For life science research only. Not for use in diagnostic procedures.
FOR *IN VITRO* USE ONLY.

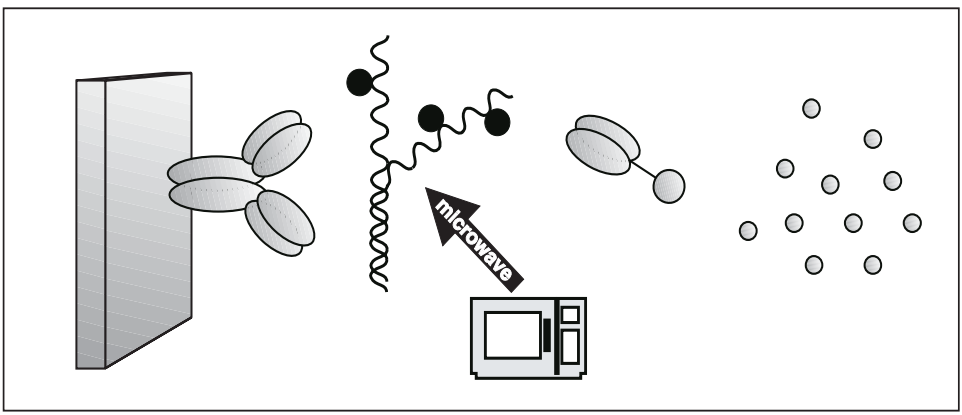
Cellular DNA Fragmentation ELISA

Photometric Enzyme-Linked ImmunoSorbent Assay (ELISA) for the detection of BrdU-labeled DNA fragments in cell lysates or in cell culture supernatants. A non-radioactive alternative to the [^{3}H]-thymidine release assay, the [^{3}H]-thymidine based DNA fragmentation assay, and the [^{51}Cr]-release assay.

Cat. No. 11 585 045 001
1 Kit for 500 tests

Instruction Manual

Version August 2004



anti-DNA coated MTP | BrdU-labeled DNA-fragments | anti-BrdU-POD Fab-fragments | substrate TMB

Roche

Table of contents

CAUTION

The following reagents, which are recommended in this instruction manual, are toxic or corrosive and should be handled with care:

- **5'-bromo-2'-deoxy-uridine**
- **TMB**
- **H_2SO_4**
- **NaOH**
- **HCl**

1. Introduction

Product description

- The **Cellular DNA Fragmentation ELISA** is a photometric enzyme-linked immunosorbent assay (ELISA) for the detection of BrdU-labeled DNA fragments in culture supernatants and cell lysates.
- 5'-Bromo-2'-deoxy-uridine (BrdU) is used as a metabolic labeling agent by the nuclear DNA of target cells. This BrdU-labeled DNA can be detected easily and quantified using a monoclonal antibody against BrdU[1-2] in an ELISA[3-5].
- This kit is a non-radioactive alternative to the [^{3}H]-thymidine release assay, the [^{51}Cr]-release assay, and the [^{3}H]-thymidine DNA fragmentation assay.

Please note: 0,01% 2-Methylisothiazolone (MIT) is used as preservative.

Number of tests

500 tests

Application

The Cellular DNA Fragmentation ELISA is used to determine cell death as key parameter in a wide variety of cell biological studies.

The assay may be applied to:

- measure **apoptotic cell death** by detection of BrdU-labeled DNA fragments in the cytoplasm of affected cells,
- measure **cell-mediated cytotoxicity** by detection of BrdU-labeled DNA fragments released from damaged target cells into the culture supernatant,
- **characterize the type of cell death** by performing kinetics and detection of BrdU-labeled DNA fragments in the cytoplasm of apoptotic cells as well as in the cell culture supernatant released from necrotic cells or at late stages of apoptosis.

Stability

The unopened kit is stable at 2-8°C until the control date printed on the kit

Quality control

The kit is function tested on HL60 or U937 cells after induction of apoptosis by camptothecin.

Advantages

- **Accurate:** results correlate to data obtained with the [^{3}H]-thymidine-based DNA fragmentation assay, [^{3}H]-thymidine- and [^{51}Cr]-release assays
- **Sensitive:** more sensitive than the [^{3}H]-thymidine-based DNA fragmentation assay and as sensitive as the [^{3}H]-thymidine- and [^{51}Cr]-release assays
- **Fast:** ELISA format allows processing of a large number of samples

2. Background Information

Cell Death: Apoptosis, Necrosis, and Cell-mediated Cytotoxicity

Introduction

Cell death can occur by two quite different mechanisms: **apoptosis** and **necrosis.**

Cell-mediated cytotoxicity by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells shows features of both mechanisms[6-7].

Apoptosis

Apoptosis (or programmed cell death) is the most common form of eukaryotic cell death[8].

It is a biological suicide mechanism preserving homeostasis and is essential in many physiological processes, such as embryogenesis, maturation of the immune system, or development of the nervous system[9-10].

The main characteristics are[11]:

- Prelytic, non-random mono-and oligonucleosomal length fragmentation of DNA ("ladder" pattern after agarose gel electrophoresis)
- Formation of membrane-bound vesicles ("apoptotic bodies")
- Cell shrinkage due to condensation of cytoplasm

Necrosis

Necrosis is also called pathological cell death because it occurs after cells have been exposed to extreme physiological conditions (*e.g.*, hypothermia) or is evoked by agents like complement or lytic viruses.

In vivo necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response[12].

The main characteristics are[11]:

- Swelling of organelles and of the cells, resulting in cell lysis due to loss of membrane integrity
- Postlytic DNA fragmentation
- Random digestion of DNA (DNA smear after agarose gel electrophoresis

Cell-mediated cytotoxicity

Cells of the immune system such as CTLs, NKs, or LAKs (lymphokine-activated killer cells) can recognize and destroy damaged, infected, and mutated target cells.

Two possible cytocidal mechanisms are involved:

- apoptosis
- lytic mechanism by which lytic molecules (*e.g.*, perforin) are secreted by the effector cell and polymerize to form lytic pores in the target cell membrane.

The mechanisms are not mutually exclusive, but complementary[6-7].

The main characteristic is:

- The fragmented DNA is released from the cytoplasm into the culture super-natant due to pore formation in the target cell plasma membrane.

3. Principle of the assay and possible application

Principle of the assay

Cells proliferating *in vitro* are incubated with the non-radioactive thymidine analogue BrdU, which is incorporated into the genomic DNA.

BrdU-labeled DNAfragments are released from the cells

- into the cell cytoplasm during apoptosis,

or

- into the cell culture supernatant during cell-mediated cytotoxicity.

These DNA fragments are detected immunologically by the ELISA technique using

- an anti-DNA-antibody bound to the MTP to capture the DNA fragments,

and

- an anti-BrdU-antibody-POD conjugate to detect the BrdU contained in the captured and subsequently denatured DNA fragments.

3. Principle of the assay and possible application, continued

Diagram

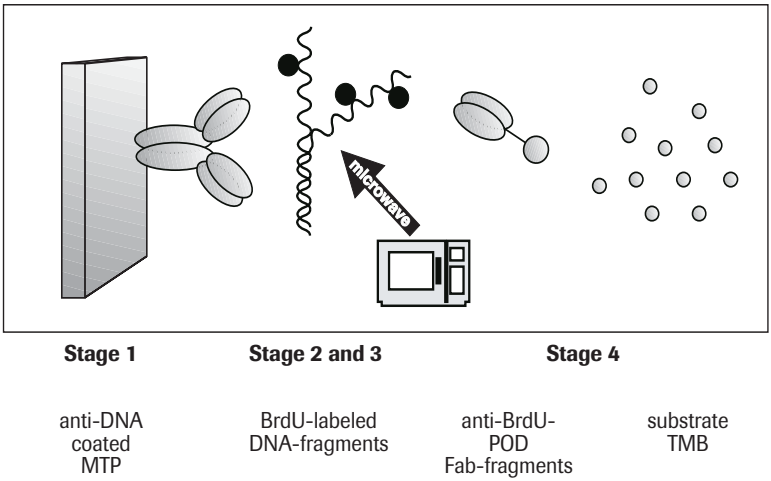


Fig.1: Principle of the assay

Stage	Description
1	Coating of the MPs with anti-DNA antibody and blocking of non-specific binding sites (*section 7.1*)*
2	Labeling of the cells with BrdU (*section 6.1*)
3	Options and possibilities:

If you want to...	then ...
characterize **the type of cell death** occurring	set up a kinetics assay and/or look for the appearance of DNA fragments in the culture supernatant as well as in the cytoplasm (*section 6.2*)*
measure **apoptosis**	look for appearance of DNA fragments in the cytoplasm only *(section 6.3)**
measure **cell-mediated cytotoxicity**	look for appearance of DNA fragments in the supernatant released from dead target cells (*section 6.4*)*
perform a **positive control**	solubilize the genomic DNA by endogenous nucleases or by NaOH treatment (*section6.5*)

4	Determine the quantity of DNA fragments in the sample by ELISA and photometric determination with TMB as substrate *(section 7.2)*

* Possible stopping points

4. Assay characteristics

Sample material	Culture supernatant and cytoplasmic lysates of cells containing DNA metabolically prelabeled with BrdU (*e.g.*, cell lines and other *in vitro* proliferating cells)
Sensitivity	• **In apoptosis,** the ELISA allows the detection of BrdU-labeled DNA fragments in the cytoplasmic fraction of 1×10^3 cells/well. • In **cell-mediated cytotoxicity,** the ELISA allows the detection of BrdU-labeled fragments in the supernatant of 2×10^3 target cells/well.
Specificity	• The **anti-DNA antibody** binds to single- and double-stranded DNA. It does not cross-react with BrdU. • **Anti-BrdU-POD, Fab fragments,** bind to BrdU incorporated into DNA after denaturation of the DNA. There is no cross-reactivity with other cellular components, such as thymidine or uridine.
Assay time	4.5–5.5 h

5. Kit contents and preparation of working solutions

Bottle/ Cap	Label	Content	Working Solution	Reconstitution/ Preparation of working solution	Stability of working solution	For use in
1 white	Anti-DNA antibody	Monoclonal antibody from mouse (clone MCA-33); lyophilized; stabilized	Solution 1	Reconstitute lyophilizate in 1 ml redist. water for 10 min at 15-25°C (RT), and mix well	stable for 6 months at 2-8°C	Solution 3
2 red	Anti-BrdU-peroxi-dase	Monoclonal antibody from mouse (clone BMG 6H8, Fab-fragment), conjugated with peroxidase; lyophilized; stabilized	Solution 2	Reconstitute lyophilizate in 1ml redist. water for 10 min at RT and mix well	stable for 6 months at 2-8°C	Solution 6
3 white	Coating buffer, 10 ×	6 ml solution		• For 1 ×: Dilute 1 ml of 10 × coating buffer with 9 ml redist. water	unstable, prepare immediately before use	Solution 3
			Solution 3	• Shortly before use, dilute 0.2 ml of reconstituted anti-DNA antibody (solution 1) with 9.8 ml 1 × coating buffer	unstable, prepare immediately before use	• Procedure for coating of MPs (Section 7.1, *table 8)*
4 green	Washing buffer, 10 ×	• 2 bottles each containing 100 ml • contains EDTA, Tween 20, and a preservative	Solution 4	• Prewarm 10× washing buffer to RT • For 1 ×: Dilute 40 ml of 10 × washing buffer with 360 ml redist. water, mix well	2 weeks at 2-8°C	• Procedure for coating of MTPs (Section 7.1, table 8) • Procedure for ELISA and photometric measurement • *(Section 7.2, table 10)*

continued on next page

5. Kit contents and preparation of working solutions, continued

Bottle/ Cap	Label	Content	Working solution	Reconstitution/ Preparation of working solution	Stability of working solution	For use in
5 red	Incubation buffer, 2 ×	• 125 ml solution • contains BSA, EDTA,Tween 20, and a preservative	Solution 5	• Prewarm 2× incubation buffer to RT • For 1×: Dilute 20 ml of 2 incubation buffer with 20 ml redist. water, mix	2 weeks at 2-8°C	• Procedure for coating of MPs *(Section 7.1, table 9)* • Procedure for characterization of cell death *(Section 6.2)* • Procedure for measuring apoptosis *(Section 6.3)* • Solution 6
			Solution 6	Dilute 0.2 ml anti-BrdU-peroxidase antibody (solution 2) with 9.8 ml 1× washing buffer (solution 4)	unstable; prepare immediately before use	• Procedure for ELISA and photometric measurement *(Section 7.2, table 11)*
6 red	Substrate solution	• 55 ml TMB solution • ready-to-use		undiluted stock solution		• Procedure for ELISA and photometric measurement *(Section 7.2, table 11)*
7 red	BrdU labeling reagent, 1000 ×	1 ml 10 mM 5'-bromo-2'-deoxy-uridine in PBS, pH 7.4, sterile	Solution 7	For 1 mM: Dilute 0.9 ml 1000 × BrdU labeling reagent with 8.1 ml sterile PBS or culture medium	• 3 months at 2-8°C, or • stable for several years at −15 to −25°C, • store protected from light!	• Procedure for labeling of the cells (*Section 6.1)*
8	Adhesive cover foils	10 sheets				

5.1 Additional required solutions and equipment

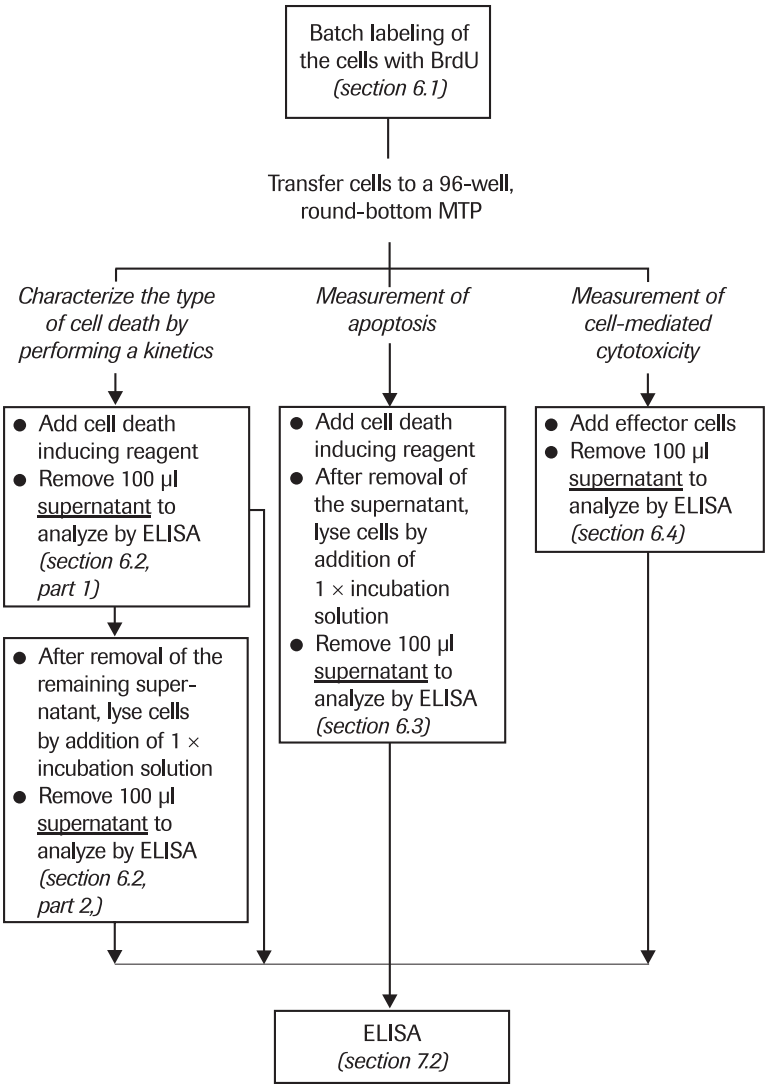
Solution	Preparation	Stability	For use in
Solution 8: Stop solution	Add 560 µl conc. H_2SO_4 (95–97 %) to 8 ml ice-cold redist. water, mix well, and make up to 10 ml	stable for a minimum of one year at 15-25°C	Procedure for ELISA and photometric measurement *(Section 7.2, table 11)*
Solution 9: Exonuclease III solution	• Prepare 1× nuclease reaction buffer: 66 mM Tris, 0.66 mM $MgCl_2$, 1 mM 2-mercaptoethanol, pH 8.0 • Dilute exonuclease III (Cat. No. 779 709) with 1 × nuclease reaction buffer to a final concentration of 10 U/ml	unstable; prepare immediately before use	Procedure for ELISA and photometric measurement (Section 7.2, table 10)
Solution 10: 1% Triton X-100	For 1×: Dilute 1 ml 10% Triton[1)] X-100 (Cat. No. 1 332 481) with 8 ml redist. water, mix well, and make up to 10 ml	unstable; prepare immediately before use	Procedures for positive control (Section 6.5, table 6)
Solution 11: 0.25 M NaOH	Dissolve 100 mg NaOH in 8 ml redist. water, mix well, and make up to 10 ml	unstable; prepare immediately before use	Procedures for positive control *(Section 6.5, table 7)*
Solution 12: 0.25 M HCl	Add 208.4 µl conc. HCl (37%) to 8 ml ice-cold redist. water, mix well, and make up to 10 ml	stable for a minimum of one year at 15-25°C	Procedures for positive control *(Section 6.5, table 7)*
Solution 13: 0.2 M K_2HPO_4	Dissolve 3.48 g K_2HPO_4 in 80 ml redist. water, mix well, and make up to 100 ml	stable for a minimum of one year when stored frozen at −15 to −25°C	Procedures for positive control (Section 6.5, table 7)
Solution 14: 0.2 M KH_2PO_4	Dissolve 2.72 g KH_2PO_4 in 80 ml redist. water, mix well, and make up to 100 ml	stable for a minimum of one year when stored frozen at −15 to −25°C	Procedures for positive control (Section 6.5, table 7)
Solution 15: 0.2 M K_2HPO_4/ KH_2PO_4 pH 7.0	To prepare solution 15, add solution 14 to solution 13 until a pH of 7 is adjusted. Adjust pH of solution 13 by adding solution 14 to a pH of 7.0.	stable for a minimum of one year when stored frozen at −15 to −25°C	Procedures for positive control (Section 6.5, table 7)

Equipment

- Microplates (MP), (*e.g.*, Nunc-1-Immuno-Maxisorp made by Nunc, clear):
- round-bottom MP (*section 6.1–6.5*)
- flat-bottom MP (*section 7.1–7.2*)
- MP reader
- MP shaker

6. Preparation of the samples

Flow chart



6.1 Procedure for labeling of the cells

Batch labeling procedure

This table describes how to perform a batch labeling of the cells with BrdU

Step	Action
1	Adjust cell number to 2–4 × 10^5 cells/ml culture medium
2	Add BrdU labeling solution (solution 7) to a final concentration of 10 μM
3	Incubate for 2 hours (up to overnight [ON]) at 37°C ***Note:*** Labeling time strongly depends on the cell type and the stage of cell culture! (recommended time: 2–20 h)
4	Centrifuge for 10 min at 250 × g
5	Carefully and thoroughly remove the BrdU-containing culture medium
6	• Resuspend cells in BrdU-free culture medium • Final concentration: 2 × 10^5 cells/ml for measuring cell-mediated cytotoxicity, or 1 × 10^5 cells/ml for all other applications

6.2 Procedure for characterization of cell death

Principle

This procedure consists of two parts:

Part 1:The supernatant is analyzed, which will contain DNA fragments

- at early stages of necrosis, and
- at late stages of apoptosis.

Part 2:The remaining cells are lysed in order to release apoptotic DNA fragments located in the cytoplasm.

Part 1

This table describes how to sample the supernatant from the labeled cells.

Step	Action
1	Pipette 100 µl of BrdU-labeled cells in culture medium (1×10^5 cells/ml, from section 6.1) into duplicate wells of a 96-well, round-bottom MP
2	Add an additional 100 µl cell culture medium, containing an appropriate amount of apoptosis inducing reagent, per well
3	Incubate at 37°C in a humidified atmosphere (5% CO_2) for an appropriate period of time (1–6 h)
4	Centrifuge for 10 min at 250 × g
5	Remove 100 µl of the supernatant to analyze in the ELISA procedure *(section 7.2)* *Note:* The sample can be stored at −15 to −25°C for up to three days

Part 2

This table describes how to continue with the remaining cells in order to obtain **Part 2**the DNA fragments from the cytoplasm.

Step	Action
1	• Carefully and thoroughly remove the remaining supernatant
2	• Add 200 µl 1 × incubation solution (solution 5) per well to lyse the cells • Incubate for 30 min at 15-25°C
3	Centrifuge for 10 min at 250 × g
4	Remove 100 µl of the supernatant to analyze in the ELISA procedure *(section 7.2)* *Note:* The sample can be stored at −15 to −25°C for up to three days

6.3 Procedure for measuring apoptosis

Procedure for extraction of cytoplasmic DNA fragments

This table describes how to extract apoptotic DNA fragments from the cytoplasm.

Note: Before applying this procedure, characterize the type of cell death occurring as apoptosis by "Procedure for characterization of the type of cell death" and/or by other methods[11] (*e.g.*, morphology of the cells, DNA ladder)!

Step	Action
1	Pipette 100 µl of BrdU-labeled cells in culture medium (1×10^5 cells/ml, from section 6.1) into duplicate wells of a 96-well, round-bottom MP
2	Add 100 µl cell culture medium containing an appropriate amount of apoptosis-inducing agent per well
3	Incubate at 37°C in a humidified atmosphere (5% CO_2) for an appropriate period of time (1–6 h)
4	Centrifuge 10 min at 250 × g
5	Carefully and thoroughly remove the supernating culture medium
6	• Add 200 µl 1× incubation solution (solution 5) per well to lyse the cells • Incubate 30 min at 15-25°C
7	Centrifuge 10 min at 250× g
8	Remove 100 µl/well of the supernatant to analyze in the ELISA procedure *(section 7.2)* *Note:* The sample can be stored at −15 to −25°C for up to three days

6.4 Procedure for measuring cell-mediated cytotoxicity

Procedure for measuring cell mediated cytotoxicity

This table describes how to extract BrdU-labeled DNA fragments from the supernatant released by dead target cells.

Step	Action
1	Pipette 100 µl BrdU-labeled target cells in culture medium (2×10^5 cells/ml, from section 6.1) into duplicate wells of a 96-well, round-bottom MP
2	• Add an additional 100 µl culture medium, containing an appropriate number of effector cells, per well (recommended ratio of effector to target cells: 0.01–10) • **Negative Control:** Pipet 100 µl culture medium into different duplicate wells of a 96-well, round-bottom MP to determine spontaneous release of DNA fragments
3	Incubate for 1–6 h at 37°C in a humidified atmosphere (5% CO_2)
4	Centrifuge for 10 min at 250 × g
5	Remove 100 µl of the supernatant to analyze in the ELISA procedure *(section 7.2)* *Note:* The sample can be stored at −15 to −25°C for up to three days

6.5 Procedures for positive control

Introduction

For determining the amount of BrdU incorporated into genomic DNA, it is imperative to denature the full-length DNA for quantitative solubilization!

Two methods may be applied:
Method 1: Solubilization of genomic DNA by endogenous nucleases
Method 2: Solubilization of genomic DNA by NaOH treatment

Method 1

This method is based on the fact that most cells contain endogenous nucleases.

After cell lysis, these nucleases will be activated by Ca^{2+}- and Mg^{2+}-ions contained in the culture medium and will partially solubilize the DNA.

Note: Depending on the cell line, the level of endogenous nucleases will vary and may result in poor fragmentation and subsequent solubilization and therefore may not be quantitative.

Step	Action
1	Pipette 100 µl of BrdU-labeled cells in culture medium (1×10^5 cells/ml, from section 6.1) into a well of a 96-well, round-bottom MP
2	Add 100 µl redist. water containing 1% Triton X-100 (solution 10)
3	Incubate cells for the same time as for the cellular assay at 37°C (*section 6.2–6.4*)
4	Centrifuge for 10 min at 250 × g
5	Remove 100 µl of supernatant for analysis by ELISA *(section 7.2)*

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6.5 Procedures for positive control, continued

Method 2

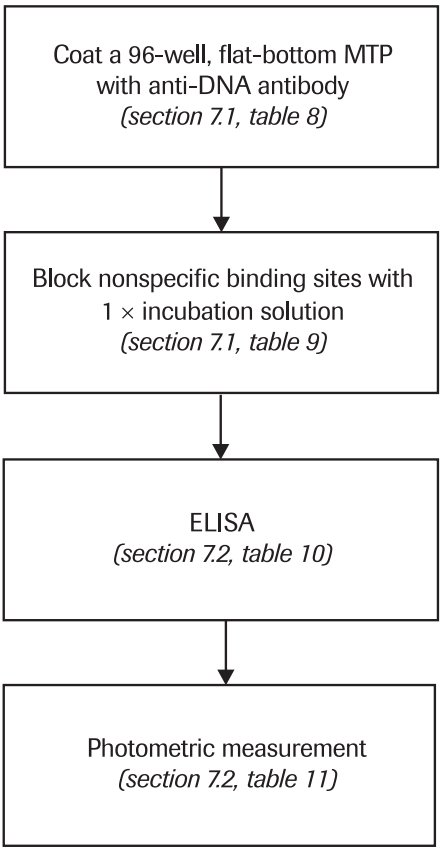
Genomic DNA is denatured and degraded by NaOH treatment.

Note: DNA solubilization by NaOH differs from all physiological nuclease cleavage during apoptosis, necrosis, and cell-mediated cytotoxicity. It provides a maximal amount of degraded DNA and a maximal value. This artificial method of degradation will not be obtained physiologically.

Step	Action
1	Transfer 500 µl of BrdU-labeled cells in culture medium (1×10^5 cells/ml, from section 6.1) to a 1.5 ml-reaction tube
2	Centrifuge for 5 min at 250 × g
3	Discard supernatant
4	• Add 125 µl 0.25 M NaOH (solution 11) • Incubate for 30 min at 15-25°C
5	• Add 125 µl 0.25 M HCl (solution 12) • Add 250 µl 0.2 M K_2HPO_4/KH_2PO_4, pH 7 (solution 15)
6	• Centrifuge 5 min at 11 000 × g
7	• Remove 400 µl of supernatant, and titrate in the ELISA *(section 7.2)* *Note:* For dilution, use the incubation solution (solution 5).

7. ELISA and photometric measurement

Flow chart



7.1 Procedure for coating of the MTPs

Coating procedure

This table describes how to coat the MTP with the anti-DNA-antibody.

Step	Action
1	Pipette 100 µl anti-DNA coating solution (solution 3) into each well of a 96-well, flat-bottom MTP
2	Performing the coating

If you want to...	then...
proceed with the assay on the same day	incubate for 1 hour at 37°C
proceed with the assay on the next day	cover the MTP with an adhesive cover foil, and incubate ON at 4°C
store the coated MTP for up to 1 week	• incubate for 1 hour at 37°C • remove the coating solution by aspirating • cover the MTP with an adhesive cover foil • store at 2-8°C • proceed with step 1 of the "Blocking procedure" *(table 9)*

3	Remove the coating solution by aspirating away the buffer. Alternatively, the MTP may be inverted and tapped gently on a paper towel. Proceed with "Blocking procedure" *(table 9)*

Blocking procedure

After the MTP has been coated with the anti-DNA-antibody, nonspecific binding sites are blocked by the following procedure.

Step	Action
1	• Add 200 µl of 1 × incubation solution (solution 5) • Cover the MTP with an adhesive cover foil • Incubate 30 min at 15-25°C
2	Remove the incubation solution by aspirating or inverting
3	Wash the wells three times with 250–300 µl of washing solution (solution 4) for 2–3 min each
4	• Remove the washing solution by aspirating or inverting • Proceed with section 7.2

7.2 Procedure for ELISA and photometric measurement

Protocol for ELISA Procedure

This table describes how to detect BrdU-labeled DNA fragments in the samples.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Transfer the 100 µl of a sample obtained in sections 6.2–6.5 into a well of the precoated 96-well, flat-bottom MTP (section 7.1)</td></tr>
<tr><td>2</td><td>• Cover the MTP tightly with an adhesive cover foil
• Incubate 90 min at 15-25°C or over night at 2-8°C</td></tr>
<tr><td>3</td><td>Remove the solution by aspirating or inverting</td></tr>
<tr><td>4</td><td>Wash the wells three times with 250–300 µl washing solution (solution 4) for 2–3 min per wash</td></tr>
<tr><td>5</td><td>Fixing and Denaturing of DNA
<table>
<tr><th>If you want to..</th><th>then...</th></tr>
<tr><td>fix and denature the DNA by microwave irradiation</td><td>• leave the washing solution in the well after the last wash-step in step 4
• place the uncovered MTP in a microwave oven
• also place a 500 ml beaker containing 300 ml water in the microwave oven
• irradiate for 5 min on medium power (500 W)
• cool down the MTP for approx. 10 min at -20°C
• remove the fluid by aspirating or inverting</td></tr>
<tr><td>fix and denature the DNA by nuclease treatment</td><td>• Pipette 100 µl exonuclease III solution (solution 9) per well
• cover the MTP tightly with an adhesive cover foil
• incubate for 30 min at 37°C
• remove the solution by aspirating or inverting
• wash the plate as described in step 4</td></tr>
</table></td></tr>
<tr><td>6</td><td>• add 100 µl of anti-BrdU-POD conjugate solution (solution 6) per well
• cover the MTP tightly with an adhesive cover foil
• incubate 90 min at 15-25°C or ON at 2-8°C</td></tr>
<tr><td>7</td><td>• Wash as described in step 4
• Continue with table 11</td></tr>
</table>

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7.2 Procedure for ELISA and photometric measurement, continued

Procedure for photometric measurement

The values can be measured either at 370 nm or at 450 nm.

Note: Measurement at 450 nm will result in a 2–3 fold increase of O.D. values, but does not allow the kinetic of color development to be followed.

Step	Action
1	Pipette 100 µl substrate solution into each MTP well used
2	Photometric measure/Taking readings

If you want to...	then...
measure at 450 nm (reference wavelength 690 nm)	• incubate in the dark on a MTP shaker until color development is sufficient • add 25 µl stop solution (solution 8) per well • incubate 1 min on the shaker • measure within 5 minutes after adding stop solution *Note:* Color will begin to fade after 5 min!
measure at 370 nm (reference wavelength 492 nm)	• DO NOT add stop solution! • measure the absorbance at specific time points after substrate solution has been added (i.e., every 30 s) to follow the kinetics of color development

8. Typical results

Introduction

The following figures show typical results when using this kit to:

- Characterize the type of cell death
- Measure apoptosis
- Measure cell-mediated cytotoxicity

Characterization of type of cell death

The Cellular DNA Fragmentation ELISA enables the measurement of DNA fragments in the cell cytoplasm (lysate) and the culture supernatant (SN).

Figure 2 shows that, upon increased time of exposure to the apoptosis-inducing agent camptothecin (CAM), DNA fragments appear first in the cell lysate. No BrdU-labeled DNA fragments were detected in the supernatant during the first 4 hours after cell death induction, indicating that DNA fragmentation occurred prior to plasma membrane lysis.

Conclusion: Cell death due to apoptosis. Necrotic cells would have released DNA fragments into the supernatant at very early stages of cell death.

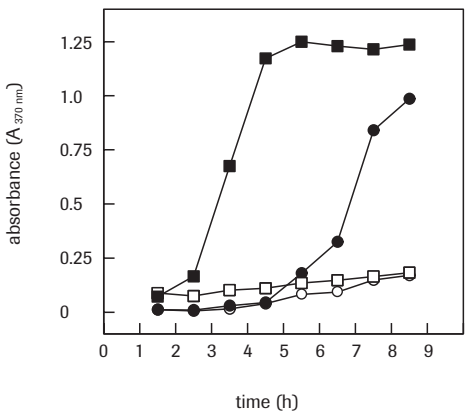

Fig.2: Kinetics of CAM-induced apoptotic cell death in HL60 cells. 10^4 BrdU-labeled cells/well were incubated either in the presence of 200 ng/ml CAM (■, ●), or in the absence of CAM (❑, ❍) for 1 to 8 h at 37°C. After the times indicated, 100 µl/well supernatant (●, ❍) and 100 µl/well lysate (■,❑) were removed and tested by ELISA.

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8. Typical results, Continued

Measuring Apoptosis

Figure 3 shows sensitive detection of nucleosomes in the cytoplasmic fractions at different cell concentrations.

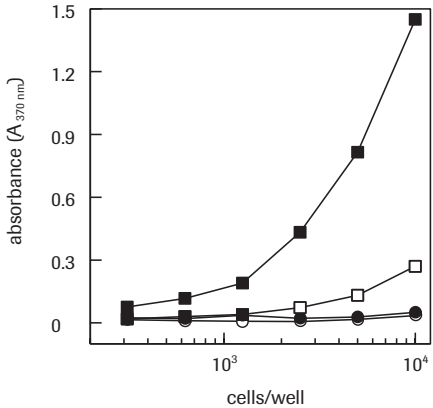

Fig. 3: Measuring apoptosis with the Cellular DNA Fragmentation ELISA. BrdU-labeled HL60 cells were cultured at different concentrations in the presence of 200 ng/ml CAM (■, ●) or in the absence of CAM (❑, ○) for 3 h at 37°C. After incubation, supernatants (●, ○) and lysates (■, ❑) were tested by ELISA.

Measuring Cell-mediated cytotoxicity

Effects of inducing agent or effector cell concentration can be measured over time. Typical results are shown in Figure 4 below.

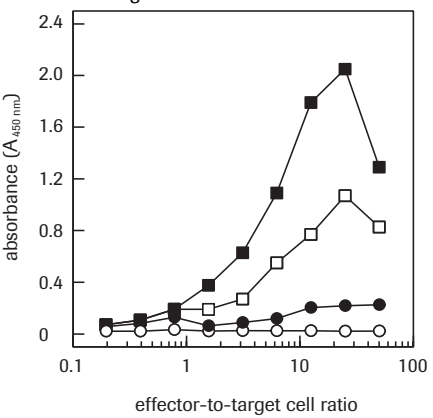

Fig. 4: Kinetics of CTL mediated cytotoxicity in P815 target cells.
2×10^4 BrdU-labled target cells /well were incubated with CTLs at different effector-to-target cell-ratios
(E/T) for 1 h (○), 2 h (●), 4 h (❑), and 6 h (■), respectively. After incubation, 100 µl/well supernatant was removed and tested by ELISA.

9. Appendix

9.1 Troubleshooting

Symptom	Possible Cause	Recommendation
Low absorbance value from positive control (*section 6.5, Method 2, Solubilization by NaOH*)	• Doubling time of cell line is >30 h	• Increase the number of cells/well to 2–3 × 10^4 • Increase labeling time to 24 h
	• Filter wavelength is not suitable	• Suitable wavelength is discussed in section 7.2
Low signal of positive control (*section 6.5, Method 1, Solubilization by endogenous nucleases)*	• Endonuleases require Ca^{2+} and Mg^{2+} for activity	• Add 5 mM $Ca^{2+}$and 10 mM Mg^{2+}
	• Cell line has low endogenous levels of nuclease	• Try solubilization by NaOH method (*section 6.5, Method 2*)
	• Microwave irradiation is too high or too low	• Try a different microwave oven • Try solubilization by NaOH (*section 6.5, Method 2*)
Low signal of samples, high signal of positive control	• No apoptosis	• Increase concentration of apoptosis-inducing agent • Prolong incubation time of apoptosis-inducing reagent
High signal in untreated samples and high signal of positive control	• Cells died spontaneously	• Check condition for cell culture
	• Cell density is too high	• Reduce number of cells per well
	• Cells have a fast doubling time	• Reduce labeling time
Low reproducibility between duplicate cultures	• Insufficient lysis of individual cells	• Cells should be completely dispersedand resuspended during lysis step
	• Nuclear DNA pellet was disturbed during supernatant removal after lysis	• Recentrifuge with higher speed, and remove supernatant more carefully
	• Solution in wells evaporating by excessive irradiation	• Use at least 300 ml water in a beaker to absorb the excess energy from microwave oven and to keep atmosphere humidified. Take care that the washing solution does not boil (check after irradiation for 2 min).

9.2 References

- **BrdU antibody**

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- **Cell death**

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[1] Tween is a trademark of ICI, Americas, Inc., USA.
[2] Triton is a trademark of Rohm & Haas Company, Philadelphia, PA,USA.

9.3 Ordering Information

Apoptosis-specific physiological change	Detection method:	Product:	Cat. No.
DNA fragmentation	• Gel Electrophoresis	• Apoptotic DNA-Ladder Kit	11 835 246 001
	• *In situ* assay	• In Situ Cell Death Detection Kit, TMR red	12 156 792 001
		• In Situ Cell Death Detection Kit, Fluorescein	11 684 795 001
		• In Situ Cell Death Detection Kit, AP	11 684 809 001
		• In Situ Cell Death Detection Kit, POD	11 684 817 001
		Single reagents for TUNEL and supporting reagents:	
		• TUNEL AP	11 772 457 001
		• TUNEL POD	11 772 465 001
		• TUNEL Enzyme	11 767 305 001
		• TUNEL Label Mix	11 767 291 001
	• ELISA	• Cell Death Detection ELISA^{PLUS}	11 774 425 001
		• Cell Death Detection ELISA^{PLUS}, 10×	11 920 685 001
		• Cellular DNA Fragmentation ELISA	11 585 045 001
Cell membrane alterations	• Microscopy or FACS	• Annexin-V-Alexa 568	03 703 126 001
		• Annexin-V-Biotin	11 828 690 001
		• Annexin-V-FLUOS	11 828 681 001
		• Annexin V FLUOS Staining Kit	11 858 777 001
Enzymatic activity	• Western Blot	• Anti-Poly(ADP-Ribose) Polymerase	11 835 238 001
	• FIENA	• Caspase 3 Activity Assay	12 012 952 001
	• In situ Assay	• M30 CytoDEATH (formalin grade)	12 140 322 001
		• M30 CytoDEATH, Fluorescein	12 156 857 001
Expression of apoptosis-related proteins	• Apoptosis Induction	• Anti-Fas	11 922 432 001
	• Assay/Western Blot	• Anti-p53 (pan)	11 810 928 001

For further information please access our web-site address at:
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or the Apoptosis special interest site:
http://www.roche-applied-science.com/sis/apoptosis/

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