Exploration of semiconductor chip-based single-molecule protein sequencing for identification of hemoglobin variants

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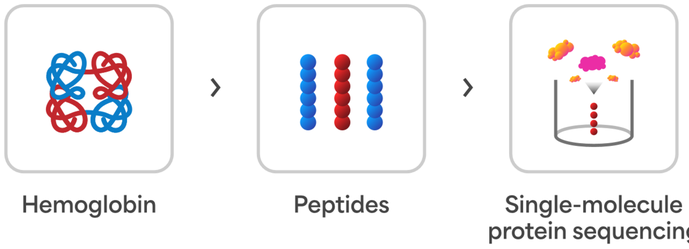
ABSTRACT

Identification of hemoglobin (Hb) variants is of significant value in the clinical diagnosis of hemoglobinopathies. Conventional methods used to identify Hb variants in clinical laboratories can narrow down the range of candidates for a Hb variant sample but are unable to pinpoint the exact Hb variant. In this study, a semiconductor chip-based single-molecule protein sequencing (SMPS) technology was explored as a novel method to identify Hb variants. Two heterozygous Hb variant samples underwent SMPS analysis. Proteotypic peptides corresponding to Hb variants were successfully detected, enabling the identification of the samples as Hb Handsworth (Hb SMPS subunit G18R) and Hb G-Accra (Hb β-G-Accra subunit D73N). Independent liquid chromatography — high-resolution mass spectrometry (LC-HR-MS) analysis confirmed the presence of both variants. This is the first demonstration of SMPS for direct identification of clinical hemoglobin variants.



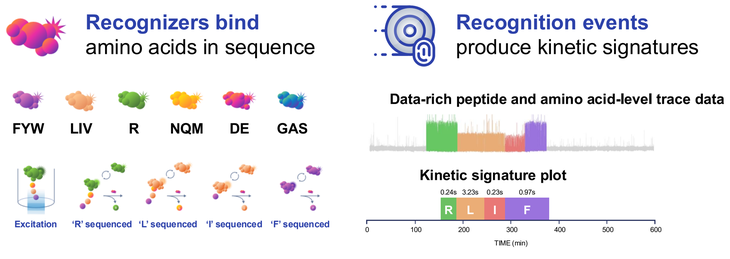
BACKGROUND

Accurate identification of hemoglobin (Hb) variants is critical for the diagnosis of hemoglobinopathies, yet conventional clinical methods such as electrophoresis and ion-exchange chromatography often fail to unambiguously resolve specific variants. While high-resolution mass spectrometry can provide definitive structural information, its adoption is limited by infrastructure and expertise requirements. Single-molecule protein sequencing technologies offer a potential alternative by directly interrogating amino acid sequences at the single-molecule level. Here, we explore the application of the Quantum-Si Platinum® benchtop platform for identifying heterozygous Hb variants in clinical samples, assessing its ability to detect variant-specific proteotypic peptides and discriminate among electrophoretically indistinguishable candidates.

INTRODUCTION TO SINGLE-MOLECULE PROTEIN SEQUENCING

- **Simple sample prep process:** Proteins are digested, functionalized, conjugated, and immobilized on the surface of a semiconductor chip
- **Unique *kinetic signature* mechanism:** Fluorescently labeled N-terminal amino acid (NAA) recognizers and aminopeptidases are added to the semiconductor chip
- **Single-molecule level data:** Fluorescent intensity and duration of each NAA binding event generate a unique kinetic signature
- **Automated analysis:** Kinetic signatures are automatically analyzed to align reads to reference peptides and compute false discovery rate (FDR)

The Platinum® Pro benchtop instrument enables single-molecule protein sequencing



METHODS

Whole blood samples from two patients with heterozygous Hb variants and a wild-type control were processed by red blood cell isolation, lysis, and proteolytic digestion with Lys-C. Peptides were chemically functionalized, immobilized in attoliter-scale reaction chambers on a semiconductor chip, and analyzed using SMPS (Quantum-Si, Library Prep Kit V2, Sequencing Kit V3). Sequential N-terminal amino acid interrogation was performed using a panel of fluorescent recognizers and aminopeptidases. Single-molecule kinetic signatures were aligned to *in silico* peptide databases comprising wild-type and candidate variant sequences. Variant identification relied on detection of proteotypic peptides with direct observation of the amino acid substitution, signal-to-noise filtering, and statistical enrichment analysis relative to wild-type controls.

RESULTS

Sample 1 (α subunit variants):

- Electrophoresis suggested multiple candidates (Hb Ottawa, Handsworth, Reims, Memphis, G Audhali, Kurdistan, Russ)
- NGPS detected α peptide (V16–K40) with G18R substitution (Figure 1)
- Strong statistical support ($p < 0.001$; highest log odds ratio) (Figure 3)
- Ruled in Hb Handsworth; ruled out other candidates
- Confirmed by independent LC–HR MS and synthetic peptide standards

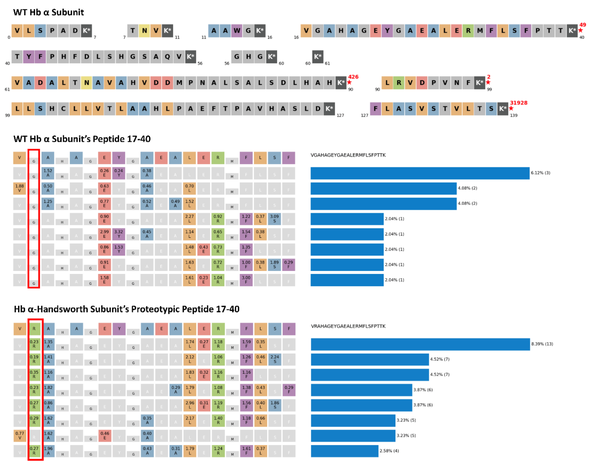


Figure 1: SMPS result of Sample 1 with peptide alignments to heterozygous Hb Handsworth. The Lys-C-digested peptides of Hb α subunit are displayed on the top and the detected amino acids are colored according to the bound recognizers (FYW: purple, LIV: orange, R: green, AS: blue, NQ: yellow, DE: red). The detected Hb α-Handsworth subunit's proteotypic peptide and its corresponding WT peptide are exhibited at the bottom.

Sample 2 (β subunit variants):

- Electrophoresis suggested Hb Kenitra, G Accra, Aalborg
- NGPS detected β peptide (V66–K82) with D73N substitution
- Significant enrichment vs. control ($p < 0.05$; positive log odds ratio)
- Identified Hb G Accra
- Confirmed by LC–HR MS and synthetic peptide standards

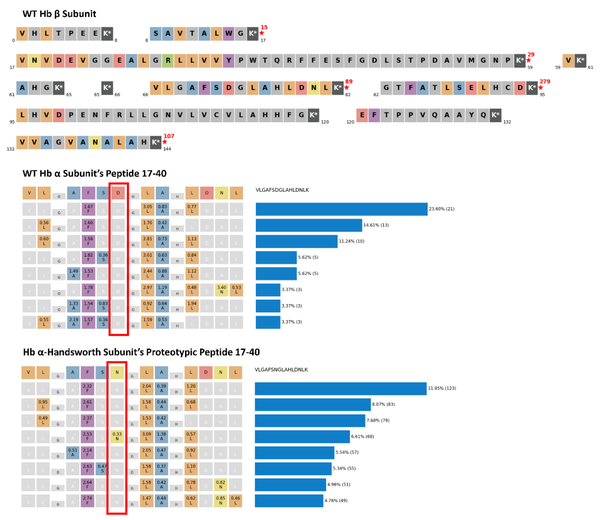


Figure 2: SMPS result of Sample 2 with peptide alignments heterozygous Hb G-Accra. The Lys-C-digested peptides of Hb β subunit are displayed on the top and the detected amino acids are colored according to the bound recognizers. The detected Hb β-G-Accra subunit's proteotypic peptide and its corresponding WT peptide are exhibited at the bottom, highlighting the eight most abundant sequence matches identified. Note: The C-terminal peptides of Hb α subunit (YR) and Hb β subunit (YH) are not displayed because NGPS measures lysine-containing peptides.

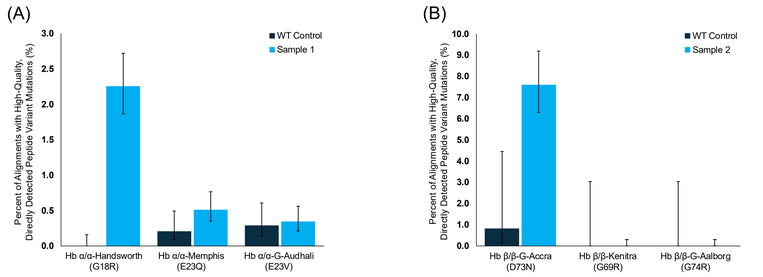


Figure 3. (A) Detection rates of high-quality, directly observed G18R, E23Q, and E23V Hb α mutations as a fraction of the total alignments to the peptide family, comparing the WT Control to Sample 1. (B) Detection rates of high-quality, directly observed D73N, G69R, and G74R Hb β mutations as a fraction of the total alignments to the peptide family, comparing the WT Control to Sample 2. Error bars show the minimum and maximum values of the 95% Wilson Score Interval.

CONCLUSIONS AND FUTURE DIRECTIONS

- **SMPS enables hemoglobin variant identification:** Single-molecule protein sequencing accurately resolved ambiguous electrophoretic results by directly detecting variant-specific amino acid substitutions.
- **High specificity and statistical confidence:** Variant peptide detection showed strong statistical support and discrimination from wild type, reinforced by synthetic peptide validation.
- **Effective with partial sequence coverage:** Even incomplete peptide coverage was sufficient for unambiguous Hb variant identification when key substitutions were captured.
- **Complementary to existing workflows:** SMPS integrates well with conventional screening (electrophoresis), narrowing candidates and enabling precise identification.
- **Potential clinical utility:** SMPS represents a **simpler, high-performance alternative** to MS-based methods for hemoglobin variant analysis, with promise for broader adoption in clinical diagnostics.
- **Future directions:** The Proteus™ instrument, launching at the end of 2026, will expand amino acid coverage to 18 at launch and 20 by 2027.

The Proteus instrument will feature automated sequencing, higher throughput, and analysis of up to 4 samples

REFERENCES

Luo, R. *et al. Analytical Chemistry* **2026** *98* (5), 3458-3465 DOI: 10.1021/acs.analchem.5c07226

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