**In situ labeling and TMT preparation**  
  
In situ treatment

1. Grow cells to 80-90% confluency in 10cm dishes in complete growth media.
2. Aspirate media and wash cells twice with cold DPBS
3. Incubate cells w/ 2 mL of serum-free media containing probe or control for 30 min at 37°C

In situ crosslinking prep

1. Aspirate media and irradiate cells for 10 min under 365-nm UV light in Stratagene UV Stratalinker 1800 at 4°C (no lid).
2. Collect cells by scraping in DPBS, transfer to eppendorf tubes and centrifuge  
   at 3000 rpm for 3 min, remove supernatant.
3. Add cold DPBS (1 mL) to each eppie, vortex to resuspend pellet.
4. Repeat steps 2 & 3 (\*cell pellets can be stored at -80°C at this stage or as lysates)
5. Add cold DPBS or NP40 based lysis buffer (~400 µL, should be ~2x pellet size) to pellet. Use DPBS for probe enrichments with LC-MS/MS as readout. Use NP40 buffer for gel-based probe experiments. For whole cell lysates used without click reaction, use NP40 buffer with protease and phosphatase inhibitor cocktail added immediately prior to experiment.
6. Lyse cells by sonication. For 10 cm plates use 10 pulses at 18% power 1 second on 1 second off. Determine protein concentrations by using the BCA protein assay on a microplate reader. Protein concentrations adjusted to ~1 mg/mL (500 µL). Note: If pellet is to be fractionated, after sonication, lysate fractionated by centrifugation (100,000g, 45min) to yield soluble and membrane proteomes.

**Click chemistry and removal of excess reagents**

1. For each sample, add the following reagents (make 'click stock' and add 55uL/sample):  
   For 22 samples = 11 ML proteome, 110 μL Biotin-Peg3, 220 μL TCEP, 660 μL TBTA, 220μL CuSO4

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|  | Volume added | Final Concentration |
| Proteome | 1 mL | 1 mg/mL |
| CuSO4 (50mM stock in water) | 20 μL | 2 mM |
| Biotin-PEG3-azide (ChemPep Inc., cat #271605) | 10 μL | 100 μΜ |
| Tris(2-carboxyethyl) phosphine 1.2mg/500m (TCEP) dal | 20 μL | 1 mM |
| Tris[(1-benzyl-1H-1,2,3-triazol-4- yl)methyl]amine (TBTA, 1.7mM in DMSO-tBuOH (1:4 v/v) | 60 μL | 100 μΜ |

1. Carry out click reaction for 1 hr at room temperature while shaking, or vortex every 15 min.
2. Transfer to a 15 mL conical on ice, add cold MeOH (2 mL) and vortex.
3. Centrifuge at 5000 rpm for 10 min, creating a protein pellet.
4. Carefully remove top. Wash pellet with 1:1 MeOH:CHCI3 (1 mL,
5. Remove washings and resuspend pellet in cold MeOH (2 mL) and sonicate resulting in a cloudy solution. Add cold CHCI3 (0.5 mL).
6. Centrifuge at 5000 rpm for 10 min to pellet protein and remove supernatant  
   Denature, Reduce and Alkylate

**Denature, Reduce and Alkylate**

1. To each sample, add freshly made 6M urea in DPBS (500 µL), followed up 10uL of 10%SDS (mixture does not have to be clear - clears after incubation)
2. Premix equal volumes of freshly prepared TCEP (200mM in DPBS) and K2CO3 (600 mM in DPBS). 50 µL of this solution added to each sample.
3. Pellet resuspended by sonication and solution incubated for 30 min at 37 C on a shaker.
4. To each sample, 70 µL of a freshly prepared 400mM (in DPBS) iodoacetamide (IAA) solution added. Solution incubated at room temperature protected from light.
5. To each solution, 130 µL of 10% SDS in DPBS added and sample diluted with 5.5 mL DPBS.

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| 6 M Urea | 1.8 g/5 mL (10 Samples) |
| 200 mM TCEP | 57 mg/mL |
| 600 mM K2CO3 | 83 mg/mL |
| 400 mM IAA | 74 mg/mL |

**Avidin enrichment and trypsinization**

1. Wash streptavidin beads (Thermo cat # 20353; 100 µL/sample) with DPBS (200 µL x3) and resuspend in 100 µL DPBS through inversion – do not vortex. Quickly add homogenously resuspended beads to each sample.
2. Incubate for 1.5 hr at room temperature while rotating. After incubation, pellet beads by centrifugation (2 min x2000 rpm).
3. Wash beads with 0.2% SDS in DPBS (5 mL), DPBS (2 x 5 mL) and milliQ water (2 x 5 mL).
4. Pellet beads and resuspend in 200 mM EPPS buffer pH 8 (0.5 mL x 2) and transfer to low-bind Eppendorf tubes.
5. Pellet beads and resuspend in 200 µL of trypsin premix: 2 mL 2M Urea in 200mM EPPS pH 8, 20 µL 100 mM CaCl2 (in DPBS), 1 vial sequencing -grade porcine trypsin (promega; 20 µg). DO NOT USE PROMEGA TYRYPSIN RECONSTITUTION BUFFER. Note\*\* 1 vial trypsin is enough to digest one TMT-10 plex experiment (10 samples).
6. Solution digest for ~14 hrs on a 37°c shaker

**TMT labeling of protein-enrichments (For standard TMT reagents)**

1. Next morning-remove sample from beads (~220 µL)
2. Add dry ACN to 30% final volume (~95 µL)
3. Add 6 ul of respective 10-plex TMT tag (for FFF probes), 3 µL (for FP  
   probes) to each sample
4. Vortex and incubate at RT for 1 hr-1 hr 15 min
5. Add 6 µL of 5% hydroxylamine to each sample, vortex and incubate 15 minutes
6. Add 4 µL formic acid and vortex
7. Dry down to ~100 µL and store at -80c

**Resuspending samples**:

1. Add 400-500ul fresh buffer A (95% Water, 5% ACN, 0.1% Formic) to the first tube
2. Pipette up and down and vortex to resuspend
3. Hard spin in microfuge and transfer to second tube-resuspend, vortex, spin and add to third tube (and so-on and soon until 10th tube is resuspended)
4. Add 200 ul buffer A to the empty tube 1- vortex and hard spin and transfer to tube 2 (repeat vortex and transfer for remaining tubes)
5. Final volume should be ~ 500ul

**Desalting using Sep-Pak C18 Cartridge**

1. Resuspend sample in ~500 ul buffer A (95% H20, 5% ACN, 0.1% formic). Add an additional 20 ul formic acid to ensure sample is acidic (can check using pH paper). Water bath sonicate for 5 minutes.
2. Condition cartridge by adding 1 mL 100% ACN (x3)
3. Equilibrate by adding 1 mL buffer A (95% H20, 5% ACN, 0.1% formic) (x3)
4. Load sample slowly, 1 drop/sec, take flow through and re-load sample (slowly) again.
5. Desalt by passing 1 mL 95% H20, 5% ACN, 0.1% formic (x3)
6. Elute by adding 1 mL 80% acetonitrile/0 1% FA. Blow cartridge dry
7. Speed vac the elution
8. Resuspend sample in 200ul 95% H20, 5% ACN, 0.1% formic to load on HPLC for offline high pH fractionation.

**High pH HPLC fractionation -- Use TMT\_wash method**

1. Resuspend sample in 50 ul buffer A (95% H20, 5% ACN, 0.1% formic). The sample loop is 50 ul total. Water bath sonicate for 5 minutes and spin down at top speed in a centrifuge for 1 minute. Inspect to ensure no particulate matter is present.
2. Install zorbax C18 extend column on the vanquish UHPLC with fraction collector (closest to door) and close all Thermo programs.
3. Open the chromeleon instrument configuration program through the start menu, and import the “FC Complete Instrument Configuration” saved on the desktop.
4. Open the Chromeleon software, wash the zorbax C18 extend column in 80% Buffer B, and then equilibrate in 100% Buffer A at a 0.5 mL/min flow rate for 30 minutes. Buffer A should be 10 mM AMBIC pH 8.5, and buffer B should be 100% ACN.
   1. Verify the pressure responds to the buffer changes. 100% A should be ~116 bar, whereas 80% B should be ~66 bar.
5. Prepare 96 well 1mL deep plates by adding 20uL of 20% formic acid.
6. Make sure the fraction collector needle is in the “above plate” position. The needle inside the fraction collector should extend from the plastic housing about 3 mm. Place plate in Red slot on the fraction collector.
7. Reset the fraction volume on the fraction collector, and make sure the Red position is selected for collection in chromeleon.
8. Put sample in Red A1 position in the autosampler and verify this position is selected in chromeleon software
9. Set up an 50 uL injection in the chromeleon software using the “HPLC\_Fractionation\_02192025” method in the c://Thermo/methods/hanigan folder.
10. Name sample with date, experiment type, and replicate number.
11. Submit sample and verify the salt peak elutes within the first 5 minutes of the run.
12. Speed vac plate (with balanced plate) and resuspend in Buffer B (80% ACN, 20% water 0.1% FA), combining down each column into a single sample.
13. Speed vac concatenated samples, which are now ready for resuspension and injection on mass spec.