Achieving Nanogram (Femtomole) Detection Limits of Human Serum Albumin from In-Gel Digestion Coupled with Next-Generation Protein Sequencing™ on Platinum® Pro

SUMMARY

The limit of detection (LOD) in protein sequencing is essential for accurate and comprehensive proteome analysis, especially for identifying low-abundance proteins in biological samples. To evaluate the LOD of the Platinum® Pro Next-Generation Protein Sequencer™, varying amounts of human serum albumin (ranging from 1 ng to 30 µg) were analyzed using SDS-PAGE. Protein bands were excised from the gel, libraries were prepared, and sequencing was performed on Platinum Pro.

Albumin was successfully sequenced and identified across all input levels. Notably, at a 10 ng input (150 fmol), albumin was the top-inferred protein with a probability of 95.60% using the entire human proteome as inference panel. At a 1 ng input, it remained the top-inferred protein using Quantum-Si's standard 10-70 kDa human proteome inference panel.

This ability to detect proteins at such low abundances marks a significant advancement in proteomic analysis, highlighting the potential for characterizing rare or low-copy proteins using Quantum-Si's Platinum Pro.

INTRODUCTION

In complex biological samples such as serum, tissues, and cells, protein abundances can vary widely – from highly abundant proteins like albumin found in blood to low-abundance proteins such as signaling molecules or biomarkers. A lower limit of detection (LOD) is essential for accurately identifying and quantifying these low-abundance proteins, which play a critical role in understanding cellular functions, discovering disease biomarkers, and developing innovative therapeutics. The ability to detect extremely low concentrations of biomarkers enhances diagnostic precision,

enables earlier interventions, and aids in treatment monitoring. Establishing an LOD is crucial for advancing both basic research and clinical proteomics applications.

The separation and enrichment of complex samples are critical components of proteomic workflows, with gel electrophoresis providing a simple and effective method for isolating specific protein bands. SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) is a widely used technique for separating protein mixtures, producing distinct bands visible upon staining. Following separation, western blotting is commonly employed to identify specific proteins by transferring them to a membrane and using antibody-based detection. While effective, antibody-based methods are often hindered by issues such as off-target binding, inefficient reagent use, and data inaccuracies, resulting in wasted time and resources. To overcome these challenges, developing alternative antibody-free methods for sensitive and accurate protein identification could significantly enhance research efficiency and reliability.

A promising approach for identifying pre-separated components without the use of antibodies is Next-Generation Protein Sequencing (NGPS™) using Quantum-Si's Platinum Pro workflow.[2] This innovative method begins with proteins excised from an SDS-PAGE gel, which are subsequently digested into peptide fragments. The peptide fragments are then linked to macromolecular linkers at the C-terminus, immobilized on Quantum-Si's semiconductor chip, and probed with dye-labeled N-terminal amino acid (NAA) recognizers. This process generates distinctive pulsing patterns with characteristic kinetic properties. Sequential cleavage of NAAs by aminopeptidases in solution reveals subsequent amino acids for recognition. Real-time data on fluorescence lifetime, intensity, and kinetics are collected and analyzed using cloud-based software, enabling peptide sequence identification and corresponding protein inference.

To integrate gel electrophoresis with the Platinum Pro workflow, we developed an in-gel digestion protocol that complements Quantum-Si's downstream library preparation and sequencing workflows. This protocol mirrors established procedures used in other proteomic workflows but is optimized for compatibility with the Platinum Pro sequencer. To demonstrate its utility, we prepared in-gel digested peptide libraries using human serum albumin (ALBU) as a model protein. Sequencing these libraries produced kinetic signatures that aligned with ALBU peptides, enabling accurate protein identification and inference. Specifically, ALBU was accurately identified and inferred with a probability of 95.60% at a 10 ng input using the entire human proteome as the inference panel. At a 1 ng input, ALBU remained the top-inferred protein with a probability of 76.28% using Quantum-Si's standard 10-70 kDa inference panel of the human proteome. These results highlight the compatibility of the in-gel digestion protocol with Quantum-Si's library preparation and sequencing workflows, emphasizing its potential for identifying low-abundance proteins in complex mixtures.

MATERIALS AND METHODS

Preparation and Sequencing of 1 ng-30 µg (15 fmol-450 pmol) of Albumin

We began by resuspending recombinant human serum albumin (ALBU) protein (Millipore Sigma Cat # A3782) as a 0.7 mg/mL stock solution in Sample Buffer (acetonitrile (MeCN) 20% v/v, HEPES 100 mM) from Quantum-Si Library

Preparation Kit – Lys-C v2 (Cat # 910-00012-02). This stock solution was diluted to obtain 10 µL solutions ranging from 1 ng to 30 µg of ALBU. These solutions, together with a negative control (0 ng input), were prepared in Novex™ Tris-Glycine SDS Sample Buffer 2X (Thermo Fisher Scientific Cat # LC2676), heated at 95°C for 5 minutes, and loaded onto a 4-20% Novex™ Tris-Glycine Mini Protein Gel (Thermo Fisher Scientific Cat # XPO4200PK2). The gel was run in Novex™ Tris-Glycine SDS Running Buffer (Thermo Fisher Scientific Cat # LC2675) at 200 V for 55 minutes. To visualize gel bands, the staining and destaining procedure was performed using SimplyBlue™ SafeStain (Thermo Fisher Scientific Cat # LC6060) following the manufacturer's microwave protocol.

ALBU bands were excised from the gel with a new razor blade and placed on a clean surface. The gel bands were diced into ~1 mm cubes and transferred into clean 1.5 mL tubes. Protein digestion and peptide extraction were performed using an in-gel digestion kit (Thermo Fisher Scientific Cat #89871), substituting the provided trypsin with LysC endoprotease from the Quantum-Si Library Preparation Kit – LysC v2 (Cat #910-00012-02). Briefly, the diced gel pieces were first destained twice with destaining solution ($(NH_4)HCO_3$, 2 mg/mL; MeCN, 50% v/v) at 37°C for 30 minutes with shaking (1,000 rpm) on a thermomixer. They were then reduced with reducing buffer ($(NH_4)HCO_3$, 2 mg/mL; TCEP, 2 mM) at 60°C for 10 minutes and alkylated with alkylation buffer ($(NH_4)HCO_3$, 2 mg/mL; iodoacetamide, 20 mg/mL) at room temperature in the dark for 1 hour.

Post-alkylation, the gel pieces were washed twice with destaining buffer at 37°C for 15 minutes with shaking (1,000 rpm) and subsequently shrunk with MeCN at room temperature for 15 minutes. Digestion was carried out by adding 100 µL of digestion buffer (MeCN, 20% v/v; HEPES, 100 mM; LysC, 20 µg/mL) and incubating overnight (16-18 hours) at 37°C with shaking (1,000 rpm). The following day, the supernatant containing ALBU-digested peptides was collected (~50 µL per sample due to gel absorption) and transferred into new collection tubes.

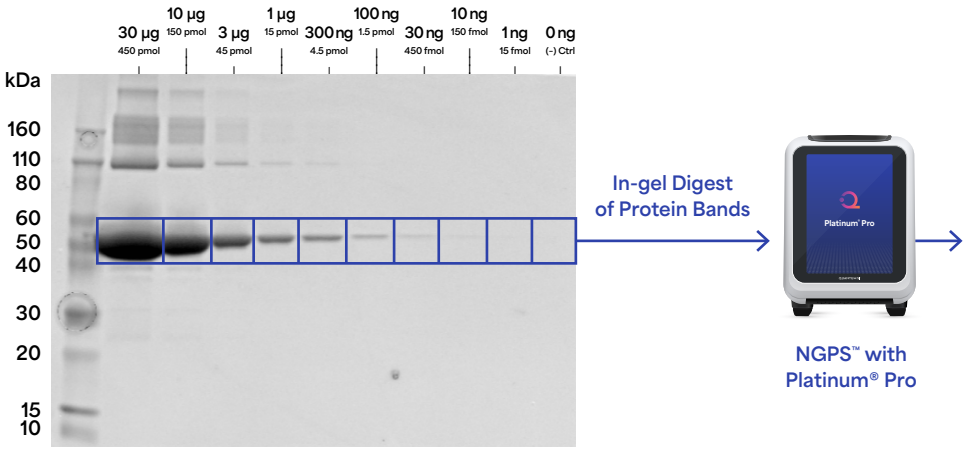
To generate the peptide library for sequencing, digested peptides were prepared using Quantum-Si's Library Preparation Kit – LysC v2 and Protocol, starting at Day 2 with the following modifications. Briefly, diazotransfer was performed by incubating 50-µL digested peptide samples with 3 µL of K_2CO_3, 6.5 µL of $CuSO_4$, and 1 µL of ISA for 90 minutes at room temperature. Before quenching, the beads tubes were briefly centrifuged, and the supernatant was removed to prevent sample dilution. The entire samples were then quenched by directly adding them to the beads tube and incubating at room temperature for 30 minutes with end-to-end mixing. Post-quenching, the samples were filtered using the provided spin columns, and 2 µL of acetic acid was added to adjust the pH. Linker conjugation was performed by adding 2 µL each of EDTA, CTAB, and K-Linker to 47 µL of the sample. The reaction was incubated at 37°C overnight (16-18 hours).

On the next day, samples were removed from 37°C and stored on ice until sequencing on Quantum-Si's Platinum Pro instrument using the Quantum-Si Sequencing Kit V3 (Cat # 910-00038-00) and Protocol. All samples were loaded at 0.15-0.2 nM via split-chip experiments (which use only one side of the chip). The data was analyzed using Peptide Alignment v2.9.0 (ALBU as reference file) and Protein Inference v2.8.0 workflows (entire human proteome as inference panel for the 10 ng-30 µg inputs, Quantum-Si's standard inference panel of 10-70 kDa human proteome for the 1 ng input).

RESULTS AND DISCUSSION

In this technical note, we aimed to determine the limit of detection (LOD) of Platinum Pro on in-gel digested protein samples. To achieve this, we performed a titration series of human serum albumin (ALBU) ranging from 1 ng (15 fmol) to 30 µg (450 pmol) and analyzed using SDS-PAGE (Figure 1). The gel bands corresponding to each input amount were digested in-gel, followed by library preparation using a modified version of Quantum-Si's library preparation workflow (see Materials and Methods).

Next, protein libraries were sequenced on the Platinum Pro instrument, and the data analyzed using the Peptide Alignment v2.9.0 workflow with ALBU as the reference. As shown in Figure 2A, the number of ALBU alignments decreased with sample input, from 14,496 alignments at 30 µg to 139 alignments at 1 ng. Similarly, the number of high-confidence peptides (FDR <10%) decreased with sample input, from 9 peptides at 30 µg to 3 peptides at 1 ng (Figure 2B). As expected, the negative control (no protein input), resulted in no high-confidence peptides (8 alignments). This demonstrates Platinum Pro's ability to accurately identify peptides and proteins, even from very low alignment counts.



Input	Top Protein Inferred
30 µg	ALBU – 99.99%
10 µg	ALBU – 99.99%
3 µg	ALBU – 99.99%
1 µg	ALBU – 99.99%
300 ng	ALBU – 99.99%
100 ng	ALBU – 99.99%
30 ng	ALBU – 99.12%
10 ng	ALBU – 95.60%
1 ng*	ALBU – 76.28%

* All inputs from 10 ng to 30 µg were inferred against the entire human proteome. The 1 ng input was inferred against QSI standard 10-70 kDa human proteome panel.

FIGURE 1

An overview of the in-gel digestion of human serum albumin bands from SDS-PAGE, followed by sequencing on the Next-Generation Protein Sequencer™, Platinum Pro. Varying amounts of human albumin were loaded onto SDS-PAGE, stained with *SimplyBlue™ SafeStain*, in-gel digested, and prepared for sequencing. The samples were then loaded onto a semiconductor chip and sequenced on Platinum Pro. Albumin was successfully sequenced and inferred at a 1 ng (~15 femtomole) protein input.

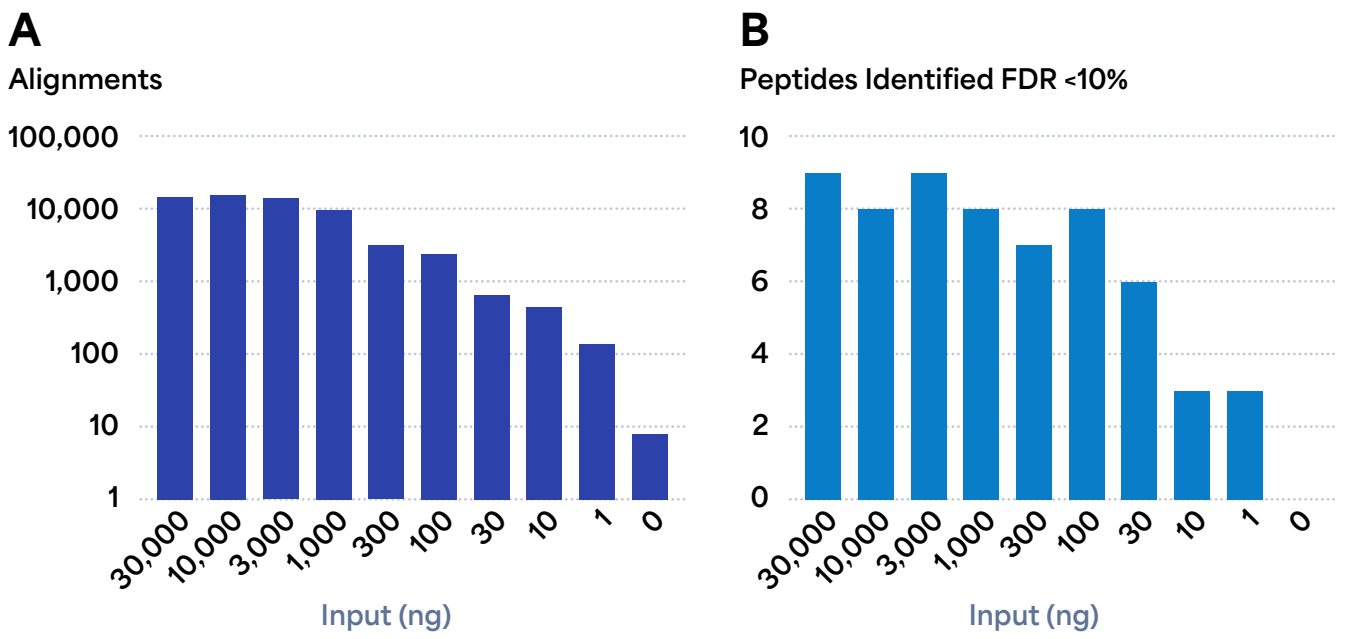


FIGURE 2

Data demonstrates the relationship between sample input and both the number of total alignments (A) and the number of high-confidence peptides (B). As expected, decreasing the sample input resulted in a decrease in the number of peptides and alignments. Notably, even at the lowest input amount of 1 ng (15 femtomole), 3 peptides and 139 alignments were identified.

To test whether albumin could be inferred without specifying the exact protein, the data was analyzed using the Protein Inference v2.8.0 workflow. For inputs of 10 ng–30 µg, the entire human proteome was used as input for the inference panel. For the 1 ng input, the 10–70 kDa human proteome was used as input for the inference panel. The results showed that ALBU was consistently the top-inferred protein, with more than 95.60% probability across input amounts at or greater than 10 ng (Table 1). This highlights Platinum Pro's ability to accurately identify an unknown protein from nanogram or femtomole levels of protein input.

At an input of 1 ng, ALBU was the second-highest inferred protein with a probability of 17.31% (data not shown) when the entire human proteome was used as the inference panel. However, when we applied Quantum-Si's standard inference panel, which is restricted to the 10–70 kDa human proteome, ALBU became the top-inferred protein with a probability of 76.28% (Table 1). This highlights a strategy for enhancing the identification of low-abundance proteins by narrowing the size of the inference panel.

IF Rank	30 µg	10 µg	3 µg	1 µg	300 ng	100 ng	30 ng	10 ng	1 ng*	(-) ctrl
1	**ALBU 99.99%**	**ALBU 99.99%**	**ALBU 99.99%**	**ALBU 99.99%**	**ALBU 99.99%**	**ALBU 99.99%**	**ALBU 99.12%**	**ALBU 95.60%**	**ALBU 76.28%**	**N/A**
2	NEBU 95.97%	CD209 99.21%	NE~BU 92.21%	CD209 96.77%	CD209 37.33%	MACF1 31.82%	MACF1 18.92%	MACF1 12.34%	K1C13 59.85%	N/A
3	CD209 93.84%	NEBU 85.53%	CD209 91.49%	DDX60 82.98%	MACF1 36.77%	ADCY2 26.54%	CHD2 11.30%	CHD2 9.03%	K2C1 55.49%	N/A
4	MACF1 79.38%	MACF1 77.89%	MACF1 79.12%	ADCY2 55.40%	ADCY2 29.75%	CHD2 22.7%	RBM44 7.78%	ADCY2 8.41%	K1C10 50.68%	N/A
5	ADCY2 59.60%	ADCY2 67.38%	DDX60 72.17%	MACF1 54.72%	CHD2 27.73%	RBM44 21.37%	PLXB3 7.78%	RBM44 7.12%	PDCD6 31.18%	N/A

TABLE 1

Shown above are the top five inferred proteins with probability scores across various sample input amounts (1 ng-30 µg). Albumin (ALBU) consistently ranked as the top inferred protein, with a notable probability of 95.60% at the 10 ng input (entire human proteome as inference panel) and (*) a 76.28% probability at the 1 ng input (10-70 kDa human proteome as inference panel).

CONCLUSION

This study highlights the remarkable sensitivity and precision of Quantum-Si's NGPS technology with Platinum Pro, enabling protein detection and identification at nanogram (femtomole) levels. Human serum albumin (ALBU) was successfully sequenced and identified at inputs as low as 1 ng (15 femtomole), providing unmatched single-molecule resolution. By integrating in-gel digestion with Quantum-Si's library preparation workflow, we established an antibody-free approach to accurately sequence and identify proteins directly from SDS-PAGE gel bands.

These findings highlight the sequencer's potential to generate valuable insights from minimal sample input, enabling the detection of low-abundance proteins such as biomarkers and signaling molecules. With its exceptional sensitivity, precision, and scalability, Platinum Pro has significant potential to advance biomarker discovery, explore cellular processes, and enhance diagnostic accuracy.

REFERENCES

1. Baker M. Reproducibility crisis: Blame it on the antibodies. *Nature*. 2015;521(7552):274-276. doi:10.1038/521274a
2. Reed BD, Meyer MJ, Abramzon V, et al. Real-time dynamic single-molecule protein sequencing on an integrated semiconductor device. *Science*. 2022;378(6616):186-192. doi:10.1126/science.abo7651