

Cell-Based Infrared ELISA

Human Cleaved Caspase-3 (Asp175) Infrared Immunoassay

Catalog Number KCBIR835

A near-infrared assay using two spectrally distinct fluorophores to measure Cleaved Caspase-3 (Asp175) in whole cells.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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
PRINCIPLE OF THE ASSAY

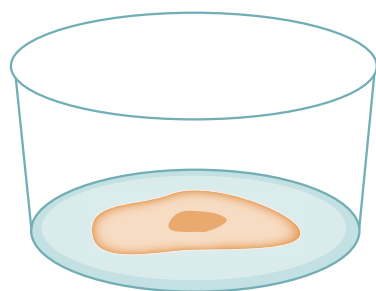
This Cell-Based Infrared ELISA contains the components required to run a near-infrared immunostaining assay to measure Cleaved Caspase-3 (Asp175) in whole cells. This simple and efficient assay eliminates the need to prepare cell lysates and can be used to investigate signaling and the effects of inhibitors on cells. Cells are grown in 96-well microplates and stimulated with ligands. Following stimulation, cells are fixed and permeabilized in the wells. The target protein amount is measured using a double immunofluorescent staining procedure. The cells are simultaneously incubated with two primary antibodies: an antibody specific for the target protein and a normalization antibody that is specific for GAPDH, a housekeeping protein. The primary antibodies are derived from different species. Two species-specific secondary antibodies labeled with two spectrally distinct near-infrared fluorophores are used for detection. The fluorescence of the target protein is normalized to that of GAPDH in each well for the correction of well-to-well variations. This two-wavelength assay results in precise analysis of Cleaved Caspase-3 (Asp175) with good reproducibility.

TECHNICAL HINTS AND LIMITATIONS

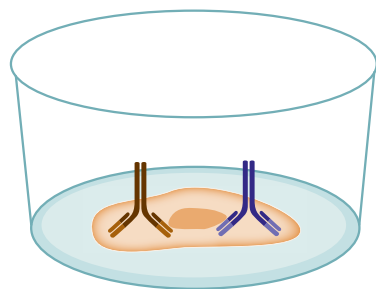
- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Cell-Based Infrared ELISA should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- A thorough and consistent wash technique is essential for proper assay performance. To minimize cell loss during the wash steps, avoid dispensing liquid directly onto the cell surface. Instead, gently dispense the liquid down the wall of the cell culture wells, always using the same side of the wells. Empty the wells by decanting and remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- To avoid cross-contamination, change pipette tips between additions of each reagent and/or sample. Also, use separate reservoirs for each reagent.
- It is recommended that all samples and controls be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8 °C.

ASSAY OVERVIEW

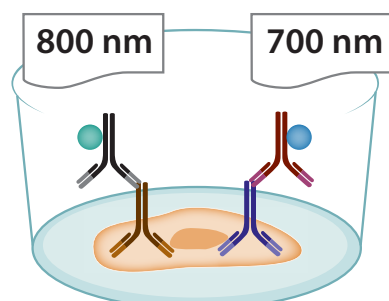
-  Rabbit Anti-Cleaved Caspase-3 (Asp175)
-  Goat Anti-Total GAPDH
-  IRDye® 800CW-Conjugated Anti-rabbit IgG
-  IRDye® 680LT-Conjugated Anti-goat IgG



1. Seed cells in a 96 well plate. Stimulate cells with ligands. Fix, permeabilize, and block cells.



2. Add primary antibodies (rabbit anti-Cleaved Caspase-3 (Asp175) and goat anti-total GAPDH).



3. Add secondary antibodies (IRDye 800CW-conjugated anti-rabbit IgG and IRDye 680LT-conjugated anti-goat IgG). The plate is imaged by scanning simultaneously at 700 nm and 800 nm with a LI-COR Odyssey® or Aeries™ instrument.

MATERIALS PROVIDED

Store the unopened kit at 2-8 °C. Do not use past kit expiration date. This kit contains sufficient materials to run one 96-well plate.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Cleaved Caspase-3 (ASP175) Antibody	967337	1 vial of lyophilized rabbit anti-Cleaved Caspase-3 (Asp175) antibody.	Store at 2-8 °C for up to 1 month or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 2 months.*
Total GAPDH Antibody	967134	1 vial of lyophilized goat anti-total GAPDH antibody.	
IRDye® 800CW-conjugated anti-rabbit IgG**	894155	22 µL of IRDye 800CW-conjugated donkey anti-rabbit IgG secondary antibody.	May be stored at 2-8 °C for up to 2 months.*
IRDye® 680LT-conjugated anti-goat IgG**	894152	22 µL of IRDye 680LT-conjugated donkey anti-goat IgG secondary antibody.	
Blocking Buffer	893235	35 mL of 10% fetal bovine serum in buffer with preservatives.	
Wash Buffer (5X)	893236	60 mL of a buffered surfactant with preservatives.	Store at room temperature.
Microplate	607582	96-well cell culture clear-bottom black microplate and cover for use as a vessel in the assay.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- 37% formaldehyde (Molecular Biology Grade; Sigma, Catalog # F8775). Refer to the MSDS prior to use.
- 1X PBS (Irvine Scientific, Catalog # 9240).
- Deionized or distilled water.
- Pipettes and pipette tips.
- Multi-channel pipette for washing.
- Cell culture incubator.
- Microfuge tubes.
- Orbital shaker.
- LI-COR Odyssey or Aeries Infrared Imaging System.

***This product is covered by one or more of the following US Patent numbers: US 6,995,274, US 7,504,089, PCT - WO0224815A1, and US Application 61/184,750 pending.*

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REAGENT PREPARATION

Cleaved Caspase-3 (Asp175) Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 μ L of 1X PBS.

Total GAPDH Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 μ L of 1X PBS.

Primary Antibody Mixture* - Immediately before use, add 100 μ L of the Cleaved Caspase-3 (Asp175) antibody and 100 μ L of the Total GAPDH Antibody to 9.8 mL of Blocking Buffer. 100 μ L of the primary antibody mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

Secondary Antibody Mixture* - Immediately before use, centrifuge vials for 30 seconds at 1000 x g prior to removing cap. Add 20 μ L of the IRDye 800CW-conjugated antibody and 20 μ L of the IRDye 680LT-conjugated antibody to 10 mL of Blocking Buffer. 100 μ L of the secondary antibody mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

1X Wash Buffer - Add 60 mL of Wash Buffer (5X) to 240 mL of 1X PBS to prepare 1X Wash Buffer.

SOLUTION PREPARATION

4% Formaldehyde (for adherent cells) - Add 1.3 mL of 37% formaldehyde to 10.7 mL of 1X PBS to prepare 4% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

8% Formaldehyde (for non-adherent cells) - Add 2.6 mL of 37% formaldehyde to 9.4 mL of 1X PBS to prepare 8% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

*Once prepared, the primary and secondary antibody mixtures cannot be stored. Prepare only enough as needed to run the assay.

GENERAL ASSAY PROCEDURE

A. Culture, Stimulate, Fix, and Block Cells

1. Seed 100 μ L of 10,000-20,000 adherent cells into each well of the black 96-well microplate with clear bottom and incubate overnight at 37 °C in a cell culture incubator.

Note: *The cell number used is dependent upon the cell line and the relative amount of target protein. Optimal cell numbers should be determined by each laboratory for each assay.*

2. Grow and treat the cells as desired.
3. Centrifuge the plate at 500 x g for 3 minutes at 2-8 °C, then remove medium. Fix cells by adding 100 μ L of 4% formaldehyde in 1X PBS. Add the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the fixed cells at 2-8 °C for up to 2 weeks.

Warning: *Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical. Refer to MSDS prior to use.*

4. Remove the formaldehyde solution and wash the cells 3 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
5. Remove the Wash Buffer, and add 100 μ L of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature.

B. Binding of Primary and Secondary Antibodies

1. Remove the Blocking Buffer and wash the cells 3 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
2. Add 100 μ L of the Primary Antibody Mixture to each well. Cover with a plate sealer and incubate for 16 hours at 2-8 °C. In cells known to generate high amounts of Cleaved Caspase-3 (Asp175), a 2-3 hour primary antibody incubation is sufficient; however, for maximum sensitivity, an overnight incubation is recommended.

Note: *Depending on the experimental design (refer to the Calculation of Results section), some wells should be incubated with Primary Antibody Mixture and some with only the Blocking Buffer as the negative controls (secondary antibody alone).*

3. Remove the Primary Antibody Mixture and wash the cells 3 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
4. Add 100 μ L of the Secondary Antibody Mixture to each well. Cover with a plate sealer and incubate for 2 hours at room temperature.

Note: *The Secondary Antibody Mixture is added to each well including the negative control wells.*

C. Near-Infrared Detection

1. Remove the Secondary Antibody Mixture from each well and wash the cells 4 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
2. Image the plate using a LI-COR Odyssey or Aeries instrument. The plate is scanned simultaneously at 700 nm and 800 nm with 169 μ m resolution, medium quality, focus offset of 3.0 mm, and intensity setting of 5 for both channels.

PROCEDURE FOR NON-ADHERENT CELLS

This protocol has been validated for use with non-adherent cells by culturing and fixing cells as follows.

1. Seed 100 μL of suspension cells into each well of the black 96-well microplate in serum-free or normal growth media at the desired cell density (seeding cells at a density of $0.2\text{-}1.0 \times 10^6$ cells/mL is recommended).
2. Incubate the plate at 37 °C. Depending on the cell line and treatment, the typical incubation time is 0.5-16 hours.
3. Treat the cells as desired. Prepare the treatment media containing a 5X final concentration of treatment. Add 25 μL of 5X treatment media to the wells and bring the total volume up to 125 μL . Mix solution by gently agitating the plate and incubate according to your treatment protocols.
4. Centrifuge the plate at 500 x g for 3 minutes at 2-8 °C and remove media.
5. Fix the cells by adding 100 μL of 8% formaldehyde in 1X PBS. Apply the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the fixed cells at 2-8 °C for up to 2 weeks.
6. Continue with section A, step 4 of the General Assay Procedure on page 5.

CALCULATION OF RESULTS

Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The fluorescence from these wells is the background fluorescence and is subtracted from all sample wells. Normalized results can be determined by dividing the Cleaved Caspase-3 (Asp175) fluorescence at 800 nm in each well by the total GAPDH fluorescence at 700 nm in each well. The normalized duplicate readings for each sample are then averaged.

KIT PERFORMANCE DATA

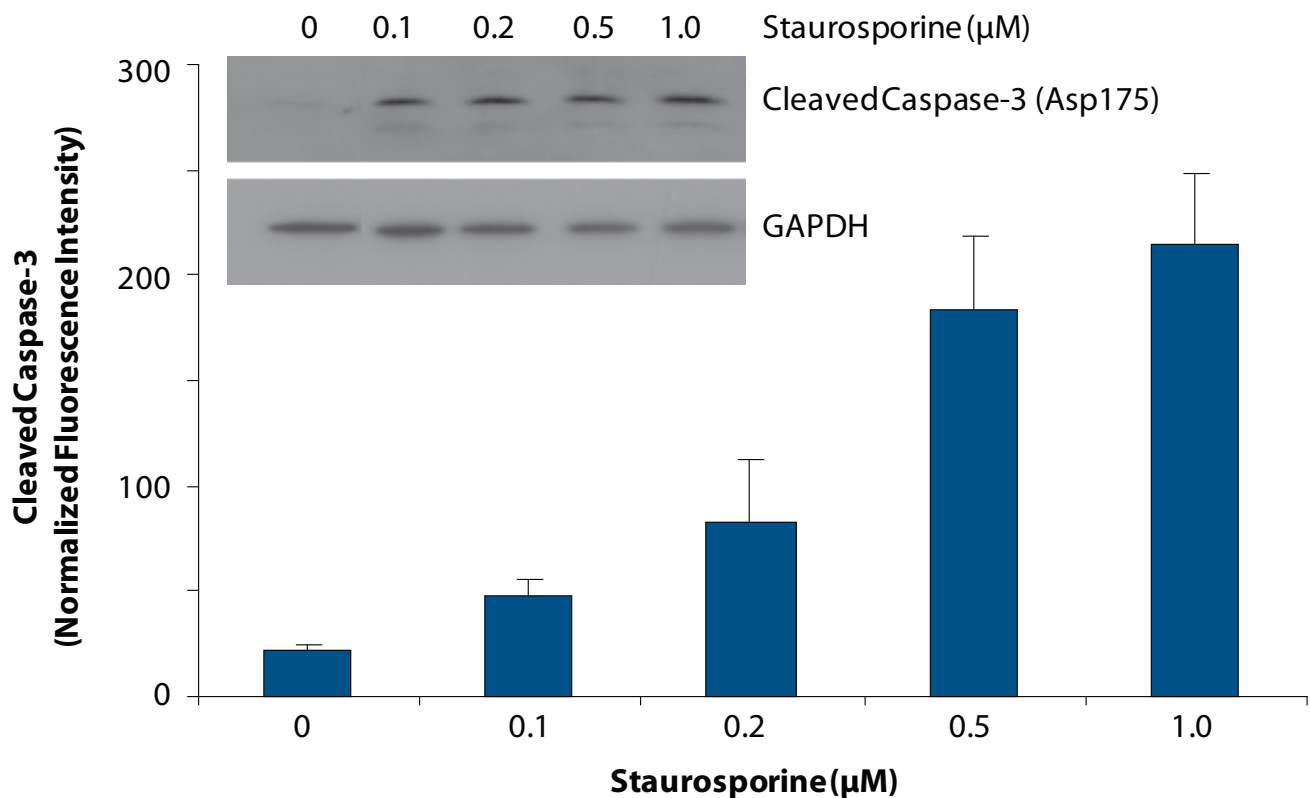


Figure 1: Staurosporine-induced Caspase-3 cleavage in non-adherent cells. Jurkat human acute T-cell leukemia cells were cultured in 96-well plates and treated with the indicated amounts of staurosporine for 4 hours. After fixation of cells in the wells, the amount of Cleaved Caspase-3 (Asp175) was determined using this Human Cleaved Caspase-3 (Asp175) Cell-Based Infrared ELISA kit. Values represent the mean ± the range of duplicate determinations. Analysis of Cleaved Caspase-3 (Asp175) and GAPDH (the normalization protein) by Western blotting using the antibodies supplied in this kit is also shown (inset).

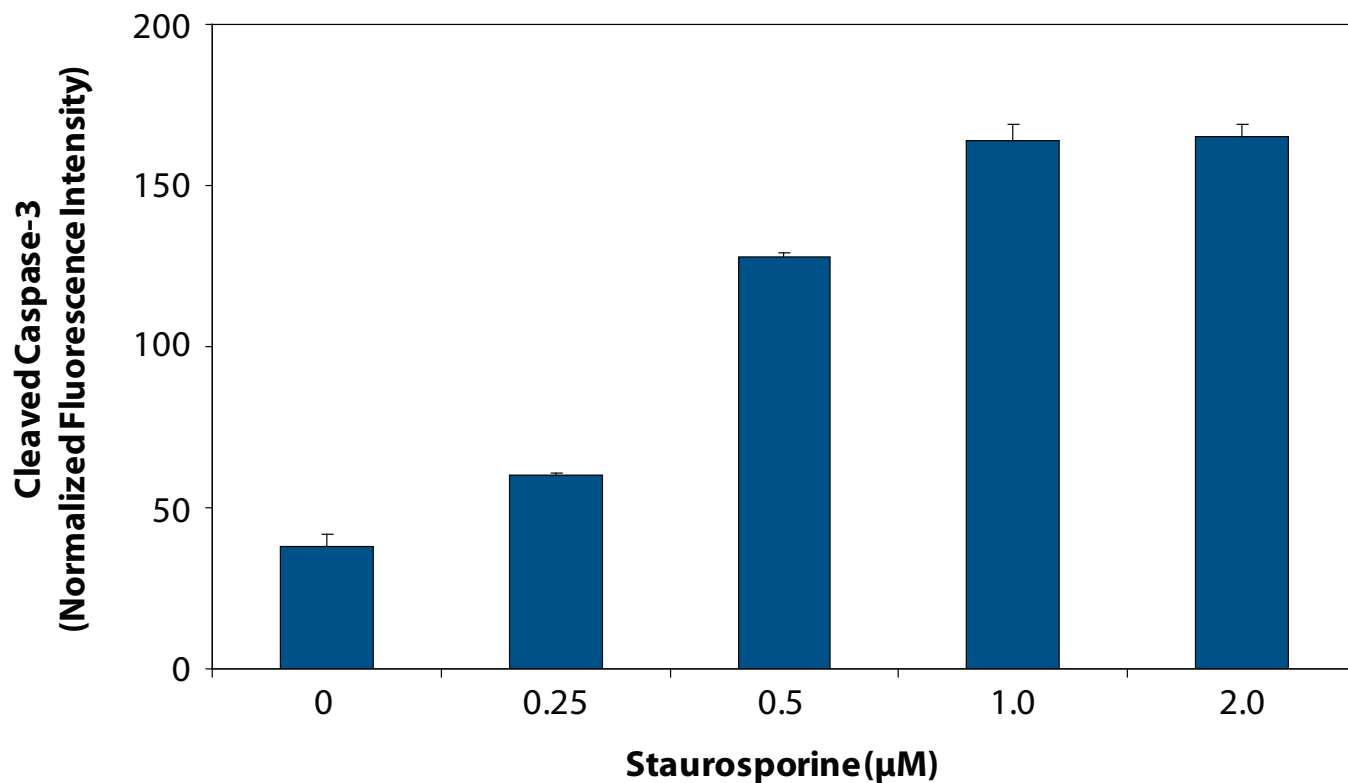


Figure 2: Staurosporine-induced Caspase-3 cleavage in adherent cells. HeLa human cervical epithelial carcinoma cells were cultured in 96-well plates and treated with the indicated amounts of staurosporine for 4 hours. After fixation of cells in the wells, the amount of Cleaved Caspase-3 (Asp175) was determined using this Human Cleaved Caspase-3 (Asp175) Cell-Based Infrared ELISA kit. Values represent the mean \pm the range of duplicate determinations.

PLATE LAYOUT

Use this plate layout as a record of samples assayed.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

