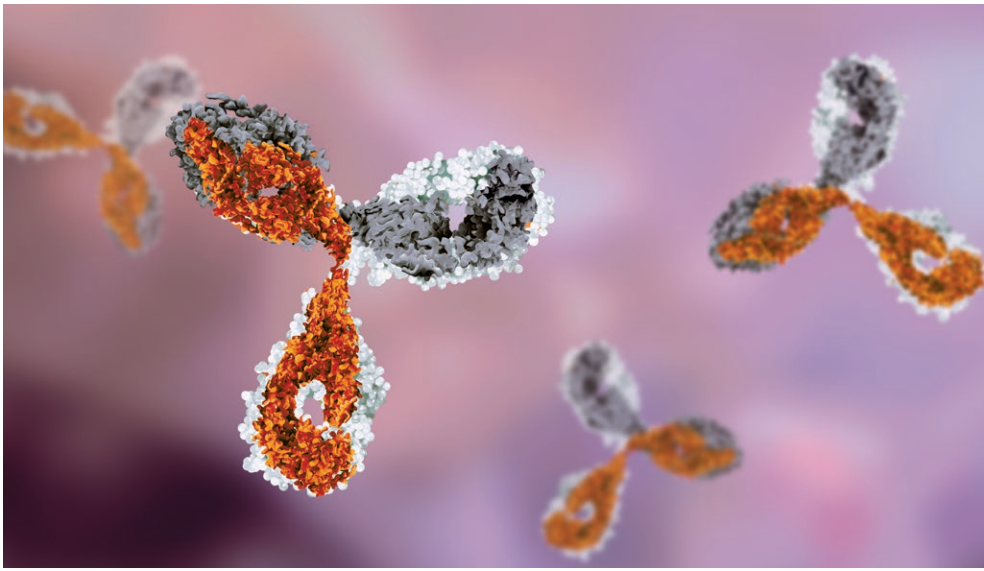
sartorius

Antibody Internalization Human Reagent

Cat No. 90564 for 1 x 96-well format
Cat No. 90565 for 1 x 384-well format

Antibody Internalization Mouse Reagent

Cat No. 90566 for 1 x 96-well format
Cat No. 90567 for 1 x 384-well format



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

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Assay Overview (1 x 96- and 1 x 384-well formats)

This overview summarizes the protocol. Detailed instructions are provided in the Assay Protocol section. Workflow and volumes are identical for both 1 x 96- and 1 x 384-well formats.

Count cells and resuspend at 2x desired concentration

↓

Conjugate antibody at 2x top concentration with Antibody Internalization Reagent and prepare dilutions

↓

Plate 20 µL of labeled antibody per well

↓

Add 20 µL of cells to conjugated antibody, mix, and incubate at 37°C for 2 hours

↓

Acquire data on Intellicyt® iQue Screener PLUS in the RL1 channel

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

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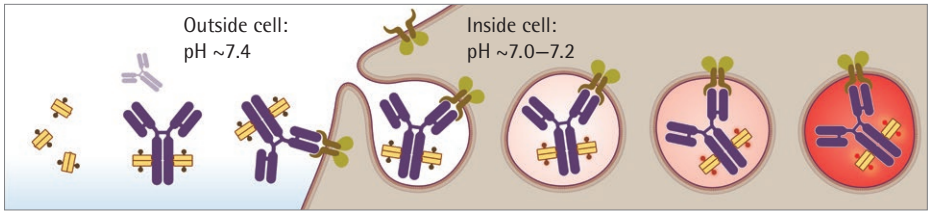
Introduction

Antibody internalization and associated kinetics are critical characteristics that impact antibody therapeutics, pharmacokinetic profiles, and influence the mechanism of action of antibody drugs. For example, antibody-drug conjugates may require rapid internalization to deliver their payload directly into cells to avoid systemic dissemination. However, rapid antibody internalization can be detrimental to immune-mediated killing mechanisms such as antibody-dependent cell cytotoxicity or complement-dependent cytotoxicity.

During biologic drug discovery, antibodies are engineered to increase their biological potencies, to optimize conjugations to non-protein components, and to increase serum half-life. These modifications can have profound effects on antibody activity and specificity, as well as antibody internalization. This necessitates thorough in vitro, high throughput screening of antibodies during the early discovery process to quickly identify the most suitable drug candidates for further development.

Antibody Internalization Reagent is a novel, pH sensitive dye that identifies antibody internalization in a simple plate-based format. The mouse and human types are designed for use with antibodies containing either mouse or human Fc regions. The assay features the flexibility to combine other validated reagents for multiplexed, no-wash protocols with high throughput capabilities. The ability to quickly profile and compare large sets of antibodies and characterize their key attributes, such as antibody internalization, can vastly reduce the time required for candidate generation and expedite the development of potential therapeutic treatments.

Assay Principles

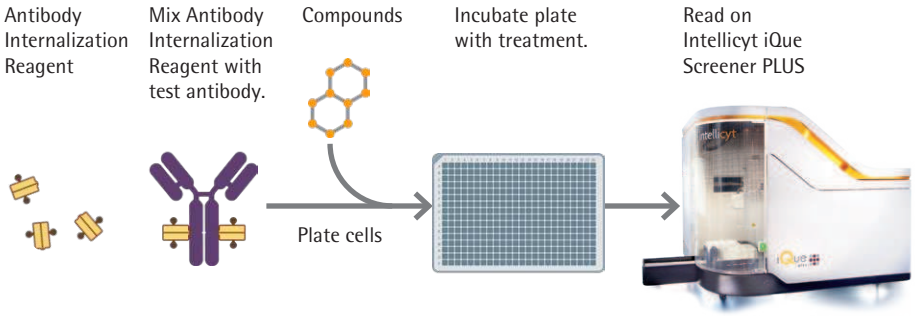


Because the reagent binds to the Fc region of the antibody of interest, it is essential to choose the appropriate type (mouse or human) of the Antibody Internalization Reagent.

This high-throughput, no-wash assay measures antibody internalization from 20 µL of sample and can be used to profile a large number of antibodies in a 96- or 384-well format.

Antibodies are labeled with the novel, pH-sensitive Antibody Internalization Reagent. The labeled antibodies have little fluorescence at neutral pH, but become highly fluorogenic at a lower pH when they are internalized and processed through the acidic lysosome/endosome pathway. After a two hour incubation, sample acquisition is performed using the Intellicyt® iQue Screener PLUS (with sampling times of less than 5 minutes for a 96-well and 20 minutes for a 384-well format). The integrated ForeCyt® software enables data analysis and visualization with plate-level analytics.

Assay Workflow Overview



1. Conjugate antibodies with Antibody Internalization Reagent and prepare dilutions.
2. Plate cells, add conjugated antibody, and incubate.
3. Acquire data RL-1

Reagents Provided

Components	1 x 96-well format (10 µg lyophilized powder)	1 x 384-well format (40 µg lyophilized powder)
Antibody Internalization Human Reagent	Cat No. 90564	Cat No. 90565
Antibody Internalization Mouse Reagent	Cat No. 90566	Cat No. 90567

NOTE: Cat No. 90564 and 90566 (1 x 96-well format) includes 10 µg of Antibody Internalization Reagent. This is enough for 1 x 96-well plate of a single test antibody (at up to 4 µg/mL top concentration, 12 replicates) with overage when reconstituted. Cat No. 90565 and 90567 (1 x 384-well format) includes 40 µg of Antibody Internalization Reagent. This is enough reagent for 1 x 384-well plate or 4 x 96-well plates with overage when reconstituted.

Storage and Stability

Intellicyt® Antibody Internalization Reagent is light sensitive; protect from light.

Store lyophilized product at 2-8°C. Rehydrate with sterile water and vortex gently until dissolved. Remaining reconstituted reagent can be stored at -80°C for > 1 year. Avoid repeated freezing and thawing. Antibody labeling should be performed immediately before use.

Materials Required but Not Provided

- Test antibody of interest (with human or mouse Fc region) at a known concentration
- Target cells of interest
- Target cell growth media
- Multichannel pipette capable of delivering 20 µL (e.g. Sartorius Tacta or Picus)
- 96-well v-bottom plate (Intellicyt #10149) or 384-well v-bottom plate (Greiner #781280)

Intellicyt iQue Screener PLUS Detector Channels (VBR)

Detector	Spectrum	Violet Laser (405 nm)		Blue Laser (488 nm)		Red Laser (640 nm)	
445/45 nm		VL1					
530/30 nm		VL2		BL1			
572/28 nm		VL3		BL2			
615/24 nm		VL4		BL3			
675/30 nm		VL5		BL4		RL1	Antibody Internalization Reagent Detection Channel
780/60 nm		VL6		BL5		RL2	

Detection Channels

The Antibody Internalization Reagent uses the RL1 detection channel. This reagent was validated for use on the Intellicyt iQue Screener PLUS, but is also compatible with the Intellicyt® and iQue Screener platforms.

Recommended Materials

We strongly recommend running a positive and negative istotype control along with the test antibody.

- For the mouse Antibody Internalization Reagent, the positive control is human anti-CD71 produced in mouse (Sigma SAB4700520-100UG, supplied at 1 mg/mL in PBS) and the negative isotype control is Mouse IgG1 (R&D Systems, MAB002).
- For the human Antibody Internalization Reagent, the negative isotype control is Human IgG1 (Absolute Antibody, AB00178-10.0).

Cell Preparation

Count target cells and adjust concentration with growth media to 2x desired final assay concentration in a volume sufficient to ensure 20 µL per well with overage. We suggest a final assay concentration of 1 x 10^6 cells/mL.

For a 96-well format, we recommend at least 3 mL of cells prepared at 2 x 10^6 cells/mL (6 million cells) to accommodate liquid handling.

For a 384-well format, we recommend at least 12 mL of cells prepared at 2 x 10^6 cells/mL (24 million cells) to accommodate liquid handling.

Preparation of Reagent for 1 x 96- and 1 x 384-well format

1. Rehydrate Antibody Internalization Reagent with 100 µL sterile water for 1 x 96-well format (or 400 µL sterile water for 1 x 384-well format). This results in a final concentration 100 µg/mL for either format. The reagent is light sensitive; protect from light.

> Prepare a 1:3 molar ratio of test antibody to Antibody Internalization Reagent. The reagent is approximately one third the size of an antibody, equal mg/mL quantities will produce a 1:3 molar ratio of test antibody to reagent.

2. Calculate the amount of labeled antibody needed using the following formula.

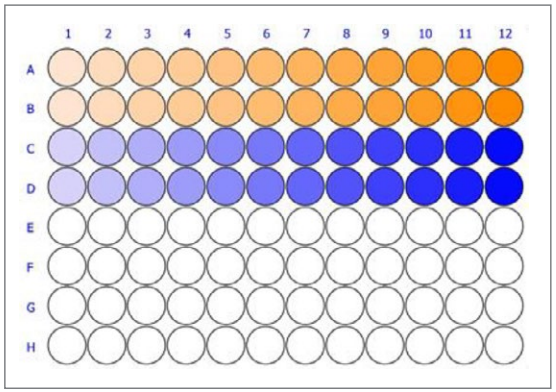
   Calculation for 2 Replicates: Mouse Antibody Labeling Using Positive Control Anti-CD71 at 1 mg/mL Stock Concentration for 1 x 96- Well Format

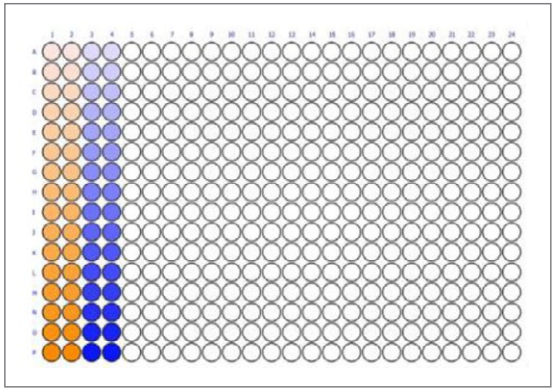
> 1. Determine final assay concentration of test antibody. 1 µg/mL for anti-CD71 is recommended for a positive control. Prepare working stock at 2x or 2 µg/mL.
>
> 2. Determine volume of test antibody required at 2x for 2 replicates of 20 µL per well top concentration as well as adequate overage for 1:2 serial dilutions:
>
>   (# of wells) x (volume per well) x (overage for serial dilutions)
>
>   **2 wells x 20 µL per well x 5 = 200 µL labeled antibody at 2x**
>
> 3. Calculate volumes of test antibody, Antibody Internalization Reagent, and media for a 2x final assay concentration of labeled antibody:
>
>   a. Determine volume of test antibody. **For a small number of replicates, dilute test antibody stock to 100 µg/mL for more accurate pipetting:**
>
>   (total volume of labeled antibody) µL x (2x final concentration of test antibody) µg/mL/(diluted stock concentration of test antibody) µg/mL
>
>   **(200 µL) x (2 µg/mL)/(100 µg/mL) = 4 µL of test antibody (at 100 µg/mL)**
>
>   a. Determine volume of Antibody Internalization Reagent:
>   (volume of test antibody) µL x (diluted stock concentration of test antibody) µg/mL/ (stock concentration of Antibody Internalization Reagent) µg/mL
>
>   **(4 µL) x (100 µg/mL)/(100 µg/mL) = 4 µL Antibody Internalization Reagent**
>
>   b. Determine volume of media:
>
>   (total labeled antibody volume) – (test antibody volume) – (Antibody Internalization Reagent volume)
>
>   (200 µL) – (4 µL) – (4 µL) = 192 µL media

Assay Protocol for 1 x 96- and 1 x 384-well format

Label test antibody and prepare dilutions. We strongly recommend running an appropriate positive and negative istotype control along with the test antibody for the mouse or human type.

1. Add test antibody and Antibody Internalization Reagent (using previous calculations) to culture media in a tube. Protect from light, and incubate for 15 minutes at 37˚C.
2. Prepare 1:2 serial dilutions of the labeled antibody in culture media. This can be done in a separate 96-well format or in tubes.
3. Plate 20 µL of dilutions (including a media-only blank) in a 96- or 384-well plate from low to high concentration. The template includes positive and negative controls run in duplicate.





4. Add target cells to plated, labeled antibody dilutions. Depending on the number of samples and replicates, you may choose to plate either horizontally or vertically.
   a. Use a multichannel pipette to add 20 µL of well-mixed target cells at 2x to the wells of a 96- or 384-well format.
   b. Mix using either the plate shake function on the Intellicyt iQue Screener PLUS (2000 RPM) or manually with a multichannel pipette. For plate Shaker instructions see Appendix A.
3. Cover plate and incubate at 37˚C for 2 hours.
4. Acquire data on the Intellicyt iQue Screener PLUS.

NOTE: The work flow and volumes are identical for 1 x 96- and 1 x 384-well formats.

System Operation

1. Launch ForeCyt® software.
2. If you have not already imported the template provided by Intellicyt, import it now, and select the correct format (96- or 384-well). Create a New Experiment using the template.

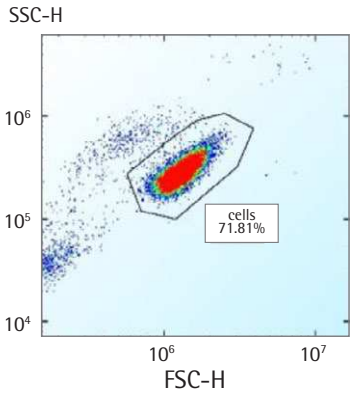
   File → New Experiment → Use Template → Experiment Name
3. If you have samples other than the recommended controls on your plate, you can set up Series dilutions in the Series tab. You can also change the concentrations or layout of Series at this time.

   Design → Series → Add Series
4. Be sure your plate is in place and click “Run” on the Menu to acquire data.

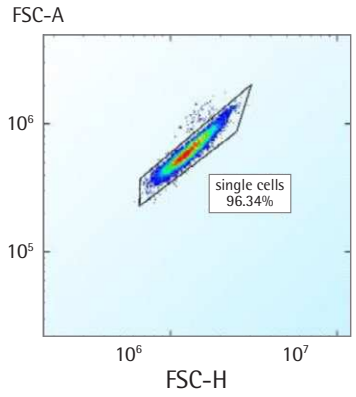
Data Analysis

1. **Identify cell populations.** The template gates are pre-set for different populations (cells, single cells, and internalized cells). Below are the gating details if you want to manually draw the gates or fine tune the existing gates from the template:
   a. In all events, FSC-H vs. SSC-H, draw gate around "cells" avoiding cell debris.
   b. Make a new plot, FSC-H vs. FSC-A, gating on "cells," and draw another gate around "single cells."
   c. Make a histogram based on "single cells," change the x-axis to "RL1-H," and draw a gate for "internalized cells" which will appear furthest to the right.

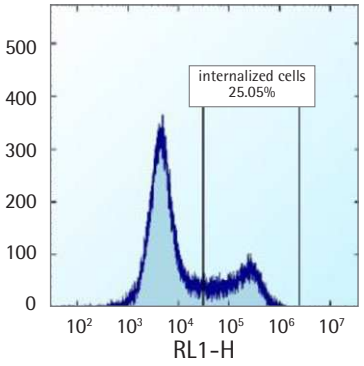
All Events



Cells



Single Cells



4. **Plot dose response curves.** The template includes a dose response curve plotted for MFI and percent internalized for a control sample. Follow these steps to create curves for additional samples:
   a. Create Primary Metric

      Metrics → Add → Primary Metric

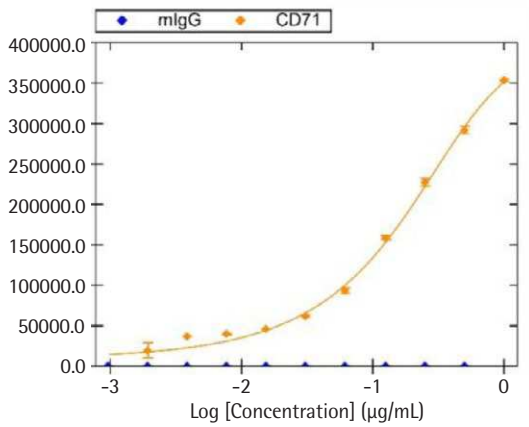
      (Primary Metrics for "median RL1-H of internalized cells" and for each antibody and/or cell type tested)
   b. Create Advanced Metric

      Metrics → Add → Advanced Metric

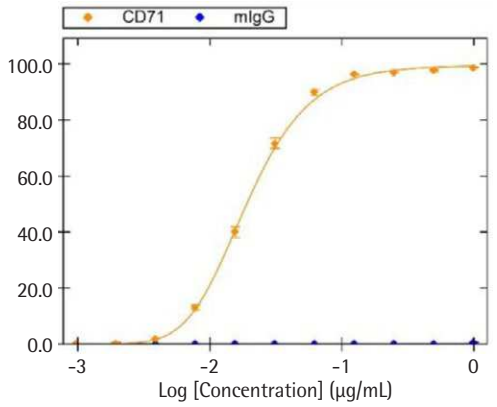
      ("Internalized cells as percent of single cells" and for each antibody and/or cell type tested)

Examples of positive and negative control antibody dose response curves showing MFI and percent internalized for Jurkat cells:

Median RL1-H of internalized cells



Internalized cells as % of single cells

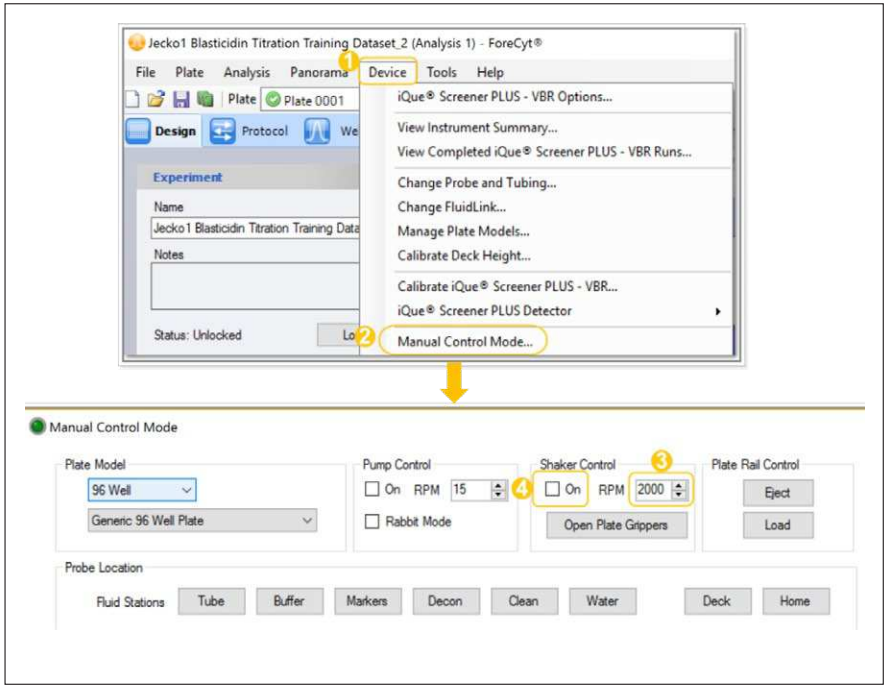


Appendix A

Mixing Samples Using Shaker

This assay requires shaking. If you don't have a separate shaker, you can use the one on the IntelliCyt screening system.

1. Click on Device in the menu bar.
2. Scroll down to Manual Control.
3. In the Manual Control window, use the arrows to set the RPM.
4. As soon as you click On, the shaker will begin to shake and continue to shake until you unclick.



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