For life science research only.
Not for use in diagnostic procedures.

Cell Death Detection ELISA

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Photometric enzyme immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death

Cat. No. 11 544 675 001 1 kit
96 tests

Store the kit at +2 to +8°C.

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1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	white	Cell Death Detection ELISA, Anti-histone	▪ Lyophilized, stabilized ▪ Monoclonal antibody from mouse (clone H11-4).	1 bottle
2	red	Cell Death Detection ELISA, Anti-DNA-POD	▪ Lyophilized, stabilized ▪ Monoclonal antibody from mouse (clone MCA-33) conjugated with peroxidase (POD). ▪ Binding of the DNA components of the nucleosomes and the color reaction with ABTS substrate.	1 bottle
3	white	Cell Death Detection ELISA, Coating buffer, 10x conc.	For preparation of Coating solution.	1 bottle, 2 ml
4	green	Cell Death Detection ELISA, Washing buffer, 10x conc.	For preparation of Washing solution.	1 bottle, 40 ml
5	red	Cell Death Detection ELISA, Incubation buffer	Ready-to-use solution.	1 bottle, 100 ml
6	white	Cell Death Detection ELISA, Substrate buffer	▪ Ready-to-use solution. ▪ Use 5 ml Substrate buffer for one ABTS tablet.	1 bottle, 15 ml
7	white	Cell Death Detection ELISA, ABTS substrate tablet	Each tablet sufficient for 5 ml Substrate solution.	1 bottle, 3 tablets, 5 mg each
8	foil bag	Cell Death Detection ELISA, Microplate	Shrink-wrapped with a desiccant capsule (12 × 8 wells).	1 strip frame, 12 modules of 8 wells each
9	–	Cell Death Detection ELISA, Self-adhesive Plate Cover Foil	Prevents evaporation. ⚠ ***Cover the Microplate modules with the Cover Foils during each incubation step.***	4 foils

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	white	Anti-histone	Store at +2 to +8°C.
2	red	Anti-DNA-POD	
3	white	Coating buffer, 10x conc.	
4	green	Washing buffer, 10x conc.	
5	red	Incubation buffer	
6	white	Substrate buffer	
7	white	ABTS substrate tablet	Store at +2 to +8°C. ⚠ ***Keep protected from light.***
8	foil bag	Microplate	Store at +2 to +8°C.
9	–	Self-adhesive Plate Cover Foil	

1.3. Additional Equipment and Reagent required

For sample preparation and ELISA assay

- Sterile disposable tubes and pipette tips
- Centrifuge
- CO_2 incubator

For the preparation of kit working solutions

- Double-distilled water

For titration of camptothecin

- Camptothecin (CAM)
- HL-60 (ATCC CCL-240) human myelogenous leukemic cell line
- For suspension cultures: cell-culture grade, round-bottomed microplates
- For adherent cells: cell-culture grade, flat-bottomed ELISA assay microplates
- Microplate shaker
- ELISA reader: The green color of the substrate ABTS can be easily detected by eye, for numeric values, however, a photometric measurement is required
- Microplate washer or multichannel pipettes for more convenient washing of the microplate
- Automated pipeting system for automated workflows

The kit contains all the reagents needed and in sufficient amounts for 96 tests.

1.4. Application

The Cell Death Detection ELISA is used to analyze histone-associated DNA fragments (mono- and oligonucleosomes) which are known to be present in the cytoplasm of cells undergoing apoptosis.

1.5. Preparation Time

Assay Time

Approximately 5 to 6 hours.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The Cell Death Detection ELISA is used with:
- Cytoplasmic fractions (lysates) from cell lines.
- Cytoplasmic fractions (lysates) from cells *ex vivo*.
- Tissue homogenates

Control Reactions

Negative control

Prepare a negative control for cell death induction (cellular assay). Depending on cell culture conditions, each exponentially growing permanent cell culture contains a certain amount of dead cells, approximately 3 to 8%. In the immunoassay, these inherent dead cells in the untreated sample (without cell death-inducing reagent) will cause a certain absorbance value.

Depending on the amount of dead cells, this value may exceed the absorbance value of the immunoassay background.

Positive control

Since the negative control of the cellular assay will cause a certain absorbance value in the immunoassay, there is no need for a positive control. However, if an extra positive control for the immunoassay is desired, prepare according to the following protocol:

1. Centrifuge an aliquot of untreated cells (5 × 10^4 cells/ tube in 500 µl, see section, **Induction of cell death**) at 1,500 rpm for 5 minutes.
2. Discard the supernatant.
3. Resuspend the cell pellet in 500 µl hypertonic buffer (10 mM Tris, pH 7.4, 400 mM NaCl, 5 mM $CaCl_2$, and 10 mM $MgCl_2$).
4. Incubate at +37°C for at least 2 hours.
5. Spin down the cell fragments at 15,000 rpm.
6. Remove supernatant carefully, dilute the supernatant 1: 5 with Incubation buffer (Bottle 5), and use this sample as a positive control for the immunoassay.

Safety Information

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

Content	Reconstitution/Preparation of Working Solution	Storage and Stability	For use in...
Anti-histone (Bottle 1)	Reconstitute the lyophilizate in 1 ml double-distilled water for 10 minutes; mix thoroughly.	Store 2 months at +2 to +8°C.	ELISA assay, preparation of Coating solution.
Anti-DNA-POD (Bottle 2)	Reconstitute the lyophilizate in 1 ml double-distilled water for 10 minutes; mix thoroughly.	Store 2 months at +2 to +8°C.	ELISA assay, preparation of Conjugate solution.
Coating solution	▪ Predilute 1 ml Coating buffer concentrate (Bottle 3) with 9 ml double-distilled water. ▪ Shortly before use, dilute 1 ml Anti-histone antibody (Bottle 1, reconstituted) with 9 ml Coating buffer.	Always prepare fresh before use; do not store.	ELISA assay, Step 1
Washing solution	▪ Warm Washing buffer concentrate (Bottle 4) at +15 to +25°C. ▪ Dilute 40 ml of the prewarmed Washing buffer in 360 ml double-distilled water; mix thoroughly.	Store 2 months at +2 to +8°C.	ELISA assay, Steps 6, 9, and 12
Sample solution	▪ Depends on the cell system used and the extent of cell death, see section, **Sample preparation.** ▪ Example: Dilute 25 µl sample in 225 µl Incubation buffer (Bottle 5).	Store 2 months at +2 to +8°C.	ELISA assay, Step 7
Conjugate solution	Dilute 1 ml Anti-DNA-POD (Bottle 2, reconstituted) with 9 ml Incubation buffer (Bottle 5).	Always prepare fresh before use; do not store.	ELISA assay, Step 10
Substrate solution	▪ Depending on the number of samples tested, dissolve 1, 2, or 3 tablets from Bottle 7 in 5, 10, or 15 ml Substrate buffer (Bottle 6). ▪ Equilibrate to +15 to +25°C before use.	Store 1 month at +2 to +8°C. ⚠ ***The ABTS solution reacts to light on exposure over a long period. Keep protected from light.***	ELISA assay, Step 13
Microplate	Use only the Microplate modules required for the particular experiment. Close the foil bag containing the remaining modules and the desiccant capsule tightly with adhesive tape. ⓘ *The Microplates are ready-to-use and do not need to be rehydrated before use.*	Once the foil bag is opened, store Microplate modules desiccated at +2 to +8°C for a minimum of 2 weeks.	ELISA assay

2.2. Protocols

Sample preparation

Dilute the cells with culture medium to obtain a suitable cell concentration. Depending on the cell type and the cell death-inducing agent, the cell number per test must be determined and optimized. The following cellular model system, in particular the cell number per test, is an example for a test procedure. As a model system for cell death, camptothecin (CAM) was used as the apoptosis-inducing drug. The tests were performed with the human myelogenous leukemic cell line HL-60 (ATCC CCL-240) as target cells (Fig. 1).

Induction of cell death (cellular assay)

1. Dilute exponentially growing HL-60 cells with culture medium to obtain a cell concentration of 1 × 10^5 cells/ml.
   – Transfer into tubes (500 µl/tube = 5 × 10^4 cells/tube).

2. Add 500 µl culture medium with different concentrations of CAM (0 µg CAM/ml to 4 µg CAM/ml).
   Use value 0 µg/ml as a negative control for the cellular assay (= viable, untreated cells).

3. Close tubes loosely to allow further exchange of gas and incubate at +37°C in a CO_2 incubator for 4 hours.

4. Centrifuge the cells at 200 × *g* for 5 minutes.

5. Discard the supernatant and resuspend the cell pellet in 1 ml culture medium.

6. Centrifuge the cells at 1,500 × *g* for 5 minutes.

7. Resuspend the cell pellet with 500 µl Incubation buffer (Bottle 5) per tube (1 × 10^5 cells/ml) and mix thoroughly.

8. Incubate the sample for approximately 30 minutes at +15 to +25°C (= lysis).

9. Centrifuge the lysate at 20,000 × *g* for 10 minutes.

10. Carefully remove 400 µl of the supernatant (= cytoplasmic fraction).
    ⚠ ***Do not shake the pellet (cell nuclei, containing high molecular weight, unfragmented DNA).***

11. Predilute the resulting supernatant 1:10 with Incubation buffer (= 1 × 10^4 cell equivalents/ml) and detect the nucleosomes in the sample by immunoassay, see **ELISA assay**.

Store the samples in aliquots at −15 to −25°C if they cannot be tested on the same day or, at the latest, one day later.

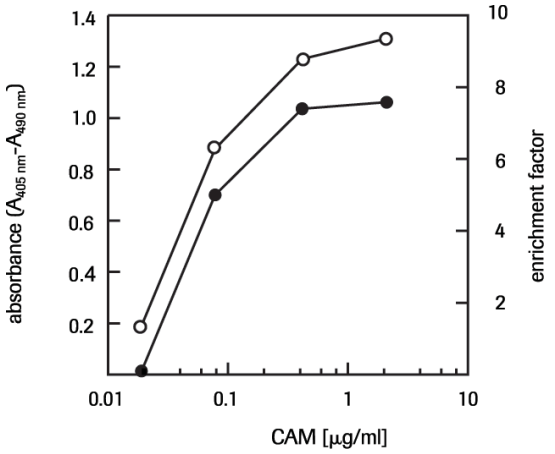

Fig. 1: Enrichment of nucleosomes in the cytoplasm of cells treated with camptothecin. HL-60 cells were exposed for 4 hours at +37°C to different CAM concentrations, see section **Induction of cell death**. After cell lysis and centrifugation, the cytoplasmic fractions were prediluted 1:10 with Incubation buffer and tested for nucleosomes by ELISA (= 1 × 10^4 cell equivalents/ml = 1 × 10^3 cell equivalents/well; for definition of cell equivalents, see section **ELISA assay**). Substrate reaction time: 15 minutes (○ absorbance [10^{-3}], ● enrichment factor).

ELISA assay

Cell Equivalent: Using 5 × 10^4 cells/well (500 µl), the sample analyzed (100 µl sample solution after a 1:10 predilution of the supernatant) corresponds to a cell equivalent of 1 × 10^4 cells/ml or 1 × 10^3 cells/well.
All samples should be analyzed in duplicates.
In addition, a negative control (cells without CAM treatment) should be analyzed, allowing calculation of an enrichment factor.

All incubation steps are performed at +15 to +25°C. To avoid contamination of the negative control during the washing and substrate incubation steps, use separate solutions.

1. Pipette 100 µl Coating solution into each well of the Microplate modules.
   – Cover Microplate modules tightly with the Self-adhesive Cover Foil.

2. Incubate for 1 hour at +15 to +25°C, or overnight at +2 to +8°C.

3. Remove Coating solution thoroughly by tapping or suction.

4. Pipette 200 µl Incubation buffer (Bottle 5) into each well of the Microplate module.
   – Cover Microplate modules tightly with the Self-adhesive Cover Foil.

5. Incubate for 30 minutes at +15 to +25°C.

6. Remove solution thoroughly by tapping or suction.
   – Rinse wells three times with 250 to 300 µl Washing solution per well; remove Washing solution carefully.

7. Pipette 100 µl of Sample solution into each well of the Microplate modules.
   – For determination of the background of the immunoassay, pipette 100 µl per well of Incubation buffer into two wells.
   – Cover Microplate modules tightly with the Self-adhesive Cover Foil.

8. Incubate for 90 minutes at +15 to +25°C.

9. Remove solution thoroughly by tapping or suction.
   – Rinse wells three times with 250 to 300 µl Washing solution per well; remove Washing solution carefully.

10 Pipette 100 µl of Conjugate solution into each well of the Microplate module, except the blank position.
– Cover Microplate modules tightly with the Self-adhesive Cover Foil.

11 Incubate for 90 minutes at +15 to +25°C.

12 Remove solution thoroughly by tapping or suction.
– Rinse wells three times with 250 to 300 µl Washing solution per well; remove Washing solution carefully.

13 Pipette 100 µl of Substrate solution into each well of the Microplate modules.

14 Incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis, approximately 10 to 20 minutes.

15 Homogenize well contents by carefully tapping the side of the Microplate before measuring at 405 nm with respect to a substrate solution blank.
– Alternatively, use 490 nm as the reference wavelength.

2.3. Parameters

Specificity

- Anti-histone antibody binds to histones H1, H2A, H2B, H3, and H4 of various species, such as human, mouse, rat, hamster, cow, opossum, and Xenopus.
- Anti-DNA-POD antibody binds to single- and double-stranded DNA. Therefore, the ELISA allows the detection of mono- and oligonucleosomes from various species and may be applied to measure apoptotic cell death in many different cell systems.

3. Results

Calculation

1. Average the values from the double absorbance measurements of the samples.

2. Subtract the background value, see section **Background value**, of the immunoassay from each of these averages.

3. Calculate the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula:

$$\text{enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding control (viable cells)}}$$

$$\text{mU} = \text{absorbance} \left[10^{3}\right]$$

Fig. 2: Enrichment factor formula.

Background value

Depending on the individual assay conditions, the background value (Incubation buffer instead of sample solution) of the immunoassay may vary. Under normal conditions, the background is below 100 mU after 15 minutes substrate reaction.

Handling very concentrated samples

Samples with values exceeding the measurement range of the photometer should be diluted and run again, see section **ELISA assay**. The corresponding control sample (viable cells) must be diluted by the same factor. Please make a note of this dilution factor when calculating the enrichment factor. Alternatively, the substrate reaction time can be decreased.

Detection limit

The exact detection limit of dying/dead cells in a particular sample strongly depends on the kinetics of cell death, the cytotoxic agent used, and the amount of affected cells in the total cell population. Using HL-60/CAM as a cellular model system for cell death, the immunoassay allows the specific detection of mono- and oligonucleosomes in the cytoplasmic fraction of 5 × 10^2 cells/ml (= 50 cell equivalents/well) (Fig. 3).

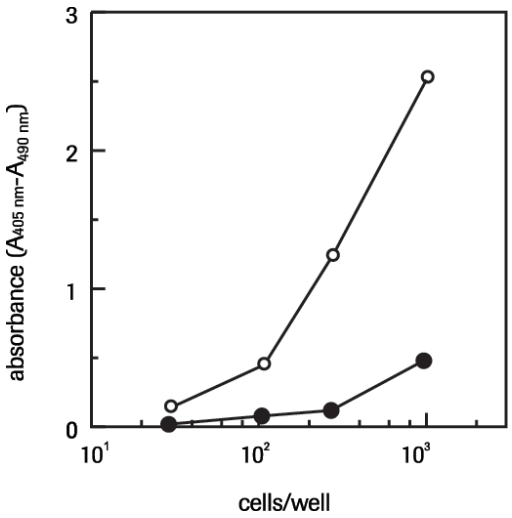

Fig. 3: Detection of nucleosomes in cytoplasmic fractions at different cell concentrations. HL-60 cells were cultured at different cell concentrations with CAM (2 µg/ml) or without CAM for 4 hours at +37°C. After cell lysis, the cytoplasmic fractions of the samples were prediluted 1:10 with Incubation buffer and tested in the immunoassay. Substrate reaction time: 10 minutes (○ with CAM, ● without CAM).

4. Additional Information on this Product

4.1. Test Principle

How this product works

The assay is based on a quantitative sandwich enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates (Fig. 4).

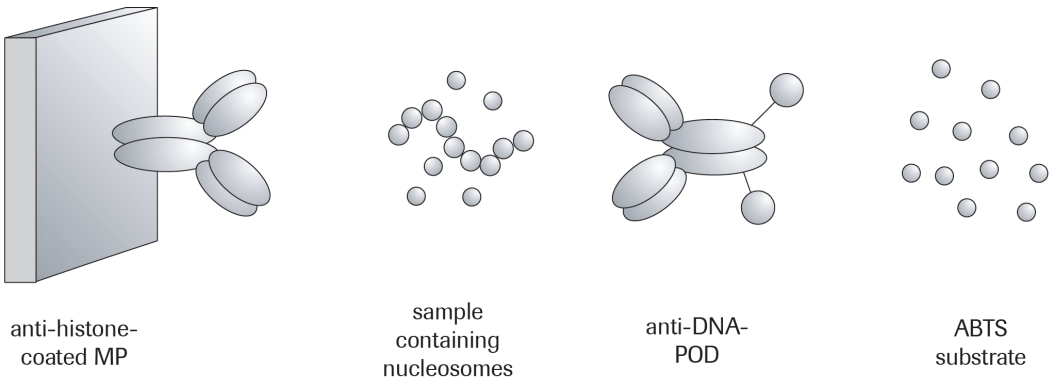

Fig. 4: Test principle

1. Fixation of Anti-histone antibody by adsorption on the wall of the Microplate module.
2. Saturation of nonspecific binding sites on the wall by treatment with Incubation buffer (= Blocking solution).
3. Binding of nucleosomes contained in the sample via their histone components to the immobilized Anti-histone antibody.
4. Addition of Anti-DNA-POD which reacts with the DNA part of the nucleosome.
5. Removal of unbound peroxidase conjugate by a washing step.
6. Determination of the amount of peroxidase retained in the immunocomplex with ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)]), as a substrate.

Eukaryotic cell death

Two distinct forms of eukaryotic cell death can be described by morphological and biochemical criteria: necrosis and apoptosis.

- Necrosis is accompanied by increased ion permeability of the plasma membrane; the cells swell and the plasma membrane ruptures within minutes (osmotic lysis).
- Apoptosis is characterized by membrane blebbing (zeiosis), condensation of cytoplasm, and the activation of an endogenous endonuclease as well as specific proteases.

Apoptosis

The endogenous endonuclease is Ca^{2+}- and Mg^{2+}-dependent and cleaves double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. In contrast, the DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3, and H4, and is therefore protected from cleavage by the endonuclease. The DNA fragments yielded are discrete multiples of an 180 bp subunit which is detected as a “DNA ladder” on agarose gels after extraction and separation of the fragmented DNA. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cell is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown.

Natural occurrence of apoptosis

Apoptosis is the most common form of eukaryotic cell death. It describes a physiological suicide mechanism that maintains tissue homeostasis.This type of cell death naturally occurs during:

- Normal tissue turnover
- Embryonic development of tissue, organs, and limbs.
- Thymic maturation: deletion of autoreactive T cells.
- Senescence of neutrophil polymorphs and following removal of specific growth factors, such as IL-2 or the addition of physiological stimuli, such as tumor necrosis factor and glucocorticoids.

Induction of apoptosis

Apoptosis is also induced by:

- Cytotoxic T lymphocytes and natural killer (NK) cells.
- Ionizing radiation
- Monoclonal antibodies, such as anti-Fas and anti-APO-1.

Physiological role of apoptosis

Inappropriate regulation of apoptosis may play an important role in many pathological conditions, such as cancer, AIDS, autoimmunity, Alzheimer disease, etc. This product is intended as a tool to increase scientific knowledge about these relationships.

i *The kit was not tested for use under hypoxic conditions.*

4.2. Quality Control

For lot-specific certificates of analysis, see section **Contact and Support**.

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Layout changes.
Editorial changes.
Update to include new safety Information to ensure handling according controlled conditions.

5.3. Trademarks

ABTS is a trademark of Roche.
All other product names and trademarks are the property of their respective owners.

5.4. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

5.5. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.6. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.7. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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