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Quantification of mRNA in Lipid Nanoparticles Using Mass Spectrometry

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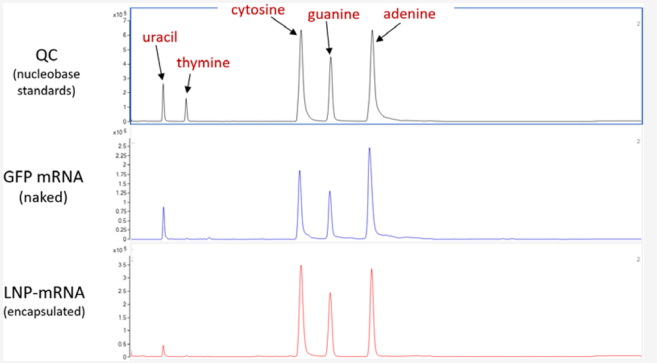
ABSTRACT: Lipid nanoparticle-encapsulated mRNA (LNP-mRNA) holds great promise as a novel modality for treating a broad range of diseases. The ability to quantify mRNA accurately in therapeutic products helps to ensure consistency and safety. Here, we consider a central aspect of accuracy, measurement traceability, which establishes trueness in quantity. In this study, LNP-mRNA is measured in situ using a novel liquid chromatography−mass spectrometry (LC-MS) approach with traceable quantification. Previous works established that oligonucleotide quantification is possible through the accounting of an oligomer's fundamental nucleobases, with traceability established through common nucleobase calibrators. This sample preparation does not require mRNA extraction, detergents, or enzymes and can be achieved through direct acid hydrolysis of an LNP-mRNA product prior to an isotope dilution strategy. This results in an accurate quantitative analysis of mRNA, independent of time or place. Acid hydrolysis LC-MS is demonstrated to be amenable to measuring mRNA as both an active substance or a formulated mRNA drug product.



INTRODUCTION

Messenger RNA (mRNA) therapeutics is an emergent platform for the treatment and prevention of diseases.[1−3] The COVID-19 pandemic has accelerated our ability to design and deliver vaccines and supply safe and effective treatments. Currently, numerous clinical trials are being investigated using diverse applications of mRNA therapeutics for infectious diseases of viral or bacterial pathogens, for cancers, and more.[4] mRNA drug product delivery in vivo is generally achieved after encapsulating mRNA in a lipid nanoparticle (LNP) vesicle to protect the nucleic acid cargo as it is shuttled through the cell membrane for the final translation into protein antigen. Unfortunately, this mRNA encapsulation step introduces a new analytical measurement challenge—ensuring full mRNA extraction from the LNP vesicle before quantification. Encapsulation also introduces the necessity to measure mRNA quantity twice within a drug development process—within and without LNP—requiring additional time and capabilities. Not surprisingly, extraction approaches and measurement platforms are not uniform within the mRNA community, and no shared reference material is currently available to harmonize approaches to bring measurements into concordance.

Therapeutics are becoming more complex—from small, pure synthetic chemical derivatives to mixtures of proteins, to nucleic acid oligomers with complex structure and increased heterogeneity, and to micrometer-sized cell-based therapies. As the production of these products gets more sophisticated, establishing measurement trueness and defining traceability have become proportionately more challenging and meaningful. For mRNA therapeutics, the quantity of active substance (i.e., naked mRNA) is commonly assessed using UV spectroscopy, RT-qPCR, or RT-dPCR, whereas the drug product (LNP-mRNA) is commonly assessed by fluorescence-based assays expressed in terms of encapsulation efficiency (free mRNA/total mRNA).[5] While UV spectroscopy is both fast and inexpensive, it lacks well-defined calibration, can be nonspecific, and depends on the estimates of molar absorptivity coefficients.[6] PCR-based techniques, while specific, are limited by RNA-to-cDNA conversion efficiency and are traceable to the enzymes, primers, or initiation steps used, which may change over time or setting. Measurement variability is commonly observed in PCR comparability studies,[7,8] and a lack of traceability is implicit because of the required enzymatic steps and nonuniform calibrators. Also, reverse transcription can result in a loss of information about the existing base modifications on the mRNA template. Correspondingly, fluorescence assays like RiboGreen, which

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are widely used to determine drug product quantity following mRNA extraction using nonionic surfactants such as Triton, lack traceability, matched calibration, and internal control (and use intercalating dyes which are potential mutagens[9,10]). While all of these approaches may be fit for their specific purpose, currently, there is no way to compare between or to evaluate their accuracy.

It is proposed here that a careful consideration of mRNA quantity includes a link through an SI-traceable measurement approach in order to ensure calibration consistency, to reduce bias, and to account for measurement drift. No such SI-traceable standard currently exists for nucleobases; however, the best available well-characterized materials are used in this work to provide a framework for establishing SI traceability, when higher-order standards do eventually become available. Previous work[11] demonstrated the use of acid hydrolysis-isotope dilution-liquid chromatography-mass spectrometry (ID-LC-MS) to quantify pure or matrix-based oligonucleotides. Here, we expand on that technique to measure the mRNA cargo within an LNP-encapsulated product. Data presented demonstrate that LNPs are lysed, and mRNA hydrolyzes into nucleobases, in a single hydrolysis step using formic acid at elevated temperatures (140 °C). Nucleobase quantity is traceable through well-characterized reference standards, and individual nucleobase mass fractions can serve as independent surrogates of the mRNA mass fraction. Additionally, this technique can concurrently monitor protein contamination (via amino acid detection) and DNA contamination (via thymine detection) during LC-MS analysis. Nucleotide monophosphates (NMPs) can be observed in parallel scans using multiple reaction monitoring mass spectrometry (MRM-MS) for assessing hydrolysis completion.

The proposed acid hydrolysis-ID-LC-MS method for oligonucleotides is analogous to amino acid analysis commonly used as the gold standard for quantifying pure proteins. ID is a method of internal standardization that enables measurement calibration to a stable, common standard using a ratiometric approach rather than relying on analytical equations and acts as a primary method to minimize uncertainty.[12] Pure, well-characterized standards (nucleobase calibrators) are used as common and consistent primary measurement anchors. The multiplexed power of MRM analysis enables consistent, traceable, and accurate measurements.

EXPERIMENTAL SECTION

Materials. LNP-encapsulated mRNA with a luciferase gene-encoding sequence was purchased through ProMab Biotechnologies, Inc. (Richmond, CA), catalogue PM-LNP-0024, and stored at 4 °C. The in vitro-transcribed mRNA product is predicted to have 2082 nucleotides including a 5′ cap and a cotranscribed polyA tail (MW = 707,681 Da). An LNP-encapsulated mRNA material with a GFP gene-encoding sequence was generously prepared and provided by colleagues at InDevR, Inc. (Boulder, CO), with an in vitro-transcribed mRNA product predicted to have 865 nucleotides (MW = 296,272 Da) including the capping and enzymatic polyA tail (expected average polyA length = 125 nucleotides). An IVT-produced 5moU-substituted Cas9 mRNA (product L-7206) that was provided aliquoted and frozen in 1 mmol/L sodium citrate buffer was purchased from TriLink BioTechnologies (San Diego, CA) and stored at −80 °C until use. The full mRNA is predicted to be of 4521 nucleotides (MW = 1,491,930 Da).

Nucleobase calibrators were purchased from Millipore Sigma. The calibrators are categorized as 'Pharmaceutical Secondary Standard Certified Reference Materials' with traceability to the USP, EP (PhEur), and BP primary standards. No further chemical purity or water analysis was performed in-house [adenine (PHR1383, traceable to USP 1012101, PhEur A0230000); cytosine (PHR1350, traceable to USP 1162148); guanine (PHR1243, traceable to BP 879 and USP 1302156); thymine (PHR1345, traceable to USP 1754532); and uracil (PHR1581, traceable to USP 1705753 and PhEur Y0000764)].

Stable isotope-labeled nucleobases were purchased through Cambridge Isotope Laboratories, Inc. (Andover, MA) for use as internal standards and are described in Supplemental Table S1. Formic acid was LC-MS-graded from Honeywell (56302). Trifluoroacetic acid (~6 mol/L, > 99% v/v) was purchased through Sigma (299537). LC-MS-grade Chromasolv water and acetonitrile were purchased from Honeywell. Stock solutions of adenine, cytosine, thymine, and uracil were prepared gravimetrically in LC-MS-grade water, approximately 4−10 mg of powder dissolved in 10 mL of water; guanine stock solutions were prepared in 0.1 mol/L HCl in LC-MS-grade water with gentle heating. Subsequent dilution stocks were made with pure LC-MS-grade water. All nucleobase stock solutions were stored at 4 °C.

Isotope Dilution. A double exact-matching isotope dilution (ID) workflow was designed for the quantitative assay with SIL-internal standard nucleobases prespiked into samples and calibrants prior to hydrolysis in order to normalize for potential bias from instrumental or sample-preparation variation. Calibration was achieved with a five-point bracketing approach using gravimetrically prepared nucleobases of known mass fractions in a mixture with SIL-nucleobases, prepared at unlabeled/labeled mass ratios of approximately 0.6, 0.8, 1.0, 1.2, and 1.4 (m/m %), where the midpoint of the calibration range is estimated as the expected nucleobase mass fraction in the mRNA. SIL internal standards were gravimetrically prepared at a ~ 1:1 molar equivalence to the expected nucleobase mass fraction in the sample and were held roughly constant among calibrators. All calibration regression lines were reported with coefficients of determination (R^2) ≥ 0.99.

Acid Hydrolysis. Samples or calibrators were gravimetrically added to 400-μL glass flat-bottomed autosampler inserts (Agilent, 5181-3377) together with SIL-nucleobase mixtures and dried to dryness in a speed-vac without heat. Glass inserts were placed in an acid-resistant, temperature-safe Teflon vessel within a steel compression pressure bomb. Three drops of neat formic acid was added using a Pasteur pipet to the bottom of the glass insert, and approximately 2 mL of formic acid was pipetted external to the glass inserts, into the Teflon vessel. The pressure bomb was tightened to ensure vapor pressure retention and placed into an oven at 140 °C. After hydrolysis, glass inserts were removed and dried to dryness in a speed-vac, and samples were subsequently reconstituted in water with no sample cleanup for downstream analysis by mass spectrometry.

LC-MS/MS Analysis. Separation of nucleobases was achieved on a mixed-mode chromatography column based on ion-exchange and reversed-phase characteristics, using a decreasing pH gradient and increasing organic concentration (acetonitrile, ACN) in the mobile phases. Gradient elution increased linearly from 99% (v/v) mobile phase A (0.5 mL/L trifluoroacetic acid (TFA)) to 10% (v/v) mobile phase B (4.5 mL/L TFA in 0.2 L/L aqueous ACN) over 10 min; linearly to

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Table 1. MRM Fragmentation Transitions for LC-MS/MS Analysis

	analyte	precursor ion $(M \pm H)^{\pm}$	product ion $(M \pm H)^{\pm}$	Fragmentor voltage (V)	collision energy (V)	polarity
nucleobases	adenine	136.1	92	100	30	positive
			119		20	positive
	SIL-adenine	141.4	95.3		30	positive
			123.4		20	positive
	cytosine	112.1	52.1	120	32	positive
			95		18	positive
	SIL-cytosine	115.4	52.5		32	positive
			97.4		18	positive
	guanine	152.1	110	140	18	positive
			135		15	positive
	SIL-guanine	157.4	113.4		18	positive
			139.3		15	positive
	thymine	127.1	54	120	22	positive
			110		12	positive
	SIL-thymine	134.4	58.5		22	positive
			116.4		12	positive
	uracil	113.00	70	125	17	positive
			96		15	positive
	SIL-uracil	119.4	74.4		17	positive
			101.5		15	positive
	5moU	143.0	98.0	125	20	positive
			70.0		20	positive
NMPs	AMP	346.2	211	140	18	negative
	GMP	362.2	211	140	18	negative
	TMP	337.2	211	140	18	negative
	UMP	323.2	211	140	18	negative
amino acids	phenylalanine	166.2	120.1	70	10	positive
	leucine/isoleucine	132.1	86.1	60	10	positive
	valine	118.1	72.1	60	10	positive
	proline	116.1	70.1	80	10	positive

40% (v/v) B over 20 min, followed by a column wash at 95% (v/v) B, and re-equilibration. The column was washed with 95% (v/v) ACN for >1 h between sample sets. A constant mobile phase flow rate of 200 μL/min was used. The PrimeSep 100 LC column (SIELC Technologies, Wheeling, IL), 2.1 × 250 mm, 3 μm particles, was maintained at 50 °C in a thermostated column compartment. Samples were kept at 5 °C in an autosampler. Fresh mobile phases were prepared weekly. For quantification, a multiple-reaction monitoring (MRM) assay was developed to target two fragmentation transitions for each nucleobase and the stable-isotope-labeled analogue (Table 1). Precursor-to-product ion fragmentation transitions and ionization conditions were optimized using purified standards of nucleobases. An Agilent 1290 Infinity liquid chromatography system (Agilent Technologies, Santa Clara, CA) was used inline with an Agilent 6460A triple quadrupole (QQQ) mass spectrometer. Electrospray ionization (ESI) was achieved in positive ion polarity for nucleobases and amino acids and in negative polarity for nucleotide monophosphate (NMP) detection. All analyses were performed with the following Agilent 6460A MS source parameters: source gas temperature = 300 °C, source gas flow = 13 L/min, nebulizer = 345 kPa (50 psi), sheath gas temperature = 250 °C, sheath gas flow = 12 L/min, capillary voltage = ± 3500 V, and nozzle voltage = ± 1500 V. Data was acquired in unit resolution for MS^1 and MS^2 with a 100 ms dwell time per transition and cell accelerator voltage of 7 V. Collision energy and Fragmentor voltage were held constant for unlabeled and SIL analog pairs (Table 1). Agilent MassHunter Workstation software (version B.10.01) was used for peak selection and integration. Peak retention times and integrated peak areas were automatically determined by MassHunter, and the samples were visually inspected with manual integration as required. Peak area ratios were exported to Microsoft Excel for quantitative analysis.

RESULTS

LNP-mRNA Quantification. An off-the-shelf, commercial LNP-mRNA product was acquired to establish precision of the proposed method of quantitative analysis using direct acid hydrolysis ID-LC-MS. The LNP-mRNA product was designed by the manufacturer as a control material having a luciferase encoding sequence in a 2082 nucleotide-long mRNA. The LNP particles were formulated with a proprietary mixture of SM-102, DSPC, cholesterol, and DMG-PEG2000 and the LNP-mRNA and was provided in a dilute PBS solution including sodium acetate and ethanol. For quantification by LC-MS/MS, a double isotope dilution strategy was employed with traceability established through freshly prepared solutions of pure nucleobase calibrators (Pharmaceutical Secondary Standard CRMs) which are traceable to USP and PhEur primary standards. Roughly 66 μL (~3.3 μg mRNA) of the commercial LNP-mRNA was gravimetrically aliquoted (in triplicate) for acid hydrolysis, dried to dryness, and directly hydrolyzed without sample cleanup at 140 °C for 24 h in a pressure bomb.

A mixture of stable-isotope-labeled (SIL) ($^{13}C/^{15}N$) nucleobases was used as an internal standard to account for potential variability arising from the hydrolysis process,

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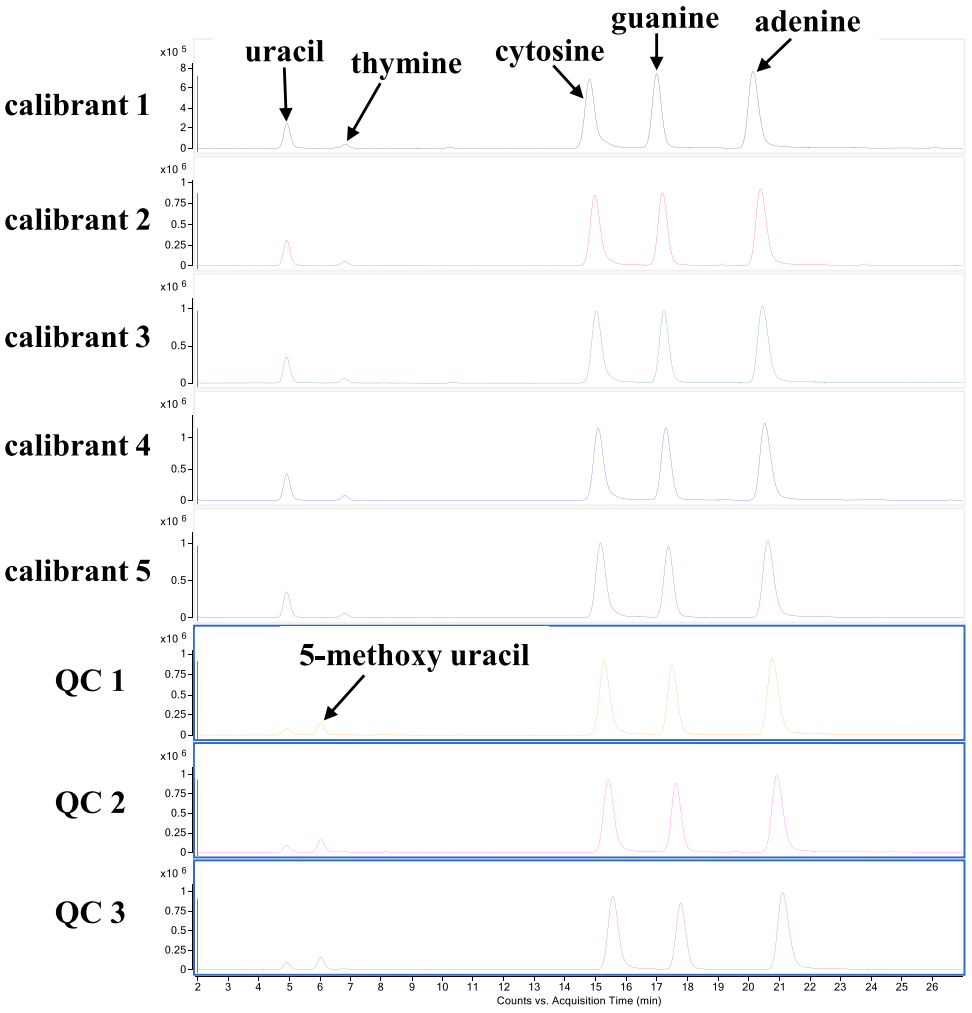

Figure 1. LC-MS/MS (MRM) TICs of acid hydrolyzed calibrators (pure nucleobases) and QCs (naked mRNA).

variability from electrospray ionization, or from autosampler sampling inconsistency, as well as to account for instrument drift over time. SIL was added gravimetrically to the calibration solutions, to the LNP-mRNA samples, and to the QCs at the earliest possible moment, notably prior to hydrolysis. A five-point calibration was designed to bracket the nucleobase mass fractions expected in the sample, with calibration points ±20% and ±40% of expected values and with one calibration point at roughly the expected mass fraction. All calibration solutions were hydrolyzed concurrently with samples and QCs. Regression analysis ($y = mx + b$) of the calibration mixtures yielded R^2 values ≥0.99 for two MRM transitions from each nucleobase. Figure 1 shows the chromatographic resolution of natural bases with detection by MRM-MS. Chromatography is consistent among calibrants, samples, and QC samples.

Table 2 presents quantitative results of the mRNA mass fraction in the LNP-mRNA sample (μg/mg) based on calculations from measurements of surrogate nucleobases. A standard deviation (σ) and coefficient of variation (relative standard deviation, % RSD) for the measurements is provided. The nucleobases adenine, cytosine, and guanine were quantified from triplicate measurements using two MRM transitions each. Uridine triphosphate (UTP) was replaced by 5-methylpseudouridine triphosphate during IVT, and was therefore not quantified (no commercial calibrator exists). One measurement for guanine was determined as an outlier using a standard z-score calculation ($z > 3$). The overall precision of the mRNA quantification is 3.9% RSD, with a mean mass fraction result of 38.83 μg/g ± 1.5 μg/g. Figure 2 provides representative extracted ion chromatogram (XIC) data from LC-MS/MS (MRM) analysis of the acid hydrolyzed LNP-mRNA.

Table 2. Quantification (μg/g) of mRNA in LNP Samples Calculated from Adenine, Cytosine, or Guanine Mass Fractions, and Combined

	adenine	cytosine	guanine	overall
mean ($\bar{x}$), μg/g	38.54	38.49	39.63	38.83
st. dev. (σ)	1.6	2.0	0.27	1.5
% RSD	4.1	5.3	0.67	3.9

A commercial, unencapsulated mRNA was used as QC for sample preparation and analytical measurement. Roughly 3 μg of the QC mRNA was measured in triplicate. QC samples were hydrolyzed concurrently with the LNP-mRNA samples and nucleobase calibrators. QC samples were prespiked using the identical internal standard mixture. Table 3 provides quantitative results for mRNA QC, having an estimated mass concentration of 1.0 mg/mL, as determined by the manufacturer using OD_{260} absorbance measurements. The overall mean mRNA concentration was determined to be 1000.9 μg/g with a measurement precision ($n = 18$) of 2.8%

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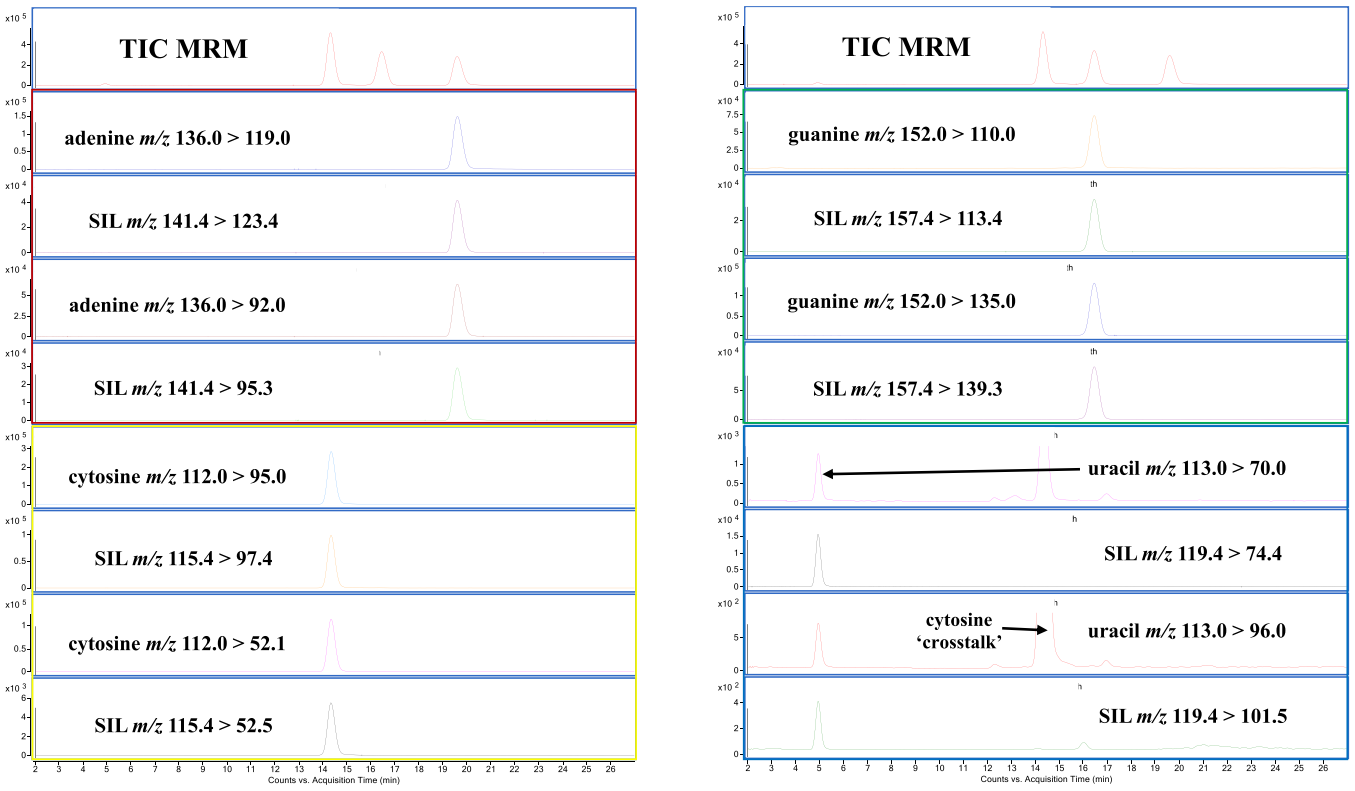

Figure 2. LC-MS/MS (MRM) XICs of acid hydrolyzed LNP-mRNA (ProMab).

Table 3. Quantification (μg/g) of mRNA in Unencapsulated (Naked) QC Samples Calculated from Adenine, Cytosine, or Guanine Mass Fractions, and Combined

	adenine	cytosine	guanine	overall
mean ($\bar{x}$), μg/g	974.0	1006.9	1021.9	1000.9
st. dev. (σ)	14.8	24.4	20.5	28.1
% RSD	1.5	2.4	2.0	2.8

RSD among the three nucleobases quantified (A, C, G). Figure 1 provides the total ion mass chromatograms obtained from the analysis of the QC samples. In addition to the standard nucleobases for A, C, and G, the QC mRNA sample was transcribed in vitro using modified 5-methoxy UTP (5moUTP), and could be detected (but not quantified) by two MRM fragmentation transitions of 5-methoxy uracil (5moU) (Table 1).

Hydrolysis Time-Course Analysis. Three sets of time-course analyses were performed to assess the robustness of the acid hydrolysis ID-LC-MS/MS method.

First, nucleobase stability was demonstrated under acid hydrolysis conditions (low pH, high temperature) to ensure calibrators are fit-for-purpose and to safeguard measurement accuracy. Supplemental Figure 1a plots raw peak areas integrated from MRM data obtained for each nucleobase over six time points (0, 1, 18, 40, 126, and 168 h) ranging from zero to 7 days. In Supplemental Figure 1b, raw peak areas are normalized to SIL (^{15}N/^{13}C) nucleobase internal standards.

Second, acid hydrolysis was performed on a pure, unencapsulated mRNA material at seven time points (1, 3, 5, 9, 15, 24, and 72 h) over 3 days to ensure mRNA hydrolysis completion. All nucleobases were observed to be completely hydrolyzed before 24 h. Supplemental Figure 2 plots the time-course of the liberation of nucleobases from an intact mRNA during acid hydrolysis. It is noted that the purines—adenine and guanine—are hydrolyzed earlier during the time-course, and the signal is saturated more rapidly relative to the pyrimidines, cytosine, and 5moU. The purines are mostly liberated by 1 h of hydrolysis, whereas pyrimidines require more hydrolysis time to reach a saturation curve inflection point.

Third, a separate [GFP] mRNA-LNP material was prepared by a collaborator encapsulated using lipids equivalent to those used in the Pfizer Comirnaty vaccine[13] and subjected to acid hydrolysis at three distinct time points (19.5, 25, and 42 h). Nucleobase mass fractions were quantified by acid hydrolysis-ID-LC-MS, and mRNA mass fraction was calculated from each nucleobase measurement. The nNucleobase signal was normalized to SIL nucleobase internal standards and standardized to pure nucleobase calibrators. Supplemental Figure 3 plots the time-course of mRNA mass fraction determined by the liberation of nucleobases from an LNP-encapsulated mRNA product after being subjected to acid hydrolysis. No statistical difference is observed between quantitative results from all hydrolysis time points, suggesting that this LNP-encapsulated mRNA does not require more than 19.5 h of hydrolysis time to reach completion. Earlier time points were not considered for this experiment out of concern for contaminating the mass spectrometer and column with intact LNPs. Because the three time points can be considered statistically equivalent, the results could be treated as sample replicates and are combined in Table 4 as triplicate measurements, with the mean (58.0 μg/mL), standard deviation (±3.4 μg/mL), and variability (5.9%) provided. A RiboGreen assay was performed by a collaborator, and the data reported the estimated mRNA concentration to be ~56.5 μg/

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Table 4. Quantification (μg/g) of GFP mRNA in LNP-Encapsulated Samples Averaged for Three Hydrolysis Time Points and Calculated from Adenine, Cytosine, or Guanine Mass Fractions

	adenine	cytosine	guanine	overall
mean ($\bar{x}$), μg/g	54.3	58.4	61.4	58.0
st. dev. (σ)	0.5	2.8	1.2	3.4
% RSD	0.9	4.7	2.0	5.9

mL (encapsulated) and ~63 μg/mL (total, Triton-extracted) in the LNP-mRNA material with no reported uncertainty.

Impurity Analysis. Three sets of impurity analyses were performed alongside the measurements of RNA-specific nucleobases.

First, nucleotide monophosphates (NMPs) were monitored by LC-MS/MS in negative polarity ionization to assess for hydrolysis completion. MRM assays using polarity switching were developed for adenosine monophosphate (AMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), and 5-methoxy uridine monophosphate (5moUMP) based on the common fragmentation of these NMPs at either the N-glycosidic bond between the ribose ring and the nucleobase (resulting in −1 charged ions of m/z = 211 and a weaker signal from negative ions of the particular nucleobase), or at the phosphodiester bond (resulting in two strong negatively charged phosphate ions, PO_3^- and $H_2PO_4^-$). These fragment ions have been previously[14] described and were observed in full scan MS^2 spectra of partially hydrolyzed mRNA samples to be the prominent diagnostic ions (Supplemental Figure 4).

Furthermore, as discussed previously, an mRNA hydrolysis time-course was performed to assess hydrolysis completion. Full-scan MS^1 TICs were acquired at each hydrolysis time point in negative polarity from 100 to 2000 m/z over an LC gradient to detect unhydrolyzed NMPs (and other unhydrolyzed mRNA fragments which were notably unobserved in a full-scan chromatographic run). Supplemental Figure 5 shows the loss of chromatographic features due to pyrimidine NMPs. Purine NMPs (AMP and GMP) were not clearly observed above noise in full-scan MS^1 even at early time points. It is shown that the contribution of the NMP signal is negligible by 9 h of hydrolysis. The samples were subsequently analyzed in the base peak chromatograms (BPC) from product ion MS^1 chromatographic analyses, and Supplemental Figure 6a,b supports previous evidence that purines are fully hydrolyzed at an earlier time point relative to pyrimidines.

Second, the acid hydrolysis-ID-LC-MS/MS method is amenable to quantifying the levels of the DNA-specific nucleobase thymine. Residual DNA contamination in mRNA therapeutic products results from the incomplete removal of plasmid DNA following, typically, DNase I digestion, LiCl precipitation, chromatographic purification, and/or tangential flow filtration steps.[15] Product-related impurity detection for dsDNA is a critical quality attribute for mRNA active substance[15] and is commonly assessed using ELISA, whereas process-related residual DNA template impurities are detected by qPCR techniques. Thymine has been shown previously[11] to be liberated from DNA following similar acid hydrolysis approaches, as described above. Supplemental Figure 7 is provided to demonstrate thymine impurity detection in hydrolyzed samples of unencapsulated mRNA and in LNP-encapsulated products. Thymine can act as a surrogate for deoxyribonucleotides and distinguishes residual DNA from the mRNA therapeutic product.

Third, residual protein impurities can be assessed contemporaneously through the detection of amino acids, which act as proxies for proteins following acid hydrolysis. Residual protein impurity is commonly suggested as a critical quality attribute for active substance impurity release testing.[5,16] Amino acids are detectable using the identical acid hydrolysis sample preparation and LC separation as those used for nucleobases, and they are resolved from nucleobases and NTPs chromatographically. Figure 3 provides extracted ion chromatograms for commonly measured amino acids (those commonly accepted as stable during hydrolysis in AAA studies[17]), detected here in a hydrolyzed LNP-mRNA (luciferase) commercial product. Five amino acids ideal for quantification were monitored by MRM (Ile, Leu, Phe, Pro, and Val) and were detectable above the LOQ. Absolute

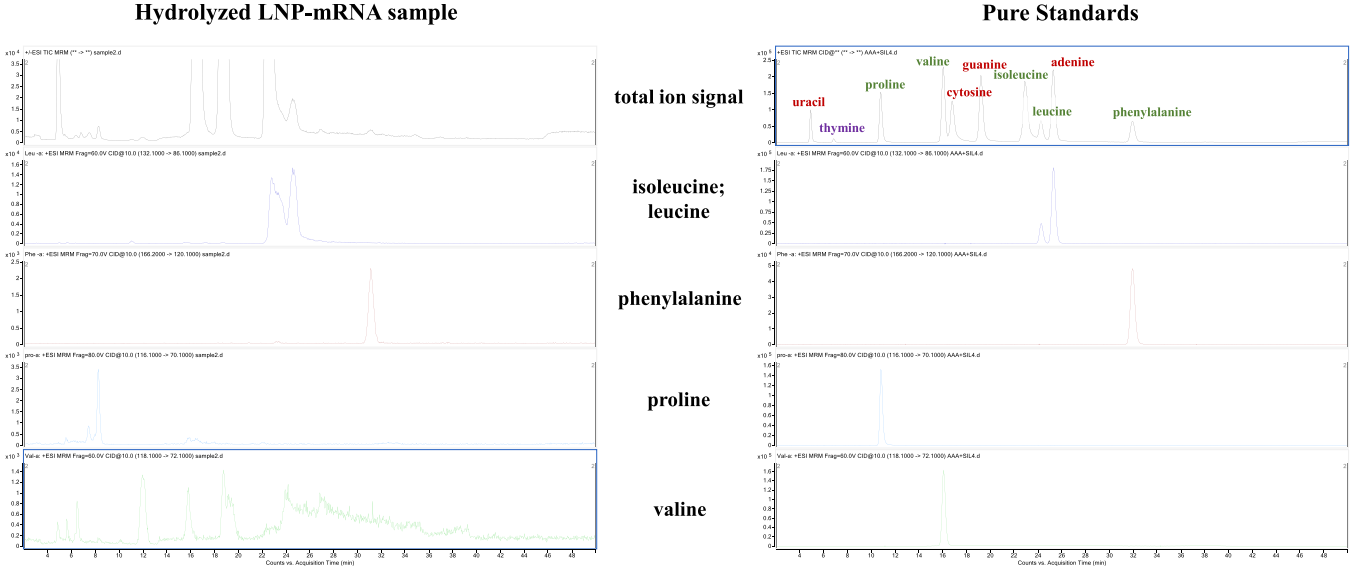

Figure 3. Residual protein impurity detected in LNP-mRNA products.

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Table 5. Comparison of Quantitative Results for mRNA Products

	TriLink Cas9 mRNA (QC)	ProMab luciferase mRNA-LNP	GFP mRNA-LNP (in-house prep)
mRNA MW	1,491,930 Da	707,680.6 Da	296,272 Da
# nucleotides	4521	2082	865
hydrolysis/ID LC-MS/MS	1000.9 μg/g ± 28.1 μg/g	38.83 μg/g ± 1.5 μg/g	58.0 μg/g ± 3.4 μg/g
RiboGreen ± Triton extraction		∼50 μg/mL	∼56.5 μg/mL (encapsulated) ∼63 μg/mL (total)
UV	∼1000 μg/mL		

quantitation of protein contamination in mRNA therapeutics requires knowledge of the makeup and ratio of all residual proteoforms within the mixture, which is unknown for these products, and use of optimized hydrolysis conditions for each proteoform.

DISCUSSION

This work demonstrates the effectiveness of direct acid hydrolysis to enable precise mRNA quantification for naked or LNP-encapsulated mRNA. Measurement precision of this assay is shown to be less than 5% RSD for a combined agreement between nucleobases, independent sample preparations, and replicates, similar to other isotope dilution methods.

No reference measurement procedures nor higher-order reference standards exist thus far for mRNA quantitation, impeding measurement comparability. Multiple factors, however, add confidence to the measurement accuracy (trueness) of this acid hydrolysis-ID-LC-MS assay and are identified as (1) agreement among nucleobases to act as independent surrogates of intact mRNA mass fraction, (2) samples and QC materials that are comparable with estimates from other analytical techniques (UV, RiboGreen), (3) evidence of complete mRNA hydrolysis and nucleobase stability, and (4) the use of well-characterized calibrators within a higher-order measurement system (isotope dilution).

Table 5 provides a summary of the experimental results for mRNA quantification using the acid hydrolysis-ID-LC-MS approach, in contrast to spectroscopy estimates. The QC material used for this work was a 4521-nucleotide mRNA transcript with the mass concentration estimated using UV absorbance at approximately 1 mg/mL. The LC-MS approach agreed well (1000.9 μg/g ± 28.1 μg/g) to the UV estimate from the manufacturer. Agreement to UV spectroscopy was anticipated for measurements of pure RNA, based on previous work[11] that measured the commercial, synthetic RNA (RNA Control 250, Ambion). The previous study also demonstrated the agreement of LC-MS with droplet digital PCR for estimating the quantity in matrix-based DNA materials (including a 7934 bp linearized DNA plasmid and whole genomic DNA prepared from buffy coat fractions).

Table 5 also compares the quantitative results with RiboGreen assays following Triton extraction using two different LNP-encapsulated mRNA products. An evaluation of the results shows modest agreement for one example of LNP-mRNA material but poorer agreement (Δ = −20%) in another example. Deviation between these platforms is most likely due to the fact that the measurement assays are calibrated very differently. Calibration for the acid hydrolysis-ID-LC-MS assay is traceable through pure nucleobase powders (well-characterized standards). External calibration to traceable materials ensures accuracy, and internal controls ensure that sample preparation losses are normalized. One recognized source of potential bias for this method, incomplete hydrolysis, is considered unlikely in light of the time-course hydrolysis analysis of LNP-mRNA, which is shown to be constant between 19.5 and 45 h.

Comparatively, RiboGreen calibration is based on the use of 16S and 23S rRNA controls provided by the manufacturer kit. This calibration approach uses different lengthed and structured RNA materials, potentially made of a different base composition and provided in a different matrix, as a nonexact-matched standard to externally calibrate quantity. More so, internal control is not normally possible for RiboGreen assays to account for the sample preparation bias. Nonspecific binding to DNA, fluorescence signal variability for homopolymers like polyA, extraction and staining efficiency of mRNA, molar extinction coefficient estimations, photodegradation, and effects from differing salt concentrations are other known potential sources of uncontrolled bias to consider.[18]

This study is a proof-of-principle demonstrating that acid hydrolysis-ID-LC-MS is capable of accurate, repeatable measurements of LNP-encapsulated mRNA. It is understood that the next important step is demonstrating reproducibility of the measurement assay between laboratories and over time. Only three nucleobases were used to independently calculate the mRNA mass fraction of the LNP-luciferase mRNA. This is due to the fact that the LNP-mRNA material was transcribed using 5-methyl pseudouridine, and currently, there is no commercially available 5-$CH_3\Psi$ nucleobase standards to use for calibration (nor a stable-isotope-labeled analogue). Similarly, there are no higher-order standards available for other modified uridine analytes such as 5-methoxy uridine, pseudouridine (Ψ), or N1-methylpseudouridine (N1-$CH_3\Psi$). Other modified bases such as 5-methyl cytosine may be important components of future RNA-based drugs and are also amenable to quantification using this approach. Developing these calibration standards is possible with modest investment, and should be a consideration for the future.

Because ID LC-MS/MS assays quantify nucleobase mass fractions as surrogates for the measurand (mRNA) mass fraction, it is necessary to know the mRNA composition (the number of each nucleobase in the sequence) and the mRNA MW to make the required calculations. The current study was limited to commercial products where this information could and would be provided by the manufacturers, upon request. However, the approach is broadly applicable where the mRNA composition and MW are knowable.

Isotope dilution strategies combine robust and accurate quantification. The proposed method for nucleic acid quantification is analogous to the current gold standard approach for absolute protein quantification, amino acid analysis (AAA), whereby a protein is hydrolyzed into amino acids that serve as protein surrogates.[17,19] There are advantages to using isotope dilution strategies, in addition to accuracy and precision. Traceability of the measurement is well-defined,

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which enables the results to be independent of the time or location. Similarly, mass spectrometry offers a direct measurement of nucleobases and nucleobase modifications. Approaches that rely on a reverse transcription step do not retain information on base modifications. Because modified uridine is commonly used in vaccine development, a measure of modified base incorporation rates might be considered for these products. An example shown in Figure 1 is provided for a QC material expected to be modified with 5-methoxy uridine. However, as demonstrated in Supplemental Figure 8, small amounts of unmodified uracil (above LOQ) are also detected in a commercial product.

ID LC-MS/MS approaches can multiplex measurements of multiple attributes in a single method. Here, we show three examples: thymine detection as a surrogate of residual dsDNA (associated with unpurified plasmid or template), amino acid detection as surrogates of residual protein contamination (often associated with T7 polymerase, the most abundant IVT reaction component), and negatively charged nucleotide monophosphate detection using polarity switching. Other mRNA-related measurements, such as lipid composition or 5′ capping, could be targeted within a similar assay and are the focus of future work.

Lastly, it is worth considering that quantitative nucleobase analysis can be used to estimate the average polyA tail length of mRNA therapeutic products. PolyA tailing is important for mRNA stability and translation[20−22] and is considered a potential critical quality attribute[15] in biomanufacturing. By calculating the intact mRNA mass fraction (based on nonadenine nucleobase mass fractions), it is possible to predict the total number of adenines on an intact mRNA product using the measured adenine mass fraction. Then, a prediction for average polyA tail length can be calculated by the difference (for enzymatically added tails). For polyA tails that are cotranscribed in the DNA template, the expected polyA tail length can be confirmed.

It is important to consider the limitations of this approach. LC-MS has inherently lower sensitivity compared with PCR-based techniques. For targets such as vaccines, however, this is not a concern considering that a multidose vial of either existing COVID mRNA vaccine contains 30 or 100 μg of mRNA, respectively, for the Pfizer Comirnaty or Moderna mRNA-1273 vaccines—well more than this is required by the acid hydrolysis-ID-LC-MS analysis. A second limitation is that this approach is not suitable for high-throughput applications. Yet, the intent here is not for day-to-day monitoring of finished products but rather for value assignment of in-house calibrators or higher-order reference materials that can link high-throughput measurements to a consistent standard. Lastly, this approach does not distinguish between nucleobases coming from sources within a mixture, from degraded mRNA, or between free and encapsulated mRNA. In the case of mixture analysis or LNP-encapsulation, a total nucleobase mass fraction can be determined.

CONCLUSIONS

This study is the first report of a bottom-up, mass-spectrometry-based quantitative approach for encapsulated mRNA. Acid hydrolysis is capable of simultaneously releasing mRNA cargo from LNPs and hydrolyzing the mRNA into constituent nucleobases for downstream quantification. Isotope dilution techniques are the gold standard approach for absolute unbiased biomolecule quantification. This work links the mRNA quantity to the international system of units (kg), assuring measurement traceability and enabling assay standardization. Nucleobase stability ensures that the hydrolysis procedure is robust under extreme conditions, and the ensuing LC-MS assay is shown to predict mRNA mass fraction accurately based on the comparability to known control materials. This technique can fill a unique niche in the biomanufacturing of LNP-mRNA products by establishing measurement traceability, by quantifying base modifications, by detecting DNA and protein contamination, and by estimating the polyA tail length. Future work aims to expand this work in a multiattribute method for LNP-mRNA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c04406.

Time-course analyses of nucleobases, mRNA, and LNP-mRNA and mass spectra of nucleotide monophosphates (PDF)

Composition of SIL nucleobase standards (PDF)

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Notes

The authors declare no competing financial interest.

Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

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