

Reagents and Equipment Required But Not Provided

(Product Codes are given where appropriate)

- Cells to undergo apoptosis. The example procedure in this bulletin uses Jurkat E6-1 cells.
- Apoptosis inducer. Apoptosis may be either spontaneous or induced. The example procedure in this bulletin uses staurosporine, Product Code S 4400.
- Phosphate buffered saline (PBS), Product Code D 8537
- DMSO, Product Code D 8418
- Bovine serum albumin (BSA), Product Code A 8022
- Spectrophotometer with quartz cuvettes
- ELISA reader and flat-bottom, 96 well plates
- Polypropylene test tubes and microcentrifuge tubes

Storage

Store the kit at -20°C .

Preparation Instructions

Note: Use 17 megohm water only (Product Code W 3888) in all the steps

- 1x Assay Buffer: 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT. Dilute 10x Assay Buffer 10-fold with 17 megohm water.
- Caspase 3 substrate (Ac-DEVD-pNA), 20 mM in DMSO. Dissolve the vial contents (15 mg) in 1.2 ml of DMSO to prepare a stock solution. Alternatively, dissolve 1 mg of substrate in 78.5 μl of DMSO. Store at -20°C .
For assays using 96 well plates dilute the 20 mM stock solution to 2 mM with 1x Assay Buffer.
- Caspase 3 inhibitor (Ac-DEVD-CHO), 2 mM in DMSO. Dissolve the vial contents (0.5 mg) in 500 μl of DMSO to prepare a stock solution. Store at -20°C .
For assays using 96 well plates dilute the 2 mM stock solution to 200 μM with 1x Assay Buffer.
- Caspase 3 positive control. Reconstitute the vial (5 μg) with 50 μl of 17 megohm water (100 $\mu\text{g}/\text{ml}$) Store in aliquots at -70°C .
Just before use, dilute an aliquot to 5 $\mu\text{g}/\text{ml}$ 20-fold in 1x Assay Buffer containing 1 mg/ml BSA or 1x Lysis Buffer containing 1 mg/ml BSA.
- 1x Lysis Buffer: 50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT. Dilute 5x Lysis Buffer 5-fold with 17 megohm water.
Note: In order to protect the cell lysate caspases from non-specific proteolysis, protease inhibitors that do not include cysteine protease inhibitors (e.g. E-64, leupeptin) may be added.

- p-Nitroaniline Standard for developing a calibration curve for assays in 96 well plate. Dissolve the vial in 0.72 of ml DMSO. Store the stock solution at -20°C . To determine the actual concentration, dilute a sample of the stock solution 100-fold in 1x Assay Buffer and determine absorbance at 405 nm using a quartz cuvette. Calculate the actual concentration of the stock solution using the molar absorptivity of $\epsilon^{\text{mM}} = 10.5$ at 405 nm.
- Staurosporine. 1 mg/ml in DMSO.

Procedure

The following procedure is an example using Jurkat cells induced to apoptosis using staurosporine and lysed prior to the determination of caspase 3 activity.

Three controls are recommended for each Caspase 3 Colorimetric Assay:

- Inhibitor-treated cell lysate control (for measuring the nonspecific hydrolysis of the substrate)
- Caspase 3 positive control
- Reagent blank (negative control)

A. Preparation of Cell Lysates from Apoptotic Cells

1. Induce apoptosis in a cell suspension of Jurkat cell (at least 10^7 cells) by addition of staurosporine to a final concentration of 1 $\mu\text{g}/\text{ml}$. Reserve a sample of non-induced cells for a zero-time control.
2. Incubate for 2.5 to 3 hours at 37°C in a 5% CO_2 atmosphere.
3. Pellet the induced cells and the control cells by centrifugation at $600 \times g$ for 5 minutes at 4°C .
4. Remove the supernatant by gentle aspiration.
5. Wash the cell pellets once with 1 ml of PBS. Centrifuge the cells and remove the supernatant completely by gentle aspiration.
6. Suspend the cell pellets in 1x lysis buffer at a concentration of 100 μl per 10^7 cells.
7. Incubate the cells on ice for 15-20 minutes.
8. Centrifuge the lysed cells at $16,000$ to $20,000 \times g$ for 10 to 15 minutes at 4°C .
9. Transfer the supernatants to new tubes.
10. Analyze the lysates immediately or freeze in liquid nitrogen and store in aliquots at -70°C .

B. 1 ml Volume Assay Method

Equipment required: test tubes, spectrophotometer, and 1 ml quartz cuvettes

- The positive control volume recommended in the reaction scheme is compatible with the expected activity found in $0.5-1.5 \times 10^6$ apoptotic Jurkat cells. The positive control and sample volumes can be increased if required.
- Use quartz cuvettes only, since plastic cuvettes attenuate the absorption at 405 nm.
- Yellowish color is visualized by the naked eye at approximately 0.2 OD at 405 nm.

- Place 10 μ l of cell lysate or Caspase 3 Positive Control in the appropriate tubes as indicated in Table 1.
- Add 1x Assay Buffer to each of the tubes as indicated in Table 1.

- Add 10 μ l of caspase 3 inhibitor to the appropriate tubes.
- Start the reaction by adding 10 μ l of caspase 3 substrate to each tube and mix gently. Cover the tubes and incubate at 37 °C for 1.5 to 2 hours. If signal is too low, continue the incubation over night. Read Absorbance at 405 nm.
- Calculate the caspase 3 activity in μ mol of pNA released per min per ml of cell lysate or positive control based on the formula:

$$\text{Activity, } \mu\text{mol pNA/min/ml} = \frac{\text{OD} \times d}{\epsilon^{\text{mM}} \times t \times v}$$

Where: $\epsilon^{\text{mM}} = 10.5$

v - volume of sample in ml

d - dilution factor

t - reaction time in minutes

Table 1.

Reaction scheme for 1 ml Volume Assay Method

	Cell lysate	Caspase 3 5 μ g/ml	Assay buffer	Caspase 3 inhibitor Ac-DEVD-CHO 2 mM	Caspase 3 substrate Ac-DEVD-pNA 20 mM
Reagent blank	----	----	990 μ l	----	10 μ l
Non-induced cells	10 μ l	----	980 μ l	----	10 μ l
Non-induced cells + inhibitor	10 μ l	----	970 μ l	10 μ l	10 μ l
Induced cells	10 μ l	----	980 μ l	----	10 μ l
Induced cells + inhibitor	10 μ l	----	970 μ l	10 μ l	10 μ l
Caspase 3 positive control	----	10 μ l	980 μ l	----	10 μ l
Caspase 3 positive control + inhibitor	----	10 μ l	970 μ l	10 μ l	10 μ l

C. 96 Well Plate Microassay Method

Equipment required: flat bottom 96 well plate and ELISA reader

- The positive control volume recommended in the reaction scheme is compatible with the expected activity found in 0.25 to 1×10^6 apoptotic Jurkat cells. The positive control and sample volumes can be increased if required.
 - Yellowish color is visualized by the naked eye at approximately 0.2 OD 405 nm.
- Place 5 μ l of cell lysate or Caspase 3 Positive Control in the appropriate wells as indicated in Table 2.
 - Add 1x Assay Buffer to each of the wells as indicated in Table 2.

- Add the Caspase 3 Inhibitor to the appropriate wells.
- Start the reaction by adding 10 μ l of caspase 3 substrate to each well and mix gently by shaking. Try to avoid forming bubbles in the wells.
- Cover the plate and incubate at 37 °C for 70 to 90 minutes. If signal is too low, continue the incubation over night.
- Read Absorbance at 405 nm.
- Calculate the results using a p-nitroaniline calibration curve. This method is recommended for accurate results to avoid miscalculations that stem from incompatibility of the ELISA reader and the plastic plates.

Table 2.

Reaction scheme for 96 Well Plate Microassay Method

	Cell lysate	Caspase 3 5 µg/ml	1× Assay buffer	Caspase 3 inhibitor Ac-DEVD-CHO 200 µM	Caspase 3 substrate Ac-DEVD-pNA 2 mM
Reagent blank	----	----	90 µl	----	10 µl
Non-induced cells	5 µl	----	85 µl	----	10 µl
Non-induced cells + inhibitor	5 µl	----	75 µl	10 µl	10 µl
Induced cells	5 µl	----	85 µl	----	10 µl
Induced cells + inhibitor	5 µl	----	75 µl	10 µl	10 µl
Caspase 3 positive control	----	5 µl	85 µl	----	10 µl
Caspase 3 positive control + inhibitor	----	5 µl	75 µl	10 µl	10 µl

D. p-Nitroaniline (pNA) Calibration Curve

1. Prepare a series of p-nitroaniline solutions at the concentration range of 10 to 200 µM by diluting the p-nitroaniline stock solution in 1× Assay Buffer.
2. Add 100 µl of each dilution to a well. Include 100 µl of assay buffer as a blank.
3. Read Absorbance at 405 nm.
4. Prepare a calibration curve of the absorbance values versus the concentrations of the p-nitroaniline solutions. Alternatively, plot the OD₄₀₅ values versus the amount of p-nitroaniline per well, in µmol, using the following table as a guideline.

µM p-Nitroaniline	µmol p-Nitroaniline per 100 µl
10	0.001
20	0.002
50	0.005
100	0.01
200	0.02

5. Calculate the caspase 3 activity in µmol pNA released per min per ml of cell lysate or positive control based on the formula:

$$\text{Activity, } \mu\text{mol pNA/min/ml} = \frac{\mu\text{mol pNA} \times d}{t \times v}$$

Where:

- v - volume of sample in ml
d - dilution factor
t - reaction time in minutes

References

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