SARTORIUS

Product Guide

Incucyte® Mouse IgG1 Fabfluor-594 Antibody Labeling Dye

For Live-Cell Immunocytochemistry

Product Information

Presentation, Storage and Stability

The Incucyte® Mouse IgG1 Fabfluor-594 Antibody Labeling Dye for cell surface marker analysis is supplied as lyophilized solid 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).

The Incucyte® Fabfluor Antibody Labeling Dyes are also available in Fabfluor-488 formats (Cat. Nos. 4743, 4744, and 4745) enabling multiplexing of live-cell immunocyto-chemistry in different colors

Product Name	Cat. No.	Ex. Max	Em. Max	**Amount**	**Labeling Suitability**	**Storage**	**Stability**
Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green | Red Optical Module							
Incucyte® Mouse IgG1 Fabfluor-594 Antibody Labeling Dye	4844	593 nm	614 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® Mouse IgG1 Fabfluor-594 Antibody Labeling Dye is designed for quick, easy labeling of Fc containing test antibodies with a red fluorophore. Once labeled, the Fabfluor-594-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. With 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. Furthermore, the Incucyte® Mouse IgG1 Fabfluor-594 Dye can be multiplexed with the Incucyte® green 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-594 Dye is prepared at stock concentration of 0.5 mg/mL by the addition of 100 µL of sterile water and triturating (not supplied, centrifuge if solution not clear). This will re-hydrate 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.

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, but not the Fab portion of mouse IgG1. No reactivity is demonstrated against IgM or other non-immunoglobulin serum proteins. The antibody may cross-react with other mouse IgG subclasses or with immunoglobulins from other species.

Example Data

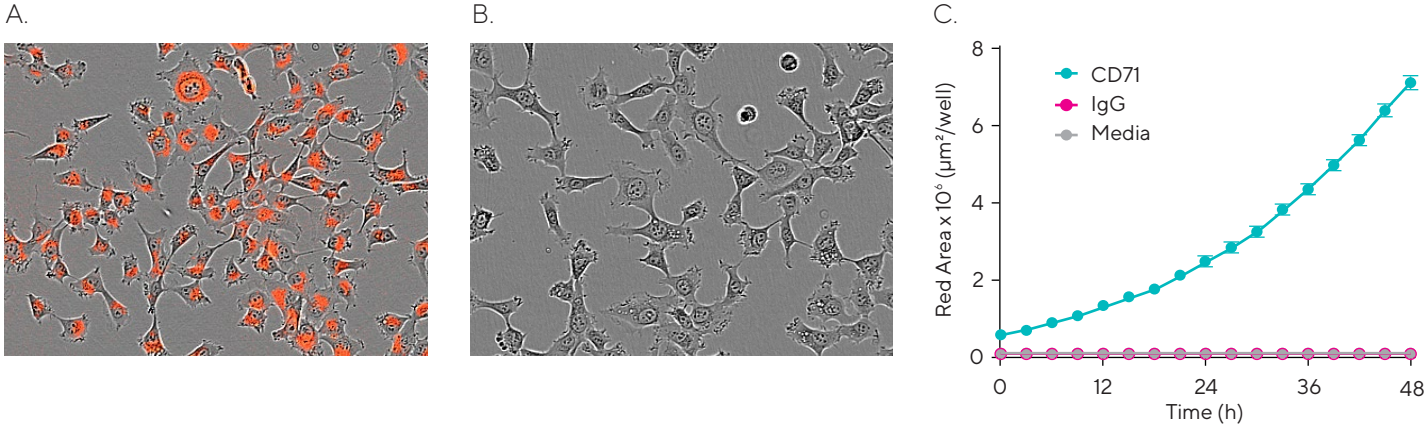
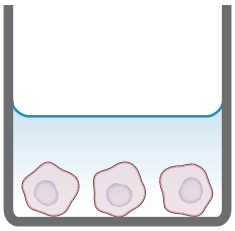


Figure 1: Use of live-cell immunocytochemistry to quantify real-time expression of CD71 surface expression on HT-1080 cells. Anti-CD71 antibody and IgG1 isotype control were labeled with Incucyte® Mouse IgG1 Fabfluor-594 using the protocol below. HT-1080 cells were incubated with Fabfluor-594-anti-CD71 antibody or Fabfluor-594-IgG1 (1 µg/mL). HD phase and red 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 red fluorescence in the presence of labeled CD71 antibody (images shown at 24 h). (B) Cells treated with labeled isotype control display no cellular fluorescence. (C) The graph shows the quantification of red fluorescence area over time, indicating an increase in CD71 expression as HT-1080 cells proliferate over time.

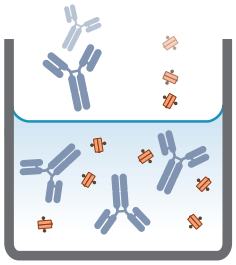
Quick Guide

1. Seed cells



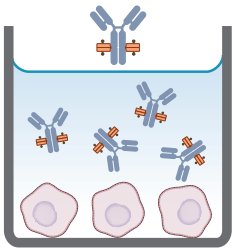
Seed cells (100 µL/well, 5–30K/well) into a 96-well plate. Note: For non-adherent cell types, PLO coat plate prior to cell seeding.

2. Label test antibody



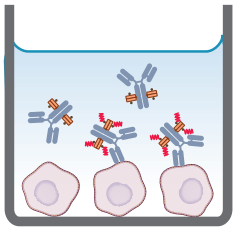
Mix antibody and Fabfluor-594 Dye in media at 3X final concentration. Incubate for 15 minutes to allow conjugation.

3. Add labeled antibody



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

4. Live-cell fluorescent imaging



Capture images with 10X or 20X objective in Incucyte® Live-Cell Analysis System.

Protocols and Procedures

Required Materials

- Incucyte® Mouse IgG1 Fabfluor-594 Antibody Labeling Dye (Sartorius Cat. No. 4844)
- 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-06333-56) 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. Anti-CD71 (transferrin receptor) marker is recommended as a positive control. Mouse IgG1 is recommended as a negative control.

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

Incucyte® Live-Cell Immunocytochemistry Assay Protocol

1a. Seed 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 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 (100 µL per well) into a 96-well flat bottom microplate.

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 in cell culture hood for 30 minutes at room temperature, then return to incubator or place in Incucyte® Live-Cell Analysis System to monitor cells.

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 h for HT-1080). Some cell types may require overnight incubation.

1b. Seed 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% PLO (Sigma Cat. No. P4957, 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 100 µL (depends on cell type used) for use in Step 3.

2. Labeling of Test Antibody

Note: It is recommended 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® Fabfluor-594 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-594 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-594.

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® Fabfluor-594 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 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-594 Dye at 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-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 wells. Working concentration = 3X, or 3 µg/mL
2. Determine volume of labeled antibody required at 3X final assay concentration:
   [# wells] x 50 µL (e.g., for 8 replicates of each labeled test antibody):
   8 x 50 µL = 400 µL minimum (500 µL used for this example).
3. Calculate volumes of test antibody, Incucyte® Fabfluor-594 Dye, and media required to provide 3X test 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 /1,000
      500 µL x 3 µg/mL / 1 mg/mL / 1,000 = 1.5 µL
   b. Determine volume of Incucyte® Fabfluor-594 Dye:
      [Volume of test antibody] µL x [Stock concentration of test antibody] mg/mL / [Stock concentration of Fabfluor-4594] mg/mL
      1.5 µL x 1 mg/mL / 0.5 mg/mL = 3.0 µL

      Note: Incucyte® Fabfluor-594 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-594 as MW of a typical antibody is ~ 3X of Fabfluor-594. In this case, the stock concentration in mg/mL of test antibody is twice that of Fabfluor-594. Therefore, 2X volume of Fabfluor-594 is required.
   c. Determine volume of media: [Total volume] – [Test antibody volume] – [Fabfluor-594 volume]
      500 µL – 1.5 µL – 3.0 µL = 495.5 µL

3. Add Incucyte® Fabflour-594 Labeled Test Antibody

Adherent Cells

3.1 Remove cell plate from incubator.

3.2 Using a multi-channel pipette, add 50 µL of labeled antibody to desired wells.

3.3 Remove any bubbles and place plate in Incucyte® Live-Cell Analysis System.

Non-Adherent Cells

3.1 Add reagents to PLO-coated plate:
   a. Add 50 µL of labelled antibody to required test wells.
   b. Add 100 µL of cell suspension to wells.
   c. Remove any bubbles.

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

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

4. Acquire Images and Analyze

4.1 Using Incucyte® integrated software, schedule repeat scanning for every 2–3 hours.
   a. Scan type: Standard.
   b. Image Channels: select "Phase" and "Red".
   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.

4.2 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-594 Dye.

4.3 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 red channel turned off.
   b. Turn red channel on and mask red 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 red 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 surface expressed protein will appear as a red ring followed by intracellular red signal, as there will be internalization of the signal over time (time depends on cell type studied). Suggested metrics for data analysis is shown below:

1. Quantification of fluorescence area.
   a. Suggested metric: Total Red Area (µm^2/image or µm^2/well).
2. Quantification of intensity integrated over the area of detectable red fluorescence.
   a. Suggested metric: Total Red Integrated Intensity (RCU x µm^2/ image, or RCU x µm^2/ well).
3. To correct for cell proliferation, it is advisable to normalize the area measurement for cell coverage (e.g., Red Area Confluence/Phase Area Confluence).

   Note: If using Cell-by-Cell Analysis, the post-classification data can be displayed as either % of cells expressing red fluorescence or mean intensity of positive red objects.

Multiplexing Guidelines

When multiplexing with green 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. 0–2% is recommended to remove Red contributing to Green.

When multiplexing with Incucyte® Fabfluor-488:

a. Follow the Fabfluor-488 Product Guide to conjugate antibody and label cells with Fabfluor-488.

   Note: Incucyte® Opti-Green (supplied with Fabfluor-488 dyes) is needed for cell staining with Fabfluor-488 conjugated antibodies for background subtraction.

b. To prevent mislabeling, the concentrations of both Fabfluor-594 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-594 and Fabfluor-488 (e.g., use Fabfluor-488 labeled antibody with IgG2a or 2b isotype to multiplex with Fabflour-594-labeled antibody with IgG1 isotype).

d. "Surface Fit" is recommended for background subtraction for both green and red fluorescence.

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