SARTORIUS

Product Guide

Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Kit

For Live-Cell Immunocytochemistry

Product Information

Presentation, Storage and Stability

The Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Kit for cell surface marker analysis contains sufficient quantity of reagents to label 50 µg of test antibody, when used at the suggested molar ratio (1:3 of test antibody to labeling Fab). The kit includes one vial of Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Dye and one vial of background suppressor Incucyte® Opti-Orange, both of which are supplied as lyophilized solids. The lyophilized solids should be stored at 2–8 °C and are stable for at least one year upon receipt. Once rehydrated, it is recommended that the solutions are used as soon as possible or aliquoted and stored at -80 °C; avoid freezing and thawing (stable for 1 year post-rehydration).

Product Name	Cat. No.	Ex. Max	Em. Max	Amount	Labeling Suitability	Storage	Stability
Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Kit	BA-04873						
▪ Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Dye		555 nm	565 nm	50 µg	Mouse IgG1 Fc containing antibody	Lyophilized 2-8 °C Rehydrated -80 °C	Lyophilized: 1 year from receipt Rehydrated: 1 year post hydration
▪ Incucyte® Opti-Orange				2 nmol		Lyophilized 2-8 °C Rehydrated -80 °C	Lyophilized: 1 year from receipt Rehydrated: 1 year post hydration

Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green | Orange | NIR or an Orange | NIR Optical Module

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

Background

Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Kit is designed for quick, easy labeling of Mouse IgG1 Fc containing test antibodies with an orange fluorophore. Once the antibody is labeled with Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Dye, the Fabfluor-555-antibody complex 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 Incucyte® Opti-Orange and the Incucyte® integrated analysis software, background fluorescence is minimized. This kit 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. Furthermore, the Incucyte® Mouse IgG1 Fabfluor-555 Dye can be multiplexed with Incucyte® Green and/or NIR fluorescent reagents, enabling measurements of different cellular events, protein localizations, or cell types in a single well.

Recommended Use

We recommend that Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Dye is prepared at a stock concentration of 0.5 mg/mL by adding 100 µL sterile water (not supplied) and triturating (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-Orange background suppressor be prepared at a stock concentration of 10 mM by adding 200 µL sterile water (not supplied) and triturating (centrifuge if solution not clear). Stock solution should be diluted in complete growth media to produce a final assay concentration of 30 µM. 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 but not the Fab portion of mouse immunoglobulins. No reactivity 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.

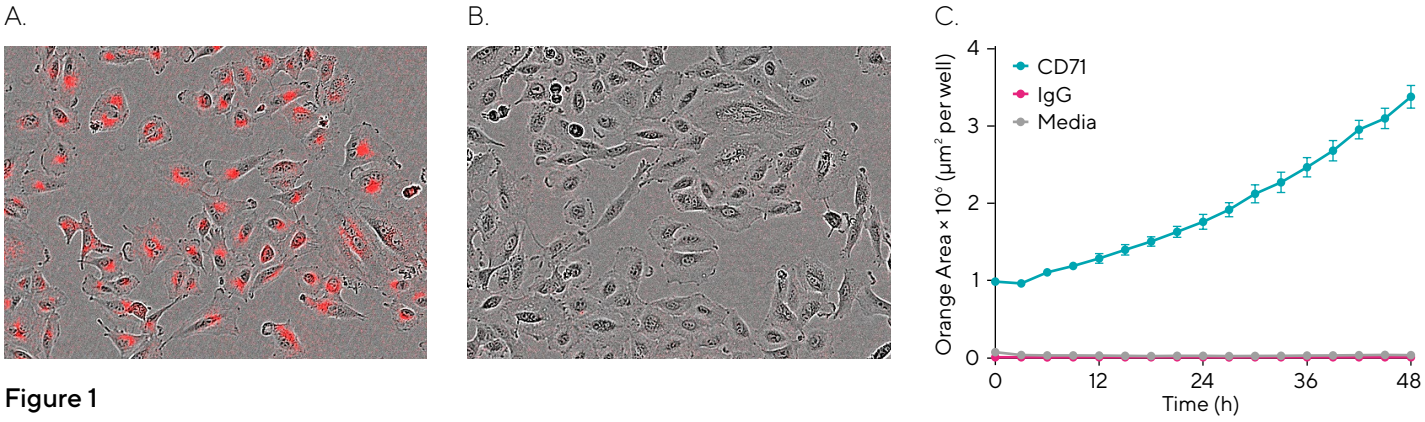


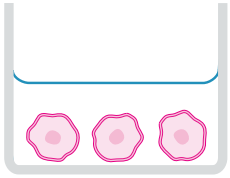
Figure 1

Example Data

Use of live-cell immunocytochemistry to quantify real-time expression of CD71 on the surface of A549 cells. Anti-CD71 antibody and IgG1 isotype control were labeled with Incucyte® Mouse IgG1 Fabfluor-555 Dye using the protocol below. A549 cells were incubated with Incucyte® Opti-Orange (30 µM) in combination with Fabfluor-555-anti-CD71 antibody or Fabfluor-555-IgG1 (1 µg/mL). HD phase contrast and orange fluorescence images were captured on the Incucyte® Live-Cell Analysis System every 3 h over 48 h using a 10X magnification. (A) Images of cells show orange fluorescence in the presence of labeled CD71 antibody (images shown at 24 h). (B) Cells labeled with mouse IgG1 isotype control display no cellular fluorescence. (C) The graph shows the quantification of orange fluorescence area over time, indicating an increase in CD71 signal as A549 cells proliferate over time.

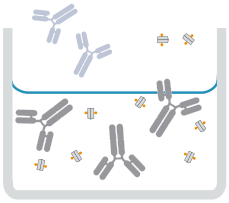
Quick Guide

1. Seed cells



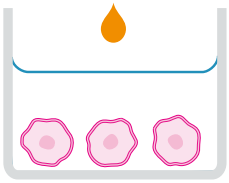
Seed cells (50 µL/well, 5–30K/well) into a 96-well plate.
Note: For non-adherent cell types, coat plate with PLO prior to cell seeding.

2. Label test antibody



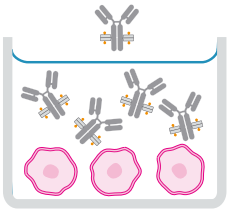
Mix antibody and Fabfluor-555 Dye at a molar ratio of 1:3 in media, 3X final concentration. Incubate for 15 minutes to allow conjugation.

3. Add Incucyte Opti-Orange



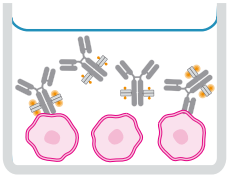
Add 50 µL/well, 3X final concentration.

4. Add labeled antibody



Add antibody-Fabfluor mix (50 µL/well) to cell 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® Mouse IgG1 Fabfluor-555 Antibody Labeling Kit (Sartorius Cat. No. BA-04873)
- Test antibody of interest (at known concentration) containing Fc region of mouse IgG1. It is strongly recommended to use low endotoxin/azide-free antibodies when available.
- Cells of interest
- Cell culture media
- 96-well flat bottom microplate (e.g., Corning Cat. No. 3595) for imaging
- 96-well round bottom plate (e.g., Corning Cat. No. 3799) or amber microtube (e.g., Cole-Parmer Cat. No. UX-06333-56MCT-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. Mouse isotype IgG1 is recommended as a negative control.

- Anti-CD71, clone MEM-189, IgG1 (e.g., Sigma Cat. No. SAB4700520-100UG)
- Mouse IgG1 isotype control (e.g., R and D Systems Cat. No. MAB002 or BioLegend Cat. No. 400124)

Incucyte® Live-Cell Immunocytochemistry Assay Protocol

1a. Seed Target Cells of Interest—Adherent Cells

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 hr. 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 0.01% poly-L-ornithine solution (not supplied). 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.

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) for use in Step 3.

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, exchange buffer using a desalting column (e.g., Zeba from Thermo Scientific).

2.1 Rehydrate Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Dye with 100 µL sterile water (final concentration = 0.5 mg/mL).

Note: Do not mix. Let the dye dissolve in the water for 15 minutes at room temperature, then mix by pipetting.

Note: A 1:3 molar ratio of test antibody to Incucyte® Fabfluor-555 Dye is recommended. The size of Fab fragment is a third of the size of a standard antibody. Therefore, equal mass (mg:mL) quantities produce a 1:3 molar ratio of test antibody to Fabfluor-555.

Note: The dye is light sensitive, keep in amber or foil wrapped tubes. Remaining rehydrated dye solution can be aliquoted and stored at -80 °C (avoid freezing and thawing, stable for 1 year).

2.2 Mix test antibody with rehydrated Incucyte® Mouse IgG1 Fabfluor-555 Antibody Labeling Dye and cell growth media in a round bottom microplate or amber tube, protected from light. Prepare sufficient quantity to enable 50 µL/ well at 3X final assay concentration.

Note: We strongly recommend using both a negative and a positive control antibody (see Recommended Materials above).

a. Add test antibody at 3X 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® Mouse IgG1 Fabfluor-555 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 at room temperature or 37 °C.

Example Calculation of Antibody Labeling Using Positive Control Anti-CD71 at 1 mg/mL Stock Concentration

1. Required final assay concentration of test antibody: 1 µg/ mL for anti-CD71 is recommended for positive wells. Working concentration = 3X, or 3 µg/mL.
2. Determine volume of labeled antibody required at 3X final assay concentration: [# wells] × 50 µL (e.g., for 8 replicates of each labeled test antibody):
   8 × 50 µL = 400 µL minimum (500 µL used for this example).
3. Calculate volumes of test antibody, Incucyte® Fabfluor-555 Dye, and media required to provide 3X test final assay concentration of labeled test antibody.
   a. Determine volume of test antibody: [Total volume] µL × [Working concentration test antibody] µg/mL / [Stock concentration test antibody] mg/mL /1,000.
   500 µL × 3 µg/mL / 1 mg/mL / 1,000 = 1.5 µL
   b. Determine volume of Incucyte® Fabfluor-555 Dye: [Volume of test antibody] µL × [Stock concentration of test antibody] mg/mL / [Stock concentration of Fabfluor-555] mg/mL
   1.5 µL × 1 mg/mL / 0.5 mg/mL = 3.0 µL
   Note: Incucyte® Mouse IgG1 Fabfluor-555 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-555 as MW of a typical antibody is ~ 3X of Fabfluor-555. In this case, the stock concentration in mg/ mL of test antibody is twice that of Fabfluor-555. Therefore, 2X volume of Fabfluor-555 is required.
4. Determine volume of media: [Total volume] – [Test antibody volume] – [Fabfluor-555 volume]
   500 µL – 1.5 µL – 3.0 µL = 495.5 µL

3. Dilution of Incucyte® Opti-Orange Background Suppressor

3.1 Dilute Opti-Orange stock in complete growth media for a final assay concentration of 30 µM (see calculations below).

Note: A final assay concentration of 30 µM 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-Orange. Human PBMCs may not require Opti-Orange for staining of highly expressed surface makers.

Example Calculation for Opti-Orange Background Suppressor

1. Required final assay concentration of Opti-Orange is 30 µM. Working concentration = 3X, or 90 µM (0.09 mM).
   Determine volume of Opti-Orange required at 3X final, i.e., dilution of 1:3 recommended upon addition to cells:
   [# wells] × 50 µL (plus additional required to prepare dilution series if desired)
   (e.g., For 96 replicates of 1:3 dilution of Opti-Orange):
   96 × 50 µL = 4800 µL minimum (5000 µL used for this example).
2. Calculate volume of Opti-Orange required to provide 3X final assay concentration.
   a. Determine volume of Opti-Orange:
   [Total volume] µL × [Working concentration Opti-Orange] mM / [Stock concentration Opti-Orange] mM
   5000 µL × 0.09 mM/10 mM = 45 µL
   b. Determine volume of media.
   [Total volume] – [Opti-Orange]
   5000 µL – 45 µL = 4955 µL

4. Add Incucyte® Mouse IgG1 Fabflour-555 Test Antibody and Opti-Orange 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-Orange to desired wells.
   b. Add 50 µL of labeled antibody to desired wells.
   c. Remove any bubbles and place plate in Incucyte® Live-Cell Analysis System.

Non-Adherent Cells

4.1 Add reagents to a PLO-coated plate:
   a. Add 50 µL of diluted Opti-Orange to desired wells.
   b. Add 50 µL of labeled antibody to desired 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 for quick settling.

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

5. Acquire Images and Analyze

5.1 Using Incucyte® integrated software, schedule repeat scanning for every 2–3 hours.
   a. Scan type: Standard.
   b. Image Channels: select "Phase" and "Orange".
   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. The use of "Surface Fit" background subtraction is also required for the Incucyte® Fabfluor-555 Dye.

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. In the Analysis Definition:
   a. Set mask for phase confluence measure with orange channel turned off.
   b. Turn orange channel on and mask orange objects. Exclude background fluorescence using "Surface Fit" background subtraction feature. The "Surface Fit" feature will subtract the background from each image; applicable for analyzing objects which change in fluorescence intensity over time.
      i. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked.
      ii. Choose a threshold in which orange 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.sartorius.com/incucyte**

Analysis Guidelines

Staining of surface-expressed protein will appear as an orange ring followed by intracellular orange 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
   a. Suggested metric: Total Orange Area (µm²/image or µm²/well).
2. Quantification of intensity integrated over the area of detectable orange fluorescence
   a. Suggested metric: Total Orange Integrated Intensity (OCU x µm²/image, or OCU × µm²/well).
3. To correct for cell proliferation, it is advisable to normalize the area measurement for cell coverage (e.g., Orange Area Confluence/Phase Area Confluence).
   Note: If using Cell-by-Cell Analysis, the post-classification data can be displayed as either % of cells expressing orange fluorescence or mean intensity of positive orange objects.

Multiplexing Guidelines

When multiplexing with green or NIR fluorescent proteins or reagents, spectral unmixing may be required to account for signal that has been contributed from one of the given channels. Spectral unmixing values must be applied prior to running an analysis job. 8–10% is recommended to remove Orange contributing to Green, 0% is recommended to remove Orange contributing to NIR.

When multiplexing with Incucyte® Fabfluor-488 Antibody Labeling Dye:

a. Follow the Incucyte® Fabfluor-488 Product Guide and Fabfluor-555 Product Guide to conjugate antibody and label cells with Fabfluor-488 and Fabfluor-555, respectively.
   Note: Incucyte® Opti-Green (supplied with Fabfluor-488 dyes) is needed for cell staining with Fabfluor-488 conjugated antibodies for background subtraction.
   Note: When multiplexing, increase working concentrations of reagents (e.g. Opti-Green, Opti-Orange, Fabfluor-488, Fabfluor-555 and cell suspension) up to 8X final assay concentrations and adjust volumes accordingly to avoid excess addition.
b. To prevent mislabeling, the concentrations of both Fabfluor-555 and Fabfluor-488 labeled antibodies need to be optimized first. The optimal concentration of the labeled antibody is dependent on the protein density on the surface of the cells and assay duration.
c. Avoid using antibodies from the same IgG isotype for Fabfluor-555 and Fabfluor-488 (e.g., use Fabfluor-488 labeled antibody with IgG2a or IgG2b isotype to multiplex with Fabflour-555-labeled antibody with IgG1 isotype).
d. "Surface Fit" is recommended for background subtraction for both green and orange fluorescence.

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

North America
Sartorius Corporation
300 West Morgan Road
Ann Arbor, Michigan 48108
USA
Phone +1 734 769 1600
Email: orders.US07@sartorius.com

Europe
Sartorius UK Ltd.
Longmead Business Centre
Blenheim Road
Epsom
Surrey, KT19 9QQ
United Kingdom
Phone +44 1763 227400
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202
Email: orders.US07@sartorius.com

Find out more: www.sartorius.com/incucyte

For questions, email: AskAScientist@sartorius.com