



***In situ* Apoptosis Detection Kit**

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In situ Apoptosis Detection Kit

1. Description

Apoptosis is a cell regulation system controlled by gene. It is defined as a physiological process for maintaining individual lives to cause death of cells which are dangerous or unnecessary. Apoptosis is much associated with cell growth, differentiation, and other physiological cellular phenomena, especially regarding immune system. It is now manifest that apoptosis plays a significant role in various stages of cancer cells development; growth, or proliferation inhibition, disappearance, differentiation induced by physical factors (like radiation, heat) and by chemical factors (ex. medicine). One of the features of apoptotic cells is fragmentation of chromatin DNA at nucleosome level (185 bp). This fragmented DNA can be detected histochemically by terminal labeling. TUNEL, an acronym for Terminaldeoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling, is an effective method for measuring the DNA fragments resulting from the apoptotic activation of intracellular endonucleases. Fluorochrome-labeled nucleotides are *in situ* incorporated onto the ends of these DNA fragments, allowing histologic localization and individual cells to be detected.

2. Principle

TUNEL method uses Terminaldeoxynucleotidyl 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 the immediate detection by viewing with a fluorescence microscope. Since incorporated fluorescence can also be detected with peroxidase-labeled anti-fluorescence antibody, it is possible to detect with a light microscope.

3. 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:* Apoptosis cells are stained more specifically than necrosis cells.
- 4. *Flexible:*
 - Both tissue section and fixed cells are applicable as a sample.
 - Both fluorescence microscope and light microscope can be used for detection.
 - Individual component can be available separately.
- 5. *Accuracy:* As a control slide is supplied, this kit is suitable for confirming an user's techniques, procedures, or for training of an inexperienced person.
- 6. *Safety:* The supplied buffer does not include hazardous reagents (cacodylic acid), allowing safe procedure.

4. Kit component

(For 20 assays)

1. Labeling Safe Buffer 2 x 500 μ l
2. TdT (Terminaldeoxynucleotidyl Transferase) Enzyme 2 x 50 μ l
3. Anti-FITC HRP Conjugate 1 x 1.5 ml
4. Control Slides* 2 slides
5. Permeabilisation Buffer 2 x 1.0 ml

* 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 "10. Protocol, C. Paraffin-embedded tissue section", page 5. After deparaffinizing and treatment with Proteinase K, please follow the protocol according to the method of the subsequent detection.

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[For detection using paraffin-embedded section]

- Glass or plastic coplin jar
- Xylene
- Ethanol (100%, 90%, 80%)
- Glass coverslip
- Proteinase K (20 µg/ml)
- 3% H₂O₂ (For endogenous peroxidase inactivation)
- Mounting medium

[For detection using frozen section]

- Slideglass pretreated to prevent exfoliation (precoated with silan)
- Fixation solution (ex. 10% neutral-buffered formalin, acetone, 4% paraformaldehyde, etc.)
- Methanol containing 0.3% H₂O₂ (for Endogenous peroxidase inactivation)

[For detection using cell]

- Slideglass pretreated to prevent exfoliation (precoated with silan)
- Fixation solution (ex. 10% neutral-buffered formalin, 4% paraformaldehyde, etc.)
- Methanol containing 0.3% H₂O₂ (for Endogenous peroxidase inactivation)
- Microcentrifuge (cytospin)

10.PROTOCOL

[A. Cultured cell]

1. Wash the collected cells with PBS, and dry in air on a silanized slideglass. Fix the cells with 4% paraformaldehyde / PBS solution (pH7.4) by leaving at room temperature for 15-30 min.
2. After washing the glass with PBS after fixation, inactivate endogenous peroxidase with methanol containing 0.3% H₂O₂ at room temperature for 15-30 min. Wash with PBS after inactivation.
Note: 1) The step of peroxidase inactivation is needed only when coloring the section. This process is omitted when performing flow cytometry analysis or only observing with a fluorescence microscope.
2) When flow cytometric detection is performed subsequently, the above two steps 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°C.
3. Apply 100 µl of Permeabilisation Buffer on ice for 2-5 min for well permeation of enzyme 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°C humidified chamber for 60-90 min. It is recommended to cover with a coverslip* to prevent drying. Terminate the reaction by washing with PBS.
Note: When performing reaction in a tube, mix gently once in every 15 min. to suspend the precipitated cells.

The slides treated as above can be applicable to detection with a fluorescence microscope or flow cytometry. When viewing with a light microscope, follow the procedure described as below.

5. Apply Anti-FITC HRP Conjugate at 37°C for 30 min, and wash with 3-4 times PBS. After coloring with DAB at room temperature for 10-15 min, terminate the reaction by washing with distilled water.
Note: When performing reaction in a tube, sometimes mix gently so that antibody can react uniformly with the cells.
6. Stain the cells with 3% methyl green. Mount and detect with a light microscope.

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[B. Frozen tissue section]

1. Freeze the fresh cells 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 (pH7.4) 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% H₂O₂) at room temperature, 15-30 min.
Note: The step of peroxidase inactivation is needed only when coloring the section. This process is omitted when only observing with a fluorescence microscope.
3. Apply 100 μ l of Permeabilisation Buffer on ice for 2-5 min. so that enzyme reaction mixture can permeate well.
4. Apply 50 μ l of labeling reaction mixture (consisting of TdT Enzyme 5 Labeling Safe Buffer 45 μ l, prepared and cooled on ice prior to use) on the slide, and incubate in a 37°C humidified incubator for 60-90 min. It is recommended to cover the slideglass with a coverslip* to prevent drying. Terminate the reaction by washing in 3 changes of PBS for 5 min each wash.

The slides treated as above can be applicable to detection with a fluorescence microscope.

5. Apply 70 μ l of Anti-FITC HRP Conjugate and incubate at 37°C for 30 min, and wash in 3 change of PBS for 5 min per wash.
Note: 1) Cover the tissue uniformly with the antibody.
2) It is recommended to cover the slideglass with a 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 the cells 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 "10. Protocol, D. Deparaffinization", page 6. Wash with distilled water. Apply 20 μ g/ml Proteinase K and leave at room temperature for 15 - 30 min. Wash with PBS.
2. Inactivate the endogenous peroxidase by applying 3% H₂O₂* for 5 min. Wash with PBS.
Note: The step of peroxidase inactivation is needed only when coloring the section. This process is omitted when only observing with a fluorescence microscope.
3. Apply 50 μ l of labeling reaction mixture (consisting of TdT Enzyme 5 Labeling Safe Buffer 45 μ l, prepared and cooled on ice prior to use) on the slide, and incubate in a 37°C humidified chamber for 60-90 min. It is recommended to cover the slideglass with a coverslip* to prevent drying. Terminate the reaction by washing the slides in 3 changes of PBS for 5 min per wash.

The above processes allows detection with a fluorescence microscope.

4. Apply 70 μ l of Anti-FITC HRP Conjugate and incubate at 37°C for 30 min, and wash in 3 changes of PBS for 5 min. per wash.
Note: 1)Cover the tissue uniformly with the antibody.
2)It is recommended to cover the slideglass with a 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.

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[D. Deparaffinization]

1. Apply the followings 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.

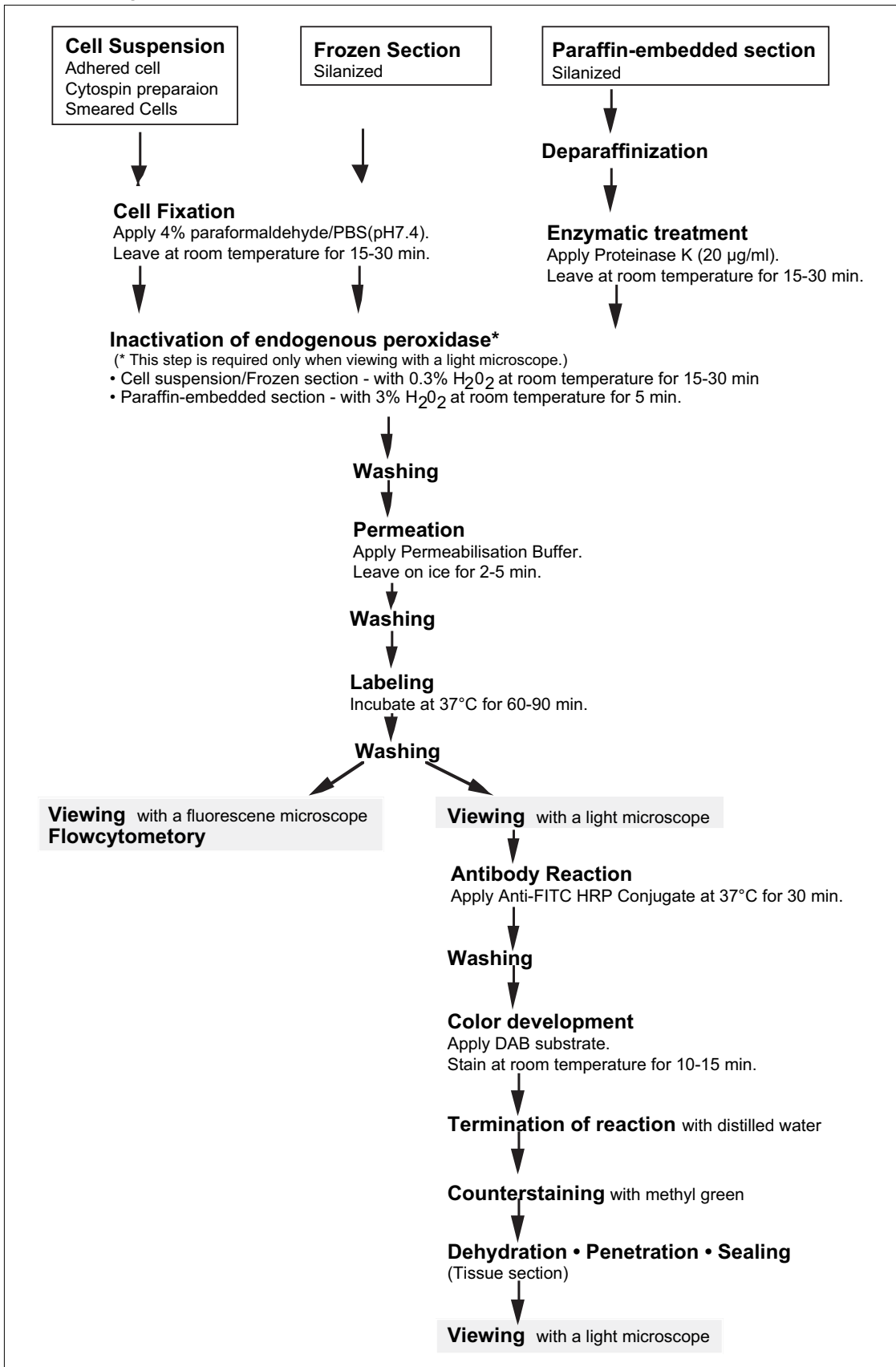
11. Note

Please read through this note prior to starting the protocol

1. It is recommended to use silanized slideglasses to prevent exfoliation.
2. Humidified chamber should be prewarmed to 37°C.
3. Covering the slides with coverslips* during reaction is useful to spread the reaction mixture uniformly (by capillary phenomenon). It also prevents evaporation during incubation.
4. When covering the slides with reaction mixture after washing with PBS, tap off the excess water with filter paper or paper towel.
5. When washing the cells with PBS, pay special attention not to pour PBS directly to the cells because cells exfoliate. When using tissue section as a specimen, wash in 3 changes for 3 min. per wash.

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12. Flow chart of procedure



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13. Control Slide Experiment

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



control slide (rat mammary gland)

DAB color development
Contrast staining 3% methyl green

14. Q&A

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 the enough permeation 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 time of coloring the substrate.

Q2: Non-apoptosis cells are stained.

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

15. Related Product

ApopLadder Ex™ (Cat.# 40-831-160020)

Note: For research use only. Not for use in diagnostic or therapeutic procedures.
