plate assay. This assay kit provides ForeCyt templates for both 96-well and 384-well formats.

Manual Pipetting Recommendation

This protocol requires pipetting 5 μ L volumes of liquid for a 384-well format. If you are pipetting manually instead of using an automatic liquid handler, be extra careful during the 5 μ L volume transfer of the prepared reagent from the reservoir to the assay well with appropriate pipettes (See [Appendix F](#)). If the plate is empty, touch the tip to the well bottom and then release all the liquid to transfer 5 μ L volume into the well. If the plate already has a reagent or sample in the wells, touch the pipette tip to the upper inner wall of the well at 45-degree angle before you release the 5 μ L liquid. Touching the wall of the well prevents the 5 μ L 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.

This protocol also requires pipetting 10, 50, 90, 100 and 180 μ L volumes of liquid for 2 different plate formats. Change tips after manual pipetting to avoid cross-contamination.

Shaking (for Quick Mixing)

This assay requires shaking to mix the sample/reagents. If you don't have a separate shaker, you can use the one on the Intellicyt iQue screening system, as shown in **Figure 3**. (1) Click on **Device** in the menu bar. (2) Scroll down to **Manual Control**. (3) In the Manual Control window, use the arrows to set the RPM to 2000 (for brief mixing) or to 3000 (for more vigorous mixing after a spin down and aspiration). (4) As soon as you click **On**, the shaker will begin to shake and continue to shake until you click again to un-check.

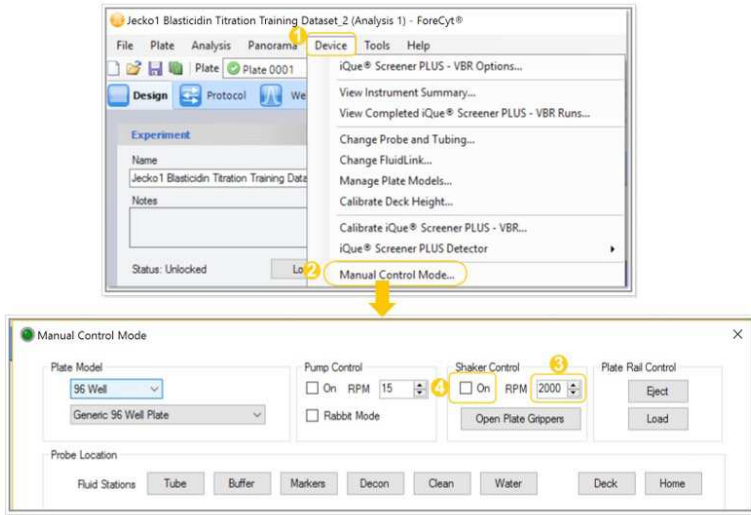


Figure 3. Steps for using the shaker on the iQue screener PLUS.

WARNING: Only perform a vigorous shake at 3000 rpm immediately after aspiration. Shaking at 3000 rpm with liquid in the wells will result in cross-contamination.

(See [Appendix D](#)) on mixing samples with the Intellicyt shaker.

Use a Plate Washer for Aspiration

For wash/aspiration steps, use an automated plate washer. Manual aspiration of plates and/or plate inversion techniques could result in severe sample loss. Aspiration programs for this assay have been tested on a BioTek ELx405 Select. If you have a different plate washer brand or model, it is possible to approximate the aspiration settings on a different system. [Appendix E](#) provides recommended plate types and aspiration settings.

Dilute Protein Standards with Fresh Culture Media

It is critical to use FRESH CULTURE MEDIA to ensure the reproducibility and reliability of your data. Inaccurate or unexpected assay results can often be attributed to not following this best practice. Use fresh culture media to dilute the combined 2 protein standards provided in the kit. This media should be the same media you used to grow your culture sample. A specific diluent for protein standards dilution is not provided with this kit.

This assay cannot be used to measure cytokines from human sera. If you need to measure the 2 cytokines from human sera, you may purchase QBeads kits from Intellicyt which provides special diluent for human sera samples and protein standards dilution.

How to Make Sure Your Sample Cytokines are within the Linear Range of Standard Curves

This kit includes a template with standard curve layouts. ForeCyt uses 4PL with $1/Y^2$ weighting for fitting the standard curves. At the log scale, ForeCyt can provide the linear range for each standard curve.

Use a 1:3 serial titration with the top concentration at 50,000 pg/mL for each cytokine. If you need to modify the top concentration, dilution factor, and the plate layout for the standard, refer to the ForeCyt Reference Guide and make the adjustment in the Design tab. If a different culture media for the standard dilution is used it may have a slight impact on the standard curve and the linear range. For example, if you use RPMI1640 with 10% fetal bovine serum as fresh culture media for the standard dilution here is the expected linear range for each cytokine:

- IFN γ : 150-20,000 pg/mL
- TNF α : 200-50,000 pg/mL

NOTE: The detection range will be even wider than the linear range.

Generally, activated T cells secrete high levels of IFN γ and TNF α , depending on patient donor, cell density, cell health and cell proliferation. Since IFN γ and TNF α have a wide linear detection range, the assay workflow should not require any sample dilution.

Here are two exceptions:

1. If you anticipate samples of IFN γ or TNF α at a level higher than the linear range, dilute your sample with your culture media before running the assay. Increase the sip time (described below) to acquire enough cell events.
2. If you anticipate your samples will have very low level of IFN γ (< 150 pg/mL) and TNF α (<200 pg/mL), refer to [Appendix B](#) to modify the assay workflow in order to extend the linear range of IFN γ and TNF α at the low end.

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. Increase it to acquire enough cell events for your data analysis to reach statistical significance of your cell population of interest. Sip volume per second varies slightly from machine to machine and even from day-to-day. Generally, it is about 1.5 μL per second. If you decide to use a longer sip time than the default 4-second sip time in the template, you also need to adjust inter-well shaking in ForeCyt Protocol. Tables 1 and 2 may help you adjust the sip time if necessary, assuming the lowest cell density in the culture plate is 1 million/mL.

If you anticipate that you will not have enough cell events even by increasing the sip time, increase the cell number acquisition from the assay plate by concentrating cells in the original culture/treatment plate (see [Appendix C](#)) before transferring cell/supernatant mixture samples to the assay plate. Refer to Appendix C to improve the precision of cell density calculation by running daily iQue volumetric calibration. If you have a cell attachment issue, Appendix C also provides recommendations about using cell-repellent plates or ultra-low binding plates to avoid cell attachment to the well bottom/wall.

Table 1: Data Acquisition Adjustments for 96-well Format

Sip Time Per Well	Cell Density in the Culture Plate	Transfer Volume	Final Volume After Resuspension in Assay Plate	Estimated Cell Density in Assay Plate	Estimated Volume Acquired (Assume 1.5 μL /second sip/well)
4s Sip (default)	1 Million/ML (Assumption: the lowest possible density ~the seeding density)	10 μL (From Culture Plate to Assay Plate)	~25 μL (20 μL + residual volume)	0.3 Million/mL (Assume wash causes 20% cell loss)	6 μL
6s Sip					9 μL
8s Sip					12 μL
10s Sip					15 μL
12s Sip					18 μL

Sip Time Per Well	Estimated Cell Events Acquired Per Well	Inter-well Shake Frequency	Inter-well Shake Duration	Aquisition Time per Plate
4s Sip (default)	1800	Every 6 Wells	4 seconds	~15 mins
6s Sip	2700	Every 6 Wells	6 seconds	~18 mins
8s Sip	3600	Every 4 Wells	8 seconds	~22 mins
10s Sip	4500	Every 3 Wells	10 seconds	~28 mins
12s Sip	5400	Every 3 Wells	12 seconds	~32 mins

Table 2: Data Acquisition Adjustments for 384-well Format

Sip Time Per Well	Cell Density in the Culture Plate	Transfer Volume	Final Volume After Resuspension in Assay Plate	Estimated Cell Density in Assay Plate	Estimated Volume Acquired (Assume 1.5 μL /second sip/well)
4s Sip (default)	1 Million/ML (Assumption: the lowest possible density ~the seeding density)	5 μL (From Culture Plate to Assay Plate)	~15 μL (10 μL + residual volume)	0.3 Million/mL (Assume wash causes 20% cell loss)	6 μL
6s Sip					9 μL
8s Sip					12 μL

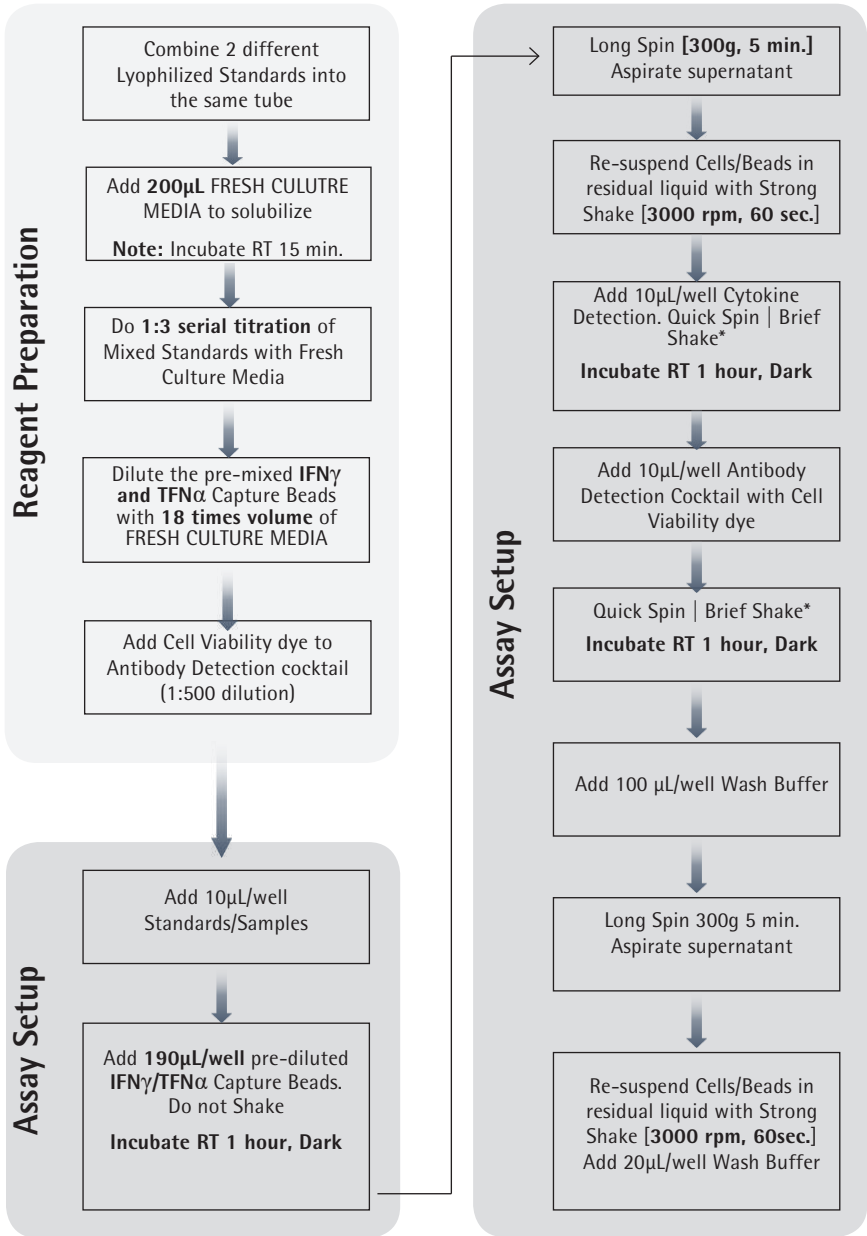
Sip Time Per Well	Estimated Cell Events Acquired Per Well	Inter-well Shake Frequency	Inter-well Shake Duration	Aquisition Time per Plate
4s Sip (default)	1800	Every 6 Wells	4 seconds	~40 mins
6s Sip	2700	Every 6 Wells	6 seconds	~60 mins
8s Sip	3600	Every 4 Wells	8 seconds	~80 mins

Cell Culture Preparation and Treatment

This assay is designed to detect T cell activation from cell culture. Before running the assay, you may need to prepare your immune cell culture with appropriate media and conditions. Low cell density may make it difficult to achieve statistical significance for the cell population of interest.

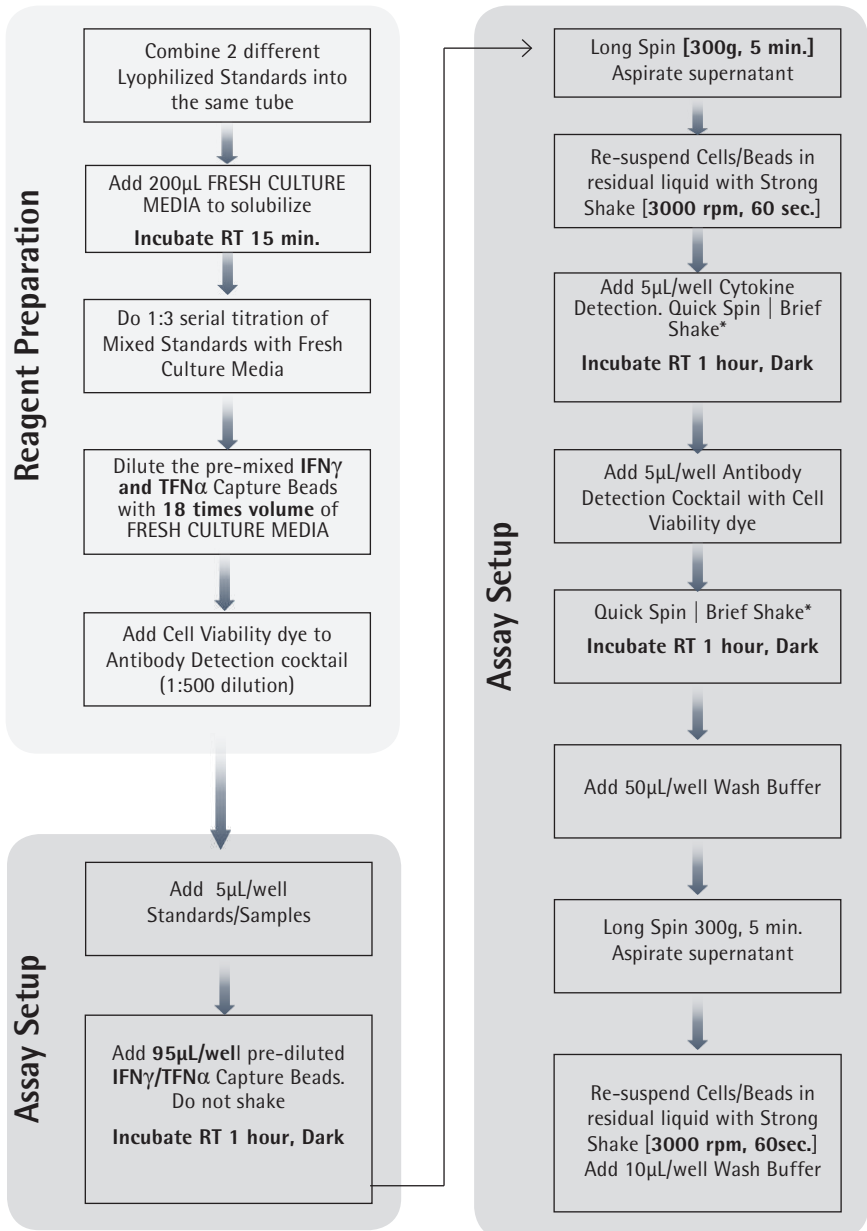
- You may include recombinant human IL-2 protein (10 ng/mL) with in the culture media to help maintain the T cell health/growth.
- Include negative and positive controls of T cell activation. You may use activation reagents such as CD3/CD28 DynaBeads or PHA or other appropriate reagents to stimulate T cell activation. The negative and positive controls will be useful to fine tune the gating of different activated T cell populations (See [Data Analysis](#) Section).
- If you plan to measure T cell proliferation, stain cells prior to cell culture using the Cell Proliferation and Encoding Dye (B/Green), provided in the kit. A detailed protocol for this optional cell staining procedure is found in [Appendix A](#).

Assay Protocol Overview (96-well Plate)



*Quick Spin = 300 g, 5 sec. -Brief Shake = 2000 rpm, 20 sec.

Assay Protocol Overview (384-well Plate)



*Quick Spin = 300 g, 5 sec. -Brief Shake = 2000 rpm, 20 sec.

Before You Begin

- Briefly centrifuge all vials before use to prevent reagent loss.
- Gently mix the dye with a pipette or briefly vortex prior to use.
- Vigorously vortex capture beads prior to use to ensure homogenous solution and consistent concentration in the assay. Beads tend to settle and aggregate over time. As with all things in life, mix them up occasionally. Ensure beads have not splashed onto the caps during this process.

The cell viability analysis of this assay is based on a different dye than traditional Trypan Blue-based viable cell measurement. The cell viability dye not only stains the necrotic cells but also the apoptotic cells. The viable cell number may be lower in this assay than that in a typical Trypan Blue-based assay.

Reagent Preparation

1. Combine and Solubilize the 2 Lyophilized Cytokine Standards (IFN γ and TNF α)

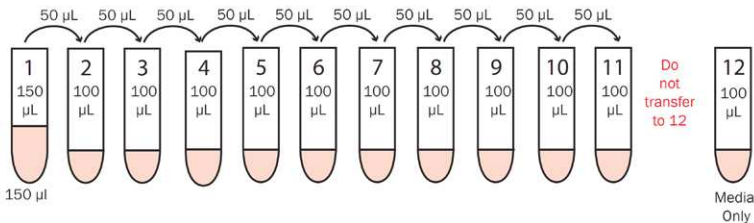
- There are 2 glass vials in the kit, one for each cytokine standard. Examine each vial to determine if the white sphere that is the lyophilized cytokine standard is at the bottom of the vial, or if it is stuck to the rubber lid (sometimes they hide up in there). If it is stuck to the lid, gently tap the glass vial against the workbench to force the sphere to fall to the bottom of the vial. Only use 1 glass vial of each cytokine for the following standard preparation on each assay day.
- In order to combine the 2 standards, slowly and very carefully, open the rubber lids of each of the 2 vials to prevent the lyophilized cytokine spheres from flying out of the vial due to the slight positive pressure inside the vial. Combine the 2 lyophilized cytokine standard spheres into one tube. Combine in a 1.5 mL centrifuge tube or 15 mL conical tube by carefully pouring the sphere from the vial to the tube. Visually confirm that each sphere fell to the tube bottom. If not, tap the tube against workbench to force the sphere to fall to the bottom of the tube.
- Add 200 μ L fresh culture media (the same media used to grow your sample culture) to the tube with the 2 lyophilized cytokine spheres.
- Do NOT mix. Let the spheres dissolve in the media for 15 minutes at room temperature.
- After 15 minutes. Gently mix the cytokine standards by manually pipetting up and down 5-6 times to prepare standards for serial dilution.

2. Prepare the 1:3 Serial Dilution of the 2 Cytokine Standards (IFN γ and TNF α)

The concentration for each cytokine in the combined cytokine solubilized mixture is 50,000 pg/mL. This will be the highest concentration in the following series of 1:3 serial dilutions.

- Prepare 12 microcentrifuge tubes (for 96-well plate assay format) or 16 micro-tubes (for 384-well plate assay format). Label them #1 –#12 (for 96-well plate assay) or #16 (for 384-well plate assay). You may also use an empty 96-well plate or 12-channel pipette reservoir instead of micro-tubes.
- To tubes # 2-12/16 add 100 μ L of FRESH CELL CULTURE MEDIA. This is the same media used to grow your sample culture. After you have added the cell culture media, set aside the tubes until you are ready to make the serial dilutions by adding the standard to the micro-tubes containing the media.
- To tube #1, add 150 μ L of the solubilized cytokine standard prepared in #2 above. It is the highest concentration standard (50,000 pg/mL for each cytokine) for the standard curve (top standard).
- From tube #1, remove 50 μ L of standard, and transfer to tube #2. Gently pipette up and down at least 6 times to completely mix the solution.
- From tube #2, transfer 50 μ L volume to tube #3 and mix by gently pipetting the mixture up and down. Continue transferring and mixing until you reach the next to the last tube. **Do not transfer any standard into tube #12 (for 96-well plate assay) or #16 (for 384-well plate assay).** Tube 12 or 16 will be the negative controls with zero cytokine standard.

96-well Plate Format



384-well Plate Format

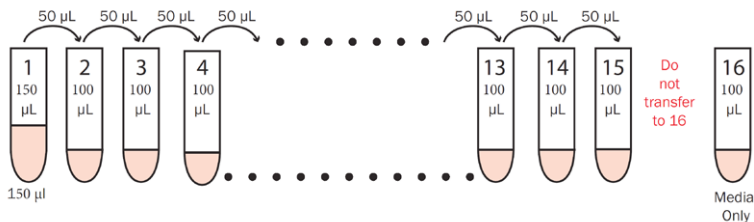


Figure 4. Preparation of Standard Curve. Dilute cytokine standards 1:3 in fresh tissue culture media. Use 12 samples (96-well) or 16 samples (384). Note that the last sample for both formats contains media alone.

About Standards

Figure 5 is an example of how to designate the cytokine standards on the plate in the Design tab of ForeCyt. Do NOT add the standard into the assay well until you are ready to begin the assay set up. When setting up your plate map in ForeCyt Design we recommend designating 1-2 rows of standard and arranging the standard wells from top to bottom and from low concentration to high concentration in 384 well plate; and, from left to right and from low concentration to high concentration in 96 well plate; and, from left to right and from low concentration to high concentration in 384 well plate. (A template with the standard design is already provided in the kit as shown in Figure 5 & 6.) You may change this configuration. Design -> Standards -> Edit Standard Set (Figure 6). Typical standard curves were also provided (Figure 7).

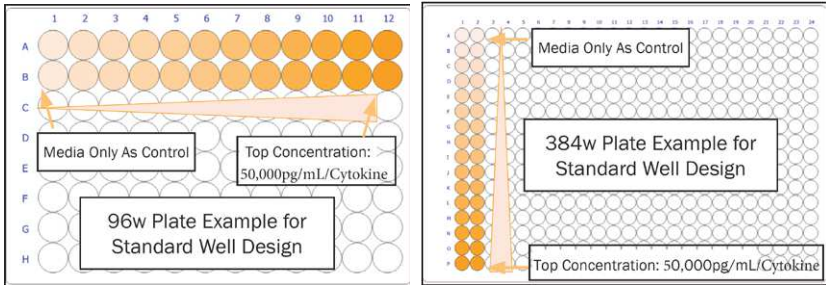


Figure 5. Arrange the standard wells from left to right (from low concentration to high concentration) in 96-well plate and from top to bottom (from low concentration to high concentration) in 384-well plate. This design is included in the assay template provided in the kit USB drive.

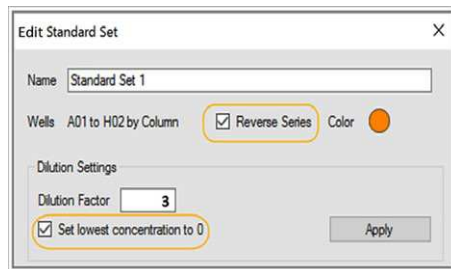


Figure 6. Reverse Series to achieve a left to right (from low concentration to high concentration) in 96-well plate and top to bottom and (from low concentration to high concentration) in 384-well plate. Check the Set lowest concentration to zero checkbox. The assay template includes the reverse series and also set the lowest concentration at 0. If you use a different direction of series or if you do not include the lowest concentration at 0 as specified in the assay template, you may edit the standard set.

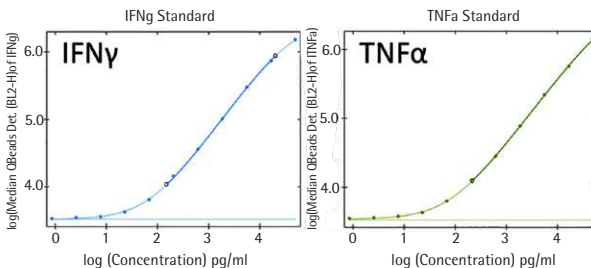


Figure 7. Representative standard curves (IFN γ and TNF α) with 1:3 serial dilutions. IFN γ linear range: 150-20,000 pg/mL; TNF α linear range: 200-50,000 pg/mL.

3. Dilute the Pre-mixed IFN γ /TNF α Capture Beads

The assay kit provides pre-mixed IFN γ /TNF α capture beads that require dilution with fresh culture media.

- Label a 50 mL conical tube, or a larger container such as bottle or reservoir, "Diluted Pre-mixed IFN γ /TNF α Capture Beads".
- Dilute the pre-mixed IFN γ /TNF α capture beads with a 18-fold volume of fresh culture media by transferring the precise volume to the appropriate container (See table below.)
- Vigorously vortex the container for 10 seconds. If you dilute the beads in a reservoir, you may use manual pipetting to mix the beads.

	Pre-Mixed IFNγ/TNFα Capture Beads	Fresh Culture Media (18-fold Volume)
1 x 96 wells	1.15 mL	20.7 mL
1 x 384 wells	2.3 mL	41.4 mL
5 x 384 wells	11.5 mL	207.0 mL

4. Add the Cell Membrane Integrity Dye (R/Red) into the Antibody Panel Cocktail

The assay kit provides a separate vial of Cell Membrane Integrity Dye (R/Red) at a 500x concentration to measure cell viability. Add the Cell Membrane Integrity Dye (R/Red) into the vial/bottle with the Antibody Panel Detection Cocktail right before the assay. Use the following table for dye amounts. After dye addition, mix the dye by manually pipetting the solution up and down 5 times.

	Cell Membrane Integrity Dye (R/Red) (500x)	Antibody Panel Detec- tion Cocktail (Volume in Original Vial/Bottle)
1 x 96 wells	2.5 μ L	1.25 mL
1 x 384 wells	5 μ L	2.5 mL
5 x 384 wells	25 μ L	12.5 mL

96-Well Plate Assay Set Up

In this one-wash assay you will use the serially diluted cytokine standards for quantitation of 2 cytokines (IFN γ and TNF α) in the sample. This will generate 2 standard curves to determine the cytokine concentration in the sample (Figure 7).

NOTE: For more accurate population gating and subsequent data analysis we strongly recommend using stimulation reagents such as CD3/CD28 DynaBeads or PHA or other appropriate reagents for positive and negative T cell stimulation in wells of the original culture plate.

Total Protocol Time: 3 hours

Total Hands-On Time: Approximately 30–60 minutes

1. Add Cell/Supernatant Mixture Sample and 2-plex Cytokine Standards

- Mix the cell/supernatant mixture in the original culture plate 10 times with a pipette to break up cell aggregates and to resuspend the cells. Be careful not to introduce bubbles in the samples. Transfer 10 μ L of cell/supernatant sample to each well of the assay plate you designated as Sample when you set up your plate in the ForeCyt Design tab.
- Transfer 10 μ L cytokine Standards prepared earlier to each well of the assay plate you designated for Standards in the ForeCyt Design tab.

2. Add the Pre-diluted Pre-mixed IFN γ /TNF α Capture Beads Prepared Earlier

- Vigorously vortex the pre-diluted pre-mixed IFN γ /TNF α capture beads prepared earlier. Transfer all the beads to a reservoir. Add 190 μ L of beads to each assay well. Agitate the beads occasionally during the transfer of the beads to the plate to prevent the beads from settling.

NOTE: During the liquid transfer, change the tip to avoid cross-well contamination.

- Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes. Do NOT shake the plate to prevent the liquid volume in assay wells from splashing into adjoining wells.

3. Spin/Aspiration/Agitation

- After incubation, spin the assay plate (300g, 5 minutes). Aspirate the supernatant with a plate washer, following the manufacturer's instructions. Agitate the sample in the residual liquid in the plate on the iQue plate shaker (3,000 rpm, 60 seconds).

4. Add Cytokine Detection Cocktail

- Transfer all the Cytokine Detection Cocktail to a reservoir. Add 10 μL /well cytokine detection cocktail to the assay plate.
- Do a quick spin of the plate (300g, 5 seconds) to ensure that all samples are at the well bottom and not attached to the well sides.
- Mix the plate for 20 seconds at 2,000 rpm using the iQue plate shaker to ensure thorough mixing.
- Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

5. Add Antibody Panel Detection Cocktail with Cell Viability Dye

- Transfer all the antibody panel detection cocktail with cell viability dye prepared earlier to a reservoir. Add 10 μL /well antibody panel detection cocktail with cell viability dye to the assay plate.
- Do a quick spin of the plate (300g, 5 seconds) to ensure that all samples are at the well bottom and not attached to the well sides.
- Mix the plate for 20 seconds at 2,000 rpm using the iQue plate shaker to ensure thorough mixing.
- Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

6. Wash/Resuspension

- Add 100 μL /well of wash buffer provided in the kit to the assay plate.
- Spin the assay plate (300g, 5 minutes). Aspirate the supernatant in the assay plate with a plate washer, following the manufacturer's instructions. Agitate the sample in the residual liquid in the well (3,000 rpm, 60 seconds) using the iQue plate shaker.
- Add 20 μL /well wash buffer. Do a quick spin of the plate (300g, 5 seconds) to ensure that all samples are at the well bottom and not attached to the well sides.
- The samples are now ready for acquisition on iQue Screener PLUS (VBR) platform.

384-Well Plate Assay Set Up

In this one-wash assay you will use the serially diluted cytokine standards for quantitation of 2 cytokines (IFN γ and TNF α) in the sample. This will generate 2 standard curves to determine the cytokine concentration in the sample (**Figure 7**).

NOTE: The following assay setup includes brief shaking in steps 4 and 5 (2,000 rpm for 20 seconds) and strong shaking in steps 3 and 6 (3,000 rpm for 60 seconds). **WARNING:** Make sure that the rpm for these shakes are correct to avoid cross-well contamination.

Total Protocol Time: 3 hours

Total Hands-On Time: Approximately 30-60 minutes

1. Add Cell/Supernatant Mixture Sample and 2-plex Cytokine Standards

- Mix the cell/supernatant mixture in the original culture plate 10 times with a pipette to break up cell aggregates and to resuspend the cells. Be careful not to introduce bubbles in the samples. Transfer 5 μ L of cell/supernatant sample to each well you designated as Sample in the assay plate when you set up your plate in the ForeCyt Design tab.
- Transfer 5 μ L cytokine Standards prepared earlier to each well designated for Standards in the assay plate.

2. Add the Pre-diluted Pre-mixed IFN γ /TNF α Capture Beads Prepared Earlier

- Vigorously vortex the pre-diluted pre-mixed IFN γ /TNF α capture beads prepared earlier. Transfer all the beads to a reservoir. Add 95 μ L of beads to each assay well in the assay plate. Agitate the beads occasionally during the transfer of the beads to the plate to prevent the beads from settling.
- Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes. Do NOT shake the plate to prevent the liquid volume in assay wells from splashing into adjoining wells.

NOTE: During the liquid transfer, change the tip to avoid cross-well contamination.

3. Spin/Aspiration/Agitation

- After incubation, spin the assay plate (300g, 5 minutes). Aspirate the supernatant with a plate washer, following the manufacturer's instructions. Agitate the sample in the residual liquid in the plate using the iQue plate shaker (3,000 rpm, 60 seconds).

4. Add Cytokine Detection Cocktail

- Add 5 μL /well cytokine detection cocktail provided in the kit to the assay plate.
- Do a quick spin of the plate (300g, 5 seconds) to ensure that all samples are at the well bottom and not attached to the well sides.
- Mix the plate for 20 seconds at 2,000 rpm using the iQue plate shaker to ensure thorough mixing.
- Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

5. Add Antibody Panel Detection Cocktail with Cell Viability Dye

- Transfer all the antibody panel detection cocktail with cell viability dye prepared earlier to a reservoir. Add 5 μL /well antibody panel detection cocktail with cell viability dye to the assay plate.
- Do a quick spin of the plate (300g, 5 seconds) to ensure that all samples are at the well bottom and not attached to the well sides.
- Mix the plate for 20 seconds at 2,000 rpm using the iQue plate shaker to ensure thorough mixing.
- Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

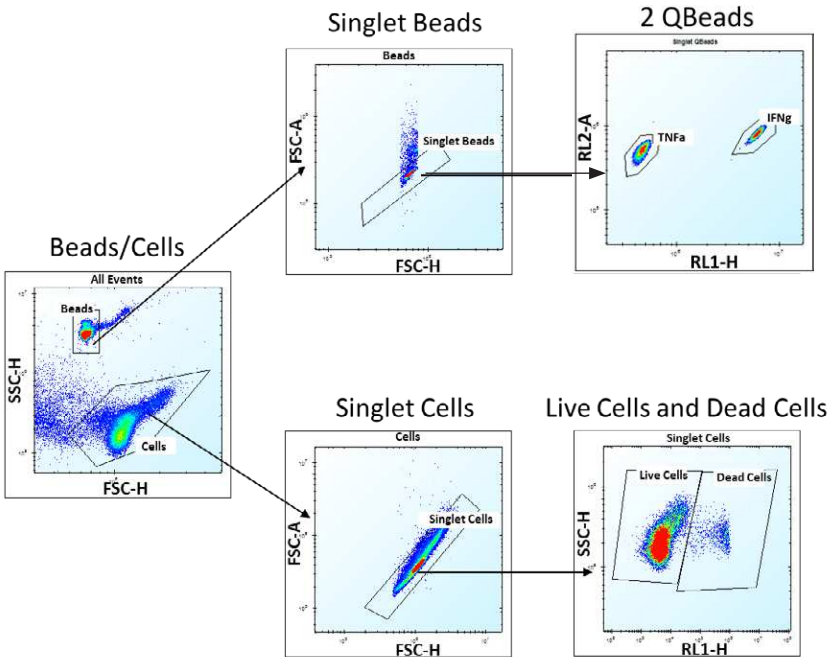
6. Wash/Resuspension

- Add 50 μL /well wash buffer provided in the kit to the assay plate.
- Spin the assay plate (300g, 5 minutes). Aspirate the supernatant with a plate washer, following the manufacturer's instructions. Agitate the sample in the residual liquid in the well (3,000 rpm, 60 seconds).
- Add 10 μL /well wash buffer to the assay plate. Do a quick spin of the plate (300g, 5 seconds) to ensure that all samples are at the well bottom and not attached to the well sides.
- The samples are now ready for acquisition on iQue Screener PLUS (VBR) platform.

Sample Acquisition and Data Analysis

1. Launch ForeCyt.
2. If you have not already imported the template provided by Intellicyt, import it now. Create a New Experiment using the template. Adjust the sip time if necessary to achieve the statistical significance for your cell population of interest. Define your T cell activation positive wells and negative wells in the Design tab in ForeCyt after importing the template. *This is important for later data analysis and for fine-tuning the gating of activated T cell populations.*
3. The template gates are pre-set for different populations. Here are the gating details if you want to manually draw the gates or fine tune the existing gates from the template:

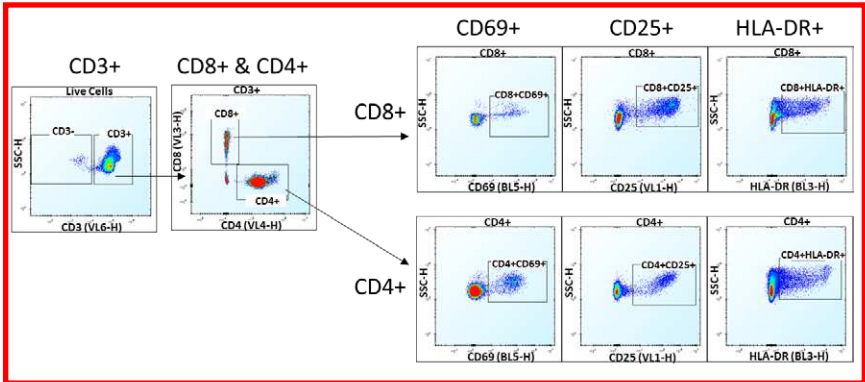
Gate Cells/Beads from All Events:



NOTE: If you have used CD3/CD28 DynaBeads in the culture, make sure not to include DynaBeads in "Cells" gate. DynaBeads are much smaller in FSC-H and in SSC-H plot than cells and beads used for cytokine detection.

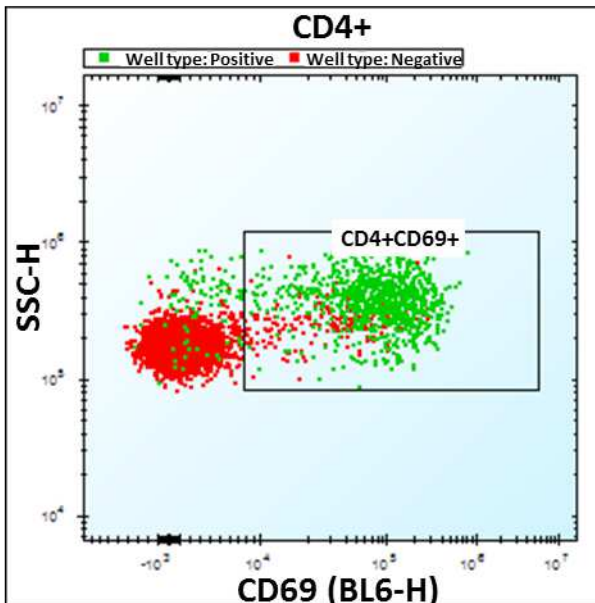
Gate Different Cell Phenotypes/Activated Cells from Live Cells:

(Manually adjust the linear range of bi-exponential scale in the dot plots, if necessary, to improve the separation of different populations)



Use the Positive/Negative Control Wells to Fine Tune the Gates of Activated Cells. (Example: Gate CD69+ Cells in CD4+ T Cells in an Overlay Plot with Positive Wells and Negative Wells)

NOTE: You have to assign positive and negative wells in the Design tab in ForeCyt.



Compensation Matrix

Primary Channel	Spillover Channel	Prolif. (Opt.) (BL1-H)	HLA-DR (BL3-H)	CD69 (BL5-H)	Cell Viability (RL1-H)	CD25 (VL1-H)	CD8 (VL3-H)	CD4 (VL4-H)	CD3 (VL6-H)
Prolif. (Opt.) (BL1-H)			6.23	0.02	0.00	0.00	2.51	0.82	0.00
HLA-DR (BL3-H)		0.24		1.80	0.16	0.00	0.82	15.43	0.20
CD69 (BL5-H)		0.40	1.47		0.08	0.01	0.31	0.22	13.56
Cell Viability (RL1-H)		0.08	0.08	0.01		0.02	0.07	0.08	0.01
CD25 (VL1-H)		0.00	0.00	0.00	0.00		4.88	1.95	0.02
CD8 (VL3-H)		0.45	10.13	0.11	0.00	4.72		97.80	1.28
CD4 (VL4-H)		0.01	5.58	0.15	0.10	1.07	5.93		3.01
CD3 (VL6-H)		0.02	0.01	3.41	0.15	3.15	0.35	0.38	

The template already includes the compensation matrix. It is not necessary to adjust any compensation matrix even if you use the optional Cell Proliferation and Encoding Dye (B/Green) in the assay.

Appendix A: Optional Proliferation and Encoder Dye Protocol

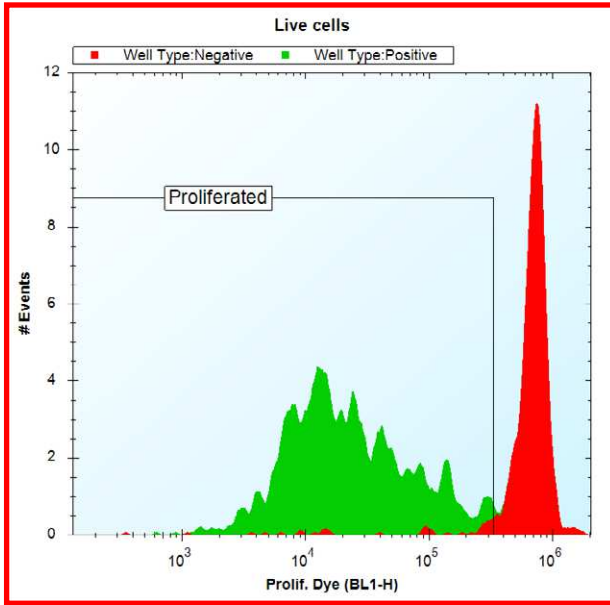
This following protocol uses the Cell Proliferation and Encoding Dye (B/Green) to multiplex T cell proliferation measurement into the assay. The assay template provided on the USB drive in the kit already includes the compensation matrix for the dye detection channel (BL1 in iQue Screener PLUS with VBR configuration). Below are instructions for using the optional proliferation/encoding dye to stain your T cells (for proliferation measurement) or to stain your target cells:

1. Before beginning, ensure the dye is completely thawed. If necessary, place the dye vial in a 37 degrees Celsius water bath for 5–10 minutes before use.
2. Prepare dye stock by diluting the Proliferation and Encoder dye provided in the kit into HBSS buffer or PBS buffer (**dilution factor 1:1250**). The HBSS or PBS buffer must be protein-free. Select one buffer and use it consistently across the assay protocol when it is required.
3. Collect your T cells or target cells in a 50 mL conical tube. Spin cells down (500g, 5 minutes) and remove the original culture media.
4. Re-suspend cells in 20 mL protein-free HBSS or PBS. Spin cells down (500g, 5 minutes). Remove the supernatant. Re-suspend cells in protein-free HBSS or PBS to 1–4 million/mL.
5. Combine an equal volume of the prepared cells and the prepared dye stock. The final dye concentration in the staining tube will be 1:2500 diluted. Mix gently and incubate cells at room temperature for 15 minutes, cover the plate to prevent evaporation and protect from light.

TIP: Thoroughly mix the cell/dye mixture carefully. Avoid cells or dye droplets attaching to the inner wall of the tube which may result in inconsistent cell staining.

6. After staining, wash by adding at least 2x volume of complete culture media (with 10% serum) to the staining sample. Spin (500g, 5 minutes). Remove the supernatant. Resuspend cells manually in the residual liquid.
7. Repeat **Step 6** two more times.

8. After the final wash, carefully re-suspend cells to the desired cell density for your culture/assay. For T cell proliferation positive control, you may need to add T cell activation reagents (such as CD3/CD28 DynaBeads (1:1 cell/bead ratio) or PHA (10 $\mu\text{g}/\text{m}$) to stimulate the T cells. The following example illustrates gating the proliferated cells acquired by iQue Screener PLUS (VBR) after 5-day culture. No compensation adjustment is required.



Appendix B: Modifying Assay Workflow to Handle Samples with Very Low Levels of IFN γ and TNF α

If you expect your samples will have very low levels of both IFN γ (< 150 pg/mL) and TNF α (<200 pg/mL), you may make adjustments in the assay workflow by simplifying the protocol to extend the linear range of IFN γ and TNF α at the low end.

Recommendations are below:

1. Eliminate Reagent Preparation: Step 3: The original assay protocol specifies diluting the pre-mixed IFN γ /TNF α capture beads with 18 times volume of fresh culture media. You should bypass this dilution step. Do **NOT** dilute the pre-mixed IFN γ /TNF α capture beads provided in the kit.
2. **Consolidate Plate Assay Setup Steps 1–3 as shown below:**
 - A. For 96-well assay format:
 - 1) Add 10 μ L/well Standards/Samples.
 - 2) Add 10 μ L/well of the pre-mixed IFN γ /TNF α capture beads to the assay plate. Quick spin (300g, 5 seconds). Brief shake (2,000rpm, 20 seconds). Incubate at room temperature for 1 hour with lid on and without light.
 - 3) Add 180 μ L/well fresh culture media to each well. Spin the plate (300g, 5 minutes). Aspirate the supernatant. Re-suspend cell/beads in the residual liquid in the assay plate by strong shake (3,000rpm, 60 seconds).
 - 4) Continue the assay by following the 96-well Plate Assay Setup Steps 4–6.
 - B. For 384-well assay format:
 - 1) Add 5 μ L /well Standards/Samples.
 - 2) Add 5 μ L/well the pre-mixed IFN γ /TNF α capture beads in the assay plate. Quick spin (300g, 5 seconds). Brief shake (2,000rpm, 20 seconds). Incubate at room temperature for 1 hour with lid on and without light.
 - 3) Add 90 μ L /well fresh culture media to each well. Spin the plate (300g, 5 minutes). Aspirate the supernatant. Re-suspend cell/beads in the residual liquid in the assay plate by strong shake (3,000 rpm, 60 seconds).
 - 4) Continue the assay by following the 384-well Plate Assay Setup Steps 4–6.

Appendix C: Options for Improving Cell Event Acquisition

Option 1: Concentrate Your Cell Samples in the Original Culture Plate if Cell Density Is Low

If after increasing the sip time in Sampling Protocol you still did not get enough cells during sample acquisition, follow the steps below before running your next assay:

- Spin your cells down (300 g, 5 minutes) in your original T cell culture plate.
- Remove half or two thirds the volume of supernatant to double or triple cell density in the culture well. Then, re-suspend your cells in the original culture plate in the remaining supernatant by manually pipetting the sample up and down (5-6 times).
- Transfer the concentrated cell samples into the assay plate before running the assay.

Option 2: Run Daily Volumetric Calibration to Get More Precise Cell Density Data

The Intellicyt iQue Screener PLUS cannot directly perform absolute cell counting due to the slight variation of acquisition volume. Variations may be a result of tubing change, tubing wear, and system cleanliness. The acquisition volume may also vary machine-to-machine and day-to-day. If you require very precise cell density information, we recommend running a daily volumetric calibration on your iQue Screener PLUS using absolute counting beads such as SPHERO™ AccuCount Particles (Spherotech, Cat#ACBP-50-10) to do the volumetric measurement on the iQue Screener PLUS. This bead has an absolute count per volume unit. Follow the Spherotech protocol to mix and transfer the beads to a testing plate. We recommend running the exact same sampling protocol with the same sip time, the same plate type, and the same volume in the well as used in this Human T Cell Activation Cell and Cytokine Profiling Kit. You may only need to run 3-4 wells of AccuCount beads at the beginning of your assay to measure the sip volume on your iQue Screener PLUS. Use this volume measurement on the same day of the experiment in which you wish to calculate the cell density. Adjust your final calculation by considering the sip time (in ForeCyt Protocol) and the dilution factor of your sample in the final assay reaction volume.

ForeCyt Advanced Metrics, Custom Function (Metrics -> Add -> Add Advanced Metric -> Custom) can automate the calculation much like entering a calculation into a spreadsheet. If you are not familiar with how to use the custom function, click ForeCyt -> Help -> Reference Guide for more information or call customer support.

Option 3: Use Cell-repellent or Ultra-low Binding Plates to Prevent Cell Attachment

T cells usually will not attach to the well bottom/wall of the Intellicyt-recommended plates (96w plate, Costar, Cat#3897; 384w plate, Greiner, Cat#781280). However, under some user defined biological conditions, some or all of your sample cells may partially attach to the assay well bottom/wall resulting in inconsistent cell count. For these situations we recommend using the Greiner cell-repellent plate (e.g., Cat#651970, Cat#781970) or Corning ultra-low binding plate (e.g., Cat#7007, Cat#4516) in order to achieve a more precise cell counting on the iQue Screener PLUS. If ForeCyt does not list this plate model, add it to the list (Device → Manage Plate Models → Add) and map it.

Appendix D: Mixing Samples with the Intellicyt Shaker

iQue Screener PLUS (VBR)

Plate Type	Well Volume	MAX RPM
96-Well	20–40 μ L	2600
96-Well	40–60 μ L	2200
96-Well	60+ μ L	A/O*
384-Well	10–30 μ L	3000
384-Well	30–50 μ L	2800
384-Well	50+ μ L	A/O*

*A/O = Additional Optimization necessary. While it is possible to run these volumes, they were not routinely tested by the assay development team. To determine ideal shake speeds for high volume assays, Intellicyt recommends starting at low RPM values and slowly increasing to higher RPM values.

Appendix E: Plate-type Recommendations and Automated Wash Protocols for Microplates

The following plate types and aspiration settings have been extensively tested with Intellicyt assay products.

Plate Type	Well Type	Manufacturer	Manufacturer Product
384-well	V-bottom	Greiner	781280
96-well	V-bottom	Costar	3897

When using the above plate types, the following aspiration programs have been tested on a BioTek ELx405 Select.

Intellicyt recommends that wash protocols use an automated plate washer. Manual aspiration of plates and/or plate inversion techniques could result in severe sample loss.

Plate Type	Height Setting	Height Offset	Rate Setting	Aspiration Rate
384-well, V-bottom	#31	3.937 mm	#6	15 mm/sec
96-well, V-bottom	#40	5.08 mm	#6	15 mm/sec

Appendix F: Liquid Handler Recommendations

Intellicyt recommends the following liquid handlers:

12-channel pipette for manual transfer of liquid to the plate:

- Manual 12-channel pipette, Tacta, 5-120 μ L (Sartorius);
- Manual 12-channel pipette, Tacta, 30-300 μ L (Sartorius);
- Electronic 12-channel pipette, Picus, 5-120 μ L (Sartorius);
- Electronic 12-channel pipette, Picus, 10-300 μ L (Sartorius).

Single-channel pipette for reagent preparation:

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

Automated liquid handler for dispensing, if necessary:

- Personal Pipettor, 96 or 384 channels (Apricot Designs);
- MicroFlow Select, 8 channels (BioTek).

Appendix G: FAQ

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

Answer: It is possible to do that in ForeCyt software. However, there is potential day-to-day variation that may affect your cytokine quantitation if you use standard curves acquired on different days. We recommend running standard curves test on each assay day and applying the standard curves to experiment run on the same day. You may include the standards in each of your assay plates, or you may run a stand-alone standard plate and then apply standard curves to the assay plates run on the same day for cytokine quantitation. If you have in-plate standards, the cytokine quantitation is automatic with the assay template. If you run a stand-alone standard plate, you can share the fit (the standard curve) before you do the cytokine calculation on your assay plate by either "copy analysis" or by using the advanced metrics in the assay plate. If you don't know how to use this feature, refer to the ForeCyt Reference Guide for information on the Share Fit feature, or contact Intellicyt customer support.

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

Answer: It is possible to fix the cell samples with fixatives such as 1% PFA. However, you may need to validate it against your biology. Don't use methanol to fix the sample as it affects bead-based cytokine detection. In contrast, 1% PFA fixation does not affect bead-based cytokine measurement. Fixation and further wash steps may cause cell loss and affect the final event acquisition of your assay. So we recommend additional optimization. If you see significant cell loss, you may transfer your samples out of your original assay plate, and perform the fixation in a new plate. A cell-repellent plate (Greiner, Cat#651970, and Cat #781970) may reduce cell loss due to fixation or fixation-related cell cross-link to the well bottom.

Q3: Do I need to dilute my samples for the assay if my samples have high level of cytokines?

Answer: The assay is built to measure relatively high level of IFN γ (as high as 20,000 pg/mL) and TNF α (as high as 50,000 pg/mL) without sample dilution. In addition, there is 1 alternative workflow that don't require sample dilution (see [Appendix B](#)) and can measure low level of IFN γ and TNF α (IFN γ < 150 pg/mL, TNF α < 200 pg/mL). If your samples have the cytokines higher than the expected linear range, consider diluting your samples. After you dilute your samples, you may need to adjust the sip time in Protocol in ForeCyt to acquire enough cells for data analysis.

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

Answer: Increase the sip time in the Protocol in ForeCyt to acquire more cell events. The default sip time in the assay template is 4 seconds. You may increase the sip time to acquire more cells (See the table/recommendation in Section- [Best Practices and Tips](#)).

If you estimate that you still can't get enough cell events, even by increasing the sip time, follow the recommendation in [Appendix C](#) to concentrate your cell samples in a future assay.

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

Answer: Yes. A 1x 384-well kit can be used for 2 assay plates in a 96-well format. The assay kit, regardless of format, provides a USB drive with assay templates for both 384-well format and 96-well format. Choose the appropriate assay format for your need. 1x 96-well kit provides 1 vial of standard protein for each cytokine. 1x 384-well kit provides 1 vials of standard protein for each cytokine. Additional standards are also available for purchase.

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

Answer: We recommend not multiplexing this assay with other cellular or cytokine endpoints. However, it is possible to use the optional proliferation/encoder dye provided in the kit to multiplex the T cell proliferation or the target cell measurement. The compensation metrics included in the assay template already considered this multiplex measurement. No additional compensation adjustment is needed.

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

Answer: Generally, if you have very few beads and cells from sample acquisition, you can always increase the sip time and re-read the plate on iQue Screener PLUS. Usually you will get more than 50 beads for each bead population in each well. If you see very few beads per bead population per well, there may be several causes: You did not agitate the original beads vial very well. The beads were not mixed in reservoir during the transfer. The sample was not agitated in the residual buffer in the assay plate after spinning/aspiration and the beads settled to the well bottom. The beads were washed away during the aspiration of the supernatant from the assay plate after spinning.

If you get significantly fewer cells than you expected, there are several causes: Biology affected cell growth/viability. The cells were not mixed before transferring cell/supernatant sample from the culture plate to the assay plate. The sample was not agitated in the residual buffer in the assay plate after spinning/aspiration and cells settled at the well bottom. Cells were washed away during the aspiration of the supernatant after spinning.

Q8: The CD4 and CD8 signal in the dot plot show as smears rather than discreet populations. Can I adjust the plot and the gating?

Answer: The assay template included a phenotype 2D plot with CD4 at the X-axis and CD8 at the Y-axis. The plot is biexponential scale. You may need to adjust the linear range of the biexponential scale manually by increasing or decreasing 2-10 times of the linear range value. Choose the one that can get the visualization of signal separation of CD4+ and CD8+ cells. Then, you could adjust the gating to include the corresponding T cell subpopulations. As a reminder, T cell activation may affect the donor-dependent expression of CD4 and CD8 proteins. This is why you might sometimes see some smears of cell populations such as CD8+ cells. You may create a plate view to visualize the well-based 2D plot to see more details in CD4/CD8 signal separation.

For Technical Support Contact Us at:

Email: support.intellicyt@sartorius.com
Telephone: 505.345.9075, Option 2

ABOUT THE COVER:

Native to the Desert Southwest, Desert Globemallow (*Sphaeralcea ambigua*) is highly drought-resistant perennial that often grows in large clumps, reaching a height of between 20 and 40 inches. It favors sandy washes and rocky hillsides and can be important food and cover for desert tortoises. Hummingbirds adore it. Desert Globemallow blooms sporadically from spring to autumn with spectacular bursts of flowering after rain. Medicinal properties are said to include toning the immune system, healing soft tissue injuries and reducing inflammation.

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