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

TUNEL Enzyme

Enzyme solution, containing **terminal deoxynucleotidyl transferase** from calf thymus recombinant in *E. coli*

One of the two components needed to perform the TUNEL reaction for detecting apoptosis (programmed cell death) *in situ*.

Cat. No. 11 767 305 001

2 × 50 µl (20 tests)

Version 08
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Store at −15 to −25°C

1. Product overview

Contents

Terminal deoxynucleotidyl transferase in an optimized concentration dissolved in reaction buffer:
60 mM K-phosphate (pH 7.2 at 4°C), 150 mM KCl, 1 mM 2-Mercaptoethanol, 0.5% Triton X-100, 50% glycerol.

Working solution

TUNEL Enzyme is used in combination with TUNEL Label to prepare the TUNEL reaction mixture.
For one test:
Mix 5 µl TUNEL Enzyme with 45 µl TUNEL Label prior to use. For negative control use 50 µl/test TUNEL Label only.

Introduction

DNA degradation is considered as the key biochemical event of apoptosis, resulting in cleavage of nuclear DNA into oligonucleosome-sized fragments (1). Therefore, this process is commonly used for detection of apoptosis by the typical "DNA ladder" on agarose gels during electrophoresis (2). 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 DNA strand breaks which occur early during apoptosis. DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) (3, 4) have been used to introduce labeled nucleotides into partially degraded DNA.

Application

The TUNEL Enzyme is used in combination with the TUNEL Label to prepare the TUNEL reaction mixture. The TUNEL reaction mixture is used to label DNA strand breaks for detecting and quantitating apoptotic cell death at single cell level in cells and tissues.

Sample material

- Cells in suspension (6)
- Cytospin and cell smear preparations
- Adherent cells cultured on chamber slides (8)
- Frozen or formalin-fixed, plastic- or paraffin-embedded tissue sections (3)

Number of tests

The reagent is sufficient for 20 tests.

Storage/Stability

Stable at −15 to −25°C until the expiration date printed on the label.
Note: The TUNEL reaction mixture (45 µl TUNEL Label with 5 µl TUNEL Enzyme for 1 test) should be prepared immediately before use and should not be stored. Keep TUNEL reaction mixture on ice until use.

Advantages

The tailing reaction using TdT, also named ISEL (*in situ* end labeling) (5) or TUNEL (TdT-mediated dUTP nick end labeling) (1, 6), 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.
- Kinetics of nucleotide incorporation is very rapid with TUNEL compared to the ISNT.
- TUNEL preferentially labels apoptotic cells compared to necrotic cells (7).

2. Procedures and required material

Introduction

The working procedure described below has been published by R. Sgonc et al. (6). The main advantage of this simple and rapid procedure is the use of fluorescein-dUTP to label DNA strand breaks. This allows the detection of DNA degradation by fluorescence microscopy or flow cytometry directly after the TUNEL reaction. It has been shown that this direct detection of DNA strand breaks is as powerful as indirect detection methods *e.g.*, using DIG-dUTP and a fluorescein-labeled anti-DIG antibody (9).

2.1 Preparation of sample material

2.1.1 Cell suspensions

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 (6)
- 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 cell samples 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 – 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 (4°C).
9	Proceed as described under 2.2.

2.1.2 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
- For the detection TUNEL POD, it is necessary to block endogenous peroxidase with Blocking solution: 3% H_2O_2 in methanol

Procedure

In the following table describes the fixation and permeabilisation of cells.
Note: Fix and permeabilisate 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 - 25°C.
2	Rinse slides with **PBS**.
(optional)	Only necessary for the detection with TUNEL POD: Incubate with **Blocking solution** for 10 min at 15 to 25°C. Rinse slides with **PBS**.
3	Incubate in **Permeabilisation solution** for 2 min on ice (4°C).
4	Proceed as described under 2.2.

2.1.3 Tissue sections

2.1.3.1 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).
***Note*:** Use Proteinase K only from Roche, 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*, nuclease free, 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 three 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).
2	Incubate tissue section for 15 – 30 min at 21 – 37°C with **Proteinase K working solution**.

Alternatives:	Treatment:
Permeabilisation solution	Incubate slides for 8 min.
Pepsin or trypsin	15 - 60 min at 37°C.
Microwave irradiation	▪ 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 2.2.

2.1.3.2 Pretreatment 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[1)] X-100, 0.1% sodium citrate, freshly prepared
- For the detection with TUNEL POD, it is necessary to block endogenous peroxidase with Blocking solution: 3% H_2O_2 in methanol

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 - 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.
(optional)	Only necessary for the detection with TUNEL POD: ▪ Incubate with **Blocking solution** for 10 min at 15 – 25°C ▪ Rinse slides with **PBS**.
3	Incubate slides in **Permeabilisation solution** for 2 min on ice (4°C).
4	Proceed as described under 2.2.

2.2 Labeling protocol

2.2.1 Before you begin

Additional reagents required

TUNEL Label*

Preparation of TUNEL reaction mixture

The TUNEL reaction mixture should be prepared immediately before use and should not be stored.

Note: Keep TUNEL reaction mixture on ice until use.

Step	Action
1	Add for 1 test: 5 µl of **TUNEL-Enzyme** solution to 45 µl **TUNEL-Label** solution to obtain 50 µl TUNEL reaction mixture.
2	Mix well to equilibrate components.

Additional reagents required

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

2.2.2 Labeling protocol for cell suspensions

Additional buffers and equipment required

- Washing buffer: PBS
- Humidified chamber

Procedure

Please refer o the following table.

Step	Action
1	Wash cells 2 times with PBS (200 µl/well).
2	Resuspend cells in 50 µl/well **TUNEL reaction mixture.** ***Note***: For the negative controls add 50 µl/well TUNEL Label solution each.
3	Add lid and incubate for 60 min at 37°C in a humidified atmosphere in the dark.
4	Wash cells 3 times in **PBS** (200 µl/well).
5	Analyse cells by fluorescence microscopy. For evaluation by fluorescence microscopy use an excitation wavelength in the range of 450 – 500 nm (*e.g.;* 488 nm) and detection in the range of 515 – 565 nm (green). ***Note***: For analysis by flow cytometry, dilute cells approx. 1:10 in PBS.

2.2.3 Labeling protocol for adherent cells, cell smears, cytospin preparations and tissues

Additional equipment and solutions required

- Washing buffer: PBS
- Humidified chamber
- Parafilm or coverslip

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 controls 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	Add lid and incubate 60 min at 37°C in a humidified chamber in the dark.
5	Rinse slide 3 times with **PBS**.
6	Samples can be analyzed in a drop of PBS under a fluorescence microscope at this state. Use an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green).

2.2.4 Labeling protocol for difficult tissue

Additional 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
- Humidified chamber

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 (20–25°C). Transfer the slide(s) into **PBS** (20–25°C). **DO NOT** perform a Proteinase K treatment!
4	Immerse the slide(s) for 30 min at 15-25°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 15-25°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. Samples can be analyzed in a drop of PBS under a fluorescence microscope. Use an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green).

2.3 Signal conversion (optional)

General

Using TUNEL AP* or TUNEL POD* the fluorescent label may be converted into a colorimetric signal. Thus, the samples may be analyzed by light microscopy.

If preparations will be analyzed by light microscopy using TUNEL AP or -POD as secondary detection system any precipitating substrate suitable for immunohistochemistry may be used.

Additional equipment and solutions required

- Washing buffer: PBS
- Humidified chamber
- Parafilm or coverslip
- TUNEL-AP*: anti-fluorescein antibody, Fab fragments from sheep, conjugated with alkaline phosphatase
- TUNEL-POD*: anti-fluorescein antibody, Fab fragments from sheep, conjugated with horse-radish peroxidase
- Substrate solution:
  - For the use of TUNEL AP: NBT/BCIP or Fast Red
  - For the use of TUNEL POD: DAB Substrate or alternative POD substrate.
- Mounting medium for light microscopy

Procedure

Please refer to the following table.

Step	Action
1	Dry area around sample.
2	Add 50 µl **TUNEL-AP** or **TUNEL-POD** on sample. ***Note***: To ensure a homogeneous spread of Converter-AP or Converter-POD across cell monolayer and to avoid evaporative loss, samples should be covered with parafilm or cover slip during incubation.
3	Incubate slide in a humidified chamber for 30 min at 37°C.
4	Rinse slide 3× with **PBS**.
5	Add 50-100 µl **Substrate solution**.
6	Incubate slide for 10 min at 15-25°C.
7	Rinse slide 3× with **PBS**.
8	Mount under glass coverslip (*e.g.*, with PBS/glycerol) and analyze under light microscope. Alternative: Samples can be counterstained prior to analysis by light microscope.

3. References

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7 Gold, R. et al. (1994) *Lab Invest* **71**, 219.,
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4. 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, TMR red	1 kit (50 tests)	12 156 792 910
	In Situ Cell Death Detection Kit, Fluorescein	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)	11 767 291 910
	TUNEL Dilution Buffer	20 ml	11 966 006 001
	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
	FIENA		
	Caspase 3 Activity Assay	1 kit	12 012 952 001
	Homogeneous 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
	ELISA		
	p53 pan ELISA	1 kit	11 828 789 001
Single reagents	DNase I recombinant, grade I	2 × 10,000 U	04 536 282 001
	Pepsin	1 g	10 108 057 001
	Trypsin Solution	100 ml	10 210 234 001

Apoptosis-specific physiological change	Detection Mode/ Product	Pack Size	Cat. No.
	Proteinase K recombinant, lyophilizate	2 × 250 mg 25 mg 4 × 250 mg 100 mg	03 115 801 001 03 115 836 001 03 115 852 001 03 115 879 001
	Proteinase K recombinant, solution	5 ml 25 ml 1.25 ml	03 115 828 001 03 115 844 001 03 115 887 001

Changes to Previous Version Editorial changes

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany