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Test Report

DIA Quantitative Proteomics Analysis

Project Name: DIA Quantitative Proteomics Test Report

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目录

1. Overview 1

2. Reagents, Consumables and Instruments 1

2.1 Suppliers List 1

2.2 Work Solution Preparation 3

2.3 Instruments 3

3. Sample Information 4

4. Experimental Methods 5

4.1 Sample Preparation 5

4.2 nanoLC-MS/MS Analysis 7

5. Protein Identification 8

5.1 Database Search (Spectronaut) 8

6. Analysis Results 10

6.1 Quality Control 10

6.2 Results Summary 13

7. Appendix 16

1. Overview

DIA proteomics technology: the theoretical sequence database is selected directly, and each experimental sample is analyzed in DIA (Data-Independent Acquisition) mode. The acquired data are processed with professional database-search software to achieve identification and quantification of the proteome, yielding information on peptides, proteins, and changes in protein expression levels.

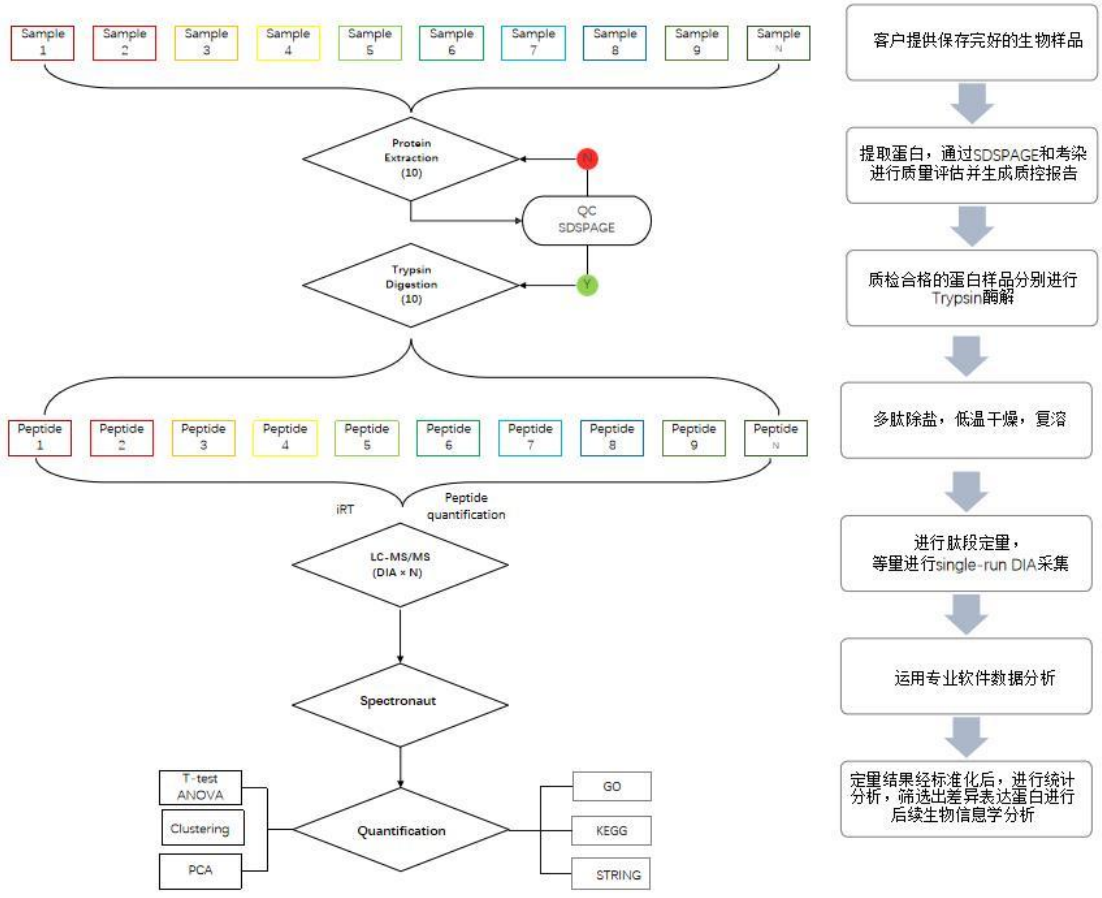


Figure 1 DIA experimental workflow

2. Reagents, Consumables and Instruments

2.1 Suppliers List

All reagents and consumables used in this study were obtained from the following

suppliers.

2.1.1 ANPEL Laboratory Technologies (Shanghai) Inc

- ACN (Acetonitrile, LC-MS)
- H_2O (LC-MS)

2.1.2 Thermo Scientific (Rockford, USA)

- FA (formic acid, LC-MS)

2.1.3 Beyotime (Shanghai, China)

- Enhanced BCA Protein Assay Kit
- RIPA

2.1.4 Biognosys (ETH Zurich, CH)

- iRT Kit

2.1.5 Omicsolution (Shanghai, China)

Table 1 Omicsolution reagents

Reagent	Description	Storage
Reagent 0	Lysis buffer, for protein lysis	Room Temp
Reagent A	Reduction and alkylation reagent, for protein reduction and alkylation	−20°C
Reagent B	Enzyme, for proteolytic digestion	−20°C
Reagent C	Stop reagent, to terminate the digestion reaction	Room Temp
Reagent D	Peptide cleanup reagent 1, for peptide washing	Room Temp

Reagent	Description	Storage
Reagent E	Peptide cleanup reagent 2, for peptide washing	Room Temp
Reagent F	Peptide elution reagent	Room Temp
Reagent G	SP3 magnetic bead cleaning solution	4°C
Reagent H	SP3 magnetic bead suspension	4°C
Reagent I	Protein bead binding buffer / desalting plate activation reagent	Room Temp
Reagent J	Digestion reaction system reagent	Room Temp
Cartridge	Peptide desalting	Room Temp

2.2 Work Solution Preparation

Working solutions were prepared as described below before the assay.

2.2.1 BCA Work Solution

- BCA protein standards of known concentration were diluted with H_2O to 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL.
- Samples were diluted by an appropriate factor according to the sampling volume.
- A : B = 50 : 1. The total volume of BCA working solution was calculated at 200 μL per well (diluted samples + blank + standards = $2 \times N_{sample} + N_{cal} + N_{blank}$).

2.3 Instruments

Instrument	Model	Manufacturer
High-performance liquid chromatograph	Vanquish neo	Thermo Scientific, USA

Instrument	Model	Manufacturer
Mass spectrometer	Astral	Thermo Scientific, USA
LC column	EASY-Spray™ HPLC column (150 µm × 15 cm)	Thermo Scientific, USA
High-speed centrifuge	Heraeus Fresco17	Thermo Scientific, USA
Thermomixer	JXH-100	Shanghai Jingxin Technology Co., Ltd., China
Vortex mixer	VORTEX-5	Qilin Instruments (Haimen) Co., Ltd., China
Microplate reader	Multiskan FC	Thermo Scientific, USA

3. Sample Information

Sample information for this test is shown in the table below. Detailed sample information is provided in the service order. Samples were stored in a −80°C freezer.

Table 2 Sample information

Sample Names	Group Names	Species
A86	A	Homo sapiens
A92	A	Homo sapiens
B96	B	Homo sapiens
B97	B	Homo sapiens
A114	A	Homo sapiens
A115	A	Homo sapiens
A116	A	Homo sapiens
A117	A	Homo sapiens
A109	A	Homo sapiens
A90	A	Homo sapiens

Sample Names	Group Names	Species
A104	A	Homo sapiens
A105	A	Homo sapiens
A102	A	Homo sapiens
A87	A	Homo sapiens
A89	A	Homo sapiens
A93	A	Homo sapiens
A73	A	Homo sapiens
A62	A	Homo sapiens
A60	A	Homo sapiens
A58	A	Homo sapiens
A52	A	Homo sapiens
A55	A	Homo sapiens
B113	B	Homo sapiens
B112	B	Homo sapiens
B111	B	Homo sapiens
B110	B	Homo sapiens
B91	B	Homo sapiens
B74	B	Homo sapiens
B103	B	Homo sapiens

4. Experimental Methods

4.1 Sample Preparation

The sample preparation procedure comprised protein extraction, BCA quantification, and

reduction, alkylation and enzymatic digestion.

4.1.1 Protein Extraction

- 200 μL of sample was taken and 50 μL of 5× RIPA lysis buffer was added.
- Cells were disrupted by ultrasonication in an ice-water bath for 20 min.
- Centrifugation at 12,000 rpm, 4°C for 10 min; the supernatant was transferred to a new EP tube.

4.1.2 BCA Assay

- BCA working solution was dispensed into a 96-well plate, 200 μL per well, with 7 standard points and 1 blank.
- 20 μL of sample (diluted accordingly) or standard protein (BSA) was added.
- Incubated with shaking at 37°C for 30 min; absorbance was measured at 562 nm.
- The standard curve was fitted from the standard protein and used to calculate the protein concentrations of the samples.

4.1.3 Reduction, Alkylation and Protein Digestion

- 30 μg of protein was precipitated with 5 volumes of acetone, centrifuged at 12,000 rpm, 4°C, the supernatant discarded, and the pellet resuspended in 60 μL of RIPA.
- 30 μg (60 μL) of protein was aliquoted into the sample plate.
- 20 μL of Reagent H was dispensed into the reaction plate.
- 100 μL of water and 100 μL of Reagent G were added to the intermediate plate, mixed, and transferred into the reaction plate.
- Incubated at 800 rpm for 1 min, magnetically attracted for 1 min, and the supernatant removed.
- 20 μL of Reagent A and 40 μL of sample were added to the reaction plate and vortexed for 1 min to mix.
- Incubated at 1,200 rpm, 95°C for 5 min.

- After cooling, 180 μL of Reagent I was added and the plate incubated at 1,200 rpm, 37°C for 10 min. The supernatant was removed.
- 200 μL of Reagent G was added and incubated at 1,200 rpm, 37°C for 1 min; the supernatant was removed. This step was repeated once.
- 20 μL of Reagent B was dispensed into the intermediate plate, followed by 100 μL of Reagent J, and mixed.
- The 120 μL of mixed reagent from the intermediate plate was added into the reaction plate.
- Incubated at 1,200 rpm, 37°C for 2.5 hours.
- 100 μL of Reagent C was added to the reaction plate and incubated at 1,200 rpm, 37°C for 1 min.
- 200 μL of Reagent I was added to the desalting plate and positive pressure was applied so that all reagent passed through the desalting plate.
- 200 μL of Reagent E was added to the desalting plate and positive pressure was applied so that all reagent passed through.
- The entire sample was loaded onto the desalting plate and positive pressure was applied so that all sample passed through.
- 100 μL of Reagent D was added to the desalting plate and positive pressure was applied so that all reagent passed through.
- 100 μL of Reagent F was added to the desalting plate and the eluate was collected.
- Vacuum drying was performed, pending reconstitution for LC-MS analysis.

4.2 nanoLC-MS/MS Analysis

- About 500 ng of total peptides from each sample were separated on a nano-UPLC system (Vanquish neo) and analyzed on an Astral mass spectrometer equipped with a nanoelectrospray ion source. Chromatographic separation was performed on a reversed-phase column (EASY-Spray™ HPLC, 150 μm ID × 15 cm, Thermo Scientific, USA). The mobile phases were an 80% acetonitrile–water–formic acid system, with mobile phase A being 0.1% formic acid in water and phase B being

0.1% formic acid in 80% acetonitrile. The column was equilibrated with 96% phase A; samples were injected directly onto the column by the autosampler and then separated by gradient elution.

- MS analysis was performed in data-independent acquisition (DIA) mode with positive ion detection. The MS1 full-scan range was 380–980 m/z at a resolution of 240k. The isolation window was 2 m/z and the normalized collision energy (NCE) was 25%. The MS2 full-scan range was 150–2,000 m/z, Absolute AGC Value: 5E4, Maximum Injection Time: 3 ms.

English Description 1: nanoLC-MS/MS analysis

For each sample, 500 ng of total peptides were separated and analyzed with a nano-UPLC (Vanquish neo) coupled to an Astral instrument (Thermo Scientific) with a nanoelectrospray ion source. Separation was performed using a reversed-phase column (EASY-Spray™ HPLC, 150 μm × 15 cm, Thermo Scientific, USA). Mobile phases were H_2O with 0.1% FA (phase A) and 80% ACN with 0.1% FA (phase B).

- Data-independent acquisition (DIA) was performed in profile and positive mode with Orbitrap analyzer at a resolution of 240K and m/z range of 380–980 for MS1; for MS2, m/z range of 150–2,000, Absolute AGC Value: 5E4, Maximum Injection Time: 3 ms. HCD fragmentation was applied with a normalized collision energy (NCE) of 25% and an isolation window of 2 m/z.

5. Protein Identification

5.1 Database Search (Spectronaut)

The raw MS files were imported into Spectronaut (version 19.0.240604.62635; Biognosys AG) for database searching, followed by qualitative analysis. The main search parameters are listed below.

Table 3 Identification parameter settings

Term	Parameter

Term	Parameter
Spectronaut version	19.0.240604.62635
Database (protein sequence database)	uniprotkb_iRT_Homo_sapiens_9606_reviewed_2025_03_05.fasta
Enzyme	Trypsin
Fixed modification	Carbamidomethyl / +57.021 Da (C)
Variable modification	Acetyl / +42.011 Da (N-Terminus); Oxidation / +15.995 Da (M)
Max missed cleavages	2
Peptide tolerance (MS1 accuracy)	20 ppm
MS/MS tolerance (MS2 accuracy)	20 ppm
Peptide and PSM FDR (confidence criterion)	≤ 0.01

English Description 2: Spectronaut database search

Vendor's raw MS files were processed using Spectronaut software (19.0.240604.62635) and the built-in Pulsar search engine. MS spectra lists were searched against their species-level UniProt FASTA databases (uniprotkb_iRT_Homo_sapiens_9606_reviewed_2025_03_05.fasta), with Carbamidomethyl [C] as a fixed modification, and Oxidation (M) and Acetyl (Protein N-term) as variable modifications.

Trypsin was used as the protease. A maximum of 2 missed cleavage(s) was allowed. The false discovery rate (FDR) was set to 0.01 at both the PSM and peptide levels. Peptide identification was performed with an initial precursor mass deviation of up to 20 ppm and a fragment mass deviation of 20 ppm. All other parameters were kept at their default values.

6. Analysis Results

6.1 Quality Control

Quality control covered iRT retention-time alignment, TIC overview, sample integrity and UPLC validity.

6.1.1 iRT / Retention Time

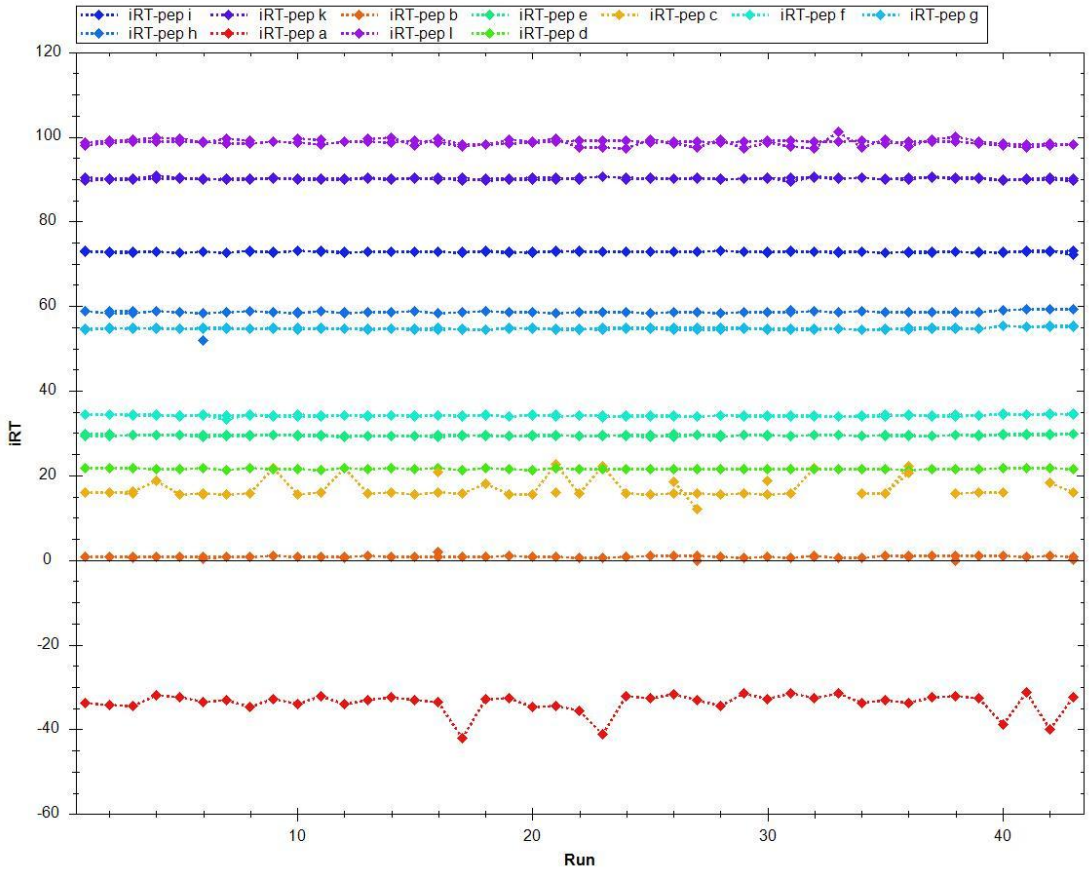


Figure 2 iRT indexed retention time / actual retention time

Indexed Retention Time (iRT) standard peptides were added for between-sample correction and chromatographic quality control. The x-axis shows the different samples, and the differently colored dots on the y-axis represent the different standard peptides.

6.1.2 TIC (Total Ion Chromatogram)

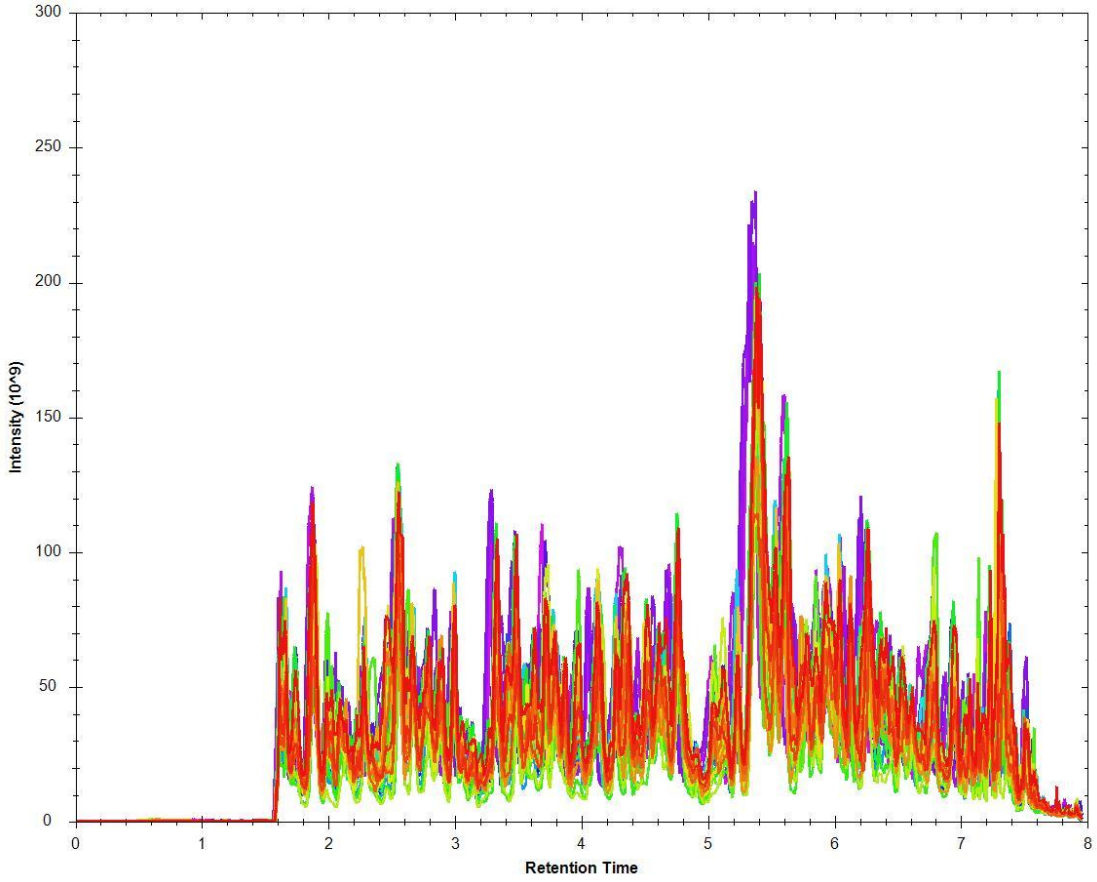


Figure 3 Total ion chromatogram (TIC) of all samples

The total ion chromatograms of all samples are shown. (Components eluted from the chromatographic separation continuously enter the mass spectrometer, which performs continuous scanning for data acquisition; each scan yields one mass spectrum, and summing the intensities of all ions in each spectrum gives a total ion current intensity.) The x-axis is the elution time and the y-axis is the peak intensity; different colors represent different samples. The chromatographic peaks are distributed as evenly as possible.

6.1.3 Sample Integrity

Biological samples must be stored at low temperature and avoid repeated freeze–thaw cycles to keep the sample state close to that at sampling, thereby ensuring the reliability

of the test results. Improper storage may cause non-specific degradation or modification of proteins, affecting data quality and even leading to erroneous conclusions.

Protein integrity is generally assessed by SDS-PAGE combined with staining; well-preserved samples should show clear, distinct bands and a uniform molecular-weight distribution.

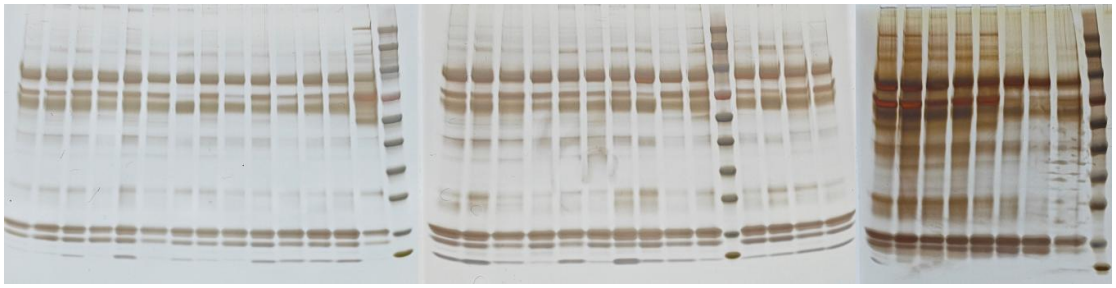


Figure 4 SDS-PAGE assessment of sample integrity

6.1.4 UPLC Validity

In LC-MS-based quantitative proteomics, each fraction is analyzed independently on the instrument, placing high demands on chromatographic repeatability and stability. A reasonable chromatographic condition should generally have the following characteristics:

- Retention times are relatively stable, reflected by good overlap of the TIC/BPC traces.
- Peptide elution times are evenly distributed, reflected by essentially consistent overall signal intensity of the TIC/BPC in the early, middle and late segments of the gradient.
- Chromatographic resolution is good, reflected by narrow, sharp and symmetrical peaks without obvious tailing; the BPC generally reflects this indicator.
- The ion source spray is stable, reflected by a smooth TIC curve without significant fluctuation.

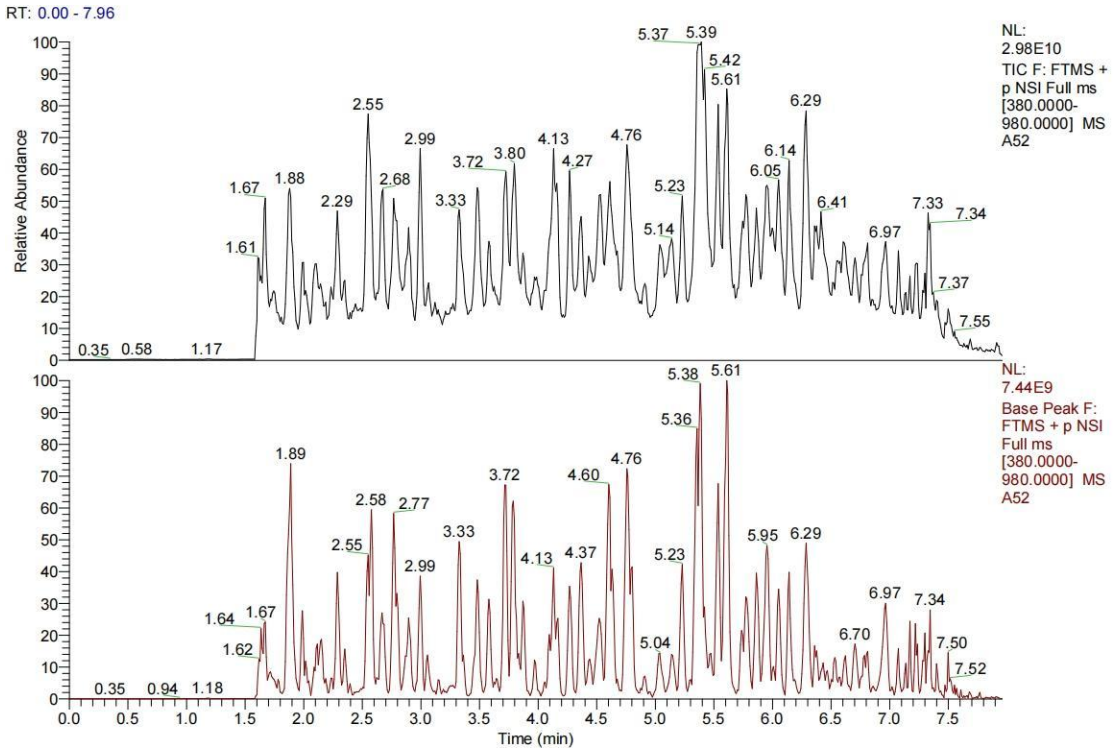


Figure 5 TIC and BPC chromatograms

6.2 Results Summary

Total protein concentrations of all samples were determined by the BCA assay.

6.2.1 Protein Concentration

Total protein concentration was determined by the BCA (bicinchoninic acid) method. Under alkaline conditions, protein peptide bonds react with copper ions in the biuret reaction; Cu^{2+} is then reduced to Cu^{+}, and Cu^{+} forms a complex with two molecules of BCA that has maximum absorbance at 562 nm.

The absorbance is linearly related to the protein concentration within a certain range. A "concentration–absorbance" equation is fitted from the absorbance values of several BSA standard solutions of known concentration, and the protein concentration of each unknown sample is calculated from its absorbance.

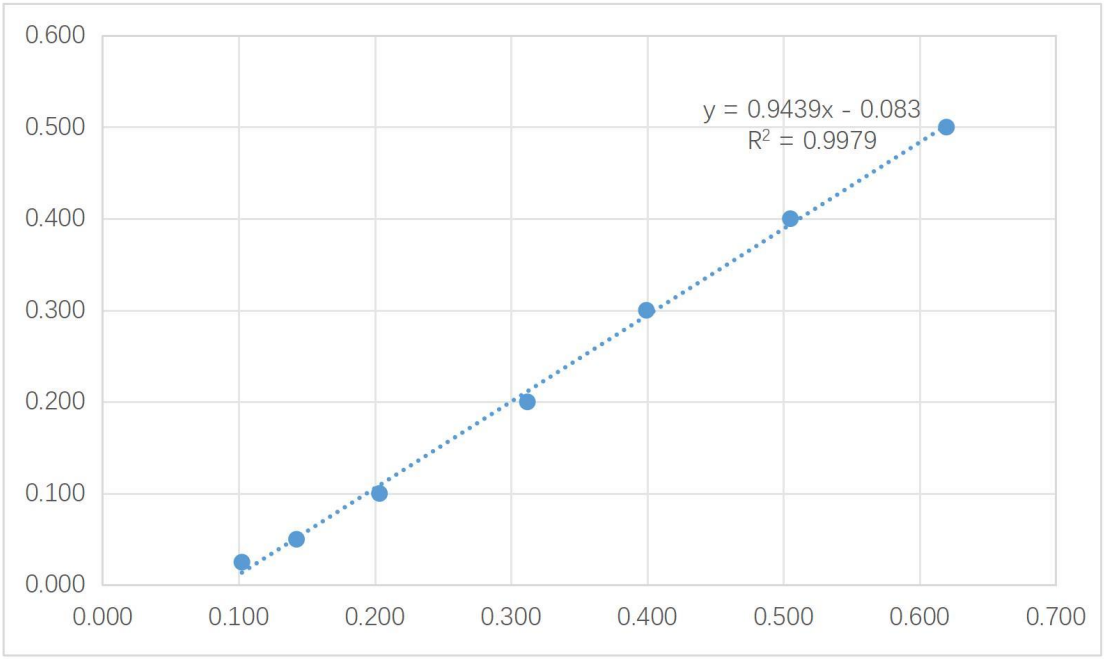


Figure 6 Protein concentration assay (BCA)

Table 4 Protein concentration assay (BCA)

sampleName	sampleType	Dilution (v/v)	OD (562 nm)	Concentration (mg/mL)
cal-01	cal	1.00	0.102	0.025
cal-02	cal	1.00	0.143	0.050
cal-03	cal	1.00	0.203	0.100
cal-04	cal	1.00	0.312	0.200
cal-05	cal	1.00	0.399	0.300
cal-06	cal	1.00	0.505	0.400
cal-07	cal	1.00	0.620	0.500
A86	spl	4.00	0.241	0.1213
A92	spl	4.00	0.264	0.1433
B96	spl	4.00	0.326	0.2013
B97	spl	4.00	0.371	0.2434

sampleName	sampleType	Dilution (v/v)	OD (562 nm)	Concentration (mg/mL)
A114	spl	4.00	0.187	0.093
A115	spl	4.00	0.187	0.093
A116	spl	4.00	0.199	0.105
A117	spl	4.00	0.182	0.089
A109	spl	4.00	0.313	0.212
A90	spl	4.00	0.150	0.058
A104	spl	4.00	0.214	0.118
A105	spl	4.00	0.209	0.114
A102	spl	4.00	0.177	0.083
A87	spl	4.00	0.197	0.102
A89	spl	4.00	0.165	0.072
A93	spl	4.00	0.220	0.124
A73	spl	4.00	0.225	0.129
A62	spl	4.00	0.201	0.106
A60	spl	4.00	0.203	0.108
A58	spl	4.00	0.257	0.159
A52	spl	4.00	0.153	0.061
A55	spl	4.00	0.198	0.103
B113	spl	4.00	0.180	0.086
B112	spl	4.00	0.222	0.127
B111	spl	4.00	0.298	0.199
B110	spl	4.00	0.326	0.224
B91	spl	4.00	0.174	0.081

sampleName	sampleType	Dilution (v/v)	OD (562 nm)	Concentration (mg/mL)
B74	spl	4.00	0.213	0.118
B103	spl	4.00	0.238	0.142

Detailed sample concentrations are provided in the QC feedback.

7. Appendix

- QC files: TIC and BPC plots
- Test results:
  - Peptide-Report-long.tsv
  - Peptide-Report-short.xlsx
  - Protein-Report-long.tsv
  - Protein-Report-short.xlsx

English translation of the original Chinese test report (Project No. BQ-WB20250512-DIA-RLY2), prepared for international colleagues. All data are consistent with the original report.