SARTORIUS

Product Guide

Incucyte® Fabfluor-488 Antibody Labeling Dyes

For Live-Cell Immunocytochemistry

Product Information

Presentation, Storage and Stability

The Incucyte® Fabfluor-488 Antibody Labeling Dyes for cell surface marker analysis 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 should be stored at 2–8° C (stable for at least 1 year). Once rehydrated, it is recommended that the solution is used as soon as possible or aliquoted and stored at -80° C; avoid freezing and thawing (stable for at least 1 year post rehydration).

Incucyte® Opti-Green background suppressor is supplied as 200 µL of 100 mM stock solution in water. The stock liquid should be stored at 2–8° C (stable for at least 1 year). Bring to room temperature and mix well (vortex) before use. Dilute on day of use in assay media.

Product Name	Cat. No.	Ex. Max	Em. Max	Amount	Labeling Suitability	Storage	Stability
Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green | Orange | NIR or Green | Red Optical Module							
Incucyte® Mouse IgG2a Fabfluor-488 Antibody Labeling Dye	4743	490 nm	516 nm	50 µg	Mouse IgG2a Fc containing Antibody	Lyophilized 2-8° C Rehydrated -80° C	1 year
Incucyte® Mouse IgG2b Fabfluor-488 Antibody Labeling Dye	4744	490 nm	516 nm	50 µg	Mouse IgG2b Fc containing Antibody	Lyophilized 2-8° C Rehydrated -80° C	1 year
Incucyte® Mouse IgG1 Fabfluor-488 Antibody Labeling Dye	4745	490 nm	516 nm	50 µg	Mouse IgG1 Fc containing Antibody	Lyophilized 2-8° C Rehydrated -80° C	1 year

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

Background

Incucyte® Fabfluor-488 Antibody Labeling Dyes are designed for quick, easy labeling of Fc containing test antibodies with a green fluorophore. Once labeled the Fabfluor-488-antibody complex, in combination with Incucyte® Opti-Green background suppressor can be used for identification of surface expressed antigens in live cells. In the absence of expressed specific antigen, little or no signal is seen on the cells. In combination with Opti-Green and the Incucyte® integrated analysis software, background fluorescence is minimized. This reagent has 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 live-cell immunocytochemistry.

Recommended Use

We recommend that Incucyte® Fabfluor-488 Antibody Labeling Dyes are prepared at stock concentrations of 0.5 mg/mL by the addition of 100 µL of sterile water and triturate (not supplied, centrifuge if solution not clear). This will rehydrate the powder to result in a buffer of 0.01 M sodium phosphate, 0.25 M NaCl at pH 7.6 with 15 mg per mL BSA (IgG and protease free). The reagent may then be diluted directly into the labeling mixture with test antibody. Do not sonicate the solution.

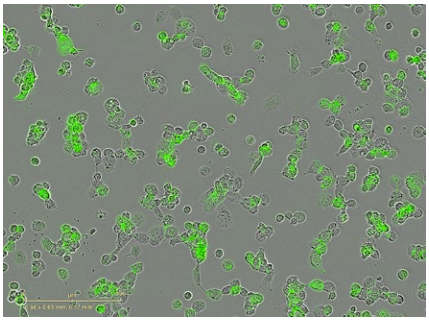
We recommend that Incucyte® Opti-Green background suppressor is mixed well before use. Stock solution should be diluted in complete growth media to produce a final assay concentration of 0.5 mM or a 1 in 200 dilution of stock. This has been shown to be suitable across a range of cell types.

Additional Information

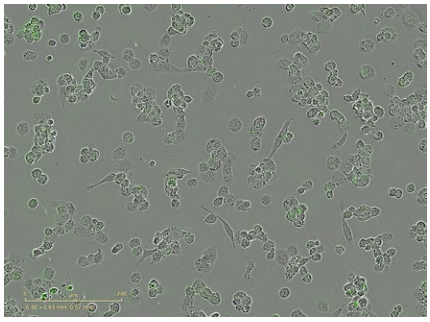
The antibody was purified from antisera by a combination of papain digestion and immunoaffinity chromatography using antigens coupled to agarose beads. Fc fragments and whole IgG molecules have been removed. Based on antigen-binding assay and/or ELISA the antibody reacts with the Fc portion of mouse IgG1, 2a or 2b 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.

Example Data

A. PMA, CD11b-Fabfluor-488



B. PMA, IgG-Fabfluor-488



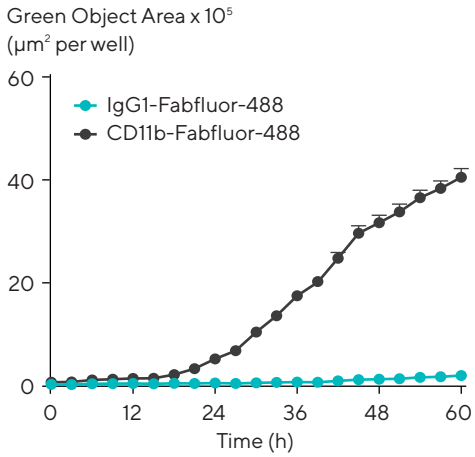
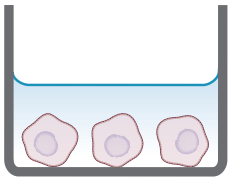


Figure 1: Use of live-cell immunocytochemistry to quantify real-time increase in CD11b surface expression induced by differentiation of THP-1 cells with PMA (100 nM). α-CD11b and IgG isotype control were labeled with Incucyte® Fabfluor-488 using the above protocol. THP-1 cells were incubated with 100 nM PMA, to induce differentiation to a macrophage morphology in combination with Opti-Green (0.5 mM) and Fabfluor-488-α-CD11b or Fabfluor-488-IgG (1 µg/mL). HD phase and green fluorescence images were captured on Incucyte® Live-Cell Analysis System, every 3 h over 60 h using a 20X magnification. Images of cells treated with PMA show green fluorescence in the presence of labeled CD11b (images shown at 48 h) (A). Cells treated with labeled isotype control display no cellular fluorescence (B). The graph shows the quantification of green fluorescence area over time, indicating an increase in CD11b expression in response to differentiation of THP-1 cells with PMA..

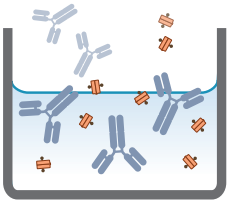
Quick Guide

1. Seed cells



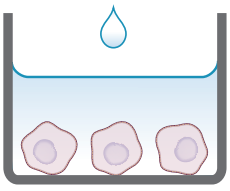
Seed cells (50 µL/well, 5–30K/well) into a 96-well plate.
Note: For non-adherent cell types, PLO coat plate prior to cell seeding.

2. Label test antibody



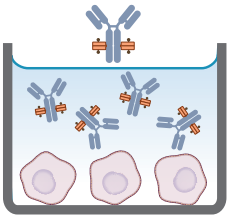
Mix antibody and Fabfluor-488 Dye at a molar ratio of 1:3 in media, 3X final concentration. Incubate for 15 minutes to allow conjugation.

3. Add Incucyte Opti-Green



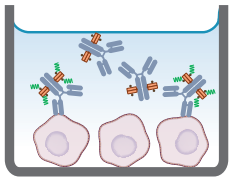
Add 50 µL/well, 3X final concentration.

4. Add labeled AB



Add antibody-Fabfluor mix (50 µL/well) to cell plate. For non-adherent cells, spin plate.

5. Live-cell fluorescent imaging



Capture images (time span and objective depend on assay and cell type, 10X or 20X) in Incucyte® Live-Cell Analysis System.

Protocols and Procedures

Required Materials

- Incucyte® Fabfluor-488 Antibody Labeling Reagent plus Incucyte® Opti-Green background suppressor
  - Mouse IgG1 (Sartorius Cat. No. 4745)
  - Mouse IgG2a (Sartorius Cat. No. 4743)
  - Mouse IgG2b (Sartorius Cat. No. 4744)
- Test antibody of interest (at known concentration) containing mouse Fc region.
  - Recommend using Azide-free antibodies when available
- Target cells of interest
- Target cell growth media
- Effector cell culture media
- PBS (w/o Ca^{2+}/Mg^{2+}, Life Tech Cat. No. 14190).
- 96-well flat bottom microplate (e.g., Corning Cat. No. 3595) for imaging
- 96-well round round bottom plate (e.g., Corning Cat. No. 3799) or amber microtube (e.g., Cole-Parmer Cat. No. MCT-150-X) for conjugation step

Additional Material for Non-Adherent Cell Types

- Poly-L-ornithine, PLO (Cat. No. Sigma P4957)

Recommended Materials

It is strongly recommended to run both a positive and negative control alongside test antibodies and cell lines. CD71 (anti transferrin receptor) marker is recommended as a positive control for the mouse Fab IgG1 or 2a. Isotype matched IgG are recommended as negative controls.

- 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., R&D Systems Cat. No. MAB002 or Biolegend Cat. No. 400124)
  - Mouse IgG2a (e.g., Biolegend Cat. No. 401501)
  - Mouse IgG2b (e.g., Biolegend Cat. No. 400322)

Incucyte® Live-Cell Immunocytochemistry Assay Protocol

1a. Seed Target Cells of Interest - Adherent Cell

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 to achieve 40–50% confluence after 2–6 h. Suggested starting range 5,000–20,000 cells/well (depends on cell type used).
Note: Seeding density must be optimized for each cell type.

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.

1.4 Remove bubbles from all wells by gently squeezing a wash bottle (containing 70–100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.5 Allow cells to settle on a level surface for 30 minutes at room temperature, then place in 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 hr for HT-1080). Some cell types may require overnight incubation.

1b. Seed Target Cells of Interest—Non-Adherent Cells

Note: For this assay, non-adherent cells will be the last addition to the plate (prepare suspension during the antibody conjugation step).

1.1 Coat a 96-well flat bottom plate with relevant coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution (Sigma Cat. No. P4957) or 5 µg/mL fibronectin (Sigma Cat. No.P4957) diluted in 0.1% BSA. Coat for 1 hour at ambient temperature, remove solution from wells, and then allow plates to dry for 30–60 minutes prior to cell addition.
Note: Some optimization of plate coatings may be required.

1.2 Count cells of interest and determine cell concentration (e.g., trypan blue + hemocytometer).

1.3 Prepare cell seeding stock in target cell growth media, suggest starting range of 20,000–40,000 cells/well in 50 µL (depends on cell type used).

1.4 Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate.

1.5 Remove bubbles from all wells by gently squeezing a wash bottle (containing 70–100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.6 Allow cells to settle on a level surface for 30 minutes at room temperature then place in Incucyte® Live-Cell Analysis System to monitor cell confluence.
Note: To reduce settling time cell plates can be centrifuged for 1 minute at 50 g.

2. Labeling of Test Antibody

Note: It is recommend to use low azide or azide-free antibodies (e.g., LEAF™ from Biolegend). Effects on cell growth from high concentrations of azide have been observed in some cell types. If this is of concern, the buffer can be exchanged using a simple desalting column (e.g., Zeba from Thermo Scientific).

2.1 Rehydrate Incucyte® Fabfluor-488 Antibody Labeling Dye with 100 µL sterile water (final concentration = 0.5 mg/mL).
Note: A 1:3 molar ratio of test antibody to Incucyte® Fabfluor-488 Dye is recommended. The labeling reagent is a third of the size of a standard antibody. Therefore, equal mg/mL quantities produce a 1:3 molar ratio of test antibody to labeling Fab.
Note: Reagent is light sensitive, keep in amber or foil wrapped tubes. Remaining rehydrated reagent can be aliquoted and stored at -80° C (avoid freezing and thawing, stable for > year).

2.2 Mix test antibody with dilute Incucyte® Fabfluor-488 Antibody Labeling Dye and target cell growth media in a round bottom microplate or amber tube to protect from light. Prepare sufficient quantity to enable 50 µL/well at 3X final assay concentration.
Note: We strongly recommend using both a negative and positive control antibody (see Recommended Materials above)

a. Add test antibody at 3X the final antibody concentration. Recommendation: A final concentration of < 1.5 µg/mL of test antibody. A reasonable starting concentration is 1 µg/mL (e.g., 3X working concentration = 3 µg/mL).
b. Add Incucyte® Fabfluor-488 Antibody Labeling Dye at a 1:3 (test antibody:Fabfluor) molar ratio. **See Example Calculations below.**
c. Add media to dilute to 3X final assay concentration. Triturate to mix.
d. Incubate for 15 minutes.

Example Calculation of Antibody Labeling Using Positive Control Anti-Cd 71 at 1 Mg/mL Stock Concentration

1. Required final assay concentration of test antibody—1 µg/ mL for anti-CD71 is recommended for positive control wells. Working concentration = 3X, or 3 µg/mL.
2. Determine volume of labeled antibody required at 3X final assay concentration, i.e., dilution of 1:3 recommended upon addition to cells: [# wells] x 50 µL (plus additional required to prepare dilution series if desired).
   (e.g., For 8 replicates of 1:3 dilution of labeled test antibody):
   8 x 50 µL= 400 µL minimum (500 µL used for this example)
3. Calculate volumes of test antibody, Incucyte® Fabfluor-488 Dye, and media required to provide 3X final assay concentration of labeled test antibody.
   a. Determine volume of test antibody: [Total volume] µL x [Working concentration test antibody] µg/mL / [Stock concentration test antibody] mg/mL /1000
      500 µL x 3 µg/mL / 1 mg/mL / 1000 = 1.5 µL
   b. Determine volume of Incucyte® Fabfluor-488: [Volume of test antibody] µL x [Stock concentration of test antibody] mg/mL / [Stock concentration of Fabfluor-488] mg/mL
      1.5 µL x 1 mg/mL / 0.5 mg/mL = 3.0 µL
      Note: Incucyte® Fabfluor-488 Dye is a third of the molecular weight of a standard antibody. Therefore, equal volumes of equal mg/mL quantities produce a 1:3 molar ratio of test antibody to Fabfluor-488 as MW of a typical antibody is ~ 3X of Fabfluor-488. In this case, the stock concentration in mg/mL of test antibody is twice that of Fabfluor-488. Therefore, 2X volume of Fabfluor-488 is required.
   c. Determine volume of media: [Total volume] – [Test antibody volume] – [Fabfluor volume]
      500 µL – 1.5 µL – 3.0 µL = 495.5 µL

3. Dilution of Incucyte® Opti-Green Background Suppressor

3.1 Dilute Opti-Green stock in complete growth media for a final assay concentration of 0.5 mM or 1:200 dilution of stock (see calculations below).

Note: A final assay concentration of 0.5 mM has proven to be suitable across a range of cell types, however some optimization may be required to assess cell proliferation in the presence of Opti-Green. For lower expressed markers, Opti-Green may be increased in some cell types (1 mM or 1:100 dilution of stock final assay concentration), allowing for a higher concentration of test antibody to be added (< 3 µg/mL). A full list of pre-assessed cell types is included at the end of this protocol.

Example Calculation for Opti-Green Background Suppressor

1. Required final assay concentration of Opti-Green is 0.5 mM. Working concentration = 3X, or 1.5 mM.
2. Determine volume of Opti-Green required at 3X final assay concentration, i.e., dilution of 1:3 recommended upon addition to cells:
   [# wells] x 50 µL (plus additional required to prepare dilution series if desired)
   (e.g., For 96 replicates of 1:3 dilution of Opti-Green):
   96 x 50 µL = 4800 µL minimum (5000 µL used for this example)
3. Calculate volume of Opti-Green required to provide 3X final assay concentration.

a. Determine volume of Opti-Green:
[Total volume] µL x [Working concentration Opti-Green] mM / [Stock concentration Opti-Green] mM
5000 µL x 1.5 mM/100 mM = 75 µL

b. Determine volume of media.
[Total volume] – [Opti-Green]
5000 µL – 75 µL = 4925 µL

4. Add Incucyte® Fabflour-488 Test Antibody and Opti-Green to Cells

Adherent Cells

4.1 Remove cell plate from incubator.

4.2 Using a multi-channel pipette:
- a. Add 50 µL of diluted Opti-Green to wells.
- b. Add 50 µL of labeled antibody to required test wells.
- c. Remove any bubbles and place plate in Incucyte® Live-Cell Analysis System.

4.3 Place plate in Incucyte® Live-Cell Analysis System.

Non-Adherent Cells

4.1 Add reagents to matrix coated plate:
- a. Add 50 µL of diluted Opti-Green.
- b. Add 50 µL of labelled antibody to required test wells.
- c. Add 50 µL of cell suspension to wells.
- d. Remove any bubbles.

4.2 Allow the plate to sit for 30 minutes at room temperature to allow even settling, or centrifuge at 50 g for 1 minute.

4.3 Place plate in Incucyte® Live-Cell Analysis System.

5. Acquire Images and Analyze

5.1 Using Incucyte® integrated software, schedule 24-hour repeat scanning for every 2–3 hours.
- a. Scan on schedule, Standard.
- b. Channel selection: select "Phase" and "Green"
- 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.

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

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

5.4 In the Analysis Definition:
- a. Set mask for phase confluence measure with green channel turned off.
- b. Turn green channel on and mask green objects. Exclude background fluorescence using the background subtraction feature. The feature "Top-Hat" will subtract local background from brightly fluorescent objects within a given radius; applicable for analyzing objects which change in fluorescence intensity over time.
  - i. The radius chosen should reflect the size of fluorescent objects 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 green objects are masked in the positive response image but low numbers in the isotype control, negative response well.

Note: For both cell types, individual cell identification can be enabled with the use of the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031). This enables the subsequent classification into subpopulations based on properties including fluorescence intensity, size and shape. For further details of this analysis module and its application see: **www.essenbioscience.com/cell-by-cell**

Analysis Guidelines

Staining of surfaced expressed protein will appear as a green ring followed by intracellular green signal as there will be internalization of the signal over time (time depends on cell type studied). Suggested metrics for data analysis are shown below:

1. Quantification of fluorescence area ("Total Object Area" or "Green Object Confluence"). Suggested metric: Analyze using "Total Green Object Area (μm^2/well)".
2. Quantification of intensity, integrated over the area of detectable fluorescence (i.e. "Total Integrated Intensity"). Suggested metric: Analyze using "Total Green Object Integrated Intensity (GCU x μm^2/ well)" metrics.
3. To correct for cell proliferation, it is advisable to normalize the area measurement for cell coverage (e.g., "Green Object Confluence"/"Phase Confluence").
   Note: If using Cell-by-Cell Analysis, post classification the data can be displayed as either % of cells expressing red fluorescence or mean intensity of positive red objects.

Table 1: Incucyte® Opti-Green Concentration Recommendations

Cell Type	Cell Line	Type	Concentration Range Tested	Recommended Concentration
Adherent	HT-1080	Epithelial (fibrosarcoma)	0.25-30 mM	1 mM
	A549	Lung		
	MDA-MB-231	Breast		
	MCF-7	Breast		
	SKOV-3	Ovary		
Non-Adherent	Jurkat	T cell leukemia	0.125-10mM	0.5 mM
	CCRF-CEM	T cell leukemia		
	Raji	B cell lymphoma		
	Ramos	B cell lymphoma		
	THP-1	Monocytic		
	HL-60	Pre-myelocytic		
	PBMC	Peripheral blood cells		

Find more information at www.sartorius.com/incucyte

For Research Use Only. Not For Therapeutic or Diagnostic Use.

Sales and Service Contacts

For further contacts, visit www.sartorius.com

Essen BioScience, A Sartorius Company
www.sartorius.com/incucyte
E-Mail: AskAScientist@sartorius.com

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Telephone +1 734 769 1600
E-Mail: orders.US07@sartorius.com

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Telephone +44 1763 227400
E-Mail:
euorders.UK03@sartorius.com

APAC
Essen BioScience K.K.
4th Floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Telephone: +81 3 6478 5202
E-Mail: orders.US07@sartorius.com