Quantum-Si's Innovative Protein Barcoding Enhances Next-Gen Screening for mRNA Therapeutics

HIGHLIGHTS

Direct protein detection: Quantify expression and differentiate functional performance of proteins

Multiplexing power: Test multiple candidates in a single model organism with up to **24×** the power of single-plex analysis

Cost-effectiveness: Enables faster, more accurate, and cost-effective mRNA therapeutic development by overcoming traditional bottlenecks in LNP delivery screening

Analytical validity: Detect low-abundance proteins and differentiate expression changes across up to 120-fold dynamic range for a half chip and 240-fold dynamic range for a full chip

WORKFLOW OVERVIEW

Generate mRNA-LNP candidates with protein barcodes

Deliver into cells or animal models

Extract cells or tissues

Enrich, functionalize, and cleave barcoded proteins (<6 hours)

Immobilize barcode libraries on the sequencing chip

Sequence using Quantum-Si Platinum® Pro

Quantify barcode abundance (expression levels)

INTRODUCTION

Nucleic acid-based therapeutics, including antisense oligonucleotides (ASO), small interfering RNA (siRNA), messenger RNA (mRNA), immunomodulatory DNA/RNA, and gene-editing guide RNA (gRNA), are promising because they can treat diseases by targeting their genetic information directly *in vivo*. These treatments can provide long-lasting or even curative effects by inhibiting, adding, replacing, or editing genes.

However, the success of these therapies in patients relies on delivery technologies that enhance their stability, facilitate internalization, and increase their ability to target the right tissue and cells.[1-3]

Lipid nanoparticles (LNPs) are a meaningful new way to deliver nucleic acid-based therapeutics, especially mRNA vaccines and gene therapies. These LNPs are stable in physiological fluids, have reduced immunogenicity, and can effectively reach cells where the therapeutic material is released. Their fusion with endosomal membranes protects nucleic acids from being broken down by enzymes during delivery.[4-6]

LNP therapeutic applications have been primarily limited to liver indications, where LNPs accumulate when administered systemically.[7] Hence, the discovery and development of mRNA vaccines and other nucleic acid-based therapeutics that target organs other than the liver are of high interest.[8] However, these often involve screening various nucleic acid and LNP materials and formulations to identify candidates that are delivered to and produce high levels of therapeutic protein within the correct tissue. This often involves screening many candidate mRNA-LNPs in animal models, a bottleneck for LNP discovery.

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This technical note introduces an innovative approach utilizing the protein barcoding application on the Quantum-Si Platinum® Pro Next-Generation Protein Sequencer.™ This method enhances and accelerates mRNA-LNP *in vitro* and *in vivo* screening by providing a cost-effective, scalable, multiplexed solution with single-molecule resolution. It addresses inefficiencies in existing workflows and allows for deeper insights into proteomics.

mRNA-LNP WORKFLOW: KEY STEPS AND CHALLENGES

The workflow for creating and screening mRNA-lipid nanoparticles (LNPs) will vary depending on the specific assay needs but generally involves the following steps:[8, 9]

1. **Select, synthesize, and characterize the mRNA payload:** Confirm the mRNA sequence after synthesis, determine purity, and characterize 5' capping and 3' poly(A).
2. **Select and formulate lipid components:** To achieve maximum efficacy, each LNP formulation must be optimized for specific biophysical characteristics such as shape, size, payload density, and surface charge.
3. **Select the screening method:** Select *in vitro* (2D or 3D cell culture) or *in vivo* (animal models) methods.
4. **Select the screening readout:** Fluorescence or next-generation sequencing, depending on the reporter used in the mRNA construct.
5. **Encapsulation of the mRNA into the LNP:** The purified mRNA is combined with the lipid nanoparticle solution, allowing the mRNA to be encapsulated within the LNP's hydrophobic core.
6. **Quality control and characterize the final product:** Perform analytical tests to assess the size, stability, encapsulation efficiency, and purity of the mRNA-LNP formulation.
7. **Perform the screen:** Based on the method and readout selected.
8. **Draw conclusions:** Identify the top candidates from the screen and feed the results back into mRNA and lipid formulations for further studies and optimization.

Various tools are available to support nanoparticle design, formulation, and quality control (QC) (steps 1-6). However, the screening step (step 7) still faces limitations with current methods, which include next-generation sequencing (NGS), mass spectrometry, flow cytometry, ELISA, and western blotting.[10-15]

For instance, using NGS for screening is efficient because it allows for the analysis of multiple mRNAs in a single animal. A significant limitation of this approach is that it measures delivery – such as the presence of mRNA – rather than translation.[13, 14] This can lead to inaccurate results and erroneous candidate selection.

On the other hand, screening that uses protein-level readout, such as mass spectrometry (MS) or flow cytometry, addresses the expression issue.[15, 16] However, limited tools are available for multiplexing at the protein level, which increases costs and reduces efficiency. In the case of flow cytometry, this also results in low sensitivity.[15] Therefore, new tools that facilitate multiplex pooled screening with a direct protein-level readout could substantially improve mRNA-LNP screening.

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SCREENING mRNA DELIVERY AND EXPRESSION USING PROTEIN BARCODING ON PLATINUM

The concept of protein barcoding has been explored in the past, but it has not gained widespread adoption due to the significant technical and operational challenges associated with traditional proteomics techniques.[17-19] Quantum-Si has developed the Quantum-Si Barcoding Kit, an innovative, cost-effective, and user-friendly technology that, when combined with the Platinum Pro protein sequencer, has the potential to make barcoding-based methods more accessible to a broader audience.

The general approach involves designing protein barcodes, which are genetically encoded short amino acid sequences that can be added to protein-coding DNA. These barcodes are crafted to be highly distinguishable and sequenceable, allowing the sequencing algorithm to easily differentiate between candidates.[20] Quantum-Si's Barcoding Kit has been validated for up to 24 peptide barcodes; each designed to minimize detection bias and maximize sensitivity across various experimental conditions. When co-expressed with a purification tag and cleavage sites, these barcodes facilitate the release and sequencing of only the barcode of interest. This enables the selection of proteins for engineering, therapeutic delivery, and screening applications. Once proteins are selected, unique barcodes associated with each expressed protein can be identified with single-molecule resolution using Next-Gen Protein Sequencing™ (NGPS™)[20] on the Quantum-Si Platinum Pro benchtop protein sequencer (Figure 1). The barcodes can be detected at a sample input concentration of 25 pmol within the 24-plex mixture and at 5 pmol for individual barcodes, enabling high sensitivity for multiple applications.[20]

The potential applications of this technology extend across various research areas. Notably, it has attracted considerable interest in the field of mRNA-LNP research as a way to expedite the development of next-generation lipid nanoparticle (LNP) delivery systems. Current methods primarily detect delivery but do not directly assess expression (for example, next-generation sequencing or NGS). In contrast, the Platinum Barcoding Kit offers the ability to conduct multiplex pooled screens with direct protein-level readouts, addressing a significant challenge existing technologies face. As a result, Platinum Pro could facilitate rapid high-throughput screening, both *in vitro* and *in vivo*, for a wide range of nanoparticle chemistries and formulations. This advancement allows for the quicker identification of cargo and delivery systems with unique targeting capabilities for extrahepatic tissues, surpassing the speed of conventional methods.

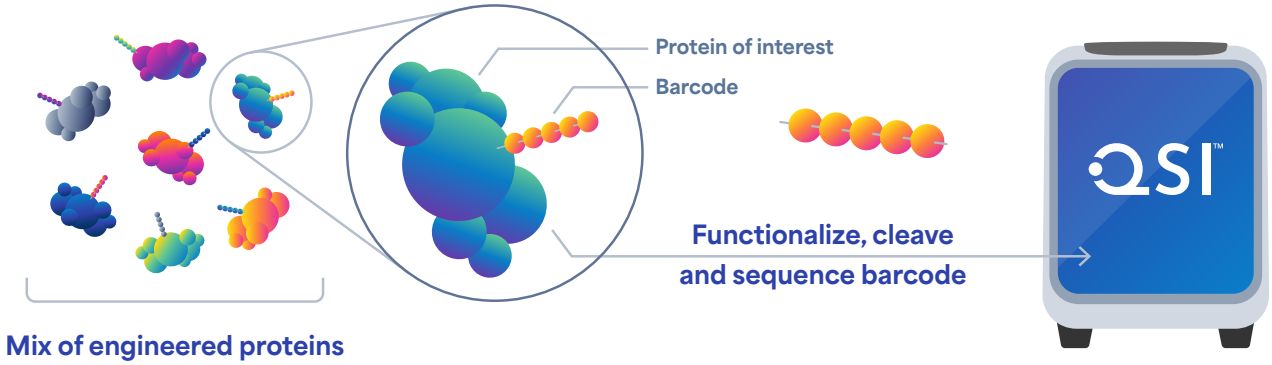

Figure 1: A schematic of engineered proteins expressing barcodes mixed together, functionalized, cleaved, and sequenced on the Quantum-Si Platinum Pro instrument.

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A

mRNA-FLAG Tag-GS Linker-LysC Cleavage-Barcode-Sortase Tag-His Tag

mRNA-DYKDDDDK-GGGGSGGGGS-K-RFEQIANFAE-LPETGG-HHHHHH

B

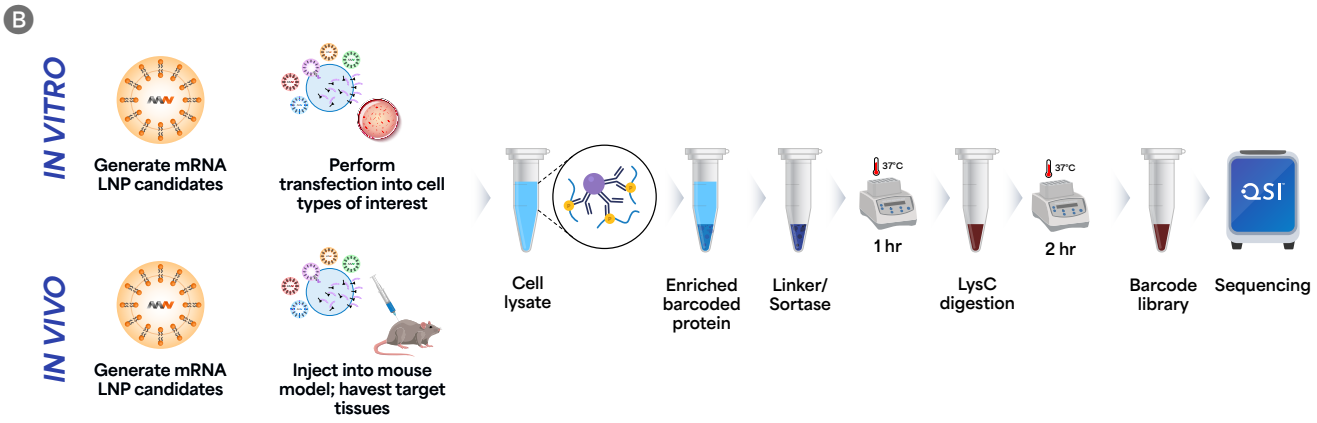

Figure 2: Overview of the Platinum barcoding design and workflow (A) Barcode construct design (B) A schematic of the mRNA-LNP delivery method using peptide barcodes.

BARCODE DESIGN

Quantum-Si has optimized barcode constructs to enable the efficient enrichment and recovery of barcodes customized for the gene of interest. Various experiments can be performed to confirm the sequenceability of our barcodes, assay sensitivity, and the limit of detection.

Barcode constructs were designed to enrich barcoded proteins expressed in cells or tissues efficiently. These constructs utilize readily available affinity tags for a single-step reaction and cleavage that can be completed in less than four hours. Each construct (Figure 2A) includes an N-terminal FLAG tag, a poly(GS) linker, the protein barcode sequence, a sortase recognition motif (LPETG), and an optional C-terminal *His* tag.[19]

In mRNA-LNP research, protein barcodes are attached to mRNA constructs, which are then packaged into lipid nanoparticles (LNPs). These mRNA-LNP complexes can be introduced into cells or injected into animal models, where they are subsequently expressed (Figure 2B). The cells or tissues are extracted and barcoded protein enriched via the FLAG tag. Peptide barcodes are modified for immobilization on the sequencing chip through a sortase reaction and are then cleaved from FLAG beads and the protein of interest using LysC digestion.

The recovered protein libraries can either be loaded for sequencing or stored at -20°C. The Platinum Barcoding Kit provides the necessary reagents to prepare peptide barcode libraries for sequencing using the Platinum Pro benchtop protein sequencer.

ORDERING INFORMATION

Product	Catalog number
Quantum-Si Barcoding Kit	910-00047-01
Protein Sequencing Kit V4	910-00038-04
Platinum Pro Next-Gen Protein Sequencer	910-10033-00

Find out more at quantum-si.com/barcoding

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PROOF OF CONCEPT USING BARCODES TO DETECT mRNA-LNP

To demonstrate the feasibility of using protein barcodes to screen for the delivery, uptake, and expression of specific mRNA constructs, we tested mRNA constructs encoding protein targets that were coupled to barcodes and delivered via LNPs *in vitro* and *in vivo*.

DETECTION OF mRNA-COUPLED BARCODES *IN VITRO*

An mRNA construct encoding the p58 protein and a unique barcode was encapsulated in a LNP and transfected into HEK293 cells. After the delivery, cell lysates were prepared, and the target protein was purified. Subsequently, the barcodes were cleaved and sequenced using NGPS.

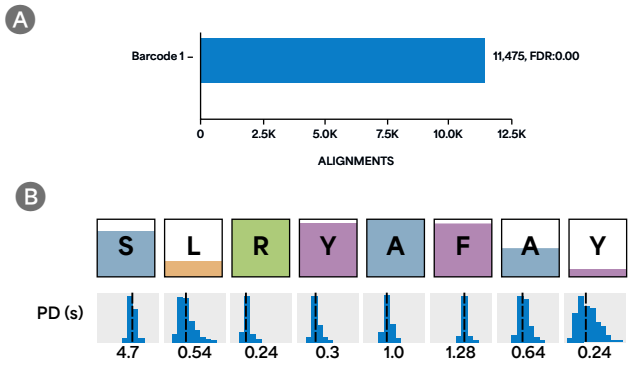

Figure 3: Sequencing results of a barcode identified from HEK293 cells. (A) The barcode was identified with high confidence. The metrics at the end of the bars represent the number of alignments and false discovery rate (FDR). FDR less than 10% indicates high-confidence barcode detection. (B) Kinetics summary plot for the recovery of a barcode by Platinum protein sequencing. PD = pulse duration. The kinetic properties are used by the Platinum Analysis software to determine the identity and order of amino acids detected during sequencing.

Upon analyzing the cell lysate from HEK293, the single barcode was identified with high confidence in the Platinum Pro sequencing data (Figure 3). No additional high-confidence alignments were generated.

DETECTION OF mRNA-COUPLED BARCODES *IN VIVO*

Equimolar amounts of short sequences encoding five unique protein barcodes, including the barcode used in the previous *in vitro* experiment, were attached to mRNA constructs that encoded the same protein. These mRNA-barcode constructs were encapsulated in LNPs of the same formulation and administered to a single mouse. The LNPs were taken up by the mouse's tissues, where the mRNAs they carried were translated into the protein featuring one of the five distinct barcodes.

Mouse spleen tissue was collected, and the target protein, containing each of the five unique barcodes as separate fusions, was purified. The barcodes were then cleaved and sequenced using NGPS on the Quantum-Si Platinum Pro instrument. The relative abundance of each barcode was quantified to determine the protein expression levels.

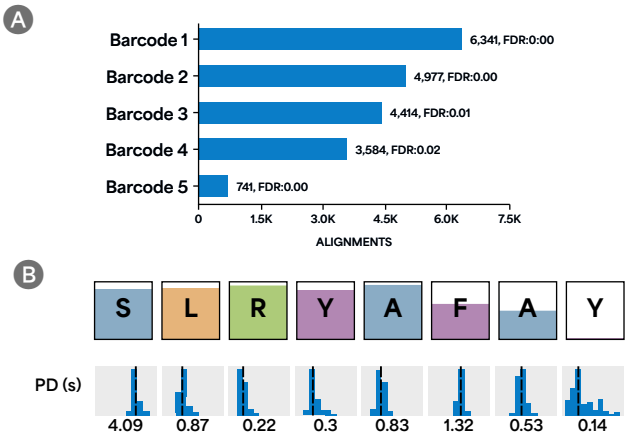

Figure 4: Sequencing results on Platinum Pro of the 5-protein-barcode mixture library. (A) The metrics at the end of the bars represent the number of alignments and false discovery rate (FDR). (B) Kinetics summary plot for the recovery of Barcode 1 by Platinum Protein Sequencing. PD = pulse duration.

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Figure 4 demonstrates that all five barcodes were successfully recovered. The software generates graphs that display the barcode alignments, highlighting the reads aligned to all identified barcodes from a given run and ranking them from highest to lowest based on the number of alignments. For each barcode, the software details the number of reads that aligned successfully to the reference, with a false discovery rate (FDR) of less than 0.05, as shown in figure 4.

The initial results from the proof-of-concept experiments demonstrate the effectiveness of using protein barcodes combined with Platinum Pro single-molecule protein sequencing for multiplexed mRNA-LNP screening using *in vitro* and *in vivo* models. Further optimization is underway to enhance the balance of barcode recovery levels.

CONCLUSION

One challenge in delivering therapeutic nucleic acids via nanoparticles is their organ and cell selectivity due to various factors influencing biodistribution.[7] By combining protein barcoding with single-molecule Next-Generation Protein Sequencing, we establish a foundation for future research to enhance the targeted tracking of therapeutic cargo uptake and functional delivery to specific tissues or cells. While DNA barcodes have been used to monitor LNP uptake.[13, 14] they often fail to confirm the encoded proteins' functional delivery and activity. In contrast, protein barcodes provide direct readouts of protein expression and localization,[17-19] resulting in a more accurate approach for assessing the success of gene therapy interventions.

Additionally, NGPS offers significant advantages over mass spectrometry for decoding protein barcodes. Unlike mass spectrometry, NGPS features straightforward, user-friendly workflows that can be conducted on a compact benchtop instrument.[20] NGPS distinguishes peptides based on the recognition of specific amino acids rather than their mass-to-charge ratio.[20] This enables researchers to create short, relatively uniform, and highly sequenceable barcodes.

Protein barcoding, when used with the Platinum Pro Next-Generation Protein Sequencer, provides an effective solution to the challenges of traditional methods, making it a valuable tool in various research settings. The benefits offered by this solution include:

- **Direct protein-level readout:** Enables pooled multiplexed screening with direct measurement of protein expression levels.
- **High sensitivity and dynamic range:** Barcodes can be read with high sensitivity where the limit of detection for each barcode in the complexity of 24 barcodes are in the sub-pmol range (~104 fmol), sample input (just 1% of mixture), and on-chip about 1 fmol, enabling precise quantification of protein expression.
- **Ease of use and scalability:** The Platinum solution is user-friendly and cost-effective, making it suitable for various applications without the technical and financial barriers often associated with mass spectrometry.
- **Reduced variability:** Barcoding minimizes sample variability by pooling multiple candidates and utilizing a single readout method, resulting in more consistent and reliable screening results.
- **Cost-effectiveness:** The ability to screen multiple candidates in a single run significantly lowers the overall cost of the screening process. This aspect is crucial for *in vivo* studies, where the cost per animal can be substantial.

Platinum Pro's protein barcoding is revolutionizing mRNA *in vivo* screening by overcoming the limitations of traditional methods. Its capability to deliver direct, sensitive, and accurate protein-level readouts makes it an essential tool for researchers working with mRNA therapeutics. By improving screening efficiency and enhancing candidate selection, Platinum Pro minimizes risks and speeds up the development of effective mRNA-based therapies.

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