Click-iT® RNA HCS Assays

Catalog nos. C10327, C10328

Table 1. Contents and storage information.

Material	C10327	C10328	Concentration	Storage*	Stability
5-ethynyl uridine (EU) (Component A)	11.3 mg	11.3 mg	NA	• 2–6°C • Desiccate • Protect from light • DO NOT FREEZE	When stored as directed, this kit is stable for at least 1 year.
Alexa Fluor® azide (Component B)	1 vial (Alexa Fluor® 488 azide)	1 vial (Alexa Fluor® 594 azide)			
Click-iT® RNA reaction buffer (Component C)	25 mL	25 mL	1X		
Copper (II) sulfate ($CuSO_4$) (Component D)	1 mL	1 mL	100 mM aqueous solution		
Click-iT® reaction buffer additive (Component E)	400 mg	400 mg	NA		
Click-iT® reaction rinse buffer (Component F)	25 mL	25 mL	1X		
HCS NuclearMask™ Blue stain (Component G)	25 µL	25 µL	2,000X aqueous solution		

*These storage conditions are appropriate when storing the entire kit upon receipt. After preparing stock solutions, optimal storage conditions may change. For storing prepared stock solutions, follow recommendations included in this product information sheet.
NA = Not applicable.

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 azide**:** 495/519 nm; Alexa Fluor® 594 azide: 590/615 nm; HCS NuclearMask™ Blue stain: 350/461 nm, bound to DNA.

Introduction

The Click-iT® RNA HCS Assay enables detection of global RNA synthesis temporally and spatially in cells and tissues.[1] The ability to detect newly synthesized RNA or changes in RNA levels resulting from disease, environmental damage, or drug treatments is an important aspect of toxicological profiling. Utilizing an alkyne-modified nucleoside, 5-ethynyl uridine (EU), and powerful click chemistry, newly synthesized RNA can be detected without the use of radioactivity or antibodies with a simple, two-step procedure. In step one, the alkyne-containing nucleoside is fed to cells or animals, and is actively incorporated into nascent RNA. The small size of the tag enables efficient incorporation of the modified nucleoside into RNA, but not into DNA. Detection utilizes the chemoselective ligation or "click" reaction

between an azide and an alkyne where the modified RNA is detected with a corresponding azide-containing dye (Figure 1). With its dimunitive "footprint", the Click-iT® detection molecule can easily penetrate complex samples and leaves open the possibility of multiplex analyses with other probes, including antibodies for the detection of RNA-interactive proteins for deeper biological insights.

Click reactions have several general characteristics: the reaction is efficient, no extreme temperatures or solvents are required, the reaction is complete within 30 minutes, the components of the reaction are bioinert, and perhaps most importantly, no side reactions occur — the label and detection tags react selectively and specifically with one another.[2–5] This final point is a key advantage of this powerful detection technique; it is possible to apply click chemistry-labeled molecules to complex biological samples and detect them with unprecedented sensitivity due to extremely low background.

The kits contain all of the components needed to label and detect newly synthesized RNA in whole cells (Figures 2–3). The kits are also supplied with HCS NuclearMask™ blue stain as a nuclear counterstain for cell demarcation or for DNA profiling. The kits include sufficient reagents for labeling two 96-well plates using 50 µL reaction volume per well. For larger quantities, inquire at www.invitrogen.com.

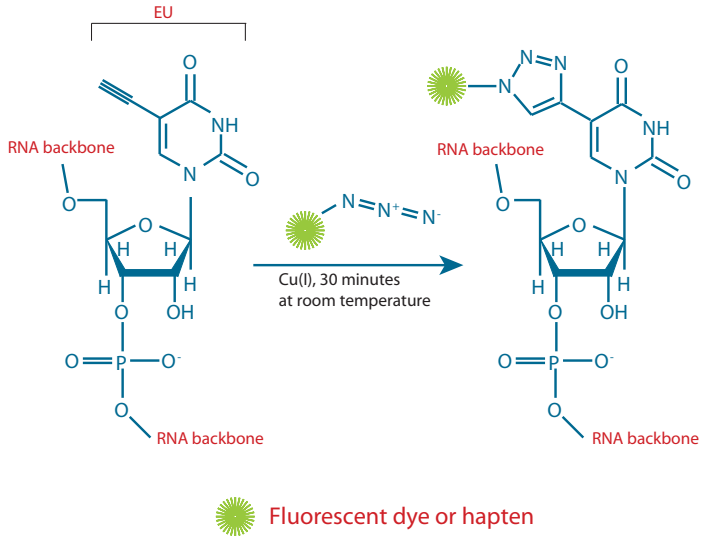

Figure 1. Click reaction between EU and azide-modified dye or hapten..

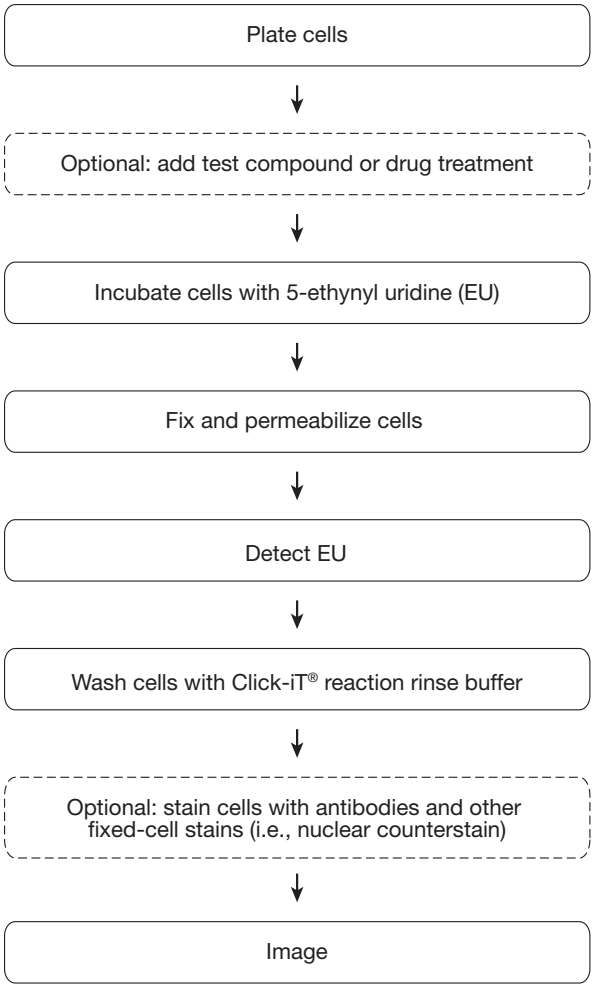

Figure 2. Workflow diagram for the Click-iT® RNA Imaging Assay.

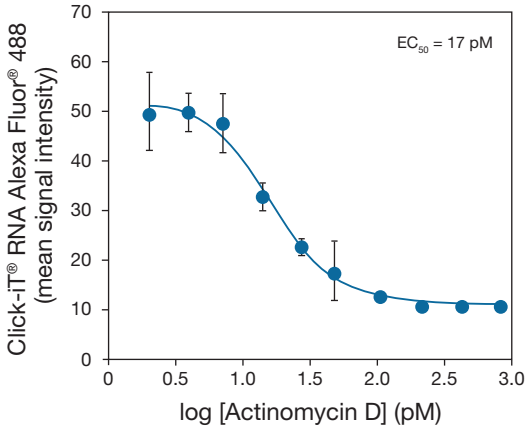

Figure 3. Dose response for actinomycin D in HeLa cells using the Click-iT® RNA Assay. HeLa cells were treated with the indicated amounts of actinomycin D for 18 hours, followed by a 1 hour incubation with 5-ethynyl uridine (EU). Cells were then fixed and permeabilized, and EU incorporated into newly synthesized RNA was detected using the green-fluorescent Alexa Fluor® 488 azide. Quantitative analysis was performed using the Thermo Scientific Cellomics® ArrayScan® VTI and Compartmental Analysis Bioapplication.

Table 2. Click-iT® detection reagent compatibility.

Molecule	Compatibility*
Qdot® nanocrystals	Use Qdot® nanocrystals **after** the Click-iT® detection reaction.
Fluorescent proteins such as Green Fluorescent Protein (GFP)	Use organic dye-based reagents, such as TC-FlAsH™ or TC-ReAsH™ reagents, for protein expression detection or anti-GFP rabbit or chicken antibodies **before** the Click-iT® detection reaction.
Organic dyes such as Alexa Fluor® dyes, fluorescein (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.
Phalloidin	Phalloidin staining is **not compatible** with the Click-iT® detection reaction. Use antibodies against other proteins, such as anti-α-tubulin, for visualization of the cytoskeleton.
Horseradish peroxidase (HRP)	Use HRP **after** the Click-iT® detection reaction.
R-phycoerythrin (R-PE) and R-PE-tandems such as Alexa Fluor® 680-R-PE	Use R-PE and R-PE tandems **after** the Click-iT® detection reaction.
Allophycocyanin (APC) and APC-tandems	**Completely compatible** with the Click-iT® detection reaction.
*Compatibility indicates whether the fluorescent molecule itself or the detection method involves components that are unstable in the presence of copper catalyst used for the Click-iT® detection reaction.	

Before You Begin

Materials Required but Not Provided

- 96-well plates (as recommended for the specific imaging instrument)
- Phosphate buffered saline (PBS), pH 7.2–7.6
- Fixative (*i.e.*, 3.7% Formaldehyde in PBS)
- Permeabilization reagent (*i.e.*, 0.1% Triton® X-100 in PBS)
- Deionized water
- Dimethylsulfoxide (DMSO)

Cautions

HCS NuclearMask™ Blue stain (Component G) is a known mutagen. Use the dye with appropriate precautions.

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.

Preparing the Stock Solutions

1.1 To prepare a 100 mM stock solution of EU (Component A), add 421 µL of deionized water to the vial and mix until fully dissolved.

After use, store any remaining stock solution at ≤–20˚C. When stored as directed, this stock solution is stable for up to 1 month.

Note: Additional EU is available separately from Invitrogen (Cat. no. E10345).

1.2 To prepare a stock solution of the Alexa Fluor® azide:

- Add 94 µL of DMSO to Alexa Fluor® 488 vial (Component B, Cat. no. C10327), and mix well by pipetting or vortexing.
- Add 85 µL of DMSO to Alexa Fluor® 594 vial (Component B, Cat. no. C10328), and mix well by pipetting or vortexing.

After use, store any remaining stock solution at ≤–20˚C. When stored as directed, this stock solution is stable for up to 1 year.

1.3 To prepare a 10X stock solution of the Click-iT® reaction buffer additive (Component E), add 2 mL of deionized water to the vial and mix until fully 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. If the solution develops a brown color, it has degraded and should be discarded.

Experimental Protocols

Labeling Cells with EU

The following protocol was developed with HeLa and NIH3T3 cells, plated at 40–50% confluency in a 96-well microplate, and fed the following day with 1 mM EU for 1 hour. Growth medium, cell density, cell type variations, and other factors may influence the assay conditions. In initial experiments, we recommend testing a range of EU concentrations to determine the optimal concentration for your cell type and experimental conditions. Sufficient material is supplied for two 96-well microplate assays based upon the protocol below. Additional EU is available separately from Invitrogen (Cat. no. E10345).

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

2.2 *Optional*: Perform cell treatments as desired. To address the potential reversibility of drug action on RNA synthesis, we recommend that you do **not** remove drug-containing media during EU treatment.

2.3 Prepare a 2X working solution of EU from the 100 mM stock solution (prepared in step 1.1) in **pre-warmed** complete medium. For example, for a 1 mM final EU treatment, prepare a 2 mM working solution. Add an equal volume of this 2X EU working solution to the media containing cells.

2.4 Incubate under normal cell culture conditions for 1 hour. The optimal incubation time depends on the cell growth rate and cell treatment, and needs to be determined experimentally.

2.5 Proceed immediately to **Cell Fixation, Permeabilization, and Click-iT® Detection**.

Cell Fixation, Permeabilization, and Click-iT® Detection

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

3.1 After EU incubation, remove media and add 50 µL of 3.7% formaldehyde in PBS to each well. Incubate for 15 minutes at room temperature.

3.2 Remove the fixative and wash each well once with PBS.

Note: If necessary, the samples can be stored overnight at 4°C.

3.3 Remove the wash solution and add 50 µL of 0.5% Triton® X-100 in PBS to each well. Incubate for 15 minutes at room temperature.

3.4 Prepare a working solution of Click-iT® reaction buffer additive by diluting the 10X solution (prepared in step 1.3; see Table 3 for volumes) 1:10 in deionized water. Prepare this solution **fresh** and use the solution on the same day.

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

Note: Add the components of the Click-iT® reaction cocktail in the order listed in Table 3, and use the cocktail immediately after preparation. The Click-iT® reaction buffer additive is susceptible to oxidation and is the limiting factor to the Click-iT® reaction cocktail's effectiveness over time.

Table 3. Click-iT® reaction cocktails.

Reaction components	Number of plates		
	0.5	1	2
Click-iT® reaction buffer (Component C)	2.14 mL	4.28 mL	8.56 mL
$CuSO_4$ (Component D)	100 µL	200 µL	400 µL
Alexa Fluor® azide (prepared in step 1.2)	9.4 µL	18.8 µL	37.5 µL
Click-iT® reaction buffer additive (prepared in step 3.4)	250 µL	500 µL	1.0 mL
Approximate total volume	2.5 mL	5 mL	10 mL

3.6 Remove the permeabilization buffer (step 3.3) and wash each well once with PBS.

3.7 Remove the wash solution and add 50 µL of Click-iT® reaction cocktail (prepared in step 3.5) to each well. Incubate for 30 minutes at room temperature, **protected from light**.

3.8 Remove the Click-iT® reaction cocktail and wash each well once with 50 µL of Click-iT® reaction rinse buffer (Component F). Then remove the Click-iT® reaction rinse buffer.

3.9 *Optional*: Perform antibody labeling on 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.

For DNA staining and cell cycle distribution, proceed to **DNA Staining**. If no additional staining is desired, proceed to **Imaging and Analysis**.

DNA Staining

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

4.1 Wash each well with PBS, then remove the wash solution.

4.2 Dilute HCS NuclearMask™ Blue stain (Component G) solution 1:1,000 in PBS.

4.3 Add 50 µL of diluted HCS NuclearMask™ Blue stain working solution to each well, and incubate for 15 minutes at room temperature, **protected from light**..

4.4 Remove the HCS NuclearMask™ Blue stain solution, and wash each well twice with PBS.

4.5 Remove the wash solution and add PBS to each well. If desired, seal the plate and proceed to **Imaging and Analysis**.

Imaging and Analysis

Scan the plate using an automated imaging platform equipped with filters appropriate for DAPI/Hoechst and FITC, or Texas Red® dyes. The nuclear region is characterized by the HCS NuclearMask™ Blue signal in the Hoechst channel. You may assess nascent RNA by determining signal intensity in the nuclear region using the FITC channel (Alexa Fluor® 488-labeled EU, Cat. no. C10327) or the Texas Red® channel (Alexa Fluor® 594-labeled EU, Cat. no. C10328).

When using the Thermo Scientific Cellomics® ArrayScan® VTI platform, use the Compartmental Analysis BioApplication. In channel 1, define the nuclear region with HCS NuclearMask™ Blue signal (the segmentation tool) as objects with Hoechst/XF93 or Hoechst/XF53 filters. In channel 2, assess the nuclear spot fluorescence intensity of Alexa Fluor® 488- or Alexa Fluor® 594-labeled EU with the FITC/XF93 filters or the Texas Red®/XF93 filters, respectively.

See Table 4 for the appropriate fluorescence excitation/emission maxima for Alexa Fluor® dyes and HCS NuclearMask™ Blue stain.

Table 4. Approximate fluorescence excitation/emission maxima.

Fluorophore	Excitation (nm)	Emission (nm)
Alexa Fluor® 488	495	519
Alexa Fluor® 594	590	615
HCS NuclearMask™ Blue stain, bound to DNA	350	461

References

1. Proc Natl Acad Sci USA 105, 15779 (2008); **2.** Chem Bio Chem 4, 1147 (2003); **3.** J Am Chem Soc 124, 3192 (2003); **4.** Angew Chem Int Ed Engl 41, 2596 (2002); **5.** 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
C10327	Click-iT® RNA Alexa Fluor® 488 HCS Assay *2-plate size*	1 kit
C10328	Click-iT® RNA Alexa Fluor® 594 HCS Assay *2-plate size*	1 kit
Related Products		
C10289	Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay *2-plate size*	1 kit
C10329	Click-iT® RNA Alexa Fluor® 488 Imaging Kit *for 25 coverslips*	1 kit
C10330	Click-iT® RNA Alexa Fluor® 594 Imaging Kit *for 25 coverslips*	1 kit
C10350	Click-iT® EdU Alexa Fluor® 488 HCS Assay *2-plate size*	1 kit
C10351	Click-iT® EdU Alexa Fluor® 488 HCS Assay *10-plate size*	1 kit
C10352	Click-iT® EdU Alexa Fluor® 555 HCS Assay *2-plate size*	1 kit
C10353	Click-iT® EdU Alexa Fluor® 555 HCS Assay *10-plate size*	1 kit
C10354	Click-iT® EdU Alexa Fluor® 594 HCS Assay *2-plate size*	1 kit
C10355	Click-iT® EdU Alexa Fluor® 594 HCS Assay *10-plate size*	1 kit
C10356	Click-iT® EdU Alexa Fluor® 647 HCS Assay *2-plate size*	1 kit
C10357	Click-iT® EdU Alexa Fluor® 647 HCS Assay *10-plate size*	1 kit
E10345	5-ethynyl uridine (EU)	5 mg
H10294	HCS NuclearMask™ Deep Red stain *250X concentrate in DMSO*	400 µL
H10325	HCS NuclearMask™ Blue stain *for 10 × 96-well plates* *2000X concentrate*	65 µL
H10326	HCS NuclearMask™ Red stain *for 10 × 96-well plates* *1000X concentrate*	125 µL

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