



ELISA PRODUCT INFORMATION & MANUAL

Caspase-3 Activity Assay Kit (Colorimetric) *NBP2-54838*

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.

Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

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1. BACKGROUND

Caspase-3 Activity Assay Kit (Colorimetric) (NBP2-54838) provides a simple and convenient means for assaying the activity of caspases that recognize the sequence DEVD.

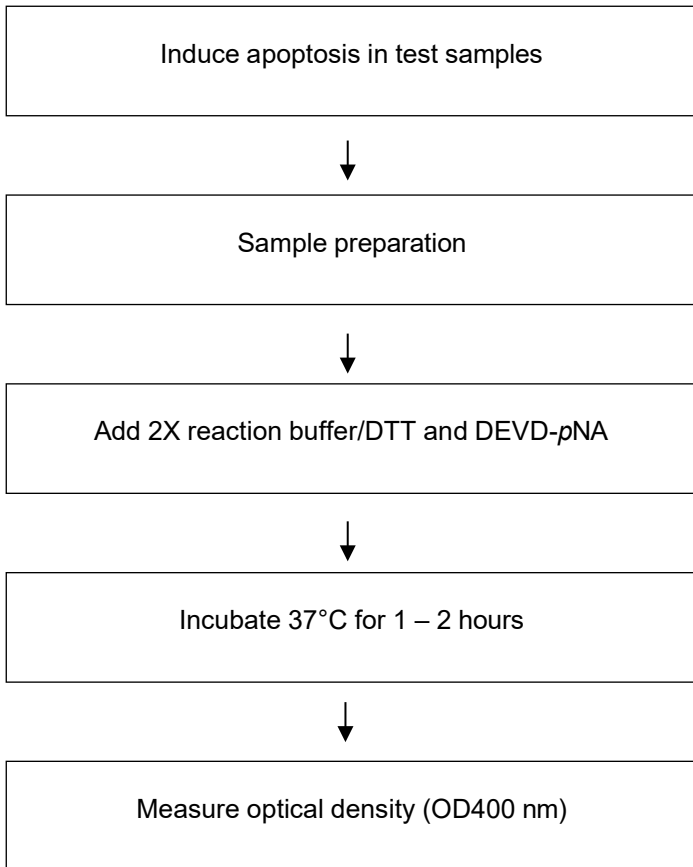
The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (p-NA) after cleavage from the labeled substrate DEVD-p-NA. The p-NA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400 or 405 nm. Comparison of the absorbance of p-NA from an apoptotic sample with an untreated control allows determination of the fold increase in Caspase-3 activity.

The caspase family of highly conserved cysteine proteases play an essential role in apoptosis.

Mammalian caspases can be subdivided into three functional groups: initiator caspases (Caspase 2, 8, 9 and 10), executioner caspases (Caspase-3, 6 and 7), and inflammatory caspases (Caspase 1, 4, 5, 11 and 12). Initiator caspases initiate the apoptosis signal while the executioner caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are rather involved in inflammatory cytokine signaling.

Initially synthesized as inactive pro-caspases, caspases become rapidly cleaved and activated in response to granzyme B, death receptors and apoptosome stimuli. Caspases will then cleave a range of substrates, including downstream caspases, nuclear proteins, plasma membrane proteins and mitochondrial proteins, ultimately leading to cell death.

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 -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

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Cell Lysis Buffer	100 mL	-20°C	4°C
2x Reaction Buffer	4x 2 mL	-20°C	4°C
DEVD-p-NA (4 mM) substrate	500 µL	-20°C	-20°C
DTT (1 M)	400 µL	-20°C	-20°C
Dilution Buffer	100 mL	-20°C	4°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Microcentrifuge
 - Pipettes and pipette tips
 - Orbital shaker
 - Dounce homogenizer (if using tissue)
 - Vortex
 - Colorimetric microplate reader equipped with filter for OD 400 nm
 - 96 well plate: clear plates for colorimetric assay
 - (Optional) Protein quantification assay
- If reading sample on a spectrophotometer:
- Spectrophotometer (alternative to microplate reader)
 - Micro quartz or regular cuvettes (if using spectrophotometer)

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 or control 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 and heat labile components and samples on ice during the assay.
- Make sure all buffers and developing solutions are at room temperature before starting the experiment.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Samples generating values higher than the highest treated sample should be further diluted in the appropriate sample dilution buffers.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the appropriate type of plate for the detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

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°.

9.2 **2x Reaction Buffer:**

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

Add DTT to the 2X Reaction Buffer immediately before use
For 10mM DTT final concentration: add 10 μ L of 1M DTT stock per 1 mL of 2X Reaction Buffer.

9.3 **DEVD-pNA Substrate (4 mM):**

Ready to use as supplied. Aliquot substrate so that you have enough to perform the desired number of assays. Store at -20°C protected from light and moisture.

9.4 **DTT (1M):**

Ready to use as supplied. Aliquot DTT so that you have enough to perform the desired number of assays. Store at -20°C.

9.5 **Dilution Buffer:**

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

10. SAMPLE PREPARATION

General Sample information:

- This product detects proteolytic activity. Do not use protease inhibitors in the sample preparation step as it might interfere with the assay.
- We recommend performing several dilutions of your samples.
- 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 Induce apoptosis in cells by desired method, concurrently incubate a control culture (untreated) without induction.
- 10.1.2 Count cells and pellet $1-5 \times 10^6$ cells.
- 10.1.3 Re-suspend cells in $50 \mu\text{L}$ of chilled Cell Lysis Buffer and incubate cells on ice for 10 minutes.
- 10.1.4 Centrifuge at $10,000 \times g$ for 1 minute.
- 10.1.5 Transfer supernatant (cytosolic extract) to a fresh tube and put on ice for immediate assay.
- 10.1.6 Measure protein concentration, and adjust to $50 - 200 \mu\text{g}$ protein per $50 \mu\text{L}$ Cell Lysis Buffer for each assay (well).

NOTE: *If not for immediate use, aliquot and store at -80°C for future use.*

11. ASSAY PROCEDURE and DETECTION

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

11.1 Set up Reaction wells:

- Sample wells = 50 μ L sample.
- Background wells = 50 μ L 2x Reaction Buffer.

NOTE: We suggest using different volumes of sample.

11.2 Reaction Mix:

Prepare Caspase Reaction Mix for each reaction:

Component	Reaction Mix (μ L)
2x Reaction Buffer	50
DTT	0.5

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

$X \mu\text{L component} \times (\text{Number samples} + \text{control} + 1)$.

- 11.3 Add 50 μ L of 2x Reaction Buffer (containing 10mM DTT) to each sample.
- 11.4 Add 5 μ L of the 4 mM DEVD-p-NA substrate (200 μ M final concentration).
- 11.5 Mix well and incubate at 37°C for 60 -120 minutes.
- 11.6 Measure output (OD 400 - 405 nm) on a microplate reader.

NOTE: Alternatively, samples can be read in a spectrophotometer in a 100 μ L or 1 mL quartz cuvette. If using 1 mL cuvette, it is necessary to dilute the samples to 1mL with Dilution Buffer. Dilution of the samples proportionally decreases the reading.

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Background reading from cell lysates and buffers should be subtracted from the readings of both treated and the untreated sample before calculating fold increase in CPP32 activity.
- Fold-increase in Caspase-3 activity can be determined by comparing sample (treated) results with the level of the untreated control.

13. TYPICAL DATA



Figure 1: Caspase-3 in Jurkat lysates (3.3×10^6 cells) following 20 hour exposure to $2 \mu\text{M}$ Camptothecin (ab120115) or 10 ng/mL anti-Fas Ab (MBL).

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 2X Reaction Buffer/10 mM DTT, cell lysis buffer and dilution buffer (if using), (aliquot if necessary); get equipment ready
- Prepare samples in duplicate. Dilute samples to protein concentration of 50 – 200 µg per 50µL Cell Lysis Buffer for each assay.
- Set up plate for samples (50 µL) and background wells (50 µL reaction buffer).
- Prepare Caspase Reaction Mix (Number samples + 1).

Component	Reaction Mix (µL)
2x Reaction Buffer	50
DTT	0.5

- Add 50 µL of 2x Reaction Buffer (containing 10mM DTT) to each sample.
- Add 5 µL of the 4 mM DEVD-*p*-NA substrate (200 µM final conc.).
- Mix and incubate at 37°C for 60 -120 mins.
- Measure plate at OD400 nm for colorimetric assay.

15. FACTORS TO CONSIDER FOR CASPASE ACTIVITY ASSAYS

Three major factors need to be taken into account when using caspase activity assays:

1. The substrate in a particular assay is not necessarily specific to a particular caspase.
Cleavage specificities overlap so reliance on a single substrate/assay is not recommended. Other assays, such as Western blot, use of fluorescent substrates e.g. FRET assays should be used in combination with caspase activity assays.
2. The expression and abundance of each caspase in a particular cell type and cell line will vary.
3. As the activation and cleavage of caspases in the cascade will change over time, you should consider when particular caspase will be at its peak concentration e.g. after 3 hours, after 20 hours etc.

The table below show the known cross-reactivities with other caspases.

Classification of caspases based on synthetic substrate preference, does not reflect the real caspase substrate preference *in vivo* and may provide inaccurate information for discriminating amongst caspase activities. Thus, caution is advised in applying the intrinsic tetrapeptide preferences to predict the targets of individual caspases.

RESOURCES

Apoptotic Executer Caspases

Caspase	Cleavage motif	Inhibitor motif	Cross-reactivity with other caspase:											
			1	2	3	4	5	6	7	8	9	10		
Caspase-3	DEVD	DEVD, LEHD*, IETD, LETD		Y							Y			
Caspase 6	VEID	DEVD, LEHD*, IETD, LETD			Y									
Caspase 7	DEVD	DEVD, LEHD*, IETD, LETD	Y		Y									

* *inhibits at high concentration*

RESOURCES

16. 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 inappropriate plate for reader	Colorimetry: Clear plates Fluorescence: Black plates (clear bottom)
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); observe for lysis under microscope
	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

RESOURCES

Problem	Cause	Solution
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 as to be in the linear range

17. FAQs

I have some lysed samples from another experiment. Can I use these lysates with this assay or is it necessary to use the lysis buffer in the kit?

As long as you are using a generic cell lysis buffer for sample prep, it should be compatible with this assay. However, please ensure that the lysates are fresh and have not undergone numerous freeze/thaws. Then dilute the lysates to 50-200 µg/50 µL using our lysis buffer and continue with step 11.2.

What positive control can be used when using this kit?

You can use an active Caspase-3 protein (Eg: ab52101) as the positive control to cleave the DEVD-pNA substrate provided in the kit or cells treated with an apoptosis inducer e.g. staurosporin.

How do I calculate the exact Caspase -3 in my samples?

This is a relative assay which will just show the fold increase of caspase-3 between your treated and untreated samples. To find the absolute levels of activated Caspase-3 in your sample, you will have to make a standard curve with active caspase-3 (Active Human Caspase-3 Full Length (ab52314)).

Can I use this kit with platelet rich plasma samples?

This kit is optimized for use with cell and tissue lysates. It cannot be used exactly the same way with plasma. If you can precipitate out the platelets from the plasma, then you can use this kit with slight optimizations.

Can this kit work with supernatant secreted from cell culture?

This kit is for use with cell and tissue lysates, but theoretically you can assay for the protein concentration in the supernatant and proceed with the assay from step 11.2.

I do not see any signal difference between the untreated and treated samples.

There can be multiple reasons for this. The DTT needs to be added to the reaction buffer right before the experiment. The caspase induction conditions need to be optimized for dosage and time points for ideal (detectable) apoptosis. If possible, ensure the apoptosis and caspase-3 induction by an alternate means as well. Ensure that the DEVD-pNA is protected from light before use.

What step is the Dilution Buffer used for? Is it for diluting the samples for the protein quantification step?

The dilution buffer is to dilute the final samples before reading their absorbance, in case of the undiluted readings being above the detection range of the instrument.

How can I 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. Auto-activation can be accounted for by using non-treated samples as a control

