

# IRDye<sup>®</sup>

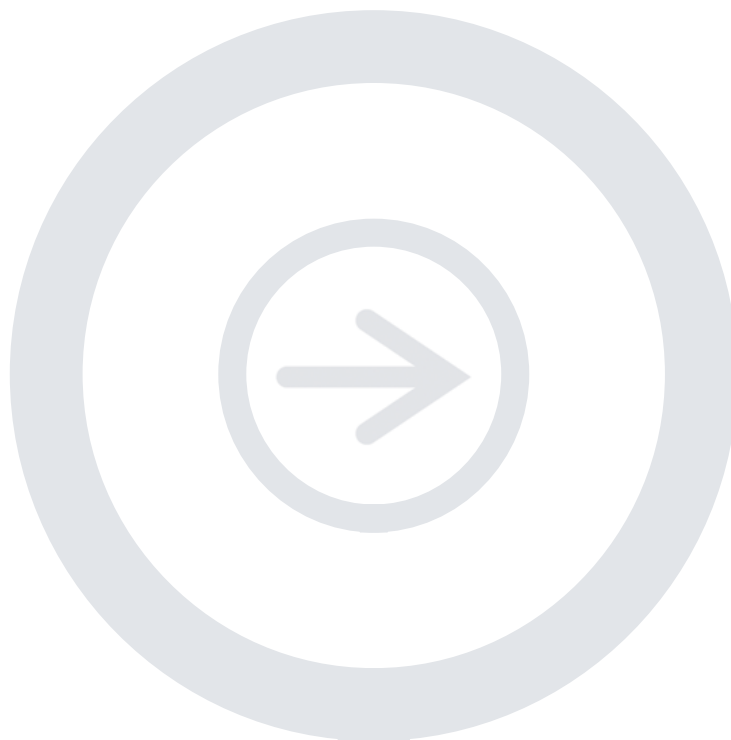
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*Infrared Dye Reagents*

## **IRDye<sup>®</sup> 800CW/QC-1 CSP-3**

Part Number: 926-08590  
Storage: -20 °C  
Stability: 6 months lyophilized  
3 months reconstituted

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<http://biosupport.licor.com/support>.



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Biosciences

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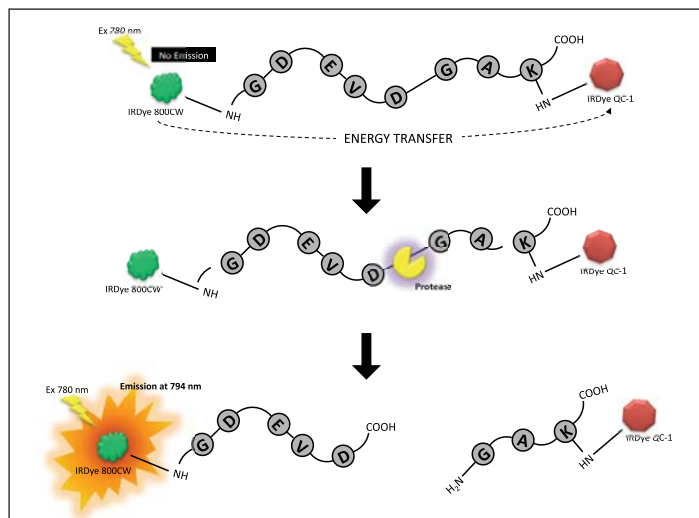
## I. Introduction

Cell apoptosis is a programmed cell death process that is implicated in a variety of diseases such as cancer, immune system diseases and nervous system disease<sup>1</sup>. Caspases, a family of intracellular cysteine proteases, are vital in the process of apoptosis. Caspase-3 functions as the most downstream executioner in the caspase-apoptosis signaling cascade<sup>2</sup>. Activation of Caspase-3 by upstream initiator caspases (Caspases 2, 8, 9) results in the final execution of cell death<sup>3</sup>.

LI-COR IRDye® 800CW/QC-1 CSP-3 substrate is a sensitive reagent to detect Caspase-3 activity, measure potency of inhibitors and screen compound collections for discovery of drug candidates. The substrate for the Caspase-3 Assay as outlined in Figure 1 is a highly-quenched NIR-FRET peptide, IRDye 800CW-GDEVDGAK(IRDye QC-1)-COOH. Cleavage of the substrate peptide separates the donor-quencher pair (IRDye 800CW/QC-1) and restores the fluorescence signal of the donor.

The Caspase-3 Assay is optimized for use on LI-COR Odyssey® and Aeries® Infrared Imaging Systems which use near-infrared excitation (780 nm) and near-infrared detection (820 ± 10 nm). Background fluorescence and compound interference in this assay are substantially reduced. The IRDye 800CW and IRDye QC-1 dyes have excellent water solubility to ensure that the dye-labeled substrate peptide is soluble in assay buffer without the need for an organic co-solvent.

**Figure 1.**  
Caspase-3 Assay using  
IRDye 800CW/IRDye QC-1  
CSP-3 substrate.



## II. Materials

### A. Provided

1. Substrate: IRDye 800CW/QC-1 CSP-3 (lyophilized, 2.4 nmoles)  
IRDye 800CW-GDEVGAK(IRDye QC-1)-COOH

### B. Not Provided

1. Fluorescence Standard: IRDye 800CW Carboxylate (lyophilized, 20 nmoles, LI-COR P/N 929-08972)
2. Inhibitor: Ac-DEVD-CHO (10 mM in DMSO, 10  $\mu$ L, Promega Cat # G5961)
3. Caspase-3 Protease: Human recombinant Caspase-3 (Calbiochem, Cat. # 235417)

*NOTE: If an alternative CSP-3 Protease is used, adjustments may be needed for enzyme activity variation.*

4. TCEP•HCl: Tris (2-Carboxyethyl) phosphine Hydrochloride (solid, 18.6 mg, Pierce Cat. # 20490)
5. 96-Well Microplate: BD Bioscience Optilux™ (Cat. # 35948) or Corning Costar (Cat. # 3603)
6. 96-Well Microplate (Cell Based Assays): Nunc™ 96 Microwell™ Plate (Cat. # 167008)

*NOTE: Other microplates with black wells and clear bottoms may work. Scan before use to determine if plates autofluoresce. Do not use white-walled plates.*

7. Sealing Foil: AlumaSeal II adhesive foil film (EXCEL Scientific, Cat. # AF-100)
8. Odyssey or Aeries Infrared Imaging System (LI-COR Biosciences)
9. Other reagents also not provided:
  - Triton X-100
  - DMSO
  - HEPES
  - EDTA
  - CHAPS
  - Glycerol
  - Potassium phosphate
  - BSA
  - Sterile water

## III. MEASURING THE IC<sub>50</sub> OF AN INHIBITOR

*NOTE: All working solutions should be prepared fresh for each experiment. Keep all reagents on ice or in a cold box.*

*NOTE: Include 5 mM of freshly prepared TCEP reducing agent in the Assay Buffer for maximum assay sensitivity.*

### A. Reagent Preparation

1. 2X Caspase-3 Assay Buffer, 30mL  
200 mM HEPES (pH 7.5) with 2mM EDTA, 0.2% CHAPS and 20% (v/v) glycerol

*NOTE: Assay Buffer does not require DMSO; however, DMSO may be necessary to dissolve the test compound(s). Up to 2% (v/v) DMSO (260  $\mu$ L in 13 mL of buffer for 100 assays using 96-well plates) may be used, as needed.*

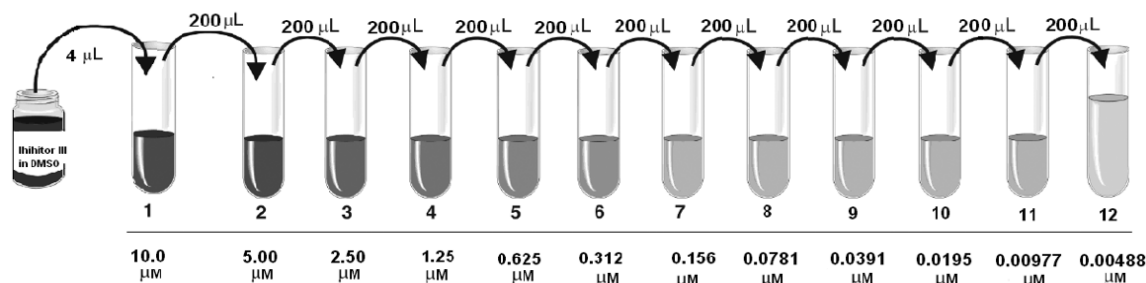
2. Stop Solution, 30 mL  
0.4 M potassium phosphate (pH 5.0) with 0.25 mg/mL BSA
3. Assay Buffer
  - a. Dissolve 18.6 mg of TCEP•HCl in 130  $\mu$ L of sterile water to make 500 mM TCEP.
  - b. Prepare Assay Buffer using the following table.

Caspase-3 Assay Buffer (2X)	6.5 mL
Sterile Water	6.2 mL
TCEP•HCl (500 mM)	130 $\mu$ L
DMSO	130 $\mu$ L
Total Volume	13.0 mL

#### 4. Inhibitor Dilution Series

*NOTE: The concentration range for the Inhibitor in reaction wells should have  $\geq 1000$ -fold difference from the highest to the lowest concentration and should span the expected  $IC_{50}$  value. For the Caspase-3 Assay, the volume of Inhibitor added is 15  $\mu$ L/well in a total reaction volume of 60  $\mu$ L/well.*

- a. Prepare 400  $\mu$ L of the highest concentration of Inhibitor in Assay Buffer.
- b. Make 2-fold serial dilutions of Inhibitor in Assay Buffer according to the following diagram.
- c. Using AcDEVDCHO inhibitor as an example:
  - Label vials 1 through 12.
  - Place 624  $\mu$ L of Assay Buffer into vial 1 and 200  $\mu$ L of Assay Buffer into vials 2 through 12.
  - Dilute 1  $\mu$ L of 10 mM Inhibitor (in DMSO) in 999  $\mu$ L of Assay Buffer to make a 10  $\mu$ M Inhibitor solution.
  - Add 1.0  $\mu$ L of 10  $\mu$ M Inhibitor into vial 1 to prepare 625  $\mu$ L of 16 nM Inhibitor; mix vial 1 thoroughly.
  - Prepare 11 additional 2-fold serial dilutions of the Inhibitor by transferring 200  $\mu$ L from vial 1 into vial 2, mixing thoroughly, subsequently transferring and mixing as shown.



5. Caspase-3 Protease Solution
  - a. Thaw an aliquot of Caspase-3 (e.g. Calbiochem, 105 U/ $\mu$ l) on ice.
  - b. Prepare 1300  $\mu$ L of Caspase-3 by diluting the enzyme with Assay Buffer to a concentration of 240 U/mL (60 U/mL in the assay).

*NOTE: The concentration of Caspase-3 may vary from lot to lot. Adjust calculation accordingly so that the final working concentration is 240 U/mL.*

6. Substrate Solution
  - a. Reconstitute lyophilized IRDye 800CW/QC-1 CSP-3 substrate with 200  $\mu$ L of sterile water to make a 12  $\mu$ M Substrate Solution; mix gently to dissolve completely.

- b. Dilute the 12  $\mu\text{M}$  Substrate Solution 1:30 with Assay Buffer to make a 400 nM Substrate Solution (200 nM concentration in the assay).

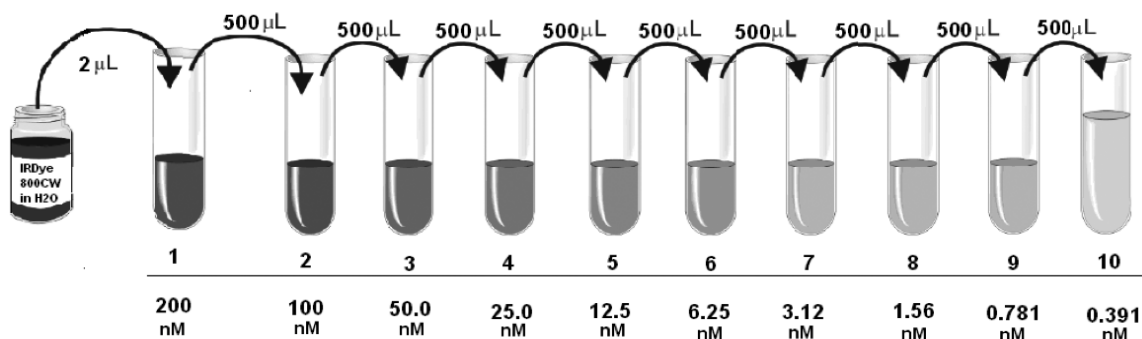
*NOTE: Dilute only enough Substrate for immediate use (30  $\mu\text{L}/\text{well}$ ).*

## 7. Fluorescence Standard

*NOTE: An IRDye 800CW Carboxylate Fluorescence Standard dilution series should be included if a fluorescence intensity calibration curve is to be generated. The dilution series should be prepared fresh for each experiment.*

- a. Using IRDye 800CW Carboxylate:

- Label vials 1 through 10.
- Add 998  $\mu\text{L}$  of Assay Buffer into vial 1 and 500  $\mu\text{L}$  of Assay Buffer into vials 2 through 10.
- To the vial of 800CW Carboxylate, add 200  $\mu\text{L}$  of sterile water to make a 100  $\mu\text{M}$  fluorescence standard; VORTEX THOROUGHLY; centrifuge briefly.
- Transfer 2  $\mu\text{L}$  of the 100  $\mu\text{M}$  standard into vial 1 to make 1000  $\mu\text{L}$  of a 200 nM standard; mix thoroughly.
- Prepare 9 additional fluorescence standards of 2-fold serial dilution by transferring 500  $\mu\text{L}$  from vial 1 into vial 2, mixing thoroughly, subsequently transferring and mixing as shown.



## B. Enzymatic Reaction (96-Well Plate)

*NOTE: Duplicate or triplicate samples and standards should be run in the experiment*

1. Reaction or Test Wells:
  - a. Place 15  $\mu\text{L}/\text{well}$  of the Inhibitor Solutions (12 solutions, serially diluted 2-fold) into the reaction wells.
  - b. Add 15  $\mu\text{L}/\text{well}$  of Caspase-3 Solution (240 U/mL) into each reaction well.
2. Control Wells:
  - a. Positive (no Inhibitor): Add 15  $\mu\text{L}/\text{well}$  of Caspase-3 Solution (240 U/mL) and 15  $\mu\text{L}/\text{well}$  of Assay Buffer into the positive (no Inhibitor) control wells.
  - b. Negative (no enzyme): Add 30  $\mu\text{L}/\text{well}$  of Assay Buffer into the negative (no enzyme) control wells.
3. Calibration Wells:
  - a. Place 60  $\mu\text{L}/\text{well}$  of the fluorescence standards (10 solutions serially diluted 2-fold) into calibration wells.
4. Pre-incubation:
  - a. Cover plate with the lid
  - b. Shake the plate gently at room temperature for 30 minutes on a plate shaker at medium speed; **protect from light.**

## 5. Substrate Addition:

- Remove the plate cover.
- Add 30  $\mu\text{L}$  of Substrate Solution (400 nM) into each reaction, positive control (no Inhibitor), and negative control (no enzyme) wells.
- Seal the plate with sealing foil.

*NOTE: To obtain good results, it is essential to seal the plate to prevent evaporation.*

- Incubate the plate at room temperature for 90 minutes on a plate shaker set at medium speed; **protect from light**.

## 6. Stop Enzymatic Reaction

- Remove the sealing foil.
- Add 60  $\mu\text{L}$  of Stop Solution to each well (including the calibration wells) using a dispenser or multichannel pipette.

*NOTE: Stop Solution should be added as quickly as possible to stop all enzymatic reactions.*

- Mix the plate on a plate shaker.

**C. Image Plate**

## 1. Image plate on Aerius or Odyssey Infrared Imaging System

- Scan the plate using the following instrument settings (these settings are a suggested starting point and may need to be optimized):

Instrument	Plate	Focus Offset (mm)	Resolution (m)	Imaging Quality Setting	Intensity Setting	
					700 Channel	800 Channel
Aerius	BD Bioscience Optilux™, Cat # 35948	3.5	200	N/A	Off	3
	Corning Costar Cat # 3603	3.5	200	N/A	Off	3
Odyssey	BD Bioscience Optilux™, Cat # 35948	3.5	169	Medium	Off	2
	Corning Costar Cat # 3603	3.5	169	Medium	Off	2

**D. Experimental Results**

Figure 2 illustrates the  $\text{IC}_{50}$  measurement of a known Caspase-3 inhibitor, Ac-DEVD-CHO. The  $\text{IC}_{50}$  value is generated by non-linear regression fitting of the experimental data to the GraphPad Prism software.

**Figure 2.**  $\text{IC}_{50}$  measurement for a known Caspase-3 inhibitor, Ac-DEVD-CHO, in a 96-well plate. The serially diluted inhibitor solutions (15  $\mu\text{L}$ /well at final concentrations from 0.00195 to 4 nM) were mixed with Caspase-3 (15  $\mu\text{L}$ /well at final concentration of 60 U/mL) for 30 minutes before adding substrate (30  $\mu\text{L}$ /well at final concentration of 200 nM). After incubating at room temperature for 90 minutes, the reactions were stopped with 60  $\mu\text{L}$ /well of stop solution. The fluorescence intensity was measured on an Aerius Infrared Imaging System.

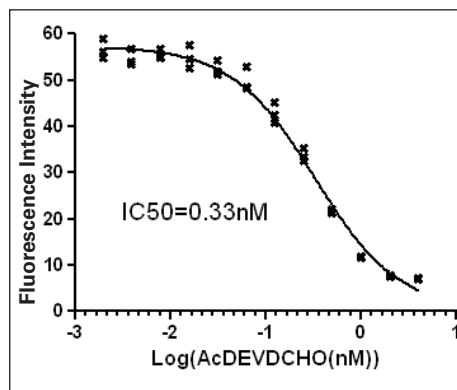
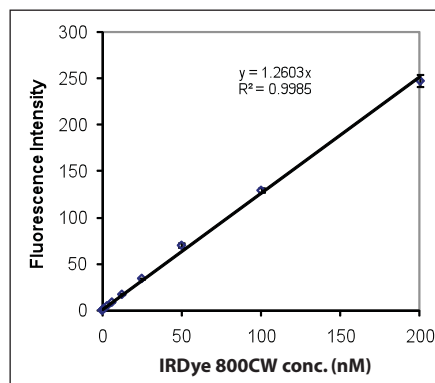


Figure 3 is the fluorescence standard calibration curve for this experiment in a 96-well format.

**Figure 3.** IRDye 800CW Carboxylate fluorescence standard curve ( $R^2=0.998$ , slope=1.26).



## IV. Measuring Caspase-3 Protease Activity

*NOTE: All working solutions should be prepared fresh for each experiment. Keep all reagents on ice or in a cold box.*

*NOTE: Include 5 mM of freshly prepared TCEP reducing agent in the Assay Buffer for maximum assay sensitivity.*

### A. Reagent Preparation

- 2X Caspase-3 Assay Buffer, 30mL  
200 mM HEPES (pH 7.5) with 2mM EDTA, 0.2% CHAPS and 20% (v/v) glycerol

*NOTE: Assay Buffer does not require DMSO; however, DMSO may be necessary to dissolve the test compound(s). Up to 2% (v/v) DMSO (260  $\mu$ L in 13 mL of buffer for 100 assays using 96-well plates) may be used, as needed.*

- Stop Solution, 30 mL  
0.4 M potassium phosphate (pH 5.0) with 0.25 mg/mL BSA
- Assay Buffer
  - Dissolve 18.6 mg of TCEP•HCl in 130  $\mu$ L of sterile water to make 500 mM TCEP solution.
  - Prepare Assay Buffer using the following table.

Caspase-3 Assay Buffer (2X)	6.5 mL
Sterile Water	6.3 mL
TCEP•HCl (500 mM)	130 $\mu$ L
Total Volume	13.0 mL

### 4. Caspase-3 Protease Dilution Series

- Thaw an aliquot of Caspase-3 (e.g. Calbiochem 105 U/ $\mu$ l) on ice.
- Label vials 1 through 12.
- Prepare 600  $\mu$ L of Caspase-3 in vial 1 by diluting the enzyme with Assay Buffer to a concentration of 1000 U/mL.
- Place 300  $\mu$ L of Assay Buffer in vials 2 through 12.
- Make 11 additional 2-fold serial dilutions of the Caspase-3 by transferring 300  $\mu$ L from vial 1 into vial 2, mixing thoroughly, and repeating the process for tubes 3 through 12 as outlined in Section III.

5. Substrate Solution

- a. Reconstitute lyophilized IRDye 800CW/QC-1 CSP-3 substrate with 200  $\mu\text{L}$  of sterile water to make a 12  $\mu\text{M}$  Substrate Solution; mix gently to dissolve completely.
- b. Dilute the 12  $\mu\text{M}$  Substrate Solution 1:30 with Assay Buffer to make a 400 nM Substrate Solution (200 nM concentration in the assay).

*NOTE: Dilute only enough Substrate for immediate use (30  $\mu\text{L}$ /well).*

6. Fluorescence Standard

*NOTE: An IRDye 800CW Carboxylate Fluorescence Standard dilution series can be included if a fluorescence intensity calibration curve is to be generated. The dilution series should be prepared fresh for each experiment.*

- a. Using IRDye 800CW Carboxylate:
  - Label vials 1 through 10.
  - Add 998  $\mu\text{L}$  of Assay Buffer into vial 1 and 500  $\mu\text{L}$  of Assay Buffer into vials 2 through 10.
  - To the vial of IRDye 800CW Carboxylate, add 200  $\mu\text{L}$  of sterile water to make a 100  $\mu\text{M}$  fluorescence standard; VORTEX THOROUGHLY; centrifuge briefly.
  - Transfer 2  $\mu\text{L}$  of the 100  $\mu\text{M}$  standard into vial 1 to make 1000  $\mu\text{L}$  a 200 nM standard; mix thoroughly.
  - Prepare 10 additional fluorescence standards of 2-fold serial dilution by transferring 500  $\mu\text{L}$  from vial 1 into vial 2, mixing thoroughly, subsequently transferring and mixing as shown in Section III.

**B. Enzymatic Reaction (96-Well Plate)**

*NOTE: Duplicate or triplicate samples and standards should be run in the experiment.*

1. Reaction Wells:

- a. Place 30  $\mu\text{L}$ /well of the Caspase-3 Dilutions into the reaction wells.

2. Negative (no enzyme) Control Wells:

- a. Place 30  $\mu\text{L}$ /well of Assay Buffer into the negative (no enzyme) control wells.

3. Calibration Wells:

- a. Place 60  $\mu\text{L}$ /well of each fluorescence standard (10 solutions, serially diluted 2-fold) into the calibration wells.

4. Substrate Addition

- a. Add 30  $\mu\text{L}$ /well of Substrate Solution (400 nM) into the reaction wells and negative (no enzyme) control wells.
- b. Seal the plate with sealing foil.

*NOTE: To obtain optimal results, it is essential to seal the plate to prevent evaporation.*

5. (Optional) Time-Course Measurement

- a. Immediately start measuring the fluorescence intensity by scanning the plate every 10 minutes using an Aerius or Odyssey Imaging System with settings outlined in the **Image Plate** section.

6. Incubation

- a. Shake the plate gently at room temperature for 90 minutes on the plate shaker set at medium speed; protect from light.

7. Stop Enzymatic Reaction

- a. Remove the sealing foil.
- b. Add 60  $\mu\text{L}$  of Stop Solution to each well (including the calibration wells) using a dispenser or multichannel pipette.

*NOTE: Stop Solution should be added as quickly as possible to stop all enzymatic reactions.*

- c. Mix the plate on a plate shaker.

### C. Image Plate

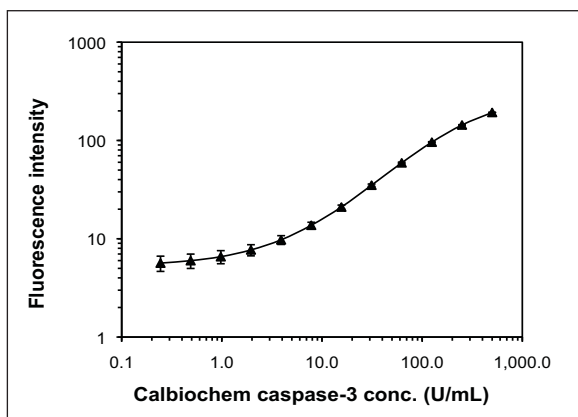
1. Image Plate on Aerius or Odyssey Infrared Imaging System
  - a. Scan the plate using the following instrument settings (these settings are a suggested starting point and may need to be optimized):

Instrument	Plate	Focus Offset (mm)	Resolution (m)	Imaging Quality Setting	Intensity Setting	
					700 Channel	800 Channel
Aerius	BD Bioscience Optilux™, Cat # 35948	3.5	200	N/A	Off	3
	Corning Costar Cat # 3603	3.5	200	N/A	Off	3
Odyssey	BD Bioscience Optilux™, Cat # 35948	3.5	169	Medium	Off	2
	Corning Costar, Cat # 3603	3.5	169	Medium	Off	2

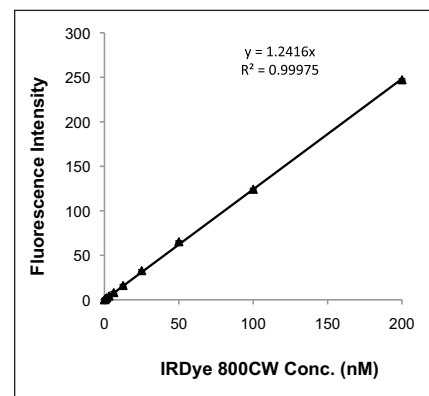
### D. Experimental Results

Figure 4 illustrates the Caspase-3 titration experimental results.

Figure 5 is the fluorescence standard calibration curve for this experiment.



**Figure 4.** Measurement of Caspase-3 activity (error bars are  $\pm$  standard deviation). IRDye 800CW/QC-1 CSP-3 (30  $\mu$ L/well at final concentration of 200 nM) was titrated with 30  $\mu$ L/well of human recombinant Caspase-3 to final concentrations of 0.24 U/mL to 500 U/mL. After incubating at room temperature for 90 minutes, the reactions were stopped with 60  $\mu$ L/well of Caspase-3 Stop Solution. The fluorescence intensity was measured on an Aerius Infrared Imaging System.



**Figure 5.** IRDye 800CW Carboxylate fluorescence standard curve ( $R^2=0.9997$ , slope=1.24).

## V. CELL-BASED CASPASE-3 ASSAY

*NOTE: Duplicate or triplicate samples and standards should be run in the experiment.*

### A. Reagent Preparation

*NOTE: All working solutions should be prepared fresh for each experiment. Keep all reagents on ice or in a cold box.*

*NOTE: It is very important to include 10 mM of TCEP reducing agent in the Caspase-3 Assay Buffer for maximum assay sensitivity.*

*NOTE: 0.2% (v/v) Triton X-100 is required for cell-based Assay Buffer.*

1. 2X Caspase-3 Assay Buffer, 30 mL  
200 mM HEPES (pH 7.5) with 2 mM EDTA, 0.2% CHAPS and 20% (v/v) glycerol
2. Stop Solution, 30 mL  
0.4 M potassium phosphate (pH 5.0) with 0.25 mg/mL BSA
3. Assay Buffer
  - a. Dissolve 18.6 mg of TCEP•HCl in 130 µL of sterile water to make 500 mM TCEP.
  - b. Prepare Assay Buffer according to the following table.

Caspase-3 Assay Buffer (2X)	6.2 mL
TCEP•HCl (500 mM)	130 µL
Triton X-100 (10%)	130 µL
Total Volume	6.5 mL

4. Substrate Solution
  - a. Reconstitute lyophilized IRDye 800CW/QC-1 CSP-3 substrate with 200 µL of sterile water to make a 12 µM Substrate Solution; mix gently to dissolve completely.
  - b. Dilute the 12 µM Substrate Solution 1:30 with Assay Buffer to make a 400 nM Substrate Solution (200 nM concentration in the assay).

*NOTE: Dilute only enough Substrate for immediate use (30 µL/well).*

### B. Cell Based Assay Procedure

*NOTE: Duplicate or triplicate samples should be run in the experiment.*

1. Adjust Jurkat cells to  $1.5 \times 10^6$ /ml in RPMI1640 with 10% serum.
2. Add anisomycin or camptothecin at a final concentration of 1 µg/ml.
3. Add 50 µl of treated cells into the wells of 96-well plate (e.g. Nunc™ 96 Microwell™ Plate).
4. Add 50 µl of untreated cells, and media into the appropriate wells of the plate as negative and background controls, respectively.
5. Incubate the plate for 4 hours at 37°C.
6. Add 50 µl of 400 nM Substrate Solution into the wells of the reaction plate and incubate for 1 hour at 37°C.

### C. Image Plate

1. Image Plate on Aeries or Odyssey Infrared Imaging System
  - a. Scan the plate using the following instrument settings (these settings are a suggested starting point and may need to be optimized):

Instrument	Plate	Focus Offset (mm)	Resolution (µm)	Imaging Quality Setting	Intensity Setting	
					700 Channel	800 Channel
Aerius Imager	Nunc™ 96 Microwell™ Plate	3.5	200	N/A	Off	3
Odyssey Imager	Nunc™ 96 Microwell™ Plate	3.0	200	N/A	Off	5

## VI. REFERENCES

- 1 N. N. Danial, S. J. Korsmeyer, Cell death: critical control points. *Cell* 116 (2004) 205-19.
- 2 N. A. Thornberry, Y. Lazebnik, Caspases: enemies within. *Science* 281 (1998) 1312-6.
- 3 E. A. Slee, C. Adrain, S. J. Martin, Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ* 6 (1999) 1067-74.

## VII. TROUBLE SHOOTING GUIDE

Problem	Possible cause	Recommendation
Low signal or failure to detect Caspase-3 activity	Low enzyme concentration	Use a minimum enzyme concentration of 0.5 U/mL for human recombinant Caspase-3 to achieve >7 signal:noise ratio; recommended enzyme concentration for the assay is greater than 30 U/mL.
	Low enzyme activity or inactive enzyme	Use human recombinant Caspase-3 (Calbiochem, Cat. #235417) for the assay; if other sources of enzyme are used, check the enzyme activity by enzyme manufacturer's test method to make sure it has the expected activity.
	Caspase-3 stability problem at low enzyme concentration	Add reducing agent, e.g. 5 mM Tris (2-Carboxyethyl) phosphine Hydrochloride (TCEP•HCl) (Pierce Cat #20490) to the assay buffer
	Nucleophiles such as DTT can damage the infrared dye	Stay below 0.5 mM DTT in assay buffer
High background from negative (no enzyme) control wells	Peptide substrate contaminated with enzyme	Prevent enzyme contamination during assay preparation
	Peptide substrate degradation during storage	Dissolve peptide substrate in 200 µL of sterile water immediately before first use, aliquot and freeze unused substrate at -20°C. Peptide substrate in water is stable for 3 months at -20°C.
High signal from concentrated inhibitor wells	Inhibitor concentration not high enough compared with its IC <sub>50</sub>	Make sure the final concentration range for the inhibitor in reaction wells has a ≥1000-fold difference from the highest to the lowest concentrations and sits around the expected IC <sub>50</sub> value.

## TROUBLE SHOOTING GUIDE *(Continued)*

Problem	Possible cause	Recommendation
	Enzyme added to the peptide substrate before mixing with inhibitor	Confirm that the enzyme is pre-incubated with inhibitor before adding peptide substrate.
Saturated signal upon scanning on Odyssey or Aeries	Intensity setting on Aeries or Odyssey at the 800 nm channel is too high	Confirm suggested channel intensity settings; adjust settings and scan plate again.
	Concentration of peptide or Carboxylate fluorescence standard is too high	Confirm the suggested dilution method was followed in the protocol for both the peptide substrate and the Carboxylate fluorescence standard.
Inconsistent readings/ poor reproducibility	Caspase-3 stability problem	Use fresh reducing agent (e.g. 5 mM TCEP) in the Assay Buffer
	Dust on bottom of plate	Clean the bottom of the plate with a delicate task wiper or dust-free cloth. 100% isopropanol may be used on most plates.
	Incorrect plate	Plates with an optical bottom are required and the suggested optimal focus offset needs to be used. Check list for recommended plates.

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