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

Roche

# Cellular DNA Fragmentation ELISA

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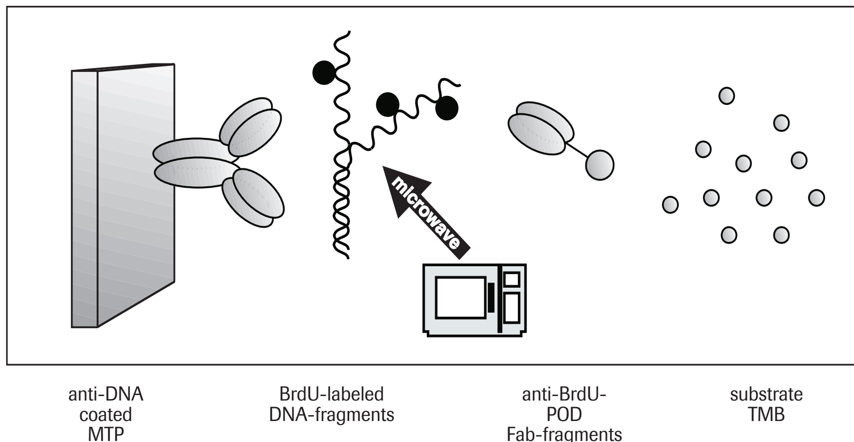
Photometric Enzyme-Linked ImmunoSorbent Assay (ELISA) for the detection of BrdU-labeled DNA fragments in cell lysates or in cell culture supernatants. A non-radioactive alternative to the [$^3$H]-thymidine release assay, the [$^3$H]-thymidine based DNA fragmentation assay, and the [$^{51}$Cr]-release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

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




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

**sigma-aldrich.com**

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## 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 to +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

**Caution**

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

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

## 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 MP 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.

**Diagram**

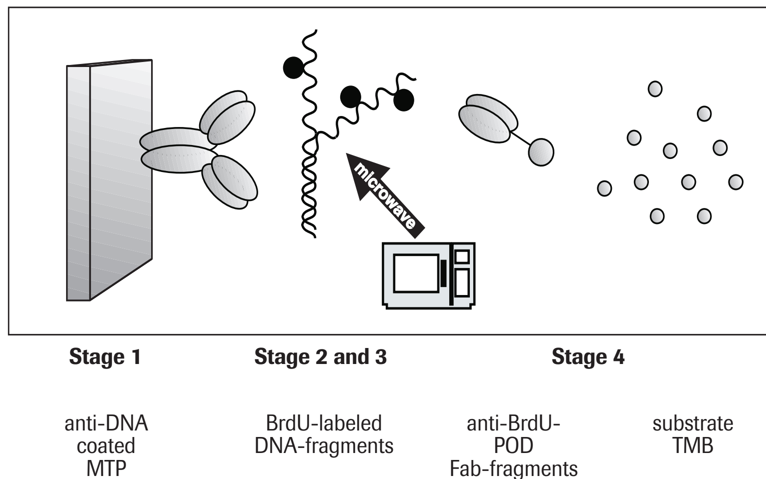



**Fig.1:** Principle of the assay

<table>
<tr><th>Stage</th><th>Description</th></tr>
<tr><td>1</td><td>Coating of the MPs with anti-DNA antibody and blocking of non-specific binding sites (section 7.1) [1]</td></tr>
<tr><td>2</td><td>Labeling of the cells with BrdU (section 6.1)</td></tr>
<tr><td>3</td><td>Options and possibilities:
<table>
<tr><th>If you want to...</th><th>then...</th></tr>
<tr><td>characterize the type of cell death occurring</td><td>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) [1]</td></tr>
<tr><td>measure apoptosis</td><td>look for appearance of DNA fragments in the cytoplasm only (section 6.3) [1]</td></tr>
<tr><td>measure cell-mediated cytotoxicity</td><td>look for appearance of DNA fragments in the supernatant released from dead target cells (section 6.4) [1]</td></tr>
<tr><td>perform a positive control</td><td>solubilize the genomic DNA by endogenous nucleases or by NaOH treatment (section 6.5)</td></tr>
</table>
</td></tr>
<tr><td>4</td><td>Determine the quantity of DNA fragments in the sample by ELISA and photometric determination with TMB as substrate (section 7.2)</td></tr>
</table>

[1] 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 $1 \times 10^3$ cells/well.
- In **cell-mediated cytotoxicity,** the ELISA allows the detection of BrdU-labeled fragments in the supernatant of $2 \times 10^3$ 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 hours

## 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 to +25°C, and mix well | stable for 6 months at +2 to +8°C | Solution 3 |
| 2 red | Anti-BrdU-peroxidase | Monoclonal antibody from mouse (clone BMG 6H8, Fab-fragment), conjugated with peroxidase; lyophilized; stabilized | Solution 2 | Reconstitute lyophilizate in 1 ml redist. water for 10 min at +15 to +25°C and mix well | stable for 6 months at +2 to +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*) |
| 4 green | Washing buffer, 10× | • 2 bottles each containing 100 ml<br>• contains EDTA, Tween 20, and a preservative | Solution 4 | • Prewarm 10× washing buffer to +15 to +25°C<br>• For 1×: Dilute 40 ml of 10× washing buffer with 360 ml redist. water, mix well | 2 weeks at +2 to +8°C | • Procedure for coating of MPs (*section 7.1*)<br>• Procedure for ELISA and photometric measurement<br>• (*section 7.2*)<br>• 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*) |
| 5 red | Incubation buffer, 2× | • 125 ml solution<br>• contains BSA, EDTA,Tween 20, and a preservative | Solution 5 | • Prewarm 2× incubation buffer to +15 to +25°C<br>• For 1×: Dilute 20 ml of 2× incubation buffer with 20 ml redist. water, mix | 2 weeks at +2 to +8°C | • Procedure for coating of MPs (*section 7.1*)<br>• Procedure for characterization of cell death (*section 6.2*)<br>• Procedure for measuring apoptosis (*section 6.3*) |
| 6 red | Substrate solution | • 55 ml TMB solution<br>• ready-to-use | | undiluted stock solution | | • Procedure for ELISA and photometric measurement (*section 7.2*) |
| 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 to +8°C, or<br>• stable for several years at −15 to −25°C,<br>• 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

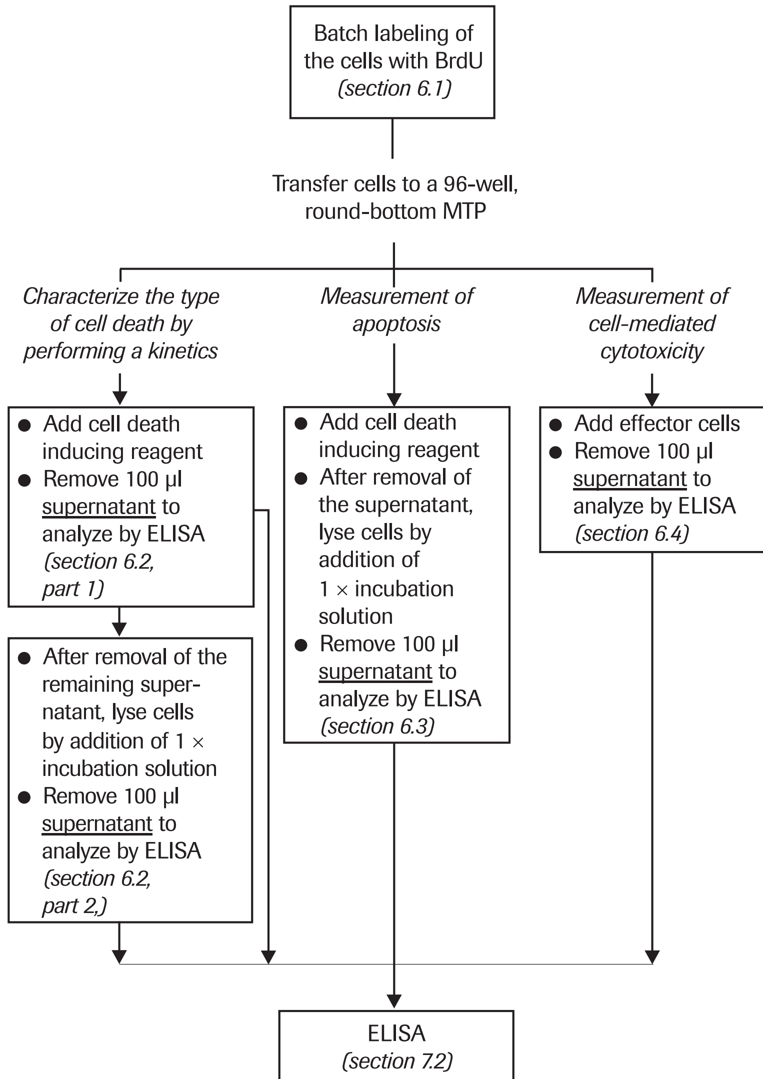
| Solution | Preparation | Stability | For use in |
|---|---|---|---|
| Solution 8: Stop solution | Add 560 μl conc. $H_2SO_4$ (95 – 97 %) to 8 ml ice-cold redist. water, mix well, and make up to 10 ml | stable for a minimum of one year at +15 to +25°C | Procedure for ELISA and photometric measurement (*section 7.2*) |
| 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<br>• Dilute exonuclease III 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*) |
| Solution 10: 1% Triton X-100 | For 1×: Dilute 1 ml 10% Triton X-100 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*) |
| 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*) |
| 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 to +25°C | Procedures for positive control (*section 6.5*) |
| 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*) |
| 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*) |
| 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*) |

**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 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) at +37°C ***Note:*** Labeling time strongly depends on the cell type and the stage of cell culture! (recommended time: 2 – 20 hours) |
| 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<br>• 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 ($1 \times 10^5$ 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 hours) |
| 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 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<br>• Incubate for 30 min at +15 to +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 ($1 \times 10^5$ 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 hours) |
| 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<br>• Incubate 30 min at +15 to +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 ($2 \times 10^5$ 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)<br>• **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 hours 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 ($1 \times 10^5$ 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)* |

**Method 2**

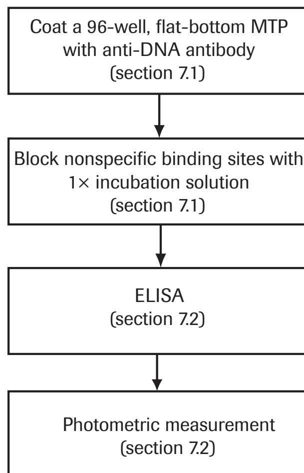
Genomic DNA is denatured and degraded by NaOH treatment.

***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 ($1 \times 10^5$ 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)<br>• Incubate for 30 min at +15 to +25°C |
| 5 | • Add 125 µl 0.25 M HCl (solution 12)<br>• 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)*<br>***Note:*** For dilution, use the incubation solution (solution 5). |

## 7. ELISA and photometric measurement

**Flow chart**




### 7.1 Procedure for coating of the MPs

**Coating procedure**

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

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Pipette 100 µl anti-DNA coating solution (solution 3) into each well of a 96-well, flat-bottom MP</td></tr>
<tr><td>2</td><td>Performing the coating
<table>
<tr><th>If you want to...</th><th>then...</th></tr>
<tr><td>proceed with the assay on the same day</td><td>incubate for 1 hour at +37°C</td></tr>
<tr><td>proceed with the assay on the next day</td><td>cover the MP with an adhesive cover foil, and incubate overnight at +2 to +8°C</td></tr>
<tr><td>store the coated MP for up to 1 week</td><td>• incubate for 1 hour at +37°C<br>• remove the coating solution by aspirating<br>• cover the MP with an adhesive cover foil<br>• store at +2 to +8°C<br>• proceed with step 1 of the ”Blocking procedure”</td></tr>
</table></td></tr>
<tr><td>3</td><td>Remove the coating solution by aspirating away the buffer. Alternatively, the MP may be inverted and tapped gently on a paper towel. Proceed with ”Blocking procedure”.</td></tr>
</table>

**Blocking procedure**

After the MP 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)<br>• Cover the MP with an adhesive cover foil<br>• Incubate 30 min at +15 to +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<br>• 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 MP (section 7.1)</td></tr>
<tr><td>2</td><td>• Cover the MP tightly with an adhesive cover foil<br>• Incubate 90 min at +15 to +25°C or overnight at +2 to +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<br>• place the uncovered MP in a microwave oven<br>• also place a 500 ml beaker containing 300 ml water in the microwave oven<br>• irradiate for 5 min on medium power (500 W)<br>• cool down the MP for approx. 10 min at –15 to –25°C<br>• 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<br>• cover the MP tightly with an adhesive cover foil<br>• incubate for 30 min at +37°C<br>• remove the solution by aspirating or inverting<br>• 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<br>• cover the MP tightly with an adhesive cover foil<br>• incubate 90 min at +15 to +25°C or overnight at +2 to +8°C</td></tr>
<tr><td>7</td><td>• Wash as described in step 4<br>• Continue with photometric measurement</td></tr>
</table>

*continued on next page*

## 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.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Pipette 100 µl substrate solution into each MP well used</td></tr>
<tr><td>2</td><td>Photometric measure/Taking readings
<table>
<tr><th>If you want to...</th><th>then...</th></tr>
<tr><td>measure at 450 nm (reference wavelength 690 nm)</td><td>• incubate in the dark on a MP shaker until color development is sufficient<br>• add 25 µl stop solution (solution 8) per well<br>• incubate 1 min on the shaker<br>• measure within 5 minutes after adding stop solution<br>Note: Color will begin to fade after 5 min!</td></tr>
<tr><td>measure at 370 nm (reference wavelength 492 nm)</td><td>• DO NOT add stop solution!<br>• 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</td></tr>
</table>
</td></tr>
</table>

## 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.

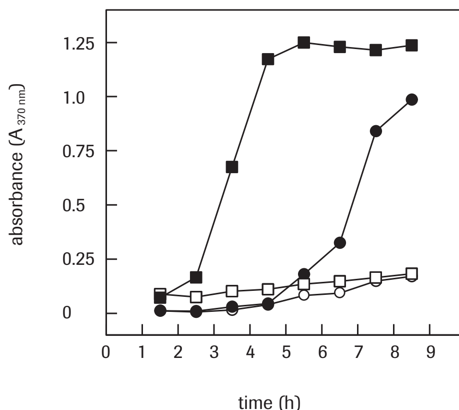



**Fig.2: Kinetics of CAM-induced apoptotic cell death in HL60 cells.** $10^4$ BrdU-labeled cells/well were incubated either in the presence of 200 ng/ml CAM (■, ●), or in the absence of CAM (❑, ❍) for 1 to 8 hours at +37°C. After the times indicated, 100 µl/well supernatant (●, ❍) and 100 µl/well lysate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

*continued on next page*

**Measuring Apoptosis**

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

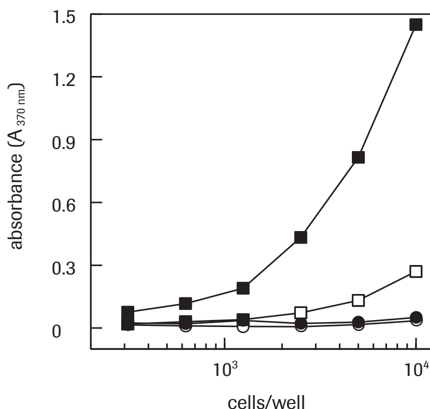



**Fig. 3: Measuring apoptosis with the Cellular DNA Fragmentation ELISA.** BrdU-labeled HL60 cells were cultured at different concentrations in the presence of 200 ng/ml CAM (■, ●) or in the absence of CAM (❑, ❍) for 3 hours at +37°C. After incubation, supernatants (●, ❍) and lysates (■, ❑) were tested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

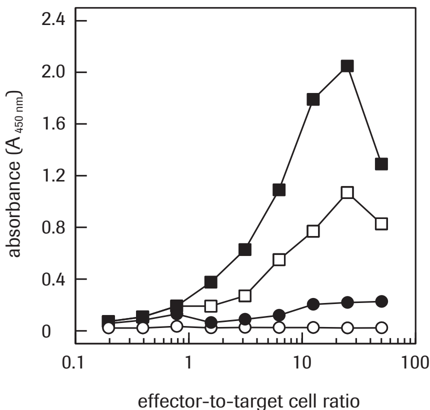



**Fig. 4: Kinetics of CTL mediated cytotoxicity in P815 target cells.**
$2 \times 10^4$ BrdU-labled target cells /well were incubated with CTLs at different effector-to-target cell-ratios
(E/T) for 1 hours (❍), 2 hours (●), 4 hours (❑), and 6 hours (■), respectively. After incubation, 100 µl/well supernatant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

# 9. Appendix

## 9.1 Troubleshooting

| Symptom | Possible Cause | Recommendation |
|---|---|---|
| Low absorbance value from positive control<br>(*section 6.5, Method 2, Solubilization by NaOH*) | • Doubling time of cell line is >30 hours | • Increase the number of cells/well to 2 – 3 × $10^4$<br>• Increase labeling time to 24 hours |
| | • Filter wavelength is not suitable | • Suitable wavelength is discussed in *section 7.2* |
| Low signal of positive control<br>(*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<br>• 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<br>• 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 dispersed and 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.<br>Take care that the washing solution does not boil (check after irradiation for 2 min). |

## 9.2 References

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## 9.3 Changes to Previous Version

- Editorial Changes

## 9.4 Ordering Information

| Apoptosis-specific physiological change | Detection method: | Product: | Cat. No. |
|---|---|---|---|
| **DNA fragmentation** | • Gel Electrophoresis<br>• *In situ* assay<br><br><br><br><br><br><br><br><br><br><br><br><br>• ELISA | • Apoptotic DNA-Ladder Kit<br>• *In Situ* Cell Death Detection Kit, TMR red<br>• *In Situ* Cell Death Detection Kit, Fluorescein<br>• *In Situ* Cell Death Detection Kit, AP<br>• *In Situ* Cell Death Detection Kit, POD<br>**Single reagents for TUNEL and supporting reagents:**<br>• TUNEL AP<br>• TUNEL POD<br>• TUNEL Enzyme<br>• TUNEL Label<br><br>• Cell Death Detection ELISA^PLUS^<br>• Cell Death Detection ELISA^PLUS^, 10×<br>• Cellular DNA Fragmentation ELISA | 11 835 246 001<br>12 156 792 910<br>11 684 795 910<br>11 684 809 910<br>11 684 817 910<br><br><br>11 772 457 001<br>11 772 465 001<br>11 767 305 001<br>11 767 291 910<br><br>11 774 425 001<br>11 920 685 001<br>11 585 045 001 |
| **Cell membrane alterations** | • Microscopy or FACS | • Annexin-V-FLUOS<br>• Annexin V FLUOS Staining Kit | 11 828 681 001<br>11 858 777 001 |
| **Enzymatic activity** | • Western Blot<br>• In situ Assay | • Anti-Poly(ADP-Ribose) Polymerase<br>• M30 CytoDEATH (formalin grade) | 11 835 238 001<br>12 140 322 001 |

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