Click EdUTP TUNEL Apoptosis Assay Kit (Red Fluorescence)

Cat #: KTA2013 Size: 20 T/50 T

	Click EdUTP TUNEL Apoptosis Assay Kit (Red Fluorescence)		
REF	**Cat #:** KTA2013	LOT	**Lot #:** Refer to product label
	Applicable samples: Flow cytometry and fluorescence detection of cell and tissue samples		
	Storage: Stored at -20℃ for 12 month, protected from light		

Assay Principle

The Click EdUTP TUNEL detection method is a technique that significantly enhances the sensitivity by modifying the nucleotide analog EdUTP based on the basic principle of the conventional TUNEL (deoxyribonucleotide terminal transferase-mediated nick end labeling method). Its fundamental principle is derived from the classic TUNEL detection method. During apoptosis, the chromosomal DNA undergoes double-strand or single-strand breaks, generating a large number of 3'-hydroxyl (3'-OH) ends with a sticky end. Under the catalysis of deoxyribonucleotide terminal transferase (TdT), these 3'-OH ends can be labeled. The traditional TUNEL technique usually uses fluorescently labeled or biotinylated deoxyuridine triphosphate nucleotides (dUTP), and incorporates them into the DNA break sites through a one-step method by TdT enzyme, thereby achieving staining of apoptotic cells. Since normal or proliferating cells lack DNA breaks and 3'-OH formation, they are rarely stained, which makes the TUNEL technique a classic method for detecting cell apoptosis.

The Click EdUTP TUNEL technology has been optimized based on the traditional method. The core lies in the application of a modified nucleotide analog EdUTP. This analog is connected to a small-volume bioorthogonal acetylenic modification group on the dUTP molecule. During the detection process, due to the much smaller molecular size of the acetylenic group compared to the fluorescent substances or other large-molecule-modified groups used in traditional TUNEL, TdT enzyme can more effectively recognize and catalyze the incorporation of EdUTP into the 3'-hydroxyl ends generated after DNA breaks. This efficient incorporation enables more thorough labeling of the break sites, thereby enhancing the potential sensitivity of the detection from the source. The subsequent signal amplification and detection steps rely on the click chemistry reaction principle. Under the catalysis of copper ions, the acetylenic group incorporated into the DNA break site can efficiently and specifically form a stable triazole ring structure with the subsequent added fluorescently labeled azide. This reaction is characterized by its rapid, efficient, and good biocompatibility, ensuring the accuracy and stability of the detection signal.

The Click EdUTP TUNEL Apoptosis Detection Kit (Red Fluorescence) is based on the above principles and provides a complete set of reagent components from labeling to detection, as well as an optimized experimental protocol. It is suitable for various sample types, including adherent cells, suspended cells, paraffin-embedded tissue sections, and frozen sections, and is compatible with both fluorescence microscopy observation and flow cytometry analysis. The fluorescence signal detection channel is the Red channel (Ex/Em = 555 nm/565 nm).

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Materials Supplied and Storage Conditions

Kit components	Size		Storage conditions
	20 T	50 T	
BSA Wash Solution(5×)	6 mL	15 mL	-20℃
TritonX-100(100%)	100 μL	100 μL	4℃
DNase I(5 U/μL)	4 μL	10 μL	-20℃
10×DNase I Buffer	0.3 mL	0.6 mL	-20℃
TdT Enzyme	40 μL	100 μL	-20℃
Equilibration Buffer	2 mL	5 mL	-20℃
EdUTP	10 μL	25 μL	-20℃, protected from light
Probe Diluent	100 μL	250 μL	-20℃
Reaction buffer(10×)	100 μL	300 μL	4℃
AbFluor 555 azide	8 μL	20 μL	-20℃, protected from light
Copper Reagent	30 μL	75 μL	4℃
Copper Protectant	70 μL	175 μL	-20℃, protected from light
Reducing Agent	100 mg	100 mg×2	-20℃, protected from light
DAPI(500×)	5 μL	12 μL	-20℃, protected from light
Tissue Autofluorescence Quencher	2 mL	5 mL	RT, protected from light,For detection in tissue samples
Proteinase K(20mg/mL)	4 μL	10 μL	-20℃

Note: For this kit in 20 T/50 T specifications when used with a fluorescence microscope, taking a 96-well plate as an example, each well contains 50 μL of the system, and it can be used for 20 and 50 detections respectively; taking a 24-well plate as an example, each well contains 200 μL of the system, and it can be used for 5 and 10 detections respectively, and so on. When used with a flow cytometer, each sample is in a 200 μL system, and it can be used for 5 and 10 detections respectively.

Materials Required but Not Supplied

• Centrifuge, fluorescence microscope,flow cytometer

• cell culture plate, phosphate-buffered saline (PBS, pH 7.4), 4% paraformaldehyde (fixative), deionized water, xylene, ethanol, an Pap Pen, and antifade mounting medium

Reagent Preparation

TritonX-100 (0.3%): According to the actual consumption, dilute 100% TritonX-100 to 0.3% TritonX-100 with PBS.

TritonX-100 (0.2%): For flow cytometry detection. According to the actual consumption, dilute 100% TritonX-100 to 0.2% TritonX-100 with PBS.

1×DNase I Buffer: According to the actual consumption, dilute 10×DNase I Buffer to 1×DNase I Buffer with deionized water.

Equilibration Buffer： Centrifuge at 800 g for 5 min before use, discard the pellet, and collect the supernatant.

EdUTP Working Solution: According to the actual consumption, dilute EdUTP 10 times with Probe Diluent to obtain EdUTP

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Working Solution, the unused EdUTP can also be stored at -20℃ and protected from light for 12 month after aliquoting to avoid repeated freezing and thawing.

BSA Wash Solution (1×): Add PBS to dilute BSA Wash Solution (5×) into 1×work solution (final concentration is 3% BSA/ PBS), and mix well. The solution can be stored stably for 6 month at -20℃ after aliquoting.

Reaction Buffer (1×): Ready to use as supplied. Equilibrate to room temperature before use. Store at 4℃.

Preparation of Reducing Agent (10×): Add 1 mL deionized water to Reducing Agent to prepare Reducing Agent (10×) with a final concentration of 100 mg/mL and mix until the compound is completely dissolved. The solution can be stored stably for 6 month at -20℃ after aliquoting. If the solution turns brown, the components have been degraded and it is not recommended to continue using it.

DAPI (1×): According to the actual consumption, dilute DAPI (500×) to DAPI (1×) with PBS.

Tissue Autofluorescence Quencher: Ready to use as supplied. Equilibrate to room temperature before use. It can significantly quench the autofluorescence caused by lipofuscin. Tighten the bottle cap after use to prevent evaporation.

Assay Procedure

A. Sample Preparation

1. For adherent cells (Analysis by Fluorescence microscope)

(1) Cells are seeded into multi-well plates (such as 96-well plates), cell culture dishes, or onto coverslips, and cultured in a CO_2 incubator at 37℃ for at least 24 h. Apoptosis is induced by the desired method, while control cells are cultured without induction. The following procedure is described using a 96-well plate as an example.

(2) Remove the medium and fix the cells with 50 μL 4% paraformaldehyde for 30 min at room temperature.

(3) Remove the fixation solution and wash with 200 μL PBS 3 times (5 min each time).

(4) Add 50 μL 0.3% Triton X-100, after the fixation, and incubate the plate for 30 min at room temperature.

(5) Wash the cells with 50 μL BSA Working Solution 3 times,5 min each time,and then wash with 200 μL PBS 3 times (5 min each time).

Note:

For coverslip-attached cells and other plate cultures, adjust the volumes of fixative and 0.3% Triton X-100 according to practical requirements, just enough to cover the cells.

(6) For the positive control, after completion of sample preparation, cells may be treated with 10 U/mL DNase I at room temperature for 10–20 min. To prepare this solution, dilute the 5 U/μL DNase I stock solution 1:500 with 1× DNase I Buffer to obtain a final concentration of 10 U/mL. After digestion, wash the samples three times with 200 μL PBS (5 min each wash), and then continue with step B.1 for fluorescence microscopy observation.

2. For non-adherent cells (Analysis by Flow Cytometry)

(1) Culture the cells to optimal density, and after trypan blue counting, ensure cells viability of over 95%. Induce apoptosis using the desired method, while simultaneously culturing untreated control cultures.

(2) Centrifuge at 300 g for 5 min and collect the cells (cell density per sample approximately 5×10^6 cells), then wash once with PBS.

Note:

1) For adherent cells, after digestion with trypsin (without EDTA), collect the cells by centrifugation. If the digestion time is too long, it may damage the cell membrane structure and lead to false-positive results due to cell necrosis; therefore, the trypsin digestion time should be carefully controlled (mild digestion solution is recommended).

2) If the washing step is likely to cause cell loss, skip the washing and proceed directly to the subsequent fixation step to minimize cell loss.

(3) For every 5×10^6 cells, add 1 mL of 4% paraformaldehyde and incubate at room temperature for 30 min. During this period, gently shake the cells to ensure that the sample is fully fixed.

(4) Centrifuge the cells at 800 g for 5 min, remove the fixative, resuspend them in 1 mL of PBS, and repeat the washing process twice.

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(5) Centrifuge the cells at 800 g for 5 min, remove the supernatant, and add 500 μL of 0.2% Triton-X 100 to each 5×10^6 cells. Incubate at 4℃ for 10 min.

(6) Centrifuge the cells at 800 g for 5 min, remove the supernatant, resuspend them in 1 mL of PBS. Repeat the washing process twice.

(7) Centrifuge the cells at 800 g for 5 min, then remove the supernatant. Seal the cells with 500 μL of BSA Working Solution for 15 min. During the sealing process, gently shake and mix the cells.

(8) Centrifuge the cells at 800 g for 5 min, then remove the blocking solution and wash twice with 1 mL of PBS.

(9) For the positive control setup, after sample preparation is completed, at room temperature, 1 mL of 10 U/mL DNase I (diluted from 5 U/μL DNase I in 1×DNase I Buffer at a ratio of 1:500) can be used to digest the cells for 10-20 min. Then, the samples are washed twice or three times with PBS, and then proceed to the flow cytometry analysis in step B.2.

Note:

The induction efficiency of DNase I is affected by the induction time, incubation temperature and cell density. It is recommended that the initial concentration of DNase I be 10 U/mL, and the incubation time be 10 min. If insufficient induction or excessive induction leads to cell loss and other phenomena, the concentration of DNase I and incubation time can be appropriately adjusted to determine the optimal conditions that can achieve a balance between "producing the maximum positive signal" and "maintaining the minimum physical loss of cells".

3. For Paraffin-Embedded Tissue (Analysis by Fluorescence microscope)

(1) Deparaffinize tissue by immersing twice in xylene for 10-20 min.

(2) Rehydrate tissue by the following washes (in the order given): two washes for 5 min each in 100% ethanol, then one wash for 3 min each successively in 95%, 70%, and 50% ethanol.

Note: It can also be operated according to the tissue dewaxing and rehydration operation system of your own laboratory.

(3) Wash the sample in 200-500 μL PBS twice for 5 min each.

(4) Drain excess PBS from tissue, after the sections were shaken dry, circle the tissue with a Pap Pen, and incubate for 15 min at room temperature in 20 μg/mL Proteinase K (in PBS, preparation before use) solution.

Note: The time of protease digestion will have to be optimized for specific tissue types and thicknesses. Over digestion by protease will result in loss of cellular structure and possible release of tissue section from slide. Under digestion will result in poor TdT labeling.

(5) Terminate the protease treatment by washing cells three times for 5 min each in PBS with gentle agitation.

(6) For the positive control, after completion of sample preparation, cells may be treated with 10 U/mL DNase I at room temperature for 10–20 min. To prepare this solution, dilute the 5 U/μL DNase I stock solution 1:500 with 1× DNase I Buffer to obtain a final concentration of 10 U/mL. After digestion, wash the samples three times with 200 μL PBS (5 min each wash), and then continue with step B.1 for fluorescence microscopy observation.

4. For Frozen tissue sections (Analysis by Fluorescence microscope)

(1) After sections have dried on the slide, fix with 200 μL 4% paraformaldehyde for 30 min at room temperature.

(2) Wash by immersing in 200-500 μL PBS twice for 5 min each.

(3) Drain excess PBS from tissue, after the sections were shaken dry, circle the tissue with a Pap Pen, and incubate for 15 min at room temperature in 20 μg/mL Proteinase K (in PBS, preparation before use) solution.

(4) Terminate the protease treatment by washing cells three times for 5 min each in 200-500 μL PBS with gentle agitation.

(5) For the positive control, after completion of sample preparation, cells may be treated with 10 U/mL DNase I at room temperature for 10–20 min. To prepare this solution, dilute the 5 U/μL DNase I stock solution 1:500 with 1× DNase I Buffer to obtain a final concentration of 10 U/mL. After digestion, wash the samples three times with 200 μL PBS (5 min each wash), and then continue with step B.1 for fluorescence microscopy observation.

B. EdUTP labeling

The EdUTP labeling reaction buffer should be prepared prior to use, based on the number of samples to be analyzed. The table below gives the amounts for a single reaction, which are provided for reference only:

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Reaction Components	Volume per well (μL) for 96-well plate	Volume per well (μL) for 24-well plate
Equilibration Buffer	45	180
EdUTP Working Solution	5	20
TdT Enzyme	2	8
Total Volume	52	208

Note: Before preparing the EdUTP labeling reaction buffer, equilibrate all components to room temperature. Due to storage at low temperature, Equilibration Buffer may exhibit slight precipitation of some components. Mix thoroughly by inverting the tube until completely dissolved before use.Equilibration Buffer contains sodium cacodylate and cobalt chloride, which are highly toxic chemicals. In case of skin contact, rinse immediately with plenty of water. If an accident occurs or if you feel unwell, seek medical attention immediately.Do not drink, eat, or smoke while handling this reagent.

1. Analysis by Fluorescence microscope

(1) Add 50 μL of Equilibration Buffer to each sample (for 96-well plates, 50 μL is recommended; for 48-well plates, 140 μL; for 24-well plates, 200 μL; for 12-well plates, 400 μL; for 6-well plates, 1 mL; for tissue sections, 50-200 μL is suggested, and the volume may be adjusted according to sample characteristics and area, ensuring that the tissue is adequately covered). Incubate at room temperature in the dark for 5 min.

(2) Remove the Equilibration Buffer. Then, add EdUTP labeling reaction buffer to each sample. The recommended volumes are: 50 μL for 96-well plates, 140 μL for 48-well plates, 200 μL for 24-well plates, 400 μL for 12-well plates, 1 mL for 6-well plates, and 50-200 μL for tissue sections. The volume may be adjusted according to sample characteristics and area, ensuring that the tissue is just covered. Incubate the samples in a humidified chamber at 37℃ in the dark for 1 h (the incubation time can be adjusted as needed for different sample types)Wash samples 3 times for 5 min each in PBS.

Note: Add an appropriate amount of ultrapure water to the bottom of the humidified chamber to maintain humidity during incubation and thereby minimize evaporation of the reaction buffer.

(3) Remove the reaction buffer, then wash the samples three times with 200 μL of BSA Wash Solution, 5 min per wash.

(4) Continue with Step C.

2. Analysis by Flow Cytometry

(1) Take 1×10^6 cells and resuspend them in 200 μL of Equilibration Buffer. Incubate at room temperature in the dark for 5 min.

(2) Centrifuge the cells at 800 g for 5 min. Remove the supernatant and resuspend in 200 μL of EdUTP labeling reaction buffer. Incubate at 37℃ for 1-2 h (the incubation time should be different depending on the samples), during which periodically mix cells gently.

(3) Centrifuge 800 g for 5 min to remove the supernatant. Resuspend in 200 μL of EdUTP-labeled reaction buffer and incubate at 37°C in the dark for 1-2 h (the incubation time can be adjusted according to different samples as needed). During the incubation, gently shake the tubes at regular intervals to mix the cells.

(4) Centrifuge the cells at 800 g for 5 min, remove the supernatant, and resuspend them in 1 mL of BSA Wash Solution. Repeat the washing process 2-3 times.

(5) Continue with Step C..

C. EdUTP detection

Prepare the Click-iT working solution according to the table below:

Note:

(1) Add the components in the order listed in the table, otherwise the reaction will not yield optimal results. The prepared Click-iT working solution must be used within 15 min.

(2) In this step, add 50 μL of Click-iT working solution to each sample. Recommended volumes are 50 μL for 96-well plates, 140 μL for 48-well plates, 200 μL for 24-well plates, 400 μL for 12-well plates, 1 mL for 6-well plates, and 50-200 μL for sections; the volume may be adjusted according to sample characteristics and area size, ensuring that the tissue is just covered. The volume of Click-iT working solution can be adjusted as needed, but the reaction components must be added in proportion.

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Reaction Components	One Sample	Five Samples	Ten Samples
Copper Reagent	1 μL	5 μL	10 μL
Copper Protectant	3 μL	15 μL	30 μL
Reaction Buffer(1×)	40.6 μL	203 μL	406 μL
AbFluor 555 Azide	0.4 μL	2 μL	4 μL
Reducing Agent(10×)	5 μL	25 μL	50 μL
Total Volume	50 μL	250 μL	500 μL

1. Analysis by Fluorescence microscope

(1) Remove the BSA Wash Solution. Then, add Click-iT working solution to each sample. The recommended volumes are: 50 μL for 96-well plates, 140 μL for 48-well plates, 200 μL for 24-well plates, 400 μL for 12-well plates, 1 mL for 6-well plates, and 50-200 μL for sections. The volume may be adjusted according to sample characteristics and area size, ensuring that the tissue is just covered. Incubate the samples in a humidified chamber at 37℃ in the dark for 30 min.

(2) Remove the Click-iT working solution, then wash the cells with BSA Wash Solution (1×) three times, each for 3-5 min. Remove BSA Wash Solution (1×), add an appropriate amount of PBS to immerse the cells, and take fluorescence microscopic photos for observation. Use the green channel (Ex/Em = 555 nm/564 nm).

Microscopy photography tips: 1) Use the DAPI channel to locate cell positions; 2) Prioritize negative control group imaging, as the fluorescence of the negative control is non-specific staining fluorescence that needs to be subtracted to no fluorescence through black and white balance; 3) When taking photos of the experimental or positive group, subtract the same black and white balance as the negative control to obtain the effective fluorescence of the experimental or positive group.

2. Analysis by Flow Cytometry

(1) Centrifuge the cells at 800 g for 5 min.Remove the BSA Wash Solution, add 200 μL of Click- iT working solution to each sample, and incubate at 37 ℃ in the dark for 30 min.

(2) Centrifuge the cells at 800 g for 5 min.Remove the Click-iT working solution, then wash the cells with BSA Wash Solution (1×) three times, each for 3-5 min.

D. Optional step: Perform DAPI nuclear staining or antibody labeling

1. Nuclear Staining

(1) After Step C, remove the BSA Wash Solution (1×) , and then stain with DAPI (1×) at room temperature in the dark for 10 min.

Important Note: It is essential to keep the samples in the dark during incubation.

(2) Remove the DAPI (1×) staining solution, then wash the samples with PBS three times for 5 min per wash.

(3) Quench the Autofluorescence of the Tissue. For tissue samples, add Tissue Autofluorescence Quencher, incubate at room temperature for 5 min, protected from light. Soak in deionized water for 5 min, and then soak in PBS 3 times for 5 min each time.

Note: 1. Tissue sections will produce spontaneous fluorescence, and only tissue samples need to quench the spontaneous fluorescence. After Tissue Autofluorescence Quencher treatment, the tissue will be stained blue, and the fluorescence intensity of Tunel will be reduced to some extent, but the fluorescence microscope photography will not be affected; 2. The incubation time of the Tissue Autofluorescence Quencher needs to be precisely determined. It can be timed for 5 min after adding the Tissue autofluorescence Quencher to the first sample. 3.Tissue Autofluorescence Quencher is volatile. During the incubation process, it needs to be replenished in time to prevent the reagent from drying out, solidifying and caking, which may affect the subsequent photography and observation.

(4) For cells grown on coverslips, place a drop of aqueous mounting medium or antifade mounting medium onto a glass slide, then mount the coverslip with the cell- side facing down onto the slide. For paraffin sections, frozen sections, and other similar samples, apply a drop of aqueous mounting medium or antifade mounting medium onto the tissue section and cover with a coverslip. Observe under a fluorescence microscope. For cells cultured in plates or dishes, add an appropriate volume of PBS to cover the cells, and then observe and capture images using a fluorescence microscope. For non- adherent cells, resuspend the

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cells in PBS and analyze directly by flow cytometry. Red channel: Ex/Em = 555 nm/564 nm; DAPI channel: Ex/Em = 360 nm/460 nm .

2. Antibody Labeling

(1) After Step C, remove the BSA Wash Solution (1×).Dilute the primary antibody according to the manufacturer's instructions at the recommended ratio, and immediately add the diluted primary antibody to the samples. Incubate at room temperature for 1 h with gentle rocking on a rocking platform, or alternatively, incubate overnight at 4 ℃ with gentle rocking.

(2) Collect the primary antibody solution. Add an appropriate volume of PBS and wash the samples for 5 min with gentle rocking on a rocking platform. Repeat this wash step twice more (for a total of three washes). If the background is high, the washing time and the number of washes may be appropriately increased.

(3) Dilute the secondary antibody at the recommended ratio according to the manufacturer's instructions.Add the diluted secondary antibody to the samples immediately. Incubate at room temperature for 1 h with gentle agitation on a rocker, in the dark.

(4) Remove the secondary antibody solution. Add an appropriate volume of PBS and wash the samples for 5 min with gentle rocking on a rocking platform. Repeat this wash step twice more (for a total of three washes). If the background is high, the washing time and the number of washes may be appropriately increased.

(5) Stain with DAPI (1×) at room temperature in the dark for 10 min.

Important Note: It is essential to keep the samples in the dark during incubation.

(6) Remove the DAPI (1×) staining solution. Then wash the samples with PBS three times for 5 min per wash.

(7) Quench the Autofluorescence of the Tissue. For tissue samples, add Tissue Autofluorescence Quencher, incubate at room temperature for 5 min, protected from light. Soak in deionized water for 5 min, and then soak in PBS 3 times for 5 min each time.

Note: 1. Tissue sections will produce spontaneous fluorescence, and only tissue samples need to quench the spontaneous fluorescence. After Tissue Autofluorescence Quencher treatment, the tissue will be stained blue, and the fluorescence intensity of Tunel will be reduced to some extent, but the fluorescence microscope photography will not be affected; 2. The incubation time of the Tissue Autofluorescence Quencher needs to be precisely determined. It can be timed for 5 min after adding the Tissue autofluorescence Quencher to the first sample. 3.Tissue Autofluorescence Quencher is volatile. During the incubation process, it needs to be replenished in time to prevent the reagent from drying out, solidifying and caking, which may affect the subsequent photography and observation.

(8) For cells grown on coverslips, place a drop of aqueous mounting medium or antifade mounting medium onto a glass slide, then mount the coverslip with the cell- side facing down onto the slide. For paraffin sections, frozen sections, and other similar samples, apply a drop of aqueous mounting medium or antifade mounting medium onto the tissue section and cover with a coverslip. Observe under a fluorescence microscope. For cells cultured in plates or dishes, add an appropriate volume of PBS to cover the cells, and then observe and capture images using a fluorescence microscope. For non- adherent cells, resuspend the cells in PBS and analyze directly by flow cytometry. Red channel: Ex/Em = 555 nm/565 nm; DAPI channel: Ex/Em = 360 nm/460 nm .

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Typical Data

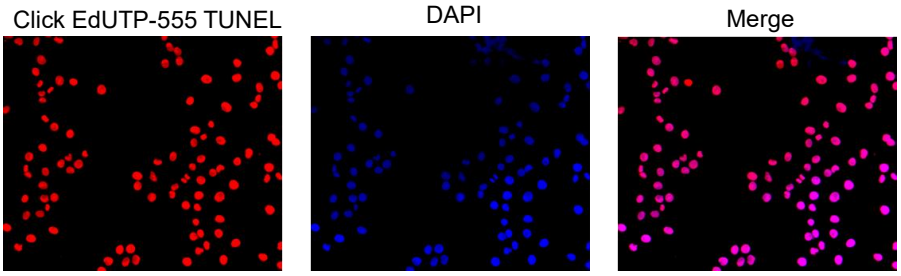


Figure 1. Representative images of apoptosis in HeLa cells induced with the inducer provided in this kit and stained with the Click EdUTP TUNEL Apoptosis Detection Kit (Red fluorescence).

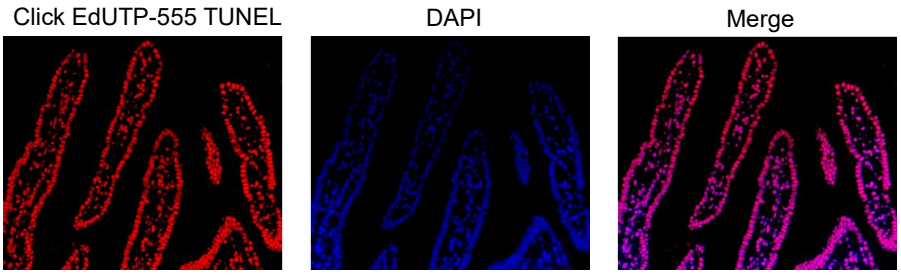


Figure 2.Representative images of apoptosis in mouse intestinal tissue sections induced with the inducer provided in this kit and stained with the Click EdUTP TUNEL Apoptosis Detection Kit (Red fluorescence).

Recommended Products

Catalog No.	Product Name
KTA0002	Annexin V-AbFluor™ 488/PI Apoptosis Detection kit
KTA2010	One-Step TUNEL Apoptosis Assay Kit (Green Fluorescence)
KTA4001	Mitochondrial Membrane Potential Assay Kit (JC-1)
KTA3020	Caspase-1 Assay Kit (Colorimetric)
KTA2012	Click EdUTP TUNEL Apoptosis Assay Kit (Geen Fluorescence)
BMU104	SuperKine™ Enhanced Antifade Mounting Medium

Disclaimer

The reagent is only used in the field of scientific research, not suitable for clinical diagnosis or other purposes.

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