SARTORIUS

Product Guide

Incucyte® Cell Cycle Lentivirus Reagents (EF1α, Puro)

Product Information

Presentation, Storage and Stability

Incucyte® Cell Cycle Lentivirus Reagents are supplied as a 0.2 mL vial of 3rd generation HIV-based, VSV-G pseudotyped lentiviral particles suspended in DMEM.

The lentivirus reagent should be stored at -80° C. When stored as described, the Cell Cycle Lentivirus Reagent will be stable for at least 6 months from the date of receipt.

Product Name	Selection	Amount	Cat. No.	Storage Conditions	Stability
Compatible with Incucyte Live-Cell Analysis Systems configured with a Green/Red optical module Green (TagGFP2): $\lambda_{abs}/\lambda_{em}$ = 483/506 nm Red (mKate2): $\lambda_{abs}/\lambda_{em}$ = 588/633 nm					
Incucyte Cell Cycle Green/Red Lentivirus Reagent	puro	0.2 mL	4779	-80° C	6 months from date of receipt
Compatible with Incucyte Live-Cell Analysis Systems configured with a Green/Orange/NIR optical module Green (TagGFP2): $\lambda_{abs}/\lambda_{em}$ = 483/506 nm Orange (TagRFP): $\lambda_{abs}/\lambda_{em}$ = 555/584 nm					
Incucyte Cell Cycle Green/ Orange Lentivirus Reagent	puro	0.2 mL	4809	-80° C	6 months from date of receipt

For viral titer and lot information please visit our web page at **www.essenbioscience.com/lentivirus-viral-titers**
Safety data sheet (SDS) information can be found on our website at **www.sartorius.com**

Background

Incucyte Cell Cycle Lentivirus Reagents enable efficient, non-perturbing, and homogeneous labeling of living mammalian cells for *in vitro* analysis of cell cycle progression. The Cell Cycle Lentivirus Reagents are single-cassette, genetically encoded ubiquitination-based indicators that take advantage of cell cycle dependent changes in the expression patterns of Geminin and Cdt1. By linking fluorescent proteins TagGFP2 and mKate2 (green/red), or TagGFP2 and TagRFP (green/orange) to fragments of Geminin and Cdt1, the G1 and S/G2/M phases can be monitored in real-time. These reagents are ideal for generating stable cell populations or clones using puromycin selection. The Cell Cycle Green/Red Lentivirus Reagent is compatible with the Incucyte Live-Cell Analysis System configured with a Green/Red optical module. The Cell Cycle Green/Orange Lentivirus Reagent is compatible with the Incucyte Live-Cell Analysis System configured with a Green/Orange/NIR optical module.

Recommended Use

We recommend thawing the Incucyte Cell Cycle Lentivirus Reagents on ice. Avoid repeated freeze/thaw cycles as this can impair transduction efficiency. The lentivirus reagents can be prepared in full media and added directly to plated cells. We recommend a multiplicity of infection (MOI) of 3 to 6 transduction units (TU) per cell depending on the cell type being transduced. The cationic polymer Polybrene® may be added to enhance transduction efficiency. Post infection, stable cell lines or clones may be generated using puromycin selection.

Example Data

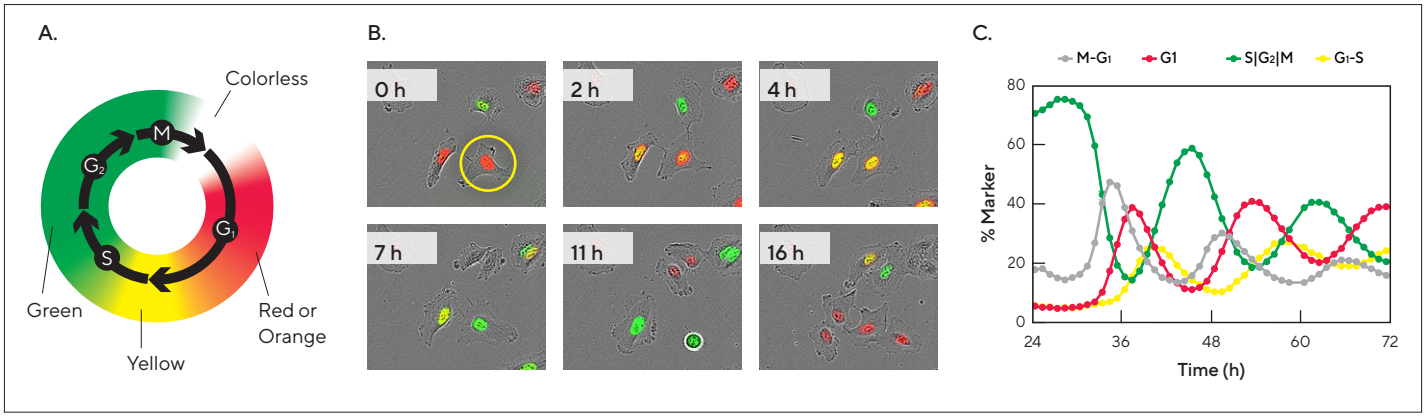
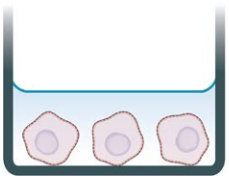


Figure 1. Visualization of cell cycle dynamics with Cell Cycle Lentivirus Reagents. Schematic (A) shows the various stages of the cell cycle with associated fluorescent signature. (B) Cell images collected on an Incucyte Live-Cell Analysis System display A549 cells transfected with the Cell Cycle Green/Orange Lentivirus Reagent as they grow and divide. Starting in G1 (orange, 0h), the marked cell progresses to S phase (yellow, 2–4h) before reaching S/G2/M phase (green, 7h) and mitosis (11h), and then returning to G1 (orange, 16h). (C) Division of HeLa cells stably expressing Cell Cycle Green/Red Lentivirus Reagent was arrested by 2.5 mM thymidine for 24 hours, causing cells to accumulate in G2/M (green). Upon removal of the thymidine block, cells begin to synchronously divide and progress through the cell cycle (24–72h). Data shows the % cells expressing each marker peaks in sequence: non-fluorescent (M–G1 transition), red (G1), green + red (G1–S transition), green (G2/M). Two full cycles of cell division are observed for this cell type between 24 and 72h.

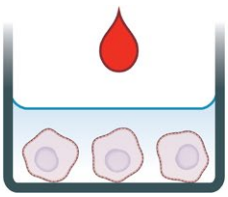
Quick Guide

1. Seed cells



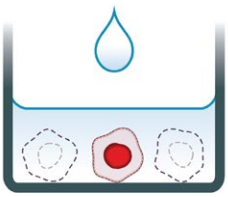
Cell Seeding
Seed cells in growth media and leave to adhere (4–24 hours). Cells should be 15–35% confluent at the time of transduction.

2. Transduce



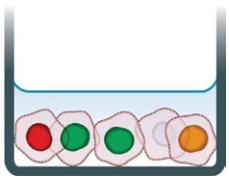
Add Cell Cycle Lentivirus Reagent
Add Cell Cycle Lentivirus Reagent diluted in media ± Polybrene. After 24 hours, replace the media with fresh growth media. Monitor expression using the Incucyte Live-Cell Analysis System.

3. Apply selection



Generate a Stable Population or Clone
Apply puromycin selection to derive a stable, homogenous cell population or clone that expresses the genetically encoded cell cycle indicator. (Optional: Freeze cells and use for future assays).

4. Live-cell fluorescent imaging



Automated Imaging and Quantitative Analysis
Capture images in the presence of treatments in an Incucyte Live-Cell Analysis System with an appropriate optical module. Analyze using integrated software.

Protocols and Procedures

Materials

Required materials

- Incucyte Cell Cycle Lentivirus Reagent (Sartorius Cat. No. 4779 or 4809)
- Flat bottom 96-well tissue culture plate (e.g. Corning Cat. No. 3595)
- Complete cell culture media for cell line of choice

Optional

- Incucyte® Cell-by-Cell Analysis Software Module (Sartorius Cat. No. 9600-0031)
- Polybrene (Sigma Cat. No. H9268)
- Poly-L-Ornithine (Sigma Cat. No. P4957)–optional, for non-adherent cell types

Suggested Transduction Protocol

If you plan to use the Cell Cycle Lentivirus Reagent to generate stably-expressing clones or populations, the puromycin selection concentration should be determined by performing a kill curve with parent cell line (see "Antibiotics Selection" section below). Optimizing MOI and transduction conditions are less important as the selection process will eliminate non- or low-expressing cells within the population.

1. Seed cells in growth media of choice at a density such that they are 15–35% confluent at time of infection. Incubate plate for 4–24 hours or until cells have attached.
2. Add the Cell Cycle Lentivirus Reagent at desired MOI diluted in media ± Polybrene. An MOI of 3–6 and Polybrene concentration of 8 µg/mL is recommended for most cell types.
3. Incubate at 37°C, 5% CO_2 for 24 hours.
4. After incubation, remove media and replace with fresh growth media. Return to incubator for additional 24–48 hours, monitoring expression using an Incucyte Live-Cell Analysis System.
5. Harvest cells and expand, freeze, or seed at desired density for subsequent experiments. For stable selection, proceed to step 6.
6. (Optional) Remove media and replace with fresh growth media containing the appropriate concentration of puromycin determined from the kill curve (see next section "Optimization Protocols, Antibiotic Selection").
7. Incubate for 72–96 hours, replacing media every 48 hours.
8. (Optional) Maintain stable population in a maintenance concentration of selection media (e.g. complete media containing 0.5 µg/mL puromycin).

Optimization Protocols

Antibiotic Selection (optional)

In order to generate a stable cell line expressing the Cell Cycle Lentivirus Reagent, determine the minimum concentration of antibiotic required to efficiently eliminate non-transduced cells using a dilution series of puromycin (typical antibiotic working concentration range for mammalian cells is 0.5–10 µg/mL, and effectiveness can be reached for most cell lines in 2–7 days).

Polybrene Concentration

The cationic polymer, Polybrene, may be used to increase the efficiency of transduction of certain cell types. Polybrene can be toxic to some cell types (e.g. primary neurons). The Incucyte® Cytotoxicity Assay can be used to evaluate the toxic effect of Polybrene on your cells.

Protocol for Primary Cells and Transient Assays

If you plan to use the Cell Cycle Lentivirus Reagent for transient assays, we recommend optimizing MOI and Polybrene concentration for each cell type used. Once these steps are complete, follow the "Suggested Transduction Protocol", steps 1 through 5.

Cell Cycle Assay Protocol

General Guidelines

- Following cell seeding, place plates at ambient temperature 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.

Adherent Cell Line Protocol

1. Seed cells transduced with the Cell Cycle Lentivirus Reagent (100 µL per well) at an appropriate density into a 96-well plate to achieve 15–35% confluency at time of assay. The seeding density will need to be optimized for the cell line used; however, we have found that 2,000 to 5,000 cells per well (20,000–50,000 cells/mL seeding stock) are reasonable starting points.
2. Allow cells to adhere for 4–20 hours at 37° C. During this time the plate can be monitored in the Incucyte Live-Cell Analysis System to evaluate cell health and baseline assessment of cell cycle progression.

   Note: If interested in modulators of cell cycle, prepare treatments in complete growth media and add to the wells at desired final assay concentration.

3. Return plate to the Incucyte Live-Cell Analysis System for continued imaging and analysis.

Non-adherent Cell Line Protocol

1. Coat a flat bottom 96-well plate with relevant coating matrix. We recommend coating with 50 µL of 0.01% Poly-L-Ornithine solution (not supplied). Coat plates for 1 hour at ambient temperature, remove solution from wells, and then allow plates to dry for 30 – 60 minutes prior to cell addition.
2. Count cells and prepare a cell seeding stock at the appropriate density. Seed your choice of cells (100 µL per well) at desired density into the coated 96-well plate. The seeding density will need to be optimized for the cell line used but we recommend 10,000 – 30,000 cells per well (100,000 – 300,000 cells/mL).

   Note: If adding modulators of cell cycle, we recommend preparing treatments and adding these to the well prior to cell seeding in order to avoid heterogeneous cell seeding.

3. Allow cells to settle at ambient temperature for 30 minutes, then place in the Incucyte Live-Cell Analysis System for imaging and analysis.

Live-cell Imaging

Using Incucyte software, schedule scans at desired frequency.

Note: Scan interval will vary depending on experimental paradigm (for cell cycle monitoring, this may require more frequent scans).

1. Scan type: Standard
2. Scan settings: Select none if using Basic Analyzer. If using Incucyte Cell-by-Cell Analysis Software Module, select either the Adherent or Non-Adherent option within the scan settings for greater ease of data analysis.
3. Channel selection: Phase, Green, Red or Orange.
4. Objective:
   a. If using Basic Analyzer, we recommend either 10X or 20X objective.
   b. If Cell-by-Cell Analysis for non-adherent cells is enabled, only 20X imaging is available.
   c. If Cell-by-Cell Analysis for adherent cells is enabled, 10X or 20X imaging is available.
5. Analysis Setup:
   Spectral Unmixing is required in order to correct for fluorescence bleed-through.
   a. Green/Red (% Red contributes to Green = 8%)
   b. Green/Orange (% Orange contributes to Green = 12 – 14%)

Data Analysis

Analysis using Cell-by-Cell Analysis Software

1. Adherent Cell Protocol: Select Adherent Cell-by-Cell analysis type to create a new Analysis Definition. Prior to processing, verify that spectral unmixing is set appropriately in order to ensure accurate data analysis.
   a. Select representative images from both treated and control wells. Ensure majority of images have ~50% cell coverage to enable accurate masking.
   b. Begin with optimizing the Cell Boundary (with fluorescent channels turned off) by determining the optimal Seed mask (goal is to have one seed per cell) using the slider controls.
   c. Evaluate the Cell-by-Cell Mask and refine the parameters accordingly. Once you are happy with the parameters, click "Preview All" to ensure that the analysis parameters are appropriate for other time points or treatments selected.
   d. Once you have previewed all of the representative images and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and Wells to be analyzed, as well as assigning an analysis definition name.

      Note: If your experiment is in progress you will have an option to check "Analyze Future Scans" to perform real-time analysis.

   e. For further in-depth guidelines to aid with analysis, please see the User Manual within the Incucyte software user interface.

2. Non-Adherent Cell Protocol: Select Non-Adherent Cell-by-Cell analysis type to create a new Analysis Definition. Prior to processing, verify that spectral unmixing is set appropriately in order to ensure accurate data analysis.
   a. Select representative images from both treated and control wells.
   b. Begin by setting an object diameter as measured with the measuring tool.
   c. Use the sliders for Threshold, Texture and Edge sensitivity to optimize the Cell-by-Cell mask to result in accurately masked cells.
   d. Once you have previewed all of the representative images and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and Wells to be analyzed, as well as assigning an analysis definition name.

      Note: If your experiment is in progress you will have an option to check "Analyze Future Scans" to perform real-time analysis.

   e. For further in depth guidelines to aid with analysis, please see the User Manual within the Incucyte software user interface.

3. Data Interpretation (Cell-by-Cell Analysis)
   a. Once cell segmentation has been completed, the data can be classified within the software based on green and red or orange fluorescence intensity using a 2D scatter plot. Classification gates can be set to determine high and low green and red or orange populations.
      i. High red or orange, low green–cells in G1 phase
      ii. Low red or orange, high green–cells in S/G2/M phase
      iii. Low red or orange, low green–M/G1 phase, and non-transfected
      iv. High red or orange, high green–G1/S phase
   b. After classification, any identified sub-population can be viewed either as count or percentage of total population.

Analysis using Basic Analyzer

1. If Cell-by-Cell Analysis Software is not available, process data using Basic Analyzer. Prior to processing, verify that spectral unmixing is set appropriately in order to ensure accurate data analysis.

   Note: Basic Analyzer method will not account for "colorless" (M/G1 and non-transfected) cells.

   a. Select Phase/Green/Red or Orange/Overlap Image channels. Overlap will mask the yellow (G1/S) transition of the cell cycle.
   b. Select representative images from both treated and control wells.
   c. Begin by optimizing the phase segmentation with fluorescent channels turned off and clicking "Preview Current". Adjust segmentation, cleanup and filter parameters as required.
   d. Subsequently optimize analysis masks for the Green and Red or Orange Image Channels, followed by Overlap.

      Note: Check overlap mask to be sure it is not over-estimating the number of "yellow" cells (you may need to filter or alter the both the Green and Red or Orange channel edge split).

   e. Once you are happy with the selected parameters for all channels, click "Preview All" to ensure these are appropriate for other time points or treatments selected.
   f. Once you have previewed all of the representative images and are satisfied with the parameters, complete the Launch wizard analysis by selecting the Scan Times and Wells to be analyzed, as well as assigning an analysis definition name.

2. Data Interpretation (Basic Analyzer)
   a. After the analysis job is complete, the Green/ Red or Orange/Overlap count (per image) may be plotted.

      Note: the Green and Red or Orange counts do not represent the pure populations because the cells masked as Overlap (yellow) will be included in these counts.

   b. In order to normalize count data, it is easiest to plot Object Count (per image) and export raw data into a data analysis program (e.g. Excel).
      i. To calculate the pure population of Green and Red or Orange cells, subtract the Overlap count from each count (e.g. Red or Orange Count minus Overlap Count = pure Red or Orange population).
      ii. To calculate the total cell number, take the sum of Green plus Red or Orange counts and subtract the Overlap count. This will correct for the cells that are counted twice because they are transitioning between G1/S and expressing both Green and Red or Orange.
      iii. Determine the Percent Total of Green/Red or Orange/Yellow counts by dividing the pure count by the total cell number. For example % Total Red or Orange cells = 100 * (Red or Orange Count – Overlap Count) ÷ [(Red or Orange Count + Green Count) – Overlap Count].
      iv. Cell cycle populations can be determined using normalized counts.
         1. % Red or Orange Object Count– cells in G1 phase
         2. % Green Object Count–cells in S/G2/M phase
         3. % Overlap Object Count– G1/S phase

Safety Considerations

Sartorius products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of a technically qualified individual experienced in handling lentivirus reagents. Please read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

The backbone of the lentivirus particles in this system has been modified to improve their safety and minimize their relation to the wild-type human HIV-1 virus. These modifications include:

- The lentiviral particles are replication incompetent and only carry the non-oncogenic gene of interest.
- A deletion in the 3′ LTR (U3) results in "self-inactivation" (SIN) of the lentivirus after transduction and genomic integration of the target cell (Yee et al., 1987; Yu et al., 1986; Zufferey et al., 1998). This alteration renders the lentiviral genome incapable of producing packageable virus following host integration.
- The lentiviral particles are pseudotyped with VSV-G from the Vesicular Stomatitis Virus rather than the native HIV-1 envelope (Burns et al., 1993; Emi et al., 1991; Yee et al., 1994).

Replication-defective lentiviral vectors, such as the 3rd generation vector provided in this product, are not known to cause any diseases in humans or animals. However, lentivirus particles still pose some biohazardous risk because they can transduce primary human cells and can integrate into the host cell genome thus posing some risk of insertional mutagenesis. For this reason, we highly recommend that you treat lentiviral stocks as Biosafety Level 2 (BSL-2, BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.

For more information about the BL-2 guidelines and lentivirus handling, we recommend referring to local documentation based on geography. The Essen BioScience 3rd generation HIV-based lentiviruses meet BL-2 requirements based on the criteria in the document, "Biosafety in Microbiological and Biomedical Laboratories", 5th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

Institutional Guidelines: Safety requirements for use and handling of lentiviruses may vary at individual institutions. We recommend consulting your institution's health and safety guidelines and/or officers prior to implementing the use of these reagents in your experiments.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

Product Label License

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