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ProductInformation

Cathepsin D Assay Kit

Product Code **CS0800**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Cathepsin D is an ubiquitous aspartic protease belonging to the A1 peptidase family with an activity profile similar to pepsin A. It is intracellularly located in the lysosomes and its biological role is varied. The enzyme has been associated with apoptotic events such as the release of cytochrome c from mitochondria and the loss of the transmembrane potential ($\Delta\psi$),[1] with aging,[2,3] Alzheimer's disease,[4] and breast cancer.[5] Cathepsin D overexpression in breast cancer cells has been found to be associated with increased risk of metastasis in patients and thus, is useful in diagnosis and prognosis of breast and other cancers.[5,6]

The kit assay is based on the hydrolysis by the enzyme of an internally quenched fluorimetric substrate.[7] This enables direct, real-time measurement of the product released by the action of the enzyme.

The Cathepsin D Assay Kit provides all the reagents required for efficient detection of cathepsin D activity in cell lysates, tissue homogenates, lysosomal isolated fractions, or purified enzyme preparations.

The kit was tested with CHO, HeLa, HEK 293, A431, HepG2, U937, and Jurkat cells; with yeast cells (*S. cerevisiae*); and with rat brain, kidney, and liver tissues.

Components

The kit is sufficient for 120 × 100 µl reactions in a multiwell plate.

Assay Buffer 1× Product Code A3855	20 ml
Cathepsin D from bovine spleen Product Code C3138	5 units
Albumin Solution Product Code A3980	1 ml
Cathepsin D Substrate Solution, 1 mM Product Code C4492	0.25 ml
7-Methoxycoumarin-4-acetic acid Standard Solution Product Code M0320	0.1 ml
Pepstatin A Solution Product Code P3749	0.6 ml

Equipment required but not provided

- Fluorimetric plate reader with temperature control
- Nunc® FluoroNunc™ 96 well plates (Product Code P8741 or equivalent)
- 37 °C incubator (optional)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Use ultrapure water for preparation of reagents.

Enzyme Dilution Buffer – For the Cathepsin D control assay, prepare 1 ml of Enzyme Dilution Buffer by adding 25 µl of Albumin Solution (Product Code A3980) to 975 µl of Assay Buffer 1× (Product Code A3855). Mix well by inversion.

MCA Standard Solution – Dilute the 7-Methoxycoumarin-4-acetic acid Standard Solution (Product Code M0320) 20-fold to 50 µM with Assay Buffer 1× (add 5 µl of the 1 mM 7-Methoxycoumarin-4-acetic acid Standard Solution to 95 µl of Assay Buffer 1×).

Cathepsin D Control Solutions – Dissolve the contents of one vial of Cathepsin D (Product code C3138) in 0.5 ml of water. This Cathepsin D Stock Solution contains ~0.7 mg-protein/ml. The Cathepsin D Stock Solution can be aliquotted and stored at –20 °C.

For assaying the Cathepsin D Control, sequentially dilute an aliquot of the Cathepsin D Stock Solution 300-fold with Enzyme Dilution Buffer: dilute a 10 µl aliquot of the Cathepsin D Stock Solution 10-fold with 90 µl of Enzyme Dilution Buffer. Then, dilute 10 µl of the 10-fold diluted enzyme solution, 30-fold with Enzyme Dilution Buffer (add 10 µl of diluted enzyme solution to 290 µl Enzyme dilution buffer). The final 300-fold diluted Cathepsin D Control Solution has a protein concentration of ~2.4 µg/ml. The Cathepsin D Control Solution may be stored at 4 °C for up to 3 hours, but should be discarded afterwards.

The Pepstatin A Solution (2 mg/ml, Product Code P3749) and Cathepsin D Substrate Solution (1 mM, Product Code C4492) are supplied ready-to-use.

Storage/Stability
The kit is shipped on dry ice and storage at –20 °C is recommended.

Procedure
The assay uses an internally quenched fluorescent substrate, MCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-NH_2 trifluoroacetate salt, that is not initially fluorescent due to quenching of the MCA (7-methoxycoumarin-4-acetyl) group by the DNP (2,4-dintrophenyl group). After cathpesin D cleavage of the peptide between the two phenylalanine residues, the quenching is relieved and the released MCA-peptide will fluoresce at 393 nm after excitation at 328 nm.

When assaying extracts of cells or tissues it is possible that other enzymatic activities may cleave the substrate. For determination of cathepsin D enzyme activity only, the aspartic protease inhibitor Pepstatin A is added to the sample at a final concentration of 0.2 mg/ml to inhibit the cathepsin D activity. The activity inhibited by Pepstatin A is equivalent to the cathepsin D activity present.

This procedure is provided as a guideline and may have to be modified depending on the instrumentation available to measure fluorescence of multiwell plate assays:

- Sensitivity – the optimal sensitivity setting for the assay on the multiwell plate reader will be a function of the specific instrument. This should be determined for each instrument using the MCA standard curve.
- Some fluorimeters may use monochromators to select wavelengths and others may use filters. The wavelengths indicated in the assay are compatible with both options. For a BioTek™ Synergy HT plate reader the excitation filter was 320±20 nm and the emission filter was 420±50 nm. The sensitivity was set to 100.
- The 96 well plates used can be white or black depending on the specific instrument. Normally black plates will give a lower background value.
- If the fluorescence of the 300-fold diluted Cathepsin D Control Solution is too high, the enzyme can be further diluted. The dilution must be sequential in order not to use all of the Assay buffer 1x supplied with the kit.

For the biological test samples, reagents present in the extraction buffer may interfere with the assay. Therefore, it is important to verify that buffer components do not affect the results. Detergents like TRITON™ X-100 and *n*-dodecyl β-D-maltoside should not be present at any concentration in the sample due to quenching of the fluorescence. CHAPS can be present in the sample at a final concentration ≤0.5%. Sodium cholate, SDS, digitonin, and Lubrol PX strongly inhibit the enzyme activity.

In most cases, the activity in biological test samples will vary greatly from 0.002-2 units of cathepsin D activity per mg of protein. It should be noted that in some sources it is difficult to obtain a measurable activity with crude extracts (1,000 × *g* supernatant), thus, it is suggested to precipitate the lysosomes from the 1,000 × *g* supernatant with centrifugation at 20,000 × *g* to ensure a more concentrated sample.

The lower limit of detection with this kit for pure cathepsin D is 750 picograms of enzyme (13 femtomoles of enzyme). An example of the fluorescence values obtained with the cathepsin D control enzyme is shown in Figure 2 (Appendix).

1. MCA Standard Curve – Place 0, 5, 10, 15, 20, and 30 µl of the prepared 50 µM MCA Standard Solution in separate wells and then add Assay Buffer 1× and water according to Table 1.

Table 1.
Preparation of MCA Standard Curve

MCA Standard Solution (ml)	Assay Buffer 1x (ml)	Water (ml)	MCA per well (nmoles)
0	20	80	0
5	20	75	0.25
10	20	70	0.50
15	20	65	0.75
20	20	60	1.00
30	20	50	1.50

2. On the same plate, prepare reaction assays (blank, control, control with Pepstatin A, test sample, and test sample with Pepstatin A) according to the scheme in Table 2:
   a. Add 5 µl of the Cathepsin D Control Solution into two wells. For the control with inhibitor, place 10 µl of Pepstatin A Solution (Product Code P3749) in one of the wells.
   b. Add the test sample (biological extract) in the range of 5-30 µl (depending on the expected activity of the biological sample) to two wells. For the test sample with inhibitor, add 10 µl of Pepstatin A Solution to one well.
   c. Add 20 µl of Assay Buffer 1× to each well.
   d. Bring the volume in each well to 98 µl with water. Mix the content of the wells by tapping lightly on the side of the plate.

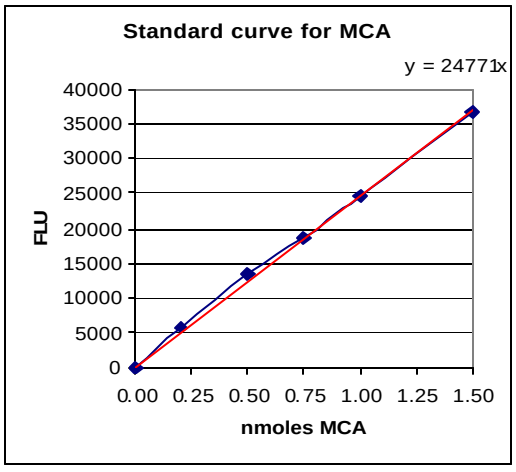
Table 2.
Reaction Scheme for Cathepsin D Enzymatic Assays

Assay	Enzyme volume (ml)	Assay Buffer 1´ (ml)	Water (ml)	Pepstatin A Solution (ml)
Blank	0	20	78	0
Cathepsin D Control Solution	5	20	73	0
Cathepsin D Control Solution + Pepstatin A	5	20	63	10
Test sample	x	20	78-x	0
Test sample + Pepstatin A	x	20	68-x	10

3. Pre-incubate the plate at 37 °C for 10 minutes to allow for inhibition of the enzyme.
4. Set the fluorescent plate reader parameters:
   Excitation wavelength at 328 nm
   Emission wavelength at 393 nm
   Adjust the sensitivity to the optimal value (see Procedure introduction).
   Set the temperature at 37 °C.
   Set the kinetic program for an assay time appropriate for the amount of enzyme added to the wells. For a 30 minute incubation (biological test samples), read at 2 minute intervals; for a 4 minute incubation (Cathepsin D Control Solution), read at 20 second intervals.
   Set an automatic shake protocol of 2 seconds before the initial reading.
5. Add 2 µl of the Cathepsin D Substrate Solution (Product Code C4492) to each well of the enzymatic assay with a multichannel pipette, **but not to the wells for the MCA Standard Curve**, and place in the programmed fluorescent plate reader. The final concentration of the Cathepsin D Substrate in the reaction is 20 µM.
   Note: If automatic shaking is not available, mix by tapping lightly on the side of the plate.
6. Incubate the plate for 2-4 minutes for the Cathepsin D Control solution or up to 30 minutes for the biological test samples.
   Note: If a fluorimeter with temperature control is not available, incubate the plate in an external incubator and read at suitable intervals.

Calculations
MCA Standard Curve - Plot fluorescence units versus the nmoles of MCA in each well. An example of the MCA Standard Curve is shown in Figure 1. From the standard curve calculate the fluorescence of 1.0 nmole of MCA.

Figure 1.
MCA Standard Curve



Calculate the activity for each sample, according to the formula shown below, for a time period that shows linearity in the fluorescence measurement.

$$\text{Units/ml} = \frac{FLU}{T} \times \frac{D}{V_{enz}} \times \frac{1}{FLU_{(1\ nmole)}}$$

FLU = increase in fluorescence observed during the time period of linearity
T = length of time in minutes, when increase of fluorescence is linear
D = dilution factor of enzyme sample
V_{enz} = volume of enzyme sample in ml
$FLU_{(1\ nmole)}$ = fluorescence of 1 nmole of MCA as calculated form the MCA standard curve.

This calculation will give a value of nmoles of MCA peptide released per minute per ml of enzyme sample. Note: For biological samples, subtract the activity in the presence of Pepstatin A from the total activity found. The resulting value is the amount of Cathepsin D activity present.

Unit definition: One unit of Cathepsin D will release the equivalent of 1.0 nmole of MCA peptide from a 20 µM solution of MCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-NH_2 trifluoroacetate salt per minute at pH 4.0 and 37 °C.

References

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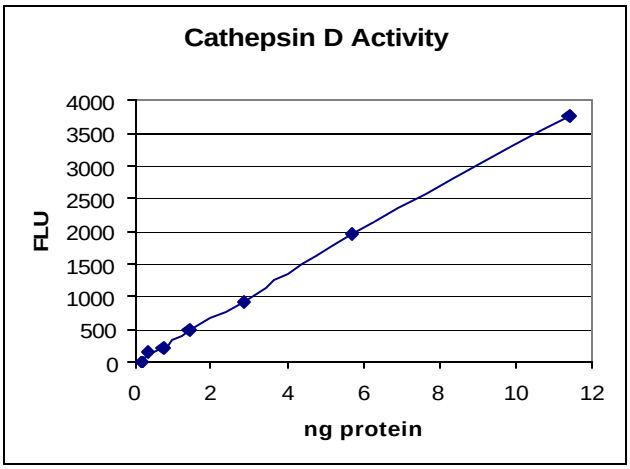
Appendix

Figure 2.
Example of fluorescence values obtained with the control enzyme (Cathepsin D from bovine spleen) as a function of the amount of protein.



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