SARTORIUS

Product Guide

iQue® Human T Cell Exhaustion Kit

Product Information

Presentation, Storage and Stability

The iQue® Human T Cell Exhaustion Kit contains enough reagents for measurement of T cell exhaustion.

Product Name	Cat. No.	Format
iQue® Human T Cell Exhaustion Kit	97069	1 x 96 wells
iQue® Human T Cell Exhaustion Kit	97070	5 x 96 wells
iQue® Human T Cell Exhaustion Kit	97071	1 x 384 wells
iQue® Human T Cell Exhaustion Kit	97072	5 x 384 wells

Table 1. Product Information

Note: The 1 x 384-well kit has enough reagents to run 4 x 96-well plates. There is enough reagent for the specific kit size and provides minimal liquid overage.

Kit Components	Cat. No. 97035 1 x 96 well	Cat. No. 97036 5 x 96 well	Cat. No. 97037 1 x 384 well	Cat. No. 97038 5 x 384 well	Storage	Stability
Human IFNγ and TNFα Capture Beads (Pre-mixed)	1 bottle 2 mL	5 bottles 2 mL	1 bottle 5.4 mL	5 bottles 5.4 mL	2–8°C	Minimum 6 month shelf life; up to one year
Human IFNγ and TNFα Cytokine Standards (Separate)	1 vial for each cytokine	5 vials for each cyto-kine	1 vial for each cytokine	5 vials for each cytokine	2–8°C	
Human IFNγ and TNFα Cytokine Detection Cocktail	1 bottle 2 mL	5 bottles 2 mL	1 bottle 5.4 mL	5 bottles 5.4 mL	2–8°C	
Antibody Panel Detection Cocktail (Pre-mixed: 6 FL antibodies)	1 bottle 2 mL	5 bottles 2 mL	1 bottle 5.4 mL	5 bottles 5.4 mL	2–8°C	
iQue® Cell Membrane Integrity (R/Red) Dye	1 vial 25 µL	5 vials 25 µL	1 vial 100 µL	5 vials 100 µL	-20°C	
iQue® Cell Proliferation and Encoding (B/Green) Dye	1 vial 25 µL	5 vials 25 µL	1 vial 100 µL	5 vials 100 µL	-20°C	
Wash Buffer	1 bottle (25 mL)	1 bottle (125 mL)	1 bottle (50 mL)	1 bottle (250 mL)	2–8°C	

Table 2. Kit Components and Storage

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

Background

The iQue® Human T Cell Exhaustion Kit enables high-throughput, multiplexed measurements of T cell phenotype, exhaustion state, health, proliferation, and secreted cytokines from the same assay well containing a mixture of cells and cytokine detection beads. The kit is designed to simultaneously measure, in each assay well, these endpoints:

- Viability
- Immune Cell Phenotypes: CD3+, CD4+ and CD8+
- T cell exhaustion markers | IRs: PD-1, Lag-3 and Tim-3
- Secreted effector cytokines: IFNγ and TNFα

This one-wash assay protocol has been validated to run on the iQue® 3 (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 (**Table 3**).

The assay detects:

- Live, proliferative cells with the optional iQue® Cell Proliferation and Encoding (B/Green) Dye, a cell permeable green fluorescent dye.
- Dead cells are detected with the iQue® Cell Membrane Integrity (R/Red) Dye, a fluorescent membrane integrity dye that enters dead cells or cells with a compromised membrane, staining the nucleic DNA by intercalation.
- Immunophenotyping of live cells occurs via a fluorescent antibody panel for:
  - CD3+ T cells
  - CD3- non-T cells
  - CD3+CD4+ T helper cells
  - CD3+CD8+ T cytotoxic cells
  - PD-1+ T cell inhibitory receptor
  - Tim-3+ T cell inhibitory receptor
  - Lag-3+ T cell inhibitory receptor
- T cell effector cytokine secretion, quanitified in a sandwich immunoassay with iQue Qbeads® | Human IFNγ and TNFα Capture Beads:
  - IFNγ (a Th1 cytokine)
  - TNFα (produced by predominantly Th1 and CD8+)

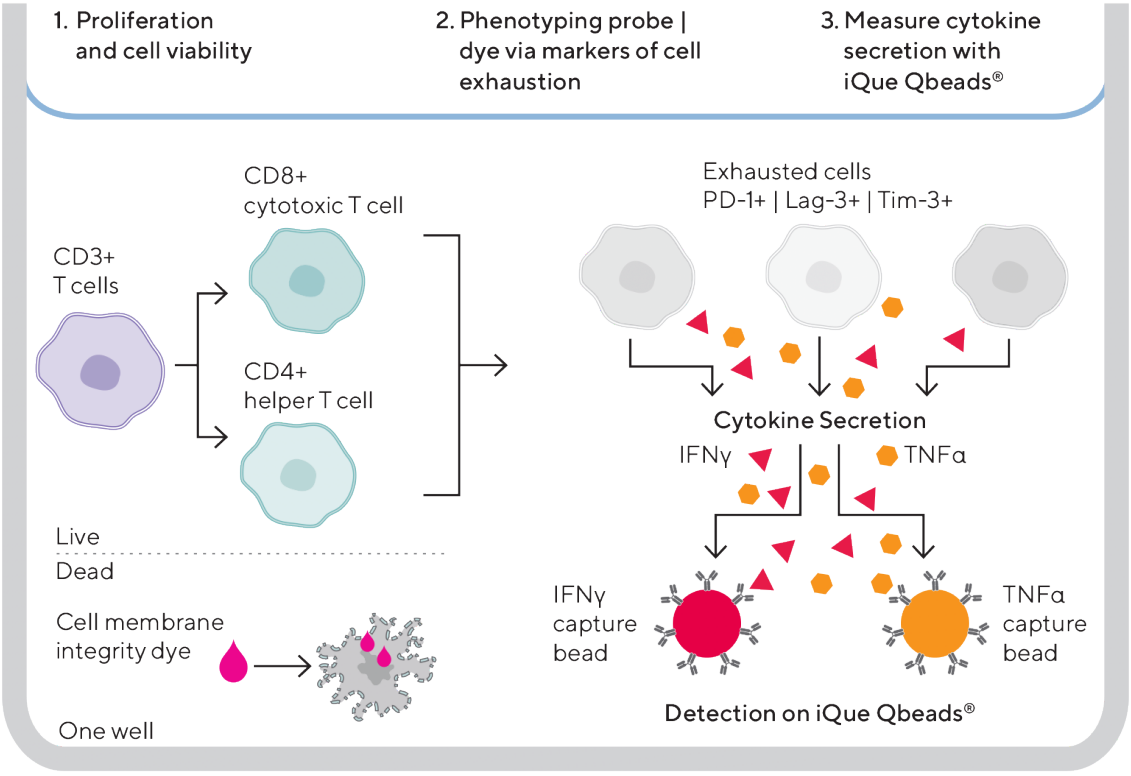


Figure 1. Illustration of the iQue® Human T Cell Exhaustion Kit assay principles. Live cells are separated from dead cells based on the staining with iQue® Cell Membrane Integrity (R/Red) Dye. Basic T cell phenotypes are measured by staining with CD3, CD4, and CD8 markers. Secreted pro-inflammatory cytokines IFNγ and TNFα are also quantified by iQue Qbeads® | Human IFNγ and TNFα Capture Beads in a sandwich immune assay format in the same assay well.

Detection Reagent	Excitation (nm)	Detector (nm)	iQue® 3 (VBR)	iQue® PLUS (VBR)
CD3	405	445/45	V/Blue	VL1
CD8	405	780/60	V/Crimson	VL6
Tim-3	405	675/30	V/Red	VL5
Lag-3	488	675/30	B/Red	BL4
PD-1	488	780/60	B/Crimson	BL5
IFNγ and TNFα	488	572/28	B/Yellow	BL2*
CD4	640	780/60	R/Crimson	RL2
iQue® Cell Membrane Integrity (R/Red) Dye	640	675/30	R/Red	RL1
iQue® Cell Proliferation and Encoding (B/Green) Dye	488	530/30	B/Green	BL1

Table 3. Laser Detection Channels for Components of the iQue® Human T Cell Exhaustion Kit
*Cytokine quantitation in B/Yellow channel and iQue Qbeads® are distinguished using the R/Red and R/Crimson channels.

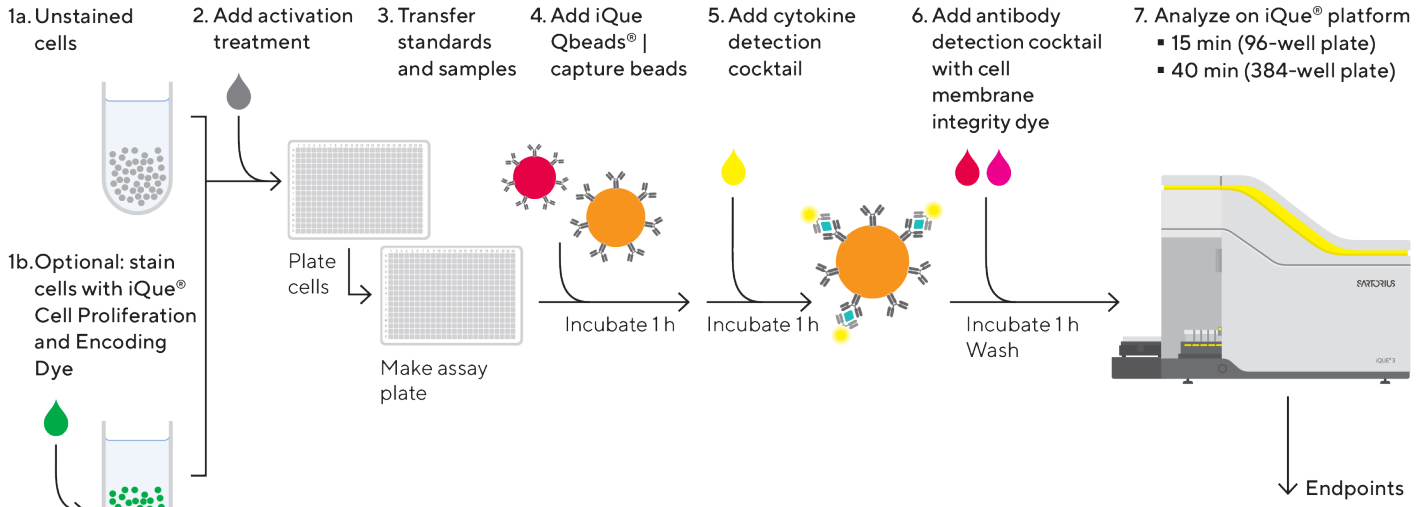
Note: This assay is only compatible with the iQue® platform using the violet, blue and red (VBR) laser configuration. Other iQue® platforms including iQue®3, iQue® PLUS (VYB lasers), and iQue® PLUS (BR lasers) will NOT work with this assay due to the detection channel limitation.

Note: Do not perform the optional iQue® Cell Proliferation and Encoding (B/Green) Dye Protocol if your cells express Green Fluorescent Protein (GFP).

Recommended Use

Assay Overview

This assay is validated for use with human T cells or PBMCs in a 96- or 384-well format.



Basic T Cell ID		IRs			Secreted Cytokines		Relative Cell Count	Cell Viability	Cell Proliferation (optional)
		PD-1	Lag-3	Tim-3	IFNγ	TNFα			
CD3+	CD4+	+ | -	+ | -	+ | -	+ | -	+ | -	+	+ | -	+
	CD8+	+ | -	+ | -	+ | -	+ | -	+ | -	+	+ | -	+

Figure 2. iQue® Human T Cell Exhaustion Kit Assay Workflow. T cells or PBMCs are activated in the cell culture plates. An aliquot of the cells | supernatant mixture from each well is transferred into assay plates along with iQue Qbeads® | Human IFNγ and TNFα Capture Beads. After incubating 60 minutes, the Human IFNγ and TNFα Cytokine Detection Cocktail (anti-IFNγ and anti- TNFα) is added to the plate. After 60 minutes incubation, a fluorescent Antibody Panel Detection Cocktail against CD3, CD4, CD8, Tim-3, Lag-3, PD-1 and the iQue® Cell Membrane Integrity (R/Red) Dye that detects cell viability is added to the assay plate. After a 60-minute incubation, the assay plates are washed once before sample acquisition on the iQue® platform using the violet, blue and red (VBR) laser configuration. T cell and T cell subtypes, 3 surface exhaustion markers expressed, 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.

Materials Required, but not Supplied

- iQue® platform with VBR Lasers
- Centrifuge capable of spinning microfuge tubes and | or 15 mL conical tubes at up to 500 x g
- Centrifuge capable of spinning microplates
- Vortex mixer
- Fresh complete cell culture media (Same media used to grow your sample | cell culture)
- Microtiter assay plates
- 96-well assay plate (Sartorius®, Cat. No. 90151) or similar
- 384-well assay plate (Greiner®, Cat. No. 781280) or similar
- Microcentrifuge tubes and | or 15 mL conical tubes
- Reagent reservoirs (VWR®, Cat. No.89094-680)
- Universal black lid (Corning®, Cat. No.3935) or foil to protect from light | evaporation
- Appropriate liquid handler or multi-channel pipette
- Human T cells or PBMCs

Optional

- CD3 | CD28 DynaBeads® Human T-Activator (ThermoFisher®, Cat. No. 111-61D)
- HBSS or PBS buffer if using the iQue® Proliferation and Encoding (B/Green) Dye
- Media + 10% serum if using the iQue® Proliferation and Encoding (B/Green) Dye
- Plate washer (such as BioTek® model ELx405)
- 12-channel pipette reservoir (Example source: VWR®, Cat. No. 80092-466) (optional for preparing serial titrations)

Cell Culture and Preparation for 96- and 384-Well Plates

1.0 Cell Culture

1.1 Culture T cells or PBMCs at a density ~ 2.0x10^6 cells/mL at 37°C 5% CO_2 in cell culture media recommended by the supplier of the cells.

Note: Cell health reagents may be added to promote the health and growth of T cells, if desired.

1.1.1 Obtain enough human T cells or PBMCs for your planned experiment. We recommend a seeding density of ≥1.0 x 10^6 cells/mL (typical range from 1–4 x 10^6 cells/mL for cells in plates that will be given any T cell exhaustion treatment(s) (**Table 4**).

Plate Format	Min. Culture Volume	Density (cells/mL)
96	50 µL	1.0x10^6 cells/mL
384	25 µL	1.0x10^6 cells/mL

Table 4. Cell culture volumes and recommended cell density for 96- and 384-well assay kit formats.

Note: Your experimental design might include an optional treatment plate(s) with positive and negative controls of T cell activation and exhaustion. Use of the treatment plate depends on your assay workflow needs. If treatment plate is used, it is the first plate made and incubated for a duration of time based on your experimental design. Typically, treatment plates include positive and negative controls for T cell stimulation and exhaustion, as well as any investigatory drug treatments or conditions. You will transfer 10 µL of the cell suspension from the treatment plate to the wells of your assay plate that will be placed on the iQue®.

2.0 Optional: T Cell Proliferation and Encoding Measurement

2.1 Dilute the iQue® Proliferation and Encoding (B/Green) Dye into a protein-free buffer such as HBSS buffer or PBS buffer at a dilution factor of 1:1250 (**Table 5**).

2.1.1 Ensure that the iQue® Proliferation and Encoding (B/Green) Dye is completely thawed before use.

2.1.2 Briefly centrifuge the vial. Gently mix the dye by pipette or vortexing before use.

Note: Use the same dilution buffer consistently across the assay protocol when it is required.

Assay Kit Format	2X Dye Solution	HBSS | PBS	iQue® Proliferation and Encoding (B/Green) Dye
96	2.0 mL	1998.4 µL	1.6 µL
384	4.625 mL	4621.3 µL	3.7 µL

Table 5. Preparing 1:1250 iQue® Proliferation and Encoding (B/Green) Dye working stock for 96- and 384-well assay kits

2.2 Wash cells with protein-free buffer

2.2.1 Spin cells down in a conical tube (500 x g, 5 minutes) and remove the original culture medium.

2.2.2 Resuspend cells in 20 mL protein-free HBSS or PBS and centrifuge cells at 500 x g, 5 minutes. Remove the supernatant.

2.2.3 Resuspend cells in buffer at 1–4 x 10^6 cells/mL.

2.3 Combine cells and the diluted iQue® Proliferation and Encoding (B/Green) Dye (**Table 6**).

Note: Avoid cells or dye droplets attaching to the inner wall of the tube which may result in inconsistent cell staining.

2.3.1 Mix an equal volume of cell suspension with the prepared 1:1250 iQue® Proliferation and Encoding (B/Green) Dye solution from **Step 2.1.2**, resulting in a final dilution of 1:2500 of the iQue® Proliferation and Encoding (B/Green) Dye.

2.3.2 Thoroughly and gently mix.

2.3.3 Incubate cells at room temperature for 15 minutes and protect from light.

Assay Kit Format	Total Volume	2X Dye Stock	Cell Suspension Volume	Final Cell Density
96	4.0 mL	2.0 mL	2.0 mL	0.5–2 x 10^6 cells/mL
384	9.25 mL	4.625 mL	4.625 mL	0.5–2 x 10^6 cells/mL

Table 6. Combining cell suspension with 2X iQue® Proliferation and Encoding (B/Green) Dye working stock for 96- and 384-well assay kits

2.4 Wash cell | dye mixture by adding at least 2X volume of complete culture medium (with 10% serum) to the staining sample.

2.4.1 Spin (500 x g, 5 minutes). Remove the supernatant.

2.4.2 Resuspend cells manually in the residual liquid.

2.4.3 Repeat wash two more times.

2.5 Resuspend cells after the final wash at a density for the assay (typically 1–4 x 10^6 cells/mL)

Note: You may wish to activate or apply other treatments to your cells, in which case you should proceed to **Step 3.1**, Stimulate T Cells, shown below.

3.0 Stimulate T Cells

3.1 Stimulate T Cells according to the design of the experiment.

Note: T cell exhaustion or stimulation reagents may require optimization based on your cells and assay design. Please review literature and product information on the chosen T cell reagents used to stimulate T cells or induce T cell exhaustion prior to performing your assay.

Note: For more accurate population gating and subsequent data analysis we strongly recommend using stimulation reagents such as CD3 | CD28 DynaBeads®, at a 1:1 cell-to-bead ratio, for positive T cell stimulation in wells of the original culture plate.

3.2 Incubate T cells in treatment plate at 37°C 5% CO2 for a duration required by your experimental design, typically 1–14 days.

4.0 Combine and Rehydrate the Human IFNγ and TNFα Cytokine Standards

4.1 Combine the two lyophilized Human IFNγ and TNFα Cytokine Standards spheres from the kit into a 1.5 mL microfuge tube or 15 mL conical tube.

Note: Ensure the lyophilized cytokine spheres are at the bottom of the glass vial. Gently tap the Human IFNγ and TNFα Cytokine Standards vials on the workbench to force the sphere to fall to the bottom of the vial. Slowly open the rubber lids of the glass vials to prevent the lyophilized cytokine spheres from flying out of the vial due to the slight positive pressure inside the vial.

Note: Use only 1 glass vial of each cytokine for the standard preparation.

4.2 Rehydrate the Human IFNγ and TNFα Cytokine Standards in fresh culture media

4.2.1 Slowly add 200 µL fresh culture media (the same media used to grow your sample culture) to the tube with the 2 lyophilized cytokine spheres.

Note: Do NOT mix. Mixing at this step causes the reagent to foam.

Note: 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.

4.2.2 Let stand for 15 minutes at room temperature to fully reconstitute.

4.2.3 Once dissolved, gently mix by pipetting up and down

Note: Do NOT vortex.

5.0 Make a 1:3 Serial Dilution of the Combined Human IFNγ and TNFα Cytokine Standards

5.1 Prepare a 1:3 serial dilution (top concentration 50,000 pg/mL) of the reconstituted Cytokine Standards with fresh culture medium, as show in (**Figure 3** for 96-well and **Figure 4** for 384-well).

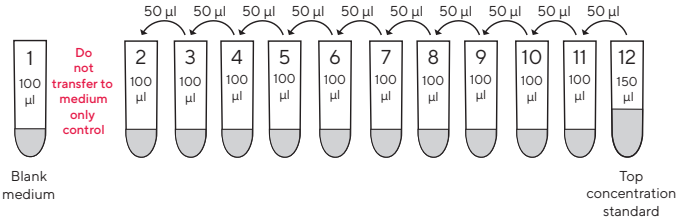


Figure 3. 96-Well Dilution Series

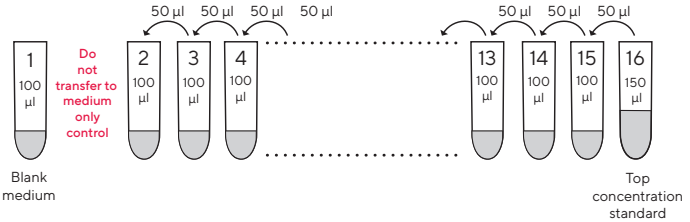


Figure 4. 384-Well Dilution Series

5.1.1 Obtain and label dilution tubes. You may also use an empty 96-well plate or 12-channel pipette reservoir instead of microtubes.

- For 96-well plate, label tubes #1–12
- For 384-well plate, label tubes #1–16

5.1.2 Add the specified volume of fresh culture media to select tubes. This is the same media used to grow your sample culture.

- For 96-well plate, add 100 µL media to tubes #1–11
- For 384-well plate, add 100 µL media to tubes #1–15

5.1.3 Add the 50,000 pg/mL solubilized cytokine standards to the indicated tube for your plate type.

- For 96-well plate protocol, add 150 µL solubilized cytokine standard to tube #12
- For 384-well plate protocol, add 150 µL solubilized cytokine standard to tube #16

5.1.4 Begin the serial titration by transferring the 50,000 pg/mL solubilized cytokine standard to dilution tubes.

- For a 96-well plate, transfer 50 µL of standard from tube #12 into tube #11. Gently pipette up and down at least 5 times to completely mix the solution.

- For a 384-well plate, transfer 50 µL of standard from tube #16 into tube #15. Gently pipette up and down at least 5 times to completely mix the solution.

5.1.5 Continue serial dilution transfer and mixing, do not transfer to the last tube (blank).

- For 96-well plate, do not transfer any solution to tube #1 (blank).
- For 384-well plate, do not transfer any solution to tube #1 (blank).

5.1.6 Set the cytokine standard serial dilutions aside for later.

6.0 Dilute the Pre-Mixed Human Human IFNγ and TNFα Capture Beads

Note: If you anticipate your samples will have very low levels of both IFNγ (< 55 pg/mL) and TNFα (<85 pg/mL), do NOT dilute the pre- mixed Human IFNγ | TNFα Capture Beads provided in the kit. Instead refer to Page 15, section "**Troubleshooting for IFNγ and TNFα Cytokine Levels Out of Linear Range in Your Samples**."

6.1 Dilute the Human IFNγ and TNFα Capture Beads at a 1:10 dilution in a 50 mL conical tube (**Table 7**).

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

6.1.2 Vortex the Human IFNγ and TNFα Capture Beads vial for at least 15 seconds.

Plate Type	Human IFNγ and TNFα Capture Beads	Culture Media (Fresh)
1 x 96 plate	2.0 mL	18.0 mL
5 x 96 plates	2.0 mL	18.0 mL
1 x 384 plates	5.4 mL	48.6 mL
5 x 384 plates	5.4 mL	48.6 mL

Table 7. Human IFNγ and TNFα Capture Beads dilution volumes for 96- and 384-well plates

Note: Recommended volumes of reagent mixtures are based on the plate type. Following this protocol will provide enough mixture for an entire assay plate with modest excess volumes.

6.2 Mix the media and Human IFNγ and TNFα Capture Beads.

6.2.1 Vigorously vortex for 10 seconds.

6.2.2 Set the pre-mixed, diluted Human IFNγ and TNFα Capture Beads aside for later.

Assay Protocol for 96- and 384-Well Plates

Total Protocol Time: Approximately 3 hours
Total Hands-On Time: Approximately 30–60 minutes

7.0 Design Assay Plate and Add Standards and Samples

Note: During liquid transfers, change pipette tips to avoid cross-well contamination.

Note: Do not add the standard into the assay well until you are ready to begin the assay set up.

7.1 Design assay plate layout (**Figure 5** for 96-well plate and **Figure 6** for 384-well plate). A template with the recommended standard design is already in provided in the kit USB drive.

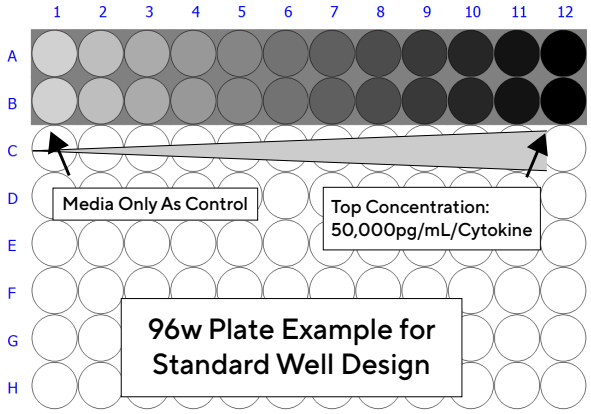


Figure 5. Recommended 96-well plate layout

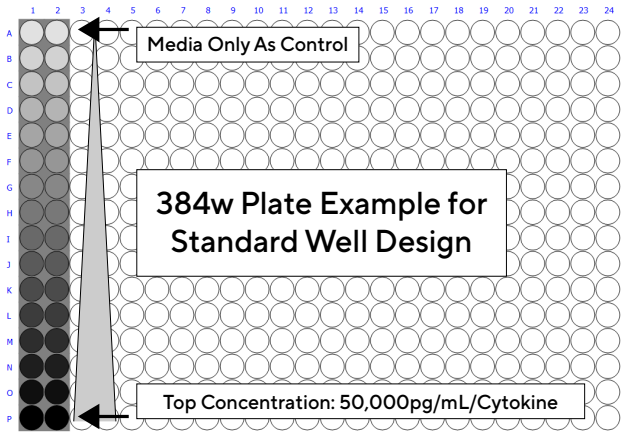


Figure 6. Recommended 384-well plate layout

Note: Cytokine standards can be run on a separate plate from assay samples if desired.

Note: Wells in white in **Figure 5 and 6** are wells to contain your samples.

Note: You may change the configuration of your plate for a non-standard layout in iQue Forecyt® Design → Standards → Edit Standard Set. This can be helpful in case you used a different direction of series layout, dilution factor, max concentration or did not include the lowest concentration at 0.

7.2 Transfer the Human IFNγ and TNFα Cytokine Standards prepared in **Step 5.1.6** to the wells in the assay plate (**Table 8**).

Plate Type	Cytokine Standards to add to wells
96-well	10 µL
384-well	10 µL

Table 8. Volume of cytokine standards to 96- or 384-well plate

7.3 Obtain your original cell culture plate and mix the cell | supernatant mixture by gentle pipetting. Repeat 5X.

Note: Do not introduce air bubbles into your cell samples during pipetting.

7.4 Transfer the cell | supernatant mixture to the wells designated for samples in the assay plate, according to the plate layout you designed in **Step 7.2** (**Table 9**).

Plate Type	Cell | Supernatant Sample to wells
96-well	10 µL
384-well	10 µL

Table 9. Volume of cell | supernatant to add to 96- or 384-well plate

8.0 Add Pre-diluted Human IFNγ and TNFα Capture Beads

8.1 Obtain the diluted, pre-mixed Human IFNγ and TNFα Capture Beads prepared from **Step 6.2.2**. Vortex vigorously for 10 seconds.

8.2 Add the diluted, pre-mixed, vortexed Human IFNγ and TNFα Capture Beads to a reservoir and then add the reagent to the assay plate (**Table 10**).

Note: Change the tip after dispensing to avoid cross-well contamination.

Note: Agitate the reservoir occasionally during the transfer of the beads to the plate to prevent the beads from settling.

Plate Type	Capture Beads to add to wells
96-well	100 µL
384-well	100 µL

Table 10. Volume of capture beads to add to each well in 96- or 384-well plate

8.3 Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

Note: Do NOT shake the plate to prevent the liquid volume in assay wells from splashing into adjoining wells.

9.0 Long Centrifugation and Aspirate Assay Plate

9.1 After the incubation, spin the assay plate (300 x g, 5 min). Aspirate the supernatant with a plate washer, following the manufacturer's instructions.

Note: Do not touch the aspiration probe to the bottom of the plate well; do NOT aspirate the samples during this step.

Note: If no plate washer is available, you may carefully aspirate by manual pipetting or inversion and a quick flick over a sink.

9.2 Agitate the sample in the residual liquid in the plate with the plate shaker (3,000 RPM, 60 seconds). If using the plate shaker feature on the iQue® platform:

9.2.1 iQue Forecyt® Menu Bar → Device → Manual Control Mode → Shaker Control → RPM → 3000

9.2.2 To begin shaking select "On," the shaker will continue to shake once this option is chosen.

9.2.3 Un-check | deselect "On" to stop shaking

Note: 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.

10.0 Add Human IFNγ and TNFα Cytokine Detection Cocktail

10.1 Add the Human IFNγ and TNFα Cytokine Detection Cocktail to the assay plate. Give the assay plate a quick spin (300 x g, 5 seconds) and a brief shake (2,000 RPM, 20 seconds) (**Table 11**).

Plate Type	Human IFNγ and TNFα Cytokine Detection Cocktail to add
96-well	10 µL
384-well	10 µL

Table 11. Volume of Human IFNγ and TNFα Cytokine Detection Cocktail to add to each well in 96- or 384-well plate

10.2 Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

11.0 Add the iQue® Cell Membrane Integrity (R/Red) Dye into the Antibody Panel Detection Cocktail

11.1 Before starting, ensure that the iQue® Cell Membrane Integrity (R/Red) Dye is completely thawed. Briefly centrifuge the vial. Gently mix the dye by pipette or vortex.

11.2 Dilute the iQue® Cell Membrane Integrity (R/Red) Dye at 1:250 into the Antibody Panel Detection Cocktail. Gently mix by pipetting up and down (**Table 12**).

Plate Type	iQue® Cell Membrane Integrity (R/Red) Dye	Antibody Panel Detection Cocktail
1 x 96 plate	8 µL	2.0 mL
5 x 96 plates	8 µL	2.0 mL
1 x 384 plates	21.6 µL	5.4 mL
5 x 384 plates	21.6 µL	5.4 mL

Table 12. iQue® Cell Membrane Integrity (R/Red) Dye dilution volumes for 96- and 384-well plates.

12.0 Add Antibody Detection Panel Cocktail to Measure Cell Viability

12.1 Obtain the mixture of the Antibody Panel Detection Cocktail containing the iQue® Cell Membrane Integrity (R/Red) Dye from **Step 11.2**. Mix gently by pipetting.

12.2 Add the mixture to a reservoir and then add the reagent to the assay plate (**Table 13**).

Note: Change the tip after dispensing to avoid cross-well contamination.

Plate Type	Volume of Antibody Panel Detection Cocktail containing the iQue® Cell Membrane Integrity (R/Red) Dye to add
96-well	10 µL
384-well	10 µL

Table 13. Volume of Antibody Panel Detection Cocktail containing the iQue® Cell Membrane Integrity (R/Red) Dye to add to each well in 96- or 384-well plate.

12.3 Give the assay plate a quick spin (300 x g, 5 seconds) to ensure that all samples are at the well bottom.

12.4 Shake the plate (2000 RPM, 20 seconds) to ensure samples are mixed thoroughly.

12.5 Cover the plate to prevent evaporation and protect from light. Incubate the plate at room temperature for 60 minutes.

13.0 Add Wash Buffer to Wash Plate

13.1 Add Wash Buffer to each well (**Table 14**).

Plate Type	Volume wash buffer to add to each well
96-well	100 µL
384-well	50 µL

Table 14. Volume of wash buffer to add to 96- or 384-well plate

13.2 Spin the assay plate (300 x g, 5 min).

13.3 Aspirate the supernatant with a plate washer.

Note: Do not touch the aspiration probe to the bottom of the plate well; do NOT aspirate the samples during this step.

14.0 Resuspend and Add Wash Buffer

14.1 Agitate the sample in the residual liquid in the plate with the plate shaker (3,000 RPM, 60 seconds). If using the plate shaker feature on the iQue® platform:

14.1.1 iQue Forecyt® Menu Bar → Device → Manual Control Mode → Shaker Control → RPM → 3000

14.1.2 To begin shaking select "On," the shaker will continue to shake once this option is chosen.

14.1.3 Un-check | deselect "On" to stop shaking

Note: 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.

14.2 Add Wash buffer to all the wells of the assay plate (**Table 15**).

Plate Type	Volume wash buffer to add to each well
96-well	20 µL
384-well	10 µL

Table 15. Volume of wash buffer to add to the 96- or 384-well plate

14.3 Give the assay plate a quick spin (300 x g, 5 seconds).

14.4 Agitate briefly to ensure homogeneous sample mixing (2000 RPM, 20 sec)

14.5 Proceed to **Steps 15 and 16: Data Acquisition and Data Analysis for 96 and 384 well plates**.

15.0 Data Acquisition for 96- and 384-Well Plates

15.1 Launch iQue Forecyt® Software.

15.2 Import the provided experiment template (included on USB key in the kit package).

15.3 Create a New Experiment using the provided template.

15.4 In the Design section, assign wells to Samples. From the wells selected, define your T cell stimulation positive wells and negative wells.

Note: This is important for later data analysis and for fine-tuning the gating of stimulated T cell populations.

15.5 In the Design section, under the Series subsection, edit | add Series to ensure proper plate layout. In the Standards subsection, edit | add Standards to ensure proper plate layout.

15.6 In the Protocol Section, adjust Sample Order if plate layout is different (i.e., horizontal instead of vertical) from the recommended plate layout in **Step 7.1**. Acquire samples from low concentration to high to minimize carryover.

- For 96-well plates see **Figure 5**.
- For 384-well plates see **Figure 6**.

Note: For samples with low cell density, adjustment to sip times may be required in the iQue Forecyt® Protocol section. See **Page 12, General Guidelines →Troubleshooting Low Cell Density →3**. Optional Protocol: Adjust Sip Time to collect sufficient cell events.

15.7 Click "Run" on the Controller window to acquire the plate.

16.0 Data Analysis for 96- and 384-Well Plates

16.1 Click on the Analysis portion of the software and review the data using tabs on the screen. Start with the "Beads and Cells" tab.

Note: Use the template with pre-set gates to observe different populations. Manually draw the gates or fine tune the existing gates from the template if needed

16.2 Review distinct populations of cells and iQue Qbeads® | Human IFNγ and TNFα Capture Beads by reviewing pre-set gates:

16.2.1 All Events → iQue Qbeads® → Singlet Beads 2 iQue Qbeads® | Human IFNγ and TNFα Capture Beads (**Figure 7**).

16.2.2 All Events → Cells → Singlet Cells → Live and Dead Cells→ CD3+ (**Figure 7**)

16.2.3 CD3+ → CD3+ & CD4+ and CD3+ & CD8+ (**Figure 8**)

16.2.3 (Optional) Live and Proliferating Cells (**Figure 9**)

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 iQue Qbeads®| Human IFNγ and TNFα Capture Beads® used for cytokine detection.

16.3 Review T cell immunophenotyping based on live cells:

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

16.3.1 CD3+ cells → CD3+ PD-1 → CD3+ Lag-3 → CD3+ Tim-3 (**Figure 8**).

16.3.2 CD3+ CD4+ cells → CD3+ CD4+ PD-1 → CD3+ CD4+ Lag-3 → CD3+ CD4+ Tim-3

16.3.3 CD3+ CD8+ cells → CD3+ CD8+ PD-1 → CD3+ CD8+ Lag-3 → CD3+ CD8+ Tim-3

16.4 Review stimulated and exhausted T cells populations of cells (**Figure 10**).

Note: Use the Positive | Negative Control Wells to Fine Tune the Gates of Exhausted cells (Example: Gate PD-1+ cells in CD3+ T Cells in an Overlay Plot with Positive Wells and Negative Wells).

16.4.1 CD3+ | PD-1+

16.4.2 CD3+ | Lag-3

16.4.3 CD3+ | Tim-3

16.5 Obtain the concentrations of IFNγ and TNFα in the Analysis section by viewing the tab labeled "2 Cytokines."

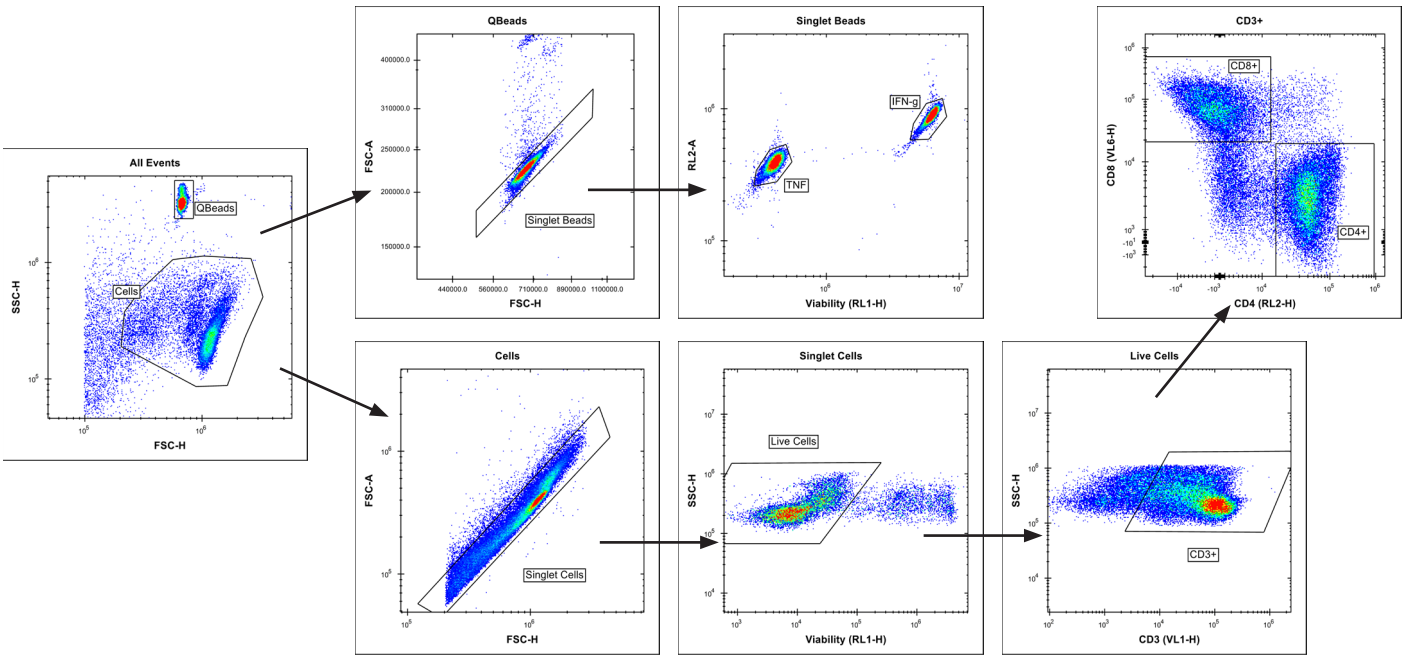


Figure 7. Gate Cell| Beads from All Events

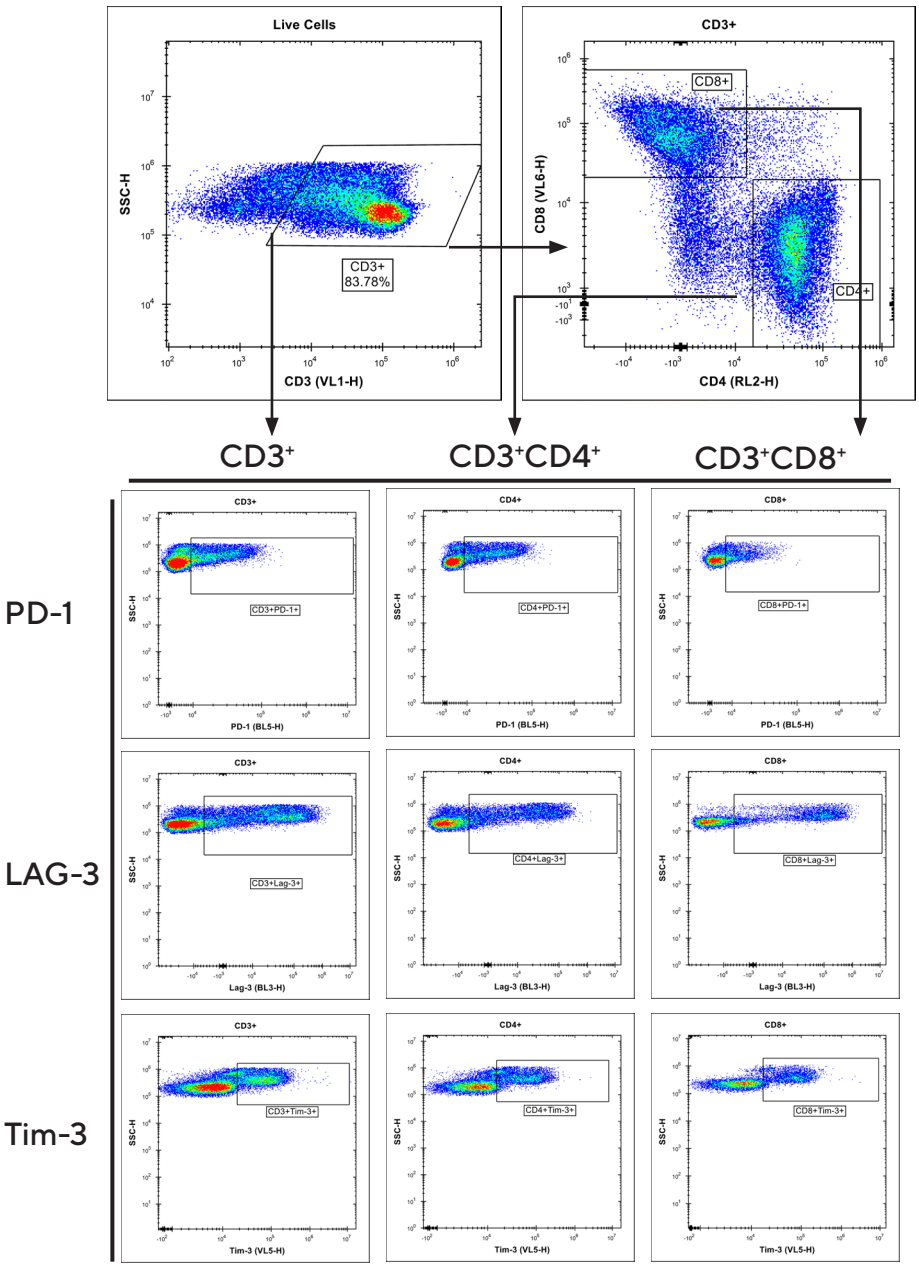


Figure 8. Gate Different T cell subsets for Inhibitory Receptors

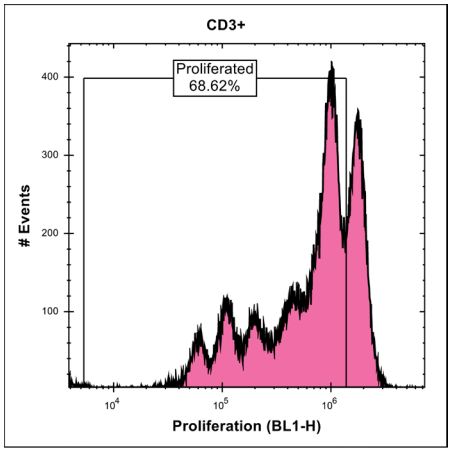


Figure 9. Histogram of Proliferating or Non-Proliferating T Cell Populations.

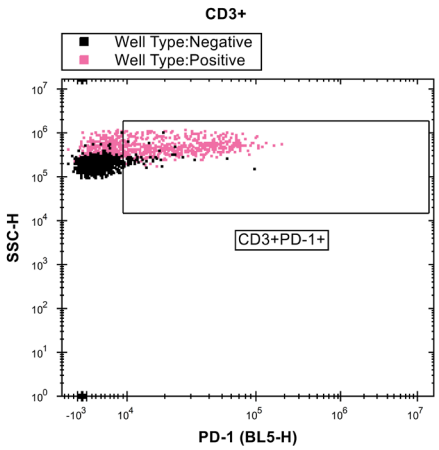


Figure 10. Positive and Negative T Cell Populations.

Cell Culture Preparation and Treatment

- Low T cell density may make it difficult to achieve statistical significance for the cell population of interest. You may need to optimize your T cell media and culture conditions prior to performing the assay. You may include recombinant human IL-2 protein (10 ng/mL) and | or other cytokine cocktails with biological activity in the culture media to help maintain or promote the T cell health | growth.
- This assay is validated in cell culture with RPMI 1640 medium with 10% fetal bovine serum. Other similar culture medium may also work in this assay.

Troubleshooting Low Cell Density

If you anticipate that you will not have enough cell events to reach statistical significance of your cell population of interest, you may have to modify your assay protocol using one or more of the optional protocols below.

1.0 Optional Protocol: Concentrate your samples.
We recommend concentrating your samples in the original culture plate if cell density is low.

1.1 Spin your cells down (300 x g, 5 minutes) in your original T cell culture plate.

1.2 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).

1.3 Transfer the concentrated cell samples into the assay plate before running the assay.

2.0 Optional Protocol: Use ultra-low binding plates to reduce cell plate attachment.
T cells usually will not attach to the well bottom/wall of the recommended plates (96w plate, Costar®, Cat. No. 3897; 384w plate, Greiner®, Cat. No. 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 an ultra-low binding plate to achieve a more precise cell count (**Table 16**). If iQue Forecyt® does not list this plate model, add it to the list (Device → Manage Plate Models → Add) and map it.

Manufacturer	Catalogue # Option 1	Catalogue # Option 2
Greiner	651970	781970
Corning	7007	4516

Table 16. Ultra Low Binding Plates Information

3.0 Optional Protocol: Adjust Sip Time to collect sufficient cell events.
You may wish to adjust the sip time to acquire enough cell events for your data analysis to reach statistical significance of your cell population of interest. 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, which assumes the lowest cell density in the culture plate is 1×10^6/mL. You may choose to increase the sip time in the assay plate to accommodate for cell densities below 1×10^6/mL/mL. 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.

4.0 Optional Protocol: Adjusting Sip Times for low cell density:

4.1 Open iQue Forecyt® under Protocol section, adjust the Sip times if necessary, by following the **Tables 17 and 18** below, to achieve the statistical significance for your cell population of interest. **Table 17** gives adjustments for 96 well plate assays and **Table 18** gives adjustments for 384 well plate assays.

4.2 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 frequency and duration in iQue Forecyt® under Protocol → Shake→ Interwell Shake → Interwell Duration. Refer to **Tables 17 and 18** for details.

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 x 10^6/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 x 10^6/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	Acquisition Time per Plate
4s Sip (default)	1800	Every 4 Wells	4 seconds	~16 mins
6s Sip	2700	Every 4 Wells	4 seconds	~20 mins
8s Sip	3600	Every 4 Wells	4 seconds	~23 mins
10s Sip	4500	Every 3 Wells	4 seconds	~27 mins
12s Sip	5400	Every 3 Wells	4 seconds	~30 mins

Table 17: Data Acquisition Adjustments for 96-well Format

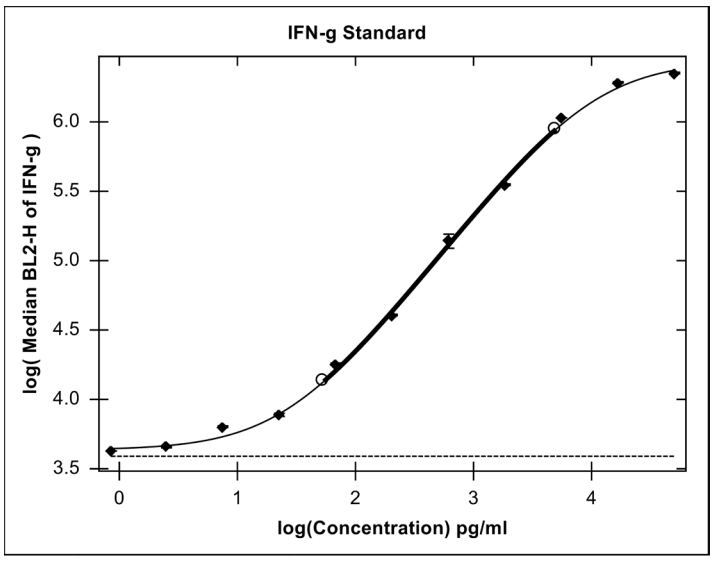
Sip Time Per Well	Cell Density in the Culture Plate	Transfer Volume	Final Volume After Resuspension in Assay Plate	Estimated Cell Density in Assay Plate	Estimated Volume Acquired (Assume 1.5 µL/second sip/well)
4s Sip (default)	1 x 10^6/mL (Assumption: the lowest possible density ~the seeding density)	5 µL (from culture plate to assay plate)	~15 µL (10 µL + residual volume)	0.3 x 10^6/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	Acquisition Time per Plate
4s Sip (default)	1800	Every 6 Wells	4 seconds	~45 mins
6s Sip	2700	Every 6 Wells	4 seconds	~57 mins
8s Sip	3600	Every 4 Wells	4 seconds	~75 mins

Table 18: Data Acquisition Adjustments for 384-well Format

Cytokine Measurements

- Dilute Protein Standards with fresh culture media.
- Only prepare cytokine standards the day of the assay.
- This assay cannot be used to measure cytokines from human sera. If you need to measure cytokines from human sera, including TNFα cytokines, you may purchase iQue Qbeads® Human Inflammation Panel Kit (Cat. No. 97097) or a custom iQue Qbeads® Human Plexscreen Kit from Sartorius which provides special diluent for human sera samples and protein standards dilution.
- Once the cytokine standards are combined and solubilized, each cytokine is 50,000 pg/mL.
- This kit includes a template with standard curve layouts. iQue Forecyt® uses 4PL with $1/Y^2$ weighting for fitting the standard curves. At the log scale, iQue Forecyt® can provide the linear range for each standard curve.
- The cytokine detection range is greater than the linear range.
- When following the recommended protocol and diluting the Human IFNγ andTNFα Cytokine Standards 1:3 in the same culture media used to grow the cell cultures and generating standard curves (**Figure 11**), the expected linear range for each cytokine is:
  - IFNγ: 55–5,000 pg/mL
  - TNFα: 85–10,000 pg/mL



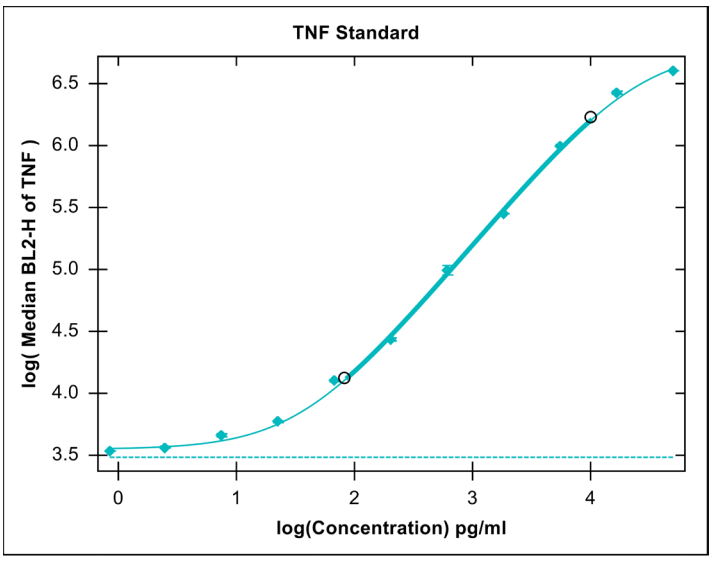


Figure 11. Representative Standard Curves (IFNγ and TNFα) with 1:3 Serial Dilutions. The IFNγ linear range 55-5,000 pg/mL is shown in the bolded black line. The TNFα linear range of 85-10,000 pg/mL is shown in the bolded teal line. The dashed line represents the fluorescent background with the standard concentration is zero.

- If you wish to multiplex multiple cytokine measurements, along with IFNγ and TNFα, you may purchase the iQue® Human T Cell Companion Kits including IL-2, IL-6, IL-13, IL-17A, GM-CSF and IL-10 (sold separately, see **Table 19**).

Cat. No.	Description
97028	iQue® Human IL-2 T Cell Companion Kit
97029	iQue® Human IL-6 T Cell Companion Kit
97030	iQue® Human IL-10 T Cell Companion Kit
97031	iQue® Human IL-13 T Cell Companion Kit
97032	iQue® Human IL-17A T Cell Companion Kit
97033	iQue® Human GM-CSF T Cell Companion Kit

Table 19. iQue® Human T Cell Companion Kits

Troubleshooting for IFNγ and TNFα Cytokine Levels Out of Linear Range in Your Samples

Generally, exhausted T cells secrete low levels of IFNγ and TNFα, depending on patient donor, cell density, cell health and cell proliferation. Since the iQue® platform has a wide linear detection range for IFNγ and TNFα, the typical assay workflow should not require any modifications such as sample dilutions. Here are two exceptions based on sample concentration:

1. **IFNγ or TNFα ABOVE linear range of detection.** If you anticipate samples of high cytokine concentrations IFNγ (> 5,000 pg/mL) or TNFα (> 10,000 pg/mL) above the linear range of detection, dilute your sample with your culture media before running the assay. Diluting your samples might result in the need to increase the sip times to acquire sufficient cell events. (**Page 12 General Guidelines: Troubleshooting Low Cell Density**)
2. **IFNγ or TNFα BELOW the linear range of detection.** If you anticipate your samples will have very low level of IFNγ (< 55 pg/mL) and TNFα (< 85 pg/mL), it is recommended you perform modifications to the standard protocol as follows:

2.1 Optional Protocol: Extend the linear range
When dealing with dilute samples by **NOT** diluting the pre- mixed Human IFNγ and TNFα Capture Beads provided in the kit. Instead do the following:

2.1.1 Skip bead dilution **(**skip **Protocol Step 6.1)**

2.1.2 Perform centrifugation and vortex of reagent vials before use **(**do perform **Steps 6.1.1 and 6.1.2)** for 96- or 384-well plate, depending on your assay.

2.1.3 Add 10 µL/well Standards | Samples (refer to **Protocol Step 7.1** and **Figures 5 or 6** depending on the type of well plate in your assay for recommended plate layout).

2.1.4 Add 10 µl/well of the premixed, **UNDILUTED** Human IFNγ and TNFα 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 covered to block the light.

Note: If using the shaker feature on the iQue® platform:

i. iQue Forecyt® Menu Bar → Device → Manual Control Mode → Shaker Control → RPM → 2000
ii. To begin shaking select "On", the shaker will continue to shake once this option is chosen.
iii. Un-check/ deselect "On" to stop shaking.

2.1.5 Add 100 µL/well fresh culture media to each well. Spin the plate (300 x g, 5 minutes). Aspirate the supernatant. Resuspend cell | beads in the residual liquid in the assay plate by strong shake (3,000 RPM, 60 seconds).

2.1.6 Prepare the iQue® Cell Membrane Integrity (R/Red) Dye into the Antibody Panel Detection Cocktail to measure cell viability **(**proceed to **Protocol Step 10.0)**

2.2 If it is not possible to extend out the linear range of cytokine detection by skipping the dilution step of the Human IFNγ and TNFα Capture Beads, perform dilution series other than a 1:3 with the Human TNFα and IFNγ Cytokine Standards.

Note: If you need to modify the top cytokine standard concentration, dilution factor, and | or the plate layout for the cytokine standards, refer to the iQue Forecyt® Reference Guide.

i. Open the webpage: https://intellicyt.com/forecyt-9.0-reference-guide/Content/Home.htm
ii. iQue Forecyt® Reference Guide →Design → Series → Edit Series.
iii. iQue Forecyt® Reference Guide → Design →Standards → Edit Standards.

Proliferation and Cell Viability Measurements

- It is optional to measure T cell proliferation in this assay. The iQue® Cell Proliferation and Encoding (B/Green) Dye is included to measure T cell proliferation. This reagent is used before preparing the cells for the microplate assay.
- The iQue® Cell Membrane Integrity (R/Red) Dye included to measure cell viability is based on a different dye than traditional Trypan Blue-based viable cell measurement. iQue® Cell Membrane Integrity (R/Red) Dye 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.

Assay Plates

- The assay plates are recommended for use with this assay kit for both a 96-well and 384- well plate format assay (**Table 20**).

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

Table 20. Recommended Assay Plates

Liquid Handling: Pipetting, Plate Washing, Shaking and Kit Usage

Plate Washers for Automated Wash and Aspiration

- We recommend a plate washer for wash and aspiration steps. Manual aspiration of plates and | or plate inversion techniques could result in severe sample loss. See **Table 21** for recommended plate washer settings. When using an automated plate washer for plate aspiration, it is recommended to optimize the plate washer settings prior to performing the iQue® Human T Cell Exhaustion Assay. To avoid removing cells and beads from your assay wells, optimize the plate washer settings for your specific plate washer instrument.

Compensation Matrix

- The template already includes the compensation matrix. It is not necessary to adjust any compensation matrix even if you use the optional iQue® Cell Proliferation and Encoding (B/Green) Dye in the assay **(Table 22)**.

Manual Pipetting

- When pipetting manually, be careful during the volume transfers to ensure the liquid in the pipette transfers completely into the assay well and does not remain on the pipette tip. This is critical during the transfer of volumes under 10 µL. It is recommended you touch the bottom of the well, the side of the well or another liquid surface in the well to ensure complete liquid transfer into the assay well. If liquid remains on the side of the assay well after pipetting, a quick spin will move all the liquid to the bottom of the well.
- Change tips after manual pipetting to avoid cross-contamination.

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

Table 21. Automated plate wash settings for BioTek ELx405 Select Plate Washer. If you have a different plate washer brand or model, it is possible to approximate the aspiration settings on a different system.

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

Table 22. Compensation Matrix

96- | 384-Well iQue® Human T Cell Exhaustion Kit Cell Culture, Cell Proliferation Labeling and T Cell Exhaustion Quick Guide

Cell Culture

Step 1.0	Culture cells at a density of ~ 2.0 x 10^6 cells/mL.	☐

Start time _____ *Stop time* _____

Optional Step 2.0: Cell Proliferation Labeling

Step 2.1	Dilute the iQue® Proliferation and Encoding (B/Green) Dye into a protein-free buffer such as HBSS buffer or PBS buffer at a dilution factor of **1:1250**		☐
⇩			
Step 2.2	Long Spin **[300 x g, 5 min.]** to wash cells with protein-free buffer. Resuspend in protein-free buffer 1–4 million/mL.		☐
⇩			
Step 2.3	Combine cells and diluted iQue® Proliferation and Encoding (B/Green) Dye. Mix.	**Incubate RT 15 min, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 2.4	Long Spin **[500 x g, 5 min.]** to wash cell | dye mixture in a 2X volume of complete media. Repeat 2X.	☐
⇩		
Step 2.5	Resuspend cells at density needed for assay, typically >1.0 x 10^6 cells/mL.	☐

T Cell Stimulation

Step 3.0	Stimulate T cells with T Cell Stimulation reagent(s) for positive control(s) and experimental treatment(s).	☐

96-Well iQue® Human T Cell Exhaustion Kit Quick Guide

Reagent Preparation

Step 4.0	Combine 2 different Lyophilized Human IFNγ and TNFα Cytokine Standards into the same tube. **Add 200 µL fresh culture media to solubilize.** ☐ **INFγ** ☐ **TNFα**	**Incubate RT 15 min.**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 5.0	Make **1:3 serial dilution** of Combined Cytokine Standards with fresh culture media.		☐
	⇩		
Step 6.0	Dilute the pre-mixed Human IFNγ and TNFα Capture Beads with **9 times volume** of fresh culture media. ☐ **INFγ and TNFα**		☐

Assay Protocol

Step 7.0	Add **10 µL/well** Standards or Samples to appropriate wells.		☐
	⇩		
Step 8.0	Add **100 µL/well** pre-diluted Human IFNγ and TNFα Capture Beads. Do not shake.	**Incubate RT 1 hour, Dark**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 9.0	Long Spin **[300 x g, 5 min.]** Aspirate supernatant. Resuspend Cells | Beads in residual liquid with Strong Shake **[3000 RPM, 60 sec.]**		☐
	⇩		
Step 10.0	Add **10 µL/well** Human IFNγ and TNFα Cytokine Detection Cocktail. Quick Spin| Brief Shake*	**Incubate RT 1 hour, Dark**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 11.0	Add iQue® Cell Membrane Integrity (R/Red) Dye to Antibody Panel Detection Cocktail (**1:250 dilution**).		☐
	⇩		
Step 12.0	Add **10 µL/well** Antibody Panel Detection Cocktail with iQue® Cell Membrane Integrity (R/Red) Dye. Quick Spin | Brief Shake *	**Incubate RT 1 hour, Dark**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 13.0	Add **100 µL/well** Wash Buffer. Long Spin [**300 x g, 5 min.]** Aspirate supernatant.		☐
	⇩		
Step 14.0	Resuspend Cells|Beads in residual liquid with Strong Shake. **[3000 RPM, 60 sec.]** Add **20 µL/well** Wash Buffer.		☐

*Quick Spin = 300 x g, 5 sec. | Brief Shake = 2000 RPM, 20 sec.

384-Well iQue® Human T Cell Activation Kit Quick Guide

Reagent Preparation

Step 4.0	Combine 2 different Lyophilized Human IFNγ and TNFα Cytokine Standards into the same tube. **Add 200 µL fresh culture media to solubilize.** ☐ **INFγ** ☐ **TNFα**	**Incubate RT 15 min.**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 5.0	Make **1:3 serial dilution** of Combined Cytokine Standards with fresh culture media.		☐
	⇩		
Step 6.0	Dilute the pre-mixed Human IFNγ and TNFα Capture Beads with **9 times volume** of fresh culture media. ☐ **INFγ and TNFα**		☐

Assay Protocol

Step 7.0	Add **10 µL/well** Standards or Samples to appropriate wells.		☐
	⇩		
Step 8.0	Add **100 µL/well** pre-diluted Human IFNγ and TNFα Capture Beads. Do not shake.	**Incubate RT 1 hour, Dark**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 9.0	Long Spin **[300 x g, 5 min.]** Aspirate supernatant. Resuspend Cells | Beads in residual liquid with Strong Shake **[3000 RPM, 60 sec.]**		☐
	⇩		
Step 10.0	Add **10 µL/well** Human IFNγ and TNFα Cytokine Detection Cocktail. Quick Spin | Brief Shake*	**Incubate RT 1 hour, Dark**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 11.0	Add iQue® Cell Membrane Integrity (R/Red) Dye to Antibody Panel Detection Cocktail (**1:250 dilution**).		☐
	⇩		
Step 12.0	Add **10 µL/well** Antibody Panel Detection Cocktail with iQue® Cell Membrane Integrity (R/Red) Dye. Quick Spin | Brief Shake *	**Incubate RT 1 hour, Dark**	☐
	Start time _____ ⇩ *Stop time* _____		
Step 13.0	Add **50 µL/well** Wash Buffer. Long Spin **[300 x g, 5 min.]** Aspirate supernatant.		☐
	⇩		
Step 14.0	Resuspend Cells|Beads in residual liquid with Strong Shake. **[3000 RPM, 60 sec.]** Add **10 µL/well** Wash Buffer.		☐

*Quick Spin = 300 x g, 5 sec. | Brief Shake = 2000 RPM, 20 sec.

Sales and Service Contacts

For further information, visit www.sartorius.com

Sartorius BioAnalytical Instruments, Inc.

www.sartorius.com/ique

Publication No. #17130 Rev B

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
Telephone +1 734 769 1600
E-Mail: AskAScientist@sartorius.com
Online Store: shop.intellicyt.com

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Telephone +44 (0) 1763 227400
E-Mail: euorders.UK03@sartorius.com

APAC
Essen BioScience K.K.
4th floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Telephone: +81 3 6478 5202
E-Mail: orders.US07@sartorius.com