Example Experiment

Measuring Apoptosis in HeLa Cells Following Anisomycin Treatment

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I. Background

Accurate quantification of protein expression and/or post-translational modifications is important for advancing both basic and translational research. Given the flexibility, reproducibility, and higher throughput of the In-Cell Western™ Assay, it offers a convenient alternative to Western blotting and is a powerful platform for meaningful *in situ* analyses. The In-Cell Western microplate format can be used to analyze:

- Protein phosphorylation and signaling (1 - 3)
- Off-target effects of drugs on signaling pathways (4)
- Timing and kinetics of signaling events (5, 6)
- Quantification of viral load (7 - 11)
- Genotoxicity assays (12, 13)
- Cell proliferation and apoptosis assays (14)
- Bacterial-induced epithelial signaling (15)
- Glycoprotein analysis (16, 17)
- Library screening (18 - 20)
- Screening of monoclonal antibody clones (21)

II. Introduction

Understanding the processes leading to programmed cell death (apoptosis) is of utmost importance in the study of many diseases, including autoimmune diseases, neurological diseases, and cancer (22). For example, damaged cells that do not enter the apoptotic pathway may continue to proliferate and become cancerous (23). Due to the importance and complexity of the apoptosis signaling pathway, it is not surprising that this pathway plays an important role in numerous diseases. Consequently, understanding how new therapies impact this complex pathway is of great importance to the drug discovery field.

III. Experimental Design

In this experiment, we illustrate how the In-Cell Western Assay can be utilized to quantify the apoptotic response after drug treatment in a human cancer cell line. Anisomycin, a protein synthesis inhibitor, has been shown to trigger apoptosis in several cancer cell lines (24 – 30). Caspases (cysteinyl aspartate-specific proteases) are a family of important signaling molecules with essential roles that are both organ and subtype dependent (31). Caspase-3 has been implicated as an “effector” caspase aswsociated with the initiation of apoptosis,

and its activity is a common marker used in cellular assays to quantify activators and inhibitors of the death cascade (32).

In the following example, caspase-3 activation is monitored using an antibody that detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3. This antibody does not recognize full length caspase-3 or other cleaved caspases. Cleaved caspase-3 levels are then normalized against CellTag™ 700 Stain (licor.com/CellTag). Therefore, we have investigated the effects of anisomycin on apoptosis as measured by caspase-3 activation in HeLa cells by In-Cell Western™.

IV. Required Reagents

LI-COR Reagents

- IRDye® 800CW Goat anti-Rabbit Secondary Antibody (LI-COR P/N 926-32211)
- CellTag™ 700 Stain (LI-COR P/N 926-41090)
- Intercept® (PBS) Blocking Buffer (licor.com/intercept)

Additional Reagents

- 1X PBS wash buffer
- HeLa cells (ATCC® CCL-2™)
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 20% Tween® 20
- Anisomycin (Sigma®, P/N A9789)
- 0.25% Trypsin/0.53 mM EDTA Solution (Sigma, P/N T-3924)
- 37% formaldehyde
- 10% Triton® X-100
- Black-sided 96-well or 384-well microplates with clear well bottoms
- Sealing film
- Cleaved Caspase-3 (Asp175) Antibody (Cell Signaling Technologies, P/N 9661)

V. Prepare Cells

1. Allow HeLa cells to grow in a T75 flask using standard tissue culture procedures until ~80% confluency is achieved (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2. Remove growth media and wash cells with sterile 1X PBS (room temperature (RT)).
3. Add 5 mL Trypsin-EDTA (Sigma) and incubate 3-5 minutes at 37 °C to displace cells.
4. Neutralize displaced cells with culture media and pellet by centrifugation.
5. Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube.

> **Note:** To maintain cell integrity, do not pipet or vortex during pellet disruption.

6. Reconstitute cells in complete media so that 50,000 cells/mL is achieved.
7. Manually mix the cell suspension thoroughly.
8. Under sterile conditions, dispense 200 µL of the cell suspension per well in a 96-well plate (10,000 cells plated per well).
9. Incubate cells at 37 °C with 5% CO_2 and monitor cell density until ~80% confluency is achieved.

VI. Treat Cells

1. Warm serum-free media (DMEM, Gibco) to 37 °C.
2. Remove cell culture media.
3. Add either serum-free media for resting cells (mock) or serum-free media containing dilution series (1:2) of Anisomycin ranging in concentration from 0.07 - 40 µM. Add 100 µL of resting or activation media per well.
4. Allow incubation at 37 °C with 5% CO_2 for 4 hours.

VII. Fix and Permeabilize Cells

Fix Cells

1. Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

2. When incubation period is complete, carefully remove activation media manually or by aspiration to avoid detaching the cells.
3. Using a multi-channel pipettor, add 150 µL of fresh Fixing Solution (RT). Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells from the well bottom.
4. Allow incubation on bench top for 20 minutes at RT with no shaking.

Permeabilize Cells

1. Prepare Triton® Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

2. Remove Fixing Solution to an appropriate waste container (contains formaldehyde).
3. Using a multi-channel pipettor, add 200 µL Triton Washing Solution (RT). Add the solution down the sides of the wells carefully to avoid detaching the cells.
4. Allow plate to shake on a rotator for 5 minutes at RT.
5. Repeat washing steps 4 more times, removing wash manually each time.

> **Important:** Do not allow cells to become dry during washing. Immediately add the next wash after manual disposal.

VIII. Block Cells

1. Using a multi-channel pipettor, add 150 µL of Intercept® Blocking Buffer to each well. Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells.
2. Allow blocking for 1.5 hours at RT with moderate shaking on a plate shaker.

IX. Primary Antibody

Dilute Primary Antibody

1. Dilute the primary antibody in Intercept® Blocking Buffer.
   Cleaved Caspase-3; rabbit (1:100 dilution)
2. Mix the primary antibody solution thoroughly before adding to wells.

Incubate with Primary Antibody

1. Remove blocking buffer and add 50 µL of the desired primary antibody in Intercept Blocking Buffer to cover the bottom of each well.
2. Make sure to include control wells without primary antibody to serve as a source for background well intensity. Only add 50 µL of Intercept Blocking Buffer to control wells.
3. Securely seal plate with sealing film and incubate with primary antibody overnight with gentle shaking at 4° C.

Wash

1. Prepare Tween® Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS + 0.1% Tween 20	1000 mL

2. Remove primary antibody solution.
3. Using a multi-channel pipettor, add 200 µL Tween Washing Solution (RT). Add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.
4. Allow wash to shake on plate shaker for 5 minutes at RT.
5. Repeat washing steps 4 more times.

X. Secondary Antibody

Dilute Secondary Antibody

1. Dilute the fluorescently-labeled secondary antibody in Intercept® Blocking Buffer as specified below. The recommended antibody dilution range is 1:200 to 1:1,200. To lower background, directly add CellTag™ 700 Stain to the antibody dilution to obtain a 1:500

dilution of CellTag 700 Stain.
IRDye® 800CW Goat anti-Rabbit (1:800 dilution)
CellTag™ 700 Stain (1:500 dilution)

> **Note:** Minimize exposure of the antibody vials to light.

2. Thoroughly mix the antibody-stain solution then add 50 µL of the solution to each well.

Incubate with Secondary Antibody

1. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

Wash

1. Remove secondary antibody solution.
2. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution at RT (see "Wash" on the previous page). Add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.
3. Allow wash to incubate on a plate shaker for 5 minutes at RT.
4. Repeat washing steps 4 more times. Protect plate from light during washing.

XI. Image

1. After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4 °C for several weeks (sealed and protected from light).
2. Before plate scanning, clean the bottom plate surface and the Odyssey® Imager scanning bed (if applicable) with moist, lint-free tissue to avoid any obstructions during scanning.
3. Scan plate with detection in both 700 and 800 nm channels.

Suggested Scan Settings

All settings may require adjustment for optimal data quality. Higher resolutions or scan qualities can be used, but the scan time will increase.

Instrument	Resolution	Scan Quality	Intensity Setting (700 nm)	Intensity Setting (800 nm)
Odyssey Classic	169 µM	lowest	5	5
Odyssey CLx	169 µM	lowest	Auto Mode	Auto Mode
Odyssey Sa	200 µM	lowest	7	7
Aerius™ Imager	200 µM	lowest	7	7

XII. Experimental Results

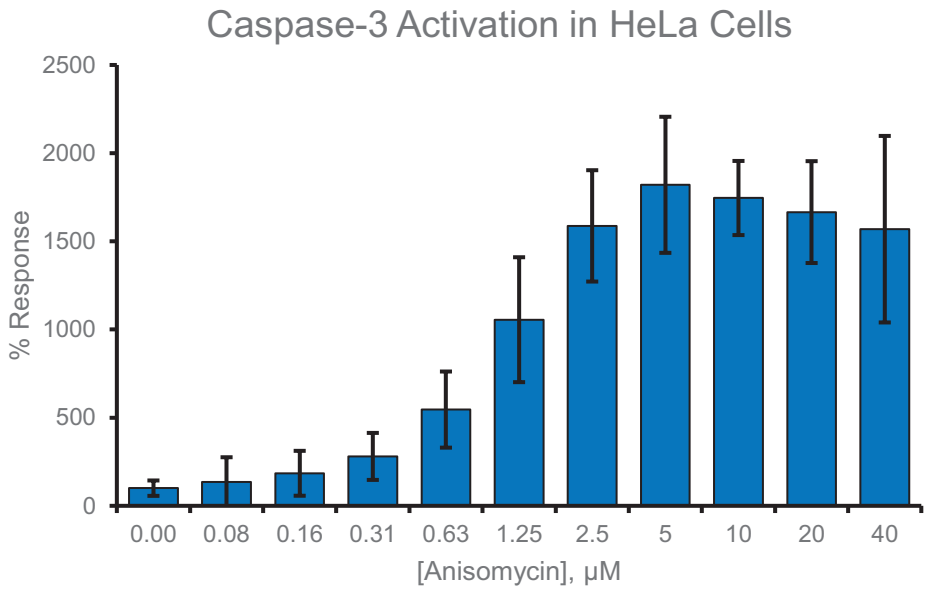

Figure 1. Induction of apoptosis in HeLa cells was achieved with increasing concentrations of anisomycin. An increase in cleaved caspase-3, a cleaved by-product indicative of apoptosis, is illustrated in the graph. Error bars represent the standard deviation from four technical replicates. The ultimate result of apoptosis induction is cell death. The reduction in cell number per well is taken into account when normalizing with CellTag™ 700 Stain. In an assay such as this, normalization is very important. See www.licor.com/ICWnormalization for more information on normalization options for an In-Cell Western™ Assay.

XIII. References

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