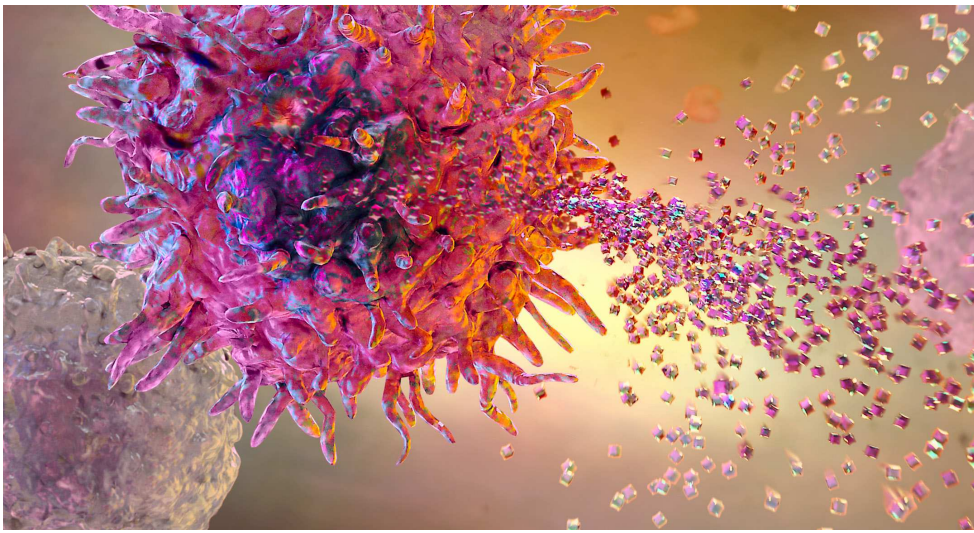
Human T Cell Memory Cell and Cytokine Profiling Kit

Cat No. 97035 1 x 96-well format
Cat No. 97036 5 x 96-well format
Cat No. 97037 1 x 384-well format
Cat No. 97038 5 x 384-well format



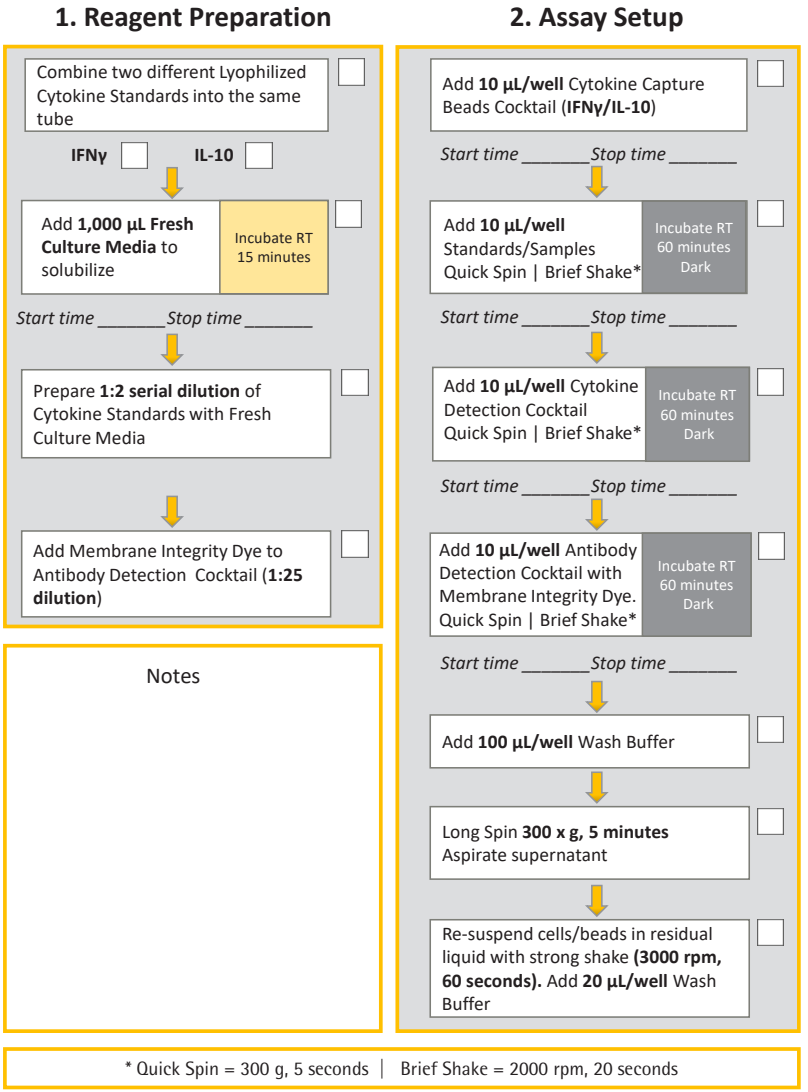
Open immediately upon arrival and store reagents at temperatures stated on labels. For research use only.

Publication No. 17022 Rev A.

Table of Contents

Quick Guide (1 x 96-well format)

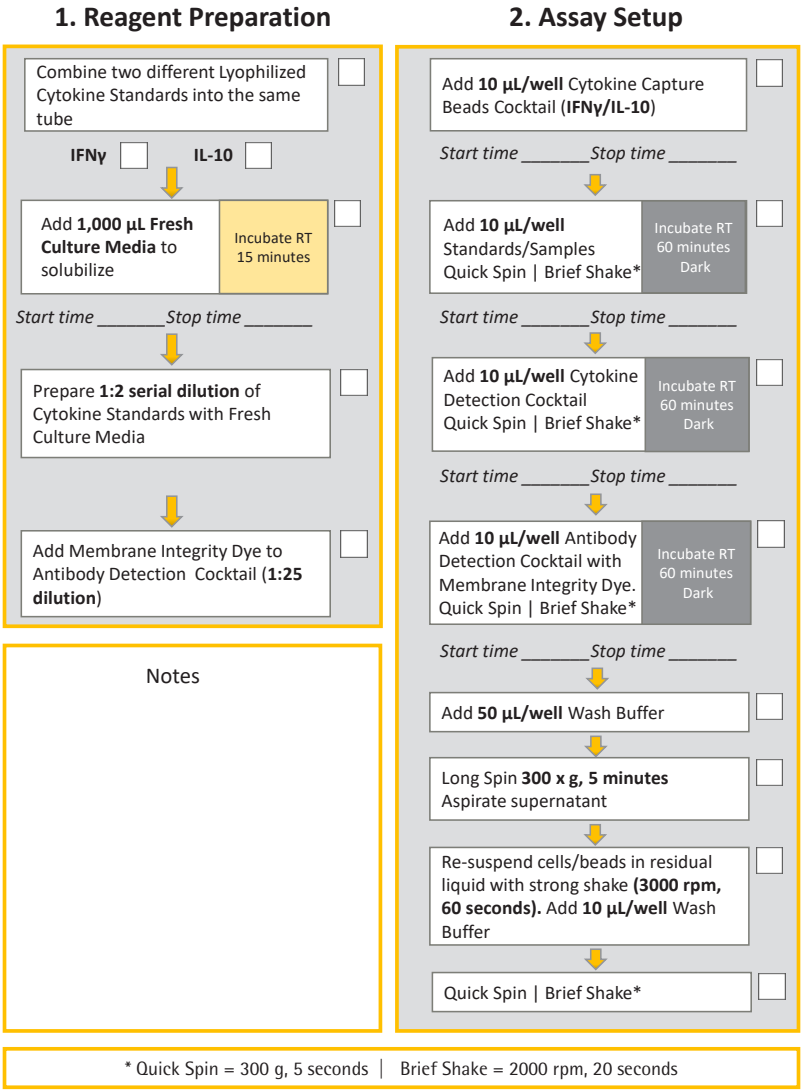
This overview summarizes the protocol. Detailed instructions are provided in the Assay Protocol section.



NOTE: For first time assay users, refer to Assay Protocol for detailed step by step procedures. The Quick Assay Guide is a tool you can utilize once familiar with the protocol.

Quick Guide (1 x 384-well format)

This overview summarizes the protocol. Detailed instructions are provided in the Assay Protocol section.



NOTE: For first time assay users, refer to Assay Protocol for detailed step by step procedures. The Quick Assay Guide is a tool you can utilize once familiar with the protocol.

Introduction

The Human T Cell Memory Cell and Cytokine Profiling Kit was designed for ease of use in multiplexing cellular information and bead-based, secreted cytokine measurements 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 format.** This assay format disrupts common immunology research workflows which generally require multiple assays, and is optimized for use on the Intellicyt iQue3 and iQue Screener Plus equipped with Violet, Blue and Red lasers.

- **Single platform and data analysis package** streamline data acquisition, analysis workflow, and solve data synchronization issues.

- **Spatial-temporal analysis of T memory cell phenotypes and functions** at different stages in a single, high content assay with a total of 11 endpoints: T naive(T_N), T stem cell-like memory (T_{SCM}), T central memory (T_{CM}), T transitional memory (T_{TM}), T effector memory (T_{EM}), T effector memory re-expressing CD45RA (T_{EMRA}), T terminal effector (T_{TE}), cell viability, cell count, secreted pro-inflammatory cytokine IFNγ, and anti-inflammatory cytokine IL-10 (see **Figure 1** for more detail).

- **Simplified 'plug-and-play' assay workflow with no additional color compensation.** Pre-mixed reagents for CD antibody staining and for cytokine detection make it possible to run the assay in a 'plug-and-play' format. Total assay time is approximately 3 hours, with a hands-on time of about 30 minutes. An included template with pre-set compensation matrices enables data acquisition of the multiplexed, phenotyping assay without the need for single stain color compensation.

- **Flexibility to choose additional cytokine measurements from a validated list of Human T Cell Companion Kits.** This assay kit comes with QBeads® for 2 different cytokine measurements: IFNγ and IL-10. However, the user may choose to multiplex the assay and measure up to 6 additional cytokines from Human T Cell Companion Kits including IL-2, IL-6, IL-13, IL-17A, GM-CSF, and TNF (sold separately, see **Appendix A**).

Assay Principles

The Human T Cell Memory Cell and Cytokine Profiling Kit is a cell and bead mixture assay that simultaneously measures these markers:

- T cell phenotype markers: CD3, CD4, and CD8
- T naive/memory/effector cell phenotype markers: CD45RA, CD45RO, CD27, CD62L, and CD95
- Secreted pro-inflammatory cytokine IFNγ and anti-inflammatory cytokine IL-10
- 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. Live cells are immunophenotyped by staining with a fluorescent antibody panel to separate CD3+ T cells, CD3- non-T cells, CD4+ T helper cells, and CD8+ T cytotoxic cells. The panel also includes 5 different T cell surface markers for T naive/memory/effector cell phenotyping: CD45RA, CD45RO, CD27, CD62L, and CD95. Of note, CD27 is a long term survival marker. Effector cytokines secreted by activated T cells, including pro-inflammatory cytokine IFNγ, and anti-inflammatory cytokine IL-10, are measured in a "sandwich" immune assay format using 2 different QBeads included in the same well.

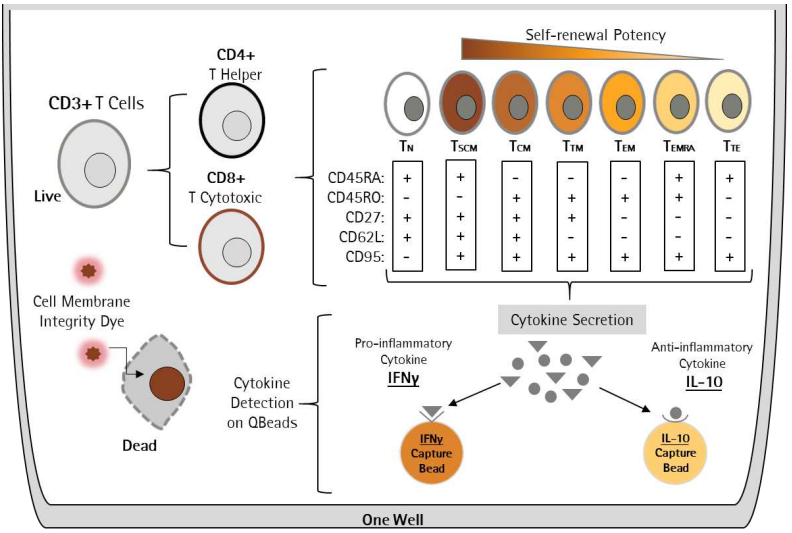


Figure 1. Illustration of the Human T Cell Memory Cell and Cytokine Profiling Kit assay principles. Live cells are separated from dead cells based on the staining of a cell permeable DNA intercalation dye. Basic T cell phenotypes are measured by staining with CD3, CD4, and CD8 markers. T cell subsets (T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, T_{EMRA}, and T_{TE}) at different stages of differentiation after activation, are measured by staining with CD45RA, CD45RO, CD27, CD62L, and CD95 markers. Secreted pro-inflammatory cytokine IFNγ and anti-inflammatory cytokine IL-10 are also quantified by QBeads in a sandwich immune assay format in the same assay well.

Assay Workflow Overview

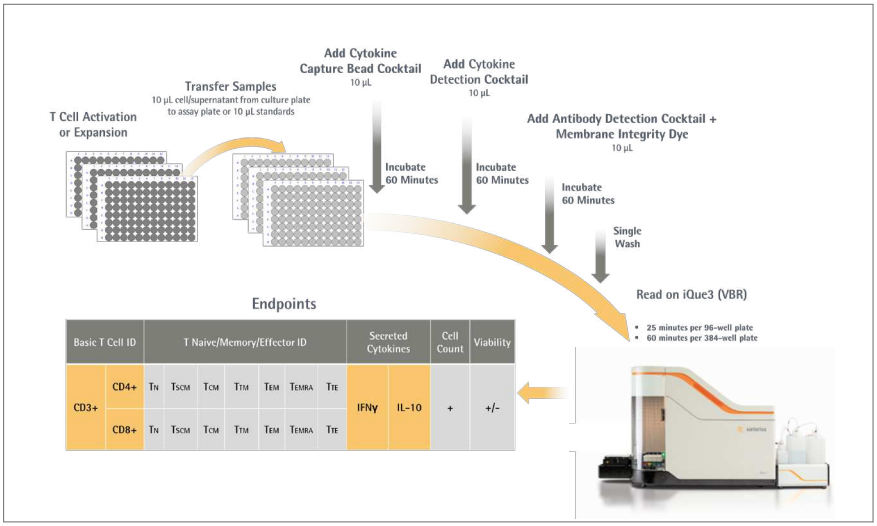


Figure 2. Assay Workflow. Miniaturized, multiplexed assay and templated analysis in either 96-well or 384-well format allows the user to get answers faster. Identification of T cells, basic T cell subtypes, T cell subtypes at different differentiation stages including T naive/memory/effector cells, and 2 secreted cytokines including inflammatory cytokine IFNγ and anti-inflammatory cytokine IL-10 are all included in the final readouts. In the table, "+" means a certain number of cells, and "+/-" means cell viability range between 0 -100%.

Reagents Provided

Components	Quantity Provided
Human Cytokine Capture Beads Cocktail (IFNγ and IL-10)	1 bottle
Human IFNγ and IL-10 2 separate standards	1 vial for each cytokine
Cytokine Detection Cocktail	1 bottle
Antibody Detection Cocktail (pre-mixed: 8 fluorescent antibodies)	1 bottle
Cell Membrane Integrity Dye (B/Red, viability)	1 vial
Wash Buffer	1 bottle

NOTE: The reagents provided will be enough for specific kit size (# of plates) and allow for minimal overage. A kit manual and a USB key with assay templates are also included in the kit package.

Storage and Stability

Store the Human T Cell Memory Cell and Cytokine Profiling Kit at 2-8° C. Product is stable for minimum of 6 months from date of purchase.

iQue3/ iQue Screener PLUS (VBR) Detector Channels

Detector	Spectrum	Violet Laser (405 nm)		Blue Laser (488 nm)		Red Laser (640 nm)	
445/45 nm		VL1	CD45RO (V/Blue)				
530/30 nm		VL2		BL1	CD62L (B/Green)		
572/28 nm		VL3	CD8 (V/Yellow)	BL2	Qbeads Detection		
615/24 nm		VL4	CD4 (V/Orange)	BL3			
675/30 nm		VL5		BL4	Cell Membrane Integrity Dye (B/Red)	RL1	CD95 (R/Red)
780/60 nm		VL6	CD3 (V/Crimson)	BL5	CD45RA (B/Crimson)	RL2	CD27 (R/Crimson)

NOTE: This assay is only compatible with theiQue3 and iQue Screener PLUS equipped with Violet, Blue and Red lasers. Other iQue platforms including iQue Screener, iQue Screener PLUS (VYB lasers), iQue Screener PLUS (BR lasers), iQue3 (VYB lasers), and iQue3 (BR lasers) will NOT work with this assay due to detection channel limitations.

RL1 and RL2 channel: Classification of QBeads (IFNγ and IL-10). In addition, RL1 is used for CD95 detection and RL2 is used for CD27 detection.

Materials Required But Not Provided

- Intellicyt iQue3 or iQue Screener PLUS equipped with Violet, Blue and Red lasers
- Centrifuge capable of spinning microcentrifuge tubes and/or 15 mL conical tubes at up to 8,000 g
- Centrifuge capable of spinning microplates
- Vortex mixer
- Fresh complete cell culture media (same media used to grow sample cell culture)
- Microcentrifuge tubes and/or 15 mL conical tubes
- 96-well v-bottom (Intellicyt, Cat. 90151) or 384-well v-bottom (Greiner, Cat. 781280) plates
- Reagent reservoirs (e.g. VWR, Cat. 89094-680)
- Black plate lid (e.g. Corning, Cat. 3935) or foil to protect from light/evaporation
- 12-channel pipette reservoir (e.g. VWR, Cat. 80092-466) (optional for preparing serial titrations)
- Appropriate liquid handler or multi-channel pipette (see **Appendix E**)
- Plate washer (such as BioTek model ELx405)
- Stimulation or expansion reagents such as CD3/CD28 Dynabeads®.

Sample Preparation

- This assay is designed to detect T naive/memory/effector cells in cell culture. Before running the assay, you may need to prepare your immune cell culture with appropriate culture media and culture conditions, including initial cell density. If the assay cell density is too low, it may be difficult to achieve statistical significance in your cell population of interest.
- The assay is validated in X-VIVO™ 15 medium with 5% human AB serum, RPMI 1640 medium with 10% fetal bovine serum, CTS™ OpTmizer™ T cell expansion medium (serum-free), and ImmunoCult™-XF T cell expansion medium (serum free). Other similar culture media may also work in this assay.
- If necessary, you may include recombinant human cytokines such as IL-4 and IL-7, and/or other cytokine cocktails with biological activity in the culture media to help maintain or promote the T memory cell frequency and the T cell health/growth.

It is strongly recommended to include negative and positive controls. You may use stimulation reagents such as CD3/CD28 Dynabeads or other appropriate reagents to stimulate or expand T cells.

Before You Begin

Reagents:

- 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.

Software:

- The assay plate design can be found in the Design tab of ForeCyt, and in the template provided (USB key in kit package). Become familiarized with the assay plate design.
- This assay uses serially diluted cytokine standards to generate 2 standard curves for quantitation of IFNγ and IL-10 in the sample.
- A template with the standard design is provided in the kit as shown in **Figure 3**. Cytokine standard placement is recommended on the Design tab. When setting up the plate map in ForeCyt Design, designate rows 1-2 for standards. Arrange the standard wells from low to high concentration, from left to right for a 96-well plate, or from top to bottom for a 384-well plate.
- To change this configuration: Design ➔ Standards ➔ Edit Standard Set (**Figure 4**).
- Representative standard curves are shown in **Figure 5**.

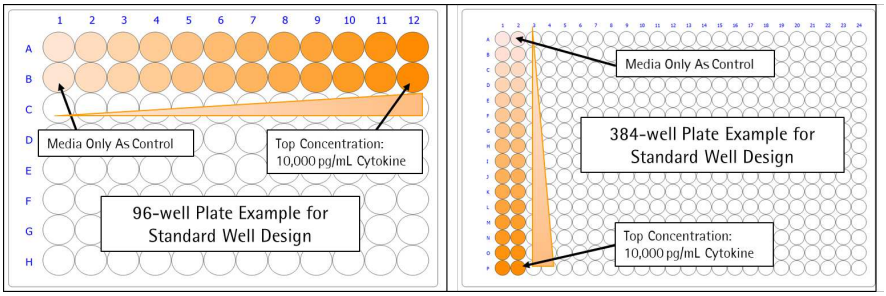


Figure 3. Standard Curve Set Up. Arrange the standard wells from left to right (from low concentration to high concentration) in 96-well format and from top to bottom (from low concentration to high concentration) in 384-well format. This design is already adopted in the assay template on the provided USB key.

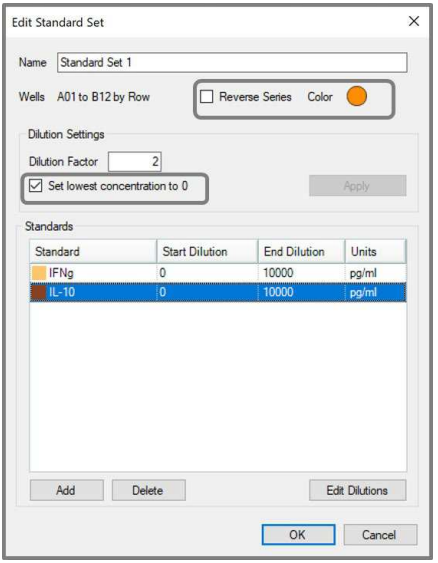


Figure 4. Edit Standard Set. In ForeCyt versions prior to 7.1, to achieve a left to right (from low concentration to high concentration) in 96-well format and top to bottom and (from low concentration to high concentration) in 384-well format, the **Reverse Series** checkbox must be selected along with the **Set lowest concentration to 0** checkbox. The provided assay templates are preset to have the standards in the low to high configuration with the lowest concentration set to zero. If a different standard orientation, or your lowest concentration is not zero as adopted in the assay template, you may edit the standard set as necessary.

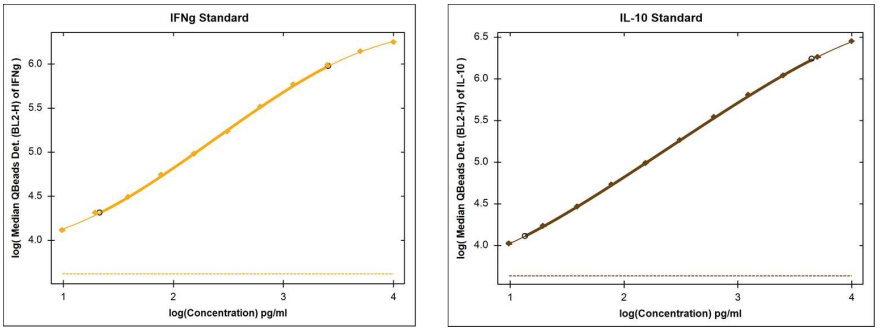


Figure 5. Representative standard curves (IFNγ and IL-10) with 1:2 serial dilutions. The linear range for IFNγ is 10-2,100 pg/mL, and 10-6,400 pg/mL for IL-10. The bold line indicates the linear range in each graph, with the detection range wider than the linear range. The dashed line represents the fluorescent background when the standard concentration is zero.

Preparation of Reagents

1. Prepare the Cytokine Standards (Human IFNγ and IL-10)

- Combine the two lyophilized cytokine standard spheres by carefully pouring them into one tube (1.5 mL centrifuge tube or 15 mL conical tube). **Only use 1 glass vial of each cytokine for the following standard preparation on each assay day.**
- Add 1,000 µL fresh culture media (the same media used to grow your sample culture) to the tube with the 2 lyophilized standard spheres. Allow the cytokine standards to dissolve in the media for 15 minutes at room temperature. Do not mix.
- Gently mix the cytokine standards by manually pipetting 5-6 times to prepare standards for serial dilution.

2. Prepare 1:2 Serial Dilution of the Cytokine Standards

Each solubilized cytokine standard is at a concentration of 10,000 pg/mL. This will be used as the top concentration in the following 1:2 serial dilutions.

> **NOTE:** Only prepare cytokine standards to be used on the day of assay.

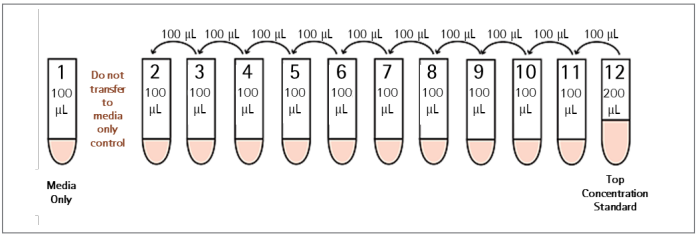
This guideline can be followed directly, or optimized for your laboratory (**Figure 6**).

- Prepare 12 tubes (96-well format) or 16 tubes (384-well format) and label #1 –12 (or 16).
- To tubes # 1-11 (or 15), add 100 µL of fresh culture media (the same media used to grow your sample culture).
- To tubes # 12 (or 16), add 200 µL of the solubilized cytokine standard prepared in step 1.

> **NOTE:** This is the top concentration (10,000 pg/mL) for each of the standard curves.

- From tube #12 (or 16), transfer 100µL of standard to tube #11 (or 15). Pipette up and down to gently mix and then transfer from tube #11 to #10 (or 15 to 14).
- Continue the dilutions until tube #2. Tube #1 will be the media only control.

For 96-well format: 12-point, 1:2 serial dilutions of cytokine standards to fill 1-2 rows of the plate.



For 384-well format: 16-point, 1:2 serial dilutions of cytokine standards to fill 1-2 columns of the plate.

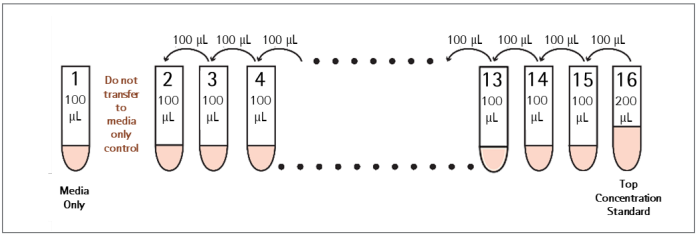


Figure 6. Preparation of Standard Curve. Dilute cytokine standards 1:2 in fresh culture media. Use 12 samples (96-well format) or 16 samples (384-well format). Note that the last sample for both formats contains media alone.

3. Add Cell Membrane Integrity Dye (B/Red) into Antibody Detection Cocktail

The assay kit provides a vial of concentrated Cell Membrane Integrity Dye (B/Red). The Cell Membrane Integrity Dye must be added into the Antibody Detection Cocktail at a 1:25 dilution **right before** the assay. You may follow the table below for appropriate volumes.

	Cell Membrane Integrity Dye (B/Red, 1:25 Dilution)	Antibody Detection Cocktail (Volume in original vial/ bottle)
1 x 96-wells	80 µL	2.0 mL
5 x 96-wells	270 µL	6.75 mL
1 x 384-wells	216 µL	5.4 mL
5 x 384-wells	1080 µL	27.0 mL

NOTE: Once prepared, the Cell Membrane Integrity Dye and Antibody Detection Cocktail mix is stable only for the day of assay. Prepare the mixture volume fresh for each day of assay and discard any leftover.

Assay Protocol

This Protocol may be used for both the 96-well and 384-well formats of this assay.

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

Total Protocol Time: 3 hours
Total Hands-On Time: Approximately 30 minutes

1. Add the Pre-mixed IFNγ/IL-10 Capture Beads to the Assay Plate

- Vigorously vortex Human Cytokine Capture Beads Cocktail and transfer to a reservoir.
- Transfer 10 µL of beads to each assay well.

> **NOTE:** Agitate the beads in the reservoir occasionally during the transfer of the beads from the reservoir to the plate to prevent the beads from settling.

2. Add Cell/Supernatant Mixture Samples and Cytokine Standards

- Ensure the cell/supernatant mixture in the original culture plate is in suspension by manual pipetting 6-8 times, then transfer 10 µL of cell/supernatant sample to each well of the assay plate designated as Sample during the plate set up on the ForeCyt Design tab.
- Transfer 10 µL cytokine standards prepared earlier (Step 2 in Preparation of Reagents) to each well of the assay plate you designated for Standards in the ForeCyt Design tab.
- Centrifuge the plate (300 x g, 5 seconds) to ensure that samples and reagents are at the bottom of the well.
- Mix the plate for 20 seconds at 2,000 RPM using the iQue3/iQue Screener PLUS 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.

3. Add Cytokine Detection Cocktail

- Transfer the Cytokine Detection Cocktail to a reservoir. Add 10 µL/well to the assay plate.
- Centrifuge the plate (300 x g, 5 seconds) to ensure that samples and reagents are at the bottom of the well.
- Mix the plate for 20 seconds at 2,000 RPM using the iQue3/iQue Screener PLUS 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.

4. Add the Prepared Cell Membrane Integrity Dye and Antibody Detection Cocktail Mixture

- Transfer the Cell Membrane Integrity Dye and Antibody Detection Cocktail mixture to a reservoir. Add 10 µL/well to the assay plate.
- Centrifuge the plate (300 x g, 5 seconds) to ensure that samples and reagents are at the bottom of the well.
- Mix the plate for 20 seconds at 2,000 RPM using the iQue3/iQue Screener PLUS 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. Wash/Resuspension

- Add 100 µL/well (96-well format) or 50 µL/well (384-well format) of Wash Buffer to the assay plate.
- Centrifuge the plate (300 x g, 5 minutes).
- Aspirate the supernatant in the assay plate.

> **NOTE:** It is recommended that this step be carried out using a plate washer following the manufacturer's recommendations. 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 can be employed; however, a plate washer is the preferred method.

- Mix the sample in the residual liquid in the well for 60 seconds at 3,000 RPM using the iQue/iQue3 plate shaker.
- Add Wash Buffer, 20 µL/well (96-well format) or 10 µL/well (384-well format) to the assay plate. Specifically for 384-well format, you may use an additional quick spin (300 g, 5 seconds) to ensure that all samples are at the well bottom, and a brief shake (2000 rpm, 20 seconds) to ensure homogeneous sample mixing.
- Secure the assay plate onto the plate loader of the iQue3 or iQue Screener Plus (VBR). The samples are now ready for acquisition.

Sample Acquisition and Data Analysis

1. Launch ForeCyt software

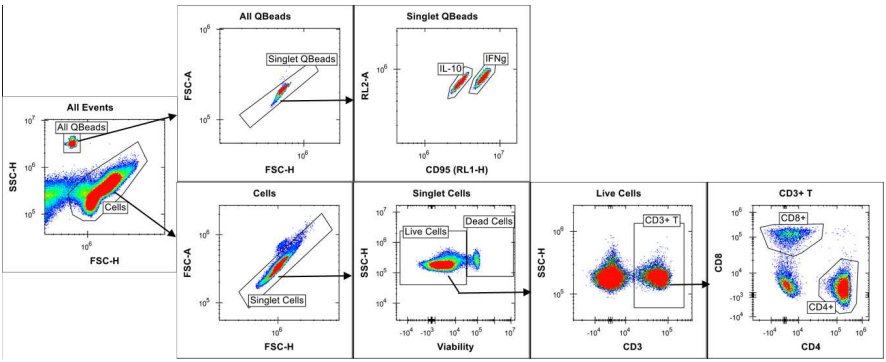
2. Create a New Experiment using the template

- If you have not already imported the template provided (USB key in the kit package), import it now.
- Adjust the sip time and inter-well shaking if necessary to achieve the statistical significance for your cell population of interest. Refer to the tables in the **Best Practices and Tips**. Define your positive and negative control wells in the Design tab in ForeCyt after importing the template.

3. Select "Run" in the Controller Window

The template gates are pre-set for different populations. If you prefer, below are the gating strategy details to manually draw the gates or fine-tune the existing gates from the template:

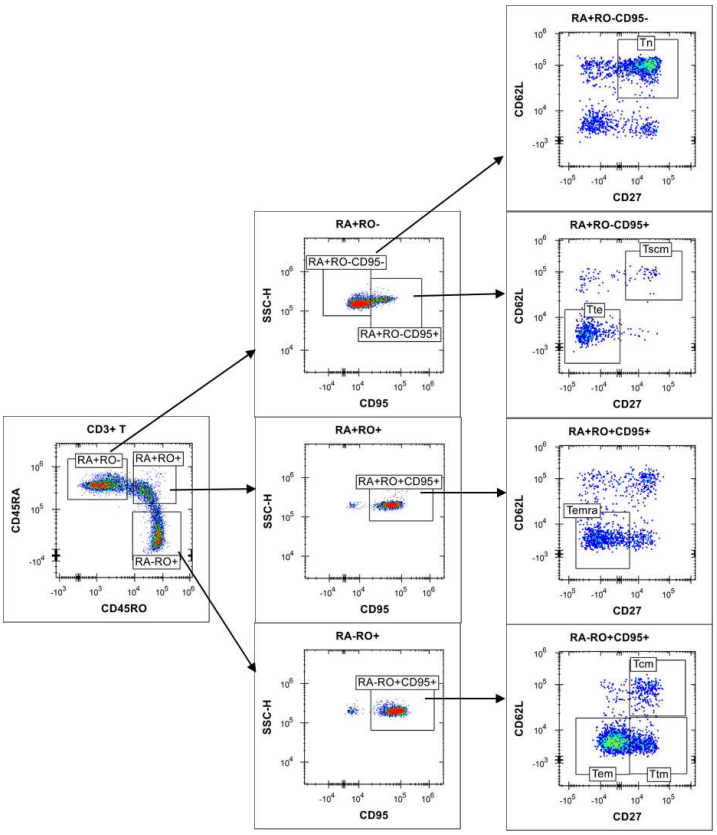
Gate Cells/Beads from All Events:



NOTE: If CD3/CD28 Dynabeads were used in your culture, make sure to exclude Dynabeads from the "Cells" gate. Dynabeads are much smaller in the FSC-H and SSC-H plot than cells and the beads used for cytokine detection.

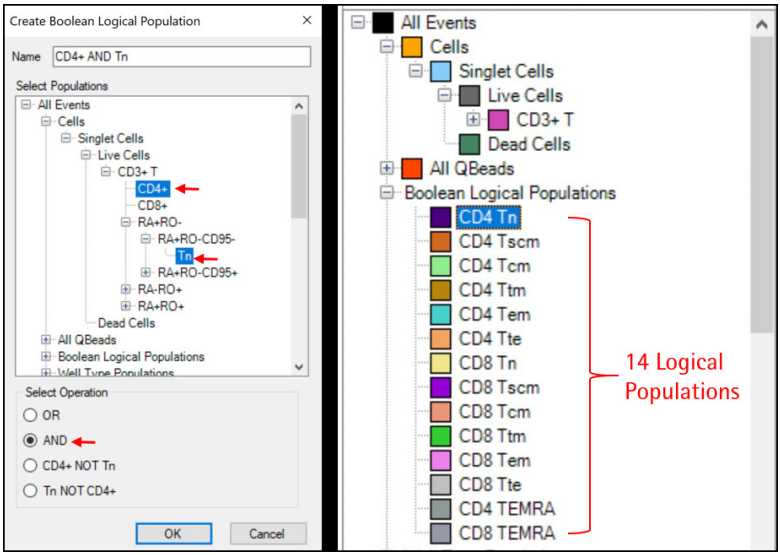
Gate T Naive/Memory/Effector Cells from CD3+ T Cells:

1. Manually adjust the linear range of bi-exponential scale in the dot plots, if necessary, to improve the separation of different populations.
2. CD95+ cells may have 2 populations (high and low expression) depending on treatments and donors.
3. T naive/memory/effector cell populations may look differently in your samples compared with the example below. This is possibly due to the donor condition, treatment, culture condition, or culture time. For example, T_{EMRA} may not be seen in every sample.
4. If you are unsure that the gating in any 2D plot is appropriate, you may right click the 2D plot and select plate view. Confirm if the gates are in the right position by comparing event distribution in different treatments such as negative and positive wells.
5. If there are too many events in any specific plot, making it difficult to visualize the population separation, you may highlight the plot and change the Bin Threshold on the left panel from 0 to 1 (or even 2).



Create 14 Logical Populations to Analyze Different CD4+ or CD8+ T Naive/ Memory/Effector Cells:

Under the ForeCyt analysis tab (populations sub-tab), create the Boolean logical populations. For information on creating the Boolean logical populations, refer to ForeCyt Reference Guide.



Compensation Metrics

Spillover Matrix

Primary Channel \ Spillover Channel	CD62L (BL1-H)	Viability (BL4-H)	CD45RA (BL5-H)	CD95 (RL1-H)	CD27 (RL2-H)	CD45RO (VL1-H)	CD8 (VL3-H)	CD4 (VL4-H)	CD3 (VL6-H)	RL2-A
CD62L (BL1-H)		1.65	0.06	0.00	0.00	0.01	1.59	0.78	0.00	0
Viability (BL4-H)	1.64		12.85	6.32	0.77	0.22	3.61	14.54	2.68	0
CD45RA (BL5-H)	0.48	0.28		0.02	22.70	0.01	0.28	0.20	13.43	0
CD95 (RL1-H)	0.00	0.77	0.04		7.15	0.00	0.01	0.10	0.32	0
CD27 (RL2-H)	0.01	0.03	0.67	5.76		0.01	0.03	0.03	4.96	0
CD45RO (VL1-H)	0.01	0.01	0.00	0.00	0.00		4.98	2.07	0.03	0
CD8 (VL3-H)	0.48	3.00	0.12	0.01	0.00	4.53		97.28	1.36	0
CD4 (VL4-H)	0.01	2.53	0.16	0.10	0.01	0.94	5.94		2.99	0
CD3 (VL6-H)	0.03	0.02	3.48	0.17	19.22	3.24	0.40	0.42		0

NOTE: The template already includes the compensation metrics. It is not necessary for you to adjust any compensation metrics in the assay.

Best Practices and Tips

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

Plate Type

The assay protocol described in this manual is designed for both a 96-well and 384-well formats. It is NOT recommended to use 1 x 384-well kit to run 4 96-well plates of assay. Intellicyt recommends the use of v-bottom plates for both 96-well and 384-well formats (Intellicyt, Cat. 90151 for 96-well plates and Greiner, Cat. 781280 for 384-well plates). This assay kit provides ForeCyt templates for both 96-well and 384-well formats.

Manual Pipetting Recommendation

This protocol requires pipetting small volumes of liquid. If you are pipetting manually, care should be taken during the liquid transfers so that volumes are fully dispensed into the wells of the assay plate with appropriate pipettes (see **Appendix E**). Avoid cross contamination by pipetting fluids onto the wall of the well. A quick spin will force the prepared reagents to the bottom of the well to mix with existing reagent/sample already in the well.

Shaking for Mixing

This assay requires shaking to mix the sample/reagents. If you don't have a separate shaker, you may use the one on the Intellicyt iQue3/iQue Screener PLUS. **Appendix C** provides a reference for well volumes and maximum shaking speed (rpm). (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 (**Only** for sample resuspension in **residual buffer** after spin/aspiration). (4) As soon as you select **On/Plate Shake**, the shaker will begin to shake and continue to shake until you deselect.

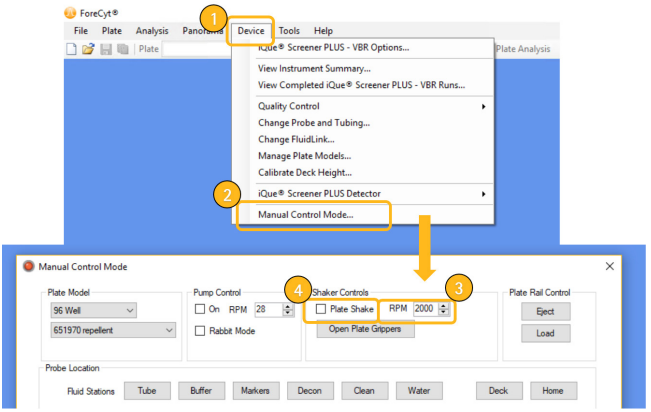


Figure 7. Steps for using the shaker on the iQue Screener PLUS and iQue3.

WARNING: Don't do brief shake with 3,000 RPM as it may cause cross-contamination.

Use a Plate Washer for Aspiration

For wash/aspiration it is best to use an automated plate washer. Manual aspiration of plates and/or plate inversion techniques may result in severe sample loss. Aspiration programs for this assay have been tested on a BioTek ELx405 Select. For a different plate washer brand or model, it is best practice to optimize your specific plate washer settings so that sample loss is avoided. **Appendix D** provides recommended plate types and aspiration settings for BioTek ELx405 Select.

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 1:2 serial dilutions with the highest concentration at 10,000 pg/mL for each cytokine. If you need to modify the top concentration, dilution factor, and/or the plate layout for the standard, refer to the ForeCyt Reference Guide and make the adjustment in the Design tab. Using a different culture media for the standard dilution may have a slight impact on the standard curve and linear range. For example, if you use X-VIVO 15 with 5% human AB serum as fresh culture media for standard dilution, below is the expected linear range for each cytokine:

IFNγ: 10-2,100 pg/mL

IL-10: 10-6,400 pg/mL

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

Generally, the secreted levels of IFNγ and IL-10 may vary depending on donor, cell density, cell health, or cell proliferation. If you anticipate secreted IFNγ or IL-10 levels much 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. If the cell count is too low after sample dilution, carry out the dilution in the original culture plate, centrifuge, and then remove an appropriate volume of liquid. The concentration of cytokine in the sample will be reduced while the cell density will be increased based on the volume of liquid removed.

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 8 seconds per well (96-well format) or 6 seconds per well (384-well format). You may increase the sip time in order to acquire enough cell events for your data analysis to reach statistical significance for your cell population of interest. Sip volume per second varies slightly from machine to machine and even from day to day. Generally, it is approximately 1.5 µL per second. To use a longer sip time than default, adjustments may be made in the ForeCyt Protocol tab.

Inter-well shaking may need to be adjusted as well, as settling will occur over time. The tables below may help you adjust the sip time and sampling protocol if necessary, assuming the lowest cell density in the culture plate is 1 million/mL.

For 96-well Format:

Sip Time per Well	Cell Density in the Culture Plate	Transfer Volume	Final Volume After Resuspension in Assay Plate	Estimated Cells Density in Assay Plate
8 sec. 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)	0.35 million/mL (assume wash causes 10% cell loss)
10 sec. sip				
12 sec. sip				
15 sec. sip				

Sip Time per Well	Estimated Volume Acquired (Assume 1.5 µL/second sip/well)	Estimated Cell Events Acquired per Well	Inter-well Shake Frequency	Inter-well Shake Duration	Acquisition Time per Plate
8 sec. sip (default)	12 µL	4,200	Every 4 Wells	8 seconds	~25 mins
10 sec. sip	15 µL	5,250	Every 3 Wells	10 seconds	~28 mins
12 sec. sip	18 µL	6,300	Every 3 Wells	12 seconds	~32 mins
15 sec. sip	22.5 µL	7,875	Every 3 Wells	15 seconds	~39 mins

For 384-well Format:

Sip Time per Well	Cell Density in the Culture Plate	Transfer Volume	Final Volume After Resuspension in Assay Plate	Estimated Cells Density in Assay Plate
6 sec. sip (default)	1 million/mL (assumption: the lowest possible density ~ the seeding density)	10 µL (from Culture Plate to Assay Plate)	~15 µL (10 µL + residual volume)	0.6 million/mL (assume wash causes 10% cell loss)
9 sec. sip				

Sip Time per Well	Estimated Volume Acquired (Assume 1.5 µL/second sip/well)	Estimated Cell Events Acquired per Well	Inter-well Shake Frequency	Inter-well Shake Duration	Acquisition Time per Plate
6 sec. sip (default)	9 µL	5,400	Every 6 Wells	6 seconds	~60 mins
9 sec. sip	13.5 µL	8,100	Every 4 Wells	9 seconds	~95 mins

If you predict you will not have enough cell events even after increasing the sip time, you may consider increasing the cell number acquisition from the assay plate by concentrating cells in the original culture/treatment plate (see **Appendix B**) before transferring cell/supernatant mixture samples to the assay plate. Refer to **Appendix B** to improve the precision of cell density calculation by running daily iQue3/iQue Screener PLUS volumetric calibration. If cells are sticky, **Appendix B** also provides the recommended information about using cell-repellent or ultra-low binding plates to avoid cells attaching to the well bottom/walls.

Appendix A: Multiplexing Additional Cytokines with Human T Cell Companion Kits

The Intellicyt Human T Cell Companion Kits are used in combination with the Human T Cell Immunology portfolio of Kits. Human T Cell Companion Kits allow the measurement of up to 6 human cytokines in addition to the cytokines already included in the Human T Cell Immunology Kits: Human IL-2 (Cat. 97028), Human IL-6 (Cat. 97029), Human IL-13 (Cat. 97031), IL-17A (Cat. 97032), GM-CSF (Cat. 97033), and Human TNF (Cat. 97034).

After choosing the desired Human T Cell Companion Kit(s), follow the detailed instructions included with the Human T Cell Companion Kit(s). A brief description is below:

1. Combine Standards - Combine the Human T Cell Companion Kit standards with the standards in the Human T Cell Immunology Kit.

For example: the Human T Cell Memory Cell and Cytokine Profiling Kit provides 2 cytokine standards: IFNγ and IL-10. If you purchased IL-2 and IL-6 Human T Cell Companion Kits, combine all 4 cytokine standards together in a single tube (IL-2, IL-6, IFNγ, and IL-10).

After combining all additional cytokine standards in a single tube, follow the protocol in the Human T Cell Immunology Kit protocol guide. Perform standard reconstitution and titration.

2. Combine Human T Cell Companion Kit Capture Beads (50x) - To the Cytokine Capture Beads Cocktail in the Human T Cell Immunology Kit, add the appropriate amount of Human T Cell Companion Kit capture beads to the kit vial.

Vortex the Human T Cell Companion Kit capture beads for 30 seconds. Use volumes listed in the tables below for your specific Human T Cell Immunology Kit type and configuration.

3. Combine Human T Cell Companion Kit Detection Reagent (50x) – To the premixed Cytokine Detection Cocktail in the Human T Cell Immunology Kit, add the appropriate amount of detection reagent to the kit vial. Use volumes listed in the tables below for your specific Human T Cell Immunology Kit type and configuration.

4. Perform the Assay – Follow the protocol for your particular Human T Cell Immunology Kit and configuration.

NOTE: The templates from the specific Human T Cell Immunology Kits do not have the appropriate gating for the companion kit beads. Use the Human T Cell Companion Kit template (see Figure 8 for the full gating of all 8 cytokine beads). The Human T Cell Companion Kits can only work with the Intellicyt Human T Cell Immunology portfolio of Kits.

The Human T Cell Companion Kits contain the reagent volumes needed to run 2 plates of 1 x 96-well format of Human T Cell Immunology Kits. Additional Human T Cell Companion Kits may be needed for use with 5 x 96-well format, 1 x 384-well format and 5 x 384-well formats of Human T Cell Immunology Kits.

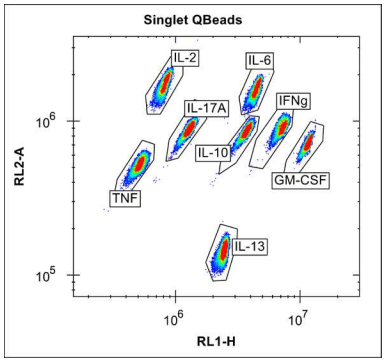


Figure 8: Human T Cell Companion Kit cytokine template.

Combine Human T Cell Companion Kit Reagents with Human T Cell Memory Cell and Cytokine Profiling Kit

	1 x 96-well	5 x 96-well	1 x 384-well	5 x 384-well
Reagents Included in the Human T Cell Memory Cell and Cytokine Profiling Kit				
Human IFNγ and IL-10 Separate Standards (# of vials)	1 IFNγ 1 IL-10	5 IFNγ 5 IL-10	2 IFNγ 2 IL-10	10 IFNγ 10 IL-10
Human Cytokine Capture Beads Cocktail (IFNγ and IL-10)	2.0 mL	6.75 mL	5.4 mL	27.0 mL
Cytokine Detection Cocktail	2.0 mL	6.75 mL	5.4 mL	27.0 mL
For each Human T Cell Companion Kit cytokine, add volumes listed below to original kit components				
Cytokine Standard (vials)	1 standard	Combine Human T Cell Companion Kit standard(s) with IFNγ and IL-10 Kit Standards fresh on each day of use.		
Companion Capture Beads (50x) Add volume to the Human Cytokine Capture Beads Cocktail (IFNγ and IL-10)	40 µL	135 µL	108 µL	540 µL
Companion Detection Reagent (50x) Add Volume to the Cytokine Detection Cocktail	40 µL	135 µL	108 µL	540 µL

Appendix B: Options for Improving Cell Event Acquisition

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

If increasing the sip time in the Sampling Protocol does not yield enough cells in sample acquisition, you have the following option: before running the assay, spin your cells down (300 x g, 5 minutes) in your original cell culture plate. Remove half or two thirds the volume of supernatant to double or triple cell density in the culture wells. Then, re-suspend your cells in the original culture plate in the remaining volume of supernatant by manual pipetting. You may then 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

The Intellicyt iQue Screener PLUS and iQue3 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 or iQue3 using absolute counting beads such as SPHERO™ AccuCount Particles (Spherotech, Cat. ACBP-50-10). 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 same sampling protocol with the identical sip time, plate type, and the volume in the well as used in this Human T Cell Memory 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 and iQue3. 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. For information on using the custom function refer to ForeCyt Reference Guide, or call customer support.

Option 3: Use Cell-Repellent or Ultra-Low Binding Plates as the Assay Plate to Prevent Cell Attachment

T cells are usually suspension cells and will not attach to the well bottom/walls of the Intellicyt-recommended plates. However, in cases of complex biology, 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 cell-repellent plates (e.g. Griener, Cat. 651970, Cat. 781970) or ultra-low binding plates (e.g. Corning, Cat. 7007, Cat. 4516) in order to achieve a more precise cell count on the iQue Screener PLUS and iQue3. If ForeCyt does not list this plate model, add it to the list (Device ➔ Manage Plate Models ➔ Add) and map it.

Appendix C: Mixing Samples Using Shaker

For iQue Screener PLUS and iQue3

Plate Type	Well Volume	MAX RPM
96-well	20–40 µL	2,600
96-well	40–60 µL	2,200
96-well	60+ µL	A/O*
384-well	10–30 µL	3,000
384-well	30–50 µL	2,800
384-well	50+ µL	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 values.

Appendix D: Plate Type Recommendations and Automated Wash Protocols for Microplates

The assay protocol described in this manual is designed for both a 96-well and 384-well plate formats. One 384-well plate format may be used to run three 96-well plate assays. Intellicyt recommends the use of 96-well v-bottom plates (Intellicyt, Cat. 90151) for 96-well plate, 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.

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

Plate Type	Well Volume	Manufacturer	Manufacturer Product #
96-well	v-bottom	Intellicyt	90151
384-well	v-bottom	Greiner	781280

When using the plate types in the table above, the following aspiration programs have been tested on a BioTek ELx405 Select. **If you have a different plate washer brand or model, it is recommended to optimize the aspiration settings on your specific plate washer system to avoid sample loss.**

Intellicyt highly recommends that wash protocols use an automated plate washer. Manual aspiration of plates or plate flicking techniques may result in severe sample loss.

Plate Type	Aspiration Height Setting	Aspiration Height Offset	Aspiration 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 E: 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 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 pipette for reagent preparation:

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

Appendix F: 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 using ForeCyt 7.0 or newer version of software. However, there is potential day-to-day variation that may affect your cytokine quantitation if you use standards curves acquired on different days. We recommend running standard curves on each assay day and applying the standard curves to the 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 Fit (of 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 use fixatives in my samples?

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, or Cat. 781970) may reduce cell loss due to fixation or fixation-related cell cross-link 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?

Answer: A 1 x 384-well format kit is optimized to run one single 384-well plate. It is not recommended to use a 1 x 384-well kit to run assay in several 96-well plates. The reagent volume provided in 1 x 384-well kit is NOT enough to run four 96-well plates in four different days.

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

Answer: We recommend not multiplexing this assay with other cellular endpoints. Select additional cytokine endpoints may be multiplexed using the Human T Cell Companion Kits (sold separately). See **Appendix A**.

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

Answer: 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 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 during aspiration steps.

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

Answer: There are several steps that may have caused well cross-contamination: pipette tips touched samples in the well and were used for reagent transfer for other wells; used the strong shake (3,000 RPM) for brief shaking (2,000 RPM is recommended).

Solution: change pipette tips as frequently as possible; use 2,000 RPM shaking speed for brief shaking.

Q7: The CD4 and CD8 signal in the dot plot looks compressed/stretched. 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 in biexponential scale. You may need to adjust the linear range of the biexponential scale manually by increasing or decreasing 2-10 times the linear range value. Choose the one that can achieve signal separation of CD4+ and CD8+ cells. Then, you could adjust the gating to include the corresponding T cell sub-populations. As a reminder, T cell activation may affect the donor-dependent expression of CD4 and CD8 proteins. This is why you may sometimes see 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.

Q8: What if I don't have access to an automated plate washer for liquid aspiration step?

Answer: If you don't 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 45 degree against the wall of assay well and try to avoid to touch the cell/bead pellet at the well bottom. Make sure to change tips after each liquid aspiration step in order to avoid well cross-contamination.

Another option to aspirate the liquid in the assay well is to flick quickly 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 viability/live cell readings from wells which only contain capture beads (e.g. wells designated for cytokine standards)?

Answer: These viability readings are usually caused by a few stray events. You may use the plate view option of live cells as a QC 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 this.

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

Answer: This assay is only optimized for cell culture samples, and is not optimized to measure cytokines in human sera. If you need to measure the same cytokines 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 any of the Human T Cell Immunology portfolio of kits.

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