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1 # DNA fragments detected in monovalent and bivalent

2 # Pfizer/BioNTech and Moderna modRNA COVID-19 vaccines

3 # from Ontario, Canada: Exploratory dose response

4 # relationship with serious adverse events.

5

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8

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20

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.

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

61   ***Conclusion:*** These data demonstrate the presence of billions to hundreds of billions of
62   DNA molecules per dose in these vaccines. Using fluorometry, all vaccines exceed the
63   guidelines for residual DNA set by FDA and WHO of 10 ng/dose by 188 – 509-fold.
64   However, qPCR residual DNA content in all vaccines were below these guidelines
65   emphasizing the importance of methodological clarity and consistency when interpreting
66   quantitative guidelines. The preliminary evidence of a dose-response effect of residual
67   DNA measured with qPCR and SAEs warrant confirmation and further investigation. Our
68   findings extend existing concerns about vaccine safety and call into question the
69   relevance of guidelines conceived before the introduction of efficient transfection using
70   LNPs. With several obvious limitations, we urge that our work is replicated under forensic
71   conditions and that guidelines be revised to account for highly efficient DNA transfection
72   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])

103 by studying the size distribution of DNA fragments as well as the DNaseI sensitivity of the

104 vaccine to determine whether the residual DNA is packaged in the LNPs.

105

106 For the purpose of this study, we are using the terms "residual DNA," "DNA mass," (or

107 similar) rather than "impurity" or "contamination" as a discussion of these regulatory terms

108 is beyond the scope of this paper.

109

110 **Methods**

111

112 ***COVID-19 Vaccines Tested***

113 Expired unopened vials of Pfizer-BioNTech BNT162b2 (n=8) and Moderna Spikevax

114 mRNA-1273 (n=16) were obtained from various pharmacies in Ontario, Canada (Figure

115 1). Three vials of in-date remnants of the same lot of Moderna XBB.1.5 vaccine were also

116 obtained. In total, 12 lots were surveyed across 27 mRNA vials: 5 lots of Moderna

117 child/adult monovalent, 1 lot of Moderna adult bivalent BA.4/5, 1 lot of Moderna child/adult

118 bivalent Wuhan-BA.1, 1 lot of Moderna XBB.1.5 monovalent, 3 lots of Pfizer adult

119 monovalent, and 1 lot of Pfizer adult bivalent Wuhan-BA.4/5 vaccines. An unopened

120 sterile injectable vial of alprostadil 66 mcg/mL in combination with papaverine 21.7mg/mL

121 and phentolamine 1 mg/mL (TriMix) was used as the negative control. The unopened

122 vials were untampered as they had intact flip-off plastic caps with printed lot numbers and

123 expiration dates. Vials had been stored in a purpose-built vaccine unit at +2-8°C in the

124 pharmacies and were transported in insulated containers with frozen gel packs and

125 placed in the testing laboratory fridge within 5 hours. Only one Moderna vial did not have

126 a printed expiration date but had a QR code that required scanning by a pharmacist. The

127 Moderna XBB.1.5 vials were similarly stored by the pharmacy. Vials were removed from

128 the refrigerator, warmed for ~20 minutes, and administered by the pharmacist to patients

129 over ~30 minutes. The remnant vials were placed in an insulated container with frozen

130 gel packs and transported to the testing laboratory fridge within 12 hours.
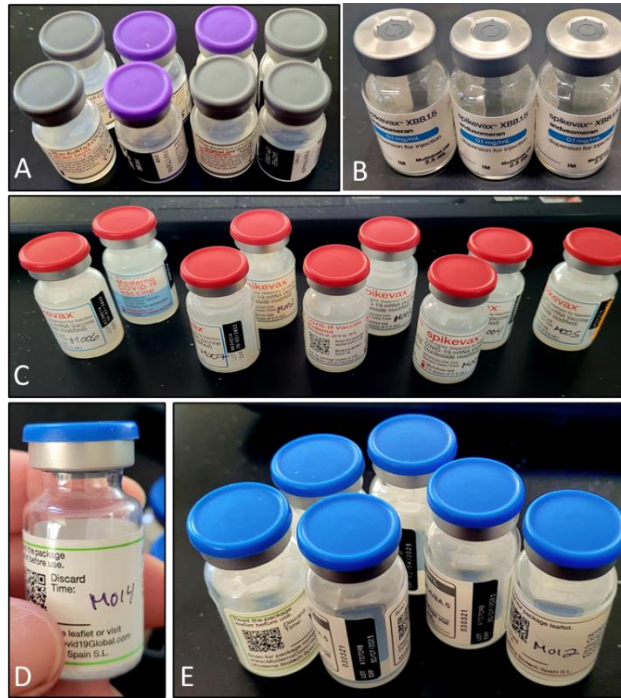
Figure 1. Vials of COVID-19 vaccine from Ontario, Canada: (A) Pfizer/BioNTech BNT162b2 adult monovalent and bivalent; Moderna Spikevax mRNA-1273 (B) adult monovalent XBB.1.5, (C) child/adult monovalent, (D) child/adult bivalent Wuhan-BA.1 and (E) child/adult bivalent Wuhan-BA.1 and adult Wuhan-bivalent 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_2O. 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_2O.

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

155

156 **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/

157

158

159 Cycling was performed on a QuantStudio 3 (ThermoFisher Scientific, Waltham, USA) with
160 an initial denaturation of 95°C for 3 minutes followed by 35 cycles of 95°C for 10 seconds
161 and 65°C for 30 seconds. Cq conversion to ng/µL was calculated using the QuantStudio
162 software v2.7.0 (ThermoFisher Scientific). Amplicon mass, as determined with the New
163 England BioLabs DNA calculator,[7] and length (105 bp for *ori*, 114 bp for spike) were used
164 to estimate the total nanograms (ng) of DNA present by adjusting for the length of the
165 plasmids (7,824bp for Pfizer and 6,777bp for Moderna). Copy number per dose was
166 adjusted for the volume of each intramuscular vaccine injection (300 µL for Pfizer and
167 500 µL for Moderna). Serial dilutions were performed on the three Pfizer lots that showed
168 the highest residual DNA concentration. to investigate PCR inhibition by the LNPs since
169 qPCR was performed directly without any treatment or extraction.

170

171 ***Qubit® fluorometry quantitation***

172 AccuGreen® HS fluorometric reagents (AccuGreen #99820 and DNA Quantification
173 Buffer #99979) and standards were acquired from Biotium (San Francisco, USA) for
174 Qubit® analysis (ThermoFisher Scientific). Fluorometric reagents (190 µL of a stock made
175 from 995 µL HS Buffer and 5 µL 200X AccuGreen dye) were vortexed with 10 µL of
176 vaccine. These samples were heated to 95°C for 8 minutes and 4°C for 5 minutes to

177 disrupt the LNPs and enable Fluorometric Dyes to access the DNA. Samples were read

178 following the manufacturer's instructions on a Qubit 3.0 Fluorometer. Qubit fluorometry

179 and qPCR data were compared.

180

181 ***Vaccine Adverse Event Reporting System (VAERS) Data***

182 The VAERS database was analyzed using the Language and Environment for Statistical

183 Computing package in R,[8] and included data spanning December 17, 2020 through

184 October 6, 2023 The VAERS data is available for download in three separate comma

185 separated values (csv) data files representing: i) general data for each report; ii) the

186 reported AEs or 'symptoms', and iii) vaccine data including vaccine manufacturer and lot

187 number.[5] A VAERS ID number is assigned to preserve confidentiality when a report is

188 filed. To assess the AEs related to a particular vaccine, it is necessary to merge the three

189 data files using the VAERS IDs as a linking variable. For this study, since we are

190 interested in the COVID-19 products, only COVID-19 vaccine type (COVID19-1

191 (monovalent) and COVID19-2 (bivalent)) were included. Other relevant variables included

192 VAERS ID*, vaccine lot (VAX_LOT), vaccine manufacturer (VAX_MANU),

193 hospitalizations (HOSPITAL) and deaths (DIED). Data were grouped by vaccine lot and

194 the total number of AE and SAE reports were counted. SAE reports included deaths,

195 hospitalizations, emergency room visits, disability reports, birth defects and life-

196 threatening reports, and individual MedDRA coded AEs, such as total deaths per lot, were

197 also counted.

198

199 The various limitations of VAERS are widely acknowledged, for example by FDA[9], and

200 include underreporting, misreporting, spontaneous reporting, and the inability to infer

201 causality. Nevertheless, to explore a possible dose-response relationship between

202 residual DNA content and SAEs, we used the ratio of the number of SAE reports to the

203 total number of AEs ("SAE reporting ratio" = SRR) as a proxy for a possible toxicological

204 effect. We used the total number of AEs reported by lot as a proxy for the total number of

205 doses administered, since this denominator is difficult to estimate. This principle is used

206 by the CDC in disproportionality signal analysis (DSA) to identify safety signals using the

207   Proportional Reporting Ratio (PRR)[10] The PRR, as devised by Evans *et al.*, is a useful

208   tool in pharmacovigilance with known limitations.[11]

209

210   It must be noted that although VAERS is a USA-based database, it accepts reports from

211   around the world. Certain categories of AEs that are reported to manufacturers outside

212   the USA, must be reported to the VAERS database. Differences in propensity for

213   underreporting as well as mandatory reporting imposed on manufacturers or medical

214   professionals within and outside the USA may introduce confounding to the estimation of

215   the SRR. Accordingly, for our exploratory dose-response analysis we only used VAERS

216   data originating outside the USA to reduce this confounding. Additionally, we have noted

217   some discrepancies in data obtained through the downloaded version of the VAERS

218   dataset, and those obtained using the VAERS WONDER front-end web-based interface

219   (https://wonder.cdc.gov/controller/datarequest/D8). We used the downloaded version as

220   it provides greater detail than the web version. The SRR was then plotted against levels

221   of DNA found in the vials to identify any association between residual DNA levels and the

222   frequency of reports of serious adverse events.

223

224   Where more than one vial was available in any lot, the average mass of residual DNA per

225   dose for that lot was used. Zero values of SRR for any given lot were only plotted if one

226   or more AEs had been identified worldwide, signifying that that lot had actually been

227   deployed. The curves were plotted on a logarithmic axis and a trend line drawn using the

228   linear function within Microsoft® Excel.

229

230   ***Oxford Nanopore Sequencing***

231   In a separate experiment using previously sequenced vaccine[4] (Pfizer children's

232   monovalent Lot# FL8095), DNA fragment size distributions were estimated using an

233   Oxford Nanopore Flongle (R.10.4.1, Oxford Nanopore Technologies (ONT), New York,

234   USA) and the Oxford Nanopore Ligation sequencing kit (SQK-LSK114) according to the

235   manufacturer's instructions. Reads were mapped to NCBI OR134577.1 with the Burrow-

236   Wheeler Aligner with maximum exact matches (BWA-MEM).[12] ONT sequencing read

237 length is unlimited, but the DNA isolation procedures can bias the length of the molecules

238 captured in the ONT ligation reaction. Single molecule reads were counted and binned

239 according to their mapped read length with BWA-MEM.

240

241 ***Nuclease sensitivity of the vaccines***

242 The same vial (Pfizer Lot# FL8095) was used to assess DNaseI sensitivity of the vaccine

243 by determining if the DNA contamination is packaged in the LNP and thus resistant to

244 DNaseI or if the DNA resides outside of the LNP and is DNaseI labile.

245

246 Nuclease protected DNA was estimated by treating 20 µL of the vaccine with 2.5 µL of

247 DNaseI-XT (2 units/µL, NEB#M0570S, New England BioLabs Inc, Ipswich, USA), 2.5 µL

248 of Grim Reefer 10X buffer (Medicinal Genomics #420123-125) and incubating at 37°C for

249 30 minutes. For the control, 2.5 µL of ddH$_2$0 was used instead of the DNaseI-XT. The

250 DNaseI-XT reaction was chemically arrested using 2.5 µL of MGC lysis buffer (Medicinal

251 Genomics #420001). After the DNaseI chemical kill step, a qPCR amplifiable internal

252 control DNA was spiked-in to verify that the DNaseI-XT had been fully inactivated
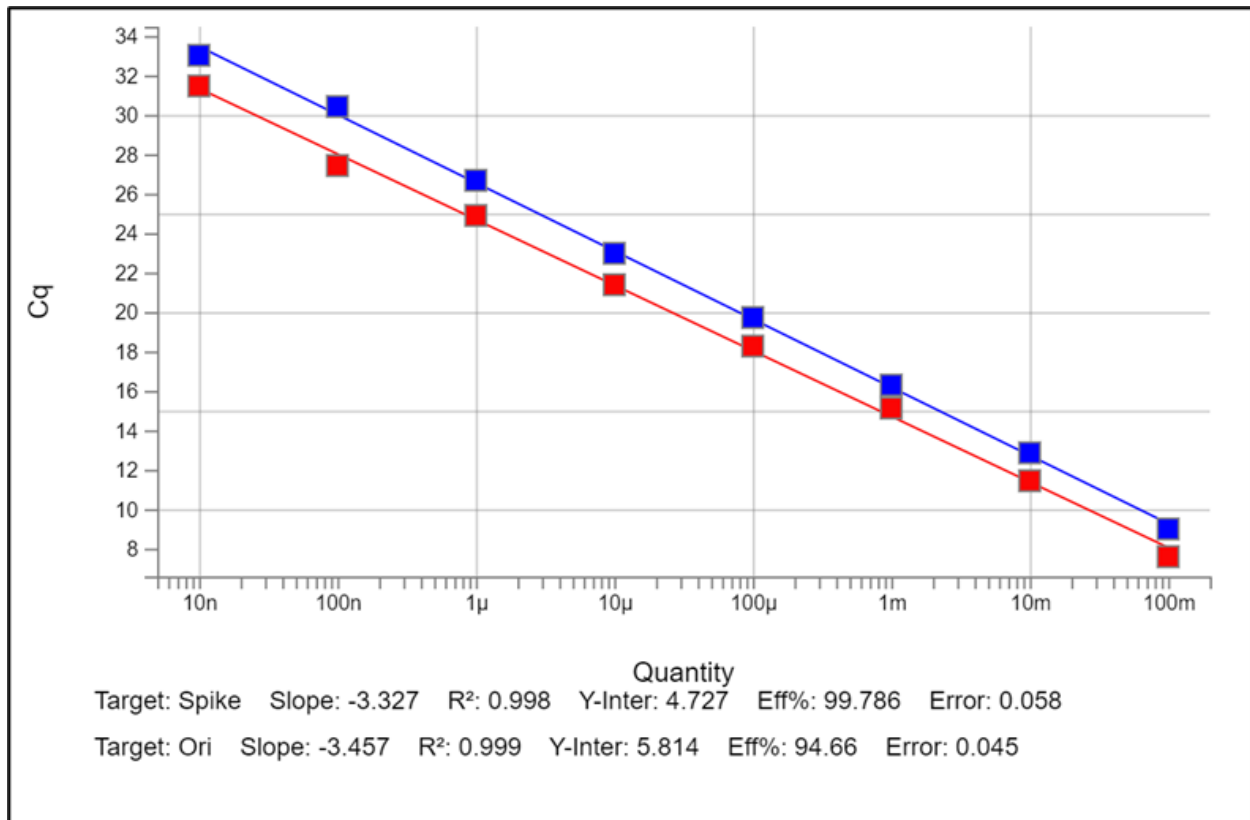
253 (Medicinal Genomics #420123-125).

254

255 After spiking in the DNaseI inactivation control, 54 µL of SenSATIVAx magnetic beads

256 (Medicinal Genomics) were used to purify DNA from the DNaseI-XT assay and the

257 DNaseI-XT negative control samples. The magnetic beads were pipette mixed 10 times

258 with the sample, incubated at room temperature for 5 minutes, magnetically separated

259 and washed twice with 70% v/v ethanol. The ethanol was removed, and the beads dried

260 for 2 minutes at room temperature. Samples were eluted in 30 µL of ddH$_2$0 and 1 µL of

261 eluate was examined by qPCR for spike and *ori* in an 18.8 µL reaction. An additional

262 DNaseI inactivation control primer and probe (0.5 µL in CY5) were added to the assay for

263 a total of 19.3 µL reaction.

264

265 # Results

266 An 8-log serial dilution standard curve was used to calibrate sample Cq values and

267 generated R^2 values of 0.998 and 0.999 for spike and *ori* amplicons, respectively. PCR

268 efficiency was 99.8% and 94.7% for spike and *ori,* respectively (Figure 2). On all plates,

269 negative controls and no template (ddH$_2$0) controls (NTC) were tested in triplicate and

270 found to be negative.

271

Target: Spike   Slope: -3.327   R²: 0.998   Y-Inter: 4.727   Eff%: 99.786   Error: 0.058

Target: Ori   Slope: -3.457   R²: 0.999   Y-Inter: 5.814   Eff%: 94.66   Error: 0.045

272

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

275 For individual vials, qPCR on Pfizer amplified at a similar time for spike, *ori*, and SV40

276 enhancer-promoter-*ori* ($_\Delta$Cq 1.48 ± 0.32) (Figure 3). Apart from Pfizer lot: FX4343, the

277 inter vial difference was small for both Pfizer (spike Cq 16.91 ± 0.52; *ori* Cq 16.91 ± 1.07;

278 SV40 promoter-enhancer-*ori* Cq 15.46 ± 2.02) and Moderna (spike Cq 20.35 ± 0.65; *ori*

279 Cq 25.34 ± 1.47) (values were based on the undiluted vials contents) (Table 2, Figure 4)

280

281 However, for all Moderna vials, except lot AS0467D, *ori* consistently amplified Cq 5-6

282 later than spike. The SV40 promoter-enhancer-*ori* was detected in all Pfizer vials but in

283 none of the Moderna vials.
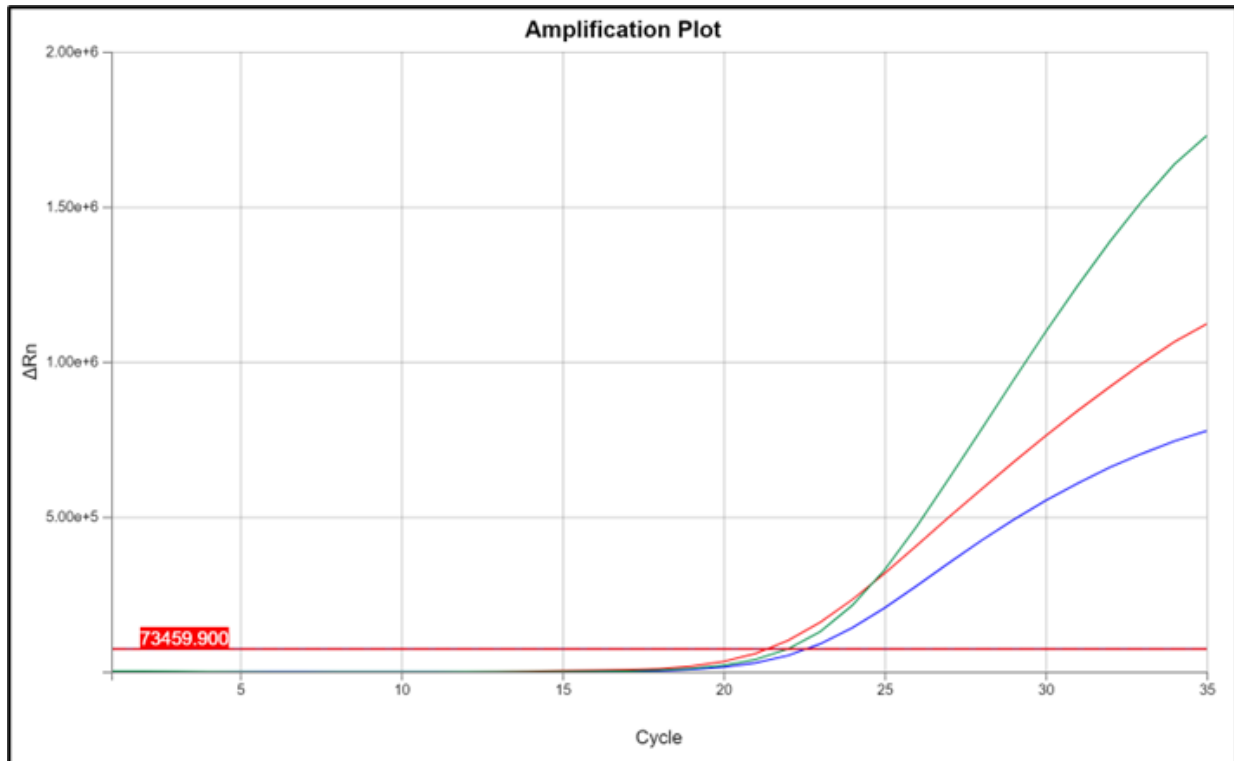
Figure 3. The amplification curve for spike (red), ori (blue), and SV40 enhancer-promoter-ori (green) in a single vial of Pfizer (Lot: Fx4343a) from two different wells of the same PCR run.
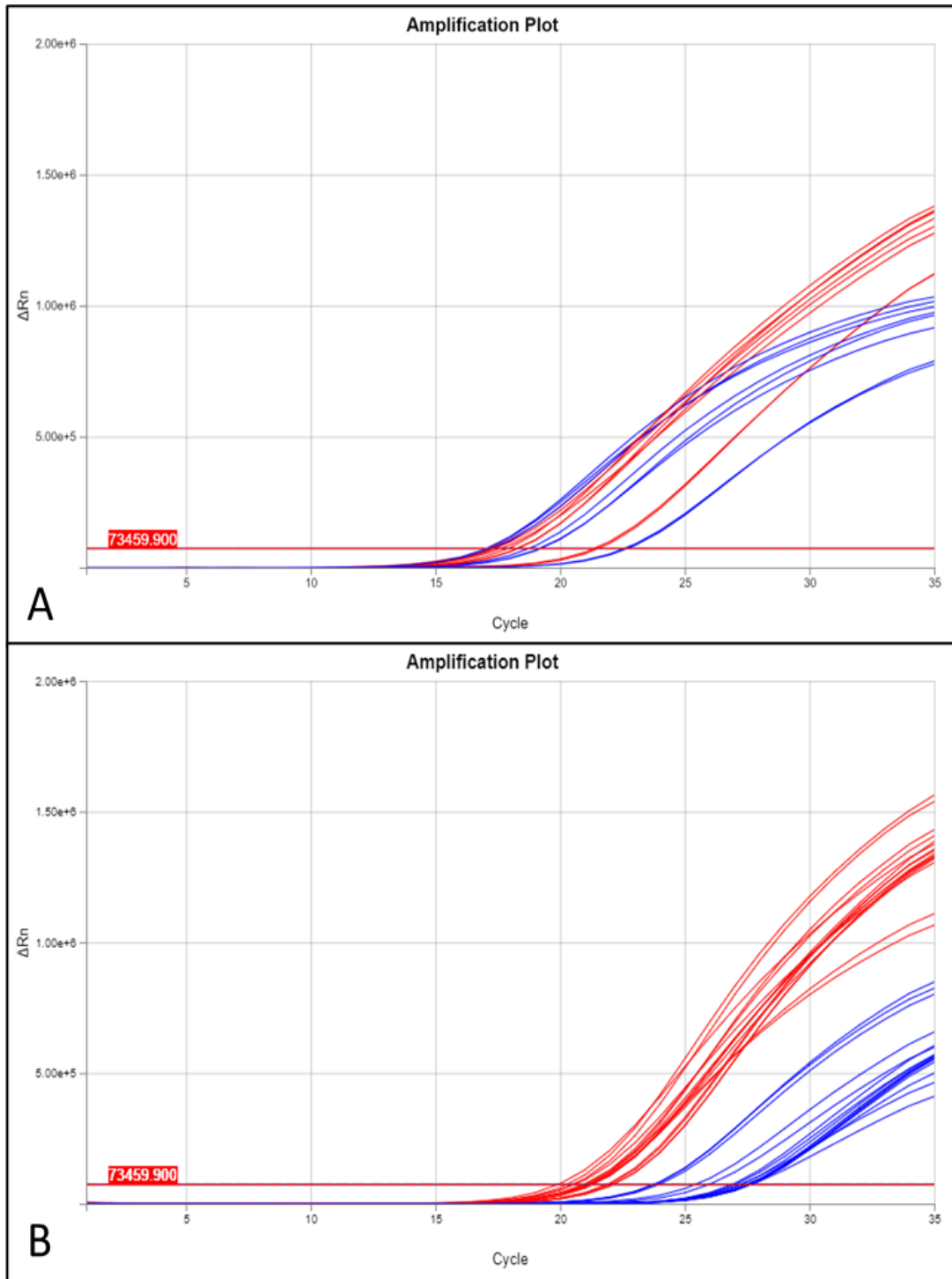
Figure 4. Amplification plot of all Pfizer (A) and Moderna (B) vials showing that spike (red) and ori (blue) amplified similarly for individual vials of Pfizer. In Moderna, inter-vial variability was consistent, but spike amplified earlier than ori (ΔCq~6).

292 **Table 2.** Details of the vaccine vials, adverse events (AEs) identified, and qPCR testing results for SARS-CoV-2 spike, ori,
293 and the SV40 promoter-enhancer-ori on all Pfizer-BioNTech and Moderna vials tested. Calculations for Pfizer and Moderna
294 were based on adult doses of 0.30 mL and 0.50 mL, respectively. Moderna is also indicated to be given to children aged
295 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
296 adjusted for the length of the amplicon (105 bp ori, 114 bp spike) only representing a fraction of the 7,824 bp Pfizer and
297 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

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

299 DNA content in none of the Moderna and three Pfizer lots exceeded 1 ng/dose for either
300 spike or plasmid *ori*. Vaccine in these three vials was diluted 10-fold serially to assess
301 LNP inhibition in qPCR (Figure 5). We observed the expected ~3.3 Cq response after
302 the 1:10 dilution (1:10, 1:100, 1:1000) suggesting that there is some LNP inhibition that
303 could impact the quantitation of DNA at these dilutions (Figure 6). Therefore, the data
304 from the 1:10 dilutions were used for further analysis. This dilution, as well as the fact
305 that some of the doses were designed to be diluted before use, was accounted for in our
306 calculations.

307
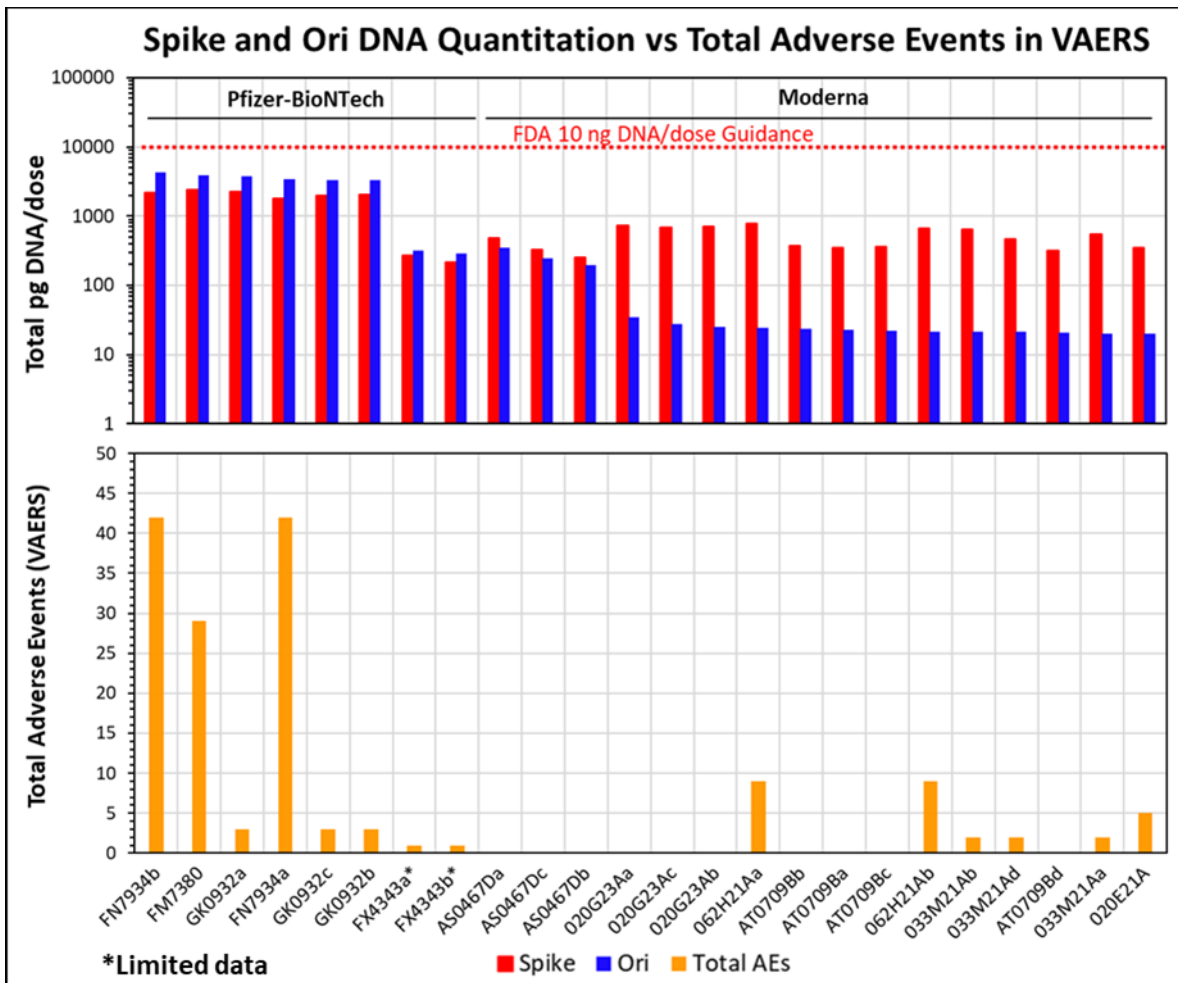
Figure 5. Comparison of residual DNA content of spike (red) and *ori* (blue) and the total
number of adverse events (orange) reported to VAERS. The FDA and WHO regulatory
guideline of 10 ng/dose[13 14] for residual DNA is shown by a red dotted line. Vials are
sorted in descending order by DNA load of 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.
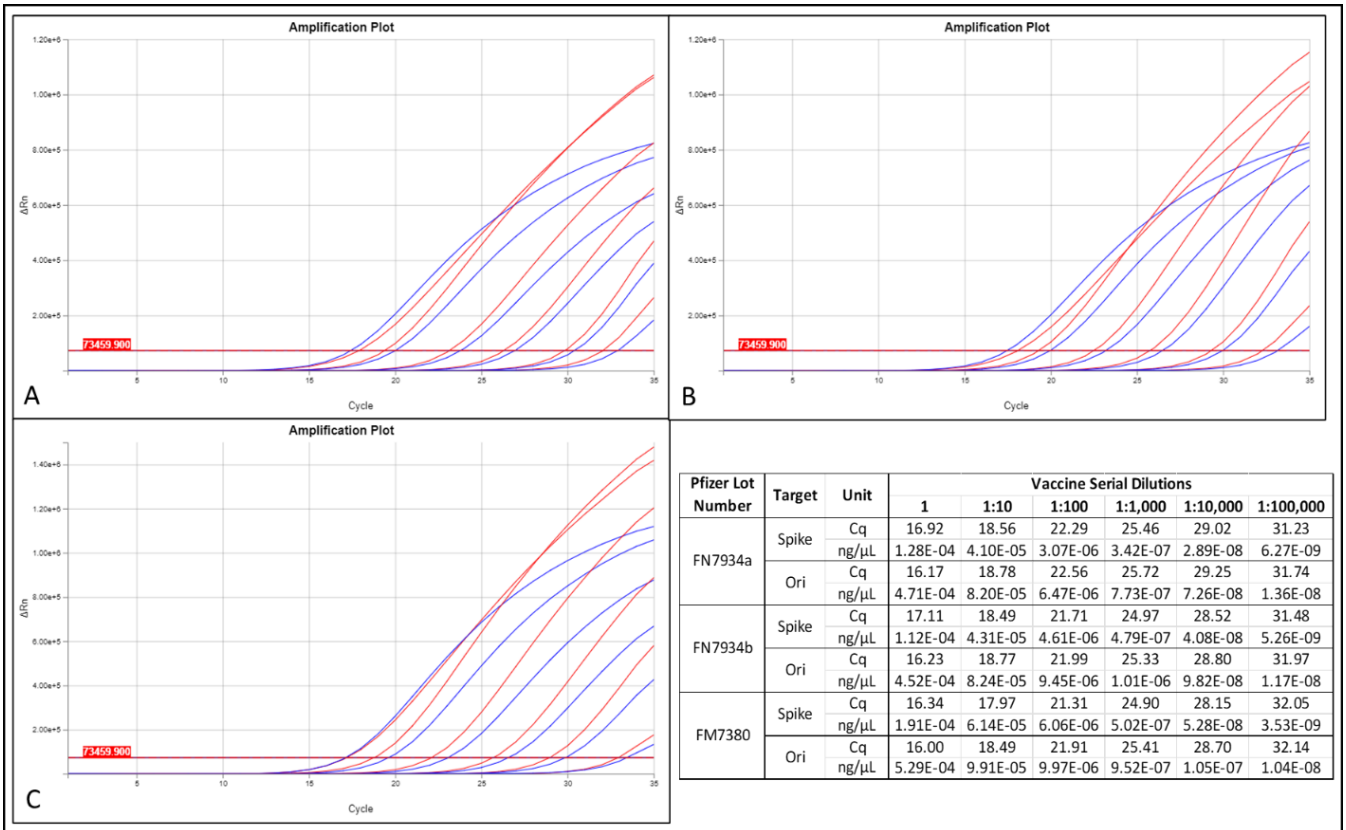
Figure 6. qPCR amplification profiles from the serial dilutions (10-fold) of the three lots containing the highest DNA loads (Pfizer lots: A, FN7934a; B, FN7934b; C, FM7380).

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*

331 values, this trendline may be influenced by three outlying values. These values were
332 obtained from vials of the Moderna BA.1-Wuhan bivalent vaccines.

333
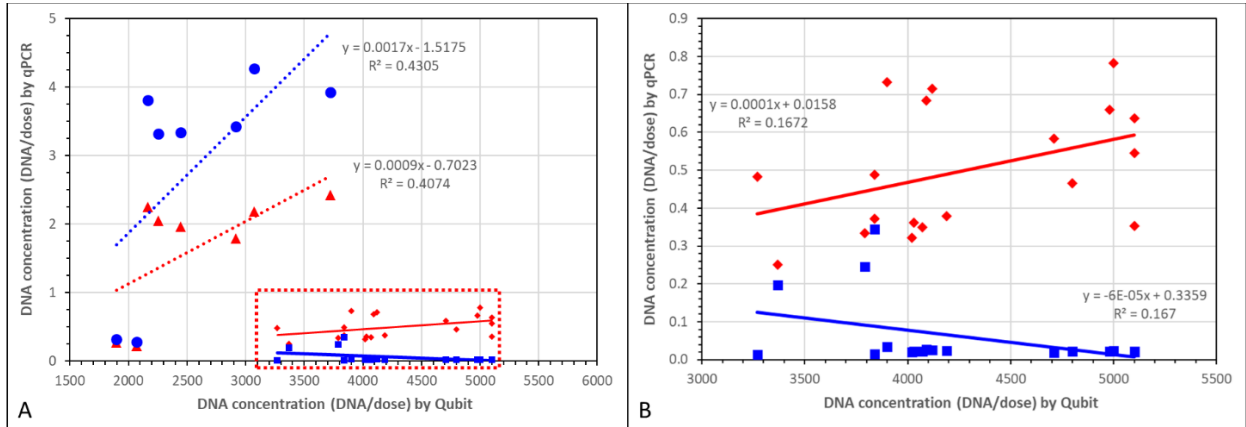
334

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

340

341 Other than Moderna lots AS0709D, AS0467D and 020G23A, VAERS reports were found
342 for all lots examined in this study (Figure 5). Of the 12 lots examined, the lots with the
343 highest numbers of reports filed to VAERS worldwide were FM7380 and FN7934 with
344 29 and 42 reports, respectively. In the case of lot FM7380, 15 individuals (52%) reported
345 an SAE, whereas for lot FN7934, 1 individual died, 2 individuals reported a disability,
346 and 18 reported being hospitalized with 21 (50%) SAEs. There were 9, 7, 5, 3, 2, and 2
347 reports filed for lots 062H21A, 020J21A, 020E21A, GK0932, 033M21A and 055K21A,
348 respectively. Of these lots, 5/7 (71%) reports for Moderna lot 020J21A involved
349 hospitalization, and there were 1/5 reports of death for Moderna lot 020E21A. In total
350 there were 100 reports of AEs filed worldwide to VAERS for these lots; 48 (48%) of these
351 were SAEs. Most of these AE (n=92) and SAE (n=44) reports originated from outside
352 the USA in similar proportion. Of these 92 AEs, 70 (76%) could be identified as
353 originating in Canada, with another 5 (5.4%) whose origin could not be determined.

354

355 In an exploratory analysis, we constructed dose-response curves by plotting (Figure 8)
356 the mass of DNA for spike (red) and plasmid *ori* (blue) found in Pfizer (upper panel) and
357 Moderna (lower panel) vials against the SAE reporting ratio (SRR). The *ori* and spike
358 curves for the Pfizer product are similar to each other and show a positive dose-response
359 relationship. The corresponding curves for the Moderna lots are shifted leftwards by one
360 to two orders of magnitude. However, the *ori* and spike curves differ in position and
361 slope.
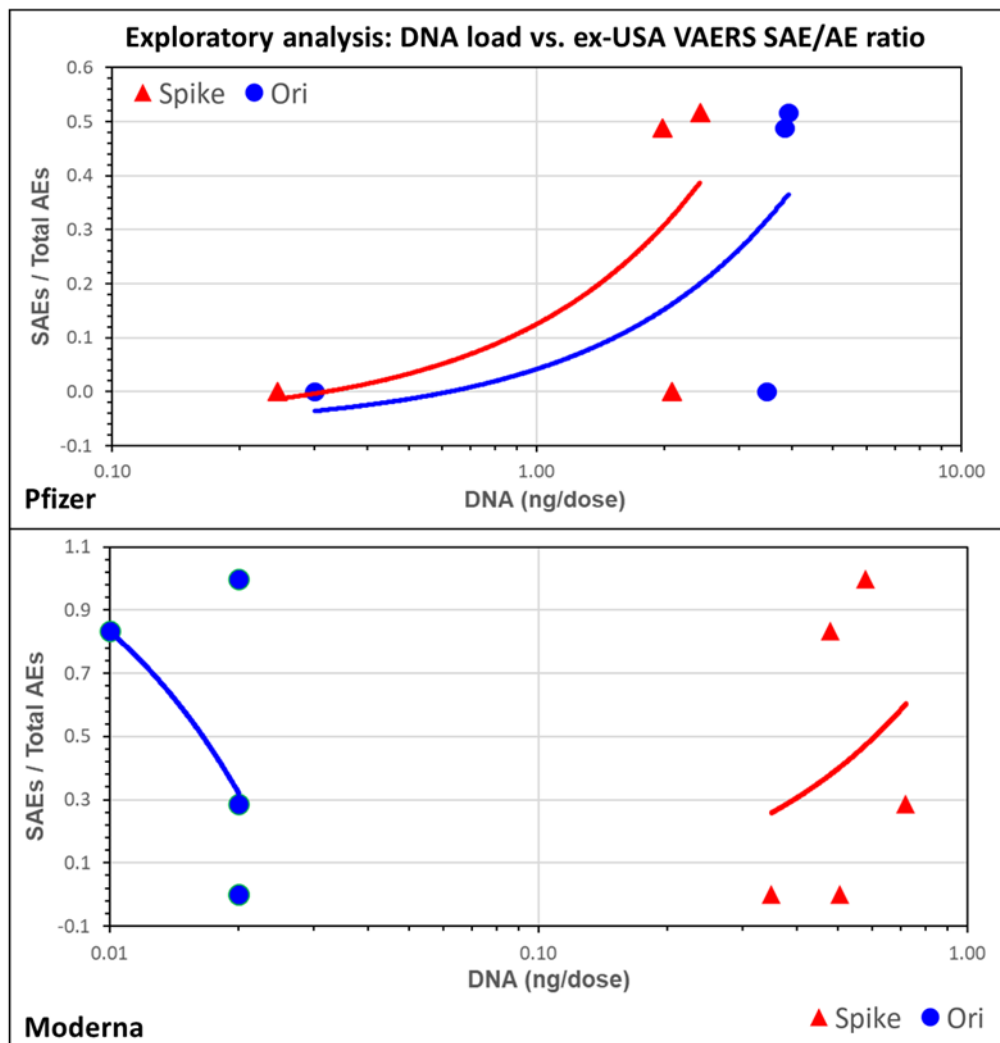
362

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

369 The corresponding plots for residual DNA estimated using fluorometry (Figure 9) yielded

370 curves with a negative slope for both the Pfizer and Moderna products.

371

372
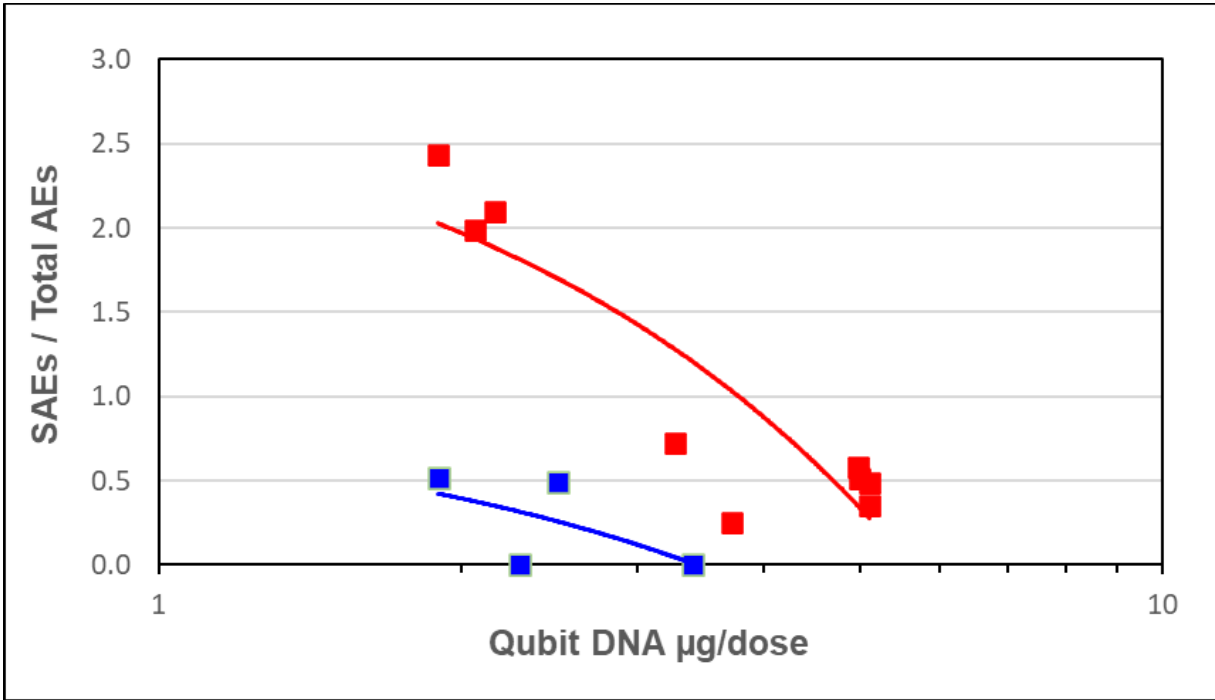
Figure 9. Exploratory dose-response analysis comparing the concentration of residual
DNA measured by Qubit fluorometry for Pfizer (blue) and Moderna (red) vaccine lots
plotted against the SRR (reports of SAEs / total number of all adverse events reported
to VAERS) from countries outside of the USA. Residual DNA mass per dose is plotted
on a logarithmic scale.

378

379 The Pfizer children's monovalent (Lot FL8095) described by McKernan *et al.*[4] was

380 sequenced with Oxford Nanopore (ONT) to assess the read length distributions after

381 mapping the reads to the reference sequence of the plasmid in NCBI (Figure 8). The

382 longest read detected in 865 reads was 3.5 kb with read mapping to most of the plasmid

383 backbone (Figure 9).

ONT Aligned Read Length BNT162b2
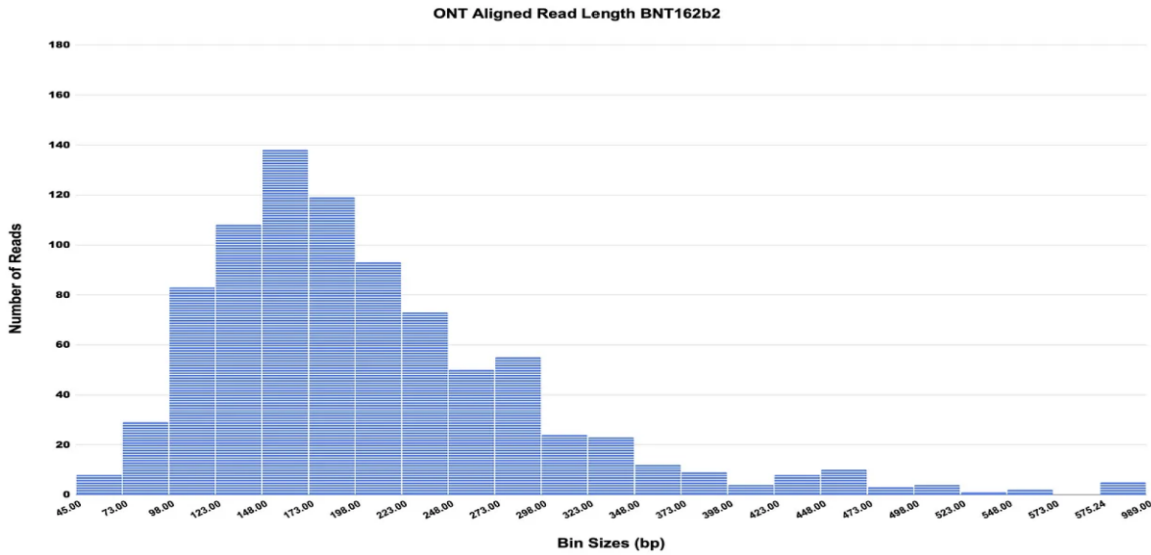
384

385 **Figure 10.** Oxford Nanopore (ONT) read length distributions from 866 reads mapped to
386 the vector sequence (NCBI OR134577.1). Mean = 214 bp. Max = 3.5 kb.
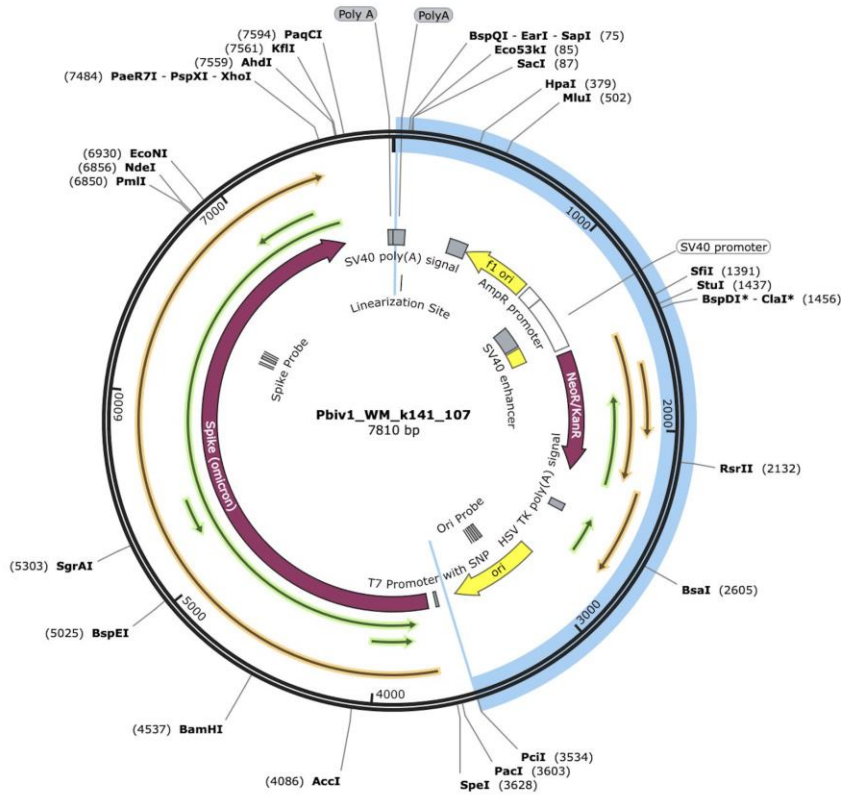
387

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

392 Nuclease sensitivity of the Pfizer vaccine was assessed using DNaseI-XT. This DNA

393 nuclease is optimized for IVT reactions rich in RNA:DNA hybrids. This treatment showed

394 $\leq$1 Cq offset while a naked DNA control spiked into LNPs was reduced from a Cq of 15

395 to undetectable under the same conditions. This indicates that the DNA present in the

396 vaccines is protected by encapsulation in the LNPs (Figure 10, Figure 11).

397
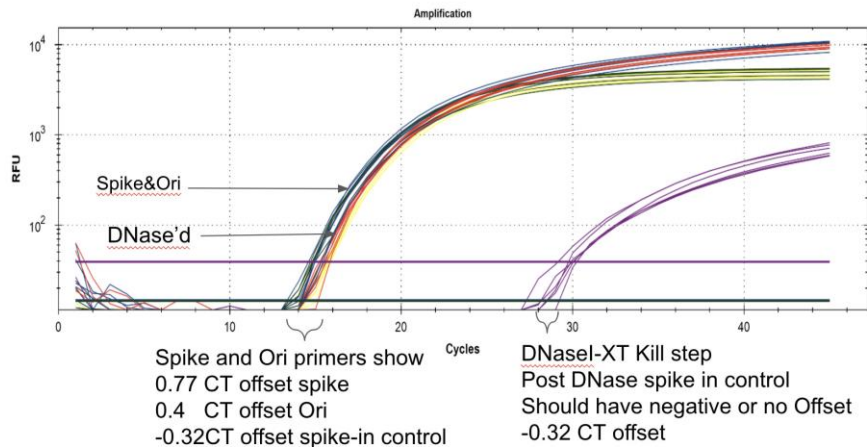
398 **Figure 12.** DNase I-XT treatment of Pfizer vaccine demonstrates nuclease resistance
399 of the DNA in the vaccines.

400
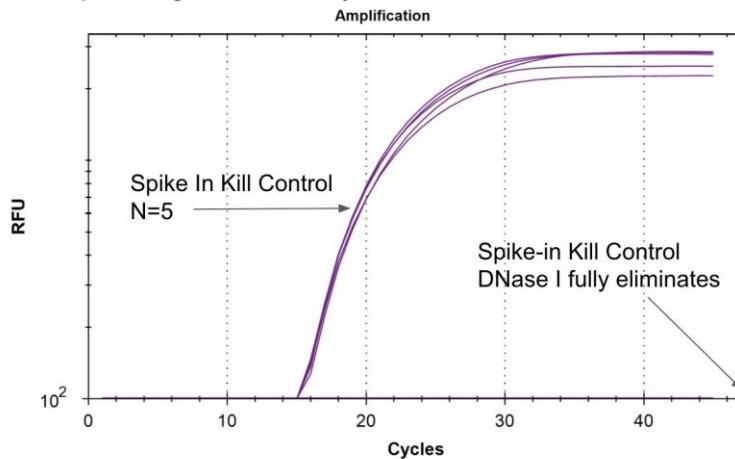
401 **Figure 13.** DNaseI-XT positive control demonstrates the digestion assay eliminates all
402 spiked in DNA under the same conditions used to assess the vaccine nuclease
403 sensitivity.

Discussion

404 Residual DNA was detected in all 27 vaccine vials surveyed. Multiple vials from the same

405 lots produced very similar loads for all targets showing assay reliability, reproducibility,

406 and consistency within the lots. These data involving vaccine vials distributed in Canada

407 are consistent with several non-peer reviewed reports of DNA contamination in modRNA

408 vaccines (McKernan, Buckhaults, Konig).[4][15][16]

409

410 Moderna had the lowest DNA concentration by qPCR but the highest concentration with

411 Qubit. The Moderna vials had the most consistent levels of DNA between vials

412 suggesting a more robust and standardized manufacturing process. In each vial of the

413 Moderna product, except for lot AS0467D, *ori* displayed lower loads than spike

414 suggesting a more effective removal of the vector DNA. Possibly, homologous modified

415 RNA may prevent digestion of template DNA by hybridization.[17]

416

417 The vials with the highest DNA concentration were from two lots of Pfizer monovalent

418 purple top vials with a phosphate buffered saline (PBS) formulation and require dilution

419 before administration. On October 29, 2021, the US FDA authorized a change of

420 formulation to a Tris/sucrose buffer; the grey topped monovalent adult vaccine and an

421 orange topped vaccine for children aged 6-11 years, This change was made to increase

422 stability, to simplify storage requirements and to provide a ready-to-use formulation.[18][19]

423 These purple-topped Pfizer lots were also associated with the highest number of AEs

424 and SAEs reported in VAERS among all the lots tested. As the actual number of doses

425 administered for each lot is unknown, we used the total number of AEs as a proxy for

426 the number of doses administered as a denominator for the number of SAEs to estimate

427 toxicologic/pharmacologic effect. This uses the same principle used by CDC[10][11] in its

428 disproportionality signal analysis (DSA).

429

430 Our exploratory analysis of the relationship between the residual DNA content and SAEs

431 reported to VAERS is preliminary and limited in sample size but warrants confirmation

432 by examining many more lots and vials. A positive dose-response relationship was

433 observed for the Pfizer lots based on qPCR estimation of residual DNA.

435 Different relationships were observed for Moderna lots for qPCR data as well as for plots
436 based on residual DNA estimated by fluorometry, for both Pfizer and Moderna lots.
437 These observations may reflect differences between the two products such as quantity
438 of DNA, the size distribution of DNA fragments, the composition and sequence of the
439 plasmid vector and composition of lipid nanoparticles. Other differences both between
440 the two products and between different lots of each product may also contribute to our
441 observations. These differences include variations in levels of contaminants or
442 impurities. One major source of impurity is fragmented mRNA for which a number of
443 toxicological mechanisms have been proposed such as its effects on miRNA
444 processes.[20] dsRNA is another type of impurity that occurs secondarily to the T7 RNA
445 polymerase promoter. dsRNA can induce pro-inflammatory cytokines[21] and has been
446 hypothesized to contribute to immune-inflammatory reactions such as myocarditis.[22]
447 Lipopolysaccharides in cells from endotoxin can bind both the S1 and S2 subunits of the
448 spike protein which may result in enhanced inflammatory responses.[23]
449
450 Wider sampling will likely reveal greater detail in terms of event types, such as death, as
451 well as comparisons with other works such as that reported by Schmeling *et al.*[24] who
452 reported a correlation of AEs to various vaccine lot numbers[24]. None of the presently
453 studied vaccine lots were included in the Schmeling study and more work is needed to
454 understand if and how this DNA contamination is related to AEs.
455
456 While the SV40 enhancer facilitates nuclear localization,[6 25] genomic integration of DNA
457 fragments has yet to be demonstrated for the COVID-19 modRNA products.[26] However,
458 it is known that DNA contamination could trigger an unwarranted innate immune
459 response and may be prothrombotic, particularly for fragments with high GC content.[27]
460 dsDNA may also be a significant factor in ischemic diseases including stroke.[28] While
461 there appears to be a correlation between high DNA contamination and SAEs more
462 research is needed to expand the sample size and elucidate any potential mechanism
463 at work.
464

465   It is important to emphasize that because qPCR cannot quantitate molecules smaller

466   than the size of the amplicon (105-114 bp), qPCR underestimates the total DNA in each

467   vaccine. This explains the large differences we have observed in residual DNA levels

468   estimated by qPCR compared with Qubit fluorometry particularly between the Pfizer and

469   Moderna products. The much larger values obtained for the Moderna product suggests

470   that there is a higher fraction of small fragmented residual DNA than in the Pfizer

471   product. This is consistent with a more thorough nuclease digestion step. This illustrates

472   the DNA contamination guidelines recommended by the FDA are highly dependent on

473   the methods used to quantitate the DNA. An alternative hypothesis to explain the high

474   fluorometric measurements is the unknown specificity of the DNA-tropic fluorometric

475   dyes when in use with samples that have high concentrations of N1-methyl-

476   pseudouridine modRNA.

477

478   This fluorometry assessment is of particular interest as fluorometry and UV

479   spectrophotometry were used to quantitate RNA in the Pfizer COVID-19 vaccines, as

480   described in EMA documents[3], while qPCR was used to quantitate DNA. This selective

481   use of different methods to quantitate RNA/DNA ratios can lead to vastly different results

482   for the ratio-metric guidelines in place at the EMA.

483

484   This elevated fluorometry quantitation compared to qPCR quantitation is consistent with

485   the ONT read length distributions that also suggest a portion of the DNA may be smaller

486   than the amplicon size. While the ONT sequencer detects molecules shorter than 100

487   bp, the methods for library construction for ONT use a 0.7X Ampure DNA purification

488   step which drastically selects against purifying molecules <150 bp in size. As a result,

489   the read length distributions for ONT reads are biased towards fragments >150 bp and

490   are not a perfect reflection of the smaller fragments that may be present and

491   undercounted by both ONT and qPCR.

492

493   Currently, the US FDA recommends manufacturers of viral vaccines to limit the amount

494   of residual DNA in the final product to below 10 ng/dose for parenteral inoculations and

495   the size of the DNA to below the size of a functional gene, or ~200 base pairs.[13] This is

496   also in keeping with recommendations from the World Health Organization (WHO).[14][29]

497   Previous residual DNA levels were set by the FDA at 10pg/dose in 1985. A 1986 WHO

498   study group concluded that the risk is negligible up to 100 pg/dose and in 1996 the WHO

499   further increased levels up to 10 ng per dose.[14]

500

501   The FDA and WHO guidelines for allowable DNA in vaccines are influenced by work

502   published by FDA scientists Sheng-Fowler *et al.*[30] This work focused on host cell

503   genomic DNA contamination and made note of the increased number of molecules

504   present when small viral vectors are the contaminating species. For these high copy per

505   nanogram contaminants, femtograms to attograms of DNA are considered the

506   equivalent of nanograms of cell substrate genomic DNA. Given the short fragment size

507   in the modRNA vaccines, the number of molecules in each dose can reach over 100

508   billion molecules. The residual DNA in these vaccines is high in copy number and rich

509   in promoters, ORFs and nuclear targeting sequences. The FDA and WHO guidelines

510   did not consider packaging of DNA in lipid nanoparticles, likely resulting in longer DNA

511   persistence as well as increased transfection efficiency. Furthermore, the guidelines did

512   not consider cumulative dosing with LNP-based modRNA. In some cases, more than

513   five doses of COVID-19 vaccines have been administered with a dose interval for

514   booster doses sometimes as short as 2 months. Moreover, the risks of cumulative dosing

515   by vaccines targeting other infections but using the same plasmid and LNP-based

516   modRNA platform has not been considered in setting the residual DNA guidelines.

517

518   The FDA guidelines are also written to only quantitate DNA fragments of 200 bp or

519   greater, in part because fragments smaller than this were not considered to be able to

520   produce a functional gene. However, Klinman *et al.,*[31] suggests that fragments as small

521   as 7bp can pose integration risks. Furthermore, the guidelines may also have considered

522   that fragments of naked DNA shorter than 200 bp would be more rapidly hydrolyzed by

523   host nucleases activity than larger molecules.[32] This accelerated destruction cannot be

524   assumed of the vaccines due to the DNA being encapsulated and protected by the LNPs.

525

526

527 Klinman *et al.*,[31] also observe that *"in evaluating the potential harm of plasmid*
528 *integration, it should be noted that the risk of introducing plasmids with strong regulatory*
529 *regions into the host genome far exceeds that associated with random point mutations."*
530

531 Finally, the guidelines do not consider if the residual DNA contains nuclear targeting
532 sequences and mammalian promoters that exist in the Pfizer vaccine.[26] Vacik *et al.*
533 demonstrated that the SV40 enhancer present in the Pfizer vector is a potent nuclear
534 targeting sequence showing promise for gene therapy.[25]
535

Conclusion
537 These data demonstrate the presence of billions to hundreds of billions of DNA
538 molecules per dose in the modRNA COVID-19 products tested. Using fluorometry, all
539 products tested exceeded the guidelines for residual DNA set by the FDA and WHO of
540 10 ng/dose by 188 – 509-fold. However, qPCR detected residual DNA content in all
541 products tested were below these guidelines emphasizing the importance of
542 methodological clarity and consistency when interpreting quantitative guidelines. The Cq
543 scores for the most recent XBB.1.5 Moderna vaccine suggest that DNA residues have
544 not been reduced from previous vaccine versions.
545

546 The preliminary evidence of a dose-response effect of residual DNA measured with
547 qPCR and SAEs warrants confirmation and further investigation. Our findings extend
548 existing concerns about vaccine safety and call into question the relevance of guidelines
549 conceived before the introduction of efficient transfection using LNPs. With several
550 obvious limitations, we urge that our work is replicated under forensic conditions and
551 that guidelines be revised to account for highly efficient DNA transfection and cumulative
552 dosing.
553

554 This work highlights the need for regulators and industry to adhere to the precautionary
555 principle, and provide sufficient and transparent evidence that products are safe and
556 effective, and disclose the details of their composition and method of manufacture.
557

Data Availability

Fastq file for the mapped ONT sequencing data:

https://mega.nz/file/UZhkiTBQ#8vjDK5JV5N5Dj2On34B6zdRObEKGBy3ZC7w8q2t9U

Vc

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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.

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