SARTORIUS

Product Guide

iQue® Human T Cell Memory Kit

Product Information

Presentation, Storage and Stability

The iQue® Human T Memory Cell Kit contains reagents for measurement of T memory cell formation.

Compatible with iQue® platform with VBR option		
Product Name	**Cat. No.**	**Format**
iQue® Human T Cell Memory Kit	97035	1 x 96 wells
iQue® Human T Cell Memory Kit	97036	5 x 96 wells
iQue® Human T Cell Memory Kit	97037	1 x 384 wells
iQue® Human T Cell Memory Kit	97038	5 x 384 wells

Table 1. Product Information
Note: The 1 x 384-well kit has enough reagents to run 2 x 96-well plates, NOT 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 5x384 well	Storage	Stability
Human IFNγ and IL-10 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
IFNγ and IL-10 2 Separate Standards	1 vial for each cytokine	5 vials for each cytokine	1 vial for each cytokine	5 vials for each cytokine	2-8°C	
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: 8 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 (B/Red) Dye	1 vial 100 µL	5 vials 100 µL	1 vial 250 µL	5 vials 250 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 Memory Kit enables high-throughput, multiplexed measurements of T cell phenotype, memory state, health and secreted cytokines from the same assay well containing a mixture of cells and cytokine detection beads (**Figure 1**). The kit is designed to simultaneously measure, in each assay well, these endpoints:

- Immune Cell Phenotypes : (CD3+, CD4+, CD8+)
- T cell naïve | memory | effector markers each immunophenotype: CD45RA, CD45RO, CD27, CD62L, and CD95
- Secreted effector cytokines: IFNγ and IL-10

The assay kit has been validated to run on the iQue® 3 (VBR configuration) which has a wide dynamic range without PMT (photomultiplier tube) 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:

- 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
  - T naive(TN)
  - T stem cell-like memory (TSCM)
  - T central memory (TCM)
  - T transitional memory (TTM)
  - T effector memory (TEM)
  - T effector memory re-expressing CD45RA (TEMRA)
  - T terminal effector (TTE)
- T cell effector cytokine secretion, quanitified in a sandwich immunoassay with iQue Qbeads® | Human IFNγ and IL-10 Capture Beads:
  - IL-10
  - TNFα (produced by predominantly Th1 and CD8+)

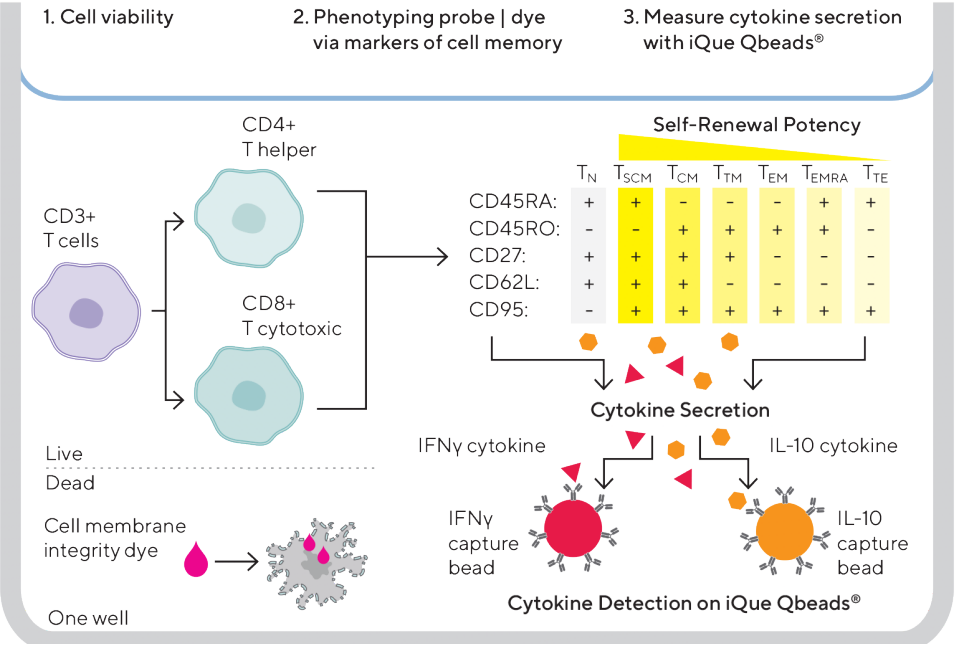


Figure 1. Illustration of the iQue® Human T Cell Memory Kit assay principles. Live cells are separated from dead cells based on the staining with iQue® Cell Membrane Integrity (B/Red) Dye. Basic T cell phenotypes are measured by staining with CD3, CD4, and CD8 markers. T cell subsets TN, TSCM, TCM, TTM, TEM, TEMRA, TTE 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 IL-10 are also quantified by iQue Qbeads® | Human IFNγ and IL-10 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)
CD45RO	405	445/45	V/Blue	VL1
CD8	405	572/28	V/Yellow	VL3
CD4	405	615/24	V/Orange	VL4
CD3	405	780/60	V/Crimson	VL6
CD62L	488	530/30	B/Green	BL1
IFNγ and IL-10	488	572/28	B/Yellow	BL2*
iQue® Cell Membrane Integrity (B/Red) Dye	488	675/30	B/Red	BL4
CD45RA	488	780/60	B/Crimson	BL5
CD95	640	675/30	R/Red	RL1
CD27	640	780/60	R/Crimson	RL2

Table 3. Laser Detection Channels for Components of the iQue® Human T Cell Memory Kit
*Cytokine quantitation in B/Yellow channel and iQue Qbeads® are distinguished using the R/Red and R/Crimson channels. In addition, R/Red is used for CD95 detection and R/Crimson is used for CD27 detection.

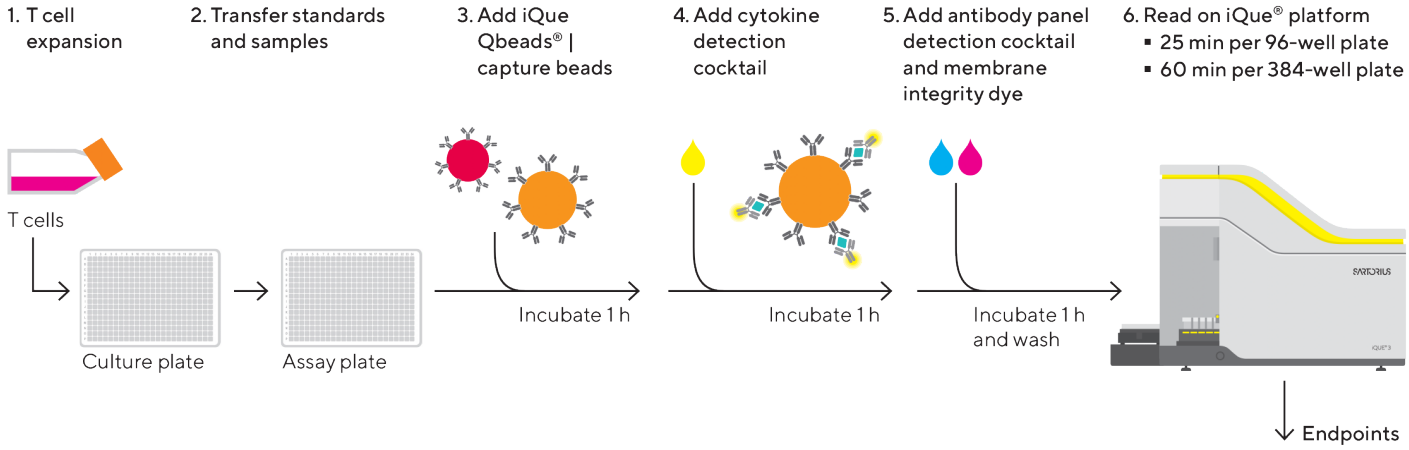
Note: This assay is only compatible with the iQue® platform using the violet, blue and red (VBR) laser configuration. Other iQue® platforms including iQue®3, iQue® PLUS (VYB lasers), and iQue® PLUS (BR lasers) will NOT work with this assay due to the detection channel limitation.

Note: This assay is not compatible for cells that 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		T Naive/Memory/Effector ID							Secreted Cytokines		Relative Cell Count	Viability
CD3+	CD4+	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{EM}	T_{EMRA}	T_{TE}	IFNγ	IL-10	+	+ | -
	CD8+	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{EM}	T_{EMRA}	T_{TE}				

Figure 2. iQue® Human T Cell Memory 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 IL-10 Capture Beads. After incubating 60 minutes, the Human IFNγ and IL-10 Cytokine Detection Cocktail (anti-IFNγ and anti-IL-10) is added to the plate. After 60 minutes incubation, a fluorescent Antibody Panel Detection Cocktail against CD3, CD4, CD8, CD45RA, CD45RO, CD27, CD62L, CD95 and the iQue® Cell Membrane Integrity (B/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, 8 surface activation markers expressed at different stages, and 2 secreted effector cytokines will be measured as the final readouts. In the table, "+" means highly expressed |secreted, "+|-"means partially expressed |secreted, "-"means low or no expression |secretion.

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 v-bottom assay plate (Sartorius®, Cat. No. 90151) or similar
  - 384-well v-bottom 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) or Phytohemagglutinin PHA (10 µg/mL)
- Plate washer (such as BioTek® model ELx405)
- 12-channel pipette reservoir (Example source: VWR®, Cat. No. 80092-466) (optional for preparing serial titrations)
- hIL-4 (PeproTech® Cat. No. 200-04)

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) (**Table 4**).

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

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

Note: Your experimental design might include an optional treatment plate(s) with positive and negative controls of T cell activation. 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 memory cell stimulation, as well as any investigatory drug treatments or conditions. You will transfer 5 µL or 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 Stimulate T Cells

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

Note: T memory cell stimulation reagents may require optimization based on your cells and assay design. Please review literature and product information on the chosen T memory cell stimulation reagents applied to T cells 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.

2.2 Incubate T cells activation treatment plate at 37°C 5% CO_2 for a duration required by your experimental design, typically 1–14 days.

3.0 Combine and Rehydrate the Human IFNγ and IL-10 Cytokine Standards

3.1 Combine the two lyophilized Human IFNγ and IL-10 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 IL-10 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.

3.2 Rehydrate the Human IFNγ and IL-10 Cytokine Standards in fresh culture media

3.2.1 Slowly add 1000 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.

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

3.2.3 Once dissolved, gently mix by pipetting up and down.

Note: Do NOT vortex.

4.0 Make a 1:2 Serial Dilution of the Combined Human IFNγ and IL-10 Cytokine Standards

4.1 Prepare a 1:2 serial dilution (top concentration 10,000 pg/mL) of the reconstituted Cytokine Standards with fresh culture medium (**Figure 3** for 96 well and **Figure 4** for 384 well).

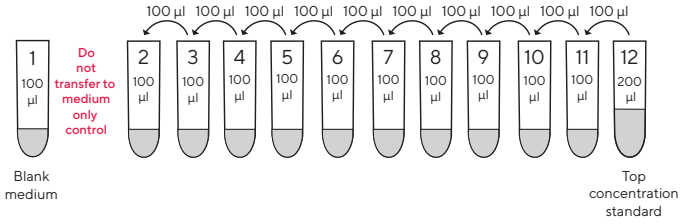


Figure 3. 96 Well Dilution Series

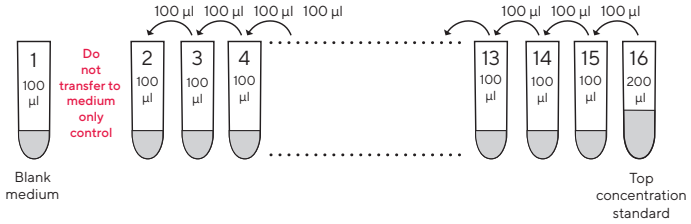


Figure 4. 384 Well Dilution Series

4.1.1 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

4.1.2 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

4.1.3 Add the 10,000 pg/mL solubilized cytokine standards to the indicated tube for your plate type.

- For 96-well plate protocol, add 200 µL solubilized cytokine standard to tube #12
- For 384-well plate protocol, add 200 µL solubilized cytokine standard to tube #16

4.1.4 Begin the serial titration by transferring the 10,000 pg/mL solubilized cytokine standard to dilution tubes.

- For a 96-well plate, transfer 100 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 100 µL of standard from tube #16 into tube #15. Gently pipette up and down at least 5 times to completely mix the solution.

4.1.5 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).

4.1.6 Set the cytokine standard serial dilutions 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

5.0 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.

5.1 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.

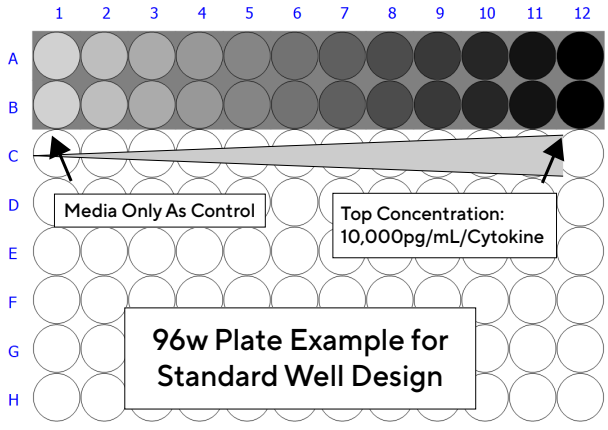


Figure 5. Recommended 96 well plate layout.

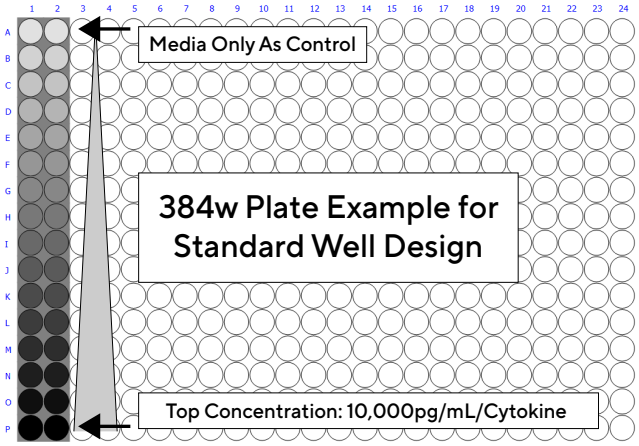


Figure 6. Recommended 384 well plate layout.

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.

Note: It is possible to run the standards and samples on separate plates if desired.

5.2 Transfer the Human IFNγ and IL-10 Cytokine Standards prepared in **Step 4.1.6** to the wells in the assay plate (**Table 5**).

Plate Type	Cytokine Standards to Add to Wells
96-well	10 µL
384-well	5 µL

Table 5. Volume of cytokine standards to 96 or 384 well plate

5.3 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.

5.4 Transfer the cell | supernatant mixture to the wells designated for samples in the assay plate, according to the plate layout you designed in **Step 5.1** (**Table 6**).

Plate Type	Cell | Supernatant Sample to wells
96-well	10 µL
384-well	5 µL

Table 6. Volume of cell | supernatant to add to 96- or 384-well plate

6.0 Add Human IFNγ and IL-10 Capture Beads

6.1 Obtain the pre-mixed Human IFNγ and IL-10 Capture Beads. Vortex vigorously for 10 seconds.

6.2 Transfer 10 mL of the pre-mixed, vortexed Human IFNγ and IL-10 Capture Beads to a reservoir.

6.3 Add the pre-mixed, vortexed Human IFNγ and IL-10 Capture Beads to a reservoir and then add the reagent to each well of the assay plate (**Table 7**).

Note: Change the tip 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.

	Human IFNγ and IL-10 Capture Beads
96-well	10 µL
384-well	10 µL

Table 7. Volume of capture beads to add to each well in 96- or 384-well plate

6.4 Spin the assay plate (300 x g, 5 sec) to ensure reagents and samples are at the bottom of the assay plate.

6.5 Agitate the sample in the residual liquid in the plate with the plate shaker (2,000 RPM, 20 seconds). If using the shaker feature on the iQue® platform:

6.5.1 iQue Forecyt® Menu Bar → Device → Manual Control Mode → Shaker Control → RPM → 2000

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

6.5.3 Un-check | deselect "On" to stop shaking.

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

7.0 Add Human IFNγ and IL-10 Cytokine Detection Cocktail

7.1 Add 10 µL Human IFNγ and IL-10 Cytokine Detection Cocktail per well 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).

7.2 Cover the plate to prevent evaporation ate at room temperature for 60 minutes.

8.0 Mix iQue® Cell Membrane Integrity (B/Red) Dye into Antibody Panel Detection Cocktail

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

8.2 Transfer the appropriate volume for a 1:25 dilution of the iQue® Cell Membrane Integrity (B/Red) Dye into Antibody Panel Detection Cocktail (**Table 8**). Gently mix by pipetting up and down.

Plate Format	iQue® Cell Membrane Integrity (B/Red) Dye	Antibody Panel Detection Cocktail
1 x 96-well	48 µL	1.20 mL
5 x 96-well	240 µL	6.0 mL
1 x 384-well	192 µL	4.8 mL
5 x 384-well	960 µL	24.0 mL

Table 8. Dilution Volumes for Antibody Detection Panel cell viability measurements.

8.3 Set aside the mixture for later.

Note: Once prepared, the iQue® Cell Membrane Integrity (B/Red) Dye and Antibody Panel 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.

9.0 Add the Antibody Panel Detection Cocktail containing iQue® Cell Membrane Integrity (B/Red) Dye Mixture into the Assay Plate

9.1 Obtain the mixture of the Antibody Panel Detection Cocktail containing the iQue® Cell Membrane Integrity (B/Red) Dye from **Step 8.3**. Mix gently by pipetting.

9.2 Transfer the mixture to a reservoir and then add the reagent to the assay plate (**Table 9**).

Plate Type	Volume Antibody Panel Detection Cocktail to add to each well
96-well	10 µL
384-well	5 µL

Table 9. Volume of Antibody Panel Detection Cocktail containing the iQue® Cell Membrane Integrity (B/Red) Dye to add to each well in 96- or 384-well plate.

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

9.4 Shake the plate (2000 RPM, 20 seconds) to resuspend cells in residual liquid.

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

10.0 Add Wash Buffer to Wash Plate

10.1 Add Wash Buffer to each well (**Table 10**).

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

Table 10. Volume wash buffer to add to 96- or 384-well plate

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

10.3 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.

11.0 Resuspend and Add Wash Buffer

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

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

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

11.1.3 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.

11.2 Add Wash buffer to all the wells of the assay plate (**Table 11**).

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

Table 11. Volume of wash buffer to add to the 96- or 384-well plate

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

Note: A brief agitation (2000 RPM, 20 sec) may ensure homogeneous sample mixing.

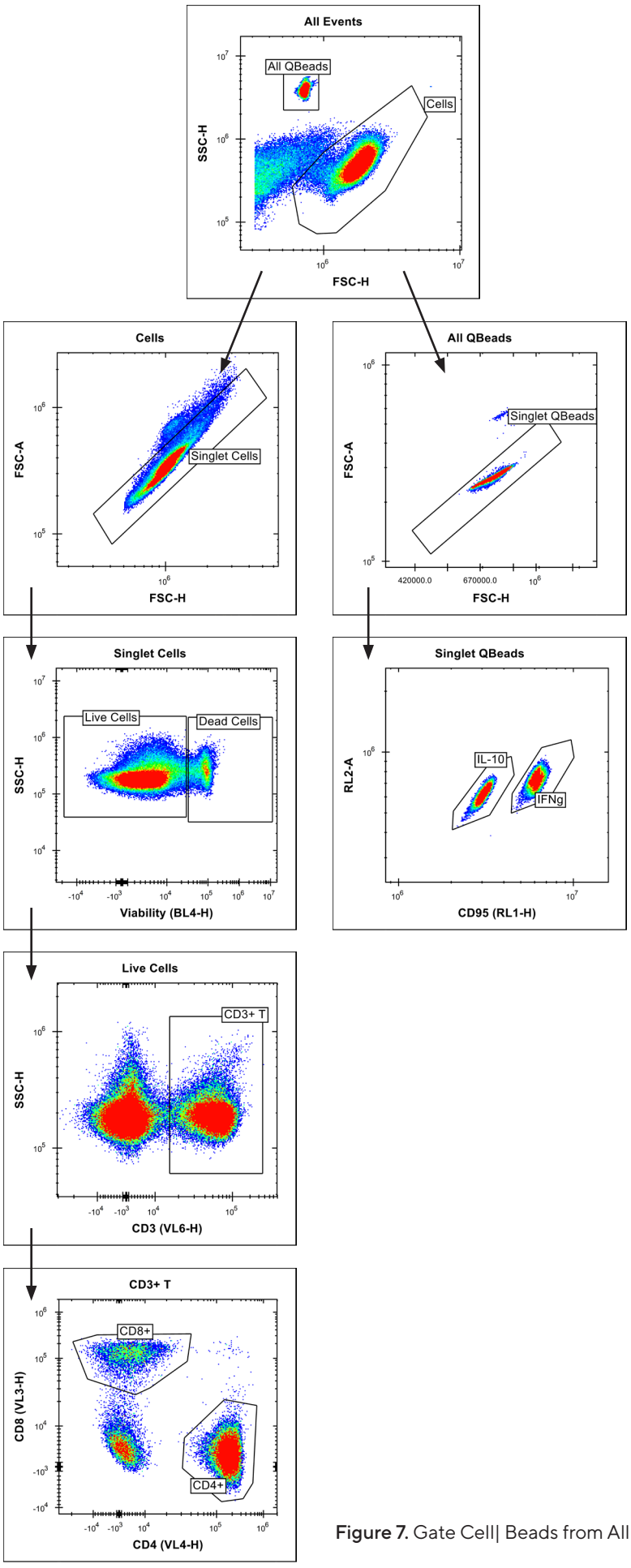


Figure 7. Gate Cell| Beads from All Events

12.0 Data Acquisition for 96- and 384-Well Plates

12.1 Launch iQue Forecyt® Software.

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

12.3 Create a New Experiment using the provided template.

12.4 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 activated T cell populations.

12.5 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.

12.6 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 5.1. 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 13, General Guidelines →Troubleshooting Low Cell Density →3. Optional Protocol: Adjust Sip Time to collect sufficient cell events**.

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

13.0 Data Analysis for 96- and 384-well plates

Note: CD95+ cells may have 2 populations (high and low expression) depending on treatments and donors. T naïve | 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, TEMRA may not be seen in every sample.

Note: 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. 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).

13.1 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.

13.2 Review distinct populations of cells and iQue Qbeads®| Human IFNγ and IL-10 Capture Beads by reviewing pre-set gates:

13.2.1 All Events → Singlet Beads → 2 iQue Qbeads®| Human IFNγ and IL-10 Capture Beads (See **Figure 7**).

13.2.2 All Events → Singlet Cells → Live and Dead Cells → CD3+ (See **Figure 7**).

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 IL-10 Capture Beads® used for cytokine detection.

13.3 Gate T Naïve | Memory | Effector Cells from CD3+ T Cells:

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

13.3.1 CD3+ cells → RA+ RO- → RA+ RO+ → RA- RO+ cells (See **Figure 8**).

13.3.2 RA+ RO- CD95- → RA+ RO- CD95+ → RA+ RO+ CD95+ → RA- RO+ CD95+

13.3.3 RA+ RO- CD95- CD62L+ CD27+

13.3.4 RA+ RO- CD95+ CD62L- CD27+

13.3.5 RA+ RO- CD95+ CD62L- CD27-

13.3.6 RA+ RO+ CD95+ CD62L- CD27-

13.3.7 RA- RO+ CD95+ CD62L+ CD27+

13.3.8 RA- RO+ CD95+ CD62L- CD27+

13.3.9 RA- RO+ CD95+ CD62L- CD27-

13.4 Create 14 Logical Populations to Analyze Different CD4+ or CD8+ T Naïve | Memory | Effector Cells (**Figure 9**):

13.4.1 Open iQue Forecyt® analysis tab → populations sub-tab

13.4.2 Create the Boolean logical populations → CD4+ Tn

Note: For information on creating the Boolean logical populations, refer to iQue Forecyt® Reference Guide.

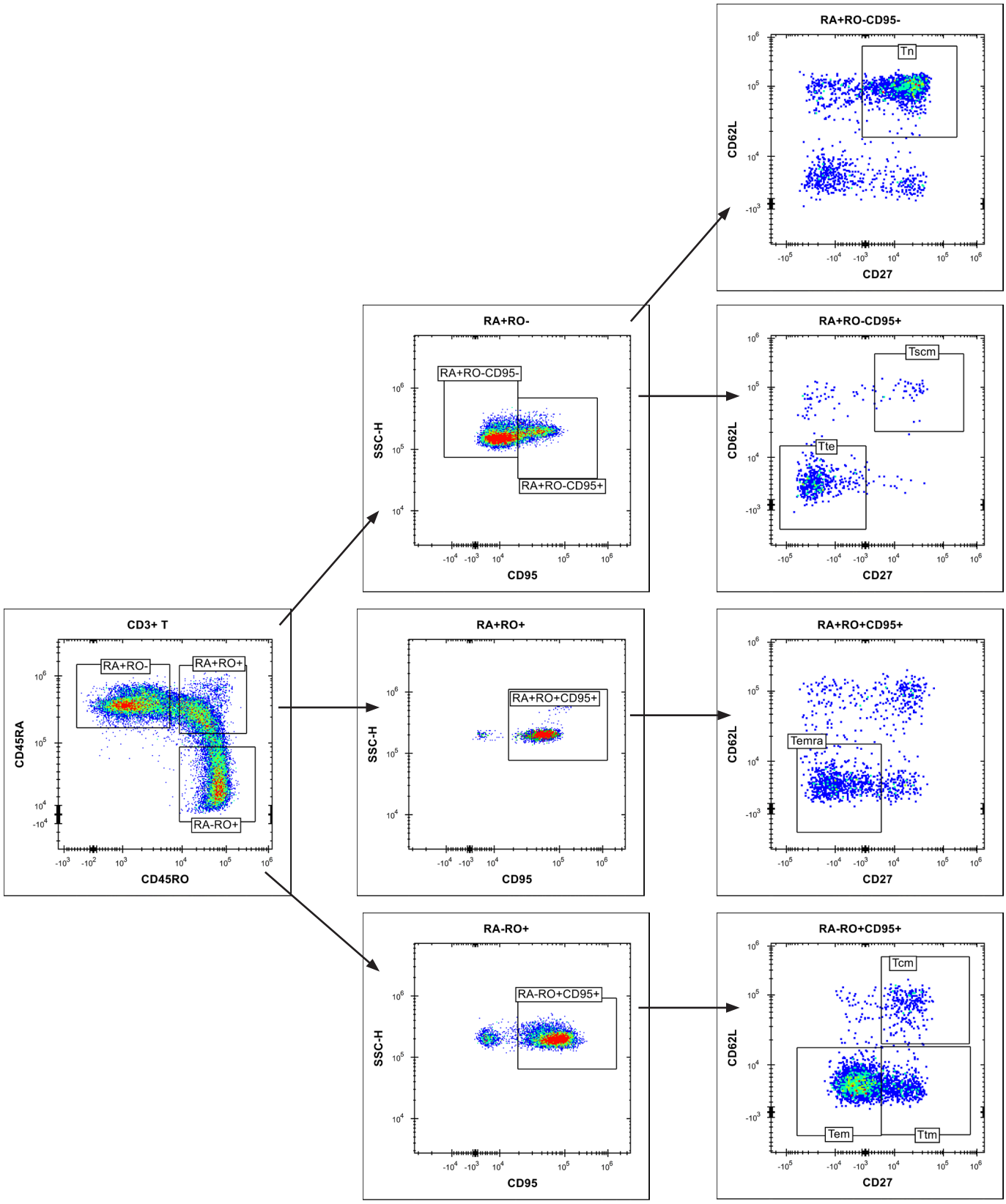


Figure 8. Gate Different Cell Phenotypes: Naïve | Memory | Effector

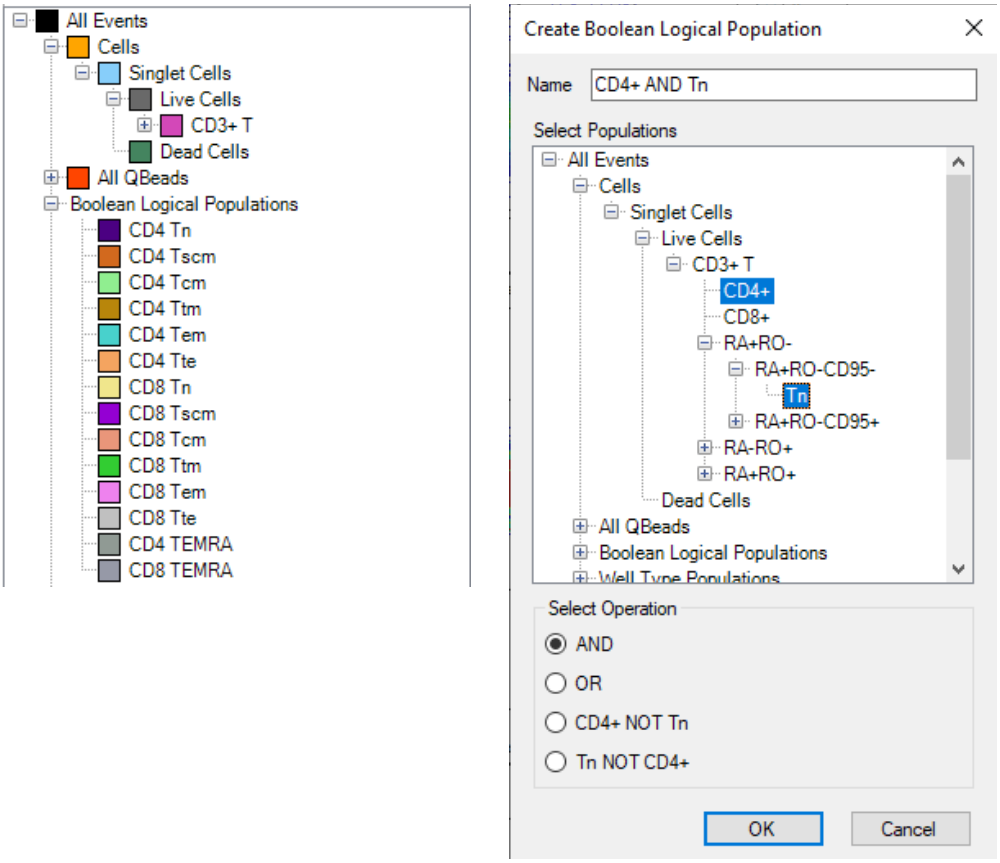


Figure 9. Creating Boolean Logical Populations

General Guidelines

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-4 or IL-7 protein (10 ng/mL) 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.
- 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.

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, resuspend 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. 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 (96-well plate, Costar®, Cat. No. 3897; 384-well 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 12**). If iQue Forecyt® does not list this plate model, add it to the list (Device → Manage Plate Models → Add) and map it.

Manufacturer	Cat. No.	Cat. No.
Greiner	651970	781970
Corning	7007	4516

Table 12. Ultra Low Binding Plates Information

3. 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. 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.

3.0 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 13 and 14**, to achieve the statistical significance for your cell population of interest. **Table 13** gives adjustments for 96 well plate assays and **Table 14** 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 13 and 14** 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)
8s 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)	0.35 x 10^6/mL (Assume wash causes 10% cell loss)	12 µL
10s Sip					15 µL
12s Sip					18 µL
15s Sip					22.5 µL

Sip Time Per Well	Estimated Cell Events Acquired Per Well	Inter-well Shake Frequency	Inter-Well Shake Duration	Acquisition Time per Plate
8s Sip (default)	4200	Every 4 Wells	8 seconds	~25 mins
10s Sip	5250	Every 3 Wells	10 seconds	~28 mins
12s Sip	6300	Every 3 Wells	12 seconds	~32 mins
15s Sip	7875	Every 3 Wells	15 seconds	~39 mins

Table 13: 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)
6s Sip (default)	1 x 10^6/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 x 10^6/mL (Assume wash causes 10% cell loss)	9 µL
9s Sip					13.5 µL

Sip Time Per Well	Estimated Cell Events Acquired Per Well	Inter-well Shake Frequency	Inter-Well Shake Duration	Acquisition Time per Plate
6s Sip	5400	Every 6 Wells	6 seconds	~60 mins
9s Sip	8100	Every 4 Wells	9 seconds	~95 mins

Table 14: 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 IL-10 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 10,000 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 and IL-10 Cytokine Standards 1:2 in the same culture media used to grow the cell cultures and generating standard curves (**Figure 10**), the expected linear range for each cytokine is:
  - IFNγ: 10–2,100 pg/mL
  - IL-10: 10–6,400 pg/mL

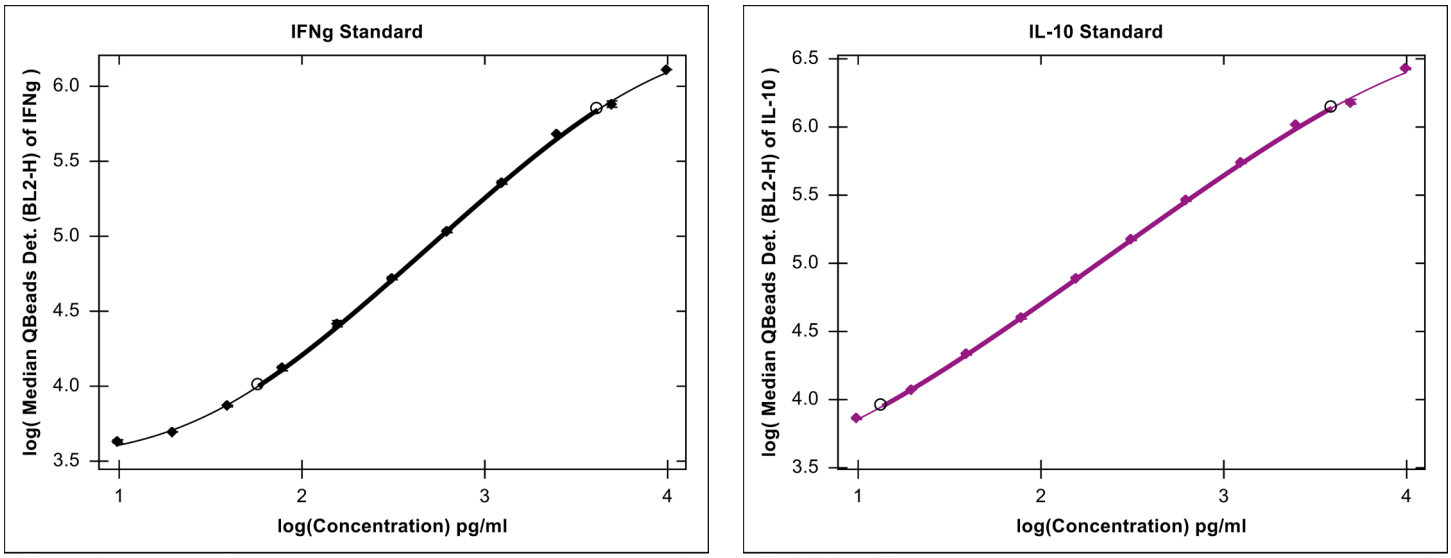


Figure 10. Representative Standard Curves (IFNγ and IL-10) with 1:2 Serial Dilutions. The IFNγ linear range 10–2,100 pg/mL is shown in the bolded black line. The IL-10 linear range of 10-6,400 pg/mL is shown in the bolded purple line. The dashed line represents the fluorescent background with the standard concentration is zero.

- If you wish to multiplex multiple cytokine measurements, along with IFNγ and IL-10, you may purchase the iQue® Human T Cell Companion Kits including IL-2, IL-6, IL-13, IL-17A, GM-CSF and TNFα (sold separately, see **Table 15**).

Catalogue #	Description
97028	Que® Human IL-2 T Cell Companion Kit
97029	Que® Human IL-6 T Cell Companion Kit
97031	Que® Human IL-13 T Cell Companion Kit
97032	Que® Human IL-17A T Cell Companion Kit
97033	Que® Human GM-CSF T Cell Companion Kit
97034	Que® Human TNF T Cell Companion Kit

Table 15. iQue® Human T Cell Companion Kits

Troubleshooting for IFNγ and IL-10 Cytokine Levels Out of Linear Range in Your Samples

Generally, stimulated T cells secrete high levels of IFNγ and IL-10, depending on patient donor, cell density, cell health and cell proliferation. Since the iQue® platform has a wide linear detection range for IFNγ and IL-10, the typical assay workflow should not require any modifications such as sample dilutions. There is an exceptions based on sample concentration:

1.0 **IFNγ or IL-10 ABOVE linear range of detection.** If you anticipate samples of high cytokine concentrations IFNγ (>2,100 pg/mL) or IL-10 (> 6,400 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. (See **Page 13, General Guidelines: Troubleshooting Low Cell Density**)

2.0 **Perform a dilution series other than a 1:2 with the Human IL-10 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.

Cell Viability Measurements

- The iQue® Cell Membrane Integrity (B/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 (B/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 16**).

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

Table 16. 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 17** 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 Memory Kit. To avoid removing cells and beads from your assay wells, optimize the plate washer settings for your specific plate washer instrument.

Shaking

- This assay requires shaking to mix the sample | reagents. If you don't have a separate shaker, you can use the one on the iQue® platform as described in **Protocol and Procedures, Page 7, Step 6.5.1**.

Compensation Matrix

- The template already includes the compensation matrix. It is not necessary to adjust the compensation matric (**Table 18**).

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 17. 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	CD62L	Viability	CD45RA	CD95	CD27	CD45RO	CD8	CD4	CD3
CD62L		1.65	0.06	0.00	0.00	0.01	1.59	0.78	0.00
Viability	1.64		12.85	6.32	0.77	0.22	3.61	14.54	2.68
CD45RA	0.48	0.28		0.02	22.70	0.01	0.28	0.20	13.43
CD95	0.00	0.77	0.04		7.15	0.00	0.01	0.10	0.32
CD27	0.01	0.03	0.67	5.76		0.01	0.03	0.03	4.96
CD45RO	0.01	0.01	0.00	0.00	0.00		4.98	2.07	0.03
CD8	0.48	3.00	0.12	0.01	0.00	4.53		97.28	1.36
CD4	0.01	2.53	0.16	0.10	0.01	0.94	5.94		2.99
CD3	0.03	0.02	3.48	0.17	19.22	3.24	0.40	0.42	

Table 18. Compensation Matrix

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

Culture Cells

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

Start time _____ ⇩ *Stop time* _____

T Cell Stimulation

Step 2.0	Stimulate T cells with reagent(s) for positive control(s) and experimental treatment(s).	☐

Reagent Preparation

Step 3.0	Combine 2 different Lyophilized Human IFNγ and IL-10 Cytokine Standards into the same tube. **Add 1,000 µL fresh culture media to solubilize.** ☐ **IFNγ** ☐ **IL-10**	**Incubate RT 15 min.**	☐

Start time _____ ⇩ *Stop time* _____

Step 4.0	Do **1:2 serial** dilution of Combined Cytokine Standards with fresh culture media.	☐

Assay Protocol

Step 5.0	Add **10 µL/well** Standards or Samples to appropriate wells.		☐
	⇩		
Step 6.0	Add **10 µL/well pre-mixed Human INFγ and IL-10 Capture Beads. Quick Spin | Brief Shake** *	**Incubate RT 1 hour, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 7.0	Add **10 µL/well** Human IFNγ and IL-10 Cytokine Detection Cocktail. Quick Spin| Brief Shake*	**Incubate RT 1 hour, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 8.0	Add iQue® Cell Membrane Integrity (B/Red) Dye to Antibody Panel Detection Cocktail (**1:25 dilution**).		☐
	⇩		
Step 9.0	Add **10 µL/well** Antibody Panel Detection Cocktail with iQue® Cell Membrane Integrity (B/Red) Dye. Quick Spin | Brief Shake	**Incubate RT 1 hour, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 10.0	Add **100 µL/well** Wash Buffer. Long Spin **[300 x g, 5 min]**. Aspirate supernatant.	☐
	⇩	
Step 11.0	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 Memory Kit Quick Guide

Culture Cells

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

Start time _____ ⇩ *Stop time* _____

T Cell Stimulation

Step 2.0	Stimulate T cells with reagent(s) for positive control(s) and experimental treatment(s).	☐

Reagent Preparation

Step 3.0	Combine 2 different Lyophilized Human IFNγ and IL-10 Cytokine Standards into the same tube. **Add 1,000 µL fresh culture media to solubilize.** ☐ **IFNγ** ☐ **IL-10**	**Incubate RT 15 min.**	☐

Start time _____ ⇩ *Stop time* _____

Step 4.0	Do **1:2 serial dilution** of Combined Cytokine Standards with fresh culture media.	☐

Assay Protocol

Step 5.0	Add **10 µL/well** Standards or Samples to appropriate wells.		☐
⇩			
Step 6.0	Add **10 µL/well** pre-mixed Human INFγ and IL-10 Capture Beads. Quick Spin | Brief Shake *	**Incubate RT 1 hour, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 7.0	Add **10 µL/well** Human IFNγ and IL-10 Cytokine Detection Cocktail. Quick Spin| Brief Shake*	**Incubate RT 1 hour, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 8.0	Add iQue® Cell Membrane Integrity (B/Red) Dye to Antibody Panel Detection Cocktail (**1:25 dilution**).		☐
⇩			
Step 9.0	Add **10 µL/well** Antibody Panel Detection Cocktail with iQue® Cell Membrane Integrity (B/Red) Dye. Quick Spin | Brief Shake	**Incubate RT 1 hour, Dark**	☐

Start time _____ ⇩ *Stop time* _____

Step 10.0	Add **50 µL/well** Wash Buffer. Long Spin **[300 x g, 5 min]**. Aspirate supernatant.	☐
⇩		
Step 11.0	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.

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