ELISA PRODUCT INFORMATION & MANUAL

Cathepsin B Assay Kit (Fluorometric)

NBP2-54841

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

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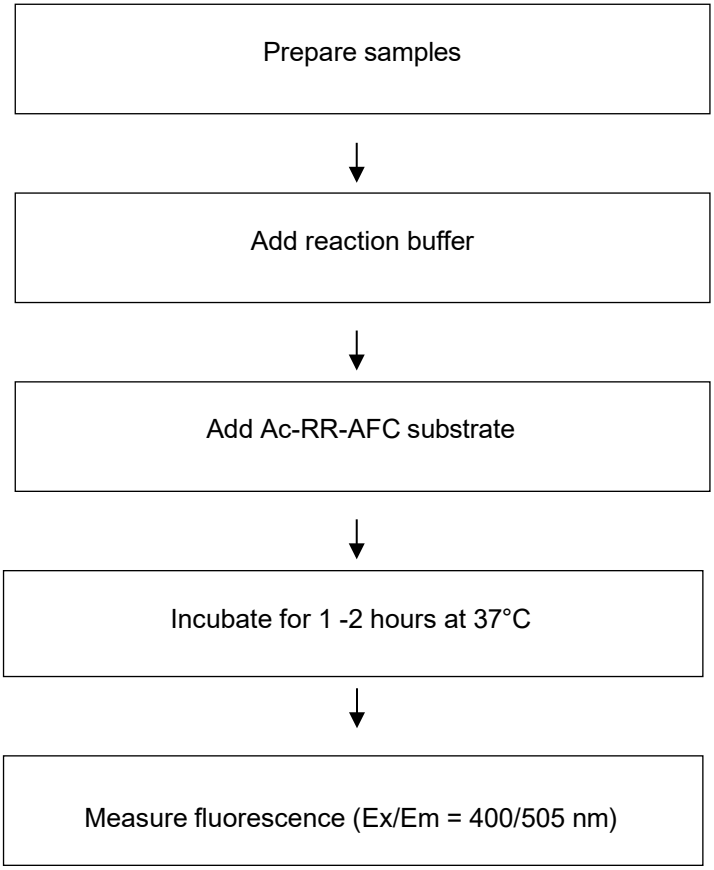
1. BACKGROUND

Cathepsin B Assay Kit (Fluorometric) (NBP2-54841) is a fluorescence-based assay that utilizes the preferred Cathepsin B substrate sequence RR labeled with AFC (amino-4-trifluoromethyl coumarin). Cell lysates or other samples that contain Cathepsin B will cleave the synthetic substrate RR-AFC to release free AFC. The released AFC can easily be quantified using a fluorometer or fluorescence plate reader.

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



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 -20ºC 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

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
CB Cell Lysis Buffer	25 mL	-20°C	4°C
CB Reaction Buffer	5 mL	-20°C	4°C
CB Substrate	200 µL	-20°C	-20°C
CB Inhibitor	20 µL	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader – equipped with filter for Ex/Em = 400/505 nm
- 96 well plate: black plates (clear bottoms)
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)
- Method for protein determination (optional) – we recommend Optiblot Bradford Reagent (ab119216)
- (Optional) ab129872 – free AFC to determine Cathepsin B units

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.
- 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 **CB Cell Lysis Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C. Once thawed, use within 6 months.

9.2 **CB Reaction Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C. Once thawed, use within 6 months.

9.3 **CB Substrate (Ac-RR-AFC):**

Ready to use as supplied. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once thawed, use within 6 months. Keep on ice while in use.

9.4 **CB Inhibitor:**

Ready to use as supplied. Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once thawed, use within 6 months. Keep on ice while in use.

10. SAMPLE PREPARATION

General Sample information:

- 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.
- **Treat cells to induce apoptosis by desired method. As a negative control, prepare a separate culture without treatment.**

10.1 Cell (adherent or suspension) samples:

10.1.1 Harvest the amount of untreated/treated cells necessary for each assay (initial recommendation = 1- 5 x 10^6 cells).

10.1.2 Wash cells with cold PBS.

10.1.3 Resuspend cells in 50 µL of chilled Cell Lysis Buffer.

10.1.4 Incubate on ice for 10 – 30 minutes.

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 Collect supernatant and transfer to a clean tube.

10.1.7 Keep on ice.

10.1.8 Use 50 – 200 µg cell lysate if protein concentration has been measured.

10.2 Tissue samples:

10.1.1 Harvest the amount of untreated/treated tissue necessary for each assay (initial recommendation = 10 mg).

10.1.2 Wash tissue in cold PBS.

10.1.3 Resuspend tissue in 100 µL of chilled Cell Lysis Buffer.

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

10.1.5 Incubate on ice for 10 – 30 minutes.

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

10.1.7 Collect supernatant and transfer to a clean tube.

10.1.8 Keep on ice.

10.1.9 Use 50 – 200 µg cell lysate if protein concentration has been measured.

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:**

- Sample treated wells = 50 µL samples from treated cells (or 50-200 µg protein, adjust volume to 50 µL/well with Cell Lysis Buffer).
- Sample untreated wells = 50 µL samples from untreated cells (or 50-200 µg protein, adjust volume to 50 µL/well with Cell Lysis Buffer).
- (OPTIONAL) Negative control wells= 50 µL samples from treated cells (or 50-200 µg protein, adjust volume to 50 µL/well with Cell Lysis Buffer). Add 2 µL CB Inhibitor.
- Background control (buffer) = 50 µL Cell Lysis Buffer

11.2 Add 50 µL CB Reaction Buffer to each well.

11.3 Add 2 µL of 10 mM CB Substrate Ac-RR-AFC (200 µM final concentration) to each well.

11.4 Incubate at 37°C for 1-2 hours protected from light.

11.5 Measure output on a fluorescent microplate reader at Ex/Em = 400/505 nm.

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

12.1 Subtract the Background control (buffer) from sample readings.

12.2 Fold-increase in Cathepsin B activity can be determined by comparing results from induced / treated cells with the level from the uninduced / untreated control.

NOTE: *If desired, Cathepsin B units can be determined by generating a standard curve using AFC (7-Amino-4-trifluoromethylcoumarin), Fluorescent marker (ab145587) under assay conditions.*

13. TYPICAL DATA

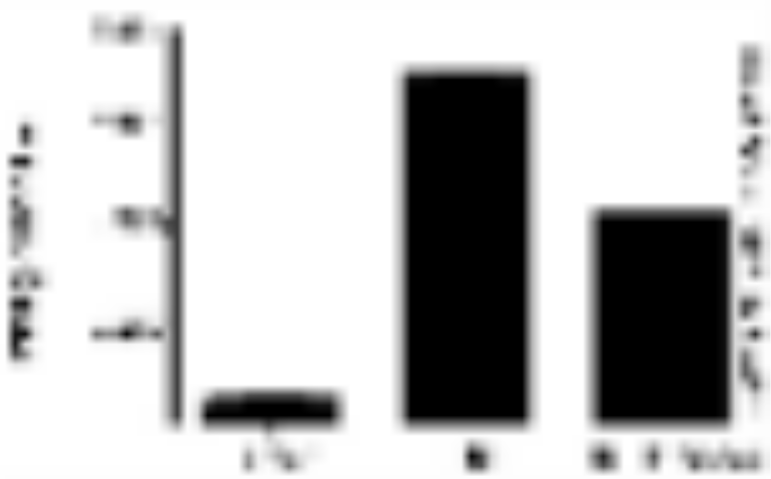


Figure 1. Cathepsin B activity in HL60 cell lysates ($2.5x10^6$ cells) with the addition of inhibitor.

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 CB Reaction Buffer, substrate (aliquot if necessary); get equipment ready.
- (Optional) Prepare standard curve and/or positive control.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for treated and untreated samples (50 µL), negative control (and positive control (50 µL) and standard (50 µL) if using).
- Add 50 µL of CB Reaction buffer to the sample and controls (and standard, if used) wells.
- Add 2 µL 10 mM substrate to all wells. For negative control, add 2 µL CB Inhibitor.
- Incubate at 37°C for 1-2 hours protected from light.
- Measure plate at Ex/Em= 400/505 nm.

15. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

16. FAQ

What is the sensitivity of the assay?

The sensitivity of this kits is 10 – 100 ng Cathepsin B/well.

17. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- RIPA: contains SDS which can destroy/decrease the activity of the enzymes.
- Protease inhibitors.

18. NOTES