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

Cell Death Detection ELISA^{PLUS}

Version: 16
Content Version: November 2020

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. 11 774 425 001 1 kit
96 tests

Store the kit at +2 to +8°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	red	Cell Death Detection ELISA^{PLUS}, Anti-histone-biotin	▪ Lyophilized, stabilized ▪ Monoclonal antibody from mouse (clone H11-4), biotin-labeled. ▪ Binding of the histone component of the nucleosomes and capturing of the immunocomplex via biotin to the coated microplate.	1 bottle
2	white	Cell Death Detection ELISA^{PLUS}, 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	blue	Cell Death Detection ELISA^{PLUS}, Positive Control	▪ Lyophilized, stabilized ▪ DNA-histone-complex	1 bottle
4	green	Cell Death Detection ELISA^{PLUS}, Incubation buffer	Ready-to-use solution.	1 bottle, 100 ml
5	red	Cell Death Detection ELISA^{PLUS}, Lysis buffer	Ready-to-use solution.	1 bottle, 100 ml
6	colorless	Cell Death Detection ELISA^{PLUS}, Substrate buffer	▪ Ready-to-use solution. ▪ To dissolve the ABTS tablets.	1 bottle, 15 ml
7	white	Cell Death Detection ELISA^{PLUS}, ABTS substrate tablet	Each tablet sufficient for 5 ml Substrate solution.	1 bottle, 3 tablets
8	colorless	Cell Death Detection ELISA^{PLUS}, ABTS Stop Solution	▪ Ready-to-use solution. ▪ Stops the ABTS substrate reaction.	1 bottle, 100 ml
9	foil bag	Cell Death Detection ELISA^{PLUS}, Microplate	▪ Streptavidin-coated ▪ Shrink-wrapped with a desiccant capsule (12 × 8 wells).	1 strip frame, 12 modules of 8 wells each
10	–	Cell Death Detection ELISA^{PLUS}, 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	red	Anti-histone-biotin	Store at +2 to +8°C.
2	white	Anti-DNA-POD	
3	blue	Positive Control	
4	green	Incubation buffer	
5	red	Lysis buffer	
6	colorless	Substrate buffer	
7	white	ABTS substrate tablet	Store at +2 to +8°C. ⚠ ***Keep protected from light.***
8	colorless	ABTS Stop Solution	Store at +2 to +8°C.
9	foil bag	Microplate	
10	–	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)
- Human lymphoma cell line U937 (ATCC CRL-1593)
- 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 ABTS substrate can easily be detected by eye; for numeric values, a photometric measurement is required
- Microplate washer or multichannel pipettes for more convenient washing of the microplate
- Automated pipetting system to perform automated workflows

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

1.4. Application

The Cell Death Detection ELISA^{PLUS} 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 3 to 4 hours.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The Cell Death Detection ELISA^{PLUS} is used with:
- Cytoplasmic fractions (lysates) from cell lines.
- Cytoplasmic fractions (lysates) from cells *ex vivo*.
- Cell culture supernatants
- Serum

Control Reactions

Negative control

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 a cell death-inducing agent) will cause a certain absorbance value (negative control).

Positive control

A DNA-histone complex serves as a positive control.

Safety Information

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

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-biotin (Bottle 1)	Reconstitute the lyophilizate in 450 µl double-distilled water for 10 minutes; mix thoroughly.	Store 2 months at +2 to +8°C.	Component of immunoreagent
Anti-DNA-POD (Bottle 2)	Reconstitute the lyophilizate in 450 µl double-distilled water for 10 minutes; mix thoroughly.	Store 2 months at +2 to +8°C.	Component of immunoreagent
Positive Control (Bottle 3)	Reconstitute the lyophilizate in 450 µl double-distilled water for 10 minutes; mix thoroughly.	Store 2 months at +2 to +8°C.	ELISA assay, Step 1
ABTS Substrate tablets (Bottle 7)	▪ 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 6
ABTS Stop Solution (Bottle 8)	If turbidity or a precipitate is visible, warm up to +37°C with shaking until the solution is clear.	Store at +2 to +8°C through the expiration date printed on the label.	ELISA assay, Step 8
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

Preparation of immunoreagent

1. Mix 1/20 volume of Anti-DNA-POD (Bottle 2, reconstituted) and 1/20 volume Anti-histone-biotin (Bottle 1, reconstituted) with 18/20 volumes Incubation buffer (Bottle 4).
   ⚠ ***Always prepare the solution shortly before use, do not store.***

2. Place the Incubation buffer (Bottle 4) into a suitable vessel.

3. The following table shows the amounts needed for 10, 20, 40, 50, and 100 tests, respectively.

Number of Tests	10	20	40	50	100
Incubation buffer	720 µl	1,440 µl	2,880 µl	3,600 µl	7,200 µl

4. Add appropriate volumes of Anti-histone-biotin and Anti-DNA-POD.

Number of Tests	10	20	40	50	100
Anti-histone-biotin (Bottle 1, reconstituted)	40 µl	80 µl	160 µl	200 µl	400 µl
Anti-DNA-POD (Bottle 2, reconstituted)	40 µl	80 µl	160 µl	200 µl	400 µl
Immunoreagent Total Amount	800 µl	1,600 µl	3,200 µl	4,000 µl	8,000 µl

5. Homogenize thoroughly.

6. Do not store the solution.
   – The solution is used in the ELISA assay, Step 2.

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 and is therefore optimized. As a model system, the human lymphoma cell line U937 (ATCC CRL-1593) and the topoisomerase I-inhibitor camptothecin (CAM) was chosen for induction of apoptosis (Fig. 1).

Induction of cell death (cellular assay)

1. For adherent cells, trypsinize and wash the cells, seed amounts of cells in the microplate wells (e.g., 1 × 10^4 or less), and let them grow for an appropriate time before starting the assay.

2. Set up a titration of camptothecin (CAM) in declining concentrations from 4 µg/ml to 2 ng/ml.
   – Prepare duplicates of 100 µl/well.
   ⚠ ***Use cell culture medium without CAM as a negative control.***

3. Dilute exponentially growing U937 cells with culture medium to a concentration of 1 × 10^5 cells/ml.

4. Add 100 µl of diluted cells (1 × 10^4 cells) to each well.

5. Incubate for 4 hours at +37°C and 5% CO_2.

6. Centrifuge the Microplate 10 minutes at 200 × *g*.

7. If you want to analyze for necrosis, carefully remove the supernatant (= necrotic fraction), and store at +2 to +8°C.
   – Proceed to Step 9 (without centrifugation).
   – If you do not want to analyze for necrosis, carefully remove the supernatant.

8. Resuspend the cell pellet in 200 µl Lysis buffer (Bottle 5).
   – Incubate for 30 minutes at +15 to +25°C (Cell lysis).
   ⚠ ***Adherent cells can be lysed directly in the well without prior removal.***

9. Centrifuge the lysate for 10 minutes at 200 × *g*.
   – Carefully transfer 20 µl from the supernatant (= cytoplasmic fraction) into the streptavidin-coated Microplate for analysis.

⚠ ***Do not shake the pellet (cell nuclei, containing high molecular weight, unfragmented DNA).***
⚠ ***Analyze samples immediately; storage at +2 to +8°C or −15 to −25°C reduces the ELISA signals.***

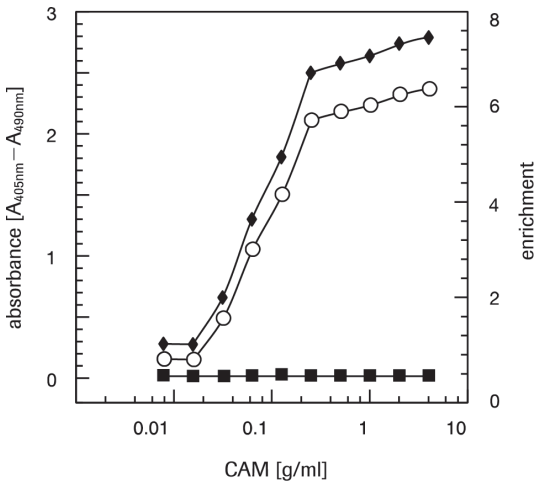

Fig. 1: Enrichment of nucleosomes in the cytoplasm of cells treated with camptothecin. U937 cells (1×10^4 cells/well = 200 µl) were exposed for 4 hours at +37°C to different CAM concentrations. Before and after lysis, cells were centrifuged and the supernatant (20 µl from 200 µl) was analyzed in the ELISA (corresponds to 1 × 10^3 cell equivalents/well = 5 × 10^4 cell equivalents/ml. For definition of cell equivalents, see section ELISA assay. Substrate reaction time: 5 minutes (♦ lysate, ■ supernatant, ○ enrichment factor of the lysate).

Sample preparation using an automated pipetting system

1. Set up a titration of camptothecin (CAM) in declining concentrations from 4 µg/ml to 2 ng/ml.
   – Prepare duplicates of 100 µl/well.
   ⚠ ***Use cell culture medium without CAM as a negative control.***

2. Dilute exponentially growing U937 cells with culture medium to a concentration of 1×10^5 cells/ml.

3. Add 100 µl of diluted cells (1×10^4 cells) to each well.

4. Incubate for 4 hours at +37°C and 5% CO_2.

5. Centrifuge the Microplate 10 minutes at 200 × *g*.

6. If you want to analyze for necrosis, carefully remove 50 µl of the supernatant (= necrotic fraction) and store it at +2 to +8°C.
   – Proceed to Step 10.
   – If you do not want to analyze for necrosis, remove 50 µl of the supernatant.
   ⚠ ***Make sure that your automated pipetting system removes the correct volume without aspirating cells.***

7. Resuspend the cell pellet in 200 µl Lysis buffer (reconstituted Bottle 5).

8. Incubate for 30 minutes at +15 to +25°C (Cell lysis).
   ⚠ ***Adherent cells can be lysed directly in the well without prior removal.***

9. Centrifuge the lysate at 200 × *g* for 10 minutes.

10. Carefully transfer 20 µl from the supernatant (= cytoplasmic fraction) into the streptavidin-coated microplate for analysis.

⚠ ***Do not shake the pellet (cell nuclei, containing high molecular weight, unfragmented DNA).***
⚠ ***Analyze samples immediately; storage at +2 to +8°C or −15 to −25°C reduces the ELISA signals.***

ELISA assay

The ELISA was developed and evaluated using 20 µl sample and 80 µl immunoreagent per Microplate well. Do not modify these proportions.

Cell Equivalent: Using 1 × 10^4 cells/well (200 µl), the sample analyzed (20 µl lysate or supernatant) corresponds to a cell equivalent of 1 × 10^3 cells/well or 5 × 10^4 cells/ml.
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. Transfer 20 µl from the following samples and controls into the Microplate:
   – Culture supernatants after centrifugation and treatment (CAM).
   – Lysates of CAM-treated cells after centrifugation.
   – Positive Control (Bottle 3).
   – Negative control (culture supernatant and lysate after centrifugation of untreated cells).
   – Background control (Incubation buffer, Bottle 4).
   ⚠ ***Due to the low volumes, pipette directly into the middle of the microplate well.***

2. Add 80 µl of the immunoreagent to each well.

3. Cover the Microplate with a Self-adhesive Cover Foil.

4. Incubate on a microplate shaker with gentle shaking (300 rpm) for 2 hours at +15 to +25°C.

5. Remove the solution thoroughly by tapping or suction.
   – Rinse each well 3 × with 250 to 300 µl Incubation buffer (Bottle 4).
   – Carefully remove solution.

6. Pipette 100 µl ABTS solution into each well.

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

8. Pipette 100 µl ABTS Stop Solution into each well.

9. Measure at 405 nm against ABTS solution plus 100 µl ABTS Stop Solution as a blank (reference wavelength approximately 490 nm).

2.3. Parameters

Specificity

- Anti-histone-biotin antibody binds to histones H1, H2A, H2B, H3, and H4 from 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 (Incubation buffer plus ABTS solution, plus ABTS Stop Solution) 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:

$$\text{enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding negative control (cells without CAM treatment)}}$$

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

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 (cells without treatment) 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.

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 treatment of 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

The Positive Control (DNA-histone-complex) included in the kit should show a signal of >600 mU after subtraction of the background within 15 minutes of the substrate reaction.

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 U937/CAM or Jurkat/CAM as a cellular model system for cell death, the immunoassay allows the specific detection of mono- and oligonucleosomes in the cytoplasmic fraction (20 µl lysate) from 150 cells/well (corresponds to 63 equivalents/ml, see Figures 3 and 4).

3. Results

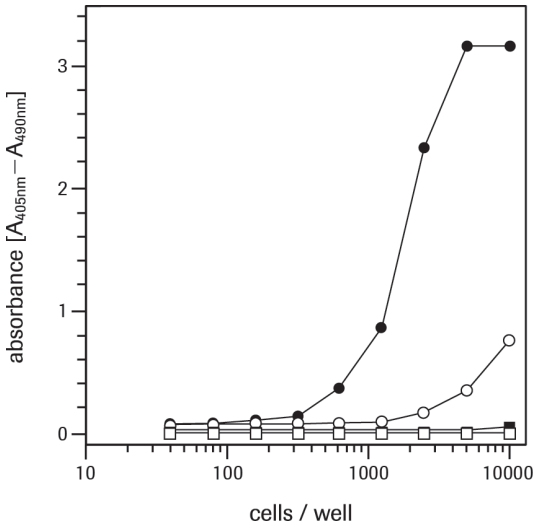

Fig. 3: Detection of nucleosomes in cytoplasmic fractions at different cell concentrations. Different cell concentrations of U937 cells were incubated with CAM (2 µg/ml) or without CAM for 4 hours at +37°C. 20 µl of cell culture supernatant and cell lysates were analyzed in the ELISA.
Substrate reaction time: 10 minutes (● lysate with CAM, ○ lysate without CAM, ■ supernatant with CAM, □ supernatant without CAM).

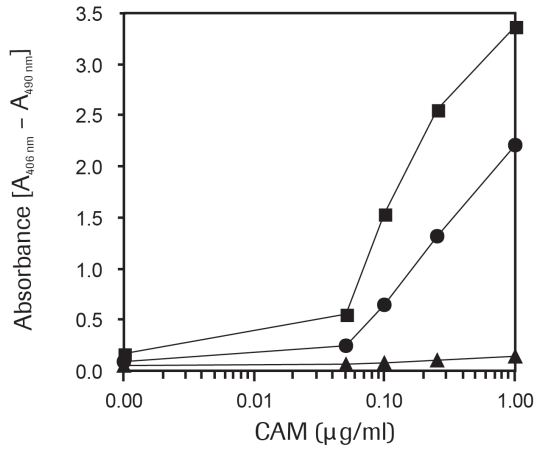

Fig. 4: Detection of nucleosomes in cytoplasmic fractions at different cell concentrations. Different cell concentrations of Jurkat cells were incubated with different CAM concentrations (0.05, 0.1, 0.25, 1 µg/ml) or without CAM for 4 hours at +37°C. 20 µl of cell lysates were analyzed in the ELISA.
Substrate reaction time: 20 minutes (■ = 1,000 cells; ●= 500 cells; ▲= 100 cells).

4. Troubleshooting

Observation	Possible cause	Recommendation
Absorbance of samples too low.	Induction of apoptosis too low.	Increase the agent concentration.
		Extend the agent incubation interval.
	Inefficient release of nucleosomes.	Extend the incubation period with Lysis buffer.
		Incubate on a plate shaker.
	Cell culture medium contains too much biotin (>25 mg/ml).	Use biotin-reduced medium or a different medium.
Absorbance of samples too high.	Induction of apoptosis too strong while negative control is in acceptable range.	Reduce agent concentration.
		Decrease incubation period.
Absorbance of negative control too high.	Cells used are in poor culture condition (many dead cells).	Use cells in exponential growth phase with a maximum of 3 to 8% Trypan Blue-positive cells.
	Too many cells used.	Reduce cell concentration.
	Too long substrate incubation period.	Shorten the substrate incubation time. Check color development 5 minutes after addition of ABTS substrate.
Absorbance of background too high.	Substrate is too old or was not protected from light and shows color development without enzymatic activity.	Use new substrate solution.
Variations too high.	Cell number/well is not homogeneous.	Prepare homogeneous cell suspension and resuspend before pipetting into wells.
	The discarded medium after agent incubation contains cells due to strong suction or too aggressive flick off.	Centrifuge for a longer period.
		Aspirate more carefully.
		Use Microplate inversion method: invert centrifuged Microplate without flicking off, and wipe off the drops in the inverted microplate position. Centrifuged cells will remain at the bottom.,
	Uneven mixing of sample and immunoreagent.	Pipette samples and immunoreagent into the middle of the well, not to the side wall.

5. Additional Information on this Product

5.1. 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. 5).

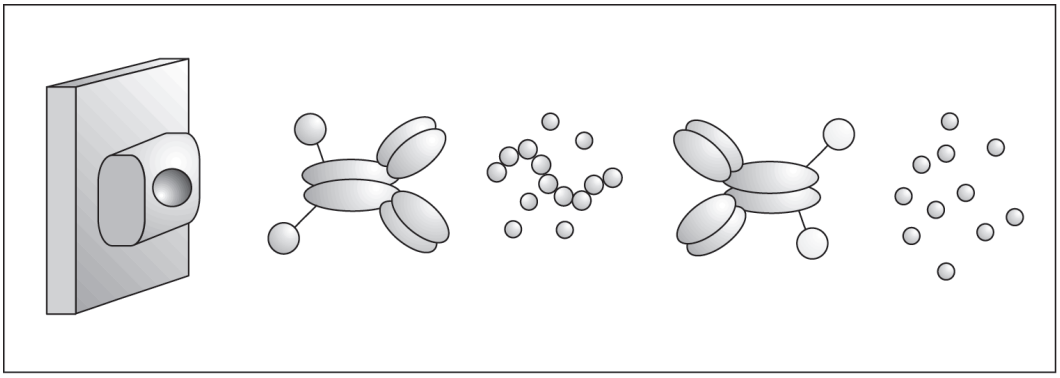

Fig. 5: Test principle

1. The sample (cell lysate, serum, culture supernatant, etc.) is placed into a streptavidin-coated Microplate.

2. A mixture of Anti-histone-biotin and Anti-DNA-POD are added and incubated.
   – During the incubation period, the Anti-histone antibody binds to the histone component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated Microplate via its biotinylation.
   – Additionally, the Anti-DNA-POD antibody reacts with the DNA component of the nucleosomes.

3. Removal of unbound components (antibodies) by a washing step.

4. Quantitative determination of the amount of nucleosomes by the POD retained in the immunocomplex.
   – POD is determined photometrically with ABTS 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 developent 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.*

5.2. Quality Control

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

6. Supplementary Information

6.1. 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.

6.2. Changes to previous version

Layout changes.
Editorial changes.
New information added related to the REACH Annex XIV.
Update to include new safety Information to ensure handling according controlled conditions.

6.3. Trademarks

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

6.4. License Disclaimer

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

6.5. Regulatory Disclaimer

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

6.6. Safety Data Sheet

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

6.7. 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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