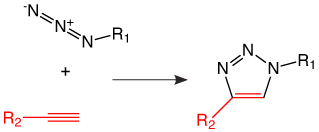
***Antonio Manetto, Simon Warncke and Thomas Frischmuth  
baseclick GmbH  
Bahnhofstrasse 9 – 15  
82327 Tutzing, Germany***[***info@baseclick.eu***](mailto:info@baseclick.eu)

***Note: All products of baseclick described in this article are patent protected and available from Glen Research Corporation, 22825 Davis Drive, Sterling, VA 20164, USA, email: support@glenres.com, in collaboration with baseclick.***

**INTRODUCTION**

In 2010, we published an article in The [**Glen Report, Volume 22, No 1**](http://www.glenresearch.com/GlenReports/GR22-1.pdf), describing the technology that baseclick has offered for Click Chemistry. In the present article, we review advances since that time and specifically highlight our new Oligo-Click Kits, which are designed to make conventional Click reactions much more user-friendly. At the same time, we position these techniques in comparison to Cu-Free Click.

The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is the most prominent example of a group of reactions named click-reactions, as shown below.



These reactions are characterized by high yields, mild reaction conditions, and by their tolerance of a broad range of functional groups.1 Typically, the reactions require simple or no workup, or purification of the product. The most important characteristic of the CuAAC reaction is its unique bio-orthogonality, as neither azide nor terminal alkyne functional groups are generally present in natural systems.

The use of this method for DNA modification has been somewhat delayed by the fact that copper ions damage DNA, typically yielding strand breaks.2 As these problems have now been overcome by the use of copper(I)-stabilizing ligands (e.g., tris(benzyltriazolylmethyl)amine, TBTA3), Carell et al. and Seela et al. discovered that the CuAAC reaction can be used to functionalize alkyne-modified DNA nucleobases with extremely high efficiency.4

In comparison to the common post synthetic labeling methods of oligonucleotides like amine/NHS-ester, thiol/iodoacetamide or maleimide labeling, modification of oligonucleotides with Click Chemistry is providing by far the highest conjugation efficiency.6

Single and multiple labeling can be performed with as little as two equivalents of label-azides resulting in complete conversion and high yields of labeled oligo. In addition, the marker azides used for click functionalization are stable to hydrolysis which allows storage in solution (in contrast to sensitive NHS esters and maleimides). Excess amounts can even be recovered after the click reaction.

**BASECLICK AND GLEN PHOSPHORAMIDITES**

It has been shown that the 5-position of pyrimidine and the 7-position of 7-deazapurine nucleosides are the ideal positions to introduce functionalities, as these sites lie in the major groove of the DNA providing steric freedom. In order to enable efficient Click Chemistry labeling of alkyne modified oligonucleotides, our nucleosides provide a 5-(octa-1,7-diynyl) side chain. Phosphoramidites of nucleosides 1-4 (Figure 1) were shown to be incorporated into DNA oligomers by solid-phase synthesis with excellent coupling efficiency (e.g., 1: > 99 %). Another feature of the octadiynyl side chain is its stabilizing effect on DNA duplexes (e.g., 1: Tm increase of 1-2 °C).

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| **FIGURE 1: STRUCTURES OF IDEAL CLICK NUCLEOSIDES** |
| Figure 1 |

Since alkyne-modified nucleoside phosphoramidites are incorporated into DNA strands during solid-phase synthesis in excellent yields and even stabilize the DNA-duplexes, Glen Research offers the dC and dT analogues, shown in Figure 2 on the following page, under license from baseclick.

**CLICK-REACTION ON OLIGONUCLEOTIDES**

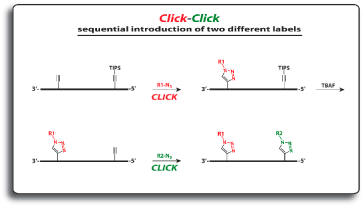
Purified oligonucleotides bearing a single alkyne moiety are usually modified with 2-5 equivalents of the corresponding marker-azide (e.g., fluorescent-dye azides). After the addition of precomplexed Cu(I), complete conversion to the labeled oligo is observed in a time span of between 30 minutes and 4 hours. After a simple precipitation step, labeled oligonucleotides can be recovered in near quantitative yields.

The Cu(I)-catalyzed Huisgen reaction enables the multiple post synthetic labeling of alkyne modified DNA as well. Complete high-density functionalization of several alkyne moieties can be achieved without the formation of by-products.

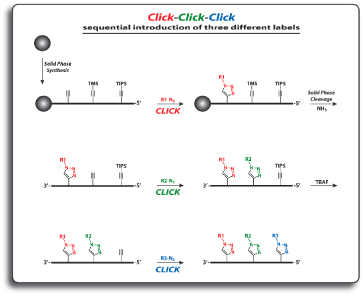
**MULTIPLE SEQUENTIAL LABELING WITH UP TO THREE DIFFERENT MARKER AZIDES**

For the attachment of up to three different labels, phosphoramidites with the alkyne groups protected with triisopropylsilyl (TIPS) and trimethylsilyl (TMS) protecting groups have been developed.5

In order to modify oligonucleotides with two sensitive molecules, two alkyne nucleosides, one with no alkyne protection and the second with TIPS protection, are incorporated into DNA strands using standard phosphoramidite chemistry. The first click reaction yields the singly modified oligonucleotide with full retention of the TIPS protecting group. For the second click, the TIPS protecting group is cleaved with tetrabutylammonium fluoride (TBAF) without causing any damage to the DNA. The second click reaction in solution yields the doubly modified oligonucleotides in excellent yields (60–90% over three steps).



For the introduction of three different labels, three alkyne nucleosides, one with no alkyne protection, the second with TIPS protection, and the third with TMS protection, are introduced into oligonucleotides. The first click reaction is performed directly on the resin. The singly modified oligonucleotide is subsequently cleaved from the support with concomitant cleavage of the TMS group and retention of the TIPS protecting group. The second click reaction is performed in solution. Precipitation of the doubly modified oligonucleotide, cleavage of the TIPS group with TBAF, and a subsequent third click reaction in solution furnishes the desired triply modified oligonucleotides in excellent overall yields.



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| **FIGURE 2: STRUCTURES OF DC AND DT CLICK PHOSPHORAMIDITES** | | |
| **[10-1540](http://www.glenresearch.com/ProductFiles/10-1540.html)** | **[10-1544](http://www.glenresearch.com/ProductFiles/10-1544.html)** | **[10-1545](http://www.glenresearch.com/ProductFiles/10-1545.html)** |
| [**C8-ALKYNE-DT**](http://www.glenresearch.com/ProductFiles/10-1540.html) | [**C8 -TIPS-ALKYNE-DT**](http://www.glenresearch.com/ProductFiles/10-1544.html) | [**C8 -TMS-ALKYNE-DT**](http://www.glenresearch.com/ProductFiles/10-1545.html) |
| **[10-1543](http://www.glenresearch.com/ProductFiles/10-1543.html)** | **[10-1541](http://www.glenresearch.com/ProductFiles/10-1541.html)** | **[10-1542](http://www.glenresearch.com/ProductFiles/10-1542.html)** |
| **[C8-ALKYNE-DC](http://www.glenresearch.com/ProductFiles/10-1543.html)** | **[C8 -TIPS-ALKYNE-DC](http://www.glenresearch.com/ProductFiles/10-1541.html)** | **[C8 -TMS-ALKYNE-DC](http://www.glenresearch.com/ProductFiles/10-1542.html)** |

**RECENT ADVANCES**

CuAAC requires the direct use of Cu(I) such as cuprous bromide (CuBr) or a source for Cu(I) such as the combination of Cu(II) salts and a reducing agent (e.g., CuSO4 and sodium ascorbate). The presence of a Cu(I)-stabilizing ligand, such as TBTA, increases the efficacy and decreases the reaction time of the CuAAC. For optimal reaction results, solutions must be freshly prepared and eventually degassed prior to use. Solubility of the ligand TBTA in diluted aqueous solutions may be an issue as well. Although this is not burdensome for regular use, occasional users can find the process troublesome.

To overcome these limitations, baseclick now offers a simple solution: the Oligo-Click Kits. These kits contain an air-stable, insoluble Cu(I) source in pellet form in a pre-loaded and ready-to-use vial. Within the kit, the TBTA ligand is replaced by an activator which is compatible with both aqueous and organic solvents. This innovative combination of catalyst and ligand/activator results in a much easier labeling work-flow including only three simple steps. The preparation of the oligonucleotide labeling via CuAAC now requires only a minimal hands-on time of a few minutes or even less and can be carried out in air without any additional precautions (Figure 3).

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| **FIGURE 3: PROCEDURE FOR LABELING WITH OLIGO-CLICK KIT** |
| Figure 1 |
| ***Click Chemistry labeling of oligonucleotides with the new Oligo-Click Kits: a simple reaction procedure and minimal hands-on time make oligolabeling even more reliable*** |

The CuAAC reaction time depends on many factors such as label size, oligonucleotide sequence, number of alkynes and other modifications within the sequence, reaction temperature, concentration of the oligonucleotide, as well as the azide in the reaction mixture. Typically, a complete conversion of a single labeled oligonucleotide using the Oligo-Click Kit is achieved in less than 1 hour when operating at 45 °C with an alkyne / azide ratio of 1:2. Lower reaction temperatures (e.g., room temperature or even 4 °C) can be efficiently applied as well in combination with longer reaction times (4 hour and overnight, respectively). The efficacy of the CuAAC reaction remains very high and in some cases even superior to the efficacy of the CuBr / TBTA system.

Labeling of oligonucleotides containing more than two alkynes normally requires the use of a larger amount of azide – up to an alkyne / azide ratio of 1:25 – as reported in the table within the kit user manual.

After the reaction, the labeling mixture is simply transferred into a new vial and the solid catalyst is discarded. No filtration is needed during this step due to the size of the catalyst pellets. Further processing of the reaction may include a precipitation step (e.g., ethanol precipitation), which removes excess of label-azide, activator and eventually organic solvents used to dissolve the azide. Thus, labeled oligonucleotides can be recovered in near quantitative yields.

**AVAILABLE OLIGO-CLICK KITS**

* Oligo-Click Kits are now available in two different sizes from baseclick:
* Oligo-Click S (optimal for labeling of up to 10 nmol single and double alkyne oligonucleotide)
* Oligo-Click M (optimal for labeling of up to 100 nmol single or multi alkyne oligonucleotide)

The Oligo-Click Reload series (S and M) provides one vial containing the activator (yellow-capped vial) along with the reactor (green-capped vial) containing the catalyst in pellet form.

Azides are available in red-capped vials within the following Oligo-Click kits:

* Oligo-Click 488 for kits containing FAM-azide
* Oligo-Click 555 for kits containing TAMRA-azide
* Oligo-Click Biotin for kits containing Biotin-azide

Figure 4 shows an example of RP-HPLC and MALDI-mass spectrum measured directly after the click reaction. In this case, an oligonucleotide containing two internal alkynes was labeled with ATTO425-azide using the Oligo-Click M Kit followed by a simple ethanol precipitation step without further purification.

The results show the outstanding efficiency of the Oligo-Click Kit and of CuAAC in general.

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| **FIGURE 4: HPLC AND MALDI ANALYSIS OF OLIGO LABELED WITH 2 ATTO DYES USING OLIGO-CLICK KIT** |
| Figure 1 |
| 18mer containin***g two internal alkynes reacted with 2.5 equivalents of ATTO425-Azide (MW = 602 g/mol), 4 h at 45 °C. MALDI-mass analysis of the crude product -> 100% oligo-dye conjugate (Calcd. 7465; Found 7461).*** |

**COMPARISON WITH CU-FREE CLICK**

It is possible to construct highly strained ring systems containing alkyne groups that will allow Click reactions to occur without the need for a copper catalyst. However, these Cu-free Click reagents tend to be simple 5'-modifiers or dT derivatives. The catalog of alkyne derivatives for CuAAC is substantially greater. At this point, no Cu-free options are available for attaching more than one type of label or tag to oligonucleotides.

Moreover, the amount of Cu ions during the CuAAC using the Oligo-Click Kits generally does not exceed 100 ng/µL (colorimetric complexation detection). This value drops to 50 ng/µL after a simple precipitation step (e.g., EtOH-precipitation) and it is further reduced to 5-10 ng/µL after RP HPLC purification. Gel purification or ion-exchange filtrations abate the Cu ions content below the detection limit of 0.01 ng/µL.

**CONCLUSION**

The easy-to-use copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction with its outstanding selectivity is in an excellent position to take over as the state-of-the-art methodology to label and modify DNA and nucleic acids in general. With baseclick's addition of the Oligo-Click Kits many factors have been radically improved such as the stability of the reagents, their solubility and the comfortable handling of CuAAC, thus providing a powerful labeling tool for chemistry and biology labs.

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**INTELLECTUAL PROPERTY RIGHTS:**

**BASECLICK GMBH HAS FILED THE FOLLOWING PATENT APPLICATIONS:**

1. WO2006/117161, New labeling strategies for the sensitive detection of analytes

***The following patents have been granted:***

EP 1877415

US 8,129,315

2. WO2008/952775, Click Chemistry for the production of reporter molecules

3. Baseclick GmbH holds a worldwide license for the research market of the “Click Chemistry” patent from “The Scripps Research Institute”:

WO03/101972, Copper-catalysed ligation of azides and acetylenes

**GLEN RESEARCH OFFERINGS**

Glen Research has collaborated with baseclick for several years on this product line. We have been offering the dC and dT analogues, shown in[**Figure 2 on Page 9**](http://www.glenresearch.com/GlenReports/GR24-26.html#fig2), for simple Click conjugations as well as sequential labeling with up to three separate azides.

We are delighted to be able to offer Oligo-Click Kits to our research customers. Our most popular scale of synthesis is 200 nmoles, so we are offering Oligo-Kit M. This kit has sufficient reagents for conjugating up to nine alkyne-containing oligonucleotides on a 100 nmole scale or a single oligonucleotide on a 1 µmole scale. The user must supply the azide and a solvent such as DMSO for dissolving the azide.

We are also offering kits for biotin, fluorescein and TAMRA labeling. Each kit has sufficient reagents for conjugating up to seven alkyne-containing oligonucleotides on a 100 nmole scale or a single oligonucleotide on a 1 µmole scale. Each kit contains all of the ingredients necessary, including the azide and DMSO solvent.

Our experience with these kits indicates that they will be of enormous help to customers carrying out click conjugation reactions on an occasional basis.