SARTORIUS

Product Guide

Incucyte® Fabfluor-pH Antibody Labeling Dyes

For Antibody Internalization Assays

Product Information

Presentation, Storage and Stability

The Incucyte® Fabfluor-pH Antibody Labeling Dyes for antibody internalization are supplied as lyophilized solids in sufficient quantity to label 50 µg of test antibody, when used at the suggested molar ratio (1:3 of test antibody to labeling Fab). The lyophilized solid can be stored at 2-8° C for one year. Once re-hydrated, any unused reagent should be aliquoted and stored at -80° C for up to one year. Avoid repeated freeze-thaw cycles.

Product Name	Cat. No.	Ex. Max*	Em. Max*	Amt.	Storage	Stability
Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green | Red optical module						
Incucyte® Human Fabfluor-pH Red Antibody Labeling Dye	4722	640 nm	664 nm	50 µg	Lyophilized: 2-8° C Rehydrated: -80° C	1 year
Incucyte® Mouse IgG1 Fabfluor-pH Red Antibody Labeling Dye	4723					
Incucyte® Mouse IgG2a Fabfluor-pH Red Antibody Labeling Dye	4750					
Incucyte® Mouse IgG2b Fabfluor-pH Red Antibody Labeling Dye	4751					
Incucyte® Rat Fabfluor-pH Red Antibody Labeling Dye	4737					
Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green | Orange | NIR or an Orange | NIR optical module						
Incucyte® Human Fabfluor-pH Orange Antibody Labeling Dye	4812	544 nm	565 nm	50 µg	Lyophilized: 2-8° C Rehydrated: -80° C	1 year

*Excitation and Emission maxima were determined at a pH of 4.5.

Background

Incucyte® Fabfluor-pH Antibody Labeling Dyes are designed for quick, easy labeling of Fc-containing test antibodies with a Fab fragment-conjugated pH-sensitive fluorophore. The pH-sensitive dye based system exploits the acidic environment of the lysosomes to quantify internalization of the labeled antibody. As Fabfluor labeled antibodies reside in the neutral extracellular solution (pH 7.4), they interact with cell surface specific antigens and are internalized. Once in the lysosomes, they enter an acidic environment (pH 4.5–5.5) and a substantial increase in fluorescence is observed. In the absence of expression of the specific antigen, no internalization occurs and the fluorescence intensity of the labeled antibodies remains low. With the Incucyte® integrated analysis software, background fluorescence is minimized. These reagents have been validated for use with a number of different antibodies in a range of cell types. The Incucyte® Live-Cell Analysis System enables real-time, kinetic evaluation of antibody internalization.

Recommended Use

We recommend that the Incucyte® Fabfluor-pH Antibody Labeling Dyes are prepared at a stock concentration of 0.5 mg/mL by the addition of 100 µL of sterile water and triturated (centrifuge if solution not clear). The reagent may then be diluted directly into the labeling mixture with test antibody. Do NOT sonicate the solution.

Additional Information

The Fab antibody was purified from antisera by a combination of papain digestion and immunoaffinity chromatography using antigens coupled to agarose beads. Fc fragments and whole IgG molecules have been removed.

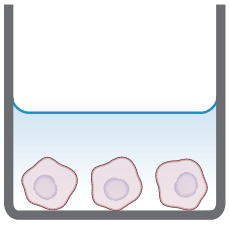
Human Red (Cat. No. 4722) or Human Orange (Cat. No. 4812)—Based on immunoelectrophoresis and/ or ELISA, the antibody reacts with the Fc portion of human IgG heavy chain but not the Fab portion of human IgG. No antibody was detected against human IgM, IgA or against non-immunoglobulin serum proteins. The antibody may cross-react with other immunoglobulins from other species.

Mouse IgG1 (Cat. No. 4723), IgG2a (Cat. No. 4750) or IgG2b (Cat. No. 4751)—Based on antigen-binding assay and/or ELISA, the antibody reacts with the Fc portion of mouse IgG, IgG2a or IgG2b, respectively, but not the Fab portion of mouse immunoglobulins. No antibody was detected against mouse IgM or against non-immunoglobulin serum proteins. The antibody may cross-react with other mouse IgG subclasses or with immunoglobulins from other species.

Rat (Cat. No. 4737)—Based on immunoelectrophoresis and/or ELISA, the antibody reacts with the Fc portion of rat IgG heavy chain but not the Fab portion of rat IgG. No antibody was detected against rat IgM, IgA or against non-immunoglobulin serum proteins. The antibody may cross-react with other immunoglobulins from other species.

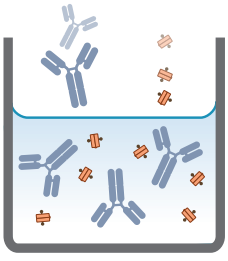
Quick Guide

1. Seed cells



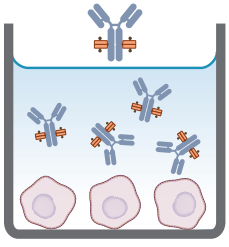
Seed cells (50 µL/well, 5,000–30,000 cells/well), into 96-well plate and leave to adhere (2–24 h, depending on cell type).

2. Label test antibody



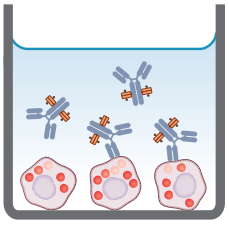
Mix antibody and Incucyte® Fabfluor-pH Dye at a molar ratio of 1:3 in media, 2x final assay concentration. Incubate for 15 minutes at 37° C to allow conjugation.

3. Add to cells



Add Fabfluor-antibody mix (50 µL/well) to cell plate.

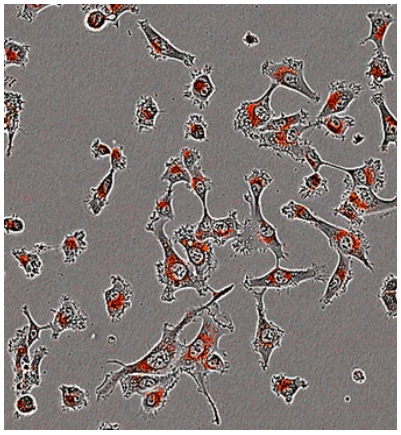
4. Live-cell fluorescent imaging



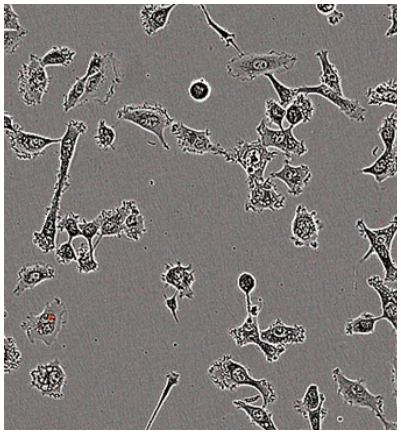
Capture images every 15–30 minutes (10X or 20X) in Incucyte® Live-Cell Analysis System with appropriate fluorescent module configured for 24-48 hours. Analyze using integrated software.

Example Data

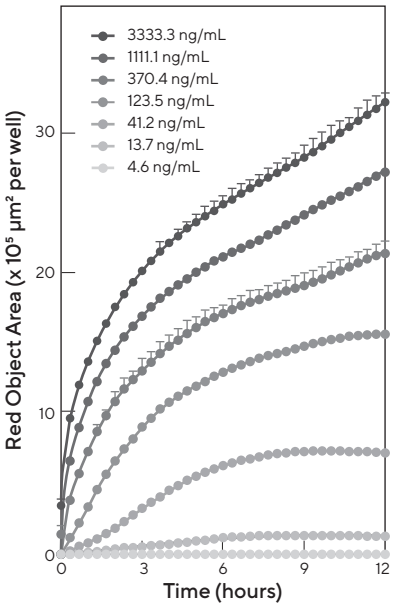
A.
CD71-Fabfluor



B.
IgG-Fabfluor



C.



D.

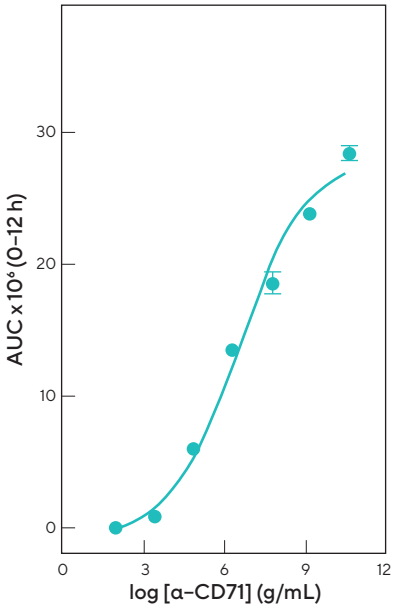


Figure 1: Concentration-dependent increase in antibody internalization of Incucyte® Fabfluor labeled-α-CD71 in HT1080 cells. α-CD71 and mouse IgG1 isotype control were labeled with Incucyte® Mouse IgG1 Fabfluor-pH Red Antibody Labeling Dye. HT1080 cells were treated with either Fabfluor-α-CD71 or Fabfluor-IgG1 (4 µg/mL); HD phase and red fluorescence images were captured every 30 minutes over 12 hours using a 10X magnification. (A) Images of cells treated with Fabfluor-α-CD71 display red fluorescence in the cytoplasm (images shown at 6 h). (B) Cells treated with labeled isotype control display no cellular fluorescence. (C) Time-course of Fabfluor-α-CD71 internalization with increasing concentrations of Fabfluor-α-CD71 (progressively darker symbols). Internalization has been quantified as the red object area for each time-point. (D) Concentration response curve to Fabfluor-α-CD71. Area under the curve (AUC) values have been determined from the time-course shown in panel C (0-12 hours) and are presented as the mean ± SEM, n=3 wells.

Protocols and Procedures

Materials

- Incucyte® Fabfluor-pH Antibody Labeling Dye
- Test antibody of interest containing human, mouse, or rat IgG Fc region (at known concentration)
- Target cells of interest
- Target cell growth media
- Sterile distilled water
- 96-well flat bottom microplate (e.g. Corning Cat. No. 3595) for imaging
- 96-well round black round bottom ULA plate (e.g. Corning Cat. No. 45913799) or amber microtube (e.g. Cole Parmer Cat. No. MCT-150-X, autoclaved) for conjugation step
- 0.01% Poly-L-Ornithine (PLO) solution (e.g. Sigma Cat. No. P4957), optional for non-adherent cells

Recommended control antibodies

It is strongly recommended that a positive and negative control is run alongside test antibodies and cell lines. For example, CD71, which is a mouse anti-human antibody, is recommended as a positive control for the mouse Fab.

- Anti-CD71, clone MEM-189, IgG1 e.g. Sigma Cat. No. SAB4700520-100UG
- Anti-CD71, clone CYG4, IgG2a e.g. BioLegend Cat. No. 334102
- Isotype controls, depending on isotype being studied—Mouse IgG1, e.g. BioLegend Cat. No. 400124, Mouse IgG2a e.g. BioLegend Cat. No. 401501

Preparation of Incucyte® Antibody Internalization Assay

1. Seed target cells of interest

1.1 Harvest cells of interest and determine cell concentration (e.g. trypan blue + hemocytometer).

1.2 Prepare cell seeding stock in target cell growth media with a cell density to achieve 40–50% confluence before the addition of labeled antibodies. The suggested starting range is 5,000–30,000 cells/well, although the seeding density will need to be optimized for each cell type.

Note: For non-adherent cell types, a well coating may be required to maintain even cell distribution in the well. For a 96-well flat bottom plate, we recommend coating with 50 µL of either 0.01% Poly-L-Ornithine (PLO) solution or 5 µg/mL fibronectin diluted in 0.1% BSA. Coat plates for 1 hour at ambient temperature, remove solution from wells and then allow the plates to dry for 30-60 minutes prior to cell addition.

1.3 Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate. Lightly tap plate side to ensure even liquid distribution in well. To ensure uniform distribution of cells in each well, allow the covered plate sit on a level surface undisturbed at room temperature in the tissue culture hood for 30 minutes. After cells are settled, place the plate inside the Incucyte® Live-Cell Analysis System to monitor cell confluence.

Note: Depending on cell type, plates can be used in assay once cells have adhered to plastic and achieved normal cell morphology e.g. 2-3 hours for HT1080 or 1-2 hours for non-adherent cell types. Some cell types may require overnight incubation.

2. Label Test Antibody

2.1 Rehydrate the Incucyte® Fabfluor-pH Antibody Labeling Dye with 100 µL sterile water to result in a final concentration of 0.5 mg/mL. Triturate to mix (centrifuge if solution is not clear).

Note: The reagent is light sensitive and should be protected from light. Rehydrated reagent can be aliquoted into amber or foil wrapped tubes and stored at -80° C for up to 1 year (avoid freezing and thawing).

2.2 Mix test antibody with rehydrated Incucyte® Fabfluor–pH Antibody Labeling Dye and target cell growth media in a black round bottom microplate or amber tube to protect from light (50 µL/well).

a. Add test antibody and Incucyte® Fabfluor–pH Antibody Labeling Dye at 2X the final concentration. We suggest optimizing the assay by starting with a final concentration of 4 µg/mL of test antibody or the Fabfluor-pH Antibody Labeling Dye (i.e. 2X working concentration = 8 µg/mL).

Note: A 1:3 molar ratio of test antibody to Incucyte® Fabfluor-pH Antibody Labeling Dye is recommended. The labeling reagent is a third of the size of a standard antibody (50 and 150 KDa, respectively). Therefore, labeling equal quantities will produce a 1:3 molar ratio of test antibody to labeling Fab.

b. Make sufficient volume of 2X labeling solution for 50 µL/well for each sample. Triturate to mix.

c. Incubate at 37° C for 15 minutes protected from light.

Note: If performing a range of concentrations of test antibody, e.g. concentration response-curve, it is recommended to create the dilution series post the conjugation step to ensure consistent molar ratio. We strongly recommend the use of both a negative and positive control antibody in the same plate.

3. Add labeled antibody to cells

3.1 Remove cell plate from incubator.

3.2 Using a multi-channel pipette, add 50 µL of 2X labeled antibody and control solutions to designated wells. Remove any bubbles and immediately place plate in the Incucyte® Live-Cell Analysis System and start scanning.

Note: To reduce the risk of condensation formation on the lid prior to first image acquisition, maintain all reagents at 37° C prior to plate addition.

4. Acquire images and analyze

4.1 In the Incucyte® integrated software, schedule to image every 15-30 minutes, depending on the speed of the specific antibody internalization.

a Scan on schedule: standard. If the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031) is available, adherent cell-by-cell or non-adherent cell-by-cell scan types can be selected.

b Channel selection: select "phase" and "red" or "phase" and "orange" (depending on reagent used).

c Objective: 10X or 20X depending on cell types used, generally 10X is recommended for adherent cells, and 20X for non-adherent or smaller cells.

NOTE: The optional Incucyte® Cell-by-Cell Analysis Software Module enables the classification of cells into sub-populations based on properties including fluorescence intensity, size and shape. For further details on this analysis module and its application, please see: www.essenbioscience.com/cell-by-cell.

4.2 To generate the metrics, user must create an Analysis Definition suited to the cell type, assay conditions and magnification selected.

4.3 Select images from a well containing a positive internalization signal and an isotype control well (negative signal) at a time point where internalization is visible.

4.4 In the Analysis Definition:

Basic Analyzer:

a. Set up the mask for the phase confluence measure with fluorescence channel turned off.

b. Once the phase mask is determined, turn the fluorescence channel on: Exclude background fluorescence from the mask using the background subtraction feature. The feature "Top-Hat" will subtract local background from brightly fluorescent objects within a given radius; this is a useful tool for analyzing objects which change in fluorescence intensity over time.

i The radius chosen should reflect the size of the fluorescent object but contain enough background to reliably estimate background fluorescence in the image; 20-30 µm is often a useful starting point.

ii The threshold chosen will ensure that objects below a fluorescence threshold will not be masked.

iii Choose a threshold in which red or orange objects are masked in the positive response image but low numbers in the isotype control, negative response well. For a very sensitive measurement, for example, if interested in early responses, we suggest a threshold of 0.2.

NOTE: The Adaptive feature can be used for analysis but may not be as sensitive and may miss early responses. If interested in rate of response, Top-Hat may be preferable.

Cell-by-Cell (if available):

a. Create a Cell-by-Cell mask following the software manual.

b. There is no need to separate phase and fluorescence masks. The default setting of Top-Hat No Mask for the fluorescence channel will enable background subtraction without generation of a mask. Ensure that the Top-Hat radius is set to a value higher than the radius of the larger clusters to avoid excess background subtraction.

c. The threshold of fluorescence can be determined in Cell-by-Cell Classification.

5. Analysis Guidelines

As the labeled antibody is internalized into the acidic environment of the lysosome, the area of fluorescence intensity inside the cells increases. This can be reported in two ways:

Ways to Report	Basic Analyzer	Cell-by-Cell Analysis
i. An increase in fluorescence area	Red | Orange Total Area (μm^2/Well)	(Post classification) High Red | Orange Intensity Object Count Per Well
	*Red | Orange Total Area / Phase Area (%)	*(Post classification) High Red | Orange Intensity Object Count / Total Object Count (%)
ii. An increase in fluorescence intensity, integrated over the area of detectable fluorescence	Red | Orange Total Integrated Intensity Per Well (RCU/OCU x μm^2/Well)	Red | Orange Total Integrated Intensity Per Well (RCU/OCU x μm^2/Well)
	*Red | Orange Total Integrated Intensity Per Well / Phase Confluence (RCU/OCU x μm^2/Well/%)	(Post classification) High Red | Orange Integrated Intensity Per Well (RCU/OCU x μm^2/Well)

* To correct for cell proliferation, it is advisable to normalize the fluorescence area to the total cell area using User Defined Metrics.

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