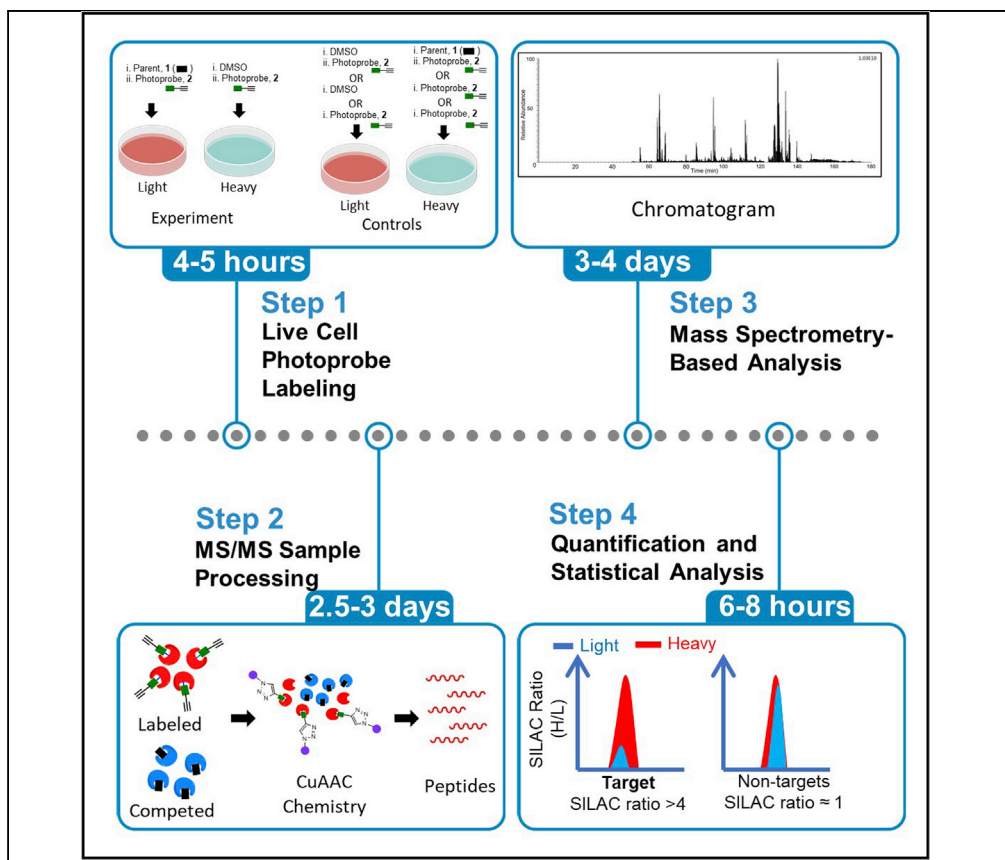


## Protocol

# Protocol for clickable photoaffinity labeling and quantitative chemical proteomics



Here, we describe a protocol for a photoaffinity labeling probe strategy for target deconvolution in live cells. We made a chemical probe by incorporation of a photoreactive group to covalently cross-link with adjacent amino acid residues upon UV irradiation. Click chemistry-based enrichment captures labeled proteins for proteomic analysis. Here, we detail specifics for finding targets of LXR $\beta$ , but the protocol can be broadly applied to other targets.

Wankyu Lee, Zhen Huang, Christopher W. am Ende, Uthpala Seneviratne

wlee@dewpointx.com (W.L.)  
Uthpala.Seneviratne@pfizer.com (U.S.)

### Highlights

Protocol detailing photoaffinity probe strategy for target deconvolution in live cells

Competition with parent compound demonstrates specific binding

Photoaffinity label provides evidence of small-molecule binding to LXR $\beta$

Click chemistry-based enrichment captures labeled proteins for proteomic analysis

Lee et al., STAR Protocols 2, 100593  
June 18, 2021 © 2021 The Author(s).  
<https://doi.org/10.1016/j.xpro.2021.100593>



## Protocol

## Protocol for clickable photoaffinity labeling and quantitative chemical proteomics

Wankyu Lee,<sup>1,3,4,\*</sup> Zhen Huang,<sup>1</sup> Christopher W. am Ende,<sup>2</sup> and Uthpala Seneviratne<sup>1,5,\*</sup><sup>1</sup>Pfizer Worldwide Research and Development, 1 Portland St, Cambridge, MA 02139, USA<sup>2</sup>Pfizer Worldwide Research and Development, Eastern Point Road, Groton, CT 06340, USA<sup>3</sup>Currently associated with Dewpoint Therapeutics<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [wlee@dewpointx.com](mailto:wlee@dewpointx.com) (W.L.), [Uthpala.Seneviratne@pfizer.com](mailto:Uthpala.Seneviratne@pfizer.com) (U.S.)  
<https://doi.org/10.1016/j.xpro.2021.100593>

## SUMMARY

Here, we describe a protocol for a photoaffinity labeling probe strategy for target deconvolution in live cells. We made a chemical probe by incorporation of a photoreactive group to covalently cross-link with adjacent amino acid residues upon UV irradiation. Click chemistry-based enrichment captures labeled proteins for proteomic analysis. Here, we detail specifics for finding targets of LXR $\beta$ , but the protocol has potential for application to other targets. For complete details on the use and execution of this protocol, please refer to Seneviratne et al. (2020).

## BEFORE YOU BEGIN

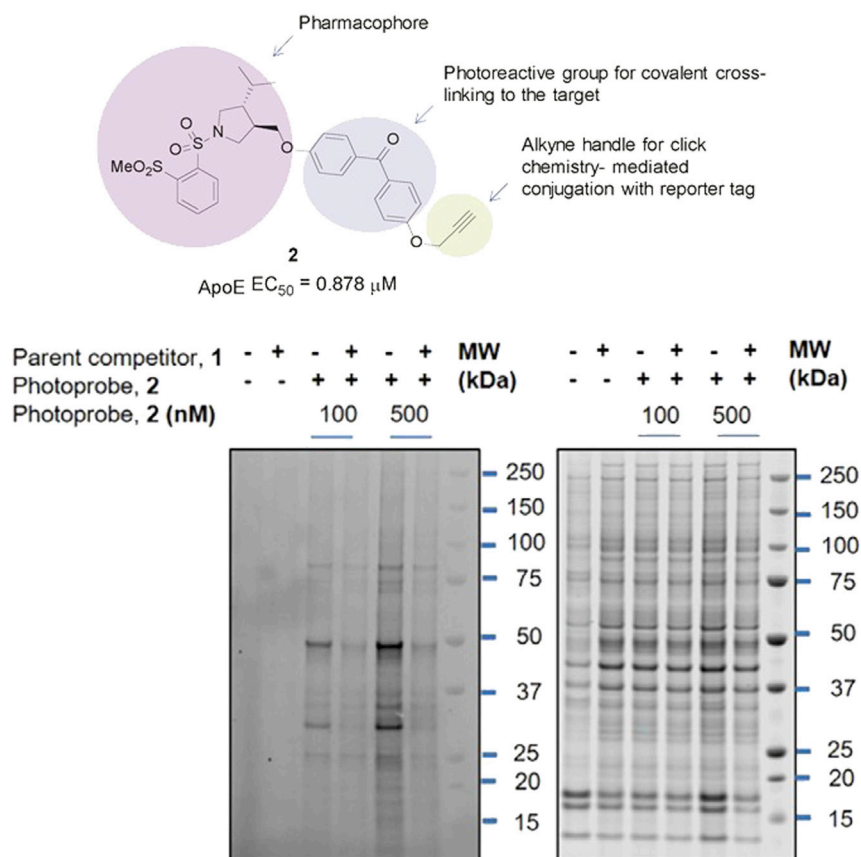
Before starting the protocol, two compounds are needed: the lead chemical matter (compound 1) that was determined to have efficacy in a phenotypic screen for enhancing astrocytic apoE, and a photoaffinity labeling (PAL) probe (compound 2) designed from 1 that still retained phenotypic activity. The hypothesis is that by retaining key chemical elements of 1 that are responsible for binding an unknown protein target of interest, a similar reversible binding to the protein target will be achieved for photoprobe 2 when treated to live cells. The attachment of a photoreactive group allows capture of protein targets of 2 by forming a covalent bond with nearby amino acid residues. (Lapinsky and Johnson, 2015, Jenmalm Jensen and Cornella Taracido, 2019). Probe 2 also contains an alkyne that can be used for copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) click chemistry to conjugate a reporter group for downstream analysis. Below, we describe the design and syntheses of both compounds. For complete details on both compounds, please refer to Seneviratne et al., 2020.

In addition to the necessary compounds, a relevant cellular system is needed. (Vincent et al., 2020) Here, we used the same cell line for the phenotypic assay for the PAL protocol: a human astrocytoma cell (CCF-STTG1) line. For quantitative mass spectrometry (MS)-based proteomics analysis, the stable isotope labeling with amino acids in cell culture (SILAC) was established for this cell line. (Mann, 2006)

## Preparation of 1 and 2

⌚ Timing: 3–6 weeks





**Figure 1. Assessment of photoaffinity probe 2**

Top: Design of photoaffinity probe 2. Left: In-gel fluorescence demonstrating dose dependence of photoprobe 2 as well as competition with parent compound 1. Right: Coomassie stain of the gel shown on the left panel.

Figure adapted with permission from [Seneviratne et al. \(2020\)](#).

1. The design and syntheses of 1 and 2 depended on the chemical matter of interest. Lead compound 1 resulted from a phenotypic screen for apoE enhancers. For photoaffinity probe 2, several design factors are critical for success:
  - a. The photoaffinity probe 2 must contain a photoreactive group such as benzophenone, diazirine or aryl azide, to enable covalent cross-linking to the target protein upon irradiation with UV light ([Preston and Wilson, 2013](#), [Lapinsky and Johnson, 2015](#)). The choice of photoreactive group can depend upon considerations for experimentally determined photochemical modification of protein targets, chemical synthetic accessibility, and minimal chemical perturbation.
  - b. Compound 2 also contains an alkyne moiety to allow CuAAC chemistry to a reporter group, such as a fluorophore for visualization by in-gel fluorescence or imaging and/or a biotin group for streptavidin enrichment of labeled proteins.
  - c. It is important that even with the modifications described in 1a and 1b that the photoprobe retains potency in the phenotypic assay and can be competed for labeling by compound 1. For compound 2, the  $EC_{50}$  was 883 nM, as compared to 57 nM for compound 1, in the already established phenotypic assay for apoE. [Figure 1](#) shows the key components and the design of photoprobe 2 that was adapted from [Seneviratne et al., 2020](#). Minimal tolerance between probes 1 and 2 is difficult to define generally since the  $OC_{50}$  values of photoaffinity probes may not correlate with photo cross-linking ([Kawamura et al., 2008](#)). For this specific case, to demonstrate that binding is dependent on the binding signatures of the lead compound 1,

competition of probe 2 with compound 1 was demonstrated in [Figure 1](#). In addition, dose dependent labeling of proteins was also determined with the probe 2, providing confidence that these signals are not the result of non-selective labeling. For further details on the protocols for the synthesis of photoprobe 2 and the in-gel fluorescence experiment, please refer to [Seneviratne et al. \(2020\)](#).

**Note:** In [Figure 1](#), the photoreactive group and alkyne handles are attached to the pyrrolidine group of lead compound 1 because this vector provided the most tolerance for large structural modifications. Modifying the compound at this vector allowed specific, productive photo cross-linking, as shown in [Figure 1](#).

- When both compounds have been synthesized and characterized, prepare concentrated stocks of each compound in DMSO to  $\geq 30$  mM concentrations, to be stored at  $-80^{\circ}\text{C}$ .

### Culture SILAC cell lines

⌚ **Timing:** 3–6 weeks

- Maintain heavy and light CCF-STTG1 cell lines in SILAC DMEM that lacks L-lysine and L-arginine, supplemented with 10% v/v dialyzed fetal bovine serum (FBS), Pen/Strep, and L-proline (100 mg/mL). Heavy cell lines contained [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ ]-L-lysine and [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ ]-L-arginine (100 mg/mL each) in the media, whereas the light cell lines included L-lysine, HCl and L-arginine, HCl (100 mg/mL each) in the media.
- After applying 3 mL of 0.25% trypsin, EDTA to detach cells, add 7 mL of appropriate media and centrifuge  $300 \times g$ , 5 min.
- Aspirate media containing trypsin and add fresh media. Make the appropriate dilution for cell passage.
- Maintain both cell lines in parallel at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for at least six passages before probe treatment up to a total cell number of  $20 \times 10^6$  per biological replicate.

**Note:** Both heavy and light cells should be passaged in parallel such that biological conditions are consistent between both cell lines.

**⚠ CRITICAL:** Six passages are typically required to achieve >99% incorporation of heavy lysine and arginine. If necessary, the incorporation of heavy amino acids can be verified through LC-MS/MS and additional passages may be required.

- Alternatively, after six passages in SILAC media, freeze cell aliquots in 10% DMSO and store in liquid  $\text{N}_2$  until necessary. When thawed, passage cells >3 times before probe treatment ([Ong and Mann, 2006](#)).
- Perform protein lysis (as described below) and trypsin digestion (as described below) to evaluate the labeling efficiency with LC-MS/MS.

TMT labels, however, have issues with co-isolation and ratio suppression, ([Bantscheff et al., 2008](#), [Karp et al., 2010](#), [Ting et al., 2011](#)). Both SILAC and TMT have low CVs (coefficient of variance) values in peptide/protein quantitation.

**Alternatives:** For MS-based quantitation, instead of SILAC, normal cell lines can be used to generate samples to be labeled by tandem mass tag (TMT) labels. TMT labels provide an increase in throughput, and thus statistical power, with up to 16 samples in a single MS run ([Li et al., 2020](#)).

**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals and peptides</b>		
Fetal Bovine Serum (FBS)	Sigma-Aldrich	Cat#F4135
DMSO	Sigma-Aldrich	Cat#D8418
SILAC DMEM	Thermo Scientific	Cat#88364
[ <sup>13</sup> C <sub>6</sub> , <sup>15</sup> N <sub>2</sub> ]-L-lysine; [ <sup>13</sup> C <sub>6</sub> , <sup>15</sup> N <sub>4</sub> ]-L-arginine	Cambridge Isotope	Cat#CNLM-291-H; Cat#CNLM-539-H
L-Lysine; -L-arginine	Cambridge Isotope	Cat#ULM-8766; Cat#ULM-8347
L-Proline	Cambridge Isotope	Cat#ULM-8333
Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies	Cat#11965
Ham's F-12 Nutrient Mixture	Life Technologies	Cat#11765
Dialyzed FBS	Gemini Bio	Cat#100-108
GlutaMAX	Life Technologies	Cat#35050
Penicillin/streptomycin (Pen/Strep)	Life Technologies	Cat#15140
Polyethyleneimine (PEI)	Polysciences	Cat#239661
Dulbecco's phosphate-buffered saline (DPBS)	Life Technologies	Cat#14190144
Sodium dodecyl sulfate (SDS)	Invitrogen	Cat#15553035
Tris(benzyltriazolylmethyl)amine (TBTA)	TCI	Cat#T2993
tert-butanol (t-BuOH)	Sigma-Aldrich	Cat#19640
Tris(2-carboxyethyl)phosphine HCl (TCEP)	Thermo Scientific	Cat#20491
Copper(II) sulfate (CuSO <sub>4</sub> )	Sigma-Aldrich	Cat#C1297
Tetramethylrhodamine (TAMRA)-biotin-azide	Huang et al., 2019	N/A
InstantBlue Coomassie Protein Stain	Abcam	Cat#ab119211
High Capacity Streptavidin Agarose Resin	Thermo Scientific	Cat#20359
Sequencing grade modified trypsin	Promega	Cat#V5111
Calcium chloride	Sigma-Aldrich	Cat#21097
Methanol (MeOH)	Fisher Scientific	Cat#A456-4
Chloroform (CHCl <sub>3</sub> )	Fisher Scientific	Cat#C297-4
Acetonitrile	Fisher Scientific	Cat#A955-4
Acetone	Fisher Scientific	Cat#A18-4
LC-MS Water	Fisher Scientific	Cat#W6-4
Distilled Water	Life Technologies	Cat#15230-162
Formic acid	Fisher Scientific	Cat#A117-50
Trifluoroacetic acid	Fisher Scientific	Cat#A116-50
Urea	Thermo Fisher	Cat#ZU10001
Dithiothreitol (DTT)	Sigma	Cat#43815
2-Iodoacetamide	Sigma	Cat#11149
Protein LoBind Tubes	Eppendorf	Cat#022431081
Micro Bio-Spin Columns	Bio-Rad	Cat#7326204
C18 ZipTips-10 μL	Millipore	Cat#ZTC18S096
C18 ZipTips-100 μL	Thermo Fisher	Cat#87784
C18 LC Trapping column	Thermo Fisher	Cat#164946
C18 EASY-Spray analytical column	Thermo Fisher	Cat#ES903
Benzonase	Millipore	Cat#71206
Compound 1	<a href="#">Seneviratne et al., 2020</a>	N/A
Compound 2	<a href="#">Seneviratne et al., 2020</a>	N/A
<b>Critical commercial assays</b>		
Bicinchoninic acid (BCA) assay	Thermo Scientific	Cat#23208
<b>Experimental models: cell lines</b>		
CCF-STTG1	ATCC	ATCC Cat# CRL-1718
<b>Software and algorithms</b>		
Image Studio 4.0	LI-COR Biosciences	<a href="https://www.licor.com/bio/image-studio-lite/">https://www.licor.com/bio/image-studio-lite/</a>
RAW Converter (v1.2)	N/A	<a href="http://fields.scripps.edu/rawconv/">http://fields.scripps.edu/rawconv/</a>

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Integrated Proteomics Pipeline (IP2) and ProLuCID	Integrated Proteomics Applications	<a href="http://www.integratedproteomics.com/">http://www.integratedproteomics.com/</a>
Proteome Discoverer (v2.1 and v2.4)	Thermo Fisher	<a href="https://www.thermofisher.com/">https://www.thermofisher.com/</a>
SEQUEST HT	Thermo Fisher	<a href="https://www.thermofisher.com/">https://www.thermofisher.com/</a>
Xcalibur™	Thermo Fisher	<a href="https://www.thermofisher.com/">https://www.thermofisher.com/</a>
Skyline	N/A	<a href="https://skyline.ms/project/home">https://skyline.ms/project/home</a>

**MATERIALS AND EQUIPMENT**

The following table describes the components for the copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) click chemistry mix for 500  $\mu$ L volume of cell lysate.

Reagent	Final concentration	Amount
TBTA (2 mM)	0.1 mM	25 $\mu$ L
CuSO <sub>4</sub> (50 mM)	1 mM	10 $\mu$ L
TCEP (100 mM)	1 mM	5 $\mu$ L
Biotin-TAMRA-azide (10 mM)	25 $\mu$ M	1.25 $\mu$ L
<b>Total</b>	<b>N/A</b>	<b>41.25 <math>\mu</math>L</b>

**△ CRITICAL:** When making the CuAAC chemistry master mix, add reagents in the order that is listed in the table above to ensure proper copper reduction and formation of copper ligand complexes.

**Note:** TBTA is solubilized first in DMSO and then diluted with *t*-butanol to achieve a 2 mM stock solution in 1:4 DMSO:*t*-butanol, to be stored at  $-80^{\circ}\text{C}$  in single use aliquots.

**Note:** Biotin-TAMRA-azide stock solution is made in DMSO and stored at  $-80^{\circ}\text{C}$  in single use aliquots. TCEP stock solution is freshly made in water.

**△ CRITICAL:** After reagents are combined into a master mix, use mix immediately.

**Note:** When TCEP is added, the color should change from light blue to yellow green. Addition of Biotin-TAMRA-azide will then result in a bright pink color.

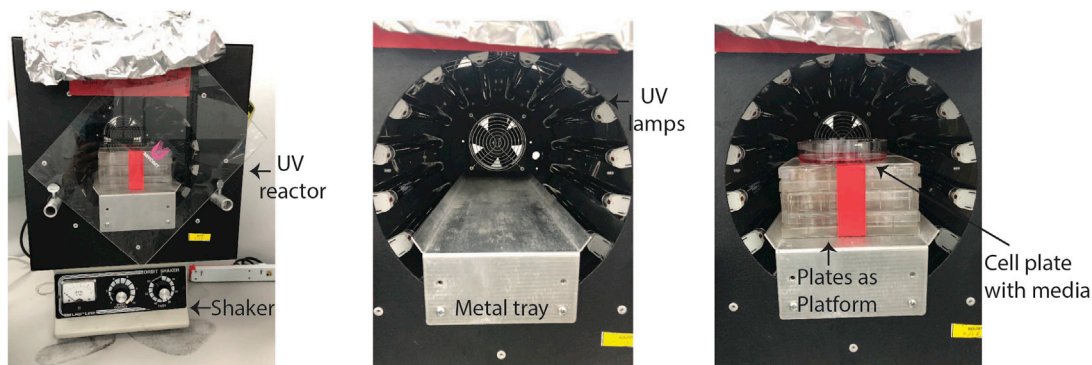
**Note:** Make enough master mix for at least two additional samples than necessary. Extra volume is necessary in case of volume loss during pipetting or evaporation of *t*-butanol.

The following table describes the components for the trypsin mix for protein digestion.

Reagent	Final concentration	Amount
Trypsin (0.5 $\mu\text{g}/\mu\text{L}$ )	0.01 $\mu\text{g}/\mu\text{L}$	4 $\mu\text{L}$
CaCl <sub>2</sub> (100 mM)	1 mM	2 $\mu\text{L}$
50 mM Tris 1.9 M Urea pH 8	-	194 $\mu\text{L}$
<b>Total</b>	<b>N/A</b>	<b>200 <math>\mu\text{L}</math></b>

**Note:** One vial of trypsin (20  $\mu\text{g}$ ) is re-suspended in 40  $\mu\text{L}$  of 50 mM Tris pH 8.0 to make stock solution.

**△ CRITICAL:** In our experience, the critical parameters for successful photo-reactivity include irradiating paired samples together, decreasing the distance between the cell plate to the UV light source to  $\sim 10$  cm, the removal of lids from cell plates and shaking at  $4^{\circ}\text{C}$ .



**Figure 2. Custom modified Rayonet apparatus for UV irradiation**

Left: Front view shows the Rayonet chamber reactor (RPR-200) that houses 16 UV lamps (RPR-3500A, 350 nm bulb) wired for 120 V, 50/60 Hz. Reactor is placed on orbital shaker for shaking. The entire unit is housed in a 4°C cold room. Middle: The inside of the reactor is shown, in which 16 UV lamps circle a metal tray. In the back, a fan acts as a cooling device. Right: When placing 10 cm or 15 cm cell plates inside the chamber, three six well plates are used as a platform to close distance between lamps and cell plate. Note that the lid of the cell plate is off. This entire unit can easily be reconstructed with the commercially available Rayonet chamber reactor, an orbital shaker and several cell plates.

△ **CRITICAL:** Wear proper personal protective equipment (PPE), such as UV goggles, lab coat and gloves, when near UV radiation. While in use, place a UV protective shield and aluminum foil over the reactor opening (Figure 2).

## STEP-BY-STEP METHOD DETAILS

### Live cell photoprobe labeling

⌚ Timing: 4–5 h

Pre-treatment with compound 1 competes the photoaffinity labeling of compound 2 for protein targets in the relevant cell line. Forward and reverse SILAC treatments are performed as label-swapping experiments to correct for potential quantitation errors. (Park et al., 2012)

1. Culture one 15 cm plate of light CCF cells and one 15 cm plate of heavy CCF cells to 90% confluence in appropriate SILAC media.
  - a. Each plate was washed twice with ice cold Dulbecco's phosphate buffered saline (DPBS, 2 × 30 mL) to remove FBS in the culture media.

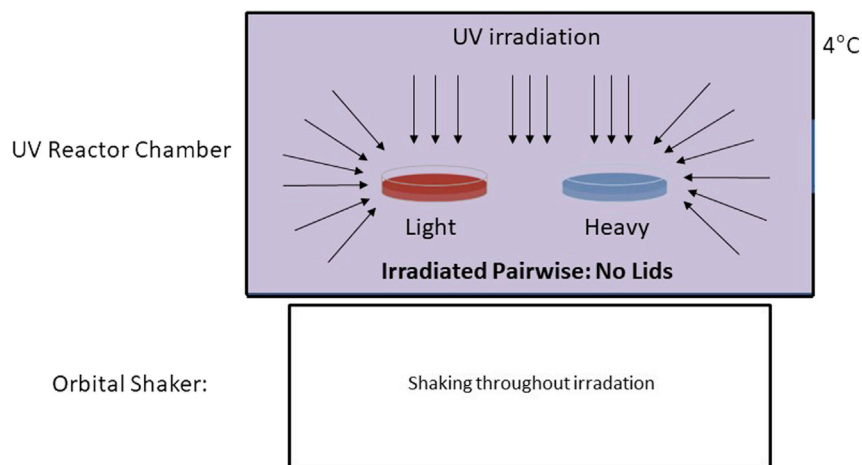
**Note:** Compounds may bind non-specifically to albumin in FBS.

2. Dissolve 1 in culture media without FBS.

**Note:** Warming to 37°C may increase solubility for certain compounds.

3. For forward SILAC:
  - a. Pre-treat light cells with compound 1 for 30 min (25 μM, 0.5% DMSO, 37°C and 5% CO<sub>2</sub>) in cell media without FBS.
  - b. Pre-treat heavy cells with DMSO for 30 min in cell media without FBS.
4. After pre-treatment, in the same media, add compound 2 (0.1% DMSO, 500 nM) to both light and heavy cells for 30 min (37°C, 5% CO<sub>2</sub>).

After 30 min treatment of compound 2, wash both cells with ice cold serum free media (2 × 25 mL).



**Figure 3. Diagram of UV irradiation**

Note pairwise irradiation, the removal of lids from cell plates and shaking at 4°C during irradiation.

△ **CRITICAL:** After photo cross-linking has occurred, cell viability should be checked to compare live cell count from prior to labeling to ensure photo cross-linking has not caused cell death.

**Note:** Here, we use 50-fold excess of 1:2, as optimized in [Seneviratne et al., 2020](#).

**Note:** At this point, both compound 1 and compound 2 will have sufficiently permeated into cells such that the interactions can be captured. Washes should be completed quickly to prevent compounds from diffusing out of cells. UV irradiation without washes results in higher background signal.

5. Perform UV irradiation for both plates using a custom modified Rayonet apparatus (360 nm, 4°C, [Figure 3](#)). In order to obtain a reproducible, efficient, and specific photo-labeling the following distance and time of labeling will be used: 10 cm distance and 10 min.
  - a. Wash both plates with ice cold DPBS (2 × 30 mL) buffer. Aspirate media during each wash.
  - b. Scrape cells in ice cold DPBS buffer (15 mL) using a cell scraper.

**Note:** Instead of cell media, cold DBPS can be used during UV irradiation. In this case, step 5a can be skipped.

**Note:** Ensure that the UV apparatus is operating correctly. A visual check that includes observing light being turned on is typically sufficient.

△ **CRITICAL:** Place both light and heavy cells into UV apparatus together such that any differences in UV irradiation are normalized between the two conditions.

△ **CRITICAL:** Shake system gently at 50 rpm and 4°C such that cells do not lift during UV irradiation.

△ **CRITICAL:** Removal of culture plate lids will provide more efficient irradiation and thus higher yield. In addition, shaking plates prevent evaporation of media.



6. Centrifuge cells (1000 × g, 10 min) to pellet.
7. Remove DPBS buffer either via pipetting or aspiration to leave minimal volume.
8. Store cells as pellets at −80°C.
9. For reverse SILAC: Repeat steps 3 to 8, but instead of pre-treating the light cells with 1, pre-treat those cells with DMSO. In addition, pre-treat heavy cells with 1.
10. For probe vs. probe controls: Repeat steps 3 to 8, but do not pre-treat either light or heavy cells prior to addition of 2.
11. For probe vs. no probe controls: Repeat steps 3 to 8 but do not pre-treat either light or heavy cells. Add 2 only to heavy cells while adding DMSO to light cells for 30 min incubation.
12. For UV vs. no-UV controls: Repeat steps 3 to 8 but 1 is added to both light and heavy cells. Only perform UV irradiation to heavy cells while leaving light cells in the dark at 4°C for 10 min.

**Note:** These control pairings in steps 9–12 provide important comparisons to eliminate non-specific-binding and UV cross-linking proteins in the MS-based quantitative proteomic analysis.

### MS/MS sample processing

⌚ **Timing:** 2.5–3 days

Samples are processed such that probe-labeled proteins are enriched via streptavidin beads and then subsequently digested into peptides for quantitative proteomic analysis.

**Note:** For all steps in MS/MS sample processing, it is important to use Protein LoBind Tubes to prevent protein from binding to tubes.

13. Re-suspend heavy and light cell pellets separately with 0.5 mL DPBS buffer pH 7.2.

⚠ **CRITICAL:** For efficiency of CuAAC chemistry, amine-based buffers like Tris should be avoided because these groups chelate Cu(I) and prevent the formation of active catalyst (Parker and Pratt, 2020).

**Note:** Adding 0.25% SDS can also work but higher SDS concentrations may induce non-specific CuAAC chemistry and thus higher background.

14. Add 1 × benzonase (25 U/mL) to break down DNA/RNA and reduce sample viscosity. Dilute benzonase (25 kU/mL) by adding 6 μL of stock to 60 μL DPBS buffer pH 7.2. Add 5 μL to each 500 μL sample. Incubate at 10 min at 20°C–25°C while shaking so that viscous samples become clear. Centrifuge at 5000 × g for 10 min at 4°C to clear cell debris and take the supernatant for subsequent steps.
15. Lyse cells at 4°C with probe sonication using a Fisherbrand Model 120 Sonic Dismembrator with the following settings: 1 s on, 1 s off pulses for 20 s at 75% amplitude.
16. Determine protein concentrations with BCA assay.
  - a. In a 96-well plate, pipet 5 μL of sample to each well and add 20 μL of DPBS buffer pH 7.2 for a 1:5 dilution. For triplicate measurements, add 16 μL sample to 64 μL DPBS buffer and then pipet 25 μL for each replicate.
  - b. For BSA protein standards, 25 μL of various protein concentration dilutions (3, 2.4, 1.9, 1.5, 1.2, 1, 0.8, 0.63, 0.5 mg/mL) made in PBS buffer was used.
  - c. Add 200 μL working reagent (A:B = 10 mL:0.2 mL) and shake for 30 min at 37°C in the dark.
  - d. Read absorbance at 562 nm using a Tecan Infinite 200 PRO plate reader.
17. Adjust lysate volumes with lysis buffer to achieve 2 mg/mL in 0.5 mL final volume.

**Alternatives:** Other protein concentration determination assays, such as DC (detergent compatible) protein quantitation assay (Bio-Rad) can also be used.

- Mix equal protein amounts of heavy and light cell lysates together to 1 mL final total volume.

**Note:** In some cases, ultracentrifugation (100,000 × g, 50 min) can be used to separate the soluble and membrane fractions for each sample. The membrane fraction is solubilized with 0.5 mL of lysis buffer before step 19. Each fraction is carried through the protocol as separate samples.

△ **CRITICAL:** Steps 16–18 are instrumental to accurate quantitation with quantitative MS-based proteomics.

- Perform CuAAC chemistry reaction by treating samples with 41 μL click chemistry master mix.
  - Incubate samples at 20°C–25°C in the dark for 1 h with end-to-end rotation using a rotisserie shaker (Thermo Scientific 415110)

**Note:** The addition of organic solvents and click chemistry reagents will cause protein precipitation and, in some cases, loss of protein. Aggregation may cause complexes that are difficult to re-solubilize in future steps. Some tips are detailed in the [troubleshooting](#) section.

**Note:** For CuAAC chemistry with <100 μL volumes, use PCR tubes. For reactions of 0.5–1 mL volumes, use either 1.5 or 2 mL Eppendorf tubes. These reaction vessels reduce empty headspace during CuAAC chemistry reactions and thus reduce oxidation of reagents.

- To confirm proper CuAAC chemistry, take 20 μL of the sample for in-gel fluorescence analysis. A control sample without probe treatment should be included to verify low background from CuAAC chemistry reagents. If not necessary, skip to step 21.
  - Add 7.5 μL of LDS loading buffer (4×) and 3 μL of reducing agent DTT (10×). Vortex and keep at 20°C–25°C for 10 min. Vortex again and centrifuge at 10,000 × g for 2 min.
  - Load 25 μL of sample to SDS-PAGE gel and run at 120 V for 1 h with 2-(N-morpholino) ethanesulfonic acid (MES) running buffer until 20 kDa band reaches the end of the gel.
  - After electrophoresis, wash with deionized water 3× before scanning gels with a Typhoon FLA 9500 Biomolecular Imager (GE Healthcare) with 532 nm laser excitation, 580 nm emission, 1000 V, 100 μm resolution, and ≥575 nm long pass filter.
  - Gel scanning with fixation.* Gels were fixed in 50% MeOH/7% Acetic acid for 15 min, then rinsed with 40% MeOH for at least 20 min before in-gel fluorescence scanning.

**Note:** Gel fixation may eliminate background signal for imaging gels.

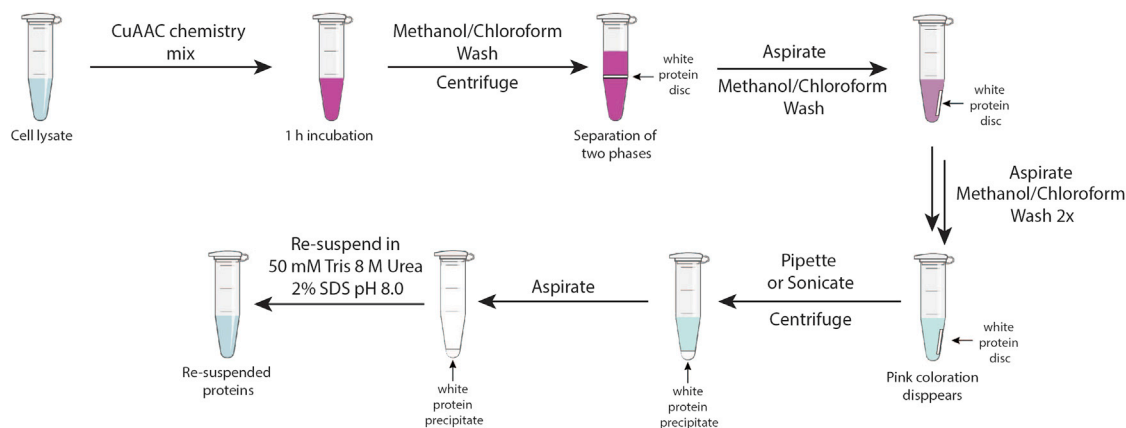
- To stain proteins, incubate gel with InstantBlue Coomassie blue reagent 14–18 h. Gels were de-stained for 1 h in water prior to scanning with Li-COR Odyssey CLx imaging system with the 700 nm channel in the Image Studio (4.0) software (LI-COR Biosciences).

- Perform protein precipitation with the leftover sample (~0.5 mL) such that excess click chemistry reagents was removed ([Figure 4](#)). [Troubleshooting 2](#)
  - Chill samples in ice and add ice-cold MeOH (0.5 mL) and cold CHCl<sub>3</sub> (0.2 mL).

**Note:** If the total volume of sample is ≥ 1 mL, transfer samples to a 15 mL falcon tube or a 5 mL Eppendorf tube. Scale all MeOH and CHCl<sub>3</sub> volumes to achieve a 4:4:1 aqueous: MeOH:CHCl<sub>3</sub> ratio for efficient precipitation.

▮ **Pause point:** Proteins can be precipitated over 14–18 h at –20°C.

- Vortex and add 0.5 mL of cold DPBS buffer.
- Vortex again.



**Figure 4. CuAAC chemistry reagent cleanup**

Workflow describing steps for protein precipitation by methanol/chloroform and subsequent washes to remove CuAAC chemistry reagents.

- d. Centrifuge to separate the phases ( $5000 \times g$ , 10 min). A white protein disc should form between the two phases.
- e. Carefully remove liquid without disturbing protein disc before adding 1:1 MeOH:  $\text{CHCl}_3$  (0.5 mL). Centrifuge at  $5000 \times g$ , 10 min.
- f. Repeat wash in step 21e twice more.
- g. Re-suspend proteins by sonication in MeOH (2 mL).
- h. Add  $\text{CHCl}_3$  (0.2 mL), vortex and pellet proteins by centrifugation ( $5000 \times g$ , 10 min).

**Note:** When washing away click chemistry reagents, note the loss of the bright pink color, leaving behind a white protein disc.

- i. Air dry proteins for approx. 2 min before re-suspension with 150  $\mu\text{L}$  of 10% SDS solution. To re-suspend, mix well.
- j. After 5–10 min, add 0.5 mL of 8 M urea in 50 mM Tris, pH 8.0 buffer. If necessary, perform sonication for protein re-suspension. Proteins should be re-solubilized at this step.

22. Reduce and alkylate proteins prior to enrichment.

- a. Add dithiothreitol (DTT) to a final concentration of 10 mM (stock made in water) and incubate samples for 30 min at  $37^\circ\text{C}$  while shaking.
- b. Alkylate samples by adding iodoacetamide to a final concentration of 12.5 mM (stock made in deionized water) and incubate for 30 min at  $20^\circ\text{C}$ – $25^\circ\text{C}$  in the dark while shaking.

**Note:** From our observations, protein coverage from MS-based proteomics is increased when reduction and alkylation is performed prior to enrichment, followed by on-bead digestion.

23. Enrich protein samples using streptavidin agarose beads. [Troubleshooting 3](#)

- a. Dilute samples by addition of 11 mL of 50 mM Tris pH 8.0 buffer.
- b. Pipet high capacity streptavidin agarose resin (120  $\mu\text{L}$  of 50% slurry per each sample) to 15 mL falcon tube. Pre-wash with 50 mM Tris, pH 8.0 buffer ( $3 \times 10$  mL).
- c. Add 100  $\mu\text{L}$  of pre-washed streptavidin agarose resin (1:1 slurry in 50 mM Tris pH 8.0) to each sample. Incubate samples 14–18 h at  $4^\circ\text{C}$  with end-to-end rotation.

24. Wash enriched proteins on beads. [Troubleshooting 4](#)

- a. Centrifuge samples at  $1,500 \times g$ , 3 min to isolate beads. Discard remaining buffer.

- b. Add 0.1% SDS in DPBS (10 mL) and incubate with beads for 10 min with end-over-end rotation. Centrifuge beads with  $1,500 \times g$ , 3 min and discard wash buffer. Repeat this step two more times.
- c. Add 50 mM Tris pH 8.0 (10 mL) and incubate with beads for 10 min with end-to-end rotation. Centrifuge beads with  $1,500 \times g$ , 3 min and discard wash buffer. Repeat this step two additional times.

**Note:** For samples that are quantitated by TMT and not SILAC, use 50 mM HEPES pH 8.5 buffer for washes instead of Tris buffers. Amine-based buffers will interfere with TMT labeling reaction.

- d. Add distilled water (10 mL) and incubate with beads for 10 min with end-to-end rotation. Centrifuge beads with  $1,500 \times g$ , 3 min and remove water.

**△ CRITICAL:** Washes in steps 24c and 24d are important for removal of SDS such that no complications arise from detergent in MS-based analysis.

25. Perform on-bead digestion by adding trypsin mix to each sample.
26. Incubate samples 14–18 h shaking at 37°C.

**Note:** Again, for samples that are quantitated by TMT, use a HEPES buffer instead of Tris.

27. Isolate digested peptides from beads by placing the entire solution on Micro Bio-Spin columns (Bio-Rad) and centrifuge ( $500 \times g$ , 3 min) to collect eluant of digested peptides.
  - a. Washes with MS-grade water (100  $\mu$ L) were also collected and pooled with eluant.
  - b. Pooled eluant was dried to near completion using a speed vacuum system. Peptides were stored at  $-80^{\circ}\text{C}$ .

**Note:** At this point, the alternative approach with TMT requires additional steps for the labeling with TMT reagent. For peptide labeling with TMT10 reagent, please refer to the protocols detailed in (Xu et al., 2019) or with TMTpro reagent in (Li et al., 2020).

**▯▯ Pause point:** Digested peptides can be stored at  $-80^{\circ}\text{C}$ .

### LC-MS/MS analysis

**⌚ Timing:** 3–4 days

Peptides processed in previous steps are now analyzed by tandem mass spectrometry (LC-MS/MS) and MS/MS spectra are analyzed for quantitative results.

28. Dissolve peptide samples in 1% TFA (40  $\mu$ L) and desalt using C18 ZipTips (Millipore, 10  $\mu$ L).
  - a. Activate the resin of C18 ZipTip with 1:1 acetonitrile:water ( $3 \times 10 \mu\text{L}$ ).
  - b. Wash the resin with 1% TFA in water ( $3 \times 10 \mu\text{L}$ ).
  - c. Load resin by pipetting in and out 30 $\times$ , vortexing sample solution every 10 $\times$ .
  - d. Wash resin with 1% TFA in water ( $3 \times 10 \mu\text{L}$ )
  - e. Elute by pipetting 30 $\times$  in 70% acetonitrile, 0.1% TFA.

**Alternatives:** Microspin columns (The Nest Group) and the Resolvex A200 (Tecan) can also be used to de-salt peptide samples.

29. Once eluted, evaporate peptide solutions to near dryness using a speed vacuum system.

**▯▯ Pause point:** Desalted peptides can be stored at  $-80^{\circ}\text{C}$  until MS/MS analysis but is recommended to only leave the peptides at this stage for at most a week.

**Table 1. Standard MS/MS parameters for Q-Exactive HF for SILAC-based approach**

Quantitation method	SILAC
Instrument	Q-Exactive HF
Experiment	Full MS/DD-MS <sup>2</sup> (TOPN)
Runtime	0–150 min
Polarity	Positive
In-source CID	0.0 eV
Default charge state	2
Inclusion	-
Exclusion	-
<b>Full MS</b>	
Resolution	120,000
AGC target	1e6
Maximum IT	50 ms
Number of scan ranges	1
Scan range	350–1600 m/z
Spectrum data type	Profile
<b>dd-MS<sup>2</sup>/dd-SIM</b>	
Microscans	1
Resolution	30,000
AGC target	5e4
Maximum IT	100 ms
Loop count	20
MSX count	1
TopN	20
Isolation window	1.4 m/z
Isolation offset	0.0 m/z
Scan range	200–2000 m/z
Fixed first mass	-
NCE/stepped NCE	23, 27, 29
Spectrum data type	Profile
<b>dd Settings</b>	
Underfill ratio	-
Minimum AGC target	5.20e3
Intensity threshold	5.2e4
Apex trigger	-
Charge exclusion	1, 6–8, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	15.0 s

30. Reconstitute peptides in 7  $\mu$ L of 5% acetonitrile, 0.1% formic acid.
31. Run peptide samples by injecting 5  $\mu$ L of sample through a trapping column (Acclaim PepMap C18 nanoViper LC column, 75  $\mu$ m ID  $\times$  20 mm, 3  $\mu$ m, 100  $\text{\AA}$ ).
32. Run captured peptides through a C18 column (EASY-Spray LC column, C18, 75  $\mu$ m ID  $\times$  50 cm, 2  $\mu$ m, 100  $\text{\AA}$ ) using an EASY-nanoLC 1200 equipped with an autosampler. The nanoLC is coupled to a Thermo Q-Exactive or Q-Exactive HF mass spectrometer.
  - a. Two buffers, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), were used in chromatography. Peptides were eluted using a linear gradient consisting of 1%–35% B at a flow rate of 300 nL/min over 120 min.
  - b. Standard mass spectrometry parameters for SILAC-based approach are described in [Table 1](#).
  - c. Standard mass spectrometry parameters for LC-MS/MS for TMTpro-based approach are described in [Table 2](#). For TMTpro, the linear gradient increased from 5 to 30% B at a flow rate of 250 nL/min over 98 min, followed by linear increase of B to 45% in 20 min and then finally to 55% B over 10 min.

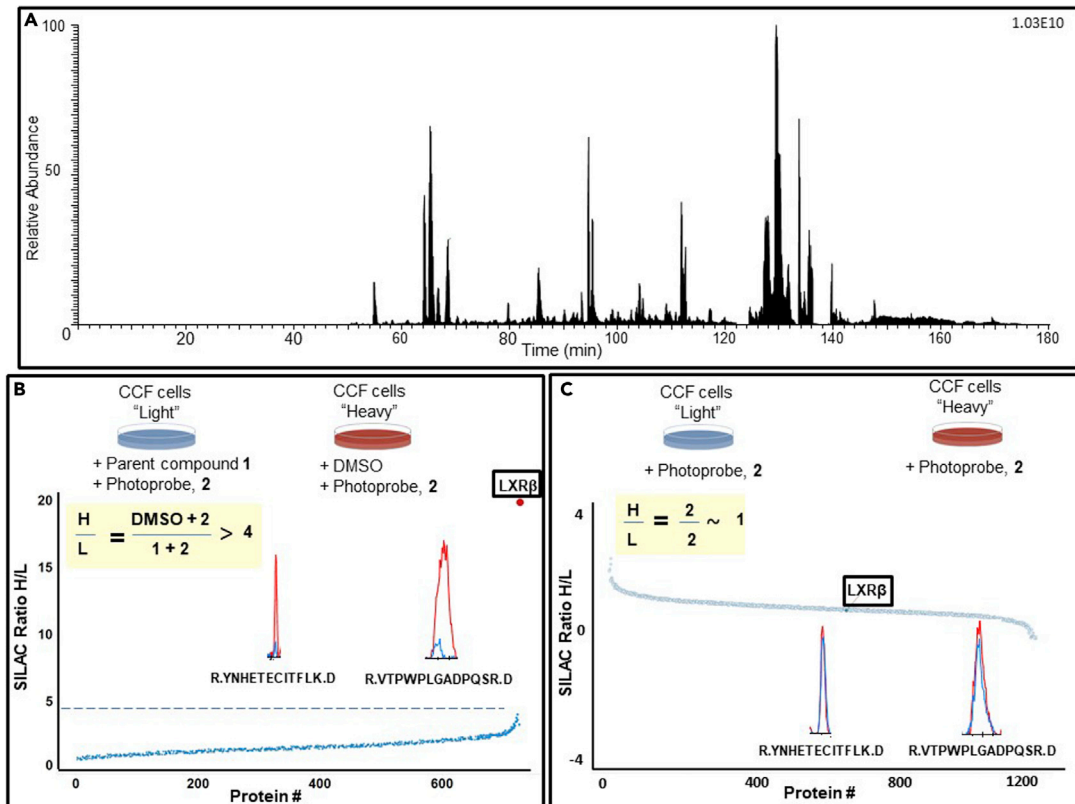
**Table 2. Standard MS/MS parameters for Fusion Lumos for TMTpro-based approach**

Quantitation method	TMTpro (16plex)
Instrument	Fusion Lumos
Experiment	Full MS OT/DD-MS <sup>2</sup> IT CID/DD-MS <sup>3</sup> OT HCD
Runtime	0–155 min
Polarity	Positive, 1500 V
In-source CID	0.0 eV
Inclusion	-
Exclusion	-
Full MS	FTMS1
Resolution	120,000
AGC target	4e5
Maximum IT	50 ms
Number of scan ranges	1
Scan range	400–1400 m/z
Spectrum data type	Profile
RF lens (%)	30
Top Speed, s	3
Dynamic exclusion properties	60s, 10 ppm (high & low), single charge, exclude with cycle
Intensity Threshold	5E3
dd-MS <sup>2</sup>	ITMS2
Mode	Top Speed
Detector type	Ion trap, normal scan range
Data type	Centroid
dd-MS <sup>2</sup> NCE %	32, activation time 10 ms
MS2 Resolution	Rapid
Quadrupole mass filter width	0.7 m/z
AGC target	1e4
Maximum IT	150 ms
Charge exclusion	1, 6–8, >8
dd-MS <sup>3</sup>	FTMS3
TopN	10
Scan range	110–500 m/z
Isolation width	2.0 m/z
AGC target	1e5
Resolution	60,000
MS3 max IT	120 ms
Spectrum data type	Profile
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	15.0 s
HCD Collision energy	55
MS3 isolation window	1.3 (+2 charge), 0.7 (+3 charge), 0.5 (+4 and +5 charge)
Detector type	Orbitrap, normal, defined

**Note:** Standard parameters shown in [Table 1](#) above are the ones used in [Seneviratne et al., 2020](#). TMTpro based standard parameters in [Table 2](#) can be used for any biotin-streptavidin pull down chemical proteomics applications.

### EXPECTED OUTCOMES

The current protocol should yield quantitative MS-based data to determine the phenotypic target of 1. LC-MS/MS chromatograms yielded multiple peaks denoting peptide elution and an ion intensity of  $\sim 10^9$  to  $10^{10}$  ([Figure 5](#)). For photoprobe 2, we observed >1000 proteins detected, as shown in [Figures 5B and 5C](#).



**Figure 5. SILAC MS data analysis**

(A) Representative chromatogram from a photolabeling experiment.

(B) Representative SILAC competition plot for the forward SILAC experiment, in which the light CCF cells are pre-treated with 1 prior to 2 treatment and irradiation while heavy CCF cells are pre-treated with DMSO prior to 2 treatment and irradiation. The H/L for the target should be >4, as demonstrated in both the competition plot and the embedded MS spectra.

(C) Representative SILAC competition plot for when both light and heavy CCF cells are treated with 2 and irradiated. The H/L for the target should be ~1, as demonstrated in both the competition plot and the embedded MS spectra. Figure adapted with permission from [Seneviratne et al. \(2020\)](#).

## QUANTIFICATION AND STATISTICAL ANALYSIS

1. Process LC-MS/MS files using Proteome Discoverer 2.1 (PD2.1, Thermo Scientific) applying SEQUEST HT for protein identification. [Troubleshooting 5](#)

**Alternatives:** Other quantitative MS analysis programs such as MaxQuant can be used for analysis. In addition, other search engines than SEQUEST can be used to provide better confidence for protein identification.

2. Search samples using a non-redundant human UniProtSP database with carbamidomethylation (+57.0214 Da) as a fixed modification and oxidation of methionine as a variable modification.
3. Include both light and heavy isotopes (heavy lysine (+8.014 Da) and heavy arginine (+10.0082 Da). Precursor mass tolerance was set to 0.02 Da for MS1 and for fragment mass tolerance, set 0.6 Da tolerance.
4. Allow up to three missed cleavages. Filter final protein lists for mass tolerances of less than 10 ppm and a false positive rate at the peptide level of less than 1% using Percolator as the node in PD2.1. Percolator has been previously demonstrated to retain many true PSMs ([Käll et al., 2007](#)). Exclude proteins that are only identified in one replicate. The number of minimal unique peptides per identified protein was set as  $\geq 3$  unique peptides.

5. For quantitative peptide area representation, process database search results and display using Skyline software according to the tutorial for MS1 full-scan filtering, ([https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial\\_ms1\\_filtering](https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_ms1_filtering)).
6. For the forward SILAC experiment, heavy to light isotope ratios (H/L) > 4 provide proteins that were competed by 1. As seen by Figure 5, plotting the H/L ratio vs. protein number will demonstrate which proteins have H/L > 4, and thus a candidate for the phenotypic target of 1.
7. For probe vs. probe controls: the phenotypic target should have H/L ~1.
8. For probe vs. no probe controls: the experiment provides a list of probe-enriched targets.
9. For UV vs. no-UV controls: the experiment provides a list of proteins that are bound to protein in UV-dependent manner.

### LIMITATIONS

A major limitation is the difficulty of drawing general guidelines for different study cases than the one of reference. More efforts may be needed in addition to what is described because several controls and optimization rounds, such as verifying competition with parent compound and dose dependent photoprobe labeling, are needed for each different photoprobe.

The protocol supports the study of only two biological replicates by SILAC quantitation and needs many experiments as internal controls (probe:probe, probe:no probe, UV:no UV). Determination of high confidence hits using this quantitation method with higher number of replicates has low sustainability. The current protocol utilizes SILAC as its quantitative method but by no means does this choice eliminate the utility of TMT16pro for such a study (Li et al., 2020). While TMT methods may have issues with ratio compression, these methods are extremely valuable for target deconvolution due to higher statistical power by including many replicates and thus higher confidence for results obtained by quantitative proteomics as compared to SILAC, where no ratio compression is present but a demanding process is required to create a suitable model with the limitation of maximum three states of labeling (light, medium, and high). Alternative quantitative proteomics techniques such as Thermal Proteome Profiling (TPP) and Proteome Integral Solubility Alteration assay (PISA) can be used for target deconvolution. Necessary data analysis pipelines and additional software licenses, however, are required for implementing these methods.

Another limitation is non-specific photo cross-linking of proteins by the photoprobe. For this reason, the appropriate control experiments must be conducted. These controls include the probe vs. probe, probe vs. no probe and UV vs. no UV controls detailed in steps 9–12 during live cell photoprobe labeling. In addition, in some cases, certain photoreactive groups, such as alkyl diazirine, may have preferential reactivity rather than non-specific labeling (West et al., 2020). Because the data analysis uses MS-based proteomics, the limitations of proteomics also hold for the overall protocol. When analyzing complex samples, depth of protein coverage may be an issue. Additional fractionation of peptides prior to reverse phase chromatography and MS/MS analysis may increase protein coverage. The current protocol uses trypsin, which in some occasional cases form tryptic peptides that are not amenable to MS/MS-based analysis, and therefore may need alternative proteolytic strategies.

The target(s) that have been deconvoluted using this protocol need to be validated using orthogonal methods. In the study with compounds 1 and 2, Seneviratne et al. further confirmed target engagement using western blot at both overexpressed and endogenous protein levels and in-gel fluorescence experiments. In addition, cellular thermal shift assays (CETSA) and identification of the photoprobe labeling site on LXR $\beta$  provided complementary orthogonal methods to confirm the target engagement with parent compound 1 (Seneviratne et al., 2020).



## TROUBLESHOOTING

### Problem 1

Low photoaffinity yields (step 5)

#### Potential solution

- Lowering the distance between the UV source and the plate may increase photoaffinity labeling.
- Increasing the UV irradiation time to 20–30 min may increase photoaffinity labeling.
- Increased concentration of photoaffinity probe can increase photoaffinity yields but also may result in non-specific photo-labeling.

### Problem 2

Inefficient CuAAC chemistry yields (step 17)

#### Potential solution:

- In some cases, using freshly made reagents for CuSO<sub>4</sub>, TBTA, TCEP and biotin-azide can help. We have also observed high background signals from CuAAC chemistry with reagents from other vendors than those we listed.
- Use CuAAC master mix immediately after combining.
- When making master mix, add reagents in the order listed in the [materials and equipment](#) section.

### Problem 3

Inefficient protein solubilization before enrichment (step 19)

#### Potential solution:

- When necessary, additional sonication at 4°C can break protein pellets for solubilization. In some cases, incubating samples at increased temperature (37°C) for 5 min can also help protein re-solubilization.
- Protein solubilization issues may result from step 18i where when protein pellets are too dry, protein may not resolubilize. Step 18i should be performed carefully such that pellets are left with minimal volumes (<10 µL) of methanol. However, leaving too much volume of methanol can also complicate protein re-solubilization.

### Problem 4

Non-specific binding of proteins (step 20)

#### Potential solution:

- Additional washes of 0.1% SDS in DPBS may remove non-specific binding of proteins to streptavidin agarose beads.
- The current protocol is designed for 1.5–2 mg protein yields from cell lysis. For situations with lower protein amounts after lysis, lowering the volume of streptavidin agarose resin would result in less non-specific binding.

### Problem 5

Data analysis revealing low protein identification (<200) (step 1)

### Potential solution:

- With low protein identification, analyzing the chromatogram can reveal what the potential problem(s) are. If the peaks on the chromatogram are low (<1e7), protein or peptide loss may have occurred in previous steps.
- For protein loss, problems typically result from re-solubilization of proteins after precipitation (step 19). Please refer to the above tips in [troubleshooting 3](#).
- Low enrichment efficiencies associated with streptavidin agarose beads can be assessed by analyzing the endogenous biotinylated proteins in the data set. A successful biotin pull down is implicated by enrichment of the following carboxylases: propionyl-CoA carboxylase (PCCA, PCCB), pyruvate-CoA carboxylase (PC), methylcrotonyl-CoA carboxylase (MCCC), acetyl-CoA-carboxylase (ACACA, ACACB). Abundances of these carboxylases should be similar between all quantitation channels.
- For potential peptide loss, trypsin digestion can be optimized by changing the ratio of trypsin to protein (w:w). Peptide quantification can be performed after trypsin digestion using a Pierce Quantitative Colorimetric Peptide Assay.
- If the chromatogram show multiple ions with mass difference between peaks of ~44 Da, detergent is present in these samples and thus prevent the detection of peptide ions. ([Katayama et al., 2001](#), [Yeung et al., 2008](#)) Careful monitoring of all possible sources of detergent must be considered. Within the current protocol, detergent is present in 1% SDS during streptavidin enrichment and can be removed by additional washes of buffer and/or water.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Uthpala Seneviratne, ([Uthpala.Seneviratne@pfizer.com](mailto:Uthpala.Seneviratne@pfizer.com)).

#### Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

#### Data and code availability

Raw proteomic data sets are available from the lead contact upon request. Processed proteomic datasets are provided in [Seneviratne et al. \(2020\)](#).

### ACKNOWLEDGMENTS

We would like to thank Lorraine F. Lanyon, Claire M. Steppan, Gayathri Ramaswamy, and Martin Pettersson for assistance in phenotypic screen strategy and implementation. We thank Todd W. Butler, Leah Cleary, Ethan L. Fisher, Edelweiss Evrard, Paramita Mukherjee, and Longfei Xie for assistance in compound synthesis and characterization. We thank Douglas S. Johnson for chemical biology leadership. We thank Robert Everley for thoughtful review of manuscript.

### AUTHOR CONTRIBUTIONS

W.L. wrote the manuscript and coordinated the project. Z.H. developed experimental methods and wrote the manuscript. C.W.a.E. contributed to the design and synthesis of compounds and wrote the manuscript. U.S. performed the experiments, supervised the project, and wrote the manuscript.

### DECLARATION OF INTERESTS

All authors are or were Pfizer employees at the time of assembling the manuscript. W.L. is an employee at Dewpoint Therapeutics.

## REFERENCES

- Bantscheff, M., Boesche, M., Eberhard, D., Matthieson, T., Sweetman, G., and Kuster, B. (2008). Robust and sensitive iTRAQ quantification on an LTQ Orbitrap mass spectrometer. *Mol. Cell. Proteomics* 7, 1702–1713.
- Jenmalm Jensen, A. and Cornella Taracido, I. (2019). Affinity-Based Chemoproteomics for Target Identification. In *Target Discovery and Validation*, A.T. Plowright (Ed.). <https://doi.org/10.1002/9783527818242.ch2>.
- Käll, L., Canterbury, J.D., Weston, J., Noble, W.S., and Maccoss, M.J. (2007). Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* 4, 923–925.
- Karp, N.A., Huber, W., Sadowski, P.G., Charles, P.D., Hester, S.V., and Lilley, K.S. (2010). Addressing accuracy and precision issues in iTRAQ quantitation. *Mol. Cell. Proteomics* 9, 1885–1897.
- Katayama, H., Nagasu, T., and Oda, Y. (2001). Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 15, 1416–1421.
- Kawamura, A., Hindi, S., Mihai, D.M., James, L., and Aminova, O. (2008). Binding is not enough: flexibility is needed for photocrosslinking of Lck kinase by benzophenone photoligands. *Bioorg. Med. Chem.* 16, 8824–8829.
- Lapinsky, D.J., and Johnson, D.S. (2015). Recent developments and applications of clickable photoprobes in medicinal chemistry and chemical biology. *Future Med. Chem.* 7, 2143–2171.
- Li, J., Van Vranken, J.G., Vaites, L.P., Schweppe, D.K., Huttlin, E.L., Etienne, C., Nandhikonda, P., Viner, R., Robitaille, A.M., and Thompson, A.H. (2020). TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples. *Nat. Methods* 17, 399–404.
- Mann, M. (2006). Functional and quantitative proteomics using SILAC. *Nat. Rev. Mol. Cell Biol.* 7, 952–958.
- Ong, S.-E., and Mann, M. (2006). A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat. Protocols* 1, 2650.
- Park, S.-S., Wu, W.W., Zhou, Y., Shen, R.-F., Martin, B., and Maudsley, S. (2012). Effective correction of experimental errors in quantitative proteomics using stable isotope labeling by amino acids in cell culture (SILAC). *J. Proteom.* 75, 3720–3732.
- Parker, C.G., and Pratt, M.R. (2020). Click chemistry in proteomic investigations. *Cell* 180, 605–632.
- Preston, G.W., and Wilson, A.J. (2013). Photo-induced covalent cross-linking for the analysis of biomolecular interactions. *Chem. Soc. Rev.* 42, 3289–3301.
- Seneviratne, U., Huang, Z., Am Ende, C.W., Butler, T.W., Cleary, L., Dresselhaus, E., Evrard, E., Fisher, E.L., Green, M.E., and Helal, C.J. (2020). Photoaffinity labeling and quantitative chemical proteomics identify LXRβ as the functional target of enhancers of astrocytic apoE. *Cell Chem. Biol.* 28, 148–157.e7.
- Ting, L., Rad, R., Gygi, S.P., and Haas, W. (2011). MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nat. Methods* 8, 937–940.
- Vincent, F., Loria, P.M., Weston, A.D., Steppan, C.M., Doyonnas, R., Wang, Y.-M., Rockwell, K.L., and Peakman, M.-C. (2020). Hit Triage and validation in phenotypic screening: considerations and strategies. *Cell Chem. Biol.* 27, 1332–1346.
- West, A., Muncipinto, G., Wu, H.-Y., Huang, A., Labenski, M.T., and Woo, C. (2020). Labeling preferences of diazirines with protein biomolecules. *J. Am. Chem. Soc.* 143, 6691–6700.
- Xu, H., Jesson, M.I., Seneviratne, U.I., Lin, T.H., Sharif, M.N., Xue, L., Nguyen, C., Everley, R.A., Trujillo, J.I., and Johnson, D.S. (2019). PF-06651600, a dual JAK3/TEC family kinase inhibitor. *ACS Chem. Biol.* 14, 1235–1242.
- Yeung, Y.-G., Nieves, E., Angeletti, R.H., and Stanley, E.R. (2008). Removal of detergents from protein digests for mass spectrometry analysis. *Analyt. Biochem.* 382, 135–137.