DATA SHEET

Jena Bioscience

CuAAC Reaction Ligand Test Kit (THPTA & BTTAA based)

Cat. No.	Amount
CLK-075	1 kit

For general laboratory use.

Shipping: shipped at ambient temperature

Storage Conditions: store at 4 °C

Short term exposure (up to 1 week cumulative) to ambient temperature possible.

Shelf Life: 12 months after date of delivery

Description:
The CuAAC Reaction Ligand Test Kit is suitable to perform Copper (Cu(I))-catalyzed Azide-Alkyne Click chemistry reactions (CuAAC) with Azide- or Alkyne- modified biomolecules and cells containing metabolically functionalized Alkyne- or Azide-modified biomolecules. It allows a direct comparison of BTTAA and THPTA to find the most suitable Cu(I)-stabilizing ligand for your experimental set-up.

1 Kit provides sufficient amounts to perform **25 CuAAC experiments each with THPTA and BTTAA à 200 µl using 2 mM $CuSO_4$ (copper source), 10 mM THPTA or 10 mM BTTAA (Cu(I)-stabilizing ligands) and 100 mM Na-Ascorbate (reduction reagent)** in 100 mM Na-Phosphate reaction buffer.

Content:
Copper source:
1 x 10 mg $CuSO_4$ (M = 159.6 g/mol), #CLK-MI004)

Cu(I) stabilizing ligands:
1 x 25 mg THPTA (M= 434.5 g/mol, #CLK-1010)
1 x 25 mg BTTAA (M= 430.5 g/mol, #CLK-067)

Reduction Reagent:
2 x 200 mg Na-Ascorbate (M = 198.1 g/mol, #CLK-MI005)

Reaction Buffer:
1 x 30 ml 100 mM Na-Phosphate Buffer, pH 7

10 ml ddH_2O

Materials required but not provided:

Alkyne-or Azide-functionalized substrates e.g.
a) fixed and permeabilized cells containing metabolically functionalized Alkyne- or Azide-modified biomolecules.
b) cell lysate containing metabolically functionalized Alkyne- or Azide-modified proteins.
c) Alkyne- or Azide-functionalized biomolecules such as DNA, RNA or oligonucleotides.

(Picolyl)-Azide or Alkyne detection reagent and appropriate solvent (e.g. DMSO)

For labeling of fixed and permeabilized cells:
Washing solutions e.g. PBS containing 3% BSA
Fixation solution e.g. PBS containing 3.7% formaldehyd
Permeabilization solution e.g. PBS containing 0.5% Triton X-100
Mounting medium for imaging
Additional labeling reagent such as nuclear stain or antibody

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1. Introduction

Copper (**Cu**(I))-catalyzed **A**zide-**A**lkyne **C**lick chemistry reactions (**CuAAC**) describe the reaction of an Azide-functionalized molecule A with a terminal Alkyne-functionalized molecule B that results in a stable conjugate A-B via a Triazole moiety.

Since terminal Alkynes are fairly unreactive towards Azides, the efficiency of CuAAC reactions strongly depends on the presence of a metal catalyst such as copper ions in the +1 oxidation state (Cu(I)).

Different copper sources, reduction reagents and Cu(I) stabilizing ligands are available however, for most bioconjugation applications the combination of the Cu(II) salt $CuSO_4$ as copper source, a water-soluble Cu(I) stabilizing ligand such as THPTA or BTTAA and sodium ascorbate as a reduction reagent is recommended.[1-3] BTTAA promotes a higher reaction efficiency under some experimental conditions.[3]

The use of Picolyl-Azide reagents instead of conventional Azide reagents can further increase the reaction efficiency and decrease the required final $CuSO_4$ concentration due to the internal copper chelating moiety.[4] Especially the combination with BTTAA as ligand may allow you to use a decreased copper concentration while maintaining similar reaction efficiencies achieved with traditional Azide reagendts.

The set-up of a CuAAC reaction is based on the following general three-step procedure:

- Prepare a mix of Alkyne- and Azide functionalized molecules in an appropriate reaction buffer.
- Prepare a $CuSO_4$: Cu(I)-ligand premix, add it to the Azide- Alkyne solution and mix briefly.
- Add Na-Ascorbate as reduction reagent at last to start the reaction.

The CuAAC Reaction Ligand Test Kit provides sufficient amounts to perform 25 CuAAC experiments each with THPTA and BTTAA à 200 µl using 2 mM $CuSO_4$, 10 mM THPTA (or 10 mM BTTAA) and 100 mM Na-Ascorbate in 100 mM Na-Phosphate reaction buffer.

General protocols for labeling of biomolecules (see 3.) and labeling of fixed and permeabilized cells containing metabolically functionalized Alkyne- or Azide-modified biomolecules (see 4.) are outlined below. Individual optimization might however be required for different CUAAC labeling experiments as well as for critical reaction parameter e.g. final $CuSO_4$ concentration, $CuSO_4$:ligand ratio, detection reagent concentration.

Hong *et al.*[2] and Presolski *et al.*[1] provide useful background information on the influence of $CuSO_4$ concentration, $CuSO_4$: ligand ratio and reaction buffer type that may be used as a starting point if optimization is required.

2. Preparation of stock solutions

Please note: *The concentration of stock solutions (2.1 to 2.3) is suitable to prepare 200 and 500 µl assays containing 2 mM $CuSO_4$, 10 mM THPTA or 10 mM BTTAA and 100 mM Na-Ascorbate (see 3.1/3.2 and 4.3/4.4 respectively). Adjustments might be required if different assay volumes or final compound concentrations are used (see 3. and 4.).*

2.1 THPTA and BTTAA stock solution (Cu(I) stabilizing ligand)

- Add an appropriate amount of ddH_2O (Tab. 1), vortex until the compound is completely dissolved and spin down briefly.
- Prepare aliquots to avoid repeated freeze-thaw-cycles and store at -20 °C.
- The solution is stable up to 1 year at -20 °C.
- BTTAA is less soluble than THPTA. If required, heat shortly up to 70°C to achieve a clear solution

Table 1 Volume of ddH_2O required for a 250 mM THPTA or 50 mM BTTAA stock solution.

THPTA	**Concentration of stock solution**	**Amount of ddH_2O**
25 mg	250 mM	230 µl
BTTAA		
25 mg	50 mM	1163 µl

2.2 $CuSO_4$ stock solution (copper source)

- Add an appropriate amount of ddH_2O (Tab. 2), vortex until the compound is completely dissolved and spin down briefly.
- Prepare aliquots to avoid repeated freeze-thaw-cycles and store at -20 °C.
- The solution is stable up to 1 year at -20 °C.

Table 2 Volume of ddH_2O required for a 100 mM $CuSO_4$ stock solution.

$CuSO_4$	**Concentration of stock solution**	**Amount of ddH_2O**
10 mg	100 mM	628 µl

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2.3 Na-Ascorbate stock solution (reduction reagent)

- Add an appropriate amount of ddH_2O (Tab. 3), vortex until the compound is completely dissolved and spin down briefly.
- Prepare aliquots to avoid repeated freeze-thaw-cycles and store at -20 °C.
- The solution is stable up to 1 year at -20 °C.

Please note: ***Do not use solutions that appear brown.*** *Freshly prepared, fully functional Na-Ascorbate solutions are colorless to slightly yellow and turn brown upon oxidization thereby losing their reduction capability.*

Table 3 Volume of ddH_2O required for a 1 M Na-Ascorbate stock solution.

Na-Ascorbate	Concentration of stock solution	Amount of ddH_2O
200 mg	1 M	1010 µl

2.4 (Picolyl)-Azide detection reagent stock solution

- (Picolyl)-Azide detection reagents are not provided within this kit.
- Add an appropriate amount of suitable solvent e.g. DMSO to achieve a stock solution concentration of 10 mM for Azide detection reagents and 500 µM for Picolyl-Azide detection reagents.
- Final concentrations of Azide or Alkyne detection reagents may range from 2 µM to 100 µM. Final concentrations of Picolyl-Azide detection reagents may range from 0.5 to 5 µM (see 3.4).
- If the molar amount of Alkyne-groups can be calculated, we recommend starting with a 10-fold molar excess of Azide-functionalized detection reagent

3. General protocol for CLICK labeling of biomolecules

The protocol below is intended as a general guideline however, individual optimization might be required.

The amount of provided reagents is sufficient to perform **25 CuAAC experiments each with THPTA and BTTAA à 200 µl** using 2 mM $CuSO_4$, 10 mM THPTA (or 10 mM BTTAA) and 100 mM Na-Ascorbate in 100 mM Na-Phosphate reaction buffer.

3.1 Prepare $CuSO_4$:THPTA - and $CuSO_4$:BTTAA -Premix

Please note: *Both the final $CuSO_4$ concentration as well as $CuSO_4$:THPTA (or BTTAA) ratio are critical parameters for CuAAC reaction efficiency. A final $CuSO_4$ concentration of 2 mM and a $CuSO_4$:THPTA (or BTTAA) ratio of 1:5 is recommended as a starting point for labeling of Azide- or Alkyne-functionalized biomolecules with a correspondingly labeled detection reagent. Individual optimization for each assay is strongly recommended. Minimum $CuSO_4$ concentration: 50 µM.*

- Prepare the $CuSO_4$:THPTA (or BTTAA)-Premix freshly for each experiment.
- Allow all solutions to warm up to room temperature.
- Mix the appropriate amount of 100 mM $CuSO_4$ and 250 mM THPTA (or 50 mM BTTAA) stock solution (Tab. 4) by vortexing and spin down briefly.
- 12 µl $CuSO_4$:THPTA (or 44 µl BTTAA)-Premix (1 Assay) is sufficient for the preparation of 500 µl CLICK reaction cocktail (see. 3.2).

Table 4A Pipetting scheme for $CuSO_4$:THPTA-Premix (ratio 1:5).

Compound	Final conc.	1 Assay
100 mM $CuSO_4$ stock solution **(see 2.2)**	33.33 mM	4 µl
250 mM THPTA stock solution **(see 2.1)**	166.66 mM	8 µl

Table 4B Pipetting scheme for $CuSO_4$:BTTAA-Premix (ratio 1:5).

Compound	Final conc.	1 Assay
100 mM $CuSO_4$ stock solution **(see 2.2)**	9.1 mM	4 µl
50 mM BTTAA stock solution **(see 2.1)**	45.45 mM	40 µl

3.2 Perform CLICK labeling

Please note: *The protocol below describes CuAAC labeling of an Alkyne-functionalized biomolecule (e.g. cell lysate containing Alkyne-functionalized proteins) with an Azide-functionalized detection reagent (e.g. Azide-functionalized fluorescent dye). It can be used vice versa as well (Azide-functionalized biomolecule and Alkyne-functionalized detection reagent).*

- Allow all solutions to warm up to room temperature.
- Final assay volume: 200 µl.
- Refer to Tab. 6 for appropriate amounts of stock solutions.
- Mix an Alkyne-functionalized biomolecule (see Tab. 5) with an appropriate amount of 100 mM Na-Phosphate reaction buffer, pH 7 to achieve a final volume of 167 µl if the $CuSO_4$:THPTA-

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Premix is used or a final volume of 135 µl if the $CuSO_4$:BTTAA-Premix is used.

Table 5 Starting amount of Alkyne-functionalized biomolecules.
Please note: *The stated amounts are intended for an orientation only. They may need to be adjusted depending on the final read-out or downstream processing after CLICK reaction.*

Substrate	Final Amount	Recommended final assay volume
Cell lysate containing Alkyne-functionalized proteins	50 µg	200 µl
Single Alkyne-functionalized oligonucleotide	5-10 nmol	20-50 µl
Multiple Alkyne-functionalized DNA or RNA generated by enzymatic incorporation of correspondingly labeled nucleotides	3-15 pmol*	20-50 µl

*e.g. 3 pmol correspond to 1.5 µg of a 1500 bp RNA fragment or 1 µg of a 500 bp DNA fragment.

- Add 1 µl of a 10 mM Azide- functionalized detection reagent stock solution (see 2.4) vortex and spin-down briefly (final concentration: 50 µM). If the molar amount of Alkyne-groups can be calculated, we recommend starting with a 10-fold molar excess of Azide-functionalized detection reagent.
- Add 12 µl $CuSO_4$:THPTA (or 44 µl BTTAA)-Premix (see. 3.1), vortex and spin down briefly.
- Add 20 µl 1M Na-Ascorbate stock solution (see 2.3) to initiate the reaction, vortex and spin down briefly.
- Incubate samples 30 – 60 min at room temperature or 37°C (protected from light).
- Alkyne-functionalized biomolecules are now CLICK-labeled and ready for downstream processing and/or analysis.

Table 6 Pipetting scheme for a 200 µl CLICK reaction assay. Please add the compounds exactly in the order described below.

Pipetting scheme using $CuSO_4$/THPTA-Premix

Compound	Final conc./amount	1 Assay (200 µl)
Alkyne-functionalized biomolecule	see tab. 5	X µl
100 mM Na-Phosphate reaction buffer, pH 7	100 mM	ad 167 µl
10 mM Azide-functionalized detection reagent stock solution **(not provided, see 2.4)**	50 µM[1]	1 µl
33.33 mM / 166.66 mM $CuSO_4$:THPTA-Premix **(see 3.1)**	2 mM / 10 mM	12 µl
1 M Na-Ascorbate stock solution **(see 2.3)**	100 mM	20 µl

Pipetting scheme using $CuSO_4$/BTTAA-Premix

Compound	Final conc./amount	1 Assay (200 µl)
Alkyne-functionalized biomolecule	see tab. 5	X µl
100 mM Na-Phosphate reaction buffer, pH 7	100 mM	ad 135 µl
10 mM Azide-functionalized detection reagent stock solution **(not provided, see 2.4)**	50 µM[1]	1 µl
9.1 mM / 45.45 mM $CuSO_4$:BTTAA-Premix **(see 3.1)**	2 mM / 10 mM	44 µl
1 M Na-Ascorbate stock solution **(see 2.3)**	100 mM	20 µl

[1]Final concentrations of Azide or Alkyne detection reagents may

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range from 2 µM to 100 µM. Final concentrations of Picolyl-Azide detection reagents may range from 0.5 to 5 µM. We recommend starting with 50 µM or 5 µM, respectively in case the molar amount of Alkyne-groups is unknown. If the molar amount of Alkyne-groups can be calculated, we recommend starting with a 10-fold molar excess of Azide-functionalized detection reagent. Concentrations can be titrated down in case of high background or up in case of low signal.

4. General protocol for CLICK labeling of fixated and permeabilized cells containing metabolically functionalized Alkyne- or Azide-modified biomolecules

The protocol below is intended as a general guideline however, individual optimization might be required.

The amount of provided reagents is sufficient to **perform 10 CuAAC experiments each with THPTA and BTTAA à 500 µl** using 2 mM $CuSO_4$, 10 mM THPTA (or 10 mM BTTAA) and 100 mM Na-Ascorbate in 100 mM Na-Phosphate reaction buffer.

4.1 Metabolically label cells with an Alkyne or Azide-functionalized substrate

- Cultivate cells on coverslips under conditions that ensure optimal growth of cell type.
- Add Alkyne- or Azide-functionalized substrate at the desired final concentration and cultivate for an appropriate time under conditions optimal for metabolic incorporation of the modified substrate.

4.2 Fixate and permeabilize cells

Please note: *The fixation with 3.7% formaldehyde in PBS and subsequent permeabilization with 0.5% Triton X-100 is a general guideline. Optimization might be required. Different reagent concentrations, different fixation and permeabilization reagents (e.g. methanol or saponin) or TBS as buffer solution intstead of PBS can be used as well. Permeabilization is not required for cell surface or lipid component labeling.*

- Remove cultivation medium
- Transfer each coverslip to a well of a 6-well plate
- Add 1 ml of 3.7% formaldehyde in PBS for fixation and incubate for 15 min. at room temperature.
- Remove fixation reagent and wash 2-3 times with PBS containing 3% BSA.
- Add 1 ml of Triton X-100 in PBS and incubate for 20 minutes at room temperature for permeabilization.

4.3 Prepare $CuSO_4$:THPTA- and $CuSO_4$:BTTAA-Premix

Please note: *Both the final $CuSO_4$ concentration as well as $CuSO_4$:THPTA (or BTTAA) ratio are critical parameters for CuAAC reaction efficiency. A final $CuSO_4$ concentration of 2 mM and a $CuSO_4$:THPTA (or BTTAA) ratio of 1:5 is recommended as a starting point for labeling of fixed and permeabilized cells containing metabolically Azide- or Alkyne-functionalized biomolecules. Individual optimization for each assay is strongly recommended. Minimum $CuSO_4$ concentration: 50 µM.*

- Prepare the $CuSO_4$:THPTA (or BTTAA)-Premix freshly for each experiment.
- Allow all solutions to warm up to room temperature.
- Mix the appropriate amount of 100 mM $CuSO_4$ and 250 mM THPTA (or 50 mM BTTAA) stock solution (Tab. 7) by vortexing and spin down briefly.
- 30 µl $CuSO_4$:THPTA (or 110 µl BTTAA)-Premix (1 Assay) is sufficient for the preparation of 500 µl CLICK reaction cocktail (see. 4.4).

Table 7A Pipetting scheme for $CuSO_4$:THPTA-Premix (ratio 1:5).

Compound	Final conc.	1 Assay	10 Assays
100 mM $CuSO_4$ stock solution **(see 2.2)**	33.33 mM	10 µl	100 µl
250 mM THPTA stock solution **(see 2.1)**	166.66 mM	20 µl	200 µl

Table 7B Pipetting scheme for $CuSO_4$:BTTAA-Premix (ratio 1:5).

Compound	Final conc.	1 Assay	10 Assays
100 mM $CuSO_4$ stock solution **(see 2.2)**	9.1 mM	10 µl	100 µl
50 mM BTTAA stock solution **(see 2.1)**	45.45 mM	100 µl	1000 µl

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4.4 Prepare CLICK reaction cocktail

Please note: *Prepare CLICK reaction cocktail* ***freshly for each experiment*** *and* ***use it immediately but definitely within 15 minutes*** *after preparation. 500 µl CLICK reaction cocktail (1 Assay) is sufficient to label one 18x18 coverslip.*

- Allow all solutions to warm up to room temperature.
- Refer to Tab. 8 for appropriate amounts of compound stock solutions.
- 500 µl CLICK reaction cocktail (1 Assay) is sufficient for a 18x18 coverslip.
- Add an appropriate amount of Azide- or Alkyne detection reagent solution to the reaction buffer, vortex and spin-down briefly.
- Add $CuSO_4$:THPTA (or BTTAA)-Premix, vortex and spin down briefly.
- Add Na-Ascorbate, vortex and spin down briefly.

Table 8 Pipetting scheme for CLICK reaction cocktail. Please add the compounds exactly in the order described below.

Pipetting scheme using $CuSO_4$:THPTA-Premix

Compound	Final conc.	1 Assay (500 µl)	10 Assays (5 ml)
100 mM Na-Phosphate reaction buffer, pH 7	100 mM	419 µl	4.19 ml
10 mM Azide or-Alkyne detection reagent stock solution **(not provided, see 2.4)**	20 µM[1]	1 µl	10 µl
33.33 mM / 166.66 mM $CuSO_4$:THPTA-Premix **(see 3.3)**	2 mM / 10 mM	30 µl	300 µl
1 M Na-Ascorbate stock solution **(see 2.3)**	100 mM	50 µl	500 µl

Pipetting scheme using $CuSO_4$:BTTAA-Premix

Compound	Final conc.	1 Assay (500 µl)	10 Assays (5 ml)
100 mM Na-Phosphate reaction buffer, pH 7		339 µl	3.39 ml
10 mM Azide or-Alkyne detection reagent stock solution **(not provided, see 2.4)**	20 µM[1]	1 µl	10 µl
9.1 mM / 45.456 mM $CuSO_4$:BTTAA-Premix **(see 3.3)**	2 mM / 10 mM	110 µl	1100 µl
1 M Na-Ascorbate stock solution **(see 2.3)**	100 mM	50 µl	500 µl

[1]Final concentrations of Azide or Alkyne detection reagents may range from 2 µM to 100 µM. Final concentrations of Picolyl-Azide detection reagents may range from 0.5 to 5 µM. We recommend starting with 20 µM or 5 µM, respectively. Concentrations can be titrated down in case of high background or up in case of low signal.

4.5 Perform CLICK labeling of fixated and permeabilized Alkyne- or Azide-labeled cells

- Remove the permeabilization buffer (see 4.2) and wash 2-3 times with PBS containing 3% BSA.
- Add 500 µl CLICK reaction cocktail (see 4.4) to each well containing one coverslip. Ensure that the coverslip is entirely covered with solution.
- Incubate samples 30 – 60 min at room temperature (protected from light).
- Remove CLICK reaction cocktail and wash cells 1-2 times with PBS containing 3% BSA.
- Remove wash solution.
- For nuclear staining with DAPI or Hoechst 33342 or antibody labeling wash once with PBS, remove PBS and proceed with staining according to the manufacturer's protocol.

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Selected References:
[1] Presolski *et al.* (2011) Copper-Catalyzed Azide-Alkyne Click Chemistry for Bioconjugation. *Current Protocols in Chemical Biology* **3**:153.
[2] Hong *et al.* (2011) Analysis and Optimization of Copper-Catalyzed Azide-Alkyne Cycloaddition for Bioconjugation. *Angew. Chem. Int. Ed.* **48**:9879.
[3] Besanceney-Webler *et al.* (2011) Increasing the Efficiacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study. *Angew. Chem. Int. Ed.* **50**:8051.
[4] Uttamapinant *et al.* (2012) Fast, Cell-Compatible Click Chemistry with Copper-Chelating Azides for Biomolecular Labeling. *Angew. Chem. Int. Ed.* **51**:5852.