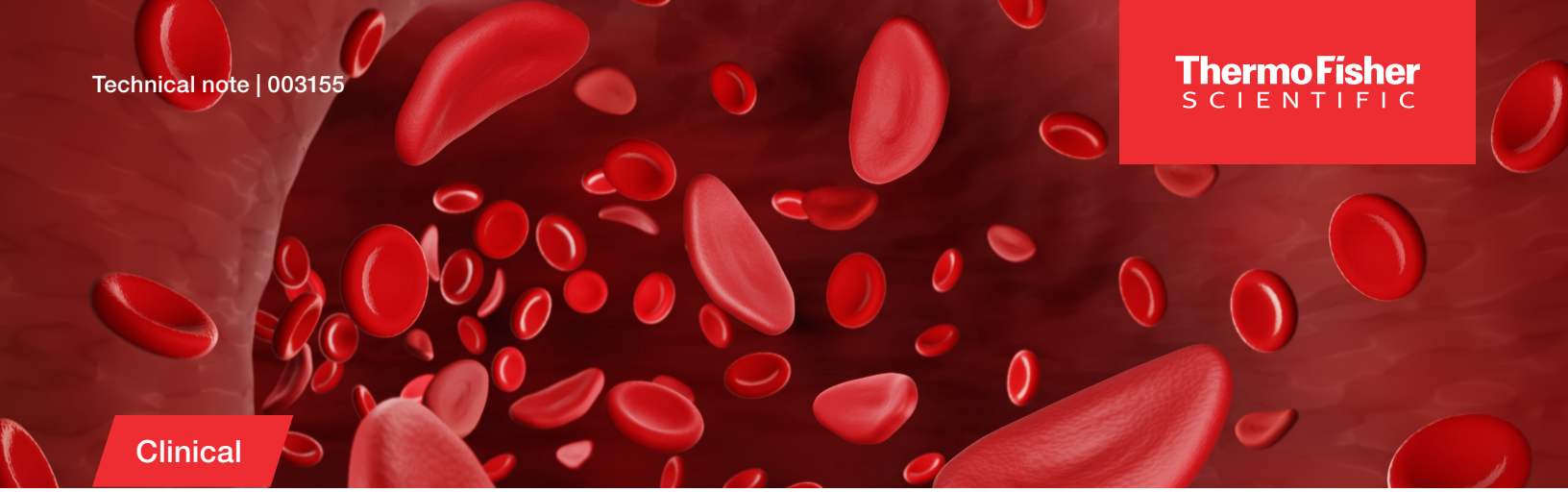

Technical note | 003155

Clinical

Enhancing detection of hemoglobin variants in clinical research using dried blood spot and high-resolution accurate mass (HRAM) Orbitrap mass spectrometry

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Keywords

Hemoglobin variants, hemoglobinopathies, sickle cell diseases, top-down protein characterization, dried blood spots, HRAM, Orbitrap Exploris 240 mass spectrometer, ProSightPD software, TraceFinder software

Application benefits

- Direct protein extraction from dried blood spots for protein identification and quantitation
- Identification: utilizing a top-down analysis approach to obtain Hb variant sequences for the characterization of structural mutation
- Quantitation: utilizing intact protein precursor isotopic *m/z* to determine ratios between target chains and variants
- Minimal sample preparation (<1 hr) in 96 well plate format, easily transferrable to automation using a robotic system
- Confident data processing and review using automated software tools, Thermo Scientific™ ProSightPD™ software, and Thermo Scientific™ TraceFinder™ software

Goal

Develop a top-down approach for hemoglobin variant detection using dried blood spots and a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer

Introduction

Hemoglobinopathies and thalassemia are the most common genetically determined disorders. They are caused by pathogenic variants in genes that control the production of hemoglobin subunits. Hemoglobin (Hb, approximately 62 kDa) is a tetramer, consisting of two alpha chains and two beta or beta-like chains. It is responsible for physiological oxygen transport in all vertebrates except for the *Channichthyidae*. Adult Hb consists of 96–98% Hb A (α2β2), 2.0–3.3% Hb A2 (α2δ2), and less than 1.0% Hb F (α2γ2).[1]

Hemoglobinopathy is a genetic defect characterized by the abnormal structure of one of the Hb subunits, usually resulting from single amino acid substitution. Thalassemia is characterized by the absence or decreased expression of one of the Hb subunits. The most common hemoglobinopathy is sickle cell disease (SCD, Hb S (β^6 Glu→Val)). Around 5.2% of the global population carries significant hemoglobinopathy-causing genes with more than 330,000 affected births occurring annually.[2]

Current techniques used in Hb analysis are electrophoretic and chromatographic assays. However, due to the lack of resolution, they are limited in differentiating isomers (i.e., between Hb C (β^6 Glu→Lys) and Hb E (β^{26} Glu→Lys)), very similar masses (i.e., <1 Da difference between normal Hb, Hb C, Hb E, and Hb D-Punjab (β^{121} Glu→Gln)), or new variants. Mass spectrometry has gained popularity in clinical research because of its robustness and versatility in analyzing a wide range of samples and analytes from small molecules to proteins. Targeted protein and peptide quantitation joined this trend by applying various proteomics approaches such as bottom-up, middle-down, and top-down methods. Recently, the top-down approach targeting intact protein biomarkers was enabled by high-resolution accurate-mass (HRAM) mass spectrometers such as Thermo Scientific™ Orbitrap™-based instruments. Here, we present the top-down analysis for enhanced detection of various hemoglobin variants using the Orbitrap Exploris 240 mass spectrometer for clinical research.

Experimental

Sample preparation

Normal hemoglobin (HB A, Sigma-Aldrich, P/N H7379) and hemoglobin S (Hb S, Sigma-Aldrich, P/N H0392) were reconstituted in water and directly injected into the mass spectrometer for intact mass confirmation before calibration curve generation. Different concentrations of Hb S calibrator stock were spiked into K2-EDTA stabilized normal human whole blood (BioIVT, Westbury, NY), which generated final concentrations of 0, 0.5, 1.0, 2.5, 5.0, 10, 20, 50, and 100 mg/mL. To demonstrate the workflow feasibility, normal beta chain was used as the internal standard to quantify Hb S beta chain. The workflow scheme is shown in Figure 1. A 10 µL aliquot of each calibrator was spotted onto Ahlstrom AutoCollect™ cards (Ahlstrom 226 grade paper, N = 3). The spotted cards were dried at room temperature for at least 3 hours. A 3.2 mm disc was punched and placed into a 96-well plate including a blank card paper punch as an assay blank.

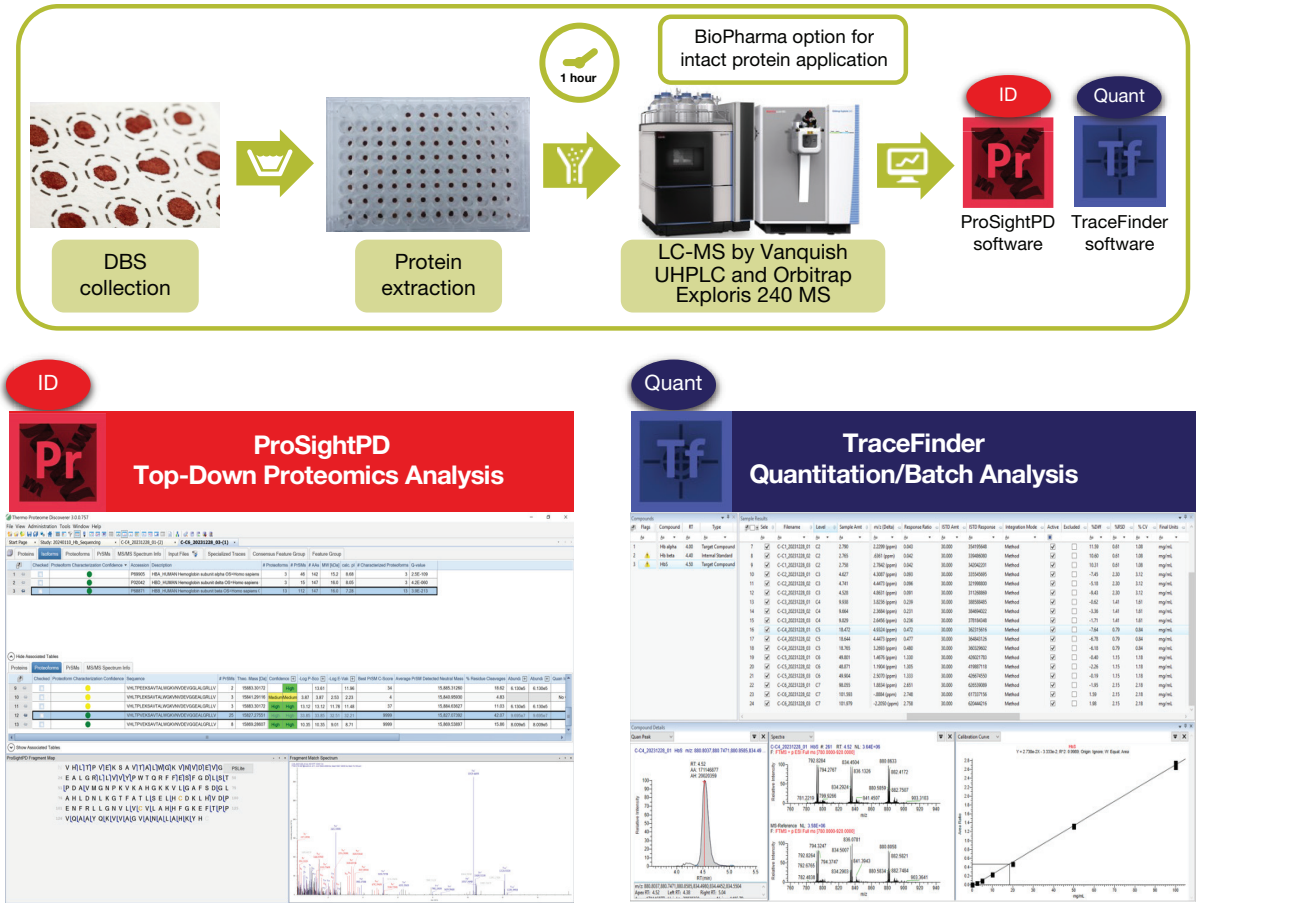


Figure 1. Experimental workflow for identification (using ProSightPD software) and quantitation (using TraceFinder software) in a single LC-MS analysis

Protein precipitation

An aliquot of 50 µL of water was added to each well to rehydrate the disc punch. The plate was sealed with a PE/PP protective sheet and agitated for 15 minutes at 1,000 rpm to facilitate extraction of hemoglobin. An aliquot of 150 µL of acetonitrile was added, and the plate was stored at -20 °C for 15 min to allow protein precipitation. After centrifugation for 5 min at 3,000 rpm, 160 µL of the supernatant was removed, followed by addition of 80 µL of water to the well to dissolve the precipitated proteins. The plate was mixed one more time and centrifuged briefly. An aliquot of 10 µL of the supernatant of each calibrator was transferred to a new plate and diluted with mobile phase A (0.1% formic acid + 0.02% trifluoroacetic acid in water) (1:8) for LC-MS analysis.

Liquid chromatography

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ Flex UHPLC system with a Thermo Scientific™ MAbPac™ RP column (1 x 100 mm, 4 µm, P/N 303183). The chromatographic gradient and other separation conditions are described in Table 1.

Table 1. LC conditions

LC gradient			
Time (min)	**% A**	**% B**	**Curve**
0.0	75	25	5
0.5	75	25	5
0.7	68	32	5
8.5	57	43	5
9.2	10	90	5
9.7	10	90	5
9.8	75	25	5
12	75	25	5
Separation conditions			
Mobile phase A	0.1% formic acid + 0.02% trifluoroacetic acid in water		
Mobile phase B	0.1% formic acid + 0.02% trifluoroacetic acid in 10: 20: 70 water: isopropanol: acetonitrile (v/v/v)		
Flow rate	0.12 mL/min		
Column temperature	80 °C		
Injection volume	2 µL		

Mass spectrometry

MS analysis was performed on an Orbitrap Exploris 240 mass spectrometer with BioPharma option installed. MS parameters are described in Table 2.

Table 2. MS conditions

MS global parameters	
Application mode	Intact protein
Pressure mode	Low pressure
Expected peak width (s)	35
Source type	Heated electrospray ionization (H-ESI)
Polarity	Positive
Spray voltage (V)	3,800
Sheath gas (Arb)	35
Aux gas (Arb)	7
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	325
Vaporizer temp. (°C)	150
Divert valve A	0.0 min: position 1-6 (waste) 0.5 min: position 1-2 (MS) 9.5 min: position 1-6 (waste)
Source position (x-y-z)	(Center-1.5-L/M)
MS scan parameters	
Orbitrap resolution	120,000
Scan range (*m/z*)	500–3,000 for intact mass confirmation 780–920 for targeted identification and quantitation
RF lens (%)	60
AGC target	Standard
Maximum injection time mode	Custom
Maximum injection time (ms)	250
Microscans	3
Intensity threshold	1.00E+04
Charge state properties	18
ddMS2 settings	
Isolation window (*m/z*)	2.5
HCD collision energies (V)	20, 22, 26, 28, 30
Orbitrap resolution	90,000
Scan range (*m/z*)	200–2,000
AGC target	Standard
Maximum injection time mode	Custom
Maximum injection time (ms)	250

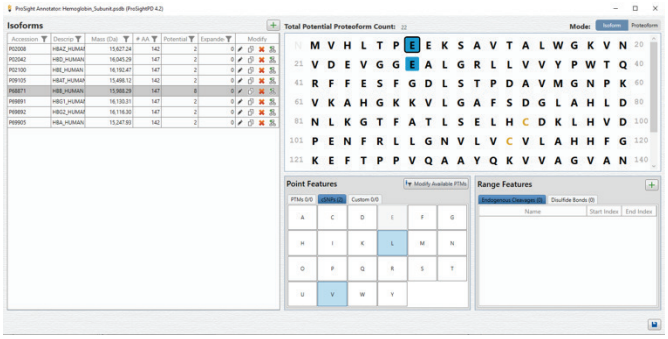
Data acquisition and processing

Sequence search was performed using ProSightPD software with a modified database. The modified database manager and workflow are shown in Figures 2A and 2B, respectively. For amino acid substitutions, cSNPs from point features were used to set up certain amino acid modifications highlighted in blue as shown in Figure 2A. Protein quantitation was performed using TraceFinder software 5.1. The three most abundant *m/z* values from charge states +18 and +19 were added to the processing method for peak extraction from different calibration points as shown in Figure 2C. Additionally, the detection algorithm as global peak settings are shown in Figure 2D.

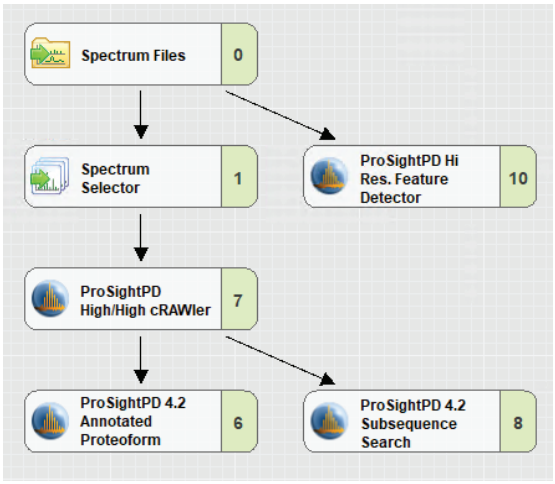
Results and discussion

The method was first developed using normal Hb and Hb S standards. Hb S was spiked into normal human whole blood and proteins were directly extracted from dried blood spots using protein precipitation. The entire sample preparation time is less than 1 hour and is performed in a high-throughput format with 96-well plates. Due to the high concentration of Hb in blood (normal Hb level is 14 to 18 g/dL for male and 12 to 16 g/dL for female),[3] one punch of a 3.2 mm disc generated enough materials for LC-MS analysis.

A

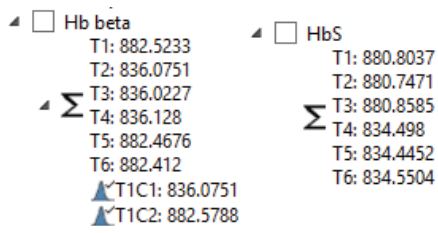


B



C

	Hb S concentration in blood (mg/mL)	Estimated amount of Hb S protein-on-column (µg)
Blank	NA	NA
C0	0	0
C1	1.0	0.010
C2	2.5	0.025
C3	5.0	0.050
C4	10.0	0.100
C5	20.0	0.200
C6	50.0	0.500
C7	100.0	1.000



D

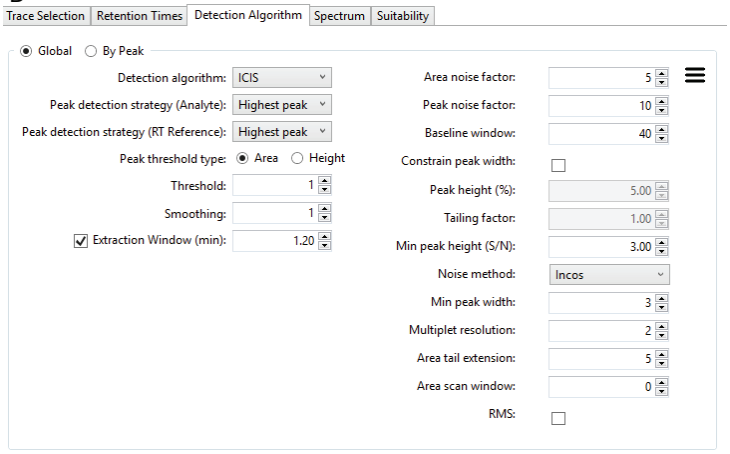


Figure 2. ProSightPD database manager (A) and processing workflow tree for high/high resolution DDA (B). Concentration levels of Hb S in the calibration samples and *m/z* values used of normal beta and Hb S beta chains for peak extraction (C) and details of detection algorithm settings in the processing method (D) in TraceFinder software.

The mass difference between normal beta and Hb S beta chains (β^6 Glu→Val) is 30 Da, which results in about 1.6 *m/z* difference at a charge state of +19. This mass difference could often be hindered by other proteins or interferences from the matrix if the mass analyzer does not have a sufficient resolving power. Utilizing a resolution of 120,000 (full width at half maximum, FWHM, at *m/z* 200) on the Orbitrap Exploris 240 mass spectrometer we were able to isolate these similar masses for accurate variant or subunit identification. The mass spectra of normal alpha, normal beta, Hb S, and delta chains are shown in Figure 4 and discussed in the next paragraph. The confirmation of the delta chain can be beneficial since Hb A2 abundance is 2.0–3.3% in adults, which shows potential for detecting low abundant variants using this method. Top-down analysis was performed using ProSightPD software and the results are shown in Figure 3. ProSightPD is a node of Thermo Scientific™ Proteome Discoverer™ software. The functions and data review panels use common features of Proteome Discoverer software as shown in Figure 3A, but with additional components such as the ProSightPD fragment map. Figures 3B to 3E show fragment maps of Hb S beta, normal beta, normal alpha, and normal delta, respectively. Sequence coverages for Hb S beta and normal beta chains are higher than 40% with the detection of confirming fragment ions of amino acid substitution, β^6 ion, indicated in red in Figures 3B and 3C. The corresponding deconvoluted spectra are also shown in Figure 3 as obtained from ProSightPD software. Additional fragment maps of alpha and delta chains confirmed the successful sampling of hemoglobin protein from DBS. Minor chains, such as epsilon and gamma chains, were identified with <10% sequence coverage, which may need further confirmation (data not shown).

The different hemoglobin chains were separated using the MAbPac column, but normal beta and Hb S beta chains coeluted as shown in Figure 4. Therefore, the chromatographic profile alone cannot differentiate these hemoglobin variants. With the Orbitrap resolution set at 120,000, these coeluted chains with a difference of only 1.6 *m/z* were fully resolved for the charge state of +19. Six of the isotopes of the +19 and +18 charge state

Figure 3. Identification results using ProSightPD software. Overview of identified proteins including mutated beta sequence (A), sequence map of Hb S beta chain with deconvoluted spectrum and indication of b ion of mutated amino acid in red (B), normal beta chain (C), alpha chain (D), and delta chain (E).

(T1 and T6, Figure 2C) were combined for Hb S beta quantitation using normal beta as the internal standard to demonstrate feasibility of this workflow. Figure 5 shows the quantitation results in TraceFinder software data review, including retention time, spectra, and the calibration curve. Analytical quantitative quality measurements were all excellent with percent difference (%diff) < 15%, and relative standard deviation of the calculated amount (%RSD) and peak area (%CV) were all under 4%. Figure 6 demonstrates good linearity of Hb S beta in this assay over three days. The data is shown in Table 3.

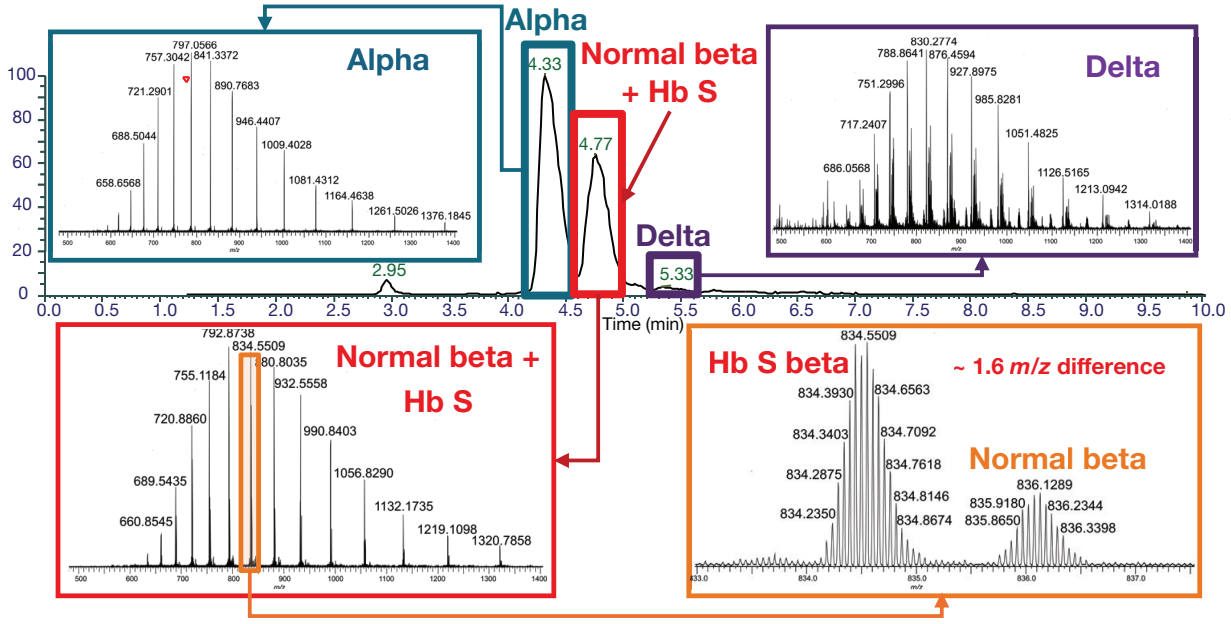


Figure 4. Detection of four chains/variants with fully resolved masses between Hb S and normal Hb A beta chains by Orbitrap MS

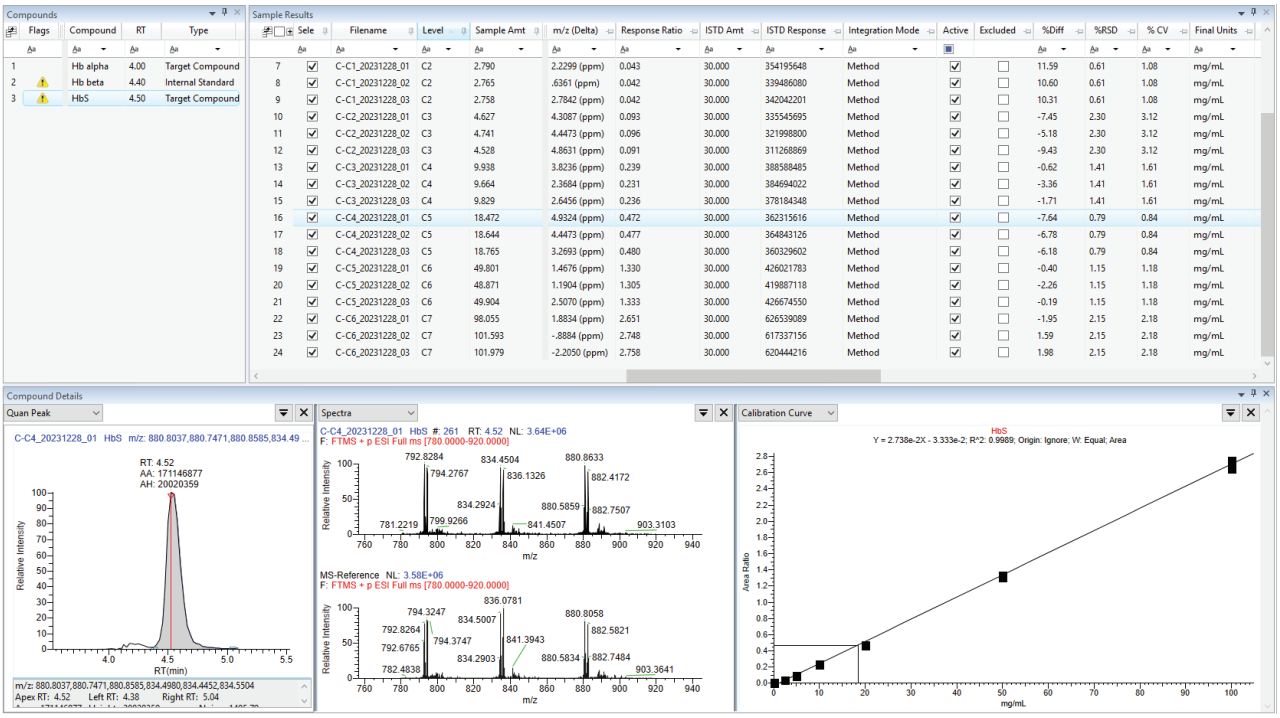


Figure 5. Example view of data review for quantitation using TraceFinder software

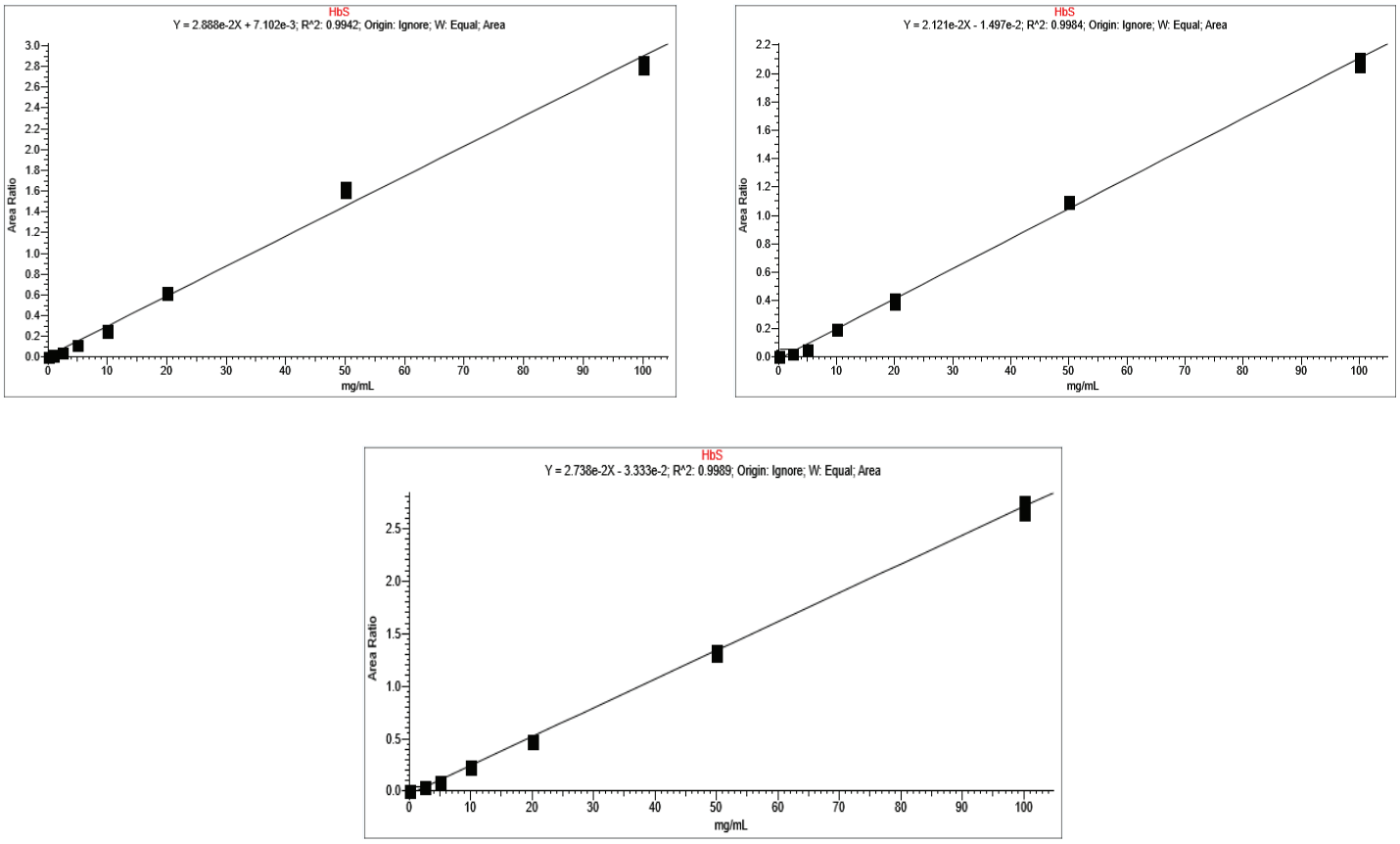


Figure 6. Calibration curves of inter-day precision analysis (N = 3)

Table 3. Inter-day precision and accuracy with calibration curve evaluation (graphs in Figure 6)

	LOD (mg/mL)	LOQ (mg/mL)	Linear range	R2	Accuracy	Precision
Day 1	1.0	2.5	2.5–100	0.9942	82–105%	<5%
Day 2	1.0	2.5	2.5–100	0.9984	73*–112%	<8%
Day 3	1.0	2.5	2.5–100	0.9989	93–111%	<4%

*One of the injections was out of specifications.

Conclusion

This study successfully demonstrates the application of an LC-MS/MS top-down approach for detecting hemoglobin variants using dried blood spots and the Orbitrap Exploris 240 mass spectrometer. The method enables direct protein extraction with minimal sample preparation, allowing high-throughput analysis. The high resolution of the Orbitrap Exploris 240 MS effectively isolates and identifies hemoglobin variants, even with minimal mass differences, and detects low-abundance variants such as the Hb delta chain. The ProSightPD software enhances data analysis by generating sequence maps for variant identification. This approach provides a robust and efficient workflow for clinical research, offering significant improvements over traditional methods. This technical note demonstrated the feasibility of hemoglobin variant detection workflow, highlighting its potential for broader clinical research applications.

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