sartorius

Protocol

IncuCyte® Antibody Internalization Assay

This protocol describes a solution for the measurement of antibody internalization by cells expressing antigen of interest. This method utilizes the IncuCyte® FabFluor-pH Red Antibody Labeling reagent and the IncuCyte® S3 Live-Cell Analysis System for image-based fluorescent measurements of antibody internalization.

Required materials

- IncuCyte® FabFluor-pH Red Antibody labeling reagent (Sartorius cat #4722 human, Sartorius Cat# 4723 mouse IgG1, Sartorius Cat# 4737 rat, Sartorius Cat#4750 mouse IgG2a, or Sartorius Cat# 4751 mouse IgG2b).
- Test antibody of interest containing human, mouse, or rat Fc region (at known concentration).
- Target cells of interest.
- Target cell growth media.
- PBS (w/o $Ca2^{+}$/$Mg2^{+}$, Life Tech 14190).
- 96-well flat bottom microplate (e.g. Corning® 3595) for imaging.
- 96-well round black round bottom ULA plate (e.g. Corning® 45913799) or amber microtube (e.g. Cole parmer® MCT-150-X) for conjugation step.

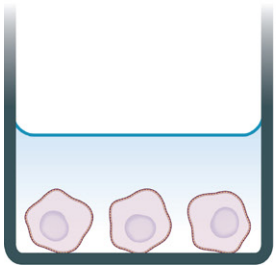
Recommended materials

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

- Anti-CD71, clone MEM-189, IgG1 e.g. Sigma SAB4700520-100UG
- Anti-CD71, clone CYG4, IgG2a e.g. Biologend 334102
- Isotype controls, depending on isotype being studied
  - Mouse IgG1, e.g. R&D Systems, MAB002 or Biolegend 400124
  - Mouse IgG2a e.g. Biolegend 401501
  - Mouse IgG2b e.g. Biolegend 400322

Quick Guide

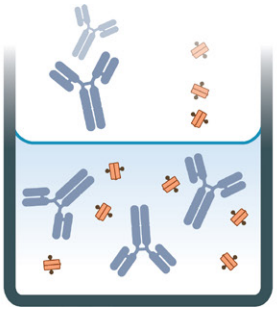
1. Seed cells



Cell Seeding

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).

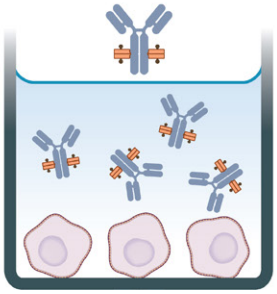
2. Label test antibody



Labeling of Test Antibody with IncuCyte® FabFluor-pH Red Reagent

Mix antibody and FabFluor Reagent at a molar ratio of 1:3 in media, 2x final assay concentration. Incubate for 15 minutes to allow conjugation.

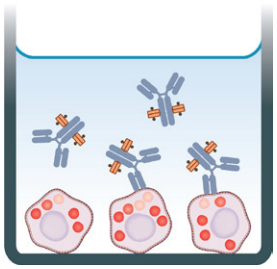
3. Add to cells



IncuCyte® FabFluor-labeled Antibody Addition

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

4. Live-cell fluorescent images



Automated Imaging andQuantitative Analysis

Capture images every 15-30 minutes (10x or 20x) in IncuCyte® S3 Live-Cell Analysis System for 24-48 hours. Analyze using integrated software.

Preparation of IncuCyte® Antibody Internalization Assay

1. Seed Target 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 to achieve 40-50% confluence after 2-6 h. Suggested starting range 5,000-30,000 cells/well (depends on cell type used).

NOTE: The seeding density will need to be optimized for each cell type. For non-adherent cell types a well coating may be required e.g. Poly-L-ornithine (PLO, Sigma P4957) to maintain even cell coverage in well (see IncuCyte Cell Proliferation Assay protocol on www.Essenbioscience.com for details).

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® S3 Live-Cell Analysis System to monitor cell confluence in well.

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 HT1080 or 1-2 h for non-adherent cell types. Some cell types may require overnight incubation.

2. Labeling of test antibody

2.1 Rehydrate IncuCyte® FabFluor-pH Red Antibody Labeling reagent with 100 µL sterile water to result in a final concentration of 0.5 mg/mL.

NOTE: A1:3 molar ratio of test antibody to IncuCyte® FabFluor-pH Red reagent is recommended. The labeling reagent is a third of the size of a standard antibody, so equal mg/ml quantities will produce a 1:3 molar ratio of test antibody to labeling Fab.

This reagent is light sensitive. It is advised to keep in amber tubes or foil wrapped tubes. Remaining re-hydrated reagent can be aliquoted and stored at -80°C (avoid freezing and thawing, stable for > year).

Example calculation for antibody labeling using positive control anti-CD71 at 1 mg/mL stock concentration

1. Determine final assay concentration of test antibody — 4 µg/mL for anti-CD71 is recommended for positive control wells. Working concentration will be 2X or 8 µg/mL.

2. Determine volume of labeled antibody required at 2X final assay concentration (dilution of 1:2 recommended upon addition to cells):
   [# wells] x 50µL (plus additional required to prepare dilution series if desired).

   For 8 replicates of highest concentration plus 8 replicates of 1:2 dilution of labeled test antibody:
   8 x 50 µL x 1.5 = 600 µL minimum (650µL used for this example)

3. Calculate volumes of test antibody, IncuCyte FabFluor-pH Red reagent, and media required to provide 2X final assay concentration of labeled test antibody.

   - Determine volume of test antibody:
     [total volume] µL x [working concentration test antibody] µg/mL / [stock concentration test antibody] mg/mL /1000
     650 µL x 8 µg/mL / 1 mg/mL / 1000 = 5.2 µL

   - Determine volume of IncuCyte FabFluor:
     [volume of test antibody] µL X [stock concentration of test antibody] mg/mL / [stock concentration of test FabFluor] mg/mL
     5.2 µL x 1 mg/mL / 0.5 mg/mL = 10.4 µL

     NOTE: IncuCyte FabFluor-pH Red reagent is a third of the molecular weight of a standard antibody, so equal volumes of equal mg/mL quantities will produce a 1:3 molar ratio of test antibody to FabFluor as MW of a typical antibody is ~3x of FabFluor. In this case, the stock concentration in mg/mL of the test antibody is twice that of FabFluor, thus the FabFluor volume should be 2X the volume of the test antibody.

   - Determine volume of media:
     [total volume] – [test antibody volume] – [FabFluor volume]
     = 634.4 µL

2.2 Mix test antibody with dilute IncuCyte® FabFluor-pH Red Antibody Labeling reagent and target cell growth media in a black round bottom microplate or amber tube to protect from light (50 ul/well).
   a. Add test antibody at 2X the final antibody concentration. We suggest optimizing the assay by testing a final concentration of 4 ug/ml of test antibody (e.g., 2X working concentration=8 ug/ml).
   b. Add IncuCyte®FabFluor-pH Red Antibody Labeling reagent at 2X the final concentration. We suggest optimizing the assay by testing a final concentration of 4 ug/ml of IncuCyte FabFluor-pH Red Antibody (e.g., 2X working concentration=8 ug/ml).
   c. Add media to bring the total volume to 50 ul/well. Triturate to mix.

NOTES: If performing a range of concentrations of test antibody e.g. concentration response-curve, it is recommended to create dilution series post conjugation step in media to ensure consistent molar ratio labeling.

We strongly recommend the use of both a negative and positive control antibody (see Recommended Materials above).

3. Add IncuCyte FabFluor-pH Red reagent to cells

3.1 Remove cell plate from incubator.

3.2 Using a multi-channel pipette, add 50 µL of labeled antibody to required test wells, remove any bubbles and immediately place plate in IncuCyte® S3 Live-Cell Analysis System.

4. Acquire images and analyze

4.1 In the IncuCyte® Software, schedule 24 hour repeat scanning for every 15-30 minute (depending on speed of internalization signal).
   a. Scan on schedule, standard.
   b. Channel selection: 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.

NOTE: If trying to achieve rapid first image acquisition, scheduling can be set up on the instrument and no start time of scan attached prior to addition of reagents to plate. The scan time can then set once the plate is placed in the instrument.

By maintaining all reagents at 37°C prior to plate addition

there is reduced risk of condensation formation on the lid and therefore no need for plate warming before first image acquisition.

For both cell types, individual cell identification can be enabled with the use of the IncuCyte Cell-by-Cell Analysis Software Module (PN 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 it's application 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:

a. Set up the mask for the phase confluence measure with red channel turned off.
b. Red channel turned on: Exclude background fluorescence from the mask by 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 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 maybe preferable.

Analysis Guidelines

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

i. An increase in fluorescence area ("total object area" or "red object confluence"). Suggested metric: Analyze using the "Total Red Object Area (µm²/well)".

ii. An increase in intensity, integrated over the area of detectable fluorescence ("Total Integrated Intensity"). Suggested metric: Analyze using "Total Red Object Integrated Intensity (RCU x µm²/well)" metrics.

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.

A complete suite of cell health applications is available to fit your experimental needs. Find more information at **essenbioscience.com**

For additional product or technical information, please e-mail us at **AskAScientist@sartorius.com**
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