



## **ELISA PRODUCT INFORMATION & MANUAL**

### **Caspase-8 Activity Assay Kit (Colorimetric) *NBP2-54817***

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. Overview

Caspase-8 Activity Assay Kit (Colorimetric) (colorimetric) (NBP2-54817) is a simple and convenient assay to quantify the activity of caspase 8 in cell lysates, based on the recognition of the sequence Ile-Glu-Thr-Asp (IETD). The assay is based on spectrophotometric detection of the chromophore p- nitroanilide (pNA) after it is cleaved from the labeled substrate IETD-pNA. The pNA light emission can be quantified using a spectrophotometer or a microtiter plate reader at OD=400 – 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an un-induced control allows determination of the fold increase in Caspase 8 activity.

The caspase family of highly conserved cysteine proteases play an essential role in programmed cell death (including apoptosis, pyroptosis and necroptosis).

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). 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.

Caspase 8 (CASP8/FLICE, EC:3.4.22.61) is the most upstream caspase involved in the activation of apoptosis through the extrinsic pathway, mediated by CD95 (Fas) receptor and TNFR. Caspase 8 is recruited to the receptors through the adapter molecule FADD, resulting in the formation of the aggregate called death-inducing signaling complex (DISC) and proteolytic activation of caspase 8. The active dimeric enzyme is then liberated from the DISC and free to activate downstream apoptotic proteases. Inhibition or inactivation of caspase 8 is required for induction of necroptosis.

## 2. Protocol Summary

Induce caspase 8 activation in test samples



Sample preparation



Add 2X reaction buffer/DTT + IED-pNA



Incubate reaction at 37°C for 1 – 2 hours



Measure optical density (OD<sub>400</sub> nm)

### 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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

### 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.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- 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.

## 6. Materials Supplied

<b>Item</b>	<b>Quantity</b>	<b>Storage Condition (Before prep)</b>	<b>Storage Condition (After prep)</b>
2X Reaction Buffer	4 x 2 mL	-20°C	-20°C/4°C
Cell Lysis Buffer	100 mL	-20°C	-20°C/4°C
Dilution Buffer	100 mL	-20°C	-20°C/4°C
DTT (1M)	400 µL	-20°C	-20°C
IETD-pNA (4mM)	500 µL	-20°C	-20°C

## 7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at OD 400 nm
- PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Cell scraper for adherent cells
- (Optional) BCA Protein Quantification Kit (ab102536) – to determine protein concentration of sample.

If reading sample on a spectrophotometer:

- Spectrophotometer (alternative to microplate reader)
- Micro quartz or regular cuvettes for spectrophotometer reading

## 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.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

## 9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

### 9.1 2X Reaction Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

### 9.2 Cell Lysis Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

### 9.3 Dilution Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

**Δ Note:** Dilution Buffer is only necessary if measuring activity in regular 1 mL cuvettes.

### 9.4 DTT (1M):

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

### 9.5 IETD-pNA Substrate (4M):

Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light.

## 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 snap freeze your samples in liquid nitrogen upon extraction and store them immediately at  $-80^{\circ}\text{C}$ . When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Induce caspase 8 in cells by desired method. Concurrently incubate a control culture without induction.

### 10.1 Adherent cell samples:

- 10.1.1 Grow cells at a 70% - 80% density.
- 10.1.2 Treat cells by desired method to induce caspase 8 activation. Have a parallel control without induction.
- 10.1.3 Gently scrape cells in culture media and spin down cell pellet at 200  $\times g$  for 10 minutes.
- 10.1.4 Wash cells in cold PBS.
- 10.1.5 Count and pellet  $1 - 5 \times 10^6$  cells.
- 10.1.6 Resuspend cells in 50  $\mu\text{L}$  of ice cold Cell Lysis Buffer.
- 10.1.7 Homogenize cells quickly by pipetting up and down a few times.
- 10.1.8 Incubate sample on ice for 10 – 30 minutes.
- 10.1.9 Centrifuge for 10 - 15 minutes at 10,000  $\times g$  using a cold microcentrifuge to remove any insoluble material.
- 10.1.10 Collect supernatant.
- 10.1.11 Transfer to a new tube.
- 10.1.12 Keep on ice.
- 10.1.13 Measure protein concentration and adjust protein concentration to 1 – 4  $\mu\text{g}$  protein/assay (50 – 200  $\mu\text{g}/50 \mu\text{L}$  Buffer for each microplate well).

## 10.2 Suspension cell samples:

- 10.2.1 Treat cells by desired method to induce caspase 8 activation. Have a parallel control without induction.
- 10.2.2 Count and spin down  $1 - 5 \times 10^6$  cells at 200 xg for 10 minutes.
- 10.2.3 Wash cells in cold PBS.
- 10.2.4 Resuspend cells in 50  $\mu$ L of ice cold Cell Lysis Buffer.
- 10.2.5 Homogenize cells quickly by pipetting up and down a few times.
- 10.2.6 Incubate sample on ice for 10 – 30 minutes.
- 10.2.7 Centrifuge for 10 - 15 minutes at 10,000 x g using a cold microcentrifuge to remove any insoluble material.
- 10.2.8 Collect supernatant.
- 10.2.9 Transfer to a new tube.
- 10.2.10 Keep on ice.
- 10.2.11 Measure protein concentration and adjust protein concentration to 1 – 4  $\mu$ g protein/assay (50 – 200  $\mu$ g/50  $\mu$ L Buffer for each microplate well).

## 11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate
- Prepare all reagents and samples as directed in the previous sections.

Add DTT to the 2X Reaction Buffer immediately before use (10mM final concentration). Add 10  $\mu$ L of 1M DTT stock to 1mL of 2X Reaction Buffer.

### 11.1 Plate Loading:

- Induced sample wells = 50 – 200  $\mu$ g protein/50  $\mu$ L (adjust volume with Cell Lysis Buffer).
- Un-Induced sample wells = 100 – 200  $\mu$ g protein/50  $\mu$ L (adjust volume with Cell Lysis Buffer).
- Background control wells = 50  $\mu$ L Cell Lysis Buffer

### 11.2 Assay Reaction:

- 11.2.1 Add 10  $\mu$ L of 1M DTT stock (Section 9.4) to 1 mL of 2X Reaction Buffer (100 mM DTT final concentration).
- 11.2.2 Add 50  $\mu$ L of 2X Reaction Buffer/DTT mixture into each reaction well (sample and background control wells.) Mix well by pipetting up and down.
- 11.2.3 Add 5  $\mu$ L of IETD-pNA substrate 4M stock (Section 9.5) into each well (sample and background control wells) (200 mM final substrate concentration). Mix well by pipetting up and down.
- 11.2.4 Incubate plate covered at 37°C for 1 – 2 hours.

### 11.3 Measurement:

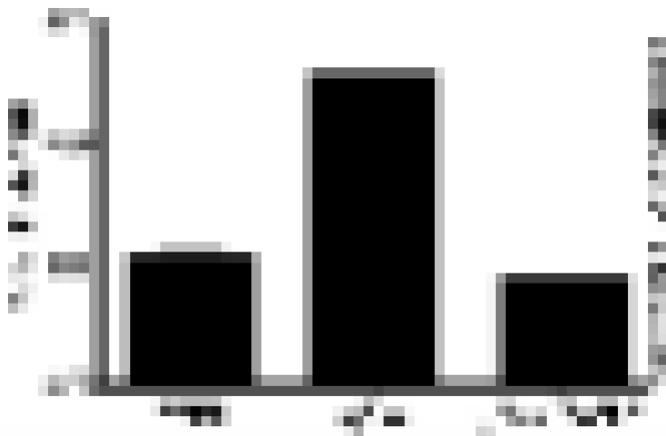
- 11.3.1 Measure output on a microplate reader at OD 400 nm.

**Δ Note:** Samples can be read in a spectrophotometer using a 100  $\mu$ L micro quartz cuvette, or by diluting sample to 1 mL with Dilution Buffer when using a regular cuvette. Keep in mind, however, that dilution of samples proportionally decreases the reading values.

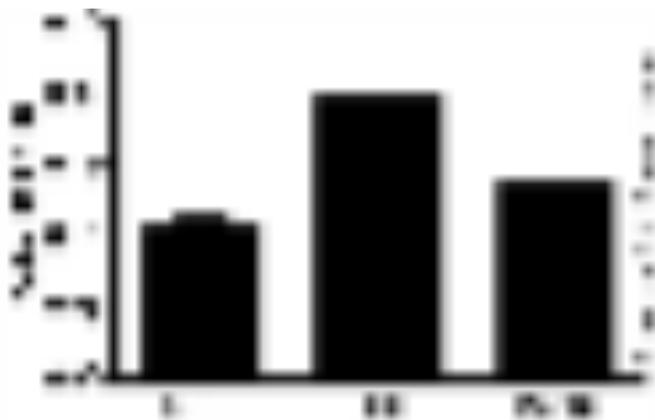
## 12. Data Analysis

- 12.1 Average the duplicate reading for each background and sample well.
- 12.2 If significant, subtract the background control (buffer) from sample reading (treated and untreated).
- 12.3 Determine fold increase in caspase 8 activity by comparing results from treated cells with untreated control.

### 13. Typical Data



**Figure 1.** Active caspase 8 in control (CTRL) Jurkat cells ( $10^6$ /mL) or in cells after four hours exposure to 50 ng/mL anti-Fas Ab (aFas) (MBL), or pretreated one hour with 50  $\mu$ M Z-VAD(OMe)-FMK (ab120487) followed by four hours with aFas. Background signal subtracted, duplicates; +/- SD.



**Figure 2.** Active caspase 8 in control (CTRL) Jurkat cells ( $10^6$ /mL) or cells treated for five hours with 10  $\mu$ g/mL Cyclohexamide (CHX) (ab120093) or four hours with 25  $\mu$ g/mL Mitomycin C (MitoC) (ab120797). Background signal subtracted, duplicates; +/- SD.

## 14. Factors to consider for caspase activity assays

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

- 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 or use of fluorescent substrates e.g. FRET assays should be used in combination with caspase activity assays.
- The expression and abundance of each caspase in a particular cell type and cell line will vary.
- 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 shows 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.

## Apoptotic Initiator Caspases

Caspase	Cleavage motif	Inhibitor motif	Cross-reactivity with other caspase:											
			1	2	3	4	5	6	7	8	9	10		
<b>Caspase 2</b>	VDVAD				Y					Y				
<b>Caspase 8</b>	IETD	IETD, LETD			Y				Y					Y
<b>Caspase 9</b>	LEHD				Y				Y		Y			Y
<b>Caspase 10</b>	AEVD				Y					Y	Y ?			

## 15. Troubleshooting

<b>Problem</b>	<b>Reason</b>	<b>Solution</b>
<b>Assay not working</b>	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
<b>Sample with erratic readings</b>	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
<b>Lower/higher readings in samples and standards</b>	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

<b>Problem</b>	<b>Reason</b>	<b>Solution</b>
<b>Standard readings do not follow a linear pattern</b>	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 $\mu$ L) 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 described in the protocol
<b>Unanticipated results</b>	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. Interferences

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

- RIPA Buffer: contains SDS which can destroy or decrease the activity of the enzyme.

## 17. FAQs

**Q. 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?**

A. 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.

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

A. 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 IETD-pNA is protected from light before use.

**Q. Can I use tissue samples with this kit?**

A. Yes, you can. Fresh tissue is preferred over frozen tissue as caspases are proteases and prone to degradation.

Homogenize tissue in 50 – 100 µL Cell Lysis Buffer and centrifuge at top speed on a cold microcentrifuge for 10 – 15 minutes. Determine protein concentration and proceed with the assay from step 11.2.

**Q. Can this kit work with supernatant secreted from cell culture?**

A. 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.

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

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

**Q. In this assay, you can compare the negative control and the sample, treated and untreated respectively. Isn't the p-NA needed only as a positive control?**

Yes, p-NA could be act as a good positive control. However, it does not give you any indication about your what is happening in your samples, it just shows that the kit components are working. Alternatively, you could use active caspase 8 as well for a positive control. Just the comparison between the treated and the untreated samples should give the answer to whether there is any induction of Caspase-8 in your samples and what fold of induction. The positive control will just help you see that the kit components are working well.

## 18. Notes





