Caspase-9 Colorimetric Assay Kit

Introduction

Cysteine-containing aspartate proteolytic enzymes (Caspase) are a family of cysteine proteases that play important roles in apoptosis, necrosis, and inflammation. Caspase-9 is a priming type of caspase that initiates endogenous apoptosis, which is apoptosis of the mitochondrial pathway. When cells are stressed, Cytochrome c is released into the cytoplasm and binds to the apoptotic protein activator 1 (Apaf-1) to form a complex. Caspase-9 was then recruited into this complex, forming the apoptotic bodies. In apoptotic bodies, caspase-9 is activated by self-cleavage. Activated caspase-9 further activates downstream effector caspases, such as caspase-3 and caspase-7.

Caspase-9 Colorimetric Assay Kit provides a convenient and simple method for the detection of LEHD-dependent caspase activity. When Caspase-9 or related caspases cleave LEHD from the labeled substrate LEHD-p-nitroaniline (LEHD-pNA), the light emission of free pNA can be measured at 405 nm or 400 nm using a microplate reader or spectrophotometer. The absorbance of free pNA in apoptotic samples was compared to an uninduced control to determine a fold increase in Caspase-9 activity.

Components and Storage

Components	K2019-25T	K2019-50T	K2019-100T
Reagent I	20 mL	20 mL	25 mL
Reagent II	30 mL	60 mL	120 mL
Reagent III	0.25 mL	0.55 mL	2 X 0.55 mL
pNA Standard (5 mM)	1 mL	1 mL	1 mL

Store the kit at -20°C, stable for 6 months. Reagent III and pNA Standard (5 mM) should be stored away from light.

Protocol

1. **Standard Diluent Preparation:** Add 1 mL of Reagent II per 9 mL of Reagent I, mixing to make the Standard Diluent.

2. **Sample Preparation:**

   1) **Cells:** Harvest cells, and discard supernatant after centrifugation. Add 100 μL of Reagent II to resuspend cells in a density of 10^7 cells/mL. Incubate on ice for 15 min. Centrifuge at 10,000 g at 4°C for 10-15 min.

2) **Tissue:** Add 1 g of tissue mass per 5-10 mL of Reagent II volume (e.g., weigh 0.1 g of tissue and add 1 mL of Reagent II). Homogenize tissue well in an ice bath, and incubate on ice for 15 minutes. Centrifuge at 10,000 g at 4°C for 10-15 min.

3. Transfer the supernatant to a new tube, place it on ice for immediate assay or store it at -80°C in aliquots for later use. At the same time, measure protein concentration Cpr using the Bradford method.

***Note:** Because Reagent II contains DTT, the Bradford method is recommended for measuring the protein concentration, and the BCA method is not recommended. At the same time, the protein concentration Cpr should be as high as possible to reach 1-4 mg/mL, which is beneficial for later detection.

4. **Standard Curve Preparation (Optional):**

1) Dilute pNA Standard (5 mM) to a series of 200, 100, 50, 25, 12.5, 0 μM standard solutions with the Standard Diluent. It is recommended to prepare fresh standard solutions every time.

2) After taking 100 μL of each concentration into a 96-well plate, immediately detect the absorbance (A) at 405 nm with a microplate reader. The absorbance value A for each standard concentration minus the absorbance value A for the blank group (0 μM) can be used to fit the actual pNA standard curve.

5. Add the following reagents to a new 96-well plate or EP tube, mix well, close the lid tightly and seal with parafilm. Incubate at 37°C for 1-2 h. When the color change of the solution is obvious to the naked eye, detect the absorbance (A) at 405 nm with a microplate reader. Calculate $\Delta A=A_{\text{detection tube}}-A_{\text{blank tube}}$. If the color change is not obvious, the incubation time can be extended appropriately or overnight.

Reagent	Detection tube	Blank tube
Reagent I	40 μL	40 μL
Sample	50 μL	
Reagent II		50 μL
Reagent III	10 μL	10 μL

6. **Data analysis:** Choose one of the following analysis methods based on your needs.

1) Caspase-9 activity = $(\Delta A_{sample}\div Cpr_{sample})/(\Delta A_{control}\div Cpr_{control})$

***Note:** The control group is the group of normal cultured cells without treatment.

2) Establish a standard curve y=ax+b, and then the Caspase enzyme activity unit definition is calculated as following:

Caspase-9 activity (U/mg prot) $=(\Delta A-b)\div a\times V_1\div(V_2\times Cpr\times T)$

***Note:** V_1: The total volume of the reaction system, 0.1 mL=10^{-4} L; V_2: The volume of the sample added, 0.05 mL; T: Reaction time, h; Cpr: Sample protein concentration, mg/mL.

Note

1. Because Reagent II contains DTT, the Bradford method is recommended for measuring the protein concentration of the sample, and the BCA method is not recommended. Try to achieve a protein concentration of 1-4 mg/mL, and the detection effect is better at this time.
2. It is recommended to select 2-3 samples with large differences expected for pre-experiments before the formal experiment. If the absorbance of sample is outside the standard curve range, it can be diluted or the sample volume can be adjusted; If the absorbance of sample is too low, it is recommended to increase the number of cells used. In addition, low absorbance values may also indicate that the observation time is not appropriate. It is recommended to set different doses and different detection time points such as 0, 2, 4, 8, 16, and 24 h to determine the optimal conditions for the experiment.
3. For your safety and health, please wear lab coats and gloves during the experiment.
4. For research use only. Not to be used in clinical diagnostic or clinical trials.

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