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DNA fragments detected in monovalent and bivalent Pfizer/BioNTech and Moderna modRNA COVID-19 vaccines from Ontario, Canada: Exploratory dose response relationship with serious adverse events.

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Abstract

Background: *In vitro* transcription (IVT) reactions used to generate nucleoside modified RNA (modRNA) for SARS-CoV-2 vaccines currently rely on an RNA polymerase transcribing from a DNA template. Production of modRNA used in the original Pfizer randomized clinical trial (RCT) utilized a PCR-generated DNA template (Process 1). To generate billions of vaccine doses, this DNA was cloned into a bacterial plasmid vector for amplification in *Escherichia coli* before linearization (Process 2), expanding the size and complexity of potential residual DNA and introducing sequences not present in the Process 1 template. It appears that Moderna used a similar plasmid-based process for both clinical trial and post-trial use vaccines. Recently, DNA sequencing studies have revealed this plasmid DNA at significant levels in both Pfizer-BioNTech and Moderna modRNA vaccines. These studies surveyed a limited number of lots and questions remain regarding the variance in residual DNA observed internationally.

Methods: Using previously published primer and probe sequences, quantitative polymerase chain reaction (qPCR) and Qubit® fluorometry was performed on an additional 27 mRNA vials obtained in Canada and drawn from 12 unique lots (5 lots of Moderna child/adult monovalent, 1 lot of Moderna adult bivalent BA.4/5, 1 lot of Moderna child/adult bivalent BA.1, 1 lot of Moderna XBB.1.5 monovalent, 3 lots of Pfizer adult monovalent, and 1 lot of Pfizer adult bivalent BA.4/5). The Vaccine Adverse Events Reporting System (VAERS) database was queried for the number and categorization of adverse events (AEs) reported for each of the lots tested. The content of one previously studied vial of Pfizer COVID-19 vaccine was examined by Oxford Nanopore sequencing to determine the size distribution of DNA fragments. This sample was also used to determine if the residual DNA is packaged in the lipid nanoparticles (LNPs) and thus resistant to DNaseI or if the DNA resides outside of the LNP and is DNaseI labile.

Results: Quantification cycle (Cq) values (1:10 dilution) for the plasmid origin of replication (*ori*) and spike sequences ranged from 18.44 - 24.87 and 18.03 - 23.83 and for Pfizer, and 22.52 – 24.53 and 25.24 – 30.10 for Moderna, respectively. These values correspond to 0.28 – 4.27 ng/dose and 0.22 - 2.43 ng/dose (Pfizer), and 0.01 -0.34 ng/dose and 0.25 – 0.78 ng/dose (Moderna), for *ori* and spike respectively measured by qPCR, and 1,896 – 3,720 ng/dose and 3,270 – 5,100 ng/dose measured by Qubit® fluorometry for Pfizer and Moderna, respectfully. The SV40 promoter-enhancer-*ori* was only detected in Pfizer vials with Cq scores ranging from 16.64 – 22.59. In an exploratory analysis, we found preliminary evidence of a dose response relationship of the amount of DNA per dose and the frequency of serious adverse events (SAEs). This relationship was different for the Pfizer and Moderna products. Size distribution analysis found mean and maximum DNA fragment lengths of 214 base pairs (bp) and 3.5 kb, respectively. The plasmid DNA is likely inside the LNPs and is protected from nucleases.

Conclusion: These data demonstrate the presence of billions to hundreds of billions of DNA molecules per dose in these vaccines. Using fluorometry, all vaccines exceed the guidelines for residual DNA set by FDA and WHO of 10 ng/dose by 188 – 509-fold. However, qPCR residual DNA content in all vaccines were below these guidelines emphasizing the importance of methodological clarity and consistency when interpreting quantitative guidelines. The preliminary evidence of a dose-response effect of residual DNA measured with qPCR and SAEs warrant confirmation and further investigation. 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.

Introduction

To produce large amounts of modified RNA (modRNA) vaccine for generalized use, Pfizer changed its manufacturing process (Process 1) used to produce material for the randomized clinical trial (RCT)[1] to a process (Process 2) similar to the one already being used by Moderna. The SARS-CoV-2 spike sequence was cloned into a plasmid containing a bacterial origin of replication (generically termed *ori*) active in *Escherichia coli*. This plasmid (7,824 base pairs (bp) and 6,777 bp for Pfizer and Moderna, respectively) also contains an aminoglycoside phosphotransferase gene (*Neo/Kan*) that allows cost effective bacterial replication in a broth containing kanamycin and a doubling of plasmid copy number every 30 minutes at 37°C. The *E. coli* cells are then harvested and lysed. DNA is extracted and linearized with the restriction enzyme *Eam*1104I. This linear DNA then acts as the template for T7 RNA Polymerase *in vitro* transcription (IVT) in the presence of N1-methyl-pseudouridine. After the IVT, DNA is hydrolyzed, reducing its prevalence in the final drug product. Documents leaked from the European Medicines Agency (EMA) and cited in the British Medical Journal[2] noted that residual DNA in modRNA products made by this process could vary significantly[3].

McKernan *et al.* performed next-generation RNA sequencing of these vaccines and, unexpectedly, found evidence of DNA derived from the expression plasmids used during manufacturing.[4] McKernan *et al.* then developed a quantitative polymerase chain reaction (qPCR) method towards the DNA contamination with primers targeting shared sequences in both Pfizer and Moderna vaccines.[4] Additionally, McKernan *et al*., found SV40 promoter-enhancer-*ori*, and SV40 polyA signal sequences in the Pfizer vaccines. To investigate the generalizability of these findings to other lots of vaccines, we obtained 24 unopened expired vials (8 Pfizer and 16 Moderna) and three vials of in-date remnants of Moderna XBB.1.5 COVID-19 vaccines that had been distributed in Ontario, Canada and examined them via Qubit® fluorometry and qPCR targeting spike, plasmid *ori*, and the SV40 promoter-enhancer-*ori*. We then queried the Vaccine Adverse Event Reporting System (VAERS) for any adverse events (AEs), including serious AEs (SAEs), associated with these lots.[5] We also extended the observations of an earlier work (McKernan *et al.*[4])

by studying the size distribution of DNA fragments as well as the DNaseI sensitivity of the vaccine to determine whether the residual DNA is packaged in the LNPs.

For the purpose of this study, we are using the terms "residual DNA," "DNA mass," (or similar) rather than "impurity" or "contamination" as a discussion of these regulatory terms is beyond the scope of this paper.

Methods

COVID-19 Vaccines Tested

Expired unopened vials of Pfizer-BioNTech BNT162b2 (n=8) and Moderna Spikevax mRNA-1273 (n=16) were obtained from various pharmacies in Ontario, Canada (Figure 1). Three vials of in-date remnants of the same lot of Moderna XBB.1.5 vaccine were also obtained. In total, 12 lots were surveyed across 27 mRNA vials: 5 lots of Moderna child/adult monovalent, 1 lot of Moderna adult bivalent BA.4/5, 1 lot of Moderna child/adult bivalent Wuhan-BA.1, 1 lot of Moderna XBB.1.5 monovalent, 3 lots of Pfizer adult monovalent, and 1 lot of Pfizer adult bivalent Wuhan-BA.4/5 vaccines. An unopened sterile injectable vial of alprostadil 66 mcg/mL in combination with papaverine 21.7mg/mL and phentolamine 1 mg/mL (TriMix) was used as the negative control. The unopened vials were untampered as they had intact flip-off plastic caps with printed lot numbers and expiration dates. Vials had been stored in a purpose-built vaccine unit at +2-8^{o}C in the pharmacies and were transported in insulated containers with frozen gel packs and placed in the testing laboratory fridge within 5 hours. Only one Moderna vial did not have 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. Vials were removed from the refrigerator, warmed for ~20 minutes, and administered by the pharmacist to patients over ~30 minutes. The remnant vials were placed in an insulated container with frozen gel packs and transported to the testing laboratory fridge within 12 hours.

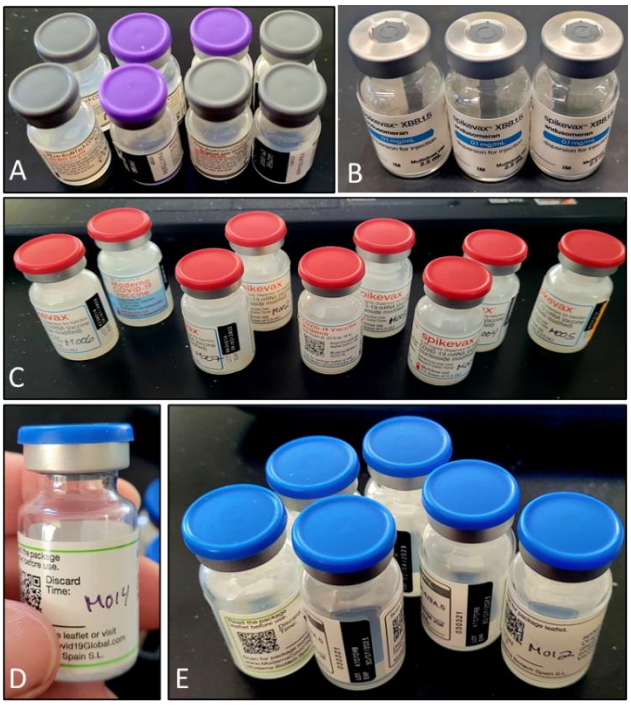


Figure 1. Vials of COVID-19 vaccine from Ontario, Canada: (A) Pfizer/BioNTech BNT162b2 adult monovalent and bivalent; Moderna Spikevax mRNA-1273 (B) adult monovalent XBB.1.5, (C) child/adult monovalent, (D) child/adult bivalent Wuhan-BA.1 and (E) child/adult bivalent Wuhan-BA.1 and adult Wuhan-bivalent BA.4/5.

qPCR Analysis of Spike, ori, and the SV40 Promoter-Enhancer-ori DNA

Each vial was tested by quantitative PCR (qPCR) for the presence of plasmid derived SARS-CoV-2 spike, *ori,* and the SV40 promoter-enhancer-*ori* DNA. Spike and plasmid *ori* were tested in duplicate with PCR primers targeting sequences shared by the Moderna and Pfizer expression plasmids (Table 1). The uniplex SV40 Enhancer assay was designed to amplify the nuclear targeting sequence unique to the Pfizer vector[6]. In brief, the 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, 0.8 µL reaction buffer and 1.0 µL of Primer-Probe mix, and 12.2 µL of ddH_20. The 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.

Spike and *ori* qPCR assays used a synthetic gDNA control (gBlock, Integrated DNA Technologies (IDT), San Diego, USA) of known concentration to generate a 10-fold serial dilution derived calibration curve. The SV40 enhancer gBlock failed initial synthesis and a standard curve could not be produced.

Table 1. Primer and probe sequences targeting spike, ori, and the SV40 promoter.

Primer-Probe Name	Sequence
MedGen-Moderna_Pfizer_Janssen_Vax-Spike_Forward	AGATGGCCTACCGGTTCA
MedGen-Moderna_Pfizer_Janssen_Vax-Spike_Reverse	TCAGGCTGTCCTGGATCTT
MedGen-Moderna_Pfizer_Janssen_Vax-Spike_Probe	/56-FAM/CGAGAACCA/ZEN/GAAGCTGATCGCCAA/3IABkFQ/
MedGen_Vax-vector_Ori_Forward	CTACATACCTCGCTCTGCTAATC
MedGen_Vax-vector_Ori_Reverse	GCGCCTTATCCGGTAACTATC
MedGen_Vax-vector_Ori_Probe	/5HEX/AAGACACGA/ZEN/CTTATCGCCACTGGC/3IABkFQ/
MedGen_SV40_Enhancer_Forward	GTCAGTTAGGGTGTGGAAAGT
MedGen_SV40_Enhancer_Reverse	GGTTGCTGACTAATTGAGATGC
MedGen_SV40_Enhancer_Probe	/5TEX615/CCAGCAGGCAGAAGTATGCAAAGC/3IAbRQSp/

Cycling was performed on a QuantStudio 3 (ThermoFisher Scientific, Waltham, USA) with an initial denaturation of 95°C for 3 minutes followed by 35 cycles of 95°C for 10 seconds and 65°C for 30 seconds. 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,[7] and length (105 bp for *ori*, 114 bp for spike) 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 for the volume of each intramuscular vaccine injection (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.

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). Fluorometric reagents (190 µL of a stock made from 995 µL HS Buffer and 5 µL 200X AccuGreen dye) were vortexed with 10 µL of vaccine. These samples were heated to 95°C for 8 minutes and 4°C for 5 minutes to

disrupt the LNPs and enable Fluorometric Dyes to access the DNA. Samples were read following the manufacturer's instructions on a Qubit 3.0 Fluorometer. Qubit fluorometry and qPCR data were compared.

Vaccine Adverse Event Reporting System (VAERS) Data

The VAERS database was analyzed using the Language and Environment for Statistical Computing package in R,[8] and included data spanning December 17, 2020 through October 6, 2023 The 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.[5] 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, since we are interested in the COVID-19 products, only COVID-19 vaccine 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) and deaths (DIED). Data were grouped by vaccine 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, and individual MedDRA coded AEs, such as total deaths per lot, were also counted.

The various limitations of VAERS are widely acknowledged, for example by FDA[9], and include underreporting, misreporting, spontaneous reporting, and the inability to infer causality. Nevertheless, to explore a possible dose-response relationship between residual DNA content and SAEs, we used the ratio of the number of SAE reports to the total number of AEs ("SAE reporting ratio" = SRR) as a proxy for a possible toxicological effect. We used the total number of AEs reported by lot as a proxy for the total number of doses administered, since this denominator is difficult to estimate. This principle is used by the CDC in disproportionality signal analysis (DSA) to identify safety signals using the

Proportional Reporting Ratio (PRR)[10] The PRR, as devised by Evans *et al.*, is a useful tool in pharmacovigilance with known limitations.[11]

It must be noted that although VAERS is a USA-based database, it accepts reports from around the world. Certain categories of AEs that are reported to manufacturers outside the USA, must be reported to the VAERS database. Differences in propensity for underreporting as well as mandatory reporting imposed on manufacturers or medical professionals within and outside the USA may introduce confounding to the estimation of the SRR. Accordingly, for our exploratory dose-response analysis we only used VAERS data originating outside the USA to reduce this confounding. Additionally, we have noted some discrepancies in data obtained through the downloaded version of the VAERS dataset, and those obtained using the VAERS WONDER front-end web-based interface (https://wonder.cdc.gov/controller/datarequest/D8). We used the downloaded version as it provides greater detail than the web version. The SRR was then plotted against levels of DNA found in the vials to identify any association between residual DNA levels and the frequency of reports of serious adverse events.

Where more than one vial was available in any lot, the average mass of residual DNA per dose for that lot was used. Zero values of SRR for any given lot were only plotted if one or more AEs had been identified worldwide, signifying that that lot had actually been deployed. The curves were plotted on a logarithmic axis and a trend line drawn using the linear function within Microsoft® Excel.

Oxford Nanopore Sequencing

In a separate experiment using previously sequenced vaccine[4] (Pfizer children's monovalent Lot# FL8095), DNA fragment size distributions were estimated 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).[12] ONT sequencing read

length is unlimited, but the DNA isolation procedures can bias the length of the molecules captured in the ONT ligation reaction. Single molecule reads were counted and binned according to their mapped read length with BWA-MEM.

Nuclease sensitivity of the vaccines

The same vial (Pfizer Lot# FL8095) was used to assess DNaseI sensitivity of the vaccine by determining if the DNA contamination is packaged in the LNP and thus resistant to DNaseI or if the DNA resides outside of the LNP and is DNaseI labile.

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

After spiking in the DNaseI inactivation control, 54 µL of SenSATIVAx magnetic beads (Medicinal Genomics) were used to purify DNA from the DNaseI-XT assay and the DNaseI-XT negative control samples. The magnetic beads were pipette mixed 10 times with the sample, incubated at room temperature for 5 minutes, magnetically separated and washed twice with 70% v/v ethanol. The ethanol was removed, and the beads dried for 2 minutes 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 DNaseI inactivation control primer and probe (0.5 µL in CY5) were added to the assay for a total of 19.3 µL reaction.

Results

An 8-log serial dilution standard curve was used to calibrate sample Cq values and generated R^2 values of 0.998 and 0.999 for spike and *ori* amplicons, respectively. PCR

efficiency was 99.8% and 94.7% for spike and *ori,* respectively (Figure 2). On all plates, negative controls and no template (ddH_20) controls (NTC) were tested in triplicate and found to be negative.

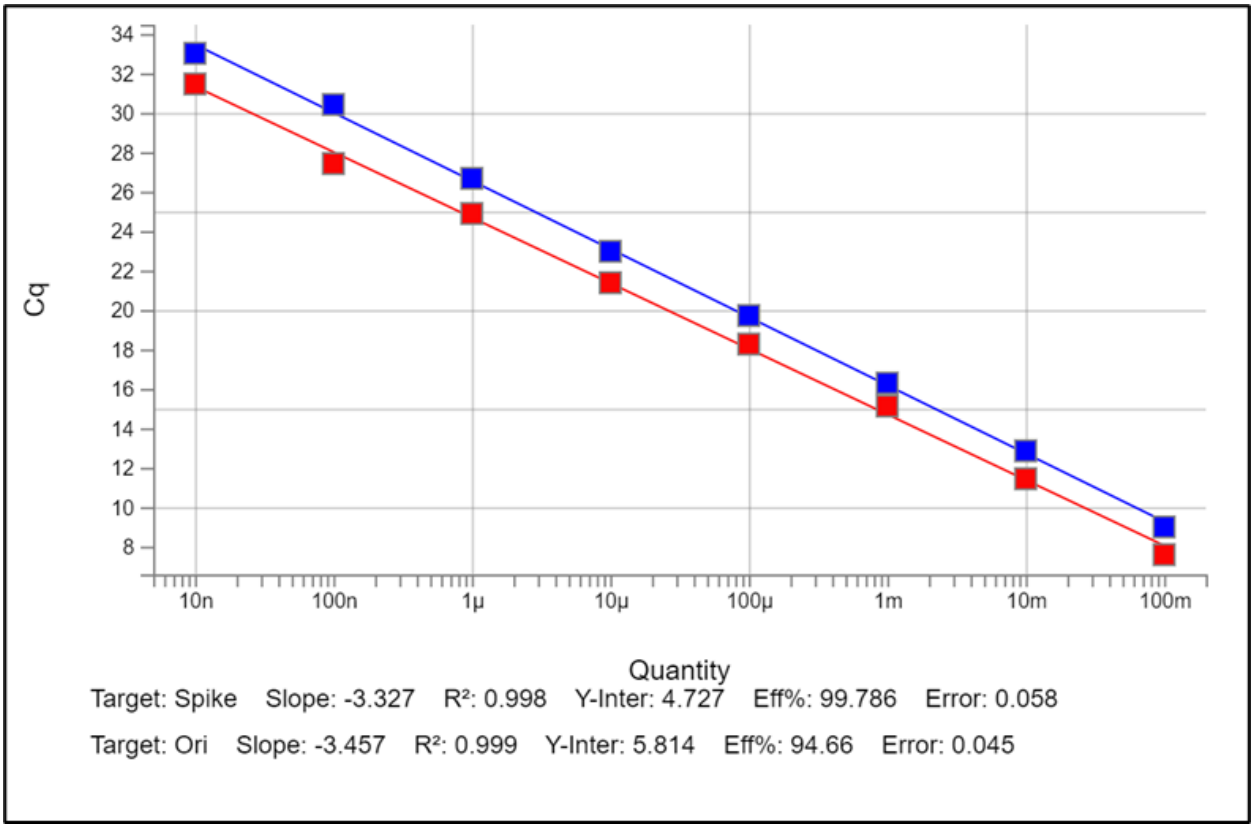


Figure 2. Calibration curves of Spike (red) and ori (blue) diluted 10-fold and tested by qPCR.

For individual vials, qPCR on Pfizer amplified at a similar time for spike, *ori*, and SV40 enhancer-promoter-*ori* ($_{\Delta}$Cq 1.48 ± 0.32) (Figure 3). Apart from Pfizer lot: FX4343, the inter vial difference was small for both Pfizer (spike Cq 16.91 ± 0.52; *ori* Cq 16.91 ± 1.07; SV40 promoter-enhancer-*ori* Cq 15.46 ± 2.02) and Moderna (spike Cq 20.35 ± 0.65; *ori* Cq 25.34 ± 1.47) (values were based on the undiluted vials contents) (Table 2, Figure 4)

However, for all Moderna vials, except lot AS0467D, *ori* consistently amplified Cq 5-6 later than spike. The SV40 promoter-enhancer-*ori* was detected in all Pfizer vials but in none of the Moderna vials.

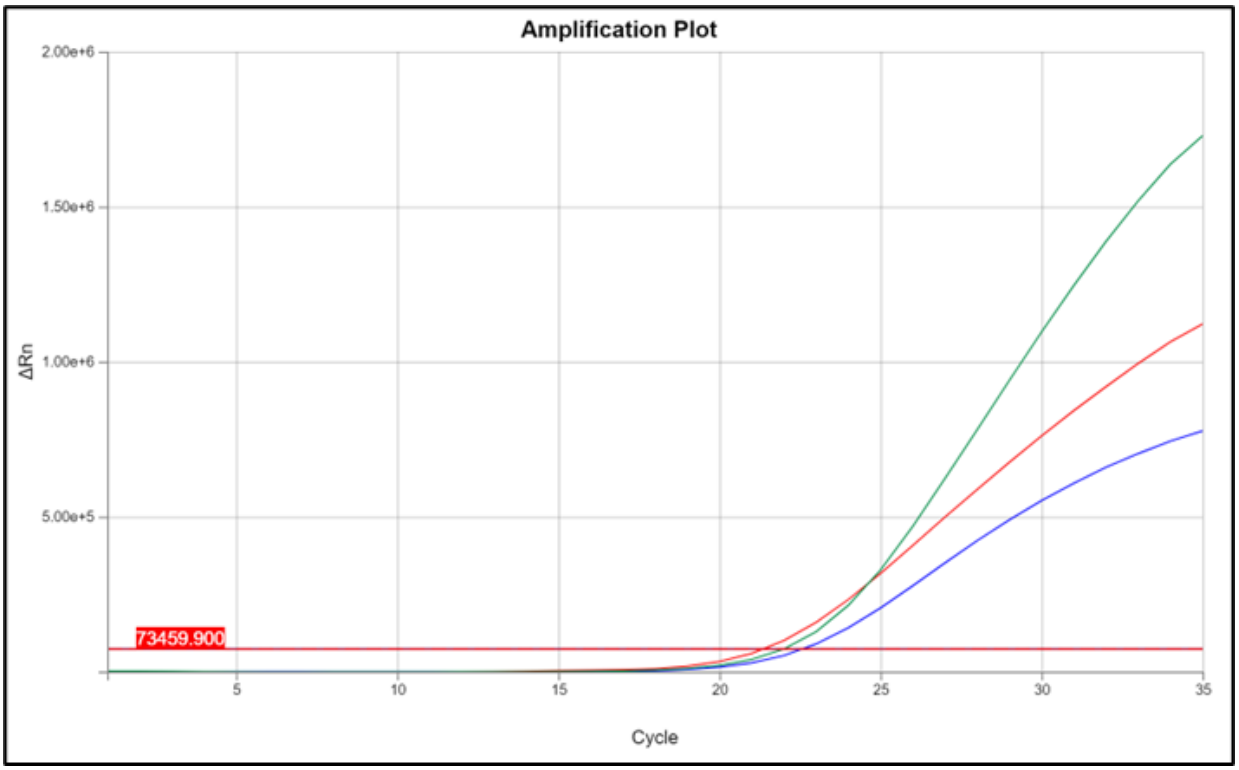


Figure 3. The amplification curve for spike (red), ori (blue), and SV40 enhancer-promoter-ori (green) in a single vial of Pfizer (Lot: Fx4343a) from two different wells of the same PCR run.

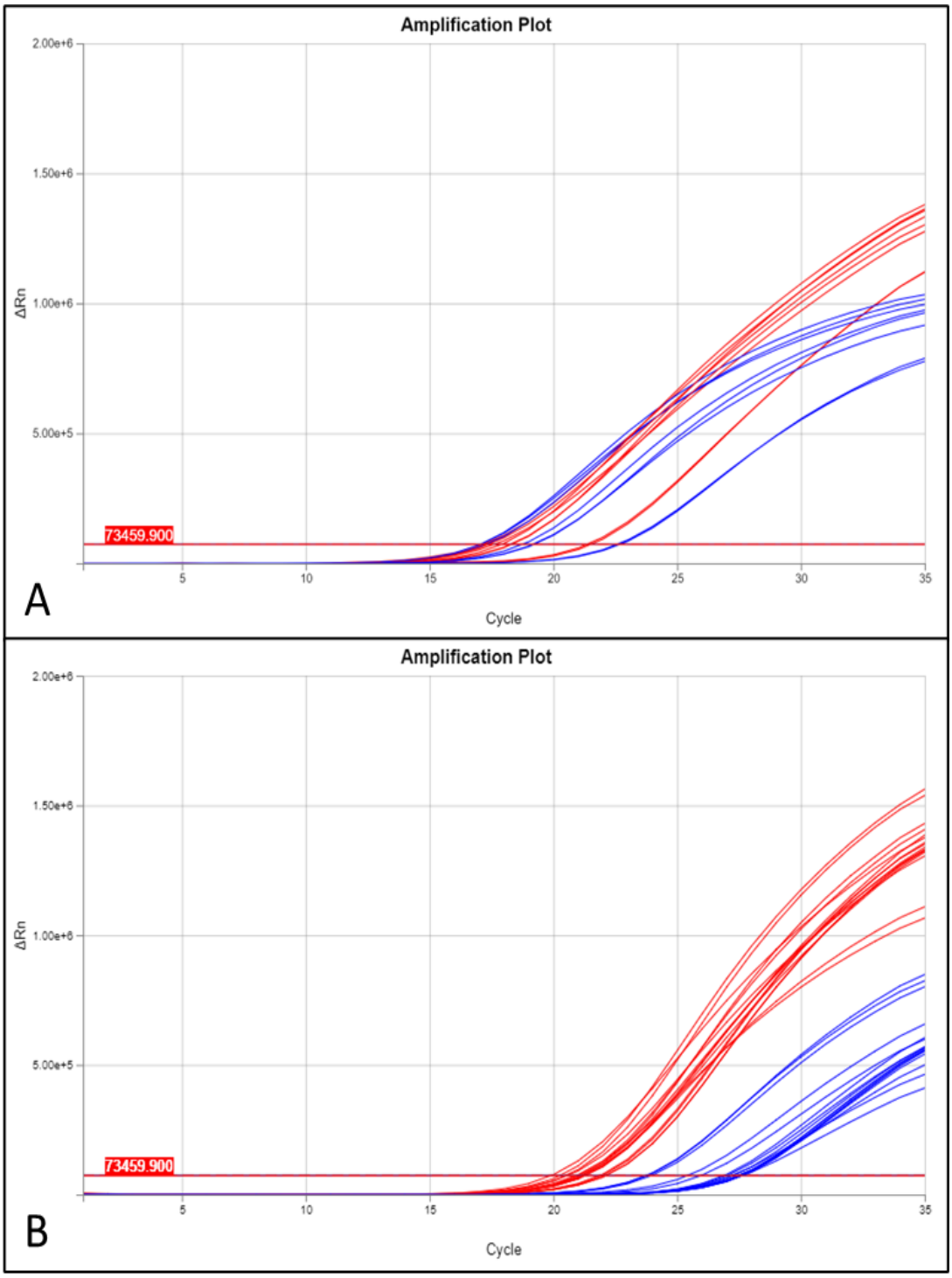


Figure 4. Amplification plot of all Pfizer (A) and Moderna (B) vials showing that spike (red) and ori (blue) amplified similarly for individual vials of Pfizer. In Moderna, inter-vial variability was consistent, but spike amplified earlier than ori (ΔCq~6).

Table 2. Details of the vaccine vials, adverse events (AEs) identified, and qPCR testing results for SARS-CoV-2 spike, ori, and the SV40 promoter-enhancer-ori on all Pfizer-BioNTech and Moderna vials tested. 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 is 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.

Vaccine Information				VAERS Data		Spike			Ori			SV40⌘
Manufacturer	Type	Lot Number *	Printed Expiry Date	Total AES	Total SAEs	Cq	Total ng/dose	Total Copies/dose	Cq	Total ng/dose	Total Copies/dose	Cq
Pfizer-BioNTech	Adult Monovalent	FM7380	02/2022	29	15	18.03	2.43	2.07E+10	18.57	3.92	1.86E+11	17.19
Pfizer-BioNTech	Adult Monovalent	FN7934a	08/2022	42	21	18.47	1.79	1.53E+10	18.77	3.43	1.62E+11	16.64
Pfizer-BioNTech	Adult Monovalent	FN7934b	02/2022			18.19	2.18	1.86E+10	18.44	4.27	3.96E+10	16.96
Pfizer-BioNTech	Adult Monovalent	FX4343a	08/2022	1	0	23.53	0.27	2.30E+09	24.71	0.32	2.94E+09	20.64
Pfizer-BioNTech	Adult Monovalent	FX4343b	07/2022			23.83	0.22	1.86E+09	24.87	0.28	2.64E+09	22.59
Pfizer-BioNTech	Adult Bivalent	GK0932a	09/2022	3	0	20.46	2.25	1.92E+10	21.01	3.81	3.54E+10	18.53
Pfizer-BioNTech	Adult Bivalent	GK0932b	09/2022			20.60	2.05	1.75E+10	21.22	3.32	3.08E+10	18.91
Pfizer-BioNTech	Adult Bivalent	GK0932c	09/2022			20.66	1.97	1.68E+10	21.21	3.33	3.09E+10	18.6
Moderna	Child/Adult Monovalent	020E21A	None Stated	5	1	23.66	0.35	3.02E+09	29.47	0.02	1.87E+08	Neg
Moderna	Child/Adult Monovalent	020J21A	30/032022	7	5	23.21	0.48	4.12E+09	30.10	0.01	1.23E+08	Neg
Moderna	Child/Adult Monovalent	033M21Aa	22/06/2022	2	1	23.04	0.54	4.65E+09	29.46	0.02	1.88E+08	Neg
Moderna	Child/Adult Monovalent	033M21Ab	30/07/2022			22.81	0.64	5.44E+09	29.38	0.02	1.99E+08	Neg
Moderna	Child/Adult Monovalent	033M21Ac	30/03/2022			23.59	0.37	3.18E+09	29.87	0.02	1.43E+08	Neg
Moderna	Child/Adult Monovalent	033M21Ad	30/07/2022			23.26	0.47	3.98E+09	29.39	0.02	1.97E+08	Neg
Moderna	Child/Adult Monovalent	055K21A	30/07/2022	2	2	22.94	0.58	4.98E+09	29.58	0.02	1.74E+08	Neg
Moderna	Child/Adult Monovalent	062H21Aa	30/07/2022	9	3	22.52	0.78	6.69E+09	29.21	0.02	2.23E+08	Neg
Moderna	Child/Adult Monovalent	062H21Ab	28/05/2022			22.76	0.66	5.64E+09	29.37	0.02	2.00E+08	Neg
Moderna	Adult Bivalent BA.4/5	AT0709Ba	30/07/2023	0	0	23.68	0.35	2.99E+09	29.30	0.02	2.09E+08	Neg
Moderna	Adult Bivalent BA.4/5	AT0709Bb	30/07/2023			23.56	0.38	3.24E+09	29.25	0.02	2.16E+08	Neg
Moderna	Adult Bivalent BA.4/5	AT0709Bc	30/07/2023			23.63	0.36	3.09E+09	29.34	0.02	2.04E+08	Neg
Moderna	Adult Bivalent BA.4/5	AT0709Bd	30/07/2023			23.80	0.32	2.74E+09	29.44	0.02	1.91E+08	Neg
Moderna	Child/Adult Bivalent BA.1	AS0467Da	02/04/2023	0	0	23.20	0.49	4.17E+09	25.24	0.34	3.20E+09	Neg
Moderna	Child/Adult Bivalent BA.1	AS0467Db	02/04/2023			24.16	0.25	2.14E+09	26.08	0.20	1.82E+09	Neg
Moderna	Child/Adult Bivalent BA.1	AS0467Dc	02/04/2023			23.75	0.33	2.85E+09	25.74	0.25	2.28E+09	Neg
Moderna	Adult Monovalent XBB.1.5	020G23Aa	29/04/2024	0	0	24.42	0.73	6.26E+09	29.42	0.03	3.18E+08	Neg
Moderna	Adult Monovalent XBB.1.5	020G23Ab	29/04/2024			24.46	0.71	6.11E+09	29.87	0.03	2.33E+08	Neg
Moderna	Adult Monovalent XBB.1.5	020G23Ac	29/04/2024			24.53	0.68	5.84E+09	29.74	0.03	2.55E+08	Neg

*Lower case letters at the end of lot numbers indicate different vials of the same lot. ⌘SV40 promoter-enhancer-*ori*

DNA content in none of the Moderna and three Pfizer lots exceeded 1 ng/dose for either spike or plasmid *ori.* Vaccine in these three vials was diluted 10-fold serially to assess LNP inhibition in qPCR (Figure 5). We observed the expected ~3.3 Cq response after the 1:10 dilution (1:10, 1:100, 1:1000) suggesting that there is some LNP inhibition that could impact the quantitation of DNA at these dilutions (Figure 6). 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.

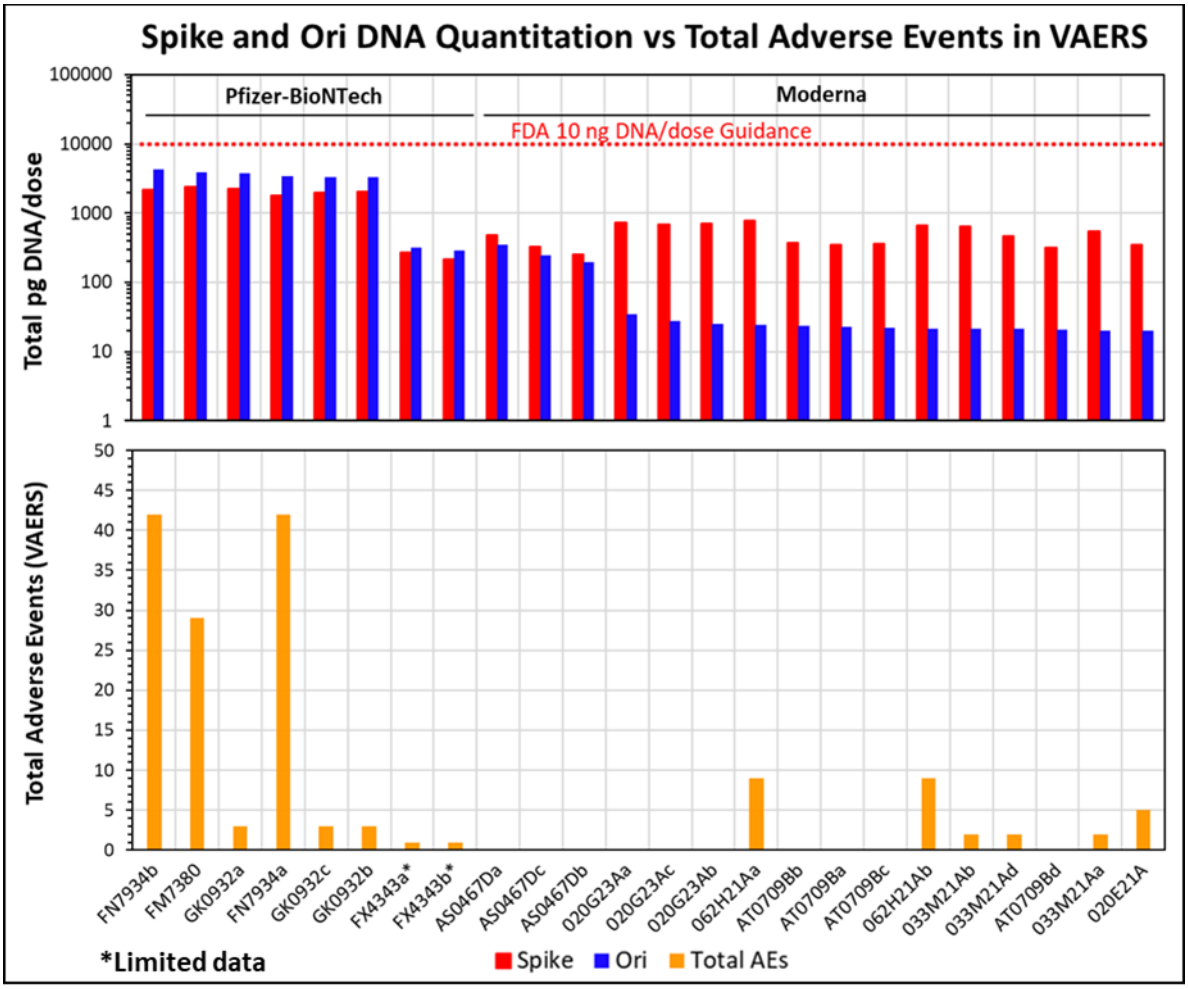
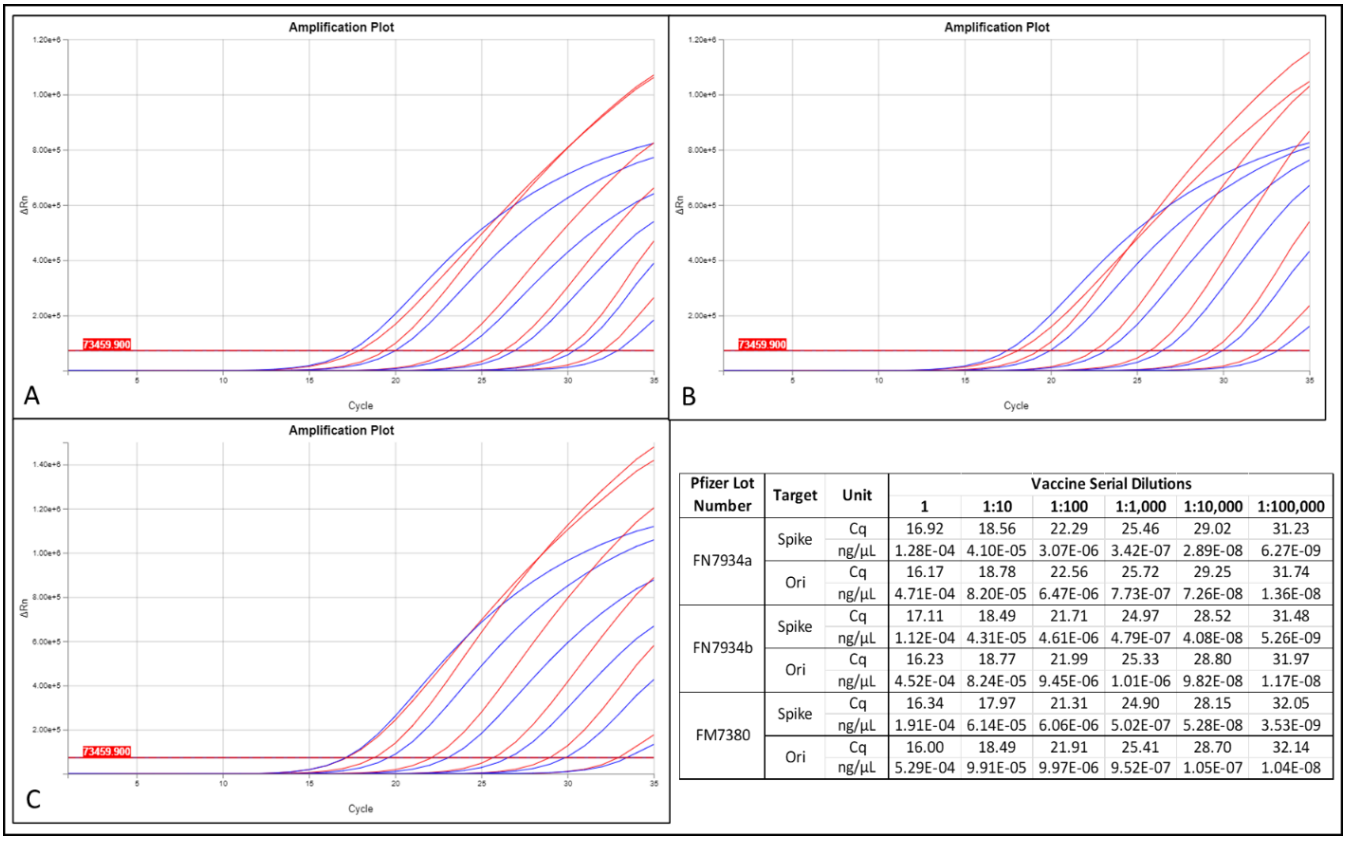


Figure 5. Comparison of residual DNA content of spike (red) and *ori* (blue) and the total number of adverse events (orange) reported to VAERS. The FDA and WHO regulatory guideline of 10 ng/dose[13 14] for residual DNA is shown by a red dotted line. Vials are sorted in descending order by DNA load of plasmid ori. 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.



Pfizer Lot Number	Target	Unit	Vaccine Serial Dilutions					
			1	1:10	1:100	1:1,000	1:10,000	1:100,000
FN7934a	Spike	Cq	16.92	18.56	22.29	25.46	29.02	31.23
		ng/µL	1.28E-04	4.10E-05	3.07E-06	3.42E-07	2.89E-08	6.27E-09
	Ori	Cq	16.17	18.78	22.56	25.72	29.25	31.74
		ng/µL	4.71E-04	8.20E-05	6.47E-06	7.73E-07	7.26E-08	1.36E-08
FN7934b	Spike	Cq	17.11	18.49	21.71	24.97	28.52	31.48
		ng/µL	1.12E-04	4.31E-05	4.61E-06	4.79E-07	4.08E-08	5.26E-09
	Ori	Cq	16.23	18.77	21.99	25.33	28.80	31.97
		ng/µL	4.52E-04	8.24E-05	9.45E-06	1.01E-06	9.82E-08	1.17E-08
FM7380	Spike	Cq	16.34	17.97	21.31	24.90	28.15	32.05
		ng/µL	1.91E-04	6.14E-05	6.06E-06	5.02E-07	5.28E-08	3.53E-09
	Ori	Cq	16.00	18.49	21.91	25.41	28.70	32.14
		ng/µL	5.29E-04	9.91E-05	9.97E-06	9.52E-07	1.05E-07	1.04E-08

Figure 6. qPCR amplification profiles from the serial dilutions (10-fold) of the three lots containing the highest DNA loads (Pfizer lots: A, FN7934a; B, FN7934b; C, FM7380).

The amount of residual DNA varied substantially between lots (0.28 - 4.27 ng/dose for Pfizer *ori*, 0.22 - 2.43 ng/dose for Pfizer spike, 0.01 - 0.34 ng/dose for Moderna *ori*, 0.25-0.78 ng/dose for Moderna spike) when tested by qPCR. Fluorometer based measurements (e.g., Qubit®) of the vaccines show 2,567 ± 618 ng/dose (range: 1,896 to 3,720 ng/dose) for Pfizer and 4,280 ± 593 ng/dose (range: 3,270 to 5,100 ng/dose) for Moderna suggesting a high fraction of the DNA is under the size range of the qPCR amplicons.

We plotted residual DNA values obtained by Qubit fluorometry against those obtained by qPCR (Figure 7). 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 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.

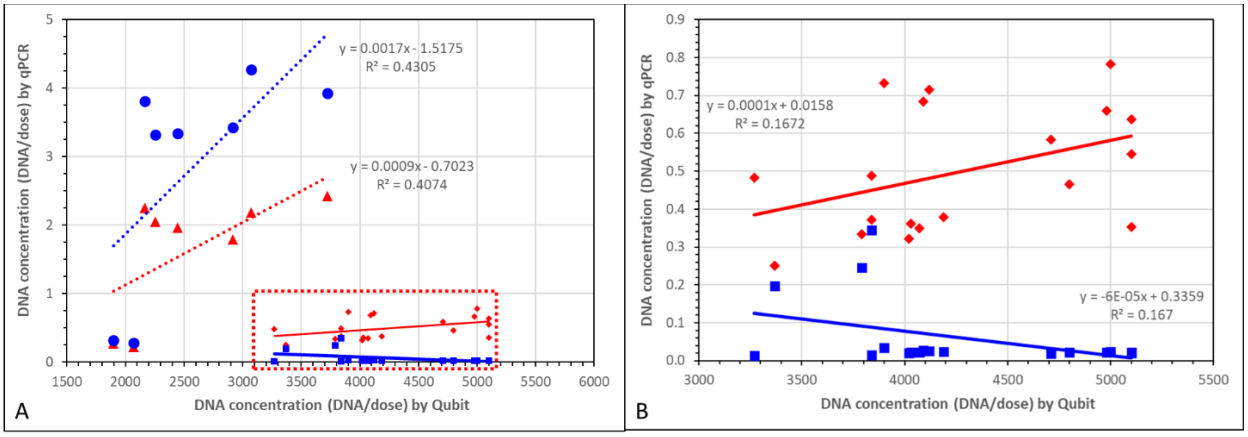


Figure 7. Graphical comparison of residual DNA concentration for spike (red) and ori (blue) determined by qPCR and total residual DNA concentration in individual vials as determined by Qubit. In panel A both Pfizer and Moderna data are plotted on the same scale. The Moderna data are enclosed in a red box and displayed separately with an enlarged scale in panel B, to display detail.

Other than Moderna lots AS0709D, AS0467D and 020G23A, VAERS reports were found for all lots examined in this study (Figure 5). Of the 12 lots examined, the lots with the highest numbers of reports filed to VAERS worldwide were FM7380 and FN7934 with 29 and 42 reports, respectively. In the case of lot FM7380, 15 individuals (52%) reported an SAE, whereas for lot FN7934, 1 individual died, 2 individuals reported a disability, and 18 reported being hospitalized with 21 (50%) SAEs. There were 9, 7, 5, 3, 2, and 2 reports filed for lots 062H21A, 020J21A, 020E21A, GK0932, 033M21A and 055K21A, respectively. Of these lots, 5/7 (71%) reports for Moderna lot 020J21A involved hospitalization, and there were 1/5 reports of death for Moderna lot 020E21A. In total there were 100 reports of AEs filed worldwide to VAERS for these lots; 48 (48%) of these were SAEs. Most of these AE (n=92) and SAE (n=44) reports originated from outside the USA in similar proportion. Of these 92 AEs, 70 (76%) could be identified as originating in Canada, with another 5 (5.4%) whose origin could not be determined.

In an exploratory analysis, we constructed dose-response curves by plotting (Figure 8) the mass of DNA for spike (red) and plasmid *ori* (blue) found in Pfizer (upper panel) and Moderna (lower panel) vials against the SAE reporting ratio (SRR). The *ori* and spike curves for the Pfizer product are similar to each other and show a positive dose-response relationship. The corresponding curves for the Moderna lots are shifted leftwards by one to two orders of magnitude. However, the *ori* and spike curves differ in position and slope.

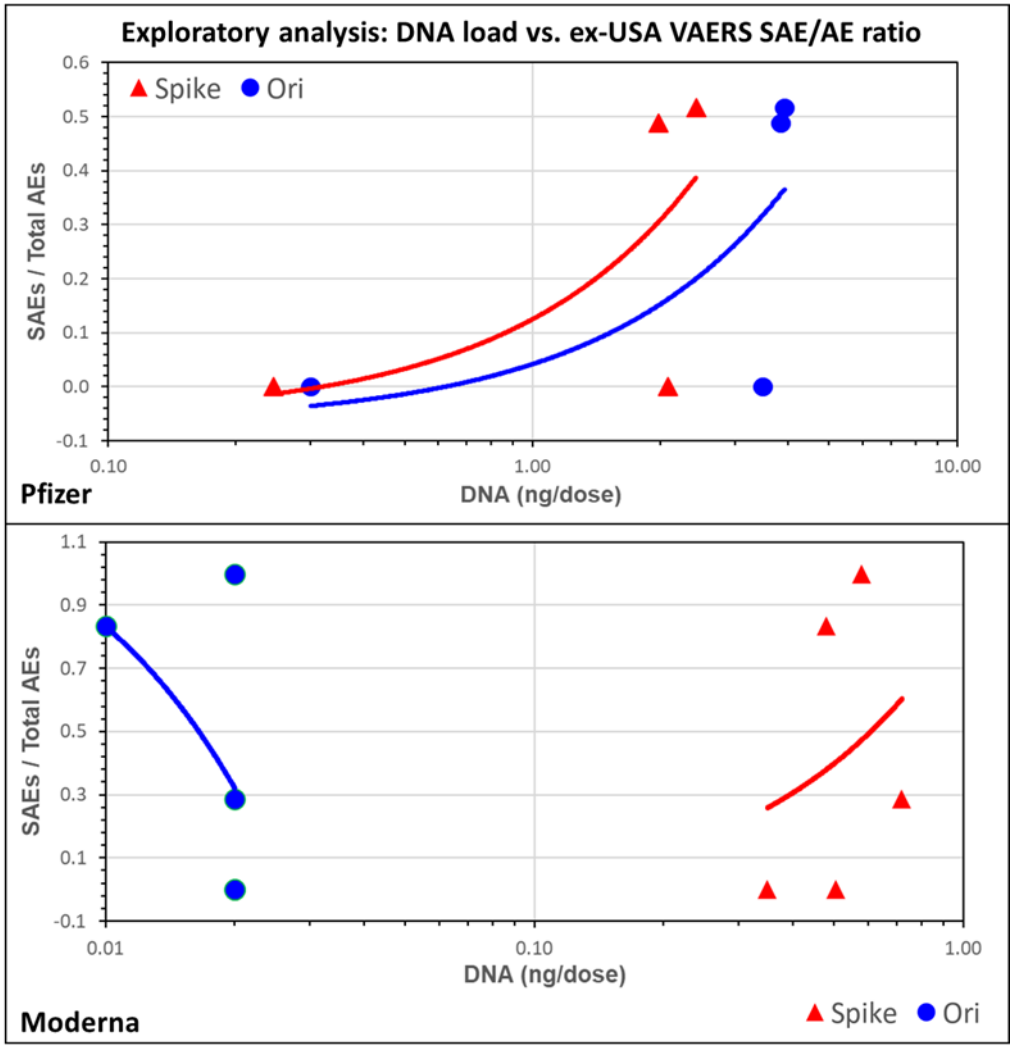


Figure 8. Exploratory dose-response analysis comparing the concentration of residual DNA measured by qPCR for spike (red) and plasmid ori (blue) found in Pfizer (A) and Moderna (B) lots plotted against the SRR (reports of SAEs / total number of all adverse events reported to VAERS) for each lot from countries outside of the USA. Residual DNA mass per dose is plotted on a logarithmic scale. Data from the 1:10 dilution were used.

The corresponding plots for residual DNA estimated using fluorometry (Figure 9) yielded curves with a negative slope for both the Pfizer and Moderna products.

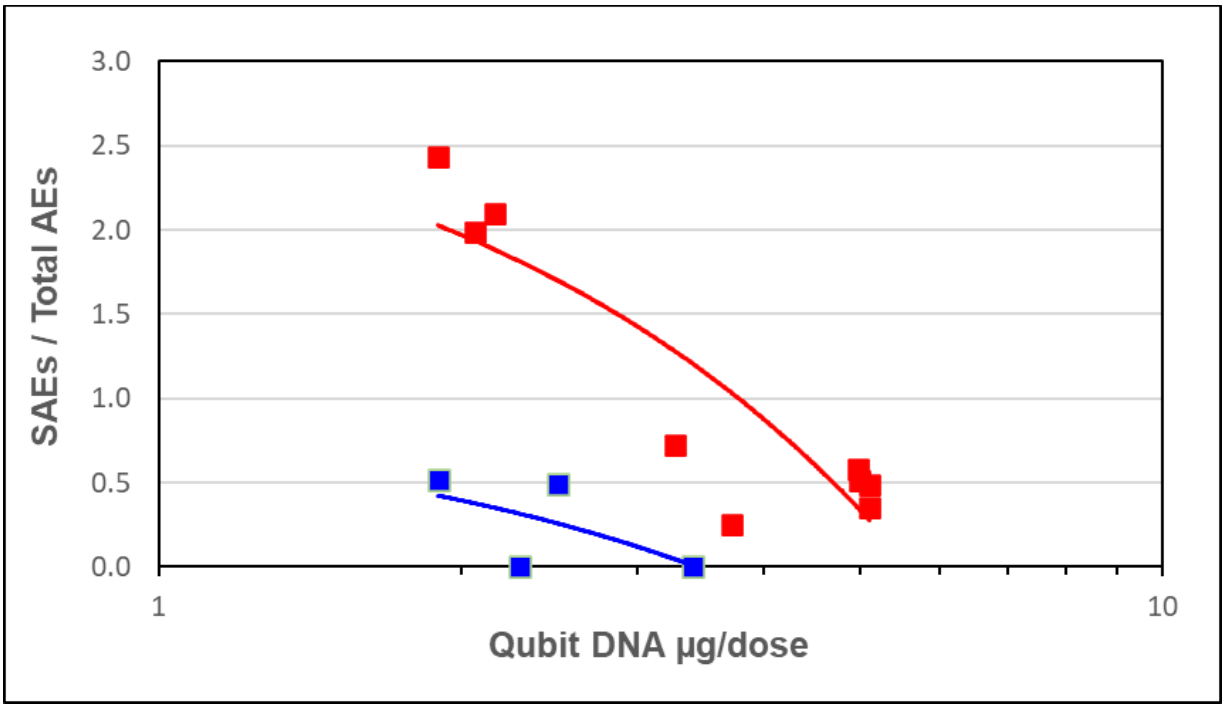


Figure 9. Exploratory dose-response analysis comparing the concentration of residual DNA measured by Qubit fluorometry for Pfizer (blue) and Moderna (red) vaccine lots plotted against the SRR (reports of SAEs / total number of all adverse events reported to VAERS) from countries outside of the USA. Residual DNA mass per dose is plotted on a logarithmic scale.

The Pfizer children's monovalent (Lot FL8095) described by McKernan *et al.*[4] 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 8). The longest read detected in 865 reads was 3.5 kb with read mapping to most of the plasmid backbone (Figure 9).

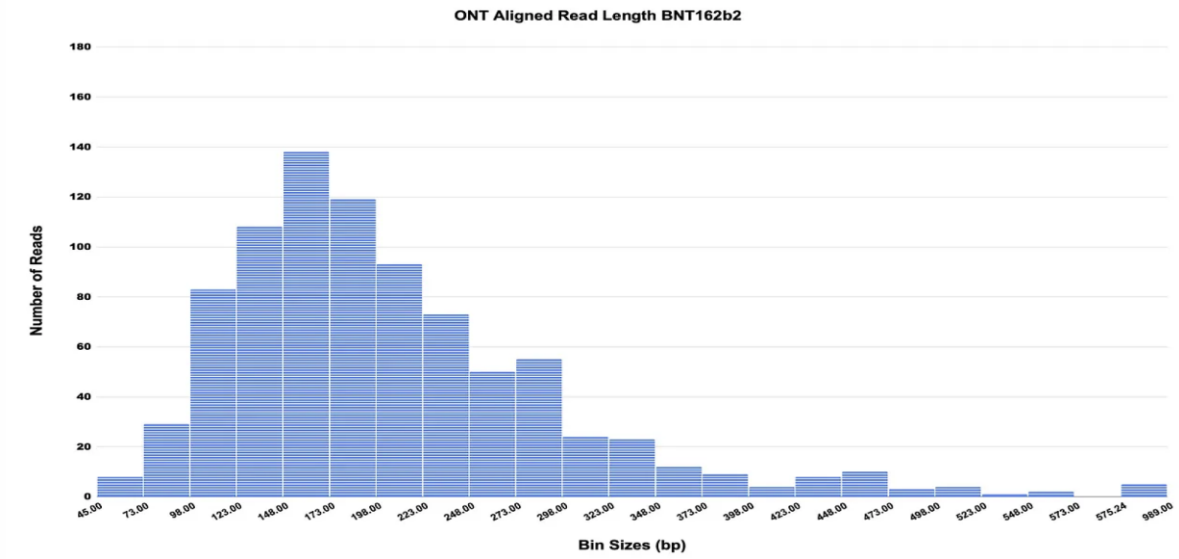


Figure 10. Oxford Nanopore (ONT) read length distributions from 866 reads mapped to the vector sequence (NCBI OR134577.1). Mean = 214 bp. Max = 3.5 kb.

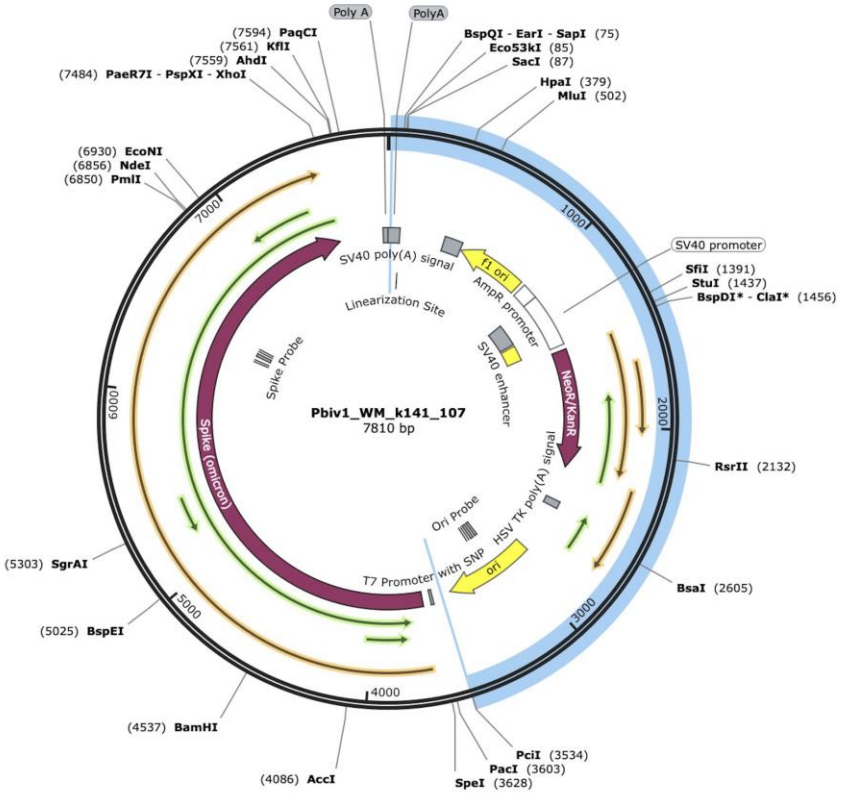


Figure 11. Longest Oxford Nanopore (ONT) read aligns to the vector region shown in blue. ori and spike primer locations are annotated on the innermost circle. Open reading frames (ORFs) are annotated in gold and green arrows. Kanamycin resistance genes were detected in a very shallow sequencing survey of the vaccine.

Nuclease sensitivity of the Pfizer vaccine was assessed using DNaseI-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 (Figure 10, Figure 11).

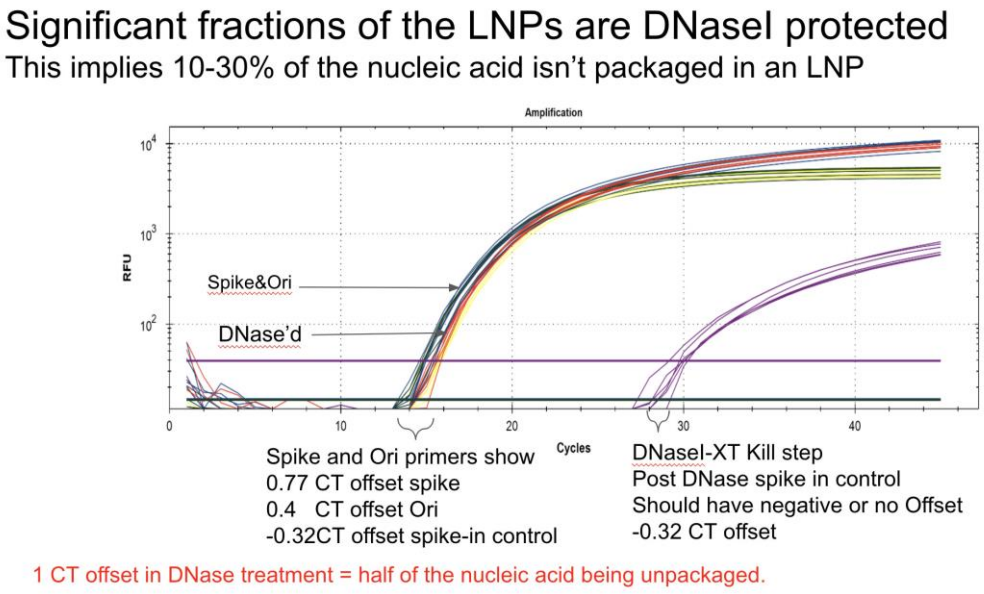


Figure 12. DNase I-XT treatment of Pfizer vaccine demonstrates nuclease resistance of the DNA in the vaccines.

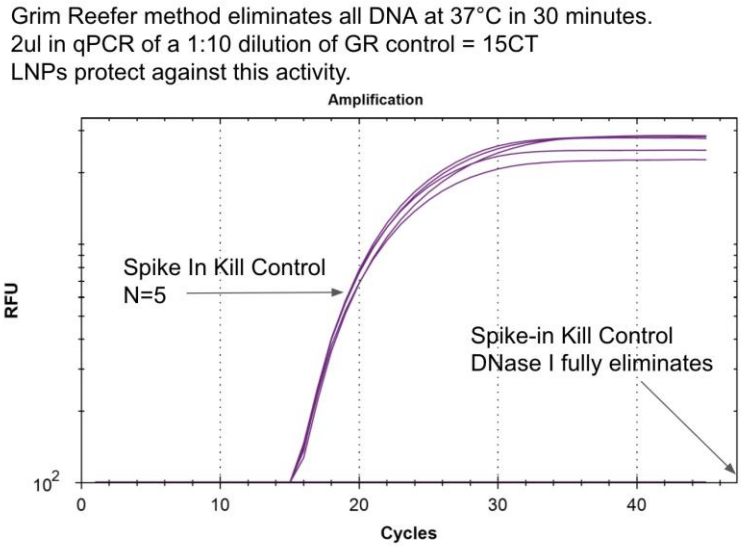


Figure 13. DNaseI-XT positive control demonstrates the digestion assay eliminates all spiked in DNA under the same conditions used to assess the vaccine nuclease sensitivity.

Discussion

Residual DNA was detected in all 27 vaccine vials surveyed. Multiple vials from the same lots produced very similar loads for all targets showing assay reliability, reproducibility, and consistency within the lots. These data involving vaccine vials distributed in Canada are consistent with several non-peer reviewed reports of DNA contamination in modRNA vaccines (McKernan, Buckhaults, Konig).[4 15 16]

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 than spike suggesting a more effective removal of the vector DNA. Possibly, homologous modified RNA may prevent digestion of template DNA by hybridization.[17]

The vials with the highest DNA concentration were from two lots of Pfizer monovalent purple top vials with a phosphate buffered saline (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.[18 19] These purple-topped Pfizer lots were also associated with the highest number of AEs and SAEs reported in VAERS among all the lots tested. As the actual number of doses administered for each lot is unknown, we used the total number of AEs as a proxy for the number of doses administered as a denominator for the number of SAEs to estimate toxicologic/pharmacologic effect. This uses the same principle used by CDC[10 11] in its disproportionality signal analysis (DSA).

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. A positive dose-response relationship was observed for the Pfizer lots based on qPCR estimation of residual DNA.

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 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 a number of toxicological mechanisms have been proposed such as its effects on miRNA processes.[20] dsRNA is another type of impurity that occurs secondarily to the T7 RNA polymerase promoter. dsRNA can induce pro-inflammatory cytokines[21] and has been hypothesized to contribute to immune-inflammatory reactions such as myocarditis.[22] Lipopolysaccharides in cells from endotoxin can bind both the S1 and S2 subunits of the spike protein which may result in enhanced inflammatory responses.[23]

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*.[24] who reported a correlation of AEs to various vaccine lot numbers[24]. 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 localization,[6 25] genomic integration of DNA fragments has yet to be demonstrated for the COVID-19 modRNA products.[26] 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.[27] dsDNA may also be a significant factor in ischemic diseases including stroke.[28] 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 because qPCR cannot quantitate molecules smaller than the size of the amplicon (105-114 bp), qPCR underestimates the total DNA in each vaccine. 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 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 DNA contamination guidelines recommended by the FDA are highly dependent on the methods used to quantitate the DNA. An alternative hypothesis to explain the high fluorometric measurements is the unknown specificity of the DNA-tropic fluorometric dyes when in use with samples that have high concentrations of N1-methyl-pseudouridine modRNA.

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[3], 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 towards fragments >150 bp and are not a perfect reflection of the smaller fragments that may be present and undercounted by both ONT and qPCR.

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.[13] This is

also in keeping with recommendations from the World Health Organization (WHO).[14 29] Previous residual DNA levels were set by the FDA at 10pg/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.[14]

The FDA and WHO guidelines for allowable DNA in vaccines are influenced by work published by FDA scientists Sheng-Fowler *et al.*[30] 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, 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. In some cases, more than five doses of COVID-19 vaccines have been administered with a dose interval for booster doses sometimes as short as 2 months. 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.,*[31] suggests that fragments as small as 7bp can pose integration risks. Furthermore, the guidelines may also have considered that fragments of naked DNA shorter than 200 bp would be more rapidly hydrolyzed by host nucleases activity than larger molecules.[32] This accelerated destruction cannot be assumed of the vaccines due to the DNA being encapsulated and protected by the LNPs.

Klinman *et al.*,[31] 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 and mammalian promoters that exist in the Pfizer vaccine.[26] Vacik *et al*. demonstrated that the SV40 enhancer present in the Pfizer vector is a potent nuclear targeting sequence showing promise for gene therapy.[25]

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, all products tested exceeded the guidelines for residual DNA set by the FDA and WHO of 10 ng/dose by 188 – 509-fold. However, qPCR detected residual DNA content in all products tested were below these guidelines emphasizing the importance of methodological clarity and consistency when interpreting quantitative guidelines. The Cq scores for the most recent XBB.1.5 Moderna vaccine suggest that DNA residues have not been reduced from previous vaccine versions.

The preliminary evidence of a dose-response effect of residual DNA measured with qPCR and SAEs warrants confirmation and further investigation. 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.

Data Availability

Fastq file for the mapped ONT sequencing data:
https://mega.nz/file/UZhkiTBQ#8vjDK5JV5N5Dj2On34B6zdRObEKGBy3ZC7w8q2t9UVc

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Author Contributions

DJ Speicher: sample management, study design, qPCR, data analysis, manuscript preparation
J Rose: VAERS analysis, manuscript preparation
LM Gutschi: data analysis, manuscript preparations
D Wiseman: data analysis, manuscript preparations
K McKernan: qPCR assay design, DNAseI and ONT experiments, manuscript preparation

Conflict of Interest Statement

Kevin McKernan is employed by Medicinal Genomics and provided qPCR reagents free of charge. The other authors declare that there are no conflicts of interest.

Revision History

2023-10-19 – version 1.0

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