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Isobaric Labeling Based LC-MS/MS Strategy for Comprehensive Profiling of Human Pancreatic Tissue Proteome

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Summary

The pancreas is an organ with both endocrine and exocrine functions, and various pathologies, such as pancreatic cancer and diabetes are associated with this organ. Owing to the limited pancreatic biopsy samples available for research, it is critical to make the best use of cadaveric pancreatic tissue for biomarker studies and mechanistic understanding of pancreas-related pathologies. Discovery-phase quantitative proteomics has attracted a lot of attention for its capabilities in large-scale protein identification and accurate protein quantification. Here, we describe a workflow using isobaric labeling (tandem mass tag or TMT) based quantitative proteomics to confidently identify and quantify human pancreatic tissue proteome, including sample preparation, isobaric tag labeling, peptide level fractionation, LC-MS/MS, database search, and statistical analysis.

Keywords

Human pancreatic tissue; Tissue proteome; Tandem mass tag; Isobaric labeling; Fractionation; LC-MS/MS; MaxQuant; Perseus

1. Introduction

Large-scale proteomic analysis of clinical tissue samples has been widely applied to uncover the molecular level changes of pathological conditions.(1, 2) Various quantitative methods such as label free, metabolic labeling, isotopic tags, and isobaric tags have been developed for protein quantification.(3) Among those methods, isobaric tag labeling provides high precision compared to the label free method. In addition, the multiplexing capability of isobaric labeling enables an increased analysis throughput, for instance, 10 samples can be analyzed simultaneously using tandem mass tag (TMT)-10plex-based peptide level labeling strategy.(1, 4-6) Furthermore, as missing value issues are common in label free proteomics, TMT10-plex strategy has the advantage of much less missing values due to the co-isolation and fragmentation of the isobaric precursor ions, which provides high quality data for

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statistical analysis and in turn better statistical power to identify the differentially expressed proteins from the limited number of clinical samples.

In this guideline, the TMT10-plex based isobaric labeling LC-MS/MS strategy is described for comprehensive identification of human pancreatic tissue proteins. This guideline covers the entire workflow of isobaric labeling-based bottom-up proteomics strategy, which include tissue homogenization and protein digestion, peptide level TMT isobaric labeling, off-line fractionation to reduce the complexity of individual proteome sample, LC-MS/MS data acquisition and database search for protein identification and quantification. Finally, the proteins with differential expressions between different pathological conditions are identified using statistical analysis.

2. Materials

All reagents and chemicals are available from Sigma Aldrich (St. Louis, MO, USA), unless otherwise specified. Protease inhibitor cOmplete® is from Roche Life Sciences (Indianapolis, IN, USA). The BCA protein assay and sequencing-grade trypsin are respectively obtained from ThermoFisher Scientific (Rockford, IL, USA) and Promega (Madison WI, USA). All solvents used are HPLC-grade. Details of reagents and materials used in each step are listed below.

2.1 For protein digestion

1. Lysis buffer: 8 M urea, 75 mM NaCl in 100 mM ammonium bicarbonate pH 7.8 with 10 mM NaF, phosphatase inhibitors cocktail 2 and 3, and protease inhibitor cOmplete® at manufacturer suggested concentrations of 1% and 4% of final volume, respectively.
2. 375 mM dithiothreitol (DTT): weigh 11.6 mg of DTT in a microcentrifuge tube, and add 200 µL DI water (*see Note 1*).
3. 1 M iodoacetamide (IAA): weigh 18.5 mg of IAA in a microcentrifuge tube, and add 100 µL DI water (*see Note 2*).
4. 500 mM CaCl₂: weigh 5.55 g CaCl₂ into a container with 100 mL DI water, store at room temperature.
5. Trypsin stock solution: prepare in 1 µg/µL in 50 mM acetic acid, and store at -20°C before use.

2.2 For TMT-10plex isobaric labeling

The amounts listed below are sufficient for labeling 10 samples/channels.

1. 1 M TEAB: prepare stock solution of TEAB (triethyl ammonium bicarbonate) by weighing 1.63 g in 15 mL plastic centrifuge tube and add DI water to a final volume of 10 mL, store at room temperature.
2. 100 mM TEAB: dilute 1 M TEAB stock solution to 100 mM TEAB before use. 150 µL of 1 M TEAB adds into a microcentrifuge tube with 1350 µL of DI water.

3. TMT labeling reagent: for the 0.8 mg vials, add 41 μL of anhydrous acetonitrile to each tube. For the 5 mg vials, add 256 μL of anhydrous acetonitrile to each tube (*see Note 3*).
4. 5% hydroxylamine: the stock solution of hydroxylamine is 50%, add 10 μL of 50% hydroxylamine into a microcentrifuge tube with 90 μL of 100 mM TEAB, freshly prepared.

2.3 For high-pH RPLC fractionation

1. Xbridge C18 analytical and guard columns are from Waters (Milford, MA, USA) or other C18 columns.
2. Buffer A: 10 mM TEAB in water (dilute from the 1 M TEAB stock solution).
3. Buffer B: 10 mM TEAB in 90% CH_3CN (*see Note 4*).

3. Methods

Seven major procedures (Figure 1) are involved in analysis of pancreatic tissue proteome. As a demonstration, tissue samples from 5 healthy subject and 5 type 1 diabetic patients are included in one TMT experiment for quantitative comparison. In the case of more than 10 samples to be compared, multiple TMT experiments will need to be performed, typically with a pooled sample serve as the common reference.(6)

3.1 Protein digestion

1. Approximately 30 mg of cadaveric pancreatic tissue from each subject is pulverized and homogenized in 500 μL of Lysis buffer.
2. The tissue sample mixture is sonicated for 3 min in an ice water bath.
3. Incubate the sample mixture with 13.7 μL 375 mM DTT (final concentration at 10 mM) for reduction of disulfide bonds at 37°C for 1 h.
4. Add 10.5 μL 1 M IAA (final concentration at 20 mM) and incubate at room temperature for 1 h in the dark.
5. The sample mixture is further diluted 8-fold with 50 mM NH_4HCO_3 to reduce the urea concentration <1 M (*see Note 5*).
6. Trypsin (0.3 mg, 1:100 ratio of enzyme to substrate) and 8.5 μL 500 mM CaCl_2 (final concentration at 1 mM) are added for digestion carried out at 37°C for 4 h.
7. A second step of trypsin digestion (add 0.3 mg, 1:100 ratio of enzyme to substrate) is performed overnight at 37°C.
8. The digestion mixture is acidified with 225 μL 20% TFA (final concentration at 1%) to stop digestion before C18 SPE desalting.

3.2 C18 SPE desalting

The SPE desalting methods may vary depending on SPE materials and manufacturer's instructions. As an example, a 1 mg/1 mL C18 SPE cartridge (SUPELCO) can be used to desalt protein digests up to 5 mg (*see Note 6*).

1. Place glass tubes at the lower section of the manual SUPELCO SPE vacuum station below “needles” for collecting solvent used for conditioning and rinsing the column (*see Note 7*).
2. Add 1 mL methanol (MeOH) to condition the column and control the flow of MeOH solvent as fast as 15-20 drops per minute by adjusting vacuum level. Repeat twice (In total 3 mL MeOH).
3. Rinse the column with 1 mL acidified water (0.1% TFA) and control the flow of 0.1% TFA solvent as fast as 10-15 drops per minute. Repeat once (In total 2 mL 0.1% TFA).
4. After rinsing, the digestion mixture from section 3.1 is loaded on to the column and flow should be controlled at a speed at least 2-fold lower than that used in the rinsing step.
5. Add 4 mL (1 mL for 4 times) of H₂O:CH₃CN (95:5) with 0.1% TFA into the column to wash away the salts. The flow should be at a speed similar to that for loading digestion mixture.
6. Take out the glass tube with solvent waste and put a new 2 mL microcentrifuge tube in the vacuum chamber for collecting desalted samples.
7. Add 1.2 mL of CH₃CN: H₂O (80:20) with 0.1% TFA into column to elute the peptides from column at a speed as slow as 8-10 drops per minute (*see Note 8*).
8. After collecting the sample, turn off vacuum and take out the sample collection tubes, and concentrate the peptide samples in vacuum concentrator before peptide concentration measurement.

3.3 TMT-10plex isobaric labeling

TMT-10plex labeling on peptides is performed according to the manufacturer's instructions, brief instructions are shown below.

1. 50 µg of peptides from each sample dissolved in 100 µL 100 mM TEAB is used for labeling, and 20 µL of the TMT reagent is used for each 50 µg peptide sample by adding the reagent into peptide solution (*see Note 9*).
2. Incubate the reaction at room temperature for 1 h.
3. Add 8 µL of 5% hydroxylamine to the sample and then vortex and incubate at room temperature for 15 min to quench the reaction.
4. Combine all samples (up to 10 samples/channels) in a new microcentrifuge tube and store at -80°C before high-pH RPLC fractionation.

3.4 High pH RPLC fractionation of peptide mixture

Many fractionation methods have been reported for peptide fractionation in order to reduce sample complexity before LC-MS/MS analysis. The high pH RPLC fractionation using C18 column is demonstrated here.

1. Xbridge C18 analytical (5 μm particles, 250 mm $\times$ 4.6 mm) and guard (20 mm $\times$ 4.6 mm) columns are used for peptide separation.
2. Prepare buffer A and B (section 2.3), and degas for those two buffers before use.
3. Aliquot the labeled samples from section 3.3 to create the quality control (QC) samples for optimizing peptide separation conditions (*see Note 10*).
4. QC samples are used to evaluate the separation performance, and further optimize the effective LC separation gradient if needed.
5. A typical starting LC gradient condition at a flow rate of 0.5 mL min⁻¹ can be 0% to 5% buffer B in 10 min, from 5% to 35% B in 60 min, from 35% to 70% B in 15 min, and hold at 70% B for an additional 10 min for washing column before using 0% B for 10 min to re-equilibrate column.
6. Collect 96 fractions of the peptides to cover the entire peptide elution time window (*see Note 11*).
7. Concatenate the 96 fractions into 24 fractions (*see Note 12*).
8. Dry the final 24 fractions in vacuum concentrator and reconstitute in 0.1% formic acid (FA) at a final concentration of 0.2 $\mu\text{g } \mu\text{L}^{-1}$ before LC-MS/MS analysis (*see Note 13*).

3.5 LC-MS/MS analysis

Protocols for LC-MS/MS analysis can vary because of diversity of LC systems (manufacturer, column, solvent composition, gradient, flow rate, etc.) and MS instruments (manufacturer, electrospray condition, fragmentation, MS parameters, analyzer, etc.). The following is the practice routinely used in our laboratory.

1. LC-MS/MS platform consists of an UltiMate 3000 RSLCnano system and a Q Exactive HF mass spectrometer coupled with an EASY-Spray ion source (ThermoFisher Scientific).
2. Peptide separation is performed on a PepMap C18 analytical column (2 μm particle, 50 cm $\times$ 75 μm , ThermoFisher Scientific). Injection volume is 2.5 μL (0.5 μg peptide amount loaded into column) per fraction (*see Note 14*).
3. A binary solvent system consisting of 0.1% FA in water (solvent A) and 0.1% FA in CH₃CN (solvent B) is used at a flow rate of 250 nL min⁻¹ (*see Note 15*).
4. LC separation is performed using the following gradient setting: hold at 4% B for 3 min (for desalting), from 4% to 8% B in 0.1 min, 8% to 40% B in 90 min (effective gradient), 40% to 90% B in 0.1 min, hold at 90% B for 10 min (for

washing column), 90% to 4% B in 0.1 min, and hold at 4% B for 17 min for re-equilibrating column (*see Note 16*).

5. MS data are acquired in profile mode and resolution for full scan (400 to 2000 m/z) is set to 120,000 (at m/z 200) with maximum ion injection time of 50 ms, and automatic gain control (AGC) target of 1e6.
6. MS/MS data are acquired with data-dependent top 15 method. An isolation window of 1.4 m/z is used to isolate precursor ions for fragmentation by higher-energy collisional dissociation (HCD) at normalized collision energy of 32 (*see Note 17*). Resolution for MS/MS spectrum is set to 60,000 (at m/z 200) with maximum ion injection time of 100 ms (*see Note 18*). AGC target for MS/MS scans is 1e5.
7. Precursor ions with single, seven and higher charge states are excluded from fragmentation, and dynamic exclusion time is set to 20 sec.

3.6 Database search for protein identification and quantification

Many database search software packages are available for this purpose. MaxQuant is demonstrated here.(7)

1. The acquired datasets (.raw files) are analyzed using MaxQuant and the built-in Andromeda search engine against a UniProt human database (*see Note 19*).
2. Variable modifications include protein N-terminal acetylation and methionine oxidation.
3. Fixed modifications contain cysteine carbamidomethylation and TMT labeled N-terminus and lysine residue.
4. A maximum of 2 missed cleavages are allowed for the search.
5. Trypsin/P is selected as the semi-specific proteolytic enzyme (*see Note 20*).
6. The false discovery rate (FDR) cut off used for both peptides and proteins is 0.01 (1%) using decoy database.
7. The precursor intensity fraction is set as 0.75 to minimize influence of the co-eluting peptides in quantification.(8)
8. Only the razor/unique peptides are used for quantitative calculations.
9. The other parameters are the default settings in MaxQuant software for processing orbitrap-type data (*see Note 21*).

3.7 Statistical analysis

The search results in ProteinGroups.txt generated by MaxQuant are directly processed by Perseus software.(9) The differentially expressed proteins are identified by statistical analysis tools built in Perseus.

1. Import the quantitative data from ProteinGroups.txt into Perseus.

2. The potential contaminants, reverse hits and proteins only identified by modification site are excluded.
3. A filtering criterion is set to keep the identified proteins with the quantified values of all ten reporter ions (no missing value) in the final identification list.
4. The protein intensities are log₂-transformed and normalized by subtracting the median intensity in each column/sample.
5. Categorize the samples into two groups: healthy subjects and patients.
6. Principal component analysis (PCA) is performed based on protein intensities to differentiate two groups.
7. Two-samples tests coupled with Benjamini-Hochberg (FDR cut off of 0.05) correction are performed to identify the differentially expressed proteins.(10)

4. Notes

1. Stock DTT solution should be freshly prepared before use.
2. Keep the IAA solution in the dark.
3. The unused reagent could be dried and stored at -20°C for future use.
4. When preparing buffer B (10 mM TEAB in 90% CH₃CN), add CH₃CN slowly to 10 mM TEAB because 100% CH₃CN can precipitate TEAB.
5. High concentration of urea affects the trypsin digestion efficiency, trypsin activity is not affected when reducing the urea concentration to <1 M.
6. The columns should not be dried in the entire experimental processes except for the last eluting step.
7. The vacuum pressure used in the chamber should not exceed 20" Hg for the specific SUPELCO SPE cartridge.
8. When the column is almost dry, increase the vacuum pressure to elute the peptide sample completely to increase the recovery.
9. Equilibrate the TMT label reagents to room temperature before use. For the 0.8mg vials, add 41 µL of anhydrous acetonitrile to each tube. For the 5mg vials, add 256 µL of solvent to each tube. Allow the reagent to completely dissolve for 5 minutes with occasional vortexing, and briefly centrifuge the tube to gather the solution. 41 µL of reagent can label 100 µg peptides, and the reagent volume can be further adjusted according to the amount of peptides used for labeling. Make sure the same amount of TMT reagents for labeling each sample.
10. The LC gradient condition may need optimization in order to have the peptides elute relative evenly during the entire LC gradient time. In the case of 500 µg of labeled peptide mixture from section 3.3, 300 µg could be used as QC samples (100 µg per injection) for optimization of LC gradient.
11. The fraction collector is set to 4°C while collecting the peptide fractions.

12. After collection of 96 fractions, 4 out of 96 fractions are pooled into one fraction, referred to as concatenation. For instance, fraction 1st, 25th, 49th, and 73rd are combined into one. Do so for the other 23 pooled fractions.
13. A rough estimation of solvent volume used for reconstitution of peptides in each fraction can be determined by the amount of peptides loaded onto column for fractionation and the number of final fractions. For instance, 100 µg divided by 24 fractions to have 4.2 µg per fraction, and to prepare sample at 0.2 µg µL⁻¹ need to add 20.8 µL solvent for reconstitution.
14. The injection volume depends on the sample loop of autosampler, column loading capacity, and MS detector, therefore injection volume need to be adjusted based on the actual set-up.
15. A flow rate of 250 nL min⁻¹ for C18 50 cm × 75 µm i.d. column results in around 550 to 600 bar column pressure when heating column at 35°C.
16. The gradient used for peptide separation can be modified depending on separation performance. However, all samples must be run under the same condition to limit variations between samples.
17. The normalized collision energy used for TMT-labeled peptides is usually higher than those for label free peptides (without labeling) in order to generate high quality reporter ions in low mass region, the normalized collision energy could be further optimized according to the number of peptides identified and the quantification accuracy.
18. To ensure 6 mDa differences between reporter ions in TMT-10plex, at least a resolution of 35,000 is needed to clearly resolve the reporter ion signals. The resolution of 60,000 is the optimal setting in Q Exactive HF MS.
19. The database information needs to include the type, sequence entry number, and releasing date of database.
20. The selection of enzyme used for search is based on the enzyme that is chosen for protein digestion in section 3.1.
21. For TMT-10plex labeling, different batches of TMT reagents have slightly different isotope impurity which needs to be included for database search to correct the reporter ion ratio. The information of isotope impurity can be found in the reagent kit.

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Fig. 1. Schematic representation of TMT10-plex based isobaric labeling proteomics workflow for tissue proteome study. Samples from 5 healthy subject and 5 patients are included in one TMT labeling experiment for quantitative comparison. Each procedure is described in the Methods section.