Specific determination of mono- and oligonucleosomes
- Cytoplasmic fractions (lysates) of cell lines,

The kit is stable at +2 to +8°C until the expiration date

**Cell Death Detection ELISA**

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

**Cat. No. 11 544 675 001**
For 96 tests

**1. Kit contents**

<table>
<thead>
<tr>
<th>Vial/ Cap</th>
<th>Label</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 white</td>
<td>Anti-histone</td>
<td>• Hybridize, stabilized&lt;br&gt;• Mouse monoclonal antibody (clone H11-4)</td>
</tr>
<tr>
<td>2 red</td>
<td>Anti-DNA-POD</td>
<td>• Hybridize, stabilized&lt;br&gt;• Monoclonal antibody from mouse (clone MCA-33) conjugated with peroxidase</td>
</tr>
<tr>
<td>3 white</td>
<td>Coating buffer</td>
<td>• 10x conc.&lt;br&gt;• 2 ml</td>
</tr>
<tr>
<td>4 green</td>
<td>Washing buffer</td>
<td>• 10x conc.&lt;br&gt;• 40 ml</td>
</tr>
<tr>
<td>5 red</td>
<td>Incubation buffer</td>
<td>• 100 ml&lt;br&gt;• Ready-to-use solution</td>
</tr>
<tr>
<td>6 white</td>
<td>Substrate buffer</td>
<td>• Ready-to-use solution&lt;br&gt;• Use 5 ml for one ABTS tablet.</td>
</tr>
<tr>
<td>7 white</td>
<td>Substrate tablet</td>
<td>• 3 x 5 mg tablets&lt;br&gt;• Each sufficient for 5 ml Substrate solution</td>
</tr>
<tr>
<td></td>
<td>Microplate</td>
<td>12 Microplate modules (8 wells, each) and frame</td>
</tr>
<tr>
<td></td>
<td>Adhesive cover foils</td>
<td>Folds to cover microplates.</td>
</tr>
</tbody>
</table>

**2. Product overview**

The assay is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively.

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

**Application**

Specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates.

**Sample material**

- Cytoplasmic fractions (lysates) of cell lines,
- cells ex vivo (see sample preparation) or
- tissue homogenates (18).

**Specificity**

Anti histone antibody reacts with the histones H1, H2A, H2B, H3 and H4 of various species, e.g., man, mouse, rat, hamster, cow, opossum, 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.

**Stability**

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

**Advantages**

- No radioisotopes are used.
- Quantitative measurement of cell death.
- No prelabeling of the cells required; this allows the quantification of apoptosis also in cells which do not proliferate in vitro, e.g., freshly isolated, ex vivo tumor cells and organ explants.
- Detection of histone-associated DNA fragments in one immunoassay, demonstrating the internucleosomal degradation of genomic DNA occurring during apoptosis.
- The antibodies used are not species specific. Determination of apoptosis in cell systems from various species.
- Results obtained correlate to those obtained by standard methods.
- Highly sensitive; less cells required to obtain results.

**Background information**

Two distinct forms of eukaryotic cell death can be classified by morphological and biochemical criteria: necrosis and apoptosis (1, 2).

Necrosis is accompanied by increased ion permeability of the plasma membrane, the cells swell and the plasma membrane ruptures within minutes (isomotic lysis). Apoptosis is characterized by membrane blebbing (enzymatic condensation of cytoplasm and the activation of an endogenous endonuclease. This Ca2+ and Mg2+ dependent nuclelease 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 (3, 4).

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 (5).

Apoptosis is the most common form of eukaryotic cell death. It occurs (e.g., during embryogenesis) in parallel with the deletion of autoreactive T cells during thymic maturation, in 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 (6, 7).

Apoptosis is also induced by cytotoxic T lymphocytes and natural killer (NK) cells (8,9) and by ionizing radiation (10) and monoclonal antibodies like anti-Fas (11) and anti-APO-1 (12, 13).

![Diagram](image-url)
3. Procedures and required material

3.1 Before you begin

Reconstitution of lyophilizates

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reconstitution</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-histone (bottle 1)</td>
<td>Reconstitute the lyophilizate in 1 ml double dist. water for 10 min Mix thoroughly.</td>
<td>2 months at +2 to +8°C</td>
</tr>
<tr>
<td>Anti-DNA-POD (bottle 2)</td>
<td>Reconstitute the lyophilizate in 1 ml double dist. water for 10 min Mix thoroughly.</td>
<td>2 months at +2 to +8°C</td>
</tr>
</tbody>
</table>

Preparation of working solutions

The kit contains all the reagents needed and in sufficient amounts for 96 tests. Double distilled water should always be used for reconstitution and dilution purposes.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reconstitution</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating solution</td>
<td>Predilute 1 ml Coating buffer concentrate (bottle 3) with 9 ml double dist. water. Shortly before use, dilute 1 ml anti-histone antibody (bottle 1, reconstituted) with 9 ml Coating buffer.</td>
<td>Prepare immediately before use!</td>
</tr>
<tr>
<td>Washing solution</td>
<td>Warm up at 15-25°C Washing buffer concentrate (bottle 4) and dilute 40 ml in 360 ml double dist. water and mix thoroughly.</td>
<td>2 months at 2–8°C+2 to +8°C</td>
</tr>
<tr>
<td>Sample solution</td>
<td>Depends on the cell system used and the extent of cell death (see below sample preparation); Example: Dilute 25 μl sample in 225 μl Incubation buffer (bottle 5).</td>
<td>2 months at +2 to +8°C</td>
</tr>
<tr>
<td>Conjugate solution</td>
<td>Dilute 1 ml anti-DNA-POD (bottle 2, reconstituted) with 9 ml Incubation buffer (bottle 5).</td>
<td>Prepare immediately before use!</td>
</tr>
<tr>
<td>Substrate solution</td>
<td>Depending on the number of samples tested, dissolve 1, 2, or 3 tablets from in 5, 10, or 15 ml Substrate buffer. Allow to come to 15 to 25°C before use. Note: The ABTS solution reacts to light on exposure over a longer period.</td>
<td>1 month, stored at +2 to +8°C, protected from light.</td>
</tr>
</tbody>
</table>

Positive control

The negative control of the cellular assay will cause a certain absorbance value in the immunoassay (see above). Therefore, a positive control for the immunoassay as an additional component is not necessary. However, if an extra positive control for the immunoassay is desired, it can be prepared following this simple procedure:

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Centrifuge an aliquot of untreated cells (5 ×10⁶ cells/tube in 500 μl, see section induction of cell death.) at 1500 rpm for 5 min.</td>
</tr>
</tbody>
</table>
| 2    | • Discard the supernatant.  
      • Resuspend the cell pellet in 500 μl hypertonic buffer (10 mM Tris, pH 7.4, 400 mM NaCl, 5 mM CaCl₂ and 10 mM MgCl₂). |
| 3    | Incubate at 37°C for at least 2 h. |
| 4    | • Spin down the cell fragments at 15,000 rpm.  
      • Remove supernatant carefully, dilute the supernatant 1:5 with Incubation buffer (bottle 5) and use this sample as a positive control for the immunoassay. |

3.2 Sample preparation

Introduction

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. The following cellular model system, in particular the cell number per test, is an example for a test procedure. As a model system for cell death, camptothecin (CAM) was used as the apoptosis-inducing drug. The tests were performed with the human myelogenous leukemic cell line HL60 (ATCC: CCL 240) as target cells (14–17).

Induction of cell death (cellular assay)

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
</table>
| 1    | • Dilute exponentially growing HL60 cells with culture medium to obtain a cell concentration of 1x10⁶ cells/ml.  
      • Transfer into Eppendorf tubes (500 μl/tube = 5 × 10⁶ cells/tube). |
| 2    | Add 500 μl culture medium with different concentrations of CAM (0 μg CAM/ml to 4 μg CAM/ml). Note: Use values 0 μg/ml as a negative control for the cellular assay (= viable, untreated cells). |
| 3    | Close tubes loosely to allow further exchange of gas and incubate at 37°C in a CO₂-incubator for 4 h. |
| 4    | Centrifuge the cells in an Eppendorf centrifuge at 200× g for 5 min. |
| 5    | Discard the supernatant and resuspend the cell pellet in 1 ml culture medium. |
| 6    | Centrifuge the cells in an Eppendorf centrifuge at 1500× g for 5 min. |
| 7    | • Resuspend the cell pellet with 500 μl Incubation buffer (bottle 5) per tube (1x 10⁶ cell/ml).  
      • Mix thoroughly. |
| 8    | Incubate the sample for approx. 30 min at 15 to 25°C (≈ lysis). |
| 9    | Centrifuge the lysate at 20,000× g for 10 min. |
| 10   | Remove 400 μl of the supernatant (= cytoplasmic fraction) carefully. Note: Do not shake the pellet (= cell nuclei, containing high molecular weight, unfragmented DNA). |
| 11   | Predilute the resulting supernatant 1:10 with Incubation buffer (= 1 × 10⁶ cell equivalents/ml) and detect the nucleosomes in the sample by immunoassay (see below). Note: It is recommended that the samples are stored in aliquots at −15 to −25°C if they cannot be tested on the same day or, at the latest, one day later. |
### 3.3 ELISA procedure

**Handling instructions**

We recommend that the cytoplasmic fractions are diluted with incubation buffer to obtain a sample solution corresponding to approx. $10^2$ – $10^4$ cell equivalents/ml. The amount of $10^3$ cell equivalents/ml is defined as the volume of cell lysate which corresponds to $10^3$ cells/ml.

**Protocol**

All incubation steps are at 15 – 25°C. **Note:** We recommend 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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
</table>
| 1 | Pipette 100 µl Coating solution into each well of the MP-modules.  
Cover MP-modules tightly with the adhesive cover foil included. |
| 2 | Incubate for 1 h at 15 – 25°C (alternatively overnight at +2 to +8°C). |
| 3 | Remove Coating solution thoroughly by tapping or suction. |
| 4 | Pipette 200 µl Incubation buffer (bottle 3) into each well of the MP-modules.  
Cover MP-modules tightly with the adhesive cover foil. |
| 5 | Incubate for 30 min at 15 – 25°C. |
| 6 | Remove solution thoroughly by tapping or suction.  
Rinse wells three times with 250 – 300 µl Washing solution per well and remove Washing solution carefully. |
| 7 | Pipette 100 µl of Sample solution into each well of the MP-modules.  
For determination of the background of the immunoassay, pipette 100 µl per well of Incubation buffer into two wells.  
Cover MP-modules tightly with the adhesive cover foil included. |
| 8 | Incubate for 90 min at 15 – 25°C. |
| 9 | Remove solution thoroughly by tapping or suction.  
Rinse wells three times with 250 – 300 µl Washing solution per well and remove Washing solution carefully. |
| 10 | Pipette 100 µl of Conjugate solution into each well of the MP-modules, except the blank position.  
Cover MP-modules tightly with the included adhesive cover foil. |
| 11 | Incubate for 90 min at 15 – 25°C. |
| 12 | Remove solution thoroughly by tapping or suction.  
Rinse wells three times with 250 – 300 µl Washing solution per well and remove Washing solution carefully. |
| 13 | Pipette 100 µl of Substrate solution into each well of the MP-modules. |
| 14 | Incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis (approx. after 10 – 20 min). |
| 15 | Homogenize well contents by careful tapping the side of the MP, before measuring at 405 nm with respect to a substrate solution blank. Optionally, use 490 nm as the reference wavelength. |

### 4. Results

**Calculation**

- Average the values from the double absorbance measurements of the samples.  
- Subtract the background value (see below) of the immunoassay from each of these averages.  
- Calculate the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula:

$$
\text{enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding control (viable cells)}} \times \frac{\text{absorbance}}{10^{-3}}
$$

**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 min substrate reaction.

**Samples exceeding measuring range**

Samples with values exceeding the measurement range of the photometer ("over") should be diluted and run again; the corresponding control sample (viable cells) 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.

**Detection limit**

The exact detection limit of dying/dead cells in a particular sample strongly depends on the kinetic of cell death, the cytotoxic agent used and the amount of affected cells in the total cell population. Using HL60/CAM as a cellular model system for cell death, the immunoassay allows the specific detection of mono- and oligonucleosomes in the cytoplasmic fraction of $5 \times 10^2$ cells/ml ($\approx 50$ cell equivalents/well) (see fig. 2).

![Absorbance vs. Cells/well](image-url)
Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page http://www.roche-applied-science.com and our Apoptosis Special Interest Site: http://www.roche-applied-science.com/apoptosis

References

1 Wylie A. H. et al. (1980) Int. Rev. of Cytol. 68, 251.

Changes to Previous Version

Editorial changes

Regulatory Disclaimer

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