

Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging

Daniela C Dieterich¹, Jennifer J Lee¹, A James Link^{2,3}, Johannes Graumann^{1,3}, David A Tirrell² & Erin M Schuman¹

¹Division of Biology, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125, USA. ²Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, USA. ³Present address: Department of Chemical Engineering and Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712, USA (A.J.L); Department of Proteomics and Signal Transduction, Max-Planck-Institute for Biochemistry, Martinsried 82152, Germany (J.G.). Correspondence should be addressed to D.C.D. (dieterd@caltech.edu).

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A major aim of proteomics is the identification of proteins in a given proteome at a given metabolic state. This protocol describes the step-by-step labeling, purification and detection of newly synthesized proteins in mammalian cells using the non-canonical amino acid azidohomoalanine (AHA). In this method, metabolic labeling of newly synthesized proteins with AHA endows them with the unique chemical functionality of the azide group. In the subsequent click chemistry tagging reaction, azide-labeled proteins are covalently coupled to an alkyne-bearing affinity tag. After avidin-based affinity purification and on-resin trypsinization, the resulting peptide mixture is subjected to tandem mass spectrometry for identification. In combination with deuterated leucine-based metabolic colabeling, candidate proteins can be immediately validated. Bioorthogonal non-canonical amino-acid tagging can be combined with any subcellular fractionation, immunopurification or other proteomic method to identify specific subproteomes, thereby reducing sample complexity and enabling the identification of subtle changes in a proteome. This protocol can be completed in 5 days.

INTRODUCTION

Cells respond to fluctuations in their environment by changing the ensemble of proteins they express. Alterations in protein synthesis, degradation and post-translational modifications enable cells to adapt to changing environmental conditions. Hence, a major endeavor in biology is the comparison of two or more protein complements in biological systems, for example, the cancerous versus non-cancerous state, the addicted versus non-addicted brain circuit or the physiology of a genetically altered mouse versus a wild-type littermate. Mass spectrometry (MS)-based proteomics has become a vital and versatile technique to characterize the expression and functional modification of proteins, complementing the genomics efforts. Unlike the substrate of genomic research, DNA, proteins are diverse and heterogeneous biomolecules lacking the possibility of amplification, a fact that impedes their characterization and identification in complex mixtures. A cornucopia of biochemical and analytical tools for protein separation, fractionation and modification have been developed and combined to enable proteomic analysis. Resolving techniques such as one- and two-dimensional gel electrophoresis and multidimensional liquid chromatography, work in conjunction with MS to decipher the protein entity of a cell or a whole organism.

Despite immense technological advances in the last decade, no single proteome of a mammalian cell or lower eukaryotic micro-organism, such as the yeast, has been completely characterized. Facing an estimated number of approximately 10,000 different proteins in a single mammalian cell¹, in-depth identification of a cell's entire proteome, let alone the comparison to another proteome, is a major challenge for modern proteomics. Although today's state-of-the-art MS instruments routinely sequence single purified proteins with subfemtomolar sensitivity, the effective identification of low-abundance proteins is orders of magnitude lower in complex mixtures owing to limited dynamic range and sequencing speed². Copy numbers of proteins from different

mammalian cells and tissues vary with a predicted dynamic range of up to six orders of magnitude, and this number is even several orders of magnitude larger in plasma samples.

Strategies to reduce proteome complexity and to increase the dynamic range of protein identification include fractionation and affinity-purification approaches at both protein and peptide levels before MS analysis. In particular, fractionation methods of whole organelles (mitochondria³ and nucleolus⁴) and compartments, such as the postsynaptic density of neurons^{5,6} as well as affinity-purified protein complexes^{5,7,8}, have been successfully used to enhance proteomic analysis. Furthermore, the combination of MS with affinity purification for different post-translational modifications^{9–13} decreases sample complexity by enrichment of a specific subpopulation of the proteome.

Quantitative knowledge of the particular set of proteins expressed in different cellular locales at different times arguably brings one close to the knowledge of a cell's phenotype. Therefore, special efforts have been dedicated to the development of differential proteomic profiling approaches to compare proteomes with one another and to obtain relative quantification of individual proteins among samples. These methods include differential two-dimensional gel electrophoresis^{14,15}, isotope-coded affinity tags¹⁶ or isobaric tags for relative and absolute quantification¹⁷, quantitative proteomic analysis using samples from cells grown in ¹⁴N or ¹⁵N-media¹⁸ and stable isotope labeling by amino acids in cell culture^{4,19}. Although post-translational modifications such as phosphorylation or ubiquitination readily provide a suitable handle for enrichment of the "phosphoproteome" or for proteins destined for degradation, reducing sample complexity by selectively enriching for newly synthesized proteins is troublesome, as all proteins—old and new—share the same pool of 20 amino acids. Nonetheless, the specific enrichment and identification of recently synthesized proteins would complement the range of differential

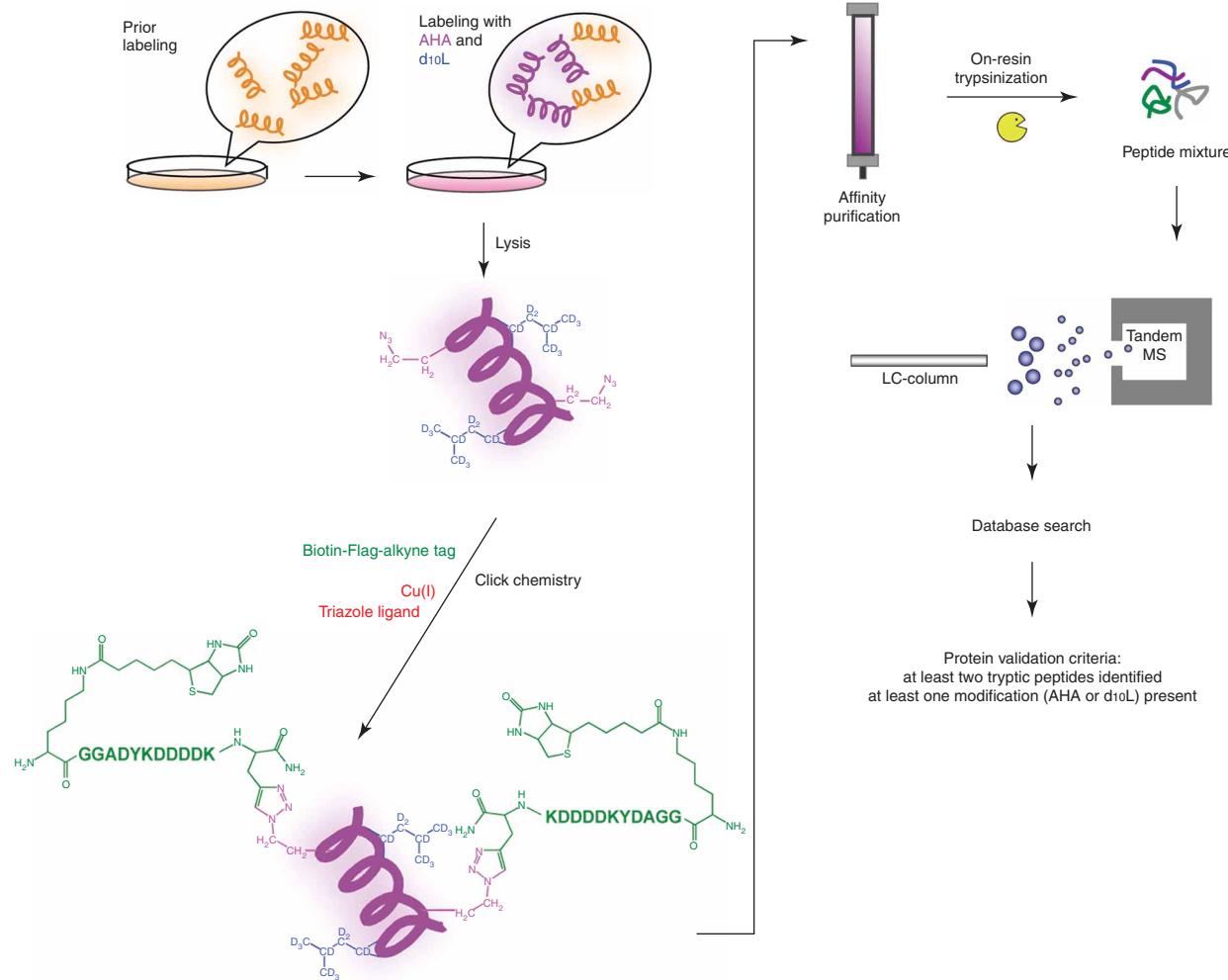


Figure 1 | The BONCAT strategy for labeling, detection and identification of newly synthesized proteins. Cells are incubated with AHA and d₁₀L to allow protein synthesis with AHA and d₁₀L incorporation. After incubation, cells are lysed or undergo a subcellular fractionation for biochemical enrichment of specific cellular compartments subsequently followed by lysis. Lysates are then coupled to an alkyne-bearing affinity tag, followed by affinity chromatography, to enrich for AHA-labeled proteins. Purified proteins are digested with a protease and the resulting peptides are analyzed by tandem MS to obtain experimental spectra. Different search programs are used to match the acquired spectra to protein sequences.

proteomic profiling methods and deepen our insights into the spatial and temporal dynamics of proteomes.

BONCAT (bioorthogonal non-canonical amino-acid tagging) was developed to specifically identify the subpopulation of newly synthesized proteins²⁰. The core of the BONCAT technique capitalizes on the manifold potential of small bioorthogonal chemoselective groups (recently reviewed by Prescher and Bertozzi²¹). These groups deliver unique chemical functionality to their target molecules, which subsequently can be tagged with exogenously delivered probes for detection or isolation in a highly selective manner. Among these chemical reporters, azides and alkynes have been used to label proteins^{22–30}, glycans^{27–30} and lipids²³ using the cell's own translation and protein modification apparatus. In the first step of BONCAT, newly synthesized proteins are labeled using the azide-bearing artificial amino acid AHA, endowing them with novel azide functionality, which distinguishes them from the pool of pre-existing proteins (see Fig. 1). Indeed, azides are abiotic in animals with the exception of an azide-metabolite found in unicellular organisms²². Moreover, despite general perception, azides are non-

toxic and stable²². Using the copper-catalyzed azide–alkyne ligation³¹, the reactive azide group of AHA is covalently coupled to an alkyne-bearing biotin-Flag tag in the second step. This tag enables subsequent detection, affinity purification and MS identification of AHA-labeled proteins. The enrichment for newly synthesized proteins decreases the complexity of the sample, fostering the identification of proteins expressed at low levels.

Although we routinely use the biotin moiety for avidin-based affinity purification, the Flag epitope provides sites for trypsin cleavage to allow direct proteolysis of proteins on the affinity matrix, bypassing the need for an elution step. In addition, the Flag epitope can be used as an alternative purification module if native biotinylation of proteins is a concern. After tryptic digestion of avidin-bound proteins, peptides bearing the tryptic remains of tagged AHA can serve as an immediate validation of candidate proteins. In the event of failed tagging, that is, unligated AHA, the mass difference between AHA and methionine marks this peptide as derived from a true newly synthesized candidate protein. To increase the chances of detecting metabolically modified peptides,

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we colabel cells with tenfold deuterated L-leucine (d_{10} L), which allows, in conjunction with the modification derived from the introduction of AHA, the validation of candidate proteins. We opted to use d_{10} L in our studies because leucine is the most abundant amino acid (9.83% of all amino-acid residues) in a human protein database as assessed by using the Python script AAEXCLUDE²⁰.

AHA is an effective surrogate for methionine, an essential amino acid, and does not require any (further) manipulations to be accepted as a substrate by the methionyl-tRNA synthetase^{24,25}. Labeling with AHA is very similar to the traditional metabolic labeling with radioactive amino acids (35 S-labeled methionine or cysteine) and has been tested in a variety of cell lines, primary neuronal cells as well as organotypic brain slice cultures (unpublished observations). The presence and incorporation of AHA is non-toxic and does not affect global rates of protein synthesis or degradation. A broad range of functionally and biochemically diverse proteins are identified²⁰. The copper-catalyzed azide–alkyne ligation³¹, also known as “click chemistry,” can be performed on denatured proteins in the presence of detergents, such as SDS, promoting the likewise identification of diverse classes of proteins, that is, membranous and soluble, acidic and basic as well as high- and low-molecular-weight proteins.

The identification of newly synthesized proteins with BONCAT is limited to proteins that possess at least one methionine residue, excluding the 1.02% of all entries in a human protein database, which do not contain a single methionine. Given that 5.08% of the human proteome possess only a single, N-terminal methionine and that this residue may be subject to removal by post-translational processing, at least 94% of the mammalian proteomes are candidates

for identification by BONCAT²⁰. Interestingly, we found no bias toward methionine-rich proteins in the proteomes we have characterized thus far, as evidenced by our identification of proteins with low methionine content²⁰. Furthermore, the methionine content of candidate proteins was found to be only slightly higher than the methionine content of the comprehensive protein database²⁰.

The core of the BONCAT technique—the chemoselective tagging of AHA-incorporated proteins—is not restricted to the mere identification of newly synthesized proteins in a shotgun proteomics approach. It also offers the possibility to work in combination with other proteomic approaches, such as isotope-coded affinity tags, isobaric tags for relative and absolute quantification or phospho- and glycoproteomic enrichment methods, to directly compare different proteomes in a single MS experiment or to facilitate the identification of even more and more specific subpopulations of the proteome, respectively. In this context, it is noteworthy that researchers may wish to choose any available mass spectrometrical identification procedure to adapt to a particular laboratory’s individual MS instrumentation environment to pursue identification of BONCAT-derived peptide mixtures. Furthermore, subcellular fractionation and immunopurification of protein complexes can be followed by BONCAT to assess the temporal and spatial dynamics of certain subcellular compartments, organelles and protein–protein interaction networks.

With regard to the specifics of the present protocol, we advise to perform a dot blot analysis mid-way through the procedure on an aliquot of the tagged (biotinylated) protein solution. This is performed to check quickly for the efficiency of the click chemistry reaction as well as to attain a crude estimate of the amount of tagged protein in the sample.

MATERIALS

REAGENTS

- Primary cells or cell lines in culture. Cell should be approximately 90% confluent
- 2× HBS (HEPES-buffered saline): 20 mM HEPES (1 M HEPES buffer; Sigma, cat. no. H0887), 238 mM sodium chloride, 10 mM potassium chloride, 4 mM calcium chloride, 4 mM magnesium chloride and 60 mM glucose, pH 7.35. Store in a refrigerator for up to 1 month after sterile filtration. Prepare 1× HBS freshly as needed
- 10× PBS (phosphate-buffered saline): 1.37 M sodium chloride, 27 mM potassium chloride, 43 mM disodium hydrogen phosphate and 14 mM potassium dihydrogen phosphate, pH 7.5
- Water, molecular biology grade (Sigma, cat. no. W4502)
- AHA (prepared as described previously³²; alternatively, researchers may obtain AHA via custom synthesis from appropriate organic synthesis companies): 20 mM stock solution in molecular biology grade water, store for up to 1 month at 4 °C; keep powder desiccated at room temperature (RT, 20–24 °C)
- L-Leucine- d_{10} (d_{10} L; Sigma, cat. no. 492949): 100 mM stock solution in molecular biology grade water, store for up to 1 month at –20 °C
- L-Methionine (Sigma, cat. no. M9625): 20 mM stock solution in molecular biology grade water, store for up to 1 month at 4 °C
- 20% (w/v)SDS in molecular biology grade water
- 20% (v/v) Triton X-100 in molecular biology grade water
- Triazole ligand (prepared as described previously³³): 200 mM stock solution in DMSO ▲ **Critical** Avoid exposure of the solution to air and water. Aliquot into small volumes and store at –20 °C. Store powder desiccated at RT
- Biotin-Flag-Alkyne (TAP) tag: NH₂-Biotinyl-**GGADYKDDDDK**-propargylglycine-CONH₂ (GenScript Corporation; amino acids bold in one-letter code): 25 mM stock solution in 1× PBS, aliquot and store at –20 °C for up to 6 months
- Copper (I) bromide, 99.999% (Aldrich, cat. no. 254185), store desiccated at RT
- Tris-(2-carboxyethyl)phosphine (TCEP; Sigma, cat. no. C4706): 0.5 M in molecular biology grade water, prepare fresh before use
- Iodoacetamide (Sigma, cat. no. I1149): 0.5 M in molecular biology grade water, prepare fresh before use
- Complete EDTA-free protease inhibitor (Roche, cat. no. 1873580)
- Benzonase (Sigma, cat. no. E1014)
- PD-10 columns (Amersham Pharmacia Bioscience, cat. no. 17-0851-01)
- ImmunoPure biotinylated BSA (Pierce, cat. no. 29130)
- Immobilized NeutrAvidin (Pierce, cat. no. 29200)
- Sequencing grade modified trypsin (Promega, cat. no. V5111)
- Endopeptidase Lys-C (Roche, cat. no. 1420429)
- Urea (Sigma, cat. no. U1250)
- Formic acid, 88% (vol/vol), ACS reagent (Sigma, cat. no. 399388)
- 10× TBS (Tris-buffered saline): 200 mM Tris/HCl, 8% (w/v) sodium chloride, pH 7.6
- 4× SDS sample buffer: 1% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 250 mM Tris/HCl pH 6.8, 0.004% (w/v) bromophenol blue
- Nitrocellulose membrane (such as Protran BA85, 0.45 μm, from Whatman) and PVDF membrane (0.2 μm, such as Immun-Blot from Bio-Rad, ImmobilonP from Millipore)
- Western blotting detection system, such as ECL from GE Healthcare
- Imaging film such as KODAK BioMax XAR Film
- Empty spin columns (such as Handee Spin Columns with screw caps and Luer-Lok adaptors from Pierce, cat. no. 69705)

EQUIPMENT

- Dot blot apparatus (such as Bio-Dot from Bio-Rad, cat. no. 170-6545)
- Rotisserie-Rotator
- Temperature-controlled shaker for microcentrifuge tubes (such as Thermomixer from Eppendorf)
- Electrospray-ionization ion-trap tandem mass spectrometer (e.g., Thermo Finnigan LCQ or LTQ)
- Software tools:
 - Search algorithms such as SEQUEST³⁴, MASCOT³⁵ and X!Tandem^{36,37}
 - DTSelect³⁸ or PeptideProphet³⁹ and ProteinProphet⁴⁰

REAGENT SETUP

All solutions for click chemistry must be free of EDTA, EGTA and other chelators to avoid inactivation of the copper (I) catalyst.

AHA/d₁₀L-HBS We use 4 mM AHA and 4 mM d₁₀L in 1× HBS (AHA-HBS) for labeling of HEK293, COS7 or primary neuronal cells. However, investigators may wish to determine the optimal concentration of AHA and d₁₀L as well as labeling time individually for metabolic labeling for each cell type on a small-scale first.

Met/d₁₀L-HBS Use same concentration of methionine in experiments as AHA and d₁₀L for the control sample.

PBS-MC 1× PBS supplemented with 1 mM magnesium chloride and 0.1 mM calcium chloride; chill on ice.

PBS-PI 1× PBS supplemented with Complete EDTA-free protease inhibitor, prepare fresh immediately before use according to the manufacturer's instruction; chill on ice until use.

PD-10-column buffer 0.05% (w/v) SDS in 1× PBS.

Biotinylated BSA standard for dot blot analysis Reconstitute ImmunoPure biotinylated BSA as a stock solution of 2 mg ml⁻¹ in water. Prepare biotinylated BSA working solutions of the following concentrations in 1× TBS: 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 ng µl⁻¹.

TBS-T 0.05% (v/v) Tween 20 (Sigma, cat. no. P1379) in 1× TBS, pH 7.6.

NeutrAvidin-binding buffer 1% (v/v) NP-40 (Nonidet P40 Substitute; Fluka, cat. no. 74385), 0.05% (v/v) SDS and 1× PBS, pH 7.5.

NeutrAvidin wash buffer A (NW-A) 1% (v/v) NP-40 and 1× PBS, pH 7.5.

NeutrAvidin wash buffer B (NW-B) 50 mM ammonium bicarbonate.

PBS-T 0.1% v/v Tween 20 in 1× PBS, pH 7.5.

Polyclonal rabbit biotin antibody (Bethyl Laboratories Inc., cat. no. A150-109A): 1:10,000 in 5% dry milk in PBS-T or TBS-T.

Secondary HRP-conjugated anti-rabbit antibody (Such as from Jackson Immuno Research) in 5% dry milk in PBS-T or TBS-T.

PROCEDURE**Cell labeling** ● **TIMING** 3.5 h

- 1| Prepare 1× HBS, AHA/d₁₀L-HBS and Met/d₁₀L-HBS (for control samples)—see REAGENTS and REAGENT SETUP—and warm to 37 °C (at least 20 min). You will need 6 ml of each solution for a round 100 mm dish.
 - 2| Rinse cells briefly in 6 ml of 1× HBS and incubate in the same volume of fresh 1× HBS for 30 min in a 37 °C, 5% CO₂ incubator. This preincubation step allows the depletion of amino acids including methionine and leucine.
 - 3| Remove 1× HBS, add 6 ml of AHA/d₁₀L-HBS or Met/d₁₀L-HBS to cells; move cells back to 37 °C, 5% CO₂ incubator for desired incubation time. In our experiments, we found an incubation of 1–2 h to be sufficient. However, shorter and longer incubation times should be selected according to the specific nature of the experiment and the cells used.
 - 4| Place cell dishes on ice and rinse cells briefly in chilled PBS-MC. (Magnesium chloride and calcium chloride are added to PBS in this step to maintain proper ionic strength in the buffer system to preserve membrane integrity. Once the cells are lysed, this is no longer necessary.)
 - 5| Harvest cells in 6 ml of chilled PBS-PI supplemented with 1 mM magnesium chloride and 0.1 mM calcium chloride; transfer the cell suspension to a 15 ml plastic tube, spin down (2,000g, 5 min, 4 °C) and carefully remove all the supernatant from the cell pellet.
- **PAUSE POINT** Cell pellets can be stored at –80 °C for several months after flash freezing in liquid nitrogen without any harmful effect on click chemistry efficiency. Note that after harvesting the cells, one may proceed to Step 6, or, alternatively carry out subcellular fractionation protocols before cell lysis. In either case, we recommend the use of a phosphate buffer system (\geq pH 7.5) for the subsequent click chemistry reaction (see also Step 6).

Click chemistry reaction ● **TIMING** 18 h; overnight step

- 6| Lyse cell pellet in 250 µl of 1% (w/v) SDS in PBS (lysis buffer) by vigorous vortexing. Add 1 µl of Benzonase (\geq 500 U) and mix well. Boil samples for 10 min at 96–100 °C to achieve complete lysis and denaturation of proteins; chill on ice.

? TROUBLESHOOTING

- 7| Adjust samples to 0.1% (w/v) SDS by adding the appropriate amount of PBS (~2,250 µl). Add 25 µl of 20% (v/v) Triton X-100 to a final concentration of 0.2% (v/v).
- ▲ **Critical Step** High concentrations of SDS (>0.2% (w/v)) will decrease the efficiency of the tagging reaction.
- 8| Spin down at 2,000g for 5 min at 4 °C; transfer the supernatant to a new test tube and allow it to warm up to RT.
- 9| Thaw TAP tag and triazole ligand; prepare a 10 mg ml⁻¹ copper bromide suspension in molecular biology grade water: transfer 10 mg of copper bromide to a microcentrifuge test tube and add 1 ml of water immediately before use. Vortex vigorously for 20 s. If more than two samples are processed, use multiple fresh copper bromide suspensions.
- ▲ **Critical Step** Fresh copper bromide suspensions must be used because of the rapid disproportionation of the catalytically active Cu(I) ion in copper bromide to Cu(0) and Cu(II) in the presence of water.
- 10| For each 1 ml of the supernatant from Step 8, add 1 µl triazole ligand, 2 µl TAP tag and 10 µl of copper bromide suspension (in the mentioned order). After the addition of the triazole ligand, a light “milky” turbidity of the sample appears, which vanishes after the addition of the TAP tag and the copper bromide suspension.
- ▲ **Critical Step** The click chemistry reaction mix has to be set up for each sample separately in the mentioned order without any delay to avoid precipitation of the triazole ligand and disproportionation of the Cu(I) ion. After the addition of each reagent, vortex vigorously for 15–20 s.

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11| Incubate samples either for 6 h at RT under constant agitation in a rotisserie-rotator, or
■ PAUSE POINT Incubate overnight at 4 °C with constant agitation.

12| Spin down at 2,000g for 5 min at 4 °C. A small turquoise pellet should be observed.

? TROUBLESHOOTING

13| Transfer the supernatant to a new test tube.

■ PAUSE POINT Samples can be stored at –20 °C at this point for short periods of time (1–2 days).

Dot blot analysis ● TIMING 5 h

14| Remove excess unreacted tag by gel filtration using PD-10 columns. Equilibrate each column with 25 ml of PD-10-column buffer. Apply 2.5 ml of sample, discard flow-through and elute sample with 3.5 ml of PD-10-column buffer; keep the 3.5 ml sample eluate fractions collected on ice until further use. Columns can be reused for larger sample volumes after washing with 30 ml of water and re-equilibration with 25 ml of PD-10-column buffer.

15| Prepare 200 µl of sample dilutions in 1× TBS. We routinely use 1:200 and 1:100 dilutions of the samples from Step 14.

16| Equilibrate nitrocellulose membrane in 1× TBS and assemble the dot blot according to the manufacturer's recommendation. Before applying the samples and the BSA standards, wash all wells once with 100 µl 1× TBS. Gently remove the buffer from the wells by applying vacuum. Apply to each well 100 µl of biotinylated BSA standard (at different concentrations; see REAGENT SETUP) and 100 µl of sample dilutions in duplicate. Remove the samples from the wells by applying vacuum. Wash all wells once again with 100 µl of 1× TBS.

17| Blocking of nonspecific protein binding to the membrane is achieved by placing the membrane in 5% dry milk in TBS-T for 45 min at RT with gentle agitation on a rocking plate. Blocking reduces “noise” and eliminates false positives in the dot blot. Incubate the membrane with the polyclonal rabbit biotin antibody dilution (1:10,000 in 5% dry milk in TBS-T) for 1 h at RT with gentle agitation. Wash the membrane three times with TBS-T for 5 min each. Incubate the membrane with the secondary antibody dilution (1:10,000 in 5% dry milk in TBS-T) for 45 min at RT with gentle agitation. Wash the membrane three times with TBS-T for 5 min each. Detect biotinylated proteins using a western blotting detection system such as ECL from GE Healthcare in combination with an imaging film such as BioMax XAR film from KODAK according to the manufacturer's instructions. The amount of biotinylated, that is, newly synthesized proteins in the samples is estimated densitometrically by comparing the intensity of the sample dots with the intensity of biotinylated BSA standard dots (see Fig. 2).

? TROUBLESHOOTING

NeutrAvidin purification and on-resin trypsinization ● TIMING 19 h; overnight step

18| Heat the samples from Step 14 for 3 min at 96–100 °C to bring any SDS precipitates back into solution.

19| Adjust samples to 1% (v/v) NP-40 and 0.05% (w/v) SDS in PBS-PI at an approximate concentration of 25 µg ml⁻¹ of biotinylated proteins. Preserve 30 µl of this sample for subsequent western blot analysis (“input”); immediately add 10 µl of 4× SDS sample buffer and store at –20 °C until further use (1–2 weeks).

20| For each 25 µg of biotinylated proteins, transfer 100 µl of NeutrAvidin resin (provided as a 50% slurry, i.e., 100 µl of the slurry corresponds to approximately 50 µl resin or “bed” volume) to a 15 ml plastic tube. Wash NeutrAvidin resin three times with ten bed volumes of NeutrAvidin-binding buffer.

21| Add the sample solution from Step 19 to the washed NeutrAvidin resin from Step 20.

■ PAUSE POINT Allow binding of biotinylated proteins to NeutrAvidin by incubating overnight (12–16 h) at 4 °C with constant agitation.

22| Spin down the resin at 2,000g for 5 min at 4 °C. Remove the supernatant carefully from the resin; preserve 30 µl of the supernatant for subsequent western blot analysis (“supernatant”); immediately add 10 µl of 4× SDS sample buffer and store at –20 °C until further use (1–2 weeks).

23| Wash resin with ten bed volumes of NW-A buffer for 10 min at RT with constant agitation, spin down (2,000g for 5 min at 4 °C) and discard the supernatant; repeat two more times.

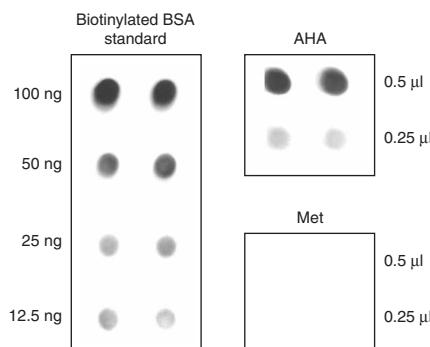


Figure 2 | Dot blot analysis of AHA versus Met-treated samples. Dot blot analysis provides a mid-procedure demonstration of proper copper(I)-catalyzed tagging as well as a crude estimate of the amount of tagged protein in the sample. Duplicates of each condition are shown. The Dot blot membrane was probed with an anti-biotin antibody.

24 Wash resin two times with NW-B buffer followed by two washes with 1× PBS in the same manner as above.

25 Wash resin two times with 50 mM ammonium bicarbonate as before, then transfer the resin to a microcentrifuge tube using a cut tip with a bigger opening. Spin down the resin (4,000g for 5 min at 4 °C). Carefully remove the supernatant. Note that researchers could alternatively proceed with SDS elution of NeutrAvidin-bound proteins followed by one-dimensional gel electrophoresis and appropriate in-gel trypsinization and tandem MS instead of MudPIT analysis, thus adapting to a particular laboratory's individual MS instrumentation environment.

26 For each initial 100 µl of Neutravidin slurry, resuspend the resin to a total volume of 87 µl in 50 mM ammonium bicarbonate. This “reduced” volume is to compensate for the later volume gain caused by dissolving urea in Step 28.

27 Heat the suspension for 10 min at 70 °C while agitating the beads using the Thermomixer.

28 Immediately, add 18 mg of urea beads for each initial 100 µl of Neutravidin slurry to the suspension and vortex the suspension until the beads have been dissolved. The final concentration of urea should be between 2 and 3 M to facilitate trypsinization of the partially denatured NeutrAvidin-bound proteins. Note that the biotin–NeutrAvidin interaction is stable at urea concentrations as high as 8 M.

29 Allow the suspension to cool down to RT before adding the reducing agent TCEP to a final concentration of 3.125 mM (for each 100 µl suspension, add 0.625 µl of 0.5 M TCEP) to reduce disulfide bonds. Incubate for 30 min at RT with constant agitation.

30 Alkylate reduced cysteine residues by adding iodoacetamide to a final concentration of 11.2 mM (for each 100 µl of suspension, add 2.25 µl of 0.5 M iodoacetamide). Incubate for 30 min at RT in the dark with constant agitation. This alkylation step is necessary for the identification of cysteine-containing peptides, promoting maximal possible sequence coverage of BONCAT-labeled candidate proteins.

31 Transfer a small aliquot (30–50 µl) of the suspension to a microcentrifuge tube for subsequent western blot analysis (“NeutrAvidin-bound proteins”). Spin down, remove the supernatant, add 20 µl of 4× SDS sample buffer and store at –20 °C until further use (1–2 weeks).

32 Add 0.1 µg endoproteinase Lys-C per 100 µl of suspension to initiate proteolysis and incubate for 4 h at 37 °C while agitating the beads. Lys-C cleaves at the C-terminal end of lysine and the resulting peptides are larger than tryptic peptides.

33 Add calcium chloride to a final concentration of 0.1 mM and trypsinize the sample adding 1 µg trypsin per 100 µl of suspension.

■ **PAUSE POINT** Incubate the reaction mixture overnight at 37 °C while agitating the beads.

34 Transfer the suspension to an empty spin column in a microcentrifuge tube. Briefly pulse-spin to separate the supernatant containing the tryptic peptides from the resin. Add formic acid to a final concentration of 5% (v/v) to the peptide solution and store at –20 °C until further use.

■ **PAUSE POINT** Samples can be stored at –20 °C for 1–2 weeks.

35 Transfer the resin back into a microcentrifuge tube and resuspend it in 50 mM ammonium bicarbonate. The total volume of the suspension should be the same as for the proteolytic digestion (see Step 31). Transfer the same aliquot as in Step 31 of this suspension to a microcentrifuge tube for subsequent western blot analysis (“NeutrAvidin after trypsinization”). Spin down, remove the supernatant, add 4× SDS sample buffer and store at –20 °C until further use (1–2 weeks).

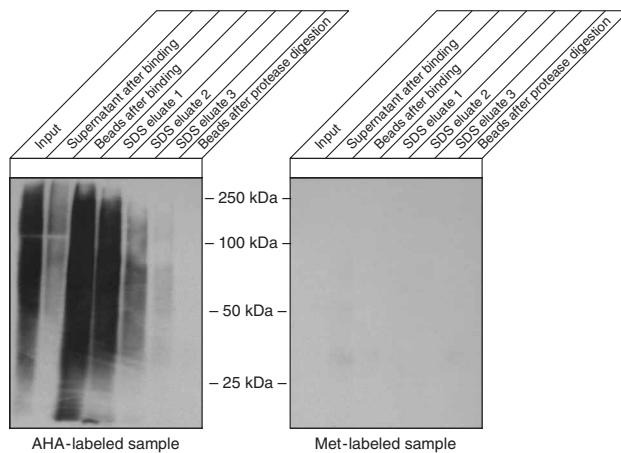


Figure 3 | Western blot analysis of AHA versus Met-treated sample purification fractions. Upon completing the procedure, a western blot analysis of the various collected fractions will reflect the efficiency of the purification and subsequent trypsinization. The western blot membrane was probed with an anti-biotin antibody.

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36 | Perform analysis of the samples collected in Steps 19, 22, 31 and 35 using the standard western blot technique to evaluate the purification and trypsinization efficiency using a biotin antibody. A typical example of western blot is shown in **Figure 3**.

Mass spectrometrical analysis • TIMING 2 days

37 | We use MudPIT^{41,42} as a shotgun proteomics approach for the identification of peptides in complex mixtures generated with BONCAT. Alternatively, researchers may wish to use either a gel-based proteomics technique (in which case, NeutrAvidin-bound proteins are eluted from the resin after Step 25) or a separate cation exchange and reverse-phase chromatography. A step-by-step MudPIT protocol has been described in detail elsewhere⁴³; therefore, the following section is focusing on the search parameters necessary for the identification of AHA- and d₁₀L-modified peptides.

38 | Search the tandem mass spectra against a protein sequence database using the SEQUEST algorithm. The following dynamic modification search parameters should be considered for the methionine residue (see Figure 4): After complete tag cleavage, the mass gain of tagged AHA over methionine is 107 AMU (atomic mass unit). In the case of incomplete tag cleavage, the resulting mass gain of tagged AHA over methionine is 695.6 AMU (with no further fragmentation of the tag moiety required), whereas in the event of failed tagging (unligated AHA), the mass loss of AHA over methionine would be 5.1 AMU. A set of stringent search constraints including the fully tryptic status of each peptide, a minimum of two valid peptides per locus and a minimum of one peptide containing an AHA-derived or d₁₀L modification, are required to ensure that identified proteins were translated during the AHA labeling step. Sample spectra can be found as supplementary material of Dieterich *et al.*²⁰ online under <http://www.pnas.org/cgi/content/full/0601637103/DC1>.

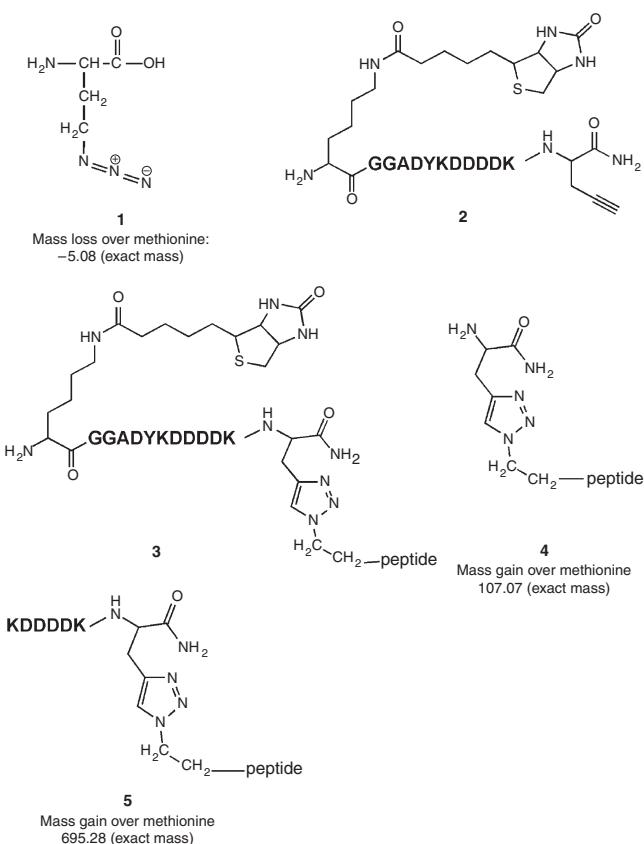


Figure 4 | Structure of AHA-based modifications. (1) Unligated AHA, (2) unligated affinity tag, (3) affinity tag coupled to AHA before trypsinization, (4) remaining moiety after complete trypsinization and (5) remaining moiety after one missed cleavage.

• TIMING

Cell labeling

Steps 1 and 2, 1 h

Step 3, Variable, that is, experiment dependent

Steps 4–5, 20 min

Click chemistry reaction

Steps 6–11, 30 min

Step 12, Variable: 6–16 h, depending on incubation temperature

Step 13, 10 min

Dot blot analysis, Steps 14–17, 5 h

Neutravidin purification

Steps 18 and 19, 10 min

Step 20, 20 min

Step 21, 12–16 h (overnight)

Steps 22–25, 2.5 h

On-resin trypsinization

Steps 26–31, 1.5 h

Step 32, 4 h

Step 33, 12–16 h (overnight)

Step 34, 10 min

Step 35, 10 min

Step 36, 8 h

Mass spectrometrical analysis, Steps 37 and 38, 2 days

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Steps	Problem	Possible reason	Solution
6	Incomplete cell lysis	Detergent concentration insufficient Total protein concentration too high Insufficient genomic DNA lysis	Increase detergent concentration up to 2% (w/v) SDS Increase sample volume Use more Benzonase to facilitate lysis of genomic DNA or shear DNA with a syringe and a needle
12	Failed click chemistry reaction	Too much detergent present Ineffective triazole ligand Bad quality of copper (I) bromide suspension pH too low	Lower detergent concentration Prepare fresh triazole ligand Use fresh copper (I) bromide Check pH of the solution and adjust to >pH 7.5
17	Low levels of tagged proteins	Failed click chemistry reaction Labeling time too short	See "Problem Step 12" Increase incubation time with AHA

ANTICIPATED RESULTS

Following a 2 h incubation time with AHA/d₁₀L and using 1.8–2.1 mg of whole mammalian cell lysates, approximately 150–200 µg of biotinylated protein is tagged with BONCAT for subsequent NeutrAvidin purification and tandem MS analysis. From these, about 200 valid candidates containing either AHA- or d₁₀L-based modified peptides can be identified with a Thermo Finnigan LCQ mass spectrometer in a shotgun approach, such as MudPIT. The use of new mass spectrometers with higher sensitivity, faster sequencing speed and higher dynamic range promises to increase the number of identifications. The rate of protein synthesis may vary from cell line to cell line and between cell lines, primary cultures and organotypic slice cultures (unpublished observations).

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