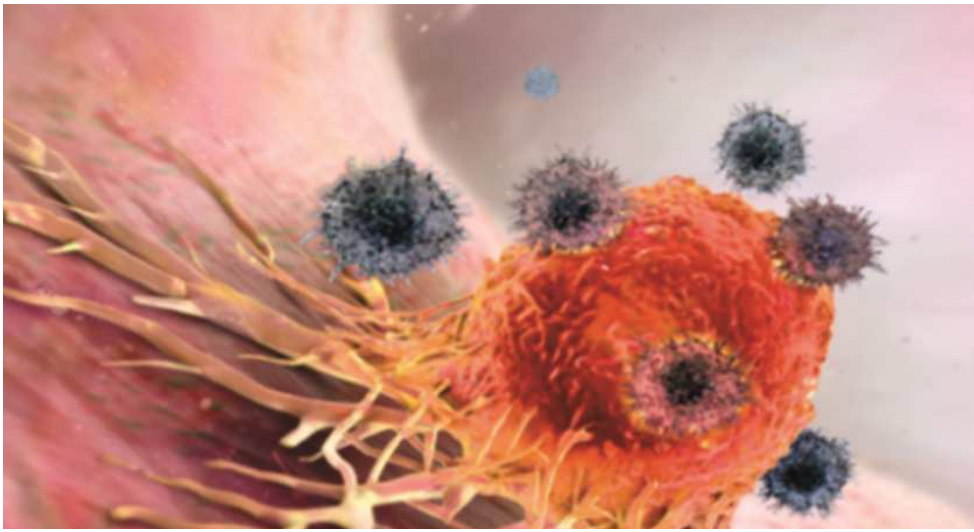
Human General Immune Cell Mediated Killing

Immune Cell Phenotype and Function Kit

Cat No. 97076 for 1 x 96-well format
Cat No. 97077 for 5 x 96-well format
Cat No. 97078 for 1 x 384-well format
Cat No. 97079 for 5 x 384-well format



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

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Section 1. Quick Guide

This Quick Guide summarizes the Human General Immune Cell Mediated Killing Kit protocol, and is valid for both 96- and 384-well formats.

NOTE: For first time assay users, refer to **Section 10** for detailed step-by-step procedures. The Quick Guide serves as an aid to utilize once the user is familiar with the protocol.

1. Encode target cells

- ☐ Wash cells in protein-free buffer (PFB) Re-suspend cells in PFB, **1-4 x 10^6/mL.**
- ☐ Dilute Cell Proliferation and Encoding Dye **1:1250** in buffer.
- ☐ Combine cells and encoding dye solution **1:1.** — Incubate RT, 15 min, Dark
- ☐ Quench with complete culture medium. Wash cells 3 times.
  Wash 1 ☐ 2 ☐ 3 ☐
- ☐ Re-suspend encoded cells in complete culture medium.

2. Reagent preparation

a.

- ☐ Solubilize Granzyme B standard in **200 µL fresh culture medium.** — Incubate RT 15 min

Start time _____ *Stop time* _____

- ☐ Prepare **1:3 serial dilution** of Cytokine Standard in fresh culture medium.

b.

- ☐ Add Cell Membrane Integrity (**1:20**) and Mitochondrial Potential (**1:1500**) dyes to Granzyme B Detection Reagent.

3. Assay Setup

- ☐ Add **10 µL/well standards/samples.** Quick spin | Brief shake*
- ☐ Add **10 µL capture beads.** Quick spin | Brief shake* — Incubate RT 1 hour, Dark

Start time _____ *Stop time* _____

- ☐ Add **10 µL combined detection reagent.** Quick spin | Brief shake* — Incubate RT 2 hours, Dark

Start time _____ *Stop time* _____

- ☐ Use included ForeCyt® template to acquire samples on Intellicyt iQue®.

Notes

* Quick spin = 300 x g, 5 seconds | Brief shake = 2000 rpm, 20 seconds

Section 2. Introduction

The Human General Immune Cell Mediated Killing Kit is designed for ease of use in multiplexing cellular function markers with bead-based, secreted protein profile measurements in the same assay. This assay is optimized for suspension cell cultures and offers unique advantages:

- Simultaneous measurement of effector and target cells, and secreted effector serine protease, Granzyme B, in a mixed cells and beads assay format.
- Improvement over common immunology research workflows that generally require multiple assays, and provides a multiplex single assay optimized for use on the Intellicyt® platform (iQue®3 and iQue® Screener PLUS) equipped with Blue and Red lasers (compatible with both VBR and BR configurations).
- Integration of a single platform and data analysis package provides rapid data acquisition, analysis workflow, and solves data synchronization issues.
- Analysis of multiple mechanisms of immune cell mediated killing in a single, high content assay, including cell count, mitochondrial membrane depolarization, loss of cell membrane integrity, and pro-apoptotic serine protease Granzyme B (see **Figure 1** for more detail).
- Simplified 'plug-and-play' assay workflow with no additional color compensation, and pre-mixed reagents for cell killing and secreted protein analyses. Total assay time is approximately 3-4 hours, with a hands-on time of about 15-30 minutes. The template includes pre-set compensation matrices and enables data acquisition of the multiplexed assay without the need for single stain color compensation.

Section 3. Assay Principles

3.1 Multiplex assay in a single well

The Human General Immune Cell Mediated Killing Kit is a cell and bead mixture assay that simultaneously measures these features:

- Target cell distinction from effector cells
- Effector cell secreted pro-apoptotic serine protease Granzyme B
- Mitochondrial membrane depolarization
- Cell membrane integrity
- Cell count and cell health (effectors and targets)

In each assay well, target cells are distinguished from effector cells by staining with a fluorescent encoder dye. Live and dead populations of the cell types are then determined by staining with a fluorescent membrane integrity dye that enters only dead cells or those with a compromised membrane, staining the nucleic DNA by intercalation. The panel also includes a dye that stains mitochondria, the retention of which is lost when cells begin the apoptotic process. The pro-apoptotic serine protease, Granzyme B, is measured in a "sandwich" immune assay format using QBeads® that are included in the same well.

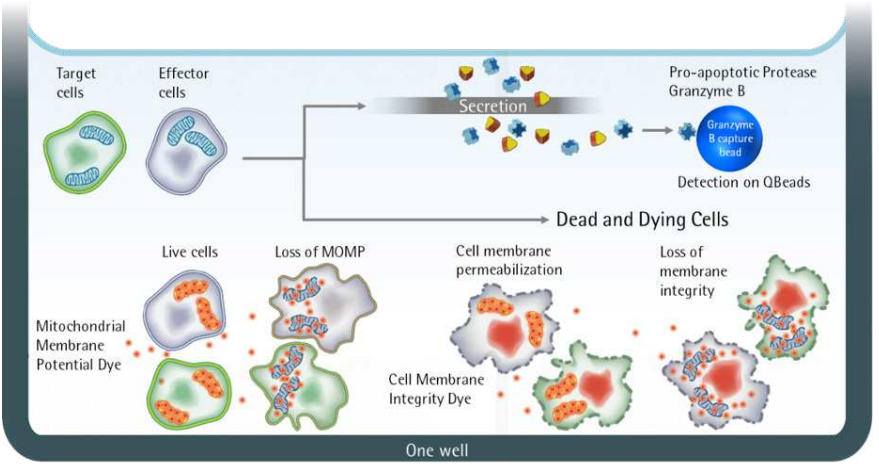


Figure 1. Simultaneous endpoint measurement in a single well. Mitochondrial outer membrane potential is also known as MOMP.

3.2 Workflow overview

Target cells are encoded with the Cell Proliferation and Encoding Dye. The encoded target cells are combined with immune cells and treatments in culture plates per the user's experimental design. After that co-culture, a sub-sample of the cells/supernatant mixture from each well is transferred into assay plates along with the Granzyme B Capture Beads. After incubating 1 hour, a combination cocktail (See **Section 10** for preparation) including Mitochondrial Membrane Potential Dye, Cell Membrane Integrity Dye, and Granzyme B Detection Reagent is added. After a final 2 hour incubation, the assay plate can be acquired on the iQue®3 or iQue® Screener PLUS (BR or VBR).

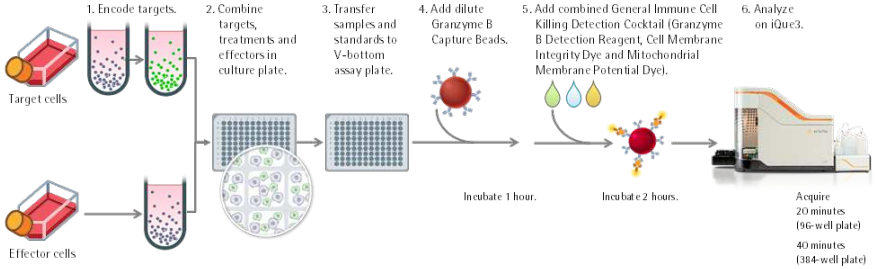


Figure 2. Assay workflow.

Section 4. Reagents Provided

Table 1. Human General Immune Cell Mediated Killing Kit contents

Reagent	Catalog No. 97076 1 x 96-well	Catalog No. 97077 5 x 96-well	Catalog No. 97078 1 x 384-well	Catalog No. 97079 5 x 384-well
Human Granzyme B Lyophilized Cytokine Standard	1 vial	5 vials	1 vial	5 vials
Cytokine Capture Beads (Granzyme B)	2 mL 1 vial	2 mL 5 vials	5.4 mL 1 vial	5.4 mL 5 vials
Cytokine Detection Reagent (Granzyme B)	2 mL 1 vial	2 mL 5 vials	5.4 mL 1 vial	5.4 mL 5 vials
Cell Proliferation and Encoding Dye (B/Green)	25 µL 1 vial	25 µL 5 vials	25 µL 1 vial	25 µL 5 vials
Cell Membrane Integrity Dye (B/Red)	100 µL 1 vial	100 µL 5 vials	500 µL 1 vial	500 µL 5 vials
Mitochondrial Membrane Potential Dye (R/Red)	20 µL 1 vial	20 µL 5 vials	20 µL 1 vial	20 µL 5 vials

Section 5. Storage and Stability

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

Section 6. iQue®3 and iQue® Screener PLUS (BR and VBR) Detector Channels

Detector	Spectrum	Violet Laser (405 nm)		Blue Laser (488 nm)		Red Laser (640 nm)	
445/45 nm		VL1					
530/30 nm		VL2		BL1	Cell Proliferation & Encoding Dye (B/Green)		
572/28 nm		VL3		BL2	Granzyme B Qbeads		
615/24 nm		VL4		BL3			
675/30 nm		VL5		BL4	Cell Membrane Integrity Dye (B/Red)	RL1	Mitochondrial Membrane Potential Dye (R/Red)
780/60 nm		VL6		BL5		RL2	Granzyme B Qbeads

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

Section 7. Materials Required but not Provided

- iQue® platform (iQue3® or iQue® Screener PLUS) configured with BR or VBR lasers.
- Cell populations of interest and appropriate complete cell culture media
- Centrifuge (up to 500 × g capability for use with microplates and microfuge tubes)
- Vortex mixer
- 96- or 384-well V-bottom assay plate (e.g., Costar® #3897 or Greiner Bio-One #781280)
- Microfuge tubes and/or 15 mL conical tubes
- Reagent reservoirs
- Universal black lid (e.g., Corning® #3935) or foil to protect from light/ evaporation
- Liquid handler or multi-channel pipettes (See **Appendix C**)

Section 8. Recommended Materials

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

- Positive control option: 10 µM staurosporine
- Negative control option: encoded target cells alone

Section 9. Cell and Reagent Preparation

9.1 Samples

a. This assay is designed to detect immune cell mediated killing in effector and target co-cultures. Prior to preparing co-cultures, the target cells must be encoded using the Intellicyt® Cell Proliferation and Encoding Dye (B/Green). See **Appendix A**. Before running the assay, prepare co-cultures in appropriate culture medium and conditions, including initial input cell density. If the assay cell density is too low, it may be difficult to achieve statistical significance for the cell population of interest. We recommend that the target cells be used at a concentration of 0.5 x 10^6 cells per mL, and the effector cells thereafter be adjusted to cell densities in accordance with the user's experimental design.

b. This assay is designed to measure the relative presence of Granzyme B in co-culture samples. If the quantitative measurement of Granzyme B is required at a specific time point, the co-culture plate should be centrifuged (5 min, 300 x g) and a sample of the supernatant (without cells) may be removed for cytokine analysis.

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

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

9.2 Assay plate design

a. The assay plate design can be found in the Design section of ForeCyt®, and in the template provided (USB flash drive in kit package).

b. This assay uses serially diluted cytokine standards to generate standard curves for the quantitation of Granzyme B in the sample.

9.3 Setting up standards in ForeCyt®

A template with the standards plate design is provided in the kit (**Figure 4**). The Standards sub-section can be located within the Design section of ForeCyt®. The Standard Set is preconfigured with the lowest value set to zero in the template provided. It is recommended to load standards in duplicate from low to high concentration in the direction that the plate will be sampled during acquisition (96-well format: left to right; 384-well format: top to bottom). For ForeCyt® version 7.1 and later this is the default setting, however, for earlier versions, this format requires the "Reverse Series" box to be checked. If necessary, the template configuration may be altered in the Design section of the experiment: Design → Standards → Edit Standard Set. A representative standard curve is shown in **Figure 5**.

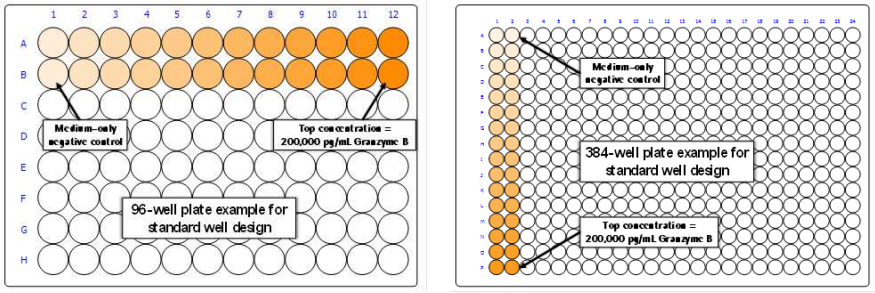


Figure 4. Configuration of the Standard Set. The Standard Set provided in the kit template is arranged from left to right for 96-well formats, and from top to bottom for 384-well formats.

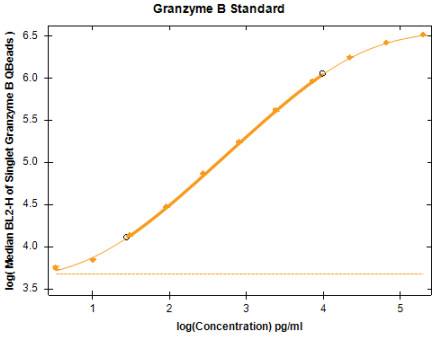


Figure 5. Representative Granzyme B standard curve with 1:3 serial dilutions. The bold line indicates the linear range; the detection range is wider than the linear range. The linear range in this example is 30-10,000 pg/mL. The dashed line represents the fluorescent background when the standard concentration is zero.

9.4 Reagents

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

b. Vortex the Granzyme B Capture Beads and Granzyme B Detection Reagent prior to use to ensure homogenous solution and consistent concentration in the assay. These reagents contain QBeads® and/or antibodies that tend to settle and aggregate over time.

9.5 Cytokine Standard preparation

a. The Cytokine Standard range for Granzyme B is 0.0 pg/mL – 200,000 pg/mL.

b. From the provided glass vial, transfer the lyophilized Granzyme B Cytokine Standard sphere into a 1.5 mL microfuge tube or 15 mL conical tube. Use only one glass vial of the Granzyme B Cytokine Standard for preparation of the standard curve on each assay day.

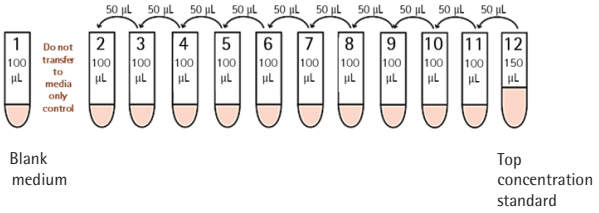
c. Add 200 µL fresh culture medium to the tube with the Granzyme B Cytokine Standard sphere. DO NOT MIX, as this causes the reagent to foam.

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

e. Once dissolved, pipette up and down to gently mix the Granzyme B Cytokine Standard.

f. Perform 1:3 serial dilutions of the Granzyme B Cytokine Standard (i.e., 50 µL of the top standard into 100 µL of culture medium serially). For a 96-well format, generate a 12-point curve, including a blank, medium-only control (**Figure 6**, top). For a 384-well format, generate a 16-point curve (**Figure 6,** bottom).

96-well format, 12-point, 1:3 serial dilutions of cytokine standards to fill rows 1-2 of the assay plate



384-well format, 16-point, 1:3 serial dilutions of cytokine standards to fill rows 1-2 of the assay plate

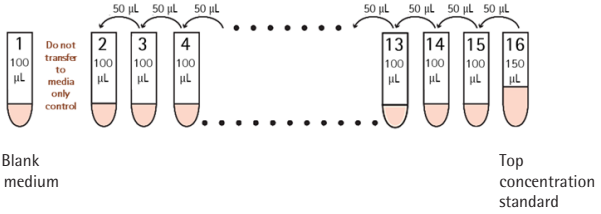


Figure 6. Serial dilution of the Granzyme B Cytokine Standard. For both 96- and 384-well formats, the Granzyme B Cytokine Standard solution is serially diluted 1:3 by adding 50 µL from the top concentration standard into 100 µL of culture media serially. A blank, medium-only tube should be included as a control.

9.6 General Immune Cell Killing Detection Cocktail preparation

Both the Cell Membrane Integrity Dye (B/Red) and Mitochondrial Membrane Potential Dye should be added to the Granzyme B Detection Reagent on the day of the assay according to the kit format. **Table 2** provides the volumes of each component used to perform the assay for one or five plates at a time.

Table 2. Volumes for Cell Membrane Integrity Dye and Mitochondrial Membrane Potential Dye addition to the Granzyme B Antibody Detection Cocktail.

Kit Format	Granzyme B Detection Reagent	Cell Membrane Integrity Dye (Final dilution = 1:20)	Mitochondrial Membrane Potential Dye (Final dilution = 1:1500)	Total Volume
1 × 96-wells	1.90 mL	100 µL	1.3 µL	2.0 mL
5 × 96-wells	7.60 mL	400 µL	5.3 µL	8.0 mL
1 × 384-wells	5.32 mL	280 µL	3.7 µL	5.6 mL
5 × 384-wells	26.60 mL	1400 µL	18.7 µL	28.0 mL

Section 10. Assay Protocol for all Formats

This protocol is applicable for both 96- and 384-well plate formats.
Total time: 3.5 hours
Hands-on time: Approximately 15-30 minutes

10.1 Add the cell/supernatant mixture and Granzyme B Cytokine Standards

a. Ensure that the cell/supernatant mixture in the original culture plate is in suspension by manual pipetting 6-8 times. Then, transfer 10 µL of samples to each well of the assay plate designated as Sample during the plate setup in the ForeCyt® Design section.

b. Transfer 10 µL of the Granzyme B Cytokine Standards (**Section 9.5**) to each well of the assay plate designated for Standards in the ForeCyt® Design section.

c. "Quick spin" the plate 5 sec, 300 x g to ensure that contents are localized at the bottom of the wells.

d. Briefly shake the plate 20 sec, 2000 rpm.

10.2 Add the Human Granzyme B Capture Beads to the assay plate

a. Vigorously vortex the Cytokine Capture Beads (Granzyme B) provided and transfer to a reservoir.

b. Transfer 10 µL of the Granzyme B Capture Beads suspension to each assay well. Agitate the reagent in the reservoir occasionally to prevent settling of the beads.

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

c. Cover the plate to prevent evaporation and protect from light.

d. "Quick spin" the plate 5 sec, 300 x g to ensure that contents are localized at the bottom of the wells.

e. Briefly shake the plate 20 sec, 2000 rpm.

f. Incubate the plate at room temperature for 1 hour.

10.3 Add the General Immune Cell Killing Detection Cocktail

a. Transfer the General Immune Cell Killing Detection Cocktail prepared earlier (**Section 9.6**) to a reservoir.

b. Add 10 µL of the cocktail per well to the assay plate.

c. Cover the plate to prevent evaporation and protect from light.

d. "Quick spin" the plate 5 sec, 300 x g to ensure that contents are localized at the bottom of the wells.

e. Briefly shake the plate 20 sec, 2000 rpm.

f. Incubate the plate at room temperature for 2 hours.

Section 11. Plate Acquisition and Data Analysis

11.1 Acquire plate

a. Launch ForeCyt® software.

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

c. In the Design section:

   i. Well Type sub-section: Assign sample wells, including positive and negative control wells.

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

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

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

e. Click "Run" on the Controller to acquire the plate.

> **NOTE:** Remove the plate lid prior to clicking "Run" on the Controller.

11.2 Data analysis and gating hierarchy

The gates within the template are pre-set for the expected populations in each well. If preferred, below are the gating details to manually draw the gates or fine-tune the existing gates in the template. Each gate can be adjusted to improve fit on populations of interest (**Figure 7**). An optimized compensation spillover matrix has also been included in the kit template (**Figure 8**).

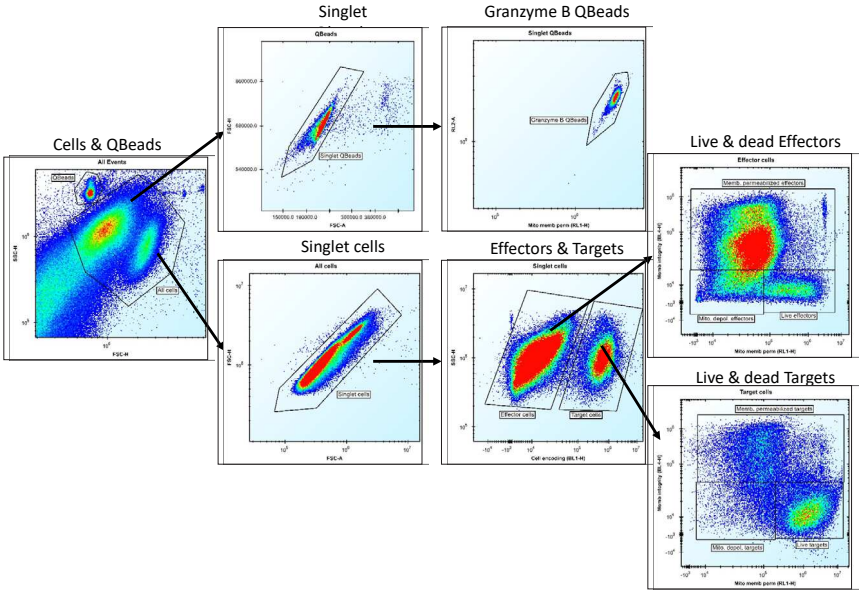


Figure 7. Gating hierarchy for the Human General Immune Cell Mediated Killing Kit. From the All Events FSC-H vs SSC-H plot, QBeads® and cell populations are identified, followed by selection of singlet populations from FSC-A vs FSC-H plots. The Granzyme B Qbeads® are identified based on RL1-H vs RL2-A scatter. The effector and target populations are designated based on fluorescence of the Cell Proliferation and Encoding Dye. Thereafter, the live and dead cell populations are gated. Live cells are those that have retention of the Mitochondrial Membrane Potential Dye (RL1-H^{high}), and no incorporation of the Cell Membrane Potential Dye (BL4-H^{low}). Mitochondrial depolarized cells are RL1-H^{low}. Membrane permeabilized cells are BL4-H^{high}. Using the Boolean Logical Population Tool in the Analysis section, the overall dead target cells can be determined with the Operation "Mito. depol. targets OR Memb. permeabilized targets". In the kit template, Boolean Logical Populations for dead target and effector cells have been created, as well as a Metric for "% dead" of each cell type.

11.3 Compensation spillover matrix

Primary Channel \ Spillover Channel	Cell encoding (BL1-H)	BL2-H	BL3-H	Memb integrity (BL4-H)	Mito Memb Perm (RL1-H)
Cell encoding (BL1-H)		0.00	0.00	0.99	0.10
BL2-H	0.00		0.00	0.00	0.00
BL3-H	0.00	0.00		0.00	0.00
Memb integrity (BL4-H)	1.25	0.00	0.00		6.74
Mito Memb Perm (RL1-H)	0.00	0.00	0.00	0.23	

Figure 8. Compensation spillover matrix. This compensation matrix is included in the ForeCyt® template and is applied when experiment templates are employed for data acquisition. There is no need to adjust any compensation metrics.

Section 12. Best practices and tips

12.1 Dilute the protein standards with fresh culture medium

It is critical to use fresh culture medium when reconstituting the Granzyme B Cytokine Standards to ensure data reproducibility and reliability. A specific diluent for protein standards is not provided with this kit, and the culture medium used here should not differ from that used in cell culture.

12.2 Plate type

The assay protocol in this manual is designed for both 96- and 384-well plate formats. The Human General Immune Cell Mediated Killing Kit has been tested with both 96- and 384-well V-bottom plates (Costar® #3897 and Greiner Bio-One #781280, respectively). This kit provides templates for both 96-well and 384-well formats.

12.3 Manual pipetting recommendation

This protocol requires pipetting small volumes (approximately 1-10 µL). Care should be taken during liquid transfers so that volumes are fully dispensed with appropriate pipettes. Avoid well cross contamination by changing pipette tips between wells. When pipetting small volumes, it is best practice to touch the bottom of the well (in an empty plate) or the side-wall of a well (when occupied with sample/reagent) to ensure release of the liquid into the assay well. Touching the wall prevents the liquid droplet from hanging on the pipette tip instead of releasing into the assay well. A quick spin (5 sec, 300 x g) will force the newly dispensed reagent to the well bottom to mix with the existing reagent/sample already in the well. For single and multi-channel pipette recommendations, see **Appendix C**.

12.4 Mixing plate contents using a shaker

The use of a plate shaker to mix plate contents is required when performing this assay. If a plate shaker is not available, the shaker on the iQue®3 or iQue® Screener PLUS may be used without exceeding the volume and speed limitations (**Table 3**). From the ForeCyt® menu bar, select Device → Manual Control Mode. In the Manual Control Mode window, set the desired RPM for shake speed (**Figure 9**). As soon as the "On/ Plate Shake" Shaker Controls checkbox is selected, the shaker will begin to shake and will continue to shake until disabled by deselecting the checkbox.

Table 3. Volume and speed limitations when using the iQue®3 (VBR) or iQue® Screener PLUS (VBR) shaker.

Plate Type	Well Volume (µL)	Maximum Speed (RPM)
96-well	20–40	2600
96-well	40–60	2200
96-well	60+	*
384-well	10–30	3000
384-well	30–50	2800
384-well	50+	*
* Larger volumes will require additional optimization. To determine ideal shake speeds for high volume assays, it is recommended to begin at a lower RPM value and gradually increase to a higher RPM value. Care should be taken to avoid well cross contamination.		

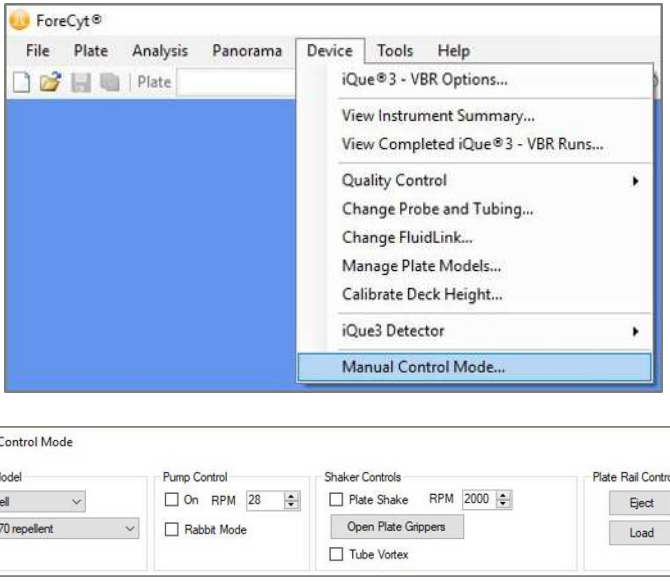


Figure 9. Steps for using the shaker on iQue®3 and iQue® Screener PLUS (BR and VBR). Set the shaker speed according to assay requirements: 2600 rpm for 96-well formats and 2800 rpm for 384-well formats. Shaking the plate with liquid in the wells at higher speeds will potentially result in cross-contamination.

12.5 Adjust the sip time to acquire enough cell events

Sip time determines how many cell events are acquired from each well. Adjust sip time as necessary to ensure that enough cell events from the population of interest are acquired to reach statistical significance during data analysis. Sip volume may vary slightly from machine to machine and day to day. Sip volume is approximately 1.5 µL per second. Adjustment of sip time from the default (10 seconds for 96-well plates; 5 seconds for 384-well plates) may be made in the ForeCyt® Protocol section. Inter-well shaking may be adjusted as well, as settling could occur over time. In this assay, for example, an initial cell density of 1 million/mL sipped for 5 seconds would result in approximately 2500 cells acquired per well.

12.6 How to ensure that sample Granzyme B is within the linear range of the standard curve

The ForeCyt® template defaults to 4PL with $1/Y^2$ weighting for the standard curves. ForeCyt® can provide the linear range for each standard curve. The template uses a 1:3 serial titration with the top concentrations of 200,000 pg/mL Granzyme B. If adjustments for concentration, dilution factor, or plate layout for the standard are necessary, refer to the ForeCyt® Reference Guide and make the adjustment in the Design section. The use of a different culture medium may have a slight impact on the standard curve linear range.

12.7 Ensure that the Mitochondrial Membrane Potential Dye is within detection limits

The protocol in **Section 9.6** refers to the Mitochondrial Membrane Potential Dye being used in the combined detection cocktail at a dilution of 1:1500. However, it is possible that some assay conditions may result in fluorescence detection that is off-scale. If this occurs, the dilution of the Mitochondrial Membrane Potential Dye may be adjusted by increasing its dilution factor, e.g., 1:1750 or 1:2000.

Section 13. Appendices

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

The following protocol uses the Cell Proliferation and Encoding Dye (B/Green) to multiplex the target cells by cell encoding. The assay template provided on the USB flash drive in the kit includes the compensation metrics for the dye detection channel (BL1 in iQue®3 and iQue® Screener PLUS). Below are instructions for encoding target cells using the Cell Proliferation and Encoding Dye (B/Green):

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

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

c. Collect target cells in a 50 mL conical tube. Centrifuge the cells (5 min, 500 x g) and remove the original culture medium.

d. Resuspend cells in 20 mL protein-free HBSS or PBS. Centrifuge the cells (5 min, 500 x g). Remove the supernatant. Resuspend cells in protein-free HBSS or PBS at 1-4 million/mL.

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

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

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

h. After the final wash, carefully resuspend cells at the desired cell density for co-culture/assay. We recommend that the target cells used at a final concentration of 0.5 x 10^6 cells/mL, and effector:target ratios of at least 1:1. **Figure 10** illustrates gating to separate the target and effector cells acquired by an iQue®3 (VBR) after co-culture.

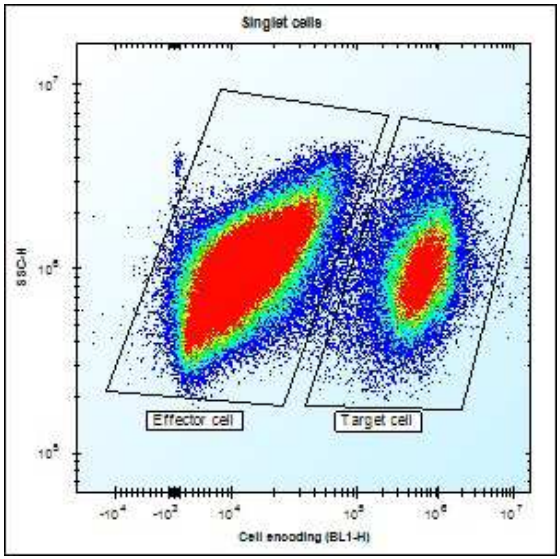


Figure 10. Example of gating to resolve target and effector cell populations from the Singlet cells population. The target cells are stained with Cell Proliferation and Encoding Dye (B/Green), while the effector cells are unstained.

13.2 Appendix B: Options for improving cell event count at acquisition

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

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

a. Centrifuge the cells (5 min, 300 × g) in the original culture plate.

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

c. Resuspend cells in the culture plate by pipetting the sample up and down (5-6 times) in the remaining supernatant.

d. Transfer the concentrated cell samples to the assay plate before running the assay.

Option 3: Use cell-repellent or ultra-low binding plates to reduce cell attachment.

Some user-defined biological conditions may cause partial attachment of cells to the assay well surface, resulting in inconsistent cell count. To achieve a more precise cell count, use cell-repellent plates (e.g., Greiner Bio-One #651970 or Greiner Bio-One #781970), or ultra-low attachment plates (e.g., Corning® #7007 or Corning® #4516). To add new plate models into ForeCyt®, click on Device → Manage Plate Models → Add.

Option 4: Run daily volumetric calibration to get more precise cell density data.

Running a daily volumetric calibration on the iQue®3 or iQue® Screener PLUS using SPHERO™ AccuCount beads (Spherotech #ACBP-50-10) is recommended if precise cell density information is required. This product has an absolute count per volume unit.

a. Follow the Spherotech protocol to mix and transfer the beads to a testing plate.

b. Mimic the run protocol in the Human General Immune Cell Mediated Killing Kit by using the same plate type, sample volume, and sip time.

c. Measure the sip volume by acquiring samples from at least three wells of AccuCount beads.

d. Use this volume measurement to calculate the cell density.

e. Adjust the final calculation by considering the sip time (in the ForeCyt® Protocol section) and the sample dilution in the final assay reaction volume.

13.3 Appendix C: Pipette recommendations

Multi-channel pipettes

- Manual 12-channel pipette, Tacta, 5-120 µL (Sartorius)
- Manual 12-channel pipette, Tacta, 30-300 µL (Sartorius)
- Electronic 12-channel pipette, Picus, 5-120 µL (Sartorius)
- Electronic 12-channel pipette, Picus, 10-300 µL (Sartorius)

Single-channel pipettes

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

13.5 Appendix D: FAQ

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

A1: Standard curves should be run on each day of the assay, and applied only to experiment plates run on the same day. This eliminates potential day-to-day variation that may affect cytokine quantitation. Standards can be included in-plate or run as a standalone plate. For in-plate standards, cytokine quantitation is automatically included in the ForeCyt® template. However, cytokine quantification can be achieved from a standalone plate by sharing the standard curve fit to other assay plates. Once the curve fit has been shared, cytokine quantitation can be performed using the Derived Concentration advanced metric. More information on the Share Fit feature and calculating a derived concentration from a shared curve can be found in the ForeCyt® Reference Guide.

Q2: Can I use fixatives in my samples?

A2: Samples may be fixed with certain fixatives (e.g., 1% PFA). However, it is important to understand how fixation may affect biological outcomes. The use of methanol for fixation is highly discouraged as it affects bead-based cytokine detection. Fixation and further wash steps may cause cell loss and affect the final event acquisition, and therefore, may warrant additional optimization. If significant cell loss is observed, perform the fixation in a cell-repellent plate (e.g. Greiner Bio-One #651970 or Greiner Bio-One #781970) to reduce cell loss due to fixation or fixation-related cell cross-linking to the well bottom.

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

A3: Yes. A 1 x 384-well kit can be used for 4 assay plates in a 96-well format. Both 1 x 96-well kits and 1 x 384-well kits provide 1 vial of each Cytokine Standard. Additional standards are also available for purchase. For all kits, both 96- and 384-well ForeCyt® templates are provided.

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

A4: Yes. The violet channels of the iQue®3 and iQue® Screener PLUS VBR instruments are not used in this kit. Be aware that the ForeCyt® template includes a compensation matrix for the specific components of this kit, and that any multiplexing may require additional compensation to account for additional fluorophores added to the assay.

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

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

- Capture beads have not been agitated adequately in their original vial.
- Capture beads were not mixed in the reservoir during transfer to the assay plate.

For low cell counts, consider the following possibilities:

- Cell proliferation/viability was affected during sample preparation
- Cells were not mixed before transferring cell/supernatant sample from the culture plate to assay plate.

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

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

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

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

A7: This assay is designed to measure relatively high levels of Granzyme B (as high as 200,000 pg/mL) without sample dilution. Diluting the samples is appropriate when cytokine levels are beyond the linear range of the standard curve. When diluting samples, consider adjusting the sip time to assure enough cellular events are collected for analyses.

Q8: Why do I sometimes get cell viability/live cell readings from wells which only contain capture beads (e.g., wells designated for cytokine standards)?

A8: These viability readings are usually caused by very few stray events. You may use the plate view option of live cells to verify that the observed cell numbers are low. These stray events can be considered background noise, and we suggest that you exclude the wells designated as standards when viewing heat maps that contain live cell data to eliminate any confusion.

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

A10: This assay is only optimized for cell culture samples, and is not optimized to measure cytokines in human sera. If you need to measure the same cytokines from human sera samples, you may purchase QBeads® kits from Intellicyt® which include a special diluent for human sera samples. The QBeads® kits for human sera samples may NOT be multiplexed with any of the Human Immune Cell Mediated Killing Kits.

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