SARTORIUS

Product Guide

pHrodo® Orange Cell Labeling Kit for Incucyte®

For Quantification of Phagocytosis of Apoptotic and Non-apoptotic Cells

Product Information

Presentation, Storage and Stability

The pHrodo® Orange Cell Labeling Kit for Incucyte® contains sufficient reagent for labeling 5 x 10^7 target cells of choice. Component A (pHrodo® Orange Cell Labeling Dye for Incucyte®) is supplied as a lyophilized solid which should be stored at -20° C (stable for at least 6 months).

Once solubilized the solution should be used as soon as possible or stored at -20° C (stable for at least 1 month). The kit contains sufficient DMSO to solubilize the dye, and buffers suitable for washing and labeling cells.

Product Name	Cat. No.	Ex. Max	Em. Max	Amt.	Storage	Stability
Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green | Orange | NIR or an Orange | NIR Optical Module						
pHrodo® Orange Cell Labeling Kit for Incucyte®	4766	558 nm	605 nm	1 kit	-20° C	6 months from date of receipt

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

Background

pHrodo® Orange Cell Labeling Dye for Incucyte® is a reagent for labeling whole cells with a pH-sensitive fluorophore. These cells are then suitable for use in downstream applications such as phagocytosis of cells.

The unique pHrodo®-based system exploits the acidic environment of the phagosome to quantify phagocytosis. As pHrodo® labeled cells residing in the neutral extracellular solution (pH 7.4) are engulfed by phagocytes and enter the acidic phagosome (pH 4.5–5.5), a substantial increase in fluorescence is observed. In the absence of phagocytes, the fluorescence intensity of the labeled cells remains low.

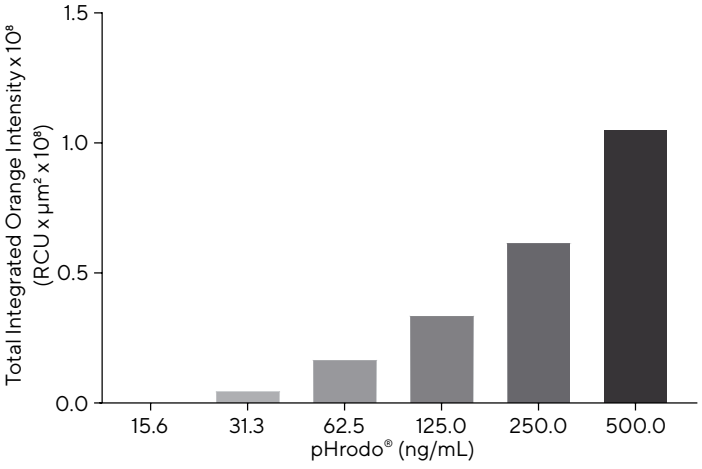
The pHrodo® Orange Cell Labeling Kit for Incucyte® has been validated for use with the Incucyte® Live-Cell Analysis System configured with a Green | Orange | NIR or an Orange | NIR Optical Module. This kit enables real-time evaluation of phagocytic regulation induced by pharmacological agents as well as genetic and environmental factors.

Recommended Use

We recommend that pHrodo® Orange Cell Labeling Dye for Incucyte® is prepared at a stock concentration of 1 mg/mL in the sterile DMSO provided. The dye may then be diluted for direct addition to cells suspended in pHrodo® Labeling Buffer for Incucyte®. Note that the dye will also bind to any primary amines present in proteins or cellular debris, therefore we recommend that a) cell lines be washed with pHrodo® Wash Buffer for Incucyte® to remove cell culture media and serum, and b) that any primary cells (such as neutrophils extracted from whole blood) be free from contamination.

Example Data

A.



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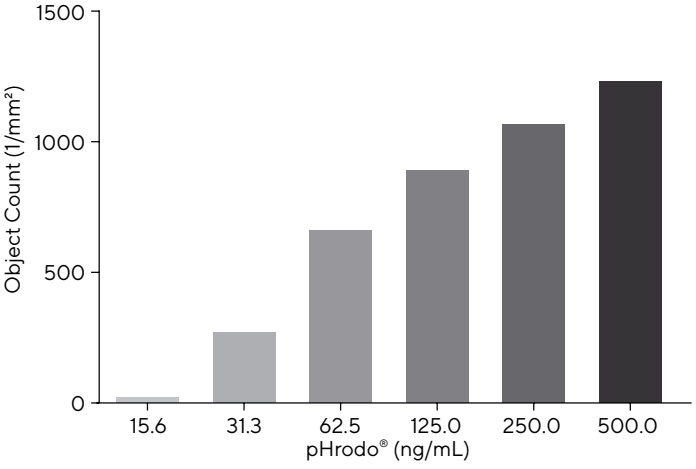
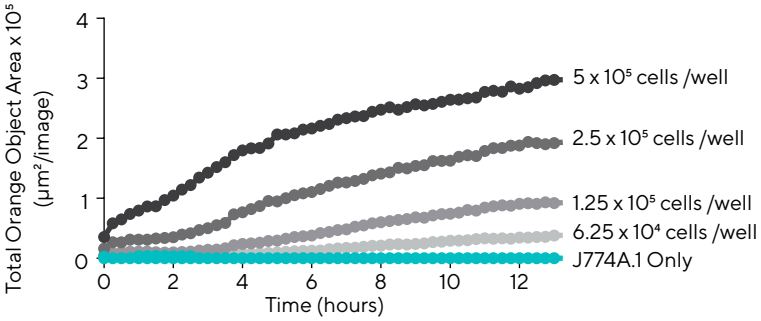


C.



Note. Proof of concept data conducted with pHrodo® Red Cell Labeling Kit for Incucyte®. (A) Total Integrated Orange Intensity of labeled Jurkats at pH 4.0 (for maximal fluorescence) increases with increasing amounts of pHrodo® Orange Cell Labeling Dye for Incucyte®. (B) The number of Orange objects increases with increasing pHrodo® Orange Cell Labeling Dye for Incucyte® until approximately 250 ng/mL, indicating that a maximal number of cells have been labeled. (C) Assay results using neutrophils labeled with pHrodo® Orange Cell Labeling Dye for Incucyte®. Neutrophils were labeled according to the protocol described, then incubated with J774A.1 macrophage cells seeded at 1 x 10^4 cells/well. Increasing numbers of labeled neutrophils in the presence of phagocytic cells yields greater increase in Total Orange Object Area as neutrophils are engulfed.

Protocols and Procedures

Materials

- pHrodo® Orange Cell Labeling Kit for Incucyte® (Sartorius Cat. No. 4766)
- Target cells of interest
- Target cell culture media
- Effector cells of interest
- Effector cell culture media
- 96-well microplate (e.g., Corning® Cat. No. 3595)
- Apoptosis inducing compound (e.g., Camptothecin) for efferocytosis protocol
- Citrate buffer, pH 4.0

General Guidelines

- Pre-warm pHrodo® Wash and Labeling Buffers (Components C and D) to 37°C in water bath.
- Following effector cell seeding, place plates at ambient temperature for 30 minutes to ensure homogenous cell settling.
- 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.
- After placing the plate in the Incucyte® Live-Cell Analysis System, acquire scans immediately.

Initial Optimization Protocols

Assay Optimization

For optimal assay results, conduct preliminary experiments to determine the following assay parameters:

1. The seeding density of the effector cells which will result in 10–20% confluence 24 hours after plating. We have found that 1×10^4 effector cells per well is a reasonable starting point to reach ~ 20% cell confluency, and recommend optimizing above and below that density (e.g., 5 - 20 x 10^3 cells/well).
2. The lowest concentration of drug treatment (e.g., camptothecin) that will induce target cell apoptosis with limited cellular debris following a 24-hour exposure. Target cell apoptosis can be measured using a cell health dye, such as Incucyte® Annexin V NIR Dye (Sartorius, Cat. No. 4768).

Target Cell Labeling Optimization

Target cells must be efficiently labeled in order to detect phagocytic events. The pHrodo® Labeling Dye concentration needs to be optimized to ensure that non-engulfed target cells have little or no fluorescence and engulfed cells have a higher fluorescence. Additionally, an analysis definition is required that can segment the high fluorescence of engulfed cells but does not segment any minimal fluorescence of non-engulfed cells. For target cell labeling optimization as per the protocol below, we recommend performing a serial dilution of the pHrodo® Cell Labeling Dye for Incucyte® in DMSO and labeling your target cells. Labeled target cells can then be added to media (pH 7.4) or citrate-based buffer (pH 4.0), which is used to mimic internalization into a low pH environment. For labeling optimization for efferocytosis assays induce target cell apoptosis prior to the optimization protocol.

		Media (pH 7.4)			Buffer (pH 4.0)								
[pHrodo®]	All	1	2	3	4	5	6	7	8	9	10	11	12
	A	pHrodo® Dye 500 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 500 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	B	pHrodo® Dye 250 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 250 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	C	pHrodo® Dye 125 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 125 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	D	pHrodo® Dye 62.5 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 62.5 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	E	pHrodo® Dye 31.25 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 31.25 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	F	pHrodo® Dye 15.63 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 15.63 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	G	pHrodo® Dye 7.81 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 7.81 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
Vehicle	H	Target Cell (1) 10K/well Media pH 7.4			Target Cell (1) 10K/well Buffer pH 4.0								

Figure 1

Target Cell Labeling Optimization

Note. Example Plate Map for testing pHrodo® labeling of target cultured cell lines.

1. Pre-warm pHrodo® Wash and Labeling Buffers (Components C and D) to 37°C in water bath.
2. Harvest target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.
3. Aspirate supernatant and resuspend cell pellet with 50 mL pHrodo® Wash Buffer (Component C).
4. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate pHrodo® Wash Buffer and resuspend cell pellet in pHrodo® Labeling Buffer (Component D) to a density of 1x10⁶ cells/mL. Separate the suspension into 8 aliquots of 1 mL.
5. Solubilize the pHrodo® Cell Labeling Dye for Incucyte® (Component A) by adding 100 µL of DMSO (Component B) to create a stock concentration of 1 mg/mL.
6. Perform a 7-point, two-fold serial dilution of the pHrodo® Cell Labeling Dye for Incucyte® in DMSO.
   a. For cultured cell lines, generate a concentration range between 50 µg/mL and 0.8 µg/mL

   Note: Suggested concentration range is based on data using the Incucyte® S3 and SX5 Live-Cell Analysis Systems. For cells extracted from blood or tissue, a higher concentration range may be required.
7. Add 10 µL of each concentration of dye, or 10 µL DMSO for control, to 1 mL cell suspension i.e., a 1:100 dilution, which will provide a final assay concentration range of
   a. 0.5 µg/mL to 8 ng/mL
8. Incubate for 1 hour at 37 °C.
9. Harvest cells by centrifugation for 7 minutes at 1000 rpm.
10. Aspirate supernatant and wash cell pellet with 1 mL complete media (cell type appropriate). Harvest cells by centrifugation for 7 minutes at 1000 rpm, aspirate supernatant and resuspend in 1 mL complete media.
11. Prepare a citrate-based buffer solution at pH 4.0. For each dilution of pHrodo® labeled cells, prepare a micro-centrifuge tube containing 400 µL of buffer, and add 40 µL of labeled cells. Mix by trituration.
12. Prepare media control conditions. For each dilution of pHrodo® labeled cells, prepare a micro-centrifuge tube containing 400 µL complete media, and add 40 µL of labeled cells. Mix by trituration.
13. Per each cell dilution, aliquot 100 µL to three wells of a 96-well plate and allow the cells to settle at ambient temperature for 30 minutes.
14. Place plate into the Incucyte® Live-Cell Analysis System and perform a scan on demand using Phase and Orange fluorescence channels.

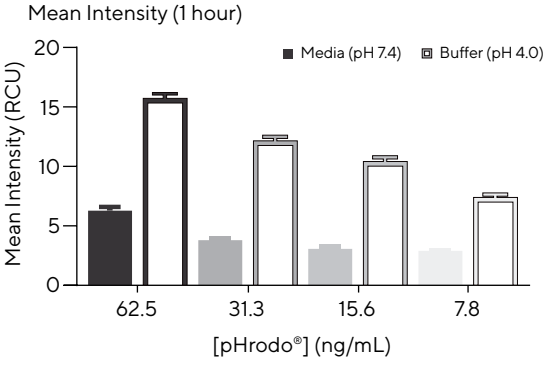
Analysis Guidelines

Refer to the Data Acquisition and Analysis Section for more detailed information on setting up an Analysis Definition.

1. Create an Analysis Definition using representative images for both media and buffer conditions. Choose a threshold in which fluorescent objects are masked in the images for the buffer conditions but not masked in the images for media conditions at the lowest pHrodo® Labeling Dye concentrations (e.g., 0.5 - 2 ROCU).

   Note: There may be some masking of fluorescent objects in the media condition for the highest pHrodo® Labeling Dye concentrations.
2. Plot the User Defined metric "Orange Mean Intensity Object Average (ROCU)" and select a pHrodo® Labeling Dye concentration that has a large difference in fluorescent intensity between media and buffer conditions, ensuring the media condition exhibits minimal fluorescence and that the cells can easily be masked in the buffer condition.

   Note: By counting the number of phase and fluorescent objects, a percentage of labeled cells may also be obtained for each concentration of dye. A percentage of labeled cells can also be obtained using the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031).



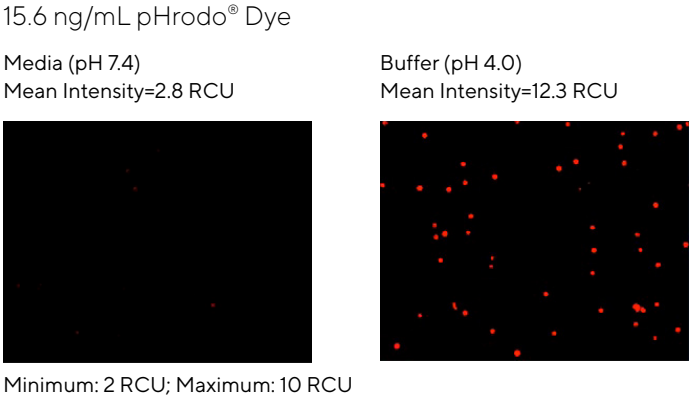


Figure 2

Target Cell Labeling Optimization Example Analysis

Note. Proof of concept data conducted with pHrodo® Red Cell Labeling Kit for Incucyte®. A serial dilution of pHrodo® Orange Cell Labeling Dye for Incucyte® (500 ng/mL – 7.8 ng/mL) was performed as per the optimization protocol using non-adherent Ramos cells. An Analysis Definition was applied using Top Hat Segmentation with a Threshold of 2 RCU. The Orange Mean Intensity Object Average (RCU) was plotted for all concentrations. Graph shows Mean Intensity for the four lowest concentrations at 1h with media conditions in closed bars and buffer conditions in open bars. The pHrodo® Dye concentration of 15.6 ng/mL was selected based on a low level of fluorescence in media and a large difference in mean fluorescent intensity between media and buffer conditions. Orange fluorescence images with user defined min – max fluorescence for 15.6 ng/mL illustrate difference in fluorescence for each condition.

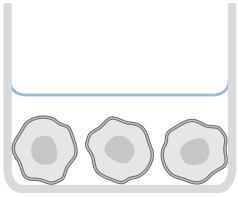
Phagocytosis of Apoptotic Cells (Efferocytosis) Protocol

This protocol provides an overview of the phagocytosis of dying cells by macrophage engulfment, known as efferocytosis. It combines the pHrodo® Cell Labeling Kit for Incucyte® with the Incucyte® Live-Cell Analysis System using your choice of target and phagocytic (effector) cells. For the measurement of the phagocytosis of non-apoptotic cells a separate protocol is available.

Important: Prior to initiating the assay, it is recommended that your experimental design includes replicate wells of each condition being tested (e.g., effector cells alone, labeled apoptotic cells alone at each density, and target:effector co-cultures at each ratio) in order to determine the assay signal window.

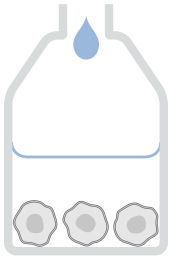
Quick Guide

1. Seed Effector Cells



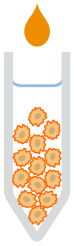
Seed phagocytic effector cells (50 µL/well). Culture overnight.

2. Treat Target Cells



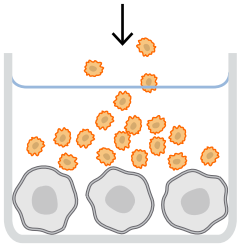
Treat target cells with apoptosis inducing reagent. Incubate for 24 hours.

3. Label Target Cells



Label apoptotic target cells with pHrodo® Labeling Dye for Incucyte®.

4. Add Target Cells



Add pHrodo® for Incucyte® labeled target cells to the effector cells (50 µL/well).

Day 0

Seed Effector Cells

1. Harvest effector cells and determine cell concentration (e.g., Trypan blue + hemocytometer).

   Note: Grow enough effector cells in advance to accommodate the different cell densities required to set up the assay (e.g., 1 x 10^6 total cells for seeding 1 x 10^4 effector cells/well).

2. Prepare cell seeding stock in culture media to achieve 10-20% confluence after 24 hours.

   Note: The seeding density will need to be optimized for each cell type used per the preliminary optimization protocol.

3. Using a multi-channel pipette, seed effector cells (50 µL per well) into a 96-well microplate.
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.
5. Allow the cells to settle on a level surface for 30 minutes, then incubate overnight at 37°C with 5% CO_2.

Treat Target Cells With Apoptotic Agent

Note: Grow enough target cells in advance to accommodate the different cell densities required to set up the assay and to account for some loss of apoptotic cells during washing steps. We recommend testing target-to-effector cell ratios by holding the effector cell constant, and creating a 7-point, two-fold serial dilution (5 to 0.08 x 10^5 cells/well) of the target cells.

1. Harvest target cells and determine cell concentration (e.g., Trypan blue staining + hemocytometer).
2. Centrifuge the cell suspension for 7 minutes at 1000 rpm.
3. Aspirate supernatant and resuspend cell pellet in 50 mL fresh growth media at a final cell density of 1x10^6 cells/mL.
4. Add apoptosis inducing compound (e.g., camptothecin) at the optimal concentration identified in the preliminary optimization experiment to the target cells.
5. Dispense cells with apoptosis inducing treatment into a T175 flask and incubate for 24 hours at 37°C with 5% CO_2.

Day 1

Label Target Cells With pHrodo® Cell Labeling Kit for Incucyte®

1. Pre-warm pHrodo® Wash and Labeling Buffers (Components C and D) to 37°C in water bath.
2. Harvest apoptotic target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.
3. Aspirate supernatant and resuspend cell pellet with 50 mL pHrodo® Wash Buffer (Component C). Gently mix cells by trituration and determine cell count using a hemocytometer (omit Trypan blue as cells are apoptotic).
4. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate pHrodo® Wash Buffer and resuspend cell pellet in pHrodo® Labeling Buffer (Component D) to a density of 1x10^6 cells/mL.
5. Reconstitute pHrodo® Cell Labeling Dye for Incucyte® (Component A) in 100 µL of DMSO (Component B) to create a stock concentration of 1 mg/mL.
6. Add the solubilized pHrodo® Cell Labeling Dye to the

target cell suspension at the concentration determined during optimization (refer to Target Cell Labeling Optimization under General Guidelines). Incubate the centrifuge tube containing cells for 1 hour at 37°C.

7. Remove excess pHrodo® Labeling Dye from cells:
   a. Centrifuge the cell labeling dye suspension at 1000 rpm for 7 minutes. Aspirate off supernatant and resuspend apoptotic target cells in 50 mL of target cell media.
   b. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate supernatant and resuspend apoptotic target cells in effector cell media to yield a cell density of 1×10^7 cells/mL.

Add Target Cells to Effector Cells

1. Prepare dilutions of the labeled apoptotic target cells by creating a 7-point, two-fold serial dilution (5 to 0.08×10^5 cells/well).
2. Immediately following target cell resuspension, remove the effector cell plate from the incubator and add the target cell suspensions to the cell plate (50 µL per well) using a multichannel pipette.
3. Remove bubbles and immediately place the microplate in the Incucyte® Live-Cell Analysis System (refer to Data Acquisition and Analysis section).

Data Acquisition and Analysis

Acquisition

Using Incucyte® integrated software, schedule repeat scanning for every 15-30 minutes, for up to 48 hours.

a. Scan type: Standard
b. Image Channels: select "Phase" and "Orange".
c. Objective: 10X or 20X depending on cell types used.
d. Scan pattern: 2 – 4 images per well.

Analysis

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

1. To help choose a threshold that ensures fluorescence of engulfed target cells only is segmented, look at images of non-engulfed cells and adjust fluorescence values to minimize any fluorescence from these cells. Select an image from a well containing a high density of target cells but no effector cells during the peak assay response (e.g., 4 - 12 hours). Under Image Channels, expand the Orange drop down and deselect the Autoscale option. Adjust the scale until no fluorescence is observed in the image and note the minimum and maximum values.
2. In the Analysis Definition:
   a. Select representative images for each condition being tested (e.g., target cells alone and target:effector co-cultures at each ratio).
   b. Deselect the Autoscale option and set the minimum and maximum values identified in Step 1.
   c. Set up the mask for the phase confluence measure with fluorescence channel turned off (optional).
   d. Turn the orange fluorescence channel on. To exclude background fluorescence from the mask, use the background subtraction feature in the Orange drop-down menu. 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. Choose a threshold in which orange objects are masked in the image without effector cells but not masked in images without effector cells (e.g., 0.5 - 2 ROCU).
3. Recommended Metrics for Phagocytosis of Cells:
   As effector cells engulf target cells, the area of fluorescence and intensity inside the effector cells increases. This can be reported in two ways:
1. Quantification of an increase in fluorescence area.
   a. Suggested metric: Total Orange Object Area (µm²/image or μm^2/well)
2. Quantification of increase in intensity integrated over the area of detectable fluorescence.
   a. Suggested metric: Total Orange Object Integrated Intensity (ROCU x μm^2/image).
3. Individual cell identification can be enabled with the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031), providing cell density is optimized for accurate segmentation of cells. 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:

Incucyte® Cell-by-Cell Analysis Software | Sartorius

   a. Post-classification data can be displayed as either % of cells expressing fluorescence or mean intensity of positive fluorescent objects.

Related Products and Applications

In addition to the pHrodo® Cell Labeling Kit for Incucyte®, a comprehensive range of Bioparticles® for phagocytosis of bacterial Gram positive, Gram negative or yeast-derived pathogens by immune cells are available for use with the Incucyte® Live-Cell Analysis System.

Product	Cat. No.	Amount	Ex. Maxima	Em. Maxima
pHrodo® Orange *E. coli* Bioparticles® for Incucyte®	4615	2 mg	560 nm	585 nm
pHrodo® Green *E. coli* Bioparticles® for Incucyte®	4616	2 mg	509 nm	533 nm
pHrodo® Orange Zymosan Bioparticles® for Incucyte®	4617	1 mg	560 nm	586 nm
pHrodo® Green Zymosan Bioparticles® for Incucyte®	4618	1 mg	506 nm	533 nm
pHrodo® Orange *S. aureus* Bioparticles® for Incucyte®	4619	2 mg	560 nm	585 nm
pHrodo® Green *S. aureus* Bioparticles® for Incucyte®	4620	2 mg	509 nm	533 nm

North America
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
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

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