Click-iT® TUNEL Alexa Fluor® Imaging Assay

Catalog nos. C10245, C10246, C10247

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage*	Stability
TdT reaction buffer (Component A)	11 mL	1X solution	• ≤–20˚C • Protect from light	When stored as directed this kit is stable for 1 year
EdUTP nucleotide mixture (Component B)	100 µL	50X solution		
TdT (terminal deoxynucleotidyl transferase) *recombinant* (Component C)	6 vials	Each vial contains 34 µL enzyme at 15 U/µL in glycerol		
Click-iT® reaction buffer (Component D)	25 mL	1X solution (contains Alexa Fluor® 488 azide for Cat. no. C10245, Alexa Fluor® 594 azide for Cat. no. C10246, Alexa Fluor® 647 azide for Cat. no. C10247)		
Click-iT® reaction buffer additive (Component E)	50 mg	Not applicable		
Hoechst 33342 (Component F)	18 µL	10 mg/mL solution in water		
DNase I (deoxyribonuclease I, Component G)	18 µL	Not applicable		
DNase I buffer (Component H)	190 µL	10X solution		

*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage of each component, see labels on individual components.

Number of assays: Sufficient material is supplied for 1 × 96-well plate or 50 coverslips based on protocols below.

Approximate fluorescence excitation/emission maxima: Alexa Fluor® 488 azide: 495/519 in nm; Alexa Fluor® 594 azide: 590/615 in nm; Alexa Fluor® 647 azide: 650/670 in nm; Hoechst 33342: 350/461 in nm, when bound to DNA.

Introduction

Since the introduction of terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay in 1992,[1] the TUNEL assay is the most widely used *in situ* test for apoptosis study.[2] 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 or bromine which can be detected directly in the case of a fluorescently-modified nucleotide (i.e., fluorescein-dUTP), or indirectly with streptavidin or

antibodies, if biotin-dUTP or BrdUTP are used, respectively. Often at late stages of apoptosis, adherent cells are known to detach or "pop" off. For a reliable and reproducible TUNEL imaging assay, the modified nucleotide must not only be an acceptable substrate for TdT, but the detection method must also be sensitive without bringing about any additional loss of cells from the sample.

System Overview

The Click-iT® TUNEL Alexa Fluor® imaging assays utilize a dUTP modified with an alkyne, a small, bio-orthogonal functional group that enables the nucleotide to be more readily incorporated by TdT than other modified nucleotides (Figures 1–2). Detection is based on a click reaction,[3-6] a copper (I) catalyzed reaction between an azide and alkyne (Figure 3). Click chemistry fills the void when methods such as direct labeling or the use of antibodies are not efficient. The small size of the Alexa Fluor® azide (MW ~1,000) compared to that of an antibody (MW ~150,000) enables effortless penetration of complex samples with only mild fixation and permeabilization required. As a result, when compared to assays using other modified nucleotides, the Click-iT® TUNEL imaging assay is fast (complete within 2 hours) and is able to detect a higher percentage of apoptotic cells under identical conditions (Figures 4–5). Furthermore, the Click-iT® TUNEL assay allows multiplexing with surface and intracellular biomarker detection. (Table 2).

The Click-iT® TUNEL Alexa Fluor® imaging assay has been tested in HeLa, A549, and CHO K1 cells with a variety of reagents that induce apoptosis including staurosporine (Figure 6). The Click-iT® TUNEL Alexa Fluor® imaging assay contains all components needed to accurately and reliably detect apoptosis on adherent cells grown on coverslips or a 96-well microplate, and includes DNase I to generate strand breaks as a positive control.

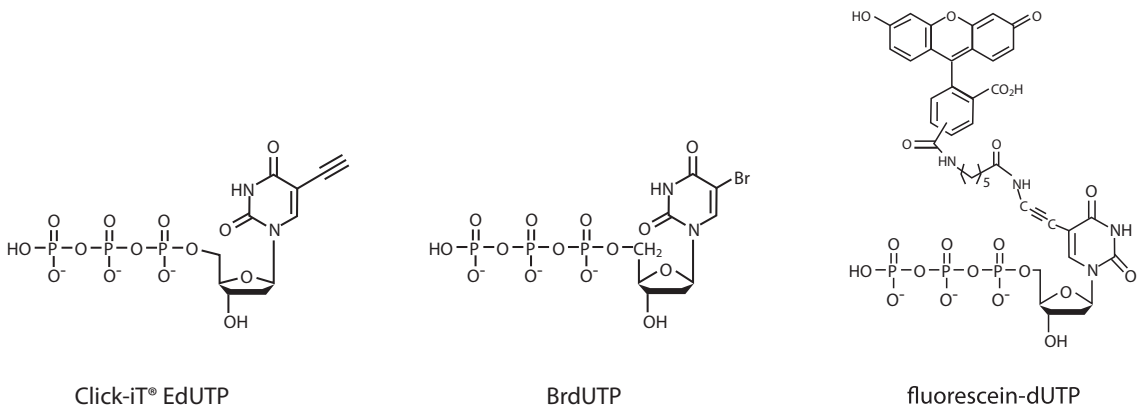

Figure 1. Modified nucleotide structures. The alkyne and bromine modifications are significantly smaller than fluorescein.

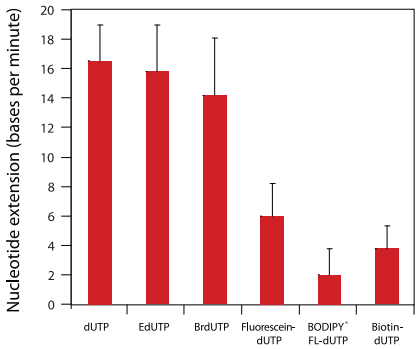

Figure 2. Comparison of TdT incorporation of several modified nucleotides. A 48-bp oligonucleotide was incubated with 30 units of TdT and an equimolar mix of the modified nucleotide with three other nucleotides for 4 hours at room temperature. The TdT reaction products were then analyzed by gel electrophoresis,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

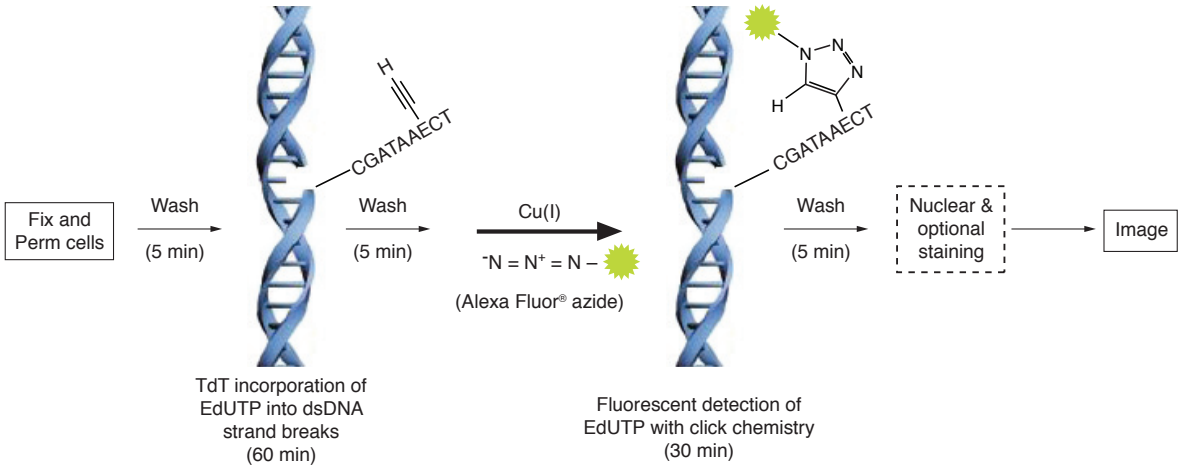

Figure 3. Detection of apoptosis with the Click-iT® TUNEL imaging assay.

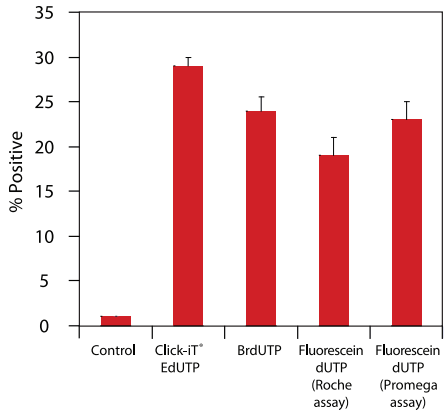

Figure 4. TUNEL assay comparison—percentage positives detected. HeLa cells were treated with 0.5 µM staurosporine for 4 hours. Following fixation and permeabilization, TUNEL imaging assays were performed according to the manufacturer's instructions. The percent positives were calculated based upon the corresponding negative control. Imaging and analysis was performed using a Thermo Fisher Scientific Cellomics® ArrayScan II.

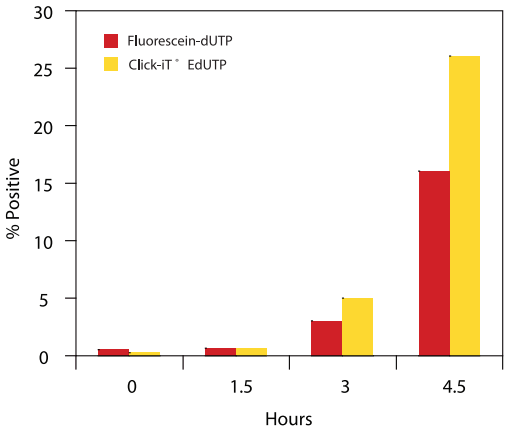

Figure 5. TUNEL assay time course comparison—percent positives detected. HeLa cells were treated with 0.5 µM staurosporine for the time points indicated. Following fixation and permeabilization, Click-iT® TUNEL imaging assays using Click-iT® EdUTP or fluorescein dUTP (Promega's DeadEnd™ Fluorometric TUNEL system) were performed according to the manufacturer's instructions. The percent positives were calculated based upon the corresponding negative control. Imaging and analysis was performed using a Thermo Fisher Scientific Cellomics® ArrayScan II.

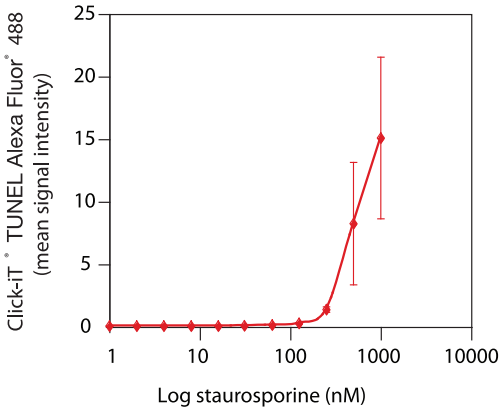

Figure 6. Dose response of staurosporine using Click-iT® TUNEL Alexa Fluor® 488 imaging assay. HeLa cells were treated with staurosporine for 18 hours at final concentrations ranging from 1 nM to 1 µM. Following fixation and permeabilization, the Click-iT® TUNEL imaging assay was performed.. Data points represent averages from 8 wells, the error bars show standard deviations.

Table 2. Click-iT® detection reagent compatibility.

Molecule	Compatibility*
Qdot® nanocrystals	Use Qdot® nanocrystals ***after*** the Click-iT® detection reaction.
Fluorescent proteins	Use organic dye-based reagents, such as TC-FlAsH™ or TC-ReAsH™ reagents for protein expression detection or anti-GFP rabbit or chicken antibodies ***after*** the Click-iT® detection reaction.
Phalloidin	Click chemistry is not compatible with phalloidin staining of F-actin. To stain the cytoskeleton, antibodies, such as anti-α-tubulin ***after*** the Click-iT® detection reaction are recommended.
Organic dyes such as Alexa Fluor® dyes or fluorescein isothiocyanate (FITC)	Completely compatible with the Click-iT® detection reaction.
TC-FlAsH™ or TC-ReAsH™ reagents	Detect the tetracysteine (TC) tag with FlAsH™ or ReAsH™ reagents ***before*** the Click-iT® detection reaction.
*Some molecules or detection methods involve components that are unstable in the presence of copper catalyst used for the Click-iT® detection reaction.	

Before You Begin

Material Required but Not Provided

- 1X Phosphate buffered saline (PBS, Invitrogen Cat. no. 14190-144 or 14190-250)
- 4% paraformaldehyde in PBS (fixative)
- 0.25% Triton® X-100 in PBS (permeabilization reagent)
- 3% Bovine serum albumin in PBS (3% BSA in PBS), pH 7.4
- Molecular biology grade water (DNase/RNase free)
- 96-well plate (as recommended for the specific, automated imaging instrument)
- 22 × 22 mm or 18 × 18 mm coverslips (for standard microscopy)

Caution

- TdT reaction buffer (Component A) contains potassium cacodylate and cobalt chloride, and is harmful if swallowed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If swallowed seek medical advice immediately. Wear appropriate laboratory protective clothing, gloves, and eye/face protection when handling this reagent.
- Hoechst 33342 (Component F) is a known mutagen. Use the dye with appropriate precautions.

Selecting Protocols

This manual includes protocols to perform the Click-iT® TUNEL imaging assay on adherent cells grown on coverslips (refer to the **Experimental Protocol for Cells Grown on Coverslips**) or a 96-well microplate (refer to **Experimental Protocol for Cells Grown in 96-well Plate**).

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.

Cell Fixation and Permeabilization

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

1.1 Remove media and wash coverslips once with PBS.

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

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

1.3 Incubate samples for 15 minutes at room temperature.

1.4 Remove 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 samples for 20 minutes at room temperature. Then wash twice with deionized water.

Preparing a Positive Control (Optional)

The DNase I generates strand breaks in the DNA to provide a positive TUNEL reaction. There is sufficient DNase I to perform ~18 positive controls.

2.1 Wash coverslips with deionized or molecular biology grade water.

2.2 Prepare DNase I solution according to Table 3 and mix well.

Note: Do not vortex the DNase I solution as DNase I denatures with vigorous mixing.

2.3 Add 100 µL of the DNase I solution (prepared in step 2.2) to each coverslip and incubate for 30 minutes at room temperature.

Table 3. DNase I solution.

Reaction Components	Number of Coverslips		
	1	2	3
Deionized water	89 µL	178 µL	267 µL
DNase I buffer (Component H)	10 µL	20 µL	30 µL
DNase I (Component G)	1 µL	2 µL	3 µL
Total Volume	100 µL	200 µL	300 µL

2.4 Wash coverslips once with deionized water, proceed to **TdT Reaction**.

TdT Reaction

This protocol uses 100 µL of the TdT reaction cocktail per coverslip. It is important to equilibrate cells to maximize efficiency of the TdT reaction.

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

Table 4. TdT reaction cocktails.

Reaction Components	Number of Coverslips				
	1	2	4	5	10
TdT reaction buffer (Component A)	94 µL	188 µL	376 µL	470 µL	940 µL
EdUTP (Component B)	2 µL	4 µL	8 µL	10 µL	20 µL
TdT* (Component C)	4 µL	8 µL	16 µL	20 µL	40 µL
Total Volume	100 µL	200 µL	400 µL	500 µL	1 mL

*Immediately after using the TdT enzyme (Component C), return the vial ≤–20°C. Do not keep the enzyme at room temperature.

3.2 Incubate coverslips for 10 minutes at room temperature.

3.3 Remove TdT reaction buffer.

3.4 Prepare the TdT reaction cocktail according to Table 4.

3.5 Add 100 µL of the TdT reaction cocktail (prepared in step 3.4) to each coverslip and allow the solution to spread completely over the surface.

3.6 Incubate coverslips for 60 minutes at 37°C. Use a humidified chamber to protect against evaporation. Remove the reaction cocktail.

Note: This reaction can be performed overnight at room temperature.

3.7 Wash coverslips twice with 3% BSA in PBS for 2 minutes each.

Click-iT® Reaction

4.1 Prepare the Click-iT® reaction buffer additive (Component E) by adding 625 µL of deionized water to the Component E vial. After use, aliquot and store any remaining solution at ≤–20°C. When stored as directed the stock solution is stable for up to 1 year.

4.2 Prepare the Click-iT® reaction cocktail according to Table 5 and mix well by vortexing.

Note: Use the Click-iT® reaction cocktail within 15 minutes of preparation.

4.3 Immediately add 100 µL of the Click-iT® reaction cocktail (prepared in step 4.2) to each coverslip and allow the solution to spread completely over the surface.

Table 5. Click-iT® reaction cocktails.

Reaction Components	Number of Coverslips				
	1	2	4	5	10
Click-iT® reaction buffer (Component D)	97.5 µL	195 µL	390 µL	487.5 µL	975 µL
Click-iT® reaction buffer additive (prepared in step 4.2)	2.5 µL	5 µL	10 µL	12.5 µL	25 µL
Total Volume	100 µL	200 µL	400 µL	500 µL	1 mL

4.4 Incubate coverslips for 30 minutes at room temperature, **protected from light**.

4.5 Remove the Click-iT® reaction cocktail and wash each coverslip with 3% BSA in 1X PBS for 5 minutes.

For antibody staining, proceed to section 5, or for DNA staining, proceed to section 6. If no additional staining is desired, proceed to **Imaging and Analysis**.

Antibody Detection (Optional)

5.1 If required, block the coverslips or wells 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 for the recommended time and temperature, **protected from light**. Remove the primary antibody solution.

5.4 Wash each coverslip or well 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 for the recommended time and temperature, **protected from light**. Remove the secondary antibody solution.

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

For DNA staining, proceed to section 6. If no additional staining is desired, proceed to **Imaging and Analysis**.

DNA Staining

6.1 Dilute Hoechst 33342 (Component F) 1:5,000 in PBS to obtain a 1X Hoechst 33342 solution.

6.2 Add 100 µL 1X Hoechst 33342 solution per coverslip or well and incubate for 15 minutes at room temperature, **protected from light**. Remove the Hoechst 33342 solution.

6.3 Wash each coverslip or well twice with PBS. Remove the wash solution.

Imaging and Analysis

The Click-iT® TUNEL stained cells are compatible with all methods of slide preparation including wet mount or prepared mounting media. See Table 6 for the approximate fluorescence excitation/emission maxima for Alexa Fluor® dyes and Hoechst 33342 bound to DNA.

Table 6. Approximate fluorescence excitation/emission maxima.

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

Experimental Protocol for Cells Grown in a 96-well Microplate

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.

Cell Fixation and Permeabilization

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

7.1 Remove media and wash wells once with PBS.

Note: If there is a chance that cells maybe lost by this wash step, proceed directly to fixation (step 7.2) without performing the wash step.

7.2 Add 100 µL fixative (4% paraformaldehyde) to completely cover each well.

7.3 Incubate plates for 15 minutes at room temperature.

7.4 Remove fixative.

7.5 Add 100 µL of the permeabilization reagent (0.25% Triton® X-100 in PBS) to completely cover each well.

7.6 Incubate plates for 20 minutes at room temperature.

7.7 Wash each well twice with deionized water.

Preparing a Positive Control (Optional)

The DNase I creates strand breaks in the DNA to provide a positive TUNEL reaction. There is sufficient DNase I to perform ~18 positive control wells.

8.1 Wash the wells with deionized or molecular biology grade water.

8.2 Prepare DNase I solution according to Table 7 and mix well.

Note: Do not vortex the DNase I solution as DNase I denatures with vigorous mixing.

8.3 Add 100 µL to each well that will be a positive control and incubate for 30 minutes at room temperature.

Table 7. DNase I solution.

Reaction Components	Number of Wells				
	1	2	4	5	8
Deionized water	89 µL	178 µL	356 µL	445 µL	712 µL
DNase I buffer (Component H)	10 µL	20 µL	40 µL	50 µL	80 µL
DNase I (Component G)	1 µL	2 µL	4 µL	5 µL	8 µL
Total volume	100 µL	200 µL	400 µL	500 µL	800 µL

8.4 Wash each well once with deionized water and proceed to **TdT Reaction**.

TdT Reaction

This protocol uses 50 µL of the TdT reaction cocktail per well. It is important to equilibrate the cells to maximize the efficiency of TdT reaction.

9.1 Add 50 µL of the TdT reaction buffer (Component A) to each well and allow the solution to spread completely over the surface.

9.2 Incubate plates for 10 minutes at room temperature. Remove the TdT reaction buffer.

9.3 Prepare the TdT reaction cocktail according to Table 8.

9.4 Add 50 µL of the TdT reaction cocktail (prepared in step 9.3) to each well and allow the solution to spread evenly in the well.

9.5 Incubate plates for 60 minutes at 37°C. Remove the reaction cocktail.

9.6 Wash wells twice with 3% BSA in PBS for 2 minutes each.

Table 8. TdT reaction cocktails.

Reaction Components	Number of Wells			
	1	8	12	100
TdT reaction buffer (Component A)	47 µL	376 µL	564 µL	4.7 mL
EdUTP (Component B)	1 µL	8 µL	12 µL	100 µL
TdT* (Component C)	2 µL	16 µL	24 µL	200 µL
Total volume	50 µL	400 µL	600 µL	5 mL

*Immediately after using the TdT enzyme (Component C), return the vial ≤–20°C. Do not keep the enzyme at room temperature.

Click-iT® Reaction

10.1 Prepare the Click-iT® reaction buffer additive (Component E) by adding 625 µL of deionized water. After use, aliquot and store any remaining solution at ≤–20°C. When stored as directed the stock solution is stable for up to 1 year.

10.2 Prepare the Click-iT® reaction cocktail according to Table 9 and mix well by vortexing.

Note: Use the Click-iT® reaction cocktail within 15 minutes of preparation.

Table 9. Click-iT® reaction cocktails.

Reaction Components	Number of Wells			
	1	8	12	100
Click-iT® reaction buffer (Component D)	97.5 µL	780 µL	1.17 mL	9.75 mL
Click-iT® reaction buffer additive (prepared in step 10.1)	2.5 µL	20 µL	30 µL	250 µL
Total volume	100 µL	800 µL	1.2 mL	10 mL

10.3 Immediately add 50 µL of the Click-iT® reaction cocktail (prepared in step 10.2) to each well and allow the solution to spread completely over the surface.

10.4 Incubate plates for 30 minutes at room temperature, **protected from light**. Remove the reaction cocktail.

10.5 Wash each well with 3% BSA in 1X PBS for 5 minutes.

10.6 **Optional:** Stain with antibodies (section 5) or other stains such as DNA staining for staining nuclei with Hoechst 33342 (section 6).

10.7 Image plate using filters appropriate for the Alexa Fluor® dye included in the Click-iT® TUNEL imaging assay (Table 6) and any other fluorophores used.

References

1. J Cell Biol 119, 493 (1992); **2.** J Surg Res 139, 143 (2007); **3.** ChemBioChem 4, 1147 (2003); **4.** J Am Chem Soc 125, 3192 (2003); **5.** Angew Chem Int Ed Engl 41, 2596 (2002); **6.** Angew Chem Int Ed Engl 40, 2004 (2001).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
C10245	Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay *for microscopy and HCS* *50–100 assays*	1 kit
C10246	Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay *for microscopy and HCS* *50–100 assays*	1 kit
C10247	Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay *for microscopy and HCS* *50–100 assays*	1 kit
Related Products		
10533-065	Terminal Deoxynucleotidyl Transferase, Recombinant	500 units
10533-073	Terminal Deoxynucleotidyl Transferase, Recombinant	3 × 500 units
14190-144	Dulbecco's Phosphate Buffered Saline 1X without Calcium Chloride without Magnesium Chloride	500 mL
14190-250	Dulbecco's Phosphate Buffered Saline 1X without Calcium Chloride without Magnesium Chloride	10 × 500 mL
18068-015	DNase I, Amplification Grade	100 units
H1399	Hoechst 33342, trihydrochloride, trihydrate	100 mg
H3570	Hoechst 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL
H21492	Hoechst 33342, trihydrochloride, trihydrate *FluoroPure™ grade*	100 mg

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