Caspase 9 Activity Assay Kit (Colorimetric Method)

Cat. No.: P-CA-389

Size: 20Assays/100Assays

Components

Name	20Assays	100Assays	Storage
Cell Lysis Buffer	50 mL	50 mL	-5~-20°C
2 × Reaction Buffer	50 mL	50 mL	-5~-20°C
Ac-LEHD-pNA (4 mM)	100 μL	500 μL	-5~-20°C, shading light
pNA (10 mM)	200 μL	1 mL	-5~-20°C, shading light
Manual	One copy		

Introduction

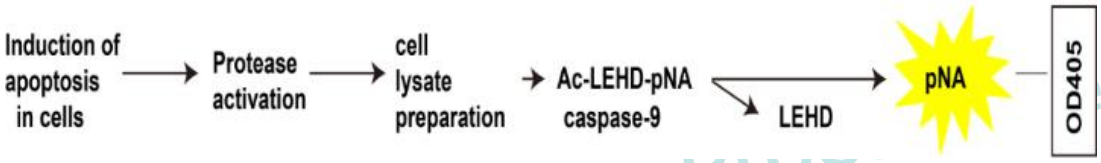
Procell Caspase 9 Activity Assay Kit uses spectrophotometry to detect the Caspase 9 activity of cells, tissue lysates or other samples.

Background

Caspase 9, also known as ICE-LAP6 or Mch6, is a relatively upstream caspase in the process of apoptosis signal transduction. After mitochondria release cytochrome c, caspase 9 can form complex with cytochrome c and Apaf1 and can be activated. Activated caspase 9 can activate caspase 3, the most critical enzyme of apoptosis, thereby promoting subsequent apoptotic signals. The activation of caspase 9 can be regulated by phosphorylation.

Detection Principle

This Caspase 9 activity assay kit is based on Caspase 9 that can catalyze the substrate Ac-LEHD-pNA to generate yellow pNA (p-Nitroaniline). The pNA has a strong absorption near 405 nm, The activity of Caspase 9 can be calculated by determining the absorbance at 405 nm.



Detection Sample Types

☑ **Cell sample** ☑ **Tissue sample**

Storage

Store at -5~-20°C for 1 year. It is recommended to aliquot the Ac-LEHD-pNA (4 mM) into smaller quantities and store in the dark. Avoid repeated freezing and thawing.

Materials Not Supplied

1. **Reagents**

PBS, Protein quantification kit (Bradford method).

2. Instrument

Spectrophotometer/microplate reader, centrifuge, incubator, pipette, mortar or homogenizer.

Notes

1. All samples need to be detected for protein concentration. Since the cell lysis buffer contain reducing agents, it is not suitable to detect protein concentration with BCA method, the Bradford method is recommended.
2. It is recommended to take 2-3 samples which expected large difference to do pre-experiment before formal experiment. If the absorbance of sample exceeds the measurement range of the standard curve, the sample needs to be diluted or the sample volume needs to be adjusted before measurement.
3. The protein concentration of the sample used for Caspase 9 activity detection should be 1-4 mg/mL, otherwise it will affect the accuracy of the experimental results.
4. It is recommended that the number of cells for one sample should not be less than 1×10^6, and the tissue sample should not be less than 50 mg, make sure that the protein concentration is 1-4 mg/mL. Otherwise, it will affect the accuracy of the experiment, resulting in low protein concentration, low activity of Caspase 9 in the reaction system, and low OD value.

Preparation

All reagents dissolved after mixing, placed in ice bath for ues.

Sample Preparation

1. Cell sample

Collect the cells with conventional methods and resuspended the cells with PBS, then count the cells, centrifuge at 600 ×g for 5 min, discard the supernatant and keep the cell pellet. Resuspended the cell pellet with pre-cooled Cell Lysis Buffer according to the ratio of 100 μL of Cell Lysis Buffer per 1 million cells, incubate on an ice bath for 30 min (Mix 3-4 times, 10s each time), and then centrifuge the lysed sample at 11000 ×g for 10-15 min at 4°C. Carefully absorb the supernatant to a new EP tube and place it on ice for use. Meanwhile, determine the protein concentration of supernatant .

Note: When detecting adherent cells, the suspension cells generated after induction of apoptosis should be collected and detected together with the subsequently collected adherent cells.

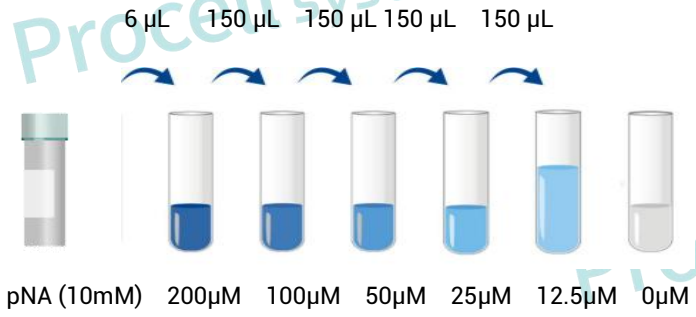
2. Tissue sample

A total of 50 mg tissue samples were washed with PBS or normal saline for 1-2 times to remove the residual blood cells in the tissue and cut into pieces with surgical scissors, add 200 μL of pre-cooled Cell Lysis Buffer, and homogenize the sample on ice (If the quality of the tissue is doubled, the pre-cold Cell Lysis Buffer needs to be doubled). Transfer the homogenized samples to a 1.5 mL tube and lysed in an ice bath for 30 min. The lysed samples were centrifuged at 11000 ×g at 4°C for 10-15 min, Carefully absorb the supernatant to a new EP tube and placed on ice for use. Meanwhile, determine the protein concentration of supernatant.

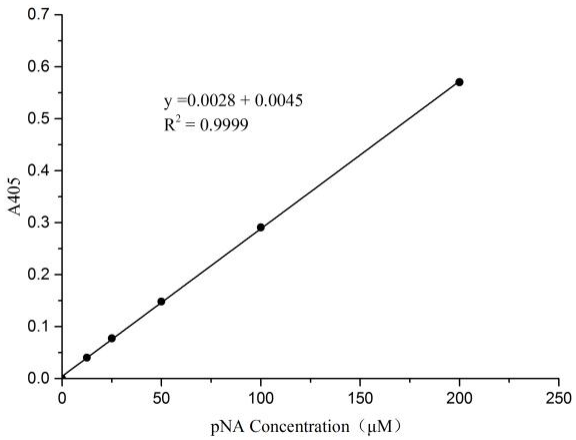
Note: The prepared sample should be determined immediately. If it cannot be determined in time, the supernatant after lysed can be stored at -80°C within 2 weeks

Experimental Operation

1. **(Optional) Preparation of pNA standard curve**
   1) Preparation of standard diluent: Take Cell Lysis Buffer and 2 × Reaction Buffer according to the ratio of 1:1 to prepare the standard diluent and mix fully.
   2) Dilution method: Take 6 EP tubes, add 294 μL of standard diluent to the first tube and 150 μL of standard diluent to the other each tube. Pipette 6 μL of pNA (10 mM) to the first tube and mix up to produce a 200 μM pNA working solution. Pipette 150 μL of the solution from the former tube into the latter one according to this step. The illustration below is for reference. **Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.**



   3) Take 100 μL of different concentrations standard solutions to the cuvettes or wells of microplate and detect the OD value at 405 nm (**In order to ensure the accuracy of experimental results, it is recommended that all samples and standards be assayed in duplicate**).
   4) Average the duplicate readings for each standard, then subtract the average zero standard OD values. Plot a standard curve with standard concentration on x-axis and absolute OD values on the y-axis. **The measured data may be different due to different experimental conditions, detection instruments, etc. and the data in the figure is for reference only.**



2. Detection of Caspase 9 activity

1) Take 50 μL of lysed sample solution and set the reaction system according to the table below.

	Blank	Experimental group
Cell Lysis Buffer	50 μL	0 μL
2 × Reaction Buffer	45 μL	45 μL
Sample	0 μL	50 μL
Ac-LEHD-pNA	5 μL	5 μL
Total Volume	100 μL	100 μL

Note: When setting the reaction system, add 2 × Reaction Buffer first, then add sample and mix gently. Finally, add Ac-LEHD-pNA and mix it. Pay attention to avoid the production of bubbles.

2) Incubate at 37°C for 1-2 h, OD405 can be detected when the color change is obvious. If the color change is not obvious, the incubation time can be appropriately extended to 4 h.

Results calculation

Method 1: Calculate according to the percentage increase of enzyme activity

$$\text{Caspase 9 activity (100\%)} = \frac{OD_{sample}-OD_{blank}}{Cpr_{sample}} \div \frac{OD_{negative}-OD_{blank}}{Cpr_{negative}} \times 100\%$$

Note: The negative is a biological control group that does not undergo apoptosis treatment.

Method 2: Calculate according to enzyme activity

1. Establish a standard curve: according to the concentration of the standard tube (x, μmol/L), and the absorbance (y, minus the blank tube with a concentration of 0) as the standard equation **y = ax+b**. The concentration of the sample can be calculated according to the formula based on the OD value of sample. If the sample have benn diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. The actual concentration is the calculated concrntration multiplied by dilution factor.
2. Calculate Caspase 9 activity:
   Definition: One unit is the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-LEHD-pNA per hour at 37°C under saturated substrate concentrations.

$$\text{Caspase 9 activity (U/mgprot)} = \frac{\triangle A-b}{a} \times \frac{V_1}{V_2 \times Cpr \times T} \times f$$

Note：

Y: $OD_{standard}$-OD_{black}

x: Concentration of standard

a: Slope of the curve

b: Intercept of the curve

△A: ODsample-ODblack

V1: Total volume of the reaction system, 0.1 mL

V2: The sample volume of the addition, 0.05 mL

T: Reaction time, h

Cpr: The protein concentration before the sample was added to the detection system, mgprot / mL

f: The dilution multiple of the sample before adding the detection system

Troubleshooting

Symptoms	Causes	Comments
OD405 is low or no signal	The drug does not activate Caspase 9.	Not all apoptosis can be detected in Caspase 9, it is necessary to set the time gradient of drug treatment, or according to the common positive control model to set the control test.
	The density of cell induction is too high.	Reduce the cell density during inducement, explore the best induction density, and the recommended induction density is 5-10 ×10^5 /mL.
	Too few cells, too many Cell Lysis Buffer.	Increase the number of cells and add 50-100 μL Cell Lysis Buffer per 1 million cells.
	Total protein content of sample to be tested is too low.	Increase the total amount of protein detected, usually not less than 50 μg. It is recommended to confirmation by pre-experiment.
	High temperature leads to inactivation of DTT in Cell Lysis Buffer and caspase.	Cell Lysis Buffer and cell lysis process were performed on ice and vortexed every 10 min to fully ensure enzyme activity.
	The poor storage conditions of cell precipitation lead to cell degradation and caspase enzyme inactivation.	Use the fresh samples as possible, or after collecting the cell precipitate, immediately put it into -5~-20°C (1 month) or -80°C (2 months) for storage and avoid repeated freeze-thaw.
	Inappropriate detection wavelength	The range of 405±20 nm can be used, the optimal detection wavelength is 405 nm.
	The incubation time of 37°C is too long.	Appropriately extend the incubation time at 37°C. It is recommended that the best incubation time is within 1-2 h. As time is extended, the background value of the control group will gradually increase, resulting in low results.
OD405 is high	Reagents or drug that treat cells are colored and have an absorption peak at 405 nm.	Increase the number of centrifugations, set the reagent as blank control and minus the impact of drug reagents when calculating the final results.
	There are many bubbles in the sample when measuring the OD values.	Detection after eliminating bubbles.
	There are impurities in the recycled 96-well plates, resulting in high results.	It is recommended to use a new disposable 96-well plates.

Notes

1. This product is only used for scientific research or further research, not for diagnosis and treatment.
2. Please take safety precautions and follow the procedures of laboratory reagent operation.
3. The pNA (4-nitroaniline) is toxic to the human body. Please be careful during operating, and pay attention to avoid direct contact with the human body or inhalation. The pNA will solidify at a lower temperature and stick to the bottom, wall or cap of centrifuge tube. It can be used until it is completely melted in a water bath at 20-25°C for a moment.

4. When the level of activated caspase in the sample is low, first confirm whether apoptosis is obvious. If cell apoptosis is obvious and it is confirmed that the caspase can be activated, the time of inducing cells can be adjusted properly to find a time point when the caspase activation is relatively strong, so as to detect the activation of the caspase. It is recommended to plot a time curve, such as 0, 2, 4, 8, 16, and 24 hours of induction, or 0, 1, 2, 4, 8, and 16 hours, or 0, 1, 2, 4, 6, and 8 hours. Specific induction time needs to be determined according to the specific situation.
5. In the reaction system of this kit, the initial concentration of the substrate is 0.2 mM. For most samples, the substrate is saturated within 2 hours of incubation at 37°C. For a few samples that the enzyme vitality is particularly high, it is necessary to use Cell Lysis Buffer to dilute the sample appropriately before determination.

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