SARTORIUS

Product Guide

Incucyte® MMP Orange Reagent Kit

For Measurement of Mitochondrial Membrane Potential

Product Information

Presentation, Storage and Stability

The Incucyte® MMP Orange Reagent Kit contains a sufficient quantity of reagent for the measurement of mitochondrial membrane potential (MMP) in 200 tests (1 test = 1 well of a 96-well microtiter plate). Included in the kit are Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) and Oligomycin A controls for mitochondrial membrane depolarization or hyperpolarization, respectively (16 tests or 16 wells of a 96-well plate).

Product Name	Cat. No.	Amount	Concentration	Max Ex/Em	Storage	Stability
Incucyte® MMP Orange Reagent	4775	30 µL	40 µM (DMSO)	552/578 nm	-20° C	6 months from date of receipt
FCCP		10 µL	10 mM (DMSO)	N/A		
Oligomycin A		10 µL	2.5 mg/mL (DMSO)	N/A		

Incucyte® MMP Orange Reagent is compatible with Incucyte Live-Cell Analysis Systems configured with a Metabolism, green/ orange/NIR or orange/NIR optical module.

Background

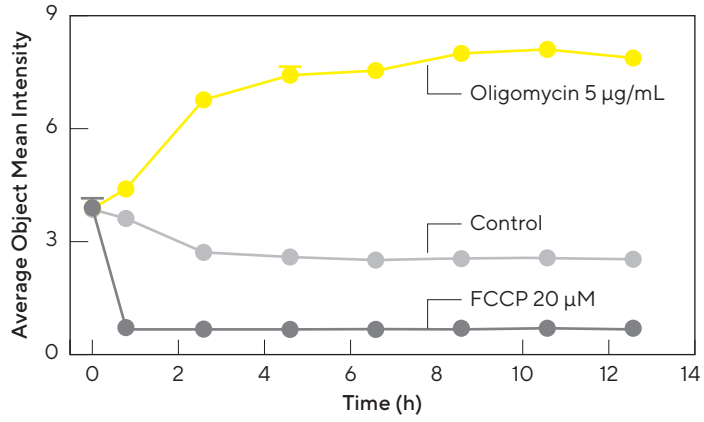
The MMP Orange Reagent Kit assesses changes in MMP, an indicator of mitochondrial health. The cell-permeable MMP Orange Reagent accumulates in active mitochondria in proportion to the MMP generated by the electrochemical gradient across the mitochondrial membrane. A drop in MMP denotes a disruption in mitochondrial function and/or integrity and can also be used as an early indicator of apoptosis. This assay kit utilizes two control compounds: FCCP, an uncoupling agent that transports H^+ ions across the mitochondrial membrane and results in depolarization (indicated by a decrease in fluorescence), and Oligomycin A, an inhibitor of ATP synthase which results in hyperpolarization (indicated by an increase in fluorescence).

Recommended Use

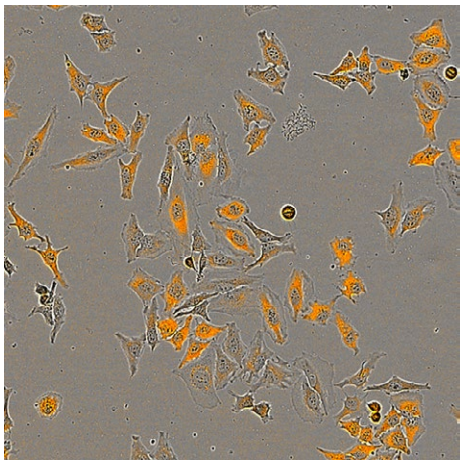
We recommend diluting the Incucyte MMP Orange Reagent in growth media (final assay concentration will be 20 nM) and adding directly to cells. Prior to adding test and control compounds (final concentrations 5 µg/mL Oligomycin A and 20 µM FCCP), it is recommended to allow the MMP reagent to load into cells (see additional details in protocols below).

The Incucyte MMP Reagent Kit is intended for use with the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031).

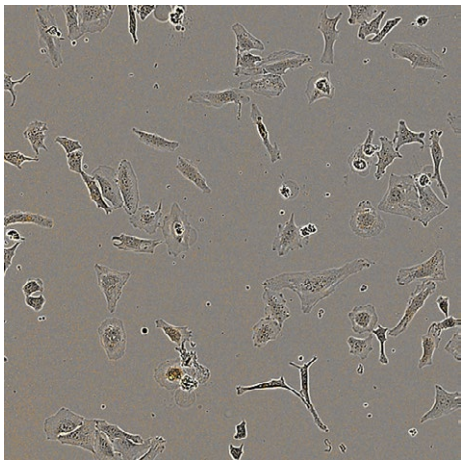
Example Data



Control



FCCP



Oligomycin A

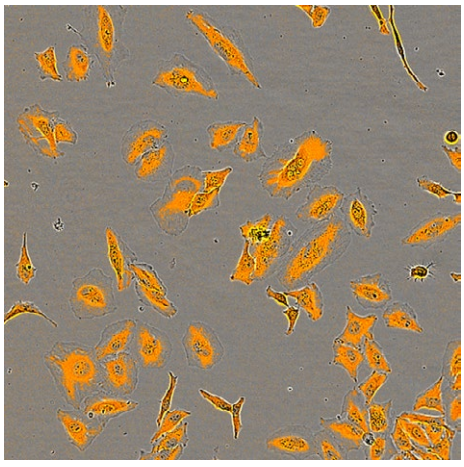
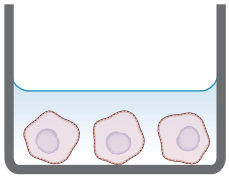


Figure 1: HeLa cells were labeled with Incucyte MMP Orange Reagent and treated with vehicle control, 5 µg/mL of Oligomycin A, or 20 µM of FCCP (compounds added between first and second time points). HD Phase and orange fluorescence images were captured every 2 hours. Images of cells treated with Oligomycin A show an increase in fluorescence intensity due to membrane hyperpolarization, whereas cells treated with FCCP show a decrease in fluorescence intensity caused by depolarization of the mitochondrial membrane.

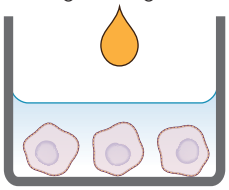
Quick Guide

1. Seed cells



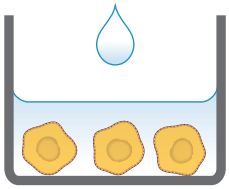
Seed cells in growth media (100 µL) and leave to adhere (4–24 hours).

2. Add Incucyte® MMP Orange Reagent



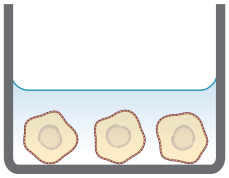
Prepare Incucyte MMP Orange Reagent, add to cells, and incubate for one hour at 37° C.

3. Add test compounds



Add compounds of interest directly to cells with MMP Orange Reagent.

4. Live-cell fluorescent imaging



Capture images and analyze fluorescence intensity data in an Incucyte System using the Incucyte Cell-by-Cell Software Module.

Protocols and Procedures

Materials

- Incucyte® MMP Orange Reagent Kit
- Flat bottom microplate (e.g. Corning® 3595) for imaging
- Incucyte® Live-Cell Analysis System equipped with a Green/Orange/NIR, Metabolism or Orange/NIR optical module
- Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031)

General Guidelines

- Following cell seeding, place plates on a level surface at ambient temperature (15 minutes for adherent cell lines and 45 minutes for non-adherent cell lines) 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.

Note: The following protocols are written to enable a simple workflow and monitoring of MMP over hours or days. The effect of some compounds (including the control compound FCCP) will occur very rapidly. Modifications to the protocol (e.g. more rapid scanning at early time points or gentle addition of compounds to pre-plated non-adherent cells) may be needed to capture early effects of such compounds.

Adherent Cell Line Protocol

Seed Cells

1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate. The seeding density will need to be optimized for the cell line used, however, we have found that 2,000–5,000 cells per well (20,000–50,000 cells/mL seeding stock) is a reasonable starting point. Allow cells to settle (15 minutes) and place in incubator until use.

   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). Some cell types may require overnight incubation.

MMP Reagent Preparation

2. Remove MMP Reagent and test compounds from freezer, allow reagents to thaw at room temperature (protected from light).
3. Dilute the MMP Reagent 1:500 to generate a 4X (80 nM) solution and add 50 µL of reagent to the cell plate, bringing the volume to 150 µL/well.
4. Incubate plate at 37° C for one hour to allow for dye loading prior to addition of test compound(s).

Add Compound(s) of Interest

5. During the MMP Reagent incubation, prepare the control and test compound(s) at 4X the final assay concentration in complete culture media.
6. Add 50 µL of the controls and test compound(s) to appropriate wells. Final assay volume is 200 µL/well, with MMP assay concentration of 20 nM.

Live-Cell Imaging of MMP

7. Place the plate into the Incucyte Live-Cell Analysis System.

   Note: Scan interval will vary depending on experimental paradigm, but typically every 1-2 hours is recommended.

   a. Scan type: Select Standard Scan with Adherent Cell-by-Cell option enabled.
   b. Image channel: select Phase and Orange
   c. Objective: 10X or 20X depending on cell types used and throughput needs.
   d. Scan pattern: 2 images per well for 10X, 4 images per well for 20X

Non-Adherent Cell Line Protocol

Notes: Non-adherent cells can be seeded on the day of assay (no need for pre-incubation).

Prepare Cell Stock

1. For non-adherent cell types, a well coating may be required to maintain even cell distribution. For a 96-well flat bottom plate, we recommend coating with 50 µL of either 0.01% poly-L-ornithine solution (Sigma P4957) or 5 µg/mL fibronectin (Sigma P4957) diluted in 0.1 % BSA. 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.
   Note: Some optimization of plate coatings may be required.
2. Remove MMP Reagent and test compounds from freezer, allow reagents to thaw at room temperature (protected from light) while counting cells.
3. Count cells and prepare a cell seeding stock at the appropriate density. The seeding density will need to be optimized for the cell line used; however, we have found that 20,000 to 40,000 cells per well (200,000–400,000 cells/mL seeding stock) is a reasonable starting point.

MMP Reagent Preparation

4. Add the MMP Reagent directly to the cell seeding stock at 2X concentration (40 nM) and gently mix solution to ensure even mixture of reagent.
5. Incubate cells at ambient temperature for 10 minutes to allow for dye loading prior to addition of test compounds.

Prepare Compounds of Interest

6. While the cells are being loaded with MMP, prepare the control and test compound(s) at 2X the final assay concentration in complete culture media.
7. Add the control and test compound(s) to the appropriate wells of the coated assay plate (100 µL per well).
8. Gently mix the cell seeding stock loaded with MMP from step 5 above.
9. Add cell seeding stock solution directly to the coated assay plate containing test compounds (100 µL per well). The assay plate now contains 200 µL per well, with a final MMP assay concentration of 20 nM.
10. Allow cells to settle on a level surface for 30 minutes at room temperature to ensure homogenous cell settling.

Live-Cell Imaging of MMP

Place the plate into the Incucyte Live-Cell Analysis System.

Note: Scan interval will vary depending on experimental paradigm, but typically every 1-2 hours is recommended.

a. Scan type: Select Standard Scan with Non-Adherent Cell-by-Cell option enabled.
b. Image channel: select Phase and Orange.
c. Objective: 20X
d. Scan pattern: 2-4 images per well

Analysis Guidelines

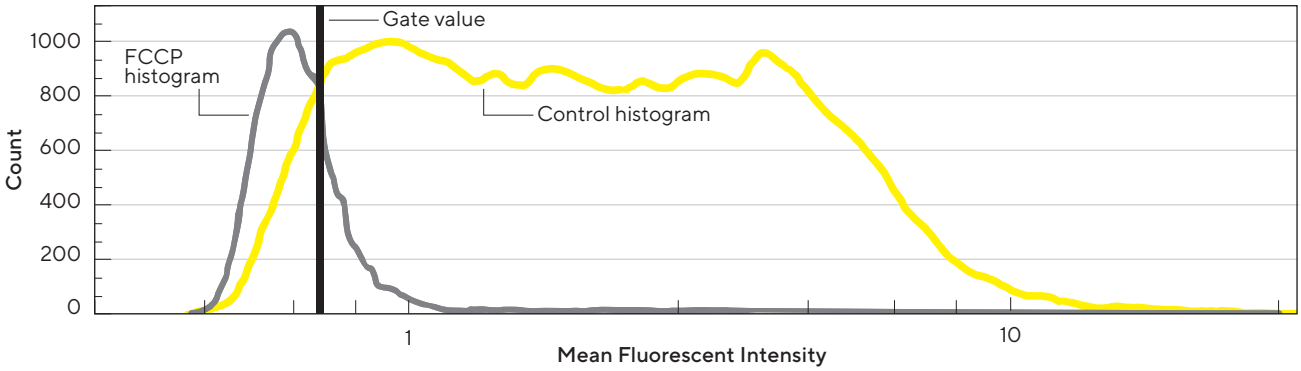
1. To generate metrics, the user must create an Analysis Definition suited to the cell type, assay conditions, and magnification selected.
2. Depending on your cell type, select Adherent or Non-Adherent Cell-by-Cell analysis type to create a new Analysis Definition.
3. Select representative images from both drug-treated and control MMP Reagent wells over time points of interest.
4. Begin by adjusting Segmentation with the fluorescent channel turned off.
5. For Adherent Cell-by-Cell mode, determine the optimal Seed mask (the goal is to have one seed per cell) using the slider controls. Viewing the Cell Boundary mask while changing the Seed Mask parameters is a convenient way to set up the mask.
6. Evaluate and refine the Cell-by-Cell Mask using Preview All to ensure segmentation is appropriate for other time points or treatments selected.
   Note: Enabling the orange fluorescence channel can be useful as another tool to differentiate cell boundaries. It is not necessary to create a fluorescence mask because Cell-by-Cell analysis enables analysis of fluorescence within the segmentation boundary.
7. Once you are satisfied with the previewed images, launch analysis: select the scan times, wells to be analyzed, and an analysis definition name. Note that if your experiment is in progress you will have an option to check Analyze Future Scans to perform real-time analysis.

Optional Classification Analysis

1. Using Incucyte Cell-by-Cell Analysis, it is possible to classify each individual cell at each time point using either one or two parameters. The division into classes is performed by setting a gate. For this assay, it is possible to gate cells based on the average mean fluorescence intensity of the MMP Reagent (cells with values above the gate fall into the High class, cells below fall into the Low class).
2. To apply classification to an analysis, open the analysis job you wish to classify and select the Launch Cell-by-Cell Classification button on the left panel. This will open Well Selection, allowing you to select wells for review. There is also a Plate Map selection method, allowing the selection of replicate wells.
3. If you are interested in monitoring a loss in MMP signal in response to compound treatment (e.g. as an early indicator of apoptosis), we recommend setting the gate directly above the peak of the FCCP control wells (see histogram image below). This will divide the cell population into High and Low MMP classes. The percentage of Low MMP cells per well can then be plotted over time.

Data Interpretation

1. The Average Mean Fluorescent (Orange) Intensity metric is used to quantify changes in MMP. This metric averages the mean intensity across each individual cell.
2. Expected results: Oligomycin A treated wells will demonstrate an increase in Avg Mean Intensity, FCCP treated wells will demonstrate a decrease in Avg Mean Intensity, and control wells should remain relatively stable over time.



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