Clinical Research

# Enhanced Detection of Hemoglobin Variants in Clinical Research by DBS and High-Resolution Accurate Mass (HRAM) MS

Yvonne E. Song, Kerry Hassell, Ed Goucher, Jingshu Guo, and Tanis Correa
Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose, CA, 95134

## Abstract

**Purpose:** to develop the top-down approach for hemoglobin variant detection in clinical research using dried blood spots and Orbitrap Exploris 240 mass spectrometer.

**Methods:** A 3.2 mm disc was punched and put into a 96-well plate for direct protein extraction and precipitation. LC-MS analysis was performed by Thermo Scientific™ Vanquish™ Flex UHPLC system with Thermo Scientific™ MAbPac™ RP column (1 x 100 mm, 4 µm, Part No. 303183) coupled to Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer with BioPharma Option installed. Sequence search was performed using ProSighPD™ with a modified database and quantitation was performed using Thermo Scientific™ TraceFinder™ software 5.1.

**Results:** Identification of variants applied top-down analysis to characterize the structural mutation while quantitation was performed by extracting isotopic m/z of precursors to quantify ratios between target chains or variants. Minimal sample preparation (< 1 hr) in 96 well plate format, easily transferrable to automation using a robotic system. Confident data processing and review was successfully developed using automated software tools, ProSightPD™ and TraceFinder™.

## 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. Common techniques used in Hb analysis are electrophoretic and chromatographic assays. However, there are challenges to differentiate isomers (i.e., between Hb C and Hb E), very similar masses (i.e., < 1 Da difference between normal beta and Hb C, Hb E, and Hb D-Punjab), or new variants due to their lack of resolution. Recently, top-down approach has emerged to target intact protein biomarkers owing to high-resolution accurate-mass (HRAM) mass spectrometry such as Orbitrap. Here, we present the top-down analysis for enhanced detection of various hemoglobin variants using Orbitrap Exploris mass spectrometry for clinical research.

## Materials and methods

### Sample Preparation

Normal hemoglobin (Sigma-Aldrich, P/N H7379) and hemoglobin S (HbS, Sigma-Aldrich, P/N H0392) were used. Different concentrations of HbS calibrator stock were spiked into 2-EDTA stabilized normal human whole blood (BioIVT, Westbury, NY), which generated final concentrations ranging from 0, 0.50, 1.0, 2.5, 5.0, 10, 20, 50, and 100 mg/mL. A 3.2 mm disc was punched and put into a 96-well plate for direct protein extraction and precipitation as shown in Figure 1.

### Test Method(s)

LC-MS analysis was performed by Thermo Scientific™ Vanquish™ Flex UHPLC system with Thermo Scientific™ MAbPac™ RP column (1 x 100 mm, 4 µm, Part No. 303183) coupled to Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer with BioPharma Option installed. Conditions are described in Table 1.

### Data Analysis

Sequence search was performed using ProSighPD™ with a modified database. The modified database manager and workflow are shown in Figure 2. Protein Quantitation was performed using TraceFinder™ software 5.1.

**Figure 1. Experimental workflow**

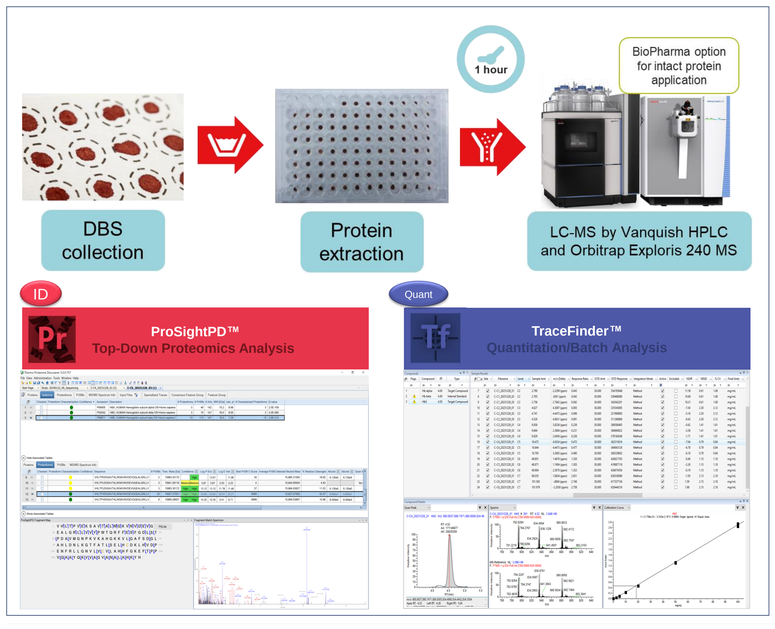



**Table 1A. LC conditions**

| 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 | | |

**Table 1B. MS conditions**

| MS global parameters | |
|---|---|
| **Application mode** | Intact protein |
| **Pressure mode** | Low pressure |
| **Source type** | Heated electrospray ionization (H-ESI) |
| **Polarity** | Positive |
| **Spray voltage (V)** | 3800 |
| **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)<br>0.5 min: position 1-2 (MS)<br>9.5 min: position 1-6 (waste) |
| **MS scan parameters** | |
| **Orbitrap resolution** | 120,000 |
| **Scan range (m/z)** | 500-3000 for intact mass confirmation<br>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** | 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** | 90000 |
| **Scan range (m/z)** | 200-2000 |
| **AGC target** | Custom |
| **Normalized AGC target(%)** | 100 |
| **Maximum injection time (ms)** | 250 |

## Results

**Figure 2. ProSightPD workflow and database manager**

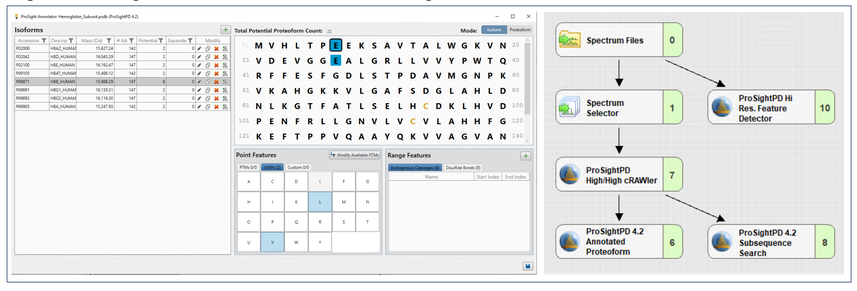



**Figure 3A. Chain/variant identification with ProSightPD**

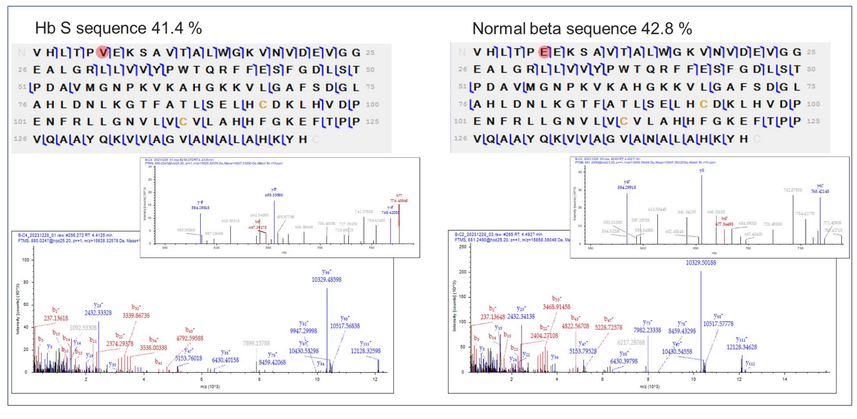
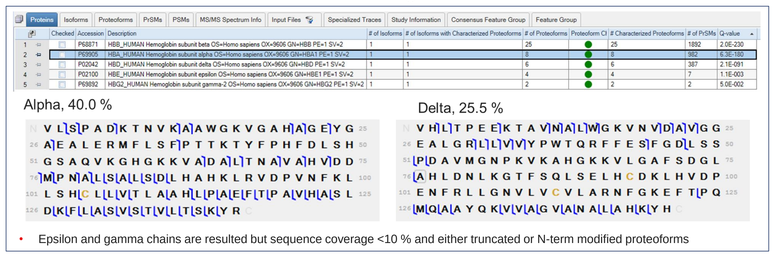



**Figure 3B. Chain/variant identification with ProSightPD**




- Epsilon and gamma chains are resulted but sequence coverage <10 % and either truncated or N-term modified proteoforms

**Figure 4. MS spectrum and chromatograms of 4 chains/variant**

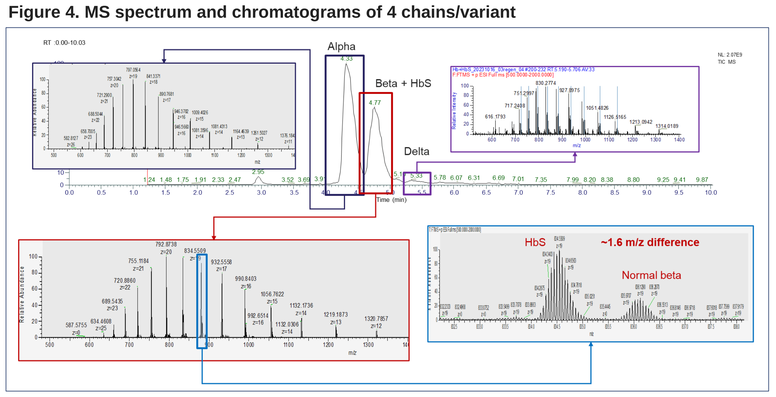



**Figure 5. Quantitation strategy and summary**

Concentration range of linear curve

| | Hb S concentration in blood (mg/mL) | Hb S protein-on-column (ug) |
|---|---|---|
| Assay Blank | NA | NA |
| C0 | 0 | 0 |
| C1 | 1 | 0.010 |
| C2 | 2.5 | 0.025 |
| C3 | 5 | 0.050 |
| C4 | 10 | 0.100 |
| C5 | 20 | 0.200 |
| C6 | 50 | 0.500 |
| C7 | 100 | 1.000 |

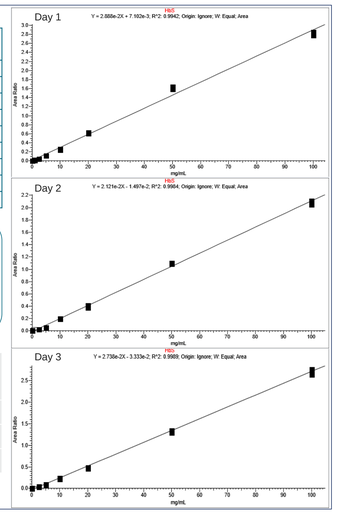
- Use normal beta as an internal standard to compare ratio between normal and Hb S beta chains
- 3 consecutive days of sample preparation and 3 injections per calibrator

Hb beta
T1: 882.5233
T2: 836.0751
T3: 836.0227
T4: 836.128
T5: 882.4676
T6: 882.412
T1C1: 836.0751
T1C2: 882.5788

HbS
T1: 880.8037
T2: 880.7471
T3: 880.8585
T4: 834.498
T5: 834.4452
T6: 834.5504

| | LOD (mg/mL) | LOQ (mg/mL) | Linear range | $R^2$ | 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% |

*lower accuracy from LOQ




## Conclusions

- Direct protein extraction from dried blood spots for protein identification and quantitation
- Identification: using top-down analysis for variant sequencing to characterize the structural mutation
- Quantitation: using intact protein quantitation by extracting isotopic m/z of precursors to quantify ratios between target chains or 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, ProSightPD and TraceFinder

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