

RNA:DNA Hybrids Survive Digestion in mRNA Vaccine Manufacturing

Kevin McKernan¹, Charles Rixey¹, Jessica Rose¹

Abstract

The process of mRNA vaccine manufacturing relies on proper DNA digestion following an in-vitro transcription reaction to remove residual contaminating DNA from the plasmid backbone from the process. To assess the quality and quantity of potential DNA impurities in mRNA vaccines, we analyzed unopened, cold-chain compliant vaccine lots for residual DNA contamination using quantitative PCR (qPCR), RNase A/Qubit fluorometry, and Oxford Nanopore sequencing from two Pfizer and three Moderna vials. We compared spike-region amplicons and plasmid-vector amplicons to distinguish between DNA contaminant as double-stranded DNA (dsDNA) versus RNA:DNA hybrids. qPCR

assays revealed more than a 100-fold discrepancy in quantitation between dsDNA with RNA:DNA hybrids consistent with uneven DNase I digestion efficiency during mRNA vaccine manufacturing. Indeed, treatment of vaccines with DNase I-XT resulted in 100-1000X higher degradation of spike DNA, particularly in plasmid regions that form RNA:DNA hybrids. Together, these results indicate that residual DNA testing which relies on a single qPCR for dsDNA fails to accurately quantify impurities, and that treating vaccine preparations with DNase I-XT during the manufacturing process may improve the quality by reducing contamination due to RNA:DNA hybrids.

Keywords: mRNA vaccines, residual DNA, RNA:DNA hybrids, DNase resistance, quality control, qPCR

Introduction

Several independent studies have raised concerns regarding residual DNA contamination in mRNA vaccines. (1-6) Konig et al. used fluorometric quantification to estimate residual DNA levels but did not include RNase A controls, leaving open the possibility of intercalating dye cross-reactivity with RNA. (2) Kammerer et al. addressed this limitation by incorporating RNase A digestion and multiple

intercalating dyes, while also demonstrating that SV40 promoter sequences from vaccine DNA persisted through several cell passages following transfection with the vaccines. (4) Wang et al. also detected substantial DNA contamination but dismissed its biological significance based solely on fragment size, without considering that lipid nanoparticles (LNPs) can alter the uptake and persistence of such fragments. (5) In contrast, Kaiser et al. attempted to refute these results, but their use of ethanol precipitation and phenol-chloroform extraction likely removed low-molecular-weight DNA, biasing the results toward apparent purity. (6)

Regulatory agencies have largely accepted sponsor-supplied data or, when performing independent analyses, relied on a single qPCR assay targeting the KAN resistance gene within the plasmid backbone. (7,8) This approach is problematic. Moderna's own patents acknowledge that qPCR cannot capture the full spectrum of plasmid DNA species. (9) Fragments shorter than the amplicon or lacking primer-

¹ Medicinal Genomics, Beverly, MA, USA

Corresponding author:

Kevin McKernan
Medicinal Genomics, Beverly, MA, USA
Email: Kevin.McKernan@medicinalgenomics.com

binding sites, such as background *E. coli*-sourced DNA, remain undetected. Because these assays amplify only a 100-200 bp region, total DNA content is inferred from one locus under the assumption that all plasmid regions persist at equivalent copy number. This assumption does not hold following DNase I treatment.

DNase I digestion is inherently non-uniform (**Figure 1**). Lenk et al. and Sutton et al. demonstrated that RNA:DNA hybrids generated during in vitro transcription (IVT) resist DNase I digestion, as the specific activity of DNase I for RNA:DNA hybrids is at least 100-fold below that for dsDNA. (10,11) Based on plasmid annotations, approximately 55% of the DNA template used for mRNA transcription corresponds to the T7 polymerase transcription product (the 4,284 bp spike insert of a 7,810 bp plasmid). These transcribed regions are expected to form RNA:DNA hybrids that are protected from DNase I cleavage, whereas fully double-stranded regions such as the KAN backbone are more readily digested. This protection may be further stabilized by the more than 800 N1-methyl-pseudouridine modifications in each mRNA transcript, (11) which promote RNA:DNA hybrid stability.

Consequently, when qPCR assays target DNase-labile regions such as KAN, they underestimate total DNA contamination by more than an order of magnitude. Pfizer's regulatory submissions to the EMA include a validated qPCR assay designed to confirm successful cloning of the spike insert into the pcDNA3.1-like plasmid, yet these results are not reported for quantitation. Instead, DNA measurements focus on the KAN locus, the region most susceptible to DNase I digestion. This methodological bias likely contributes to the discrepancy between regulatory measurements and independent reports describing persistent spike nucleic acids detected beyond 48 hours post-vaccination. (13–20)

Results

Use of RNase A in fluorometric analysis revealed residual DNA levels 15–48 times higher than the FDA's recommended limit of 10 ng per dose (**Figure 2**). (23,24) Both 95°C heat treatment and 1% Triton X-100 increased measurable DNA for both vaccine brands. Fluorometry performed in the absence of RNase A produced apparent DNA concentrations approximately an order of magnitude higher. While cross-reactivity between RNA and DNA is a known artifact of minor-groove binding fluorescent dyes, manufacturers typically report this effect as being under 7% at 10ng/ul DNA and

100ng/ul RNA. (2)

However, these cross-reactivity studies were conducted using natural single-stranded RNA and did not account for the extensive secondary structure engineered into N1-methyl-pseudouridine-modified mRNAs. Codon-optimization algorithms used in the modRNA field intentionally promote rod-like RNA folding and high double-stranded content, increasing the likelihood of minor-groove dye interaction. (18) The manufacturer's stated cross-reactivity specifications may therefore require recalibration when applied to highly structured or chemically modified RNA molecules that exhibit elevated melting temperatures.

qPCR analysis using DNase I and DNase I-XT on Triton X-100-treated vaccine preparations demonstrated a distinct DNase I-resistant region within the plasmid spike insert (**Figure 3**). This region is expected to form RNA:DNA hybrids due to the abundance of complementary mRNA in each dose. Standard DNase I cannot efficiently digest DNA within RNA:DNA hybrids, whereas DNase I-XT is specifically engineered for this purpose. In all five vaccine lots tested, DNase I produced only marginal reductions in spike-region DNA, while consistently degrading DNA originating from the plasmid's origin of replication. In contrast, DNase I-XT achieved more complete digestion across both regions, supporting the interpretation that much of the residual spike DNA persists in RNA-hybridized form.

This differential digestion significantly affects quantitative estimates of DNA contamination, making qPCR results highly dependent on the choice of assay target.

RNaseA-qPCR was also performed in triplicate and at 3 different dilutions (1X, 1:10, 1:100) to record 9 datapoints for each channel across 2-3 different assays (**Figures 4A & B**). These qPCR assays contained a final concentration of 0.5% TritonX-100 to enable dissolution of the LNPs and RNaseA activity prior to qPCR. In addition to heat killing the RT reaction we have also added RNaseA to ensure no interference with off-target nucleic acids.

Qubit and qPCR results were compared with 1:10 dilution qPCR data versus the final RNaseA treated Qubit results. The Cq scores and nanogram quantitation for these more recent vials are more contaminated than was observed with Speicher et al. using the same qPCR assay. (1) Both Pfizer lots are over the limit in the context of both qPCR and Qubit methods. All 3 Moderna lots are over the limit via Qubit but Moderna 025G23A passed the Ori qPCR

while exceeding the limit with the Spike qPCR assay (**Figure 5**). This demonstrates that qPCR is target dependent and highly influenced by the selection of the assay used and Qubit fluorometry, while not immune to RNA:DNA dye intercalation bias, provides more consistent quantitation across samples when compared to various qPCR assays (**Figure 5**).

Oxford Nanopore (ONT) sequencing further revealed numerous fragments exceeding 200 bp, including one read 5,284 bp in length that encompassed a large portion of the spike gene. Although qPCR showed large differences in quantitation depending on which assay was used, sequencing confirmed that both spike and Ori DNA persists in the vaccines, and the presence of long DNA fragments highlights the limitations of inferring fragment lengths from Cq values alone (**Figure 6**). Long DNA fragments may be underestimated by qPCR if they are not efficiently amplified, yet such fragments are detectable by sequencing and may have greater biological relevance due to their potential for cellular uptake and genomic integration. These findings emphasize the importance of using complementary methods — RNaseA-Fluorometry, multi-loci qPCR for quantitative screening and sequencing for detailed characterization — when assessing residual DNA in mRNA therapeutics.

Methods

RNase-Qubit Fluorometric DNA Quantification

DNA quantification was performed using the AccuGreen High Sensitivity DNA Quantitation Reagent (Biotium) following the manufacturer's protocol, with modifications to assess RNase sensitivity and detergent-mediated lipid nanoparticle disruption.

For each sample, 1 μ L of vaccine material was added to 199 μ L of AccuGreen® reagent. In parallel, a 1% Triton X-100 treatment was prepared by mixing 10 μ L of 10% Triton X-100 with 90 μ L of vaccine prior to dilution in the AccuGreen® reagent. The mixture was vortexed and immediately measured using a Qubit fluorometer (ThermoFisher Scientific).

To evaluate heat and RNase sensitivity, the vaccine-reagent mixture was heated to 95 °C for 1 minute, cooled immediately on ice, vortexed, and measured again using the Qubit fluorometer. Following the heat step, 1 μ L of RNase A (20 mg/mL, New England Biolabs) was added to the same sample, incubated at 37 °C for 10 minutes, and fluorescence was measured a final time.

RNase-qPCR Assay

Quantitative PCR (qPCR) assays were performed using a modified RNase pretreatment protocol to distinguish DNA-derived signals from potential RNA contamination. All polymerase mixtures were heat-treated at 95 °C for 5 minutes to deactivate RT activity prior to sample addition.

Following heat treatment, each vial 420 μ L polymerase vial received 42 μ L of 50 μ M primer (targeting either Spike/Ori or SV40 in **Table 1**), 42 μ L of 10% Triton X-100 (final in PCR is 0.5% TritonX-100), 235 μ L of nuclease-free water, and 10.5 μ L of RNase A (20 mg/mL). The samples were mixed by gentle pipetting. Polymerase reagents used are sourced from Medicinal Genomics PathoSEEK amplification mix (MGC part#420207). Primers and Probes were previously published by Speicher *et al.* (1) Spike and Ori are multiplexed in FAM and HEX respectively and SV40 is singleplex in Texas Red. Samples were cycled at 95 °C for 1 minute followed by 39 more cycles of 95 °C for 10 seconds and 65 °C for 40 seconds on a BioRad CFX for 3 channel detection.

DNase I and DNase I-XT qPCR Assay

To evaluate the impact of DNase treatment on nucleic acid detection, DNase I and DNase I-XT (New England Biolabs, NEB #M0303S, NEB #M0570) were each diluted 1:50 in their respective reaction buffers and added to vaccine preparations pretreated with 1% Triton X-100.

For each reaction, 1 μ L of diluted DNase I or DNase I-XT was added directly to the 17 μ L qPCR master mix containing primers and probes targeting the vaccine Spike and bacterial origin of replication (Ori) loci. Reactions were incubated for 10 minutes at 37 °C to allow nuclease activity, followed by heat inactivation at 75 °C for 10 minutes. Amplification was subsequently carried out using the same thermocycling protocol and polymerase reagent treatment as described for the RNase-qPCR assay.

Vaccine Preparation Protocol for ONT sequencing

Vaccine extractions were performed using a modified single-tube protocol optimized for nucleic acid recovery from lipid nanoparticle formulations. All steps were conducted using nuclease-free reagents and disposables.

Briefly, two identical extraction tubes were prepared in parallel and combined at the final elution step to yield a total volume of 50 μ L. For each tube, 400 μ L of vaccine material was mixed with 40 μ L

of 10% Triton X-100 (final concentration 1%) to disrupt lipid nanoparticles and facilitate nucleic acid accessibility.

Next, 800 μ L of SenSATIVAX® reagent was added to each tube, and the samples were mixed thoroughly by vortexing or gentle pipette agitation to ensure homogeneity. The mixture was incubated at room temperature for 10 minutes to allow binding of nucleic acids to the magnetic beads present in the SenSATIVAX® reagent. Following incubation, the beads were captured using a magnetic separation stand, and the supernatant was carefully discarded.

The beads were washed twice with 1 mL of 70% ethanol, taking care to avoid bead disruption between washes. Following the final wash, all residual ethanol was removed, and the pellet was air-dried for 5 minutes at room temperature to ensure complete evaporation of ethanol. Nucleic acids were eluted in 25 μ L of nuclease-free water. To remove residual RNA, 1 μ L of RNase A (20 mg/mL, New England Biolabs) was added to each eluted sample and incubated according to the manufacturer's instructions. The two parallel extractions were then pooled to yield a final volume of approximately 50 μ L, which was used as input for Oxford Nanopore library preparation.

Oxford Nanopore Sequencing

Sequencing libraries were prepared using the Oxford Nanopore Technologies (ONT) Ligation Sequencing Kit V14 according to the manufacturer's protocol, with four modifications to optimize yield from fragmented DNA samples.

First, the initial solid-phase reversible immobilization (SPRI) purification after end repair was performed using 90 μ L of magnetic beads instead of the standard 60 μ L to enhance DNA recovery. The second SPRI cleanup after ligation also used increased bead volumes proportional to sample input. Second, the end-repair and dA-tailing reaction time was extended to 20 minutes to accommodate the high number of molecular termini present in fragmented DNA. Finally, the adapter ligation step was prolonged to 30 minutes to maximize adapter ligation efficiency on short DNA fragments. Libraries were sequenced on an R10.4.1 flow cell using a MinION device and basecalled using the Durado basecalling model
model dna_r10.4.1_e8.2_400bps_sup@v5.2.0_4mC_5mC
@v1 and
dna_r10.4.1_e8.2_400bps_sup@v5.2.0_6mA@v1.

Reads were aligned using minimap2 to NCBI reference OR134577.1 and PV602126.1. SNAPgene

was used to generate **Figure 6** from read ID >073d6bcb-ceea-4679-a12a-4c8ca4209a06 Len=5283 run on flow cell FBE77080.

Discussion and Conclusions

Our findings reveal that residual DNA quantification in mRNA vaccines varies by more than two orders of magnitude depending on the choice of assay target and nuclease treatment conditions. This discrepancy is not merely a technical artifact but reflects a fundamental biochemical reality: RNA:DNA hybrids formed between vaccine mRNA and template DNA resist standard DNase I digestion. When regulatory assays rely exclusively on amplifying DNase-sensitive loci, they systematically underestimate the total burden of residual plasmid DNA.

The data demonstrate that DNase I-XT, an engineered nuclease capable of degrading RNA:DNA hybrids, substantially improves DNA removal and detection accuracy across all plasmid loci tested. (10) This highlights a critical gap in current manufacturing processes: DNase I, the enzyme specified in regulatory guidance for template DNA removal, is inadequate for mRNA vaccines because it cannot efficiently cleave the very regions most likely to be transcribed -- and therefore most likely to form protective hybrids with the product mRNA.

One surprising finding in our results is that the SV40 amplicon provides lower CTs (more DNA) than the Ori amplicon in the Pfizer plasmids. This may be due to the GC-box in the SV40 amplicon creating R-Loops or secondary structures that also resist DnaseI. (23) It is possible that there are several microhomologies with this GC-box and the GC codon optimized BNT162b2 sequence that could be further stabilized with N1-methyl-pseudouridine RNA. Further work is required to understand this differential signal but this underscores the vulnerability in using a single assay to survey DNA concentration post DnaseI treatment.

Fluorometric analysis with RNase A corroborated the qPCR findings, revealing DNA quantities 15–48 times greater than the FDA's stated 10 ng per dose limit. The fact that Triton X-100 and heat pretreatment increased fluorescence supports the interpretation that residual DNA is at least partially sequestered within lipid nanoparticles.

Oxford Nanopore sequencing provided independent confirmation of the presence of long DNA molecules, including one fragment exceeding 5 kb and containing a large portion of the spike sequence.

While sequencing alone does not yield precise quantitation, it verifies that qPCR-amplifiable sequences are indeed present and that at least some DNA molecules are far longer than the 200 bp fragments often assumed in regulatory assessments. Such long fragments may be more biologically relevant due to their higher likelihood of expression if taken up by host cells.

We believe these results are more parsimonious than previous Qubit vs qPCR studies published by Speicher *et al.*, (1) likely because these vials are less fragmented. When fragmentation pushes the bulk of the DNA below the amplicon size, we expect to see large discrepancies in Fluorometry vs qPCR quantitation and more discordance with studies that use DNA purification kits prior to quantitation as these kits are designed to eliminate small DNA.

Together, these findings underscore the need for a multi-locus, multi-method approach to residual DNA quantitation in mRNA therapeutics. (1–6) Reliance on a single amplicon-based assay is insufficient when different regions of the same plasmid exhibit vastly different DNase susceptibilities. Regulatory agencies should consider requiring quantitation of both DNase-labile and DNase-resistant regions, alongside orthogonal methods such as fluorometry with appropriate RNase controls and fragment-length analysis by sequencing.

From a regulatory perspective, existing EMA documentation acknowledges the availability of validated qPCR assays targeting the spike insert. (7) However, these assays are not routinely used for quantitation." The rationale for this omission deserves scrutiny.

If a spike-targeting assay exists and has been validated, questions arise about why it would not be similarly employed for DNA quantitation, particularly when qPCR is known to be amplicon-dependent and the cross-reactivity with RNA fluorometry is measurably worse than DNA fluorometry. (2,26,27) The present study suggests that such dual quantitation would reveal DNA levels significantly higher than those currently reported and even exceeding accepted thresholds.

Guetzkow *et al.* raised concerns regarding process changes that occurred between clinical trial material and commercial vaccines. (24) It is also perplexing that one would use a KAN assay for Process 1 DNA contamination quantitation as this region of the plasmid is not amplified with PCR in that process. This would make an invalid estimate of any post PCR estimate of DNA as the KAN gene is not part of your amplified target.

A common criticism of using fluorometry for DNA measurements in RNA vaccines is addressed with adequate use of RNase A. It should also be reconciled that Fluorometry is used to measure the dose of the mRNA in the vaccines according to EDQM protocols which also include the addition of TritonX-100 to disrupt the LNPs. (2,25) If this is a validated procedure for measuring the mRNA dose, it is unclear why it would not be similarly employed for DNA quantitation, particularly when qPCR is known to be amplicon-dependent and the cross-reactivity with RNA fluorometry is measurably worse than DNA fluorometry.

Fluorometry is used to measure the dose of the RNA with RiboGreen® and Jones *et al.* has demonstrated that RiboGreen® has more cross-reactivity with DNA than DNA staining dyes have with RNA. The cross-reactivity considerations should be applied consistently across both RNA and DNA quantitation methods. (26) RiboGreen® provides twice the amount of signal for 10ug/ml of DNA compared to 10ug/ml RNA, whereas the cross talk with DNA staining dyes like PicoGreen® is reported to be only 7% at 10 ng of DNA with 100 ng of RNA. (2) Our RNase A studies show a higher cross-reactivity with modRNA but it is addressable with nucleases. Thus, the use of Fluorometry to measure the dose of the RNA should also be used to measure the dose of the DNA. Inconsistent application of different assays could compromise the ratio metric guidelines used by the EMA.

It should be further noted that Georgiou *et al.* demonstrated that DNA treated with DNaseI only provides 30% of the signal of the same mass of DNA that is left undigested due to a DNA size dependent but non-linear fluorescence from intercalating dyes. (27) Based on this finding, our fluorometry readings may underestimate DNA levels present in these vaccines.

In conclusion, the data presented here demonstrate that residual plasmid DNA in mRNA vaccines is unevenly degraded by standard DNase I treatment, and that qPCR assays targeting different regions of the same plasmid can yield results differing by more than 100-fold ($>7\text{CT}$). These findings call for revised quality control strategies that incorporate multiple amplicon targets, orthogonal quantitation methods, and nucleases capable of degrading RNA:DNA hybrids. Only through such comprehensive testing can regulatory agencies and manufacturers ensure that residual DNA levels are accurately measured and appropriately controlled, thereby safeguarding vaccine quality and recipient safety.

The biological implications of LNP-encapsulated DNA fragments, particularly those derived from transcribed regions that resist standard nuclease digestion, warrant further investigation in the context of potential long-term effects, including genomic integration, immune activation, and unintended expression of plasmid-encoded sequences. (13–19,28) Given the potential for LNP-mediated transfection of residual DNA and the documented persistence of spike-derived nucleic acids beyond 48 hours post-vaccination, a comprehensive reassessment of current DNA quantitation standards and manufacturing controls for modRNA-LNP therapeutics is warranted to ensure patient safety and product quality.

Given these biological products were mandated in many jurisdictions — often liability free — and reached billions of people, the attention to quality control and GMPs must exceed the standards of pharmaceuticals targeting a subset of people. These products were administered universally to the elderly, infirm, pregnant women and infants. The transition from Process 1 to Process 2 manufacturing that occurred post-clinical trial represents a significant process change that warrants careful evaluation.

These modRNA products have generated substantial safety signals in post-market surveillance databases while being one of the more profitable pharmaceutical products ever released. In contrast, regulatory DNA quantitation quality control has relied predominantly on a single qPCR assay. The regulators had no problem demanding multi-loci qPCR for the SARS-CoV-2 virus to ensure it was never missed with S gene target failure, but they became quite comfortable relying on just one assay target for vaccine sponsor quality control.

One limitation of our study is the limited number of vials we have access to. We also cannot use the ONT data to quantitate the smaller fragments due to the limitations of nanopore sequencing. This study does not measure ssDNA which is expected to be present at equal molar concentrations in the Spike RNA:DNA hybrids and R-Loops. We also do not measure dsRNA which is an equally important side product expected with the codon optimizations used.

Our data indicate that residual DNA quantitation methodologies may require re-evaluation in the production process. A comprehensive assessment of the modRNA-LNP platform in the context of our findings appears warranted, with consideration of implementing improved quality control measures to address the issues identified in this study.

Summary/Impact Statement

Regulatory filings indicate that residual DNA testing typically relies on a single qPCR assay targeting the kanamycin (KAN) resistance gene within the plasmid backbone. Because this region resides in a DNase-sensitive portion of the vector, such testing underestimates residual DNA in sequences that remain RNA hybridized and DNase I-resistant, including the spike insert.

Data Availability

Fastq files are available:

Oxford Nanopore Data Pfizer LN2588 and GK0936 dataset 1:
<https://mega.nz/folder/oVJgDJiJ#dXUUCMmqIUF8RoZ8PC7Oug>

Oxford Nanopore Data Pfizer and Moderna pooled:
https://mega.nz/folder/9ExkxJbK#XUUYT_0uZ-unJ_HmJGc5VUA

Oxford Nanopore Data Pfizer LN2588 and GK0936 dataset 2:
<https://mega.nz/folder/5JZjyYYD#s1aabtu4ugsNvHbqh9rwug>

Qubit Data Sheet and qPCR Data Sheet:
https://mega.nz/file/YVJB2bTD#Qo59sbg-SiR4fMNYOAob-fPHGXPsl53M9q_1PKMWIb5ZE

Conflict of Interest Statements

Kevin McKernan is a founder and CSO of Medicinal Genomics and some of the reagents used in this study are manufactured by Medicinal Genomics.

Author Contributions

KJM - Lab work, analysis and manuscript preparation and final review

JR - Lab work, analysis and manuscript preparation and final review

CR - Lab work and final review

ChatGPT 5.0 and Claude Sonnet 4.5 were used to edit this article into Submission ready format.

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influence on the study design or conclusions of the study. The 3 authors donated their time and expertise for the project during holiday hours.

Table 1. Summary of various studies. Limitations and Strengths are the subjective opinion of these authors. * Speicher Over limits are Qubit measurement only.

1st Author	Prep	Detection	Over Limit (Fold)	Limitations	Strengths
Speicher	Direct:TritonX + 95C	RNaseA Qubit, qPCR, ONT	37-628X*	Replicas Qubits done on different days	Largest study but low SAEs lots
Konig	Direct:TritonX	No RNaseA Qubit	360-534X	No RNaseA/Limited Lots	1st Published Survey. Good summary of the regulations
Kammerer	Direct:TritonX	RNaseA Qubit 3X dyes & HEK293 SV40 PCR & ELISA & MS	3-4X	Limited lots, No Heat	3 different dyes & cell transfection tracks SV40 over many cell passages
Raoult	Direct:TritonX	Qubit & Illumina	516X	No RNaseA/No Heat/ Limited Lots	Illumina Sequence of monovalent lots
Kaiser	Size Selected:2X EtOH & Phenol CHCl3	RNaseA-Qubit & LCMS	At Limit	DNA prep may have lost small fragments- No Heat- Conflicts (DFG funding)	Multi-platform but all downstream of a poor prep
Wang	NEB Monarch-Size Selected: DNA prep	RNaseA Qubit & Uvspec & Agilent & Sanger	6-470X	DNA prep may have lost small fragments- No Heat	Multi platform. High Schoolers can find it.
Achs	Size Selected:2 DNA preps/TritonX	qPCR, Illumina, CE	Under Limit	DNA prep may have lost small fragments- Flawed qPCR inhibition testing, Large amplicons, errors in KAN primers, 100ul RNase- (Sensible Biotech)	Multi-platform but all downstream of a poor prep. High variance between amplicons. ILMN sequence = variable coverage
Buckhaults	Direct qPCR	8X qPCR and ONT	N/A	Not Published	confirms more Spike than Kan detection
TGA	N/A	Kan gene qPCR	N/A	Redacted, No Methods	Redacted, No Methods
Fleming	QiaGen	qPCR, Sanger	0.3X-100X	No SV40 Primers used yet claimed no SV40 detected	Also observes large Spike/Ori delta CT
This study	Direct: TritonX + 95C	RNaseA Qubit, 3X qPCR, ONT	15-43X	5 vials, No ssDNA or dsRNA measured with Qubit	{3X qPCR} {3X Targets} {3X Dilutions}, ONT, DNase1-XT

Table 2. qPCR Primer and Probe Sequences

Assay	Primer/Probe	Sequence (5' to 3')	Part Number
Spike	Forward	AGATGGCCTACCGGTTCA	MGC #100030
	Reverse	TCAGGCTGTCCTGGATCTT	
	Probe	/56-FAM/CGAGAACCA/ZEN/GAAGCTGATCGCCAA/3IAB-kFQ/	
Vector Origin	Forward	CTACATACCTCGCTTGCTAATC	MGC #100030
	Reverse	GCGCCTTATCCGGTAACATAC	
	Probe	/5HEX/AAGACACGA/ZEN/CTTATCGCCACTGGC/3IABkFQ/	
SV40 En-hancer/Pro-moter	Forward	GTCAGTTAGGGTGTGGAAAGT	MGC #100032
	Reverse	GGTGCTGACTAATTGAGATGC	
	Probe	/5TEX615/CCAGCAGGCAGAAGTATGCAAAGC/3IAbRQSp/	

Figure 1. Depiction of differential nuclease sensitivity with RNA:DNA hybrids

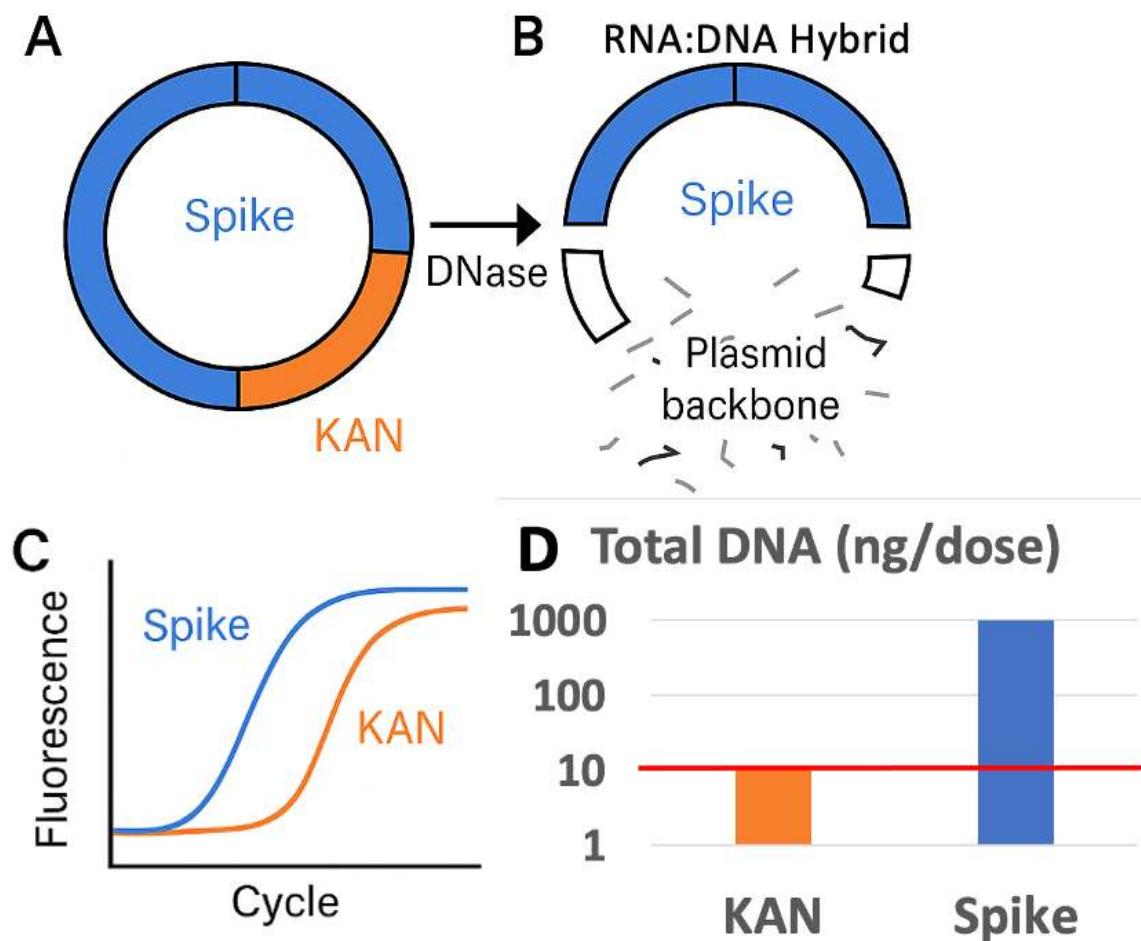


Figure 2. Qubit fluorometry of 2 Pfizer lots (LN2588 & GK0936) and 3 Moderna lots (025G23A, AW4694B, AT0709B). Samples were tested directly (Neat), after TritonX-100 treatment, 95 °C treatment and RNase A.

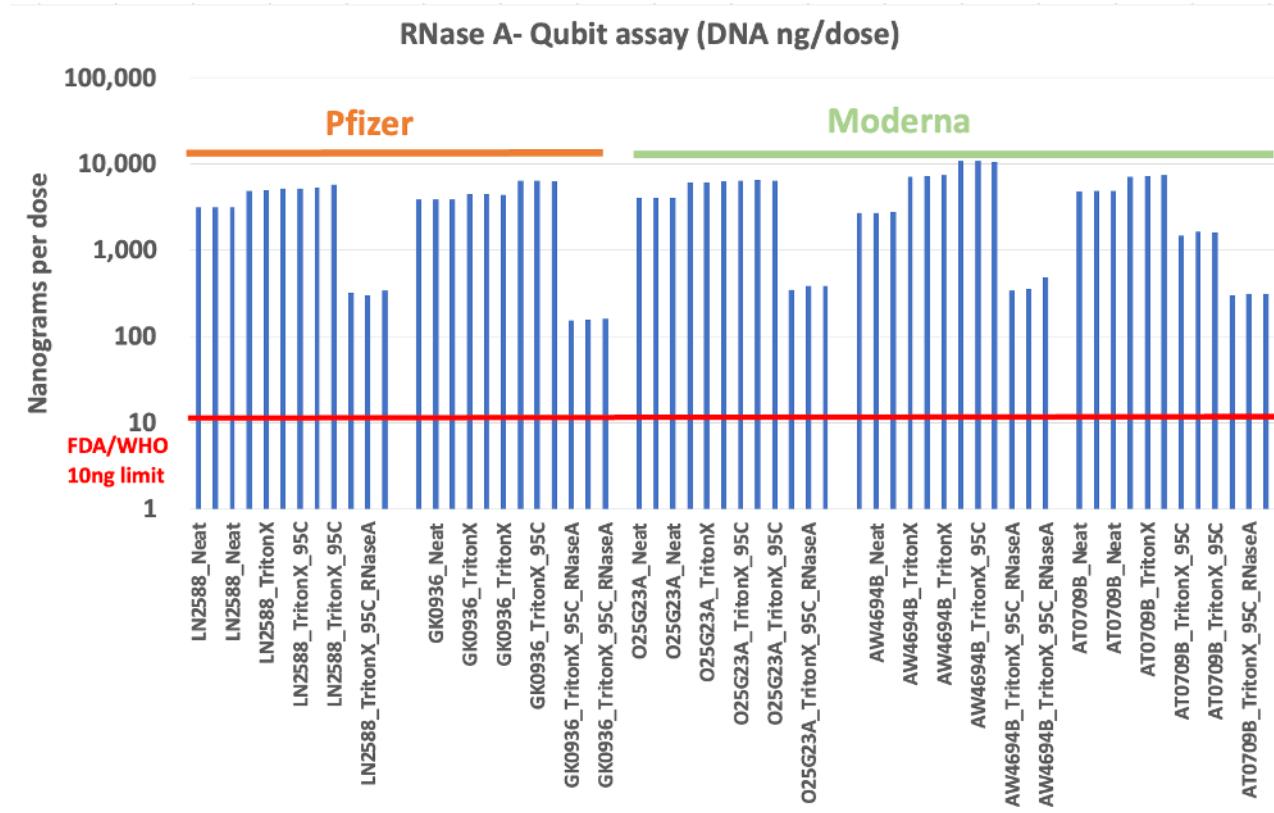


Figure 3. DNaseI/DNaseI-XT qPCR demonstrate differential nuclease sensitivity at 2 different loci in the plasmid (Spike, Ori)

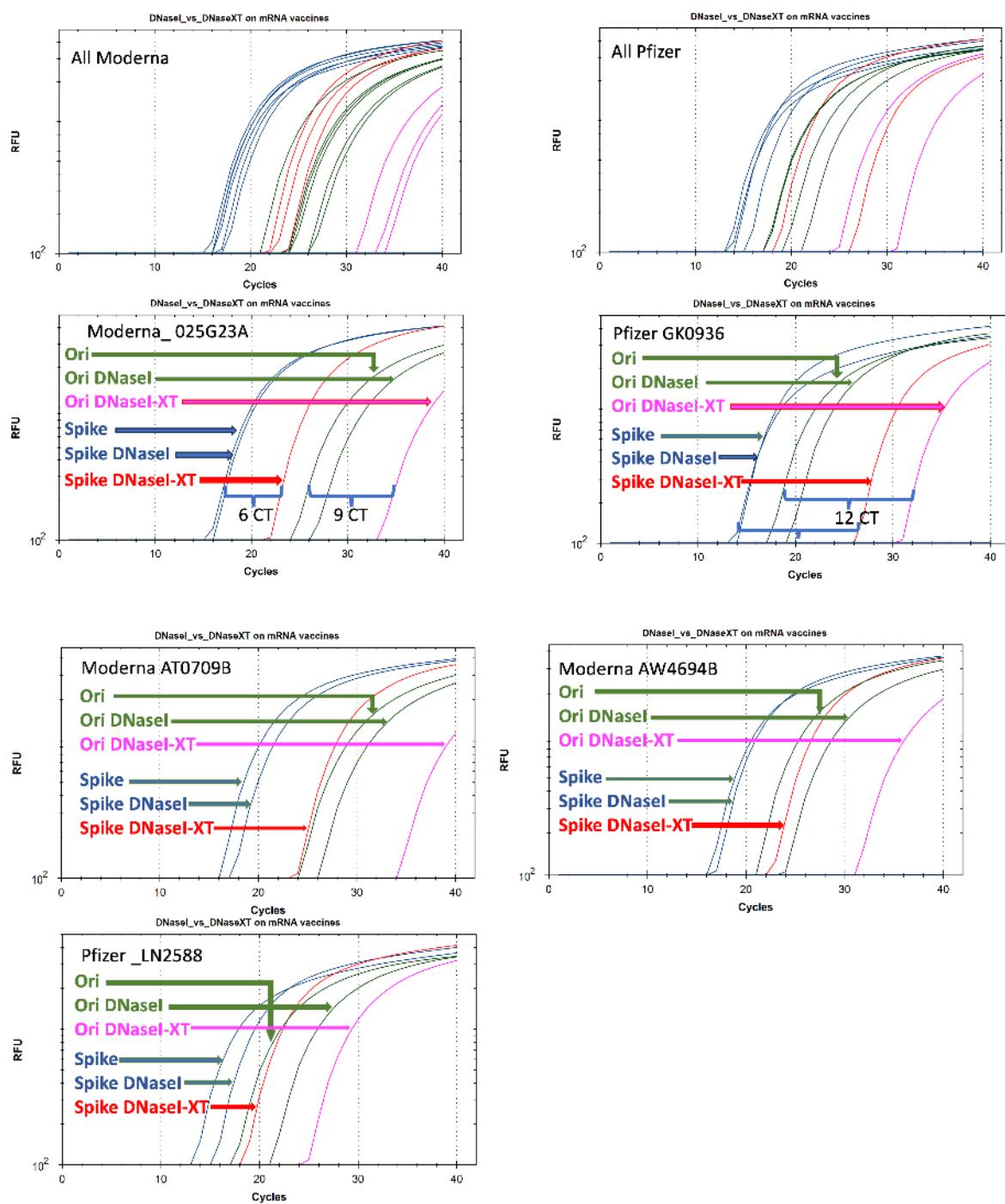


Figure 4A. qPCR performed in triplicate at 1X, 1:10 dilution and 1:100 dilution. 1:10 dilutions were used for further analysis.

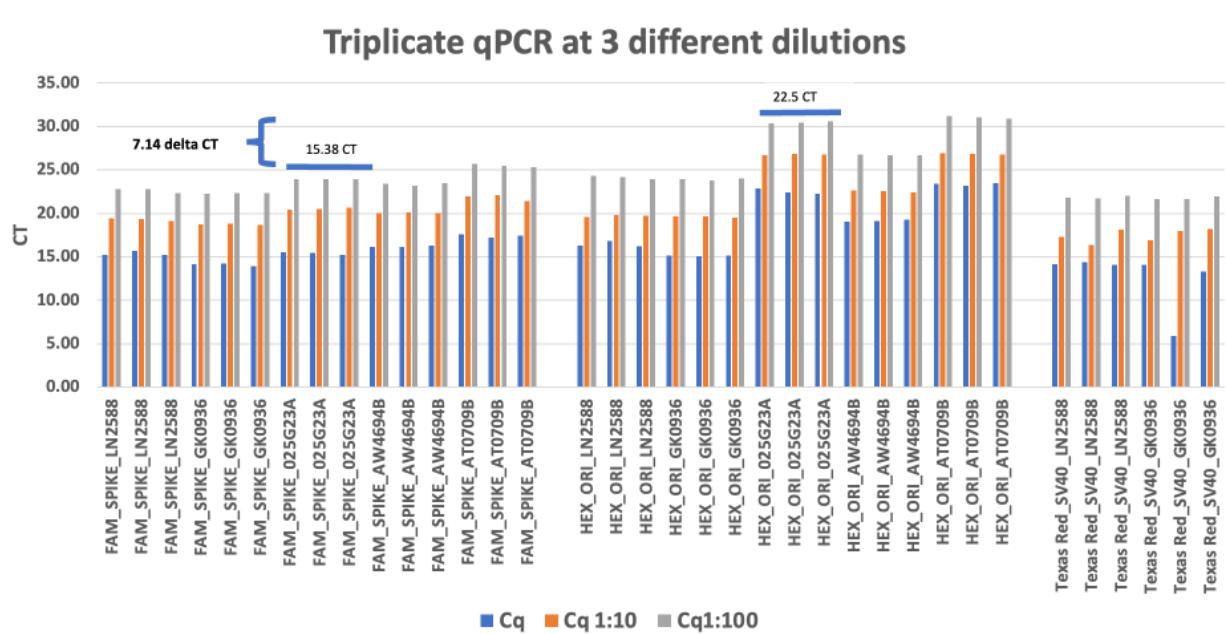


Figure 4B. Standard curve performed in triplicate across 5 Log scales for each assay with their respective efficiency, R^2 , slope and equation

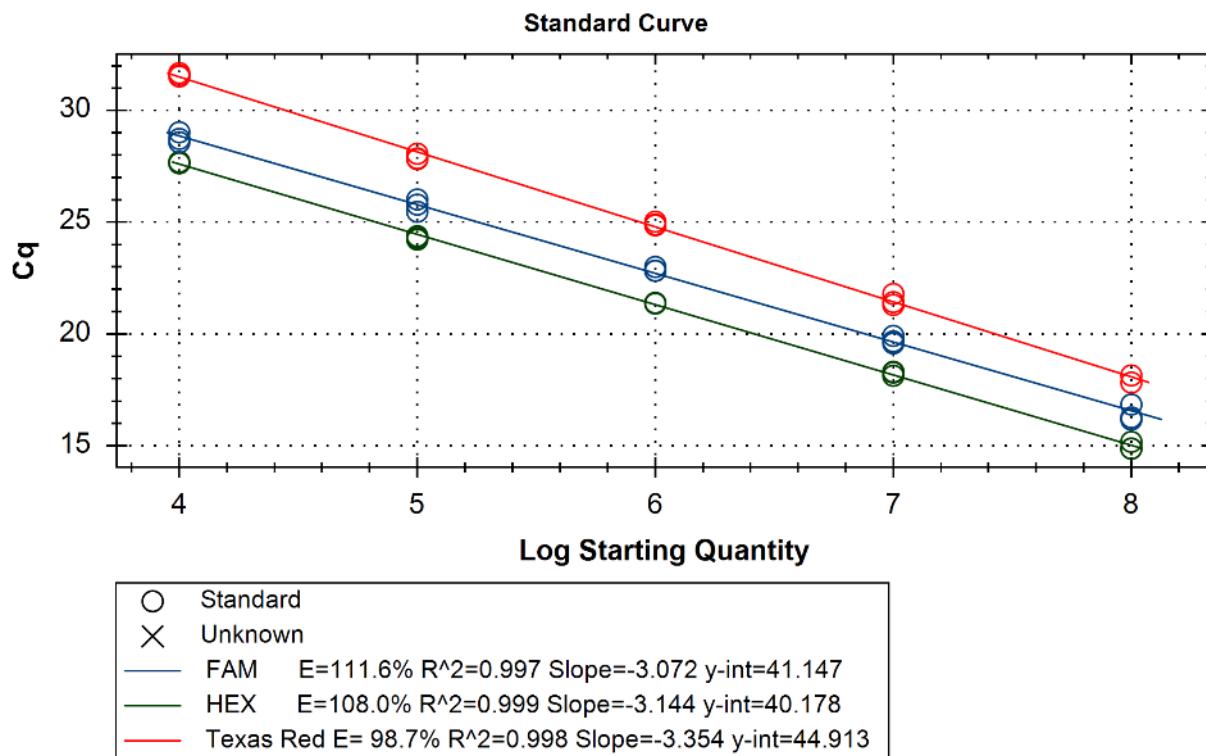


Figure 5. Comparison of Qubit versus qPCR quantitation. Qubit provides more consistent quantitation while qPCR is highly dependent on the assay used to quantitate the DNA.

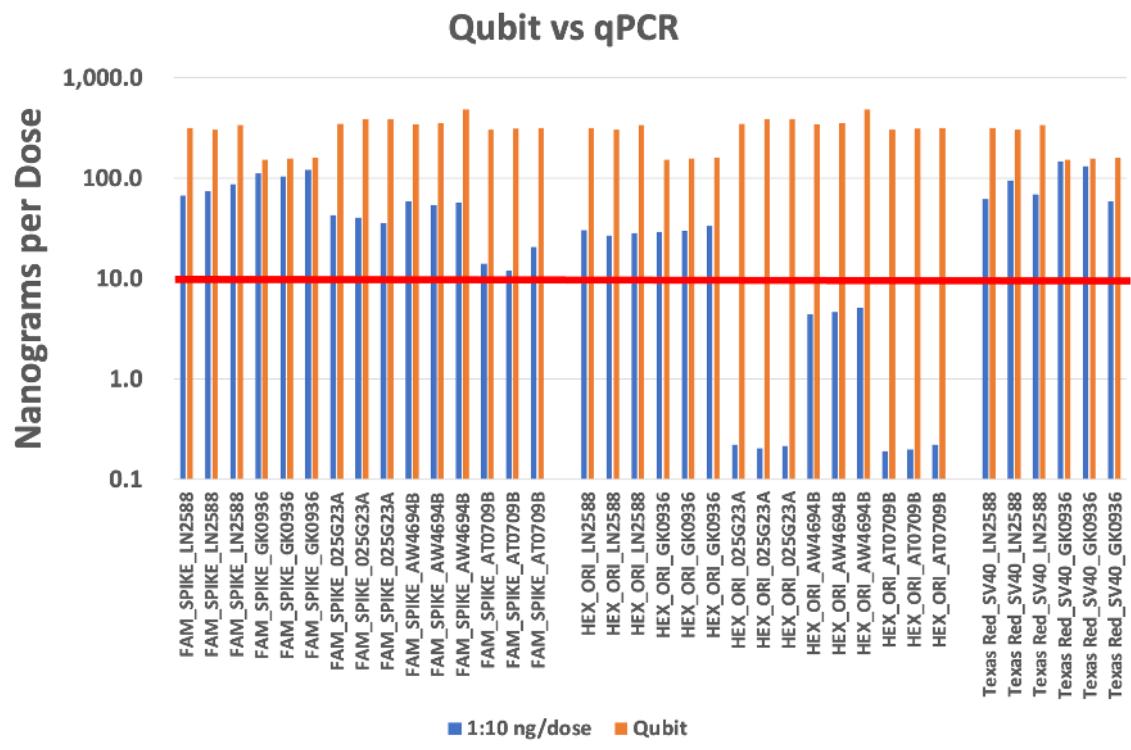
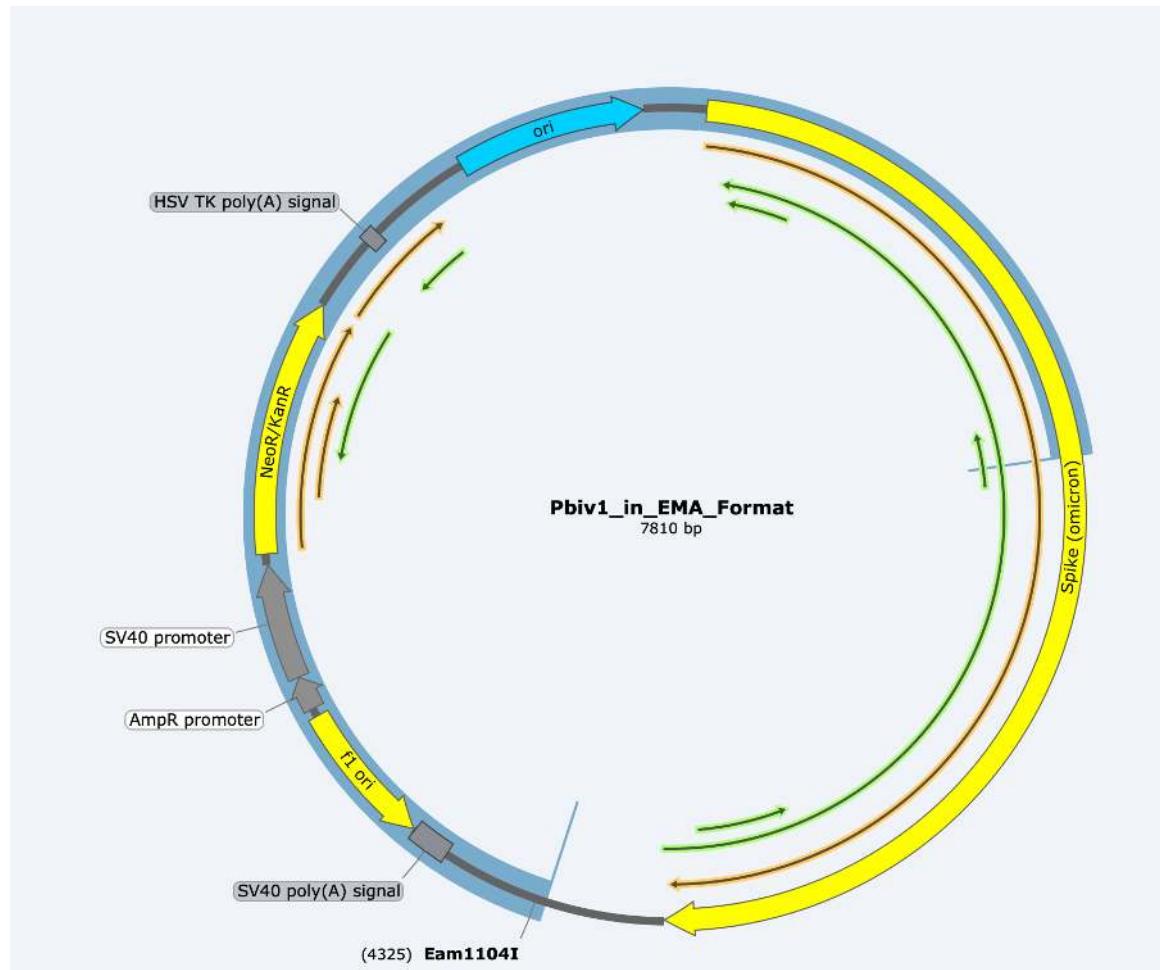


Figure 6. 5,283 base pair Oxford Nanopore read (blue highlight) from Pfizer lot



References

1. Speicher DJ, Rose J, McKernan K. Quantification of residual plasmid DNA and SV40 promoter-enhancer sequences in Pfizer/BioNTech and Moderna mRNA COVID-19 vaccines from Ontario, Canada. *Autoimmunity*. 2025;58(1):2551517. doi: 10.1080/08916934.2025.2551517
2. Konig B, Kirchner JO. Methodological Considerations Regarding the Quantification of DNA Impurities in the COVID-19 mRNA Vaccine Comirnaty. *Methods Protoc*. 2024;7(3):41. doi: 10.3390/mps7030041
3. König B, Kirchner JO. Communication on Methodological Considerations Regarding the Quantification of DNA Impurities in the COVID-19 mRNA Vaccine Comirnaty®. *Preprints*. 2024. doi: 10.20944/preprints202411.1912.v2
4. Kammerer U, Schulz V, Steger K. BioNTech RNA-Based COVID-19 Injections Contain Large Amounts Of Residual DNA Including An SV40 Promoter/Enhancer Sequence. *Science, Public Health Policy and the Law*. 2024;5:1-22. Available from: <https://publichealthpolicyjournal.com/biontech-rna-based-covid-19-injections-contain-large-amounts-of-residual-dna-including-an-sv40-promoter-enhancer-sequence/>
5. Wang TJ, Kim A, Kim K. A rapid detection method of replication-competent plasmid DNA from COVID-19 mRNA vaccines for quality control. *J High School Sci*. 2024;8(4):427-439. doi: 10.64336/001c.127890
6. Kaiser S, Kaiser S, Reis J, Marschalek R. Quantification of objective concentrations of DNA impurities in mRNA vaccines. *Vaccine*. 2025;55:127022. doi: 10.1016/j.vaccine.2025.127022
7. European Medicines Agency. Rapporteur's Rolling Review Report Quality - COVID-19 mRNA Vaccine BioNTech. 2021. Page 81. Available from: <https://www.covid-truths.co.uk/2021/04/ema-leaked-papers/>
8. Therapeutic Goods Administration. FOI 5286 - TGA KAN PCR documents. 2024. Page 5. Available from: <https://www.tga.gov.au/sites/default/files/2024-09/FOI%205286.PDF>
9. Moderna patent US10898574B2. Methods and compositions for treatment of disease. Available from: <https://patents.google.com/patent/US10898574B2/en>
10. Lenk R, Kleindienst W, Szabó GT, Baiersdörfer M, Boros G, Keller JM, Mahiny AJ, Vlatkovic I. Understanding the impact of in vitro transcription byproducts and contaminants. *Front Mol Biosci*. 2024;11:1426129. doi: 10.3389/fmolsb.2024.1426129
11. Sutton DH, Conn GL, Brown T, Lane AN. The dependence of DNase I activity on the conformation of oligodeoxynucleotides. *Biochem J*. 1997;321(Pt 2):481-486. doi: 10.1042/bj32104811
12. Parr CJC, Wada S, Kotake K, Kameda S, Matsuura S, Sakashita S, et al. N(1)-Methylpseudouridine substitution enhances the performance of synthetic mRNA switches in cells. *Nucleic Acids Res*. 2020;48(6):e35. doi: 10.1093/nar/gkaa070
13. Ota N, Itami M, Aoki T, Sakurai A, Fujisawa T, Okada Y, Noda K, Arakawa Y, Tokuda S, Tanikawa R. Expression of SARS-CoV-2 spike protein in cerebral Arteries: Implications for hemorrhagic stroke Post-mRNA vaccination. *J Clin Neurosci*. 2025;136:111223. doi: 10.1016/j.jocn.2025.111223
14. Castruita JAS, Schneider UV, Mollerup S, Leineweber TD, Weis N, Bukh J, et al. SARS-CoV-2 spike mRNA vaccine sequences circulate in blood up to 28 days after COVID-19 vaccination. *APMIS*. 2023;131(3):128-132. doi: 10.1111/apm.13294
15. Krauson AJ, Casimero FVC, Siddiquee Z, Stone JR. Duration of SARS-CoV-2 mRNA vaccine persistence and factors associated with cardiac involvement in recently vaccinated patients. *NPJ Vaccines*. 2023;8(1):141. doi: 10.1038/s41541-023-00742-7
16. Gonzalez VJ, Li L, Buarpung S, Prahl M, Robinson JF, Gaw SL. Minimal mRNA uptake and inflammatory response to COVID-19 mRNA vaccine exposure in human placental explants. *iScience*. 2023;26(8):107549. doi: 10.1016/j.isci.2023.107549
17. Hanna N, Hossain I, Nimeri N, Freitag A, Rahman S, McCoy C, et al. Detection of messenger RNA COVID-19 vaccines in human breast milk. *JAMA Pediatr*. 2022;176(12):1268-1270. doi: 10.1001/jamapediatrics.2022.3581
18. Roltgen K, Nielsen SCA, Silva O, Younes SF, Zaslavsky M, Costales C, et al. Immune imprinting, breadth of variant recognition, and germinal center response in human SARS-CoV-2 infection and vaccination. *Cell*. 2022;185(6):1025-1040.e14.

doi: 10.1016/j.cell.2022.01.018

19. Fertig TE, Chitoiu L, Marta DS, Ionescu VS, Cismasiu VB, et al. Vaccine mRNA Can Be Detected in Blood at 15 Days Post-Vaccination. *Biomedicines*. 2022;10(7):1538. doi: 10.3390/biomedicines10071538
20. US Food and Drug Administration. Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications. 2010. Available from: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/characterization-and-qualification-cell-substrates-and-other-biological-materials-used-production>
21. World Health Organization. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. WHO Technical Report Series No. 978, Annex 3. 2010. Available from: https://cdn.who.int/media/docs/default-source/biologicals/cell-substrates/trs_978_annex_3.pdf
22. Zhang H, Zhang L, Lin A, Xu C, Li Z, Liu K, et al. Algorithm for optimized mRNA design improves stability and immunogenicity. *Nature*. 2023;621(7978):396-403. doi: 10.1038/s41586-023-06127-z
23. Murchie AI, Carter, WA, Portugal J, The tertiary structure of the four-way DNA junction affords protection against DNaseI. *Nucleic Acids Research*. 1990;18(9):2599-2606.
24. Guetzkow J, Levi R. Evidence of Pfizer vaccine DNA contamination and manufacturing process changes. *BMJ* 2022;378:o1731 (Rapid Response, 6 July 2022). <https://www.bmjjournals.org/content/378/bmjjournals.o1731/rr-2>
25. Council of Europe. 5.14. Gene transfer medicinal products for human use, version legally binding until 31 March 2025, Free access to Supportive Pharmacopoeial Texts in the Field of Vaccines for Human Use during the Coronavirus Disease (COVID-19) Pandemic, Updated Package—October 2020, Published in Accordance with the Convention on the Elaboration of a European Pharmacopoeia (European Treaty Series No. 50). 2020: 2.6.35. Available online: <https://www.edqm.eu/en/d/99080> (accessed on 25 June 2024).
26. Jones L, Yue ST, Cheung CY, Singer VL. RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. *Anal Biochem*. 1998;265(2):368-374. doi: 10.1006/abio.1998.2914
27. Georgiou CD, Papapostolou I, Grintzalis K. Protocol for the quantitative assessment of DNA concentration and damage (fragmentation and nicks). *Nat Protoc*. 2009;4(2):125-131. doi: 10.1038/nprot.2008.222
28. Roncati L, Ghaleb N, Ghaleb J, Kfouri K. pDNA Impurities in mRNA Vaccines. *Microorganisms*. 2025;13(9):1975. doi: 10.3390/microorganisms13091975

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