This step-by-step protocol provides guidance for 10 µg, 100 µg, and 500 µg lysates. Optimal coverage is achieved with 250 µg lysate using 2.5 µL of 5% Fe-NTA per 10 µg. Increasing this amount showed minimal improvement, as tests with 5 µL and 7.5 µL per 10 µg did not significantly enhance capture.

Notes: This SP3-TMT-based protocol was optimized on panels of human cancer cells and tissues. This protocol can be completed before or after TMT labeling and before or after fractionation. Process each sample in 2 sets (**Phosphoproteome**) and (**Proteome**), as each fraction requires different settings on the equipment.

Caution:

1. After R&A and Digestion - clear lysates at 15-30k gs to remove lipids and Sp3 beads. Aliquot supernatant into new tube before c18 clean.
2. Use protein Lo-bind tubes to reduce sample loss.
3. If using too much TMT could compete-off the phospho from Serine (S), Threonine (T), and Tyrosine (Y). Search for TMT on these residues to calculate over-labeling, if necessary.
4. If %B is too high on HPLC, phos will come off of the column quickly. We recommend 0-1-12%B over 90 min or 180 min (always better if it is possible to optimize in shorter gradient with as much or more representative spectra captured).
5. Immediately dry down enriched phosphopeptides after elution into pre-aliquoted acid which acts as a neutralizing solution.
6. Check pH every step of the way.
7. Consider using detergent-less lysis buffer and/or creating spectral libraries for either DIA or DDA workflows to enhance precursor isolation amid any background noise.

MagBead Info:

- Delivered at 25% suspension.
- From R2P2 protocol, may need to spin peptide digests down to remove any contaminants.

TFA handling:

- Wear a labcoat
- Use glass syringe
- Be very careful

General Proteomics

Notes - Try to keep sample preps at 1mg/mL and pH 8.5

a. **Lysis** (Urea, NP40, RIPA, **Detergent-less GentleLys Dissolve**)
   i. GentleLys Dissolve is thermostable and solubilizes a big portion of the membrane proteome, increasing your peptide and protein IDs.
   ii. Avoid protein degradation by using protease and phosphatase inhibitors.
b. **Sonication** or DNase, Benzoase, etc.
   i. Covaris to sheer DNA and remove viscosity.
c. **BCA**
   i. Protein quantification
d. **OPTIONAL - Additional step enabled by GentleLys Stabilize**: Upstream characterization of **stabilized membrane proteins** enabling structure determination, PPIs, functional assays, enzymatic activity assays, and much more before loading the same detergent-less sample onto the mass spectrometer. Let us know if you need advice on this!
e. **R&A** (DTT & IAA)
   i. 5mM DTT 30 min @RT
   ii. 20mM IAA 30 min @RT in dark
   iii. 15mM DTT 30 min @RT
f. **Normalize** based on protein quantification and aliquot Sp3.
   i. Sp3 beads are used for concentrating proteins.
g. **Digestion** (LysC & Trypsin)
   i. Recommended Enzyme:Protein Ratio,
   1. LysC (1:50) for 16 hours at RT
   2. Trypsin (1:100) for 6 hours at 37C
      a. Consider promega rapid digestion or other enzymes to reduce digestion time.
h. **TMT Labeling or LFQ**
   i. Optional: Phosphopeptide enrichment before or after TMT labeling.

Below is a surface-level overview of the process:

Phosphoproteomics:

- Below is an updated protocol for our purecube magbeads with consideration for TMT workflows along with a stripping protocol for re-use. This protocol can be adapted to our Fe-NTA, Al-NTA, Zr-NTA, and Ti-NTA magbeads which all give robust and distinct phosphoproteomic profiles with a plethora of non-redundant Class I, II, & III phosphosites. Optimization may be necessary based on sample type(s). Some of our users perform combined (1:1) and sequential phos-enrichment techniques to help enhance or normalize results.

Gradients:

- When running samples on the mass spec, we recommend offline fractionation for deep coverage. When running on HPLC MS, we recommend %B gradients of 0%-4%-24% for the **Proteome** and 0%-1%-12% for the hydrophilic **Phosphoproteome**.

Benefits & Features of our IMAC for PE:

- **Enhanced Sensitivity & Specificity**: Fe-NTA MagBeads offer superior sensitivity and specificity for identifying phosphorylated peptides, enabling more precise analysis of cell signaling events.
- **Automation-Friendly**: Fe-NTA MagBeads streamline phosphoproteomics workflows with easy automation, reducing wait times while boosting productivity. Dr. Mario Leutert's work in the Villén lab at University of Washington illustrates this well: Leutert et al., 2019.
- **Proven Superiority**: In the 2019 study by Leutert et al., PureCube Fe-NTA MagBeads outperformed other IMAC beads or TiO_2 microspheres for phosphopeptide enrichment. Figures 1A, 1B, & 1C highlight the high efficiency and unique phosphopeptide enrichment achieved with Fe-NTA MagBeads.

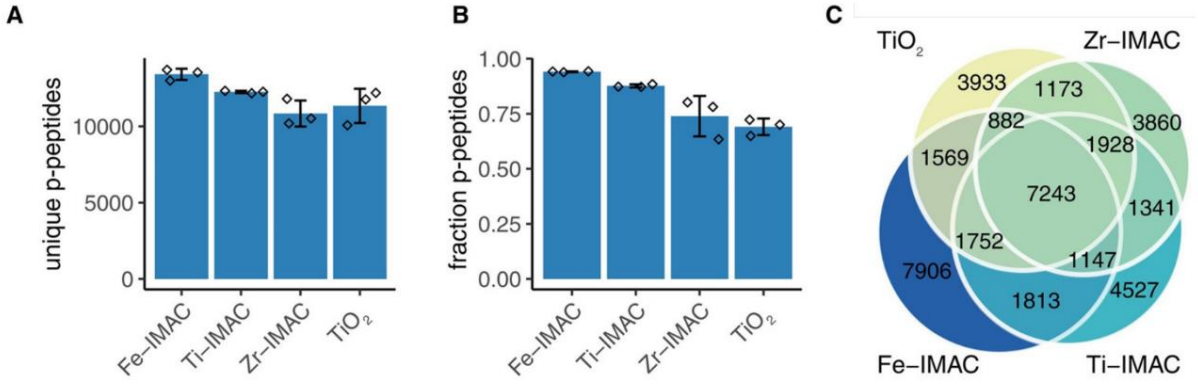

Figure 1: Comparison of phosphopeptide enrichment performance between four different products/methods. A: Number of unique phosphopeptides identified by the different enrichments (mean +/- SD, n = 3). B: Phosphopeptide enrichment efficiency is shown as the fraction of phosphorylated peptides over total peptides (mean +/- SD, n = 3). C: Venn diagram of identified phosphopeptides by the different phosphopeptide enrichment methods. Source: Leutert et al. (2019)

Example 1 (10ug)

For 10ug = 2.5uL 5% Fe-NTA MagBeads. Total 25% beads needed = 500nL

1) Prepare/Equilibrate Beads:
   - Dilute aliquot to 5% suspension - 80% ACN + 0.1% TFA (1:5)
     - 500nL 25% beads diluted in 2uL 80%ACN + 0.1%TFA
     - Starting Volume - **2.5uL** 5% iMAC
   - x3 1mL washes of beads with 80% ACN + 0.1% TFA
     - **Note**: Use 1mL or less of 80% ACN + 0.1% TFA for equilibrating beads; depending on sample size
     - Resuspend in starting volume (**2.5uL**)

2) Add beads to peptides:
   - Resuspend dried down peptides in the **2.5uL** magbead mixture.
     - When processing low input, optional to add additional 80% ACN + 0.1% TFA to increase volume and complete pH check.
   - Incubate for 30 min on a thermoshaker, vortexer, or rotator (250 rpm, 25°C).

3) Wash and Collect Flowthrough:
   - Place on magnetic stand.
   - Collect flowthrough in 2mL tube ---> **PROTEOME**
   - Perform x3 wash with 200uL 80% ACN + 0.1% TFA
     - Collect flowthrough of each wash in 2mL tube ---> **PROTEOME**

4) Elution:
   - **Pre-aliquot 200uL 75% ACN + 10% FA into labeled elution tubes**
   - **Elute with 200uL 50% ACN, 2.5% NH4OH (1:10)**
     - 200uL fresh = 100uL 100% ACN + 20uL of NH4OH (28% stock) + 80uL HPLC-H2O
   - Elute into to tubes/plates pre-loaded with 200 µl 75% ACN, 10% formic acid
     - Dry down immediately.
     - Expect ~1% phosphopeptide recovery, for this 10ug enrichment expect ~100ng enriched phosphopeptides to be recovered.

Example 2 (100ug)

For 100ug = 25uL 5% Fe-NTA MagBeads. Total 25% beads needed = 5uL

1) Prepare/Equilibrate Beads:
   - Dilute aliquot to 5% suspension - 80% ACN + 0.1% TFA (1:5)
     - 5uL 25% beads diluted in 20uL 80%ACN + 0.1%TFA
     - Starting Volume - **25uL** 5% iMAC
   - x3 1mL washes of beads with 80% ACN + 0.1% TFA
     - **Note**: Use 1mL or less of 80% ACN + 0.1% TFA for equilibrating beads; depending on sample size
     - Resuspend in starting volume (**25uL**)

2) Add beads to peptides:
   - Resuspend dried down peptides in the **25uL** magbead mixture.
     - When processing low input, optional to add additional 80% ACN + 0.1% TFA to increase volume and complete pH check.
   - Incubate for 30 min on a thermoshaker, vortexer, or rotator (250 rpm, 25°C).

3) Wash and Collect Flowthrough:
   - Place on the magnetic stand.
   - Collect flowthrough in 2mL tube ---> **PROTEOME**
   - Perform x3 wash with 200uL 80% ACN + 0.1% TFA o Collect flowthrough of each wash in 2mL tube ---> **PROTEOME**

4) Elution:
   - **Pre-aliquot 200uL 75% ACN + 10% FA into labeled elution tubes**
   - **Elute with 200uL 50% ACN, 2.5% NH4OH (1:10)**
     - 200uL fresh = 100uL 100% ACN + 20uL of NH4OH (28% stock) + 80uL HPLC-H2O
   - Elute into tubes/plates pre-loaded with 200 µl 75% ACN, 10% formic acid
     - Dry down immediately.
     - Expect ~1% phosphopeptide recovery, for this 100ug enrichment expect ~1ug enriched phosphopeptides to be recovered.

Example 3 (500ug)

For 500ug = 125uL 5% Fe-NTA MagBeads. Total 25% beads needed = 25uL

1) Prepare/Equilibrate Beads:
   - Dilute aliquot to 5% suspension - 80% ACN + 0.1% TFA (1:5)
     - 25uL 25% beads diluted in 100uL 80%ACN + 0.1%TFA
     - Starting Volume - **125uL** 5% iMAC
   - x3 1mL washes of beads with 80% ACN + 0.1% TFA
     - **Note**: Use 1mL or less of 80% ACN + 0.1% TFA for equilibrating beads; depending on sample size
     - Resuspend in starting volume (**125uL**)

2) Add beads to peptides:
   - Resuspend dried down peptides in the **125uL** magbead mixture.
     - When processing low input, optional to add additional 80% ACN + 0.1% TFA to increase volume and complete pH check.
   - Incubate for 30 min on a thermoshaker, vortexer, or rotator (250 rpm, 25°C).

3) Wash and Collect Flowthrough:
   - Place on magnetic stand.
   - Collect flowthrough in 2mL tube ---> **PROTEOME**
   - Perform x3 wash with 200uL 80% ACN + 0.1% TFA
     - Collect flowthrough of each wash in 2mL tube ---> **PROTEOME**

4) Elution:
   - **Pre-aliquot 200uL 75% ACN + 10% FA into labeled elution tubes**
   - **Elute with 200uL 50% ACN, 2.5% NH4OH (1:10)**
     - 200uL fresh = 100uL 100% ACN + 20uL of NH4OH (28% stock) + 80uL HPLC-H2O
   - Elute into to tubes/plates pre-loaded with 200 µl 75% ACN, 10% formic acid
     - Dry down immediately.
     - Expect ~1% phosphopeptide recovery, for this 500ug enrichment expect ~5ug enriched phosphopeptides to be recovered.

Storage of used MagBeads:

1. After R2-P2, beads can be recollected before they dry out.
2. Wash once with 1 ml 50% ACN, 50% MeOH, 0.01% acetic acid.
3. Store beads in 1 ml of the same buffer at 4 °C until further use.

Stripping and reloading of MagBeads:

1. Wash beads three times with 1 ml of water.
2. Wash once with 1 ml 40-100 mM EDTA, pH 8.
3. Resuspend beads in 1 ml 40-100 mM EDTA, pH 8 and incubate for 30 minutes while shaking or rotating the tubes. Ensure that the beads remain in solution.
4. Wash beads three times with 1 ml of water.
5. Wash once with 1 ml 10 mM FeCl3.
6. Resuspend in 1 ml 10 mM FeCl3 and incubate for 30 min, while shaking or rotating in tubes. Ensure that the beads remain in solution.
7. Wash beads three times with 1 ml of water.
8. Wash beads three times with 1 ml 80% ACN, 0.1% TFA.
9. Resuspend in 1 ml 80% ACN, 0.1% TFA.
10. Beads are ready to use

Reference Literature:

- Read here for more on our reagents in phosphoproteomics.
  https://pmc.ncbi.nlm.nih.gov/articles/PMC6920700/
- Read here for more on our copolymer nanodiscs in membrane proteomics.
  https://cube-biotech.com/our-science/membrane-protein-stabilization/copolymer-nanodisc/