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

Cell Death Detection ELISA^{PLUS}

Version 15

Content version: March 2016

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

Kit for 96 tests

Store the kit at +2 to +8°C

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Table of Contents

PROTOCOL

PROTOCOL

1. What this Product Does

Number of Tests The kit is designed for 96 tests.

Kit Contents

Vial/Bag Cap	Label	Contents / Function
1 red	Anti-Histone-Biotin	• Monoclonal antibody from mouse (clone H11-4) • Biotin-labeled • Lyophilized • Stabilized • For the binding of the histone component of the nucleosomes and capturing of the immunocomplex via biotin to the coated microplate (MP)
2 white	Anti-DNA-POD	• Monoclonal antibody from mouse (clone MCA-33) • Peroxidase conjugated • Lyophilized • Stabilized • For the binding of the DNA components of the nucleosomes and the color reaction with ABTS Substrate
3 blue	Positive Control	• DNA-Histone-Complex • Lyophilized • Stabilized
4 green	Incubation Buffer	• 100 ml • Ready-to-use
5 red	Lysis Buffer	• 100 ml • Ready-to-use
6 colorless	Substrate Buffer	• 15 ml • Ready-to-use • For dissolving the ABTS tablets
7 white	ABTS Substrate Tablet	• 3 tablets • Each sufficient for 5 ml substrate solution
8 colorless	ABTS Stop Solution	• 100 ml • Ready-to-use • For stopping the ABTS substrate reaction
9	Microplate	• 12 Microplate modules (8 wells each) • Streptavidin-coated • Frame
10	Adhesive Cover Foils	• 4 adhesive cover foils • To cover microplates

Storage and Stability

The unopened kit is stable at +2 to +8°C until the expiration date printed on the label.

Additional Equipment and Reagents Required

To perform assays with this ELISA Kit, you will need the following equipment for the sample preparation and the ELISA Assay:

Sample preparation

- Centrifuge
- CO_2 Incubator

For the titration of camptothecin, dependent on the cell type, the following is needed

- For suspension cultures use microplate (cell culture grade), round bottomed
- For adherent cells use microplate (cell culture grade), flat bottomed ELISA Assay
- 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 multi channel pipettes allow a more convenient washing of the microplate.
- Automated Pipetting System to perform automated workflow

Application

Measurement of apoptotic cell death in cellular systems.

Assay Time

3 – 4 hours

2. How To Use this Product

2.1 Before You Begin

Sample Material

- Cytoplasmic fractions (lysates) from cell-lines
- Cytoplasmic fractions (lysates) from cells *ex vivo*
- Cell culture supernatants
- Serum

Control Reactions

Prepare negative control (cells without CAM treatment) and positive control (bottle 3, see Procedure)

Preparation of Working Solutions

Beside the ready-to-use solutions supplied with this kit, you will need to prepare the following working solution:

⚠ We strongly recommend to use only double distilled water for reconstitution of lyophylizates

Content	Preparation	For use in	Storage and Stability
1 Anti-Histone Biotin	Reconstitute the lyophilizate in 450 µl double dist. water for 10 min and mix thoroughly.	Part of the Immunoreagent	At +2 to +8°C for 2 months
2 Anti-DNA POD	Reconstitute the lyophilizate in 450 µl double dist. water for 10 min and mix thoroughly.	Part of the Immunoreagent	
3 Positive Control	Reconstitute the lyophilizate in 450 µl double dist. water for 10 min and mix thoroughly.	ELISA Step 1	
7 ABTS Tablets	Dependent on the number of samples tested, dissolve 1, 2, or 3 tablets from bottle 7 in 5, 10, or 15 ml Substrate Buffer (vial 6).	ELISA Step 5	1 month Store protected from light! Allow to come to +15 to +25°C before use.
8 ABTS Stop Solution	If turbidity or a precipitate is visible warm up to +37°C, with shaking, until the solution is clear.	ELISA Step 6	At +2 to +8°C through the expiration date printed on the label

Preparation of the Immunoreagent

The Immunoreagent is prepared by mixing of 1/20 volume Anti-DNA-POD (bottle 2, reconstituted) and 1/20 volume Anti-histone-biotin (bottle 1, reconstituted) with 18/20 volumes Incubation Buffer (bottle 4).

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

⚠ Always prepare the solution shortly before use, do not store.

Place the Incubation Buffer (bottle 4) into a suitable vessel.

Use the following table for the amount which is needed:

# of tests	10	20	40	50	100
Incubation Buffer	720 µl	1440 µl	2880 µl	3600 µl	7200 µl

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

# 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
Immuno-reagent total amount	800 µl	1,600 µl	3,200 µl	4,000 µl	8,000 µl

• Homogenize thoroughly.

⚠ Do not store the solution. The solution is used in the ELISA Assay in step 2.

The following cellular model system, in particular the cell number per test, is an example for a test procedure and is optimized therefore.

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

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 has to be determined and optimized.

For adherent cells we recommend to trypsinize and wash the cells, seed amounts of cells in the MP wells (*e.g.,* 10^4 or less) and let them grow for an appropriate while before starting the assay.

❶	Set up a titration of camptothecin (CAM) in declining concentrations from 4 µg/ml to 2 ng/ml. Duplicates of 100 µl/well are recommended. ⚠ Use cell culture medium without CAM as negative control.
❷	Dilute exponentially growing U937 cells with culture medium to a concentration of 1×10^5 cells/ml.
❸	Add 100 µl of diluted cells (10^4 cells) to each well.
❹	Incubate for 4 h at +37°C and 5% CO_2.
❺	Centrifuge the MP 10 min, with 200 × *g*.
❻	• If you want to analyze necrosis remove the supernatant (= necrotic fraction) carefully, and store it at +2 to +8°C. Proceed with step 8 (without centrifugation). • If you don't want to analyze the necrosis, simply remove the supernatant carefully.
❼	• Resuspend the cell pellet in 200 µl Lysis Buffer (bottle 5). • Incubate for 30 min at +15 to +25°C (Cell lysis). ⚠ Adherent cells can be lysed directly in the well without prior removal.
❽	• Centrifuge the lysate at 200 × *g* for 10 min. • Transfer 20 µl from the supernatant (= cytoplasmic fraction) carefully into the streptavidin coated MP for analysis. ⚠ Do not shake the pellet (cell nuclei, containing high molecular weight, unfragmented DNA). ⚠ Samples should be analyzed immediately, because storage at +2 to +8°C or –15 to –25°C reduces the ELISA signals.

Sample preparation using an Automated Pipetting System

❶ to ❺	Perform all steps of the Sample preparation protocol above up to step 6
❻	• If necrosis analysis is desired, carefully remove 50 µl of the supernatant (= necrotic fraction) and store it at +2 to +8°C. Proceed with step 8 (without centrifugation). • If necrosis analysis is not desired, simply remove 50 µl of the supernatant ⚠ Make sure that your automated pipetting system removes the correct volume without aspirating cells.
❼	• Resuspend the cell pellet in 200 µl Lysis Buffer (reconstituted bottle 5). • Incubate for 30 min at +15 to +25°C (Cell lysis). ⚠ Adherent cells can be lysed directly in the well without prior removal.
❽	• Centrifuge the lysate at 200 × *g* for 10 min. • Transfer 20 µl from the supernatant (= cytoplasmic fraction) carefully into the streptavidin coated MP for analysis. ⚠ Do not shake the pellet (cell nuclei, containing high molecular weight, unfragmented DNA). ⚠ Samples should be analyzed immediately, because storage at +2 to +8°C or -15 to -25°C reduces the ELISA signals.

ELISA Assay

The ELISA was developed and evaluated with the use of 20 µl sample and 80 µl Immunoreagent per MP-well. It is recommended not to change these portions.

Cell Equivalent: Using 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.

⚠ It is recommended to analyze at least duplicates of the samples. Also, a negative control (cells without CAM treatment) should be analyzed, which allows calculation of an enrichment factor. Working temperature +15 to +25°C.

❶	Transfer 20 µl from • 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) into the MP. ⚠ It is important, due to low volumes, to pipette into the middle of the microplate well.
❷	Add to each well 80 µl of the Immunoreagent.

❸	• Cover the MP with an adhesive cover foil. • Incubate on a MP shaker under gently shaking (300 rpm) for 2 h at +15 to +25°C.
❹	• Remove the solution thoroughly by tapping or suction. • Rinse each well 3× with 250–300 µl Incubation Buffer (bottle 4). • Remove solution carefully.
❺	• Pipette to each well 100 µl ABTS solution. • Incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis (approx. after 10–20 min.)
❻	Pipette to each well 100 µl ABTS Stop Solution.
❼	Measure at 405 nm against ABTS solution + 100 µl ABTS Stop Solution as a blank (reference wavelength approx. 490 nm).

Calculation

In the following table the calculation is described

❶	Average the values from the double absorbance measurements of the samples.
❷	Substract the background value (Incubation Buffer + ABTS Solution + ABTS Stop Solution) of the immunoassay from each of these averages.
❸	Calculate the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula: $\text{enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding negative control (cells without CAM treatment)}}$ $\text{mU} = \text{absorbance } [10^{-3}]$

Handling very Concentrated Samples

If values of samples are exceeding the measurement range of the photometer dilute these samples and run again the ELISA (section 2.2). The corresponding control sample (cells without treatment) has to be diluted by the same factor. Please note this dilution factor when calculating the enrichment factor. Alternatively, the substrate reaction time can be decreased.

3. Results

Background of the Immunoassay

Depending on 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 min substrate reaction.

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 (normally approx. 3-8%). 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 of 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 min of the substrate reaction.

Enrichment of Nucleosomes after CAM Treatment

The results of the experiment after analysis of the samples by the ELISA are shown in Fig 1.

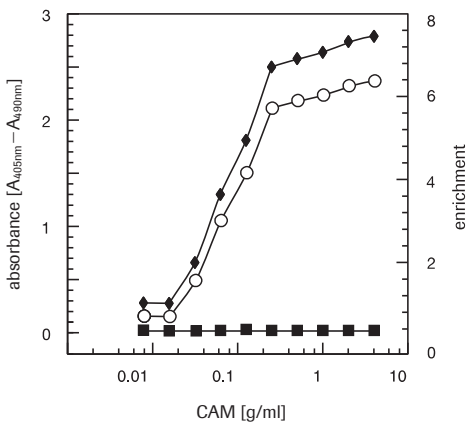

Fig. 1: Enrichment of nucleosomes in the cytoplasm of cells treated with camptothecin. U937 cells (1×10^4 cells/well = 200 µl) were exposed for 4 h at +37°C to different concentrations of CAM. Before and after lysis, cells were centrifuged and the supernatant (20 µl from 200 µl) was analyzed in the ELISA (corresponds to 1×10^3 cell equivalents/well = 5×10^4 cell equivalents/ml, for definition of cell equivalents see 2.2). Substrate reaction time: 5 min (♦ lysate, supernatant, ○ enrichment factor of the lysate).

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 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 cell equivalents/ml; see Fig. 2):

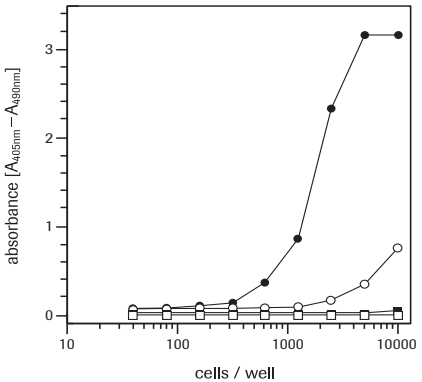

Fig. 2: Detection of nucleosomes in cytoplasmic fractions at different cell concentrations. Different cell concentrations of U 937 cells were incubated with CAM (2 µg/ml) or without CAM for 4 h at +37°C. 20 µl of cell culture supernatant and cell lysates were analyzed in the ELISA. Substrate reaction time: 10 min. ● lysate with CAM, ❍ lysate without CAM, ■ supernatant with CAM, ❑ supernatant without CAM.

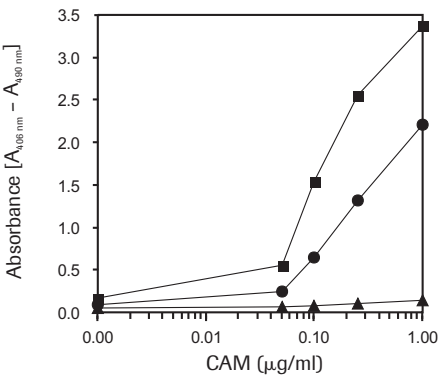

Fig. 3: Detection of nucleosomes in cytoplasmic fractions at different cell concentrations. Different cell concentrations of Jurkat cells were incubated with different CAM concentrations (0.05, 0.1, 0.25, 1 µg/ml) or without CAM for 4 h at +37°C. 20 µl of cell lysates were analyzed in the ELISA. Substrate reaction time: 20 min (■ = 1000 cells; ● = 500 cells; ▲ = 100 cells).

4. Troubleshooting

Different cellular systems could exhibit big variances during cultivation, incubation intervals, response to cytotoxic agents etc. Most problems will occur because of too high or too low absorbance values. The following table tries to give solutions for correction of mistakes, which could appear by wrong application of the kit or the cellular system used:

	Possible Cause	Recommendation
Absorbance of samples too low	Induction of apoptosis too low	• Increase the agent concentration • Prolong the agent incubation interval
	Inefficient release of nucleosomes	• Prolong the incubation period with Lysis Buffer • Incubate on a shaker
	Cell culture medium contains too much biotin (>25 mg/ml)	Use biotin-reduced or different medium.
Absorbance of samples too high	Induction of apoptosis too strong while negative control in acceptable range	• Reduce agent-concentration. • Decrease incubation period.
Absorbance of negative control too high	Cells used are in bad culture condition (a lot of dead cells)	Use cells in exponential growth phase with a max. of 3 – 8% trypanblue-positive cells.
	Too many cells used	Reduce cell concentration.
Absorbance of the background too high	Substrate is too old or was not light protected 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 because of too strong suction or too aggressive flick off	• Centrifuge for a longer period. • Aspirate more carefully. • Use MP-inversion-method: invert centrifuged MP without flicking off and wipe off the drops in inverted MP position. Centrifuged cells will remain at the bottom.
	Uneven mixing of sample and Immunoreagent	Pipet samples and immunoreagent into the middle of the well and not to the wall.

5. Additional Information on this Product

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.

Test Principle

❶	The sample (cell-lysate, serum, culture-supernatant etc.) is placed into a streptavidin-coated MP.
❷	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 MP via its biotinylation. Additionally, the Anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes.
❸	Removal of unbound components (antibodies) by a washing step.
❹	Quantitative determination of the amount of nucleosomes by the POD retained in the immunocomplex. POD is determined photometrically with ABTS Substrate.

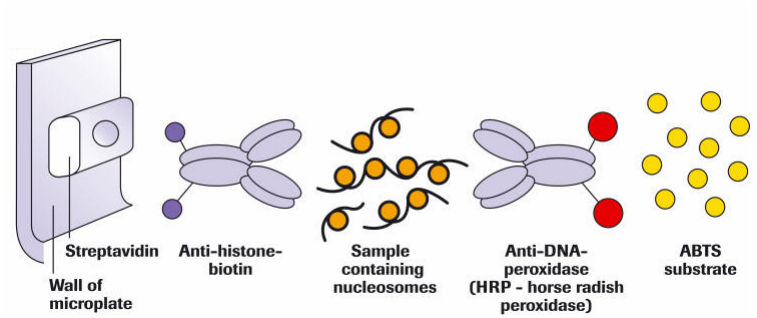

Fig. 4: Scheme shows the principle of the Cell Death Detection ELISA^{PLUS}

Specificity

- The Anti-histone-biotin-antibody binds to histones H1, H2A, H2B, H3 and H4 from various species, *e.g.,* man, mouse, rat hamster, cow, opossum and xenopus.
- The Anti-DNA-POD-antibody reacts with single and double stranded DNA.

Eucaryotic Cell Death

Two distinct forms of eucaryotic cell death can be described by morphological and biochemical criteria: necrosis and apoptosis (2, 3).

- Necrosis is accompanied by increased ion permeability of the plasma membrane; the cell 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 therefore is protected from cleavage by endonuclease (4, 5). 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 cells is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown (6-8).

Natural Occurrence of Apoptosis

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

- normal tissue turnover (9)
- embryonic development of tissue
- organs and limbs (10)
- thymic maturation: deletion of autoreactive T-cells
- senescence of neutrophil polymorphs and following removal of specific growth factors like IL-2 or the addition of physiological stimuli like tumor necrosis factor and glucocorticoids (11, 12).

Induction of Apoptosis

Apoptosis is induced by

- cytotoxic T-lymphocytes and natural killer (NK) cells (13, 14),
- ionizing radiation (15)
- monoclonal antibodies like anti-Fas (16) and anti-APO-1 (17, 18).

Physiological Role of Apoptosis

Inappropriate regulation of apoptosis may play an important role in many pathological conditions like cancer, AIDS, autoimmunity, Alzheimer disease etc. (19–22). This product is intended as a tool to increase scientific knowledge about these relationships.

Note

This kit was not tested for use under hypoxia conditions.

5.2 References

1 Darzynkiewicz Z *et al. Cytometry* (1992);**13**:795.
2 Wyllie A H *et al. Int. Rev. Of Cytol.* (1980);**68**:251.
3 Duvall E & Wyllie A H. *Immunol. Today* (1986);**7**:115.
4 Burgoyne L A *et al. Biochem J.* (1974);**143**:67.
5 Stach R W *et al. J. Neurochem.* (1979);**33**:257.
6 Duke R C & Cohen J J. *Lymphokine Res.* (1986);**5**:289.
7 Terui Y *et al. J. Cellular Physiology* (1995);**164**:74.
8 Bonfoco E *et al. Proc. Natl. Acad. Sci. USA* (1995);**92**:7162.
9 Shi B *et al. J. Clin. Invest.* (1996);**98**:1979.
10 Kerr J F R *et al. Br. J. Cancer* (1972);**26**:239.
11 Clarke P G H. *Anat. Embryol.* (1990);**181**:195.
12 Scanlon M *et al. Proc. Natl. Acad. Sci. USA* (1989);**86**:182.
13 Arends M J *et al. Am. J. Pathol.* (1990);**136**:593.
14 Sanderson C J. *Biol. Rev.* (1981);**56**:153.
15 Wyllie A H. *Int. Rev. Cytol.* (1987);**17** (Suppl): 755.
16 Yamada T & Ohyama H. *Int. J. Radiat. Biol.* (1988);**53**:65.
17 Yonehara S *et al. J. Exp. Med.* (1989);**169**:1747.
18 Trauth B C *et al. Science* (1989);**245**:301.
19 Oehm A *et al. J. Biol. Chem.* (1992);**267**:10709.
20 Carson D A & Ribeiro J M. *Lancet* (1993),**341**:1251.
21 Edgington S M. *Biotechnology* (1993);**11**:787.
22 Gougeon M L & Montagnier L. *Science* (1993);**260**:1269.
23 Chien-Ying Liu *et al.* Broad-spectrum caspase inhibition paradoxically augments cell death in TNF-α–stimulated neutrophils. *BLOOD* (2003);**101**(1):296-304.
24 Gozal E *et al. S*ilica-Induced Apoptosis in Murine Macrophage. Involvement of Tumor Necrosis Factor-B Activation; *Am. J. Respir. Cell Mol. Biol.* **27**: 91-98.

5.3 Quality Control

The kit is function tested using a cellular model (U937 cells treated with camptothecin).

6. Supplementary Information

6.1 Conventions

Text Conventions

To make information consistent and memorable, the following text conventions are used in this document:

Text Convention	Usage
Numbered stages labeled ①, ② etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷ etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Diagnostics.

Symbols

In this document, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

- Editorial Changes

6.3 Ordering Information

	Product	Pack Size	Cat. No.
DNA fragmentation			
Gel Electrophoresis	Apoptotic DNA-Ladder Kit	20 tests	11 835 246 001
In situ assay	*In Situ* Cell Death Detection Kit, TMR red	1 kit (50 tests)	12 156 792 910
	In Situ Cell Death Detection Kit, Fluorescein	1 kit (50 tests)	11 684 795 910
	In Situ Cell Death Detection Kit, AP	1 kit (50 tests)	11 684 809 910
	In Situ Cell Death Detection Kit, POD	1 kit (50 tests)	11 684 817 910
Single reagents for TUNEL and supporting reagents	TUNEL AP	70 tests (3.5 ml)	11 772 457 001
	TUNEL POD	70 tests (3.5 ml)	11 772 465 001
	TUNEL Enzyme	2 × 50 ml (20 tests)	11 767 305 001
	TUNEL Label	3 × 550 ml (30 tests)	11 767 291 910
	TUNEL Dilution Buffer	20 ml	11 966 006 001

	Product	Pack Size	Cat. No.
ELISA	Cell Death Detection ELISA	1 kit	11 544 675 001
	Cell Death Detection ELISA^{PLUS}, 10×	1 kit (10 × 96 tests)	11 920 685 001
	Cellular DNA Fragmentation ELISA	1 kit (500 tests)	11 585 045 001
Cell membrane alterations			
Microscopy or FACS	Annexin-V-FLUOS	250 tests	11 828 681 001
	Annexin-V-FLUOS Staining Kit	50 tests 250 tests	11 858 777 001 11 988 549 001
Enzymatic activity			
	Homogenous Caspase Assay, fluorometric	100 tests 1000 tests	03 005 372 001 12 236 869 001
In situ Assay	M30 CytoDEATH (formalin grade)	50 tests 250 tests	12 140 322 001 12 140 349 001
	M30 CytoDEATH, Fluorescein	250 tests	12 156 857 001
Expression of apoptosis related proteins			
ELISA	p53 pan ELISA	1 kit	11 828 789 001

6.4 Regulatory Disclaimer

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

6.5 Trademarks

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All other product names and trademarks are the property of their respective owners.

7. Quick Reference Procedure

ELISA Assay

❶	Transfer 20 µl from • 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) into the MP. ⚠ It is important, due to low volumes, to pipette into the middle of the microplate well.
❷	Add to each well 80 µl of the Immunoreagent.
❸	• Cover the MP with an adhesive cover foil. • Incubate on a MP shaker under gently shaking (300 rpm) for 2 h at +15 to +25°C.
❹	• Remove the solution thoroughly by tapping or suction. • Rinse each well 3× with 250 – 300 µl Incubation Buffer (bottle 4). • Remove solution carefully.
❺	• Pipette to each well 100 µl ABTS Solution. • Incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis (approx. after 10 – 20 min.)
❻	Pipette to each well 100 µl ABTS Stop Solution.
❼	Measure at 405 nm against ABTS Solution + 100 µl ABTS Stop Solution as a blank (reference wavelength approx. 490 nm).

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