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Quantification of residual plasmid DNA and SV40 promoter-enhancer sequences in Pfizer/BioNTech and Moderna modRNA COVID-19 vaccines from Ontario, Canada

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ABSTRACT

For some of the COVID-19 vaccines, the drug substances released to market were manufactured differently than those used in clinical trials. Manufacturing nucleoside-modified mRNA (modRNA) for commercial COVID-19 vaccines relies on RNA polymerase transcription of a plasmid DNA template. Previous studies identified high levels of plasmid DNA in vials of modRNA vaccines, suggesting that the removal of residual DNA template is problematic. Therefore, we quantified the DNA load in a limited number of Pfizer-BioNTech and Moderna COVID-19 modRNA vaccine vials using two independent methods. Total DNA and specific DNA targets were quantified by Qubit fluorometry and quantitative polymerase chain reaction (qPCR), respectively on 32 vials representing 16 unique vaccine lots. RNase A treatment was used to assess the impact of RNA crosstalk in DNA fluorometry. A preliminary assessment of DNA fragment length and DNase I sensitivity were also performed. Total DNA ranged 371–1,548 ng/dose and 1,130–6,280 ng/dose in Pfizer and Moderna products, respectively. Specific DNA of multiple plasmid DNA targets ranged 0.22–7.28 ng/dose for Pfizer, and 0.01–0.78 ng/dose for Moderna. The SV40 promoter-enhancer-*ori* (0.25–23.72 ng/dose) was only detected in Pfizer vials. Oxford Nanopore sequencing of one vial found mean and maximum DNA fragment lengths of 214 bp and 3.5 kb, respectively. These data demonstrate the presence of 1.23×10^{8} to 1.60×10^{11} plasmid DNA fragments per dose encapsulated in lipid nanoparticles. Using fluorometry, total DNA in all vials tested exceeded the regulatory limit for residual DNA set by the US Food & Drug Administration (FDA) and the World Health Authorization (WHO) by 36–153-fold for Pfizer and 112–627-fold for Moderna after accounting for nonspecific binding to modRNA. When tested by qPCR, all Moderna vials were within the regulatory limit, but 2/6 Pfizer lots (3 vials) exceeded the regulatory limit for the SV40 promoter-enhancer-*ori* by 2-fold. The presence of the SV40 promoter-enhancer element in Pfizer vials raises significant safety concerns. This study emphasizes the importance of methodological considerations when quantifying residual plasmid DNA in modRNA products, considering increased LNP transfection efficiency, and cumulative dosing presents significant and unquantified risks to human health.

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Introduction

Chemical synthesis of large RNA (>200 bp) is expensive and error prone. Therefore, a T7 polymerase is used to generate long modified RNAs (modRNA) from DNA templates. DNA templates are easier to synthesize but need to be removed from the final drug product. Due to the limitations of chemical synthesis of DNA, fragments or oligos of DNA are first chemically synthesized, stitched together with enzymatic ligation and polymerization, and finally cloned into replication competent plasmids as their full-length products. Purifying plasmid DNA from *Escherichia coli (E. coli)* can be done through direct lysis of the *E. coli* and plasmid DNA purification or through purification *via* Polymerase Chain Reaction (PCR) of the targeted plasmid insert. These two different methods of purification can have

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dramatic differences in the amount and type of residual DNA in the vaccines. These purification steps are emphasized in Moderna's patent US10898574B2 and rely on a PCR-free method that directly purifies plasmid DNA from *E. coli* cultured in a kanamycin broth. Moderna's patent states that a genetically engineered DNA (e.g. naked plasmid DNA) packaged in a lipid nanoparticle (LNP) is directly injected into a living host and could cause insertional mutagenesis and activation of oncogenes or the inhibition of tumor suppressor genes[1].

After the US Food & Drug Administration (FDA) approval and rollout of the COVID-19 vaccine, Pfizer changed its manufacturing process from PCR amplification of a DNA template (Process 1) for the randomized clinical trial (RCT) to a PCR-free purification process using DNA plasmids (Process 2) like that used by Moderna [2]. BioNTech patent US20230183769A1 provides a method for the large-scale production of pharmaceutical grade RNA using *in vitro* transcription reactions and reduces the process-related contaminant dsRNA at the same time [3]. However, as Process 2 used a 7.8Kb bacterial plasmid instead of the 4.2Kb PCR amplicon used in Process 1 for the in-vitro transcription it is probable that there was a large increase of the residual DNA in the final drug product [1].

Due to the different DNA backbones between Process 1 and Process 2, the single qPCR assay used by Pfizer to detect a specific portion of the DNA may not be sufficient for accurate quantification. The PCR based methods used in Pfizer's clinical trial contain very different background DNA compared to the vaccines given to the public as the PCR process only amplified the portion of the plasmid encoding the spike mRNA sequence. The Process 2 *E. coli* purification method contains the plasmid vector sequence with the spike sequence cloned insert. Therefore, residual DNA quantification methods that assess both the plasmid and the insert, unlike Pfizer's process that only performed PCR on the KAN gene (i.e. kanamycin resistance gene) from the plasmid vector, are required for complete quantitation. Pfizer disclosed to the regulators a qPCR assay for the spike region to confirm the presence of the spike insert but provided no quantification from this validated second assay. This is relevant as DNase I is known to fail to digest RNA:DNA hybrids. These hybrids will exist in the spike sequence after *in vitro* transcription (IVT) and may also exist in the form of residual RNA from the plasmid expression of the AmpR-SV40-KAN gene as it is propagated in *E.coli*. Thus, the DNase I activity may vary in different locations of the plasmid. Assays only measuring one location of the plasmid will fail to accurately quantify the residual DNA in the final drug product [4].

The Pfizer and Moderna plasmids share many features but most notably differ in that Pfizer's plasmid is a derivative of pcDNA3.1, which is a shuttle vector capable of both bacterial and mammalian replication *via* SV40 origins of replication, SV40 enhancers, SV40 promoters and SV40 polyA signals [5]. Plasmid DNA template used by Pfizer (7,824 bp) and Moderna (6,777 bp) both contain a *ColE1* bacterial origins of replication (generically termed *ori*) active in *E. coli*. Both plasmids also contain an aminoglycoside phosphotransferase gene (*Neo/Kan*) that allows for selection of bacteria carrying the plasmid in media containing kanamycin. The *E. coli* cells are then harvested and lysed. The plasmid DNA is extracted, purified, and then linearized with the restriction enzyme *Eam*1104I. This linear DNA then acts as the template for IVT using T7 RNA polymerase in the presence of N1-methyl-pseudouridine. It is the addition of N1-methyl-pseudouridine that makes these products bioengineered nucleoside-modified mRNA (i.e. modRNA) vaccines. After the IVT, DNA is enzymatically hydrolyzed with DNase I, reducing its prevalence in the final drug product. Documents supplied by Pfizer to the European Medicines Agency (EMA) [6] noted that residual DNA in modRNA products made by this process could vary significantly [7].

In 2023, McKernan et al. subjected COVID-19 modRNA vaccine vials to next-generation RNA sequencing and, found unexpected evidence of DNA derived from the template plasmids used during manufacturing [8]. This showed that both Pfizer and Moderna vaccines contained high copy plasmid components with different residual DNA sequences. Additionally, McKernan et al. found that all Pfizer vaccines contained SV40 promoter-enhancer-ori and SV40 polyA signal sequences being utilized as the promoter for the Kan/Neo selectable marker. These residual DNA components were absent from the required disclosures in EMA regulatory documents (pg 24 Rapporteur Rolling Review 3) [7].

Based on this information, McKernan et al. developed a qPCR method to quantify the level of residual plasmid DNA in the vaccine vials targeting multiple loci in the Moderna and Pfizer plasmids [8]. Therefore, the aim of this research was to verify the presence and quantity of residual plasmid

DNA and the SV40 promoter-enhancer-ori in the Pfizer and Moderna COVID-19 modRNA vaccine and compare the results to adverse events (AEs) reported to the Vaccine Adverse Event Reporting System (VAERS). To investigate this, we obtained 32 vials COVID-19 modRNA vaccines that had been distributed in Ontario, Canada. The quantity of specific DNA targets, i.e. spike, plasmid *ori*, and the SV40 promoter-enhancer-*ori*, were determined by qPCR and total DNA loads by Qubit® fluorometry. We also extended the observations of an earlier work (McKernan et al.) by studying the size distribution of DNA fragments as well as the DNase l sensitivity of the vaccine to determine whether the residual DNA is packaged in the LNPs. We then queried VAERS for any AEs, including serious AEs (SAEs), associated with these lots [9]. This study is an expanded version of a previous preprint [10].

Materials and methods

For this study, we used the terms "residual DNA," "DNA mass," or "impurities," (or similar) rather than "contamination" in keeping with regulatory definitions of impurities which arise from the manufacturing process [11,12]. Based on the FDA and US Pharmacopeia (USP) guidelines "impurities" are unwanted substances that are inherent to the manufacturing process or raw materials used in vaccine production. Impurities are typically expected, predictable, and arise from the materials, reagents, or intermediates used during production. Impurities would include the dsRNA, RNA:DNA hybrids, short RNA fragments, and the residual plasmid DNA fragments. Whereas "contamination" refers to unintended, extraneous substances introduced into the vaccine product through external sources, such as environmental factors, equipment, or human error, that are not part of the intended manufacturing process. Contamination is typically unexpected and can include microbial contaminants (e.g. bacteria, fungi), adventitious agents (e.g. viruses), or foreign particulates.

COVID-19 vaccine batches

Vials of Pfizer-BioNTech BNT162b2 (10 vials; 6 lots) and Moderna Spikevax mRNA-1273 (22 vials, 10 lots) were obtained from various pharmacies in Ontario, Canada (Figure 1 and Table 1). An unopened sterile injectable vial of alprostadil 66 mcg/mL in combination with papaverine 21.7 mg/mL and phentolamine 1 mg/mL (TriMix) was used as the negative control. The unopened vials were untampered with as they had in-tact flip-off plastic caps with printed lot numbers and expiration dates. Vials had been stored in pharmacies in purpose-built vaccine storage units at +2–8 °C and were transported in insulated containers with frozen gel packs prior to being placed in the testing laboratory fridge within 5 h of receipt. One Moderna vial lacked a printed expiration date but had a QR code that required scanning by a pharmacist. The Moderna XBB.1.5 vials were similarly stored by the pharmacy. The remnants of recently used vials (within 30 min of administration) were placed in an insulated container with frozen gel packs and transported to the testing laboratory fridge within 12 h.

qPCR analysis of spike, ori, and the SV40 promoter-enhancer-ori DNA

qPCR assays targeted spike and plasmid *ori* sequences shared by the Moderna and Pfizer expression plasmids (Table 2). These primers were also used to test COVID-19 modRNA vaccine vials in Australia [15]. The uniplex SV40 Enhancer assay was designed to amplify the nuclear targeting sequence unique to the Pfizer plasmid [16]. All qPCR assays used 1 µL from each vial directly added to 17.8 µL of master mix. qPCR kits were sourced from Medicinal Genomics (Part# 420201, Beverly, USA) with the master mix containing 8.8 µL reaction consisting of 3.8 µL polymerase enzyme (Medicinal Genomics Part# 420201), 0.8 µL reaction buffer and 1.0 µL of Primer-Probe mix, and 12.2 µL of ddH_20. A primer-probe mix was assembled using 12.5 µL 100 µM *ori* probe, 12.5 µL of 100 µM spike probe, 25 µL of 100 µM spike forward primer, 25 µL of 100 µM spike reverse primer, 25 µL of 100 µM *ori* forward primer, 25 µL 100 µM *ori* reverse primer, and 75 µL of ddH_20. All

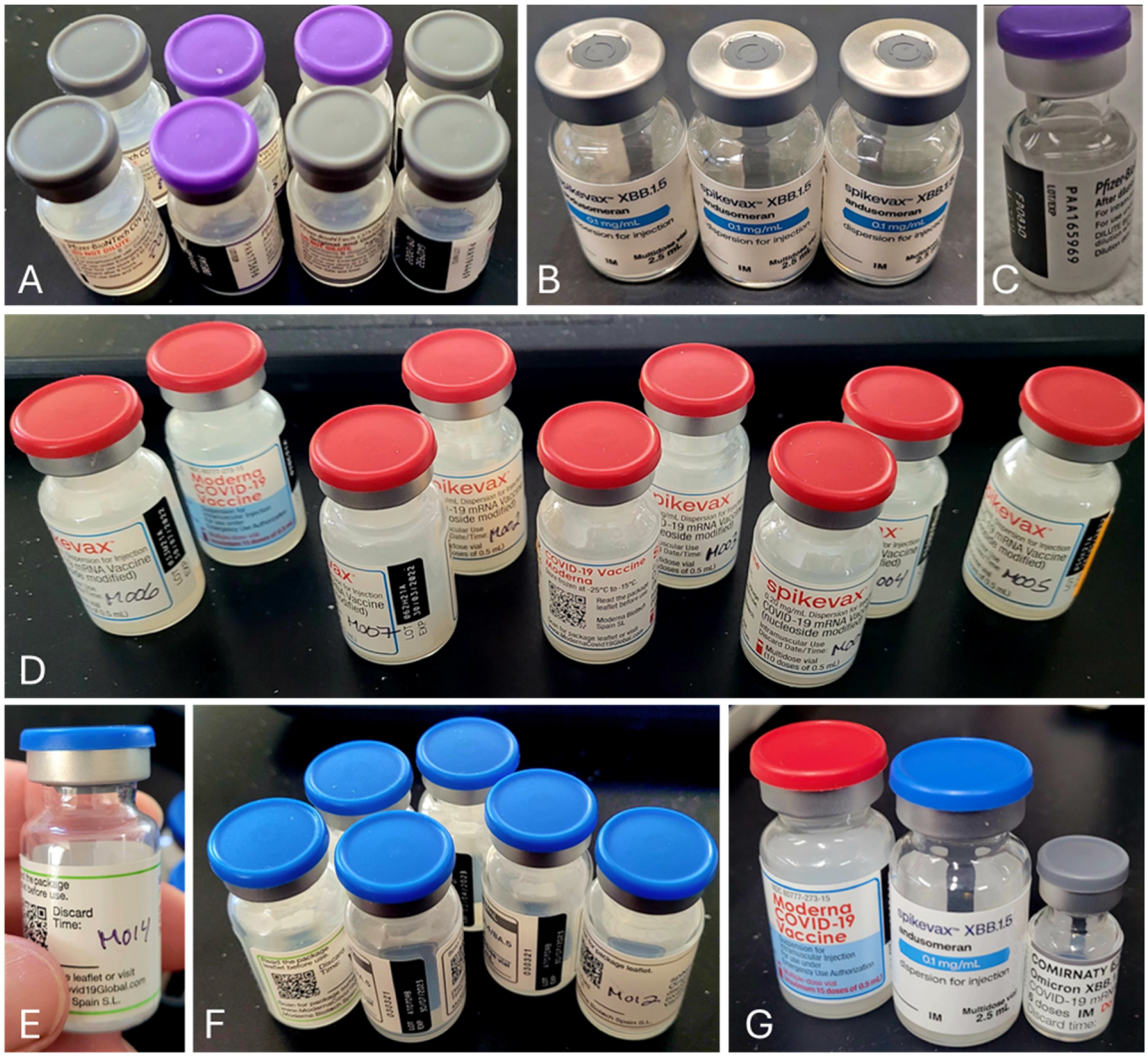

Figure 1. Vials of Pfizer/BioNTech BNT162b2 and Moderna Spikevax mRNA-1273 COVID-19 vaccine from Ontario, Canada: (A) Pfizer/BioNTech adult monovalent and bivalent; (B) Moderna adult monovalent XBB.1.5; (C) Pfizer-BioNTech adult monovalent; (D) Moderna child/adult monovalent; (E) child/adult bivalent Wuhan-BA.1; (F) child/adult bivalent Wuhan-BA.1 and adult Wuhan-bivalent BA.4/5; and (G) mix of Moderna child/adult monovalent and adult monovalent XBB.1.5, and Pfizer-BioNTech adult monovalent XBB.1.5. One vial of Moderna child/adult monovalent wasn't photographed.

qPCR assays used a synthetic gDNA control (gBlock, Integrated DNA Technologies (IDT), San Diego, USA) of known concentration (1 ng/µL) to generate a 10-fold serial dilution derived calibration curve.

Cycling was performed on a QuantStudio 3 (ThermoFisher Scientific, Waltham, USA) with an initial denaturation of 95 °C for 3 min followed by 35 cycles of 95 °C for 10 s and 65 °C for 30 s. Cq conversion to ng/µL was calculated using the QuantStudio software v2.7.0 (ThermoFisher Scientific). Amplicon mass, as determined with the New England BioLabs DNA calculator [17] and length (105 bp for *ori*, 114 bp for spike, and 152 bp for the SV40 promoter-enhancer-*ori*), were used to estimate the total nanograms (ng) of DNA present by adjusting for the length of the plasmids (7,824bp for Pfizer and 6,777bp for Moderna). Copy number per dose was adjusted first for the dilutions (e.g. 1:5 dilution for the Pfizer Adult Monovalent) and then for the volume of each adult injection dose (300 µL for Pfizer and 500 µL for Moderna). Serial dilutions were performed on the three Pfizer lots that showed the highest residual DNA concentration to investigate PCR inhibition by the LNPs, since qPCR was performed directly without any treatment or extraction.

Table 1. Description of mRNA COVID-19 vaccine lots examined in this study.

Vaccine manufacturing information							VAERS data	
Manufacturer	Mono/bivalent	Lot #	Sealed	HC approval date	Expiry date	Expired	Total AES	Total SAES
Pfizer-BioNTech	Adult Monovalent	FD0810	Sealed	2021-07-29	2022-05-31	Expired	941	154
Pfizer-BioNTech	Adult Monovalent	FM7380	Sealed	2022-01-05	2022-11-30	Expired	64	25
Pfizer-BioNTech	Adult Monovalent	FN7934a	Sealed	2022-01-11	2022-10-31	Expired	50	25
Pfizer-BioNTech	Adult Monovalent	FN7934b	Sealed					
Pfizer-BioNTech	Adult Monovalent	FX4343a	Sealed	2022-04-13	2023-07-31	Expired	4	1
Pfizer-BioNTech	Adult Monovalent	FX4343b	Sealed					
Pfizer-BioNTech	Adult Bivalent	GK0932a	Sealed	2022-10-19	2024-02-29	In code	20	2
Pfizer-BioNTech	Adult Bivalent	GK0932b	Sealed					
Pfizer-BioNTech	Adult Bivalent	GK0932c	Sealed					
Pfizer-BioNTech	Adult Monovalent XBB.1.5	HD9867	Sealed	2023-09-28	2024-10-31	In code	14	5
Moderna	Child/Adult Monovalent	092D21A	Sealed	2021-06-13	2022-02-13	Expired	49	41
Moderna	Child/Adult Monovalent	020E21Aa	Sealed	2021-07-08	2022-03-04	Expired	6	2
Moderna	Child/Adult Monovalent	020E21Ab	Sealed					
Moderna	Child/Adult Monovalent	062H21Aa	Sealed	2021-10-27	2022-05-31	Expired	10	3
Moderna	Child/Adult Monovalent	062H21Ab	Sealed					
Moderna	Child/Adult Monovalent	020J21A	Sealed	2021-11-18	2022-06-30	Expired	7	5
Moderna	Child/Adult Monovalent	055K21A	Sealed	2021-12-16	2022-07-31	Expired	2	2
Moderna	Child/Adult Monovalent	033M21Aa	Sealed	2022-02-11	2022-09-30	Expired	2	1
Moderna	Child/Adult Monovalent	033M21Ab	Sealed					
Moderna	Child/Adult Monovalent	033M21Ac	Sealed					
Moderna	Child/Adult Monovalent	033M21Ad	Sealed					
Moderna	Child/Adult Bivalent BA.1	AS0467Da	Sealed	2022-09-23	2023-04-02	Expired	0	0
Moderna	Child/Adult Bivalent BA.1	AS0467Db	Sealed					
Moderna	Child/Adult Bivalent BA.1	AS0467Dc	Sealed					
Moderna	Adult Bivalent BA4/5	AT0709Ba	Sealed	2022-11-28	2023-10-30	Expired	0	0
Moderna	Adult Bivalent BA4/5	AT0709Bb	Sealed					
Moderna	Adult Bivalent BA4/5	AT0709Bc	Sealed					
Moderna	Adult Bivalent BA4/5	AT0709Bd	Sealed					
Moderna	Adult Monovalent XBB.1.5	020G23Aa	Unsealed	2023-09-15	2024-07-29	In code	0	0
Moderna	Adult Monovalent XBB.1.5	020G23Ab	Unsealed					
Moderna	Adult Monovalent XBB.1.5	020G23Ac	Unsealed					
Moderna	Adult Monovalent XBB.1.5	025G23A	Unsealed	2023-09-15	2024-08-10	In code	0	0

Sorted by health Canada lot approval date as recorded in the Canadian national vaccine catalog [13]. adverse events (AEs) and serious AEs and SAEs sourced from Canada) were extracted from the VAERS database available for public download (https://vaers.hhs.gov) [14]. Lower case letters at the end of lot numbers indicate different vials of the same lot.

Table 2. Primer and probe sequences targeting spike, *ori*, and the SV40 promoter-enhancer-*ori*.

Primer/probe name	Sequence (5′-3′)[a]	Amplicon Length
MedGen_Moderna_Pfizer_Janssen_Vax-Spike_Forward	AGATGGCCTACCGGTTCA	114 bp
MedGen_Moderna_Pfizer_Janssen_Vax-Spike_Reverse	TCAGGCTGTCCTGGATCTT	
MedGen_Moderna_Pfizer_Janssen_Vax-Spike_Probe	5′6-FAM-CGAGAACCA-ZEN-GAAGCTGATCGCCAA-3′IABkFQ[b]	
MedGen_Vax-Vector_Ori_Forward	CTACATACCTCGCTCTGCTAATC	105 bp
MedGen_Vax-Vector_Ori_Reverse	GCGCCTTATCCGGTAACTATC	
MedGen_Vax-Vector_Ori_Probe	5HEX-AAGACACGA-ZEN-CTTATCGCCACTGGC-3′IABkFQ[c]	
MedGen_SV40-promoter-enhancer-ori_Forward	GTCAGTTAGGGTGTGGAAAGT	152 bp
MedGen_SV40-promoter-enhancer-ori_Reverse	GGTTGCTGACTAATTGAGATGC	
MedGen_SV40-promoter-enhancer-ori_Probe	5TEX615-CCAGCAGGCAGAAGTATGCAAAGC-3′IAbRQSp[d]	

[a]Pfizer bivalent expression vector BNT162b2 (OR134577.1) and Modern mRNA1273 expression vector (OR134578.1) sequences were used for primer design.
[b]Probe had 5′ terminus labeled with a 6-FAM (6-carboxy-fluorescein) fluorophore, a ZEN™ internal quencher, and 3′ terminus labeled with 3′ Iowa Black® FQ.
[c]Probe had 5′ terminus labeled with a 5HEX (Hexachlorofluorescein) fluorophore, a ZEN™ internal quencher, and 3′ terminus labeled with 3′ Iowa Black® FQ.
[d]Probe had 5′ terminus labeled with a TEX 615 fluorophore and 3′ terminus labeled with 3′ Iowa Black® RQ.

Qubit® fluorometry quantitation

AccuGreen® HS fluorometric reagents (AccuGreen #99820 and DNA Quantification Buffer #99979) and standards were acquired from Biotium (San Francisco, USA) for Qubit® analysis (ThermoFisher Scientific). Fluorometry readings were initially performed directly on the vaccines in a 200 µL mixture containing fluorometric reagents (190 µL of a stock made from 995 µL HS Buffer and 5 µL 200X AccuGreen® dye) and 10 µL of vaccine. Samples were read following the manufacturer's instructions on a Qubit® 3.0 Fluorometer. These samples were then heated to 95 °C for 8 min and cooled to 4 °C for 5 min to disrupt the LNPs and enable fluorometric dyes to access the DNA. To assess potential

signal derived from AccuGreen® binding to modified RNA, 1 µL of 20 mg/mL RNase A (NEB# T3018L) was added to the samples after the heating/cooling step and incubated at 37 °C, during which time a time series of fluorescent measurements was collected. Fluorometry measurements were collected before RNase A addition and 1, 2, 3, 5 and 10 min after RNase A addition.

Vaccine adverse event reporting system (VAERS) data

The VAERS database was analyzed using the Language and Environment for Statistical Computing package in R [18] and included data spanning December 17, 2020 through October 6, 2023. The U.S.-based VAERS data is available for download in three separate comma separated values (csv) data files representing: i) general data for each report; ii) the reported AEs or "symptoms," and iii) vaccine data including vaccine manufacturer and lot number [9]. A non-U.S.-based (foreign) VAERS data set is also available for download and contains AE reports filed to regulatory authorities from non-U.S. countries worldwide. A VAERS ID number is assigned to preserve confidentiality when a report is filed. To assess the AEs related to a particular vaccine, it is necessary to merge the three data files using the VAERS IDs as a linking variable. For this study, only COVID-19 VAX_TYPE (COVID19-1 (monovalent) and COVID19-2 (bivalent)) were included. Other relevant variables included VAERS ID*, vaccine lot (VAX_LOT), vaccine manufacturer (VAX_MANU), hospitalizations (HOSPITAL), disability (DISABLE), emergency room visits (ER_ED_VISIT), birth defects (BIRTH_DEFECT), life-threatening occurrences (L_THREAT) and deaths (DIED). Data were grouped by VAX_LOT and the total number of AE and SAE reports were counted. SAE reports included deaths, hospitalizations, emergency room visits, disability reports, birth defects and life-threatening reports. The US-and non-U.S.-based data were queried and for the purposes of this study, only Canadian lot counts are reported in the primary results. Canadian reports were distinguished from non-U.S.-based (foreign) reports using the SPLTTYPE variable which provides the country code - in the case of Canada "CA" - as the first 2-character place holders. Correlations between VAERS data and DNA levels for spike, *ori* and SV40 were plotted and calculated using the Pearson correlation coefficient.

Oxford nanopore sequencing

DNA fragment size distributions were estimated using a previously sequenced vaccine lot (Pfizer children's monovalent Lot# FL8095) [8] as a standard using an Oxford Nanopore Flongle (R.10.4.1, Oxford Nanopore Technologies (ONT), New York, USA) and the Oxford Nanopore Ligation sequencing kit (SQK-LSK114) according to the manufacturer's instructions. Reads were mapped to NCBI OR134577.1 with the Burrow-Wheeler Aligner with maximum exact matches (BWA-MEM) [19]. ONT sequencing read length is unlimited, but DNA isolation procedures required to run ONT sequencing can bias the length of the molecules captured in the ONT ligation reaction. Shorter fragments may be lost with the SPRI based DNA capture used in these methods. Single molecule reads were counted and binned according to their mapped read length with BWA-MEM.

Assessment of nuclease sensitivity

Pfizer Lot# FL8095 was also used as a standard to assess DNase l sensitivity of vaccine material. Packaged DNA in LNP should be inaccessible and thus resistant to DNase I, while DNA outside of the LNP should be DNase I labile.

Nuclease protected DNA was estimated by treating 20 µL of the vaccine with 2.5 µL of DNase I-XT (2 units/µL, NEB#M0570S, New England BioLabs Inc, Ipswich, USA) in Grim Reefer 10X buffer (Medicinal Genomics #420123-125) and incubating at 37 °C for 30 min. For the control, 2.5 µL of ddH_20 was used instead of the DNase I-XT. The DNase I-XT reaction was terminated using 2.5 µL of MGC lysis buffer (Medicinal Genomics #420001). After the DNase I kill step, a qPCR-amplifiable internal control DNA was spiked-in to verify that the DNase I-XT had been fully inactivated (Medicinal Genomics #420123-125). Subsequently, 54 µL of SenSATIVAx magnetic beads (Medicinal Genomics) were used to

purify DNA from the DNase I-XT assay and the DNase I-XT negative control samples. The magnetic beads were pipette-mixed 10 times with the sample, incubated at room temperature for 5 min, separated magnetically and washed twice with 70% v/v ethanol. The ethanol was removed, and the beads dried for 2 min at room temperature. Samples were eluted in 30 µL of ddH_20 and 1 µL of eluate was examined by qPCR for spike and *ori* in an 18.8 µL reaction. An additional DNase I inactivation control primer and probe (0.5 µL CY5 labeled probe) were added to the assay for a total of 19.3 µL reaction.

Results

qPCR testing of residual DNA

We validated the accuracy of the qPCR quantification method using an 8-log serial dilution standard curve that was used to calibrate sample Cq values and generated R^2 values of 0.998, 0.999, 0.906 and efficiencies of 99.8%, 94.7%, and 93.6% for spike, *ori*, and SV40 promoter-enhancer-*ori* amplicons, respectively. On all plates, negative controls and no template (ddH_20) controls (NTC) were tested in triplicate and found to be negative.

Initial testing, performed on undiluted vaccine samples showed that all Pfizer vials were positive for spike, *ori*, and SV40 promoter-enhancer-*ori*. For individual vials, qPCR for Pfizer amplified at similar times for spike, *ori*, and SV40 enhancer-promoter-*ori* (average $_{\Delta}$Cq 0.89 ± 0.28). Despite Pfizer lot FX4343 amplifying slightly later than the other vials, the inter-vial difference was small (spike $_{\Delta}$Cq 17.86 ± 1.58; *ori* $_{\Delta}$Cq 17.91 ± 2.05; SV40 promoter-enhancer-*ori* $_{\Delta}$Cq 15.46 ± 2.02). The inter-vial difference of each of the targets was also small for Moderna (spike Cq 20.65 ± 0.88; *ori* Cq 25.48 ± 1.32). However, for all Moderna vials, except lot AS0467D (Bivalent BA.1), *ori* consistently amplified 2.95 ± 0.30 cycles later than spike. The SV40 promoter-enhancer-*ori* was detected in all Pfizer vials but in none of the Moderna vials.

To assess LNP inhibition on the qPCR reaction efficiency, vials of lots FM7380 and FN7934 were subject to 10-fold serial dilution. The expected ~3.3 quantification cycle (Cq) response was observed only after the 1:10 dilution suggesting that there is some LNP inhibition that could impact the quantitation of DNA at these dilutions. Therefore, the data from the 1:10 dilutions were used for further analysis. This dilution, as well as the fact that some of the doses were designed to be diluted before use, was accounted for in our calculations.

Amount of specific residual DNA fragments determined by qPCR

qPCR testing of the vials diluted 1:10 produced Cq values for *ori* and spike of 18.44–24.87, and 18.03–23.83 for Pfizer, and 25.24–30.10 and 22.35–24.53 for Moderna, respectively (Table 3). The SV40 promoter-enhancer-*ori* assay produced Cq values of 17.46–24.65 in the Pfizer vials and did not amplify in the Moderna vials. The reproducibility of the spike, *ori* and SV40 assays was 0.72 ± 0.14, 0.74 ± 0.23, and 0.65 ± 0.16 for Pfizer, and 0.83 ± 0.14, 0.89 ± 0.18, and negative for Moderna, respectively. After calculations, the amount of residual DNA varied substantially between the Pfizer lots analyzed [0.22–2.43 ng/dose for spike, 0.28–7.28 ng/dose for *ori*, and 0.25 to 23.72 ng/dose for SV40 promoter-enhancer-*ori*] but was more consistent in the Moderna lots [0.25–0.78 ng/dose for spike and 0.01–0.34 ng/dose]. While Pfizer had a substantially higher amount of residual DNA than Moderna, only the SV40 promoter-enhancer-*ori* DNA from lots FM7380 and FN7934 were over the FDA 10 ng DNA/dose guidance (Figure 2). Lots FM7380 and FN7934 were Pfizer monovalent purple top vials with a PBS formulation.

Total DNA determined by fluorometry

Fluorometer-based measurements (e.g. Qubit®) of the vaccines showed that the Moderna vials consistently had a much greater amount of total DNA than the Pfizer vials (Figure 3). The total DNA detected in the neat vaccine (i.e. directly from the vials) was 621 ± 282 ng/dose (range: 262 to

Table 3. qPCR Testing on all Pfizer-BioNTech and moderna vials diluted 1:10 with results for SARS-CoV-2 spike, *ori*, and the SV40 promoter-enhancer-*ori* on.

		Spike			Plasmid Origin of Replication (*ori*)			SV40 promoter-enhancer-*ori*		
Manufacturer	Lot #	Cq	Total ng/dose	Total Copies/ dose	Cq	Total ng/dose	Total Copies/ dose	Cq	Total ng/dose	Total Copies/ dose
Pfizer-BioNTech	FD0810	20.17	0.43	3.69E + 09	19.90	2.80	2.60E + 10	20.76	0.43	2.92E + 09
Pfizer-BioNTech	FM7380	18.03	2.43	2.07E + 10	18.57	3.92	3.64E + 10	18.18	16.06	1.09E + 11
Pfizer-BioNTech	FN7934a	18.47	1.79	1.53E + 10	18.77	3.43	3.18E + 10	17.46	23.72	1.60E + 11
Pfizer-BioNTech	FN7934b	18.19	2.18	1.86E + 10	18.44	4.27	3.96E + 10	17.96	18.10	1.22E + 11
Pfizer-BioNTech	FX4343a	23.53	0.27	2.30E + 09	24.71	0.32	2.94E + 09	24.59	0.26	1.73E + 09
Pfizer-BioNTech	FX4343b	23.83	0.22	1.86E + 09	24.87	0.28	2.64E + 09	24.65	0.25	1.68E + 09
Pfizer-BioNTech	GK0932a	20.46	2.25	1.92E + 10	21.01	3.81	3.54E + 10	20.27	2.61	1.77E + 10
Pfizer-BioNTech	GK0932b	20.60	2.05	1.75E + 10	21.22	3.32	3.08E + 10	20.49	2.35	1.59E + 10
Pfizer-BioNTech	GK0932c	20.66	1.97	1.68E + 10	21.21	3.33	3.09E + 10	20.29	2.58	1.74E + 10
Pfizer-BioNTech	HD9867	21.24	0.99	8.46E + 09	20.81	7.28	6.75E + 10	20.12	2.84	1.92E + 10
Moderna	092D21A	22.35	0.66	5.64E + 09	27.88	0.04	4.16E + 08	Negative		
Moderna	020E21Aa	23.66	0.35	3.02E + 09	29.47	0.02	1.87E + 08	Negative		
Moderna	020E21Ab	24.52	0.50	4.24E + 09	29.99	0.09	8.26E + 08	Negative		
Moderna	062H21Aa	22.52	0.78	6.69E + 09	29.21	0.02	2.23E + 08	Negative		
Moderna	062H21Ab	22.76	0.66	5.64E + 09	29.37	0.02	2.00E + 08	Negative		
Moderna	020J21A	23.21	0.48	4.12E + 09	30.10	0.01	1.23E + 08	Negative		
Moderna	055K21A	22.94	0.58	4.98E + 09	29.58	0.02	1.74E + 08	Negative		
Moderna	033M21Aa	23.04	0.54	4.65E + 09	29.46	0.02	1.88E + 08	Negative		
Moderna	033M21Ab	22.81	0.64	5.44E + 09	29.38	0.02	1.99E + 08	Negative		
Moderna	033M21Ac	23.59	0.37	3.18E + 09	29.87	0.02	1.43E + 08	Negative		
Moderna	033M21Ad	23.26	0.47	3.98E + 09	29.39	0.02	1.97E + 08	Negative		
Moderna	AS0467Da	23.20	0.49	4.17E + 09	25.24	0.34	3.20E + 09	Negative		
Moderna	AS0467Db	24.16	0.25	2.14E + 09	26.08	0.20	1.82E + 09	Negative		
Moderna	AS0467Dc	23.75	0.33	2.85E + 09	25.74	0.25	2.28E + 09	Negative		
Moderna	AT0709Ba	23.68	0.35	2.99E + 09	29.30	0.02	2.09E + 08	Negative		
Moderna	AT0709Bb	23.56	0.38	3.24E + 09	29.25	0.02	2.16E + 08	Negative		
Moderna	AT0709Bc	23.63	0.36	3.09E + 09	29.34	0.02	2.04E + 08	Negative		
Moderna	AT0709Bd	23.80	0.32	2.74E + 09	29.44	0.02	1.91E + 08	Negative		
Moderna	020G23Aa	24.42	0.73	6.26E + 09	29.42	0.23	2.13E + 09	Negative		
Moderna	020G23Ab	24.46	0.71	6.11E + 09	29.87	0.03	2.33E + 08	Negative		
Moderna	020G23Ac	24.53	0.68	5.84E + 09	29.74	0.03	2.55E + 08	Negative		
Moderna	025G23A	24.38	0.54	4.62E + 09	29.30	0.13	1.20E + 09	Negative		

Calculations for Pfizer and moderna were based on adult doses of 0.30 mL and 0.50 mL, respectively. Moderna is also indicated to be given to children aged 6–12 years of age with a dose 0.25 mL making the resultant total ng/dose half of that given to adults. Total ng/dose was adjusted for the length of the amplicon (105 bp *ori*, 114 bp spike) only representing a fraction of the 7,824 bp Pfizer and 6,777 bp moderna plasmid. Lower case letters at the end of lot numbers indicate different vials of the same lot. ⌘SV40 promoter-enhancer-*ori*.

1,116 ng/dose) for Pfizer and 1,661 ± 793 ng/dose (range: 379 to 3,430 ng/dose) for Moderna. Boiling of the fluorometric solution containing the vaccine caused the lysis of the lipid nanoparticles, releasing both residual DNA and modRNA, and greatly increasing the amount of total DNA detected to 1,696 ± 987 ng/dose (range: 590 to 3,180 ng/dose) for Pfizer and 5,210 ± 2,095 ng/dose (range: 2,980 to 9,700 ng/dose) for Moderna. This increase in fluorometry was also due to crosstalk between the modRNA and the fluorometric dyes [21]. The addition of RNase A quickly degraded the modRNA and reduced the fluorometry, which stabilized after 1 min. After 10 min of RNase A the total DNA detected in the vaccine vials was 775 ± 448 ng/dose (range: 371 to 1,548 ng/dose) for Pfizer and 2,821 ± 1,244 ng/dose (range: 1,130 to 6,280 ng/dose) for Moderna. This high amount of total DNA in both the vaccines suggests that a high fraction of the DNA is under the size range of the qPCR amplicons (i.e. is heavily degraded), and that Moderna used had a substantially higher amount of plasmid DNA.

Due to method development and acquiring of the vaccines at various times the vaccines were tested several times by fluorometry (Figure 4). The average variance directly from the vial (pre-boil) was 317 ± 278 ng/dose (range: 43–727 ng/dose) and 456 ± 121 ng/dose (range: 199–675 ng/dose), after boiling was 1,145 ± 533 ng/dose (range: 519–1,949 ng/dose) and 1,554 ± 1,023 ng/dose (range: 176–3,169 ng/dose), and after RNase A treatment was 297 ± 217 ng/dose (range: 100–765 ng/dose) and 1,065 ± 624 ng/dose (range: 113–2,365 ng/dose) for Pfizer and Moderna, respectively.

We plotted residual DNA values obtained by Qubit fluorometry against those obtained by qPCR (Figure 5). For the Pfizer product, the trend lines for *ori* and spike estimates both had a positive slope. The graph for the Moderna product differs from that of the Pfizer product with little overlap

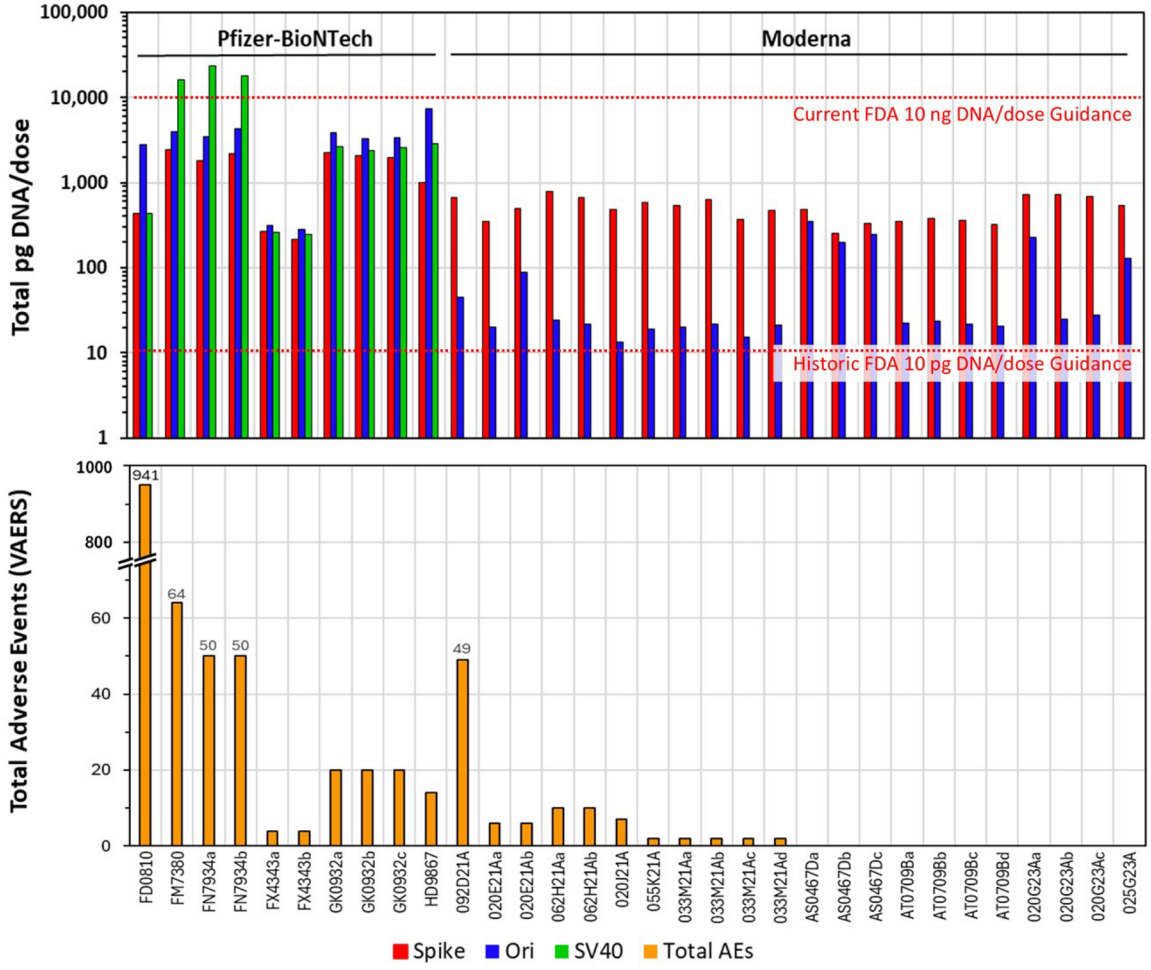

Figure 2. Comparison of residual DNA content of spike (red) *ori* (blue), SV40 promoter-enhancer-*ori* (green) as measured by qPCR, and the total number of adverse events (orange) reported to VAERS. The historic (10 pg/dose) and current (10 ng/dose) FDA and WHO regulatory guideline [12,20] for residual DNA is shown by a red dotted line. Vials are sorted by health Canada lot approval date as recorded in the Canadian national vaccine catalog [13]. Lower case letters at the end of lot numbers indicate different vials of the same lot. The total number of AEs was determined per lot and reproduced for each vial in the same lot.

of values in either axis, with much shallower slopes. Although a detailed view of the Moderna plots suggests a negative slope for the *ori* values, this trendline may be influenced by three outlying values. These values were obtained from vials of the Moderna BA.1-Wuhan bivalent vaccines.

Other than Moderna lots AS0709D, AS0467D and 020G23A, VAERS reports were filed for all lots examined in this study (Table 2 and Figure 2). Of the 16 lots examined, the lots with the highest numbers of reports filed to VAERS worldwide were FD0810, FM7380, FN7934 and 092D21A with 944, 64, 50 and 49 reports, respectively. In the case of these lots, 157 (17%), 25 (39%), 25 (50%) and 42 (86%) of individuals reported an SAE. These percentages far exceed the upper limit for the expected percentage of SAEs in any given VAERS data set as per the VAERS data use guide [22]. Also of note, 1/6 (17%) reports of SAEs involved death for Moderna lot 020E21A.

Regarding Canadian VAERS reports, the same trend was seen where FD0810, FM7380, FN7934 and 092D21A had 134, 27, 48 and 40 reports, respectively. In the case of these lots, 77 (57%), 16 (59%), 23 (48%) and 38 (95%) of individuals reported an SAE. These percentages far exceed the upper limit for the expected percentage of SAEs in any given VAERS data set as per the VAERS data use guide[22]. In the case of Moderna lot 092D21A almost all reports were SAEs. The percentage of SAEs for all lots combined originating from Canada is very high at 58%.

Lot FD0180 is the only lot in this study that was primarily distributed and administered in the United States. Of the total reports for lot FD0810 ($n = 944$), 682 (72%) were reported in various states

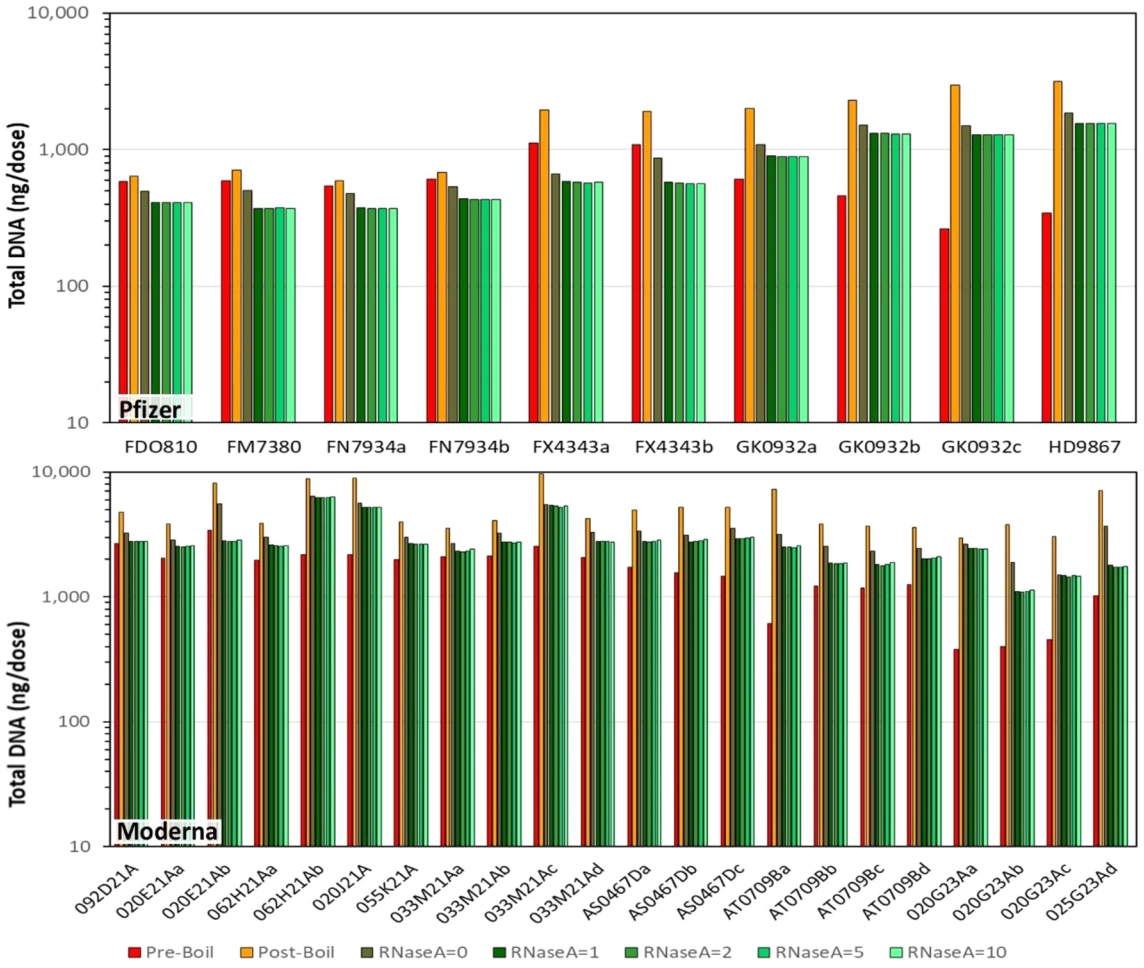

Figure 3. Total DNA was measured by Qubit® fluorometry directly from the vial (pre-boil), after a 95 °C boil step (post-boil) and at 5 different time points with RNase A digestion.

in the USA and more specifically, 546 (58%) were reported in Colorado alone (Supplementary Table 3). Of the non-USA-based (foreign) lots reported to VAERS for this lot, 83% were reported in Canada. 84% of all foreign reports (non-USA-based) were reported in Canada indicating that these vials were primarily distributed and administered in Canada.

Fragment length analysis

The Pfizer children's monovalent (Lot FL8095) described by McKernan et al. [8] was sequenced with Oxford Nanopore (ONT) to assess the read length distributions after mapping the reads to the reference sequence of the plasmid in NCBI (Figure 6). The longest read detected in 865 reads was 3.5 kb with read mapping to most of the plasmid backbone (Figure 7).

Nuclease sensitivity of the Pfizer vaccine was assessed using DNase I-XT. This DNA nuclease is optimized for IVT reactions rich in RNA-DNA hybrids. This treatment showed ≤1 Cq offset while a naked DNA control spiked into LNPs was reduced from a Cq of 15 to undetectable under the same conditions. This indicates that the DNA present in the vaccines is protected by encapsulation in the LNPs (Figures 8 and 9).

Discussion

Residual DNA was detected in all 32 vaccine vials surveyed. Multiple vials from the same lots produced very similar loads for all targets showing assay reliability, reproducibility, and consistency within

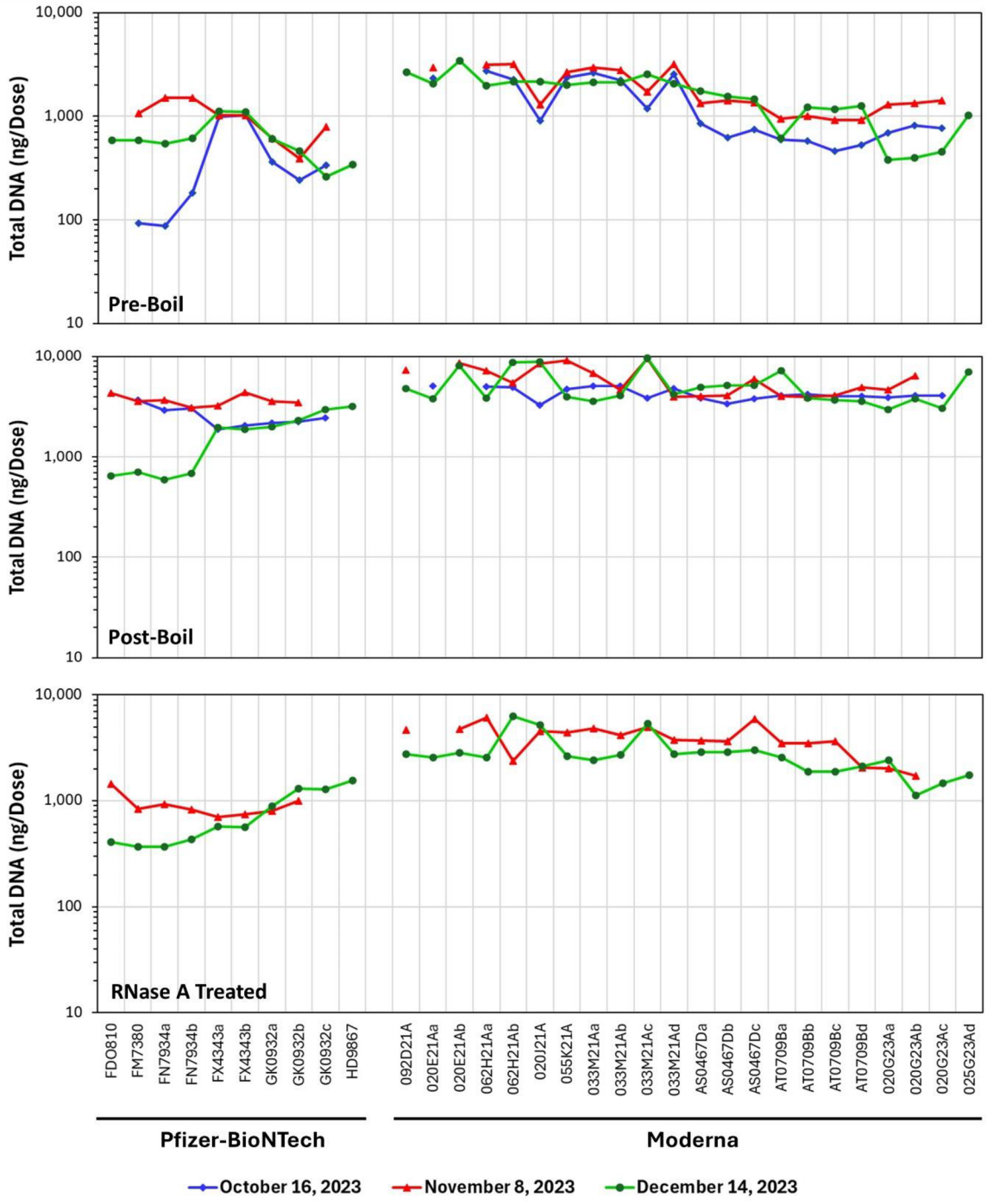

Figure 4. Qubit measurements were repeated after a freeze-thaw cycle to understand the variance one may observe shipping frozen vials lab to lab. Qubit® measurements were taken at three different time points (October, November and December 2023) directly from the vial (pre-boil), after a 95 °C boil step (post-boil) and at three time points after RNase A digestion.

the lots. These data involving vaccine vials distributed in Canada are consistent with several non-peer reviewed and peer reviewed reports of DNA contamination in modRNA vaccines (McKernan, Buckhaults, Konig, Raoult, Kämmerer, Wang) [8,23–26].

Moderna had the lowest DNA concentration by qPCR but the highest concentration with Qubit. The Moderna vials had the most consistent levels of DNA between vials suggesting a more robust and standardized manufacturing process. In each vial of the Moderna product, except for lot AS0467D, *ori* displayed lower loads (Ct > 6) than spike suggesting a more effective removal of the vector DNA.

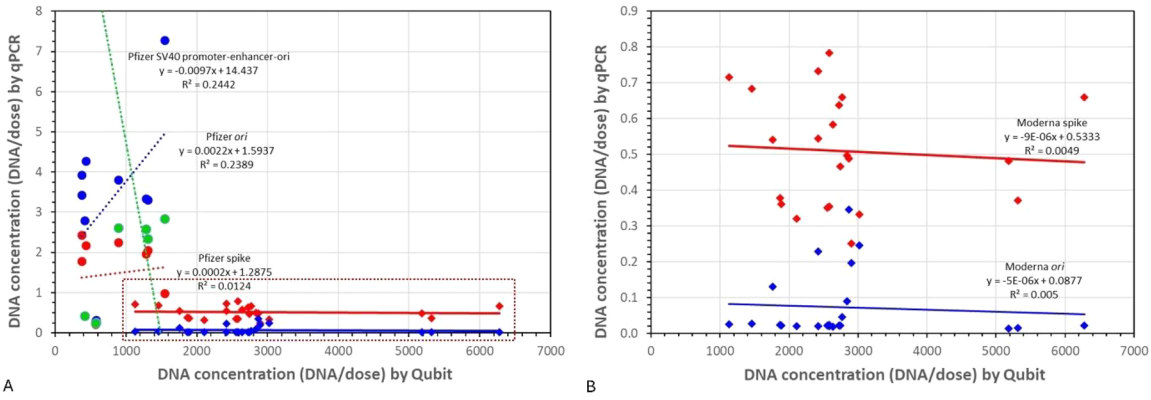

Figure 5. Graphical comparison of residual DNA concentration for spike (red), *ori* (blue), and SV40 promoter-enhancer-*ori* (green) determined by qPCR and total residual DNA concentration in individual vials as determined by qubit®. In panel a both Pfizer and Moderna data are plotted on the same scale. The Y-axis was adjusted to see all samples, except the three SV40 promoter-enhancer-*ori* results. The Moderna data are enclosed in a red box and displayed separately with an enlarged scale in panel B, to display detail.

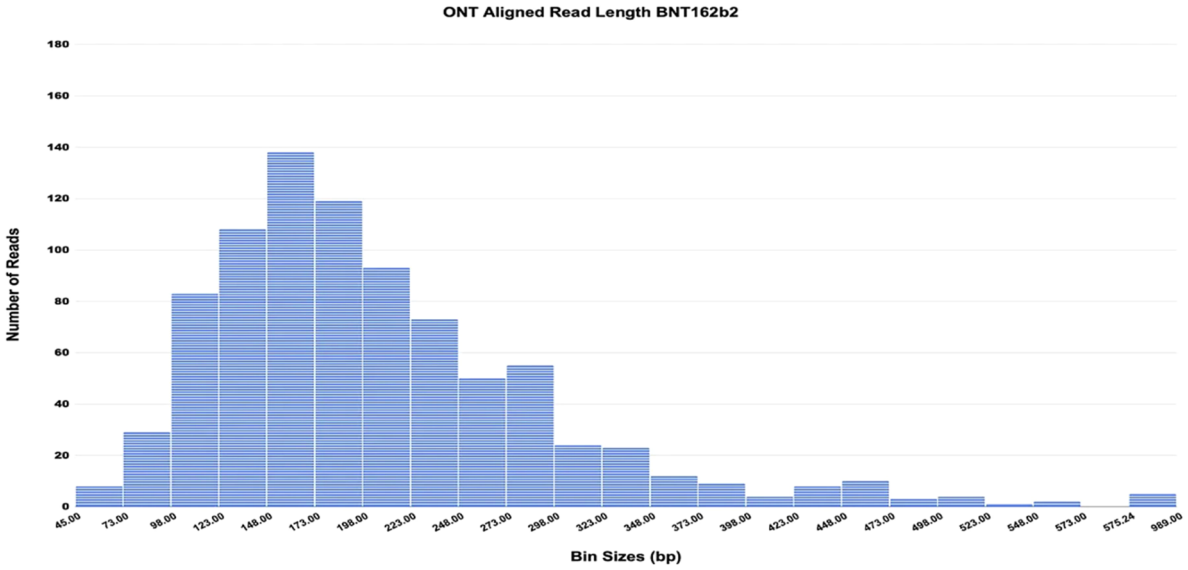

Figure 6. Oxford nanopore (ONT) read length distributions from 865 reads mapped to the vector sequence (NCBI OR134577.1). Mean = 214 bp. Max = 3.5 kb.

Possibly, homologous modified RNA may prevent digestion of template DNA by hybridization [4]. Of note, AS0467D represents bivalent BA.1 vaccine, which was noted to consist of two separate LNPs containing different purities of mRNA that were mixed together in the final product [27]. As DNase I is inhibited by RNA-DNA hybrids, the different modRNA constructs may present different secondary structure or hybridization potential with DNA and alter DNase I activity compared to other Moderna vaccines. This extreme difference in the spike and ori genetic targets points to a manufacturing variance that should not exist and highlights the importance that the regulators should not rely on a single PCR assay to determine the load of the residual plasmid DNA in the final drug product.

The vials with the highest DNA concentration were from two lots of Pfizer monovalent purple top vials with a PBS formulation and require dilution before administration. On October 29, 2021, the US FDA authorized a change of formulation to a Tris/sucrose buffer; the grey topped monovalent adult vaccine, and an orange topped vaccine for children aged 6–11 years. This change was made to increase stability, to simplify storage requirements and to provide a ready-to-use formulation [28,29]. These purple-topped Pfizer lots were also associated with some of the highest number of AEs and SAEs reported in VAERS among all the lots tested. The reason for this difference is not known, but

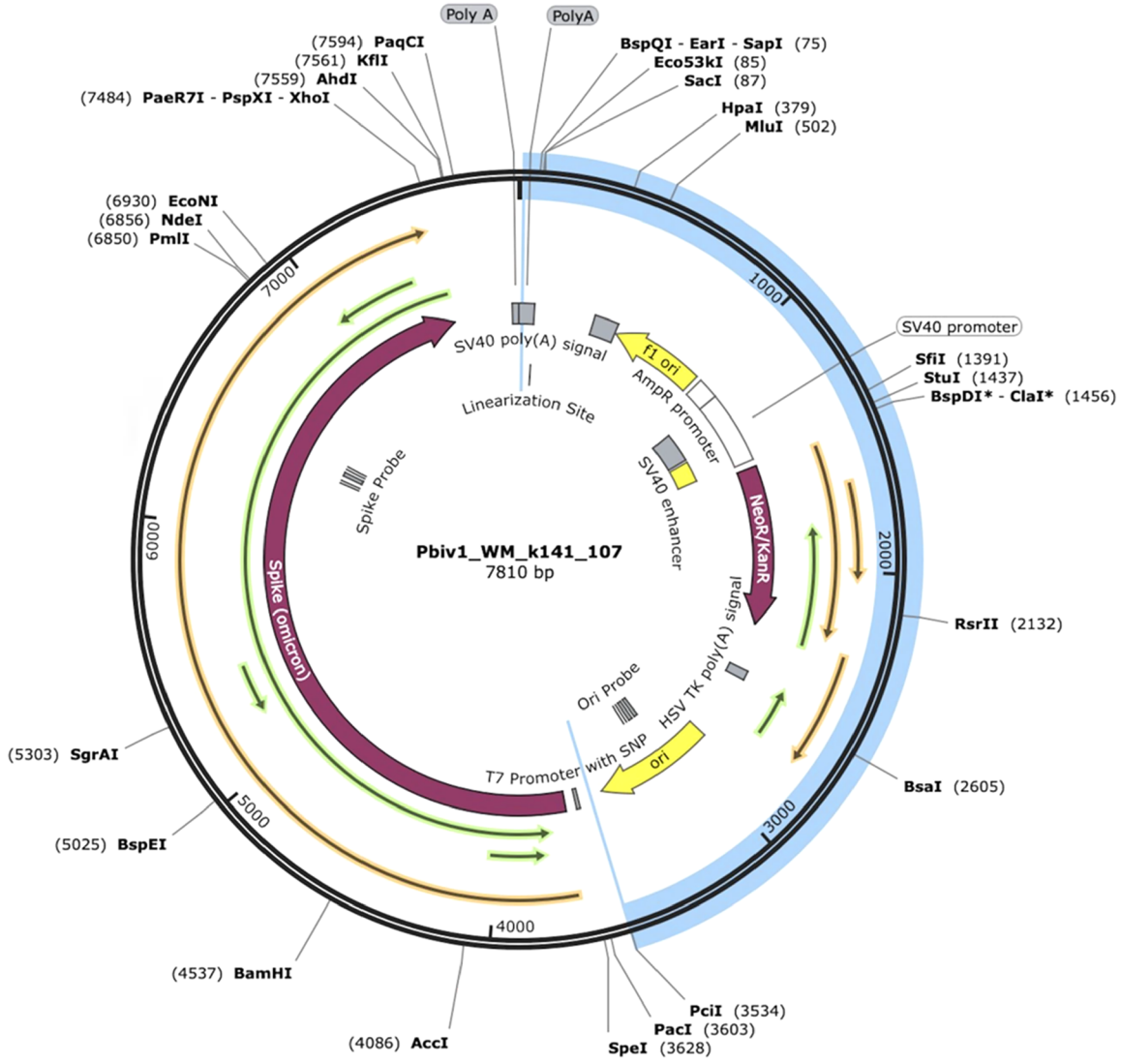

Figure 7. The longest oxford nanopore (ONT) read aligns to the vector region shown in blue. *ori* and spike primer locations are annotated on the innermost circle. Open reading frames (ORFs) are annotated in gold and green arrows. Kanamycin resistance genes were detected in a very shallow sequencing survey of the vaccine.

the use of PBS buffer resulted in visible particles in the final drug product which may be associated with toxicities including microemboli and inflammatory reactions [30]. Furthermore, lipid impurities due to lipid hydrolysis and aldehyde formation were observed resulting in lipid adducts which may have toxicological implications. The use of a Tris buffer acts as an "aldehyde sink" reducing LNP impurities as detected by Moderna scientists [31]. Adducts result in the loss of modRNA function and may affect the higher order structure of mRNA and changes to mRNA kinetics or other unknown factors may be responsible [32].

The inter and intra-lot variability in residual DNA continues to be demonstrated even after 3 years of manufacturing experience. This continued variability, especially in the Pfizer vials, is perhaps more fundamental than achieving a low level of impurities since it demonstrates an inconsistent manufacturing process and likely occurs with other process and product-related impurities. DNase purity and robustness may differ between vendors or lots of the DNase enzyme [33]. Small differences in the IVT process or other factors may also play a role in the variability of residual DNA. The DNase digestion step of the Pfizer/BioNTech vaccine was subject to a recommendation by the EMA to enhance the robustness of the method at the time of approval [34]. However, a FOIA obtained in Germany showed ongoing issues with the consistency of residual DNA levels within and among Pfizer/BioNTech manufacturing sites, the use of in-house "standards," and a lack of

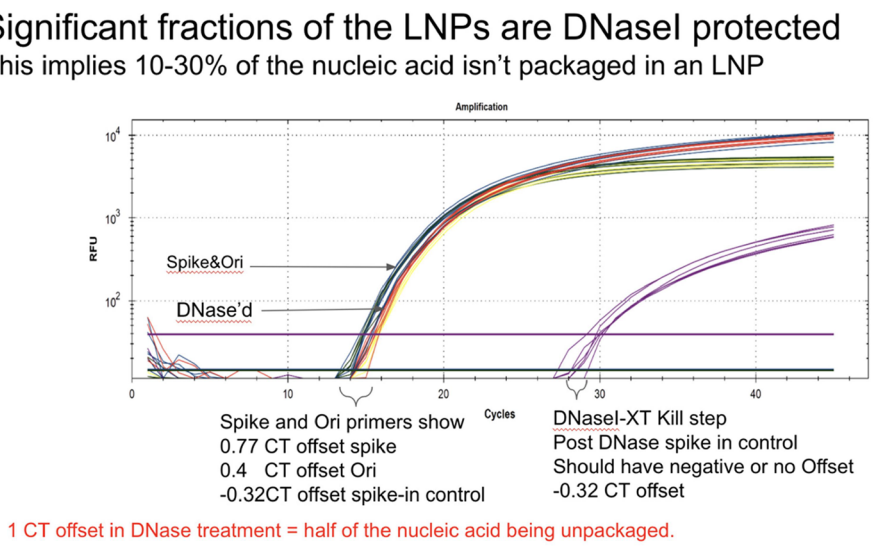

Figure 8. DNase I-XT treatment of Pfizer vaccine demonstrates nuclease resistance of the DNA in the vaccines.

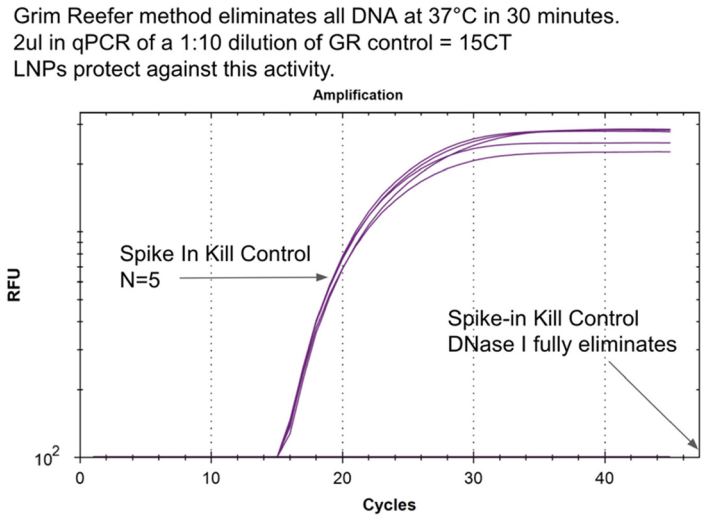

Figure 9. DNase I-XT positive control demonstrates the digestion assay eliminates all spiked in DNA under the same conditions used to assess the vaccine nuclease sensitivity.

correlation between the activity of the DNase enzyme and residual DNA a full 15 months after authorization [35]. This demonstrates ongoing efforts to remove DNA fragments were met with limited success.

Our exploratory analysis of the relationship between the residual DNA content and SAEs reported to VAERS is preliminary and limited in sample size but warrants confirmation by examining many more lots and vials. This preliminary analysis appears to show a correlation between the adverse events reported for each lot and the levels of DNA, especially in the context of the *ori*. More testing of vials from other independent laboratories, including prospective long-term monitoring, is required to confirm our results and confirm whether a correlation exists. When tested by qPCR, Pfizer lot FD0810, the oldest Pfizer lot examined, exhibited lower levels of spike and SV40 promoter-enhancer-ori with comparable ori DNA levels to other adult monovalent (purple-topped) vials, yet it was associated with the highest reported total AEs in VAERS. We only tested a single vial, which may not represent the entire lot. Without knowing the lot sizes or how many people received doses from each lot, correlating SAEs to specific lots is challenging with date from just 16 lots. If a lot associated with high SAEs was administered to significantly more people it could skew the SAEs-per-person ratio, further complicating efforts to establish a clear correlation. However, our findings suggests that beyond

plasmid DNA levels, issues related to the LNPs, toxicity, autoimmunity and/or aberrant immune responses due to ribosomal frameshifting may attribute to AEs, including Complement Activation-Related Pseudoallergy (CARPA), may contribute to AEs shortly after vaccination [36–41]. The random composition of DNA fragments in these vials complicates direct correlation between plasmid DNA levels and specific adverse reactions because the genotoxic effects of each fragment vary unpredictably depending on their sequence and interaction with the host genome. This variability makes it impossible to precisely estimate adverse effects based solely on DNA quantity. Higher DNA loads might also contribute to long-term health risks, such as cancer from genomic integration events. VAERS primarily captures short-term AEs and may underreport long-term effects. Further vial testing is needed to establish a dose-response curve using VAERS data. Long-term prospective monitoring is essential to assess the impact of genetic vaccines, especially since the spike protein has been detected up to 704 days post-vaccination [42].

Different relationships were observed for Moderna lots for qPCR data as well as for plots based on residual DNA estimated by fluorometry for both Pfizer and Moderna lots. These observations may reflect differences between the two products such as dose volume, quantity of DNA, the size distribution of DNA fragments, the composition and sequence of the plasmid vector and composition of lipid nanoparticles. Other differences, both between the two products and between different lots of each product, may also contribute to our observations. These differences include variations in levels of contaminants or impurities. One major source of impurity is fragmented mRNA for which several toxicological mechanisms have been proposed, such as its effects on miRNA processes [43]. dsRNA is another type of impurity that occurs secondarily to the T7 RNA polymerase promoter. dsRNA can induce pro-inflammatory cytokines [44] and has been hypothesized to contribute to immune-inflammatory reactions such as myocarditis [45]. Lipopolysaccharides in cells from endotoxin can bind both the S1 and S2 subunits of the spike protein which may result in enhanced inflammatory responses [46].

Wider sampling will likely reveal greater detail in terms of event types, such as death, as well as comparisons with other works, such as that reported by Schmeling et al. who reported a correlation of AEs to various vaccine lot numbers [47]. None of the presently studied vaccine lots were included in the Schmeling study and more work is needed to understand if and how this DNA contamination is related to AEs.

While the SV40 enhancer facilitates nuclear targeting [16,48], genomic integration of DNA fragments has yet to be demonstrated for the COVID-19 modRNA products [49]. However, it is known that DNA contamination could trigger an unwarranted innate immune response and may be prothrombotic, particularly for fragments with high GC content [50]. dsDNA may also be a significant factor in ischemic diseases including stroke [51]. Cytosolic DNA is also known to trigger cGAS-STING and chronic stimulation of this pathway is believed to be pro-oncogenic [52]. Moderna US patent #10,898,574 also emphasizes the insertional mutagenesis risk of residual DNA in their vaccines. While there appears to be a correlation between high DNA contamination and SAEs more research is needed to expand the sample size and elucidate any potential mechanism at work.

It is important to emphasize that as qPCR cannot quantitate molecules smaller than the size of the amplicon (105–114 bp), qPCR underestimates the total DNA in each vaccine. The failure for qPCR to fully quantitate residual DNA in mRNA vaccines is described in Moderna patent #US 10,077,439. It is important to emphasize that the use of a single amplicon for qPCR can lead to 100-fold different estimates in some vaccines. The Spike DNA in some Moderna vaccines is ~100 fold more prevalent (~6CTs) than the non-spike DNA suggestive of DNase I inhibition with complementary RNA [4].

This explains the large differences we have observed in residual DNA levels estimated by qPCR compared with Qubit® fluorometry particularly between the Pfizer and Moderna products. The much larger values obtained for the Moderna product could partially be due to the larger dose volume but also suggests that there is a higher fraction of small fragmented residual DNA than in the Pfizer product. This is consistent with a more thorough nuclease digestion step. This illustrates the residual DNA guidelines recommended by the FDA are highly dependent on the methods used to quantitate the DNA. Furthermore, PCR assay robustness and reliability needs to be established given the actions of N-1-methylpseudouridine, the presence of RNA-DNA hybrids.

Konig et al. also found high DNA contamination using Qubit® fluorometry [24]. However, this study lacked an RNase A treatment and is likely over-estimating the amount of the DNA present due to the cross talk of DNA specific dyes intercalating with RNA. While the specificity of these DNA specific fluorometric dyes is well published, little is known about the impact on their specificity when in use with samples that have high concentrations of N1-methyl-pseudouridine modRNA or GC rich RNA prone to secondary structure and minor groove formation. Our Qubit® data showed that variance between sampling and freeze-thaw cycles do occur. This variance could be due to crude nature of the fluorometric assays, freeze-thaw cycles, or due to sampling error especially as sample vortexing and multiple pipetting can introduce air bubble that will alter the LNP size and settling [53]. However, our RNase A data demonstrate that Qubit® fluorometry must be coupled with the removal of the vaccine modRNA to properly quantitate the residual DNA when high concentrations of RNA are co-present.

This fluorometry assessment is of particular interest as fluorometry and UV spectrophotometry were used to quantitate RNA in the Pfizer COVID-19 vaccines, as described in EMA documents [7], while qPCR was used to quantitate DNA. This selective use of different methods to quantitate RNA/DNA ratios can lead to vastly different results for the ratio-metric guidelines in place at the EMA.

This elevated fluorometry quantitation compared to qPCR quantitation is consistent with the ONT read length distributions that also suggest a portion of the DNA may be smaller than the amplicon size. While the ONT sequencer detects molecules shorter than 100 bp, the methods for library construction for ONT use a 0.7X Ampure DNA purification step which drastically selects against purifying molecules <150 bp in size. As a result, the read length distributions for ONT reads are biased toward fragments >150 bp and are not a perfect reflection of the smaller fragments that may be present and undercounted by both ONT and qPCR.

Similarly, Georgiou et al. demonstrate that Fluorometric dyes produce 70% lower signal after DNase I treatment as the dyes provide non-linear signal for longer molecules of the same mass of DNA [54]. These data have not been compensated for this effect and likely reflect an underestimate of the actual quantity in the fluorometric assay. These smaller DNA molecules are also frequently lost during commonly used DNA isolation procedures such as Phenol/Chloroform purification or Ethanol precipitation. Kaiser et al. measured much lower amounts of DNA in their vaccines, but their methods did not ascertain the loss of short DNA fragments after multiple ethanol precipitations [55]. As DNA fragments <100bp can be lost during purification methods, including ethanol precipitation, it is best to quantify the total DNA directly on the final drug product without any purification steps.

Currently, the US FDA recommends manufacturers of viral vaccines to limit the amount of residual DNA in the final product to below 10 ng/dose for parenteral inoculations and the size of the DNA to below the size of a functional gene, or ~200 base pairs [12]. This is also in keeping with recommendations from the World Health Organization (WHO) [20,56], and is for "naked" DNA, i.e. DNA that is not associated with proteins, lipids, or other molecules for protection or packaging. Previous residual DNA levels were set by the FDA at 10 pg/dose in 1985. A 1986 WHO study group concluded that the risk is negligible up to 100 pg/dose and in 1996 the WHO further increased levels up to 10 ng per dose [20]. However, as the residual DNA is encapsulated in the LNPs and is transfected efficiently directly into cells the DNA guidelines for modRNA vaccines need to be re-evaluated.

The FDA and WHO guidelines for allowable DNA in vaccines are influenced by work published by FDA scientists Sheng-Fowler et al. [57]. This work focused on host cell genomic DNA contamination and made note of the increased number of molecules present when small viral vectors are the contaminating species. For these high copy per nanogram contaminants, femtograms to attograms of DNA are considered the equivalent of nanograms of cell substrate genomic DNA. Given the short fragment size in the modRNA vaccines, the number of molecules in each dose can reach over 100 billion molecules. The residual DNA in these vaccines is high in copy number and rich in promoters, SV40 mammalian origins of replication, ORFs and nuclear targeting sequences. The FDA and WHO guidelines did not consider packaging of DNA in lipid nanoparticles, likely resulting in longer DNA persistence as well as increased transfection efficiency. Furthermore, the guidelines did not consider

cumulative dosing with LNP-based modRNA. Data have shown that LNPs are capable of increasing RNA or DNA cell entry by 10–100-fold [58–60].

In Japan, 13% of the population received seven doses of COVID-19 vaccines with a booster dose interval sometimes as short as 2 months [61]. Moreover, the risks of cumulative dosing by vaccines targeting other infections but using the same plasmid and LNP-based modRNA platform has not been considered in setting the residual DNA guidelines.

The FDA guidelines are also written to only quantitate DNA fragments of 200 bp or greater, in part because fragments smaller than this were not considered to be able to produce a functional gene. However, Klinman et al. suggests that fragments as small as 7 bp can pose integration risks [62]. Furthermore, the guidelines may also have considered that fragments of naked DNA shorter than 200 bp would be more rapidly hydrolyzed by host nuclease activity than larger molecules [63]. This accelerated destruction cannot be assumed of the vaccines due to the DNA being encapsulated and protected by the LNPs.

Klinman et al. also observe that "*in evaluating the potential harm of plasmid integration, it should be noted that the risk of introducing plasmids with strong regulatory regions into the host genome far exceeds that associated with random point mutations.*"

Finally, the guidelines do not consider if the residual DNA contains nuclear targeting sequences, mammalian origins of replication and mammalian promoters that exist in the Pfizer vaccine [49]. Vacik et al. demonstrated that the SV40 enhancer present in the Pfizer vector is a potent nuclear targeting sequence showing promise for gene therapy [48]. Senigl et al. demonstrate the SV40 enhancer is a somatic hypermutability element with tumorogenic potential [64]. Finally, Drayman et al. demonstrate the SV40 Enhancer in the Pfizer vaccine binds to P53, reminding us that the simple nanogram limits of residual DNA need to be reconsidered based on the functionality and replicative capacity of that DNA [65].

Conclusion

These data demonstrate the presence of billions to hundreds of billions of DNA molecules per dose in the modRNA COVID-19 products tested. Using fluorometry coupled with RNase A digestion, all products tested exceeded the guidelines for residual DNA set by the FDA and WHO of 10 ng/dose by 36–627-fold. qPCR testing showed that all Moderna vials were within the regulatory limit and that 3 Pfizer vials exceeded the regulatory limit for the SV40 promoter-enhancer-ori and showed much greater intra- and inter-lot variability. qPCR underestimates the total DNA with results varying greatly by genomic target emphasizing the importance of using more than one assay to accurately determine the DNA load. It is important that regulators produce clear and consistent guidelines on how to quantify mRNA and plasmid DNA in modRNA vaccines. The PCR results for the most recent XBB.1.5 Moderna and Pfizer vaccines suggest that DNA residues have not been reduced from previous vaccine versions.

Our findings extend existing concerns about vaccine safety and call into question the relevance of guidelines conceived before the introduction of efficient transfection using LNPs. With several obvious limitations, we urge that our work is replicated under forensic conditions and that guidelines be revised to account for highly efficient DNA transfection and cumulative dosing.

This work highlights the need for regulators and industry to adhere to the precautionary principle and provide sufficient and transparent evidence that products are safe and effective, and disclose the details of their composition and method of manufacture.

Author contributions

CRediT: **David J. Speicher**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing; **Jessica Rose**: Formal analysis, Methodology, Software, Writing – review & editing; **Kevin McKernan**: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing.

Disclosure statement

Kevin McKernan is employed by Medicinal Genomics and provided qPCR and Qubit® reagents free of charge. No potential conflict of interest was reported by the author(s).

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Data availability statement

FASTQ file for the mapped ONT sequencing data: https://mega.nz/file/UZhkiTBQ#8vjDK5JV5N5Dj2On34B6zdROb EKGBy3ZC7w8q2t9UVc

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