Click-iT™ Plus TUNEL Assay

for In Situ Apoptosis Detection with Alexa Fluor™ Dyes

Catalog Numbers C10617, C10618, C10619

Pub. No. MAN0010877 **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 determinate 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 streptavidin or antibodies.

The Click-iT™ Plus TUNEL Assays utilize EdUTP (a dUTP modified with a small, bio-orthogonal alkyne moiety), which is incorporated at the 3'-OH ends of fragmented DNA by the TdT enzyme. Detection is based on a click reaction (Breinbauer and Köhn, 2003; Wang et al., 2003; Rostovtsev et al., 2002; Kolb et al., 2001), a copper catalyzed covalent reaction between an Alexa Fluor™ picolyl azide dye and an alkyne (Figure 1). Because of the small size of the alkyne moiety, the EdUTP nucleotide is more readily incorporated by TdT than other modified nucleotides (Figure 2).

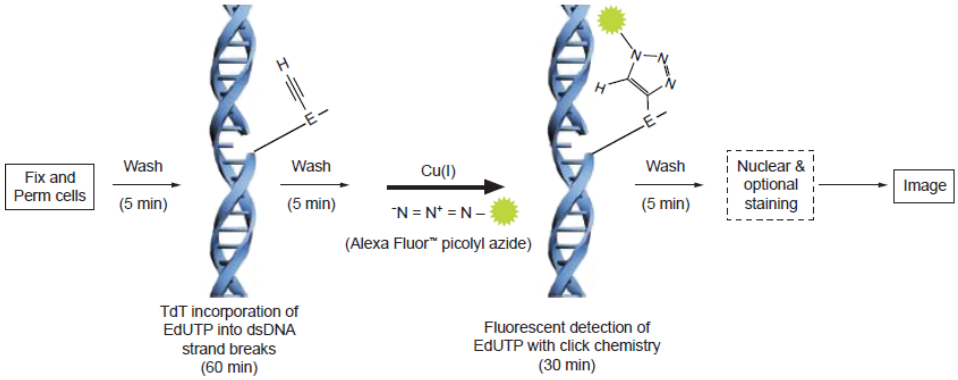

Figure 1 Apoptosis detection with the Click-iT™ Plus TUNEL Assay.

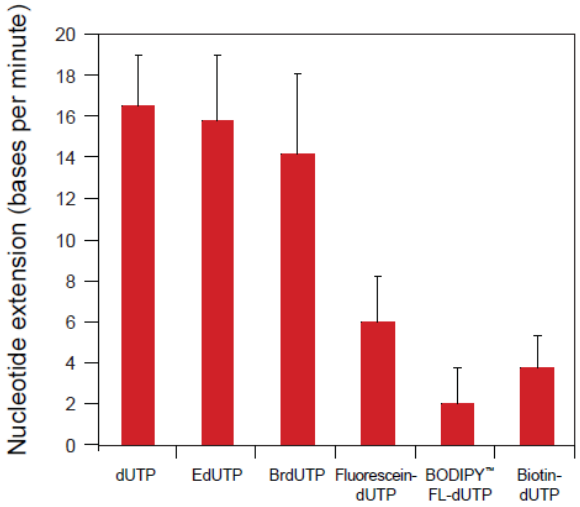

Figure 2 Comparison of TdT incorporation of several modified nucleotides.
A 48-bp oligonucleotide was incubated with 30 units of TdT and an equimolar mix of the modified nucleotide with three other nucleotides for 4 hours at room temperature. The TdT reaction products were then analyzed by gel electrophoresis;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

The mild reaction conditions for the Click-iT™ Plus TUNEL Assay have been demonstrated to preserve cell morphology, the binding properties of phalloidin, and the signal from fluorescent proteins such as GFP and RFP (Figure 3 and Figure 4).

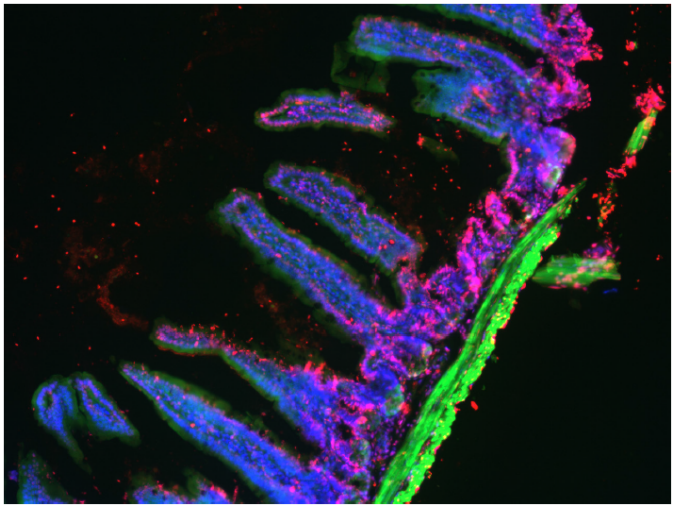

Figure 3 Click-iT™ Plus TUNEL staining in tissue.
Formalin-fixed, paraffin embedded (FFPE) mouse intestine was treated with DNAse to fragment the DNA. After treatment, the Click-iT™ Plus TUNEL Assay with the Alexa Fluor™ 594 dye (red) was utilized to detect the fragment DNA. After the TUNEL reaction, actin was stained with Alexa Fluor™ 647 phalloidin (pink). The cells were counterstained with Hoechst™ 33342 (blue). The GFP expressing epithelial cells are clearly visible (green).

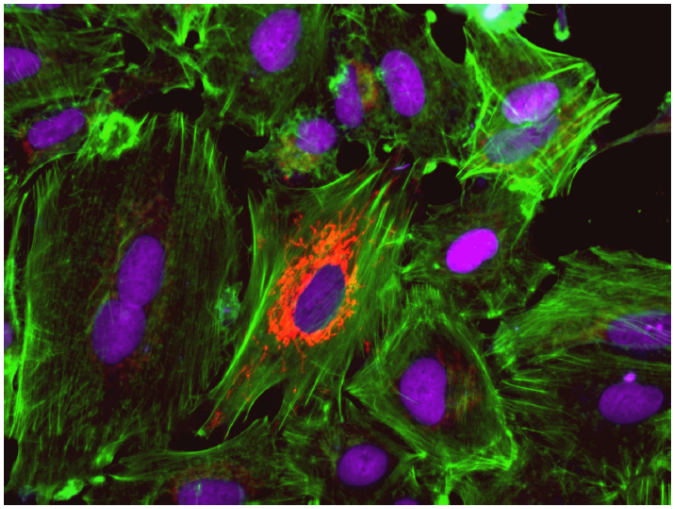

Figure 4 Click-iT™ Plus TUNEL staining in adherent cells.
HeLa cells were transduced with CellLight™ Mitochondria-RFP, BacMam 2.0 and treated with DNase to induce TUNEL positive DNA strand breaks. After the treatment, the Click-iT™ Plus TUNEL Assay with the Alexa Fluor™ 647 dye was utilized to detect the fragment DNA (purple). After the TUNEL reaction, actin filaments were stained with ActinGreen™ 488 ReadyProbes™ Reagent (green). The RFP has localized to the mitochondria, resulting in the red fluorescent signal.

The Click-iT™ Plus TUNEL Assays have been optimized and contain all the components needed to label and detect apoptotic cells from formalin-fixed, paraffin embedded (FFPE) tissue samples or on adherent cells grown on coverslips. 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	Cat. No. C10617	Cat. No. C10618	Cat. No. C10619	Concentration	Storage[1]
TdT Reaction Buffer (Component A)	8.0 mL	8.0 mL	8.0 mL	1X Solution	≤–20°C Protect from light
EdUTP nucleotide mixture (Component B)	55 µL	55 µL	55 µL	50X Solution	
TdT (terminal deoxynucleotidyl transferase) *recombinant* (Component C)	3 x 34 µL	3 x 34 µL	3 x 34 µL	15 U/µL in glycerol	
Click-iT™ Plus TUNEL Reaction Buffer, 10X (Component D)	500 µL	500 µL	500 µL	10X Solution in Tris-buffered saline	
Click-iT™ Plus TUNEL Reaction Buffer Additive (Component E)	400 mg	400 mg	400 mg	Not applicable	
Copper Protectant (Component F)	100 µL	100 µL	100 µL	Not applicable	
Alexa Fluor™ picolyl azide dye (Component G)	25 µL of Alexa Fluor™ 488 picolyl azide	25 µL of Alexa Fluor™ 594 picolyl azide	25 µL of Alexa Fluor™ 647 picolyl azide	DMSO Solution	
Proteinase K (Component H)	500 µL	500 µL	500 µL	25X Solution	
Number of assays: Sufficient material is supplied for 50 coverslips based on the protocol below.					
Approximate fluorescence excitation and emission maxima, in nm: Alexa Fluor™ 488 picolyl azide: 495/519 nm; Alexa Fluor™ 594 picolyl azide: 590/615 nm; Alexa Fluor™ 647 picolyl azide: 650/670 nm; Hoechst™ 33342: 350/461 nm, when bound to DNA.					
Note: The insert allows for the convenient storage of all the reagents, including the 1X Click-iT™ Plus TUNEL Supermix, at ≤–20°C. A 15-mL conical tube containing the 1X Click-iT™ Plus TUNEL Supermix will fit into the I slot.					

[1] These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions for each component, see labels on individual components.

Before you begin

Required materials not supplied

- 1X Phosphate-buffered saline (PBS) (Cat. No. 14190-144 or 14190-250)
- 4% Paraformaldehyde in PBS (fixative)
- 0.25% Triton™ X-100 in PBS (permeabilization reagent)
- 5 mg/mL Bovine serum albumin and 0.1% Triton™ X-100 in PBS, pH 7.4
- 1X solution of Hoechst™ 33342
- 3% Bovine serum albumin in PBS (3% BSA in PBS), pH 7.4
- Molecular biology grade water (DNase/RNase free)
- Coverslips for standard microscopy (22 mm × 22 mm or 18 mm × 18 mm)
- DNase I (Cat. No. 18068-015)

Prepare the stock solutions

Allow vials to warm to room temperature before opening.

1. Prepare a working solution of 1X Click-iT™ Plus TUNEL Reaction Buffer (Component D): Transfer all of the solution (500 µL) in the Component D vial to 4.5 mL of deionized water. Rinse the Component D vial with some of the diluted Click-iT™ Plus TUNEL Reaction Buffer to ensure the transfer of all of the 10X concentrate.

   To make smaller amounts of 1X Click-iT™ Plus TUNEL Reaction Buffer, dilute volumes from the Component D 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. Prepare a working solution of 1X Click-iT™ Plus TUNEL Supermix according to Table 1.

   Table 1 Click-iT™ Plus TUNEL Supermix.

Supermix components	Alexa Fluor™ 488 picolyl azide	Alexa Fluor™ 594 picolyl azide	Alexa Fluor™ 647 picolyl azide
1X Click-iT™ Plus TUNEL Reaction Buffer (from step 1)	2630 µL	2625 µL	2625 µL
Copper Protectant (Component F)	67 µL	67 µL	67 µL
Alexa Fluor™ picolyl azide (Component G)	3.7 µL	8.3 µL	8.3 µL
Total volume	2.7 mL	2.7 mL	2.7 mL

   After use, store any remaining 1X solution at ≤–20°C. When stored as directed, this 1X solution is stable for up to 6 months.

3. To prepare a 100X stock solution of the Click-iT™ Plus TUNEL Reaction Buffer Additive (Component E): Transfer 2 mL of deionized water to the contents of the vial (400 mg), then mix until fully dissolved. After use, aliquot any remaining stock solution and store at ≤–20°C.

4. *For tissue samples only:* Prepare a 1X Proteinase K solution by diluting Component H 1:25 in PBS. After use, aliquot any remaining stock solution and store at ≤–20°C.

Experimental protocol for tissue sections

The following protocol was developed using FFPE tissue sections of mouse intestine, kidney, liver, heart, and colon. The tissue type and treatment may influence the number of apoptotic cells detected.

1 Deparaffinize tissue sections

Deparaffinize tissue sections in Coplin jars at room temperature according to Table 2.

Table 2 Tissue deparaffinization procedure.

Solution	Incubation time
Xylenes	5 min
Xylenes	5 min
50%:50% Xylenes:EtOH	3 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 tissue sections

2.1. Immerse the slides in fixative (4% paraformaldehyde) for 15 minutes at 37°C.

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

2.3. Add sufficient volume of the permeabilization reagent (Proteinase K solution from step 4) to completely cover the tissue section.

2.4. Incubate the samples for 15 minutes.

Note: A coverslip or humidified chamber is recommended to protect against evaporation.

Note: Optimization of the Proteinase K incubation time may be necessary depending on the thickness of the tissue section.

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

2.6. Immerse the slides in fixative (4% paraformaldehyde) for 5 minutes at 37°C.

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

2.8. Rinse the slides in 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 unit 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, then proceed to “Perform the TdT reaction” on page 5.

4 Perform the TdT reaction

4.1. Add 100 µL of TdT Reaction Buffer (Component A) to each slide and allow the solution to spread completely over the tissue.

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

4 Perform the TdT reaction *(continued)*

4.3. Prepare the following TdT reaction mixture:

Table 3 TdT reaction mixtures.

Reaction components	Number of slides					
	1	2	5	10	25	50
TdT Reaction Buffer (Component A)	47 µL	94 µL	235 µL	470 µL	1,175 µL	2,350 µL
EdUTP (Component B)	1 µL	2 µL	5 µL	10 µL	25 µL	50 µL
TdT enzyme (Component C)	2 µL	4 µL	10 µL	20 µL	50 µL	100 µL
Total volume	50 µL	100 µL	250 µL	500 µL	1,250 µL	2,500 µL

4.4. Remove the TdT Reaction Buffer from the samples.

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

Note: A coverslip or humidified chamber is recommended to protect against evaporation.

4.6. Rinse the slides in deionized water.

4.7. Wash the slides with 3% BSA and 0.1% Triton™ X-100 in PBS for 5 minutes.

4.8. Rinse the slides in 1X PBS.

5 Perform the Click-iT™ Plus reaction

5.1. Prepare a 10X Click-iT™ Plus TUNEL Reaction Buffer Additive by diluting the 100X solution (from step 3) 1:10 in deionized water. Prepare this solution fresh and use the solution on the same day.

5.2. Prepare the Click-iT™ Plus TUNEL reaction cocktail according to Table 4, then mix well by vortexing.

IMPORTANT! Use the Click-iT™ Plus TUNEL reaction cocktail within 15 minutes of preparation.

Table 4 Click-iT™ Plus TUNEL reaction cocktails.

Reaction components	Number of slides					
	1	2	5	10	25	50
Click-iT™ Plus TUNEL Supermix (from step 2)	45 µL	90 µL	225 µL	450 µL	1,125 µL	2,250 µL
10X Click-iT™ Plus TUNEL Reaction Buffer Additive (from step 5.1)	5 µL	10 µL	25 µL	50 µL	125 µL	250 µL
Total volume	50 µL	100 µL	250 µL	500 µL	1,250 µL	2,500 µL

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

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

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

5.5. Remove the Click-iT™ Plus TUNEL reaction cocktail, then wash each slide with 3% BSA in PBS for 5 minutes.

5.6. Rinse the slides in 1X PBS.

For antibody staining, proceed to "(Optional) Stain with antibodies" on page 7; for DNA staining, proceed to "(Optional) Stain DNA" on page 7. If no additional staining is desired, proceed to "Image and analyze" on page 7.

6 *(Optional)* Stain with antibodies

6.1. If required, block the samples with 3% BSA in 1X PBS for the recommended time, **protected from light**. Remove the blocking solution.

6.2. Prepare and add the primary antibody solution as recommended by the manufacturer.

6.3. Incubate the samples for the recommended time and temperature, **protected from light**. Remove the primary antibody solution.

6.4. Wash samples twice with 3% BSA in PBS. Remove the wash solution.

6.5. Prepare and add the secondary antibody solution as recommended by the manufacturer.

6.6. Incubate the samples for the recommended time and temperature, **protected from light**. Remove the secondary antibody solution.

6.7. Wash each sample twice with 3% BSA in PBS. Remove the wash solution.

For DNA staining, proceed to "(Optional) Stain DNA" on page 7. If no additional staining is desired, proceed to "Image and analyze" on page 7.

7 *(Optional)* Stain DNA

7.1. Add 100 µL of 1X Hoechst™ 33342 solution per sample, then incubate for 15 minutes at room temperature, **protected from light**. Remove the Hoechst™ 33342 solution.

7.2. Wash each sample twice with PBS. Remove the wash solution.

8 Image and analyze

The cells stained with the Click-iT™ Plus TUNEL reaction are compatible with all methods of slide preparation, including wet mount or prepared mounting media. See Table 5 for the approximate fluorescence excitation and emission maxima for Alexa Fluor™ dyes and Hoechst™ 33342 bound to DNA.

Table 5 Approximate fluorescence excitation/emission maxima.

Fluorophore	Excitation (nm)	Emission (nm)
Alexa Fluor™ 488 picolyl azide dye	495	519
Alexa Fluor™ 594 picolyl azide dye	590	615
Alexa Fluor™ 647 picolyl azide dye	650	670
Hoechst™ 33342 bound to DNA	350	460

Experimental protocol for cells grown on coverslips

The following protocol was developed using HeLa cells treated with 0.5 µM staurosporine for 4 hours to induce apoptosis. Cell type and treatment may influence the number of apoptotic cells detected.

1 Fix and permeabilize cells

This protocol was optimized with a fixation step using 4% paraformaldehyde in PBS followed by a permeabilization step with 0.25% Triton™ X-100, but it is amenable to other fixation and permeabilization reagents such as 70% ethanol.

1.1. Remove media and wash the coverslips once with PBS.

Note: If there is a chance that cells may be lost by this wash step, proceed directly to fixation (step 1.2) without performing the wash step.

1 Fix and permeabilize cells *(continued)*

1.2. Add a sufficient volume of fixative (4% paraformaldehyde) to completely cover the coverslips.

1.3. Incubate the samples for 15 minutes at room temperature.

1.4. Remove the fixative.

1.5. Add sufficient volume of the permeabilization reagent (0.25% Triton™ X-100 in PBS) to completely cover the coverslips.

1.6. Incubate the samples for 20 minutes at room temperature, then wash twice with deionized water.

2 *(Optional)* Prepare a positive control

2.1. To induce DNA strand breaks (i.e., TUNEL positive cells), incubate fixed and permeabilized cells with 1 unit 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.

2.2. After incubation, wash once with deionized water, then proceed to “Perform the TdT reaction” on page 8.

3 Perform the TdT reaction

3.1. Add 100 µL of TdT Reaction Buffer (Component A) to each coverslip and allow the solution to spread completely over the surface.

3.2. Incubate the coverslips for 10 minutes at 37°C.

3.3. Prepare the TdT reaction mixture according to Table 6:

3.4. Remove the TdT Reaction Buffer from the samples.

Table 6 TdT reaction mixtures.

Reaction components	Number of coverslips					
	1	2	5	10	25	50
TdT Reaction Buffer (Component A)	47 µL	94 µL	235 µL	470 µL	1,175 µL	2,350 µL
EdUTP (Component B)	1 µL	2 µL	5 µL	10 µL	25 µL	50 µL
TdT enzyme (Component C)	2 µL	4 µL	10 µL	20 µL	50 µL	100 µL
Total volume	50 µL	100 µL	250 µL	500 µL	1,250 µL	2,500 µL

3.5. Add 50 µL of the prepared TdT reaction mixture (from step 3.3) to each coverslip, then incubate for 60 minutes at 37°C.

Note: A coverslip or humidified chamber is recommended to protect against evaporation.

3.6. Wash the coverslips twice with 3% BSA in PBS for 5 minutes each.

4 Perform the Click-iT™ Plus reaction

4.1. Prepare a 10X Click-iT™ Plus TUNEL Reaction Buffer Additive by diluting the 100X solution (from step 3) 1:10 in deionized water. Prepare this solution fresh and use the solution on the same day.

4.2. Prepare the Click-iT™ Plus TUNEL reaction cocktail according to Table 7, then mix well by vortexing.

IMPORTANT! Use the Click-iT™ Plus TUNEL reaction cocktail within 15 minutes of preparation.

Table 7 Click-iT™ Plus TUNEL reaction cocktails.

Reaction components	Number of coverslips					
	1	2	5	10	25	50
Click-iT™ Plus TUNEL Supermix (from step 2)	45 µL	90 µL	225 µL	450 µL	1,125 µL	2,250 µL
10X Click-iT™ Plus TUNEL Reaction Buffer Additive (from step 4.1)	5 µL	10 µL	25 µL	50 µL	125 µL	250 µL
Total volume	50 µL	100 µL	250 µL	500 µL	1,250 µL	2,500 µL

4.3. Immediately add 50 µL of the Click-iT™ Plus TUNEL reaction cocktail (from step 4.2) to each cover slip and allow the solution to spread completely over the surface.

4.4. Incubate the samples for 30 minutes at 37°C, protected from light.

4.5. Remove the Click-iT™ Plus TUNEL reaction cocktail, then wash each coverslip with 3% BSA in PBS for 5 minutes.

For antibody staining, proceed to “(Optional) Stain with antibodies” on page 9; for DNA staining, proceed to “(Optional) Stain DNA” on page 9. If no additional staining is desired, proceed to “Image and analyze” on page 10.

5 *(Optional)* Stain with antibodies

5.1. If required, block the coverslips with 3% BSA in 1X PBS for the recommended time, **protected from light**. Remove the blocking solution.

5.2. Prepare and add the primary antibody solution as recommended by the manufacturer.

5.3. Incubate the samples for the recommended time and temperature, **protected from light**. Remove the primary antibody solution.

5.4. Wash each coverslip twice with 3% BSA in PBS. Remove the wash solution.

5.5. Prepare and add the secondary antibody solution as recommended by the manufacturer.

5.6. Incubate the samples for the recommended time and temperature, **protected from light**. Remove the secondary antibody solution.

5.7. Wash each coverslip twice with 3% BSA in PBS. Remove the wash solution.

For DNA staining, proceed to “(Optional) Stain DNA” on page 9. If no additional staining is desired, proceed to “Image and analyze” on page 10.

6 *(Optional)* Stain DNA

6.1. Add 100 µL 1X Hoechst™ 33342 solution per coverslip, then incubate for 15 minutes at room temperature, **protected from light**. Remove the Hoechst™ 33342 solution.

6.2. Wash each coverslip twice with PBS. Remove the wash solution.

7 Image and analyze

The cells stained with the Click-iT™ Plus TUNEL reaction are compatible with all methods of slide preparation, including wet mount or prepared mounting media. See Table 8 for the approximate fluorescence excitation and emission maxima for Alexa Fluor™ dyes and Hoechst™ 33342 bound to DNA.

Table 8 Approximate fluorescence excitation/emission maxima.

Fluorophore	Excitation (nm)	Emission (nm)
Alexa Fluor™ 488 picolyl azide dye	495	519
Alexa Fluor™ 594 picolyl azide dye	590	615
Alexa Fluor™ 647 picolyl azide dye	650	670
Hoechst™ 33342 bound to DNA	350	460

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

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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. MAN0010877

Revision	Date	Description
C.0	15 April 2022	The format and content were updated.
B.0	06 November 2017	The temperature for tissue deparaffinization was changed to room temperature. The branding and legal/regulatory language were updated.
A.0	12 September 2014	New document for the Click-iT™ Plus TUNEL Assay.