In-Cell Western™ Assay

FAQs for Suspension Cells

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR®

Revised September, 2010. The most recent version of this protocol is posted at https://www.licor.com/bio/support/

1. Handling Suspension Cells

1.1 How do you culture suspension cells?

For instructions on culturing suspension cells, look up your cell line at http://www.atcc.org/ and follow the guidelines.

1.2 How do you make non-adherent cells (suspension cells) attach to plates?

A simple trick is to replace your complete media containing 10% serum (usually fetal bovine serum) with the same media minus the serum. Then allow the cells to sediment, forming a monolayer of cells within 10 minutes. *Caution: Although cells appear attached to the plates, they are relatively loosely attached; therefore, extreme caution is required during solution-changing steps.*

1.3 How do I know that I have a monolayer?

Method #1 – Examine cells in the round bottom 96-well plates under a light microscope. The center of the wells should all have a small flat circular surface area where all the cells in that field are "in focus". Moving the plane of focus up or down will cause cells to be "off focus".
Method #2 – Hold the round bottom 96-well plate under a light source. The monolayer should look opaque rather than transparent. Cells will not attach on top of the cell monolayer, so the opaqueness is due only to the monolayer.

1.4 I cannot get a monolayer of cells. I get lots of spaces between cells. Is seeding 200,000 cells/well enough?

Seeding 200,000 cells/well is more than enough to form a complete cell monolayer. It is necessary to allow the cells in serum-free media to sediment in the T75 flask (or other tissue culture plates) for approximately 30 minutes before counting cells using a hemacytometer. When cells in serum-free media are placed, for example, in a T7 tissue culture flask, a monolayer of cells will immediately begin to form on the bottom of the flask. This will dramatically decrease the number of cells in suspension that are available for plating. *NOTE: Once a complete monolayer has formed on the plate, the rest of the cells will remain in suspension. Count these cells in suspension and the cells attached to the T75 flask can be discarded later.*

1.5 During my washing steps, cells are coming off the plates.

1.5.1 Are you using the recommended round bottom 96-well plate (BD Bioscience, P/N 353077)?

If no, cells will more easily detach from the flat bottom plates than the round bottom plates. The multi-channel pipettors will generate enough pressure when expelling liquid from the pipet to cause cell detachment when using flat bottom plates. Cells will detach even when pipetting down the sides of the wells.

If yes, make sure you pipet down the sides of the wells and not directly onto the cells. If this doesn't help, you may need to change your multi-channel pipettor because different brands of pipettors have different amount of pressure required to expel the liquid from the pipet. The recommended multi-channel pipettor is the 12-channel Finnpipette (Thermo Electron Corp, P/N 4610050).

1.5.2 Are you shaking or rotating the plates at a moderate-to-high speed?

If yes, gentler shaking/rotating is needed to prevent cells from detaching. Cells will detach. Set shaking or rotating speed to very low speed.

If no, are you dumping the solutions straight from the plates? Dumping causes cells to detach. Either aspirate very slowly or manually pipet using the sides of the wells.

2. Round vs. Flat Bottom 96-well Plates

2.1 Why can't I use the flat bottom 96-well plates?

LI-COR Biosciences recommends using the round bottom 96-well plates. For an explanation, see 1.5.

2.2 When I scan an empty round bottom 96-well plate, I get lots of background noise. The round bottom plate shows some background autofluorescence. The background fluorescence is relatively small compared to signal (about 200-fold difference, depending on the intensity of the signal) and can be subtracted from the signal. It is necessary to include background wells containing cells and only the secondary antibodies in order to completely subtract away the background noise originating from the plate as well as from the non-specific binding of the secondary antibodies.

3. Scan Settings

3.1 Why does my scanned image look so weak?

Assuming that you followed the protocol correctly and your antibodies work, did you set the focus offset to 3.0 to 3.5 mm for the BD Bioscience round bottom plates (P/N 353077)? If using the Nunc® round bottom plates (P/N 16332), the default setting for the flat bottom 96-well plates (3.0 mm) will not produce much signal. The focus offset for the Nunc round bottom plates should be set to 3.5 to 3.95mm. For maximum signal strength with the Odyssey® Infrared Imager, BD Bioscience round bottom plates are rec- ommended. Both BD Biosci ence and Nunc round bottom plates work well with the Odyssey Sa instrument.

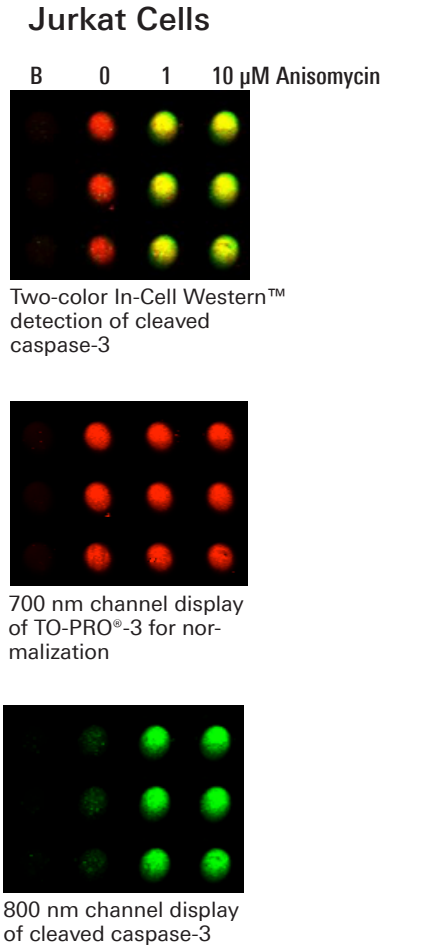
4. Other Suspension Cell Lines and Different Pathways

4.1 Have you tested other suspension cell lines?

Yes. Suspension cell lines tested include Jurkat, K-562 and THP-1. A sample protocol can be downloaded from http://biosupport.licor.com.

4.2 Have you tested other pathways?

Yes. Pathways tested include ERK activation and apoptosis using cleaved caspase3 as a marker (Figure 1). A sample protocol can be downloaded from http://biosupport.licor.com.



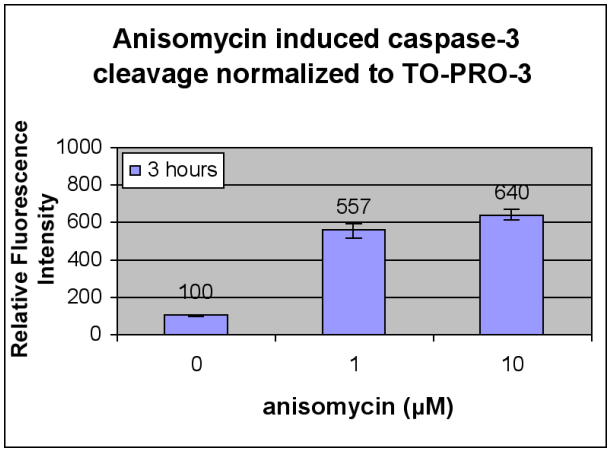


Figure 1. Anisomycin-induced apoptosis in Jurkat cells. The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 nm channels detecting TO-PRO-3 DNA staining and cleaved caspase-3 (Asp175), respectively. The image was scanned using the Odyssey® Sa Infrared Imaging System with scan setting of 200 µm resolution, focus offset of 3.5, and intensity of 3.5 (700 channel) and 4 (800 channel). Background (B) wells were incubated with a secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the increase in caspase-3 cleavage in response to anisomycin treatment for 3 hours in Jurkat cells.

LI-COR®

Global Headquarters, Serving the United States
4647 Superior Street • Lincoln, NE 68504 • Phone: +1-402-467-0700 • Toll free: 800-645-4267 • Fax: +1-402-467-0819
biosales@licor.com

Regional Offices
LI-COR GmbH, Germany, Serving Europe, Africa, and the Middle East.
LI-COR Biosciences GmbH • Siemensstraße 25A • 61352 Bad Homburg • Germany • Phone: +49 (0) 6172 17 17 771
Fax: +49 (0) 6172 17 17 799 • bio-eu@licor.com

LI-COR Ltd., United Kingdom, Serving Denmark, Finland, Iceland, Ireland, Norway, Sweden, and UK.
LI-COR Biosciences UK Ltd. • St. John's Innovation Centre • Cowley Road • Cambridge • CB4 0WS • United Kingdom
Phone: +44 (0) 1223 422104 • Fax: +44 (0) 1223 422105 • bio-eu@licor.com

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988-11461 09/10