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Technical Note

Quantitative Proteomics Approach to Characterize Cellular Reprogramming

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ABSTRACT: Tandem mass tag (TMT)-based proteomics facilitate multiplexing in mass spectrometry (MS)-based quantification and identification of proteins and their post-translational modifications. The use of TMT isobaric tags can enable multiplexing of up to 18 samples using commercially available kits. A single TMT experiment can quantify proteome, serine, threonine phosphorylation, and tyrosine phosphorylation. Of note, tyrosine phosphorylation is of low abundance, and identification/quantification can be improved using two complementary strategies. First, by employing SH2 superbinder which increases the number of identified sites. The SH2 Superbinder is more cost-effective than the commonly used phosphotyrosine antibodies. Second, by employing phosphotyrosine booster strategy, a pervanadate-treated channel to boost the signal of low-abundant phosphotyrosine. Noteworthy, pervanadate boost increases the likelihood of low abundant peptide to be selected for MS2, and facilitating the detection of > 6000 proteins, 10,000 unique pS/T and 1000 unique pY sites.

Keywords: Proteomics; Phosphoproteomics; TMT; Phosphotyrosine enrichment; SP3

1. INTRODUCTION

Bottom-up proteomics commonly uses tryptic digests to quantify proteins and post-translational modifications across different conditions to improve our understanding of signalling. Different strategies in proteomics can be used to quantify proteins and PTMs, including label-free, stable isotopic labelling using amino acids in cell culture (SILAC) or isobaric tags [1]. Isobaric tags have the advantage of multiplexing up to 18 samples from commercially available kits and up to 21 samples currently in literature [2, 3]. Multiplexing using isobaric tags reduces the required instrument time and run-to-run variation [1]. Multiple optimizations in recent years have improved isobaric labelling, proteomics and phosphoproteomics workflows. With the recent optimization of TMT to peptide ratio, the significant reduction in cost in TMT labelling has made it more accessible [4].

The development of Solid-Phase-enhanced Sample Preparation (SP3) has created a consistent protocol for sample clean-up with improved recovery [5, 6]. Using SP3, a more stringent lysis buffer can be used than conventional urea lysis. Additionally, SP3 outperforms other protocols in the low microgram range if this protocol ever needs to be scaled down due to low sample quantities [7].

Low abundant targets such as phosphotyrosine have been previously hard to detect without large starting amounts of material, making certain sample types previously not possible and/or TMT labelling cost prohibitive. With the recent development of the boost channel, using a pervanadate treated sample loaded into one of the TMT channels to increase the signal of phosphotyrosine over nonphosphotyrosine peptide and improve MS2 identification, the number of phosphotyrosine detected has been largely increased [8]. A similar carrier channel approach is used in single-cell proteomics, and much of the optimization of the carrier channel has been applied to the PV boosting [9–12]. Another strategy to improve phosphotyrosine identifications is using SH2 superbinder, which has been

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shown to be more cost effective and increase the number of identifications when compared to phosphotyrosine antibodies such as 4G10 [13]. Modification to this protocol can be made with small adjustments to increase plexing up to 18 samples or decrease starting amounts to quantify proteome and phosphoprotome.

2. MATERIALS

All solutions are prepared using mass spectrometry grade reagents and HPLC grade water. Certain microcentrifuge tubes have caused poor recovery with this protocol. In this protocol we have used either Corning™ Costar™ Low Binding Plastic Microcentrifuge Tubes (Thermo scientific, cat. No. 07-200-184) or Protein LoBind Tubes (Ependorpf, cat. No. 0030108442) where possible.

2.1. Preparing pervanadate boost

1. 100 mM orthovanadate stock solution: To prepare the sodium orthovanadate stock solution increase pH to 10, the solution will turn yellow. Boil until solution turns clear then cool to room temperature. Repeat increasing solution to pH 10 and boiling until solution remains clear at pH 10. This can be aliquoted and stored at –20°C until use.
2. 100mM Hydrogen Peroxide solution. To prepare dilute 10.2µL of 30% hydrogen peroxide into 989.8µL of water.

2.2. Reduction, Alkylation and Protein Digestion

1. Lysis buffer: 50mM HEPES, pH 8, 1%(wt/vol) SDS, 1% (vol/vol) Triton X-100, 1% (vol/vol) NP-40, 1% (vol/vol) Tween 20, 1% (wt/vol) deoxycholate, 5 mM EDTA, 50 mM NaCl, 1% (vol/vol) glycerol, 1× cOmplete protease inhibitor
2. 1M tris(2-carboxyethyl) phosphine solution (TCEP)
3. 500mM Indole-3-Acetic Acid (IAA)
4. 1M dithiothreitol (DTT)
5. SP3 beads: 1:1 mixture of Sera-Meg beads (GE Healthcare, cat. No. 45152105050250 and 65152105050250)
6. Anhydrous Ethanol
7. 80% Ethanol
8. 50mM EPPS, pH 8.0
9. Lysyl Endopeptidase (LysC)
10. Sequence Grade Trypsin

2.3. TMT labeling

1. BCA protein quantification kit (Peirce™, cat no. 23225)
2. TMT11plex Isobaric Label Reagent Set (ThermoFisher, cat no. A34808)
3. hydroxylamine 5% solution
4. ZipTip with 0.6 µL C18 resin (Millipore sigma, ZTC18S096)
5. HPLC grade water
6. HPLC grade ACN
7. HPLC grade 70% ACN

2.4. Superbinder Enrichment

1. SH2S-Magic Agarose beads (Precision Proteomics Inc.)
2. 50mM Ammonium Bicarbonate
3. 200mM Ammonium Bicarbonate
4. 0.4% TFA

2.5. Phosphopeptide Enrichment

1. High-Select™ TiO2 Phosphopeptide Enrichment Kit

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2. HPLC grade water
3. HPLC grade water with 0.1% FA
4. 2mL low binding collection tubes

2.6. High pH Reverse Phase Fractionation

1. Pierce™ High pH Reversed-Phase Peptide Fractionation Kit
2. HPLC grade water
3. HPLC grade TFA
4. HPLC grade ACN
5. 2mL low binding collection tubes

2.7. Mass Spectrometer Injects

1. Compatible nLC Vials for low volume pickup
2. HPLC grade water with 0.1% FA

2.8. Equipment

1. 1.5 mL magnetic rack
2. ThermoShaker
3. SpeedVac Vacuum Concentrators
4. Thermo Scientific EASY-nLC 1000 or equivalent nano LC
5. EASY-Spray 75µm x 500mm
6. Q Exactive™ Plus Hybrid Quadrupole-Orbitrap or newer orbitrap instrument

3. METHODS

3.1. Preparing pervanadate Boost

1. Mix equal volumes of 100 mM orthovanadate and 100 mM H_2O_2 to make 50 mM pervanadate.
2. Wash cells with serum-free media and add fresh serum-free media to cells.
3. Add 500µM of pervanadate to cell media and incubate for 10 minutes
4. Harvest cell pellet and flash freeze until processing rest of the experimental samples

3.2. Proteomics sample preparation

1. Spin down 3.0x10^6 cells per sample. (*see* **Note** 1)
2. Add lysis buffer to cell pellet. (*see* **Note** 2)
3. Sonicate three 5 second pulses with 15 seconds off at an amplitude of 35% on ice.
4. Spin at max speed for 10 min at 4°C, transfer supernatant to a new tube.
5. Preform BCA assay to measure protein. (*see* **Note** 3)
6. Add TCEP 5mM (made fresh) to each sample and incubate for at least 30 minutes at room temperature. (*see* **Note** 4)
7. Add IAA 15mM (made fresh) to each sample and incubate for 45 minutes at room temperature in dark.
8. Quench the alkylation by adding 5 mM DTT. (*see* **Note** 5)
9. According to an ideal bead to protein ratio of 10:1 (wt/wt), for 150 µg protein, 1.5 mg beads for each sample will be used.
10. Take the required amount of SP3 beads from both stock solutions, place it on a magnetic rack until all the beads have attached to the wall, then remove the supernatant.
11. Add 1 ml water to wash the beads, repeat once.
12. Reconstitute the beads at the concentration of 50mg/mL in water. (*see* **Note** 6)
13. After the reduction and alkylation of the cell lysate, take 150 µg protein to a new tube and dilute it with lysis buffer to a final volume of 130µL.
14. Add 20 µL prepared SP3 bead, which should contain 1.5 mg beads, to 150 µg protein, reaching a final volume of 150 µL.

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15. Add 150 µL 100% ethanol to the sample to reach at least 50% ethanol in binding solution.
16. Incubate the mixture in a shaker at room temperature for 10 min. (*see* **Note** 7)
17. After the binding is finished, gentle spinning can be performed if a lot of beads attach to the cap. Place the tube on the magnetic rack until all the beads have settled down to the wall.
18. Discard the supernatant without interrupting the beads or keep the supernatant for future analysis.
19. Remove the tube from the magnetic rack and wash the beads with 300µL of 80% ethanol. Add back to the magnet and remove supernatant. Repeat this step twice more. (*see* **Note** 8)
20. After the last washing, take care to remove the ethanol as much as possible.
21. Resuspended the magnetic beads at 1mg/ml of protein in 50mM of EPPS buffer, pH 8.5 (i.e., 150µg of protein in 150µL of EPPS).
22. Sonicate the beads on water bath for 30 s to disaggregate the beads
23. Add LysC at 1 mAu per 100 µg of protein or ratio of lysC to protein at 1:100 µg
24. Leave rotating at 37°C for 2 hours.
25. Incubate with trypsin at a ratio of trypsin to protein 1:50, overnight shaking at 37°C (ie. 16-18 hours)
26. The following day, centrifuge the tube at max speed for 1 min, and place it on a magnetic rack until all the beads have migrated to the wall. Carefully transfer the supernatant containing peptides to the new tube.
27. Wash beads with 50µL of water and combine with elution, repeat this with 50µL of 30% Acetonitrile.
28. Spin at max speed again for 5 minutes to get rid of any possible magnetic bead residues. Move the supernatant to the fresh tube.

3.3. TMT labeling

29. Measure peptide using either BCA or pierce peptide quantification, colorimetric or Fluorometric. Quantification for BCA can be improved using Peptide Digest Assay Standard from ThermoFisher (Cat#23295). (*see* **Note** 9 & 10)
30. Optional, inject 100-250ng to test digestion efficiency
31. Aliquot 100µg of peptide, diluting with 50mM EPPS to 100µL and Speed-vac the peptides dry.(*see* **Note** 11)
32. Resuspend peptide samples at 5 g/L in water (i.e., 20uL for 100ug)
33. Allow TMT reagent to equilibrate to room temp for 5 minutes and spin down and resuspend in 41µL of acetonitrile for 0.8mg vials.
34. Add 6.8µL of the respective TMT reagent to their sample, vortex and incubate for 1 hour either.
35. Dilute sample to 1µg/µL of water and take 1µL from each sample to test for labeling efficacy and channel normalization.
36. Desalt using ZipTip (ZTC18S096/Millipore sigma) or equivalent
37. To validate the labeling efficiency, setup a MaxQuant search using a label-free search with variable TMT N-term and Lysine modification search using MaxQuant. Using the evidence file count the number of fully labeled peptides over the total number. (*see* **Note** 12)

Table 1: List of Fully labeled TMT peptides of the possible locations TMT can be labeled in.

N-term	Lysine	C-term
TMT-labeled	TMT-labeled	Lysine
Actyl	TMT-labeled	Lysine
TMT-labeled	None	Arginine

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38. Preform a second search using MaxQuant in the group specific parameters tab set type to Reporter ion MS2 and select 11plex TMT.
39. Under the combine folder, then txt folder using the peptide.txt file. Divid all genes by the row intensity and then measure the column median. Use these values to normalize the mixing of TMT channels.
40. Add 2.7µL of hydroxylamine 5% to each sample and incubate for 15 minutes
41. Combine all the TMT labeled samples, but PV treated sample and acidify sample to below pH 3 for desalting.
42. Activate 100mg C18 SepPak 3mL column with 3 mL of 100% ACN. Repeat this once.
43. Equilibrate column with 3mL of 0.1% FA in water, repeat this step once
44. Load sample onto the C18 column and load the flow through onto the column.
45. Wash using 3mL of 0.1% FA in water, repeat twice more.
46. Elute sample using 700µL of 70% ACN with 0.1% FA, repeat this once
47. Remove 5% of the sample for proteome to be later fractionated.
48. SpeedVac until the volume is reduced to approximately 20uL

3.4. SH2 Superbinder enrichment

1. Prepare 200µL of SH2 superbinder beads for each plex plus an additional for boost channel. (*see* **Note** 13)
2. Equilibrate in 1mL 50mM ammonium bicarbonate (ABC). Aspirate volume to just above the beads and avoid drying the beads.
3. Vortex and spin down at 1000g for 30 seconds. (*see* **Note** 14)
4. Load the TMT plex sample and PV-Boost into separate superbinder beads mixtures and incubate in a rotating incubator for 30 minutes.
5. Spin down at 1000g for 30 seconds and aspirate liquid to just above the beads. Retain supernatant for Phosphopeptide enrichment
6. Wash beads with 1mL of 200mM ABC by shortly vertexing and rotating for 1 minute. Repeat once. Retain the first two washes for pST enrichment
7. Repeat step 6, but use 50mM ABC instead and repeat twice
8. Elute phosphopeptide by incubating in 300µL of 0.4% TFA for 10 minutes. Repeat this once.
9. Remove 30 µL of TMTplex and mix with 3 µL boost channel for test injection and ensure you are not carrying over beads. Dry the rest of the samples.
10. Calculate the median sample intensity to PV reporter intensity.
11. Adjust mixing to a 20x ratio of PV to median sample intensity.
12. The elution can be loaded directly into the reverse phase spin column.

3.5. Phospho-peptides enrichment

1. Combine the flow through and the first two washes of the superbinder and SpeedVac sample.
2. Follow High-Select™ TiO2 Phosphopeptide Enrichment Kit for enrichment of pST peptides, alternatively you can use any commercially available Ti-IMAC, Fe-IMAC or TiO2 kits.
3. Optional, further phosphopeptide enrichment can be performed by using Fe-IMAC on the Flow through of TiO_2. We use protocol from Swaney&Villén, 2016 for Fe-IMAC. (*see* **Note** 15)

3.6. Reverse phase high pH

1. Use the pierce reverse phase spin column instruction manual with modification. Briefly, for the superbinder and IMAC enriched fractions (phsophoproteome) follow the unlabeled gradient table in the datasheet and for and the TMT follow the labeled

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gradient table for proteome analysis. After each gradient add additional fraction using 50% ACN with 0.1% FA, additional peptides eluted in acidic conditions. (*see* **Note** 16)

3.7. Mass spectrometer injections

1. The settings used in this protocol are based on Q Exactive Plus and EasyLCn-1000 with EASY-Spray 75µm x 500mm at 45°C, adjust setting according to instrument available.

Table 2: Setting up parameters for proteome analysis on Q Exactive Plus.

Parameter	Setting
Full MS	
Resolution	70,000
AGC target	3e6
Maximum IT	50 ms
Scan range	375 to 1300 m/z
dd-MS^2	
Resolution	35,000
AGC target	1e5
Maximum IT	110
MSn	15
Isolation window	0.7
Fixed first mass	120 m/z
(N)CE	32
Intensity threshold	1.0e4
Charge exclusion	1, 6-8, >8

Table 3: Setting up parameters for proteome analysis for LC gradient on EasyLCn-1000.

Time	%B (100% ACN)	Flow Rate
0	5	300nL/min
6	5	300nL/min
10	7	300nL/min
210	25	300nL/min
250	40	300nL/min
254	95	300nL/min
259	5	300nL/min
264	95	300nL/min
269	5	300nL/min
274	95	300nL/min
285	95	300nL/min

Table 4: Setting up parameters for phosphoproteome analysis on Q Exactive Plus.

Parameter	Setting
Full MS	
Resolution	70,000
AGC target	3e6
Maximum IT	50 ms
Scan range	375 to 1300 m/z
dd-MS^2	
Resolution	70,000
AGC target	5e5
MSn	10
Maximum IT	240
Isolation window	0.7

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Fixed first mass	120 m/z
(N)CE	32
Intensity threshold	1.0e4
Charge exclusion	1, 6-8, >8

Table 5: Setting up parameters for phosphoproteome analysis for LC gradient on EasyLCn-1000.

Time	%B (100% ACN)	Flow Rate
0	5	100nL/min
5	5	100nL/min
160	25	100nL/min
180	40	100nL/min
183	95	100nL/min
186	95	100nL/min
189	5	100nL/min
192	5	100nL/min
195	95	100nL/min
198	95	100nL/min
201	5	100nL/min
204	5	100nL/min
207	95	100nL/min
225	95	100nL/min

Table 6: Setting up parameters for Superbinder Enriched Samples on Q Exactive Plus.

Parameter	Setting
Full MS	
Resolution	140,000
AGC target	3e6
Maximum IT	50 ms
Scan range	375 to 1300 m/z
dd-MS^2	
Resolution	140,000
AGC target	2e5
MSn	6
Maximum IT	750
Isolation window	0.7
Fixed first mass	120 m/z
(N)CE	32
Intensity threshold	1.0e4
Charge exclusion	1, 6-8, >8

Table 7: Setting up parameters for LC gradient on EasyLCn-1000 for superbinder enriched sample.

Time	%B (100% ACN)	Flow Rate
0	5	100nL/min
5	5	100nL/min
105	25	100nL/min
125	40	100nL/min
128	95	100nL/min
131	95	100nL/min
133	5	100nL/min
136	5	100nL/min
139	95	100nL/min
142	95	100nL/min
145	5	100nL/min
148	5	100nL/min

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151	95	100nL/min
165	95	100nL/min

3.8. Data Processing

1. When using fragpipe ensure all parent folders and files do not contain spaces use underscore in place. (*see* **Note** 17 & 18)
2. Convert RAW files to mzML using ProteoWizard MSConvert. Go to Peak picking in Filter and set msLevel to 1 (See Figure_)
3. Have all mzML files of each plex within subfolders, we name our parental folder A, B, C.. ect.
4. Load files and set biological replicates based on parent folder.
5. Load pre-set of TMT10-Bridge for proteome and TMT10-Phosho-Bridge. If all samples are on single plex use non-bridge pre-sets.
6. In MSFragger tab adjust minimum peptide length to 6 for superbinder enriched fraction.
7. Under Quant(isobaric) tab adjust TMT10 to TMT11 and set normalization to All
8. Under the Quant(Isobaric) tab set the plex layout click Edit/Create for each TMT plex assigning each sample to each reporter in all TMT plexes
9. Move to the Run tab and set directory creating a new folder, then click run
10. After FragPipe run is complete the quantification results will be in the directory folder created under TMT-report.
11. Files can be loaded into Perseus or R (including MSstatsTMT package).
12. Analysis can be performed using ANOVA or t-test, and then plotted on either hierarchical clustering or volcano plots, respectively. The significant features from T-test or clusters from hierarchical clustering can be used to look for enriched pathways using Fishers exact test, GSEA, KSEA, String, Panther, ect...

4. Notes

1. The number of cells will vary by cell type and can be scaled down when the yield of protein is known.
2. The lysis buffer can be different depending on your needs, see chart in Hughes et al, 2014
3. Reduction and alkylation from steps 6 and 7 can be performed while waiting for BCA to develop.
4. TCEP can be done simultaneously as IAA.
5. The beads prep can be done during the reduction and alkylation
6. The prepared beads can be stored at 4°C for one month.
7. The aggregation of the beads may be observed, which indicates the binding is induced.
8. For each washing, make sure all the beads are suspended properly, avoid pipetting as beads are sticky and aggressive mixing including vortexting.
9. The peptide recovery should be close to 100%, so there should be 100 μg peptides per sample
10. BCA kit is compatible up to 10% acetonitrile and Pierce peptide quantification kits are compatible up to acetonitrile.
11. The dried EPPS and peptide when resuspended at 4g/L for TMT labeling will lead to 200mM EPPS.
12. Video tutorials for setting up MaxQuant and adding new modifications are available on MaxQuant YouTube channel.
13. The 200μL of Superbinder has been tested up to 2.25mg of TMT samples
14. To make ABC fresh each time.
15. Different metal chemistries can enrich different phosphosite motifs

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16. Alternatively, if there is access to an HPLC capable of high pH fractionation performance that would give better performance.
17. Based on our experience we have found that Fragpipe database performed better across multiple TMTplex compared to MaxQuant.
18. Tutorials for setting up and using Fragpipe are available at https://fragpipe.nesvilab.org.

FIGURE LEGEND

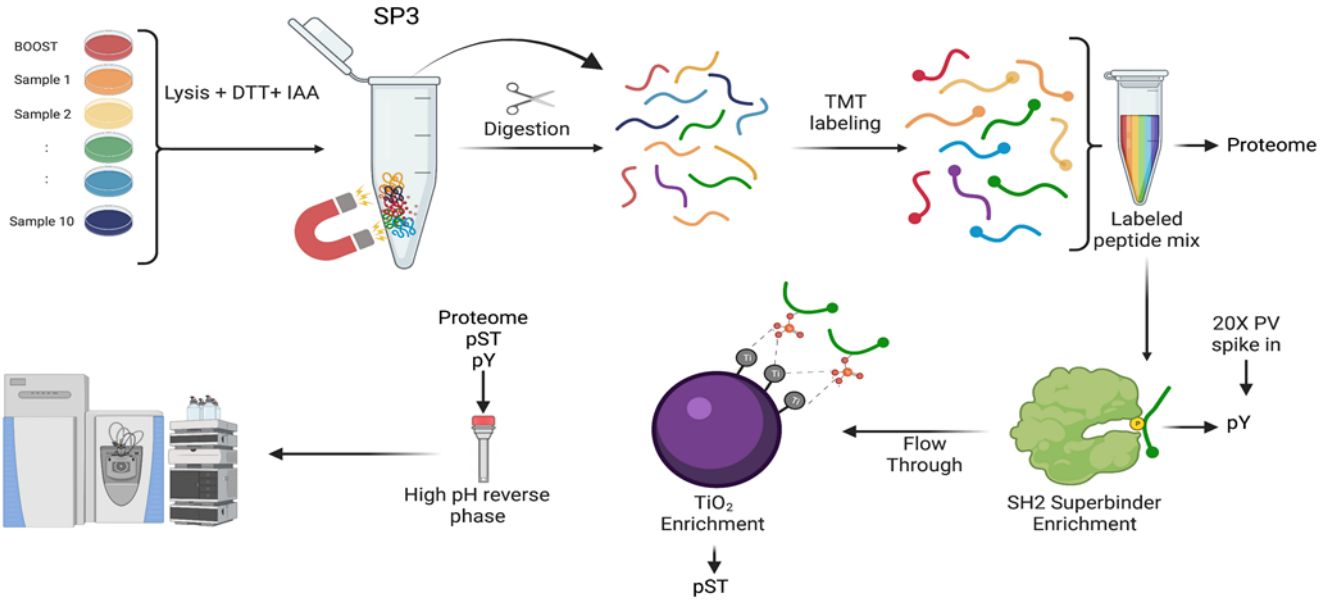

Figure 1: Workflow of sample processing of TMT proteomics and phosphoproteomics. The Samples are first lysed, reduced and alkylated, and then cleaned up through SP3. Samples are then digested, and TMT labeled. The labeled peptides can then be mixed and enriched for phosphotryorsine, and phosphoserine and threonine. Samples are finally fractionated and then injected onto the mass spectrometer.

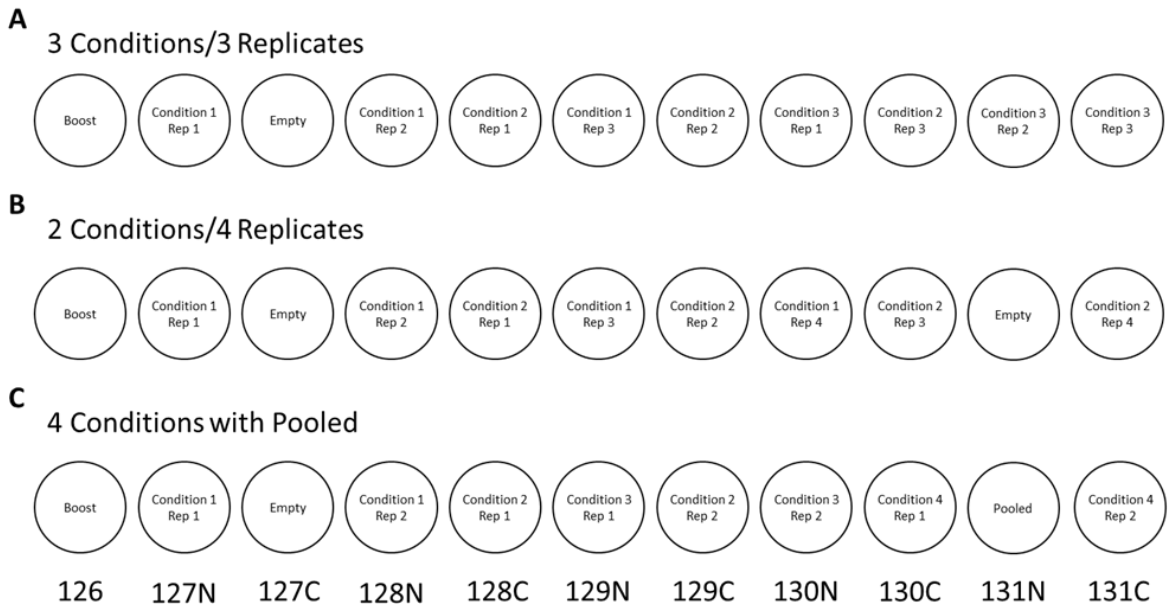

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Figure 2: Location of samples and boost samples in TMT plex. Shown here comparison of three conditions with each three replicates, two conditions with four replicates each or four conditions with 2 replicates each with a pooled sample for normalization across multiple plexes. Location of samples can be altered if proper considerations are taken

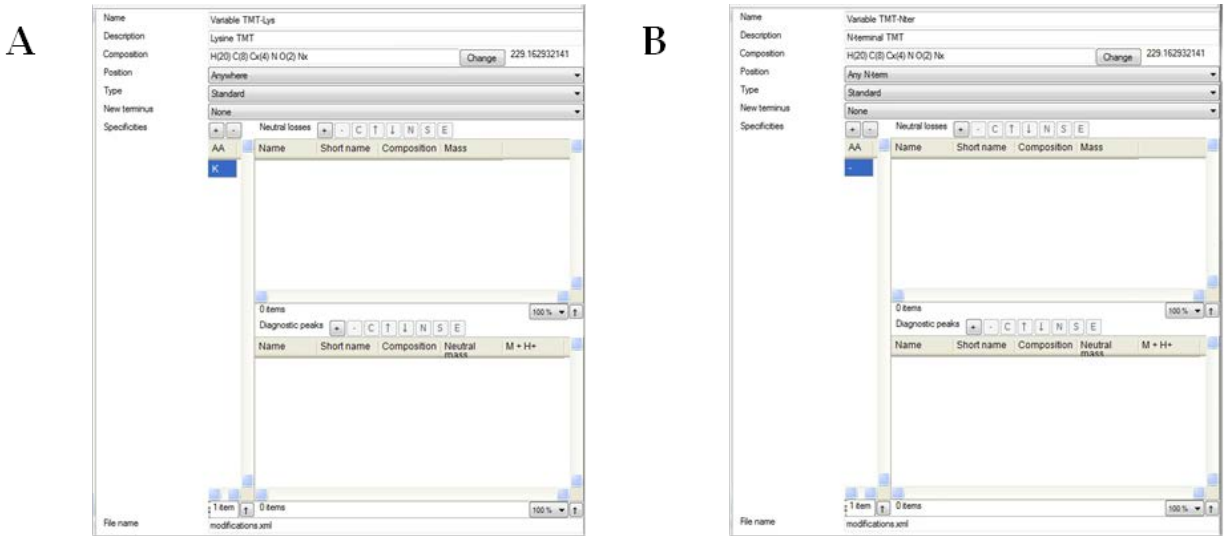

Figure 3: Setup of MaxQuant modifications for labeling efficiency search. Set of modification of (A) variable TMT labeled Lysine and (B) variable TMT labeled N-terminus.

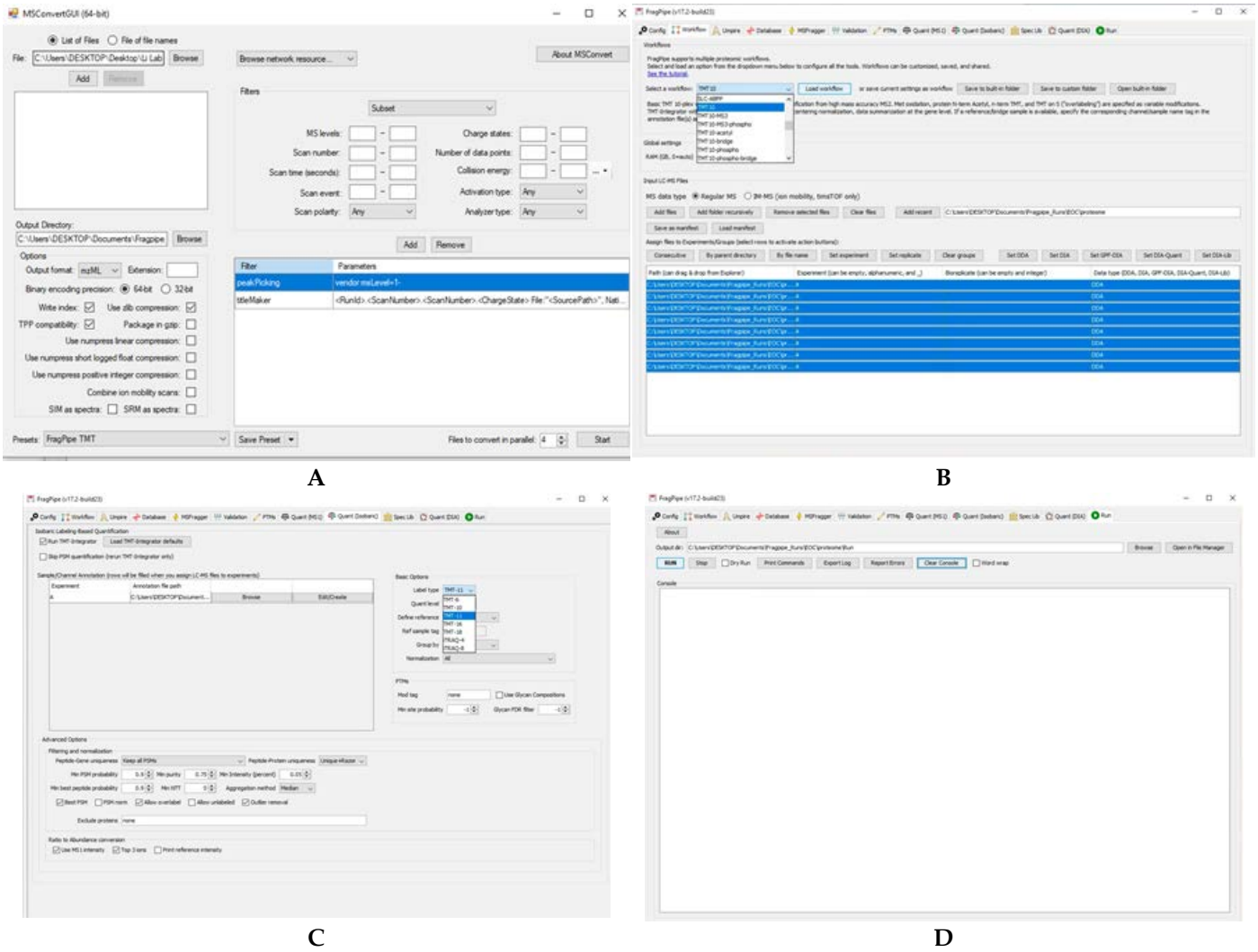

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Figure 4: Setup of Fragpipe for database search of Mass spectrometry files. (A) First Raw files need to be converted into mzML files for search in Fragpipe. (B) The appropriate TMT workflow can be selected. Files can then be loaded into fragepipe and experiment name set per plex. (C) Changing to TMT11 for label type and setting up of the TMT annotation files need to be selected. (D) in the run tab, output directory needs to be set and run selected.

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