# Detection of protein-synthesizing microorganisms in the environment via bioorthogonal non-canonical amino acid tagging (BONCAT)

**R. Hatzenpichler and V. J Orphan**

## Abstract

This protocol provides a detailed description of how to design and perform BONCAT experiments using two different bioorthogonal amino acids, L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG), which are both surrogates of L-methionine. It illustrates how incorporation of these noncanonical amino acids into new proteins can be detected in whole cells via copper-catalyzed or strain-promoted azide-alkyne click chemistry and outlines how the visualization of translational activity can be combined with the taxonomic identification of cells via FISH. Last, the protocol discusses potential problems that might be encountered during BONCAT experiments with environmental samples and how they can be overcome.

Book chapter describing this BONCAT protocol is published in:

**Detection of Protein-Synthesizing Microorganisms in the Environment via Bioorthogonal Noncanonical Amino Acid Tagging (BONCAT)**

**Authors: Roland Hatzenpichler and Victoria J. Orphan**

T.J. McGenity et al. (eds.), Hydrocarbon and Lipid Microbiology Protocols, Springer Protocols Handbooks, DOI 10.1007/8623_2015_61, © Springer-Verlag Berlin Heidelberg 2015

**Citation:** R. Hatzenpichler and V. J Orphan Detection of protein-synthesizing microorganisms in the environment via bioorthogonal non-canonical amino acid tagging (BONCAT). **protocols.io**

dx.doi.org/10.17504/protocols.io.drp55m

**Published:** 24 Jul 2016

## Before start

There are two variants of azide-alkyne click reactions:

(1) a Cu (I)-catalyzed cycloaddition reaction and

(2) a strain-promoted version that exploits the high reactivity of a cyclooctyne system that allows the click reaction to take place in the absence of a catalyst.

Both labeling reactions are simple with only a few inexpensive chemicals required. The protocol can be preformed within 1–2 hours to identify transcriptionally active microorganisms in a variety of environmental samples after incubation with a methionine surrogate.  In our experience, the BONCAT reaction is generally not influenced by the presence of complex organic (e.g., an extracellular matrix) or inorganic (e.g., minerals or sediment particles) substances. This results in a low level of background noise when applied to environmental samples. While copper-catalyzed click chemistry can be employed to detect both AHA- and HPG-tagged proteins, the strain-promoted click reaction is restricted to the visualization of AHA uptake (because HPG does not contain an azide group).

Solutions required for BONCAT labeling (Unless indicated otherwise, all reagents can be stored at room temperature).

For L-2-amino-4-azidobutanoic acid (L-azidohomoalanine, AHA): Dissolve in nano-pure water, adjust to pH 7.0, filter sterilize (0.2 µm), and store in the dark at 4ºC. Prepare stock solutions (typically in the range between 1–100 mM).

For L-2-amino-5-hexynoic acid (L-homopropargylglycine, HPG): Dissolve in nano-pure water, adjust to pH 7.0, filter sterilize (0.2 µm), and store in the dark at 4ºC. Prepare stock solutions (1–100 mM).

Once samples have been incubated with either HPG or AHA, paraformaldehyde fixation (PFA) and preparation of microbial cells on a teflon coated slide or polycarbonate filter should be done following a standard protocol for FISH (e.g. Ishii et al. 2004 or Daims et al 2005).  After PFA fixed samples are prepared, the copper-catalyzed (Cu I) click reaction for fluorescence detection of newly synthesized proteins in whole cells requires the following solutions:

Phosphate-buffered saline (PBS): 130 mM NaCl, 5% (v/v) phosphate buffer in nano-pure water, adjust to pH 7.4, and sterilize filter (0.2 µm).

20 mM Copper sulfate solution ($CuSO_4 \cdot 5 H_2O$) in nano-pure water, sterilize filter (0.2 µm), and store at room temperature or 4ºC.

50 mM THPTA : Tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine in nano-pure water, sterilize filter (0.2 µm), and store in aliquots at -20ºC.

100 mM freshly prepared sodium ascorbate in 1X PBS (this reducing agent is added to ensure the copper sulfate solution has a sufficient amount of copper I to catalyze the azide-alkyne reaction). Should be made fresh each time and used the same day.

100 mM Aminoguanidine hydrochloride in 1X PBS (this reagent should also be made fresh each time)

1–10 mM stock solutions in either dimethylsulfoxide (DMSO) or dimethylformamide (DMF) for preparation of the dye solution following manufacturer's instructions (Alexa dyes or Cy dyes)

for HPG incubations, use azide-conjugated fluorophores

for AHA incubations, use alkyne-conjugated fluorophores

See following references for additional information on copper-catalyzed and strain promoted Click and FISH sample preparation.

Ishii, K., M. Mußmann, B. J. MacGregor, and R. Amann. 2004. An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. FEMS Microbiology Ecology 50:203-212.

Daims H, Stoecker K, Wagner M (2005) Fluorescence in situ hybridization for the detection of prokaryotes. In: Osborn AM, Smith CJ (eds) Molecular microbial ecology. Bios Advanced Methods, Abingdon, pp 213–239

Bagert JD, Xie YJ, Sweredoski MJ, Qi Y, Hess S, Schuman EM, Tirrell DA (2014) Quantitative, time-resolved proteomic analysis by combining bioorthogonal noncanonical amino acid tagging and pulsed stable isotope labeling by amino acids in cell culture. Mol Cell Proteomics 13(5):1352–1358.

Dieterich DC, Link AJ, Graumann J, Tirrell DA, Schuman EM (2006) Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). Proc Natl Acad Sci U S A 103:9482–9487

Torne CW, Christensen C, Meldal M (2002) Peptidotriazoles on solid phase: [1,2,3]- friazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J Org Chem 67:3057–3064

Agard NJ, Prescher JA, Bertozzi CR (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. J Am Chem Soc 126:15046–15047

## Materials

- L-2-amino-4-azidobutanoic acid (L-azidohomoalanine, AHA) by Contributed by users
- L-2-amino-5-hexynoic acid (L-homopropargylglycine, HPG) by Contributed by users
- 20% Paraformaldehyde by Contributed by users

Tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine (THPTA) by Click Chemistry Tools

Sodium ascorbate by Sigma Aldrich

Aminoguanidine hydrochloride by Sigma Aldrich

carboxyrhodamine 110 by Click Chemistry Tools

Citifluor AF-1 anti-fading solution by Electron Microscopy Sciences

- Teflon-coated slides by Contributed by users

dimethylsulfoxide (DMSO) by Sigma Aldrich

## Protocol

### Incubation with Bioorthogonal Amino Acid

**Step 1.**
Directly add AHA or HPG using a sterile-filtered (0.2 µM), pH- adjusted (pH 7.0) stock solution yielding a final concentration of 1 nM to 1 mM. Additionally, perform replicate experiments and include replicated incubations without AHA/HPG.

*For L-2-amino-4-azidobutanoic acid (L-azidohomoalanine, AHA): Dissolve in nano-pure water, adjust to pH 7.0, filter sterilize (0.2 µm), and store in the dark at 4 C. Prepare stock solutions of 1–100 mM.*

*For L-2-amino-5-hexynoic acid (L-homopropargylglycine, HPG): Dissolve in nano-pure water, adjust to pH 7.0, filter sterilize (0.2 µm), and store in the dark at 4 C. Prepare stock solutions of 1–100 mM.*

### Cell Fixation

**Step 2.**
Fix cells according to standard protocols [34] immediately after sampling either by (1) fixation in 3% formaldehyde (PFA) in PBS **(See Steps 3-6)** or (2) by resuspending pelleted biomass in a 1:1 mix of PBS:EtOH. **(See Step 7)**

NOTES
**Victoria Orphan** 25 May 2016

If working with marine microorganisms, use of 3X PBS is recommended.

### Cell Fixation (PFA ONLY)

**Step 3.**
For fixation with PFA, pellet the biomass, remove the supernatant (SN), and resuspend cells in 3% PFA in PBS. For aqueous samples, directly add PFA to reach a final concentration of 3% PFA.

### Cell Fixation (PFA ONLY)

**Step 4.**

Fix for either 3 h on ice or 1 h at RT.

Cell Fixation (PFA ONLY)

**Step 5.**

After fixation, Pellet the biomass by centrifugation or filter onto 0.2 µm filters.

Cell Fixation (PFA ONLY)

**Step 6.**

Wash with PBS to remove remaining PFA before resuspending biomass in 1:1 PBS: EtOH. Store at -20°C. Make sure to deposit PFA in the chemical waste.

Cell Fixation (EtOH ONLY)

**Step 7.**

For EtOH fixation, pellet biomass, remove supernatant, resuspend in 1:1 PBS:EtOH, and store at -20°C.

Preparing the Samples for Click Labeling

**Step 8.**

Immobilize biomass either on glass slides or filters. Dry at 46°C or, if not available, at 37°C or RT.

Preparing the Samples for Click Labeling

**Step 9.**

Dehydrate and permeabilize cells by sequentially placing slides or filters for 3 min into 50 mL tubes that contain 50, 80, and 96% ethanol. Dry biomass using pressurized air.

Preparing the Samples for Click Labeling

**Step 10.**

Pellet sample via centrifugation (16,100g or max. setting for 5 min at RT) and resuspend in 250 µL 80% EtOH.

Preparing the Samples for Click Labeling

**Step 11.**

Mix by vortex and incubate for 3 min at RT.

Preparing the Samples for Click Labeling

**Step 12.**

Add 1.5 mL 96% EtOH, mix by vortex, and incubate for 3 min at RT.

Preparing the Samples for Click Labeling

**Step 13.**

Afterwards, pellet sample via centrifugation and resuspend in 221 µL PBS. Removing small volumes of leftover EtOH is not necessary as it does not interfere with the click reaction.

Cu(I) Click Chemistry

**Step 14.**

If using immobilized biomass, after dehydration of the sample, prepare the dye premix by mixing 1.25µL of 20 mM $CuSO_4$ solution with 2.50µL of 50 mM THPTA and 0.30µL of alkyne dye. Allow to react for 3 min at RT in the dark.

*We recommend to perform Cu(I)-catalyzed click chemistry at a dye concentration of 1-5µM (final concentration) to guarantee for best signal-to-noise ratios, but substantially lower or higher concentrations can be used, if necessary. We successfully tested concentrations as low as 10 nM and as high as 50µM.*

DURATION

00:03:00

Cu(I) Click Chemistry (Immobilized)

**Step 15.**
If using immobilized biomass after dehydration, continue. Otherwise, skip to Step 18.

Add 12.5 µL of each 100 mM sodium ascorbate and 100 mM aminoguanidine hydrochloride to 221 µL PBS. Then, add the dye premix and invert the tube once (do not mix by vortex to maintain reducing conditions).

Cu(I) Click Chemistry (Immobilized)

**Step 16.**
Cover the sample with 20 µL of the click solution, transfer the slide into a humid chamber (water on tissue paper), and incubate in the dark at RT for 30 min. Increasing the incubation time is possible, but typically does not increase fluorescence signal.

DURATION
00:30:00

Cu(I) Click Chemistry (Immobilized)

**Step 17.**
Wash the slide or filter three times for 3 min each in PBS-filled 50 mL tubes before dehydrating it by incubating it for 3 min in 50% EtOH at RT.

Cu(I) Click Chemistry (Solution)

**Step 18.**
If the biomass is in solution, all reagents (sodium ascorbate and aminoguanidine, followed after 3 min by the dye premix, final concentrations as described above) are added directly to the sample. Invert tubes once and incubate in the dark at RT for 30 min. Afterwards, wash samples three times with PBS and then one time in 50% EtOH (RT). Between washing steps, pellet samples via centrifugation for 5 min at 16,100g (or highest setting) at RT. Finally, resuspend biomass in a 1:1 mix of PBS:EtOH, transfer onto a glass slide, and air-dry

DURATION
00:30:00

**Step 19.**