UNIVERSITY of GUELPH
ONTARIO VETERINARY COLLEGE
DEPARTMENT OF PATHOBIOLOGY

September 9, 2024

I have received and undertaken the testing of the three vials you delivered to me at the University of Guelph and in accordance with your letter of instruction dated May 13, 2024. Per your request, please find below the final report.

A. Executive Summary of Findings

Background: Previous work in Canada, conducted by colleagues and myself, showed that the Pfizer and Moderna COVID-19 modRNA vaccines contained residual plasmid DNA. While the DNA when quantified by quantitative PCR (qPCR) is slightly lower than the TGA limit of 10 ng/dose, when the vaccine vials were tested by fluorometry the total DNA levels greatly exceeded the regulatory limit by 7 to 145-fold. The Pfizer COVID-19 modRNA vaccines also contained an SV40 promoter-enhancer-ori that was not initially disclosed to the National Regulatory Agencies, namely the USA FDA, Health Canada the European Medicines Agency, and the Therapeutic Goods Administration (TGA) in Australia.

Objective: Ms. Ashby-Koppens provided 3 vials of COVID-19 modRNA vaccines (2 Pfizer: 1 adult monovalent, Lot# FN0565 and 1 child monovalent, Lot# FR4268; 1 Moderna child/adult monovalent; Lot# 2100695) and requested the following testing.

1. Quantitative real-time PCR for Spike, Origin of Replication (ori), and SV40 promoter-enhancer-ori DNA.
2. Fluorometry via Qubit as per the protocol in the Speicher *et al*, 2023 preprint (https://doi.org/10.31219/osf.io/mjc97), including using RNase A.
3. Complete the chain of custody report and schedule accompanying this letter.

Methods: In each vial the amount of spike, ori and SV40 promoter-enhancer-ori DNA was detected and quantified by qPCR. Total DNA was determined by Qubit® fluorometry directly on the vaccines and then repeated following the use of boiling to open the lipid nanoparticles and treatment with RNase A to reduce the potential cross talk from modified RNA with AccuGreen® Testing was repeated on all vials to confirm the initial results.

Results: All samples were found to contain spike and ori sequences, but only the Pfizer samples contained the SV40 promoter-enhancer-ori sequence. The Pfizer Lot# FN0565 exceeded the TGA limit of 10 ng/dose for all targets and Pfizer Lot# FR4268 exceeded the limit only for spike. The total DNA, as determined by fluorometry, exceeded the TGA limit by 7 to 145-fold.

				Quantitative PCR			Qubit Fluorometry
Sample ID	Manufacturer	Lot #	Run #	Spike (ng/dose)	Ori (ng/dose)	SV40 promoter-enhancer-ori (ng/dose)	Total DNA (ng/dose)
AP001	Pfizer	FN0565	1	163.68	12.97	9.79	494
			2	156.85	7.68	14.69	848
AP002	Pfizer	FR4268	1	76.69	1.48	3.70	108
			2	68.70	0.76	5.21	78
AM001	Moderna	2100695	1	6.46	0.76	NEGATIVE	1460
			2	8.10	0.54	NEGATIVE	1221

Conclusion: All Australian vials contain synthetic DNA that exceed the TGA limit of 10 ng/dose by fluorometry and all Pfizer vials contain the SV40 promoter-enhancer-ori sequence. Residual DNA levels tested by PCR exceeded the TGA regulatory limit for both Pfizer lots.

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Full Detailed Report

B. Scientific Background

1. In October 2023, I, Dr. David J. Speicher, co-authored a preprint[1] on the high levels of residual plasmid DNA present in the Pfizer and Moderna COVID-19 modified mRNA (modRNA) vaccine. This study confirmed the earlier work of Kevin McKernan[2] (Medicinal Genomics) and Dr. Philip Buckhaultz[3]. Our October 2023 paper independently tested 27 modRNA vials, the greatest number of unopened vials of COVID-19 vaccine to date.
2. The vials for this Canadian study[1] were obtained in Canada from 12 unique lots. Spike and ori DNA sequences were detected in all Pfizer and Moderna COVID-19 modRNA vaccine vials by quantitative PCR (qPCR). 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 highly fragmented (<100bp) and unable to be detected by qPCR.
3. The Australian Therapeutic Goods Administration (TGA)[4], USA Food and Drug Administration (FDA)[5], and the World Health Organization (WHO)[6] regulatory body guidelines allow up to 10 ng DNA/dose in the vaccines. These guidelines are for naked DNA fragments ≤200 bp and not for protected synthetic DNA inside lipid nanoparticles (LNPs). The guidelines also do not account for multiple dosing of the same vaccine or platform, the risk of regulatory sequences, integration of small DNA fragments (7bp to 200 bp), or nuclear entry/integration. As this report relates to COVID-19 vaccine vials distributed in Australia, throughout this document "the 10 ng/dose" guidelines" will be referred to as "TGA 10 ng DNA/dose Guidance".
4. Only the Pfizer-BioNTech COVID-19 modRNA vaccines contain an SV40 enhancer-promoter-ori, which is known to promote nuclear localization and host genomic integration when fragments containing the SV40 enhancer are inserted cytoplasmicly.[7]
5. A 1999 study by David Dean *et al*[7] showed that as few as 3 to 10 copies of DNA fragments with a 72bp SV40 enhancer injected cytoplasmicly (e.g. how the DNA fragments inside the LNPs in the COVID modRNA vaccines are inserted into the cells) in non-dividing cells, greatly increases their ability to be transported into the nucleus.
6. To date, preliminary work conducted in Germany has found evidence of genomic integration of the whole COVID-19 vaccine spike DNA open reading frame. After human ovarian cancer cells (OVCAR-3) were exposed in cell culture overnight to the Pfizer modRNA vaccine, the whole SARS-CoV-2 spike DNA as sequenced in the Pfizer vaccine was found to have integrated into the genome at chromosomes 9 and 12.[8] This study highlighted that integration of the DNA fragments in the Pfizer COVID-19 modRNA vaccine

into the human genome is possible, and it is important to investigate whether integration can take place in primary cells in the vaccinated population.

7. Residual plasmid DNA has been found in vials from multiple countries.[1-3 9] I am not aware of any other COVID-19 vaccine vials from Australia being tested, apart from the three vials described in this report, that have been independently examined for the presence of residual plasmid DNA.

C. Methods

1. COVID-19 Vaccine Vials Received

On May 14, 2024, I received three Australian vials of COVID-19 modRNA vaccines at the University of Guelph (Table 1; from Left to Right in Figure 1). These vials were shipped on 15kg of dry ice, but when the package was received there was no dry ice in the package and contents were cool to the touch, but not warm. Temperature of the package was not recorded. The vials were immediately placed in a laboratory fridge (+2-8°C) until tested. The Pfizer vials were unopened were untampered as they had intact flip-off plastic caps with printed lot numbers and expiration dates. The Moderna vial did not have an intact flip-off plastic cap and appears to have been used as the septum appeared to be punctured, and the contents of the vial was at half volume.

Table 1: List of COVID-19 modRNA vaccines from Australia that were received at the University of Guelph for testing.

Sample ID	Manufacturer	Lot #	Mono/Bivalent	Cap Colour	Expiry Date
AP001	Pfizer-BioNTech	FN0565	Adult Monovalent	Purple	06/2022
AP002	Pfizer-BioNTech	FR4268	Child Monovalent	Orange	08/2022
AM001	Moderna	2100695	Child/Adult Monovalent	Missing	25/06/2022

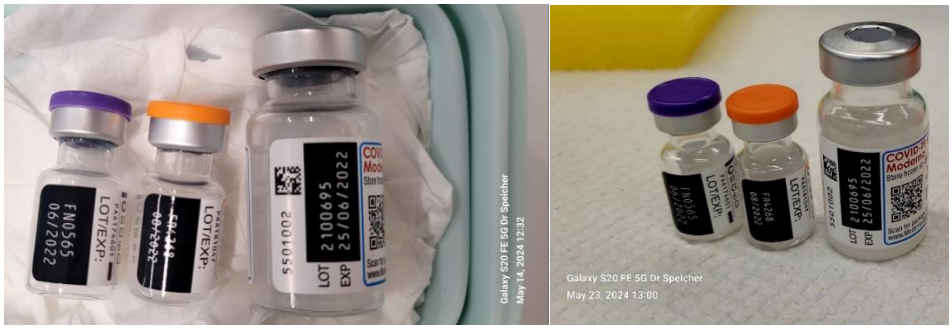

Figure 1: Pfizer (adult monovalent, purple capped and child monovalent, orange capped vials) and Moderna (no cap, larger vial) COVID-19 modRNA vaccine vials received at the University of Guelph on May 14, 2024 (left). The May 23, 2024 photo (right) was taken immediately prior to testing and shows the top of the vials.

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2. Quantitative PCR Testing

Each vial was tested by qPCR for the presence of plasmid derived SARS-CoV-2 spike, ori, and the SV40 promoter-enhancer-ori DNA sequences. Each sample was tested in duplicate with PCR primers targeting sequences shared by the Moderna and Pfizer expression plasmids (Table 2).

Table 2: 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/

In brief, the qPCR assays used 2 µL from each vial directly added to 8 µL of master mix. qPCR kits were sourced from Medicinal Genomics (PathoSEEK® RT-qPCR Master Kit v2; Part# 420207, Beverly, USA) with the master mix containing 10 µL reaction consisting of 5 µL polymerase enzyme, and 1.0 µL of Primer-Probe mix, and 2 µL of ddH20. The vaccine was tested at 1:10 dilution as previous testing showed that this was the highest residual DNA concentration to investigate PCR inhibition by the LNPs since qPCR was performed directly without any treatment or extraction.[1]

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

Cycling was performed on a QuantStudio 3 (ThermoFisher Scientific, Waltham, USA) with an initial denaturation of 95°C for 1 minutes followed by 40 cycles of 95°C for 5 seconds and 65°C for 30 seconds (Figure 2). As a calibration curve was used QuantStudio software v2.7.0 (ThermoFisher Scientific) produced Cycle of quantitation (Cq) scores ng/µL for each sample. Amplicon mass, as determined with the New England BioLabs DNA calculator,[10] and length (105 bp for ori, 114 bp for spike, 72 bp for SV40 promoter-enhancer-ori) were used to estimate the total nanograms (ng) of DNA present by adjusting for the length of the plasmids (7,824bp for Pfizer and 6,777bp for Moderna). The PCR copy number/dose and the total DNA as determined by fluorometry was adjusted first for the dilutions (1:5 dilution for the Pfizer adult monovalent and 1:2 for the child monovalent) and then for the volume of each intramuscular vaccine injection dose used clinically (300 µL for Pfizer Adult Monovalent, 200 µL for Pfizer Child Monovalent, and 500 µL for Moderna) to provide a final ng/dose value.

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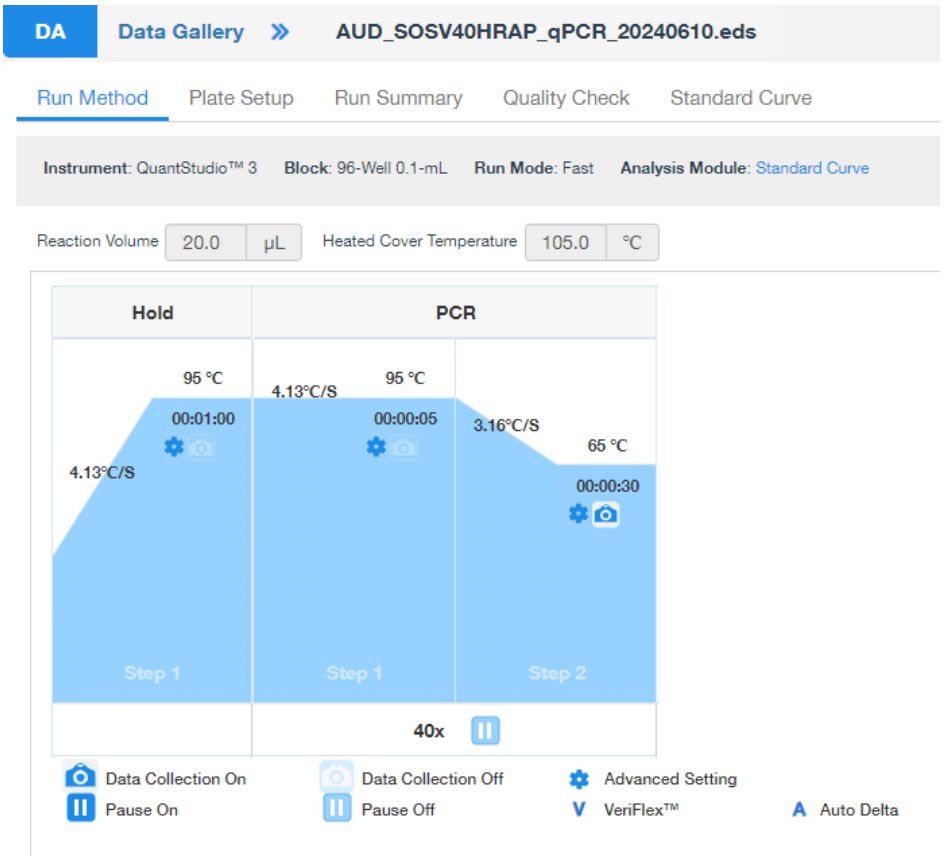

Figure 2: PCR cycling conditions used to test the COVID-19 modRNA vaccines for Spike, Ori and SV40 promoter-enhancer-ori DNA.

3. 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. To reduce the cross talk from AccuGreen® with modified RNA the samples were then treated with 1 µL RNase A (New England BioLabs, #T3018-2) and then read over a period of 10 minutes (T=0, 1, 2, 5, 10 minutes).

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D. Results

1. Quantitative PCR Testing

qPCR testing for spike, ori, and SV40 promoter-enhancer-ori DNA sequences was performed on June 10, 2024. All samples were found to contain sequences for COVID-19 vaccine spike and ori, but only the Pfizer samples contained sequences for the SV40 promoter-enhancer-ori (Figure 3). The levels of all targets were found to exceed the TGA 10 ng DNA/dose guidance in Pfizer FN0565.[4] In Pfizer FR4268 only the spike DNA exceeded the TGA limit. The DNA concentration between vials varied greatly with the levels of spike DNA in both Pfizer vials being the highest of those reported globally to date.[1-3] The high degree of variance between the genomic targets (e.g. spike vs ori) is possibly due to incomplete digestion of the plasmid. Whilst the level of vaccine spike DNA in the Moderna vial was 6.5 – 8.1 ng/dose; just below the TGA 10 ng DNA/dose guidance this equates to ~30 billion DNA fragments per dose. While the number of these fragments entering a cell is unknown, it is known from Dean et al (1999) that only 3-10 copies of these spike DNA fragments containing the SV40 enhancer are needed to be inserted into a single cell for the risk of insertional mutagenesis to exist.[7]

As the DNA loads yielded in the initial testing were the highest seen globally to date, PCR testing was repeated on July 5, 2024, on a new aliquot of the vaccine and all new reagents to rule out any contamination or sources of error. The vaccine was also tested in duplicate. Two Pfizer vials and one Moderna vial from the Canadian study[1] were included in the run to rule out variability between the runs. Repeat testing of the new aliquot using new reagents produced very similar results as the previous run and the two Canadian vials run as a positive control produced the same Cq values as previously tested (data not shown). Again, for the Pfizer vials levels of spike and the SV40 promoter-enhancer-ori exceeded the TGA 10 ng/dose guideline, but ori was below. Therefore, the PCR assay performed optimally, and the DNA yield determined by the testing is valid and true.

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Table 3: Quantitative PCR results of testing three Australian vials of COVID-19 modRNA vaccines for vaccine spike, origin of replication (ori), and the SV40 promoter-enhancer-ori (SV40).

Vaccine Spike DNA								
Sample ID	**Manufacturer**	**Lot #**	**Run #**	**Spike (Cq)**	**Spike (ng/µL)**	**Spike (Copies/µL)**	**Total ng/dose**	**Total Copies/dose**
AP001	Pfizer	FN0565	1	10.69	3.98E-03	1.70E+07	163.68	6.99E+11
			2	10.88	3.81E-03	1.63E+07	156.85	6.70E+11
AP002	Pfizer	FR4268	1	12.33	1.12E-03	4.77E+06	76.69	3.28E+11
			2	12.80	1.00E-03	4.28E+06	68.70	2.93E+11
AM001	Moderna	2100695	1	18.47	2.17E-05	9.28E+04	6.46	2.76E+10
			2	18.32	2.73E-05	1.16E+05	8.10	3.46E+10
Vaccine Origin of Replication (ori) DNA								
Sample ID	**Manufacturer**	**Lot #**	**Run #**	**Ori (Cq)**	**Ori (ng/µL)**	**Ori (Copies/µL)**	**Total ng/dose**	**Total Copies/dose**
AP001	Pfizer	FN0565	1	17.26	3.12E-04	2.89E+06	12.97	1.23E+11
			2	17.57	1.85E-04	1.71E+06	7.68	7.27E+10
AP002	Pfizer	FR4268	1	18.56	2.14E-05	1.98E+05	1.48	2.06E+10
			2	18.99	1.09E-05	1.01E+05	0.76	1.05E+10
AM001	Moderna	2100695	1	23.40	2.34E-06	2.17E+04	0.76	7.02E+09
			2	24.58	1.67E-06	1.55E+04	0.54	5.00E+09
Vaccine SV40 promoter-enhancer-ori DNA								
Sample ID	**Manufacturer**	**Lot #**	**Run #**	**SV40 (Cq)**	**SV40 (ng/µL)**	**SV40 (Copies/µL)**	**Total ng/dose**	**Total Copies/dose**
AP001	Pfizer	FN0565	1	14.87	2.35E-03	1.59E+07	9.79	5.29E+11
			2	14.18	3.53E-03	2.39E+07	14.69	7.94E+11
AP002	Pfizer	FR4268	1	17.27	3.56E-04	2.41E+06	3.70	2.00E+11
			2	17.35	5.01E-04	3.39E+06	5.21	2.82E+11
AM001	Moderna	2100695	1	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
			2	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

Cq = cycle of quantitation

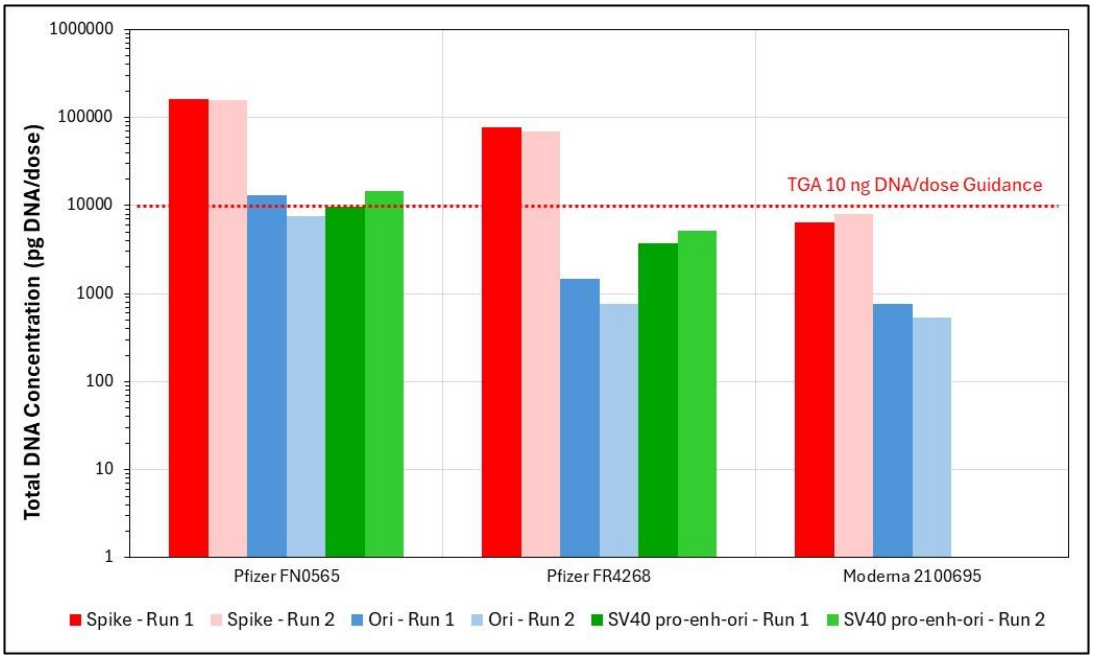

Figure 3: Graphical analysis of the DNA loads for spike, ori and SV40 as quantitated by qPCR. The dotted red line denotes the TGA 10 ng DNA/dose guidance.

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2. Qubit® Fluorometry Quantitation

As per instruction #2, the initial Qubit® Fluorometry testing was performed on May 28, 2024. For all vials the total residual DNA exceeded the TGA guideline of 10 ng/dose (Table 4, Figure 4). In the initial run (May 28, 2024) the total DNA ranged from 434 ng/dose (Pfizer FR4268) to 2803 ng/dose (Pfizer FR0565). Boiling of the samples increased the DNA yield because the boiling disrupted the LNPs and the AccuGreen® dye could contact both the modRNA and plasmid DNA protected inside the LNPs. Treatment with RNase A degraded the modRNA and reduced the cross talk from AccuGreen® with modRNA thus reducing the fluorescent signal. The concentration of only DNA still exceeded the TGA guideline of 10 ng/dose by 7 to 145-fold. The total DNA in Pfizer FR4268 is much lower than Pfizer FN0565 as this is a child monovalent vaccine and 200 µL per dose is administered. Whereas Pfizer FN0565 is adult monovalent and 300 µL is administered.

Table 4: Total DNA concentration of the vaccine vials as determined by Qubit® fluorometry. The RNase= values equate to the time since RNase A was added to the sample. Values displayed are in ng/dose.

				RNase A					
Sample ID	**Lot #**	**Run #**	**Pre-Boil**	**Post-Boil**	**RNase=0**	**RNase=1**	**RNase=2**	**RNase=5**	**RNase=10**
AP001	Pfizer FN0565	1	2803	3552	1094	533	499	499	494
		2	1219	1325	1104	864	845	849	848
AP002	Pfizer FR4268	1	434	442	224	112	106	103	108
		2	276	264	99	77	77	77	78
AM001	Moderna 2100695	1	1610	2050	1730	1460	1460	1460	1460
		2	1710	1760	1340	1210	1220	1222	1221

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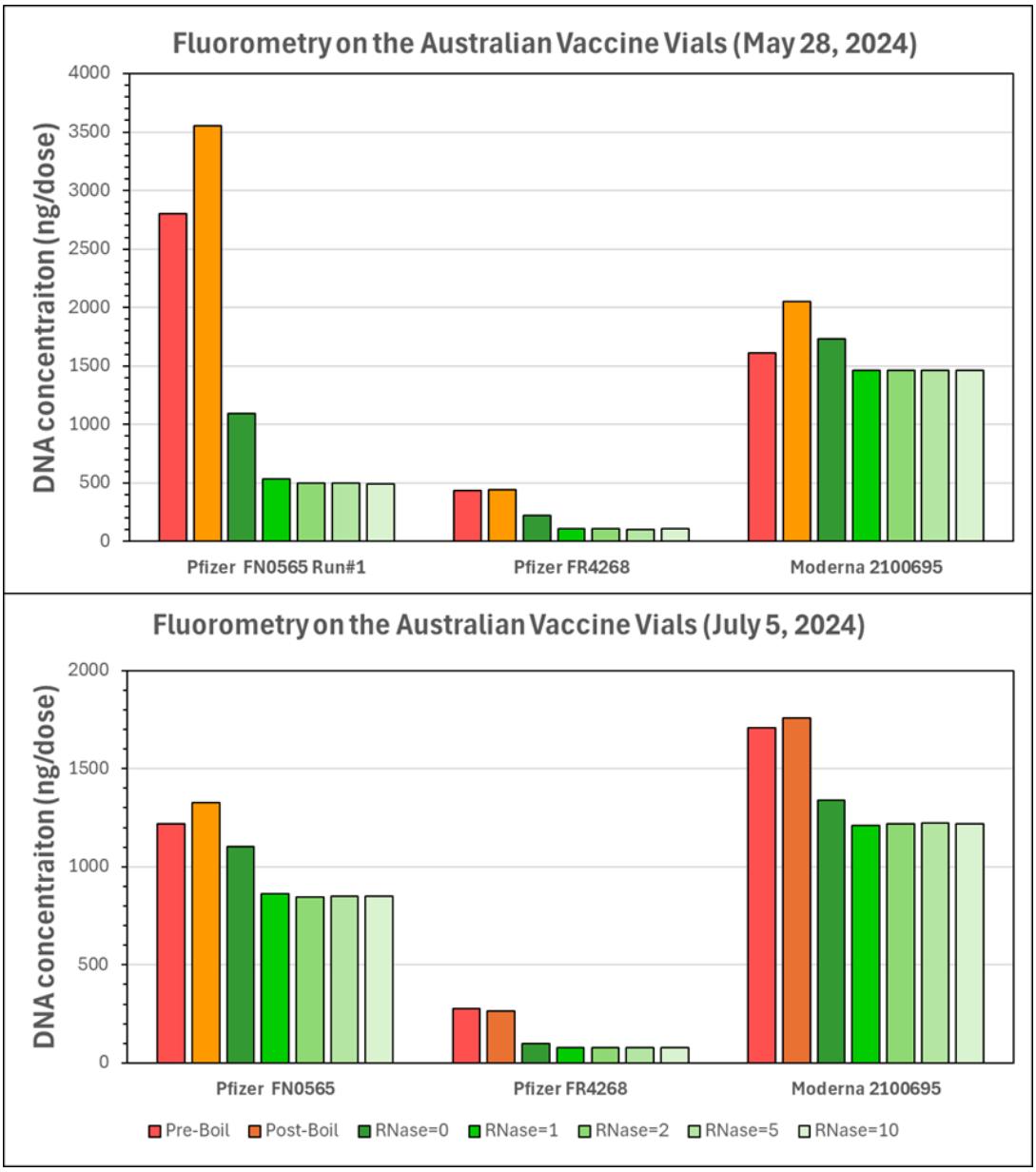

Figure 4: Graphical analysis of the total DNA loads as determined by Qubit® fluorometry. The RNase= values equate to the time since RNase A was added to the sample. Values are in ng/dose and the TGA 10 ng/dose guidance would be slightly above the X-axis.

As the total DNA of the vials determined by Qubit® Fluorometry for both Pfizer and Moderna were lower than the Canadian vials (i.e. 1,896 to 3,720 ng/dose for Pfizer and 3,270 to 5,100 ng/dose for Moderna)[1] the testing was repeated on July 5, 2024. Very similar results were produced for Pfizer FR4268 and Moderna 2100695 with the slight variance due to error in pipetting and handling the LNPs. For Pfizer FN0565 testing of the vaccine vial contents pre- and post-boil produced values only half of those in the initial testing but following the use of

RNase A the total DNA values are comparable (i.e. 494 ng/dose in Run 1 and 848 ng/dose in Run 2). The variability in the pre- and post-boil samples is possible due to sampling error and in increase in cross-talk due to a higher amount of modRNA present in the sample. Variability in pipetting of the LNPs would also attribute to the differences in test results.

E. Conclusions:

1. Both Pfizer vials had spike DNA loads above the TGA 10 ng DNA/dose guidance when tested by qPCR. The spike, ori and SV40 promoter-enhancer-ori DNA sequences in Pfizer FN0565 were all above the TGA limits with the spike DNA being the highest concentration levels seen in vials independently tested globally to date.
2. Despite the extremely high DNA loads the results were repeatable suggesting the result is true and valid.
3. The DNA concentration varied greatly depending on the target highlighting the need for PCR assays assessing the residual plasmid DNA load in the COVID-19 vaccines to target multiple regions when determining DNA loads, and then extrapolating the total DNA for the whole plasmid and not individual regions.
4. The Moderna vial had DNA loads, determined by qPCR, that were below the TGA 10 ng DNA/dose guidance.
5. The total DNA concentration in all Australian vials when tested by Qubit® fluorometry far exceeded the TGA 10 ng DNA/dose guidance with Moderna having the highest total DNA levels.

Sincerely,

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