Protease Activity Assays

References

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Kit

1. Calbiochem Calpain Activity Assay Kit, Fluorogenic, Cat# QIA1201KIT

Solutions

Assays using cultured cells/tissue lysates

Calpain

1. Homogenization Buffer: 170 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA) in water
2. Extraction Buffer: 5 mM EDTA, 150 mM NaCl, 0.5 % Triton X-100, 20 mM Tris-HCl, pH 7.4
3. 10X reaction buffer 1X: 50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 30 mM NaCl, and 5 mM dithiothreitol (DTT)

Caspase 3

1. Extraction Buffer: 50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.4
2. Assay Buffer: 100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10 % glycerol 0.1% CHAPS, pH 7.4

Caspase 2

1. Extraction Buffer: 50 mM HEPES, 10 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4
2. Assay Buffer: 100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1mM EDTA, 10 % glycerol 0.1% CHAPS, pH 7.4

AEP

1. Assay Buffer: 20 mM citric acid, 60 mM Na_2HPO_4, 1 mM EDTA, 0.1% CHAPS and 1 mM DTT, pH 6.0

In Vitro Assays

Calpain

1. Dissolving Buffer: 20 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, DTT 10 mM, 2 mM phenylmethylsulfonyl fluoride (PMSF)
2. Reaction Buffer: 50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 30 mM NaCl, and 5 mM DTT
3. Calpain Solvent: 20 mM imidazole, 5 mM beta mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 30% glycerol, pH to 6.8

Caspase 2, Caspase 3, and AEP

1. Assay Buffer: 100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4
2. AEP buffer: 50 mM Sodium Citrate, 5 mM DTT, 0.1% CHAPS, and 0.5% Triton X-100, pH 6.0

Protocol

Protease Activity Assays using cultured cells or tissue lysates

Calpain Activity Assay

1. Harvest cells (2 x 10^6 cells) in 150 µL of homogenization buffer.
2. Pellet cells by centrifugation at 500 x g for 5 min at 4°C.
3. Resuspend cells in 100 µL of extraction buffer and incubate on ice for 20 min. Gently mix the samples by pipetting up and down.
4. Centrifuge at 10,000 x g for 5 min in a microcentrifuge at 4°C.
5. Transfer the supernatant to a new Eppendorf tube and place on ice.
6. Determine protein concentration by the modified Lowry method.
7. Dilute the cell lysate (50-200 µg) in 85 µL of extraction buffer. For positive controls, use 1-2 µL of active calpain (Calbiochem, 1 µg/µL) and bring the volume to 85 µL with extraction buffer. For negative controls, add 1µL of 1 mM calpain inhibitor Z-LLY-FMK to the treated cell lysate.
8. Add 10 µL of 10X reaction buffer and 5 µL of 2 mM calpain substrate Ac-LLY-AMC to each well in a 96-well plate (fluorescence friendly).
9. Incubate at 37°C for 1 h in the dark.
10. Read the fluorescence at an excitation wavelength of 360-380 nm and an emission wavelength of 440-460 nm using a fluorescence microplate reader.

Caspase 3 Activity Assay

1. Harvest cells (2 x 10^6 cells) in phosphate buffered saline (PBS).
2. Centrifuge at 500 x g x 5 min.
3. Resuspend the pellet in Extraction Buffer.
4. Incubate on ice for 10 min.
5. Centrifuge at 10,000 x g x 5 min.

6. Transfer the supernatant to a new Eppendorf tube and place on ice.
7. Determine protein concentration using the modified Lowry method.
8. Dilute 100-200 µg of cytosolic extract into 50 µL of assay buffer. Transfer to a 96 well plate.
9. Add 50 µL of assay buffer/well and 10 µL /well of caspase-3 fluorometric substrate (0.3 mM Ac-DEVD-AMC, Calbiochem. Final concentration: 30 µM).
11. Cover the plate and incubate at 37°C for 2 hrs.
12. Read the plate using a fluorescence plate reader measuring excitation at 360 nm and emission at 460 nm.

Caspase 2 Activity Assay

1. Harvest cells (2 x 10^6 cells) in PBS.
2. Centrifuge at 500 g x 5 min.
3. Resuspend the pellet in chilled Extraction Buffer.
4. Incubate on ice for 10 min.
5. Centrifuge at 10,000 x g x 5 min.
6. Transfer supernatant (cytosolic extract) to a fresh tube and keep on ice.
7. Determine protein concentration using a modified Lowry method.
8. Dilute 100-200 µg of the cytosolic extract into 50 µL assay buffer. Transfer to a 96 well plate.
9. Add 50 µL Assay Buffer.
10. Add 5 µL /well of caspase-2 fluorometric substrate (4 mM VDVAD-AMC, Abcam, Cambridge, MA) (Final concentration: 200 µM).
11. Cover the plate and incubate at 37°C for 1-2 hrs.
12. Read samples using a fluorescence plate reader measuring excitation at 360 nm and emission at 460 nm.

AEP Activity Assay

1. Harvest cells (2 x 10^6 cells) in cold PBS.
2. Centrifuge at 500 g x 5 min.
3. Resuspend the pellet in Assay Buffer.
4. Incubate on ice for 10-30 min.
5. Centrifuge at 10,000 x g x 5 min.
6. Transfer supernatant (cytosolic extract) to a fresh tube and keep on ice.
7. Determine protein concentration using the modified Lowry method.
8. Dilute 100-200 µg of the cytosolic extract in 50 µL of Assay Buffer. Transfer to a 96 well plate.
9. Add 50 µL Assay Buffer (final volume 100 µL).
10. Add AEP substrate Z-Ala-Ala-Asn-AMC (Bachem, Torrance, CA) at a final concentration of 20 µM.
11. Cover the plate and incubate at 37°C for 1-2 hrs.
12. Quantify AMC released by substrate cleavage by measuring at 535/587 nm in a fluorescence plate reader.

In Vitro Assays

Calpain Activity Assay

1. Dissolve tau recombinant proteins (6 µg) in 50 µL of Dissolving Buffer.
2. Add 10 µL of reaction buffer and 1 µg/U calpain (EMD Bioscences, Inc., San Diego, CA) diluted in calpain solvent.
3. Incubate for 1 h at 30°C in water bath.
4. Add equivalent volume of 2X Laemmli buffer to stop the reaction and boil for 10 min (ref 5).
5. Separate tau fragments by SDS-PAGE followed by Western blot analysis using specific tau antibodies (i.e., clone tau5, diluted 1:1000, Abcam) (ref 4, ref 5).

Caspase 2, Caspase 3, and AEP assays

1. Incubate recombinant tau proteins (6 µg) diluted as described earlier with recombinant caspase 2 (5 µg/mL), caspase 3 (5 µg/mL) (G-Biosciences) in assay buffer or with recombinant AEP protein (Novoprotein, 5 µg/mL) in AEP buffer.
2. Incubate for 1 h at 37°C.
3. Add equivalent volume of 2X Laemmli buffer to stop the reaction and boil for 10 min (ref 5).
4. Separate tau fragments by SDS-PAGE followed by Western blot analysis using specific tau antibodies (i.e., clone tau5, diluted 1:1000, Abcam) (ref 4, ref 5).