ab65302
Cathepsin D Activity Assay Kit (Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of cathepsin D activity in various samples.

This product is for research use only and is not intended for diagnostic use.
1. **BACKGROUND**

Cathepsin D Activity Assay Kit (fluorometric) (ab65302) is a fluorescence-based assay that utilizes the preferred cathepsin-D substrate sequence GKPILFFRLK(Dnp)-D-R-NH₂, labeled with MCA. Cell lysates or other samples that contain cathepsin-D will cleave the synthetic substrate to release fluorescence, which can then easily be quantified using a fluorometer or fluorescence plate reader at Ex/Em = 328/460 nm.

Apoptosis can be mediated by mechanisms other than the traditional caspase-mediated cleavage cascade. There is growing recognition that alternative proteolytic enzymes such as the lysosomal cathepsin proteases may initiate or propagate proapoptotic signals. Cathepsins are lysosomal enzymes that are also used as sensitive markers in various toxicological investigations.
2. **ASSAY SUMMARY**

Sample preparation

Add reaction mix and incubate at 37°C for 1-2 hrs

Measure fluorescence (Ex/Em = 328/460 nm)
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 6 months.**
5. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD Cell Lysis Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>CD Reaction Buffer</td>
<td>5 mL</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>CD Substrate (1mM)</td>
<td>200 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

6. **MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully perform this assay:

- PBS
- Fluorescent microplate reader – equipped with filter for Ex/Em = 328/460 nm
- 96 well plate: black plates (clear bottoms) for fluorometric assay
- Microcentrifuge
- Pipettes and pipette tips
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)
7. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Keep enzymes, heat labile components and samples on ice during the assay.

- Make sure all buffers and solutions are at room temperature before starting the experiment.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure the heat block/water bath and microplate reader are switched on.
9. **REAGENT PREPARATION**

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Cell Lysis Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C once opened.

9.2 **CD Reaction Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C once opened.

9.3 **CD Substrate (1 mM):**

Ready to use as supplied. Store at -20°C away from light.
10. **SAMPLE PREPARATION**

**General Sample information:**

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

10.1 **Cell (adherent or suspension) samples:**

10.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1 x 10⁶ cells).

10.1.2 Wash cells with ice cold PBS.

10.1.3 Resuspend cells in 100 µL of PBS.

10.1.4 Homogenize cells quickly by pipetting up and down a few times.

10.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

10.1.6 Resuspend cells in 200 µL of chilled CD Cell Lysis Buffer. Incubate cells on ice for 10 minutes.

10.1.7 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

10.1.8 Transfer the clear cell lysate into a labeled new tube.
10.2 **Tissue samples:**

10.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 100 mg).

10.2.2 Wash tissue in cold PBS.

10.2.3 Resuspend tissue in 1 mL of CD Cell Lysis Buffer.

10.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

10.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

10.2.6 Transfer the clear tissue lysate into a labeled new tube.

**NOTE:** We suggest using different volumes of sample to ensure readings are within the Standard Curve range.
11. **ASSAY PROCEDURE and DETECTION**

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

11.1 **Set up Reaction wells:**

- Treated Sample wells = 5 – 50 µL samples (adjust volume to 50 µL/well with Cell Lysis Buffer).
- Untreated Sample wells = 5 – 50 µL samples (adjust volume to 50 µL/well with Cell Lysis Buffer).

11.2 **Reaction Mix:**

Prepare CD Reaction Mix for each reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Fluorometric Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Buffer</td>
<td>50</td>
</tr>
<tr>
<td>Substrate</td>
<td>2</td>
</tr>
</tbody>
</table>

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

\[ X \, \mu L \, \text{component} \times (\text{Number samples} + \text{Standards} + 1) \]

11.3 Add 52 µL of Reaction Mix to each well.
11.4 Incubate at 37°C for 1 - 2 hours, protected from light.
11.5 Measure output on a microplate reader.

- Fluorometric assay: measure Ex/Em = 328/460 nm.
12. **CALCULATIONS**

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Cathepsin D activity can be expressed by the relative fluorescence units (RFU) per million cells, or RFU per microgram protein of your sample, or RFU fold increase of treated samples vs the untreated control or the negative control sample.

13. **TYPICAL DATA**

**TYPICAL STANDARD CURVE** – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

![Typical Standard Curve](image)

**Figure 1:** Cathepsin D levels were measured in standard control samples from ab119586.
DATA ANALYSIS

Figure 2: Cathepsin D measured in mouse tissue lysates (mg of extracted protein).

Figure 3: Cathepsin D measured in cell lysates.
14. **QUICK ASSAY PROCEDURE**

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare Lysis Buffer, Reaction Buffer and Substrate; (aliquot if necessary); get equipment ready.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for treated samples (50 µL) and untreated samples (50 µL).
- Prepare CD Reaction Mix (Number samples + 1)
- Add 2 µL CD Reaction Mix to all sample wells.
- Incubate plate 37°C 1-2 hrs.
- Measure plate at Ex/Em= 328/460 nm for fluorometric assay.

<table>
<thead>
<tr>
<th>Component</th>
<th>Fluorometric Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Buffer</td>
<td>50</td>
</tr>
<tr>
<td>Substrate</td>
<td>2</td>
</tr>
</tbody>
</table>
### 15. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use PCA precipitation protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
16. FAQ

How can we control auto-activation during the lysis and assay procedure?

The Cell Lysis buffer will eventually lyse everything, however, only activated form can cleave the substrate. Autoactivation can be accounted for by using non-treated samples as a control.

What is the sensitivity of this kit?

10 – 100 ng/assay.

What is the MCA used in this kit?

MCA is 7-methoxycoumarin-4-acetic acid.
17. INTERFERENCES
18. **NOTES**
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