A112

TUNEL BrightGreen
Apoptosis Detection Kit

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Manual Version 6.1

Catalog

1/Introduction

Background

Apoptosis is a basic biological phenomenon of cells. It plays an important role in evolution, homeostasis and system development of organism. Some morphological, physiological or biochemical changes will happen during the apoptosis of cells, such as cell shrinkage, loss of contact between neighboring cells, loss of mitochondrial membrane potential, abnormal membrane permeability, chromatin condensation, nuclear fragmentation, DNA degradation; formation of membrane protrusions; phosphatidylserine's turning out of membrane while the structure of cell membrane is complete. Finally, the cell breaks apart into multiple vesicles called apoptotic bodies, which will undergo phagocytosis. The above described morphological changes occur at different stages of the apoptosis.

A landmark of apoptosis is degradation of chromosome DNA. This specific and regular degradation produces DNA fragments in different lengths of an integer multiple of 180 bp to 200 bp. This is exactly the length of DNA strand that wraps histone. It suggests that the chromosomal DNA is cleaved at the junction between nucleosomes, producing oligonuclear fragments of different lengths. Experiments have shown that the regulated degradation of DNA is a result of an endogenous endonuclease, which cleaves chromosomal DNA at the junction of nucleosomes. Agarose gel electrophoresis shows a specific ladder pattern in apoptotic cells but a diffuse continuous pattern in dead cells.

Principle of the Detection

The TUNEL BrightGreen Apoptosis Detection Kit is designed to detect DNA fragmentation, which represents a characteristic of late stage apoptosis. This kit is based on a terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling (TUENL) method. This kit utilizes an optimized FITC-12-dUTP TUNEL Assay to provide simple, accurate, and rapid detection of apoptotic cells. TdT catalyzes the incorporation of FITC-12-dUTP at the 3'-OH terminus of fragmented DNA in apoptotic cells. FITC-12-dUTP-labeled DNA can be directly observed under fluorescence microscopy or quantified by flow cytometry. The BrightGreen Labeling Mix in this kit contains FITC-12-dUTP and a patented Bright factor. This unique small molecule compound can be non-covalently combined with FITC to enhance its stability and amplify its signal. It makes the marker lighter and more resistant to quenching.

2/Components

Components	A112-01 (20 rxn)	A112-02 (50 rxn)	A112-03 (100 rxn)
5 × Equilibration Buffer	1.25 ml	1.25 ml × 2	1.25 ml × 3
BrightGreen Labeling Mix	100 µl	250 µl	250 µl × 2
Recombinant TdT Enzyme	20 µl	50 µl	50 µl × 2
Proteinase K (2 mg/ml)	40 µl	100 µl	100 µl × 2
DNase I (1 U/µl)	5 µl	13 µl	25 µl
10 × DNase I Buffer	100 µl	260 µl	500 µl

3/Storage

Store at -20 ℃. The BrightGreen Labelling Mix should be protected from light and stored at -20 ℃.

4/Protocol

4.1 Brief Summary

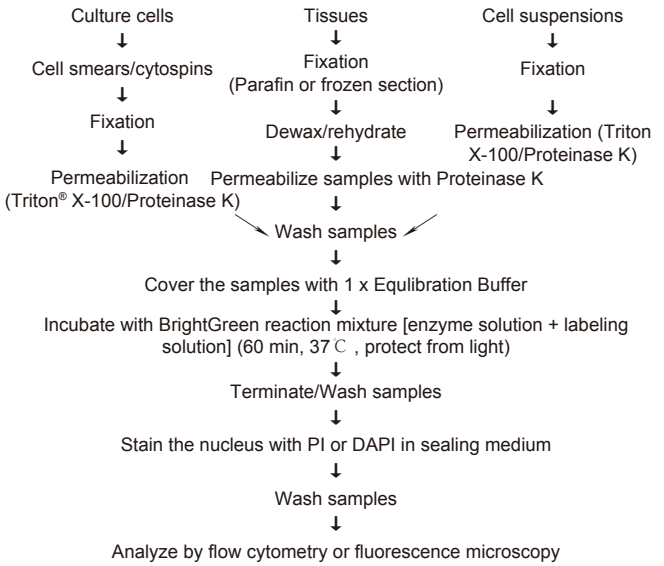

Figure 1: Workflow schematic

4.2 Sample Pretreatment

A.Paraffin-embedded tissue sections

1. Deparaffinize sections by immersing the paraffin-embedded tissue sections in xylene for 5 minutes at room temperature. Replace with fresh xylene and repeat to achieve complete deparaffinization.
2. Wash the samples by immersing the slides in 100% ethanol for 5 minutes at room temperature. Replace with fresh 100% ethanol and repeat.
3. Rehydrate the samples by sequentially immersing the slides through graded ethanol washes (90%, 80%, 70%) for 3 minutes each at room temperature.
4. Immerse and wash the samples with PBS. Remove the excess liquid around samples on the slide with filter papers. The contours of sample distribution can be plotted around the samples with a crayon or hydrophobic pen for convenience of the followed permeability treatment and balance marking operation. Keep samples wet during the entire experiment in a wet box.
5. Prepare a 20 μg/ml Proteinase K solution from the Proteinase K stock (2 mg/ml) by diluting 1:100 in PBS. Each sample requires 100 μl of Proteinase K solution.
6. Add 100 μl of the 20 μg/ml Proteinase K to each slide to cover the tissue section. Incubate slides for 20 minutes at room temperature.

Note: Proteinase K assists the staining reagents to permeate tissues in subsequent steps. With prolonged Proteinase K incubations, the risk of releasing the tissue sections from the slides increases in subsequent wash steps. However, short incubation time may cause inadequate permeability treatment which can affect marking efficiency. For best results, optimization of the incubation time of Proteinase K may be needed.

7. Immerse and wash the samples with PBS for 2-3 times. Remove the excess liquid around samples on the slide with filter papers. Keep samples wet in a wet box.

B.Frozen tissue sections

The protocol is similar to that of paraffin-embedded tissue sections except the replacing of deparaffinization step with a brief warm-up step and a 10 minutes' reduction in Proteinase K treatment step. Frozen tissues should be fixed before the experiment. To avoid samples shedding in the cleaning step, we recommend to immerse slides 2-3 times in PBS rather than clean with spritz bottle.

Note: Keep samples wet during the entire experiment.

1. Immersing slides in 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes at room temperature.
2. Remove the excess liquid around samples on the slide with filter papers.
3. Immerse the slides in PBS for 15 minutes at room temperature. Repeat this step to do PBS washing twice in total.
4. Remove the excess liquid around samples on the slide with filter papers. The contours of sample distribution can be plotted around the samples with a crayon or hydrophobic pen for convenience of the followed permeability treatment and balance marking operation. Keep samples wet during the entire experiment in a wet box.
5. Prepare a 20 μg/ml Proteinase K solution from the Proteinase K stock (2 mg/ml) by diluting 1:100 in PBS. Each sample requires 100 μl of the Proteinase K solution.
6. Add 100 μl of the 20 μg/ml Proteinase K solution to each slide to cover the tissue section. Incubate slides for 10 minutes at room temperature.

Note: Proteinase K assists the staining reagents to permeate tissues in subsequent steps. With prolonged Proteinase K incubations, the risk of releasing the tissue sections from the slides increases in subsequent wash steps. However, short incubation time may cause inadequate permeability treatment which can affect marking efficiency. For best results, optimization of the incubation time of Proteinase K may be needed.

7. Wash samples 2-3 times in an open beaker containing PBS solution.
8. Remove the excess liquid around samples on the slide with filter papers. Keep samples wet in a wet box.

C.Cell samples

Preparation of cytospins:

Culture adherent cells in Lab-Tek® Chamber Slides. After inducing apoptosis, wash the slides twice with PBS.

Preparation of cell smears:

Preparation of poly-lysine coated slides: Absorb 50-100 μl 0.01% (W/V) poly-lysine aqueous solution (Sigma Cat. # P9155 or Sigma Cat. # P8920, diluting 1:10 in water) and drop onto the surface of each pre-cleaned glass slide. Disperse the poly-lysine solution into a thin layer in the area to be used for immobilizing cells. After the slide turned to dry, wash rapidly with deionized water and dry it in the air for 30-60 minutes. The coated slides can be stored at room temperature for several months.

Resuspend cells in PBS to about 2 × 107 cells/ml. Absorb 50-100 μl of cell suspension and drop onto a poly-glycine coated slide. Smear the cell suspension gently over the slide with another clean slide.

1. Fix samples by immersing slides in freshly prepared 4% paraformaldehyde solution in PBS in a Coplin jar for 25 minutes at 4 ℃.
2. Wash the samples by immersing in fresh PBS for 5 minutes. Repeat this PBS wash step.
3. Remove the excess liquid around samples on the slide with filters. The contours of sample distribution can be plotted around the samples with a crayon or hydrophobic pen for convenience of the followed permeability treatment and balance marking operation. Keep samples wet during the entire experiment in a wet box.
4. Prepare a 20 μg/ml Proteinase K solution from the Proteinase K (2 mg/ml) by diluting 1:100 in PBS. Each sample requires 100 μl of Proteinase K solution.

5. Add 100 µl of the 20 µg/ml Proteinase K to each slide to cover the tissue section. Incubate slides for 5 minutes at room temperature. (It can also be immersed in 0.2% Triton® X-100 solution in PBS and incubated at room temperature for 5 minutes for permeabilization.)

Note: Proteinase K assists the staining reagents to permeate tissues in subsequent steps. With prolonged Proteinase K incubations, the risk of releasing the tissue sections from the slides increases in subsequent wash steps. However, short incubation time may cause inadequate permeability treatment which can affect marking efficiency. For best results, optimization of the incubation time of Proteinase K may be needed.

6. Wash samples 2-3 times in an open beaker containing PBS solution.

7. Remove the excess liquid around samples on the slide with filter papers. Keep samples wet in a wet box.

4.2.1 Optional protocol of DNase treatment for positive controls

A positive control of DNA cleavage can be treated as described below. After samples permeabilization, cells were treated with DNase I to prepare a positive control slide.

Note: DNase I treatment of fixed cells can cause cleavage of chromosomal DNA, resulting in many markable DNA 3' ends. The protocol described below usually causes green fluorescence in most of the treated cells.

1. Dilute 10 × DNase I Buffer with deionized water by 1:10. Each sample requires 200 µl of 1 × DNase I Buffer, which can be prepared by mixing 20 µl of 10 × DNase I Buffer with 180 µl of deionized water. Add 100 µl to the permeablized sample and incubate at room temperature for 5 minutes. Add 1 µl of DNase I (1 U/µl) to the remaining 100 µl of 1 × DNase I Buffer to a final concentration of 10 U/ml.

2. Gently knock off the liquid, add 100 µl of the buffer containing 10 U/ml DNase I and incubate for 10 minutes at room temperature.

3. Gently knock off the liquid, wash the slide in a Coplin jar filled with deionized water for 3-4 times.

Note: Positive control slides must use a separate Coplin jar. Otherwise the residual DNase I on positive control slides may introduce a high background on experimental slides.

4.3 Labeling and detecting

1.Dilute 5 × Equilibration Buffer with deionized water by 1:5. Each sample requires 100 µl of 1 × Equilibration Buffer, which can be prepared by mixing 20 µl of 5 × Equilibration Buffer with 80 µl of deionized water.

2. Add 100 µl of 1 × Equilibration Buffer to cover all areas of the sample to be tested and incubate at room temperature for 10-30 minutes. Optionally, put the slides in a Coplin jar containing 1 × Equilibration Buffer, and make sure the slides is totally immersed by the liquid in the jar. Thaw BrightGreen Labeling Mix on ice while cells being equilibrated. Prepare TdT incubation buffer for all experiments and optional positive controls according to Table 1. For a standard reaction with an area of less than 5 cm2, the required volume is 50 µl. Determine the total volume of TdT incubation buffer needed by multiplying the number of all experiments and optional positive controls with 50 µl. For samples with larger surface areas, the reagent volume can be increased proportionally.

Components	Volume (µl/ 50 µl)	Number of samples (experiments + optional positive controls)	Total (volume (µl)
ddH_2O	34		
5 × Equilibration Buffer	10		
BrightGreen Labeling Mix	5		
Recombinant TdT Enzyme	1		

Table 1. TdT incubation buffer for experiments and optional positive controls
For negative control: Prepare a control incubation buffer containing no TdT Enzyme and replace TdT Enzyme with ddH2O.

3.Absorb most of the 100 µl of Equilibration Buffer with filter papers around the equilibrated area. Add 50 µl of TdT incubation buffer to cells in the 5 cm2 area. Keep cells wet.

4. Cut the parafilm into the same size as the tissue or cytospins. Cover it on samples to ensure the average distribution of the reagents gently. Place a towel with water soaked in the bottom of the wet box. Place the slides in the wet box and incubated at 37 ℃ for 60 minutes. Wrap the wet box with aluminum foil to avoid light.

Note: The edges of parafilm can be folded for removal and operation. Slides should be protected from light after step 3.

5. Remove the parafilm and incubate the slides in PBS solution for 5 minutes at room temperature.

6. Gently remove excess liquid and incubate with fresh PBS solution for 5 minutes at room temperature. Repeat this step once.

7. Wipe the PBS solution around or on back of the samples with filter papers.

Note: The slides can be washed three times with PBS containing 0.1% Triton® X-100 and 5 mg/ml BSA for 5 minutes each time after washed by PBS for a cleaner background. Free unreacted marker can be cleared in this way.

8. Stain the samples in a Coplin jar. Immerse the slides into the Coplin jar containing PI solution in darkness for 5 minutes. The PI solution is freshly prepared with PBS and diluted to 1 µg/ml. (Optional protocol: Immerse the slides into the Coplin jar containing DAPI solution in darkness for 5 minutes. The DAPI solution is freshly prepared with PBS and diluted to 2 µg/ml.)

9. Wash the samples in deionized water for 5 minutes at room temperature. Repeat twice for three times in total.

10. Wipe the excess water on slides and add 100 µl of PBS to the sample areas to keep samples wet.

11. Immediately analyze samples by a fluorescence microscope with standard light filters. The green fluorescence should be observed at 520±20 nm; The red fluorescence of PI at >620nm, the blue fluorescence of DAPI at 460 nm. If necessary, the slides can be stored overnight at 4 ℃ in dark conditions.

Note: All cells can be stained into red/blue by PI/DAPI. Only apoptotic nuclei produce green fluorescence caused by incorporation of BrightGreen.

4.4 Protocol for suspended cells detection by flow cytometry

1. Wash 3-5 × 106 cells twice by PBS and centrifuge at 300 g for 10 minutes each time at 4℃. Resuspend cells with 0.5 ml of PBS.

2. Fix samples by adding 5 ml of 1% paraformaldehyde solution in PBS on ice for 20 minutes.

3. Centrifuge cells at 300 g for 10 minutes at 4℃. Remove the supernatant and resuspend cells in 5 ml of PBS. Repeat the washing and resuspend the cells in 0.5 ml of PBS.

4. Permeate cells with 5 ml of pre-cooled 70% ethanol on ice and incubate at -20 ℃ for 4 hours. Cells can be stored in 70% ethanol at -20 ℃ for one week. 0.2% Triton® X-100 in PBS can also be used to permeate cells with an incubation time of 5 minutes at room temperature.

5. Centrifuge cells at 300 g for 10 minutes. Remove the supernatant and resuspend cells in 5 ml of PBS. Repeat the washing and resuspend the cells in 1 ml of PBS.

6. Transfer 2 × 106 cells to a 1.5 ml microcentrifuge tube.

7. Centrifuge cells at 300 g for 10 minutes. Remove the supernatant and resuspend cells in 80 µl of 1 × Equilibration Buffer (Dilute 5 × Equilibration Buffer with deionized water by 1:5). Incubate at room temperature for 5 minutes

8. Thaw BrightGreen Labeling Mix on ice while cells being equilibrated. Prepare TdT incubation buffer for all experiments and optional positive controls based on Table 1. For a standard reaction with 2 × 106 cells, the required volume is 50 µl. Determine the total volume of TdT incubation buffer needed by multiplying reaction numbers by 50 µl.

9. Centrifuge cells at 300 g for 10 minutes. Remove the supernatant and resuspend cells in 50 µl of TdT incubation buffer. Incubate at 37 ℃ for 60 minutes in dark condition. Gently resuspend cells with a micropipette every 15 minutes.
10. Add 1 ml of 20 mM EDTA to terminate the reaction. Gently mix by a micropipette.
11. Centrifuge cells at 300 g for 10 minutes. Remove the supernatant and resuspend cells in 1 ml of 0.1% Triton® X-100 in PBS containing 5 mg/ml BSA. Repeat this step to wash twice in total.
12. Centrifuge cells at 300 g for 10 minutes. Remove the supernatant and resuspend cells in 0.5 ml of 5 µg/ml PI in PBS, which contains 250 µg of DNase-free RNase A.
13. Incubate cells for 30 minutes at room temperature in a dark condition.
14. Analyze cells with a flow cytometry detecting FITC-12-dUTP green fluorescence at 520±20 nm and PI red fluorescence at >620nm.

Note: All cells can be stained into red/blue by PI/DAPI. Only apoptotic nuclei produce green fluorescence caused by incorporation of BrightGreen.

5/Trouble Shooting

Q. High background (E.g. Strong green fluorescence background of non-apoptotic cells)

A. That is due to nonspecific incorporation of BrightGreen. To solve this problem, please keep the cells or slides wet during the whole experiment. Extra washing steps after the labeling reaction can be performed: slides may be washed three times for 5 minutes with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA.

Q.Weak fluorescence signal

A.Insufficient permeabilization with Triton X-100 or Proteinase K. Please optimize the permeabilization step by adjusting incubation time with permeabilization reagent.

Q.Loss of tissue section from the slides

A.Insufficient coating of the slides prior to attachment of tissue section. Coating microscopic slides with 3-aminopropyl triethoxysilane (Sigma Cat.# A3648) is better than poly-L-lysine before spreading the tissue sections.

Q.Few cells remaining for the final microscopy or flow cytometry analysis

A.Large amount of cells were lost during the procedure: Start with a higher number of cells. When prepare a cell suspension for attachment to microscope slides, please wash cells with PBS containing 1% BSA during centrifugation. When preparing cells in suspension, please wash cells with PBS containing 1% BSA during centrifugation.

6/Buffers and Solutions

1 × PBS (pH 7.4)
137 mM NaCl
2.68 mM KCl
1.47 mM KH2PO4
8.1 mM Na2HPO4

PI (1 mg/ml):

10 mg PI was dissolved in 10 ml PBS. Store at 0-4 ℃ and protect from light. Proper dilution is needed for using.

DAPI (1 mg/ml):

10 mg DAPI was dissolved in 10 ml PBS. Store at 0-4 ℃ and protect from light. Proper dilution is needed for using.

DNase I buffer:

40 mM Tris-HCl (pH 7.9)
10 mM NaCl
6 mM MgCl2
10 mM CaCl2

1% Formaldehyde solution

Mix 90 ml of PBS with 6.25 ml of 16% methanol-free formaldehyde. Adjust the pH to 7.4 with serval drops of 1N NaOH. Add PBS up to 100 ml. This solution should be freshly prepared before use every time.

4% Formaldehyde solution

Mix 70 ml of PBS with 25 ml of 16% methanol-free formaldehyde. Adjust the pH to 7.4 with serval drops of 1N NaOH. Add PBS up to 100 ml. This solution should be freshly prepared before use every time.

4% Paraformaldehyde solution

Weigh 4 g of paraformaldehyde in the fume hood, add PBS to 100 ml. Pack in a sealed container, then heat in water bath at 65 ℃ for 2 hours. It can be stored at 4 ℃ for at least 2 weeks.

10% Triton® X-100 solution

Mix 85 ml of deionized water and 10 ml of Triton® X-100 solution in a beaker on a magnetic stirrer. Add water up to 100 ml.