For research purposes only. Not for use for *in vitro* diagnostic procedures for clinical diagnosis.

In Situ Cell Death Detection Kit, TMR red

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 Store at −15 to −25°C
1 Kit (50 tests)

Instruction Manual

Version December 2004

Roche

1. Preface

1.1 Table of Contents

1.2 Kit Contents

Caution

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

When using 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	• Terminal deoxynucleotidyl transferase from calf thymus *(EC 2.7.7.31)*, recombinant in *E. coli*, in storage buffer • 10× conc. • 5 × 50 µl
2 red	Label Solution	• Nucleotide mixture in reaction buffer • 1× 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 in front of each procedure.

Procedure	Equipment	Reagents
Preparation of sample material (section 3.2)		
• Cell suspension (section 3.2.1) • Adherent cells, cell smears and cytospin preparations (section 3.2.2.) • Cryopreserved tissue (section 3.2.3.2)	• Shaker • V-bottomed 96-well microplate	• Washing buffer: Phosphate buffered saline (PBS*) • Blocking solution: 3% H_2O_2 in methanol • 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)
Paraffin-embedded tissue (section 3.2.3.1)		• Xylene and ethanol (absolute, 95%, 90%, 80%, 70%, diluted in double distilled water) • Washing buffer: PBS* • Proteinase K*, nuclease free, working solution: [10–20 µg/ml in 10 mM Tris/HCl, pH 7.4–8] Alternative treatments • Permeabilisation solution: (0.1% Triton[1)] X–100, 0.1% sodium citrate), freshly prepared • Pepsin* (0.25%–0.5% in HCl, pH 2) or trypsin*, 0.01 N HCl, nuclease free • 0.1 M Citrate buffer, pH 6 for microwave irradiation
Labeling protocol (section 3.3)		
Positive control (section 3.3.1)		• Micrococcal nuclease or • DNase I, grade I*
• Cell suspensions (section 3.3.2) • Adherent cells (section 3.3.3)	• Parafilm or coverslips • Humidified chamber	Washing buffer: PBS*
Difficult tissue (section 3.3.4)	• Plastic jar • Microwave • Humidified chamber	• 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.

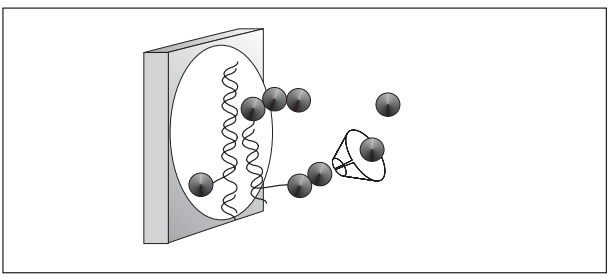


Fig. 1: DNA of fixed cells labeled by the addition of TMR red dUTP at strand breaks by terminal transferase.

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

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.

False positive results: Extensive DNA fragmentation may occur in certain forms of necrosis (38).

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.

To confirm 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.

Sample Material

- Cell suspensions from
  - 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

The unopened kit is stable at −15 to −25°C through the expiration date printed on the label.

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

Advantage

Please refer to the following table.

Benefit	Feature
Sensitive	Detection of apoptotic cell death at single cell level via fluorescence microscope 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	• 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	• 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). Inappropriate regulation of apoptosis may play an important role in many pathological conditions like ischemia, stroke, heart disease, cancer, AIDS, autoimmunity, hepatotoxicity and degenerative diseases of the central nervous system (18–20).

In oncology, extensive interest in apoptosis comes from the observation, that this mode of cell death is triggered by a variety of antitumor drugs, radiation and hyperthermia, and that the intrinsic propensity of tumor cells to respond by apoptosis is modulated by expression of several oncogenes and may be a prognostic marker for cancer treatment (21).

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:

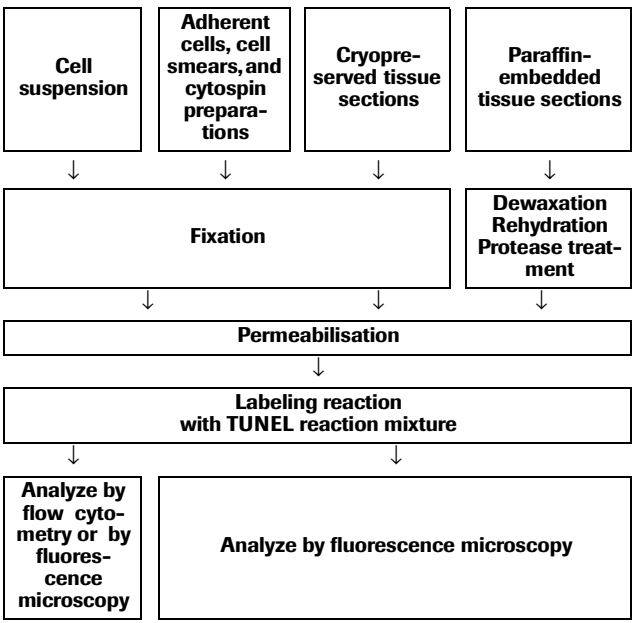
- Label intensity of apoptotic cells is higher with TUNEL compared to ISNT, resulting in an increased sensitivity (2, 4).
- Kinetics of nucleotide incorporation is very rapid with TUNEL compared to the ISNT (2, 4).
- TUNEL preferentially labels apoptosis in comparison to necrosis, thereby discriminating 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 tetramethyl- 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 permeabilisate 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 µl/well cell suspension into a V-bottomed 96-well microplate.
3	Add 100 µl/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, microplate should be incubated on a shaker during fixation.
5	Centrifuge microplate at 300 g for 10 min and remove fixative by flicking off or suction.
6	Wash cells once with 200 µl/well **PBS**.
7	Centrifuge microplate at 300 g for 10 min and remove **PBS** by flicking off or suction.
8	Resuspend cells in 100 µl/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 permeabilisate 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 Tissue Sections

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 only from Roche Applied Science, because it is 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, nuclease free
- 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.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>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).</td></tr>
<tr><td>2</td><td>Incubate tissue section for 15–30 min at +21 to +37°C with Proteinase K working solution.
<table>
<tr><th>Alternatives:</th><th>Treatment:</th></tr>
<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>• 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>
</table></td></tr>
<tr><td>3</td><td>Rinse slide(s) twice with PBS.</td></tr>
<tr><td>4</td><td>Proceed as described under 3.3.</td></tr>
</table>

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 permeabilisate 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 µl TUNEL reaction mixture per sample and 2 negative controls by using 50 µl 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 µl **Label Solution** (vial 2) for two negative controls.
2	Add total volume (50 µl) of **Enzyme Solution** (vial 1) to the remaining 450 µl Label Solution in vial 2 to obtain 500 µl TUNEL reaction mixture.
3	Mix well to equilibrate components.

Additional Reagents Required

- Micrococcal nuclease or
- DNase I, grade I*

Controls

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

Negative control:	Incubate fixed and permeabilized cells in 50 µl/well **Label Solution** (without terminal transferase) instead of TUNEL reaction mixture.
Positive control:	Incubate fixed and permeabilized cells with **micrococcal nuclease or DNase I,** grade I (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 Applied Science*

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 µl/well).
2	Resuspend in 50 µl/well **TUNEL reaction mixture**. ***Note***: For the negative control add 50 µl 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 µl 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 µl **TUNEL reaction mixture** on sample. ***Note***: For the negative control add 50 µl 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× 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	• 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	• 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	• 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 µg/ml, 4 h at +37°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.

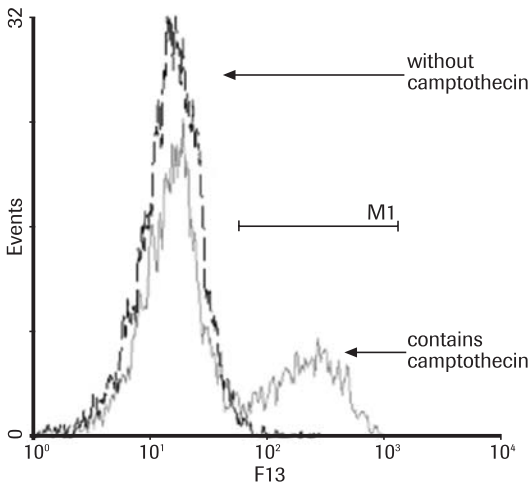


Fig. 2: Analysis of camptothecin induced apoptosis in U937 cell by flow cytometry
Dotted line: Cells cultured in the absence of camptothecin.
Solid line: Cells cultured in the presence of camptothecin (2 µg/ml, 4 h).
Cells analyzed under marker M1 are apoptotic (TUNEL positive)

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 (*e.g.,* methacrylate) leads to DNA strand breaks	Try different embedding material or different polymerization reagent.
	Fixation	Acidic fixatives (*e.g.,* methacarn, Carnoy's fixative)	• 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 (*e.g.,* smooth muscles) show DNA strand breaks very soon after tissue preparation.	• 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, *e.g.*, 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 mamma carcinoma.	Reduce concentration of labeling mix to 50% by diluting with TUNEL Dilution Buffer.

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** available from Roche Applied Science*

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)	• Try 4% buffered paraformaldehyde. • Try formalin or glutaraldehyde.
		Extensive fixation leads to excessive cross-linking of proteins	• Reduce fixation time. • Try 2% buffered paraformaldehyde.
	Permeabilisation	Permeabilisation too short so that reagents can't reach their target molecules	• 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	• 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).

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5.1 Troubleshooting, continued

Problem	Step/ Reagent of Procedure	Possible Cause	Recommendation
No signal on positive control	DNase treatment	Concentration of DNase is too low	• For cryosections apply 3 U/ml DNase I, grade I. • For paraffin-embedded tissue sections apply 1500 U/ml DNase I, grade I. • In general, use 1 U/ml DNase I, grade I, dissolved in 10 mM Tris-HCl, pH 7.4 containing 10 mM NaCl, 5 mM $MnCl_2$, 0.1 mM $CaCl_2$, 25 mM KCl and incubate 30 min at +37°C. • Alternative buffer: Tris- HCl pH 7.5 containing 1 mM $MgCl_2$ and 1 mg/ml BSA.
Counter-staining 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 acquired, apoptosis is too far	Change apoptosis inducing procedure: 2-3 Clusters should be visible in the FSC/SSC histogram: 1. debris and apoptotic bodies 2. whole cells 3. shrinked cells gate should delete 1.): clearly separated peaks.
		No signal for apoptosis	Time depends on cell line and inducing agents and should be optimized.

** available from Roche Applied Science*

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5.3 Ordering Information

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**		
	In Situ Cell Death Detection Kit, Fluorescein (also usable for FACS)	1 kit (50 tests)	11 684 795 910
	In Situ Cell Death Detection Kit, AP	1 kit (50 tests)	11 684 809 910
	In Situ Cell Death Detection Kit, POD	1 kit (50 tests)	11 684 817 910
	Single reagents for TUNEL and supporting reagents		
	TUNEL AP	70 tests (3.5 ml)	11 772 457 001
	TUNEL POD	70 tests (3.5 ml)	11 772 465 001
	TUNEL Enzyme	2× 50 µl (20 tests)	11 767 305 001
	TUNEL Label	3× 550 µl (30 tests)	11 767 291 001
	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-Alexa 568	250 tests	03 703 126 001
	Annexin-V-Biotin	250 tests	11 828 690 001
	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

Apoptosis-specific physiological change	Detection mode/Product	Pack size	Cat. No.
Enzymatic activity	**Western Blot**		
	Anti-Poly (ADP-Ribose) Polymerase	100 µl	11 835 238 001
	FIENA		
	Caspase 3 Activity Assay	1 kit	12 012 952 001
	Fluorimetric microplate Assay		
	Homogenous Caspases Assay, fluorometric	100 tests 1000 tests	03 005 372 001 12 236 869 001
	***In situ* Assay**		
	M30 CytoDEATH (formalin grade)	50 tests 250 tests	12 140 322 001 12 140 349 001
	M30 CytoDEATH, Fluorescein	250 tests	12 156 857 001
Expression of apoptosis-related proteins	***In situ* Assay/Western Blot**		
	Anti-p53-Protein pan (BMG 1B1)	200 µg	11 810 928 001
	ELISA		
	p53 pan ELISA	1 kit	11 828 789 001

Single reagents

Product	Pack Size	Cat. No.
DNase I, grade I	20 000 U	10 104 132 001
Pepsin	1 g	10 108 057 001
Trypsin, solution	100 ml, sterile	10 210 234 001
Proteinase K, PCR grade	1250 µl	11 964 364 001

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/apoptosis

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