Overview

**Purpose:** Create an integrated, high-throughput analytical method to perform hemoglobinopathies for clinical research.

**Methods:** Incorporate CX plates for sample preparation and direct loading onto an SEC column for HR/AM MS and AIF hemoglobin profiling using a Thermo Scientific™ Q Exactive™ Focus mass spectrometer.

**Results:** Incorporation of CX plates for sample preparation facilitated rapid profiling analysis of 135 samples in ca. 2 hrs. The method robustly detected low level spikings of either human or bovine hemoglobin based on precursor charge state distribution as well as confirmatory fragment ion detection.

Introduction

Hemoglobin profiling for clinical research is used for glycohemoglobin analysis and hemoglobinopathies focus on detecting abnormalities. A desired workflow requires methods to support high-throughput analysis with confident identification of hemoglobin variants and relative quantitation and the entire method must be robust and rugged with limited instrument down-time for cleaning. The approach presented here integrates routine whole blood preparation with fast and routine profiling based on HRAM MS and all ion fragmentation (AIF) acquisition schemes.

**Methods**

A stock sample of whole blood was obtained from an in-house donor and bovine hemoglobin was purchased from Sigma (St. Louis, MO). WB samples were diluted 100-fold using 90:10 (water/MeOH) and mixed at estimated ratios (assuming human hemoglobin concentration of 200 μg/mL) of 10:0, 9:1, 7:3, 5:5, 3:7, 1:9, 0:10 (human/bovine). A total of 25 samples per ratio was loaded into 96 well Thermo Scientific™ SOLA™ Retain CX plates, washed using water and organic (1 min. per step) and the targeted MW range was displaced using an ammonium formate solution. The extraction plate was loaded into the autosampler for direct injection onto an SEC column for introduction into the Q Exactive Focus mass spectrometer. Data acquisition utilized alternating HRAM MS and AIF methods with 10 μscans for each. Automated data processing was performed using the Pinnacle software (Optys Technologies, Inc, Boston, MA) based on reported sequences for all hemoglobin chains. Search parameters considered truncations, modifications, and SNPs/error tolerant searching and matches were scored based on accurate mass analysis for MS and fragment ions.

**Results**

**Figure 1.** Representative data acquisition strategy for the routine hemoglobin profiling experiment where 1A shows the chromatographic profile and alternating (1B) HRAM data acquisition between MS and (1C) AIF.

### 1A

**Directed to Mass Spectrometer**

### 1B

**Directed to Waste**

### 1C

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**Thermo**

**Scientific**
Incorporation of CX plates for sample preparation and direct hemoglobin profiling experiment where A shows the facilitated rapid profiling analysis of 135 samples in ca. 2 hrs. The hemoglobin concentration of 200 µg/mL of 10:0, 9:1, 7:3, 5:5, 3:7, ... and bovine. The spiked bovine levels were ca. 1:1 with estimated human amounts. The inset shows an narrow mass range to demonstrate the precursor distribution between the different alpha and beta chains.

**Figure 2.** Automated protein sequence determination in Pinnacle based on user-defined Uniprot sequence and enabling partial cleavage assumptions for human alpha and beta chains. The insets show the matched isotopic profile based on refined sequence.

**Figure 3.** AIF data analysis for the identified alpha and beta chains. The matched product ions are marked in red triangles and based on 10 ppm mass tolerance, charge state, and isotopic distribution for the originally matched protein sequence. The insets show the protein sequence coverage where the red ticks indicate matched b- and y-type fragments.

**Figure 4.** HRAM MS analysis of a hemoglobin mixture from human and bovine. The spiked bovine levels were ca. 1:1 with estimated human amounts. The inset shows an narrow mass range to demonstrate the precursor distribution between the different alpha and beta chains.

**Figure 5.** HRAM MS analysis of different spiking levels between human and bovine hemoglobin levels for alpha and beta chains.

**Figure 6.** Comparative AIF spectra covering different spiking levels of human:bovine. The labeled fragment ions are matched based on sequences identified in Figure 4 and 5. Nomenclature used to label fragment ions indicate species, chain, fragment type, and position.

**Figure 7.** Repetitive AUC values for each hemoglobin chain across 25 replicates. Reported AUC values results from summing individual charge states. The human/bovine spiked ratios shown in each graph. Measured CVs listed by each chain.
Introduction

We present a robust, high-throughput workflow for automated hemoglobinopathies and based on the incorporation of routine sample preparation, data acquisition, and processing for clinical research. Key features include:

- SOLA CX plate for batch sample preparation to isolate hemoglobin compounds.
- Directly loading onto an SEC column for a 6 min isocratic introduction into the Q Exactive Focus mass spectrometer.
- HRAM MS and AIF data facilitated automated qualitative and quantitative analysis.

Figure 8. Summary report for quantitative hemoglobin spiking analysis. Histogram reports AUC values and error bars for each chain. In addition, the AUC ratios and %RSDs is reported between human and bovine. The ratios were corrected based on the estimated 1:1 ratios.