molecular probes®
by life technologies™

Click-iT® Plus Alexa Fluor® Picolyl Azide Toolkit

Catalog nos. C10641, C10642, C10643

Table 1 Contents and storage

Material	Amount	Concentration	Storage*
Alexa Fluor® picolyl azide (Component A)	100 µg	1 vial, lyophilized solid	• 2–8°C • Desiccate • Protect from light • DO NOT FREEZE
Click-iT® reaction buffer (Component B)	4 mL	10X solution in Tris-buffered saline, pH 7.4	
$CuSO_4$ (Component C)	1.5 mL	100 mM aqueous solution	
Copper protectant (Component D)	0.5 mL	100 mM $CuSO_4$ plus chelate in aqueous solution	
Click-iT® buffer additive (Component E)	400 mg	1 vial, lyophilized solid	

*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions for each component, see vial labels. When stored as directed, the product is stable for up to 1 year after receipt.

Number of assays: Sufficient material is supplied for at least 30 cell or tissue assays based on the cocktail protocol below (see Tables 5 and 6) at a total reaction volume of 0.5 mL.

Approximate fluorescence excitation/emission maxima, in nm: Alexa Fluor® 488 picolyl azide (Cat. no. C10641): 495/519; Alexa Fluor® 555 picolyl azide (Cat. no. C10642): 555/565; Alexa Fluor® 647 picolyl azide (Cat. no. C10643): 650/670.

Introduction

The Click-iT® Plus kits minimize the deleterious effects of copper in click reactions by the use of a modified azide and a copper protectant. These two components permit the utilization of a much lower copper concentration without sacrificing reaction efficiency. The amount of copper required for a classic click reaction limits the utility of this labeling method for multiplexing applications or for labeling live cells. Free copper ions, in either valency or as a redox agent, can inhibit the activity of various enzymes (e.g., horseradish peroxidase), denature proteins, cleave the phosphodiester bonds of nucleic acid strands, promote the precipitation of various biomolecules, or generate radicals (e.g., reactive oxygen species, ROS) by numerous pathways. Copper can also quench quantum dots, fluorescent proteins, and various organic dyes (e.g., Phen Green™ dye and calcein).

The picolyl azide incorporates a copper-chelating group to the azide structure to allow co-diffusion and concentrate copper at the reaction site. The copper protectant includes a chelator that limits the concentration of free copper ions, accelerates the cycloaddition reaction, and acts as a non-toxic reducing agent to limit further reduction of copper, thus limiting the generation of reactive oxygen species (ROS). The copper protectant is

an aqueous solution of 100 mM copper sulfate plus chelator; this solution provides the lowest level of free copper ions necessary for the click reaction. To moderate the amount of free copper ions in the click reaction, Click-iT® Plus Alexa Fluor® picolyl azide toolkits also provide copper sulfate ($CuSO_4$) separately. A classic click reaction would utilize copper sulfate alone. By adjusting the ratio of copper and copper protectant, one may control the amount of free copper in the click reaction and optimize it for the sample (see Table 5, page 7).

The Click-iT® Plus Alexa Fluor® picolyl azide toolkits are compatible with PerCP, phycobiliproteins (R-PE, APC, and tandems), and fluorescent proteins such as GFP, RFP, and mCherry (see Table 2, below). Click-iT® Plus labeling can be also multiplexed with dye- or enzyme-conjugated antibodies against surface and intracellular markers.

Table 2 Compatibility of Click-iT® Plus and classic Click-iT® reactions with various fluorescent molecules and labeling methods

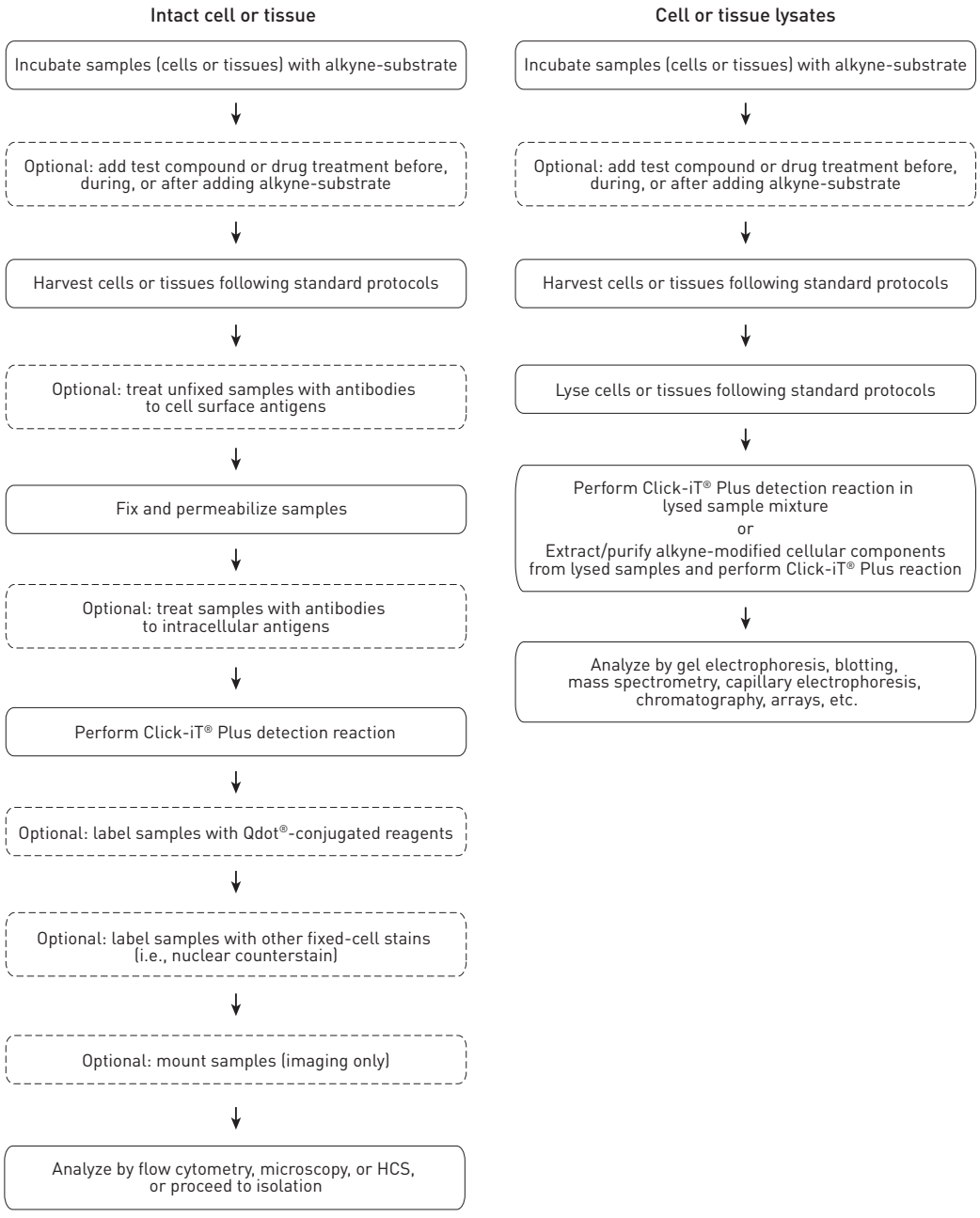
Molecule	Click-iT® Plus compatibility*	Classic Click-iT® compatibility*
Fluorescent proteins such as GFP, RFP, and mCherry	Compatible	Not compatible
Horseradish peroxidase (HRP)	Use HRP **after** the Click-iT® Plus detection reaction, because azide inhibits HRP activity.	Use HRP **after** the Click-iT® detection reaction, because azide inhibits HRP activity.
Organic dyes such as Alexa Fluor® dyes, fluorescein, and Cy® dyes (Cy®3, Cy®5, etc.)	Compatible	Compatible
PerCP and PerCP tandems such as PerCP-Cy®5.5	Compatible	Compatible
Phalloidin	Phalloidin staining is compatible with the Click-iT® Plus detection reaction **only with low free copper concentration** in the final reaction mix (i.e., maximum modulation).	Not compatible
Allophycocyanin (APC) and APC-tandems such Alexa Fluor® 750-APC.	Compatible	Compatible
R-phycoerythrin (R-PE) and R-PE-tandems such as Alexa Fluor® 680-R-PE	Compatible	Use R-PE and R-PE tandems **after** the Click-iT® detection reaction.
Qdot® nanocrystals	Use Qdot® nanocrystals **after** the Click-iT® Plus detection reaction.	Use Qdot® nanocrystals **after** the Click-iT® Plus detection reaction.

* Compatibility indicates whether the fluorescent molecule itself or the detection method involves components that are unstable in the presence of copper used in the Click-iT® Plus detection reaction.

Experiment workflows

The Click-iT® Plus Alexa Fluor® picolyl azide toolkits can be used for labeling and detecting alkyne-tagged molecules of interest in intact cultured cells and tissue samples, as well as lysates (Figure 1, below).

Figure 1 Workflow diagrams for Click-iT® Plus detection reaction using intact cell/tissue samples or lysates



Guidelines for Use

Final concentration of Alexa Fluor® picolyl azide

For click labeling, final concentrations of Alexa Fluor® picolyl azide (PCA) may range from 0.5 µM to 5 µM. Final concentrations below or above this range are also possible. The final concentration of Alexa Fluor® PCA should be optimized per the specific application. We recommend starting with a final concentration of 5 µM Alexa Fluor® PCA, and titrating this amount down in case of high background.

Free copper level in the reaction

The Click-iT® Plus kits allow the modulation of free copper levels in the click reaction by varying the amounts of $CuSO_4$ (Component C) and copper protectant (Component D), thus minimizing the deleterious effects of copper without sacrificing reaction efficiency. The amount of free copper required for an efficient click reaction is sample-dependent and should be optimized for each sample type.

To optimize the amount of free copper in the click reaction for your sample, adjust the ratio of $CuSO_4$ (Component C) and copper protectant (Component D) in the reaction cocktail, using Table 3, below, as a general guideline and starting point. A classic click reaction would contain $CuSO_4$ (Component C) alone in the reaction cocktail without any copper protectant (0% modulation, maximum copper level), whereas a click reaction requiring moderate amounts of free copper would use a $CuSO_4$-to-copper protectant ratio of 2:1 (~30% modulation, medium copper level). Refer to Table 5 (page 7) to prepare the $CuSO_4$-copper pre-mix that offers the desired level of free copper modulation in the final click reaction cocktail.

Table 3 Recommended levels of free copper and copper protectant in the Click-iT® Plus detection reaction

Free copper concentration		
Low	**Medium**	**High**
DNA synthesis	DNA synthesis	DNA synthesis
Oligonucleotides	Nascent RNA imaging	Nascent RNA imaging
Nascent RNA imaging	Peptides	Peptides
RNA isolation	Proteins	Proteins
Peptides	APC and APC-tandems	APC and APC-tandems
Proteins	RPE and RPE-tandems	Small molecules
APC and APC-tandems	GFP	
RPE and RPE-tandems	Small molecules	
GFP		
Phalloidin		
Small molecules		
High	**Medium**	**Low**
Copper protectant level		

Final reaction volume

Final reaction volumes may be scaled up or down depending on the total number of labeling reactions or the amount of sample to be labeled. Table 6 (page 8) provides an example of a single click reaction with a total reaction volume of 500 µL that would be suitable for a monolayer of adherent cells on an 18 × 18-mm coverslip or for 100 µL of suspension cells at a cell density of 1×10^7 cells/mL.

Sample treatment

Samples may be treated with drugs, chemicals or other treatments before, during or after the addition of the alkyne-substrate. If adding any drug or chemical additive at the same time with the alkyne substrate, these reagents should not degrade, bind to or promote precipitation of the alkyne substrate.

Permeabilization

Permeabilization is not recommended for fatty acid or lipid alkyne substrates that may be incorporated either into the plasma membrane, organelle membranes or lipid droplets/inclusion bodies. Exposure to detergents (i.e., Triton® X-100, saponin), surfactants (i.e., Tween), acetone, and alcohol (including glycerol in mounting media) may promote dissociation of lipophilic cellular entities.

Before You Begin

Materials required but not provided

- DMSO (dimethylsulfoxide)
- Deionized water
- Coverslips/microscope slides, culture dishes, microplates, or tubes to culture cells and to perform subsequent extraction, purification, and the click reaction.

  For imaging, coverslips/slides or other surfaces should be treated to promote attachment of cells/tissues. Examples of suitable surface treatments include APTES, poly-lysine, gelatin, etc.

- Alkyne-substrate (see Product List, page 9) added to media/buffer or normal saline:
  - » For cultured cells, suitable growth medium (e.g., HPG requires methionine-free media)
  - » For *in vitro* assay, suitable reaction buffer for enzymatic incorporation
  - » For *in vivo* injection, normal saline or other delivery solution

For cultured cells or tissue processing

- Fixative (e.g., 3.7% Formaldehyde in PBS)
- Blocking agent such as 1–5% Bovine serum albumin (Fraction V, defatted BSA) in PBS, pH 7.4, or 5–10% animal serum in PBS, pH 7.4
- Wash buffer such as PBS, HBSS, or TBS (pH 7.2–7.6)
- *Optional:* Permeabilization reagent (e.g., 0.5% Triton® X-100 in PBS, saponin)

  Note: Permeabilization reagent is not required for surface labeling or labeling of lipid components.

- *Optional:* Labeling reagents such as antibodies, avidin/streptavidin, or stains, as well as suitable diluents
- *Optional:* Mounting medium (for imaging)

For extraction/purification of the alkyne-incorporated cellular components or from *in vitro* reactions

- Solvents, buffers, and other components required to extract/purify the component of interest such as lysis buffers, detergents, imidazole, NH_4Cl, RNase or RNase inhibitors, DNase or Benzonase, trypsin (protease digestion) or protease inhibitors, sonicators, homogenizers, disruption beads, chloroform/phenol, hexane or similar organic solvents, alcohol, chromatography column resins and buffers, gel electrophoresis materials, blotting materials, etc.

IMPORTANT! For any cellular or non-cellular processing during the click reaction and after the attachment of the dye-azide, avoid extremes of pH, high salt concentrations, strong oxidizing or reducing agents, heavy metals, and quenching agents.

Handling and disposal

Dispose of materials in compliance with all applicable local, state, and federal regulations. For more information on the composition of these materials, consult the Safety Data Sheets (SDSs), which are available at **www.lifetechnologies.com/sds**.

Be sure to take the appropriate precautions (wear a laboratory coat, disposable gloves, and eye protection) when handling cells and tissue samples. Dispose of cells and tissue samples as biohazardous waste.

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 DMSO-containig reagents in compliance with all pertaining local regulations

Prepare solutions

Alexa Fluor® picolyl azide stock solution (500 µM)

1.1 Prepare a 500 µM stock solution of the Alexa Fluor® picolyl azide (PCA) (Component A) by adding the appropriate volume of anhydrous DMSO to Component A (see Table 4, below), and mix well. After use, store any remaining stock solution at ≤–20°C. When stored as directed, this stock solution is stable for up to 1 year.

Note: For applications requiring lower dye concentrations, dilute the Alexa Fluor® PCA stock solution accordingly.

Table 4 Volume of DMSO required for a 500 µM Alexa Fluor® picolyl azide (PCA) stock solution

Reagent	Nominal molecular weight	Amount of DMSO
Alexa Fluor® 488 PCA	~950	210 µL
Alexa Fluor® 555 PCA	~1350	148 µL
Alexa Fluor® 647 PCA	~1370	146 µL

IMPORTANT! When using live cells, minimize DMSO and copper concentrations in the final reaction cocktail.

Click-iT® Plus reaction buffer working solution (1X)

1.2 To prepare a working solution of 1X Click-iT® Plus reaction buffer (Component B), transfer 4 mL of the solution in the Component B bottle to 36 mL of deionized water (see Note below).

After use, store the 1X Click-iT® reaction buffer solution at 2–8°C. When stored as directed, the 1X solution is stable for 6 months.

Note: To prepare smaller amounts of 1X Click-iT® reaction buffer, dilute the appropriate volume from the Component B bottle 1:10 with deionized water. Store undiluted 10X Click-iT® reaction buffer at 2–8°C. The 10X solution is stable for 1 year.

Click-iT® buffer additive stock solution (10X)

1.3 To prepare a 10X stock solution of the Click-iT® buffer additive (Component E), add 2 mL of deionized water to the vial, then mix until fully dissolved. After use, store any remaining stock solution at ≤–20°C.

When stored as directed, the 10X stock solution is stable for up to 1 year. If the solution develops a brown color, it has degraded, and should be discarded.

$CuSO_4$-copper protectant pre-mix

We recommend pre-mixing the $CuSO_4$ (Component C) and copper protectant (Component D) before adding them to the Click-iT® Plus reaction cocktail (page 8) to obtain the desired level of free copper modulation for your sample in the final reaction. Note that the amount of free copper required for an efficient click reaction is sample-dependent and should be optimized for each sample type.

1.4 Prepare $CuSO_4$ and copper protectant pre-mix according to Table 5, below. For general guidelines and recommendations on the levels of free copper and copper protectant in the final Click-iT® Plus reaction cocktail for various sample types and assays, refer to Table 3 (page 4).

Table 5 Recommended levels of free copper and copper protectant in the Click-iT® Plus reaction

Component	Free copper level										
	Low			Medium					High		
$CuSO_4$ (Component C)	none	1 µL	2 µL	3 µL	4 µL	5 µL	6 µL	7 µL	8 µL	9 µL	10 µL
Copper protectant (Component D)	10 µL	9 µL	8 µL	7 µL	6 µL	5 µL	4 µL	3 µL	2 µL	1 µL	none
	High (100–80%)			Medium (70–30%)					Low (20–0%)		
	Copper protectant level										

Experimental Protocols

Label cells with alkyne substrate

In initial experiments, we recommend testing a range of alkyne substrate concentrations to determine the optimal concentration for your cell type and experimental conditions. Growth medium, cell density, cell type variations, and other factors may influence labeling.

2.1 Grow cells in the appropriate culture medium under optimal conditions for cell growth. Disturbing the cells by temperature changes or washing prior to incubation with the alkyne substrate may alter the cellular incorporation of the substrate.

2.2 Add the alkyne substrate to the culture medium at the desired final concentration and mix well. For *in vivo* applications, deliver the alkyne-substrate with a suitable delivery solvent (normal saline, buffer, etc.).

For a negative staining control, include cells from the same population that have not been treated with the alkyne substrate.

2.3 Incubate the cells for the desired length of time under conditions optimal for the cell type and experimental conditions.

2.4 Harvest cells or tissues per standard methods. For paraffin-embedded samples, deparaffinize samples and equilibrate into PBS or other physiological buffers prior to the click reaction.

Fix and permeabilize cells

The protocol below provides guidelines for fixation using 3.7% formaldehyde in PBS, followed by permeabilization with 0.5% Triton® X-100 reagent. However, other fixation/permeabilization protocols with reagents such as methanol and saponin can also be used.

3.1 *Optional:* If desired, treat unfixed sample with antibodies against cell surface antigens.

3.2 Remove media from sample and add an appropriate amount 3.7% formaldehyde in PBS. Incubate for 15 minutes at room temperature.

3.3 Remove the fixative and wash sample twice with 3% BSA in PBS.

3.4 Remove the wash solution and add an appropriate amount of 0.5% Triton® X-100 in PBS and incubate for 20 minutes at room temperature.

Perform Click-iT® Plus detection reaction

The following steps describe the preparation of the Click-iT® Plus reaction cocktail and the subsequent Click-iT® Plus detection reaction. We recommend pre-mixing the $CuSO_4$ and copper protectant (see page 7) before adding them to the Click-iT® Plus reaction cocktail to obtain the desired level of free copper in the final reaction. Note that the amount of free copper required for an efficient click reaction is sample-dependent and should be optimized for each sample type.

4.1 Prepare 1X Click-iT® buffer additive by diluting the 10X solution (prepared in step 1.3) 1:10 in deionized water. Prepare only as much of this solution as necessary for that day's experiments, and use on the same day. For details, see Table 6, below.

4.2 Prepare $CuSO_4$ and copper protectant pre-mix as described on page 7.

4.3 Prepare the Click-iT® Plus reaction cocktail according to Table 6, below, adding the reaction components **in the order given**. Note that the volumes given in Table 6 are for a single reaction volume of 500 µL using a final concentration of 5 µM Alexa Fluor® PCA. For other reaction volumes, adjust the volumes up or down accordingly. For multiple samples of the same type, you may prepare a master mix.

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

Table 6 Click-iT® Plus reaction cocktail per a single reaction volume of 500 µL using a final concentration of 5 µM Alexa Fluor® PCA

Component*	Volume
1X Click-iT® reaction buffer (prepared in step 1.2)	435 µL
500 µM Alexa Fluor® PCA solution (prepared in step 1.1)	5 µL
$CuSO_4$-copper protectant pre-mix (prepared in step 1.4)	10 µL
1X Click-iT® buffer additive (prepared in step 4.1)	50 µL
Total volume	500 µL

* Add the reaction components in the order listed in the table.

4.4 Remove the permeabilization buffer (step 3.4) and wash sample twice with 3% BSA in PBS. Remove the wash solution.

4.5 Add the Click-iT® Plus reaction cocktail to the sample. Ensure that the reaction cocktail is evenly distributed over the sample.

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

4.7 Remove the reaction cocktail and wash sample once with 3% BSA in PBS. Remove the wash solution.

4.8 If desired, label sample with Qdot®-conjugated reagents or other fixed-cell stains (e.g., nuclear counterstain) following manufacturer's recommendations.

If no further staining is desired, proceed to analyzing the sample by flow cytometry, microscopy, or HCS methods, or isolate the labeled sample for analysis by gel electrophoresis, blotting, mass spectrometry, capillary electrophoresis, chromatography, arrays, etc.

References

1. ChemBioChem 4, 1147 (2003); **2.** J Am Chem Soc 125, 3192 (2003); **3.** Angew Chem Int Ed Engl 41, 2596 (2002); **4.** Angew Chem Int Ed Engl 40, 2004 (2001); **5.** Proc Natl Acad Sci 105, 2415 (2008); **6.** J Am Chem Soc 130, 11576 (2008); **7.** Proc Natl Acad Sci 103, 9482 (2006); **8.** Angewandte Comm 51, 5852 (2012).

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

Cat. no.	Product Name	Unit Size
C10641	Click-iT® Plus Alexa Fluor® 488 picolyl azide toolkit	1 kit
C10642	Click-iT® Plus Alexa Fluor® 555 picolyl azide toolkit	1 kit
C10643	Click-iT® Plus Alexa Fluor® 647 picolyl azide toolkit	1 kit
Related Products		
A10044	EdU (5-ethynyl-2'-deoxyuridine)	50 mg
C10186	Click-iT® HPG (L-homopropargylglycine) *for nascent protein synthesis*	5 mg
C10264	Click-iT® fucose alkyne (tetraacetylfucose alkyne)	5 mg
C10447	Click-iT® LAA (Linoleamide alkyne) *for lipid peroxidation detection*	5 × 0.4 mg
C10459	OPP (O-propargyl-puromycin)	5 mg
D12345	DMSO, anhydrous	10 × 3 mL
E10187	EdU (5-ethynyl-2'-deoxyuridine)	500 mg
E10345	5-ethynyl uridine (EU)	5 mg

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