molecular probes®
by life technologies™

Click-iT® Lipid Peroxidation Detection with Linoleamide Alkyne (LAA)

Catalog nos. C10446, C10447

Table 1 Contents and storage

Click-iT® Lipid Peroxidation Imaging Kit – Alexa Fluor® 488 (Catalog no. C10446)			
Material	**Amount**	**Storage**	**Stability**
Click-iT® LAA (Linoleamide Alkyne) (Component A)	5 × 0.4 mg	• ≤–20°C • Protect from light • Dessicate	When stored as directed, the product is stable for 6 months from the date of receipt.
Alexa Fluor® 488 Azide (Component B)	1 × 180 µL		
Click-iT® Reaction Buffer (Component C)	1 × 7.5 mL	Room temperature	
Copper(II) sulfate ($CuSO_4$) (Component D)	1 × 5.5 mL		
Click-iT® Buffer Additive (Component E)	1 × 400 mg		
Cumene hydroperoxide (Component F)	1 × 100 µL		
Click-iT® LAA (Linoleamide alkyne) *for lipid peroxidation detection* (Catalog no. C10447)			
Material	**Amount**	**Storage**	**Stability**
Click-iT® LAA (Linoleamide Alkyne) (Component A)	5 × 0.4 mg	• ≤–20°C • Protect from light • Dessicate	When stored as directed, the product is stable for 6 months from the date of receipt.
Number of Assays: Sufficient material is supplied for 100 coverslips or 5 × 96-well plates, based on protocol below.			
Approximate excitation/emission maxima: AlexaFluor® 488 azide, 495/519 nm.			

Introduction

Click-iT® LAA (Linoleamide alkyne) leverages copper-catalyzed click chemistry and the linoleamide alkyne (LAA) reagent (alkyne-modified linoleic acid) for the detection of lipid-peroxidation-derived protein modifications in fixed cells. Linoleic acid is the most abundant polyunsaturated fatty acid found in mammals and its lipid peroxidation products likely account for the majority of lipid-derived protein carbonyls[1].

When incubated with cells, Click-iT® LAA incorporates into cellular membranes. Upon lipid peroxidation, LAA is oxidized and produces 9- and 13-hydroperoxy-octadecadienoic acid (HPODE). These hydroperoxides decompose to multiple α,β-unsaturated aldehydes, which readily modify proteins at nucleophilic side chains (Figure 1, page 2). These alkyne-containing modified proteins can be subsequently detected using Click-iT® chemistry and multiplexed with other probes appropriate for fixed cells. Click-iT® LAA reagent can be used for various applications when combined with appropriate azide-modified detection reagents and other related reagents.

- The Click-iT® LAA assay for lipid peroxidation detection is amenable to traditional fluorescence microscopy, high content screening (HCS), and flow cytometry utilizing a simple workflow (Figure 2, below).
- The Click-iT® Lipid Peroxidation Imaging Kit – Alexa Fluor® 488 (Cat. no. C10446) is provided as a complete kit containing sufficient reagents for five 96-well plates or 100 coverslips.
- Click-iT® LAA (Linoleamide alkyne) for lipid peroxidation detection (Cat. no. C10447) is a stand-alone reagent that may be combined with a variety of azide-modified detection reagents and other related reagents.

Figure 1 Mechanism of action of Click-iT® LAA

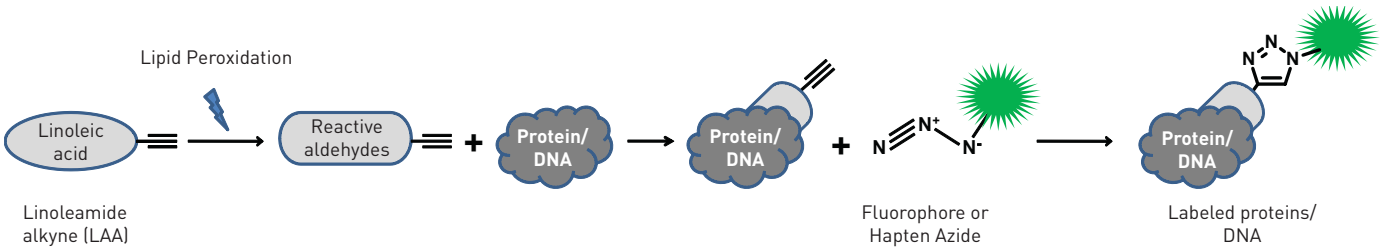


Figure 2 Workflow for Click-iT® LAA lipid peroxidation detection

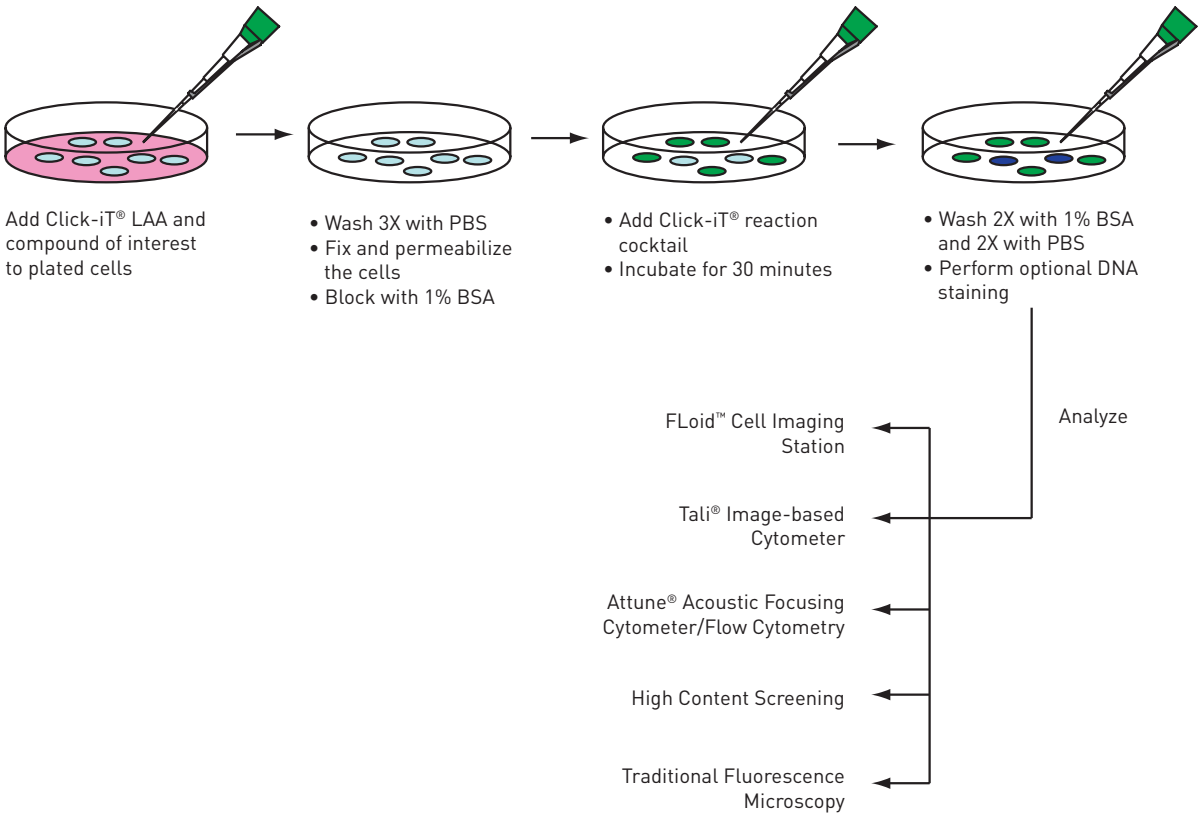
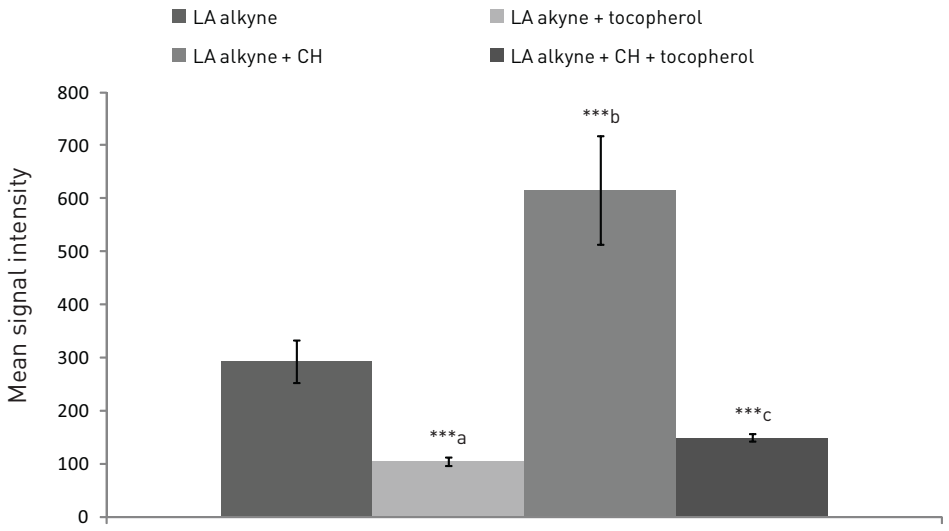


Figure 3 BPAE cells were plated on 35-mm glass bottom dishes (MatTek) and incubated in complete growth medium at 37°C. For tocopherol treatment, the cells were pre-treated with 150 µM α-tocopherol for 30 minutes. The cells were then treated with vehicle (DMSO) or 100 µM cumene hydroperoxide (CH) and then immediately fed with 50 µM linoleamide alkyne and incubated for 2 hours at 37°C. The cells were then fixed with 4% formaldehyde for 15 minutes at room temperature. The cells were washed 3X with PBS and then permeabilized with 0.05% Triton® X-100 for 10 minutess. The cells were then blocked with 1% BSA for 30 minutes. The cells were washed and the click reaction was performed with 5 µM Alexa Fluor® 488 azide for 30 minutes. The cells were washed 1X with 1% BSA and 2X with PBS, and then imaged on a Zeiss Axiovert inverted microscope using a 40X objective. The signal intensity was quantitated using SlideBook™ 5.0 software. The intensity values were background subtracted and plotted. The statistical analysis was performed by Students t-test (***a = the values are significantly different from controls without tocopherol with P ≤0.0001; ***b = values were significantly different from drug treated cells with P ≤0.0001 and ***c = values were significantly different from drug treated and tocopherol treated samples).



Before Starting

Materials required but not provided

- Cells and culture medium
- Phosphate buffered saline (PBS, pH 7.2–7.6)
- Fixation/Permeabilization solution (e.g., Image-iT® Fixation/Permeabilization Kit)
  or
  Fixative (i.e., 3.7% formaldehyde in PBS) and permeabilization solution (i.e., 0.5% Triton® X-100)
- Deionized water

Caution

DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Always wear suitable laboratory protective clothing and gloves when handling this reagent.

Cumene hydroperoxide (Component F) is corrosive and toxic in contact with skin, and fatal if inhaled. Use appropriate precautions and always wear suitable laboratory protective clothing and gloves when handling this reagent. Cumene hydroperoxide is flammable; keep away from heat, sparks, open flames, and hot surfaces.

Preparing stock solutions

1.1 To prepare a 50-mM Click-iT® LAA stock solution, add 25 µl of anhydrous DMSO to a vial of Click-iT® LAA (Component A) and mix it well.

Note: Click-iT® LAA is provided as clear neet oil, and might not be visible at the bottom of vial.

Caution: The Click-iT® LAA is supplied in an oxygen-scavenging pouch. Make sure to put all unused vials immediately back in the pouch and then seal the pouch.

1.2 To prepare a 10X stock solution of Click-iT® Buffer Additive, add 2 mL of deionized water to the bottle of Click-iT® Buffer Additive (Component E) and mix well. When stored at –20°C, this stock solution is stable for 1 year. If the solution develops a brown color, it has degraded and should be discarded.

1.3 To prepare a 100-mM (1000X) stock solution of cumene hydroperoxide, add 1 µL of cumene hydroperoxide (Component F, 5.4 M) to 54 µL of 100% ethanol.

Caution: Cumene hydroperoxide is toxic. Use appropriate precautions when using this compound.

Preparing working solutions

Prepare these solutions fresh and use on the same day.

2.1 To prepare 1X working solution of Click-iT® Reaction Buffer (Component C), add 1 mL of the buffer to 10 mL of deionized water.

2.2 To prepare 1X working solution of Click-iT® Buffer Additive (Component E), add 1 mL of the 10X stock solution (prepared in step 1.2) to 10 mL of deionized water.

Experimental Protocols

Labeling the cells with Click-iT® LAA

The following protocol was developed using BPAE, RAW, HepG2, and U-2OS cells with an optimized concentration of 50 µM Click-iT® LAA, but it can be adopted to any other cell type. Growth medium, cell density, and cell type variation can influence labeling with Click-iT® LAA. In initial experiments, we recommend using a range of Click-iT® LAA concentrations to determine the optimal concentration for your cell type and experimental conditions.

Following protocol can be easily adapted for 96-well or other plate formats by proportionally adjusting the volumes to the format used.

3.1 Grow the cells and let them recover overnight at 37°C.

3.2 Add Click-iT® LAA stock solution (prepared in step 1.1) to the cells in complete growth medium at a final concentration of 50 µM (i.e., a dilution of 1000X).

3.3 Treat the cells with the compound of interest for the desired time. For positive controls with cumene hydroperoxide (CH), add CH (Component F in Cat. no. C10446) to a final concentration of 100 µM and incubate for 2 hours.

Note: The compound of interest can be added immediately after the addition of Click-iT® LAA to the growth medium.

3.4 Wash the cells three times with PBS to remove free Click-iT® LAA from the cells.

3.5 Proceed immediately to cell fixation and permeabilization, page 5.

Cell fixation and permeabilization

This protocol was optimized with a fixation step using 3.7% formaldehyde in PBS, followed by a 0.5% Triton® X-100 permeabilization step. Alternatively, you may use the Image-iT® Fixation/Permeabilization Kit following the instructions supplied with the kit.

4.1 For convenient processing, transfer the coverslips into a 6-well plate such that each well contain a single coverslip.

4.2 After incubation, remove the media and add 1 mL of 3.7% formaldehyde in PBS to each well containing the coverslips. Incubate for 15 minutes at room temperature.

4.3 Remove the fixative and wash the cells in each well three times with 1 mL of PBS.

4.4 Remove the wash solution. Add 1 mL of 0.5% Triton® X-100 in PBS to each well, then incubate at room temperature for 10 minutes.

4.5 Block by adding 1% BSA in PBS solution to the cells and incubating at room temperature for 30 minutes.

Click-iT® LAA detection

This protocol uses 500 µL of Click-iT® reaction cocktail per coverslip. A smaller volume can be used as long as the remaining reaction components are maintained at the same ratios.

5.1 Prepare Click-iT® reaction cocktail according to Table 2, below. It is important to add the ingredients in the order listed in the table; otherwise, the reaction will not proceed optimally. Use the Click-iT® reaction cocktail within 15 minutes of preparation.

Table 2 Click-iT® reaction cocktails

Reaction components*	Number of coverslips							96-well plate
	1	2	4	5	10	25	50	1 plate
1X Click-iT® reaction buffer (prepared in step 2.1)	430 µL	860 µL	1.8 mL	2.2 mL	4.3 mL	10.7 mL	21.4 mL	10.3 mL
$CuSO_4$ (Component D)	20 µL	40 µL	80 µL	100 µL	200 µL	500 µL	1 mL	480 µL
Alexa Fluor® 488 azide (Component B)	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL	30 µL
1X Click-iT® buffer additive (prepared in step 2.2)	50 µL	100 µL	200 µL	250 µL	500 µL	1.25 mL	2.5 mL	1.2 mL
Total volume	500 µL	1 mL	2 mL	2.5 mL	5 mL	12.5 mL	25 mL	12 mL

*__Note__: Add the ingredients in the order listed in the table.

5.2 Remove the blocking solution (step 4.5) and wash the cells twice with PBS to completely remove the BSA before the click reaction.

5.3 Add 0.5 mL of Click-iT® reaction cocktail to each well containing a coverslip (add 125 µL, if using a 96-well plate). Rock the plate briefly to insure that the reaction cocktail is distributed evenly over the coverslip.

5.4 Incubate the plate for 30 minutes at room temperature, protected from light.

5.5 Remove the reaction cocktail, then wash each well twice with 1% BSA in PBS and twice with PBS only. Remove the wash solution.

For nuclear staining, proceed to DNA staining. If no additional staining is desired, proceed to imaging and analysis.

5.6 *Optional*: Perform antibody labeling of the samples at this time, following the recommendations from the manufacturer of the primary and secondary antibody. It is important to keep the samples protected from light during incubations.

DNA staining

6.1 Wash each well with 1 mL of PBS. Remove the wash solution.

6.2 Dilute the Hoechst 33342 dye to a final concentration is 5 µg/mL.

Note: A range between 2–10 µg/mL of Hoechst 33342 has been shown to work.

6.3 Add 1 mL of 5 µg/mL Hoechst 33342 solution per well. Incubate for 30 minutes at room temperature, protected from light. Remove the Hoechst 33342 solution.

6.4 Wash each well twice with 1 mL of PBS. Remove the wash solution.

6.5 Proceed to imaging and analysis.

Reference

1. Chem Res Toxicol 23, 557 (2010).

Product List **Current prices may be obtained at www.invitrogen.com or from our Customer Service Department.**

Catalog no.	Product Name	Unit Size
C10446	Click-iT® Lipid Peroxidation Imaging Kit – Alexa Fluor® 488	1 kit
C10447	Click-iT® LAA (Linoleamide alkyne) *for lipid peroxidation detection*	5 × 20 µL
Related Products		
C10422	CellROX® Deep Red Reagent *for oxidative stress detection*	5 × 50 µL
C10423	CellEvent® Caspase-3/7 Green Detection Reagent *2 mM solution in DMSO*	100 µL
C10443	CellROX® Orange Reagent *for oxidative stress detection*	5 × 50 µL
C10444	CellROX® Green Reagent *for oxidative stress detection*	5 × 50 µL
C10448	CellROX® Reagent Variety Pack *for oxidative stress detection*	1 kit
C6827	CM-H_2DCFDA (5-(and-6)-chloromethyl- 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) *mixed isomers* *special packaging*	5 × 50 µg
A14291DJ	Live Cell Imaging Solution	500 mL
A10266	Alexa Fluor® 488 azide (Alexa Fluor® 488 5-carboxamido-(6-azidohexanyl), bis(triethylammonium salt))	0.5 mg
A20012	Alexa Fluor® 555 azide, triethylammonium salt	0.5 mg
A10270	Alexa Fluor® 594 azide (Alexa Fluor® 594 carboxamido-(6-azidohexanyl), bis(triethylammonium salt))	0.5 mg
A10277	Alexa Fluor® 647 azide, triethylammonium salt	0.5 mg
B10184	Biotin azide	1 mg
I10188	Iodoacetamide azide	1 mg
O10180	Oregon Green® 488 azide (Oregon Green® 488 6-carboxamido-(6-azidohexanyl), triethylammonium salt)	0.5 mg
T10182	Tetramethylrhodamine (TAMRA) azide (tetramethylrhodamine 5-carboxamido-(6-azidohexanyl)) *5-isomer*	0.5 mg
R37602	Image-iT® Fixation/Permeabilization Kit	1 kit
R37603	BackDrop™ Background Suppressor *for live cells*	1 kit
R37605	NucBlue™ Live Cell Stain *Hoechst 33342 special formulation*	1 kit
R37606	NucBlue™ Fixed Cell Stain *DAPI special formulation*	1 kit

Related Platforms

Attune® Acoustic Focusing Cytometer
(Cat. no. 4469120)

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(Cat. no. T10796)

FLoid™ Cell Imaging Station
(Cat. no. 4471136)

Purchaser Notification

Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
USA
Phone: +1 760 603 7200
Fax: +1 760 602 6500
Email: techsupport@lifetech.com

European Headquarters
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF
UK
Phone: +44 141 814 6100
Toll-Free Phone: 0800 269 210
Toll-Free Tech: 0800 838 380
Fax: +44 141 814 6260
Tech Fax: +44 141 814 6117
Email: euroinfo@invitrogen.com
Email Tech: eurotech@invitrogen.com

Japanese Headquarters
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Japan
Phone: +81 3 5730 6509
Fax: +81 3 5730 6519
Email: jpinfo@invitrogen.com

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