Cathepsin D, Rapid Format ELISA KIT

**Cat. No.:** DEIA7175  
**Pkg.Size:** 96T

### Intended use

The Cathepsin D, Rapid Format ELISA KIT is designed to measure cathepsin D in tissue cytosols, extracts and culture fluids and extracts.

### General Description

Cathepsin D (CD) is a normal lysosomal aspartyl proteinase found in all tissues. It is synthesized as a 52 kDa inactive precursor (pro-CD). Proteolytic removal of the amino-terminal, 43 amino acid pro fragment, and cleavage at an internal site, results in an enzymatically active 48 kDa heterodimer consisting of two chains of 14 and 34 kDa. CD has a catalytic pH optimum of between 3 and 5 and is specifically inhibited by pepstatin. Two N-linked oligosaccharides on CD contain terminal mannose-6-phosphate residues which are responsible for localizing CD to the lysosomes via the mannose-6-phosphate receptor. The normal function of CD is to degrade proteins in the lysosome at acidic pH.

The observation that the 52 kDa pro form of CD was secreted by the hormone-dependent breast cancer cell line, MCF7, led to investigations which examined the significance of this protein in carcinogenesis. In vitro studies support a role for CD in cell transformation. The level of CD synthesized by cells is increased in response to mitogenic signals from estrogen, EGF, FGF and IGF-I. In addition, CD is capable of digesting extracellular matrix proteins in in vitro models and transfection of the CD gene into rat cells was observed to increase their tumorigenicity when injected into nude mice.

A number of clinical studies have investigated the role of CD in breast cancer and support its usefulness as a prognostic marker for overall and relapse-free survival in node negative patients. Other studies indicate that elevated CD is not a useful prognostic marker or is of prognostic value only for node positive patients.

### Principle Of The Test

The Cathepsin D, Rapid Format ELISA KIT is a sandwich-type immunoassay. Plates are pre-coated with a mouse monoclonal anti-cathepsin D IgG2a antibody. The Detector Antibody is a purified rabbit polyclonal antibody which recognizes human CD. Both of these reagents have been raised against mature CD purified from human liver.

To perform the test, the sample and biotinylated detector antibody (rabbit polyclonal) are pipetted into the wells and allowed to incubate simultaneously for four hours at 37°C to allow binding of the antigen by the capture antibody. The amount of detector antibody bound to antigen is measured by binding it with a streptavidin/horseradish peroxidase conjugate, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantitated by spectrophotometry and reflects the amount of CD protein in the sample when compared to the standards supplied in the kit.

The Cathepsin D, Rapid Format ELISA KIT is designed to accurately determine the nanogram quantity of CD in tissue cytosols and extracts, as well as in cell culture-derived cell extracts and fluids. Careful attention to extraction methods and the assay protocol will provide the investigator with a reliable tool for the quantitative measurement of CD.

### Reagents And Materials Provided

Samples and standards should be assayed in duplicate. A standard curve must be included each time samples are analyzed. The following components are supplied and are sufficient for the one 96-well plate kit.
1. Anti-Cathepsin D Coated 96-Well Plate - 1 microplate supplied ready to use, with 96 wells (12 strips of 8) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-CD monoclonal antibody.
2. CD Standards - 2 separate vials containing CD standards at 100 ng/ml. Reconstituted standards should be discarded after one use.
3. Cathepsin D Sample/Detector Diluent - 1 bottle containing 25 ml of buffer containing BSA and 0.1% sodium azide.
4. Detector Antibody - 1 bottle supplied ready to use, containing 12 ml of biotinylated rabbit anti-CD polyclonal antibody in 0.2 M Tricine (pH 8.5), a protein stabilizer, and 0.1% sodium azide.
5. Conjugate Diluent – 1 bottle containing 12 ml of 0.01 M PBS (pH 7.4), BSA.
6. Conjugate Concentrate - 1 vial containing 0.2 ml of 400X streptavidin conjugated horseradish peroxidase in buffer. Must be diluted to 1X with Conjugate Diluent to make Working Conjugate.
7. TMB Substrate - 12 ml of the chromogenic substrate, tetra-methylbenzidine (TMB Substrate).
8. AEA - 1 vial containing 3 ml of Antigen Extraction Agent containing Zwittergent KCl, sufficient for 18 ml of extract.
9. Stop Solution - 1 bottle supplied ready to use, containing 12 ml of 2.5 N H2SO4.
10. Plate Wash Concentrate - (20X) - Dilute with water to 1X prior to use.

Materials Required But Not Supplied

2. Precision repeating pipettor.
3. Wash bottle or multichannel dispenser for plate washing.
5. Vortex mixer.
6. Plate reader or spectrophotometer capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/595 nm. Reference filters of 540, 560 and 595 can be used. A single wavelength spectrophotometer can be used at 450 nm, which will give a somewhat higher reading.
7. 500 or 1000 ml graduated cylinder.
8. Reagent reservoirs.
9. Deionized water of high quality.
11. Liquid household bleach for inactivating clinical specimens and decontamination of plate washer.
12. Disposable paper towels.
13. Receptor Buffer.
15. Cell Resuspension Buffer: 50 mM NaF, 50 mM Tris (pH 8.0), containing 5 mM EDTA, 0.2 mM PMSF, 1 µg/ml pepstatin, and 0.5 µg/ml leupeptin.

Storage

Upon receipt, standards must be stored at –20°C. All other components may be stored at –20°C or 4°C (do not refreeze after thawing). Do not expose reagents to excessive light. Allow kit reagents to warm to room temperature before use.

Specimen Collection And Handling

Cell Lysate Protocol

Numerous extraction protocols can be used. The following protocol has been shown to work with a number of cell lines. It is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the method of choice. Users may wish to experiment with extraction procedures that work best in their hands.
1. For suspension cells, pellet by centrifugation, remove supernatant, wash with PBS and proceed to step #3. For attached cells, remove supernatant from cells.
2. Wash cells once with PBS, harvest cells by scraping and gentle centrifugation.
3. Aspirate PBS leaving an intact cell pellet (at this point the cell pellet can be frozen at -80°C and lysed at a later date).
4. Resuspend pellet in Resuspension buffer (approximately 1 ml for every 1 x 107 cells).
5. Add 20 µl of Antigen Extraction Agent (AEA; provided) for every 100 µl of cell suspension.
6. Incubate 30 min on ice with occasional vortexing.
7. Centrifuge at 1000 x g for 5 min, remove supernatant for measurement of CD.

**Sample Tissue Protocol:**
Steps 1-4 (following) are commonly performed on tissue specimens to prepare a cytosol for the measurement of steroid receptors using dextran-coated charcoal binding assays. This cytosol preparation is suitable for measurement of total cathepsin D as well. Follow steps 1-7 to prepare samples for assays. Preparation of a detergent extract of cathepsin D from a tissue homogenate preparation is also possible and provides an equivalent recovery of cathepsin D from the specimen. (In this case, it is important to report results in picomoles of CD per mg of whole tissue extract versus per mg of cytosol.) Tissue homogenate can be detergent extracted for the measurement of cathepsin D after processing as described below using the alternative steps 4 and 5 for detergent extract. Steps 1-3, 6 and 7 are equivalent for either method selected.

1. Weight the frozen specimen and slice the tissue into small pieces, or grind the frozen specimen in liquid nitrogen using a mortar and pestle.
2. Add cold (4°C) Receptor Buffer to the pieces of tissue. Use a buffer:tissue ratio 10:1 (v/w); for example, add 10 ml of buffer to 1 gm of tissue. Receptor Buffer contains 10 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 10% glycerol and 0.1% sodium azide. An effective cocktail of proteinase inhibitors consists of 0.5 µg/ml leupeptin, 1 µg/ml pepstatin and 0.2 mM pA-PMSF. Just before use, 0.1% monothioglycerol (MTG) is added to Receptor Buffer for ligand binding assays. The MTG is not necessary for the CD Assay but does not interfere in the assay. Molybdate (10 mM) is added to Receptor Buffer in some laboratories. It also has no effect of the measurement of cathepsin D using the CD Assay.
3. Homogenize on ice using a mechanical tissue homogenizer such as a Brinkmann Polytron, Braun Mikro-Dismembrator, or equivalent.

**Cytosol Method**
4. For Cytosol preparation, centrifuge the homogenized tissue at 105,000 x g for 1 h at 4°C in an ultracentrifuge. If necessary, adjust the volume of each preparation with additional receptor buffer prior to centrifugation.
5. Recover the supernatant (cytosolic fraction) from the tubes.

**Alternative Method: Detergent Extract**
4. Mix 1 volume of Antigen Extraction Agent (AEA) with 5 volumes of homogenate in a microcentrifuge tube (a 1:6 dilution). Mix for 5 min at room temperature by drawing the sample up and down in a pipet tip. Incubate for 30 min on ice with occasional vortexing.
5. Centrifuge at 15,000 rpm (about 14,000–16,000 x g) for 10 min in a benchtop microcentrifuge (max speed). Recover the supernatant (detergent extract). These specimens may be stored frozen at -70°C
6. Measure the protein concentration in the supernatant with the Micro-BCA assay or the Bio-Rad Protein Microassay. At least a 1:10 dilution of the sample must be made to prevent interference by AEA (if used) in the Bio-Rad Protein Microassay. A 1:10 dilution will work with the Pierce Micro-BCA protein assay. DO NOT use the Pierce Micro-BCA protein assay if receptor buffer contains MTG. The same solution used to dilute the sample for protein measurement should be used to prepare the protein standards and the assay blank. PBS usually works well. Sample Diluent cannot be used in protein measurement because it contains BSA. Receptor Buffer is also an inappropriate diluent for protein assays since it contains 10% glycerol.
7. Prior to measurement of cathepsin D levels in the CD Assay, the sample may need to be diluted in Sample Diluent to a suitable protein concentration (i.e. 20 µg/ml).
**Assay Steps**

NOTE: It is critical that all reagents required to perform this procedure are allowed to come to room temperature prior to use.

The Rapid Format Cathepsin D ELISA is provided with removable strips of wells so the assay can be carried out on two separate occasions. Since conditions may vary, a standard curve MUST be determined each time the assay is performed. Standards should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Warm kit to room temperature for about 1 h.
2. Remove the appropriate number of wells from the foil pouch and place them into the empty well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
3. Prepare a working solution (1X) of Wash Buffer by adding 25 ml of the 20X concentrated solution (provided), to 475 ml of deionized water. Mix well.
4. Each time an assay is performed, reconstitute a Lyophilized Standard by carefully and accurately pipetting dH2O and Sample Diluent, as described on the lyophilized Cathepsin D STANDARD vial label to give a concentration of 100 ng/ml. Let the reconstituted standard sit for 15 minutes, with occasional swirling. Avoid excessive agitation of the standard, avoid vigorous vortexing. After reconstituting the Cathepsin D Lyophilized Standard it should be diluted with Sample Diluent. Obtain six tubes and label them 100, 50, 25, 12.5, 6.25, and 0 ng/ml. Add 300 µl of Sample Diluent into each tube except the 100 ng/ml tube (first tube) which gets “undiluted” reconstituted standard. Remove 600 µl from the original vial of lyophilized material and add it to the first tube. Remove 300 µl from the first tube (100 ng/ml) and add it to the second tube (50 ng/ml) and mix gently. Repeat this procedure until you reach the fifth tube (6.25 ng/ml). The last tube (0 ng/ml) should just be Sample Diluent. Reconstituted standards should be discarded after one use.
5. Prepare all samples (diluted with Sample Diluent as needed). Samples should be at room temperature when assayed.
6. Pipette 100 µl of the Detector Antibody to each well
7. Add samples and each of the Cathepsin D standards (in duplicate) by pipetting 100 µl into appropriate wells on top of the Detector Antibody using clean pipette tips for each sample.
8. Cover wells with a plate sealer and incubate at 37°C for 4 h.
9. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
10. Dilute a sufficient amount of the 400X Conjugate 1:400 in Conjugate Diluent to provide 100 µl of 1X solution for each of the sample and standard wells, mix gently. For example: add 30 µl to 12 ml of Conjugate Diluent. Filter the solution with a 0.2 µm syringe filter.
11. Pipette 100 µl of the 1X Streptavidin Conjugate into each well, cover with a plate sealer and incubate at room temperature for 30 min. Discard any unused 1X Conjugate.
12. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
13. FLOOD ENTIRE PLATE WITH dH2O. Remove contents of wells by inverting over sink and tapping on paper towels.
14. Add 100 µl of Substrate Solution to each well and incubate IN THE DARK at room temperature for 30 min.
15. Add 100 µl of Stop Solution to each well in the same order as the previously added Substrate Solution.
16. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/595 nm. Reference wavelengths of 560 or 595 can also be used. Samples can also be measured at a single wavelength of 450 nm, which will give a somewhat higher reading. Wells must be read within 30 min of adding the Stop Solution.

**Typical Standard Curve**

Typical Standard Curve

Figure 1:
Evaluation

A. Concentration of Standards
The Rapid Format Cathepsin D ELISA quantitates CD protein relative to the number of CD molecules having binding sites for both the capture antibody and the detector antibody. The standards are calibrated in nanograms (ng) of CD protein per ml.

B. Concentration of Unknowns
Average the absorbance values for each Standard and specimen dilution to obtain the mean absorbance.
1. Subtract the mean absorbance of the 0 ng/ml Standard (background absorbance) from the mean absorbance of each Standard and sample dilution.
2. On graph paper, plot the mean corrected absorbance for each Standard on the y-axis versus the concentration of CD (in ng/ml) on the x-axis.
3. Determine the concentration of CD for each specimen dilution by interpolation from the standard curve. Software packages are available (such as Softmax, Molecular Devices, Menlo Park, CA and KinetiCalc, BioTek Instruments, Inc. Winooski, VT) which can simplify this process.
4. Results for cell culture fluid samples can be expressed per ml in the original sample by correcting the value obtained from the standard curve for the dilution factor.
5. For tissue specimens, the ng/ml value should be divided by the protein concentration of the particular dilution assayed to give nanograms of CD per µg of sample protein assayed. A simple conversion enables reporting of samples in picomoles CD/mg of protein. More than one dilution of a particular sample should be tested. When the sample signals fall on the standard curve and respond in a parallel manner with that of the standard curve, the CD concentration in the sample, calculated for each dilution, should produce the same value.
Specificity

No cross reactivity was observed.

Precautions

1. Store standards at –20°C. All other components may be stored at –20°C or 4°C (do not refreeze after thawing). Do not expose reagents to excessive light. Warm kit reagents to room temperature before use (let sit at room temperature approximately 30 min before use.)
2. Allow kit to warm to room temperature for 1 h just before use.
3. Use only the wells provided with the kit.
4. Rinse all detergent residue from glassware.
5. Use deionized water of the highest quality.
6. Do not mix reagents from different kits.
7. The buffers and reagents used in this kit contain either sodium azide or chloroacetamide as preservatives. Care should be taken to avoid direct contact with these reagents.
8. Do not mouth pipet or ingest any of the reagents.
9. Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
10. Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols.
   Wear protective gloves and dispose of biological samples properly.
11. Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
12. Wear disposable gloves and eye protection when handling Stop Solution (2.5 N sulfuric acid).

Analyte Gene Information

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REFERENCES
