Immunoprecipitation of IL-6 from Human Serum for Next-Generation Protein Sequencing on Platinum®

August 14, 2024

SUMMARY

The enrichment of low-abundant proteins from complex biological matrices is a key step for many proteomic workflows. In this technical note, we present an immunoprecipitation protocol compatible with Quantum-Si's next-generation protein sequencing workflow. This process utilizes an on-bead digestion protocol that forgoes the sample elution step and proceeds directly to the preparation of digested peptide libraries. We demonstrate the feasibility of this approach by specifically enriching IL-6 through immunoprecipitation from human serum, followed by protein sequencing and identification on Platinum®.

INTRODUCTION

The human blood proteome is a complex mixture of high and low abundant proteins with a concentration range that spans over 10 orders of magnitude.[1] Low-abundant proteins, such as cytokines, chemokines, and growth factors, are of particular interest in biomedical research due to their role as indicators of disease states. A notable example is Interleukin-6 (IL-6), a multifunctional cytokine that plays a critical role in immune system activation. In healthy individuals, IL-6 is present in the blood at relatively low concentrations, between 1 and 5 pg/mL. However, IL-6 production rapidly increases in response to infections, tissue injuries, and other immune challenges, making it an early biomarker for diseases.[2]

Q-SI TECHNOLOGY

Quantum-Si's benchtop Platinum® instrument enables protein sequencing from biological samples in a simple user-friendly workflow. Our technology utilizes dye-tagged N-terminal amino acid recognizers and semiconductor chip technology to detect the binding characteristics and binding order of N-terminal amino acids, resulting in unique kinetic signatures that can be used to differentiate and identify amino acid residues and PTMs. A more detailed overview of the workflow and technology can be found in our Science Paper.

To study low abundant proteins within biological samples, specialized techniques to enrich and concentrate proteins of interest are essential for effective analysis. One widely used technique in molecular biology and biochemistry for this purpose is immunoprecipitation (IP). The underlying principle of IP is both simple and effective. Antibodies with specificity for the protein of interest are first immobilized on a solid matrix, such as agarose or magnetic beads. Monoclonal antibodies, exhibiting high affinity and specificity for target protein, are typically employed to ensure efficient antigen-antibody interactions. The sample containing proteins of interest (e.g., a cell lysate or a biological fluid such as serum) is then incubated with the antibody-coated beads. The antibodies selectively bind to the target protein, forming an antibody-protein complex. Through subsequent washing steps, unwanted proteins and contaminants are removed, leaving the specific protein(s) of interest entrapped with the beads. Finally, the captured protein is eluted from the antibody-protein complex, preparing it for subsequent downstream protein analysis.

Once the immunoprecipitated protein has been isolated, the identity of the proteins within the sample can be analyzed using proteomic analysis techniques, such as mass spectrometry or immunoassays. However, these techniques either require complex equipment and analysis or do not provide amino acid level sequencing information. One alternative to identifying proteins following immunoprecipitation with single amino acid resolution in a simple benchtop workflow is through next-generation protein sequencing using Quantum-Si's Platinum®. This innovative method entails digesting the isolated proteins into peptide fragments and attaching them to macromolecular linkers at the C-terminus. The peptides are then immobilized on Quantum-Si's semiconductor chip and probed with dye-labeled N-terminal amino acid (NAA) recognizers, resulting in distinctive pulsing patterns with characteristic kinetic properties. Individual NAAs are removed sequentially by aminopeptidases in solution, exposing the NAA of subsequent amino acids for recognition. Fluorescence lifetime, intensity, and kinetic data are collected and analyzed with Cloud-based software to identify the peptide sequence and corresponding protein.

To demonstrate the efficacy of this approach, we enriched IL-6 from human serum using immunoprecipitation and utilized Quantum-Si's Platinum instrument for next-generation protein sequencing of the IL-6 peptide libraries. Sequencing of the digested peptide libraries produced single-molecule peptide traces that aligned with peptides from IL-6, enabling the successful enrichment and identification of the protein from human serum. These results demonstrate the complementary nature of immunoprecipitation of target proteins from complex samples, followed by protein identification analysis on Quantum-Si's Platinum instrument.

METHODOLOGY

We began by preparing 100 µg of three anti-IL-6 antibodies, a Human/Primate IL-6 Antibody (Bio-Techne Cat # MAB206), a mouse monoclonal anti-IL-6 high affinity antibody (Sigma Cat # SAB4200734) and a recombinant anti-IL-6 antibody (Abcam Cat # ab233706), along with a mouse IgG-1 Isotype control (Bio-Techne Cat # MAB002), in 100 µL of PBS. Next, the antibody was biotinylated by adding a 40-fold molar excess of freshly prepared Biotin-PEG4-NHS ester in PBS (BroadPharm Cat # BP-20566). The biotinylation reaction was allowed to proceed for 30 min at room

temperature and was quenched by desalting with a 40-K MWCO Zeba™ Spin Desalting Column (Thermo Scientific Cat # 87766). The biotinylated antibody solution was then diluted to 400 µL volume in PBS and added to 100 µL of PBS-washed Pierce™ Streptavidin Agarose (Thermo Scientific Cat # 20353), where it was allowed to bind for one hour at room temperature with rotation to ensure the resin remained suspended in the solution. After one hour, the combined solution was centrifuged at 1,000 rcf for one min to collect the supernatant, and the resin coupling efficacy was determined by measuring the A280 absorbance of the flow-through fraction. The antibody-coupled resin was then washed 2X with 400 µL PBS and stored at 4°C until ready for use.

To demonstrate immunoprecipitations, we spiked in either 1, 5, 10 or 15 µg of Recombinant Human IL-6 Protein (Bio-Techne Cat # 206-IL) into PBS or depleted human serum (Innovative Research Cat # ISERABHI100ML). The human serum was depleted of highly abundant proteins using the PureProteome Human Albumin/Immunoglobin Depletion Kit (MilliporeSigma Cat # LSKMAGHDKIT). For depleting the human serum, 900 µL of magnetic beads slurry was washed three times with PBS, and 25 µL of serum was diluted to a final volume of 100 µL. Diluted serum was incubated with the washed beads, and after one hour, the depleted serum supernatant was collected. In addition, the depleted serum was further cleared by incubating the coupled mouse IgG antibody resin in the serum for one hour at room temperature. For each protein input, a three-fold excess of anti-IL-6-conjugated resin was incubated at 4°C overnight to allow the formation of the antibody-IL-6 complex. After the overnight incubation, beads were washed several times with PBS to remove unbound proteins and contaminants and then subjected to an on-bead digestion protocol to facilitate ease of use with Quantum-Si's library preparation workflow.

During on-bead digestion, the reduction, alkylation, and LysC endopeptidase digestion were performed directly on the antibody resin complex using Quantum-Si's Library Preparation Kit (Cat # 910-10011-00) and Protocol (Document # 950-10007-00) with some modifications. Briefly, 200 µL of Sample Buffer was used to transfer resins to a 2.0 mL collection tube. The volume of kit reagents used during "Activation", "Quench", and "Solution Adjustment" was increased two-fold to account for the initial starting volume. Other than the volume changes, all other steps were followed according to the protocol. During the "Library Reaction" step, we assumed an input protein concentration of 0.24, 1.2, 2.4, and 3.6 µM based on the starting inputs of the 1, 5, 10, and 15 µg samples respectively. Next, we added 1 µL of Additive N and 1 µL of Solution K with 48 µL of the derivatized peptide solution. Reaction tubes were wrapped in parafilm and incubated overnight (16 hrs) at 37°C. On the next day, samples were removed from the heat block and stored on ice until sequencing on Quantum-Si's Platinum instrument using the Protein Sequencing Kit (Cat # 910-0003-00) and Protocol (Document # 950-10006-00).

RESULTS AND DISCUSSION

To explore the applicability of the on-bead digestion and IP procedure in tandem with Quantum-Si's library preparation and protein sequencing workflows (Figure 1A), we began by optimizing IP performance using recombinant IL-6 protein spiked into PBS. To ensure the success of immunoprecipitation, the selected antibody should display high specificity and affinity to the target protein and exhibit minimal cross-reactivity with other proteins. We therefore tested three different commercially available monoclonal IL-6 antibodies for their ability to pull-down the recombinant IL-6 from PBS while retaining efficient capture of the bound protein during subsequent wash steps.

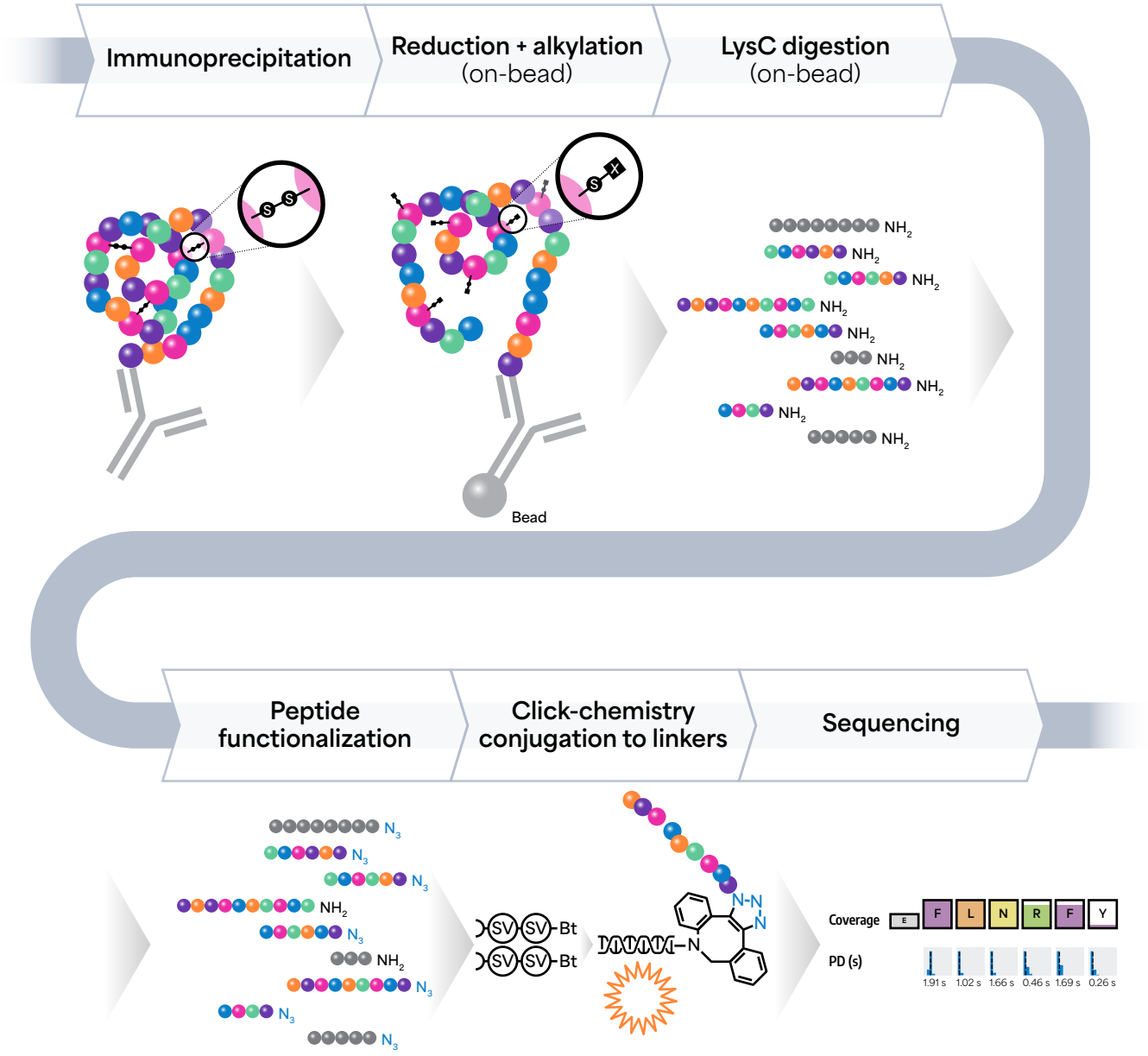

FIGURE 1A

Workflow for on-bead digestion and immunoprecipitation of proteins for next-generation protein sequencing.

We found that while all the antibodies were specific for IL-6 pull-down, most exhibited significant loss of the bound material during the wash steps. As shown in Coomassie-stained SDS-PAGE gel in Figure 1B, the high affinity mouse IL-6 antibody demonstrated a high level of retention of the target protein during testing, with no loss of the bound IL-6 detected during the wash steps. Thus, we employed this antibody for all subsequent experiments.

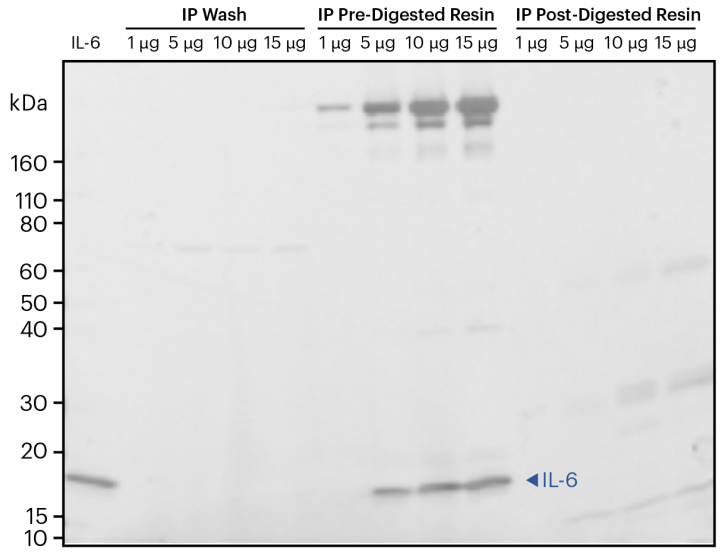

FIGURE 1B

Coomassie-stained SDS-PAGE demonstrating the successful on-bead digestion of the captured IL-6 protein from the antibody-coupled resin using the mouse monoclonal anti-IL-6 high affinity antibody. Pre-digested resin lanes indicate the presence of both IgG and IL-6, demonstrating strong IL-6 binding with anti-IL-6 conjugated resin. Post-digested resin lanes indicate loss of both IgG and IL-6 from the resin band after performing the on-bead reduction, alkylation, and endopeptidase digestion of the samples.

We prepared protein libraries from the IL-6 in PBS titration series obtained through IP with the high affinity mouse IL-6 antibody using the on-bead digestion workflow in combination with Quantum-Si's Library Preparation Kit. As shown in Figure 1B, this workflow resulted in the release and proteolytic digestion of the proteins bound to the streptavidin agarose resin, enabling their downstream use with Quantum-Si's Library Preparation Kit. The libraries were then sequenced on the Platinum instrument, generating single molecules traces that positively identified multiple IL-6 peptides within the sample (Figure 2).

After successfully demonstrating the ability to perform IP and sequencing of IL-6 from PBS, our next objective was to explore the feasibility of applying this workflow to a more complex biological sample. To demonstrate this, we performed a similar IL-6 spike-in and IP titration series using human serum as the biological matrix. To minimize non-specific background binding, we first depleted the serum of highly abundant proteins, such as albumin and immunoglobins, followed by an additional round of clearing using a mouse IgG antibody coupled to streptavidin-agarose resin. Subsequently, we spiked an IL-6 titration series into the depleted serum and performed IP using the IL-6 antibody-conjugated

resin. Following the IP process, the samples underwent the same processing steps as previously described to produce protein libraries, which were then sequenced using the Platinum instrument.

Similar to the IP results in PBS, we successfully demonstrated the enrichment of IL-6 from serum, followed by its identification by Platinum sequencing (Figure 3). As expected, we observed a positive correlation between the amount of IL-6 spike-in and the number of alignments. With increasing amounts of IL-6 spike-in, the number of alignments obtained also increased. This result is most likely due to the fact that the estimated starting protein sample is below the recommended sample input of 5 µM for Quantum-Si's Library Preparation Kit. Despite this limitation, both the 10 and 15 µg samples yielded a significant number of alignments from the immunoprecipitated sample, enabling the positive identification of the protein from the sequencing results.

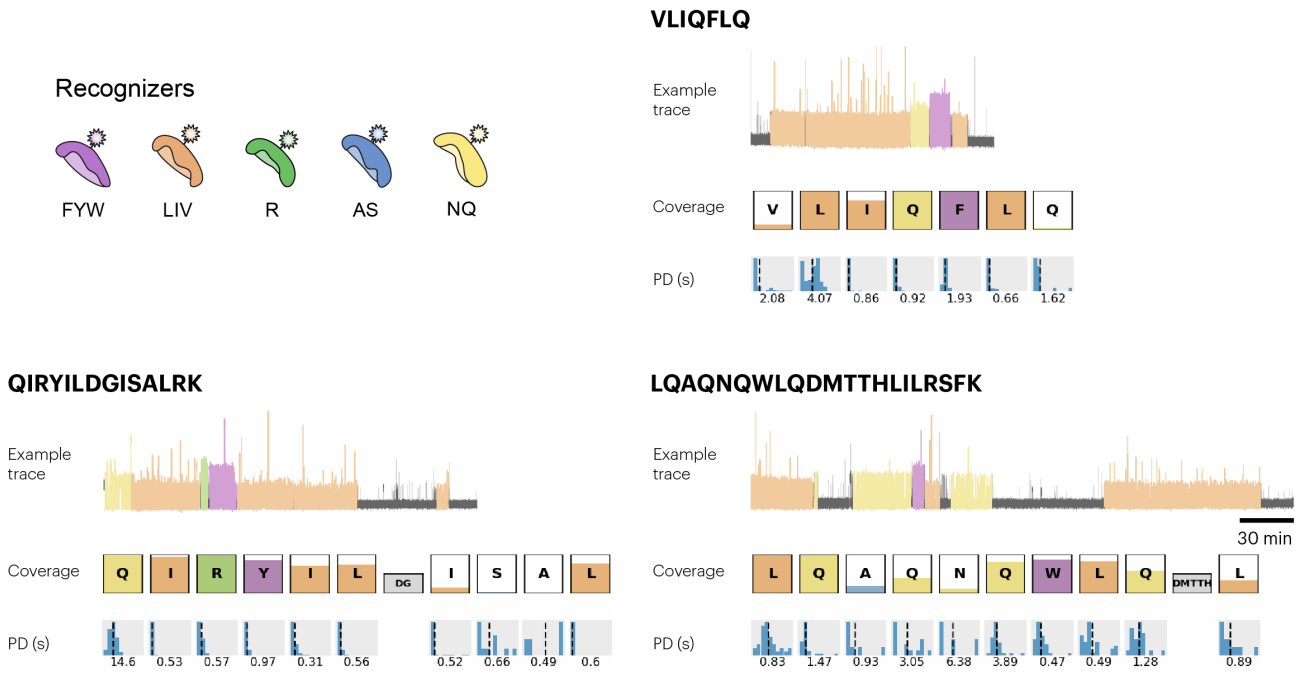

FIGURE 2

Example Traces, Coverage, and Pulse Duration Data for the Peptides Identified in IL-6 Immunoprecipitated from PBS. Five recognizers were used to identify 12 amino acids (F, Y, W, L, I, V, A, S, N, Q, R, and K), enabling identification of 3 peptides as shown in the figure.

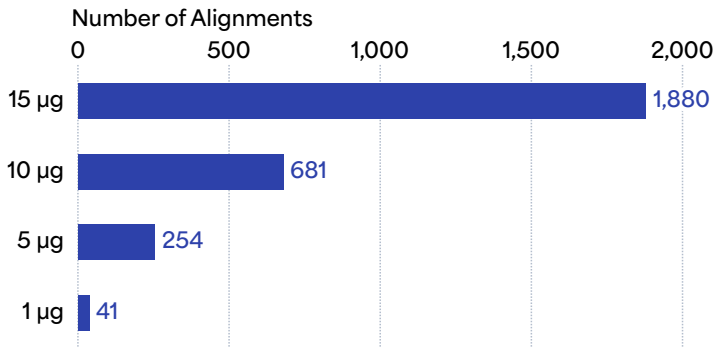

FIGURE 3

Bar Graph Demonstrating the Number of Aligned IL-6 Peptides Found in Each Immunoprecipitated Serum Sample from a Full-Chip Platinum Run. The number of aligned reads increases as a function of the starting input concentration of the protein target. It should be noted that only the 15-µg sample input is expected to be close to the minimum suggested input material for library preparation.

CONCLUSION

In this application note, we presented an immunoprecipitation workflow using a biotinylated antibody coupled to streptavidin agarose to enrich proteins from a biological matrix for next-generation protein sequencing. A key aspect of this workflow was the development of an on-bead digestion procedure that was compatible with Quantum-Si's downstream library preparation workflow. The protocols presented here for on-bead digestion and library preparation of immunoprecipitated proteins offer valuable tools to explore the intricacies of more complex biological samples such as macromolecular complexes derived from various biological sources using next-generation protein sequencing.

REFERENCES

1. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects [published correction appears in Mol Cell Proteomics. 2003 Jan;2(1):50]. *Mol Cell Proteomics*. 2002;1(11):845-867. doi:10.1074/mcp.r200007-mcp200
2. Jones SA, Takeuchi T, Aletaha D, Smolen J, Choy EH, McInnes I. Interleukin 6: The biology behind the therapy. *Considerations in Medicine*. 2018 Nov 1;2(1):2-6.