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BAF_Protocol_014_TMT-Based proteomics: Isobaric isotope labeling quantitative method

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Protocol status: Working
We use this protocol and it's working

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Abstract

Labeling samples with TMT, digestion, cleanup and fractionation by high pH. Note that generally you need on the order of 25-100ug protein per plex channel for this to work well.

Materials

All reagents listed are from Thermo Scientific:

Pierce™ Quantitative Colorimetric Peptide Assay Kit - 23275
Anhydrous acetonitrile (Acetonitrile, LC-MS Grade) - 51101
50% Hydroxylamine - 90115
TEAB - part #
TMTpro™ 16plex Label Reagent Set, 1 x 0.5 mg - A44521 (Thermo Scientific)
Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit - 84868 (Thermo Scientific)
Pierce™ High pH Reversed-Phase Peptide Fractionation Kit - 84868
Thermo Orbitrap Exploris 480 - BRE725533
Thermo Easy Spray Ion Source - ES081
Thermo nLC 1200 - LC140
Thermo Acclaim PepMap 100, 75um x 2cm - 164946
Thermo Easy Spray 75um x 150mm, 3um particle - ES900
Thermo Optima 0.1% FA (formic acid) in water - LS118-500
Thermo Optima 80% ACN (acetonitrile) / 0.1% FA in water - LS122-500
Thermo Proteome Discoverer 2.5 - OPTON31040/CPQ00507094 (now 3.1 cloud-based)
Proteome Scaffold Q+S 5.3.3 - Q+S (replacing with Scaffold DDA 6.3+)

Generate tryptic peptides

1 Refer to BAF_Protocol_007 Solution Digest with Protein Precipitation for protein extraction, clean-up and trypsin digestion.

2 After digestion, samples should be desalted, follow BAF_Protocol_003 Desalting.

3 Add 100 uL of 100 mM TEAB pH 8.5 to the dried samples, resuspend well and perform a BCA quantification. Use 25–100 µg of protein digest per labeling reaction, per TMT tag.
Mandatory: use same amount of each digested sample per TMT tag.

TMT-Labeling - protocol follows TMTprot 16plex, 1 x 0.5 mg (#A44521), user guide

4 For complete labeling of lysine and N-termini, use a minimum ratio of 1:5-1:10, sample to tag (w:w).

5 Immediately before use, equilibrate the TMTpro label reagents to room temperature in the foil pouch.

6 Add anhydrous acetonitrile to each vial, 20 uL (for 0.5 mg kit), then allow the reagent to dissolve for 5 min with occasional vortexing.

7 Briefly centrifuge the tube to gather the solution.

8 Transfer the quantified peptides (100 uL containing the same protein digest amount raging 25-100 ug) to each TMTpro reagent vial

9 Incubate the reaction for 1 h at room temperature.

10 Add 5 uL of 5% hydroxylamine to the sample, then incubate for 15 minutes to quench the reaction.

11 Combine **equal** amounts of each sample (110 uL) in a new microcentrifuge tube, then speedvac to dry the labeled peptide sample.

High pH fractionation (Pierce #84868)

12 Prepare 10 pre-washed 2 mL tubes to be used during sample collection: washes and fractions.

13 **A. Conditioning of the spin columns** – note: do not exceed recommended centrifugation speeds.

14 Remove the protective white tip from the bottom of the column and discard. Place the column into a 2.0 mL sample tube

15 Centrifuge at 5000xg for 2 minutes to remove the solution and pack the resin material. Discard the liquid

16 Centrifuge at 5000xg for 2 minutes to remove the solution and pack the resin material. Discard the liquid

17 Remove the top screw cap and load 300 uL of ACN into the column. Replace the cap, place the spin column back into a 2.0 mL sample tube and centrifuge at 5000xg for 2 minutes.

18 Wash the spin column twice with 0.1% TFA solution, as described in Step 3. The column is now conditioned and ready to use.

19 **B. Fractionation of digest samples**

20 Prepare elution solutions according to Table 02 (vendor instructions) and use pre-washed 2.0 mL tubes.

21 Dissolve dried labeled peptide in 600 uL of 0.1% TFA solution.

22 Place spin column into new 2.0 mL sample tube. Load 300 uL of the sample solution onto each column, replace the top cap and centrifuge at 3000xg for 2 minutes. Retain eluate as “flow-through” fraction.

23 Place the column into a new 2.0 mL sample tube. Load 300 uL of water onto the column and centrifuge again to collect the wash. Retain eluate as “wash01” fraction.

24 Place the column into a new 2.0 mL sample tube. Load 300 uL of 5% CAN, 0.1% TFA onto the column and centrifuge again to collect the wash. Retain eluate as “wash02” fraction. Repeat this step once.

25 Place the column into a new 2.0 mL sample tube. Load 300 uL of the appropriate elution solution (10% ACN, 0.1% TFA) onto the column and centrifuge again to collect the wash. Retain eluate as fraction 01.

26 Repeat the step 06 for the remaining step gradient fractions using the appropriate elution solutions from table 02 in new 2.0 mL tubes numbered 02 to 09.

27 Evaporate the liquid contents of each sample to dryness.

28 Dried samples were stored at -80C until LC-MSMS analysis.

29 Re-suspended each fraction in 25 uL of 0.1 % F.A (adjust this volume to the starting amount of protein digestion used per TMT tag)

LC-MS/MS analysis in a Orbitrap 480 with nano EASY-LC 1200

30 **nLC parameters- 2 h for complex samples:**
--> Sample pickup: 5 uL (loop size 20 uL) with a flow of 10 uL/min.
--> Sample loading: 20 uL, max. pressure 500 bar, no flow defined.
--> Gradient: Flow = 300 nl/min. 0 min 2%B; 3 min 2% B; 3-105 min 2-25% B; 105-125 25-40% B; 125-126 min 40-95%B; 126-131 min 95%B.
--> Pre-column equilibration: 10 uL, max. pressure 500 bar, no flow defined.
--> Analytical column equilibration: 8 uL, max. pressure 500 bar, no flow defined.
--> Autosampler wash: standard wash with 100 uL of flush volume.
--> EASY-spray: column heating on, column temperature: 40°C.

General instrument parameters:
--> Spray Voltage 1700V, Ion Transfer Tube 285C, All gases 0.

Full scan parameters:
--> Scan 375-1500 m/z, resolution 120K, time 60ms, normalized AGC 300%, RF lens 40%, 1 microscan

MS/MS parameters:
--> Min intensity 1E4, Charge +2 to +9, Dynamic exclusion - 1 time, 20s, 10 ppm, isotopes, 10 MS2 scans, isolation 2 m/z, resolution 60K, HCD NCE 30%, first mass 100, time 60ms, normalized AGC 30%, 1 microscan.

Database search in Proteome Discoverer 2.5

31 Thermo RAW files are set up to search in PD 2.5 software (Proteome Discoverer) to produce an output MSF file. The RAW files for an individual project are placed in a folder and a sub folder is created with the MSF files produced by PD 2.5 inside. The MSF files will be loaded for display/analysis in Scaffold 5.3+ software.

31.1 Open PD 2.5 and start a new study.

Choose:
Study name - will create sub folder with this name for MSF (and associated) files
Root Directory - where your RAW files are
Processing Workflow -
PWF_QE_Reporter_TMTpro16_Based_Quant_SequestHT_Percolator.pdProcessingWF
Consensus Workflow -
CWF_Comprehensive_Enhanced_Annotation_Reporter_Quant.pdConsensusWF

31.2 Tab Study definition:
Add quantification Method --> choose TMTpro 16plex

Tab Input files:
Click 'Add Files' to add your RAW files. They will then be displayed under the input files tab.
On each file, click Quan Method --> TMTpro 16plex
Select all files and add to processing step

Tab Workflows --> Processing Workflow:
Spectrum Files - No parameters just to get files
Spectrum Selector (set to just take every scan)Precursor Selection - Use MS1 Precursor
Provide Profile Spec - Automatic
RT, Scan, Charge State - all 0
Min Precursor Mass - 350 Da
Max Precursor Mass - 5000 Da
Total Intensity - 0
Min Peak Count - 1
S/N FT - 1.5
Reporter Ions Quantifier:
Integration Tolerance: 4.5 ppm
Integration Method: Most Confident Centroid
Mass Analyzer - FTMS
MS order - MS2
Activation Type - HCD
Min. Collission Energy - 0

Max. Collision Energy - 1000
Sequest HT:
Database - FASTA of your species (must be parsed in PD before can choose - restart PD after parsing and before search set up)
Enzyme - Trypsin (Full)
Missed Cleavage - 2
Min length - 6
Max length - 144
Precursor Tolerance - 10 ppm
Fragment Tolerance - 0.02 Da
Averages set to false
Neutral loss a,b,y and flanking ions - true
Weight b,y = 1; rest 0
Max equal modifications = 3
Dynamic modification oxidation M
Static modifications: cabamidomethyl C, TMTpro 16plex/+340.207 Da K
Peptide N-terminus: TMTpro 16plex/ +304.207 Da (Any N-terminus)
Target Decoy PSM Validator:
Target/Decoy - concatenated
Strict 0.01
Relaxed 0.05

Tab Workflows --> Consensus Workflow:
Just set to defaults as using Scaffold later to display and parse the data. If you want to see specific display in PD 2.5, then you would need to set parameters here.
Protein annotation - select protein database used for search.

Data filtering, display and relative quantization in Scaffold Q+S 5.3.3

32 Run Scaffold 5.3.3 and choose new analysis.
Select Quantitative Technique: Check TMTpro 16plex -->next
New TMT-16plex Sample --> Check MudPit Experiment (to combine the 8 fractions (8 MSF files) into one analysis output) --> next
Queue Search Engine for Landing --> select all 8 MSF files and add to Import Queue.
Load and Analyze data --> Enter the database which was used in PD for the search. You will have to index in Scaffold just as you did in PD before you can use a database. Use Legacy LFDR, protein cluster analysis, and pre-compute FDR.
Click 'Load Data' and allow to run.

Once all data is loaded, apply filters using a FDR, Peptide/Protein Prophet or XCorr - or some combination. For our general settings in proteomics we use - min peptide 1, protein prophet 90%, peptide prophet 60%, DeltaCN 0, Xcorr - +1>1.8, +2>2.0, +3>2.2, +4>3.0.

At the sample view, by default it displays one sample column and quantitative value for each identified proteins, in order to get the TMT tag intensities, enter sample grouping and apply statistics do the following.
Click on Q bottom: Launch Q+ Quantitation (iTRAQ, SILAC, TMT, Label-free) Module in a new window.
Analysis type: intensity-based --> next
Experiment type: Between-subjects (Independent groups). --> next
Edit sample names and categories --> edit sample name of each tag and for treatment groups --> next
Organize Quant samples --> drag and drop each sample to its group --> next
Settings --> use non-exclusive peptides: false, Calculation type: Median, Blocking level: Unique peptides, Use protein average as reference: True, Spectrum Quality Filter: no filter. --> Finish

Once data is loaded, it displays all tags with sample names as edited and log2 fold changes for each quantified protein.
Quantitative analysis --> apply specific statistics in accordance with the study design and generate graphics and GO to be exported or export data to excel and apply statistics with algorithm of choice to detect disregulated proteins and enriched pathways.

Protocol references

TMTpro Mass Tag Labeling Reagents and Kits - User Guide, ThermoScientific.
https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0018773_TMTproMassTagLabelingReagentsandKits_UG.pdf

Pierce High pH Reversed-Phase Peptide Fractionation Kit
https://assets.fishersci.com/TFS-Assets/LSG/manuals/84868_highph_rp_peptidefract_UG.pdf