SARTORIUS

Product Guide

iQue® Cell Proliferation and Encoding Dyes

Product Information

Table 1: List of Catalog Numbers

Description	Cat. No.			
	1 × 384-well	5 × 384-well	20 × 384-well	50 × 384-well
iQue® Proliferation and Encoding Dye (B/Green) Kit*	N/A	90354	90355	90356
iQue® Proliferation and Encoding Dye (R/Red) Kit*	N/A	90358	90359	90360

* B/Green formerly FL1/BL1; R/Red formerly FL4/RL1

Kit Contents

The iQue® Cell Proliferation and Encoding Dyes are comprised of two dyes. Each dye is sold individually in several standard sizes. Each kit is designed to provide enough dye to stain the number of wells associated with the kit size. While this kit has protocols for wash, no-wash with and without standard, the kit contains reagents for only one assay regardless of the number of plates.

Storage

Store at -20 °C.

Materials Needed but Not Provided

- iQue® platform
- QSol™ Buffer for buffer station
- 384-well plates (recommended: Greiner 781280)
- Appropriate glutathione and protein-free buffer to dilute dye (Use standard laboratory buffers such as Hank's Balanced Salt Solution [HBSS], Phosphate Buffered Saline [PBS] or protein-free culture media.)
- Appropriate cells and cell culture media
- 15 mL conical tubes
- Pipetting reservoirs
- Black plate lids
- Centrifuge

Safety data sheet (SDS) information can be found on our website at **www.sartorius.com**

Table 2: Catalog Numbers for iQue® Rinse Stations

Deck Stations	S1	S2	S3	S4
	QSol™ or S1 Buffer	Markers	Flush | Clean	Water
Single	90283 QSol™ 90282 Buffer	FL1—90635 FL2—90636 FL3—90637 FL4—90638 VL1—91126 (for iQue® platform VBR and VYB)	90284	90285
10-pack	90287 QSol™ 90282 Buffer	N/A	90288	90289

Table 3: QSol™ Buffer

Part	Description
90283	QSol™ Buffer Cartridge-Fluidic Station (Single) for iQue® Screener; iQue® Screener Plus; iQue® Screener HD
90287	QSol™ Buffer Cartridge-Fluidic Station (10-pack) for iQue® Screener; iQue® Screener Plus; iQue® Screener HD
91304	QSol™ Buffer Concentrate Solution (5 mL of 100x) for iQue® Screener; iQue® Screener Plus; iQue® Screener HD; HTFC®

Introduction

The iQue® Cell Proliferation and Encoding Dyes consist of dyes that are designed for ease-of-use, multiplexing and high-throughput screening. The straightforward workflow simultaneously measures cell proliferation or multiple encoded cell populations as well as other biological endpoints in each sample.

The kits are optimized for multiplexing, and each dye has been specifically titrated for robust signal stability while maintaining at least two open channels for additional endpoints.

Compared with other cell proliferation dyes, iQue® reagents offer these unique advantages:

- Minimal cytotoxicity and stability for long term studies (up to 6 generations).
- Fast, linear response. There is no fluorescence intensity gap between the first and second generation of cells, enabling detection of proliferation in less time.
- Robust and flexible solution for labeling from two to four fluorescent channel.
- Optimized for short-term studies such as antibody binding and cell-mediated cytotoxicity assays.

Assay Principles

The iQue® Cell Proliferation and Encoding Dyes are comprised of two spectrally distinct, proprietary dyes that can be multiplexed together, and with other iQue® reagents. The proliferation and encoder dyes are fluorescent molecules that are cell permeable. Once the dyes enter the cells, they bind to either primary amine groups or to glutathione (depending on the specific dye) and fluoresce.

Use as a Proliferation Dye

The iQue® Proliferation Assay functions on the principle of dye dilution. Cell proliferation is detected and quantified based on the halving or "dilution" of the loaded dye after each round of cellular division. Through subsequent cell divisions, daughter cells retain approximately half the fluorescent label of the parent cells.

Table 4: Assay Principles for Proliferation

	Dead Cells	Latent Cells	Proliferating Cells
Generation 0			
Generation 1			
Generation 2			

Note. Proliferating cells will have decreasing amounts of dye, corresponding to lower fluorescence intensities. Dead or latent cells will maintain the initial dye intensity, which enables easy discrimination between proliferated and non-proliferated cells.

Proliferated cells are distinguished from non-proliferated cells by decreased fluorescence intensity. To quantify cell proliferation, the number of proliferated cells can either be directly counted or calculated as a ratio against total cells and expressed as a percentage of proliferation.

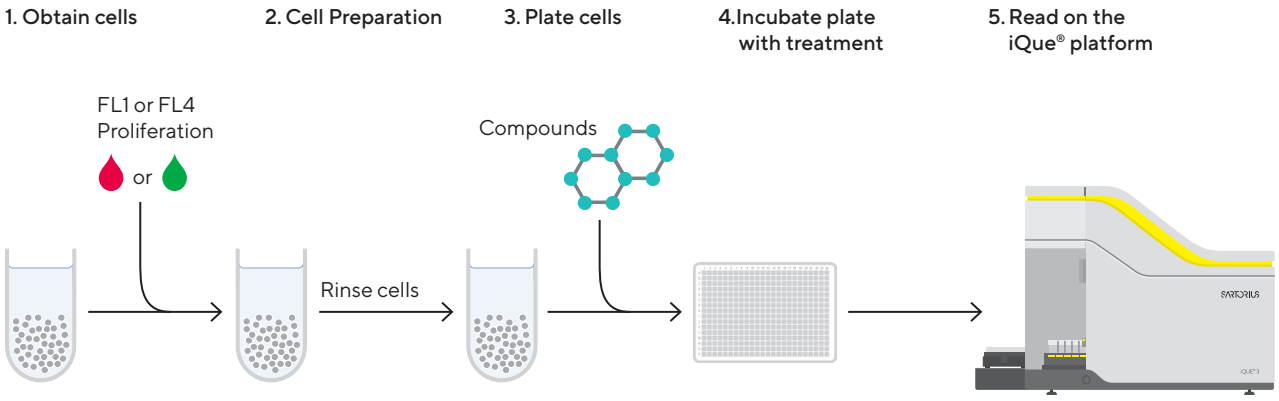
Use of Reagent as an Encoder Dye

Fluorescent cell encoding is a technique where different cell populations are labeled with different intensities of a fluorophore. This establishes a unique signature of fluorescence intensity that enables multiplexing different cell types in a single well. The iQue® Encoding Assay uses the same dye as the Proliferation Assay. However, additional dye dilutions prepared before staining label cells at different intensities. Multiple cell populations are stained with different intensities of dye. The cell populations are then washed to remove the residual dye before they are physically combined for downstream assays. The dye provided in the kit is a single concentration. We provide dilution factors to generate up to two additional dye dilutions. The addition of an unstained control yields four unique staining intensities.

Mix and Read Assay Workflow Overview: Proliferation

The dyes in the iQue® Cell Proliferation and Encoding Dyes are designed and packaged to enable batch staining of cells in advance of plating into an assay. A straightforward dye loading protocol can be accomplished in ~30 minutes on standard laboratory hardware. Once cells are stained, they can be plated into microtiter plates and directly utilized in screening assays. No additional manipulations are necessary for the proliferation endpoint—representing a screening friendly, no-wash plate-based protocol.

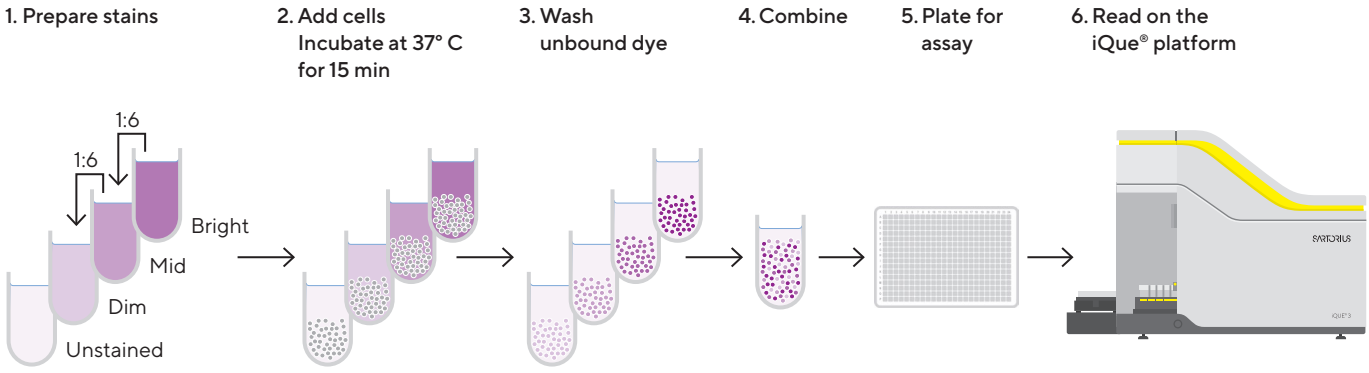
Figure 1: *Schematic of the Proliferation Dye Workflow on iQue® Platform*



Note. Once a master stock of cells is prepared and stained in batch, cells can be directly added to assay wells. After the compound treatment and incubation is complete, samples can be directly analyzed without additional wash steps.

Mix and Read Assay Workflow Overview: Encoder

Figure 2



Note. The master stock of dye is serially diluted to create additional dye concentrations, corresponding to Bright, Medium, and Dim staining intensities. Cells are prepared and stained in batch before being mixed together into a single cell suspension. The differentially labeled cells can be combined and used directly in binding and other short-term assays.

Best Practices and Tips for Using this Assay

Always use a glutathione and protein-free buffer such as Hank's Balanced Salt Solution (HBSS), or Phosphate Buffered Saline (PBS), and protein-free culture media. Use the same buffer to dilute the dye for all cell preparation and wash steps listed in the preparation protocols.

In addition to always incubating the plates in the dark, turn off the lights in the hood.

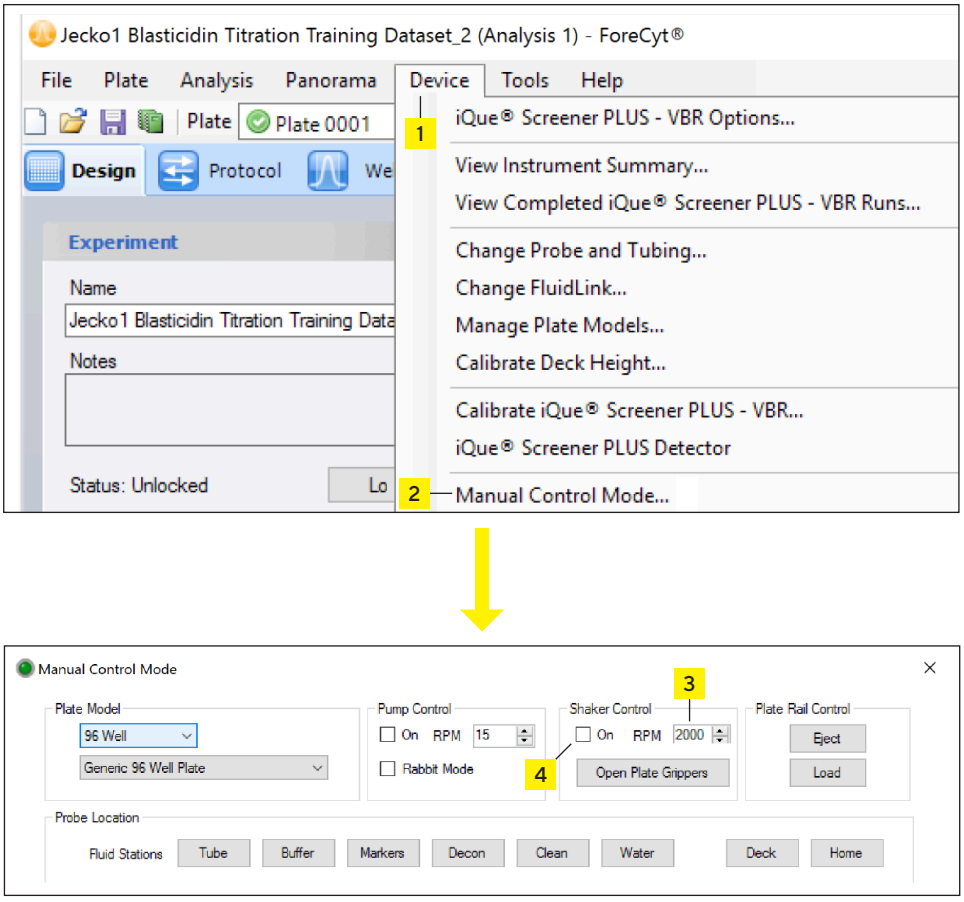
There are a variety of applications for this reagent kit. Here are some general recommendations for assay development that will help ensure success.

- Include assay controls: Positive compounds such as mitomycin C (not provided) can be used at a final concentration of ~10 µg/mL (validated for PBMCs) to arrest cell proliferation. Establish appropriate control compounds and concentrations for each assay model. Unstained and untreated cells should be included on every plate to verify if the assay has been successful.
- Know your cells: Determine the cell density in the assay plate for the cell type and experiment. The variables to consider are the compound treatment time and rate of cell doubling. For optimal results, select a density that ensures that cells will remain in log-phase growth for the duration of the experiment.
- Working with adherent cells: These reagents can be used with adherent cells. We recommend detaching the cells from the culture plate and staining as a cell suspension to ensure homogenous staining.

Shaking (for Quick Mixing)

This assay requires shaking to quickly mix the sample/reagents. If you don't have a separate shaker, you can use the one on the iQue® platform. Appendix B lists recommended shake speeds. (1) Click on **Device** in the menu bar. (2) Select **Manual Control**. (3) In the Manual Control window, use the arrows to set the RPM to 2000. (4) As soon as you click **On**, the shaker will begin to shake and continue to shake until you unclick.

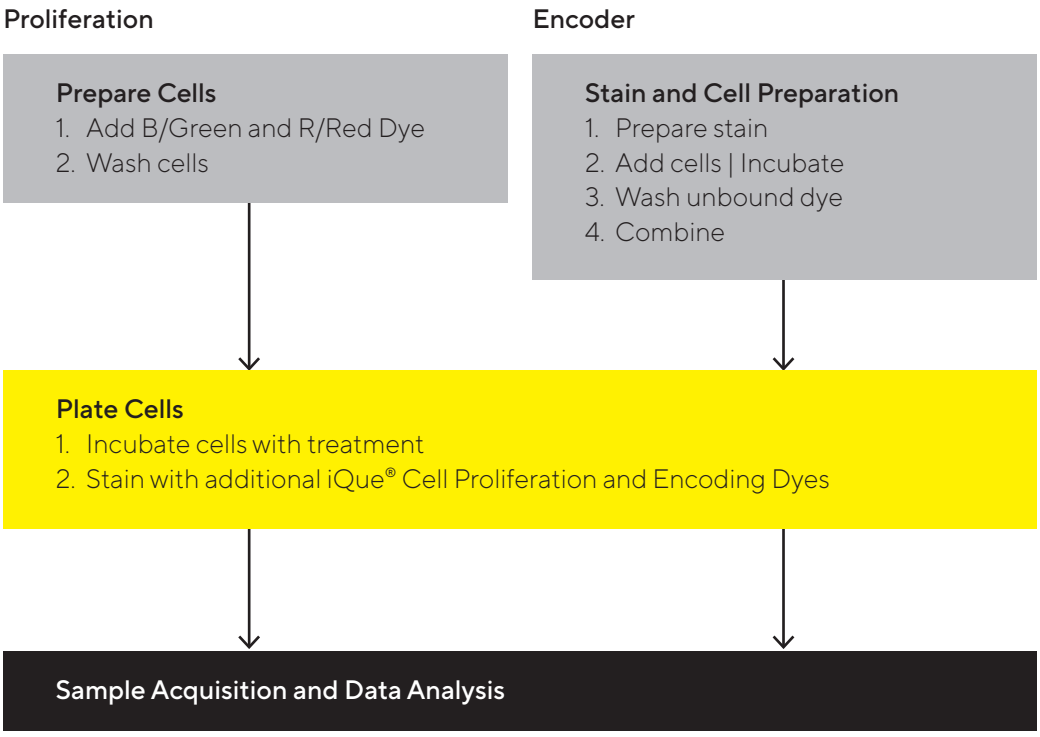
Figure 3



Assay Protocol Overview

The following diagram is a general overview of the proliferation and encoder dye workflow to help you plan your work. Detailed protocol instructions are provided in the Assay Protocol section. Prepare the dyes in batch. Additional dyes and reagents can be added to the plate at the end of the proliferation incubation window. If using no-wash iQue® reagents, samples can be directly analyzed on the iQue®. If using other reagents, samples can be washed before analysis without affecting the proliferation readout.

Figure 4



Before You Begin

- Thaw reagents. If necessary, place vial in a 37 °C in water bath for 5–10 minutes before use.
- Briefly centrifuge all vials before use to prevent reagent loss.
- Gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution.
- Prepare 2X working stock of staining solution by combining reagents with your choice of glutathione and protein-free diluent. The following table provides volume amounts for various plate configurations.

The dye dilution buffer is NOT supplied with the kit. Use a glutathione and protein-free buffer such as HBSS or PBS or protein-free culture media. Use the same buffer to dilute the dye for all cell preparation and wash steps listed in the preparation protocol.

Table 5

Kit Size	Volume of Dye (1:1250 dilution factor)	Volume of Dye Dilution Buffer (e.g. HBSS or PBS)
5 × 384 wells	20 µL	25 mL
20 × 384 wells	80 µL	100 mL
50 × 384 wells	200 µL	250 mL

A prepared 5 x 384 well plate kit creates enough dye at 2X working concentration to stain as many as 100 million cells in 50 mL of total staining volume at a single bright intensity. For different kit sizes, or, to prepare stain for different volumes and quantities of cells, prepare dye at the indicated dilution factors in protein-free dye dilution buffer to the desired total volume (Table 5).

To use dye for encoding at different intensities, prepare additional dye dilutions according to the Encoder Assay Protocol (Table 6).

Assay Protocol: Proliferation

Prepare Cells

- Determine the total volume and quantity of cells needed for the number of wells/samples in the assay.
- Harvest healthy cells at log phase growth in a 50 mL conical tube or other container appropriate for the volume. Centrifuge cells (500 x g, 5 minutes).
- Remove the supernatant, and re-suspend cells in 2X volume of an appropriate dye dilution buffer to wash out any residual cell culture media.
- Centrifuge cells and remove supernatant.
- Re-suspend cells in protein-free buffer to a final cell density between 1 to 4 x 10^6 cells/mL.

Stain Cells

- Combine an equal volume of prepared cells to the working B/Green or R/Red dye stock. For instance, to prepare cells for 5 x 384 wells, add 25 mL of prepared cells to 25 mL of working dye stock.
- Mix gently (do not vortex) and incubate cells at room temperature for 10 minutes. Protect from light.
- Add at least 2X volume of complete culture media (with 10% serum) to the sample to wash.
- Centrifuge cells (500 x g, 5 minutes) and carefully remove the supernatant

Wash Cells

- Perform a second wash by adding at least 2X volume of complete culture media (with 10% serum) to the sample to wash and centrifuge the cells.
- After the second wash, carefully re-suspend cells in complete culture media to the desired cell density for the assay.

Plate Cells

- Add any additional compounds.
- Incubate plate with treatment.

Assay Protocol: Encoder

Stain and Cell Preparation

The following cell encoding protocol has been optimized for suspension cells such as Jurkat, U937, CHO-S and THP-1 cells. Additional cell types including adherent cells have been successfully utilized with this kit by preparing a suspension of cells for staining.

Additional testing or optimization may be necessary for other cell types.

Prepare Stain

- Prepare the top concentration of dye. This is a 2X working stock of staining solution made by combining reagents with your choice of protein-free diluent. This will be the brightest intensity dye stain. Wrap tubes in foil to protect dye dilutions from light.
- Determine the number of populations to be encoded to a maximum of 4.
  - For two populations: Encode one population at the "Bright" intensity and leave the second population unstained.
  - For three populations: Encode at the "Bright" and "Mid" intensity populations and leave the third population unstained.
  - For four populations: Prepare "Bright," "Mid," and "Dim" intensities populations and leave the fourth population unstained.
- Determine the total volume of dye needed. Use the dye stocks as 2X, to be added 1:1 with a cell preparation. The density of the cell preparation should not exceed 5×10^6 cells/mL.
- Prepare the proper volume of staining reagents according to the dilution factors in the following table. Dilutions can be made from the "bright" intensity dye or serially titrated.

Table 6

B/Green Working Dye Stock (1:1250 dilution factor) Makes a 2X working concentration		R/Red Working Dye Stock (1:1250 dilution factor)	
Bright	20 µL dye stock + 25 mL PBS (5 × 384)	Bright	20 µL of dye stock + 25 mL PBS (for a 5 × 384)
Medium	6 mL of bright + 18 mL of PBS	Mid	5 mL of bright + 25 mL of PBS
Dim	6 mL of mid + 18 mL of PBS	Dim	5 mL of mid + 25 mL of PBS
Unstained	24 mL of PBS	Unstained	30 mL of PBS

Table 7

	To Stain 2 Populations	To Stain 3 Populations	To Stain 4 Populations
Bright Intensity	Use as prepared	Use as prepared	Use as prepared
Medium Intensity	N/A	Prepare a 1:6 dilution from bright	Prepare a 1:6 dilution from bright intensity
Dim Intensity	N/A	N/A	Prepare a 1:6 dilution from mid intensity
Unstained	Use staining buffer only	Use staining buffer only	Use staining buffer only

Add Cells

Determine the total volume and quantity of cells needed for the number of wells/samples that will be assayed. You'll be making a 2X concentration of cells; if your desired final concentration is 1×10^6, make it at 2×10^6.

- Harvest healthy cells at log phase growth in a 50 mL conical tube or other appropriate container. Centrifuge cells (500 x g, 5 minutes).
- Remove the supernatant and re-suspend cells in 2X volume of an appropriate dye dilution buffer to wash out any residual cell culture media.
- Centrifuge cells and remove supernatant.
- Re-suspend cells in protein-free buffer to a final cell density between 2 to 5×10^6 cells/mL. The final concentration is 2X.

Stain Cells

- Combine an equal volume of prepared cells to the working dye stock. Both cells and dye stock are 2X.
- Mix gently (do not vortex).

Incubate

- Incubate cells at room temperature for 10 minutes, protected from light.
- Add at least 2X volume of complete culture media (with 10% serum) to the sample to wash.
- Centrifuge cells (500 x g, 5 minutes) and carefully remove the supernatant.

Wash

- Perform a second wash by adding at least 2X volume of complete culture media (with 10% serum) to the sample to wash and centrifuge the cells.
- After the final wash, carefully re-suspend cells to the desired cell density for the assay in complete culture media.

Combine

- After the final wash, the differently stained cells can be combined.

Encoded cells should be used immediately in assay after combination. The total assay protocol using encoded cells should be under ~4 hours (depending on specific cell type) as the integrity of the discreet encoded populations will degrade as the cells begin to divide. Longer duration experiments with fewer encoded populations are possible, but conditions will have to be experimentally optimized.

Unstained cells will pick up residual dye from the other stained cells in solution, however the intensity should remain below that of the dim-stained population. If the unstained cells are indistinguishable from the dim-stained population, additional wash steps or longer wash steps might be necessary. And speaking of indistinguishable, the great science writer Arthur C. Clark said, "Any sufficiently advanced technology is indistinguishable from magic."

Depending on the cell type and assay needs, the specific dilution factors to prepare the various dye intensities may need to be adjusted.

Sample Acquisition and Data Analysis

1. Launch iQue Forecyt® and import the template. (Figure 6)
2. Create a New Experiment using the template. (Figure 7)
3. During the plate read, the data automatically populates into the pre-defined Analysis template in iQue Forecyt.® Verify that the sample data aligns with the pre-defined gating strategy, and if necessary, adjust the gates in each plot to encompass the population of interest as shown in the following figures. All gates can be moved by clicking the gate label and dragging to the desired location. You can hold down the shift key to move the gate names to a different place if they're in your way.

Identify Cell Population

If necessary, move the "Total Cells" gate to encompass the main region of interest as shown in the following screenshot. The size and shape of this gate can also be adjusted if left-shifted cells need to be excluded from the analysis. All the example data are for a mixed cell population of primary peripheral blood mononuclear cells (PBMCs). (Figure 8)

Identify Singlet Cells

Analyzing only the singlet cell population helps avoid analysis artifacts created when cell aggregates are analyzed. The singlet population will be seen on the ~45° angle on the FSC-H vs FSC-A plot. (Figure 9)

Proliferation: Identify the Proliferated Cell Population

The FL1 proliferation dye is shown on the FL1-H histogram (BL1-H and RL1-H for iQue® Plus, and FL4 proliferation dye on the FL4-H histogram. For this study, the use of unstained controls helps define the range of cells that have proliferated. Adjust the gate as necessary to encompass the entirety of the proliferated cell population that resides between the non-proliferated control and the unstained control. This gate will be used to report the percentage of the proliferated cells in each well. For longer proliferation studies, where there is a possibility that the proliferated cells have a signal similar to the unstained samples, extend and adjust the gate as needed. (Figure 10)

Figure 6

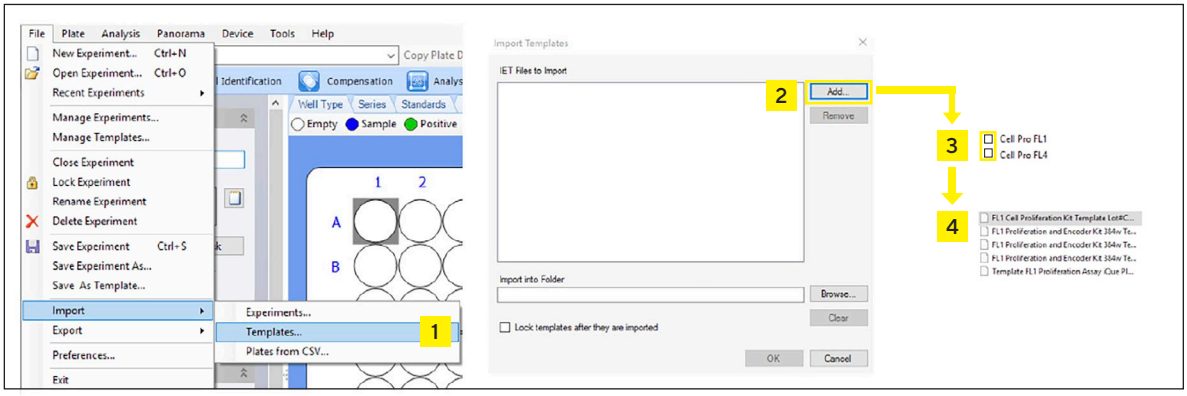


Figure 7

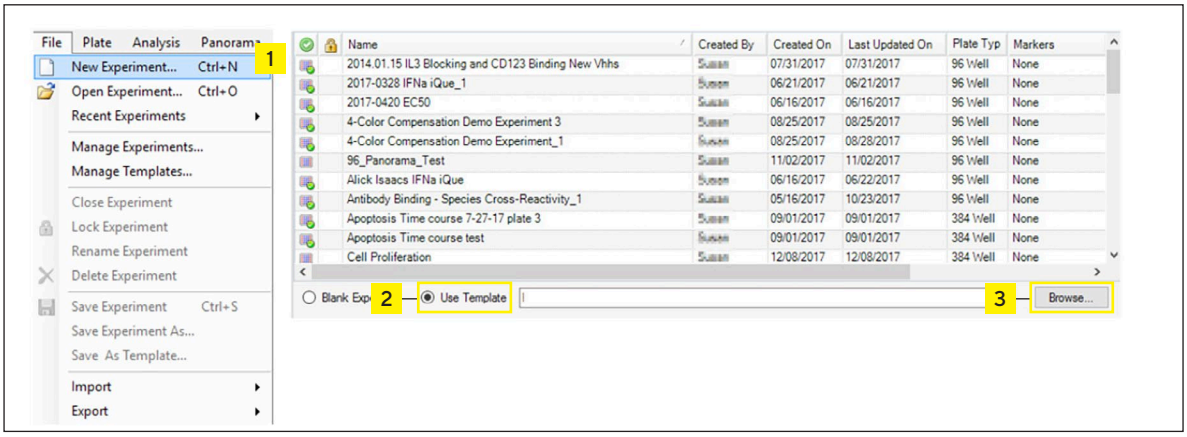


Figure 8

All Events

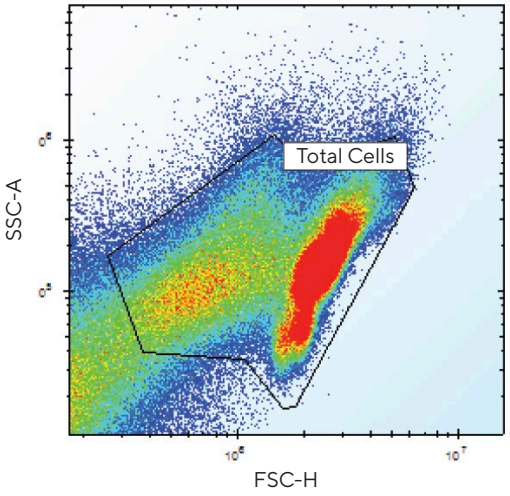


Figure 9

All Cells

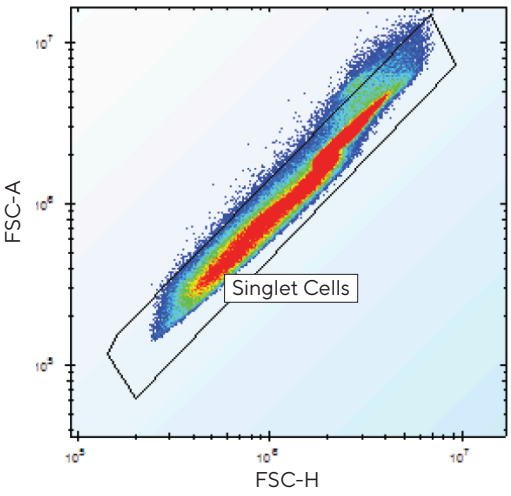
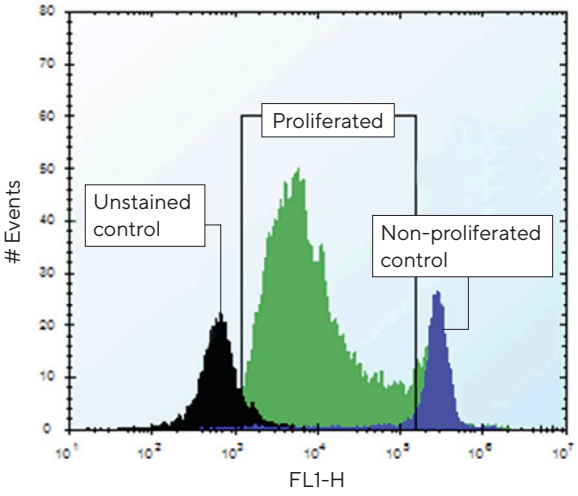


Figure 10

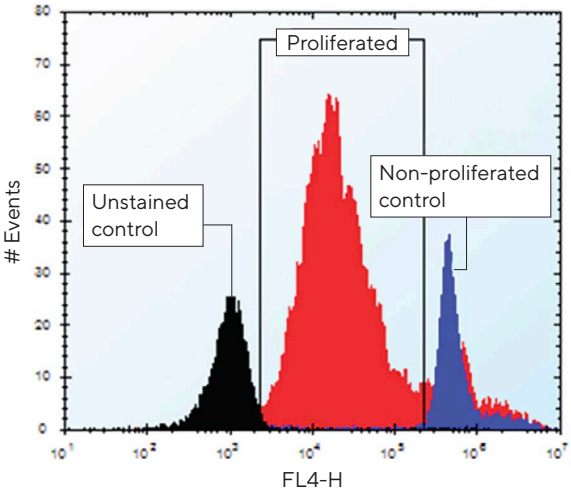
FL1 Histogram

Singlet Cells



FL4 Histogram

Singlet Cells



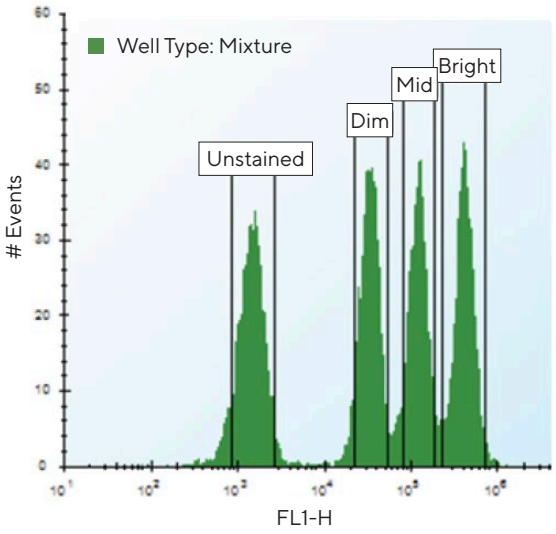
For iQue® Screener, the gating strategy is slightly different. Create a FSC-A vs SSC-A plot with the singlet beads population. Gate mouse IgG2b capture beads first (the population on the right), and then gate other 3 beads. Next, create FL4-A vs. FL3-A plot from "Other 3 beads" population, and gate mouse IgG1, IgG2a, IgG3 population from the left to the right.

Encoding: Identify the Various Encoded Cell Populations
The FL1 proliferation and encoder dye is shown on the FL1-H histogram, and FL4 proliferation and encoder dye on the FL4-H histogram. Adjust the gates as necessary to encompass the entirety of the encoded cell populations. It is possible to identify the encoded populations on either a 1D histogram or 2D plot. (Figure 11)

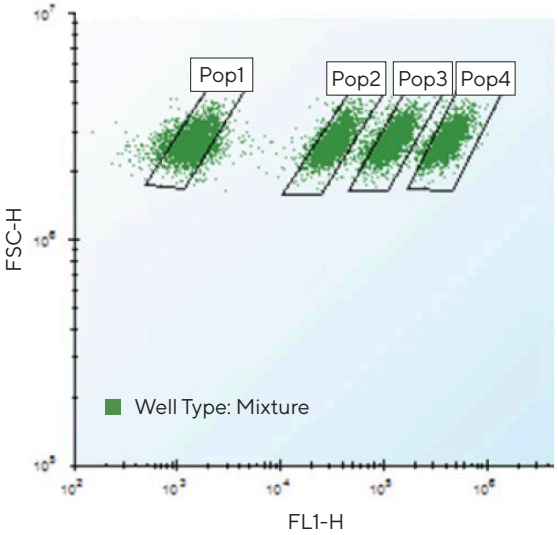
Figure 11

FL1 Dye Gating

FL1-H 1D Histogram
1:4 Serially titrated dye

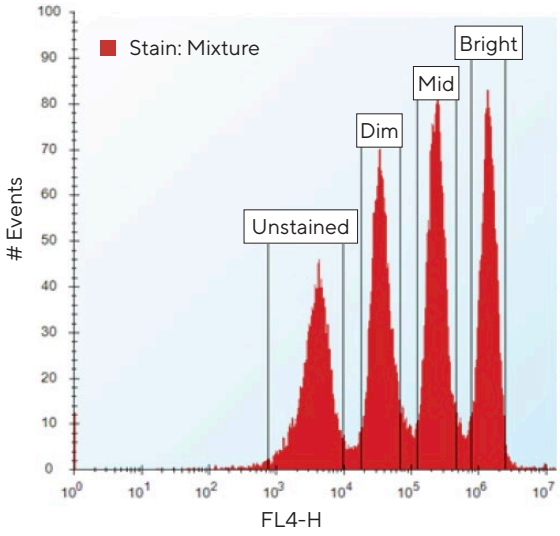


FL1-H vs. FSC-H 2D Plot
1:4 Serially titrated dye

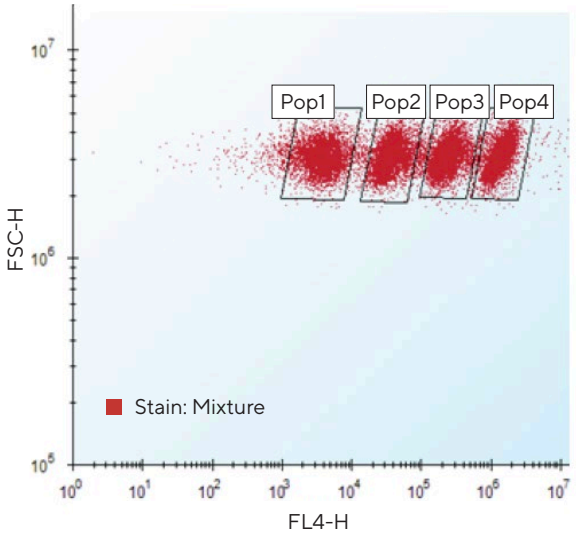


FL4 Dye Gating

FL4-H 1D Histogram
Singlet cell encoder lot #CE4-070914-01



FL4-H vs. FSC-H 2D Plot
1:6 Serially titrated

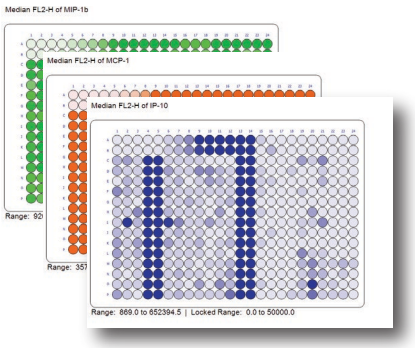


Visualization of Screening Results

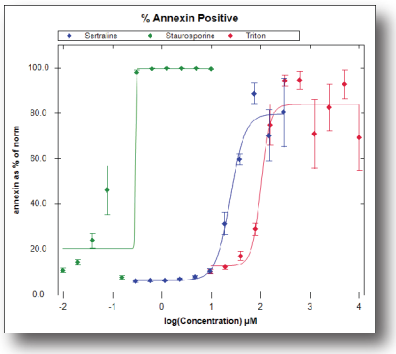
After all the gates have been verified and adjusted as necessary for the plate-level data set, you can generate additional analyses including heat maps, dose responses, standard curves, and Panoramas.

Figure 12

Heat Maps

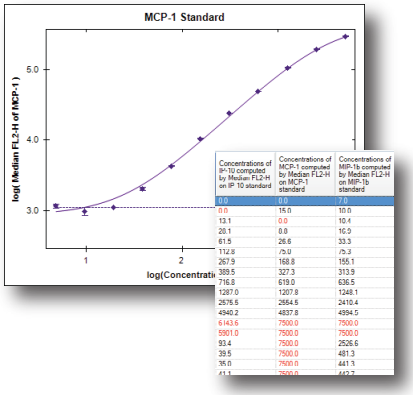


Dose Response Curves

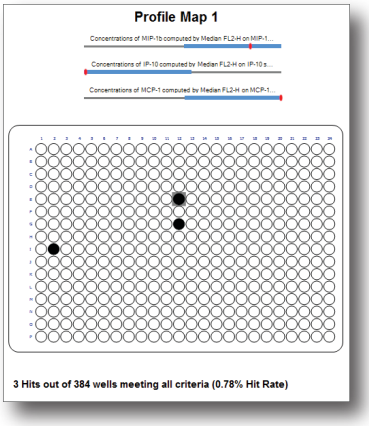


PDF Data Reports

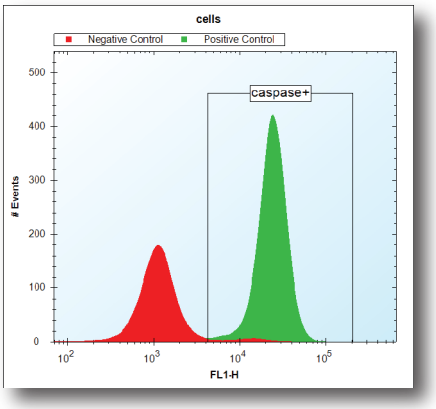
Standard Curves



Profile Maps



Overlay Plots



Appendix A: Additional Information and Example Data

This reagent has been extensively tested for screening applications using both suspension, adherent cells and primary PBMCs.

Cell Lines/Types Validated:

Suspension Cells

- Jurkat (T lymphocytic)
- Ramos (B lymphocytic)
- U937 (Leukemic monocytic)
- PBMCs (Peripheral blood mononuclear cells, from primary donors)

Adherent Cells

- HeLa (human cervical cancer)
- A549 (human lung adenocarcinoma)
- H4 (human neuroglioma)

For the use of adherent cells, establish proper detachment before use. Use QSol™ buffer to prevent clogs and ensure better data acquisition. Some modifications and additional optimizations may be required for use with other cell types.

Appendix B: Mixing Samples with the iQue® Shaker

Table 8: Maximum Fill Volumes and Shake Speed for the iQue® Screener and iQue® Screener PLUS

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

Table 9: Maximum Fill Volumes and Shake Speed for the HTFC Screening System

Plate Type	Well Volume	Recommended MAX RPM
96-Well	20–40 µL	2,800
96-Well	40–60 µL	2,400
96-Well	60+ µL	A | O*
384-Well	10–30 µL	3,500
384-Well	30–50 µL	3,000
384-Well	50+ µL	A | O*

Table 10: Maximum Fill Volumes & Shake Speed for the iQue® Screener HD

Plate Type	Well Volume	Recommended MAX RPM
96-Well	20–40 µL	3,200
96-Well	40–60 µL	2,400
96-Well	60+ µL	A | O*
384-Well	10–30 µL	3,500
384-Well	30–50 µL	3,100
384-Well	50+ µL	A | O*
1536-Well	up to 5 µL	5,000

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

Appendix C: Microplate Recommendations and Wash Protocols

The following plate types have been extensively tested with the iQue® wash protocols:

Table 11

Plate Type	Well Type	Manufacturer	Manufacturer Product #
384-well	V-bottom	Greiner	781280
96-well	V-bottom	iQue®	10149

When using the above plate types, the following aspiration programs have been tested on a BioTek ELx405 Select. If you have a different plate washer brand or model, it is possible to approximate the aspiration settings on a different system.

It is highly recommended that wash protocols utilize the aid of an automated plate washer. Manual aspiration of plates and/or plate inversion techniques could result in severe

sample loss.

Table 12

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

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