ab65308
Calpain Activity Assay Kit (Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Calpain activity in cell and tissue lysates.

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

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Version 5 Last Updated 7 June 2023

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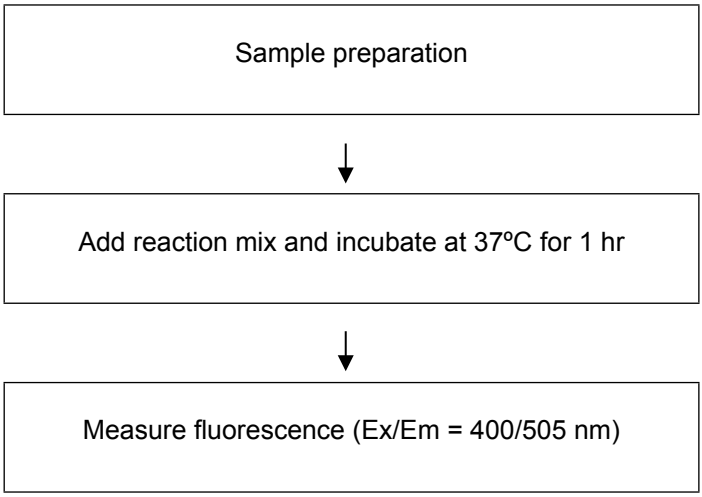
1. BACKGROUND

Calpain Activity Assay Kit (fluorometric) (ab65308) provides optimized buffers and reagents for a convenient measurement of calpain activity. The Extraction Buffer provided with the kit specifically extracts cytosolic proteins without contaminations of cell membrane and lysosome proteases. The Extraction Buffer also prevents auto-activation of calpain during the extraction procedure. Thus, the kit detects only activated calpain in cytosol upon treatment of cells with inducers (e.g., chemicals or drugs). The fluorometric assay is based on the detection of cleavage of calpain substrate Ac-LLY-AFC.

Ac-LLY-AFC emits blue light (λ_{max} = 400 nm); upon cleavage of the substrate by calpain, free AFC emits a yellow-green fluorescence (λ_{max} = 505 nm), which can be quantified using a fluorometer or a fluorescence plate reader. Comparison of the fluorescence intensity from a treated sample with a normal control allows determination of the changes in calpain activity.

Activation of calpain is involved in many forms of physiological and pathological processes (e.g., apoptosis). Calpain activation requires cell membrane and Ca^{2+}, and activated calpain is released into cytosol.

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 -80ºC 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)
Extraction Buffer III/Extraction Buffer	25 mL	- 80°C	4°C
10X Reaction Buffer	1.5 mL	-80°C	4°C
Calpain Substrate (Ac-LLY-AFC)	500 µL	-80°C	-80°C
Active Calpain I (Positive Control)	10 µL	-80°C	-80°C
Calpain Inhibitor (Z-LLY-FMK)	10 µL	-80°C	-80°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
- 96 well plate: black plates (clear bottoms)
- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent or colorimetric microplate reader
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)
- Method for measuring protein concentration e.g. ab102535

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 **Extraction Buffer III/Extraction Buffer:**

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

9.2 **10X Reaction Buffer:**

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

9.3 **Calpain Substrate:**

Ready to use as supplied. Aliquot substrate so that you have enough to perform the desired number of assays. Store at -80°C protected from light.

9.4 **Active Calpain I (positive control):**

Ready to use as supplied. Aliquot positive control so that you have enough to perform the desired number of assays. Store at -80°C.

9.5 **Calpain Inhibitor:**

Ready to use as supplied. Aliquot inhibitor so that you have enough to perform the desired number of assays. Store at -80°C.

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 1-2 x 10^6 cells).

10.1.2 Wash cells with cold PBS.

10.1.3 Resuspend cells in 100 µL of Extraction Buffer III/Extraction Buffer and incubate samples on ice for 20 minutes. Gently mix the samples by tapping several times during incubation.

10.1.4 Centrifuge for 1 min in a microcentrifuge (10K x g) and transfer supernatant to a clean tube5. Keep on ice

10.1.5 Measure protein concentration.*

10.2 Tissue Samples:

10.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

10.2.2 Wash tissue in cold PBS.

10.2.3 Resuspend tissue in 100 µL of Extraction Buffer III/Extraction 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 Collect supernatant and transfer to a clean tube.

10.2.7 Keep on ice.

10.2.8 Measure protein concentration*.

***NOTE:** Due to the high reducing agent content in the Extraction Buffer III/Extraction Buffer, dilute about 10-fold then use a Coomassie-based protein assay such as ab102535. Typically you would expect to see ~50 – 200 µg of protein.*

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.**

12.1 **Set up Reaction wells:**

- Sample well (treated cells) = Dilute the cell lysate ~ 50 – 200 µg, (adjust volume to 85 µL/well with Extraction Buffer III/Extraction Buffer).
- Positive control wells = 1-2 µL Active Calpain I Positive Control /Active Calpain (adjust volume to 85 µL/well with Extraction Buffer III/Extraction Buffer).
- Negative Control = Use untreated lysate or add 1 µL of Calpain Inhibitor to the treated cell lysate; adjust volume to 85 µL/well with Extraction Buffer III/Extraction Buffer).

12.2 Add 10 µL of 10X Reaction Buffer to each well.

12.3 Add 5 µL of Calpain Substrate to each well.

12.4 Incubate at 37ºC for 60 minutes protected from light.

12.5 Measure output on a microplate reader.

- Fluorometric assay: measure Ex/Em = 400/505 nm.

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- The changes in calpain activity can be determined by comparing results of treated samples and negative control. Alternatively, the activity can be expressed as Relative Fluorescent Unit (RFU) per milligram protein of each sample.

13. TYPICAL DATA

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

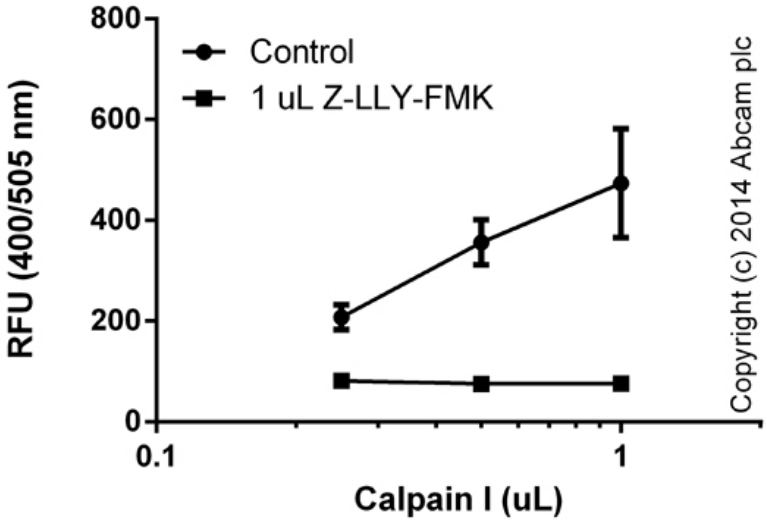


Figure 1. Different amounts of positive control (Calpain I) treated with 1 µL of inhibitor (Z-LLY-FMK).

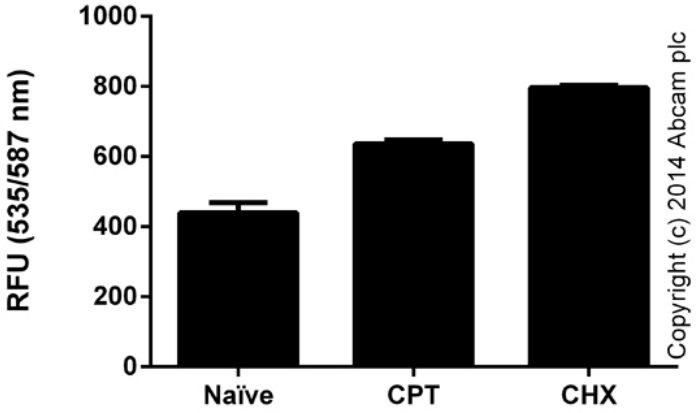


Figure 2: 10^7 Jurkat cells (in 10 mL) were cultured in the absence or presence of 10 µM Camptothecin (CPT) (ab120115) or 10 µg/mL Cyclohexamide (CHX) (ab120093) for 4 hours. Pelleted cells were lysed in 0.5 mL of Extraction Buffer III/Extraction Buffer and tested directly.

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 Extraction Buffer III/Extraction Buffer, Reaction Buffer, Calpain Substrate, Active Calpain I Positive Control/Active Calpain, Calpain Inhibitor (aliquot if necessary); get equipment ready
- Set up plate for samples (85 µL), positive control (85 µL) and negative control (85 µL).
- Add 10 µL 10X Reaction Buffer to all wells.
- Add 5 µL Calpain substrate to all wells.
- Incubate plate 37ºC 60 mins.
- Measure plate at Ex/Em= 400/505 nm for fluorometric assay.

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

We have used increasing amounts of Active Calpain I Positive Control/active Calpain 1 with this assay, but our readings are not linear. Why?

Substrate saturation can account for the non-linear signal.

Calpains will automatically be activated by calcium as soon as the plasma membrane is broken to collect cellular proteins. Does the kit take this into consideration?

Yes this is taken into consideration. Pro-calpain activation requires cell membrane, and after activation the active calpain is released into cytosol.

Firstly, ab65308 utilizes mild detergent in the Extraction Buffer III/Extraction Buffer to extract only activated calpain present in cytosol, but not inactivated calpain which is still associated with cell membrane.

Secondly, to prevent the auto-activation of calpain, the Extraction Buffer III/Extraction buffer is supplied with optimal concentrations of EDTA and EGTA to chelate the calcium to avoid any autoactivation of calpain during the extraction procedure.

Does this kit measure activity of Calpain 1 or 2 or both?

It will measure activity from any active Calpain isoform in the sample. Calpain I and II (mu-Calpain and m-Calpain respectively) are activated by micro- and nearly millimolar concentrations of Ca2+ within the cell. To determine Calpain I activity it is necessary to make sure that the calcium concentration is in micromolar range in the sample or purify Calpain I specifically to test their activity.

The protocol included with the kit does not describe how to perform the assay directly on cells cultured in a 96 well plate. How do I perform the assay in the plate?

It would be difficult to perform the assay directly in the same 96-well plate, as you would need to extract the activated Calpain and transfer the extract into a new plate before performing the analysis.

The Calpain that we are interested in, is connected to Talin, which forms, along with other proteins, a large focal adhesion complex that is attached to membranes (mostly plasma membrane). In the lysis procedure, it says: "The Extraction Buffer III/extraction buffer provided with the kit specifically extracts cytosolic proteins without contamination by cell membrane". Would you think that this lysis will be effective enough to include Calpain in complexes?

The kit is designed for detecting activated cytosolic Calpain after translocate from cell membrance to cytosol. If you want to detect all Calpains that associated with the cell membrane, you can add 1% Triton X-100 into the Extraction Buffer III/extraction buffer, which will extract the all Calpains from the cells.

Since the assay does not come with a "stop solution" to use before the O.D. reading, is it correct to assume that during the 1 hour incubation that the sample and the Calpain substrate is consumed during the enzymatic reaction? If it takes several minutes to get the color reaction for actual reading, will the color of the reaction maintain itself?

The active enzyme is still in the reaction, and the reaction is still going on. The color will be there for quite long time. If you want to stop the reaction, you may add 1% SDS to inactivate active Calpain.

If I increase number of cells, how should I adjust volume of Extraction Buffer III/extraction buffer?

When you are increasing the number of cells you are aiming for higher concentrations. The adjustment of the volume will defeat that purpose. Increase the amount of lysate and keep everything else the same.

Is the Calpain inhibitor specific only for Calpain I?

This Calpain inhibitor inhibits both Calpain I and II (μ- calpain and m-calpain respectively).

17. INTERFERENCES

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

- DMSO – concentration of more than 1-2% will interfere with the assay.
- SDS – inactivates calpain.

18. NOTES

For all technical and commercial enquires please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)