Cat. # MK500

For Research Use

TaKaRa

In situ Apoptosis Detection Kit

Product Manual

v201609Da

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I. Description

Apoptosis (programmed cell death) is a physiological process in which cells that are unnecessary or threaten the welfare of an organism are destroyed in a tightly regulated manner. Apoptosis is an essential component of many biological processes, including the differentiation and maintenance of various tissue types, and immune system development and function. It is also well understood that disruption of apoptosis can lead to the development and proliferation of cancer cells. Consequently, many forms of cancer treatment involve the induction of apoptosis via radiation and/or chemotherapy.
A key feature of apoptotic cells is fragmentation of chromatin DNA into internucleosomal fragments of approximately 185 bp. This kit is designed to detect fragmented DNA histochemically by terminal labeling. The TUNEL method (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) is an effective method for measuring the DNA fragments that result from the apoptotic activation of intracellular endonucleases. Fluorescein-labeled nucleotides are incorporated onto the ends of these DNA fragments *in situ*, allowing for histologic localization and detection of individual cells.

II. Principle

The TUNEL method uses terminal deoxynucleotidyl transferase to label 3'-OH ends of DNA fragments that are generated during the process of apoptosis. The cells undergoing apoptosis are specifically labeled with fluorescein-dUTP with high sensitivity, allowing immediate detection of these cells by fluorescence microscopy or flow cytometry. Since incorporated fluorescein can also be detected with a peroxidase-labeled anti-fluorescein antibody, it is also possible to detect apoptotic cells with a light microscope.

<Feature>

1. *Ready to use* :	This kit allows speedy detection.
2. *High sensitivity* :	This kit allows detection of the cells at the primary stage of apoptosis at the single-cell level.
3. *Specific* :	Apoptotic cells are stained more specifically than necrotic cells.
4. *Flexible* :	・Both tissue sections and fixed cells are applicable as samples. ・Both fluorescence and light microscopy can be used for detection. ・Individual components are available separately.
5. *Accuracy* :	The supplied control slide can be used to confirm that the procedure was performed correctly, or for training new users.
6. *Safety* :	The supplied buffer does not include hazardous reagents such as cacodylic acid, ensuring user safety.

III. Components (For 20 assays)*1

(1) Labeling Safe Buffer	500 µl x 2
(2) TdT (Terminal deoxynucleotidyl Transferase) Enzyme*2	50 µl x 2
(3) Anti-FITC HRP Conjugate*3	1.5 ml
(4) Control Slides*4	2 slides
(5) Permeabilization Buffer	1.0 ml x 2

*1 Individual components are available separately with the following catalog numbers.

1. Labeling Safe Buffer (Cat. #MK501)	500 µl x 10
2. TdT Enzyme (Cat. #MK502)	50 µl x 10
3. Anti-FITC HRP Conjugate (Cat. #MK503)	1.5 ml x 5
4. Control Slides (Cat. #MK504)	2 slides x 5
5. Permeabilization Buffer (Cat. #MK505)	1.0 ml x 10

*2 TdT enzyme is recombinant protein produced in *E. coli*.

*3 Rabbit polyclonal antibody

*4 Control slide is a paraffin-embedded tissue section of rat mammary gland. When it is used as a positive control slide, deparaffinization of the section is needed at first. Please refer for deparaffinization procedure to "**V. Protocol, 4. Procedure, [C. Paraffin embedded tissue section]**." After deparaffinization and treatment with Proteinase K, please follow the detection protocol for paraffin-embedded tissue.

IV. Storage

Shipped at -20℃.
Store components separately

Components	Storage
(1), (2),(5)	-20℃
(3)	4℃*1
(4)	room temperature*2

*1 Store at 4℃ once thawed.
*2 Store at room temperature after delivered, though the kit is shipped at -20℃.

V. Protocol

1. Compatible Specimens

・Cell :	Adherent cells	(Cultured on a chamber slide)
	Non-adherent cells	(Cytospin preparation on a slide glass, or smeared and collected in a microtube)
・Tissue section :		Frozen section, paraffin-embedded section

2. Preparation of Labeling Reaction Mixture

1) For one sample, prepare the labeling reaction mixture by adding 5 μl of TdT Enzyme to 45 μl of Labeling Safe Buffer. Labeling Safe Buffer also contains enough buffer for 2 negative control reactions (50 μl x 2).
2) Mix the prepared mixture gently but well enough to ensure uniformity. The reaction mixture should be prepared just before use, and should be stored on ice until use. Do not store the prepared mixture for long-term use. If it is left for a long period, the enzyme in the mixture might be inactivated.

   Note : Anti-FITC HRP Conjugate needs no preparation prior to use.

3. Reagents and Instruments Required other than This Kit

Please refer to the list specific to your application to determine exactly what is needed.

・Distilled water
・Washing buffer (PBS or TBS)
  e.g., PBS (Phosphate Buffered Salts) Tablets (Cat. #T900)
  TBS (Tris-Buffered Saline) powder (Cat. #T903)*
・Coloring substrate (DAB)
  e.g., TaKaRa DAB Substrate (Cat. #MK210)
・H_2O_2
・Counterstain solution
  e.g., Methyl Green (Dako, Code. S196230)
・Micropipette and microtubes (autoclaved)
・Incubation chamber
  e.g., slide humidity incubation box (LabScientific)
・Incubator (37℃)
・Plastic coverslip or Parafilm
  e.g., Apop Tag Plastic Coverslip (Merck Millipore, Code. S7117)
・Silanecoated glass slides
  e.g., Silanized Slides (Dako, Code. S30330),
  Silane Coated Microscope Slides (LabScientific)
・Cover glasses
・Microscope (fluorescent or light)

* Not available in all geographic locations. Check for availability in your area.

[For detection using paraffin-embedded section]

・Glass or plastic coplin jar
・Xylene
・Ethanol (100%, 90%, and 80%)
・Coverslip or Parafilm
・Proteinase K (Cat. #9034)
・3% H_2O_2 (For endogenous peroxidase inactivation)
・Mounting medium

[For detection using frozen section]

- Glass slide (precoated with silan)
- Fixation solution
  e.g., 10% neutral-buffered formalin, acetone, or 4% paraformaldehyde, etc.
- Methanol containing 0.3% H_2O_2 (for endogenous peroxidase inactivation)

[For detection using cells]

- Glass slide (precoated with silan)
- Fixation solution
  e.g., 10% neutral-buffered formalin, or 4% paraformaldehyde, etc.
- Methanol containing 0.3% H_2O_2 (for endogenous peroxidase inactivation)
- Microcentrifuge (cytospin)

4. Procedure

[A. Cultured cells]

(1) Wash the collected cells with PBS, and dry in air on a silanized glass slide. Fix the cells with 4% paraformaldehyde / PBS solution (pH 7.4) by leaving at room temperature for 15 - 30 min, and wash with PBS.

Note : When flow cytometric detection is performed subsequently, this step should be done in a conical tube or microtube. The treated cells can be stored for 1 - 2 months when stored in 70% ethanol at -20℃.

(2) Inactivate endogenous peroxidase with methanol containing 0.3% H_2O_2 at room temperature for 15 - 30 min. Wash with PBS after inactivation.

Note : The peroxidase inactivation step is needed only when coloring the section. This process is omitted when performing flow cytometry analysis or when only observing with a fluorescene microscope.

(3) Apply 100 μl of Permeabilization Buffer on ice for 2 - 5 min to allow penetration of the labeling reaction mixture. Wash with PBS.

(4) Apply 50 μl of labeling reaction mixture (consisting of TdT Enzyme 5 μl + Labeling Safe Buffer 45 μl, prepared and cooled on ice prior to use) on the slide, and incubate in a 37℃ humidified chamber for 60 - 90 min. It is recommended to cover with a plastic coverslip to prevent drying. Terminate the reaction by washing with PBS.

Note : When performing the reaction in a tube, mix gently once every 15 min to suspend the precipitated cells.

Cells treated as above can be analyzed with a fluorescent microscope or flow cytometry. When viewing with a light microscope, follow the procedure as described below.

(5) Apply Anti-FITC HRP Conjugate at 37℃ for 30 min, and wash with PBS 3 - 4 times. After coloring with DAB at room temperature for 10 - 15 min, terminate the reaction by washing with distilled water.

Note: When performing the reation in a tube, mix gently on occasion so that antibody can react uniformly with the cells.

(6) Stain the cells with 3% methyl green. Mount and detect with a light microscope.

[B. Frozen tissue section]

(1) Freeze the fresh tissues immediately in an OTC compound. Slice the frozen tissue with a cryostat stick onto a silanized slide. Fix the cells with freshly prepared 4% paraformaldehyde/PBS solution (pH 7.4) or acetone at room temperature for 15 - 30 min. Wash with PBS for 20 - 30 min.

(2) Wash the slides with PBS, and inactivate the endogenous peroxidase using methanol (containing 0.3% H_2O_2) at room temperature, 15 - 30 min.

Note : The peroxidase inactivation step is needed only when coloring the section. This process is omitted when samples are observed with a fluorescent microscope.

(3) Apply 100 μl of Permeabilization Buffer on ice for 2 - 5 min so that the labeling reaction mixture can permeate well.

(4) Apply 50 μl of labeling reaction mixture (consisting of TdT Enzyme 5 μl + Labeling Safe Buffer 45 μl, prepared and cooled on ice prior to use) on the slide, and incubate in a 37℃ humidified incubator for 60 - 90 min. It is recommended to cover the glass slide with a plastic coverslip to prevent drying. Terminate the reaction by washing the slide 3 times in PBS for 5 min each time.

Tissue sections treated as above can be analyzed with a fluorescent microscope.

(5) Apply 70 μl of Anti-FITC HRP Conjugate and incubate at 37℃ for 30 min, and wash in PBS 3 times for 5 min each time.

Note : 1) Cover the tissue uniformly with the conjugate.
2) It is recommended to cover the glass slide with a plastic coverslip to prevent drying.

(6) After coloring with DAB at room temperature for 10 - 15 min, terminate the reaction by washing with distilled water.

(7) Stain with 3% methyl green. Observe using a light microscope after dehydration, penetration, and sealing.

[C. Paraffin-embedded tissue section]

(1) Deparaffinize the section following the procedure described in "Deparaffinization," (see below). Wash with distilled water.
Apply 10 - 20 μg/ml Proteinase K and leave at room temperature for 15 min.
Wash with PBS.

Note : When the intensity of staining of apoptosis cells is low, change the reaction condition of Proteinase K (400 μg/ml, 5 min). When the incubation time is too long, the tissue may be disrupted.

(2) Inactivate the endogenous peroxidase by applying 3% H_2O_2 for 5 min. Wash with PBS.

Note : The peroxidase inactivation step is needed only when coloring the section. This process is omitted when samples are observed with a fluorescent microscope.

(3) Apply 50 μl of labeling reaction mixture (consisting of TdT Enzyme 5 μl + Labeling Safe Buffer 45 μl, prepared and cooled on ice prior to use) on the slide, and incubate in a 37℃ humidified chamber for 60 - 90 min. It is recommended to cover the glass slide with a plastic coverslip to prevent drying. Terminate the reaction by washing the slide 3 times in PBS for 5 min each time.

Tissue sections treated as above can be analyzed with a fluorescent microscope.

(4) Apply 70 μl of Anti-FITC HRP Conjugate and incubate at 37℃ for 30 min, and wash 3 times in PBS for 5 min each time.

Note : 1) Cover the tissue uniformly with the conjugate.
2) It is recommended to cover the glass slide with a plastic coverslip to prevent drying.

(5) After coloring with DAB at room temperature for 10 - 15 min, terminate the reaction by washing with distilled water.

(6) Stain with 3% methyl green. Observe with a light microscope after dehydration, penetration, and sealing.

<Deparaffinization>

1. Apply the following in order.

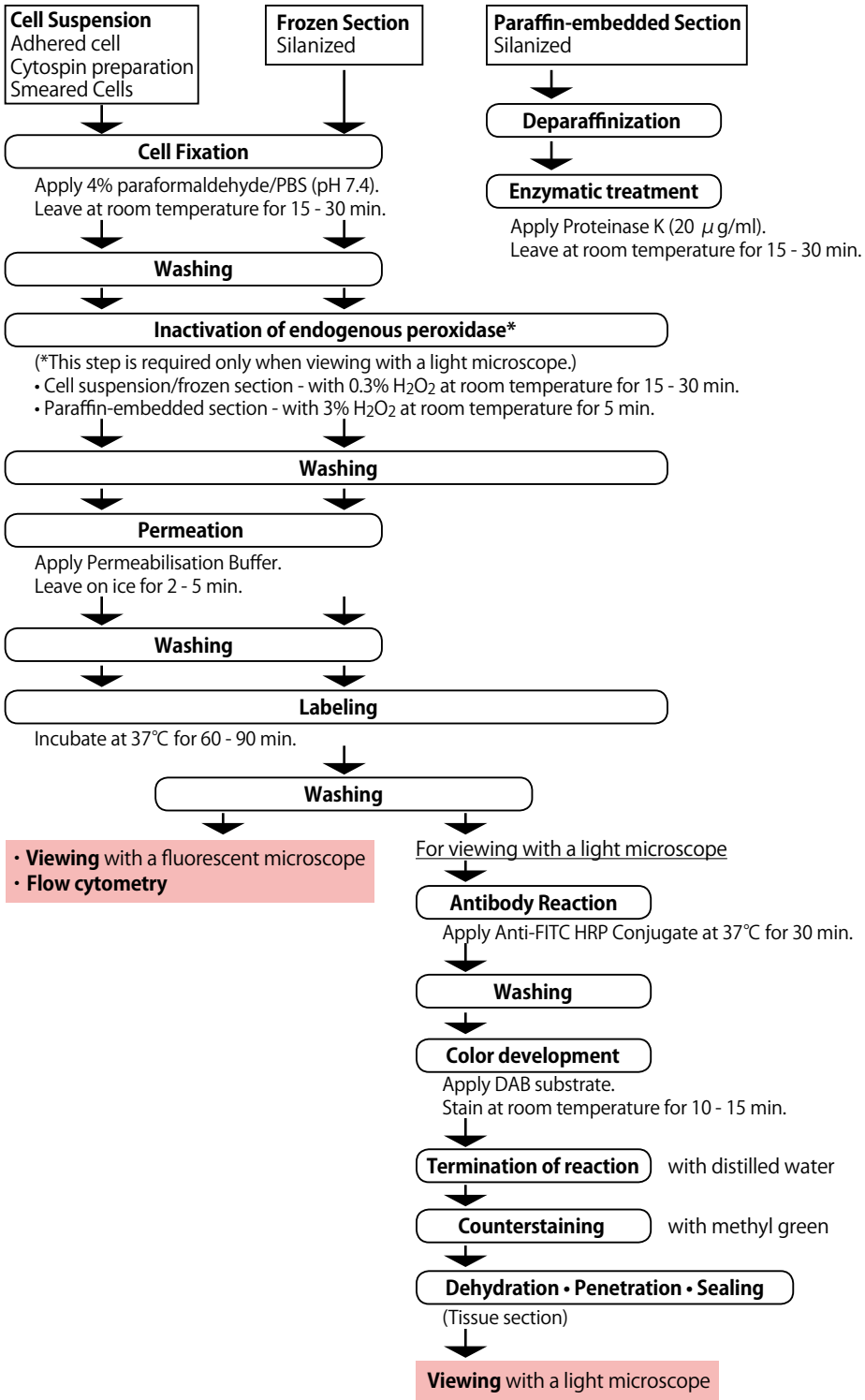
Xylene I	for 5 min
Xylene II	for 5 min
Xylene III	for 5 min
100% ethanol	for 5 min
100% ethanol	for 5 min
90% ethanol	for 5 min
80% ethanol	for 5 min

2. Wash with flowing water for 2 min.

3. Immerse in distilled water.

* Please read through this note prior to starting the protocol.

VI. Protocol Summary



【Control slide experiment】

Optical-microscope observation of the stained paraffin-embedded tissue section.

Control slide (rat mammary gland)
DAB color development
Counter staining with 3% methyl green

【Note】

1. It is recommended to use silanized glass slides to prevent exfoliation.
2. The humidified chamber should be prewarmed to 37℃.
3. Covering the slides with plastic coverslips during the reaction is useful to spread the reaction mixture uniformly (by capillary action). It also prevents evaporation during incubation.
4. When covering the slides with the reaction mixture after washing with PBS, tap off the excess water with filter paper or a paper towel.
5. When washing the cells with PBS, pay special attention not to pour PBS directly on to the cells because the cells will exfoliate. When using tissue sections as a specimen, wash them 3 times for 5 min each time.

VII. Troubleshooting

Q1 : The intensity of staining of apoptosis cells is low.

A1 : i) The reaction mixture might not have permeated well in tissues or cells due to steric hindrance. For adequate permeabilization of the mixture, please adjust the treatment time with Proteinase K or Permeabilization Buffer.
ii) Extend the enzymatic reaction time.
iii) Extend the antibody reaction time or substrate coloring time.

Q2 : Non-apoptotic cells are stained.

A2 : Non-specific binding might have occurred. Repeat the washing steps or add the blocking reagent into the washing buffer, e.g., 1%(w/v) BSA, or skimmed milk.

Q3: Can hematoxylin staining be used in place of methyl green staining for light microscopy?

A3: Methyl green staining is recommended to evaluate nuclear size and position of apoptosis-positive cells. The methyl green stain provides good contrast with the brown DAB stain of apoptosis-positive cells, but the hematoxylin stain is not sufficient.

VIII. Reference

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IX. Related Product

ApopLadder Ex ™ (Cat. #MK600)