In situ Apoptosis Detection Kit

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Also available from TaKaRa

ApopLadder Ex™ ..... **Cat.#MK400**

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 inhibiton, 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 kit is designed to detect fragmented DNA histochemically by terminal labeling. TUNEL, method (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) is an effective method for measuring the DNA fragments resulting from the apoptotic activation of intracellular endonucleases. Fluorescein labeled nucleotides are *in situ* incorporated onto the ends of these DNA fragments, allowing histologic localizaion and individual cells to be detected.

2. Principle

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 the immediate detection by viewing with a fluorescein microscope. Since incorporated fluorescein can also be detected with peroxidase-labeled anti-fluorescein antibody, it is possible to detect with an 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 l 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 μl
2. TdT (Terminal dexoynucleotidyl Transferase) Enzyme *[1] ..... 2 x 50 μl
3. Anti-FITC HRP Conjugate ..... 1 x 1.5 ml
4. Control Slides *[2] ..... 2 slides
5. Permeabilisation Buffer ..... 2 x 1.0 ml

*[1] TdT enzyne is produced recombinant in *E.coli*.
*[2] Control slide is a paraffin-embeded 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-embeded tissue section**", page 5. After deparaffinizing and treatment with Proteinase K, please follow the protocol according to the method of the subsequent detection.

**Individual component is available separately with the following catalog numbers.
1. Labeling Safe Buffer (Cat.#MK501) ..... 10 x 500 μl
2. TdT Enzyme (Cat.#MK502) ..... 10 x 50 μl
3. Anti-FITC HRP Conjugate (Cat.#MK503) ..... 5 x 1.5 ml
4. Control Slides (Cat.#MK504) ..... 5 packs x 2 slides
5. Permeabilisation Buffer (Cat.#MK505) ..... 10 x 1.0 ml

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5. Storage

Shipped at -20℃ .
Store the component separately 1, 2, 5 .....at -20℃
3 .....at 4℃ *
4. .....at room temperature**

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

6. Reference:

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5. R. Gold, M. Schmied, G. Rothe, H.Zischler, H. Breitschopf, H.Wekerle, and H. Lassmann (1993) *The Journal of Histochemistry and Cytochemistry*, **41**, No.7, 1023-1030.
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7. Jorn Strater, Andreas R, Gunthert, Silke Bruderlein, Peter Moller (1995) *Histochemistry* **103**, 157-160.
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10. Xun Li, Jianping Gong, Eric Feldman, Karen Seiter, Frand Traganos and Zbigniew Darzynkiewicz (1994) *Leukema and Lymphoma*, **13,** Suppl. 1, 65-70.
11. Alexander Dolzhanskiy, Ross S. Basch (1995) *Journal of Immunological Methods*, **180**, 131-140.
12. Gold R., Schmied M. *et al*. (1994) *Laboratory Investigation* **71** No.2, 219-225.

7. Available specimen form:

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

8. Preparation of labeling reaction mixture

1) Per one sample, prepare the reaction mixture by adding 5 μl of TdT Enzyme to 45 μl of Labeling Safe Buffer. One tube of Labeling Safe Buffer also contains buffer in the amount for 2 negative control reactions (50 μl x 2).
2) Mix the prepared mixture gently but well to be mixed uniformly. Reaction mixture should be prepared each time use, and should be stored on ice until use. Do not store the prepared mixture. 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.

9. Reagents and instruments requried other than this kit

Please refer to specific protocols to determine applicalibity.

- Distilled water
- Washing buffer (PBS or TBS)
  ex. Phosphate Buffered Salts (Cat.#T900)
  TBS (Tris-Buffered Saline) powder (Cat.#T903)
- Coloring substrate (DAB)
  (ex. SIGMA Chemical Co. (Code.#D-5905), DAKO(Code.#S3000))
- H_2O_2
- Counterstain solution (methyl green)
- Micropipette and microtube (autoclaved)
- Humidified chamber
- Incubator (37℃)
- Glass coverslip, Guard
  ex. Takara Slide Seal for in situ PCR (Cat.#9066, #9067, #9068)
- Cover glass
- Slide glass (precoated with silan)
- Microscope (Both fluorescence and light one are applicable.)

[For detection using paraffin-embedded section]
- Glass or plastic coplin jar
- Xylene
- Ethanol (100%, 90%, 80%)
- Glass coverslip
  ex. Takara Slide Seal for in situ PCR (Cat.#9066, #9067,#9068)
- Proteinase K (Cat.#9033) (20 μg/ml)
- 3% H_2O_2 (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, aceton,
  4% paraformaldehyde, etc.)
- Methanol containing 0.3% H_2O_2 (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% H_2O_2 (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% H_2O_2 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 fluorescene 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℃ .
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℃ 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.

   *For coverslip, Takara Slide Seal for in situ PCR (Cat.#9066, 9067, 9068) is available.

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

5. Apply Anti-FITC HRP Conjugate at 37℃ 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 reation 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.

[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 (pH7.4) or aceton 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 step of peroxidase inactivation is needed only when coloring the section. This process is omitted when only observing with a fluorescene microscope.
3. Apply 100 μl of Permeabilisation Buffer on ice for 2-5 min. so that enzyme 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 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 fluorescene microscope.

5. Apply 70 ml of Anti-FITC HRP Conjugate and incubate at 37℃ 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 with 3% methyl green. Observe using a light microscope after dehydration, penetration and sealing.

   *For coverslip, Takara Slide Seal for in situ PCR (Cat.#9066, #9067, #9068) is available.

[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 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, 5min). When the incubation time is too long, the disruption of the tissue may be caused.
2. Inactivate the endogenous peroxidase by applying 3% H_2O_2 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 fluorescene 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 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 μl of Anti-FITC HRP Conjugate and incubate at 37℃ 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.

   * For coverslip, Takara Slide Seal for in *situ* PCR (Cat.#9066, 9067, 9068) is available.

[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.

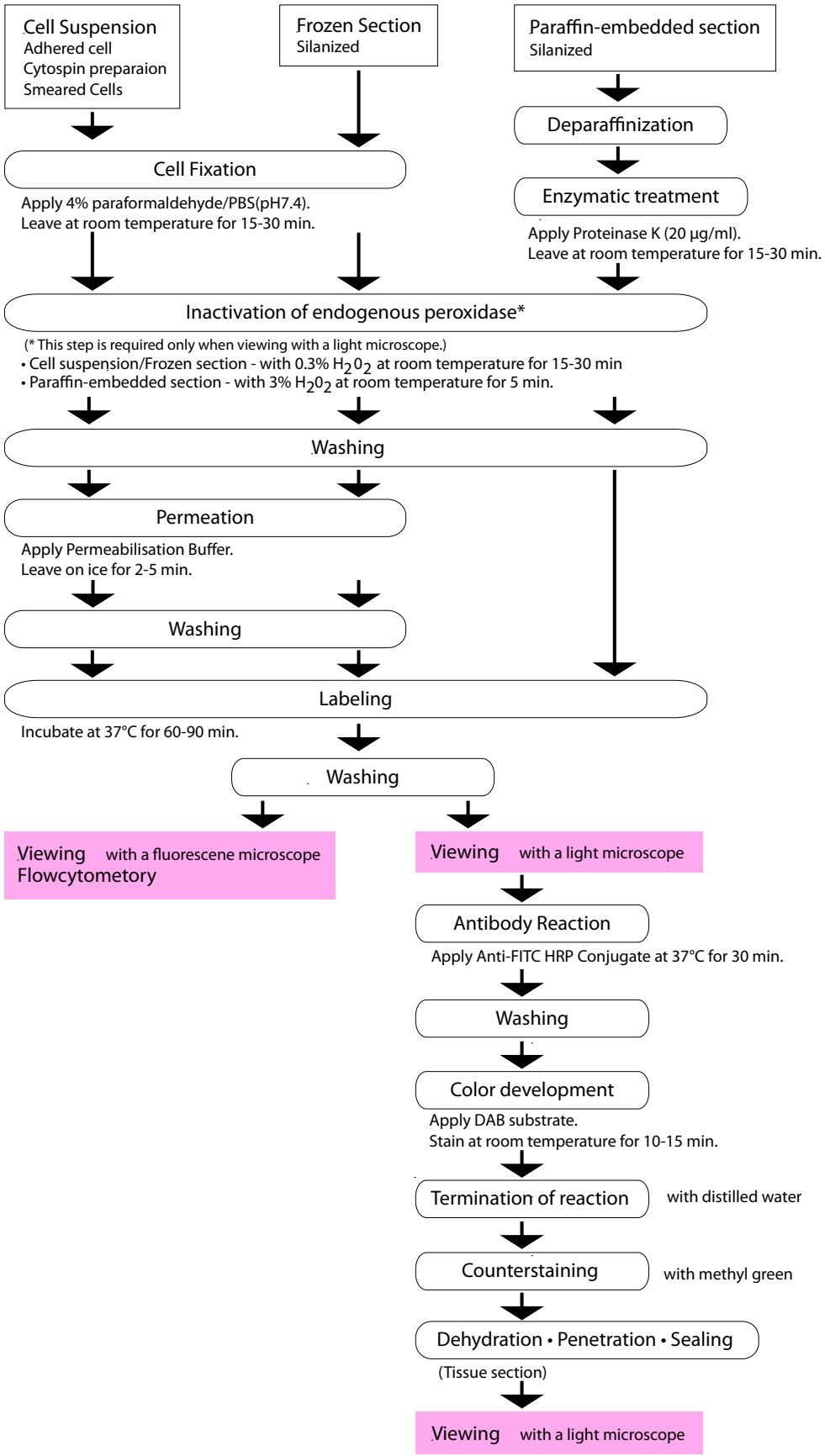
11. Note

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℃ .
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.

   * For coverslip, Takara Slide Seal for in *situ* PCR (Cat.#9066, 9067, 9068) is available.

12. Flow chart of procedure



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.#MK400)