

# IncuCyte® pHrodo Red Phagocytosis Assay

For quantification of phagocytosis of apoptotic and non-apoptotic cell

This protocol is intended for the measurement of both apoptotic (efferocytosis) and non-apoptotic phagocytosis (antibody-dependent cellular phagocytosis) of cells by macrophages. This method utilizes the IncuCyte® pHrodo® Red Cell Labeling Kit and the IncuCyte® Live-Cell Analysis System for image-based fluorescent measurements of phagocytosis.

## Required materials

- IncuCyte® pHrodo® Red Cell Labeling Kit for Phagocytosis (Sartorius Cat # 4649)
- Target cells of interest
- Target cell culture media
- Effector cells of interest
- Effector cell culture media
- 96-well microplate (e.g., Corning® Cat # 3595)

## Initial optimization experiment

### 1 Assay Optimization

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

- 1.1 The seeding density of the effector cells which will result in 10–20% confluence 24 hours after plating. We have found that 10,000 effector cells per well is a reasonable starting point to reach ~20% cell confluency, and recommend optimizing above and below that density (e.g., 6, 7, 8, 9, 10, 11, 12 and 13k cells/well).
- 1.2 The lowest concentration of drug treatment (e.g., camptothecin or staurosporine) that will induce target cell apoptosis with limited cellular debris following a 24-hour exposure. Target cell apoptosis can be measured using the IncuCyte® Annexin V Reagent (Cat. No. 4642).

### 2 Target Cell Labeling Optimization

Target cells in the IncuCyte® pHrodo Red Phagocytosis Assay must be efficiently labeled in order to detect phagocytic events. We recommend performing a serial dilution of the IncuCyte® pHrodo® Red Cell Labeling Dye in DMSO and labeling your target cells per the optimization protocol below:

- 2.1 Suspend target cells at a density of  $1 \times 10^6$  cells/mL in IncuCyte® pHrodo® Cell Labeling Buffer (component D). Separate the suspension into aliquots of 1 mL.
- 2.2 Solubilize the IncuCyte pHrodo Red Cell Labeling Dye (component A) by adding 100  $\mu$ L of DMSO (component B) to create a stock concentration of 1 mg/mL.

2.3 Perform a serial dilution of the IncuCyte pHrodo Red Cell Labeling Dye in DMSO.

- a. For cells extracted from blood or tissue, generate a concentration range between 1 mg/mL (stock) and 100  $\mu$ g/mL.
- b. For cultured cell lines, generate a concentration range between 100  $\mu$ g/mL and 10  $\mu$ g/mL.

2.4 Add 10  $\mu$ L of each concentration of dye to 1 mL cell suspension i.e., a 1:100 dilution, which will provide a final assay concentration range of

- a. 0  $\mu$ g/mL to 1  $\mu$ g/mL,
- b. 1  $\mu$ g/mL to 100 ng/mL

2.5 Incubate for 1 hour at 37 °C. Harvest cells by centrifugation for 7 minutes at 1000 rpm.

2.6 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.

2.7 Prepare a citrate-based buffer solution at pH 4.0. For each dilution of IncuCyte pHrodo Red Labeled Cells, prepare a micro-centrifuge tube containing 300  $\mu$ L of buffer, and add 30  $\mu$ L of labeled cells. Mix by trituration.

2.8 Per each buffered cell dilution, aliquot 100  $\mu$ L to three wells of a 96-well plate and allow the cells to settle at ambient temperature. Scan the plate in phase and red fluorescence. By counting the number of phase and fluorescent objects, a percentage of labeled cells may be obtained for each concentration of dye.

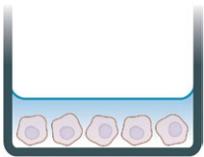
## Apoptotic Phagocytosis Protocol (Efferocytosis)

This protocol provides an overview of the phagocytosis of dying cells by macrophage engulfment, known as efferocytosis. It combines the IncuCyte pHrodo Red Cell Labeling Kit with the IncuCyte® Live-Cell Analysis System using your choice of target and phagocytic (effector) cells.

**Prior to initiating the assay, it is important 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.**

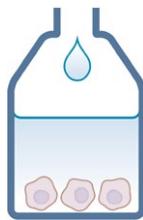
### Quick Guide

#### 1 SEED EFFECTOR CELLS



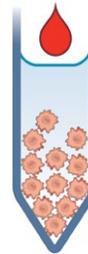
Seed phagocytotic effector cells (50 µL/well). Culture overnight.

#### 2 TREAT TARGET CELLS



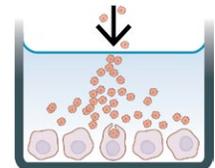
Treat target cells with apoptosis inducing reagent. Incubate for 24 hrs.

#### 3 LABEL TARGET CELLS



Label apoptotic target cells with IncuCyte pHrodo Labeling Dye.

#### 4 ADD INCUCYTE PHRODO LABELED TARGET CELLS



Add IncuCyte pHrodo labeled target cells to treated wells (10 µg/well, 25 µL/well).

### Day 0

#### 1 Seed effector cells

1.1 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.  $1 \times 10^6$  total cells for seeding 10,000 effector cells/well).

1.2 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.

1.3 Using a multi-channel pipette, seed effector cells (50 µL per well) into a 96-well 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 the cells to settle on a level surface for 30 minutes, then incubate overnight at 37 °C with 5% CO<sub>2</sub>.

#### 2 Treat target cells with apoptotic agent

2.1 Harvest target cells and determine cell concentration (e.g. Trypan blue staining + hemocytometer).

**NOTE:** Grow enough target cells in advance to accommodate the different cell densities required to set up the assay. We recommend testing target-to-effector cell ratios by holding the effector cell constant, and creating a 7-point, two-fold serial dilution (500,000 cells/well to 7,812 cells/well) of the target cells.

2.2 Centrifuge the cell suspension for 7 minutes at 1000 rpm.

2.3 Aspirate supernatant and resuspend cell pellet in 50 mL fresh growth media at a final cell density of  $1 \times 10^6$  cells/mL.

2.4 Add apoptosis inducing compound (e.g., camptothecin or staurosporine) at the optimal concentration identified in the preliminary optimization experiment to the target cells.

2.5 Dispense cells with apoptosis inducing treatment into a T175 flask and incubate for 24 hours at 37 °C with 5% CO<sub>2</sub>.

### Day 1

#### 3 Label target cells with IncuCyte pHrodo Red Cell Labeling Kit

3.1 Harvest apoptotic target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.

3.2 Aspirate supernatant and resuspend cell pellet with 50 mL IncuCyte® pHrodo® Wash Buffer (component C). Gently mix cells by trituration and determine cell count using a hemocytometer (omit Trypan blue as cells are apoptotic).

- 3.3 Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate wash buffer and resuspend cell pellet in IncuCyte pHrodo Cell Labeling Buffer (component D) to a density of  $1 \times 10^6$  cells/mL.
- 3.4 Reconstitute IncuCyte pHrodo Red Cell Labeling Dye (component A) in 100  $\mu$ L of DMSO (component B) to create a stock concentration of 1 mg/mL.
- 3.5 Add the solubilized IncuCyte pHrodo Red 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.
- 3.6. Remove excess pHrodo reagent 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  $1 \times 10^7$  cells/mL.

- 4 **Add target cells to effector cells**
  - 4.1 Prepare dilutions of the labeled apoptotic target cells by creating a 7-point, two-fold serial dilution (500,000 cells/well to 7,812 cells/well).
  - 4.2 Immediately following target cell resuspension, remove the effector cell plate from the incubator and add the target cell suspensions to the cell plate (50  $\mu$ L per well) using a multichannel pipette.
  - 4.3 Remove bubbles and immediately place the microplate in the IncuCyte Live-Cell Analysis System (refer to Data Acquisition and Analysis section).

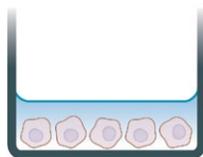
### Non-apoptotic phagocytosis protocol (antibody-dependent cellular phagocytosis)

This protocol provides an overview of the phagocytosis of antibody-treated cells by macrophage engulfment, referred to as antibody-dependent cellular phagocytosis (ADCP). It combines the IncuCyte pHrodo Red Cell Labeling Kit with the IncuCyte Live-Cell Analysis System using your choice of target and phagocytic (effector) cells.

**Prior to initiating the assay, it is important that your experimental design includes replicate wells of each condition being tested (e.g. labeled target cells alone as well as target:effector co-cultures at each ratio  $\pm$  antibody, isotype, or vehicle controls).**

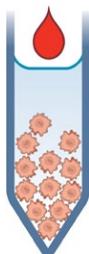
#### Quick Guide

**1 SEED EFFECTOR CELLS**



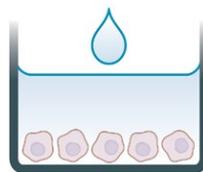
Seed phagocytotic effector cells

**2 TREAT TARGET CELLS**



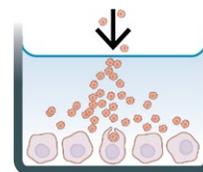
Label target cells with IncuCyte pHrodo Labeling Dye.

**3 TREAT EFFECTOR CELLS**



Treat effector cells with compounds prior to phagocytosis (0.5-24 h pretreatment, 25  $\mu$ L/well).

**4 ADD INCUCYTE PHRODO LABELED TARGET CELLS**



Add IncuCyte pHrodo labeled target cells to treated wells (10  $\mu$ g/well, 25  $\mu$ L/well).

## Day 0

### 1 Seed effector cells

1.1 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.  $1 \times 10^6$  total cells for seeding 10,000 effector cells/well).

1.2 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.

1.3 Using a multi-channel pipette, seed effector cells (50  $\mu$ L per well) into a 96-well 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 the cells to settle on a level surface for 30 minutes, then incubate overnight at 37 °C with 5% CO<sub>2</sub>.

## Day 1

### Prepare treatment plate

Using effector cell media, prepare 4x the final desired concentration of antibody, isotype control, and vehicle control in a separate 96-well plate (minimum volume per well should be 50  $\mu$ L). Set plate aside.

### 2 Label target cells with IncuCyte pHrodo Red Cell Labeling Kit

**NOTE:** Grow enough target cells in advance to accommodate the different cell densities required to set up the assay. We recommend testing target-to-effector cell ratios of 20:1, 10:1 and 5:1.

2.1 Harvest target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.

2.2 Aspirate supernatant and resuspend cell pellet with 50 mL IncuCyte pHrodo Cell Wash Buffer (component C). Gently mix cells by trituration and determine cell count using a hemocytometer.

2.3 Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate wash buffer and resuspend cell pellet in IncuCyte pHrodo Cell Labeling Buffer (component D) to a density of  $1 \times 10^6$  cells/mL.

2.4 Reconstitute IncuCyte pHrodo Red Cell Labeling Dye (component A) in 100  $\mu$ L of DMSO (component B) to create a stock concentration of 1 mg/mL.

2.5 Add the solubilized IncuCyte pHrodo Red 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 h at 37 °C.

2.6 Remove excess IncuCyte pHrodo Red Labeling Dye from cells:

- 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.
- 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  $1 \times 10^7$  cells/mL.

### 3 Add treatment to effector cells

3.1 During target cell incubation, use a multichannel pipette to transfer 25  $\mu$ L from the treatment plate containing the antibody, isotype control, and vehicle control to the effector cell plate. Incubate for 15-30 minutes at 37 °C.

### 4 Add IncuCyte® pHrodo® labeled target cells to effector cells

4.1 Create two-fold serial dilutions of the labeled target cells by creating a 7-point, two-fold serial dilution (500,000 cells/well to 7,812 cells/well).

Target Cell Seeding Density	Cell no. Per Well	Volume Per Well
$1 \times 10^7$ cells/mL	250,000	25 $\mu$ L
$5 \times 10^6$ cells/mL	125,000	25 $\mu$ L
$2.5 \times 10^6$ cells/mL	62,500	25 $\mu$ L

4.2 Immediately following target cell resuspension, remove the effector cell plate from the incubator and add the target cell suspensions to the cell plate (25  $\mu$ L per well) using a multichannel pipette.

4.3 Remove bubbles and immediately place plate in the IncuCyte Live-Cell Analysis System (refer to Data Acquisition and Analysis section).

## Data acquisition and analysis

### 1 Acquisition

In the IncuCyte® software, schedule 24 hour repeat scanning for every 15-30 minutes, for up to 48 hours.

- a. Objective: Ensure 10x or 20x objective is installed
- b. Channel Selection: Select "Phase" and "Red" (800 ms acquisition time)
- c. Scan Mode: Select "Standard" scan type and Scan Pattern of 1 image per well if using 10x, and 4 images per well if using 20x.

### 2 Analysis

To generate the metrics, the user must create a Processing Definition suited to the cell type, assay conditions and magnification selected, then apply the Processing Definition to the data set as an Analysis Job.

2.1 Select an image from a well containing a high concentration of treated target cells (e.g., 250,000 cells/well) but no effector cells during the peak assay response (e.g., 24 hours). Under Image Channels, expand the Red drop down and deselect the AutoScale option. Adjust the scale until no red is observed in the image. Note the minimum and maximum values. Add the image to a New Image Collection by selecting "Create or Add to Image Collection."

2.2 Select an image from a well containing both effector cells and the same concentration of treated target cells selected in step 1 (e.g., 250,000 cell/well) during the peak assay response (e.g., 24 hours). Save the image to the Image Collection.

2.3 With this image collection, create a new Processing Definition:

- a. Deselect the AutoScale option and set the minimum and maximum values as identified in step 1.
- b. To exclude background fluorescence from the mask, use the background subtraction feature in the Parameters 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, as in this case.
- c. The radius chosen should reflect the size of the cells; 20 µm is often a useful starting point.
- d. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked. Choose a threshold in which red objects are masked in the image with effector cells but not masked in the image without effector cells.

2.4 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: as an increase in fluorescence area ("Total Object Area") or increase in intensity, integrated over the area of detectable fluorescence ("Total Integrated Intensity"). Analyze using the "Total Red Object Area (µm<sup>2</sup>/image)" or "Total Red Object Integrated Intensity (RCU x µm<sup>2</sup>/image)" metrics.

## Related products and applications

In addition to the pHrodo Red Cell Labeling Kit, 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
IncuCyte® pHrodo® Red E. coli Bioparticles®	4615	2 mg	560 nm	585 nm
IncuCyte® pHrodo® Green E. coli Bioparticles®	4616	2 mg	509 nm	533 nm
IncuCyte® pHrodo® Red Zymosan Bioparticles®	4617	1 mg	560 nm	585 nm
IncuCyte® pHrodo® Green Zymosan Bioparticles®	4618	1 mg	506 nm	533 nm
IncuCyte® pHrodo® Red S. aureus Bioparticles®	4619	2 mg	560 nm	585 nm
IncuCyte® pHrodo® Green S. aureus Bioparticles®	4620	2 mg	509 nm	533 nm

A complete suite of cell health applications is available to fit your experimental needs. Find more information at [essenbioscience.com/oncology](https://essenbioscience.com/oncology)

- Immune Cell Killing
- Immune Cell Proliferation and Clustering
- Chemotaxis Cell Migration and Invasion
- Scratchwound Cell Migration and Invasion

For additional product or technical information, please e-mail us at [AskAScientist@sartorius.com](mailto:AskAScientist@sartorius.com) visit our website at [essenbioscience.com](https://essenbioscience.com) or call

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