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A rapid detection method of replication-competent plasmid DNA from COVID-19 mRNA vaccines for quality control

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Abstract

Despite the rapid development of SARS-CoV-2 mRNA vaccines to combat the Coronavirus infectious disease 2019 (COVID-19) pandemic, vaccine hesitancy gained traction as the pandemic continued. Among the widely discussed topics related to the COVID-19 mRNA vaccines, DNA contamination cast doubt on the quality of the product and may have undermined public trust. Here, we report a simple method to detect residual replication-competent plasmid DNA that is present in mRNA vaccines as impurities. Using 4 vials of experimental mRNA vaccines, we found that two out of four vials of those experimental mRNA vaccines contained residual plasmid DNA that transformed *Escherichia coli* cells. We subsequently applied our method to assess 2 separate lots of Pfizer COVID-19 mRNA vaccines and found no replication-competent plasmid DNA. However, these authorized vaccines do contain residual DNA to a level that exceeds 10 ng per dose. Our results suggest that stringent and transparent monitoring of DNA impurity may aid in the buildup of public trust in mRNA vaccines.

Keywords

COVID-19, mRNA vaccine, DNA impurity, Spike protein, Replication competent, Plasmid DNA, Residual DNA, Vaccine hesitancy, DNA fragment, Adverse events

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Introduction

The revolution in messenger RNA (mRNA) technology has enabled the rapid development of coronavirus disease 2019 (COVID-19) mRNA vaccine (1). The award of the 2023 Nobel Prize in Medicine to Drs. Katalin Karikó and Drew Weissman for their work of modifying mRNA to make it a therapeutic enabling a platform catapulted the technology into the limelight. COVID-19 mRNA vaccines are made of an antigen-encoding messenger RNA encapsulated into lipid nanoparticles (2). The mRNA is transcribed from a DNA plasmid *in vitro* by T7 RNA polymerase and then purified after DNase I digestion to remove the original plasmid DNA (pDNA) (Figure 1). The purified mRNAs are subsequently formulated to be packaged within a lipid envelope. The mRNA-based vaccines are easy to manufacture and update. Upon administration, the mRNA is translated into the spike protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), inducing anti-Spike antibodies and hence giving the immune system a head start over the virus. Despite millions of lives being saved, the mRNA vaccine is not perfect; just as all other medical products. One critique of the COVID-19 mRNA vaccine is that there may be DNA present in the vaccine and some even associate mRNA vaccines with the possibility of inducing genetic mutations in vaccinees (3). Questions like this, if not addressed, may greatly influence the receptiveness of the mRNA vaccine by the public (4-7).

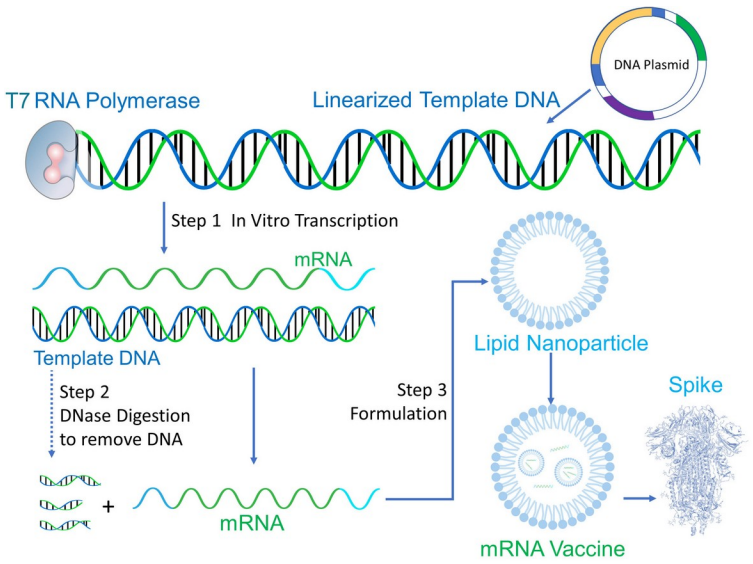


Figure 1. A simplified workflow for mRNA vaccine production. A DNA plasmid containing the gene sequence of the SARS-CoV-2 Spike protein is linearized and used as the template for *in vitro* transcription by T7 RNA polymerase. The reaction mixture then undergoes DNase digestion to remove the original plasmid DNA. The derived mRNA is subsequently purified and encapsulated within a mixture of lipids that form lipid nanoparticles (LNP). Delivery of mRNA-LNP into human cells leads to the production of Spike protein and triggers specific immune responses.

By design, mRNA does not integrate into human chromosomal DNA, thus eliminating the possibility of inducing mutations in host DNA. The manufacturing of the mRNA vaccines, however, does involve the template pDNA because the flow of genetic information follows the Central Dogma, i.e., from DNA to RNA, and then to protein. Despite extensive efforts, it is impossible to completely remove all DNA impurities from the final product (8, 9). In other words, trace amount of pDNA is expected to be found in the COVID-19 mRNA vaccines as impurities. For quality control purposes, it is reasonable to set an allowable DNA limit in mRNA vaccines. Current recommended limits (<10 ng/dose, length<200 bp), which are frequently cited in the literature, are set for continuous cell line-derived DNA by the World Health Organization and adopted by U.S. and European regulatory agencies (10-12). A few recent studies reported that the licensed Pfizer and Moderna mRNA vaccines may contain greater amounts of DNA impurities (13-15).

Compared to smaller DNA fragments, which are anticipated to undergo subsequent degradation once being taken up by human cells, those larger DNA pieces with genetic elements that enable active replication (replicative or replication-competent) are potentially more harmful. To this end, we designed experiments to examine whether there was residual DNA present in COVID-19 mRNA vaccines that was capable of transforming *Escherichia coli* (*E. coli*) cells. We further characterized the amount and size of DNA fragments from various sources of mRNA vaccines.

Methods

This work was conducted in the BSL-1 research facility at the FDA white oak campus.

mRNA vaccines

The in-house mRNA vaccine contains the SARS-CoV-2 variant XBB.1.5 spike sequence. The mRNA was *in vitro* transcribed from a pcDNA3.1(+) vector containing the relevant spike sequence flanked by a 5'-UTR and 3'-UTR and was formulated with SM-102, DSPC, cholesterol, and DMG-PEG 2000 at a molar ratio of 50:10:38.5:1.5, sold as LipidLaunch™ LNP-102 exploration kit from Cayman Chemical, Ann Arbor, MI. The Pfizer and Moderna biosimilar COVID-19 biosimilar vaccines were obtained from BEI resources, which is an NIH supported program managed by the American Type Culture Collection (ATCC), Manassas, VA, (NR-59449 and NR-59450, respectively). COMIRNATY (COVID-19 Vaccine, mRNA) BNT162b2 Original (NR-59604) and BNT162b2 Bivalent (NR-59605) were acquired from BEI resources.

DNA extraction and quantification

Residual DNA from 5 to 50 µg mRNA vaccines in the volume of 50 to 300 µl was extracted using Monarch Plasmid DNA Miniprep kit (New England Biolabs, Ipswich, MA) following the manufacturers' instructions. The RNA component in the vaccine was presumably removed during the extraction because the Neutralization Buffer (B3) in the kit contains RNase A. At the final step, 30 µl nuclease free water was added to each column for elution. The eluted DNA was quantified on a NanoDrop Microvolume Spectrophotometer or Qubit Fluorometer using the Qubit dsDNA

HS Assay Kit (ThermoFisher Scientific, Carlsbad, CA).

Agarose gel electrophoresis

5 µl residual DNA was mixed with 1 µl 6x DNA loading buffer with dye before loading to a 1% agarose gel containing TAE and GelRed Nucleic Acid Stain (Biotium, Fremont, CA). Electrophoresis was run at ~ 100 volts on a PowerPac Basic Power Supply (Bio-Rad, Hercules, CA) till DNA fragments were well separated on the gel. Gel images were captured under UV light in a Gel Doc system (Thermo Fisher Scientific).

Ligation and transformation

The ligation reaction was set up at room temperature in a volume of 20 µl consisting of 10 µl residual DNA, 2 µl 10x ligation buffer, 7 µl nuclease free water, and 1 µl T4 DNA ligase (New England Biolabs). The ligated product was transformed into DH5α competent cells using the 5x KCM (0.5 M KCl, 0.15 M $CaCl_2$, and 0.25 M $MgCl_2$) method. In brief, 100 µl competent cells were thawed on ice. 20 µl ligated product was added to 20 µl 5x KCM buffer plus 60 µl nuclear free water and then mixed with thawed cells. After 20 minutes incubation on ice, the cells were left at room temperature for 10 minutes. 500 µl SOC medium was added to each tube containing the cells and left in a 37 ^{o}C bacterial shaker for 45 minutes at 225 rpm. Recovered cells were spread on Luria broth (LB) agar plates with 100 µg/ml ampicillin or 50 ug/ml kanamycin. After 16 hours incubation at 37 ^{o}C, bacterial colonies were visually inspected using a white light box.

DNA size analysis

8 µl of residual DNA isolated from Pfizer mRNA vaccines was submitted to the Facility for Biotechnology Resources for size analysis on the Agilent 2100 Bioanalyzer using the High Sensitivity DNA Assay kit (Agilent, Santa Clara, CA). A ladder including 15 size markers ranging from 35 to 10380 bp was included in the analysis.

Statistical analysis

Standard unpaired T-Test was used to calculate statistical significance using GraphPad Prism (8.4.2) software for Windows, GraphPad Software, San Diego, CA.

Results

A simple strategy to detect replication-competent DNA in mRNA vaccines

First, we designed a strategy to rapidly quantify the number of undigested or linearized near full-length pDNA in the mRNA vaccines. Shown in Figure 2, undigested pDNA can be readily transformed into *E. coli* cells and form antibiotics-resistant colonies. Linearized pDNA, depending on whether it contains the bacterial replication origin and the antibiotic resistance marker, may be ligated into a circular DNA that can be transformed into competent *E. coli* cells for enumeration. Since mRNA manufacturing includes a DNase I treatment step that would in theory cleave plasmid DNA into much smaller fragments that are not transformable, the presence of transformable DNA, i.e., replication-competent DNA in *E. coli* cells, would be a strong indicator for inefficient pDNA removal and hence poor product purity. To test the feasibility of this method, we extracted DNA from an experimental mRNA vaccine (50 µg)

containing the XBB.1.5 Spike protein coding sequence (Figure 3). The concentrations of the DNA (in 30 µl), determined by a NanoDrop Spectrophotometer, were in the range of 10-100 nanograms per microliter. The presence of DNA was confirmed by agarose gel electrophoresis with the majority DNA content migrating ~ 100 bp. We subsequently performed ligation using T4 DNA ligase and transformed the ligated product into chemically prepared DH5α cells. Our first trial yielded only one colony on Ampicillin (Amp)-containing LB plate (Figure 3A). Interestingly, DNA extracted from the colony-derived culture revealed a size slightly over 2 kb (Figure 3B), which contained only a portion of the original plasmid (Amp +) as revealed by Sanger sequencing (Figure 3C).

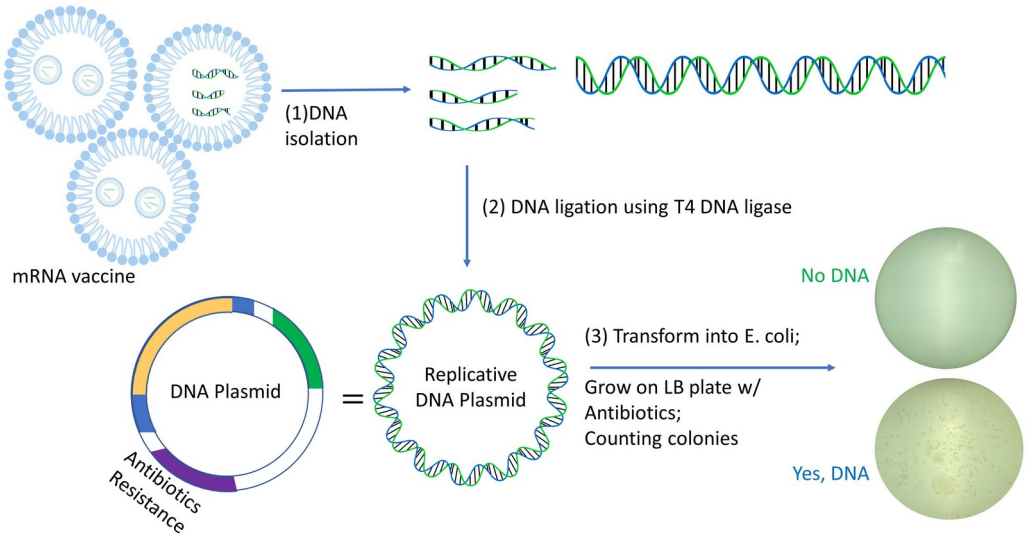


Figure 2. A simple strategy to detect replication-competent (transformable) DNA from mRNA vaccines: isolate DNA from mRNA vaccines, ligate into a circular form, transform DNA into E. Coli Cells, grow on a LB plate in the presence of an antibiotic. Only those bacteria that obtain the antibiotic-resistant gene from the template plasmid DNA will form colonies in the presence of antibiotic.

Detection of replication-competent DNA from biosimilar mRNA vaccines

Next, we extracted DNA from a Moderna biosimilar (5 µg) and a Pfizer biosimilar (5 µg) mRNA vaccine in addition to our experimental mRNA vaccine (50 µg). Once again, DNA was readily detected by gel electrophoresis and concentrations ranged from 10-100 ng/µl (30 µl in total) (Figures 4A, 4B). After ligated DNA was transformed into *E. coli* cells, we only detected two colonies from the Pfizer biosimilar vaccine-derived DNA. Both colonies were culturable (Figure 4C), and DNA extracted from the colonies-derived cultures consisted of 2 or 3 fragments of different sizes (Figure 4D).

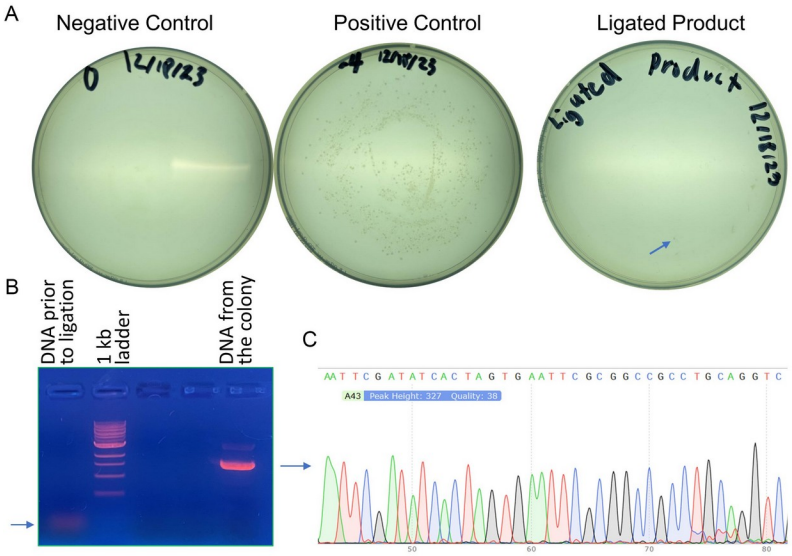
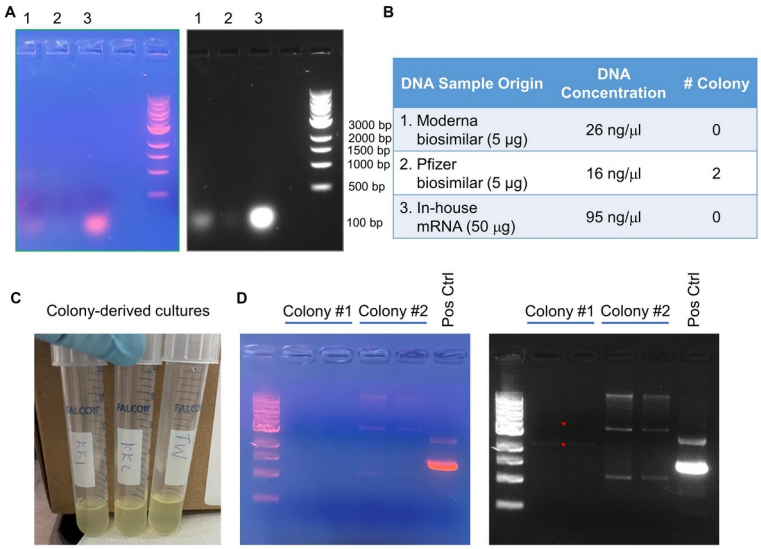


Figure 3. Detection of replication-competent DNA from an in-house made mRNA vaccine. A, colony growth after transformation. B &C, colony-derived DNA is visualized on an agarose gel and then sequenced. A portion of the detected sequence from the template pDNA is shown here.



DNA Sample Origin	DNA Concentration	# Colony
1. Moderna biosimilar (5 µg)	26 ng/µl	0
2. Pfizer biosimilar (5 µg)	16 ng/µl	2
3. In-house mRNA (50 µg)	95 ng/µl	0

Figure 4. Detection of replication-competent DNA from biosimilar mRNA vaccines. A, gel electrophoresis images of DNA isolated from three different experimental mRNA vaccines. B, summary of DNA concentrations (measured by Nanodrop) and the numbers of colonies after transformation. C, propagation of colonies in LB broth with antibiotics. D, gel electrophoresis images of DNA extracted from colony-derived cultures. Red star signs indicate two bands that are barely visible.

DNA impurity in Pfizer mRNA vaccines

Having established this method, we performed another experiment using two lots of commercial Pfizer mRNA vaccine (Lot PAA194854, monovalent; and PAA184098, bivalent). Three separate vials of mRNA from each lot were used for DNA extraction by three different operators (Figure 5A). Once again, agarose gel electrophoresis confirmed the presence of small DNA fragments (~ 100 bp) (Figure 5B). After transformation, the ligated product did not yield any colonies (Figure 5C). These results supported a complete removal of replication-competent pDNA from the analyzed vials of the Pfizer mRNA lots. Since smaller DNA fragments cannot be resolved by agarose gel electrophoresis, we submitted DNA samples for size analysis on an Agilent 2100 Bioanalyzer. Shown in Figures 5D and 5E, all six samples displayed a prominent band close to the 35-bp DNA ladder with very little product above this size. To quantify the amount of DNA in the commercial mRNA vaccines, we measured the DNA concentration from six trial samples and then calculated the amount of DNA in 30 µg mRNA (an equivalent of one human dose). Because NanoDrop spectrophotometer detects DNA of all forms, we also measured the DNA using Qubit dsDNA HS assay.

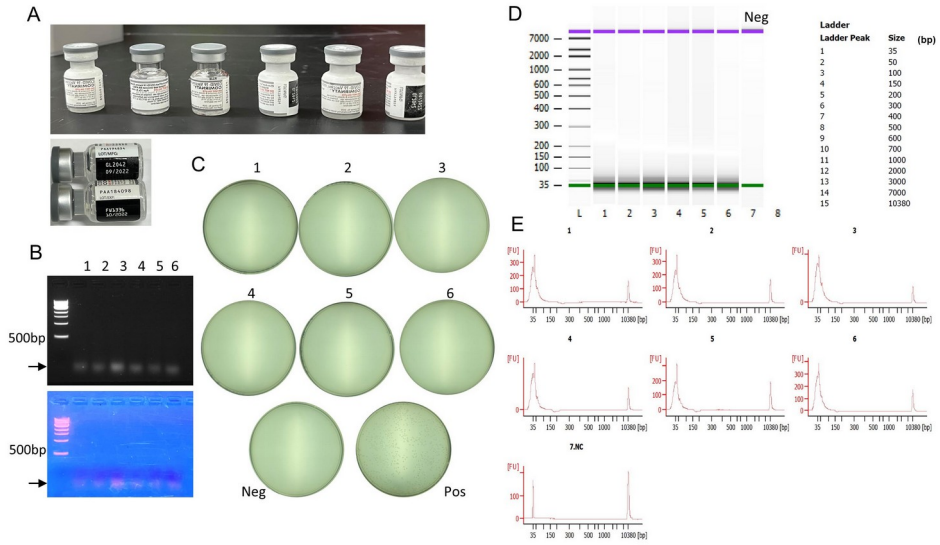


Figure 5. DNA analysis of Pfizer COVID-19 mRNA vaccines. A, six samples from two different Pfizer mRNA vaccine lots (Lot PAA194854, monovalent; PAA184098, bivalent). B, gel electrophoresis images of DNA extracted from one human dose (30 µg) of Pfizer vaccines. C, colony formation. D, high-sensitivity DNA assay by Agilent 2100 bioanalyzer. The DNA ladder contains 15 DNA fragments with indicated sizes. E, DNA size analysis on bioanalyzer. The 35- and 10380-bp DNA ladders were added to every sample as controls.

Shown in Table 1, the amount of total DNA, detected by NanoDrop, from an equivalent of one human dose mRNA vaccine fell into the range of several thousand nanograms. The amount of total DNA, quantified by the more accurate Qubit dsDNA HS assay, ranged from 40-110 nanograms. Overall, we concluded that there was significant amount of DNA from the

Pfizer mRNA vaccine equivalent to one human dose (Figure 6). However, the DNA was nearly all composed of small fragments of ~ 35 bp in length. Once again, this finding was consistent with a fully executed DNase I treatment of the commercial mRNA vaccine.

Table 1. Amounts of residual DNA in Pfizer COVID-19 mRNA vaccines

Sample #	Lot	Nanodrop (ng/µL)[1]	Total DNA (ng)[1]	Qubit dsDNA HS assay (ng/µL)[2]	Total dsDNA (ng)[2]	Fragment Size (Agilent 2100 Bioanalyzer)[3]	#colony
1	mono	69	3450	1.34	67	Peaks around 35bp, <100bp	0
2	bi	74	3700	0.968	48.4		
3	mono	131	6550	2.19	109.5		
4	bi	95	4750	1.37	68.5		
5	mono	82	4100	0.828	41.4		
6	bi	72	3600	1.34	67		

[1]Nanodrop is a spectrometer that measures DNA concentration based on UV absorbance, which does not distinguish DNA or RNA. It's also easily affected by free nucleotides, salts, and organic compounds. [2]Qubit uses fluorometric dye for specific quantification of dsDNA. [3]Refer to Figure 5D. Monovalent (mono) vaccine contains the Wuhan variant spike protein sequence, whereas the bivalent (bi) vaccine contains both the Wuhan variant and Omicron BA.5 variant spike sequences.

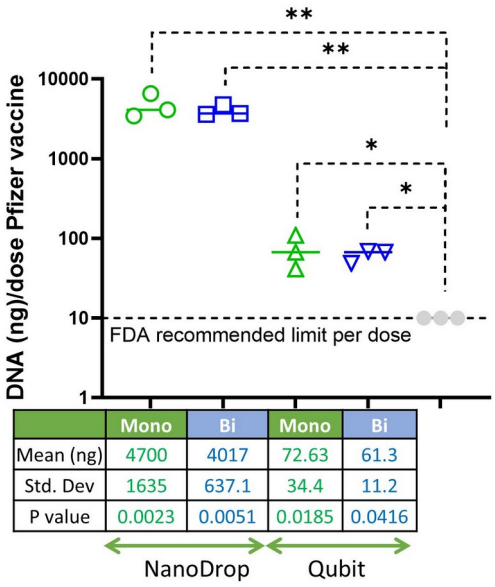


Figure 6. DNA amounts from 30 µg Pfizer mRNA vaccines measured by NanoDrop and Qubit.

Discussion

Not more than 10 ng/dose (<10 ng/dose) is a limit of cell substrate derived residual DNA recommended by the WHO guideline for parenteral viral vaccines produced using continuous non-tumorigenic cell lines

(https://cdn.who.int/media/docs/default-source/biologicals/cell-substrates/cells.final.mtgrep.ik.26_sep_07.pdf?sfvrsn=3db7d37a_3&download=true). The residual DNA in the COVID-19 mRNA vaccines, however, is derived from digested and undigested plasmid DNA (pDNA) template from which the mRNA is transcribed. When the pDNA template does not contain eukaryotic promoters or replication origins, the potential harm caused by the pDNA fragments may be lower than that derived from cellular DNA. Nonetheless, establishing a limit for pDNA in each dose of the mRNA vaccine ensures manufacturing consistency. A recent report suggested that the quantitative PCR (qPCR), commonly used by industry to detect residual DNA, may have underestimated the actual amount because smaller fragments may not be readily amplified during PCR (16). The authors reported that there appeared to be a correlation between the amount of DNA and the number of self-reported severe adverse events (SAEs). Depending on the location of the primers and probes, qPCR amplicons are typically between 100-200 bp. If the size of the amplicon decreases by 10-fold (i.e., 10-20bp), qPCR is anticipated to miss a significant amount of DNA. The relationship between DNA impurity per dose and the number of SAEs certainly warrants further investigation.

In this study, residual DNA was detected from six vials of two different lots of Pfizer COVID-19 mRNA vaccines. The estimated amount of residual DNA in one human dose appears to be 6 to 470 times 10 ng. This amount is slightly less than reported by another group (16). Different DNA extraction and quantification methods may account for the discrepancy. In support, we noted a significant difference between the amounts of DNA measured by NanoDrop or Qubit dsDNA HS assay. Nanodrop is a spectrometer that measures DNA concentration based on UV absorbance, which does not distinguish DNA or RNA. It is also easily affected by free nucleotides, salts, and organic compounds. Although we performed DNA extraction using a commercial kit that includes RNase in the reagent, we were unable to verify the extent of removal of RNA. Hence, it is possible that there was a measurable amount of RNA (from the mRNA vaccine) or free nucleotides, which increased the readings for the NanoDrop method. By contrast, Qubit uses a fluorometric dye for specific quantification of dsDNA, which is more specific for DNA quantification, although Qubit could also lead to overestimation of the amount of DNA if the dye intercalates into the mRNA or samples are improperly prepared, which has been reported by others (17). In our study, a Pfizer biosimilar mRNA vaccine at a DNA concentration of 16 ng/uL contained transformable pDNA, whereas the Moderna biosimilar and in-house mRNA standard did not, despite DNA concentrations of 26 and 95 ng/uL, respectively. It should be noted that the ability to form antibiotic-resistant colonies is not necessarily correlated with the concentration of DNA in the samples. One product may contain more DNA, but if the DNA is thoroughly digested (linearized), it would not form colonies.

What was reassuring was that with both gel electrophoresis and with the Agilent 2100 bioanalyzer, we found that the size of the

residual DNA in Pfizer mRNA vaccines was well below 100 bp. Our finding contrasts with the reported longest read of 3.5 kb, detected by nanopore sequencing, from the Pfizer children's monovalent Lot FL8095 (18), highlighting that the method of detection could lead to significant differences. In our study, no replication-competent DNA was recovered from the Pfizer mRNA vaccines, although it was detected sporadically from an in-house mRNA vaccine and a biosimilar vaccine. These findings highlight the rigorousness of the commercial manufacturing process in that large pDNA templates appear to be completely cleaved into smaller pieces, incapable of replication.

The potential health risk posed by residual small DNA fragments is currently unknown. Theoretically, DNA fragments can be directly integrated into host genome, increasing the risk of insertional mutagenesis. Alternatively, DNA fragments may contain oncogenes that may induce carcinogenesis if ingressed into host cells. Although it is possible for a DNA fragment as short as 7 bp to integrate into the genome, there has been no evidence of integration to a sensitivity of about one copy/microgram DNA, which is at least three orders of magnitude below the spontaneous mutation frequency (19). Although some investigators have reported the presence of larger DNA fragments with SV40 promoter/enhancer from the commercial mRNA vaccines (18), our results showed the efficient digestion of plasmid DNA in Pfizer COVID-19 mRNA vaccines. The SV40 promoter-ori DNA consists of a 17-bp A-T-rich sequence, three copies of a G-C-rich 21-bp repeat, and two copies of a 72-bp repeat (20). Since we only detected DNA fragments < 35 bp in our study, it is practically unlikely for these broken pieces of SV40 promoters to be functional. The plasmid DNA template does not contain oncogenes. Therefore, it is less likely that these DNA fragments will be oncogenic or infectious. Smaller DNA fragments can be immunostimulatory, contributing to local reactions after vaccination. Future research is warranted to address such a concern.

Our study is limited by sample size. It is conceivable that testing more commercial mRNA vaccines will build public trust and accumulate data to enable setting a statistically confident limit for pDNA.

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