Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

REVIEW ARTICLE

Development of mRNA manufacturing for vaccines and therapeutics: mRNA platform requirements and development of a scalable production process to support early phase clinical trials

JILL WHITLEY, CHRISTOPHER ZWOLINSKI, CHRISTIAN DENIS, MAUREEN MAUGHAN, LEONIE HAYLES, DAVID CLARKE, MEGHAN SNARE, HONG LIAO, SEAN CHIOU, TINA MARMURA, HOLLY ZOELLER, BEN HUDSON, JOHN PEART, MONICA JOHNSON, AMELIA KARLSSON, YUNFEI WANG, CYNTHIA NAGLE, CHERELL HARRIS, DANIEL TONKIN, STEPHANIE FRASER, LIEZA CAPIZ, CHRISTINA L. ZENO, YVONNE MELI, DIANA MARTIK, DANIEL A. OZAKI, AMY CAPARONI, JASON E. DICKENS, DREW WEISSMAN, KEVIN O. SAUNDERS, BARTON F. HAYNES, GREGORY D. SEMPOWSKI, THOMAS N. DENNY, and MATTHEW R. JOHNSON

DURHAM, NORTH CAROLINA; AND PHILADELPHIA, PENNSYLVANIA

The remarkable success of SARS CoV-2 mRNA-based vaccines and the ensuing interest in mRNA vaccines and therapeutics have highlighted the need for a scalable clinical-enabling manufacturing process to produce such products, and robust analytical methods to demonstrate safety, potency, and purity. To date, production processes have either not been disclosed or are bench-scale in nature and cannot be readily adapted to clinical and commercial scale production. To address these needs, we have advanced an aqueous-based scalable process that is readily adaptable to GMP-compliant manufacturing, and developed the required analytical methods for product characterization, quality control release, and stability testing. We also have demonstrated the products produced at manufacturing scale under such approaches display good potency and protection in relevant animal models with mRNA products encoding both vaccine immunogens and antibodies. Finally, we discuss continued challenges in raw material identification, sourcing and supply, and the cold chain requirements for mRNA therapeutic and vaccine products. While ultimate solutions have yet to be elucidated, we discuss approaches that can be taken that are aligned with regulatory guidance. (Translational Research 2022; 242:38–55)

From the Duke Human Vaccine Institute, Duke University School of Medicine, Durham, North Carolina; Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

Submitted for Publication August 17, 2021; revision submitted November 22, 2021; Accepted for Publication November 29, 2021.

Reprint requests: Thomas N. Denny, Duke Human Vaccine Institute, Duke University School of Medicine, 2 Genome Court MSRB II Building, Room 4077 DUMC Box 103020, NC 27710. e-mail: thomas.denny@duke.edu.

1931-5244/$ - see front matter

© 2021 Published by Elsevier Inc.

https://doi.org/10.1016/j.trsl.2021.11.009

INTRODUCTION

Over the past ten years, substantial improvements have been made to mRNA-based vaccines and therapies to enhance protein translation, modulate innate and adaptive immunogenicity and improve delivery, especially when coupled with developments in lipid nanoparticle (LNP) technology.[1] Before the SARS-CoV-2 pandemic, there was significant progress in making mRNA-based vaccines that elicit potent immunity against infectious disease targets in animal models of influenza virus, Zika virus, rabies virus, and HIV-1.[1-3] The progress made with the Pfizer and/or BioNTech SARS-CoV-2 mRNA-LNP vaccine,[4] and a similar vaccine from Moderna,[5] have substantiated the potential of mRNA-LNP technology and have amplified the need for robust, consistent, and rapid production of mRNA to expand the evaluation of additional vaccine and therapeutic targets.[6]

There are 2 main approaches to RNA vaccines and therapeutics. The conventional non−replicating mRNA encoding the gene of interest along with 5' and 3'untranslated regions (UTR) to enhance gene expression and the self-amplifying RNA that in addition to the gene of interest, encode specific RNA virus replication genes to enable abundant intracellular RNA production.7 Both technologies utilize the host cell machinery to translate the mRNA encoded target immunogen or therapeutic protein. An important component of these RNA systems is the delivery mechanisms utilized to stabilize, protect, and target the RNA for cellular uptake and delivery to the cytosol. LNP formulations containing novel ionizable cationic lipids are the current leaders in the RNA delivery field with successful application in the delivery of small interfering RNA (siRNA), as well as mRNAs encoding vaccine antigens and therapeutic proteins.[8,9] The work described here utilizes the non−replicating mRNA technology developed by Kariko and Weissman with the incorporation of a modified nucleoside, as well as optimized 5' and 3 UTR sequences, improved codon usage, a defined poly(A) tail length, and incorporation of a cap analogue.[10] This modified mRNA system coupled with LNP delivery have demonstrated induction of T follicular helper cells and germinal center formation resulting in robust and sustained immune responses in preclinical animal models further demonstrating the necessity to develop this platform for early phase clinical evaluation.[11]

Although mRNA production is available from contract manufacturing organizations, the timelines to obtain cGMP material, as well as production and analytical release testing costs, can be prohibitive for small scale, early clinical applications. Additionally, we have found it desirable to not only have mRNA manufacturing processes that can be conducted by research teams at scales appropriate for initial animal studies, but that can then also be scaled up to phase I through commercial production scale without changing product quality attribute profiles. Several methods to purify in vitro transcribed mRNAs have been described, including LiCl precipitation, commercially available spin columns and ion pair reverse phase HPLC,[12] however, these methods are not suitably scalable, affordable, and may not be amenable to cGMP operations. In addition, utilizing different raw materials and processes at small and production scale can create differences in product quality attributes, and manufacturing process performance between scales that must be addressed in regulatory submissions pose a potential risk to clinical development.

To address the need for broadly scalable mRNA production, we have developed a platform process utilized for both lab scale needs and under GMP-conditions for early phase clinical studies. In addition, characterization and release assays have been developed to enable the production and release of early phase GMP mRNA products. Here we describe a straightforward, scalable, reproducible production and purification platform that provides mRNA with the quality, purity, and safety profile required for clinical trial use. The in vitro transcription reaction conditions were optimized using commercial reagents to provide consistent mRNA yields, while downstream operations focused on removal of process residuals and reaction byproducts such as double stranded RNA (dsRNA) using scalable filtration and chromatography systems. Analytical assays critical to support development and manufacturing activities were established and qualified to enable rapid assessment of the process operations and to release the mRNA drug substance for LNP encapsulation and fill and/or finish activities. mRNA produced by this platform is suitable for LNP encapsulation, and the resulting mRNA-LNP have demonstrated protein expression, robust immune responses, and efficacy in numerous animal studies.

PROCESS DEVELOPMENT

Design of the DHVI mRNA drug substance manufacturing platform was broken into 2 key areas of development, upstream enzymatic processes and downstream chromatography and ultrafiltration-based purification. The resulting purified naked mRNA is then suitable for encapsulation into lipid nanoparticles (LNP) and final formulation. The mRNA construct was designed to incorporate a number of factors that aid in

decreasing non−specific immune activation and improving translation efficiency. To decrease activation of the innate immune system, N1-Methylpseudouridine modified nucleosides were incorporated.[13,14] To improve translatability, the plasmid was designed to encode the target coding sequence along with 5 and 3' untranslated regions (UTR) and the entire 101-base Poly(A) tail, ensuring an optimal poly(A) tail length for translation in human cells. In addition, CleanCap from TriLink Biotechnologies (San Diego, CA) was incorporated to allow for the cotranscriptional incorporation of the natural type I capping structure. Fig 1. Plasmid map encoding an example vaccine immunogen for mRNA expression shows a typical antigen-encoding plasmid map that encodes the 5'-UTR, coding sequence, 3'-UTR, and poly(A) tail.

The upstream process involves 3 enzymatic steps: plasmid linearization, mRNA transcription, and DNA template digestion. Development of the upstream process involved selection of available raw materials and determination of process parameters to meet the target drug substance quality profile and desired yield. For optimal in vitro transcription yields, it is critical to start with high quality template DNA. Some key quality attributes that are required before use include the presence of only a single band of linearized DNA, a supercoiled ratio of at least 70%, and a sequencing-verified correct poly(A) tail length. To date, all plasmids that DHVI has used for IVT reactions have been manufactured by commercial vendors (Puresyn, PA; GenScript, NJ; Aldevron, ND) and have encoded for transcripts between 1600 and 2900 bases. For plasmid linearization, we used molecular-grade WFI water (Intermountain Life Sciences, UT), NotI-HF enzyme (New England Biolabs, MA), and IVT buffer (Promega, WI). The use of IVT buffer for NotI-based plasmid linearization was designed and tested for optimal compatibility with the subsequent transcription reaction. Following the linearization step, an aliquot of the linearized DNA template was added to a separate reaction vessel followed by the addition of IVT buffer (Promega, WI), nucleotide triphosphates (ATP, GTP, CTP) (Promega, WI), T7 enzyme mixture (Promega, WI), pseudouridine (N1-Methylpseudouridine-5′-Triphosphate) (TriLink, CA),

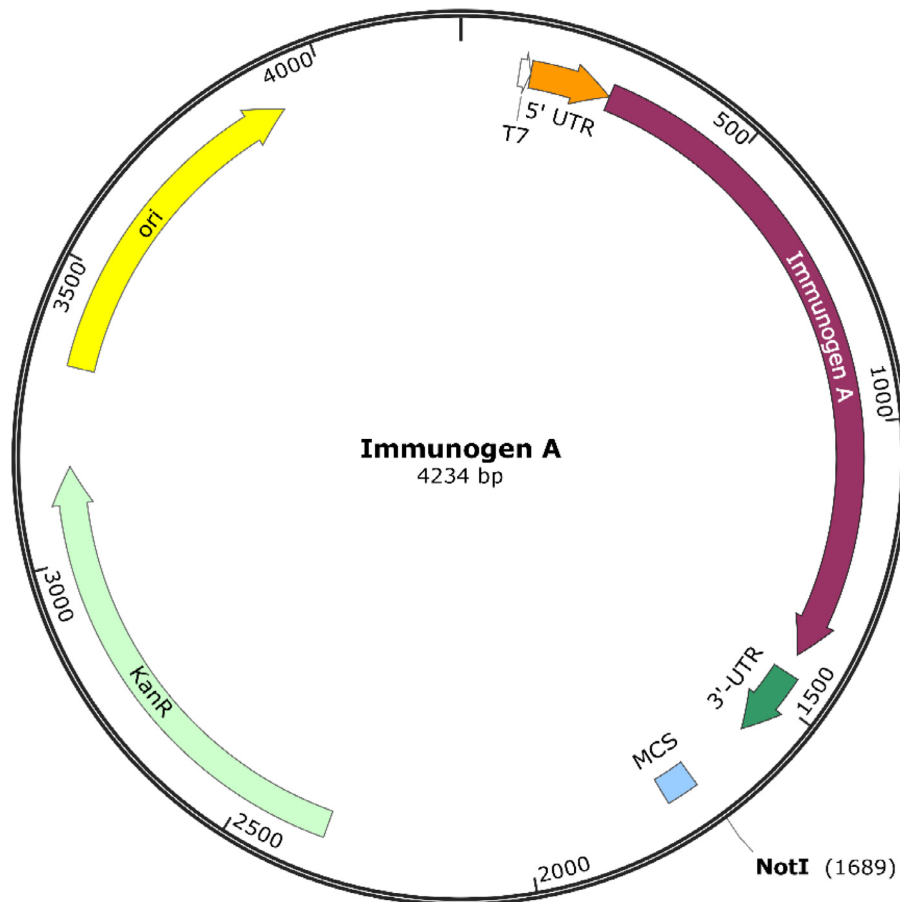

Fig 1. Plasmid map encoding an example vaccine immunogen for mRNA expression.

and CleanCap capping reagent from (TriLink, CA). After RNA transcription, the linearized DNA template was digested with the addition of DNase enzyme and a DNase buffer supplement (New England Biolabs, MA). Finally, EDTA was added to quench the reactions. Raw material selection across suppliers for each synthetic step was governed by the ability to meet the desired mRNA drug substance quality profile, the availability in support of both process development and early phase GMP manufacture, and the grade of the material. Although GMP grade materials were selected, when possible, non−GMP research grade materials were also utilized in certain circumstances as long as the ultimate quality profile was not affected. We found that endotoxin control was the primary raw material risk due to its variability in raw material lots and the difficulty of its removal in subsequent downstream purification steps. Endotoxins can be introduced to the product primarily from IVT reaction components and large volume buffers used in purification. Reaction agitation, temperature, incubation time, and reactant concentrations were also carefully examined for each reaction step in order to yield a robust upstream platform process across multiple mRNA constructs. Ultimately, all steps were incubated in shaking reaction flasks (Corning, NY) at 37°C, and 225 rpm. We found that varying nucleotide or CleanCap concentrations had significant effects on the IVT yield, while varying the amount of linearized DNA did not. Final concentrations in the IVT reaction are 4mM CleanCap, 5 mM of each nucleotide (including pseudouridine), and 0.05 mg/mL linearized DNA. Plasmid linearization, *in vitro* transcription, and DNase digestion were each incubated for 2 hours, while EDTA quenching was performed for 15 minutes at 37°C. This optimized process consistently yielded 3−5 mg/mL of full-length mRNA drug substance and low residual double stranded DNA. Moreover, it has been demonstrated across multiple mRNA constructs of varying sequence, and length.

De novo design of the downstream platform process entailed precipitation mitigation, resin selection, and selection of buffers suitable for purification. This design included an initial RNA reaction dilution (25X) and a chromatography step bracketed by 2 tangential flow filtrations (TFF or ultrafiltration (UF) and diafiltration (DF)). These unit operations were implemented to remove enzyme reactants, residual DNA, and undesired high molecular weight (HMW) species. To assist in resin, filter, and parametric selections, a size exclusion chromatography (SEC) analytical method was developed as an in-process tool to assess the purity of the mRNA drug substance throughout downstream development. Anion exchange, hydrophobic, and mixed-mode resins were first evaluated in bind and elute mode, which proved insufficient to produce the desired mRNA quality profile and purification yields. Core bead resins were then evaluated and shown to provide sufficient purification performance, in particular the removal of enzyme reactants, and residual DNA. The focus of development then shifted to selection of TFF membranes and appropriate operating volumes and parameters to eliminate reaction components and impurities. . This downstream platform process results in approximately 3 mg/mL of purified bulk drug substance per mL of IVT reaction. The cGMP scalable downstream platform process yielded approximately 80% purified mRNA bulk drug substance relative to the non−scalable lithium chloride purification process.[15] Multiple sequences have been purified using this platform, ranging from 1118 nt to 2869nt with process yields ranging from 72.7% to 94.8%. Table I shows the average step yields and intermediate concentrations during purification of various RNA constructs at GMP production scale.

The mRNA drug substance process and in-process testing are summarized in the flow diagram in Fig 2. The Upstream mRNA Process contains 2 (2) unit operations. The first unit operation is DNA Linearization, where plasmid DNA is digested by NotI in a single container, and incubated at 37°C. Prior to performing the Linearization step, a brief preconditioning of the linearization reaction components must occur. The Nuclease-free Water, 5X IVT Buffer, Supercoiled non−linear plasmid DNA, and NotI restriction enzyme pre-conditioning entails pre-warming of all materials. During the plasmid DNA digest incubation, the NotI enzyme will specifically target and cleave the plasmid DNA. After incubation is finished, the linearized DNA template is sampled, and processed forward into the next unit operation. Alternatively, linearized DNA can be stored frozen or held at room temperature overnight.

The second unit operation of the Upstream process is the IVT reaction. This unit operation is a combination of 3 steps; mRNA IVT reaction + plasmid DNA Digestion + EDTA Quenching. mRNA is transcribed by combining the water, 5X IVT buffer, NTPs (ATP, CTP, GTP), pseudouridine, linearized DNA template,

Table I. Average step yields and mRNA concentration for downstream purification

Downstream process step	Average step yield	Average concentration (mg/mL)
UFDF 1	89.2%	0.61
Chromatography	94.5%	0.30
UFDF 2	101.5%	1.07
Filtration and/or Bulk Fill	93.4%	1.09

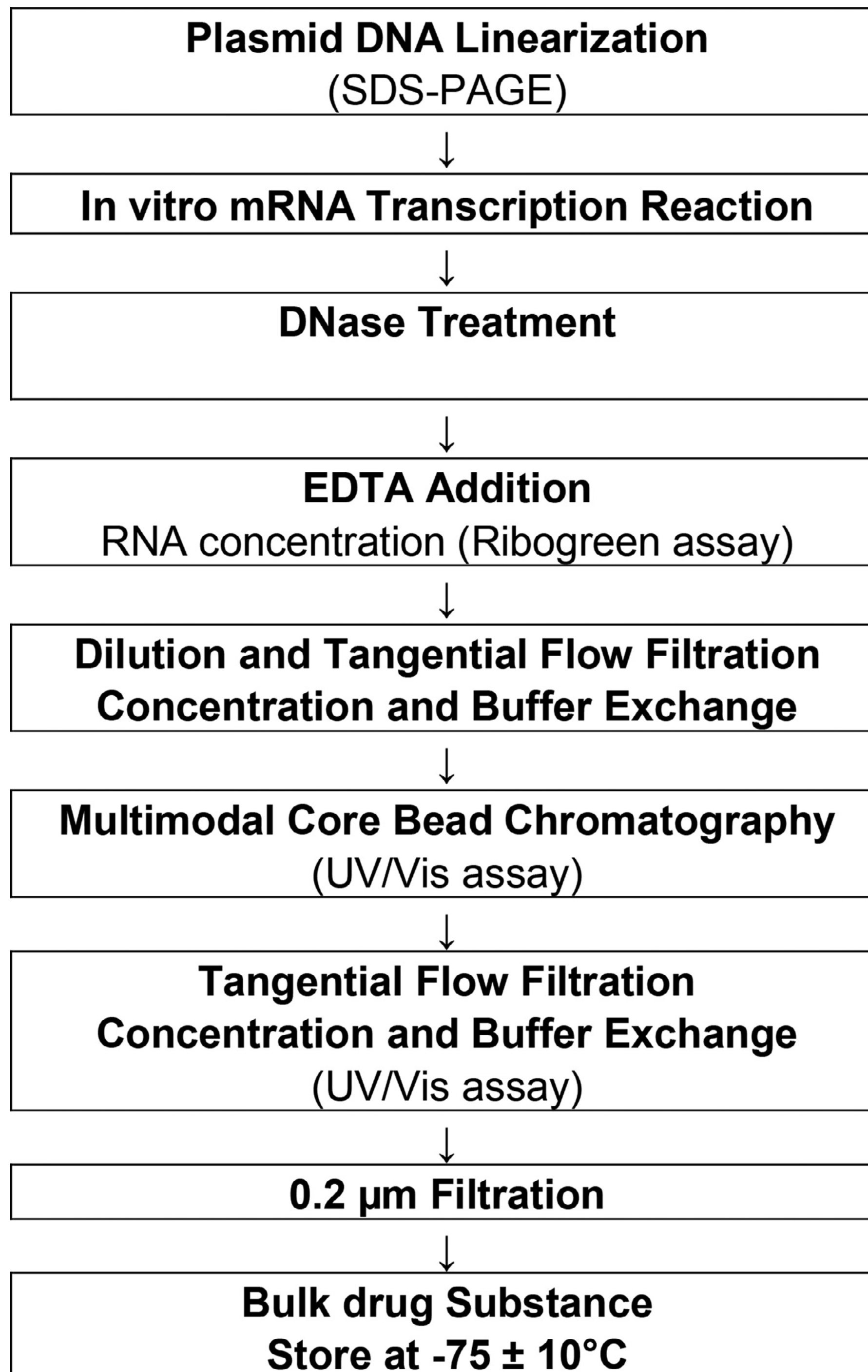

Fig 2. Scalable mRNA production process flow diagram and in-process testing.

CleanCap, and T7 enzyme mixture in a single container and mixing and/or incubating at 37˚C.

At the end of the RNA IVT incubation, DNase I enzyme and 10X DNase I buffer are added to the RNA IVT reaction and incubated at 37˚C in order to degrade the DNA template. After DNase incubation, EDTA is added to the reaction vessel to quench the DNase activity, stabilize the mRNA, and mitigate against precipitation of the mRNA. After EDTA incubation is finished, the mRNA IVT is sampled, and processed forward into the downstream unit operations.

The Downstream Process contains 4 unit operations. The downstream operations begin with receipt of IVT reaction product, followed by a dilution with nuclease free water. The purpose of this operation is 2-fold: to dilute EDTA concentration from IVT reaction and to prevent and/or reverse precipitation of mRNA. This material enters into a UFDF step to concentrate and buffer exchange the diluted IVT. The concentration reduces the total volume to allow for faster processing time in the chromatography unit operation. The buffer exchange provides purification from small IVT reaction components and normalizes chromatography loading conditions. The UFDF filter is assembled, flushed with nuclease free water, and flushed with buffer. The product is then concentrated to a target volume before being diafiltered into buffer. The product is recovered by performing a plug flush of the UFDF assembly.

The second step in the downstream unit operations is the multimodal core bead chromatography step. In this purification step, the mRNA flows through the resin as the pore size will not allow interaction of ligand with the mRNA. This step removes IVT reaction component contaminants. The prepacked column is sanitized, equilibrated, loaded, and washed; the flow-through and wash are collected as a single bulk fraction before proceeding to the next unit operation.

The third step of the mRNA purification process is a UFDF step to concentrate to a target concentration and diafilter the chromatography Flow-Through and/or Wash pool into the formulation buffer. The UFDF filter is assembled, flushed with buffer, then flushed with nuclease free water. The product stream is concentrated before being dialfiltered into nuclease free water. Next, the product is buffer exchanged to the final formulation buffer (1.0 mM citrate, pH 6.4). The product is recovered by performing a plug flush of the UFDF assembly.

The fourth and final step in the downstream process is a 0.2 μm filtration and then closed bulk drug substance fill into sterile fill containers. It should be noted any tubing, chromatography or filtration components that cannot be tested as nuclease-free are held with 0.5M NaOH for a minimum of 1 hour at ambient conditions before being flushed with nuclease-free water and the appropriate buffer for the unit operation.

The mRNA bulk drug substance produced from the above upstream and downstream platform process is then suitable to be encapsulated into LNP. These platforms are both scalable and transferrable across mRNA constructs where consistent drug substance and LNP encapsulated mRNA final drug product quality profiles have been produced by the upstream, downstream and LNP encapsulation processes, both at pilot and cGMP production scale to sufficiently supply early phase clinical trials.

SCALE UP AND TRANSFER FOR GMP MANUFACTURE

Due to a lack of regulatory guidance and established expectations available at the time of mRNA platform development, initial frameworks derived from the vaccine regulatory landscape were adopted for technology transfer and process scale up into GMP production. This framework approach enabled the team to predefine acceptance criteria for platform success through the lens of a highly conservative, known regulatory path, laying a foundation for scale-up design, and quality controls at the outset.

Due to the novel nature of the mRNA platform, having a consensus around a conservative approach to scale-up design and quality controls proved to be invaluable in this new manufacturing landscape. Unlike established platforms for vaccine manufacturing, with a large, reliable, fit-for-purpose, well-established supply chain at the ready, the mRNA platform by nature required new components that were often only available research-grade and lacked established quality designations such as USP, EP or JP grade often seen with established commercial reagents.

To address the lack of established quality standards faced with mRNA platform materials, we utilized the pre-defined acceptance criteria and employed rigorous, in-depth risk assessments for each proposed material to determine the required controls for this category of materials. Impact to patient safety, operator safety, facility cross-contamination potential, product quality, and regulatory hurdles are a few of the categories that were assessed for each new material. All mRNA platform material vendors were assessed and qualified by the Quality Unit. This process involved obtaining information through questionnaire and/or audit, relating to the vendors' quality systems and operations as they pertained to manufacturing and/or testing of the materials. Additional controls, such as release testing of incoming materials to detect endotoxin, vendor testing for nucleases, and sequencing to verify material prior to use are a few examples of outcomes derived and implemented from the risk assessments.

For Phase I manufacturing we utilized a GMP scale up approach that was focused on single use assemblies and components, tight control of small volumes, and, where possible, closed system processing. The mRNA production process utilized for GMP production was built from a library of single-use assemblies and components sourced from qualified vendors, inclusive of sterile validation packages, with back-up qualified suppliers for many critical components.

While again we aimed for Phase I manufacturing, the use of these single use components is ideal for even large-scale production due to their inherent reduced

risk for microbial or RNase contamination and batch to batch carryover of nucleic acid material. Additionally, the use of single-use components allowed for quick adaptation to process development changes and removed the requirement for cleaning verification and validation in GMP production.

Typical Phase I bioprocess single-use manufacturing components operate at an upstream scale ranging from 125 mL to 200 L, and downstream processing volumes ranging from 2 L to 10 L. The mRNA platform described above, in contrast, operates at an upstream scale ranging from 300 mL to 900 mL, typically yielding a volume of bulk drug substance ranging from 1 L to 4 L. The contrast in lower end volumetric operations presented a challenge due to the very high cost of raw materials utilized and the negative impact that can occur from line hold up volume losses at small scale.

To address these concerns, the team designed new assemblies and components for this smaller scale space, focusing on tight control of the smaller processing volumes and closed processing operations, when possible. Due to the nature of the product, additional focus was given to certifying the single-use assemblies and components as RNase-free. At the time of the platform design and development, with few exceptions, vendors did not certify assemblies and components as RNase-free. In collaboration with key suppliers, we established testing and qualified a library of components and assemblies that are certified RNase-free, and therefore destined for use for mRNA manufacturing.

Prototypes of the new assemblies and components were then tested via water runs, first item by item to identify potential issues and refine the designs, and then coupled together with other items within and across unit operations. The water runs resulted in multiple benefits for the design and scale-up of the mRNA platform, inclusive of: (1) materials were successfully tested and deemed ready for manufacturing; (2) operators repeatedly conducted hands-on training for process steps well ahead of manufacturing runs; and (3) batch documents were created for the water runs and red-lined throughout to have a final draft ready for manufacturing. This approach proved to de-risk the overall process, materials, and operations well ahead of costly engineering and GMP runs.

The final mRNA manufacturing process was split into 6 primary unit operations based from the above-described process development: a DNA Linearization step, an IVT reaction step, an Ultrafiltration Diafiltration (UFDF1) step, a Chromatography step, a second Ultrafiltration Diafiltration (UFDF2), and finally Bulk Drug Substance filtration and fill. The process developed was based off of a 300 mL IVT reaction platform that is scalable in increments of 300 mL. The results shown in Table II and Table III below demonstrate that the products produced via the developed process meet acceptance criteria for release and stability testing when executed both within the development environment and when transferred into production in GMP-compliant conditions.

ANALYTICAL CHARACTERIZATION AND RELEASE ASSAY METHOD DEVELOPMENT

An additional challenge with mRNA vaccine therapies is the lack of a consolidated and transferrable package of analytical characterization and release assays that will meet regulatory agency expectations for a well-characterized product. Analytical methods for characterization, lot

Table II. Lot-to-lot comparability of mRNA drug substance at 300 mL IVT reaction scale

Assay	Immunogen A (development 300 mL scale)	Immunogen A (GMP suite 300 mL scale)
Immunogen Design	HA Ferritin Nanoparticle	HA Ferritin Nanoparticle
Predicted mRNA length	1570 bases	1570 bases
Appearance	Not tested	Clear, colorless solution, essentially free from visible particulates
Concentration	1.04 mg/mL	1.03 mg/mL
Product Yield	0.98 g	1.09 g
Calculated mRNA length	1508 bases	1541 bases
mRNA purity	90.4%	90.4%
dsRNA content	<0.5%	<0.5%
Residual plasmid DNA content	<90.2 pg DNA/mg RNA	<91.1 pg DNA /mg RNA
Residual protein content	<30.3 ppm	<30.3 ppm
Capping efficiency	98%	96%
Poly-A tail length	>100 nt	>100 nt
Potency	Expected antigenicity	Expected antigenicity
Bioburden USP <61>	Not tested	<1 CFU/mL
Endotoxin USP <85>	0.036 EU/mL	<0.5 EU/mL

Table III. Stability evaluation at accelerated temperature conditions for mRNA-LNP non−GMP drug product

Assay	Immunogen A (225 mg scale) T = 0	Immunogen A (225 mg scale) 5 d at 25°C	Proposed specification
% Encapsulation	95.8	96.2	≥80%
Particle size	74.2 nm	75.3 nm	75 ± 25 nm
mRNA content (mg/mL)	0.510 mg/mL	0.518 mg/mL	0.5 ± 0.2 mg/mL
pH	7.11	7.16	7.4 ± 0.5
mRNA integrity %	97.4	96.2	≥70%

release and stability testing of mRNA and mRNA-LNP candidates were developed and performed to monitor critical quality attributes, process-related residuals and safety (Table II and Table III), and the proposed specifications were based on regulatory requirements and feedback from pre-IND discussions with the FDA and government sponsors. As with more established therapeutic modalities, the testing panel includes evaluation of quality attributes such as appearance, pH and osmolality, and safety tests for measuring endotoxin, bioburden and sterility. Key attributes that are unique to the evaluation of mRNA include characterization of the 5' cap and poly-A tail regions to ensure appropriate stability and translation of the molecule *in vivo,* as well as assessment of dsRNA content for its potential to activate an innate immune response. Additionally, as the mRNA products we currently have under development require lipid formulation for delivery, analytical methods to measure lipid purity and content are required to support lot release and stability testing for the mRNA-LNP drug product.

We have produced a number of mRNA-encoded vaccine antigen and monoclonal antibody candidates from small-scale research IVT reactions utilizing the above-described purification approach. Batch sizes have ranged from 5-10 mL for animal-model studies to larger-scale 300-900 mL reactions that have been performed in GMP manufacturing. Analytical characterization has shown lot-to-lot comparability regardless of whether the mRNA was synthesized and purified in development or in GMP manufacturing (Table II), thereby demonstrating successful scale up, and tech transfer of the production process. The purity of the mRNA drug substance was evaluated using capillary gel electrophoresis (CGE) under denaturing conditions where the relative amount of full-length mRNA transcript was found to be >90% in both process development and in production in the GMP suite. As judged by the CGE assay, the remaining impurities in the mRNA drug substance are likely truncated mRNA species. Clearance of residual protein impurities was assessed by an Enzyme-linked Immunosorbent Assay (ELISA) specific for the detection of T7 polymerase as this protein is the most abundant IVT reaction component, while qPCR was used to monitor the removal of residual plasmid DNA. As judged by the ELISA and qPCR assays, both protein and DNA process impurities were successfully cleared to acceptable limits for clinical use.

Assessment of dsRNA content is also required for mRNA therapeutics given the ability of this impurity to trigger an innate immune response. We have monitored the presence of dsRNA by ELISA using monoclonal antibody reagents, which are specific for the detection of dsRNA longer than ∼30−40 bp.[16] Using this method, dsRNA content has been shown to comprise less than 0.5% of the product mass. Other inherent quality attributes of the mRNA, such as the 5' cap and poly-A tail region, have been evaluated by ultra-performance liquid chromatography (UPLC) and CGE methods, respectively. To assess capping efficiency, a small oligonucleotide fragment containing the 5' region of interest was isolated for ion-pair reversed phase UPLC analysis using a biotinylated cleavage probe, as previously described.[17] The inclusion of a mRNA control sample, which contains pseudouridine, but is synthesized in the absence of the 5' cap reagent, facilitates discrimination between capped, and uncapped species in the chromatogram. With this method, the observed capping efficiencies have been >95%, in line with the manufacturer's expected value for the use of the CleanCap reagent during the IVT reaction. The DNA template for mRNA transcripts currently in development encodes for a poly-A tail with a length of greater than 100 nucleotides as a critical quality attribute described previously.[9] Following enzymatic digestion with RNase A to isolate the poly-A region and subsequent analysis of the fragment by CGE, the length of the poly-A region has been confirmed to be of the correct size, which is expected to confer sufficient stability, and translation efficiency of the molecule *in vivo*.

In addition to the aforementioned test methods, which have enabled a platform approach to analytical development, product-specific evaluation of the protein encoded by the mRNA has been evaluated by cell-based expression assays. Functional binding

characterization of the expressed protein from transfected cells has been performed to confirm both the integrity of the mRNA molecule and to demonstrate that key epitopes of the expressed protein are preserved upon translation. ELISA methods have been developed as a standard approach to characterization, although other ligand binding platforms, such as Luminex bead array and biolayer interferometry (BLI) and surface plasmon resonance (SPR), have been utilized.

To date, the formulation of the mRNA drug product has been performed in partnership with outsourced manufacturing vendors. Analytical methods for the evaluation of the mRNA-LNP drug product (Table I) are utilized for the purified product and for in-process samples, as appropriate, to ensure the quality of the product throughout the manufacturing formulation process. Lot-to-lot comparability has been demonstrated between small-scale formulations used in early exploratory pre-clinical studies and formulations of larger-scale. CGE analysis of mRNA-LNP products has shown that the mRNA released from the lipid formulation has a purity that is comparable to that of the mRNA drug substance, with the percentage of main drug product peak typically exceeding 85%−90%. The particle size and polydispersity of mRNA-LNP products has been measured by dynamic light scattering and shown to be within the size limits of 75 ± 25 nm with the polydispersity index below the target criteria of 0.1. Encapsulation efficiencies have been measured by fluorescence-based RiboGreen assay. A comparison of the concentration of free mRNA in LNP samples vs the concentration in LNP samples disrupted by detergent has found the percentage of encapsulated material to be consistently >90%. Identity and content analysis of the 4 lipid components within the nanoparticle has been performed by the outsourced manufacturer and analyzed by UPLC equipped with either a charged aerosol (CAD) or evaporative light scattering detector (ELSD). The lipid content ratio has met the target criteria for the given concentration of encapsulated RNA. As with the mRNA drug substance, potency of the mRNA-LNP has been demonstrated by functional binding assay of the expressed protein from transfected cells. mRNA and mRNA-LNP candidates produced to date are intended for storage at ≤-65°C. Long-term stability studies, as well as evaluations conducted at accelerated temperature conditions of 5°C, 25°C and -20°C, are currently ongoing. Thus far, stability at 25°C for the mRNA-LNP has been demonstrated for up to 1 week (Table III). Additional studies of diluted mRNA-LNP drug product will be conducted, as necessary, to support clinical dosing.

IN VIVO EVALUATION OF MRNA PRODUCTS FROM THE PLATFORM PROCESS

mRNA encoding antibodies. Evaluation in animal models was key to evaluating the suitability of the above-described process for mRNA production and characterization. For mRNA products encoding antibodies, we partnered with the Defense Advanced Research Projects Agency (DARPA) Pandemic Prevention Program (P3). This US Department of Defense program has played a key role in funding and driving public and/or private development of end-to-end platforms to rapidly develop antibody countermeasures, including mRNA-based therapies. The DARPA P3 program at Duke University (Duke P3) aims to identify and propagate viral pathogens, isolate human neutralizing antibody sequences, develop effective approaches to deliver neutralizing antibodies as nucleic acids, and develop good manufacturing practices for a final drug product for submission to the Agency for Phase I approval within 60 days of receiving an outbreak blood specimen.[18] Key to the success of this program is delivery of a scalable and compliant GMP manufacturing process for mRNA encoding the heavy and light chain of antibodies encapsulated in LNP for prophylactic-delivery.

mRNA-LNP delivered human antibodies against influenza virus. As proof-of-concept, the Duke P3 program isolated 2 different human antibodies that neutralize H1 and H3 influenza virus strains and used these sequences to develop and optimize the end-to-end platform for rapid isolation, manufacture and mRNA delivery of antibodies. Using the above-described mRNA production process and analytical characterization assays, mRNA encoding the heavy and light chains for these antibodies was produced. For each antibody sequence to be tested, the mRNA heavy and light chain sequences were mixed at a 1:1 molar ratio, encapsulated into lipid nanoparticles by Acuitas Therapeutics (Vancouver, BC) and tested for acceptable endotoxin levels for testing in mice and non−human primates (NHP).

Serum levels of the antigen-specific human antibody in ug/mL were used to monitor expression and kinetics of the mRNA-launched antibody in pre-clinical animal models (Fig 3 and Table IV). Two independent IV administered dose response studies in wild-type BALB/C mice with CH65 (anti−H1 influenza) mRNA in optimized Acuitas LNP are shown in Fig 3A and Fig 3B, respectively. A similar dose study was run in wild-type mice with an anti−influenza H3 Hemagglutinin antibody mRNA (Ab700364) in optimized Acuitas LNP (Fig 3C). Shown in Fig 3D is an IV administered dose response study in non−human primates (rhesus macaques) with Ab700364 mRNA in optimized Acuitas LNP.

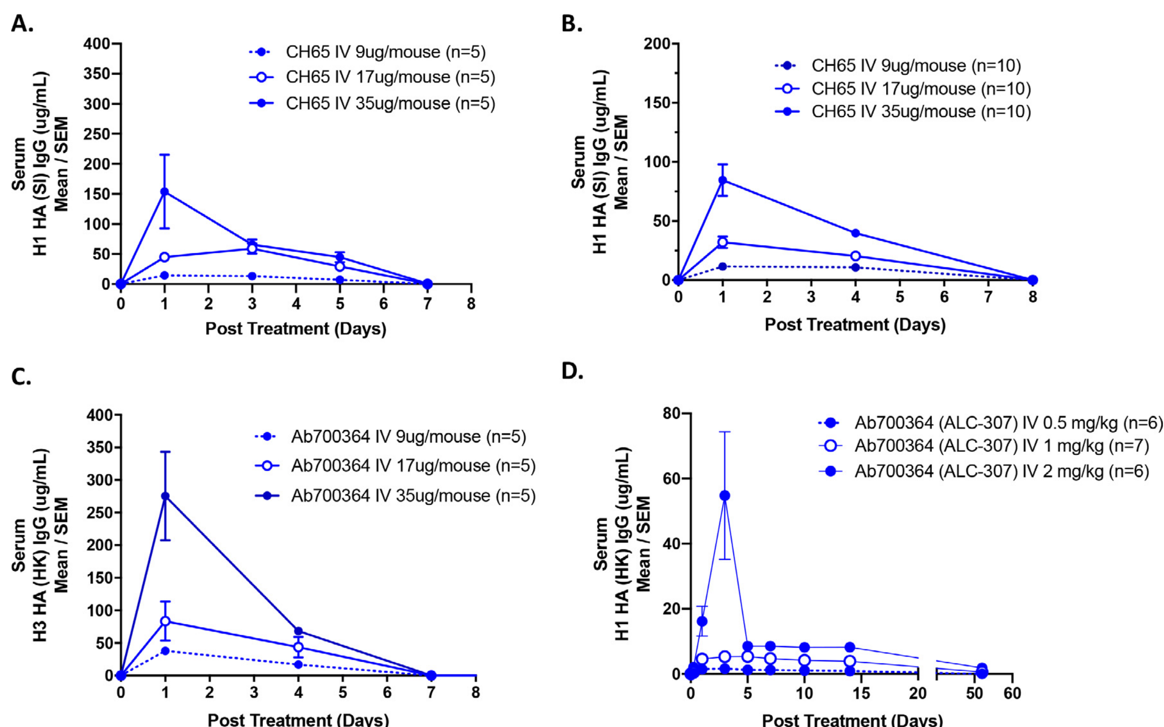

Fig 3. Influenza HA-specific human antibody in serum following IV administration of the mixture of the heavy and light chain mRNA in LNPs. A, Dose response in wild-type BALB/C mice with CH65 mRNA in optimized Acuitas LNP (study 1). B, Dose response in wild-type BALB/C mice with CH65 mRNA in optimized Acuitas LNP (study 2). C, Dose response in wild-type BALB/C mice with Ab700364 mRNA in optimized Acuitas LNP. D, Dose response in non−human primates (rhesus) with Ab700364 mRNA in optimized Acuitas LNP.

For each study the in vivo expressed maximum concentration (Cmax), time to reach maximum concentration (Tmax), and the human antibody half-life in either a wild-type mouse or NHP was calculated (Table IV). Across all mouse studies (IV administered antibody mRNA in LNP) the average time to reach maximum expression level was 1.3 days and the half-life of the human antibody in a wild-type mouse is 2.5 days. The average Cmax in mice was 21.8, 59.3 and 128.2 mcg/mL at 0.5, 0.9 and 1.9 mg/kg, respectively. In non−human primates the average time to reach maximum expression following IV administration was 2.8 days and the half-life of the human antibody in NHPs is 15.8 days. The average Cmax in NHPs was 1.6, 6.2, and 58.8 mcg/mL at 0.5, 1.0 and 2.0 mg/kg, respectively.

Table IV. Pre-clinical PK − Duke P3 proof-of-concept developmental antibody studies

Antibody mRNA	Model	Dose (mg/kg)*	T half (D)	Cmax (ug/mL)	Tmax (D)
CH65 (H1 Ab)	BALB/C Mouse (Study1)	0.5	2.9	14.8	1.8
		0.9	3.7	60.7	2.2
		1.9	2.3	154.0	1.0
CH65 (H1 Ab)	BALB/C Mouse (Study 2)	0.5	3.2	12.4	1.6
		0.9	2.6	32.2	1.3
		1.9	2.4	87.1	1.3
Ab700364 (H3 Ab)	BALB/C Mouse	0.5	2.0	38.1	1.0
		0.9	2.3	84.9	1.6
		1.9	1.7	275.7	1.0
Ab700364 (H3 Ab)	NHP (Rhesus)	0.5	13.9	1.6	0.7
		1.0	14.8	6.2	1.3
		2.0	18.7	58.5	3.3
Ab700364 (H3 Ab)	BALB/C Mouse	1.9	2.3	37.6	1.0

*Dose adjusted from mcg/mouse to mg/kg based on average mouse weight per study.

The Duke P3 sponsor Go/No Go metric for the platform was to exceed 10 mcg/mL within 72 hours, and we have accomplished this with all test doses of formulated mRNA in mice and at the highest dose in NHPs. It is important to note that no changes in animal body weight or temperature were observed in any of the preclinical mouse and NHP studies with IV administration of mRNA-LNP.

To establish in vivo potency and/or efficacy of the Duke P3 isolated H3 influenza antibody (Ab700364) and the mRNA delivery approach, the mouse adapted X-31 lethal challenge model was used. Animals were treated IV 24 hours prior to challenge with a dose range of Ab700364 mRNA-LNP. Animals were then challenged intranasally with 1e6 FFU of virus and monitored daily for survival and/or humane endpoints in accordance with Duke Institutional Animal Care and Use Committee approved protocols. Eighty to 100% protection was observed in these proof-of-concept studies (Fig 4).

mRNA encoding vaccine immunogens. The mRNA-LNP vaccine platform has also been employed by our group to address difficult to make vaccines, such as a preventative HIV-1 vaccine candidate. A key goal of HIV-1 vaccine development is to induce long-lasting broadly neutralizing antibodies (bnAbs) that can inhibit HIV-1 infection.[19,20] BnAbs tend to develop by extensive somatic mutation of rare germline antibodies. These rare bnAb precursors also have highly selective reactivity with Env immunogens. Therefore, we hypothesize that immunization with a series of immunogens engineered to initiate bnAb lineages, and to select for affinity maturation is needed[19,21] —an approach we have termed B cell lineage immunogen design.[20] mRNA-LNP vaccines are advantageous here because the series of Env immunogens can be manufactured in a relatively short time period. Thus, our Integrated Pre-Clinical and/or Clinical AIDS Vaccine Development (IPCAVD) Program aims to produce 2 mRNAs encoding stabilized Env immunogens that can initiate and affinity mature bnAb B cell lineages.

The bnAb B cell lineage from an infected individual called DH270 has served as a vaccine template, allowing us to develop 2 trimeric Env immunogens called CH848.10.17DT and CH848.10.17 to initiate and select for affinity maturation, respectively.[22] These 2 protein designs were selected for mRNA vaccine production after extensive *in vitro* characterization to confirm mRNA expression resulted in trimeric, near-native HIV-1 envelope. To evaluate the above-described mRNA production approach, mRNA encoding the CH848.10.17DT was produced using a standard LiCl precipitation method[23] as well as the mRNA production method outlined in the sections above. Subsequently, these mRNAs were encapsulated in LNPs (Acuitas Therapeutics, Vancouver, BC). A mouse model was engineered to express the precursor of the DH270 lineage, called the DH270 UCA, to demonstrate the ability of the CH848.10.17DT mRNA-LNP vaccine to initiate the DH270 bnAb B cell lineage. Mice were immunized intradermally (ID) 4 times with 20 mcg of mRNA-LNPs produced by either of 2 different purification methods or LNPs alone. The immunogenicity of the Env trimers expressed from these mRNAs *in vivo* was evaluated by ELISA and virus neutralization assays. CH848.10.17DT mRNA-LNP

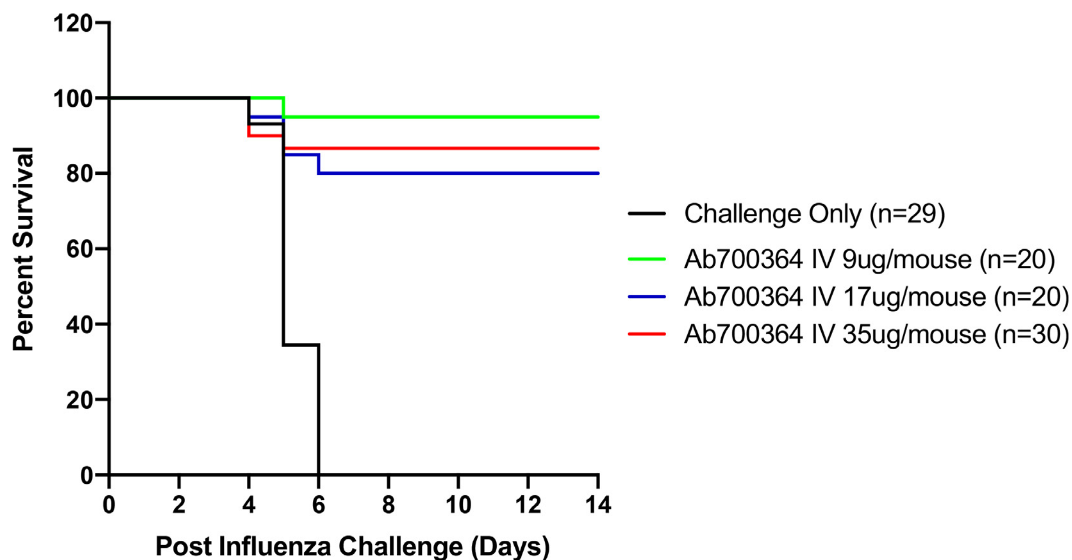

Fig 4. Pre-treatment with Ab700364 mRNA-LNP protects mice from lethal challenge with X-31 influenza virus (1e6 FFU; mouse adapted A/Aichi/2/1968) 24 hours later.

immunization elicited serum IgG that bound to CH848.10.17DT gp120 and Env trimer at a higher magnitude than empty LNPs. There was no substantial difference in the binding titers of serum IgG induced by mRNA-LNPs produced by the 2 different purification methods, suggesting that both purification methods produced high-quality mRNA (Fig 5). These results indicate that the newly developed large-scale mRNA production and purification method generates immunogenic mRNA vaccines that encode trimeric HIV-1 envelope.

DISCUSSION

The growing exploration of mRNA platforms for delivery of vaccines and therapeutics drove the need to

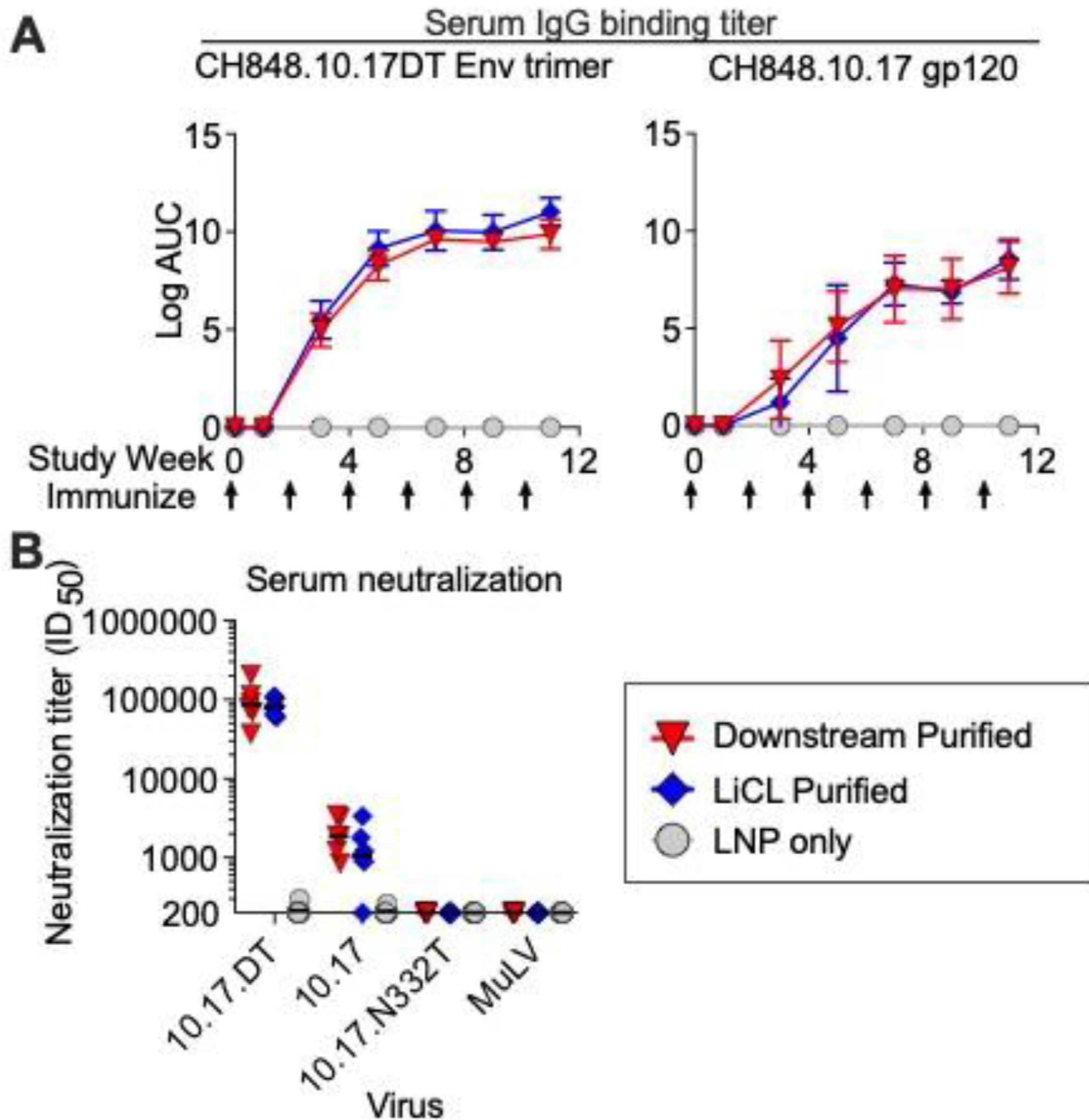

Fig 5. Binding and neutralizing antibody responses elicited by mRNA-LNP immunization of heterozygous DH270 broadly neutralizing antibody precursor mice. A, Serum IgG binding titer to the HIV-1 envelope trimer (left) that matches the vaccine or a related HIV-1 gp120 subunit of envelope (right). Arrows indicate timepoints of immunizations. Group mean and standard error are shown. Binding titer is shown as area under the log transformed curve (log AUC). B, Serum neutralizing antibody titer against variants of the CH848.10.17 pseudovirus. DT indicates the virus engineered to bind to the HIV-1 bnAb precursor of the DH270 antibody lineage. N332T indicates the knockout mutant version of the CH848.10.17 virus, which lacks recognition by the DH270 lineage. MuLV indicates the murine leukemia virus used as a negative control virus.

develop scalable production approaches for mRNA to support pre-clinical studies and early phase clinical trials along with analytical methods to deliver product characterization and release testing. Here we describe a scalable, robust process for mRNA production and purification using accessible manufacturing operations. The aqueous-based chromatography and ultrafiltration are extremely scalable processes as we have seen displayed in the production of monoclonal antibodies at metric ton scale[24] and, given the need to produce orders of magnitude less product per dose, are highly amenable to global-scale production.

Another important benefit of the production and purification processes, as well as the analytical assays described, is that they are readily shared across the multiple mRNA products. Using off the shelf in vitro transcription reaction components, single use systems, and defined reaction parameters, we have demonstrated consistent mRNA yields across a large-scale range and with many different size transcripts. The purification process steps were developed to be compatible with readily available GMP equipment and procedures and have been successfully scaled from 5 mL to 900 mL RNA bulk reactions. The overall production process has been successfully demonstrated so far with mRNA transcripts ranging from 1000 to 3000 bases. The purification operations eliminate process residuals and product contaminants including dsRNA. Importantly, the production, and purification processes are shared across the multiple mRNA products. Analytical assays were developed to demonstrate mRNA purity and integrity and to control process operations. The biological function of the mRNA has been demonstrated in both in vitro and in vivo assessments, as shown above. Additional challenges that do remain for mRNA products are supply chain constraints, proprietary lipid access, and cold storage and delivery requirements.[6] Raw material shortages and the lack of approved suppliers can be addressed through risk-based strategies that have been recommended by the FDA Q9 Quality Risk Management Guidance (https://www.fda.gov/media/71543/download). In our take on this approach, the impact to patient safety, operator safety, product quality, and regulatory hurdles can be assessed for each new material, along with adding additional controls such as release testing of incoming materials to detect endotoxin, vendor-based testing for RNase, and sequencing to verify materials prior to use.

In addition, manufacturing scale up can be accelerated by executing risk assessments in parallel to process development activities, which also aid in the selection of optimal raw materials and components for process performance and low compliance risk. Two-way engagement with material vendors was a critical part of risk assessments in our experience, supporting a deep-dive approach to fully understand manufacturing constraints and capabilities, and identifying additional control measures that could be employed by material vendors. Exploratory work conducted in process development (pre-GMP) can be used to further bolster the understanding of risk and additional quality controls necessary. Where possible, alternate fit-for-purpose, qualified suppliers can be explored and secured for a small sub-set of materials (ie buffers).

Finally, the stability of mRNA-LNP vaccines, and therapies does remain a challenge for distribution. Although some work has been done with vaccine candidates, showing up to a week of stability at ambient temperatures,[25] much longer stability duration would be needed to cover the process of quality assurance release of drug product, shipping to distribution sites and then to healthcare providers, and administration to patients. Novel formulations and approaches, such as lyophilization of mRNA-LNP, are being explored to address these concerns.[26] Based from our experience delivering mRNA-based vaccines and therapies, we expect at least 6 months of shelf life would be required for clinical and commercial use.

Although at a larger scale, the processes used to produce the Moderna, and Pfizer and/or BioNTech COVID vaccines likely follow similar manufacturing operations. Optimized plasmid templates include 5’ and 3’ UTR elements to increase half-life and expression of the target sequence. A 5’ cap structure and long poly(A) tail to enhance RNA translation and stability. Codon optimization and modified nucleoside substitution for translation efficiency and to prevent activation of innate immune sensors.[8,9] Larger scale IVT reactions may be conducted in bioreactors using similar reaction components as described here. The downstream operations may include new types of purification strategies such as cellulose-based chromatography to remove dsRNA impurities.[27] Despite the advances in mRNA-LNP vaccine technologies, large scale IVT mRNA synthesis and purification as well as LNP encapsulation operations remain challenging due to high reagent costs, raw material availability and slow production time. A recent review article by Rosa, et al., describes potential new production approaches to overcome these challenges including alternative purification strategies and a continuous manufacturing process with enzyme and NTP recycling to reduce operation time and costs. The operations would include a microfluidic formulation step to encapsulate the mRNA into LNP providing start to finish product manufacturing.[28]

MATERIALS AND METHODS

Plasmid production. The DNA plasmid is manufactured and purified by a commercial manufacturer. *E. coli* cells are transfected with 1−5 ng of cDNA plasmid. Transformed cells are inoculated into animal-derived component free growth media containing kanamycin. Following fermentation at 30˚C for approximately 16 hours, bacterial cells are harvested by centrifugation. The cell paste is subjected to alkaline lysis with RNase A. The cell lysate is then clarified by centrifugation and adjusted to chromatography loading conditions through the addition of endotoxin removal buffer. Plasmid DNA is purified by anion exchange chromatography and hydrophobic interaction chromatography. Supercoiled plasmid DNA is ethanol precipitated, resuspended in endotoxin free water, processed through a 0.2-micron filter and stored at -20˚C. Each lot of plasmid DNA is analyzed prior to release by a standard battery of analytical tests to confirm its identity, purity, and quality (Table V).

Upstream enzymatic and transcription materials. Table VI summarizes the synthesis steps, incubation conditions, and material components for the IVT reaction.

Downstream purification materials. Table VII summarizes each downstream purification process step, components of the step, and purpose for the purification step.

Analytical methods. Table VIII and Table IX defines the quality profiles for the mRNA drug substance and LNP-mRNA encapsulated drug product, respectively. The sections summarize each test method utilized for drug substance and drug product quality release.

UV spectroscopy. Absorbance values were measured at 260 nm and normalized to a 1.0 cm path length using an M5 SpectraMax plate reader equipped with a SpectraDrop micro-volume microplate (Molecular Devices, San Jose, CA). The concentration of RNA was determined by multiplying the normalized absorbance value at 260 nm by the concentration factor, 40, which is based on an A260 reading of 1.0 being equivalent to ~ 40 μg/mL single-stranded RNA, and the appropriate dilution factor.

Table VI. Upstream synthesis steps and components

Synthesis step	Component	Reaction conditions
Template DNA Linearization	Plasmid NotI enzyme 5X Promega IVT buffer Nuclease free water	37˚C for 2 hours
Transcription (IVT)	NTPs (ATP, CTP, GTP) Pseudouridine Linearized plasmid CleanCap 5X Promega IVT buffer T7 Promega Express enzyme mix Nuclease free water	37˚C for 2 hours
Template DNA Digestion	DNase 10X NEB DNase buffer	37˚C for 2 hours
Quenching	EDTA	Room Temp for 15 minutes

Capillary gel electrophoresis. Experiments were run under denaturing conditions using a LabChip GXII HT Touch system (PerkinElmer, Waltham, MA) and the manufacturer's DNA 5K/RNA/CZE LabChip and RNA reagent kit. Samples were diluted in RNA sample buffer supplied by the kit which had been supplemented with DTT, to mitigate RNAse activity, and

Table V. Plasmid release assays and specifications

Test	Method	Specification
Appearance	Visual examination	Clear, colorless
Purity	$A_{260/280}$	1.8−2.0
Concentration	A_{260}	≥1.0 mg/mL
DNA homogeneity	Agarose gel electrophoresis	≥80 % supercoiled
Residual host RNA	SYBRGold agarose gel electrophoresis	≤5 %
Residual host genomic DNA	Quantitative PCR	≤5 %
Residual host protein	Micro BCA	≤2 %
Endotoxin	Kinetic turbidimetric limulus amoebocyte lysate	≤30 EU/mg
Bioburden	Bioburden assay, USP <23>	No growth
Identity	Restriction enzyme analysis with agarose gel electrophoresis	Consistent with reference standard
Plasmid Identity	Sanger Sequencing	Matches reference standard

Abbreviations: EU, endotoxin units.

Table VII. Downstream purification process overview

Downstream step	Primary components	Primary purpose
Dilution	Nuclease-free water	Resolubilize any precipitates
UF/DF 1	mPES-based Tangential Flow Filter, HEPES Equilibration Buffer	Exchange IVT reaction buffer for chromatography buffer, begin removing reaction components
Chromatography	Multimodal Core Bead Resin	Remove remaining impurities (rNTPs, DNA fragments, etc) from mRNA
UF/DF 2	mPES-based Tangential Flow Filter, HEPES Equilibration Buffer	Exchange chromatography buffer for final formulation buffer
Sterile Filtration	0.2 μm Sartopore 2 filter	Sterile filter final mRNA product

formamide at final concentrations of 0.05M and 49.8% (w/v), respectively. Following an incubation at 70°C for 3 minutes, samples were immediately cooled on ice for at least 5 min prior to loading on the chip. mRNA-LNP samples were similarly prepared except that Triton X-100 at 2% (w/v) was also included in the RNA sample buffer to release the encapsulated mRNA. RNA ladder samples were prepared in RNA sample buffer containing DTT but no formamide. Procedures for chip preparation, gel matrix reconstitution with fluorescent dye and post-run chip cleaning and storage were performed according to the manufacturer's instructions.

Residual protein content by T7 ELISA. ELISAs were performed by coating rabbit anti−T7 RNA polymerase polyclonal antibody (Creative Biolabs, New York, NY) at 3 μg/mL in 1X PBS (Thermo Fisher Scientific, Waltham, MA) on high-binding 96-well microplates (Greiner Bio-OneNorth America, Monroe, NC) overnight at 5°C. Plates were washed with 1X PBS containing 0.05% Tween-20 (Thermo Fisher Scientific, Waltham, MA) and then blocked with Blocker Casein in PBS (Thermo Fisher Scientific, Waltham, MA) for 1.5 hours at room temperature with shaking at 500 rpm. Following a wash step, purified T7 RNA polymerase (Aldevron, Fargo, ND) and test samples were diluted in 1X PBS with 0.05% Tween and 0.1% BSA (Thermo Fisher Scientific, Waltham, MA), added to the microplate and incubated for 2 hours at room temperature with shaking at 500 rpm. After washing, a horseradish peroxidase-conjugated rabbit anti−T7 RNA polymerase antibody (Creative BioLabs, New York, NY) was diluted to 3.3 μg/mL in Blocker Casein in PBS, added to the plate and incubated for 1 hour at room temperature with shaking at 500 rpm. The plate was then washed and 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA) was added to the plate. Following incubation with the substrate, the absorbance values were read at

Table VIII. Release and characterization testing for mRNA drug substance

Parameter	Assay
Appearance	Visual Inspection
RNA Concentration	Absorbance (UV)
Purity	RNA integrity by CGE
Quality	pH
	dsRNA content by ELISA
	Residual DNA contamination by qPCR
	Residual protein contamination by T7 Polymerase ELISA
	Characterization of capping efficiency by IP-RP-UPLC
	Characterization of poly-A tail length by CGE
Identity	RNA size by CGE and RNA Sequencing
Potency	Cell-based assay for functional binding assessment of expressed protein
Safety	Bioburden USP <61>
	Endotoxin USP <85>

Table IX. Release testing for mRNA-LNP drug product

Attribute	Assay
Appearance	Visual Inspection
Concentration	RNA encapsulation by RiboGreen assay
Quality	Particle size and polydisperity index by dynamic light scattering
	Zeta potential
	Total Lipid Content
	RNA: Lipid Ratio
	Particulates USP <788>
	Residual ethanol USP <467>
	pH
	Osmolality
Identity and Purity	Lipid's identity and content by UPLC-ELSD assay - UPLC-CAD/UV/ELSD
	RNA size and integrity by CGE
Potency	Cell-based assay for functional binding assessment of expressed protein
Safety	Sterility USP <71>
	Endotoxin USP <85>

450 nm on an SpectraMax M5 plate reader (Molecular Devices, San Jose, CA). The concentration of T7 RNA polymerase in the test sample was determined from a standard curve prepared from purified T7 polymerase, where the response of the standard concentrations was fit to a 4-parameter logistic equation.

dsRNA ELISA. ELISAs were performed by capturing anti−dsRNA monoclonal antibody, K1 IgG2a (Nordic MUbio, Susteren, The Netherlands), diluted to a concentration of 300 ng/mL in SuperBlock blocking buffer (Thermo Fisher Scientific, Waltham, MA) on protein A coated 96-well microplates (Thermo Fisher Scientific) overnight at 5˚C. Plates were then washed with 1X PBS containing 0.05% Tween-20 (Thermo Fisher Scientific, Waltham, MA). Samples were diluted in buffer prepared by combining equal volumes of a 0.1M NaCl in 1X TE solution and The RNA Storage Solution (Thermo Fisher Scientific, Waltham, MA), added to the plate and incubated for 2 hours at room temperature with shaking at 500 rpm. After washing, 50 μL of neat K2 IgM hybridoma supernant (Nordic MUbio, Susteren, The Netherlands) was added to the plate and incubated for 1 hour at room temperature with shaking at 500 rpm. Following a wash step, HRP-conjugated goat anti−mouse IgM, μ chain specific polyclonal antibody (Jackson ImmunoResearch, West Grove, PA) was diluted 1:16,000-fold in SuperBlock blocking buffer, added to the microplate and incubated for 1 hour at room temperature with shaking at 500 rpm. The plate was then washed and 1 -Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA) was added to the plate. Following incubation with the substrate, the absorbance values were read at 450 nm on an SpectraMax M5 plate reader (Molecular Devices, San Jose, CA). The concentration of dsRNA in the test sample was determined from a standard curve prepared from a 142-bp dsRNA standard (Nordic MUbio, Susteren, The Netherlands), where the response of the standard concentrations was fit to a 4-parameter logistic equation.

Residual plasmid DNA. qPCR experiments to measure residual plasmid DNA content were performed using an Applied Biosystems 7500 Fast Real Time PCR system (Thermo Fisher Scientific, Waltham, MA) and a kit with primers and probe designed to amplify a fragment of DNA sequence at the origin of replication (Creative Biolabs, New York, NY). Reactions were assembled and run using the thermal cycling conditions described by the kit manufacturer. Linearized plasmid DNA was used to generate a standard curve to quantitate the residual plasmid DNA content in test samples.

Capping efficiency. Capping efficiency was evaluated by ion-pair reversed-phase UPLC analysis using an H-Class Bio UPLC System and an ACQUITY UPLC Oligonucleotide BEH C18 column, 130Å, 1.7 μm, 2.1 mm x 50 mm (Waters Corp, Milford, MA). Isolation of the 5'end fragment was performed similarly to the method described by Beverly *et. al.* [17] using a biotinylated cleavage with the following sequence, *5'- TAT GTT mGmUmG mUmUmG mAmGmA mCmUmU mUmUmA mUmGmC /3BioTEG/* -3' (Integrated DNA Technologies, Coralville, IA). UPLC analysis of the isolated 5' end fragment was performed with a gradient elution at a flow rate of 0.2 mL/min with mobile phase A and B consisting of 400 mM hexafluoroisopropanol (Sigma-Aldrich, St. Louis, MO) 15 mM trimethylamine (Sigma-aldrich, St. Louis, MO) in water, pH 8.0, and 400 mM hexafluoroisopropanol, 15 mM triethylamine in methanol (Thermo Fisher Scientific, Waltham, MA), respectively. The gradient initiated at 19% B with a linear ramp to 26.5% over 15 minutes. Following a wash with 50% B for 1 minute to clean the column, the column was re-equilibrated to initial conditions for 5 minutes. Samples were maintained at 10˚C in the auto-sampler rack prior to injection at a volume of 10 μL. The column temperature was set to 60 ˚C with detection at a wavelength of 260 nm.

Poly A tail length. To characterize the distribution of poly-A tail length, the 3' region of interest was isolated by incubating mRNA samples with RNase A (New England BioLabs, Ipswitch, MA). mRNA (25 μg at 1.0 mg/mL) was heated to 90˚C for 30 − 60 s and quickly cooled to room temperature in a bead bath. 500 ng of RNase A at 40 ng/μL and nuclease free water were added to a total volume of 50 μL. Digestions were carried out for 30 minutes at 37˚C. Following digestion, the samples were immediately placed on ice for analysis by CGE analysis using a LabChip GXII HT Touch system (PerkinElmer, Waltham, MA) and the manufacturer's DNA 5K/RNA/CZE LabChip and RNA Reagent kit. RNase A digested mRNA samples and RNA ladder were incubated at 70˚C for 3 minutes, cooled on ice for $\geq$5 minutes, and diluted in 5.6-fold 1X RNA sample buffer supplied by the kit. Procedures for chip preparation, gel matrix reconstitution with fluorescent dye and post-run chip cleaning and storage were performed according to the manufacturer's instructions.

Cell-based expression analysis. HepG2 cells (ATCC, Manassas, VA) were cultured in Minimum Essential Media (EMEM) (Corning Inc, Corning, NY), containing 10% fetal bovine serum (Corning). For transfections with mRNA samples, cells were seeded at 1.0×10^6 cells and/or well in a 12 well tissue culture plate (Corning Inc, Corning, NY) 16 − 24h prior to the transfection. Cells were transfected with 4 μg of RNA using the Lipofectamine MessengerMax reagent

(Thermo Fisher Scientific, Waltham, MA) and Opti-MEM (Gibco, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The transfected cells were then incubated at 37˚C and 5% CO2 in a humidified incubator for 24 − 72 hours. After 24−72 hours, the supernatant containing the expressed protein was clarified by centrifugation at maximum rpm for 1−2 minutes. Clarified supernatants were stored at -80˚C if not used immediately. For transfections with mRNA-LNP samples, cells were seeded as described above. Cells were transfected with a total of 1.25 μg mRNA-LNP in Opti-MEM (Gibco, Thermo Fisher Scientific, Waltham, MA) where transfection of the LNP was facilitated by the inclusion of ApoE3 (Sigma-Aldrich, St. Louis, MO), diluted to a final concentration of 1 μg/mL in the transfection media. After addition of the transfection media, cells were incubated at 37˚C and 5% CO2 in a humidified incubator for 3 − 4 hours. Complete growth media was then added to each well and the plates incubated at 37˚C and 5% CO2 in a humidified incubator for 24−48 hours. The supernatants were clarified and stored as described above. Functional binding assessments of the expressed proteins from clarified supernatants were performed by ELISA.

Encapsulation efficiency. Encapsulation efficiency was measured using the Quant-it RiboGreen kit (Thermo Fisher Scientific, Waltham, MA). To determine the concentration of free mRNA, mRNA-LNP samples were diluted in 1X TE buffer (Ambion, Thermo Fisher Scientific, Waltham, MA) in a total volume of 2000 μL and then mixed with 1000 μL of Ribogreen solution. To measure the concentration of total mRNA, Triton X-100 was included in the sample preparation at a final concentration of 0.15% to release the encapsulated mRNA. Sample preparations were performed in a 4 mL disposable cuvette (Sarstedt, Newton, NC) and fluorescence response was measured at l_{ex} = 480 nm and l_{em} = 520 nm using the cuvette port of a SpectraMax M5 microplatereader (Molecular Devices, San Jose, CA). The concentration of the samples was determined from calibration curves that were generated in the presence and absence of detergent and fit to a linear regression model with 1/x weighting.

Particle sizing. Particle size and polydispersity index of mRNA-LNP samples were measured by dynamic light scattering using a Zetasizer Ultra (Malvern Panalytical, Malvern, UK). Samples and PBS (Sigma-Aldrich, St.Louis, MO) were filtered using a 0.2 μm syringe filter (Pall, Port Washington, NY). Samples were combined with PBS in a total volume of ~1500 μL to a target LNP concentration of ~7 μg/mL0. mg/mL and were prepared immediately prior to analysis. Measurements were performed with a backscatter angle of 173˚C and dispersant refractive index and viscosity parameters were set at 1.332 and 0.9073 cP, respectively.

Influenza antibody ELISA. H1 and H3-antigen specific antibody levels in mouse or NHP serum were determined by a standard ELISA as previously described.[29] Recombinant H1 and H3 hemagglutinin were obtained from Protein Sciences (Meriden, CT). Recombinant CH65 and Ab700364 antibodies were used to generate a standard curve.

Influenza virus challenge. All animal studies were completed in accordance with ethical guidelines for research utilizing animals. C57BL/6 mice (Jackson Labs) were treated intravenously (IV) with 35, 17, 9 or 4.5 ug of Ab700364 mRNA-LNP/mouse then challenged intranasally 24 hours later with 1e6 FFU of A/Aichi/2/1968 X-31. Animals were monitored daily for survival, body weight, and body condition, as previously described.[30]

All authors have read the journal's authorship agreement and that the manuscript has been reviewed by and approved by all named authors. All editorial support for preparation of the manuscript was completed by the named authors. In accordance with the University of Pennsylvania policies and procedures and our ethical obligations as researchers, we report that Drew Weissman is named on patents describing the use of nucleoside-modified mRNA in lipid nanoparticles as a vaccine platform and for therapeutics. We have disclosed those interests fully to the University of Pennsylvania, and we have in place an approved plan for managing any potential conflicts arising from licensing of our patents. Similarly, Kevin Saunders, and Barton Haynes have patents submitted for the HIV envelope immunogens referenced in this article. All other authors have read the Translational Research policy on disclosure of potential conflicts of interest and have none to declare.

ACKNOWLEDGMENTS

We acknowledge the generous funding and technical support from our collaborators at the US Department of Defense Advanced Research Projects Agency (DARPA; HR0011-17-2-0069), The Division of AIDS (CHAVD #UM1-AI144371 and IPCAVD UM1-AI135902, AI142596) and the Division of Microbiology and Infectious Diseases (CIVICs Contract 75N93019C00053) at the National Institutes of Health, and the collaborative support and supply of reference mRNAs from Norbert Pardi at the University of Pennsylvania Perelman School of Medicine and Acuitas Therapeutics in Vancouver, BC for lipid nanoparticle

encapsulation of candidate products. Some pre-clinical animal studies were performed in the Duke Regional Biocontainment Laboratory, which received partial support for construction from the NIH (UC6AI058607).

CONFLICT OF INTEREST

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

REFERENCES

1. Sahin U, Karikó K, Türeci Ö. mRNA-based therapeutics — developing a new class of drugs. Nat Rev Drug Discov 2014;13:759–80.
2. Pardi N, Hogan MJ, Pelc RS, et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. Nature 2017;543:248–51.
3. Saunders KO, Pardi N, Parks R, et al. Lipid nanoparticle encapsulated nucleoside-modified mRNA vaccines elicit polyfunctional HIV-1 antibodies comparable to proteins in nonhuman primates. NPJ Vaccines 2021;6:50.
4. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. N Engl J Med 2020;383:2603–15.
5. Baden LR, El Sahly HM, Essink B, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. N Engl J Med 2020;384:403–16.
6. Rele S. COVID-19 vaccine development during pandemic: gap analysis, opportunities, and impact on future emerging infectious disease development strategies. Hum Vaccin Immunother 2021;17:1122–7.
7. Bloom K, van den Berg F, Arbuthnot P. Self-amplifying RNA vaccines for infectious diseases. Gene Ther 2021;28:117–29.
8. Jackson NAC, Kester KE, Casimiro D, Gurunathan S, DeRosa F. The promise of mRNA vaccines: a biotech and industrial perspective. NPJ Vaccines 2020;5:11.
9. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines — a new era in vaccinology. Nat Rev Drug Discov 2018;17:261–79.
10. Karikó K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 2005;23:165–75.
11. Pardi N, Hogan MJ, Naradikian MS, et al. Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses. J Exp Med 2018;215:1571–88.
12. Weissman D, Pardi N, Muramatsu H, Karikó K. HPLC purification of in vitro transcribed long RNA. Methods Mol Biol 2013;969:43–54.
13. Karikó K, Muramatsu H, Ludwig J, Weissman D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res 2011;39:e142.
14. Pardi N, Muramatsu H, Weissman D, Karikó K. In vitro transcription of long RNA containing modified nucleosides. Methods Mol Biol 2013;969:29–42.
15. Cathala GUY, Savouret J-F, Mendez B, et al. A method for isolation of intact, translationally active ribonucleic acid. DNA 1983;2:329–35.
16. Schönborn J, Oberstrass J, Breyel E, Tittgen J, Schumacher J, Lukacs N. Monoclonal antibodies to double-stranded RNA as probes of RNA structure in crude nucleic acid extracts. Nucleic Acids Res 1991;19:2993–3000.
17. Beverly M, Dell A, Parmar P, Houghton L. Label-free analysis of mRNA capping efficiency using RNase H probes and LC-MS. Anal Bioanal Chem 2016;408:5021–30.
18. Sempowski GD, Saunders KO, Acharya P, Wiehe KJ, Haynes BF. Pandemic preparedness: developing vaccines and therapeutic antibodies for COVID-19. Cell 2020;181:1458–63.
19. Capone S, Meola A, Ercole BB, et al. A novel adenovirus type 6 (Ad6)-based hepatitis C virus vector that overcomes preexisting anti-ad5 immunity and induces potent and broad cellular immune responses in rhesus macaques. Journal of virology 2006;80:1688–99.
20. Haynes BF, Mascola JR. The quest for an antibody-based HIV vaccine. Immunol Rev 2017;275:5–10.
21. Haynes BF, Kelsoe G, Harrison SC, Kepler TB. B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. Nat Biotechnol 2012;30:423–33.
22. Bonsignori MKT, Fera D, Meyerhof RR, et al. Staged induction of HIV-1 glycan-dependent broadly neutralizing antibodies. Sci Transl Med 2017;9(381):1–12.
23. Walker SE, Lorsch J. Chapter Nineteen - RNA Purification – Precipitation Methods editor. In: Lorsch J, ed. Methods in Enzymology, Academic Press, 2013:337–43.
24. Kelley B. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. MAbs 2009;1:443–52.
25. Zhang NN, Li XF, Deng YQ, et al. A thermostable mRNA vaccine against COVID-19. Cell 2020;182:1271–1283.e16.
26. Sahin U, Karikó K, Türeci Ö. mRNA-based therapeutics−developing a new class of drugs. Nat Rev Drug Discov 2014;13:759–80.
27. Baiersdörfer M, Boros G, Muramatsu H, Mahiny A, Vlatkovic I, Sahin U, et al. A facile method for the removal of dsRNA contaminant from in vitro-transcribed mRNA. Mol Ther Nucleic Acids 2019;15:26–35.
28. Rosa SS, Prazeres DMF, Azevedo AM, Marques MPC. mRNA vaccines manufacturing: challenges and bottlenecks. Vaccine 2021;39:2190–200.
29. Samo M, Choudhary NR, Riebe KJ, Shterev I, Staats HF, Sempowski GD, et al. Immunization with the Haemophilus ducreyi trimeric autotransporter adhesin DsrA with alum, CpG or imiquimod generates a persistent humoral immune response that recognizes the bacterial surface. Vaccine 2016;34:1193–200.
30. Junkins RD, Gallovic MD, Johnson BM, et al. A robust microparticle platform for a STING-targeted adjuvant that enhances both humoral and cellular immunity during vaccination. J Control Release 2018;270:1–13.