Chapter 6

QUANTIFYING ENRICHMENT OF AZIDE-TAGGED PROTEINS

6.1 Abstract

We describe the development of SPIQE (Spike Peptide In to Quantify Enrichment), a novel method of quantifying the gain in enrichment of azide-tagged proteins from untagged proteins. The SPIQE peptide represents a small fraction of the proteomic samples but can be used to validate the method of enrichment of samples through various bio-conjugation strategies. We compare the enrichment values obtained by SPIQE to those obtained by stable isotope labeling of amino acids in culture (SILAC). Similar values indicated that SPIQE would be a useful addition to samples that do not have other characteristics to quantify enrichment.

This work was a collaborative effort with Judy Shon, Michael J. Sweredoski, Annie Moradian, Roxana Eggleston-Rangel, and Sonja Hess.
6.2 Introduction

Shotgun proteomics has revolutionized our ability to globally profile protein expression in complex cellular environments (1). This bottom-up proteomics strategy requires digestion of whole proteins within the cellular lysate, separation of the resulting peptides by liquid chromatography (LC), and subsequent detection by tandem mass spectrometry (MS/MS) (2). To broadly survey the entire complex lysate, the mass spectrometer selects the top precursor ions detected in the first MS scan (which are often the most abundant, since intensity and abundance are correlated) and subjects them to a second MS/MS fragmentation scan centered on that mass-to-charge ratio (m/z). The masses of these peptides are then compared to a theoretical digest of the organism’s proteome using computational tools (3). Because of the nature of shotgun proteomics and its limited dynamic range, highly abundant proteins in the sample are often the most likely to be found (4). Sometimes investigators are interested in identifying only a small subset of these proteins, such as proteins that have been newly-synthesized, proteins with a particular post-translational modification (PTM), or proteins that are in an active enzymatic state. In these cases, if too many peptides from other proteins are in the sample, their signal will drown out the signal of the proteins of interest (Fig 6.1).
Figure 6.1: Scheme depicting shotgun proteomics and the difficulty in finding proteins of interest (orange) when they are not in high abundance compared to the background proteome (blue).

One way to enrich for these proteins of interest is to chemically append a tag that can subsequently be used to “fish out” the proteins of interest from the rest of the proteome (5). Many groups use the azide group as a tag because it is small, absent in biological systems, and able to specifically react with both terminal alkynes in the presence of Cu(I) (Copper-catalyzed alkyne-azide cycloaddition, or CuAAC) or strained cyclooctynes (Strain-promoted alkyne-azide cycloaddition, or SPAAC), which are also known as “click” reactions (6, 7). Once an azide has been introduced into a protein of interest, it can then be conjugated to a fluorophore or enrichment handle (such as biotin or directly to a resin) for detection and enrichment. In this manner, azides have been used to identify...
newly-synthesized proteins (8-11), sites of post-translational modification (12-15), and active enzymes (16, 17).

After using CuAAC or SPAAC to append a linker attached to an affinity-handle (such as biotin) or directly to a resin, stringent washes remove the untagged proteins. The azide-tagged proteins can then be digested directly on the resin and eluted to collect their peptide fragments, or cleaved and eluted intact if the chosen linker is cleavable (18). While cleavable linkers are useful for finding the precise sites of azide incorporation into the peptide, they are sometimes unnecessary for the experiment. Identifying the site of azide-tagging on the protein is challenging, since any peptide with a tag adduct represents a very small fraction of the proteome – difficult to detect with a global search. Furthermore, azides are often heterogeneously incorporated into peptides; thus, if a cleavable resin is used the total ion current of each peptide may be distributed over multiple species and become more difficult to detect. If the goal of an experiment is to find newly-synthesized proteins in a sample, digesting the entire protein leads to more peptides that can be detected by the MS and higher confidence protein identifications. The drawback of this method is that it is more difficult to prove that the peptide came from an azide-tagged protein.

Enrichment of azide-tagged proteins from untagged ones is not a perfect process and depends on numerous factors such as the fraction of the proteins that are azide-tagged, and the relative rates of the click reaction to non-specific background reactions. Samples that
lack introduced azide tags when combined with resin will often elute nonspecifically bound proteins, which the mass spectrometer will detect. Running a “mock” enrichment alongside any experiment is often necessary to compare the amount of protein eluted from the resin in the absence or presence of azide-tagging. The ratio of these relative peptide concentrations can be used as a semi-quantitative method to determine the “strength” or level of enrichment gain. A quantitative method to determine the gain of an enrichment method on the mass spectrometer itself would be useful, which is why we developed SPIQE (Spike Peptide In to Quantify Enrichment) for reporting the gain of any azide-based proteomic enrichment. Herein we report the development of this peptide method and compare it to other methods often used to quantify enrichments.

Quantitative mass spectrometry

Mass spectrometry is not inherently quantitative; peptides do not ionize with identical efficiencies/rates in an ion trap and thus, the reported intensities of peptides with different sequences cannot be directly compared. Furthermore, the triggering of an MS/MS fragmentation is often stochastic for all but the highest abundance proteins. If a given peptide is not fragmented in one sample it does not indicate its absence from the sample, only that the peptide’s rank order was not high enough to trigger fragmentation. Quantitative MS techniques seek to solve this problem by using isotope labels for valid comparisons of peptides between samples. For example, stable isotope labeling of amino acids in culture (SILAC) relies on the use of “light” (normal) or “heavy” amino acids within proteins (19). Heavy amino acids contain isotopes such as $^{15}$N or $^{13}$C, which are
indistinguishable from light amino acids within the cell, but easily identifiable using MS due to the change in their mass-to-charge (m/z) ratio. The light and heavy peptides elute at the same time during the LC separation, and the ratios can be directly compared as a means to quantify proteins between samples. Furthermore, if one of the peptides does not trigger an MS/MS fragmentation, the first MS scan can be used to estimate the abundance of the pair.

Both Eichelbaum *et al.* and Yuet *et al.* showed that combining SILAC with azide-based metabolic labeling methods allows for both quantification of enrichment and determination of whether a given protein came from an azide-tagged sample (10, 11). In this strategy, one sample contains only light amino acids and no azide tagging, and another sample contains the heavy amino acid(s) and azide-tagged proteins. Mixing these samples together 1:1 and subjecting them to a click-based enrichment step increases the fraction of peptides with heavy amino acids (20-22). The gain of the enrichment step can be sampled for each heavy:light peptide pair observed by the MS, resulting in a high confidence measure of enrichment (Fig 6.2). Another strategy called Heavy Isotope Labeled Azidohomoalanine Quantification (HILAQ) uses a heavy-isotope labeled azide-containing amino acid, which can both identify and quantify labeled sites (23). SILAC and HILAQ are valuable tools to quantify labeling when combined with azide-enrichment methods, but some samples are not amenable to this type of metabolic tagging or prohibitively expensive to label, such as in studies using animals.
6.3 General Approach

Our approach relies on synthetic peptides with two distinct features (Fig 6.2): One version of the peptide has no azide tag and a light arginine, while the other has an azide-containing amino acid in place of methionine and a heavy arginine. Both peptides share an internal lysine that a proteolytic enzyme (i.e. trypsin, Lys-C) can recognize and cleave. A sample spiked with these peptides models an experiment that combines an azide-tagging method with SILAC, but the sample does not need to be SILAC labeled. In a typical experiment, the light and heavy peptides are mixed 1:1 and spiked in low amounts into the sample to be enriched. During enrichment, the light peptide will not bind the resin since it has no azide, while the heavy peptide will conjugate to the resin via the click reaction. After proteolytic digestion, the released light or heavy peptides are then identified using MS and their ratios quantified to measure the gain of an enrichment method without having to label samples prior using SILAC. We call this peptide mixture SPIQE (Spike Peptide In to Quantify Enrichment).
We aimed to compare the enrichment gains obtained from the SPIQE peptide to the SILAC strategy of quantifying enrichment, so we used SILAC-labeled lysates that had also been tagged with azides using bioorthogonal noncanonical amino acid tagging (BONCAT) (20, 21). BONCAT tags newly-synthesized proteins with azido-amino acids in a residue-specific manner and does not require the expression of any mutant aminoacyl-
tRNA synthetases or tRNAs. The method relies on the cell’s endogenous machinery stochastically incorporating azidohomoalanine (Aha) instead of methionine during translation. Using a 30:1 mixture of Aha:Met removes any artifacts in quantitation associated with total methionine replacement (21). Samples tagged with azides were also fully labeled with light lysine, while untagged samples were labeled with heavy lysine. This experimental design allows us to measure the gain of our enrichment in two ways: 1) Calculating the light:heavy peptide ratios observed from the lysate using the traditional SILAC method 2) Calculating the heavy:light ratio of our SPIQE peptide within this complex mixture (Fig 6.2). We then compared the values obtained by each method to see if SPIQE could be used to measure enrichment gain when samples are not labeled by SILAC.

6.4 Results

Synthesis of SPIQE peptide. A model peptide from green fluorescent protein (GFP) (GGXQHDFFKSAMPEGYVQER, where X is methionine or azidohomoalanine, and R is light or heavy arginine (Fig 6.2), was synthesized with an N-terminal acetyl group, purified by high-performance liquid chromatography (HPLC), and characterized by MS (JPT Technologies; Berlin, Germany). We chose this peptide from GFP because it has been found often in standard MS experiments, indicating efficient ionization. The synthesized peptides were dissolved in 10% acetonitrile (ACN) and mixed together in a ~1:1 ratio.
**Preparation of SILAC+BONCAT labeled lysates.** *E. coli* DH10B cells were labeled in M9 minimal media with 18 amino acids (-Met, -Lys) and either 30:1 azidohomoalanine/methionine (Aha/Met) + Lys0 (light, tagged sample) or Met + Lys8 (heavy, untagged sample) for three days, with 3 separate 1:100 refreshes into new media to ensure quantitative replacement of lysine (21, 24). We verified the cells were tagged with azides using in-gel fluorescence (Fig 6.3), then digested the samples using trypsin, ran the resulting peptides on LC-MS/MS, and used MaxQuant quantitative proteomic software to search the *E. coli* proteome (UniProt) for peptides that include light or heavy lysine (3). The SILAC values were normalized and each evidence count (n = 1299) was plotted from lowest to highest SILAC ratios, which confirmed quantitative incorporation of light lysine in the Aha-tagged samples and heavy lysine in the methionine only samples. These light and heavy lysates were then mixed in a 1:1 “light + heavy” ratio and used for subsequent quantifications of enrichment gain to compare to those observed with the SPIQE peptide.

In addition to these samples, we also performed a “label-swap” experiment where cells were growth with 30:1 Aha/Met + Lys8 (heavy, tagged sample) or Met + Lys0 (light, untagged sample). We likewise confirmed quantitative incorporation of light or heavy lysine in each of the samples (Fig S6.1A). This label swap condition was used to determine the enrichment strategy that obtained the most peptides with low (or zero) SILAC ratios. By using the SILAC ratio and the number of light or heavy peptides, we found that DBCO-based resins and linker performed best for these azide-tagged samples.
with enrichments of light peptides 100 to 1000-fold compared to pre-enriched samples (Fig S6.1B, S6.1C). We consequently used DBCO-agarose to test the SPIQE peptide enrichment.

**MS analysis of unenriched SPIQE peptide mix in complex lysate.** The 1:1 SPIQE peptide mix was added to 400 μL of the “light + heavy” *E. coli* lysate described above at either 25 μg peptide/mg of lysate or 2.5 μg peptide/mg of lysate. Prior to enrichment, we took 5% of this sample and reduced and alkylated the proteins prior to digestion and desalting. We then ran this sample using a directed search with a QTRAP mass spectrometer, acquiring MS/MS for the specific mass of the peptides (Fig 6.3B). The set-up and analysis was performed using Skyline, an application for both proteomic method creation and quantitative data analysis (25). By integrating the intensity of each peptide peak, we determined that this initial mixture without any enrichment was 44.4% “heavy” and 55.6% “light” (Fig 6.3C).

**MS characterization of enrichment using a targeted search.** We enriched the samples using DBCO-agarose, as described in the supplemental methods. Importantly, we reduced and alkylated all proteins prior to enrichment to prevent thiol-yne addition (26). The enriched peptides were digested using trypsin, desalted using stage tips, and run on a QTRAP using the same method previously used to characterize the 1:1 peptide mixture. Using Skyline software to integrate the area under the “light” and “heavy” peaks revealed that in the 25 μg peptide/mg lysate, we found >99.99% heavy peptide, and in the 2.5 μg
peptide/mg lysate, we found 99.95\% heavy peptide (Fig 6.3C). Starting with a 44.44\% “heavy” peptide mixture and bringing it to these levels indicate 8000-fold and 1600-fold enrichment, respectively.

**MS characterization of enrichment using data-dependent acquisition.** Shotgun proteomics experiments often do not use targeted searches, but instead rely on data-dependent acquisition methods to analyze peptides without prior knowledge of their sequences. We injected 100 ng of the enriched 2.5 \( \mu \)g peptide/mg lysate onto an Orbitrap Elite MS (Thermo Fisher Scientific) coupled to a Proxeon Easy-nLC 1000 (Thermo Fisher Scientific) and collected the top ions for fragmentation over a 1 hour run (See Experimental Procedures for more detailed information). We then used Skyline to search for the SPIQE peptide within this run and integrating the area under both curves revealed an enrichment factor of >10,000 (Fig 6.3D). This number may represent the limit of detection in this type of experiment; due to the lack of any signal for the light SPIQE peptide, we integrated the background noise to achieve this number so the confidence in this actual value is low.
Figure 6.3: A) *E. coli* lysate labeled with either Met+Lys0 or 30:1 AHA:Met+Lys8 and clicked to TAMRA-alkyne. Note: these lysates are not the ones used for the enrichment experiments with the SPIQE peptide as we used the label-swapped version of Met+Lys8 and 30:1 AHA:Met+Lys0 B) Traces of SPIQE peptide before and after enrichment shows the removal of the light version of the peptide. C) Table quantifying the area under the curve (AUC) from the trace in B) as well as AUCs from additional experiments described in the text.

Comparing the enrichment value of the spike-in peptide to SILAC values.

To characterize the enrichment factor using SILAC, we used MaxQuant to search the *E. coli* proteome for peptides that include light or heavy lysine. We took the light to heavy ratio of all the peptides identified by MaxQuant. After normalizing the data, we verified that while the 1:1 mix had a median value of 0.494, the enriched sample had a median value of 0 (Fig 6.4A). Because 99.99% of the peptides had SILAC values of <0.494, we
concluded that our enrichment factor is >1000, which is in agreement with the enrichment value obtained by the SPIQE peptide (Figure 6.4C).

Figure 6.4: A) *E. coli* lysate that had AHA + Lys0 or Met + Lys8 mixed 1:1 can be enriched for light peptides by using DBCO-agarose and quantified using SILAC. B) The enrichment factor using the SILAC method is similar to the enrichment factor quantified by SPIQE. C) The same *E. coli* lysate mixed 50:1 does not enrich as well using the same method.

Overall, the spike-in peptide confirmed that these azide-tagged proteins can be enriched >1000-fold following these protocols with DBCO-agarose. Furthermore, the method uses a very small (0.25% of protein lysate) amount of the spike-in peptide added to the sample that does not take up too much of the LC-MS/MS run or appreciably affect the available DBCO for reaction (Fig S6.2).

We also used MaxQuant to search for the sequence of the spike-in peptide (3). By adding the FASTA file of this spike-in peptide directly to the MaxQuant search, we hoped to be
able to quantify enrichment during any standard proteomic analysis without additional MS runs. While this search found four counts of the heavy-labeled SPIQE peptide, it did not find any of the light-labeled version so we could not quantify an enrichment gain using this method.

**Characterizing a cleavable resin to find the site of incorporation.** For some azide-tagged proteomic experiments, the site of the azide tag must be identified. The DBCO-agarose resin used in the prior experiments retains the azide-tagged peptide after digestion because there is no cleavable moiety; thus the azide-tagged peptide will not be found in subsequent MS (Fig S6.2C). Cleavable resins and linker with groups that are photocleavable, acid-labile, protease sites, or able to be reduced have been developed to release the entire protein from the resin or enrich for tagged sites (12, 27, 28). We used our spike-in peptide to quantify enrichment gain using an alkyne-agarose resin with a \( N-(1-(4,4\text{-dimethyl-2,6-dioxocyclohexylidene})\text{ethyl}) \) (Dde) linker (Dde-alkyne-agarose, Click Chemistry Tools), which releases the clicked peptide upon addition of hydrazine (Fig 6.5A). This released peptide reveals a charged alkyl amine that increases ionization efficiency, but is only a small addition to the molecular mass of the peptide so it does not exceed the ideal mass range of MS. We then used Skyline to search for the SPIQE peptide within this run and integrating the area under both curves revealed an enrichment gain of 540 (Fig 6.3D, 6.5B). Skyline also revealed the presence of the released SPIQE peptide with the alkyl amine adduct, indicating this resin would be useful to identify sites of labeling (Fig 6.5C). While the released cleaved SPIQE peptide was found 20-fold
enriched over the untagged version, this number cannot be used for comparison since they are not identical peptides and do not ionize identically.

**Figure 5:** A) Dde resin can be cleaved with hydrazine (N$_2$H$_4$) to release the azide-tagged peptide/protein either before trypsin digest to find all peptides from azide-tagged proteins or after to find only the site of azide-incorporation into the peptide respectively. B) Trace of cleaved SPIQE peptide indicates enrichment (quantified in Fig 6.3). C) Traces of peptide after enrichment using cleavable Dde resin reveals the site of azide-tagging

**Enrichment of 50:1 mix of SILAC+BONCAT labeled lysates.** We also mixed the quantitatively labeled lysates in a 50:1 heavy:light ratio to test the ability to enrich with lightly labeled samples. We verified that the samples were 98% heavy labeled prior to enrichment. After enrichment, 84% of the sample was heavy labeled, indicating an
enrichment gain of only 8.7 (Fig S7.3). This enrichment gain is much lower than the values calculated for the 1:1 mixture.

6.5 Discussion

We have developed SPIQE, a method to quantify enrichment gain in the absence of other labels. The values obtained by SPIQE matched values calculated by SILAC quantification. Thus, we envision this peptide could be “spiked-in” to samples without SILAC labels as a method of quantifying enrichment without additional MS runs or other wet-lab procedures. Using heavy:light labeled lysates, we also quantified and compared the ability of various resins and linkers to enrich azide-tagged proteins, concluding that DBCO-based linkers worked better than terminal alkynes. We also found that Dde-alkyne-agarose resin can be used to find sites of azide-tagging. Finally, we determined that samples with a low abundance of tagged proteins (50:1 labeled/unlabeled lysate) have much lower enrichment gains. We envision this method could be used to quickly optimize specificity and gain of enrichment techniques.

Enrichment of azide-tagged proteins is always a battle between specificity and reactivity. Click reactions that are 100- to 1000- fold more selective for azides over other moieties (27, 29) will still show high levels of background in samples that have few azide-tagged molecules. Various strategies have been employed to decrease background reactivity in lightly labeled samples: For example, many research groups have discovered the importance of blocking reactive cysteine groups within proteins prior to the SPAAC
reaction (26, 30). In order to continue pushing the limits of azide-tagged protein
detection, increasing the specificity of click reactions while maintaining high rates of
reactivity will be necessary.

**Limitations of the method**

Although the SPIQE method indicates whether the conditions of the enrichment enabled
successful click and pull-down of azide-tagged molecules, it cannot directly report on
whether any particular enriched peptide came from an azide-tagged protein as SILAC
does. The simple model peptide may not behave exactly as full-length proteins do in
solution. It also cannot be used in samples that have GFP expressed as it is based on a
peptide from GFP, which would obfuscate enrichment values. Finally, SPIQE enrichment
values >1000 may not have much meaning, since integration of the light peptide at this
point is often in the noise regime.

**6.6 Future Work**

While this peptide is a useful reporter for enrichment, other peptides may be synthesized
to compare their enrichment factors. Adding amino acids such as cysteine may be useful
to see if peptides containing certain amino acids are enriched more within the system
chosen. Furthermore, one could ascertain if the presence of the azide within the peptide
chain affects cleavage of the reaction depending on how close it is to the site of cleavage.
Using additional peptides in a similar manner would allow investigators to determine the
error associated with these enrichment gains.
6.7  Experimental Procedures.

**Preparation of SILAC+BONCAT labeled lysates.** *E. coli* strain KY14, that is auxotrophic for methionine and lysine (KY14) was grown in M9 minimal media with 18 amino acids (-Met, -Lys) and either 30:1 azidohomoalanine/methionine (Aha/Met) + Lys0 or Met + Lys8 ($^{13}$C$_6$$^{15}$N$_2$H$_{14}$$^{16}$O$_2$) (21, 24). The cells were refreshed three times 1:100 after growth to stationary phase into the respective media to ensure quantitative replacement of lysine with either its light or heavy version. After growth to OD600 = 1.0, cultures were spun down for 10 minutes at 3500 rcf at 4 °C. The supernatant was removed and the cells were resuspended in 500 μL 1% SDS in PBS. Benzonase (0.5 μL, 250 units/μL, Sigma) was added for 5 minutes and the cells were boiled at 95 °C for 10 minutes. After determination of protein concentration using Pierce BCA protein quantification kit, 10 μg of this lysate was conjugated to alkyne-TAMRA following the instructions of the Click-It Kit (Thermo Scientific, previously Life Technologies). The clicked samples were mixed in Laemmli sample buffer, run on an SDS-PAGE gel, washed, and visualized using a Typhoon gel scanner to assay tagging (Fig 6.3). After confirming Aha incorporation, all samples were digested with trypsin, then desalted using C18 StageTips as previously described (31) before being subjected to respective LC-MS/MS (detailed below).

**MS analysis of unenriched SPIQE peptide mix in complex lysate.** The 1:1 SPIQE peptide mix was added to 400 μL of the 1:1 “light + heavy” *E. coli* lysate at either 25 μg peptide/mg of lysate or 2.5 μg peptide/mg of lysate. This unenriched mixture (10 μL) was diluted with 90 μL AmBi to bring the final SDS concentration to 0.1%. These samples
were digested with trypsin overnight at 37 °C and the peptides were extracted using 10% ACN in 10 mM AmBi. Leftover SDS was removed using HiPPR detergent removal resin (Thermo Scientific). Samples were then desalted using C18 StageTips (31), before being resuspended in 0.1% formic acid (FA). This mixture was analyzed in a targeted manner on a QTRAP 6500 LC-MS/MS system (ABSciex) coupled to an Eksigent ekspert nanoLC 425 pump, ekspert nanoLC400 autosampler and ekspert cHiPLC. Samples were separated using a 45 min linear gradient of acetonitrile in 0.2% FA from 0% to 40% at a flow rate of 300 nL/min on a cHiPLC Chrom XP C18-CL 3 μm trap column, 120 Å (200 μm × 0.5 mm), in line with a cHiPLC Chrom XP C18-CL 3 μm column, 120 Å (75 μm × 150 mm). The transitions used to monitor the SPIQE peptide are in Table S7.4. The initial generation of multiple reaction monitoring (MRM) transitions and subsequent analysis was performed using Skyline. Up to five singly charged y-ions with an m/z greater than the precursor were selected for monitoring. Collision energies and declustering potentials were calculated using Skyline models for the QTRAP.

**Enrichment of peptide from labeled lysate and trypsin digestion.** Each sample was diluted with 400 μL of 8 M urea, reduced with 10 mM dithiothreitol (DTT) for 30 min, alkylated with 100 mM iodoacetamide for 45 min, then rotated overnight with 20 μL of washed DBCO-agarose (Click Chemistry Tools). The resin was placed in filter columns and washed with 10 x 5 mL SDS wash buffer (0.8% SDS, 150 mM NaCl, 100 mM Tris, pH 8.0), 10 x 5 mL 8 M urea in 100 mM Tris (pH 8.0), and 10 x 5 mL 20% ACN in water.
Washed resin was resuspended in 100 μL 10% ACN in 100 mM AmBi and 100 ng of trypsin was added for overnight digest at 37 °C.

**Dde-alkyne-agarose.** We followed the manufacturer’s instructions to use Dde-alkyne-agarose (Click Chemistry Tools). After adding the 2x copper catalyst solution to the lysates, SPIQE peptides, and resin, we allowed the click reaction to proceed overnight while rotating end over end. The resin was washed with the same wash buffers used for the DBCO-agarose, then whole proteins were eluted using hydrazine, according to the manufacturer’s protocol. These whole proteins were then digested using trypsin, and desalted using C18 StageTips before being loaded on to an Orbitrap Elite MS. We used Skyline to search the raw files and found both the clicked and released peptide and the unclicked version as well as its corresponding methionine version. These values cannot be quantitatively compared since the methionine version does not necessary ionize similarly to the clicked version.

**Isolation and preparation of peptides for MS.** After removing the supernatant from the overnight digest, the resin was washed three times with 100 μL 10% ACN in 100 mM AmBi to extract peptides and each wash was collected and combined. These extracted peptides were dried on a speed-vac, then desalted using C18 StageTips. The desalted samples were resuspended in 0.1% formic acid prior to injection on the MS.

**MS Analysis.** We injected 100 ng of the enriched 2.5 μg peptide/mg lysate onto a Proxeon Easy-nLC 1000 (Thermo Fisher Scientific) coupled to an Orbitrap Elite MS (Thermo
Fisher Scientific), equipped with a nano-electrospray ion source (Thermo Fisher Scientific). For LC, solvent A consisted of 97.8% H₂O, 2% ACN, and 0.2% formic acid and solvent B consisted of 19.8% H₂O, 80% ACN, and 0.2% formic acid. The chromatographic separation was achieved using a 60 min elution gradient from 2% to 30% Solvent B at a flow rate of 220 nL/minute. The mass spectrometer was operated in data-dependent mode to switch automatically between MS and MS/MS scans as described by Kalli and Hess (32). Survey full scan mass spectra were acquired in the Orbitrap (400–1600 m/z) with a resolution of 60,000 at 400 m/z. The top 20 most intense ions from the survey scan were isolated and fragmented in the linear ion trap by collisionally-induced dissociation (CID). Precursor ion charge state screening rejected all singly charged and unassigned charge states. The dynamic exclusion list was set with a max retention time of 90 sec and a relative mass window of 15 ppm.

**MaxQuant.** MaxQuant (v. 1.5.3.30) was used to process the Thermo RAW files and used to search the *E. coli* proteome (UniProt), and an in-house contaminant database (259 sequences) including human keratins and proteases. All default parameters were used, except the multiplicity was set to search for peptides with heavy Lysine (+8.0142), and up to two missed cleavages were allowed. Aha (-4.9863) and L-2,4-diaminobutanoate (-30.9768), a product of reduction of Aha, were specified as variable modifications for Met.
Figure S6.1. A) Prior to mixing, the samples were labeled quantitatively with either 30:1 Met/AHA:Lys8 or Met:Lys0. B) After mixing, the evidences had a median SILAC ratio of 0.5, indicated 1:1 mixing. Decreasing this value indicates successful enrichment. C) Chemical structures of each of the resins or linkers tested using these lysates. If the linker has a biotin handle, then Pierce Streptavidin Agarose resin was incubated with the clicked lysates.
Figure S6.2 A) LC trace of *E. coli* labeled lysates with SPIQE peptide. B) Expanding the LC trace at elution of the SPIQE peptide reveals a small peak in comparison to the rest of the peptides from the lysate.
Figure S6.3. *E. coli* lysates labeled with Met+Lys8 (heavy) or Aha+Lys0 (light) were mixed 50:1. They were enriched using DBCO-agarose beads as described for the 1:1 lysates in the methods section. After starting with 98.1% heavy labeled lysates, they were brought to 85.6% heavy labeled, which is an enrichment factor of 8.7.

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### Table S6.4: MRM transitions used to monitor SPIQE peptide on the QTRAP

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<th>DP</th>
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<td>88.4</td>
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References


