TUNEL BrightGreen Apoptosis Detection Kit

A112

Vazyme

Instruction for Use

Version 22.1

Vazyme

Contents

01/Product Description

During apoptosis, intracellular specific endonucleases are activated, chromatin DNA is specifically cleaved between nucleosomes, and DNA is degraded into integer multiples of about 180 - 200 bp fragments. The 3'-hydroxyl (3'-OH) end generated by DNA fragmentation can bind to fluorescein-12-deoxyuridine triphosphate (FITC-12-dUTP) under the action of terminal deoxynucleotidyl transferase (TdT). FITC-12-dUTP-labeled DNA can be directly observed by fluorescence microscopy or quantitatively analyzed by flow cytometry to reflect the level of apoptosis.

The BrightGreen Labeling Mix contains FITC-12-dUTP and Bright factors. This unique small molecule compound can non-covalently bind to FITC, enhance its stability and amplify its signal, resulting in brighter labeled fluorescence and stronger anti-quenching ability.

02/Components

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

03/Storage

Store at -30 ~ -15℃ and transport at ≤0℃.
BrightGreen Labeling Mix: Store at -30 ~ -15℃ and protect from light.

04/Applications

It is applicable for apoptosis detection of paraffin sections, frozen sections, adherent and suspension cultured cells.

05/Materials

Reagent: 4% paraformaldehyde, DAPI/PI, Triton X-100, PBS, absolute ethanol.
Other materials: coverslips/slides, wet box.

06/Notes

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

07/Mechanism & Workflow

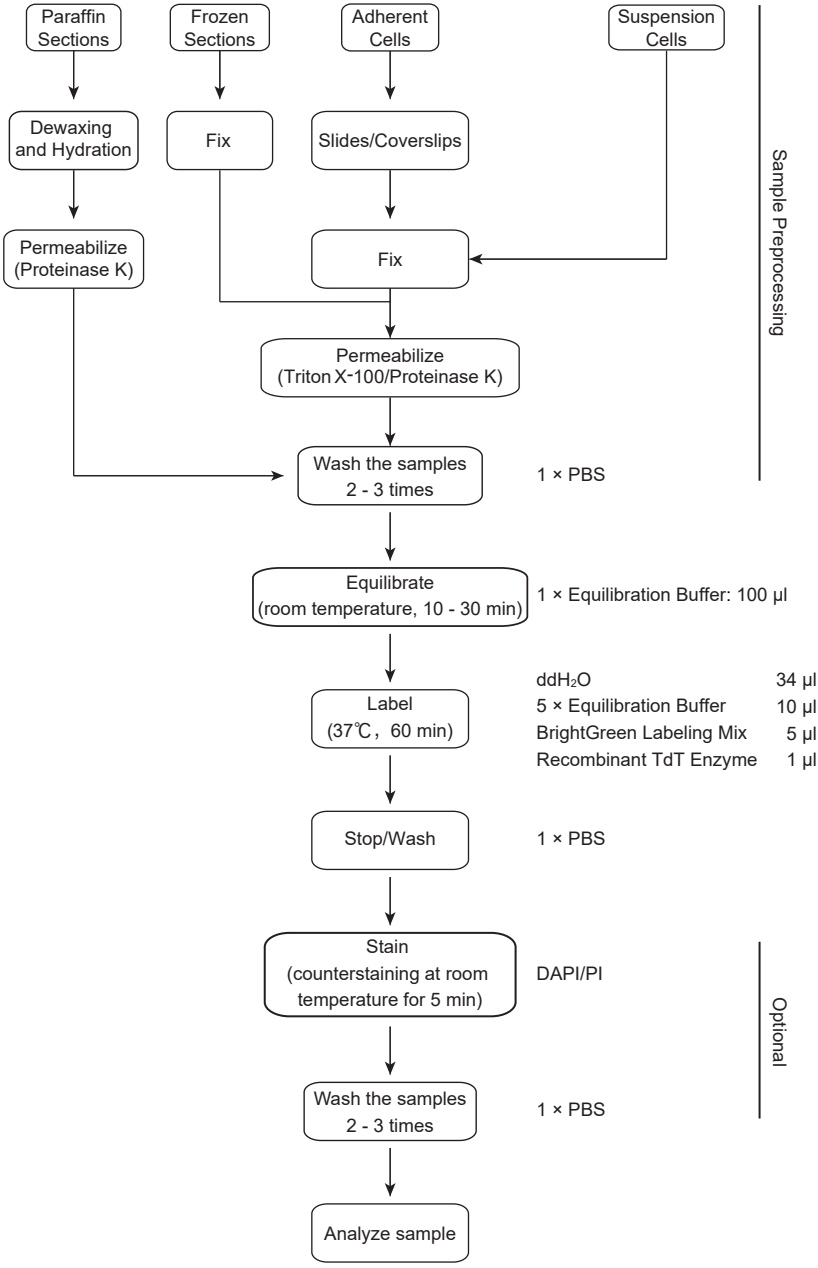

Fig 1. Workflow of the experimental procedure

08/Tissue Section/Adherent Cell Experiment Process

Please read this instruction carefully before the experiment. This protocol is suitable for the apoptosis detection of paraffin-embedded tissue sections, frozen tissue sections, and adherent cells. Each sample should be carried out in strict accordance with its operating procedures. For suspension cell detection, please refer to 09/Suspension Cell Experiment Process.

08-1/Sample Preprocessing

◇**Paraffin-embedded tissue sections**

1. Immerse the paraffin tissue sections in xylene at room temperature to completely remove the paraffin (two times for 5 min each).
   ▲Xylene is a toxic and volatile substance, please carry out in a fume hood.
2. Immerse and rinse the sections with absolute ethanol at room temperature (two times for 5 min each).
3. Immerse and rinse with gradient ethanol (90%, 80%, 70%) at room temperature (one time for 3 min each).
4. Rinse the sections with 1 × PBS and carefully blot excess fluid around the specimen on the slide with absorbent paper.
   ▲ The outline of the sample distribution can be drawn around the sample with a paraffin crayon or a hydrophobic pen, which is convenient for downstream permeabilization, equilibration and labeling. During the experiment, do not allow the sample to dry. Keep the treated samples moist in a wet box.
5. Dilute the Proteinase K solution (2 mg/ml) 1:100 in 1 × PBS to a final concentration of 20 μg/ml. Drop 100 μl of diluted Proteinase K solution on each sample to make the solution cover the entire sample area, and incubate at room temperature for 20 min.
   ▲ Proteinase K permeabilization time that is too long will increase the risk of tissue sections falling off the slide during subsequent washing steps, while too short a time may result in insufficient permeabilization and affect labeling efficiency. For best results, Proteinase K incubation time may need to be optimized.
6. Immerse and rinse the samples with 1 × PBS (2 - 3 times for 3 - 5 min each time). Gently remove excess fluid and carefully blot the fluid around the sample on the slide with absorbent paper. Keep the treated samples moist in a wet box.

08-2/Positive Treatment (only the positive control is performed this step, and other samples are directly subjected to 08-3/Labeling and Detection)

After permeabilization, samples can be treated with DNase I to prepare positive controls.

▲DNase I treats fixed cells will cause chromosomal DNA breaks, resulting in many labelable DNA 3' ends. The following processes generally results in green fluorescence in most cells treated.

1. Dilute 10 × DNase I Buffer with ddH_2O at a ratio of 1:10 to 1 × DNase I Buffer for use.
   ▲ Prepare 200 μl of 1 × DNase I Buffer for each sample.
2. Add 100 μl 1 × DNase I Buffer dropwise to the permeabilized sample and equilibrate at room temperature for 5 min.

3. Dilute DNase I (2 U/μl) with 1 × DNase I Buffer to a final concentration of 20 U/ml.
4. Gently aspirate off excess liquid, add 100 μl of 20 U/ml DNase I solution, and incubate at room temperature for 10 min.
5. Gently aspirate off the excess liquid, then thoroughly immerse and rinse the coverslips/slides in a staining jar with 1 × PBS for 2 - 3 times.
   ▲Positive control slides must use a separate staining jar. Residual DNase I on positive control slides may cause false positive signals in experimental groups.

08-3/Labeling and Detection

1. Dilute 5 × Equilibration Buffer to 1 × Equilibration Buffer with ddH_2O at a ratio of 1:5.
2. Add 100 μl 1 × Equilibration Buffer dropwise to make it completely cover the sample area to be tested, and equilibrate at room temperature for 10 - 30 min.
3. Prepare the TdT reaction mixture according to the table below in the dark during equilibration.

Components	Negative Control	Positive Control/Sample
ddH_2O	35 μl	34 μl
5 × Equilibration Buffer	10 μl	10 μl
BrightGreen Labeling Mix	5 μl	5 μl
Recombinant TdT Enzyme	0 μl	1 μl

▲ For small paraffin sections, the required volume is 50 μl, multiply 50 μl by the number of experimental and positive control reactions to determine the total volume of TdT reaction mixture required. For samples with larger surface areas, the reagent volume can be proportionally increased.

4. After equilibration, remove 1 × Equilibration Buffer with absorbent paper, and then drop 50 μl of TdT reaction mixture on the sample.
   ▲Do not let the sample dry out. Do not touch cells or tissues when using absorbent paper. Slides/Coverslips need to be protected from light.
5. Cut the parafilm to the same size as the tissue or slide, and lightly cover the sample to ensure even distribution of reagents. Place a paper towel dampened with water on the bottom of the wet box. Place the slides in a wet box and incubated at 37℃ for 60 min.
   ▲Fold up the edges of the parafilm for easy removal and handling. Wrap the wet box in tin foil to protect it from light.
6. Gently remove excess liquid, wash twice with fresh 1 × PBS solution, and incubate at room temperature for 5 min each time.
   ▲To reduce the background, after washing the sample with 1 × PBS, it can be washed again with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA (three times for 5 min each), which can remove the free unreacted label.
7. Gently wipe off the 1 × PBS solution around and behind the sample with absorbent paper.
8. Counterstain the sample in the dark with a freshly prepared 1 μg/ml PI solution in 1 × PBS or a 2 μg/ml DAPI solution freshly prepared in 1 × PBS. Place at room temperature for 5 min during staining.

9. Wash the samples, immerse the slides in 1 × PBS solution at room temperature (three times for 5 min each).
10. Gently aspirate off excess liquid and add 100 µl of 20% glycerol in 1 × PBS to the sample area to keep the sample moist.
11. Analyze samples immediately under a fluorescence microscope using a standard fluorescence filter to observe green fluorescence at 520 ± 20 nm; red fluorescence at >620 nm for PI, or blue fluorescence at 460 nm for DAPI.
    ▲If necessary, the samples can be mounted and stored overnight at 4℃ in the dark. PI/DAPI stains both apoptotic and non-apoptotic cells red/blue. The green fluorescence resulting from the incorporation of BrightGreen will be found only in apoptotic nuclei.

08-4/Sample Preprocessing

◇**Frozen tissue sections**

1. Place the frozen sections on a rack and dry at room temperature for 20 min.
2. Immerse the slides in 4% paraformaldehyde solution (dissolved in 1 × PBS) and fix for 30 min at room temperature.
3. Gently remove excess fluid and carefully blot the fluid around the sample on the slide with absorbent paper.
4. Wash by immersing the slides in PBS (two times for 5 min each).
5. Gently remove excess fluid and carefully blot the fluid around the sample on the slide with absorbent paper.
    ▲A paraffin or hydrophobic pen can be used to outline sample distribution around the sample for downstream permeabilization, equilibration and labeling. During the experiment, do not let the sample dry. Keep the treated samples moist in a wet box.
6. Prepare a solution of Triton X-100 at a concentration of 0.2% in 1 × PBS. Add 100 µl of Triton X-100 solution dropwise to each sample so that the solution covers the entire sample area and incubate for 15 min at room temperature. If the permeabilization effect is not satisfactory, dilute the Proteinase K solution at a concentration of 2 mg/ml in 1 × PBS at a ratio of 1:100 to a final concentration of 20 µg/ml. Drop 100 µl of diluted Proteinase K solution on each sample to make the solution cover the entire sample area, and incubate at room temperature for 10 min.
    ▲Permeabilization times that are too long will increase the risk of tissue sections falling off the slides during subsequent washing steps, while permeabilization times that are too short may result in insufficient permeabilization and affect labeling efficiency. For best results, the incubation time may need to be optimized.
7. Wash the samples with 1 × PBS (2 - 3 times for 3 - 5 min each). Gently remove excess fluid and carefully blot the fluid around the sample on the slide with absorbent paper. Keep the treated samples moist in a wet box.

08-5/Positive Treatment (only the positive control is performed this step, and other samples are directly subjected to 08-6/Labeling and Detection)

After permeabilization, samples can be treated with DNase I to prepare positive controls.

▲ DNase I treats fixed cells will cause chromosomal DNA breaks, resulting in many labelable DNA 3' ends. The following processes generally results in green fluorescence in most cells treated.

1. Dilute 10 × DNase I Buffer with ddH_2O at a ratio of 1:10 to 1 × DNase I Buffer for use.
   ▲ Prepare 200 µl of 1 × DNase I Buffer for each sample.
2. Add 100 µl 1 × DNase I Buffer dropwise to the permeabilized sample and equilibrate at room temperature for 5 min.
3. Dilute DNase I (2 U/µl) with 1 × DNase I Buffer to a final concentration of 20 U/ml.
4. Gently aspirate off excess liquid, add 100 µl of 20 U/ml DNase I solution, and incubate at room temperature for 10 min.
5. Gently aspirate off the excess liquid, then thoroughly immerse and rinse the coverslips/slides in a staining jar with 1 × PBS for 2 - 3 times.
   ▲ Positive control slides must use a separate staining jar. Residual DNase I on positive control slides may cause false positive signals in experimental groups.

08-6/Labeling and Detection

1. Dilute 5 × Equilibration Buffer to 1 × Equilibration Buffer with ddH_2O at a ratio of 1:5.
2. Add 100 µl 1 × Equilibration Buffer dropwise to make it completely cover the sample area to be tested, and equilibrate at room temperature for 10 - 30 min.
3. Prepare the TdT reaction mixture according to the table below in the dark during equilibration.

Components	Negative Control	Positive Control/Sample
ddH_2O	35 µl	34 µl
5 × Equilibration Buffer	10 µl	10 µl
BrightGreen Labeling Mix	5 µl	5 µl
Recombinant TdT Enzyme	0 µl	1 µl

▲ For small frozen sections, the required volume is 50 µl, multiply 50 µl by the number of experimental and positive control reactions to determine the total volume of TdT reaction mixture. For larger surface area samples, proportionally increase the reagent volume.

4. After equilibration, remove 1 × Equilibration Buffer with absorbent paper, and then drop 50 µl of TdT reaction mixture on the sample.
   ▲ Do not let the sample dry out. Do not touch cells or tissues when using absorbent paper. Slides/Coverslips need to be protected from light.
5. Cut the parafilm to the same size as the tissue or slide, and lightly cover the sample to ensure even distribution of reagents. Place a paper towel dampened with water on the bottom of the wet box. Place the slides in a wet box and incubated at 37℃ for 60 min.
   ▲Fold up the edges of the parafilm for easy removal and handling. Wrap the wet box in tin foil to protect it from light.

6. Gently remove excess liquid, wash twice with fresh 1 × PBS solution, and incubate at room temperature for 5 min each time.
   ▲To reduce the background, after washing the sample with 1 × PBS, it can be washed again with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA (three times for 5 min each), which can remove the free unreacted label.
7. Gently wipe off the 1 × PBS solution around and behind the sample with absorbent paper.
8. Counterstain the sample in the dark with a freshly prepared 1 μg/ml PI solution in 1 × PBS or a 2 μg/ml DAPI solution freshly prepared in 1 × PBS. Place at room temperature for 5 min during staining.
9. Wash the samples, immerse the slides in 1 × PBS solution at room temperature (three times for 5 min each).
10. Gently aspirate off excess liquid and add 100 μl of 20% glycerol in 1 × PBS to the sample area to keep the sample moist.
11. Analyze samples immediately under a fluorescence microscope using a standard fluorescence filter to observe green fluorescence at 520 ± 20 nm; red fluorescence at >620 nm for PI, or blue fluorescence at 460 nm for DAPI.
    ▲If necessary, the samples can be mounted and stored overnight at 4℃ in the dark. PI/DAPI stains both apoptotic and non-apoptotic cells red/blue. The green fluorescence resulting from the incorporation of BrightGreen will be found only in apoptotic nuclei.

08-7/Sample Preprocessing

◇**Adherent cells**

Preparation of cell coverslips:

Culture Adherent cells on Lab-Tek chamber slides or TC-treated cell coverslips. After the apoptosis-inducing treatment, rinse the slide twice with PBS for subsequent experiments.

Preparation of cell smears:

Resuspend cells in 1 × PBS at a concentration of 2 × 10^6 cells/ml. Pipette 50 - 100 μl of the cell suspension onto a poly-L-lysine coated slide. Use a clean slide to gently spread the cell suspension for subsequent experiments.

1. Immerse the coverslips/smears in 4% paraformaldehyde (freshly prepared in 1 × PBS) and place at 4℃ for 25 min for cell fixation.
2. Wash by immersing the coverslips/smears in 1 × PBS at room temperature (two times for 5 min each).

3. Gently remove excess fluid and carefully blot the fluid around the specimen on the coverslips/smears with absorbent paper.
   ▲The outline of the sample distribution can be drawn around the sample with a paraffin crayon or a hydrophobic pen, which is convenient for downstream permeabilization, equilibration and labeling.
   ▲During the experiment, do not let the sample dry. Keep the treated samples moist in a wet box.

4. Dilute 2 mg/ml Proteinase K solution 1:100 in 1 × PBS to a final concentration of 20 μg/ml. Drop 100 μl of diluted Proteinase K solution on each sample to cover the entire sample area and incubate for 5 min at room temperature; it can also be immersed in 0.2% Triton X-100 solution prepared with 1 × PBS at room temperature. Incubate for 5 min for permeabilization.
   ▲The cell coverslips/smears are easy to fall off during processing. It is recommended to use 0.2% - 0.5% Triton X-100 solution for permeabilization to avoid falling off.
   ▲Proteinase K permeabilization time that is too long will increase the risk of tissue sections falling off the slide in subsequent washing steps, while too short a time may result in insufficient permeabilization and affect labeling efficiency. For best results, the Proteinase K incubation time may need to be optimized.

5. Rinse the sample 2 - 3 times with 1 × PBS. Gently remove excess fluid and carefully blot the fluid around the sample on the slide with absorbent paper. Keep the treated samples moist in a wet box.

08-8/Positive Treatment (only the positive control is performed this step, and other samples are directly subjected to 08-9/Labeling and Detection)

After permeabilization, samples can be treated with DNase I to prepare positive controls.

▲ DNase I treatment of fixed cells will cause chromosomal DNA breaks, resulting in many labelable DNA 3' ends. The following procedures generally results in green fluorescence in most cells treated.

1. Dilute 10 × DNase I Buffer with ddH_2O at a ratio of 1:10 to 1 × DNase I Buffer for use.
   ▲ Prepare 200 μl of 1 × DNase I Buffer for each sample.

2. Add 100 μl 1 × DNase I Buffer dropwise to the permeabilized sample and equilibrate at room temperature for 5 min.

3. Dilute DNase I (2 U/μl) with 1 × DNase I Buffer to a final concentration of 20 U/ml.

4. Gently aspirate off excess liquid, add 100 μl of 20 U/ml DNase I solution, and incubate at room temperature for 10 min.

5. Gently aspirate off the excess liquid, then thoroughly immerse and rinse the coverslips/slides in a staining jar with 1 × PBS for 2 - 3 times.
   ▲ Positive control slides must use a separate staining jar. Residual DNase I on positive control slides may cause false positive signals in experimental groups.

08-9/Labeling and Detection

1. Dilute 5 × Equilibration Buffer to 1 × Equilibration Buffer with ddH_2O at a ratio of 1:5.

2. Add 100 µl 1 × Equilibration Buffer dropwise to make it completely cover the sample area to be tested, and equilibrate at room temperature for 10 - 30 min.

3. Prepare the TdT reaction mixture according to the table below in the dark during equilibration.

Components	Negative Control	Positive Control/Sample
ddH_2O	35 µl	34 µl
5 × Equilibration Buffer	10 µl	10 µl
BrightGreen Labeling Mix	5 µl	5 µl
Recombinant TdT Enzyme	0 µl	1 µl

▲For small cell coverslips/slides, the required volume is 50 µl, multiply 50 µl by the number of experimental and positive control reactions to determine the total volume of TdT reaction mixture required. For samples with larger surface areas, the reagent volume can be proportionally increased.

4. After equilibration, remove 1 × Equilibration Buffer with absorbent paper, and then drop 50 µl of TdT reaction mixture on the sample.
   ▲Do not let the sample dry out. Do not touch cells or tissues when using absorbent paper. Slides/ Coverslips need to be protected from light.

5. Cut the parafilm to the same size as the tissue or slide, and lightly cover the sample to ensure even distribution of reagents. Place a paper towel dampened with water on the bottom of the wet box. Place the slides in a wet box and incubated at 37℃ for 60 min.
   ▲Fold up the edges of the parafilm for easy removal and handling. Wrap the wet box in tin foil to protect it from light.

6. Gently remove excess liquid, wash twice with fresh 1 × PBS solution, and incubate at room temperature for 5 min each time.
   ▲To reduce the background, after washing the sample with 1 × PBS, it can be washed again with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA (three times for 5 min each), which can remove the free unreacted label.

7. Gently wipe off the 1 × PBS solution around and behind the sample with absorbent paper.

8. Counterstain the sample in the dark with a freshly prepared 1 µg/ml PI solution in 1 × PBS or a 2 µg/ml DAPI solution freshly prepared in 1 × PBS. Place at room temperature for 5 min during staining.

9. Wash the samples, immerse the slides in 1 × PBS solution at room temperature (three times for 5 min each).

10. Gently aspirate off excess liquid and add 100 µl of 20% glycerol in 1 × PBS to the sample area to keep the sample moist.

11. Analyze samples immediately under a fluorescence microscope using a standard fluorescence filter to observe green fluorescence at 520 ± 20 nm; red fluorescence at >620 nm for PI, or blue fluorescence at 460 nm for DAPI.
    ▲If necessary, the samples can be mounted and stored overnight at 4℃ in the dark. PI/DAPI stains both apoptotic and non-apoptotic cells red/blue. The green fluorescence resulting from the incorporation of BrightGreen will be found only in apoptotic nuclei.

09/Suspension Cell Experiment Process

1. Wash 3 × 10^6 to 5 × 10^6 cells with PBS twice, centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min each time. Then resuspend with 0.5 ml 1 × PBS.
2. Add 5 ml paraformaldehyde solution (concentration of 1%) prepared in 1 × PBS, and incubate at 4℃ for 20 min for cell fixation.
3. Centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min, discard the supernatant, and resuspend the cells with 5 ml of 1 × PBS.
4. Repeat centrifugation and resuspend cells with 0.5 ml 1 × PBS.
5. Add 5 ml Triton X-100 solution (concentration of 0.2%) prepared in 1 × PBS, and permeabilize for 5 min at room temperature, or add 5 ml ice-cold 70% ethanol and incubate at -20℃ for 4 h.
   ▲ Cells can be stored in 70% ethanol at -20℃ for one week.
   ▲ The refractive index of the permeabilized cells will decrease, and the turbidity of the cells is difficult to observe. Care should be taken during the operation to avoid cell loss.
6. Centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min, discard the supernatant, and resuspend the cells with 5 ml of 1 × PBS. Repeat centrifugation and resuspend cells with 1 ml 1 × PBS.
7. Transfer 2 × 10^6 cells to a new 1.5 ml centrifuge tube.
8. Centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min, discard the supernatant, and resuspend the cells with 1 × Equilibration Buffer (dilute 5 × Equilibration Buffer with ddH_2O at a ratio of 1:5). Incubate for 5 min at room temperature.
9. During the equilibration of the cells, prepare the TdT reaction mixture. Melt BrightGreen Labeling Mix on ice and prepare TdT reaction mixture according to the table below.
   ▲ For a standard reaction of 2 × 10^6 cells, the required volume is 50 µl. Multiply 50 µl by the number of reactions to determine the total volume of TdT reaction mixture required.

Components	Negative Control	Positive Control/Sample
ddH_2O	35 µl	34 µl
5 × Equilibration Buffer	10 µl	10 µl
BrightGreen Labeling Mix	5 µl	5 µl
Recombinant TdT Enzyme	0 µl	1 µl

10. Centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min, discard the supernatant, and resuspend the cells with 50 µl of TdT reaction mixture. Incubate at 37℃ for 60 min and protect from light. Flick the tube or gently resuspend the cells with a micropipette every 15 min.
11. Add 1 ml of 20 mM EDTA to stop reaction, and mix gently with a pipette.
12. Centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min, discard the supernatant, and resuspend the cells with 1 ml Triton X-100 solution (concentration of 0.1%) prepared in 1 × PBS (contain 5 mg/ml BSA). Repeat once for a total of two washes.

13. Centrifuge at 1,800 rpm (300 × g) for 5 min at 4℃, discard the supernatant, and resuspend the cells with 0.5 ml of 5 μg/ml PI solution prepared in 1 × PBS, which contains 250 μg DNase-free RNase A.
14. Incubate cells for 30 min at room temperature in the dark room.
15. Use flow cytometry to analyze Cells. Or observe the green fluorescence of BrightGreen at a fluorescence of 520 ± 20 nm and the red fluorescence of PI at a fluorescence of >620 nm with a standard fluorescence filter device.
    ▲ PI stains both apoptotic and non-apoptotic cells red. The green fluorescence resulting from the incorporation of BrightGreen will be found only in apoptotic nuclei.

10/TUNEL and Immunofluorescence Co-staining Experiment Processes

10-1/Paraffin Section Co-staining Steps

1. Heat-bake paraffin sections at 65℃ for 30 min, deparaffinize with xylene, and hydrate with gradient alcohol.
2. Antigen retrieval: Place the slices in 0.1 mol/L citric acid repair solution with pH = 6.0, heat in a microwave oven for 6 min on medium heat to a slight boil, then maintain on medium-low heat for 10 min, stop heating and cool down naturally for 20 - 30 minutes.
3. Wash with PBS (three times for 5 min each).
4. Add 0.2% Triton X-100 solution to permeabilize the membranes for 10 min. It should be noted that the integral protein on the cell membrane surface cannot be added with Triton X-100 solution, otherwise it will be washed off.
5. Blocking: Prepare 5% BSA with PBS and block for 1 h at room temperature.
6. Wash with PBS (three times for 5 min each).
7. (Optional) For positive treatment, dilute 10 × DNase I Buffer to 1 ×, add 100 μl 1 × DNase I Buffer to each sample, incubate at room temperature for 5 min; dilute DNase I with 1 × DNase I Buffer, aspirate the DNase I Buffer, add 100 μl DNase I to each sample, incubate for 10 min at room temperature, and wash with PBS (three times for 5 min each).
8. Equilibration: Equilibrate with 100 μl of 1 × Equilibration Buffer for 20 min at room temperature.
9. Labeling: Remove the equilibration solution, drop 50 μl of TdT reaction mixture on each coverslips to completely cover the sample area, incubate at 37℃ for 60 min in a wet box, and wash with PBS (three times for 5 min each).
10. Add the suitable concentration of primary antibody to the wet box and incubate overnight at 4℃.
11. Wash with PBS (three times for 5 min each).

12. Secondary antibody incubation: Incubate the fluorescent secondary antibody for 1 h at room temperature; wash with PBS for three times to remove excess secondary antibody.
13. Counterstain nuclei: Add 100 μl of 2 μg/ml DAPI to counterstain nuclei, incubate at room temperature for 5 min; wash with PBS (three times for 5 min each).Microscopic examination: Photographed by a fluorescence microscope.

10-2/Frozen Section Co-staining Steps

1. Keep the frozen sections at room temperature for more than 30 min, and then perform immunofluorescence.
2. Immerse the slides in 4% paraformaldehyde solution (dissolved in 1 × PBS) and fix for 30 min at room temperature.
3. Wash with 1 × PBS (three times for 5 min each).
4. Add 0.2% Triton X-100 solution to permeabilize the membranes for 10 min. It should be noted that the integral protein on the cell membrane surface cannot be added with Triton X-100 solution, otherwise it will be washed off.
5. Blocking: Prepare 5% BSA with PBS and block for 1 h at room temperature.
6. Wash with PBS (three times for 5 min each).
7. (Optional) For positive treatment, dilute 10 × DNase I Buffer to 1 ×, add 100 μl 1 × DNase I Buffer to each sample, incubate at room temperature for 5 min; dilute DNase I with 1 × DNase I Buffer, aspirate the DNase I Buffer, add 100 μl DNase I to each sample, incubate for 10 min at room temperature, and wash with PBS (three times for 5 min each).
8. Equilibration: Equilibrate with 100 μl of 1 × Equilibration Buffer for 20 min at room temperature.
9. Labeling: Remove the equilibration solution, drop 50 μl of TdT reaction mixture on each coverslips to completely cover the sample area, incubate at 37℃ for 60 min in a wet box, and wash with PBS three times, 5 min each time.
10. Add the suitable concentration of primary antibody to the wet box and incubate overnight at 4℃.
11. Wash with PBS (three times for 5 min each).
12. Secondary antibody incubation: Incubate the fluorescent secondary antibody for 1 h at room temperature; wash with PBS for three times to remove excess secondary antibody.
13. Counterstain nuclei: Add 100 μl of 2 μg/ml DAPI to counterstain nuclei, incubate at room temperature for 5 min; wash with PBS Wash with PBS (three times for 5 min each).

14. Microscopic examination: Photographed by a fluorescence microscope.

10-3/Cell Coverslips Co-staining Steps

1. Prepare the 0.2% Triton X-100 solution in 1 × PBS. Incubate (5 min) at room temperature for permeabilization.
2. Blocking: Prepare 5% BSA with PBS and block for 1 h at room temperature.
3. Equilibration: Equilibrate with 100 µl of 1 × Equilibration Buffer for 20 min at room temperature.
4. Labeling: Remove the equilibration solution, drop 50 µl of TdT reaction mixture on each coverslips to completely cover the sample area, incubate at 37℃ for 60 min in a wet box. Wash with 2 ml PBS (three times for 5 min each) and aspirate the PBS.
5. Primary antibody incubation: add 200 µl antibody (1:1,000 dilution with 5% BSA) to each coverslips and incubate overnight at 4℃ in a wet box; wash the cells with PBS to remove excess primary antibody (three times for 5 min each).
6. Secondary antibody incubation: Incubate the fluorescent secondary antibody for 1 h at room temperature; wash with PBS for three times to remove excess secondary antibody.
7. Counterstain nuclei: Add 100 µl of 2 µg/ml DAPI to counterstain nuclei, incubate at room temperature for 5 min; wash with PBS (three times for 5 min each).
8. Microscopic examination: Photographed by a fluorescence microscope.

11/FAQ & Troubleshooting

◇ High background (e.g. strong green fluorescence background of non-apoptotic cells)

Nonspecific incorporation of FITC-12-dUTP

Processing method: Keep the cells moist during operation. After labeling, wash the slides once with PBS and then wash three times with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA for 5 min each time.

◇ Insufficient number of positive cells

If the permeabilization is insufficient, the permeabilization step can be optimized by adjusting the incubation time of Proteinase K or Triton X-100.

◇ The tissue section falls off the slide

Inadequate coating of tissue sections prior to adhesion. Coating microscope slides with 3-aminopropyl triethoxysilane (TESPA; Sigma Cat.#A3648) prior to mounting is more effective than poly-L-lysine better.

◇ Microscopic or flow cytometric analysis detects very few cells

A large number of cells are lost during operation.

① Increase the initial amount of cells.

② When preparing the cell suspension, wash the cells with PBS containing 1% BSA during centrifugation.

◇ Low labeling rate

① If the labeling efficiency of samples fixed with ethanol or methanol is low (reason: chromatin failed to cross-link with proteins during fixation, therefore lost during operation), use 4% paraformaldehyde or formalin or glutaraldehyde, dissolved in 1 × PBS (pH 7.4) fixation.

② Excessive fixation leads to excessive cross-linking with proteins, which will cause low labeling efficiency. The fixation time can be shortened appropriately; or fixed with 2% paraformaldehyde dissolved in 1 × PBS.

◇ False positive

If the frozen section of some muscle tissue is not fixed with paraformaldehyde during the production process, it is prone to endogenous nuclease cleave DNA, resulting in false positives. After the tissue is taken out, it should be fixed with paraformaldehyde in time, and Proteinase K should not be used for permeabilization.

The processing time and temperature of Proteinase K solution need to explore the most suitable conditions. If the temperature is too high or the time is too long, it will easily damage the nucleic acid structure and cause false positives.

12/Appendix

◇ Preparation of poly-L-lysine coated slide

Pipette 50 - 100 μl of 0.01% (W/V) polylysine aqueous solution (1:10 dilution with ddH_2O) dropwise to the pre-cleaned glass surface. Spread the polylysine solution in a thin layer over the area that will be used to fix the cells. After the slides are dry, rinse quickly with deionized water, then put the coated slides stand in air for 30 - 60 minutes until dry. Coated slides can be stored at room temperature for three months.

◇ Buffer and solution components

1 × PBS (pH 7.4)

137 mM NaCl

2.68 mM KCl

1.47 mM KH_2PO_4

8.1 mM Na_2HPO_4

DAPI solution (1 mg/ml)

Weigh 10 mg of DAPI and dissolve it in 10 ml of PBS; store at 0 ~ 4℃ in the dark; dilute appropriately when using.

DNase I buffer

40 mM Tris-HCl (pH 7.9)

10 mM NaCl

6 mM $MgCl_2$

10 mM $CaCl_2$

4% paraformaldehyde solution

Weigh 4 g of paraformaldehyde in a fume hood and add 1 × PBS to 100 ml. Packed in an airtight container, then dissolved in a water bath at 65℃ for 2 h. Store solutions at 4℃ (stable at least two weeks).

10% Triton X-100 solution

Mix 85 ml of ddH_2O and 10 ml of Triton X-100 solution in a beaker (mix with magnetic stirring). Make up to 100 ml with ddH_2O.

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