Click-iT™ TUNEL Colorimetric IHC Detection Kit

Catalog Number C10625

Pub. No. MAN0015677 Rev. C.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

Understanding the mechanisms of programmed cell death or apoptosis can represent a critical aspect of toxicological profiling and drug discovery. Based on the cellular changes during programmed cell death, apoptosis is often classified into early, middle, and later stages. The later stages of apoptosis are characterized by changes in nuclear morphology, chromatin condensation, nuclear envelope degradation, and DNA fragmentation.

Since the introduction of terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay in 1992 (Gavrieli et al., 1992), the TUNEL assay has become the most widely used *in situ* test for the study of apoptosis (Huerta et al., 2007). The TUNEL assay is based on the incorporation of modified dUTPs by the enzyme terminal deoxynucleotidyl transferase (TdT) at the 3′-OH ends of fragmented DNA, a hallmark as well as the ultimate determinant of apoptosis. The modifications are fluorophores or haptens, including biotin, which can be detected directly in the case of a fluorescently-modified nucleotide (i.e., fluorescein-dUTP) or indirectly with HRP (horseradish peroxidase) conjugated to streptavidin or antibodies.

The Click-iT™ TUNEL Colorimetric IHC Detection Kit utilizes an EdUTP (a dUTP modified with a small, bioorthogonal alkyne moiety) nucleotide, which is incorporated at the 3′-OH ends of fragmented DNA by the TdT enzyme. After the incorporation of the modified nucleotide at the site of DNA fragmentation, biotin azide is added to the sample. After a brief click reaction (Breinbauer and Köhn, 2003; Wang et al., 2003; Rostovtsev et al., 2002; Kolb et al., 2001), a copper catalyzed covalent reaction between the azide and alkyne moiety, the biotin is attached to the modified nucleotide. Next, Streptavidin-Peroxidase (horseradish peroxidase) is added to the sample and attaches to the biotin group. Finally, addition of the DAB (peroxidase) substrate results in the colorimetric detection of apoptotic cells (Figure 1).

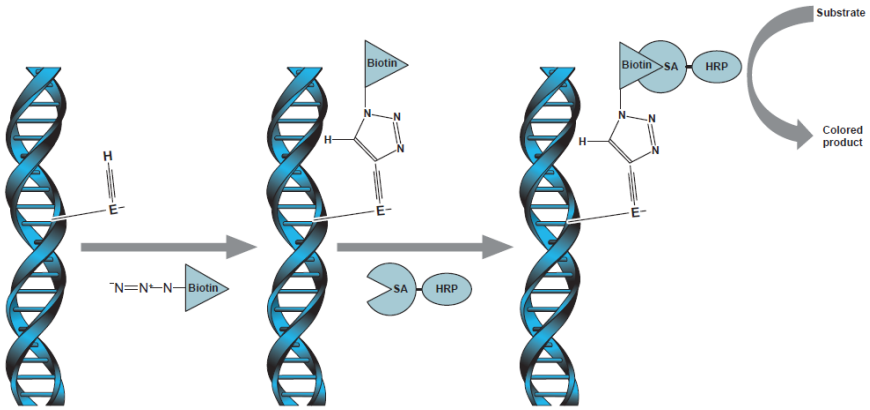

Figure 1 Detection of apoptosis with the Click-iT™ TUNEL Colorimetric assay.

Because of the high degree of labeling specificity inherent in the click technology and the small size of the alkyne moiety, the EdUTP nucleotide is more readily incorporated by TdT than other modified nucleotides (Figure 2). The benefits of labeling specificity and increased incorporation efficiency are low background and improved detection of apoptotic cells as demonstrated in Figure 3.

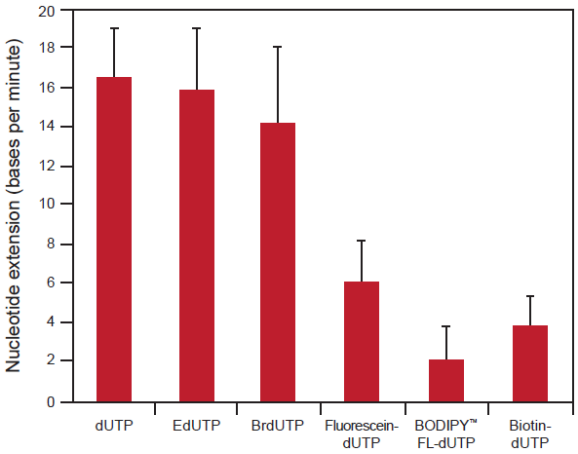

Figure 2 Comparison of TdT incorporation of several modified nucleotides.
A 48-bp oligonucleotide was incubated with 30 units of TdT and an equimolar mix of the modified nucleotide with three other nucleotides for 4 hours at room temperature. The TdT reaction products were then analyzed by gel electrophoresis using a 20% TBE pre-cast gel and subsequent staining with SYBR™ Gold nucleic acid gel stain.

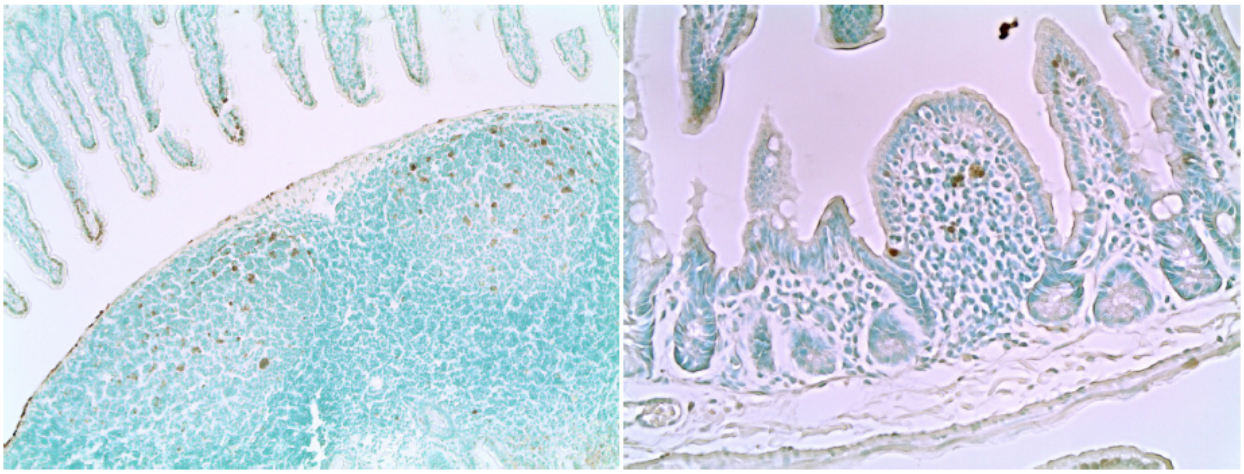

Figure 3 Click-iT™ TUNEL Colorimetric staining in tissue.
The Click-iT™ TUNEL Colorimetric IHC Detection Kit was used to detect apoptotic cells (nuclei, brown) in a sample of formalin-fixed, paraffin-embedded (FFPE) mouse intestines that were counter stained with methyl green (nuclei, green).

The Click-iT™ TUNEL Colorimetric IHC Detection Kit has been optimized and contains all of the components needed to label and detect apoptotic cells from formalin-fixed, paraffin-embedded (FFPE) tissue samples. The kits include sufficient reagents for labeling fifty (50) 18-mm × 18-mm coverslips using 50 µL of reaction reagent per test. The kits are flexible and can be configured for 50 independent TUNEL apoptosis tests.

Contents and storage

Component	Amount	Concentration	Storage[1]
Proteinase K (Component A)	10 mL	1X solution	2°C to 8°C Desiccate Protect from light DO NOT FREEZE
Click-iT™ TUNEL Colorimetric reaction buffer (Component B)	4 mL	10X TBS	
Copper II sulfate ($CuSO_4$) (Component C)	1 mL	100 mM aqueous solution	
Click-iT™ TUNEL Colorimetric reaction buffer additive (Component D)	400 mg	Not applicable	
Click-iT™ TUNEL Colorimetric wash buffer (Component E)	60 mL	100X solution	
Streptavidin-Peroxidase Conjugate (Component F)	10 mL	1X solution	
DAB substrate buffer (Component G)	20 mL	1X solution	
DAB chromogen (Component H)	1 mL	40X reagent	
UltraPure™ SSC (Component I)	50 mL	20X	
TdT reaction buffer (Component J)	8 mL	1X solution	≤–20°C Protect from light
EdUTP nucleotide mixture (Component K)	55 µL	50X solution	
TdT (terminal deoxynucleotidyl transferase), recombinant (Component L)[2]	4 x 34 µL	15 U/µL in glycerol	
Biotin azide (Component M)	50 µg	Not applicable	
Dimethylsulfoxide (DMSO) (Component N)	200 µL	Not applicable	
Wash chamber[3]	1 unit	Not applicable	Room temperature
Number of assays: Sufficient material is supplied for 50 coverslips based on the protocol below.			

[1] These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions, see the labels of individual components.
[2] **IMPORTANT!** TdT (Component L) is VERY temperature sensitive. Keep it at –20°C until ready to use.
[3] The Wash chamber has been included in the kit for your convenience. We recommend using it for step 4.11 and step 5.6.

Before you begin

Required materials not supplied

- Xylene
- 100% Ethanol
- 0.85% NaCl solution
- 1X Phosphate-buffered saline (PBS) (Cat. No. 14190-144 or 14190-250)
- 4% Paraformaldehyde in PBS (fixative reagent)
- 1X Tris-buffered saline (TBS) (Cat. No. 28358)
- 3% H_2O_2 in PBS
- Molecular biology grade, deionized water (DNase/RNase free)
- Coverslips for standard microscopy (22 mm × 22 mm or 18 mm × 18 mm)
- DNase I (Cat. No. 18068-015)

IMPORTANT! Do not use azide. Sodium azide negatively affects the click reaction.

Prepare solutions

Allow vials to warm to room temperature before opening.

1. **1X Click-iT™ TUNEL Colorimetric reaction buffer (Component B) working solution:** Transfer all of the solution (4 mL) in the Component B vial to 36 mL of deionized water. Rinse the Component B vial with some of the diluted Click-iT™ TUNEL Colorimetric reaction buffer to ensure the transfer of all of the 10X concentrate.

   To make smaller amounts of 1X Click-iT™ TUNEL Colorimetric reaction buffer, dilute volumes from the Component B bottle 1:10 with deionized water. After use, store any remaining 1X solution at 2–8°C. When stored as directed, this 1X solution is stable for up to 6 months.

2. **10X Click-iT™ TUNEL Colorimetric reaction buffer additive (Component D) stock solution:** Add 2 mL of deionized water to the Component D vial, then mix until fully dissolved. After use, aliquot the 10X Click-iT™ TUNEL Colorimetric reaction buffer additive stock solution and store remaining stock solution at ≤–20°C.

   When stored as directed, this stock solution is stable for up to 1 year. If the solution develops a brown color, it has degraded and should be discarded.

3. **Biotin azide (Component M) stock solution:** Add 32 µL of DMSO (Component N) to the Component M vial and mix well. Aliquot the stock solution and store at ≤–20°C.

4. **2X SSC solution:** Dilute Component I 1:10 into deionized water.

5. **1X Click-iT™ TUNEL Colorimetric wash solution:** Dilute the Click-iT™ TUNEL Colorimetric wash buffer (Component E) 1:100 into 1X TBS.

Experimental protocol for tissue sections

The following protocols describe how to perform the Click-iT™ TUNEL Colorimetric imaging assay on FFPE tissue samples.

1 Deparaffinize tissue sections

To deparaffinize tissue sections, place the slides in a rack and perform the following washes in a Coplin staining jar:

Note: Perform all deparaffinization steps at room temperature.

Table 1 Tissue deparaffinization procedure.

Solution	Incubation time
Xylene	5 min
Xylene	5 min
1:1 (v/v) Xylene : 100% EtOH	5 min
100% EtOH	5 min
100% EtOH	3 min
95% EtOH	3 min
85% EtOH	3 min
75% EtOH	3 min
50% EtOH	3 min
0.85% NaCl	5 min
1X PBS	5 min

2 Fix and permeabilize tissues

2.1. Immerse the slides in fixative solution (4% paraformaldehyde) for 15 minutes at room temperature.

2.2. Wash the slides by immersing them in PBS for 5 minutes.

2.3. Add sufficient volume of Proteinase K (Component A) to completely cover the tissue sections.

Note: Use less than 200 µL of Proteinase K per tissue section. Otherwise, you will run out of Proteinase K.

2 Fix and permeabilize tissues *(continued)*

2.4. Incubate the samples for 10–20 minutes at room temperature.

Note: We recommend using a coverslip or a humidified chamber to protect against evaporation.

Note: Depending on the tissue type, thickness, and degree of fixation, it may be necessary to optimize the Proteinase K incubation time.

2.5. Wash the slides by immersing them in PBS for 5 minutes.

2.6. Immerse the slides in fixative solution (4% paraformaldehyde) for 5 minutes at room temperature.

2.7. Rinse slides with PBS.

2.8. Wash the slides twice by immersing them in PBS for 5 minutes each.

2.9. Rinse the slides with deionized water.

3 *(Optional)* Prepare a positive control

3.1. To induce DNA strand breaks (i.e., TUNEL positive cells), incubate fixed and permeabilized cells with 1 U of DNase I (Cat. No. 18068-015) diluted into 1X DNase I Reaction Buffer (20 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, 50 mM KCl) for 30 minutes at room temperature.

3.2. After incubation, wash once with deionized water and proceed to “Perform the TdT reaction” on page 5.

4 Perform the TdT reaction

4.1. Add 100 µL of TdT reaction buffer (Component J) to each slide and allow the solution to spread completely over the tissue.

Note: We recommend using a coverslip or a humidified chamber to protect against evaporation.

4.2. Incubate the slides for 10 minutes at 37°C.

4.3. Prepare the TdT reaction mixture as shown in Table 2.

Table 2 TdT reaction mixture.

Reaction component	Number of coverslips		
	2	10	50
TdT reaction buffer (Component J)	93 µL	465 µL	2325 µL
EdUTP (Component K)	2 µL	10 µL	50 µL
TdT enzyme (Component L)	5 µL	25 µL	125 µL
Total volume	100 µL	500 µL	2500 µL

IMPORTANT! TdT enzyme (Component L) is highly temperature sensitive. Keep the enzyme in the –20°C freezer until ready to use. Gently pipette the reaction mixture to incorporate the TdT enzyme. Stirring by vortex is not recommended.

4.4. Remove the TdT reaction buffer from the samples by gently blotting with a paper towel. Make sure that the tissue sample does not dry.

4.5. Add 50 µL of the prepared TdT reaction mixture (from step 4.3) to each slide and incubate for 60 minutes at 37°C.

Note: We recommend using a coverslip or a humidified chamber to protect against evaporation.

4.6. Rinse the slides with PBS.

4.7. Immerse the slides in 2X SSC (from step 4) for 15 minutes to fully quench the TdT reaction.

4.8. Wash the slides twice by immersing them in PBS for 5 minutes each.

4 Perform the TdT reaction *(continued)*

4.9. To quench endogenous peroxidase enzymes, immerse the slides into a solution of 3% H_2O_2 for 5 minutes at room temperature.

4.10. Wash the slides twice by immersing them in PBS for 5 minutes each.

4.11. Wash the slides twice with 1X Click-iT™ TUNEL Colorimetric wash solution (from step 5) for 5 minutes each.

Note: For this step, we recommend using the Wash chamber included in the kit.

5 Perform the Click-iT™ reaction

5.1. Prepare 1X Click-iT™ TUNEL Colorimetric reaction buffer additive working solution by diluting the 10X solution (from step 2) 1:10 in deionized water. Prepare this solution fresh and use it on the same day. Discard any unused 1X solution.

5.2. Prepare the Click-iT™ TUNEL Colorimetric reaction cocktail according to Table 3.

IMPORTANT! Add the reaction components in the order listed; otherwise, the reaction will not proceed optimally.

Table 3 Click-iT™ TUNEL Colorimetric reaction cocktail.

Reaction component[1]	Number of coverslips		
	2	10	50
1X Click-iT™ Colorimetric reaction buffer (from step 1)	85 µL	425 µL	2125 µL
$CuSO_4$ (Component C)	4 µL	20 µL	100 µL
Biotin azide (from step 3)	1 µL	5 µL	25 µL
1X Click-iT™ Colorimetric reaction buffer additive (from step 5.1)	10 µL	50 µL	250 µL
Total volume	100 µL	500 µL	2500 µL

[1] Add the reaction components in the order listed.

Note: Use the Click-iT™ TUNEL Colorimetric reaction cocktail within 15 minutes of preparation.

5.3. Immediately after preparation, add 50 µL of the Click-iT™ TUNEL Colorimetric reaction cocktail (from step 5.2) to each slide and allow the solution to spread completely over the surface of the tissue section.

5.4. Incubate for 30 minutes at 37°C, protected from light.

Note: We recommend using a coverslip or a humidified chamber to protect against evaporation.

5.5. Rinse the slides with 1X PBS.

5.6. Wash each slide 2 times with the 1X Click-iT™ TUNEL Colorimetric wash solution (from step 5) for 5 minutes each.

Note: For this step, we recommend using the Wash chamber included in the kit.

5.7. Rinse the slides with deionized water.

5.8. Add a sufficient amount of the 1X Streptavidin-Peroxidase Conjugate (Component F) to cover the tissue (~200 µL) and incubate at room temperature for 30 minutes in a humidified chamber.

Note: Covering the tissue with a coverslip during incubation steps will allow the tissue to be covered uniformly by the reaction components. Dry the slide edges prior to adding the Streptavidin-Peroxidase Conjugate to prevent wicking.

5.9. Remove unbound Streptavidin-Peroxidase Conjugate by washing the slides 3 times for 5 minutes with 1X PBS each at room temperature. If using a coverslip, tip the slide to remove the coverslip before proceeding with the wash step.

5 Perform the Click-iT™ reaction *(continued)*

5.10. Rinse briefly in deionized water, and remove residual water without allowing the tissue to dry out.

5.11. For each slide to be developed, prepare 100 µL of 1X DAB reaction mixture by combining 5 µL of DAB chromogen (Component H) with 95 µL of DAB substrate buffer (Component G) in a centrifuge tube **immediately before use**. This results in a 1:20 dilution of the DAB chromogen in DAB substrate buffer.

IMPORTANT! Prepare the 1X DAB reaction mixture immediately before use. Discard any unused 1X solution.

Note: Depending on the desired signal strength, you may need to optimize the 1:20 recommended DAB chromogen dilution. Dilutions of 1:100 to 1:200 for the DAB chromogen may be needed, if the signal develops too rapidly.

5.12. Add 100 µL of the 1X DAB reaction mixture (from step 5.11) to each tissue section and incubate at room temperature for 1–10 minutes depending on desired signal intensity.

5.13. Wash each tissue section thoroughly with deionized water and image.

Related products

Product	Cat. No.	Unit size
Click-iT™ TUNEL Alexa Fluor™ 488 Imaging Assay, for Microscopy and HCS	C10245	50–100 assays
Click-iT™ TUNEL Alexa Fluor™ 594 Imaging Assay, for Microscopy and HCS	C10246	50–100 assays
Click-iT™ TUNEL Alexa Fluor™ 647 Imaging Assay, for Microscopy and HCS	C10247	50–100 assays
Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 488 dye	C10617	50 assays
Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 594 dye	C10618	50 assays
Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 647 dye	C10619	50 assays
Click-iT™ TUNEL Colorimetric IHC Detection Kit	C10625	50 assays

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0015677

Revision	Date	Description
C.0	15 April 2022	The format and content were updated.
B.0	10 January 2017	The dilution instructions for DAB Chromogen in DAB Substrate Buffer were corrected.
A.0	15 March 2016	New document for the Click-iT™ TUNEL Colorimetric IHC Detection Kit.