

# Peptide Barcodes for Next-Generation Protein Sequencing™

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## INTRODUCTION

Barcoding technologies that take advantage of the high information content that can be encoded in oligonucleotide sequences have enabled a wide array of applications in biotechnology, particularly when coupled with next-generation DNA sequencing (NGS) to decode this information in a high-throughput, cost-effective manner. For example, DNA barcoding combined with NGS read-out is employed to track sample identity in multiplexed libraries, provide single-cell or spatial resolution in transcriptomic studies, and to track the enrichment of genotypes in directed evolution methods for protein engineering.<sup>1-3</sup> Peptide barcodes, with their capacity to encode extensive information in short sequences, easy genetic encoding, and chemical versatility, present cutting-edge opportunities. Applications of peptide barcoding that use mass spectrometry for decoding, such as nanobody screening based on flycodes,<sup>4</sup> have been developed. However, there remains a critical need for accessible methods to directly read peptide barcode sequences and to identify peptide barcodes with single-molecule resolution. Next-Generation Protein Sequencing™ (NGPS) on Quantum-Si's Platinum® instrument offers researchers the ability to directly sequence peptide barcodes with single-molecule resolution for the first time. This user-friendly benchtop platform combines the ease-of-access of DNA-based methods with the exciting, innovative capabilities of peptide-based barcoding approaches.

Quantum-Si's workflow for real-time, single-molecule protein sequencing begins with attachment of peptides to macromolecular linkers at the C-terminus for immobilization on a semiconductor

## 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.

chip. Following peptide immobilization, dye-labeled N-terminal amino acid (NAA) recognizers and aminopeptidases are added to the chip to initiate the sequencing run. The recognizers repetitively bind and unbind the immobilized peptides when their cognate NAAs are exposed at the N-terminus. This activity generates a distinct series of pulses, termed a recognition segment (RS), for each recognized NAA with characteristic fluorescence and kinetic properties. Aminopeptidases in solution sequentially remove individual NAAs, exposing subsequent residues for detection. This dynamic process repeats until the peptide has been completely sequenced. The temporal order of NAA recognition and associated kinetic properties over the time course of sequencing are highly characteristic for a given peptide and are termed its kinetic signature. Kinetic signatures are analyzed with Platinum Analysis Software to provide high confidence alignments to individual peptide sequences.<sup>5</sup>

Here, we present a novel protein barcoding approach using NGPS on Platinum. We designed and sequenced a set of synthetic peptides to demonstrate that they can be used as distinguishable barcodes. Statistical analysis of these sequences allowed us to establish criteria for peptide barcode design and scalability estimation. We then developed a method for enzymatic library preparation of recombinantly expressed peptide barcodes and demonstrated the relative quantitation of a mixture of peptide barcodes on Platinum. Finally, we demonstrated the use of peptide barcodes to select for proteins with characteristic properties. In this experiment, we observed a 300-fold enrichment in the relative abundance of an anti-GFP nanobody after positive selection using peptide barcodes enzymatically cleaved from the enriched nanobody. This application note provides criteria for peptide barcode design, methods for generating peptide barcode libraries, and example applications that illustrate how peptide barcodes can be used for screening proteins with desired properties.

## METHODS

### SYNTHETIC PEPTIDE PRODUCTION

Peptide barcodes were synthesized by Innopep using solid-phase peptide synthesis. All peptides were prepared with an azido-lysine

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modification at the C-terminus. Peptides were confirmed to be 95% pure by HPLC and mass spectrometry analysis.

## BARCODE EXPRESSION AND PURIFICATION

Each peptide barcode was cloned into a plasmid containing an N-terminal HaloTag, a TEV protease site, a SUMO Tag, the peptide barcode sequence, a Sortase A recognition motif (LPETGG), and a C-terminal 6x-Histidine tag. Following cloning, the barcode plasmids were transformed into SHuffle T7 express competent *E. coli* (New England Biolabs, Catalog No. C3029J) and cultivated overnight on the appropriate antibiotic selection media. The sequence of the barcode plasmids was confirmed by Sanger sequencing. Next, 10 mL of Terrific Broth was inoculated with bacteria overnight with shaking at 37°C. Barcode expression was induced with the addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at OD 0.6–0.8. The bacteria were harvested by centrifugation, and the resulting pellet was washed with 50 mM HEPES pH 7.3, 150 mM NaCl, and stored at –80°C until ready for purification.

For purification of the peptide barcodes, the thawed cell pellets were resuspended in 0.2 mL of 50 mM HEPES, pH 7.3, 150 mM NaCl. Cells were lysed by adding 1 mL of NEB Express lysis buffer and mixing for 30 min at room temperature. The bacterial lysate was then centrifuged at 10,000 x g for 10 min to pellet cell debris, and the supernatant was collected for purification. Next, 200  $\mu$ L of Ni-NTA resin was pre-equilibrated by washing 3x with 50 mM HEPES, pH 7.3, 150 mM NaCl, 10 mM imidazole buffer. The bacterial supernatant was diluted with 1 volume of 50 mM HEPES, pH 7.3, 150 mM NaCl, 10 mM imidazole buffer, and the supernatant solution was applied to the pre-equilibrated Ni-NTA resin and incubated for 30 min at room temperature with mixing. The resin was then pelleted by centrifugation at 1,000 x g for 2 min, and the supernatant was collected. Next, the resin was washed 3x with 400  $\mu$ L of 50 mM HEPES, pH 7.3, 150 mM NaCl, 10 mM imidazole buffer. Elution was performed by adding 500  $\mu$ L 50 mM HEPES, pH 7.3, 150 mM NaCl, and 300 mM imidazole buffer to the resin, followed by 5 min of incubation at room temperature. The resin was then pelleted by centrifugation at 1,000 x g for 2 min.

## **SORTASE A REACTION**

For the Sortase labeling reaction, 10 µg of purified barcode peptide was introduced into a solution containing 1 µM of Sortase A pentamutant (BPS Biosciences), 1 mM of a synthetic tri-glycine-azide (GGG-azide) peptide (Click Chemistry Tools), and 1X Sortase Buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl<sub>2</sub>). The final reaction volume of 50 µL was incubated for 1 hr at 37°C to facilitate the incorporation of the GGG-azide motif at the C-terminus of the barcode peptide.

For the purification of the azide-labeled peptide barcode product, 20 µL of Magne HaloTag beads (Promega) were used per peptide barcode. The HaloTag beads were first washed 4x for 5 min with 200 µL of HEB Buffer (HEPES 50 mM pH 7.3, 0.005% IGEPAL CA-630, NaCl 150 mM) using a magnetic stand for bead collection during the wash steps. Next, 50 µL of HEB buffer was added to the 50 µL Sortase reaction (1:1), and the combined solution was added to the beads. This mixture was incubated with end-over-end mixing for 1 hr at room temperature. After incubation, the beads were magnetically separated, and the flow-through was collected for SDS-PAGE analysis. Next, the beads were washed 3x for 5 min with end-over-end mixing using HEB buffer. The beads were then stored in the final wash buffer until ready to proceed to the click chemistry reaction.

## **CLICK CHEMISTRY REACTION**

The click reaction was performed with the azide-labeled peptide barcodes still attached to the beads. After the supernatant solution was removed from the beads, 22 µL of HEB buffer was introduced. Next, 0.5 µL of Cetyltrimethylammonium bromide (CTAB) and 1 µL of K-Linker from the Library Preparation Kit - Lys-C (Quantum-Si, Cat. No. 910-00012-00) were added to the mixture. The reaction was allowed to proceed overnight at 37°C in a Thermomixer (Eppendorf) at 1,400 rpm.

The next day, the beads were washed 3x with HEB buffer, as previously described. To elute the peptide barcodes from the HaloTag, 25 µL of HEB Buffer supplemented with 1 mM DTT, and 1 U of Sumo Protease (ThermoFisher) was added to each peptide barcode. The reaction was incubated for 1 hr at 37°C at 1,400 rpm in a Thermomixer. The beads were then collected using the magnetic stand, and the supernatant containing the eluted peptide barcode

conjugated to the K-Linker was retrieved. The concentration of the eluted peptide barcodes was determined by UV-Vis absorbance and gel densitometry.

## NANOBODY SELECTION

The nanobodies targeting MBP (Sb\_MBP#1) and GFP (LaG-16) have been previously characterized.<sup>6,7</sup> These constructs were inserted between the TEV protease site and the SUMO Tag in the peptide barcoding plasmid described above. Specifically, the MBP nanobody was fused to Barcode A (RLIFAA), and the GFP nanobody was fused to Barcode B (FLRAA). The barcoded nanobodies were then expressed at 22°C in *E. coli*, purified, functionalized with Sortase A, and conjugated via GGG-azide moiety with the macromolecular K-Linker for peptide immobilization on chip after proteolysis. A pre-selection sample fraction of this library was stored for sequencing on Platinum.

The barcoded model nanobody library underwent affinity purification selection using commercially purchased GFP (Sigma-Aldrich) immobilized on M-280 tosylactivated Dynabeads (ThermoFisher), adhering to manufacturer's instructions. Briefly, the barcoded nanobody library (10 nM) was incubated with the GFP-coated Dynabeads for 5 min at room temperature in 50 mM Tris pH 7.5, 0.5 % Tween-20. Subsequently, the beads were magnetically separated and washed 5x with buffer. Barcodes from the post-selection sample associated with the nanobodies that remained on the beads after washing were then harvested by proteolysis, resulting in a nanomolar barcode solution ready for NGPS.

## NEXT-GENERATION PROTEIN SEQUENCING ON PLATINUM

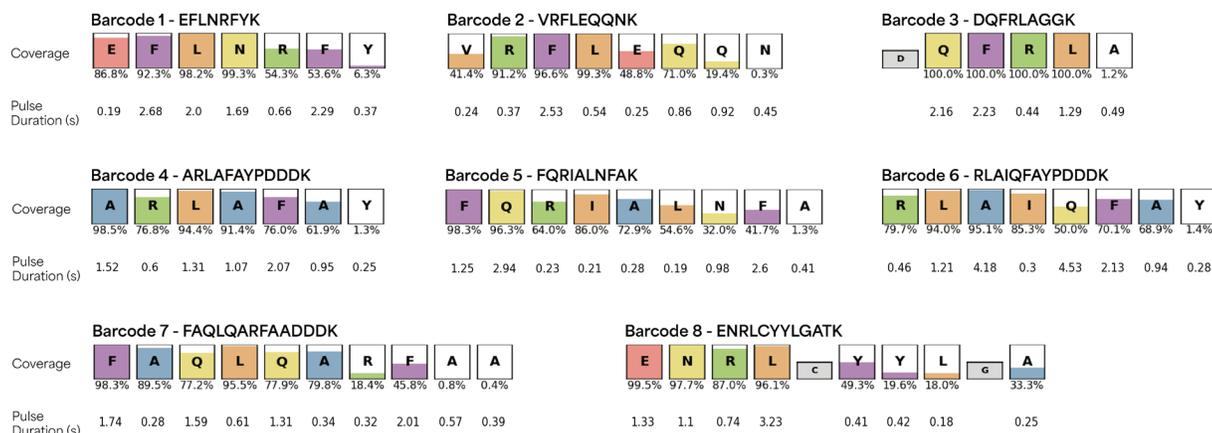
Single-molecule sequencing of peptide barcodes was performed according to Quantum-Si's user protocols. Briefly, conjugated peptide libraries were loaded onto Quantum-Si's semiconductor chip (Catalog No. 910-00011-00) via 15 min incubation. A solution containing dye-labeled recognizers was prepared using Quantum-Si's Sequencing Kit (Catalog No. 910-00011-00) and added to the chip. The chip was then installed in the Platinum instrument (Catalog No. 910-10904-00) and data was collected for 15 min. Next, a solution containing aminopeptidases from Quantum-Si's

Sequencing Kit was added to the chip with mixing and data was collected for 10 hours. Following completion of sequencing runs, data was analyzed using Quantum-Si's automated Platinum Software Analysis pipeline.

## RESULTS & DISCUSSION

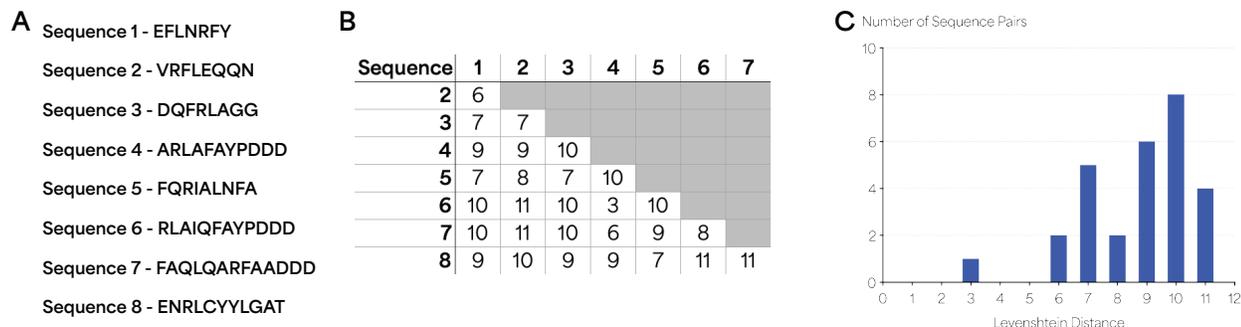
### SHORT PEPTIDE SEQUENCES CAN BE USED AS BARCODES ON PLATINUM AND ALLOW BUILDING DIVERSE BARCODE SETS

Data from previously sequenced proteins was evaluated, and the following peptide sequences were identified as candidate peptide barcodes: EFLNRFY, VRFLEQQN, DQFRLAGG, ARLAFAYPDDD, FQRIALNFA, RLAIQFAYPDDD, FAQLQARFAADDD, and ENRL-CYYLGAT. To test whether this group of peptides had the required kinetic and sequence properties to enable use as a barcode set, we generated synthetic peptides and analyzed the sequencing performance of these synthetic peptides individually. Kinetic signature plots for the 8 different peptides are shown in **Figure 1**. The signals from each of these runs were also aligned to the entire set of 8 peptides to measure the false discovery rate (FDR), defined as the fraction of off-target alignments for a given peptide. The maximum FDR of all 8 peptides was 0.2%. These results highlight the strength of Platinum's NGPS in generating distinct patterns of RSs with characteristic kinetic properties that enables the detection of each peptide barcode with high-confidence using Quantum-Si's NAA recognizers.

**FIGURE 1**

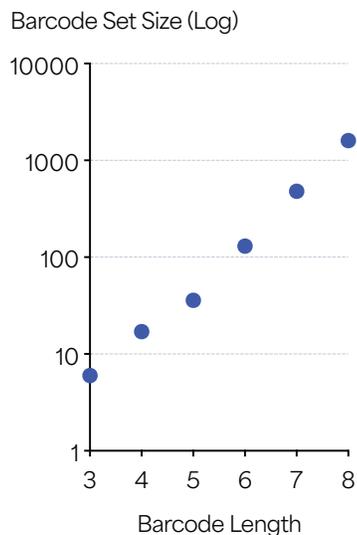
A set of peptide barcodes displaying unique patterns of RSs and on-chip pulse durations, enabling accurate identification with Platinum. The color fill in each box represents the total percent coverage of observed amino acids in each peptide sequence with the numerical value reported under each box. The mean pulse duration for each observed residue, measured in seconds, is given under each peptide sequence coverage box.

Having characterized each peptide individually on Platinum, we next sought to establish that these peptides are distinguishable from one another when sequenced together in a mixture. The Levenshtein distance (**L**) is a common measure of sequence similarity in bioinformatics. It is defined as the minimum number of edit operations (insertions, deletions, and substitutions) needed to transform one sequence into another.<sup>8</sup> For our set of eight sequences, we computed the Levenshtein distance for every pair of sequences and observed  $L \geq 3$  for all pairs, with a mean  $L = 8.7$  (**Figure 2**). This analysis, combined with the distinct kinetic signatures and low FDRs observed in NGPS, suggested that  $L \geq 3$  in all pairwise comparisons is a suitable threshold to generate barcode sets with highly distinguishable sequences. We used this threshold next to estimate how the sizes of peptide barcode sets could be increased.

**FIGURE 2**

Levenshtein distance analysis of peptide sequences. (A) Sequences of peptides used in the Levenshtein distance analysis. (B) Levenshtein distance values for all pairs of sequences, showing  $L \geq 3$  for all pairs. (C) Histogram of orthogonal pairwise Levenshtein distances between all members of the barcode set.

Many applications require extension of a barcode set to hundreds or even thousands of sequences. To assess the scalability of peptide barcodes on Quantum-Si's Platinum, we constructed barcode sets in silico with  $L \geq 3$  for 6-amino-acid-character sets (L, F, R, N, A, and E), ranging in length from 3 to 8 residues, while restricting repetitive sequences to ensure an amino acid could only repeat every 5 residues. We successfully constructed barcode sets ranging from 6-member sets for 3-residue barcodes to 1,600-member sets for 8-residue barcodes (**Figure 3**). Depending on the specific application, one could consider other design parameters to align with the desired throughput, sensitivity, and accuracy. These findings underscore the scalability of peptide design needed for applications requiring thousands of unique barcodes.

**FIGURE 3**

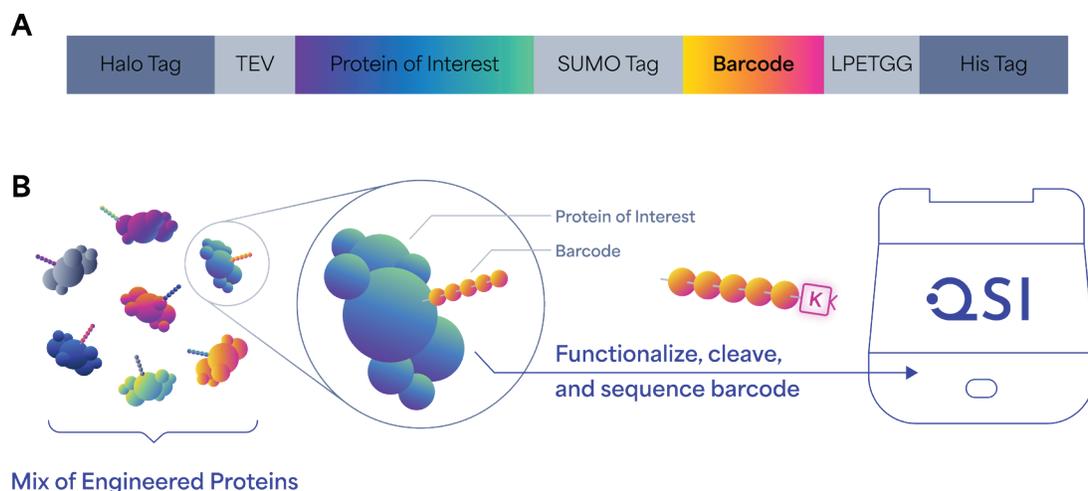
Predicted barcode set sizes at  $L > 3$  for barcode length of 3-8.

### PEPTIDE BARCODES CAN BE FUSED TO FUNCTIONAL ELEMENTS, RECOMBINANTLY EXPRESSED, AND SEQUENCED IN MIXTURES

The previous section discussed sequencing libraries based on C-terminal azido-lysine modified synthetic peptides. To assess their performance when translated, we prepared libraries from barcode peptides expressed recombinantly in *E. coli*. The standard Quantum-Si library preparation workflow involves digesting samples with endoproteinase LysC, producing peptides containing C-terminal lysine residues for conjugation to the K-linker. While this digestion process is necessary for proteomics workflows in which the production of multiple peptides from within the same protein is typically necessary for high-confidence identification of the target, barcoding applications require only the recovery of a single barcoded peptide sequence to identify the associated protein of interest. As such, digestion of samples that contain multiple proteins or protein variants may result in the production of an excess of non-barcoded peptides to barcoded ones. If both barcode and non-barcode peptides are subsequently sequenced on Platinum, this will significantly reduce the detection sensitivity of the assay as the barcode peptides will represent only a minor portion of the available peptides for immobilization into the reaction chambers of the semiconductor chip. We therefore developed an innovative solution to enrich for and specifically conjugate the K-linker to the barcode peptide from a complex sample.

Our novel library preparation approach utilized a Sortase A enzyme mediated transpeptidation reaction<sup>9</sup> (**Figure 4A**). This reaction employs a two-step mechanism in which Sortase A initially catalyzes the cleavage of a substrate, a short C-terminal LPETGG motif, between the threonine and glycine residues via a thioacyl intermediate. The intermediate is then resolved via nucleophilic attack in trans from a glycine containing substrate, resulting in ligation via amide bond formation. To apply this approach in our workflow, we appended the LPETGG motif immediately C-terminal to the peptide barcode sequence. In addition, we used a Picolyl-Azide-Gly-Gly-Gly tripeptide to introduce the required nucleophilic attack. This substrate also serves a dual role in the reaction by providing the azide functional group for the subsequent click chemistry reaction to the K-linker.

With the Sortase A mediated transpeptidation enabling the digestion-free conjugation of barcode peptides, our next task was to develop a method to specifically enrich and elute the peptide barcodes from a co-expressed protein of interest, as digestion is no longer part of the workflow. We addressed this by incorporating a SUMO tag at the N-terminus of the peptide barcode sequence. After the sample is enriched via an affinity tag or a selection method, the SUMO tag enables precise cleavage of the barcode sequence from the protein coding sequence using the SUMO protease ULP1. The enzymatic cleavage results in a free peptide barcode starting at the desired NAA of the peptide sequence. When combined with the Sortase A transpeptidation approach, this method establishes an enzymatic library preparation workflow that specifically elutes K-linker conjugated peptide barcodes from enriched or selected samples, making them ready for sequencing on Platinum.

**FIGURE 4**

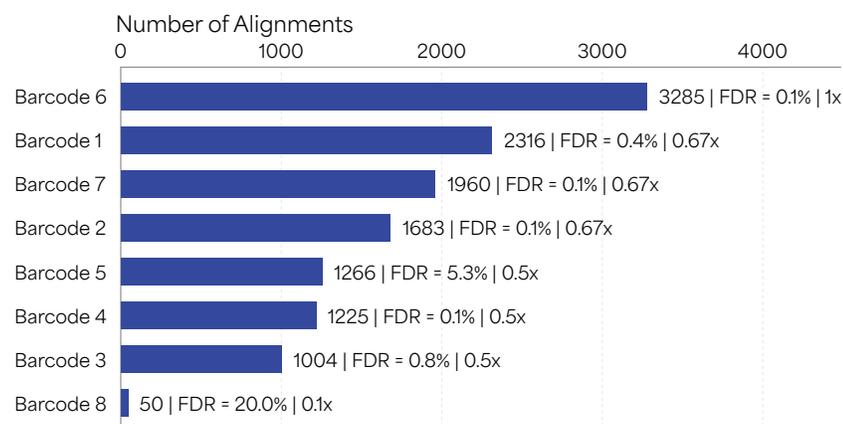
Design, generation, and sequencing workflow of barcoded protein libraries. (A) Schematic of barcode construct design, which includes a HaloTag for capturing on magnetic beads, TEV protease site to cleave the protein of interest with the barcode, a SUMO tag to specifically recover barcodes, a Sortase A recognition motif (LPETGG) for the enzymatic ligation of GGG-N3 peptide onto barcodes, and the His tag used for Ni-NTA/Talon purification of barcoded protein with all the tags. (B) Schematic representation of the generation of barcoded protein libraries. Barcoded protein libraries are expressed *in vitro* or *in vivo* and subject to screening or selection. Barcodes are then cleaved and conjugated to K-linker before sequencing on Platinum.

We next tested this fully enzymatic library preparation workflow on barcodes recombinantly expressed in *E. coli*, with the goal to demonstrate that these barcodes can be identified with minimal False Discovery Rate (FDR) in a controlled mixture of peptide barcodes with different ratios. We prepared and sequenced an 8-peptide barcode mixture library in which each peptide barcode was added to the mixture at the indicated relative amounts shown in **Table 1**. As seen in **Figure 5**, each of the peptide barcodes was identified in the mixture using NGPS, with the number of alignments decreasing with relative abundance. 7 out of 8 peptides in the mix yielded FDR values of < 10%. Of these 7 peptides, 6 yielded FDR values of < 1%, and one with ~5% FDR. This result demonstrates the capability of peptide barcodes to undergo recombinant expression, purification, and sequencing on Platinum.

	Relative Abundance
Barcode 6	1x
Barcode 1	0.67x
Barcode 7	0.67x
Barcode 2	0.67x
Barcode 5	0.5x
Barcode 4	0.5x
Barcode 3	0.5x
Barcode 8	0.1x

**TABLE 1**

Relative abundance of barcodes.

**FIGURE 5**

Sequencing results on Platinum of an 8-peptide barcode mixture library. Each peptide barcode was added to the mixture at controlled relative amounts. The metrics at the end of the bars represent Number of Alignments | FDR | Relative Abundance. For example, Barcode 1 was added to the mixture at a relative abundance of 0.67x, yielding 2,316 alignments with an FDR of 0.4%.

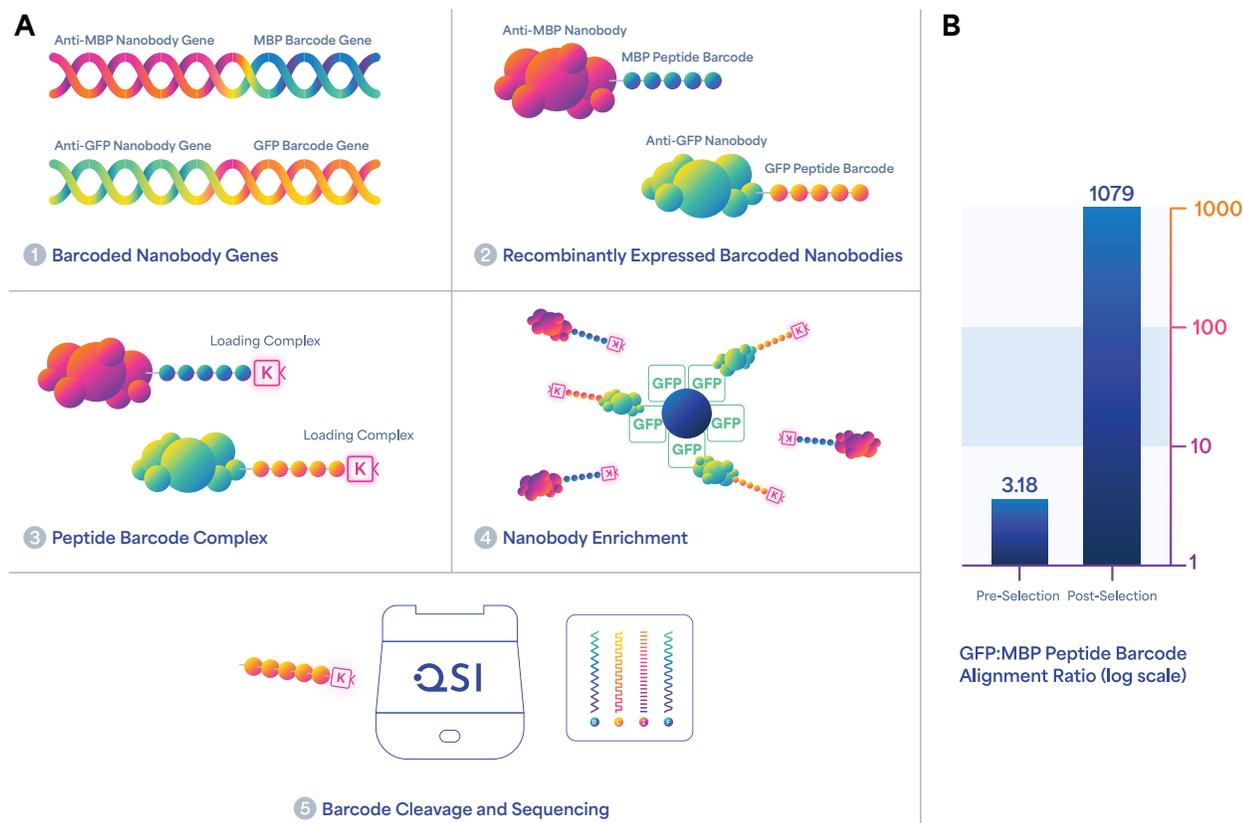
## NANOBODY ENRICHMENT SCREENING BY SINGLE-MOLECULE PEPTIDE SEQUENCING

Next, we sought to demonstrate the application of Quantum-Si's peptide barcodes in a differential enrichment assay. We focused on nanobodies: single-domain antibodies from camelids that have

gained prominence in various applications, such as targeted drug therapy.<sup>10</sup> Nanobodies are often selected from large synthetic libraries via ultra-high-throughput methods such as ribosome or phage display. However, these methods have limitations due to the requirement to maintain the phenotype-genotype linkage. By removing this constraint, peptide barcodes offer a streamlined solution for the screening of nanobody variants.

In our experiment, we used a model library with two nanobodies, an anti-MBP Nanobody (targeting maltose binding protein, MBP) and an anti-GFP nanobody (targeting green fluorescent protein, GFP).<sup>6,7</sup> At the C-terminus, the nanobodies were fused with MBP peptide barcode and GFP peptide barcode, respectively (**Figure 6**). The barcoded nanobodies were then prepared for sequencing based on the targeted sequencing approach described in the previous section. During the affinity selection process, the bar-coded nanobody library was added to a solution with GFP-coated magnetic beads. Subsequently, the peptide barcodes were proteolytically eluted from the nanobodies bound to the beads. The post-selection library was then sequenced on Platinum alongside a pre-selection control library, and we compared the relative number of each barcode alignment pre- and post-selection.

As seen in the graph in **Figure 6**, the ratio of GFP:MBP peptide barcode alignments was approximately 3.18:1 in the pre-selection control library. In the post-selection library, this ratio increased to 1079:1, indicating approximately 300-fold enrichment of the anti-GFP nanobody over the MBP nanobody. This result demonstrates the ability to measure the differential enrichment of nanobody clones in a library through direct single-molecule sequencing of protein-associated barcodes. This experiment also demonstrates the advantage of targeted sequencing in specific applications. Shotgun sequencing of these two nanobodies would have produced 11 individual digested peptides (9 from the two nanobodies + the two barcodes). In contrast, our targeted sequencing approach, which increases barcode sequencing capacity approximately 5-fold, was applied to two small proteins (15 kDa each). This effect would be even more pronounced in applications targeting larger proteins of interest, in which shotgun sequencing would produce substantially more total peptides.



**FIGURE 6**

Workflow for enrichment and sequencing of barcoded nanobodies. (A) Schematic representation of the differential enrichment of an anti-GFP nanobody by peptide barcoding. Model anti-GFP and anti-MBP nanobody genes were encoded with unique peptide barcodes and expressed recombinantly. The proteins were purified, labeled with an azide tag, and conjugated to macromolecular linkers. The nanobodies were enriched using GFP immobilized on magnetic beads. After selection, the barcodes were eluted by proteolysis and sequenced on Platinum. (B) Bar graph quantification of pre- and post-selection barcoded nanobody libraries indicating a > 300-fold enrichment for the anti-GFP nanobody post-selection based on the ratio of GFP to MBP peptide barcode alignments.

## CONCLUSION

Peptide barcodes hold immense promise across various fields such as proteomics, synthetic biology, protein engineering, and drug discovery. In this application note, we demonstrated the development and utilization of peptide barcodes with NGPS on Platinum. Our work reveals that short peptide sequences yield information-rich and distinctive kinetic signatures that can be

accurately recognized. Moreover, our optimized peptide barcode designs ensure reliable relative quantification. The introduction of an enzymatic approach to targeted sequencing allows barcodes to be easily and specifically retrieved from complex matrices such as an *E. coli* lysate, a crucial capability for future applications. To explore the applicability of peptide barcodes to nanobody engineering, we used peptide barcodes to reliably monitor the enrichment of an anti-GFP nanobody from a model selection. By offering accessible and user-friendly instrumentation for decoding peptide barcodes, Quantum-Si's Platinum stands ready to facilitate widespread adoption and maximize the potential impact of peptide barcodes, paving the way for a new era of innovation in proteomics and beyond.

# QUANTUM SI™

29 Business Park Drive, Branford, CT 06405

[www.quantum-si.com](http://www.quantum-si.com) | 866.688.7374