SARTORIUS

Product Guide

Incucyte® CytoATP Lentivirus Reagent Kit (EF1α, Puro)

Product Information

Presentation, Storage and Stability

The Incucyte® CytoATP Lentivirus Reagent Kit contains two 0.2 mL vials of 3rd generation HIV-based, VSV-G pseudotyped lentiviral particles suspended in DMEM (one vial contains CytoATP Lentivirus Reagent, and one vial contains CytoATP Non-binding Control Lentivirus Reagent). The lentivirus reagents should be stored at -80° C. When stored as described, these reagents 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 an SX5 Metabolism Optical Module					
Incucyte® CytoATP Lentivirus Reagent	puro	0.2 mL	4772	-80° C	6 months from date of receipt
Incucyte® CytoATP Non-binding Control Lentivirus Reagent	puro	0.2 mL	4772	-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

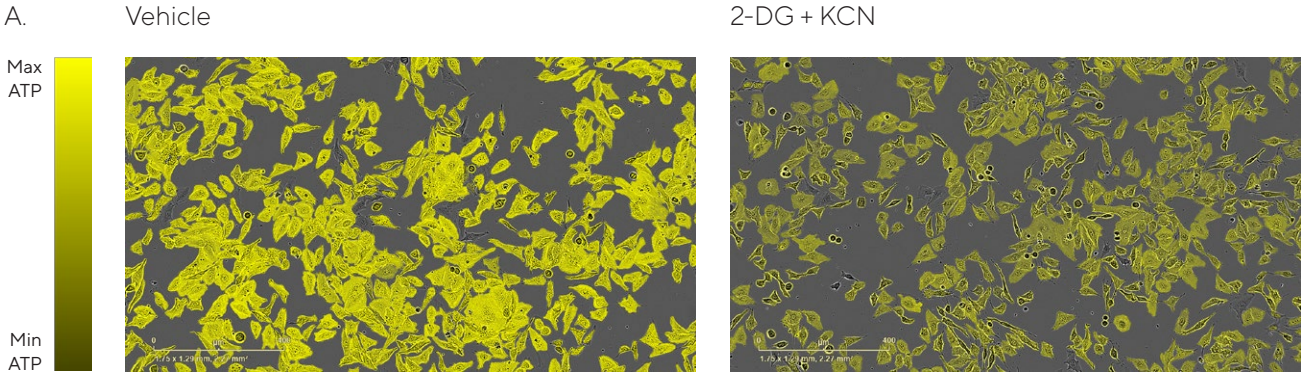
The Incucyte® CytoATP Lentivirus Reagent Kit enables efficient, non-perturbing, and homogenous labeling of mammalian cells for in vitro analysis of dynamic changes in cytoplasmic ATP. The kit contains two live-cell labeling reagents, ATP binding (CytoATP Lentivirus) and ATP non-binding control (CytoATP Non-binding Control Lentivirus). Both lentiviruses are driven by an EF-1α promotor with Puromycin selection to allow generation of cell lines or clones which stably express their respective single cassette, genetically encoded, dual fluorescent (cpmEGFP and mKOκ) indicators.

The CytoATP Lentivirus Reagent Kit has been validated for use with the Incucyte® SX5 Live-Cell Analysis System configured with an SX5 Metabolism Optical Module (Cat. No. 4820) and is not compatible with Incucyte® instruments configured with Green/Orange/NIR or Green/Red Optical Modules. The Incucyte® CytoATP Lentivirus Reagent Kit is intended for use with the Incucyte® ATP Software Module (Cat. No. 9600-0033). The SX5 Metabolism Optical Module and ATP Analysis Software utilize dual excitation and single emission fluorescence acquisition and analysis for direct measurements of ATP. The combination of this purpose-built optical module and software allows for normalized analysis of relative ATP levels independent of cell number and indicator expression level. Images of cells expressing the dual fluorescent CytoATP indicators are acquired at a fixed emission of 578 nm for both the 485 nm and 535 nm excitation wavelengths. The ratio of fluorescence intensity of those images (485X | 578M) | (535X | 578M) is used to evaluate dynamic population changes in ATP over time. This direct ratio is referred to as the Uncorrected ATP Ratio. The CytoATP Non-binding Controls enable calculation of the Corrected ATP Ratio, minimizing contribution of experimental artifacts that may affect fluorescence output and setting a limit of detection.

Recommended Use

We recommend thawing the Incucyte® CytoATP 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 for most cell types, but MOI should be optimized for 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



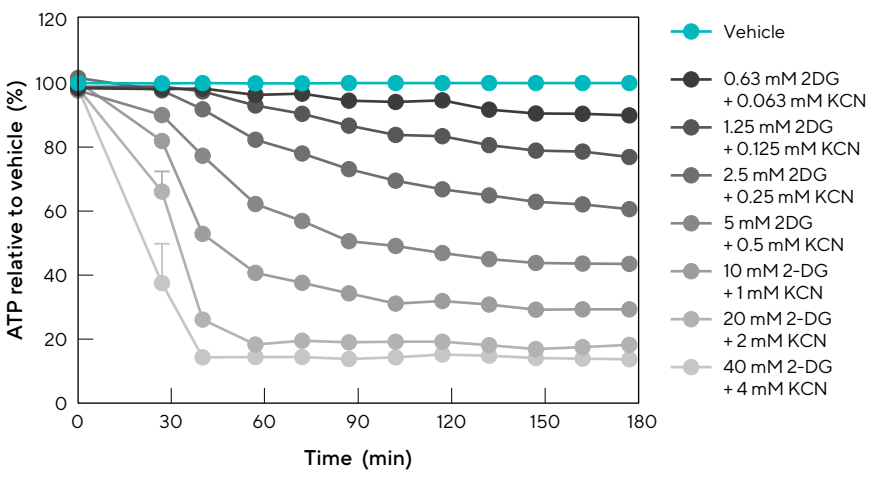
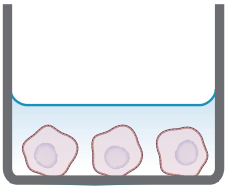


Figure 1. Visualization of ATP dynamics with Incucyte® CytoATP Lentivirus Reagents. HeLa cells stably expressing CytoATP indicators were treated with 2-Deoxy-D-glucose (2-DG) and potassium cyanide (KCN) to inhibit glycolysis and OXPHOS, respectively. Images were analyzed using the integrated Incucyte® ATP Analysis Software. (A) Color scaled ratio images provide visualization of ATP depletion in HeLa cells treated with 20 mM 2-DG + 2 mM KCN (dim yellow) compared to vehicle (bright yellow, 1 h time point). (B) Analysis demonstrates a rapid, concentration-dependent decrease in ATP following combined 2-DG and KCN treatment. Cells were treated between the first and second time points.

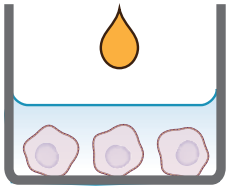
Quick Guide

1. Seed cells



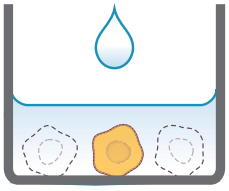
Seed cells in growth media and leave to adhere (4–24 hours). Cells should be 15–35% confluent at the time of transduction.

2. Transduce



Add CytoATP or CytoATP Non-binding Control Lentivirus diluted in media ± Polybrene. After 24 hours, replace the media with fresh growth media. Monitor expression using the Incucyte® Live-Cell Analysis System.

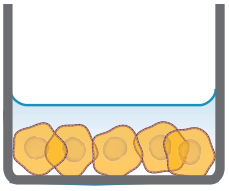
3. Apply selection



Apply Puromycin selection to derive a stable, homogenous cell population or clone that expresses the genetically encoded CytoATP Binding or Non-binding Control indicator.

Optional: Freeze cells and use for future assays.

4. Live-cell fluorescent imaging



Capture images in the presence or absence of treatments in an Incucyte® Live-Cell Analysis System configured with the Metabolism Optical Module. Analyze data using the ATP Software Module.

Protocols and Procedures

Materials

Required Materials

- Incucyte® CytoATP Lentivirus Reagent Kit (Cat. No. 4772)
- Flat bottom tissue culture plates or flasks for lentivirus infection (e.g., Corning 3516) and imaging (e.g., Corning 3595)
- Complete cell culture media for cell line of choice

Optional

- Polybrene® (Sigma H9268) to enhance lentiviral infection
- Puromycin (Thermo A1113803) for making stable cell lines

Suggested Transduction Protocol for Stable Expression in Immortalized Cell Lines

If you plan to use the CytoATP Lentivirus Reagent Kit to generate stably-expressing clones or populations, please perform Antibiotic Selection optimization first (see Optimization Protocols 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 two sets of cells in growth media of choice at a density such that they are 15–35% confluent at time of infection. Incubate plate(s) for 4–24 hours or until cells have attached.

   Note: It is necessary to generate both CytoATP (binding) and Non-binding Control-expressing cells. Non-binding Controls may only be omitted if a negligible effect of experimental treatments on Non-binding Control readout has been well established.

2. Add CytoATP Lentivirus Reagent to one set of cells and Non-binding Control Lentivirus to the second set of cells. Add lentivirus reagents at desired MOI diluted in media ± Polybrene® (a 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. Remove media and replace with fresh growth media containing the appropriate concentration of Puromycin determined from the kill curve (see "Optimization Protocols, Antibiotic Selection" below).
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).

   Note: It is recommended to allow cells to recover from Puromycin selection prior to experimentation.

Suggested Transduction Protocol Modifications for Primary Cells and Transient Assays

If you do not plan to use the Incucyte® CytoATP Lentivirus Reagent Kit to create stably expressing cells, then we recommend optimizing MOI and concentration for each cell type used (see Optimization Protocols below). Once these steps are complete, follow the "Suggested Transduction Protocol for Immortalized Cell Lines", steps 1 through 5.

Optimization Protocols Antibiotic Selection

In order to generate stable cell lines expressing CytoATP or CytoATP Non-binding Control Indicators, determine the minimum concentration of antibiotic required to efficiently eliminate non-transduced cells using a dilution series of Puromycin (typical 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).

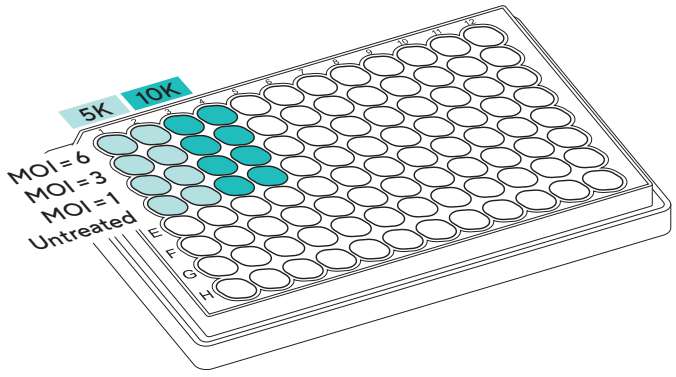
Multiplicity of Infection (MOI)

The optimal MOI for your cells can be determined empirically in a 96-well plate.

1. Plate at least two densities of cells in a 96-well plate in appropriate medium.

   Note: Passage number can have a significant effect on lentiviral transduction efficiency. Low passage cells should be used in all experiments.

2. Incubate cells overnight in a 37° C, 5% CO_2 incubator.
3. Prepare transduction media, containing lentivirus at a range of MOI ± appropriate concentration of Polybrene®.
4. Remove growth media and replace with transduction media.
5. After 24 hours, replace transduction media with growth media and return cells to incubator.
6. 48–72 hours after infection, schedule a single scan in an Incucyte® configured with an SX5 Metabolism Optical Module to evaluate the efficiency of transduction. ATP or standard (select orange channel) scan types may be used to assess expression.



Polybrene® Concentration

The cationic polymer, Polybrene®, may be used to increase the efficiency of transduction of certain cell types. Optimal Polybrene® concentration will vary depending on the cell type used (typical working concentration range for mammalian cells is 3–8 µg/mL). Note: Polybrene® can be toxic to some cell types (e.g., primary neurons).

ATP Assay Protocol

General Guidelines

- Following cell seeding, place plates at ambient temperature to ensure homogenous cell settling (15–30 minutes).
- 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.
- If using non-adherent cells, we recommend coating plates with 0.01% poly-L-ornithine solution (not supplied). For a 96-well plate, add 50 µL of 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.
- After placing the plate in the Incucyte® Live-Cell Analysis System, allow the plate to warm to 37° C prior to scanning.

Note: If the plate has significantly cooled prior to scanning, condensation may interfere with image processing. If immediate scanning is required (e.g., a test compound has a rapid effect), we recommend scheduling 2–3 consecutive scans to promote clearing of condensation.

1. Prepare cell seeding stock at an appropriate density to achieve 15–35% confluency at time of assay. The seeding density will need to be optimized for the cell line and experimental conditions used; however, we have found that 1,000 to 5,000 cells per well (10,000–50,000 cells/mL) for a 96-well plate is a reasonable starting point.

   Note: It is highly recommended to run a set of CytoATP and Non-binding Control-expressing cells in the same plate for each experimental condition (see Example Plate Map below).

2. Seed a set of cells into vessel of choice (e.g., 100 µL per well for a 96-well flat bottom microplate).
3. Allow cells to adhere for 4–20 hours at 37° C.
4. Suggested compound testing protocol: Prepare 6X concentrations of treatments and add 20 µL per treatment condition to wells containing cells in media (100 µL).

   Note: It is best practice to perform at least one baseline scan prior to any compound addition to ensure equal starting ratios between treatment conditions.

5. Place plate in Incucyte® configured with an SX5 Metabolism Optical Module and acquire images using the Incucyte® ATP Software Module.
    a. Scan Type: ATP
    b. Image channels: "Phase" is selected as default and is recommended to visualize morphology. 485X | 578M and 535X | 578M are both required for ATP analysis.
    c. Objective: 10X (recommended for adherent) or 20X (recommended for non-adherent). Scanning at 20X will generate higher quality images but will slightly increase scan time.
    d. Scan interval: determine preferred frequency of scans for your experiment (every 2 hours is a good starting point for most experimental paradigms).

Data Analysis

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

1. Select ATP Analysis Type to create a new Analysis Definition. At the Channel prompt, all three channels will be selected (Phase, 485X | 578M, and 535X | 578M). Phase analysis is optional.
2. Select representative images from both CytoATP and Non-binding Control wells (e.g., with and without treatment).
3. The fluorescence segmentation mask for this assay is based on parameters set for 535X | 578M fluorescence. The segmentation mask generated from the 535X | 578M channel is automatically used to analyze 485X | 578M.
    a. Turn on 535X | 578M and set mask based on orange objects.
    b. The Segmentation Top Hat radius feature will subtract local background from fluorescent objects within the selected radius. The radius should be chosen to reflect the size of fluorescent objects but contain sufficient background to reliably estimate background fluorescence; 60–100 µm is a useful starting point.
    c. The Segmentation Threshold feature determines the masked area of 535X | 578M fluorescence objects. Fluorescence intensity below the set threshold will not be masked. Choose a threshold which results in masking the maximum cell area without extending beyond cell borders or picking up unwanted fluorescent background.
    d. The filter feature can be helpful to avoid masking cellular debris (e.g., selecting a minimum size filter).
4. Evaluate the 535X | 578M mask and refine the parameters accordingly. Once you are happy with the parameters set, click Preview All to ensure these are appropriate for other time points or treatments selected.
5. (Optional) Set mask for the phase confluence with fluorescent channels turned off. Once you are satisfied with the parameters set, click "Preview All" to ensure these are appropriate for the other time points or treatments selected.
6. Complete the Launch Wizard analysis to select the Scan Times and wells to be analyzed. You will also be prompted to assign an analysis definition name. Note that if your experiment is in progress you will have the option to check "Analyze Future Scans" to perform real-time analysis.

Data Interpretation

1. When visualizing the acquired images, the CytoATP and Non-binding control signal will appear as 485X | 578M fluorescence (green) overlaid on 535X | 578M fluorescence (orange).
2. Once the analysis job is completed, ATP Ratio Images color-coded to denote relative ATP measurement will appear. Images are scaled from bright yellow (highest ATP) to dark yellow (lowest ATP) based on the ratio of 485X | 578M and 535X | 578M fluorescence output and are inclusive only of those pixels contained in the segmentation mask (see example data section above).

    Note: Non-binding Control values represent the lower limit of ATP detection. The Corrected ATP Ratio metric utilizes Non-binding Control measurements to account for contributions of experimental treatments or artifacts that may alter the ATP ratio.

3. In order to link the CytoATP and Non-binding Control wells, it is necessary to select the appropriate "Indicator" designations in the specialized ATP plate map editor (see example below). If this is not done, Corrected ATP Ratio images and metrics will not be enabled (only Uncorrected Ratio).

    Note that all other information (Compounds, Cells, and Growth Conditions) must be identical between corresponding wells in order for the software to perform the Corrected ATP Ratio analysis.

4. When graphing data, we recommend using the Corrected ATP Ratio metric and plotting only the wells containing CytoATP-expressing cells.

    Note: The uncorrected ATP Ratio metric will generate data directly from either the CytoATP or Non-binding Control indicator wells. This metric does not link the control indicator to the CytoATP readout for correction. This method may be acceptable for use once it is confirmed that treatments do not affect Non-binding Control readouts (e.g., feeding-induced changes in ATP). Not recommended for compound treatments that induce toxicity.

Optional Protocol for Mitochondrial Toxicity Assay (Modified from Marroquin *et al.*, 2007)

Base Media for Glucose and Galactose media:

- DMEM no Glucose (Life Tech 11966025)
- 1 mM Sodium Pyruvate (Life Tech 11360070)
- 5 mM HEPES (Life Tech 15630080)
- 10% FBS (Sigma F2442)
- 1% GlutaMax (Life Tech 35050061)

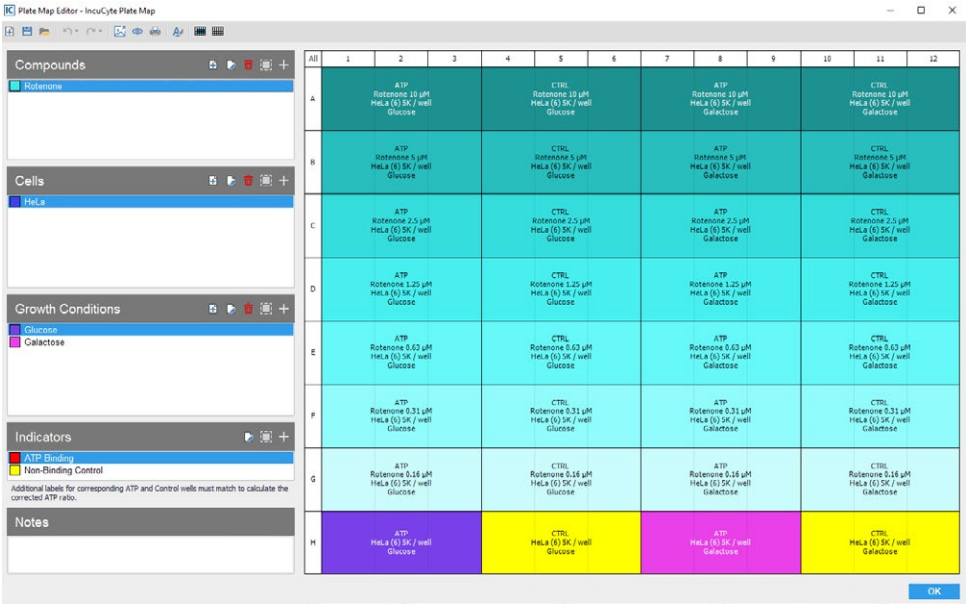
Supplement base formulation with 25 mM Glucose (Sigma G8270) or 10 mM Galactose (Sigma G0750).

1. Generate cell lines expressing CytoATP and Non-binding Control Indicators as described in the Suggested Transduction Protocol for Immortalized Cell Lines section above.
2. Prepare Base media (recipe above).
3. Split media in half, and add 25 mM glucose to one half and 10 mM galactose to the other half to complete the media formulations needed for this assay.
4. Split CytoATP and Non-binding Control cell lines into two flasks each (4 flasks in total), maintaining cells in preferred growth media of choice overnight.
5. Following overnight incubation, remove and replace media to generate a pair of Glucose and Galactose-grown cells for both CytoATP and Non-binding Control-expressing cell lines.
6. Split cells at sufficiently low density in Glucose or Galactose media to maintain 3–4 days between passages. Allow cell lines to acclimate in their respective media for at least two more passages following the switch.
7. Once cells are acclimated, plate a set of four cell lines for each assay: Glucose-grown CytoATP, Glucose-grown Non-binding Control, Galactose-grown CytoATP, and Galactose-grown Non-binding Control (see ATP Assay Protocol above). Suggested seeding is shown in Example Plate Map below.
8. Acquire images and analyze according to the ATP Assay Protocol outlined above.
   a. We recommend frequent scanning (e.g., every 20 min) at the beginning of the assay to catch rapid changes in ATP levels and separation between concentrations of potent compounds that may converge at later time points.
   b. You may scan less frequently (e.g., every 1–4 hours) for the duration of the experiment to monitor for recovery of ATP levels.

Note: Use the "Add Scans at Intervals" function to schedule the initial set of scans (e.g., every 20 minutes for 3 hours), then right click on the last scan to "Add Scans at Intervals" again for the second set of scans (e.g., every 2 hours for the remaining 21 hours of the recurring 24-hour pattern).

Example Plate Map

Designate wells containing cells expressing the CytoATP or Non-Binding Control indicators by applying labels from the pre-filled options in the Indicator box. In order to calculate the Corrected ATP Ratio (recommended), all additional labels must be identical between corresponding CytoATP and Non-binding Control wells.



Reference

Marroquin LD, *et al.* **Circumventing the Crabtree Effect: Replacing Media Glucose with Galactose Increases Susceptibility of HepG2 Cells to Mitochondrial Toxicants.** *Toxicol. Sci.*, 97(2); 539-547 (2007)

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 third 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.

Licenses and Warranty

Incucyte® CytoATP Lentivirus Kit contains proprietary nucleic acid(s) coding for proprietary fluorescent protein(s) and binding regions being, including its derivatives or modifications, the subject of pending patent applications and/or patents owned by Medical & Biological Laboratories Co., Ltd and Kyoto University. The purchase of Essen BioScience® products incorporating these nucleic acids coding for proprietary proteins conveys to the buyer the non-transferable right to use the reagent only for research conducted by the buyer. No rights are conveyed to modify or clone the gene encoding fluorescent protein contained in this product.

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