Experiment time: 2025-04-02, 17:00 - 2025-04-07, 13:00

Sample Preparation for Proteome Sequencing (I)

I. Objective of the experiment

The cell precipitation samples of T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines were prepared and sent for proteome sequencing. The resulting data will be used for subsequent analysis of differential protein expression and investigation of drug resistance mechanisms.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Number of repetitions: 3 samples per group.

2.2 Measurement principle

Label-free proteomics sequencing achieves protein relative quantification by directly comparing MS1 (first-level) and MS2 (second-level) signal intensities or spectral counts in different samples through mass spectrometry. The core mechanism involves peptide separation via liquid chromatography, followed by detection of peptides based on four dimensions: mass-to-charge ratio (m/z), retention time, ion intensity, and ion mobility (a newly added dimension). Ion mobility enhances selective detection of low-abundance peptides by leveraging differences in ion migration within the electric field, thereby reducing background interference and improving detection sensitivity and quantification accuracy. Quantification is performed by establishing a positive correlation between signal intensity and protein abundance through either integrating the chromatographic peak area (Label-free quantification, LFQ intensity) or by counting the number of fragmentation spectra (Spectral Count) matched to a peptide. This technique eliminates the need for isotope labeling, simplifies experimental procedures, and is particularly suitable for high-throughput analysis of complex samples.

III. Materials and reagents

3.1 Materials

Pipette tips and 15ml centrifuge tube were purchased from Axygen

Serological pipettes was purchased from a domestic company

Micropipettes: Eppendorf

T25 culture flasks were purchased from Corning

10-cm cell culture dishes (Corning)

Liquid nitrogen, dry ice

Human bladder transitional cell carcinoma cell line (T24)

Low, medium, and high drug-resistant cell lines T24-RC48

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

McCoy's 5A medium is from Shanghai Yuanpei Biotechnology Co., Ltd., China

Fetal bovine serum (FBS, Gibco)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

V. Experimental steps

5.1 Low, medium, high drug-resistant and wild-type T24 cell lines were seeded in 10-cm cell culture dishes until the cells grew to more than 80%.

5.2 Discard the culture medium and add 4℃ pre-cooled PBS. Shake gently for 1 minute on a flat surface and discard the PBS. Repeat the above operation three times to fully wash off the culture medium.

5.3 Place the culture dish on ice and add 1ml of 4℃ pre-colded PBS into the culture dish. Quickly scrape the cells with a clean cell scraper onto one side of the culture dish. Place the culture dish at an angle on ice so that the buffer flows to one side.

5.4 Use a pipette to aspirate the cell suspension into a pre-cooled 1.5ml EP tube and centrifuge to remove the supernatant.

5.5 Label the sample number. Rapidly frozen in liquid nitrogen, and then stored at -80°C.

5.6 Dry ice packaging, fill in the handover form of the sequencing company (with sample

information sheet), and send it to the sequencing company.

VI. Photos of experimental operation

Figure 1 Samples prepared for protein sequencing

VII. Experimental results

7.1 Analysis of Significantly Differentially Expressed Proteins

As shown in Table 1, significant protein expression differences were observed among the drug-resistant groups and the control group (wild-type) as well as between the drug-resistant groups themselves: The highly drug-resistant group showed 2,556 differentially expressed proteins compared to the control group (1,229 upregulated and 1,327 downregulated); the moderately drug-resistant group had 1,943 differentially expressed proteins (894 upregulated and 1,049 downregulated); the low-drug-resistant group contained 943 differential proteins (424 upregulated and 519 downregulated). Notably, the number of differentially expressed proteins increased significantly with the progression of drug resistance across all groups.

Table 1 Analysis of significant differences

Comparisons	Up	Down	All
high VS control	**1229**	**1327**	**2556**
low VS control	**424**	**519**	**943**
moderate VS control	**894**	**1049**	**1943**
high VS moderate	**1094**	**1081**	**2175**
high VS low	**1260**	**1266**	**2526**
moderate VS low	**723**	**820**	**1543**

7.2 Quantitative repeatability assessment (RSD analysis)

The relative standard deviation (RSD) of protein quantification values across samples showed that the median RSD within groups was lower than 15%, but there were still outliers.

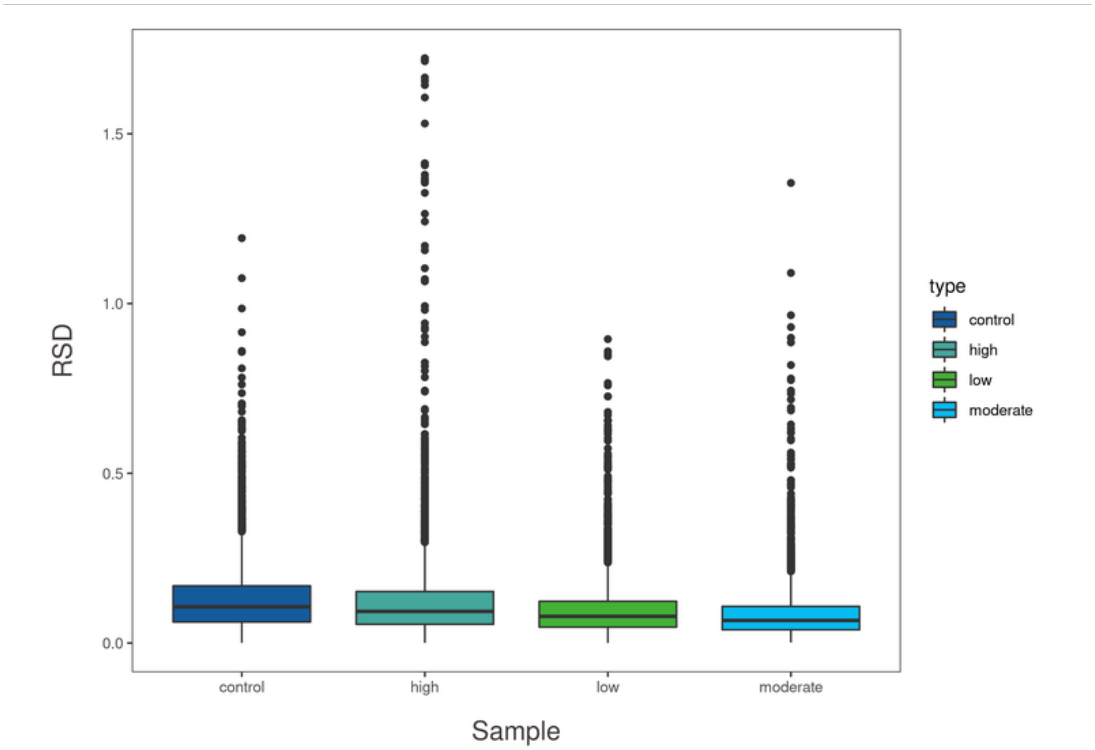

Figure 2 Box plot of relative standard deviation (RSD) of protein quantification values

7.3 Sample clustering tree and correlation analysis

The wild-type group and each drug-resistant group formed independent branches, and the samples in the groups were closely clustered.

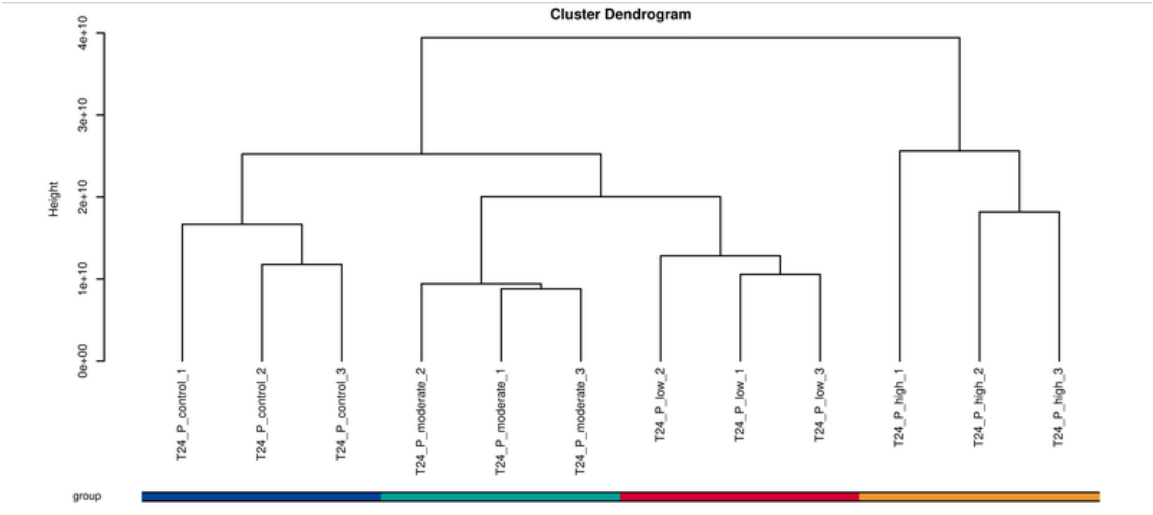

Figure 3 Results of cluster tree analysis

The Pearson correlation heatmap revealed that intra-group correlation coefficients were greater than 0.94, and the inter-group correlation coefficient decreased with the increase of drug resistance difference. The correlation coefficient between most samples in the high drug resistance group and the control group was <0.9.

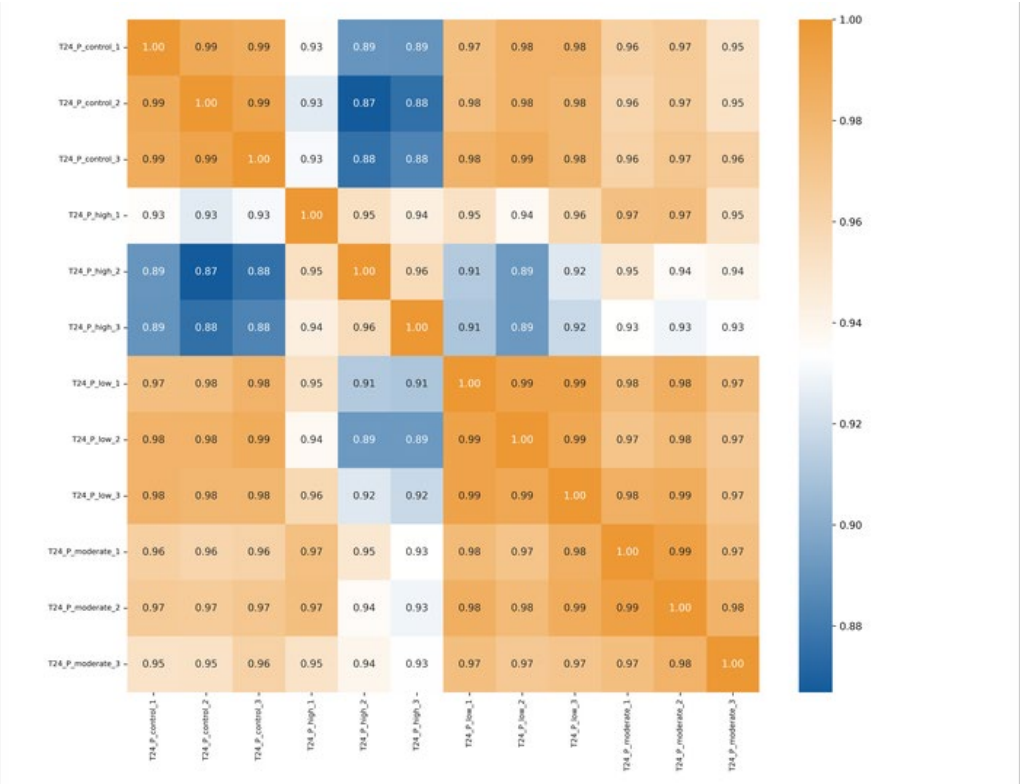

Figure 4 Correlation analysis

7.4 Principal component analysis (PCA)

In the PCA diagram, samples in the same group are closely distributed on PC1/PC2 axes with good repeatability. The wild-type group and the drug-resistant group are completely separated along PC1 axis, and the drug-resistant groups show a gradient distribution (low → medium → high drug resistance).

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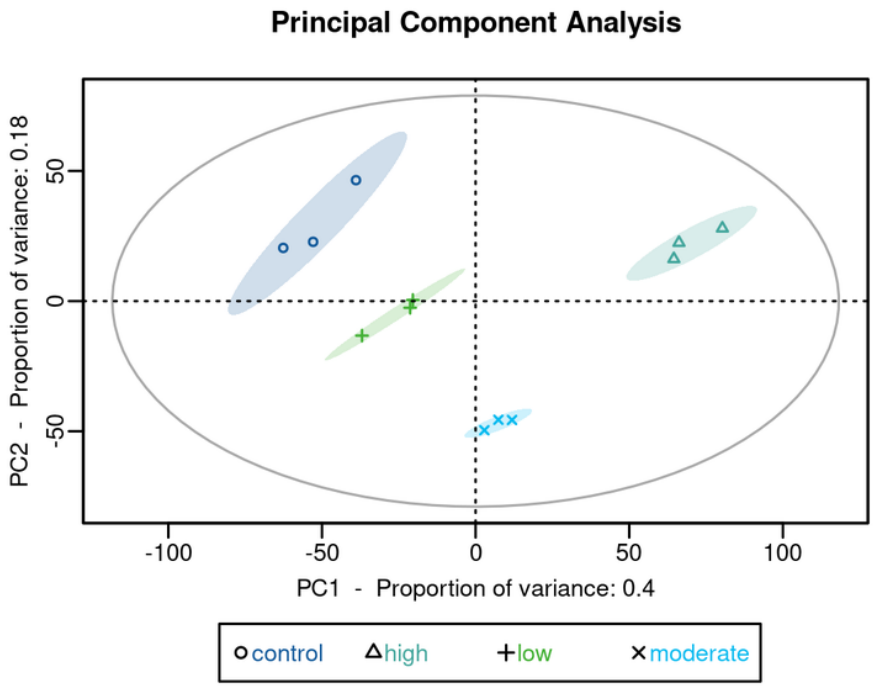

Figure 5 PCA analysis results

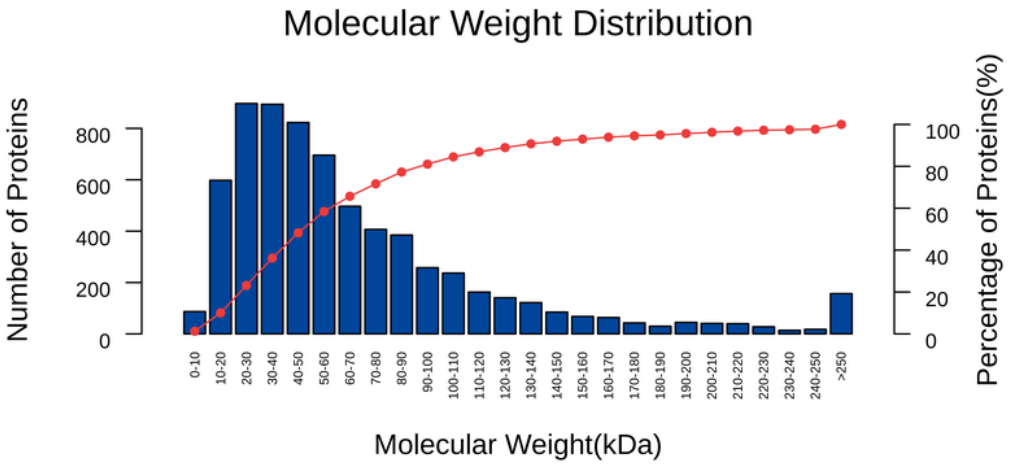

Figure 6 Distribution of relative molecular mass of proteins

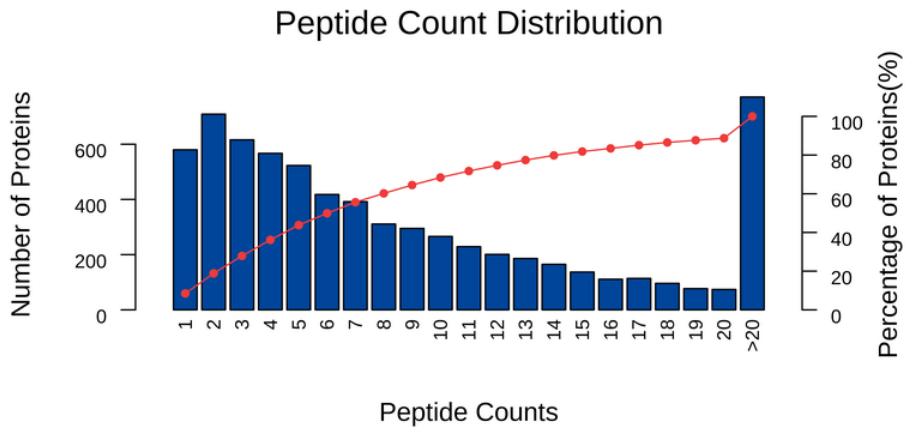

Figure 7 Distribution of identified peptide segments

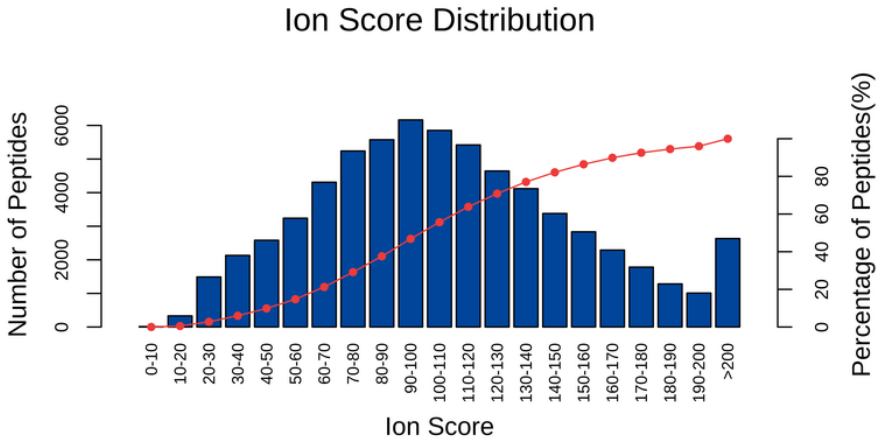

Figure 8 Distribution of peptide ion scores

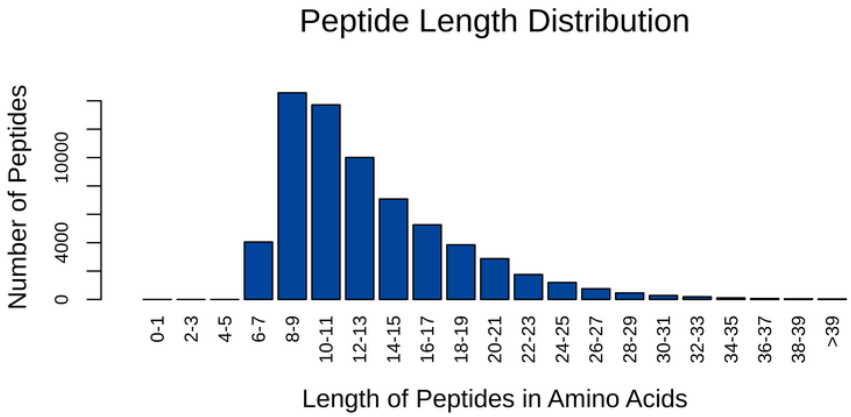

Figure 9 Peptide sequence length distribution

VIII. Results analysis

A positive correlation was observed between the level of drug resistance and the number of differentially expressed proteins. The most significant difference was observed in the highly resistant group compared to the control group (2,556 proteins), suggesting that the resistance mechanism may be related to dynamic regulation of multiple proteins. This highly resistant state likely involves the coordinated action of multiple proteins through mechanisms such as metabolic reprogramming, enhanced drug efflux, or inhibition of apoptosis. Differential proteins between resistance groups (e.g., 2,175 proteins in the highly resistant vs. moderately resistant group) may contain key biomarkers for maintaining or upgrading drug resistance.

In RSD analysis, a smaller relative standard deviation (RSD) between samples indicates better reproducibility in proteomic quantification. In this study, low intra-group RSD values validated the standardization of experimental procedures and consistency in sample preparation, thereby supporting the statistical validity of subsequent differential analysis. Some abnormally high RSD proteins may require biological replicates for further validation to exclude technical errors affecting specific protein quantification.

The primary objective of cluster analysis is to group and classify data based on similarity. By calculating pairwise similarities between samples, a dendrogram that visualizes these relationships is generated. In the clustering tree structure, original data points from different clusters form the base layer, while the topmost level represents the root node of each cluster. This experiment employs a bottom-up approach: first calculating pairwise similarity between samples, then merging the two most similar samples into a single Cluster through iterative rounds. After merging adjacent samples into a Cluster, the distance matrix between remaining samples and this merged Cluster is recalculated until complete clustering is achieved. This process establishes a hierarchical tree structure. The dendrogram provides intuitive insights into both inter-group and intra-group sample similarities, while also revealing any outliers or anomalous cases. Both the dendrogram and heat map in this study demonstrate global protein expression pattern changes induced by drug resistance, with resistance levels showing positive correlations to proteomic characteristics. The high intra-group correlation indicates reliable biological reproducibility, effectively eliminating batch effects from interfering with the results.

Principal Component Analysis (PCA) reveals both intra-group and inter-group differences in samples. In the PCA plot, higher clustering intensity within a group indicates more similar omics

characteristics among samples, suggesting better parallelism or repeatability within the group. Conversely, improved separation between groups demonstrates more pronounced omics characteristic variations. The PCA separation trend aligns with drug-resistant phenotypes, indicating that proteomic data can effectively distinguish different drug resistance stages.

In conclusion, the label-free ion mobility-based approach demonstrates exceptional repeatability and sensitivity in complex sample analysis, effectively enhancing data reliability and analytical capabilities. Meanwhile, high-throughput sequencing data, with its comprehensive coverage and precision, reveals molecular mechanisms underlying complex biological processes and drug resistance phenotypes, establishing a robust foundation for in-depth elucidation of drug resistance mechanisms.