

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



In Situ Cell Death Detection Kit, TMR red

 **Version 12**

Content version: March 2016

Kit for detection and quantification of apoptosis (programmed cell death) at single cell level, based on labeling of DNA strand breaks (TUNEL technology): Analysis by fluorescence microscopy or flow cytometry.

Cat. No. 12 156 792 910

1 Kit (50 tests)

Store at –15 to –25°C

sigma-aldrich.com

1. Preface

1.1 Table of Contents

1.	Preface	2
1.1	Table of Contents	2
1.2	Kit Contents	3
2.	Introduction	5
2.1	Product Overview	5
2.2	Background Information	8
3.	Procedures and Required Materials	10
3.1	Flow Chart	10
3.2	Preparation of Sample Material	11
3.2.1	Cell Suspension	11
3.2.2	Adherent Cells, Cell Smears, and Cytospin Preparations	12
3.2.3	Tissue Sections	13
3.2.3.1	Treatment of Paraffin-Embedded Tissue	13
3.2.3.2	Treatment of Cryopreserved Tissue	15
3.3	Labeling Protocol	16
3.3.1	Before you Begin	16
3.3.2	Labeling Protocol for Cell Suspensions	17
3.3.3	Labeling Protocol for Adherent Cells, Cell Smears, Cytospin Preparations, and Tissues	18
3.3.4	Labeling Protocol for Difficult Tissue	19
4.	Typical results	20
5.	Appendix	21
5.1	Troubleshooting	21
5.2	References	24
5.3	Ordering Information	25
	Changes to previous version	26
	Regulatory Disclaimer	26
	Disclaimer of License	26

1.2 Kit Contents

Caution

The Label Solution contains cacodylate, toxic when inhaled or swallowed and cobalt dichloride, which may cause cancer by inhalation. Avoid exposure and obtain special instructions before use.

When using the solution do not eat, drink or smoke. After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell seek medical advice immediately (show label where possible).

Collect the supernatants from the labeling reactions in a tightly closed, non-breakable container and indicate contents. Discard as regulated for toxic waste.

Kit Contents

Please refer to the following table for the contents of the kit.

Vial/ Cap	Label	Contents
1 blue	Enzyme Solution	<ul style="list-style-type: none">Terminal deoxynucleotidyl transferase from calf thymus (<i>EC 2.7.7.31</i>), recombinant in <i>E. coli</i>, in storage buffer10× conc.5 × 50 µl
2 red	Label Solution	<ul style="list-style-type: none">Nucleotide mixture in reaction buffer1× conc.5 × 550 µl

Additional Solutions Required

In addition to the reagents listed above, you have to prepare several solutions. In the table you will find an overview about the equipment which is needed for the different procedures.

Detailed information is given before each procedure.

Procedure	Equipment	Reagents
<i>Preparation of sample material (section 3.2)</i>		
<ul style="list-style-type: none">Cell suspension (section 3.2.1)Adherent cells, cell smears and cytopsin preparations (section 3.2.2.)Cryopreserved tissue (section 3.2.3.2)	<ul style="list-style-type: none">ShakerV-bot-tomed 96-well micro-plate	<ul style="list-style-type: none">Washing buffer: Phosphate buffered saline (PBS*)Fixation solution: 4% Paraformaldehyde in PBS, pH 7.4, freshly preparedPermeabilisation solution: 0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared (6)

<p>Paraffin-embedded tissue (section 3.2.3.1)</p>		<ul style="list-style-type: none"> ▪ Xylene and ethanol (absolute, 95%, 90%, 80%, 70%, diluted in double-distilled water) ▪ Washing buffer: PBS* ▪ Proteinase K*, PCR Grade, working solution: [10–20 µg/ml in 10 mM Tris/HCl, pH 7.4–8] <p><u>Alternative treatments</u></p> <ul style="list-style-type: none"> ▪ Permeabilisation solution: (0.1% Triton¹) X-100, 0.1% sodium citrate), freshly prepared ▪ Pepsin* (0.25%–0.5% in HCl, pH 2) or trypsin*, 0.01 N HCl ▪ 0.1 M Citrate buffer, pH 6 for microwave irradiation
<p><i>Labeling protocol (section 3.3)</i></p>		
<p>Positive control (section 3.3.1)</p>		<ul style="list-style-type: none"> ▪ Micrococcal nuclease or ▪ DNase I recombinant*
<ul style="list-style-type: none"> ▪ Cell suspensions (section 3.3.2) ▪ Adherent cells (section 3.3.3) 	<ul style="list-style-type: none"> ▪ Parafilm or coverslips ▪ Humidified chamber 	<p>Washing buffer: PBS*</p>
<p>Difficult tissue (section 3.3.4)</p>	<ul style="list-style-type: none"> ▪ Plastic jar ▪ Microwave ▪ Humidified chamber 	<ul style="list-style-type: none"> ▪ Citrate buffer, 0.1 M, pH 6.0. ▪ Washing buffer: PBS* ▪ Tris-HCl, 0.1 M pH 7.5, containing 3% BSA* and 20% normal bovine serum

2. Introduction

2.1 Product Overview

Test Principle

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks ("nicks") in high molecular weight DNA.

Those DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction.

Stage	Description
1	Labeling of DNA strand breaks by Terminal deoxynucleotidyl transferase (TdT) which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL-reaction).
2	TMR red labeled nucleotides, incorporated in nucleotide polymers, are detected and quantified by fluorescence microscopy or flow cytometry.

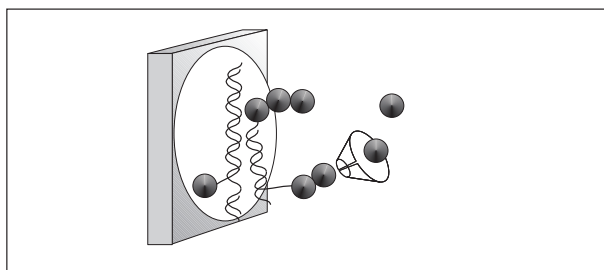


Fig. 1: DNA of fixed cells labeled by the addition of TMR red dUTP at strand breaks by terminal transferase.

Application

The *In Situ* Cell Death Detection Kit is designed as a precise, fast and simple, non-radioactive technique to detect and quantify apoptotic cell death at single cell level in cells and tissues. Thus, the *In Situ* Cell Death Detection Kit can be used in many different assay systems. Examples are:

- Detection of individual apoptotic cells in frozen and formalin-fixed tissue sections in basic research.
- Determination of sensitivity of malignant cells to drug-induced apoptosis in cancer research.
- Typing of cells undergoing cell death in heterogeneous populations by double staining procedures (6, 7).

Specificity	The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. This allows discrimination of apoptosis from necrosis and from primary DNA strand breaks induced by cytostatic drugs or irradiation (3, 4).
Test Interference	<p>False negative results: DNA cleavage can be absent or incomplete in some forms of apoptotic cell death (37). Sterical hindrance such as extracellular matrix components can prevent access of TdT to DNA strand breaks. In either case false negative results may be obtained.</p> <p>False positive results: Extensive DNA fragmentation may occur in certain forms of necrosis (38).</p> <p>DNA strand breaks may also be prominent in cell populations with high proliferative or metabolic activity. In either case false positive results may be obtained.</p> <p>To confirm the apoptotic mode of cell death, the morphology of respective cells should be examined very carefully. Morphological changes during apoptosis have a characteristic pattern. Therefore evaluation of cell morphology is an important parameter in situations where there is any ambiguity regarding interpretation of results.</p>
Sample Material	<ul style="list-style-type: none">▪ Cell suspensions from<ul style="list-style-type: none">▪ permanent cell lines (2, 27, 35),▪ lymphocytes and leukemic cells from peripheral blood (4),▪ thymocytes (1, 6),▪ bone marrow cells▪ fine needle biopsies (5)▪ Cytospins and cell smear preparations▪ Adherent cells cultured on chamber slides (31)▪ Frozen or formalin-fixed, paraffin-embedded tissue sections (1, 25, 26, 29, 30, 32–34, 36, 39)
Assay Time	1-2 hours, excluding culture, fixation and permeabilisation of cells and preparation of tissue sections.
Number of Tests	The kit is designed for 50 tests.
Kit Storage/ Stability	<p>The unopened kit is stable at –15 to –25°C until the expiration date printed on the label.</p> <p>Note: The TUNEL reaction mixture should be prepared immediately before use and should not be stored. Keep the TUNEL reaction mixture on ice until use.</p>

Advantage

Please refer to the following table.

Benefit	Feature
Sensitive	Detection of apoptotic cell death at single cell level via fluorescence microscopy and at cell populations via FACS analysis at very early stages (1, 2, 6).
Specific	Preferential labeling of apoptosis versus necrosis (3, 4).
Fast	Short assay time (1–2 h).
Convenient	<ul style="list-style-type: none"> ▪ No secondary detection system required. ▪ One incubation and one washing step only. ▪ Reagents are provided in stable, optimized form. ▪ No dilution steps required. ▪ Application in combination with fluorescein label possible
Flexible	<ul style="list-style-type: none"> ▪ Suitable for fixed cells and tissue. This allows accumulation, storage and transport of samples (2, 5). ▪ Double staining enables identification of type and differentiation state of cells undergoing apoptosis (6).
Function-tested	Every lot is function-tested on apoptotic cells in comparison to a master lot.

2.2 Background Information

Cell Death

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological, biochemical and molecular changes of dying cells.

Programmed cell death or apoptosis is the most common form of eukaryotic cell death. It is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during normal tissue turnover (8, 9). In general, cells undergoing apoptosis display a characteristic pattern of structural changes in nucleus and cytoplasm, including rapid blebbing of plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA-cleavage into oligonucleosomal length DNA fragments after activation of a calcium-dependent endogenous endonuclease (10, 11). However, very rare exceptions have been described where morphological features of apoptosis are not accompanied with oligonucleosomal DNA cleavage (37).

Apoptosis

Apoptosis is essential in many physiological processes, including maturation and effector mechanisms of the immune system (12, 13), embryonic development of tissue, organs and limbs (14), development of the nervous system (15, 16) and hormone-dependent tissue remodeling (17).

Identification of Apoptosis

Several methods have been described to identify apoptotic cells (22–24). Endonucleolysis is considered as the key biochemical event of apoptosis, resulting in cleavage of nuclear DNA into oligonucleosome-sized fragments. Therefore, this process is commonly used for detection of apoptosis by the typical “DNA ladder” on agarose gels during electrophoresis. This method, however, can not provide information regarding apoptosis in individual cells nor relate cellular apoptosis to histological localization or cell differentiation. This can be done by enzymatic *in situ* labeling of apoptosis-induced DNA strand breaks.

DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) (1–6, 25–36) have been used for the incorporation of labeled nucleotides to DNA strand breaks *in situ*. The tailing reaction using TdT, which was also described as ISEL (*in situ* end labeling) (5, 35) or TUNEL (TdT-mediated dUTP nick end labeling) (1, 6, 31, 33) technique, has several advantages in comparison to the *in situ* nick translation (ISNT) using DNA polymerase:

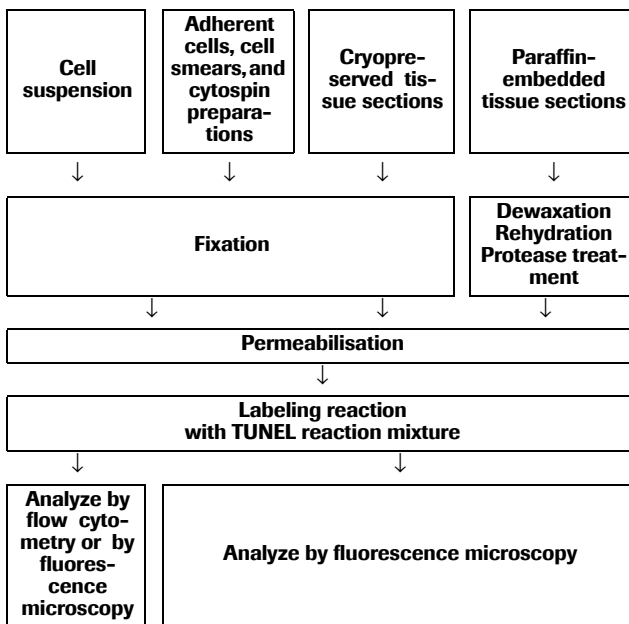
- Label intensity of apoptotic cells is higher with TUNEL compared to ISNT, resulting in an increased sensitivity (2, 4).
 - Kinetics of nucleotide incorporation is very rapid with TUNEL compared to the ISNT (2, 4).
 - TUNEL preferentially labels apoptosis in comparison to necrosis, thereby discriminating apoptosis from necrosis and from primary DNA strand breaks induced by antitumor drugs or radiation (3, 4).
-

3. Procedures and Required Materials

The working procedure described below was published by R. Sgonc and colleagues (6). The main advantage of this kit is the use of tetra-methyl- rhodamine- dUTP to directly label DNA strand breaks with red fluorescence. This allows the **direct detection** of DNA fragmentation in the red channel and secondary labeling with fluorescein in the green channel by flow cytometry or fluorescence microscopy.

3.1 Flow Chart

Assay Procedure The assay procedure is explained in the following flow chart.



3.2 Preparation of Sample Material

3.2.1 Cell Suspension

Prelabeling

For dual parameter flow cytometry with fluorescein-conjugated antibodies, incubate the cells prior to fixation with the cell surface marker.

Additional Buffers and Equipment Required

- Washing buffer: Phosphate buffered saline (PBS)
- Fixation solution: Paraformaldehyde (4% in PBS, pH 7.4), freshly prepared
- Permeabilisation solution: 0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared
- Shaker
- V-bottomed 96-well microplate

Note: Use of a V-bottomed 96-well microplate minimize cell loss during fixation, permeabilisation and labeling and allows simultaneous preparation of multiple samples.

Procedure

Please find in the following protocol the procedure for cell fixation and permeabilisation.

Note: Fix and permeabilise two additional cells for the negative and positive labeling controls.

Step	Action
1	Wash test sample 3 times in PBS and adjust to 2×10^7 cells/ml.
2	Transfer 100 μ l/well cell suspension into a V-bottomed 96-well microplate.
3	Add 100 μ l/well of a freshly prepared Fixation solution to cell suspension (final concentration 2% PFA).
4	Resuspend well and incubate 60 min at +15 to +25°C. Note: To avoid extensive clumping of cells, the microplate should be incubated on a shaker during fixation.
5	Centrifuge the microplate at 300 g for 10 min and remove fixative by flicking off or suction.
6	Wash cells once with 200 μ l/well PBS .
7	Centrifuge the microplate at $300 \times g$ for 10 min and remove PBS by flicking off or suction.
8	Resuspend cells in 100 μ l/well Permeabilisation solution for 2 min on ice (+2 to +8°C).
9	Proceed as described under 3.3.

3.2.2 Adherent Cells, Cell Smears, and Cytospin Preparations

Additional Solutions Required

- Washing buffer: Phosphate buffered saline (PBS)
 - Fixation solution: 4% Paraformaldehyde in PBS, pH 7.4, freshly prepared
 - Permeabilisation solution: 0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared (6)
-

Procedure

The following table describes preparations of adherent cells, cell smears and cytospin.

Note: Fix and permeabilise two additional cell samples for the negative and positive labeling controls.

Step	Action
1	Fix air dried cell samples with a freshly prepared Fixation solution for 1 h at +15 to +25°C
2	Rinse slides with PBS .
3	Incubate in Permeabilisation solution for 2 min on ice (+2 to +8°C).
4	Proceed as described under 3.3.

3.2.3.1 Treatment of Paraffin-Embedded Tissue

Pretreatment of Paraffin Embedded Tissue

Tissue sections can be pretreated in 4 different ways. If you use Proteinase K the concentration, incubation time and temperature have to be optimized for each type of tissue (1, 29, 33, 36, 40, 41).

Note: Use Proteinase K tested for absence of nucleases which might lead to false-positive results!

The other 3 alternative procedures are also described in the following table (step 2).

Additional Solutions Required

- Xylene and ethanol (absolute, 95%, 90%, 80%, 70%, diluted in double distilled water)
- Washing buffer: PBS
- Proteinase K, PCR Grade*, working solution: [10-20 µg/ml in 10 mM Tris/HCl, pH 7.4-8]

Alternative treatments

- Permeabilisation solution: 0.1% Triton X-100, 0.1% sodium citrate, freshly prepared
 - Pepsin* (0.25% - 0.5% in HCl, pH 2) or trypsin*, 0.01 N HCl
 - 0.1 M Citrate buffer, pH 6 for the microwave irradiation
-

Procedure

In the following table the pretreatment of paraffin-embedded tissue with Proteinase K treatment and 3 alternative procedures are described.

Note: Add additional tissue sections for the negative and positive labeling controls.

Step	Action								
1	Dewax and rehydrate tissue section according to standard protocols (e.g., by heating at +60°C followed by washing in xylene and rehydration through a graded series of ethanol and double dist. water) (1, 33, 36).								
2	<p>Incubate tissue section for 15–30 min at +21 to +37°C with Proteinase K working solution.</p> <table border="1"> <thead> <tr> <th>Alternatives:</th> <th>Treatment:</th> </tr> </thead> <tbody> <tr> <td>1. Permeabilisation solution</td> <td>Incubate slides for 8 min.</td> </tr> <tr> <td>2. Pepsin* (30, 40) or trypsin</td> <td>15–60 min at +37°C.</td> </tr> <tr> <td>3. Microwave irradiation</td> <td> <ul style="list-style-type: none"> ▪ Place the slide(s) in a plastic jar containing 200 ml 0.1 M Citrate buffer, pH 6.0. ▪ Apply 350 W microwave irradiation for 5 min. </td> </tr> </tbody> </table>	Alternatives:	Treatment:	1. Permeabilisation solution	Incubate slides for 8 min.	2. Pepsin* (30, 40) or trypsin	15–60 min at +37°C.	3. Microwave irradiation	<ul style="list-style-type: none"> ▪ Place the slide(s) in a plastic jar containing 200 ml 0.1 M Citrate buffer, pH 6.0. ▪ Apply 350 W microwave irradiation for 5 min.
Alternatives:	Treatment:								
1. Permeabilisation solution	Incubate slides for 8 min.								
2. Pepsin* (30, 40) or trypsin	15–60 min at +37°C.								
3. Microwave irradiation	<ul style="list-style-type: none"> ▪ Place the slide(s) in a plastic jar containing 200 ml 0.1 M Citrate buffer, pH 6.0. ▪ Apply 350 W microwave irradiation for 5 min. 								
3	Rinse slide(s) twice with PBS .								
4	Proceed as described under 3.3.								

3.2.3.2 Treatment of Cryopreserved Tissue

Additional Solutions required

- Fixation solution: 4% Paraformaldehyde in PBS, pH 7.4, freshly prepared
 - Washing buffer: PBS
 - Permeabilisation solution: 0.1% Triton X-100, 0.1% sodium citrate, freshly prepared
-

Cryopreserved Tissue

In the following table the pretreatment of Cryopreserved tissue is described.

Note: Fix and permeabilise two additional samples for the negative and positive labeling controls.

Step	Action
1	Fix tissue section with Fixation solution for 20 min at +15 to +25°C.
2	Wash 30 min with PBS . Note: For storage, dehydrate fixed tissue sections 2 min in absolute ethanol and store at -15 to -25°C.
3	Incubate slides in Permeabilisation solution for 2 min on ice (+2 to +8°C).
4	Proceed as described under 3.3.

3.3 Labeling Protocol

3.3.1 Before You Begin

Preparation of TUNEL Reaction Mixture

One pair of tubes (vial 1: Enzyme Solution, and vial 2: Label Solution) is sufficient for staining 10 samples by using 50 μ l TUNEL reaction mixture per sample and 2 negative controls by using 50 μ l Label Solution per control.

Note: The TUNEL reaction mixture should be prepared immediately before use and should not be stored. Keep TUNEL reaction mixture on ice until use.

Step	Action
1	Remove 100 μ l Label Solution (vial 2) for two negative controls.
2	Add total volume (50 μ l) of Enzyme Solution (vial 1) to the remaining 450 μ l Label Solution in vial 2 to obtain 500 μ l TUNEL reaction mixture.
3	Mix well to equilibrate components.

Additional Reagents Required

- Micrococcal nuclease or
- DNase I recombinant *

Controls

Two negative controls and a positive control should be included in each experimental setup.

Negative control:	Incubate fixed and permeabilized cells in 50 μ l/well Label Solution (without terminal transferase) instead of TUNEL reaction mixture.
Positive control:	Incubate fixed and permeabilized cells with micrococcal nuclease or DNase I recombinant (3000 U/ml– 3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 min at +15 to +25°C to induce DNA strand breaks, prior to labeling procedures.

* available from Roche Diagnostics

3.3.2 Labeling Protocol for Cell Suspensions

Additional Equipment and Reagents Required

- Washing buffer: PBS
- Humidified chamber

Procedure

Please refer to the following table.

Step	Action
1	Wash cells twice with PBS (200 μ l/well).
2	Resuspend in 50 μ l/well TUNEL reaction mixture . Note: For the negative control add 50 μ l Label solution.
3	Add lid and incubate for 60 min at +37°C in a humidified atmosphere in the dark.
4	Wash samples twice in PBS .
5	Transfer cells in a tube to a final volume of 250-500 μ l in PBS .
6	Samples can directly be analyzed by flow cytometry or fluorescence microscopy. For evaluation by fluorescence microscopy use an excitation wavelength in the range of 520-560 nm (maximum 540 nm; green) and detection in the range of 570-620 nm (maximum 580 nm, red).

3.3.3 Labeling Protocol for Adherent Cells, Cell Smears, Cytospin Preparations, and Tissues

- Additional Equipment and Reagents Required**
- Washing buffer: PBS
 - Parafilm or coverslips
 - Humidified chamber

Procedure

Please refer to the following table.

Step	Action
1	Rinse slides twice with PBS .
2	Dry area around sample.
3	Add 50 μ l TUNEL reaction mixture on sample. Note: For the negative control add 50 μ l Label solution each. To ensure a homogeneous spread of TUNEL reaction mixture across cell monolayer and to avoid evaporative loss, samples should be covered with parafilm or coverslip during incubation.
4	Incubate slide in a humidified atmosphere for 60 min at +37°C in the dark.
5	Rinse slide 3 \times with PBS .
6	Samples can directly be analysed under a fluorescence microscope or embedded with antifade prior to analysis. Use an excitation wavelength in the range of 520-560 nm (maximum 540 nm; green) and detection in the range of 570-620 nm (maximum 580 nm, red).

3.3.4 Labeling Protocol for Difficult Tissue

Additional Equipment and Solutions Required

- Citrate buffer, 0.1 M, pH 6.0.
- Washing buffer: PBS
- Tris-HCl, 0.1 M pH 7.5, containing 3% BSA and 20% normal bovine serum
- Plastic jar
- Microwave

Procedure

Please refer to the following table.

Step	Action
1	Dewax paraformaldehyde- or formalin-fixed tissue sections according to standard procedures.
2	Place the slide(s) in a plastic jar containing 200 ml 0.1 M Citrate buffer , pH 6.0.
3	<ul style="list-style-type: none">▪ Apply 750 W (high) microwave irradiation for 1 min.▪ Cool rapidly by immediately adding 80 ml double dist. water (+20 to +25°C).▪ Transfer the slide(s) into PBS (+20 to +25°C). DO NOT perform a Proteinase K treatment!
4	Immerse the slide(s) for 30 min at +15 to +25°C in Tris-HCl, 0.1 M pH 7.5, containing 3% BSA and 20% normal bovine serum .
5	<ul style="list-style-type: none">▪ Rinse the slide(s) twice with PBS at +15 to +25°C.▪ Let excess fluid drain off.
6	Add 50 µl of TUNEL reaction mixture on the section. Note: For the negative control add 50 µl Label solution.
7	Incubate for 60 min at +37°C in a humidified atmosphere in the dark.
8	<ul style="list-style-type: none">▪ Rinse slide(s) three times in PBS for 5 min each.▪ Evaluate the section under a fluorescence microscope.

4. Typical results

- Assay Procedures**
- Incubate U937 cells at a density of 10^6 cells/ml in the presence of camptothecin ($2 \mu\text{g/ml}$, 4 h at $+37^\circ\text{C}$) to induce apoptosis.
 - As control for non-apoptotic population, an aliquot of the cells is incubated in normal culture medium without camptothecin.
 - Harvest cells and proceed as described under 3.2.1.

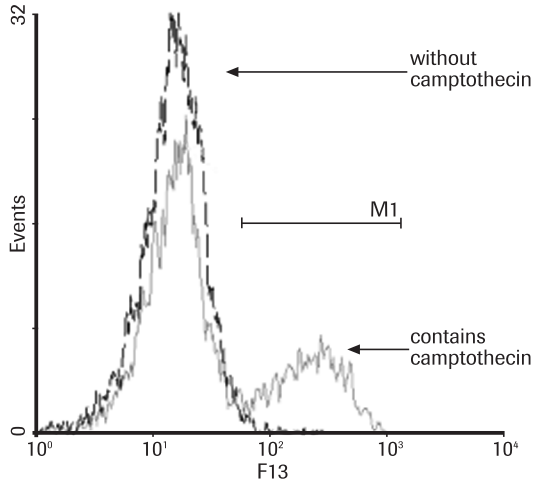


Fig. 2: Analysis of camptothecin induced apoptosis in U937 cell by flow cytometry
Dotted line: Cells cultured in the absence of camptothecin.
Solid line: Cells cultured in the presence of camptothecin ($2 \mu\text{g/ml}$, 4 h).
Cells analyzed under marker M1 are apoptotic (TUNEL positive)

5. Appendix

5.1 Troubleshooting

This table describes various troubleshooting parameters.

Problem	Step/ Reagent of Procedure	Possible Cause	Recommendation	
Nonspecific labeling	Embedding of tissue	UV-irradiation for polymerization of embedding material (<i>e.g.</i> , methacrylate) leads to DNA strand breaks	Try different embedding material or different polymerization reagent.	
	Fixation	Acidic fixatives (<i>e.g.</i> , methacarn, Carnoy's fixative)	<ul style="list-style-type: none"> Try 4% buffered paraformaldehyde. Try formalin or glutaraldehyde. 	
	TUNEL reaction	TdT concentration too high	Reduce concentration of TdT by diluting it 1:2 up to 1:10 with TUNEL Dilution Buffer*.	
	Nucleases, Polymerases		Some tissues (<i>e.g.</i> , smooth muscles) show DNA strand breaks very soon after tissue preparation.	<ul style="list-style-type: none"> Fix tissue immediately after organ preparation. Perfuse fixative through liver vein.
			Some enzymes are still active.	Block with a solution containing ddUTP and dATP.
High background	Sample	Mycoplasma contamination	Mycoplasma Detection Kit*	
		Highly proliferating cells	Double staining, <i>e.g.</i> , with Annexin-V-Fluos*. Note: Measuring via microplate reader not possible because of too high background.	
	Fixation	Formalin fixation leads to a yellowish staining of cells containing melanin precursors.	Try methanol for fixation but take into account that this might lead to reduced sensitivity.	
	TUNEL reaction	Concentration of labeling mix is too high for research in mamma carcinoma.	Reduce concentration of labeling mix to 50% by diluting with TUNEL Dilution Buffer.	

continued on next page

* available from Roche Diagnostics

Problem	Step/ Reagent of Procedure	Possible Cause	Recommendation
Low labeling	Fixation	Ethanol and methanol can lead to low labeling (nucleosomes are not cross-linked with proteins during fixation and are lost during the procedure steps)	<ul style="list-style-type: none"> ▪ Try 4% buffered paraformaldehyde. ▪ Try formalin or glutaraldehyde.
		Extensive fixation leads to excessive cross-linking of proteins	<ul style="list-style-type: none"> ▪ Reduce fixation time. ▪ Try 2% buffered paraformaldehyde.
	Permeabilisation	Permeabilisation too short so that reagents can't reach their target molecules	<ul style="list-style-type: none"> ▪ Increase incubation time. ▪ Incubate at higher temperature (e.g., +15 to +25°C). ▪ Try Proteinase K (concentration and time has to be optimized for each type of tissue). ▪ Try 0.1 M sodium citrate at +70°C for 30 min.
	Paraffin-embedding	Accessibility for reagents is too low	<ul style="list-style-type: none"> ▪ Treat tissue sections after dewaxing with Proteinase K (concentration, time and temperature have to be optimized for each type of tissue). ▪ Try microwave irradiation at 370 W (low) for 5 min in 200 ml 0.1 M Citrate buffer pH 6.0 (has to be optimized for each type of tissue).

continued on next page

Problem	Step/ Reagent of Procedure	Possible Cause	Recommendation
No signal on positive control	DNase treatment	Concentration of DNase is too low	<ul style="list-style-type: none"> ▪ For cryosections apply 3 U/ml DNase I recombinant. ▪ For paraffin-embedded tissue sections apply 1,500 U/ml DNase I recombinant. ▪ In general, use 1 U/ml DNase I recombinant, dissolved in 10 mM Tris-HCl, pH 7.4 containing 10 mM NaCl, 5 mM MnCl₂, 0.1 mM CaCl₂, 25 mM KCl and incubate 30 min at +37°C. ▪ Alternative buffer: Tris- HCl pH 7.5 containing 1 mM MgCl₂ and 1 mg/ml BSA.
Counterstaining diminishes TUNEL staining	DNA stain	Too high concentrations of DNA dye	Use 0.1–1 µg/ml BOBO-1 from Molecular Probes for counterstaining.
Equivocal signals	Double staining	Earlier stage of apoptosis than stage detected by TUNEL reaction	For additional measurement of apoptosis: M30 CytoDEATH* is suitable or Annexin V – Fluos*.
Problems with interpretation of results	FACS Analysis	Positive and negative peaks are not distinguishable, because too many apoptotic bodies were acquired, apoptosis is too far advanced	Change apoptosis inducing procedure: 2-3 Clusters should be visible in the FSC/SSC histogram: <ol style="list-style-type: none"> 1. debris and apoptotic bodies 2. whole cells 3. shrunk cells gate should delete clearly separated peaks.
		No signal for apoptosis	Time depends on cell line and inducing agents and should be optimized.

* available from Roche Diagnostics

5.2 References

- 1 Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992) *J. Cell Biol.* **119**, 493-501.
 - 2 Gorczyca, W., Gong, J. & Darzynkiewicz, Z. (1993) *Cancer Res.* **53**, 1945-1951.
 - 3 Gorczyca, W. et al. (1993) *Leukemia* **7**, 659-670.
 - 4 Gold, R. et al. (1994) *Lab. Invest.* **71**, 219
 - 5 Gorczyca, W. et al. (1994) *Cytometry* **15**, 169-175.
 - 6 Sgonc, R. et al. (1994) *Ternds Genetics* **10**, 41-42.
 - 7 Schmieid, M. et al. (1993) *Am. J. Pathol.* **143**, 446-452.
 - 8 Wyllie, A. H. et al. (1980) *Int. Rev. Cytol.* **68**, 251.
 - 9 Kerr, J. F. R. et al. (1972) *Br. J. Cancer* **26**, 239-257.
 - 10 Duvall, E. & Wyllie, A. H. (1986) *Immunol. Today* **7**, 115.
 - 11 Compton, M. M. (1992) *Canc. Metastasis Rev.* **11**, 105-119.
 - 12 Allen, P. D., Bustin, S. A. & Newland, A. C. (1993) *Blood Reviews* **7**, 63-73.
 - 13 Cohen, J. J. & Duke, R. C. (1992) *Annu. Rev. Immunol.* **10**, 267-293.
 - 14 Clarke, P. G. H. (1990) *Anat. Embryol.* **181**, 195-213.
 - 15 Johnson, E. M. & Deckwerth, T. L. (1993) *Annu. Rev. Neurosci.* **16**, 31-46.
 - 16 Batistatou, A. & Greene, L. A. (1993) *J. Cell Biol.* **122**, 523-532.
 - 17 Strange, R. et al. (1992) *Development* **115**, 49-58.
 - 18 Carson, D. A. & Ribeiro, J. M. (1993) *Lancet* **341**, 1251-1254.
 - 19 Edgington, S. M. (1993) *Biotechnology* **11**, 787-792.
 - 20 Gougeon, M.-L. & Montagnier, L. (1993) *Science* **260**, 1269-1270.
 - 21 Hickman, J. A. (1992) *Cancer Metastasis Rev.* **11**, 121-139.
 - 22 Afanasyev, V. N. et al. (1993) *Cytometry* **14**, 603-609.
 - 23 Bryson, G. J., Harmon, B. V. & Collins, R. J. (1994) *Immunol. Cell Biology* **72**, 35-41.
 - 24 Darzynkiewicz, Z. et al. (1992) *Cytometry* **13**, 795-808.
 - 25 Ando, K. et al. (1994) *J. Immunol.* **152**, 3245-3253.
 - 26 Berges, R. R. et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8910-8914.
 - 27 Gorczyca, W. et al. (1992) *Int. J. Oncol.* **1**, 639-648.
 - 28 Gorczyca, W. et al. (1993) *Exp. Cell Res.* **207**, 202-205.
 - 29 Billig, H., Furuta, I. & Hsueh, A. J. W. (1994) *Endocrinology* **134**, 245-252.
 - 30 MacManus, J. P. et al. (1993) *Neurosci. Lett.* **164**, 89-92.
 - 31 Mochizuki, H. et al. (1994) *Neurosci. Lett.* **170**, 191-194.
 - 32 Oberhammer, F. et al. (1993) *Hepatology* **18**, 1238-1246.
 - 33 Portera-Cailliau, C. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 974-978.
 - 34 Preston, G. A. et al. (1994) *Cancer Res.* **54**, 4214-4223.
 - 35 Weller, M. et al. (1994) *Eur. J. Immunol.* **24**, 1293-1300.
 - 36 Zager, R.A. et al. (1994) *J. Am. Soc. Nephrol.* **4**, 1588-1597.
 - 37 Cohen, G. M. et al. (1992) *Biochem. J.* **286**, 331-334.
 - 38 Collins, R. J. et al. (1992) *Int. J. Rad. Biol.* **61**, 451-453.
 - 39 Sei, Y. et al. (1994) *Neurosci. Lett.* **171**, 179-182.
 - 40 Ansari, B. et al. (1993) *J. Pathol.* **170**, 1-8.
 - 41 Negoescu, A. et al. (1998) *Biochemica* **3**, 34-41
-

Apoptosis-specific physiological change	Detection mode/Product	Pack size	Cat. No.	
DNA fragmentation	Gel Electrophoresis			
	Apoptotic DNA-Ladder Kit	20 tests	11 835 246 001	
	In situ assay			
	<i>In Situ</i> Cell Death Detection Kit, Fluorescein (also usable for FACS)	1 kit (50 tests)	11 684 795 910	
	<i>In Situ</i> Cell Death Detection Kit, AP	1 kit (50 tests)	11 684 809 910	
	<i>In Situ</i> 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 µl (20 tests)	11 767 305 001	
	ELISA			
	Cell Death Detection ELISA	1 kit	11 544 675 001	
	Cell Death Detection ELISA ^{PLUS}	1 kit (96 tests)	11 774 425 001	
	Cell Death Detection ELISA ^{PLUS} , 10×	1 kit	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	Western Blot			
	Anti-Poly (ADP-Ribose) Polymerase	100 µl	11 835 238 001	
	Fluorimetric microplate Assay			
	Homogenous Caspases Assay, fluorometric	100 tests 1,000 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	
Expression of apoptosis-related proteins	ELISA			
	p53 pan ELISA	1 kit	11 828 789 001	

Single reagents

Product	Pack Size	Cat. No.
DNase I recombinant	2 × 10,000 U	04 536 282 001
Pepsin	1 g	10 108 057 001

Changes to Previous Version

Editorial changes

Regulatory Disclaimer

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

Trademarks

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

Disclaimer of LicenseFor patent license limitations for individual products please refer to: [List of biochemical reagent products](#)

Contact and Support

If you have questions or experience problems with this or any Roche product for Life Science, please contact our Technical Support staff. Our scientists are committed to providing rapid and effective help.

Please also contact us if you have suggestions for enhancing Roche product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to the research community worldwide.

To ask questions, solve problems, suggest enhancements or report new applications, please visit our [Online Technical Support Site](#).

Visit [sigma-aldrich.com](#), to download or request copies of the following materials.

- Instructions for Use
- Safety Data Sheets
- Certificates of Analysis
- Information Material

To call, write, fax, or email us, visit [sigma-aldrich.com](#), and select your home country to display country-specific contact information.

