Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay

Catalog no. C10289

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage*	Stability
Click-iT® AHA (L-azidohomoalanine) reagent (Component A)	30 µL	50 mM in DMSO	• ≤–20˚C • Desiccate • Protect from light	When stored as directed this product is stable for 1 year.
Click-iT® AHA supermix** (Component B)	21.6 mL	Not applicable		
Click-iT® AHA buffer additive (Component C)	200 mg			
Hoechst 33342 (Component D)	25 µL	10 mg/mL in water		
*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage of each component, see vial labels. **Component B contains Alexa Fluor® 488 alkyne.				
Number of assays: Sufficient material is supplied for 2 × 96-well plates, based on the protocol below.				
Approximate fluorescence excitation/emission maxima: Alexa Fluor® 488 alkyne: 495/519 in nm; Hoechst 33342: 350/461 in nm bound to DNA.				

Introduction

The ability to detect and characterize newly synthesized proteins or changes in protein expression resulting from disease, drug treatments, or environmental changes is an important parameter in cytotoxicity measurements. The Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay provides a fast, sensitive, non-toxic, and non-radioactive method for the detection of nascent protein synthesis[1,2] utilizing fluorescence microscopy and high-throughput imaging (HCS).

Click-iT® AHA (L-azidohomoalanine) is an amino acid analog of methionine containing an azide moiety (Figure 1). Similar to ^{35}S-methionine, Click-iT® AHA is added to cultured cells and the amino acid is incorporated into proteins during active protein synthesis. Detection of the incorporated amino acid utilizes a chemoselective ligation or click reaction between an azide and alkyne, where the azido-modified protein is detected with an Alexa Fluor® 488 alkyne.

The Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay has been successfully tested in A549 and U-2 OS cells with a variety of reagents that inhibit protein synthesis including the translational elongation inhibitor, cycloheximide, the peptidyl transferase inhibitor, anisomycin, and the protein synthesis inhibitor, puromycin in dose response (Figure 2) and Min/Max format (Table 2). The product contains sufficient reagents to perform assays for 2 plates in a 96-well plate format. For larger quantities, inquire at www.invitrogen.com.

Table 2. Assay reproducibility from Min/Max plates*.

Cell type	CV of treated samples
U-2 OS	9.5 ± 3.8%
A549	12.1 ± 4.6%

*Cells were treated in L-methionine-free media containing 50 µM AHA and either 150 µM cyclohexamide or DMSO for 30 minutes. Cells were washed, fixed, permeabilized, and nascent protein synthesis was detected following a click reaction with Alexa Fluor® 488 alkyne. Imaging and analysis was performed using the Thermo Fisher Scientific Cellomics® ArrayScan® VTI platform. The average and standard deviation were calculated of the CV's obtained from treated samples (Max) for 9 Min/Max plates.

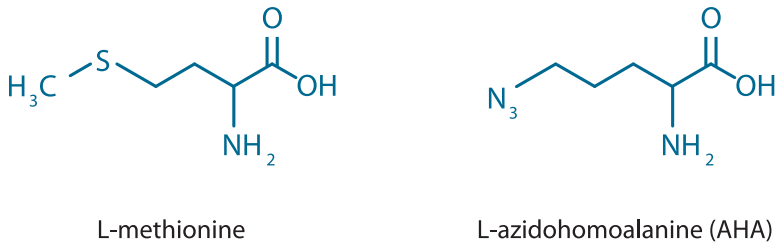

Figure 1. Structures of L-methionine and Click-iT® AHA (L-azidohomoalanine).

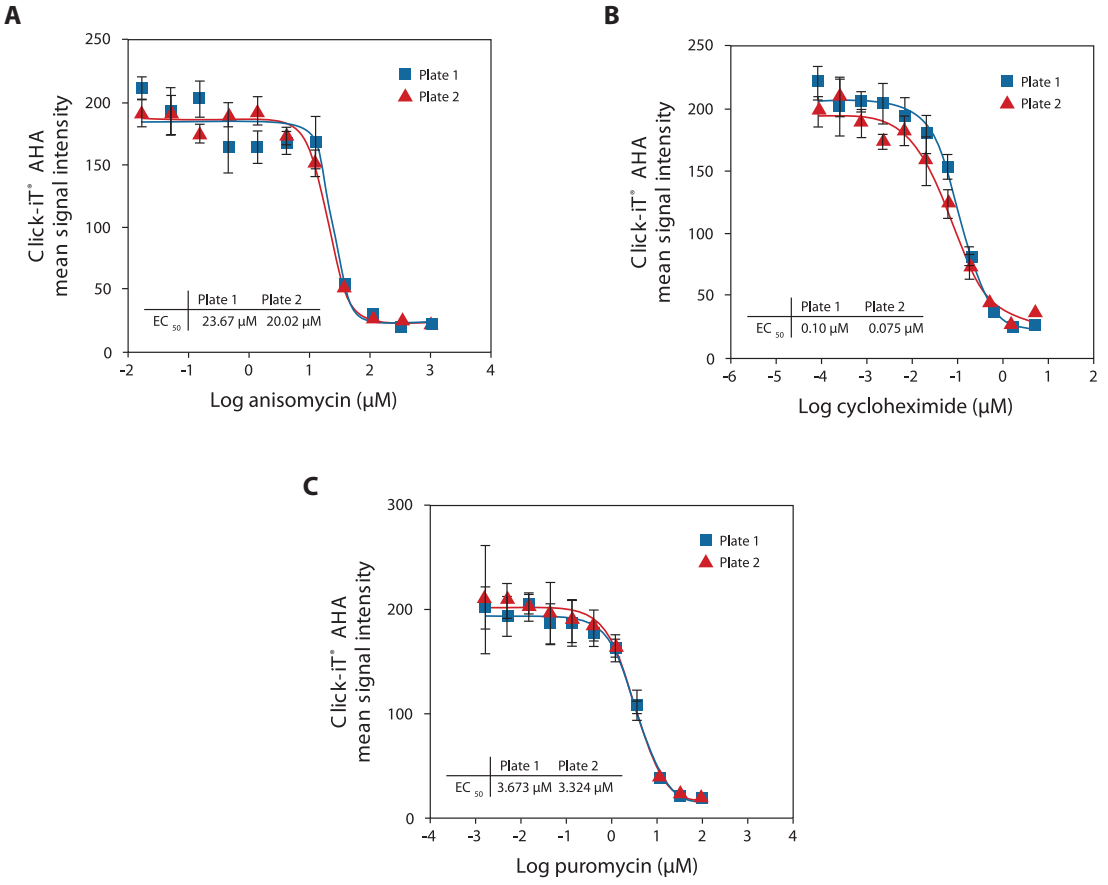

Figure 2. Dose response curves performed in duplicate. U-2 OS cells were treated in L-methionine-free media and 50 µM Click-iT® AHA for 30 minutes with anisomycin (17 pM–1 µM), cycloheximide (85 pM–5 µM), or puromycin (1.7 nM–100 µM). Cells were washed, fixed, permeabilized, and nascent protein synthesis was detected following a click reaction with Alexa Fluor® 488 alkyne. Imaging and analysis was performed using the Thermo Fisher Scientific Cellomics® ArrayScan® VTI platform.

Before Starting

Materials Required but Not Provided

- 96-well plates (as recommended for the specific imaging instrument)
- PBS (phosphate buffered saline, Invitrogen Cat. no. 70011-044 or 70011-069)
- 3.7% formaldehyde in PBS
- 0.5% Triton® X-100 in PBS
- 3% Bovine serum albumin (BSA) in PBS (3% BSA in PBS), pH 7.4
- Deionized water or 18 megaohm purified water
- Methionine-free media (examples are listed below)
  - Dulbecco's Modified Eagle Medium (D-MEM contains 4,500 mg/L D-glucose, without L-glutamine, sodium pyruvate, L-methionine, and L-cystine, Invitrogen Cat. no. 21013)
  - RPMI Medium 1640, contains no L-methionine (Invitrogen Cat. no. 0050001DJ)

Caution

- Hoechst 33342 (Component D) is a known mutagen. Use the dye with appropriate precautions.
- DMSO (in Component A), 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. Always wear protective laboratory clothing and gloves when handling this reagent.

Preparing Stock Solutions

1.1 Allow vials to completely thaw and warm to room temperature before opening.

Note: Component B may take several hours to thaw the solution due to the large vial size.

1.2 Prior to use, briefly centrifuge Click-iT® AHA reagent (Component A) and Hoechst 33342 (Component D) to maximize reagent recovery.

1.3 To make a 10X stock solution of the Click-iT® AHA buffer additive (Component C), add 1 mL deionized water to the vial and mix until completely dissolved. After use, store any remaining stock solution at ≤–20°C. When stored as directed, this stock solution is stable for up to 1 year.

1.4 Add any missing, but necessary amino acids for cell culture to the L-methionine-free medium.

Experimental Protocols

Labeling Cells with AHA

The following protocols were developed with A549 and U-2 OS cells, using an optimized Click-iT® AHA reagent (Component A) concentration of 50 µM in L-methionine-free medium supplemented to contain 200 µM L-cystine, 2 mM L-glutamine, and 10 mM HEPES, but can be adapted for any adherent cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. For initial experiments, we recommend testing a range of Click-iT® AHA reagent concentrations to determine the optimal concentration for your cell type and experimental conditions. Although sufficient material is included for standard dose response studies, additional Click-iT® AHA (L-azidohomoalanine,

Invitrogen Cat. no. C10102) is available.

Method 1—Drug Pre-incubation

2.1 Plate cells at desired density and allow cells to recover overnight before additional treatment.

2.2 Treat cells as desired.

2.3 Prepare a working stock solution of Click-iT® AHA (Component A) by diluting 1:1,000 in pre-warmed L-methionine-free medium (prepared in step 1.4) for a 50 µM final working solution.

2.4 Remove drug-containing medium (not methionine-free) and add 100 µL/well of medium with 50 µM Click-iT® AHA working solution.

2.5 Incubate for 30 minutes under conditions optimal for the cell type.

2.6 Proceed to **Cell Fixation and Permeabilization** followed by **Click-iT® AHA Detection**.

Method 2—Click-iT® AHA and Drug Co-incubation

3.1 Plate cells at desired density and allow cells to recover overnight before additional treatment.

3.2 Prepare a working stock solution of Click-iT® AHA (Component A) by diluting 1:1,000 in pre-warmed L-methionine-free medium (prepared in step 1.4) for a 50 µM final working solution.

3.3 Add desired drug to working stock solution of Click-iT® AHA (prepared in step 3.2).

3.4 Remove medium and add 100 µL/well of medium with 50 µM Click-iT® AHA and the drug.

3.5 Incubate for 30 minutes under conditions optimal for cell type.

3.6 Proceed to **Cell Fixation and Permeabilization** followed by **Click-iT® AHA Detection**.

Cell Fixation and Permeabilization

This protocol is optimized with a fixation step using 3.7% formaldehyde in PBS followed by a permeabilization step using 0.5% Triton® X-100, but is amenable to other fixation/permeabilization reagents such as ethanol and methanol.

4.1 After incubation, remove medium containing Click-iT® AHA and wash cells once with PBS. Remove PBS.

4.2 Add 100 µL/well 3.7% formaldehyde in PBS. Incubate for 15 minutes at room temperature. Remove fixative.

4.3 Wash cells twice with 3% BSA in PBS. Remove the wash solution.

4.4 Add 100 µL/well 0.5% Triton® X-100 in PBS and incubate for 20 minutes at room temperature.

Click-iT® AHA Detection

Table 3. Click-iT® reaction cocktails.

Reaction Components	Number of plates		
	0.5	1	2
Click-iT® supermix (Component B)	5.0 mL	10 mL	20 mL
Click-iT® AHA buffer additive (prepared in step 5.1)	0.6 mL	1.2 mL	2.4 mL
Total volume	5.6 mL	11.2 mL	22.4 mL

5.1 Prepare 1X Click-iT® AHA buffer additive (Table 3) by diluting the 10X solution (prepared in step 1.3) 1:10 in deionized water. Prepare this solution **fresh** and use the solution on the same day.

5.2 Prepare Click-iT® reaction cocktail according to Table 3.

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

5.3 Remove the permeabilization buffer (step 4.4) and wash cells twice with 100 µL/well 3% BSA in PBS. Remove the wash solution.

5.4 Add 100 µL/well Click-iT® reaction cocktail (prepared in step 5.2) to each well and mix well.

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

5.6 Remove the reaction cocktail and wash wells once with 3% BSA in PBS.

DNA Counterstaining

6.1 Dilute Hoechst 33342 (Component D) solution 1:1,000 in PBS to obtain a 1X Hoechst 33342 solution (final concentration is 10 µg/mL).

Note: A range between 2–10 µg/mL of Hoechst 33342 is recommended.

6.2 Remove the wash solution.

6.3 Add 100 µL/well 1X Hoechst 33342 solution. Incubate for 30 minutes at room temperature, **protected from light**. Remove the Hoechst 33342 solution.

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

Imaging and Analysis

7.1 Add PBS to each well. Seal the plate with plate sealing film, if desired.

7.2 Scan plate using automated imaging platform with filters appropriate for DAPI/Hoechst and FITC. Nascent protein synthesis is assessed by determining signal intensity in the FITC channel in the ring around the nucleus as defined by Hoechst.

References

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

Cat. no.	Product Name	Unit Size
C10289	Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay *2-plate size*	1 kit
Related Products		
C10202	Click-iT® AHA (L-azidohomoalanine) *for nascent protein synthesis*	5 mg
C10045	CellMask™ Orange plasma membrane stain *5 mg/mL solution in DMSO*	100 µL
C10046	CellMask™ Deep Red plasma membrane stain *5 mg/mL solution in DMSO*	100 µL
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
H10295	HCS Mitochondrial Health Kit *2-plate size*	1 kit
H32711	HCS CellMask™ Red cytoplasmic/nuclear stain *5 mM solution in DMSO* *for high content screening* *for cellular imaging*	125 µL
H34558	HCS CellMask™ Blue cytoplasmic/nuclear stain *for high content screening* *for cellular imaging*	1 set
H34560	HCS CellMask™ Deep Red cytoplasmic/nuclear stain *for high content screening* *for cellular imaging*	1 set
H34157	HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high content screening* *for cellular imaging* *2-plate size*	1 kit
H34158	HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high content screening* *for cellular imaging* *2-plate size*	1 kit
H34350	HCS LipidTOX™ Green phospholipidosis detection reagent *1000X aqueous solution* *for cellular imaging* 10-plate size*	each
H34351	HCS LipidTOX™ Red phospholipidosis detection reagent *1000X aqueous solution* *for cellular imaging* 10-plate size*	each
H34475	HCS LipidTOX™ Green neutral lipid stain *solution in DMSO* *for cellular imaging*	each
H34476	HCS LipidTOX™ Red neutral lipid stain *solution in DMSO* *for cellular imaging*	each
H34477	HCS LipidTOX™ Deep Red neutral lipid stain *solution in DMSO* *for cellular imaging*	each
I10291	Image-iT® DEAD Green™ viability stain *1 mM solution in DMSO*	each
70011-044	Phosphate-buffered saline (PBS) 7.4 (10X) liquid	500 mL
70011-069	Phosphate-buffered saline (PBS) 7.4 (10X) liquid	10 × 500 mL
21013-024	Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (high glucose)	500 mL
0050001DJ	RPMI Medium 1640 (Custom)	500 mL

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