molecular probes®
by life technologies™

Click-iT® HPG Alexa Fluor® Protein Synthesis Assay Kits

Catalog nos. C10428, C10429

Table 1 Contents and storage

Material	C10428	C10429	Concentration	Storage*
Click-iT® HPG (Homopropargylglycine) reagent (Component A)	30 µL	30 µL	50 mM in water	• 2–8°C • Desiccate • Protect from light • DO NOT FREEZE
Alexa Fluor® azide (Component B)	70 µL (Alexa Fluor® 488)	70 µL (Alexa Fluor® 594)	DMSO solution	
Click-iT® HPG reaction buffer (Component C)	4 mL	4 mL	10X solution in Tris-buffered saline	
Copper (II) Sulfate ($CuSO_4$) (Component D)	1 vial	1 vial	100 mM aqueous solution	
Click-iT® reaction buffer additive (Component E)	400 mg	400 mg	N/A**	
Click-iT® reaction rinse buffer (Component F)	25 mL	25 mL	1X	
NuclearMask™ Blue Stain (Component G)	25 µL	25 µL	2000X	

*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage of each component, see vial labels. When stored as directed this product is stable for 1 year. **Not applicable.

Approximate fluorescence excitation/emission maxima: Alexa Fluor® 488 azide: 495/519 in nm; Alexa Fluor® 594 azide: 590/617 in nm.

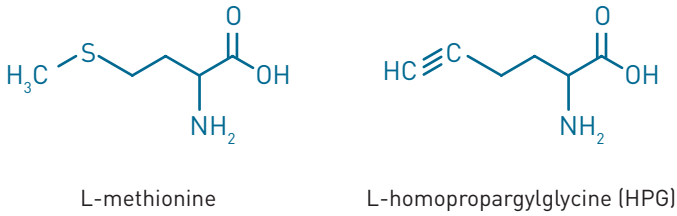
Introduction

The ability to detect and characterize newly synthesized proteins, changes in protein expression, or protein degradation resulting from disease, drug treatments, or environmental changes is an important parameter in cytotoxicity measurements. The Click-iT® HPG Alexa Fluor® Protein Synthesis Assays provide a fast, sensitive, non-toxic, and non-radioactive method for the detection of nascent protein synthesis[1–4] utilizing fluorescence microscopy and high-throughput imaging (HCS).

Click-iT® HPG (L-homopropargylglycine) is an amino acid analog of methionine containing an alkyne moiety (Figure 1, page 2). Similar to ^{35}S-methionine, Click-iT® HPG 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 alkyne-modified protein is detected with either Alexa Fluor® 488 or Alexa Fluor® 594 azide.

The Click-iT® HPG Alexa Fluor® Protein Synthesis Assays have been successfully tested in A549, HeLa, and U-2 OS cells with a variety of reagents that inhibit protein synthesis including the translational elongation inhibitor cycloheximide (Figures 2 and 3). In addition, drugs that inhibit protein clearance, such as MG134 or Bortezomib, show a dose-dependent inhibition of clearance (Figure 4). The product contains sufficient reagents to perform assays for 25 coverslips or 2 plates in a 96-well plate format. For larger quantities, inquire at www.lifetechnologies.com.

Figure 1 Structures of L-methionine and Click-iT® HPG (L-homopropargylglycine)



L-methionine L-homopropargylglycine (HPG)

Figure 2 HPG is incorporated into synthesizing proteins and the inhibition of the protein decreases the Click-iT® HPG signal in U-2 OS cells. The images below show Click-iT® HPG fluorescence (Alexa Fluor® 488 and Alexa Fluor® 594) in U-2 OS cells in the presence of either the vehicle or the test compound (cyclohexamide or ansiomycin) and the graph quantifies the minimum and maximum Click-iT® HPG signal in these cells.

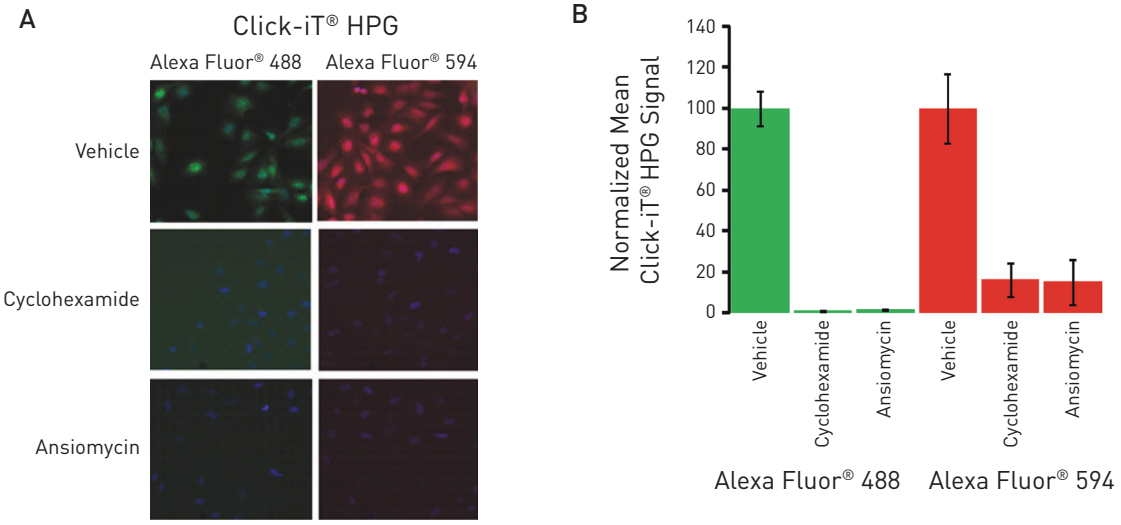


Figure 3 Using Click-iT® HPG to monitor the inhibition of the protein synthesis with structurally unrelated molecules. The dose-dependent decrease was monitored using the Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit using two drugs and three different cell types.

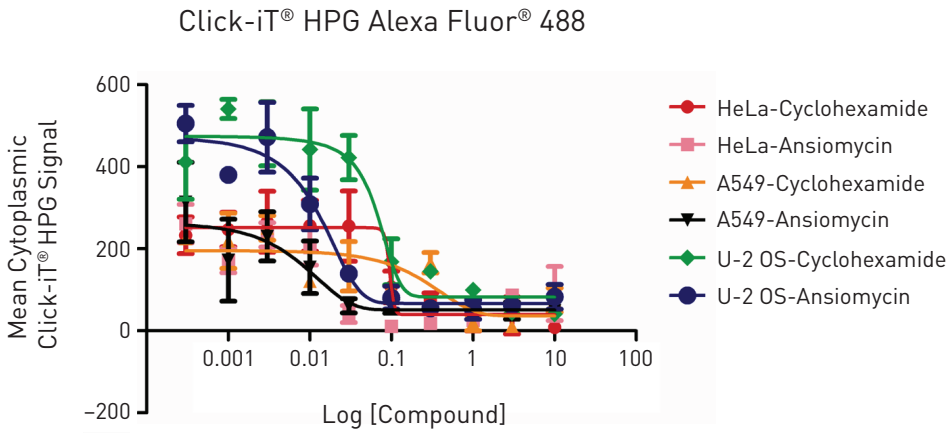
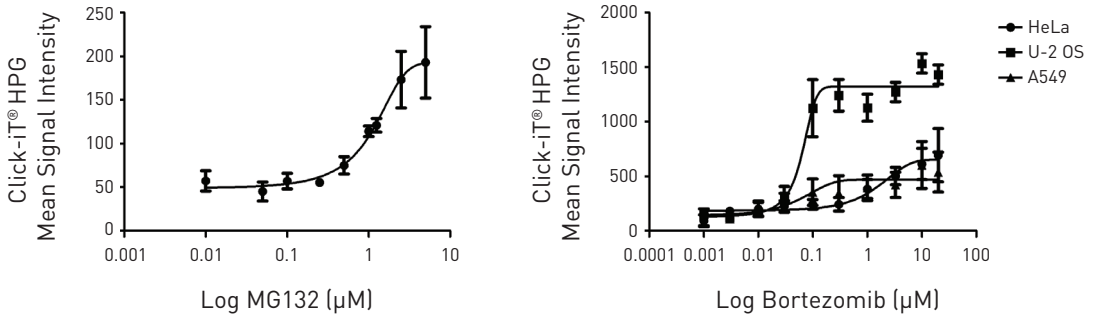


Figure 4 Inhibition of the proteasome leads to a dose-dependent increase the Click-iT® HPG signal. Blockade of protein clearance via the proteasome with either MG132 (left) or Bortezomib (right) causes a dose-dependent accumulation of HPG-labeled proteins in HeLa (triangle), A549 (circle), and U 2-0S cells (square).



Before Starting

Materials Required but Not Provided

- 96-well plates (as recommended for the specific imaging instrument)
- PBS (phosphate buffered saline, 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, Cat. no. 21013)
  - RPMI Medium 1640, contains no L-methionine (Cat. no. 0050001DJ)

Caution

- NuclearMask™ Blue Stain (Component G) is a known mutagen. Use the dye with appropriate precautions.
- DMSO (in Component B), 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® HPG reagent (Component A) and NuclearMask™ Blue Stain (Component G) to maximize reagent recovery.

1.3 To make a 10X stock solution of the Click-iT® HPG reaction buffer additive (Component E), add 2 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 Prepare 40 mL of 1X Click-iT® HPG reaction buffer by transferring all of the solution (4 mL) in the Component C bottle to 36 mL of deionized water. Rinse the Component C bottle with some of the diluted Click-iT® HPG Reaction buffer to ensure the transfer of all of the 10X concentrate.

Note: To prepare smaller amounts of 1X Click-iT® HPG reaction buffer, dilute the volumes from the Component C bottle 1:10 with deionized water. After use, store any remaining 1X solution at 2–8°C. When stored as directed, 1X Click-iT® HPG reaction buffer is stable for 6 months.

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

Experimental Protocols

Labeling Cells with HPG

The following protocols were developed with A549 and U-2 OS cells, using an optimized Click-iT® HPG 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® HPG 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® HPG (Cat. no. C10186) 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® HPG (Component A) by diluting 1:1000 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 1 mL per coverslip or 100 µL/well of medium with 50 µM Click-iT® HPG working solution.

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

2.6 Proceed to **Cell Fixation and Permeabilization** (page 5) followed by **Click-iT® HPG Detection** (page 5).

Method 2—Click-iT® HPG 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® HPG (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® HPG (prepared in step 3.2).

3.4 Remove medium and add 1 mL per coverslip or 100 µL/well of medium with 50 µM Click-iT® HPG 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® HPG 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® HPG and wash cells once with PBS. Remove PBS.

4.2 Add 1 mL per coverslip or 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 1 mL per coverslip or 100 µL/well of 0.5% Triton® X-100 in PBS and incubate for 20 minutes at room temperature.

Click-iT® HPG Detection

5.1 Prepare 1X Click-iT® HPG reaction buffer additive (Table 2, page 6) 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 2 (page 6).

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 1 mL per coverslip or 100 µL/well 3% BSA in PBS. Remove the wash solution.

5.4 Add 1 mL per coverslip or 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 once with 1 mL per coverslip or 100 µL per well of Click-iT® reaction rinse buffer (Component F). Remove the Click-iT® reaction rinse buffer.

Table 2 Click-iT® reaction cocktails

Reaction Components	Amount needed (for 2 plates or 25 coverslips)
1X Click-iT® HPG reaction buffer (prepared in step 1.4)	21.5 mL
Copper (II) Sulfate ($CuSO_4$) (Component D)	1.0 mL
Alexa Fluor® azide (Component B)	62.5 µL
1X Click-iT® HPG buffer additive (prepared in step 5.1)	2.5 mL
Total volume	25 mL

DNA Staining

The following protocol based upon 50 µL of HCS NuclearMask™ Blue Stain working solution per well.

6.1 Dilute HCS NuclearMask™ Blue Stain (Component G) solution 1:2000 in PBS to obtain a 1X HCS NuclearMask™ Blue Stain working solution.

6.2 Remove the wash solution.

6.3 Add 1 mL per coverslip or 100 µL/well of 1X HCS NuclearMask™ Blue Stain working solution (prepared in step 6.1). Incubate for 30 minutes at room temperature, **protected from light**.

6.4 Remove the HCS NuclearMask™ Blue Stain solution and wash twice with PBS. Remove the wash solution and proceed to **Imaging and Analysis** (below).

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 for Alexa Fluor® 488 or Texas Red® for Alexa Fluor® 594. Nascent protein synthesis is assessed by determining signal intensity in the fluorescent channel in the ring around the nucleus as defined by NuclearMask™ Blue Stain.

References

1. Science 332, 966 (2011); 2. Nature Neurosci 13, 897 (2010); 3. Angew Chem Int Ed Eng 45, 7364 (2006); 4. Bioorg Med Chem Letter 18, 5995 (2008).

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

Cat. no.	Product Name	Unit Size
C10428	Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit	1 kit
C10429	Click-iT® HPG Alexa Fluor® 594 Protein Synthesis Assay Kit	1 kit
Related Products		
C10202	Click-iT® HPG (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
C10186	Click-iT® HPG (L-homopropargylglycine) *for nascent protein synthesis*	5 mg
C10289	Click-iT® HPG Alexa Fluor® 488 Protein Synthesis HCS Assay *2-plate size*	1 kit
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* *10-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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