Protocol

IncuCyte® CytoLight Rapid Reagent Cell Labeling Protocol

For the cytoplasmic labeling of cells

This protocol provides an overview of the IncuCyte® CytoLight Rapid Reagent Cell Labeling methodology. It is compatible with the IncuCyte® Live-Cell Analysis System configured with a Green/Red Optical Module and describes how IncuCyte CytoLight Rapid Reagents can be used to fluorescently label the cytoplasm of living cells without perturbing cell function or biology. In addition, these dyes can be multiplexed with IncuCyte® Annexin V, Caspase-3/7 or Cytotox reagents for simultaneous readouts of apoptosis or cytotoxicity in the same well.

Required materials

- IncuCyte CytoLight Rapid Green Reagent (Sartorius Cat. No. 4705) *or* IncuCyte CytoLight Rapid Red Reagent (Sartorius Cat. No. 4706)
- 0.01% Poly-L-ornithine solution (Sigma Cat. No. P4957)—optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning Cat. No. 3595)

General guidelines

- Protect IncuCyte CytoLight Rapid Reagent from light at all times.
- We recommend medium with low levels of riboflavin to reduce the green fluorescence background. EBM, F12-K, and Eagles MEM have low riboflavin (<0.2 mg/L). DMEM and RPMI have high riboflavin (>0.2 mg/L).
- Following cell seeding, place plates at ambient temperature (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, allow the plate to warm to 37° C for 30 minutes prior to scanning.
- If using non-adherent cells (e.g. immune cells) we recommend coating plates with 0.01% poly-L-ornithine solution (as supplied by Sigma) or 5 µg/mL fibronectin diluted in 0.1% BSA/PBS (Ca^{2+}/Mg^{2+} - free) to prevent cell aggregation at well edges. For a 96-well plate, add 50 µL of chosen matrix solution to each well, incubate for 1 hour at ambient temperature, remove solution from wells and then allow plates to dry for 30-60 minutes prior to cell addition. Plates may be coated the day before and stored, once dried, overnight at 4° C.
- For optimal results, it is recommended to utilize the highest non-perturbing concentration of the IncuCyte CytoLight Rapid Reagent when labeling cells. To determine this concentration, perform an initial optimization experiment as described below. It is recommended to perform an optimization experiment each time a new cell type is used, as the optimal final assay concentration will vary.

Preparation of IncuCyte® CytoLight Rapid Reagent

1 Prepare stock concentration

1.1 Prior to harvesting cells, bring one or more vials of IncuCyte CytoLight Rapid Reagent (green or red) to room temperature and briefly centrifuge to ensure the reagent is located in the bottom of the vial.

1.2 Solubilize CytoLight Rapid Reagent with high-quality DMSO.

If using CytoLight Rapid Red, dilute reagent in 20 µL DMSO to yield a stock concentration of 1 mM.

If using CytoLight Rapid Green dilute the reagent in 21.5 µL DMSO to yield a stock concentration of 5 mM.

2 Prepare working concentration

2.1 General use

Dilute the stock solution in PBS (Ca^{2+}/Mg^{2+} - free) to yield a concentration 100X higher than the desired final assay concentration (e.g., prepare a 10 µM working concentration for desired assay concentration of 0.1 µM).

NOTE: Highest recommended 100X working concentration is 300 µM for a final assay concentration of 3 µM.

2.1 Initial optimization

When performing initial cell labeling optimization, dilute the stock in PBS (Ca^{2+}/Mg^{2+} - free) to yield a 100X working concentration of 300 µM

- For CytoLight Rapid Red, add 6 µL stock solution to 14 µL PBS.
- For CytoLight Rapid Green, add 1.2 µL stock solution to 18.8 µL PBS.

2.2 Initial optimization

Perform a 3-fold serial dilution of the CytoLight Rapid Reagent working stock, adding 6 µL concentrated solution to 12 µL PBS (Ca^{2+}/Mg^{2+} - free) to create seven test concentrations.

NOTE: Recommended 100X test concentrations are 300 µM, 100 µM, 33 µM, 11 µM, 3.7 µM, 1.2 µM and 0 µM (non-labeled control).

Cell Labeling Protocol

Quick guide

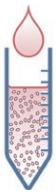
1. Harvest cells

Harvest cells. Wash with dPBS. Count and resuspend in dPBS (1 x10^5 cells/ml).

2. Label cells

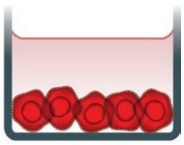
Add IncuCyte® CytoLight Rapid Live-Cell Labeling Reagent. Incubate for 20 minutes at 37° C.

3. Bind excess reagent



Bind excess reagent by adding complete medium. Centrifuge and aspirate supernatant.

4. Live-cell fluorescent analysis



Resuspend cells in complete medium and seed at desired density. Acquire images every hour (10X or 20X) in the IncuCyte® System.

1.

Harvest cells

1.1 Harvest cells using a suitable dissociation solution, then neutralize with 5 mL full growth media and centrifuge to create a pellet.

NOTE: Grow enough cells in advance to accommodate the different conditions required to set up the experiment (refer to step 4.1 for recommended seeding densities). It is recommended to prepare 50% more cells than needed in order to accommodate for cell loss during the wash step. When performing the **initial reagent optimization** experiment, 1 x 106 total cells are required.

1.2 Aspirate the media/dissociation solution and wash cells with 10 mL ambient temperature PBS (Ca^{2+}/Mg^{2+} - free).

1.3 Centrifuge cells and aspirate the supernatant.

1.4 Resuspend the cell pellet in 10 mL PBS (Ca^{2+}/Mg^{2+} - free) and determine the cell concentration to yield a final cell density of 1 x 10^5 cells/mL.

NOTE: If optimizing cell labeling concentration, aliquot 1 mL of cell suspension into seven 15 mL conical tubes.

2.

Label cells

2.1 Add 1 part working concentration of CytoLight Rapid Reagent (100X) to 99 parts of the 1 x 10^5 cells/mL suspension.

NOTE: When performing cell labeling optimization experiments, add 10 µL of each 100X CytoLight Rapid Reagent test concentration to the appropriately labeled tube from step 1.4 (above).

2.2 Incubate for 20 minutes at 37° C, mixing by inversion every 10 minutes.

3.

Bind excess reagent

3.1 Remove excess dye by adding 6-fold excess volume of cell culture medium containing serum to (e.g., add 6 mL to 1 mL cell/reagent suspension).

3.2 Centrifuge and aspirate the supernatant.

4.

Live-cell fluorescent analysis

4.1 Resuspend cells to the desired density in complete cell culture medium and seed the labeled cells at an appropriate density into a 96-well plate in triplicate. The seeding density will need to be optimized for the cell line used; however, we have found that 2-5,000 cells per well for adherent cell types or 20-30,000 cells per well for non-adherent cell types are reasonable starting points.

4.2 Place the plate into the IncuCyte Live-Cell Analysis System to monitor both cell proliferation using the phase contrast confluence metric and labeling efficiency using the appropriate fluorescence channel.

a. Objective: 10X or 20X
b. Channel selection: Phase + Fluorescence
c. Scan type: Standard
d. Scan interval: Typically, every 2 hours

Evaluating Results to Determine Optimal Dye Concentration

Use the IncuCyte System HD phase images and confluence metrics to compare the cell morphology and growth rates for each concentration of dye and compare to the non-labeled control cells. The optimal concentration of IncuCyte CytoLight Rapid Reagent is the highest concentration that does not cause significant changes to growth rate or morphology while providing efficient cell labeling.

Please note, you may find that there is some variability in the initial confluence values for cells at different dye concentrations. This is due to the independent preparation and seeding of each test condition. We recommend normalizing the confluence values for each test condition to the first-time point. Comparison between the fold-increase in confluence over time can then be used to identify optimal dye concentration.

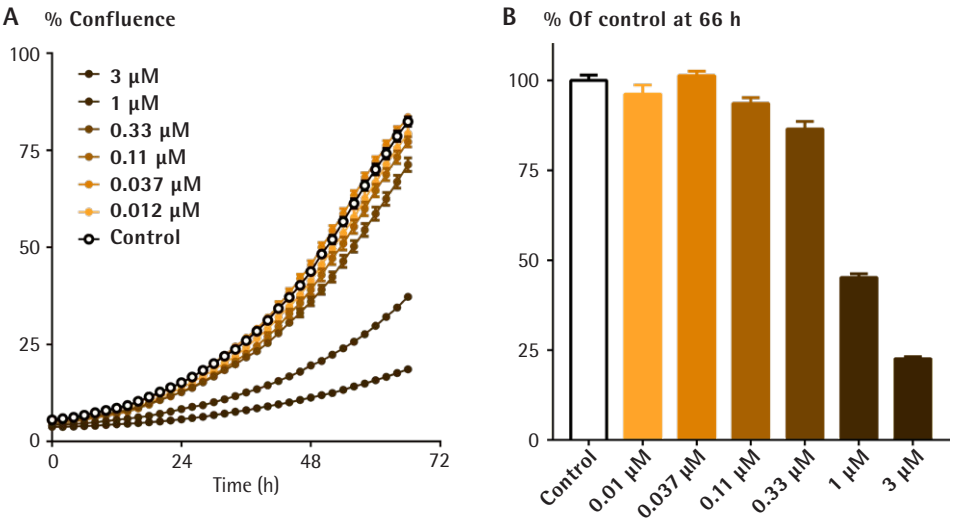


Figure 1. Selecting the optimal dye concentration. IncuCyte phase contrast image analysis was used to determine (A) cell growth time-courses and, (B) % of control values for HT-1080 (adherent) cells labeled with the IncuCyte CytoLight Rapid Green Dye. Data is presented as the mean ± SEM from 6 replicate wells. In this experiment, the optimal dye concentration was 0.11 µM.

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