SARTORIUS

Product Guide

Incucyte® Annexin V Dyes

For Detection of Phosphatidylserine Exposure

Product Information

Presentation, Storage and Stability

Incucyte® Annexin V Dyes are supplied as single vial lyophilized solids, with each vial providing sufficient quantity capable of performing 100 tests (1 test = 1 well of a 96-well microtiter plate). The lyophilized solid should be stored at -20° C and once solubilized, the solution should be stored at +4° C and protected from light.

Product Name	Cat. No.	Ex. Max	Ex. Min	Amount	Storage	Stability
Compatible with Incucyte® Live-Cell Analysis Systems configured with Green | Orange | NIR or Green | Red Optical Module						
Incucyte® Annexin V Green Dye	4642	490 nm	515 nm	100 tests	-20° C	Lyophilized – 2 years Reconstituted – 1 week
Compatible with Incucyte® Live-Cell Analysis Systems configured with Green | Red Optical Module						
Incucyte® Annexin V Red Dye	4641	593 nm	614 nm	100 tests	-20° C	Lyophilized – 2 years Reconstituted – 1 week
Compatible with Incucyte® Live-Cell Analysis Systems configured with Green | Orange | NIR or Orange | NIR Optical Module						
Incucyte® Annexin V Orange Dye	4759	562 nm	584 nm	100 tests	-20° C	Lyophilized – 2 years Reconstituted – 1 week
Incucyte® Annexin V NIR Dye	4768	662 nm	682 nm	100 tests	-20° C	Lyophilized – 2 years Reconstituted – 1 week

Background

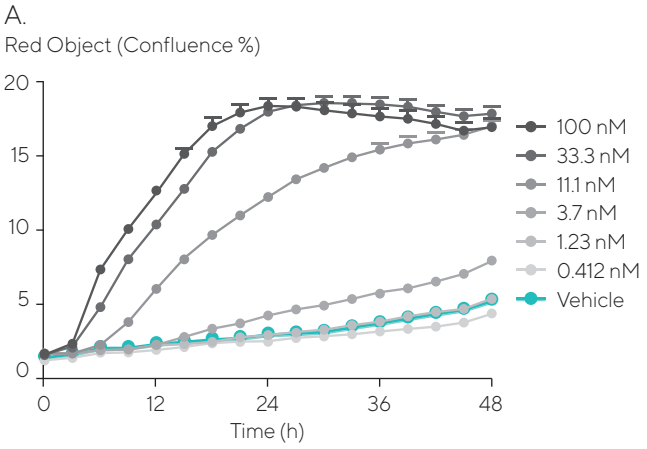
The Incucyte® Annexin V Dyes are specially formulated, highly-selective cyanine-based fluorescent dyes ideally suited for a simple mix-and-read, real-time quantitative assay of apoptosis. Addition of the Incucyte® Annexin V Dyes to normal healthy cells is non-perturbing to cell growth or morphology and yields little or no intrinsic fluorescent signal. Once cells become apoptotic, plasma membrane phosphatidylserine (PS) asymmetry is lost leading to exposure of PS to the extracellular surface and binding of the Incucyte® Annexin V Dye, yielding a bright and photostable fluorescent signal. With the Incucyte® integrated analysis software, fluorescent objects can be quantified and background fluorescence is minimized.

These pre-aliquoted reagents have been specially formulated and validated for use with the Incucyte® Live-Cell Analysis System and enable real-time monitoring of cell membrane integrity and apoptosis in response to pharmacological, biological, or other factors in mono- or complex co-cultures. Furthermore, the Incucyte® Annexin V Dyes can be combined with the Incucyte® Confluence Metric, Incucyte® Caspase-3/7 Dyes, Incucyte® Nuclight Nuclear Labeling Reagents or Incucyte® Cytotox Dyes for multiplexed measurements of apoptosis, cell proliferation (label-free or fluorescently labeled) or cytotoxicity in every assay well. Additionally, the Incucyte® Annexin V Dye can be combined with the Incucyte® Cell Cycle Lentivirus for multiplexed read outs of cell function.

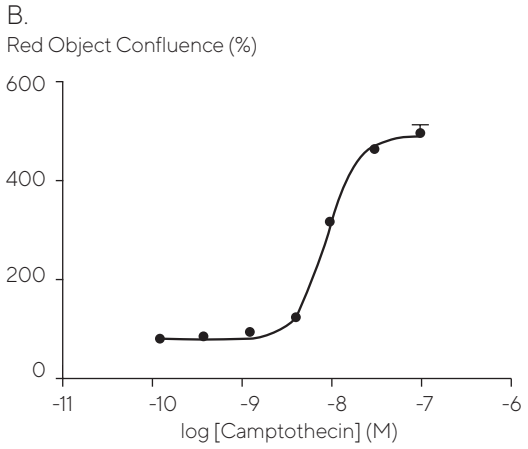
Recommended Use

We recommend solubilizing Incucyte® Annexin V Dyes by adding 100 µL of full media or PBS. The reagents may then be diluted in full media containing at least 1 mM $CaCl_2$ for direct addition to cells seeded in a 96-well plate to yield a final dilution of 1:200. For Incucyte® Annexin V NIR Dye, further optimization may be required to account for spectral unmixing during multiplexing experiments (see "Multiplexing with Annexin V NIR Dye" section). When used in an Incucyte® Live-Cell Analysis System, we recommend data collection every 2–3 hours.

Example Data



Time-course for the effects of camptothecin on Jurkat cell death (Red Object Confluence (%) presented as the mean ± SEM, n = 3 wells).

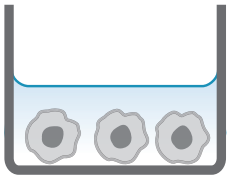


Concentration response curve to camptothecin. Area under the curve (AUC) values have been determined from the time-course shown in panel A (0–36 hours) and are presented as the mean ± SEM, n = 3 wells. Average AUC values were used to calculate pIC_{50} values (camptothecin pIC_{50} = 8.01)

Figure 1. Concentration- and time-dependent PS binding by Incucyte® Annexin V Red Dye following addition to Jurkat human T cell leukemia cells treated with camptothecin (A). Concentration response curve to camptothecin (B).

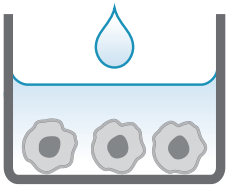
Quick Guide

1. Seed cells



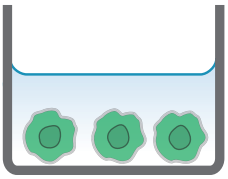
Seed cells (100 µL/well) into a 96-well plate and incubate overnight.

2. Prepare apoptosis reagent and treat cells



Prepare the desired treatments at 1X in medium containing Incucyte® Annexin V Dye and add treatment. Keep plate at ambient temperature for 15 minutes.

3. Live-cell fluorescent analysis



Capture images every 2–3 hours (20X or 10X) in the Incucyte® Live-Cell Analysis System for 24–120 hours. Analyze using integrated software.

Protocols and Procedures

Materials

- Incucyte® Annexin V Dye
- Flat bottom tissue culture plate (e.g., Corning Cat. No. 3595 , TPP Cat. No. 92096 for neuronal cell health)
- 0.01% Poly-L-ornithine solution (Sigma Cat. No. P4957)
  - optional, for non-adherent cells
- Fibronectin (Sigma Cat. No. A7906)
  - optional, for non-adherent cells

General Guidelines

- 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 (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.
- 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 monitoring apoptosis in primary neuronal cultures, we recommend the use of Incucyte® Annexin V Orange, Annexin V Red, or Annexin V NIR Dye to eliminate the risk of cell perturbation when using the green channel excitation in these sensitive cell types.

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, such that by Day 1 the cell confluence is approximately 30%. The seeding density will need to be optimized for the cell line used. We have found that 1,000 to 5,000 cells per well (10,000 – 50,000 cells/mL seeding stock) are reasonable starting points.

   Note: Monitor cell growth using the Incucyte® Live-Cell Analysis System to capture phase contrast images every 2 hours and analyze using the integrated confluence algorithm.

Prepare Apoptosis Reagent and Treat Cells

2. Solubilize Annexin V Dyes by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:200. Additional optimization to determine the proper dilution for the Incucyte® Annexin V NIR Dye may be required for multiplexing experiments (refer to "Multiplexing with Annexin V NIR Dye" section).

   Note: All treatments will be performed in this medium containing the Annexin V Dye, thus prepare a volume that will accommodate all treatment conditions. The volumes | dilutions added to cells may be varied. A volume of 100 µL per well is generally sufficient for the duration of the assay.

3. Remove the cell plate from the incubator and aspirate off culture medium.
4. Add treatments and controls to the appropriate wells of the 96-well plate.

Live-Cell Imaging

5. Place the cell plate into the Incucyte® Live-Cell Analysis System to monitor apoptosis using the appropriate fluorescent channel.
   a. Objective: 10X or 20X
   b. Channel selection: Phase Contrast and Green | Red | Orange | NIR (depending on apoptosis reagent used)
   c. Scan type: Standard (2-4 images per well)
   d. Scan interval: Typically, every 2 hours, until your experiment is complete

Non-Adherent Cell Line Protocol

Coat Plate

1. Prepare recommended coating matrix of either 0.01% poly-L-ornithine solution (Sigma Cat. No. P4957) or 5 µg/mL fibronectin (Sigma Cat. No. A7906) diluted in 0.1% BSA.
2. Aliquot 50 µL of coating matrix per well and incubate plate for 1 hour at ambient temperature.
3. Remove solution from wells and allow plates to dry for 30-60 minutes prior to cell addition.

Prepare Apoptosis Reagent and Treat Cells

4. Solubilize Annexin V Dyes by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:200 solution to ensure even mixture of reagent. Additional optimization to determine the proper dilution for the Incucyte® Annexin V NIR Dye may be required for multiplexing experiments (refer to "Multiplexing with Annexin V NIR Dye" section).

   Note: All treatments and cell seeding stocks will be prepared in this medium containing the Annexin V Dye, thus prepare a volume that will accommodate all treatment conditions. The volumes | dilutions added to cells may be varied. A volume of 200 µL per well is generally sufficient for the duration of the assay.

5. Prepare cell treatments at 2X final assay concentration in enough cell culture medium containing the Annexin V Dye to achieve a volume of 100 µL per well.

Seed Cells and Add Treatments

6. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate in medium containing the Annexin V Dye. The seeding density will need to be optimized for the cell line used. We have found that 5,000 to 25,000 cells per well (50,000–250,000 cells/mL seeding stock) are reasonable starting points.
7. Immediately add treatments and controls to appropriate wells of the 96-well plate containing cells.
8. Triturate to mix the treatments with cells evenly.

Live-Cell Imaging

9. Place the cell plate into the Incucyte® Live-Cell Analysis System to monitor apoptosis using the appropriate fluorescent channel.
   a. Objective: 10X or 20X
   b. Channel selection: Phase Contrast and Green | Red | Orange | NIR (depending on apoptosis reagent used)
   c. Scan type: Standard (2–4 images per well)
   d. Scan interval: Typically, every 2 hours, until your experiment is complete

Optional Apoptotic Analysis

An apoptotic index can be calculated on Incucyte® Live-Cell Analysis System using the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031). This enables individual cell identification and subsequent classification into subpopulations based on properties including fluorescence intensity. These subpopulations can then be expressed as a percentage of the total population to generate the cytotoxic index. To use this module, the following settings should be used:

a. Scan type: Standard | Adherent Cell-by-Cell or Non-Adherent Cell-by-Cell
b. Objective: 10X (for adherent cells) or 20X (for non-adherent cells).

For further details of this analysis module and its application see **www.essenbioscience.com/cell-by-cell**.

Multiplexing Optimization

When multiplexing with multiple fluorescent reagents, spectral unmixing may be required to account for signal that has been contributed from one of the given channels. Spectral unmixing values must be applied prior to running an analysis job.

Annexin V Green: No spectral unmixing required
Annexin V Red: 6-8% recommended to remove Red contributing to Green
Annexin V Orange: 5-7% recommended to remove Orange contributing to Green
Annexin V NIR: 1-2% recommended to remove NIR contributing to Orange. In some cases, Orange bleed through signal may be still be high, at which point additional optimization may be required to determine ideal dilution of Annexin V NIR Reagent

Multiplexing with Annexin V NIR Dye

When multiplexing Orange and NIR fluorescent reagents, it is possible for the NIR fluorescence to bleed into the Orange channel. Spectral unmixing can be used within the software to remove this bleed through signal.

Note: When multiplexing Annexin V NIR with green and orange fluorescent reagents in the Incucyte® SX5 Live-Cell Analysis System configured with a Green | Orange | NIR Optical Module, there is no contribution of Annexin V NIR signal to the green channel. Spectral unmixing will only need to be applied to remove Annexin V NIR contribution to the orange channel.

When setting up a multiplexed experiment for the first time, including wells containing only the Annexin V NIR Dye is recommended. For the Annexin V NIR Dye, 2% NIR should be removed from Orange using the spectral unmixing tool as shown in Figure 2.

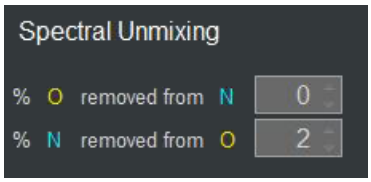


Figure 2. 2% spectral unmixing of NIR removed from Orange

There should be minimal orange fluorescence observed in the Annexin V NIR only control wells after spectral unmixing is applied.

In some cases, the Annexin V NIR signal may have a very high intensity and still be visible in the Orange channel, even after applying spectral unmixing. In this case, it may be necessary to dilute the reagent for use in a multiplex assay. The protocol below can be used to determine the optimal Annexin V NIR Dye concentration.

Annexin V NIR Concentration Optimization for Multiplex Assays

When optimizing Annexin V NIR concentration for multiplexing, we recommend performing a serial dilution of the Annexin V NIR concentration independently in order to assess the effectiveness of spectral unmixing (Figure 3).

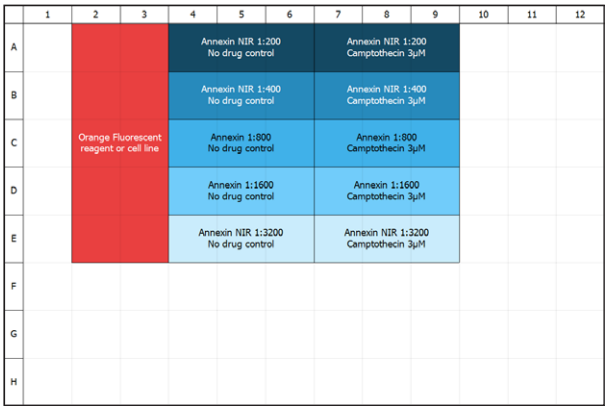


Figure 3. Plate map for optimization assay to determine optimal Annexin NIR concentration

A high concentration of a positive control compound, such as Camptothecin, should be used to induce cell death and ensure that a robust Annexin V NIR signal is observed.

Wells containing only the orange reagent or cell line to be multiplexed should be included. This allows the use of vessel autoscaling to compare the intensity of Annexin V NIR bleed through to the desired orange signal.

Example experimental procedures:

- Begin by plating 100 µL of cell suspension into Columns 2-9 as shown in the plate map shown in Figure 3.
- Using a separate compound microplate, prepare reagent(s) and compound(s) to be added to cells. Add orange fluorescent reagent or cell lines of choice to Columns 2-3 only.
- Solubilize Annexin V NIR in 100 µL of media, then dilute 1:50 to a volume of 5 mL.
- Add 120 µL of Annexin V NIR solution into Wells A4 to A9 of the compound microplate.
- Add 60 µL of media into Rows B to E in Columns 4-9 and perform a 2-fold serial dilution by transferring 60 µL down the compound plate.
- Add 60 µL of 12 µM Camptothecin (or another positive control compound) into Columns 7-9. Add 60 µL of media to Columns 4-6 as a negative control.
- Transfer 100 µL from the compound plate to the cell plate.
- Add sufficient media to orange fluorescent wells to reach a total volume of 200 µL.

Determining the Optimal Concentration

The optimal Annexin V NIR concentration for multiplexing with an orange fluorescent reagent is a concentration where the fluorescent signal is bright enough to be masked in the NIR channel while any bleed through into the Orange channel is sufficiently removed by spectral unmixing.

To determine this concentration:

- Identify the time point of the maximal Annexin V NIR response.
- View this time point in the vessel view and set color autoscale to “vessel”.
- Deselect the “Phase” and “NIR” channels to view the Orange channel only and set the spectral unmixing to remove 2% NIR from Orange.
- Select the concentration that shows minimal bleed through into the Orange channel after spectral unmixing is applied. Orange fluorescence should appear very dim or absent when scaled with the orange fluorophore wells on the plate.
- Finally, turn on the NIR channel and ensure that the intensity of the Annexin V NIR signal is bright enough to be identified and masked at the selected concentration.

In the example shown in Figure 4, HT-1080 cells were treated with Camptothecin at the suggested dilution range of Annexin NIR. The images were taken from the peak response at 24 hours. At the 1:200 concentration, substantial bleed through into the orange channel remains after spectral unmixing. At the 1:1600 dilution, the bleed through signal remaining after spectral unmixing is negligible compared to the orange fluorophore control

well on the right. The intensity of the NIR fluorescence at this concentration is still bright enough to be easily masked and analyzed. Based on this observation, it is determined that the 1:1600 dilution is the optimal concentration for Annexin V NIR assays in HT-1080 cells.

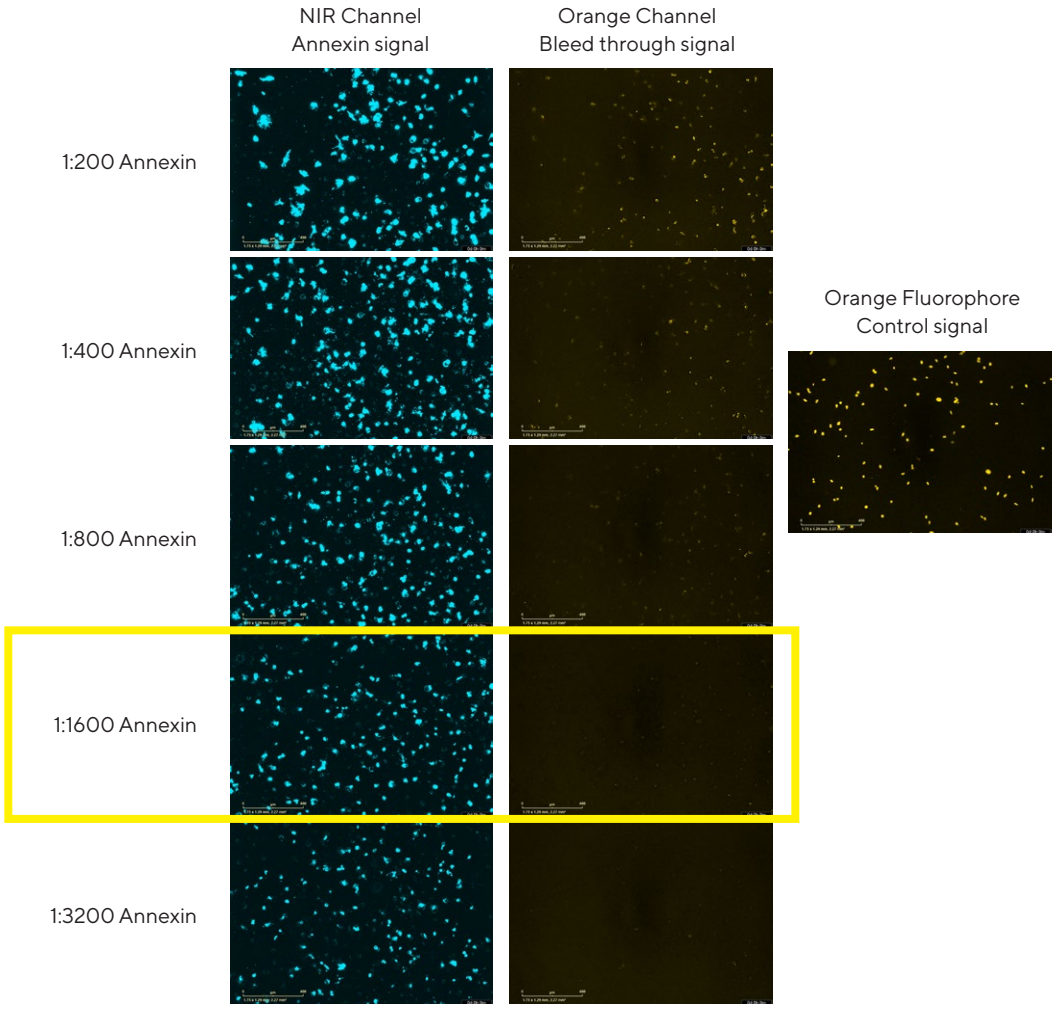


Figure 4. Determination of optimal concentration of Annexin NIR from optimization assay images

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

Sales and Service Contacts

For further contacts, visit www.sartorius.com

Essen BioScience, A Sartorius Company
www.sartorius.com/incucyte

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Telephone +1 734 769 1600
E-Mail: AskAScientist@sartorius.com

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Telephone +44 (0) 1763 227400
E-Mail: euorders.UK03@sartorius.com

APAC
Essen BioScience K.K.
4th floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Telephone: +81 3 6478 5202
E-Mail: orders.US07@sartorius.com