SARTORIUS

Product Guide

Incucyte® Kinase Akt Green/Red Lentivirus (EF1α, Puro)

Product Information

Presentation, Storage and Stability
The Incucyte® Kinase Akt Green/Red Lentivirus is supplied as one vial containing 0.2 mL 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, this reagent will be stable for at least 6 months from the date of receipt.

Product Name	Selection	Amount	Cat. No.	Storage	Stability
Compatible with Incucyte® Live-Cell Analysis Systems configured with a Green/Red Optical Module					
Incucyte® Kinase Akt Green/Red Lentivirus	puro	0.2 mL	BA-04868	-80° C	6 months from date of receipt

Background

The Incucyte® Kinase Akt Green/Red Lentivirus enables efficient, non-perturbing, and homogenous labeling of mammalian cells for *in vitro* analysis of dynamic changes in Akt kinase activity. The lentivirus reagent is a single-cassette, genetically encoded sensor based on a green fluorescent protein (TagGFP2)-tagged Akt substrate whose subcellular localization is phosphorylation-dependent, and a red fluorescent protein (mKate2) nuclear marker to denote the nuclear/cytoplasmic boundary. Upon phosphorylation by Akt, the green fluorescent sensor will translocate from the nucleus to the cytoplasm. Inhibition of Akt will prevent phosphorylation of the sensor and cause it to be retained in the nucleus. It is driven by an EF-1α promotor with puromycin selection to allow generation of cell lines or clones which stably express the genetically encoded dual fluorescent (TagGFP2 and mKate2) indicator.

The Incucyte® Kinase Akt Green/Red Lentivirus has been validated for use with the Incucyte® SX5, S3, or SX1 Live-Cell Analysis Systems configured with a Green/Red Optical Module. It is not compatible with Incucyte instruments configured with a Green/Orange/NIR or an Orange/NIR Optical Module.

Recommended Use

We recommend thawing the Incucyte® Kinase Akt Lentivirus on ice. Avoid repeated freeze/thaw cycles as this can impair transduction efficiency. The lentivirus reagent 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 (see "Optimization Protocols" on page 3). The cationic polymer Polybrene may be added to enhance transduction efficiency. Post infection, stable cell lines or clones may be generated using puromycin selection.

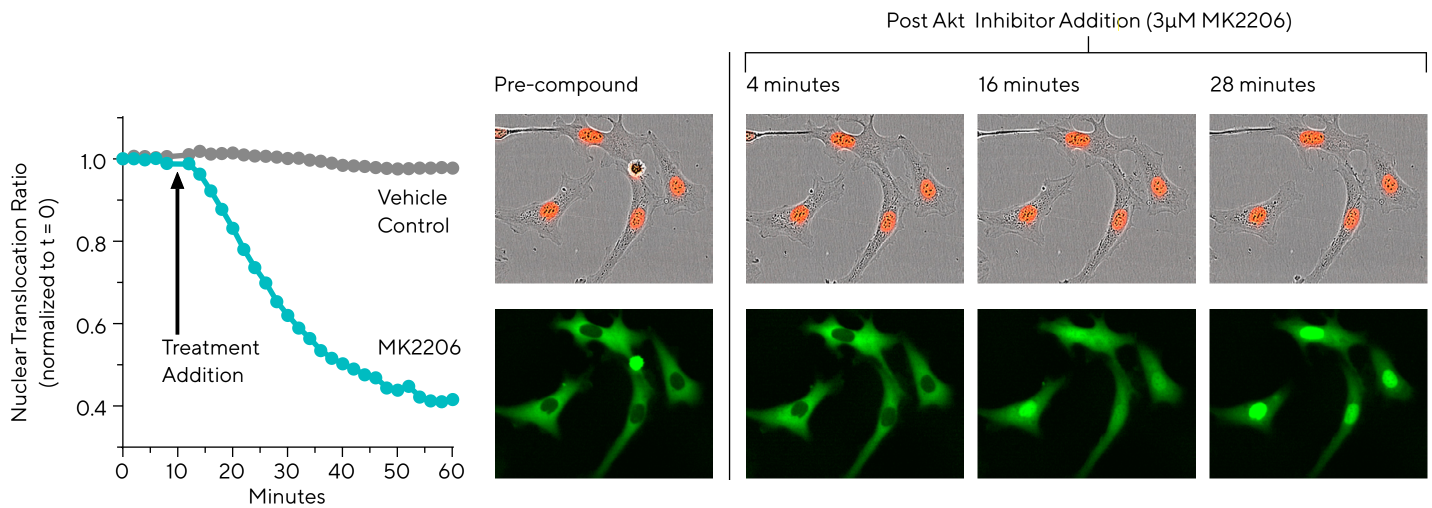
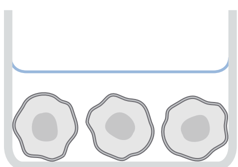


Figure 1

Note. SK-OV-3 cells stably expressing the Incucyte® Kinase Akt Green/Red indicator were treated with Akt selective inhibitor MK2206, resulting in translocation of the green fluorescent sensor from the cytoplasm to the nucleus. The kinetic graph on the left shows a decrease in the Nuclear Translocation Ratio over time, indicating Akt inhibition. The image panel shows the phase and red fluorescence image channels on the top and the green fluorescence channel on the bottom. Movement of the green fluorescent sensor from the cytoplasm to the nucleus can be seen over 28 minutes, while localization of the red fluorescent nuclear marker does not change.

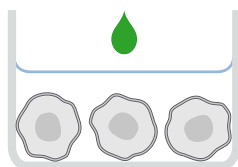
Quick Guide

1. Seed Cells



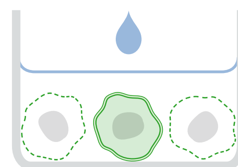
Seed cells in growth media and leave to adhere (4-24 hours). Cells should be 15-35% confluent at the time of transduction.

2. Transduce



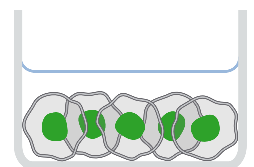
Add Kinase Akt Lentivirus diluted in media ± Polybrene®. After 24 hours, replace the media with fresh growth media. Monitor expression using the Incucyte® Live-Cell Analysis System.

3. Apply Selection



Apply puromycin selection to derive a stable, homogenous cell population or clone that expresses the genetically encoded Kinase Akt Lentivirus (Optional: Freeze cells and use for future assays).

4. Live-cell Fluorescent Imaging



Capture images in the presence of treatments in an Incucyte® Live-Cell Analysis System with a Green/Red Optical Module. Analyze using integrated software.

Protocols and Procedures

Required Materials

- Incucyte® Kinase Akt Lentivirus Reagent (Cat. No. BA-04868)
- 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 planning to use the Incucyte® Kinase Akt Lentivirus to generate stably-expressing clones or populations, please perform antibiotic selection optimization first (see "Optimization Protocols"). 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(s) for 4-24 hours or until cells have attached.
2. Add Incucyte® Kinase Akt Lentivirus 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. Remove media and replace with fresh growth media containing the appropriate concentration of puromycin determined from the kill curve (see "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).

   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 not planning to use the Incucyte® Kinase Akt Lentivirus to create stably expressing cells, then we recommend optimizing MOI and Polybrene® 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 Incucyte® Kinase Akt Lentivirus, 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).

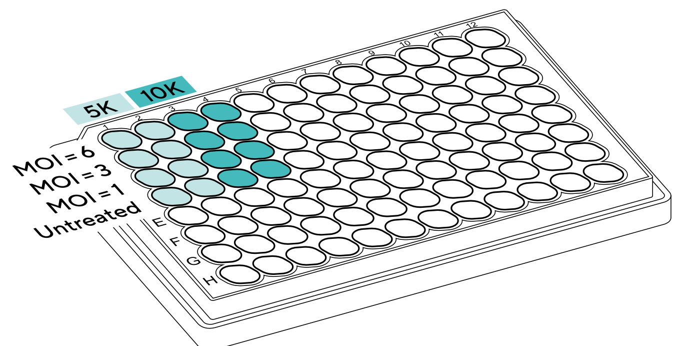
Multiplicity of Infection (MOI)

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 a Green/Red Optical Module. Both green and red fluorescence can 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).

Kinase Akt Assay Protocol

General Guidelines

- The Incucyte® Kinase Akt Lentivirus is designed for use in adherent cell types with a well-defined 2-dimensional cytoplasm. For this reason, it is not compatible with non-adherent or semi-adherent cell types.
- 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.
- 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: As cells approach confluence, the Nuclear Translocation Ratio in vehicle-treated wells may change due to factors unrelated to Akt activity, such as cell death in regions of high cell density.
2. Seed cells into vessel of choice (e.g., 100 µL per well for a 96-well flat bottom microplate).
3. Allow cells to adhere overnight at 37° C.

   NOTE: It is important to allow the cells to fully adhere and attain typical morphology before making measurements of kinase activity.
4. If interested in testing modulators of Akt activity, prepare treatments in complete growth media and add to wells at desired final assay concentration.

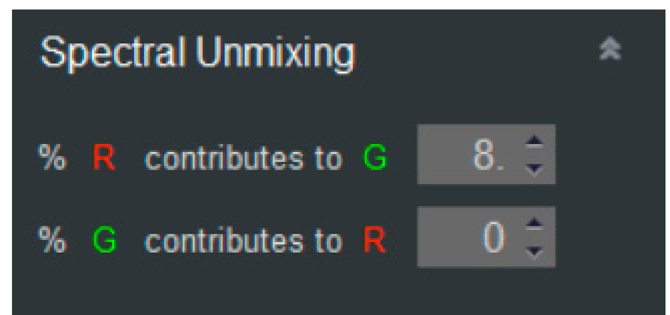
   NOTE: Changes in Akt activity after compound treatments are often rapid. It is recommended to perform at least one baseline scan prior to any compound addition to accurately assess treatment-induced changes in the assay readout.
5. Place plate in Incucyte® configured with a Green/Red Optical Module and acquire images.
   a. Scan Type: Standard
   b. Image channels: "Phase" is selected as default and is recommended to visualize morphology. Green and Red channels are required for visualization and quantification of the Kinase Akt Lentivirus.
   c. Objective: 10X or 20X. 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 1 hour is a good starting point for most experimental paradigms.

   NOTE: If rapid changes in Akt activity are expected, we recommend frequent scanning (e.g. every 15 minutes) at the beginning of the assay to catch these changes.

Data Analysis

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

1. Prior to creating an analysis definition, Spectral Unmixing must be set to 8% Red contributes to Green. This will remove the small amount of green fluorescence contributed by the red fluorescent protein.



2. Select Basic Analyzer Analysis Type to create a new Analysis Definition. At the Channel prompt, all three channels will be selected (Phase, Green, and Red). Overlap must also be selected. Set the overlap slider to "yes". Phase analysis is optional.

   NOTE: Overlap is necessary to quantify results obtained using the Incucyte® Kinase Akt Lentivirus. The Nuclear Translocation Ratio (NTR) metric is only available when overlap has been selected. Select representative images from wells with and without treatments.
3. The fluorescence segmentation mask for this assay is based on parameters set individually for Green and Red fluorescence.
   a. The Surface Fit Segmentation feature will subtract local background from fluorescent objects. No additional settings are required for using Surface Fit.
4. Green Fluorescence Segmentation

   The green fluorescence will be located primarily in the nucleus or the cytoplasm depending on Akt activity levels. It is important to mask all green fluorescence within the cell. This includes low intensity areas in the cytoplasm or nucleus. Choose a threshold that results in masking the maximum cell area without extending beyond cell borders or picking up unwanted fluorescent

background. Thresholds below the default 2 GCU are often necessary.

NOTE: Edge Split should be left on. Edge Sensitivity should be decreased to create uniform segmentation throughout the cells and prevent fragmentation of the masked area. A value of -40 is a good starting point.

5. Red Fluorescence Segmentation
The red fluorescence will be constrained to the nucleus regardless of treatments or Akt activity levels. The red fluorescence is used to define the nuclear/cytoplasmic boundary. Choose a threshold that results in masking the entire area of red fluorescence within the nucleus of the cells without picking up unwanted fluorescent background.

NOTE: The Green + Red Mask is generated automatically from overlap of the Green and Red segmentation masks. The Overlap mask should look very similar to the Red channel mask, in which case, no further adjustments are necessary.

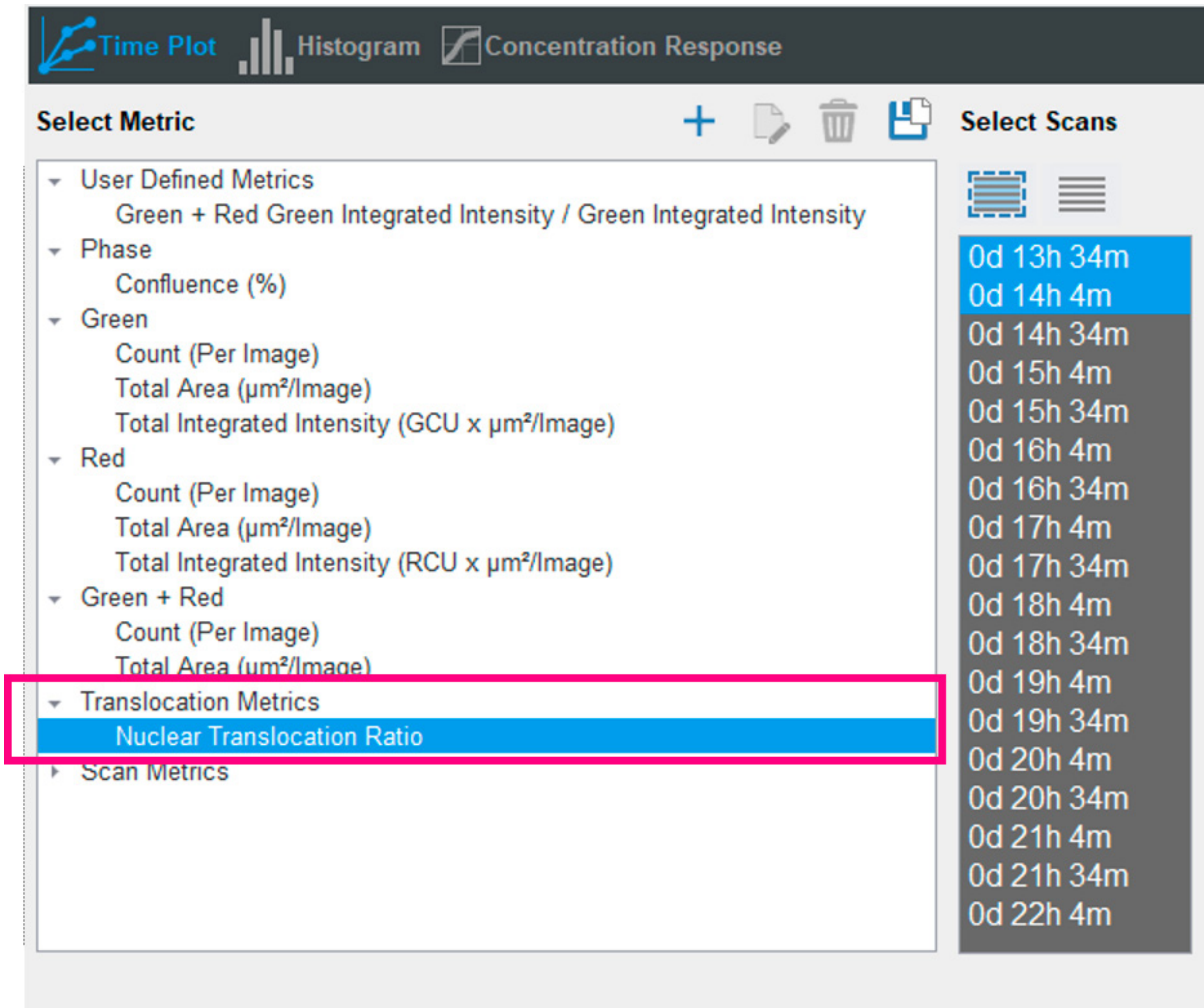
6. Evaluate the fluorescence masks and refine the parameters accordingly. Once you are happy with the parameters set, click Preview All to ensure these are appropriate for other time points or treatments selected.

7. (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.

8. 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. Once the analysis job is completed, you can view images with the Green and Red Object masks, as well as the Green + Red Overlap mask.
2. The Incucyte® Kinase Akt indicator response can be evaluated using the integrated Nuclear Translocation Ratio (NTR) metric found in the graphing window under the Translocation Metrics drop down menu as shown below.



3. The NTR quantifies shuttling of the sensor between the cytoplasm and the nucleus by measuring the green fluorescence coming from the nuclear area (as defined by the overlap mask) divided by that coming from the total cell area (as defined by the green segmentation mask). The formula for the NTR metric is below.

$$1 - \left(\frac{\textbf{Red+Green Overlap Channel:}\ \text{Green Integrated Intensity per Image}}{\textbf{Green Channel:}\ \text{Green Integrated Intensity per Image}} \right)$$

NOTE: The "one minus" in the formula is used to invert the (nuclear / total) ratio value. This makes the metric more intuitive in that a decrease or increase in Akt activity is reflected in a corresponding change in the same direction in the NTR metric.

4. When Akt activity is inhibited, the green fluorescent sensor moves from the cytoplasm to the nucleus, which causes a corresponding decrease in the NTR (Figure 2). When activation of Akt occurs, the green fluorescent sensor moves from the nucleus to the cytoplasm, which causes a corresponding increase in the NTR (Figure 3).
NOTE: Quantification of the Incucyte® Kinase Akt requires accurate segmentation of the cytoplasm and nucleus. Significant changes in the morphology of the cells can have an impact on the Nuclear Translocation Ratio metric. If compound treatments cause the cells to round up and begin to die, this will cause an artificial decrease of the NTR that may not be caused by inhibition of Akt. We recommend using the Red Object Count to identify compound concentrations that impact cell proliferation. Changes in morphology can then be confirmed by inspection of the images. Scans that contain cells with an altered morphology should be excluded from the analysis.

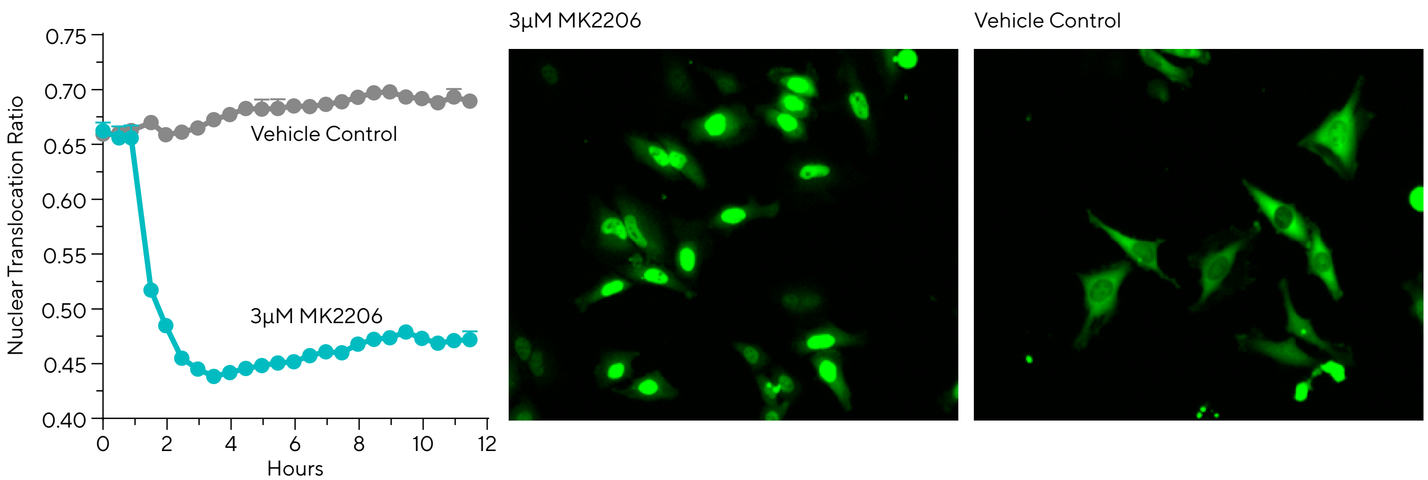


Figure 2

Inhibition of Akt in HeLa Cells Expressing the Incucyte® Kinase Akt Indicator

Note. The selective Akt inhibitor MK2206 was added to cells in the presence of 10% serum, causing the translocation of the sensor from the cytoplasm to the nucleus. The NTR metric shows a rapid decrease over time, indicating inhibition of Akt activity.

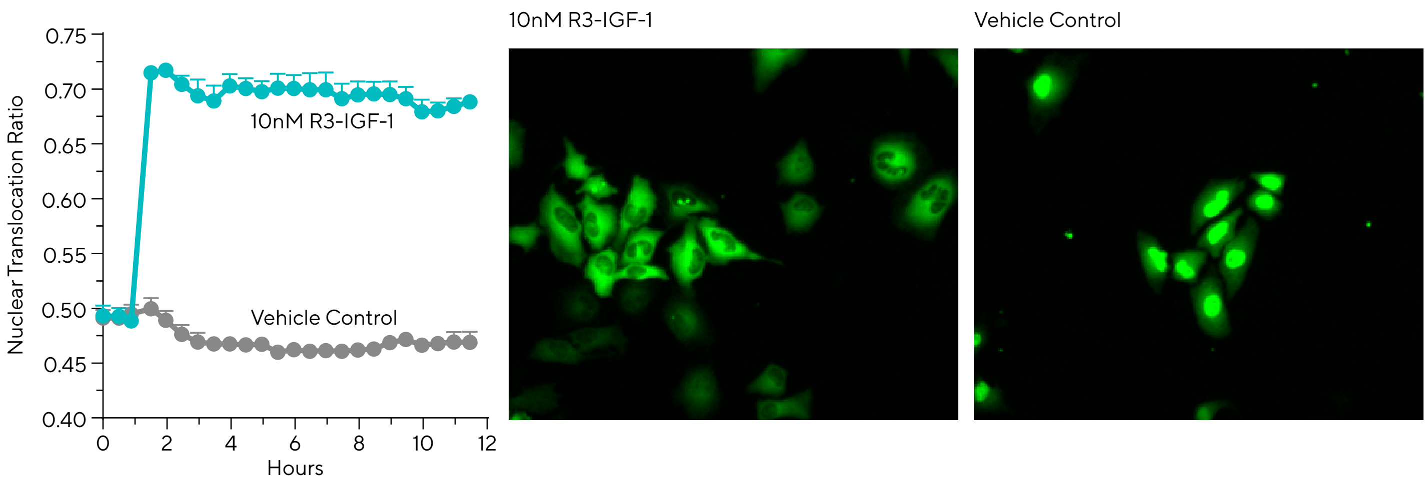


Figure 3

Activation of Akt in HeLa Cells Expressing the Incucyte® Kinase Akt Indicator

Note. Shifting the cells from 10% serum to serum free media results in a decrease in basal Akt activity and movement of the sensor to the nucleus (data not shown). Addition of a recombinant analog of insulin-like growth factor (R3 IGF-1) in the absence of serum activates Akt and causes the translocation of the sensor from the nucleus to the cytoplasm. The NTR metric increases rapidly, reflecting the activation of Akt.

Safety Considerations

Sartorius products are high-quality reagents and materials intended for research purposes only; not for therapeutic or diagnostic use.

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 (U_3) 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 envelope is psueudotyped with the VSV-G gene from 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 http://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® Kinase Akt Lentivirus contains proprietary nucleic acid(s) coding for proprietary fluorescent protein(s) the subject of pending patent applications and/or patents owned by Evrogen. The purchase of Sartorius 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.

For additional product or technical information, please e-mail us at **AskAScientist@sartorius.com**
or visit our website at **www.sartorius.com/incucyte**

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