Peer-review

Wang, Tyler J, Alex Kim, and Kevin Kim. 2024. "A Rapid Detection Method of Replication-Competent Plasmid DNA from COVID-19 mRNA Vaccines for Quality Control." *Journal of High School Science* 8 (4): 427–39.

1.Assuming that the spec of 10 ng/dose was set by qPCR, this reference, found that the DNA was below this limit for vaccine lots when tested by qPCR (https://osf.io/preprints/osf/mjc97), but that smaller fragments are not detectable by this technique due to the size range of approx 100 bp of the qPCR amplicons. Can the size range of amplicons be decreased 10X without interfering with their ability to amplify DNA? This is an interesting point to discuss and is even more relevant when the same reference found a correlation between DNA per dose and SAE.

2.You state "….After ligated DNA was transformed into E. coli cells, we only detected two colonies from the Pfizer biosimilar vaccine-derived DNA….." Did you perform experiments where you tried to transform DNA without the ligation step into E.Coli Cells? In other words, does linear DNA transform E.Coli and if not, how do you know that the DNA you detected using Gel electrophoresis and/or the Nanodrop method was not ligated to begin with?

3.You state "….These results support a complete removal of replication-competent pDNA from the Pfizer mRNA lots…." Please avoid statistically incorrect generalization based on small sample sizes. Please restate to read " These results support a complete removal of replication-competent pDNA from the analyzed vials of the Pfizer mRNA lot". Please check for these errors throughout the manuscript.

4.You state "….However, the DNA is nearly all composed of small fragments of around 35 bp in length. Once again, this finding is consistent with a fully executed DNase I treatment of the commercial mRNA vaccine…." That may be so but how then do you explain that a Pfizer biosimilar demonstrated growth at a DNA concentration of 16 ng/uL, whereas the Moderna biosimilar and in-house mRNA standard did not, at DNA concentrations of 26 and 95 ng/uL ? Were the bp dis-similar between these samples? Please include an average bp for all these samples in the table as well as the method used to measure the DNA concentrations. Please note that this reference Plasmid DNA vaccines: assay for integration into host genomic DNA. Ledwith BJ 1 , Manam S , Troilo PJ , Barnum AB , Pauley CJ , Griffiths TG 2nd , Harper LB , Schock HB , Zhang H , Faris JE , Way PA , Beare CM , Bagdon WJ , Nichols WW, Developments in Biologicals, 01 Jan 2000, 104:33-43, states that is is possible for bp as low as 7 to integrate into the genome. Please discuss in the manuscript. Also discuss the fact that the plasmid derived ori, the SV40 promoter-ori DNA can be detected in the Pfizer vaccine and its implications on reverse integration into the human genome. This is counter to your stated claim in the manuscript "…As the pDNA template does not contain eukaryotic promoters or replication origins, the potential harm caused by the pDNA fragments may be lower than that from cellular DNA….." Please check and provide references.

5.You state that "….By contrast, Qubit uses 8fluorometric dye for specific quantification of dsDNA, which is more specific for DNA quantification…." However, some references have stated that there is possibility of intercalation between the modified RNA residues of the mRNA and the dye, that may lead to overestimation. Please discuss in the manuscript.

6.You state "….The plasmid DNA template does not contain oncogene or any eukaryotic promoters/enhancers, it is also not known that these small DNA fragments can integrate into host DNA. Therefore, it is less likely that these DNA fragments will be oncogenic…." Please re-evaluate and rephrase this statement considering the evidence provided in point 4.

7.Your title "….A Rapid Detection Method of Plasmid DNA from COVID-19 mRNA Vaccines for Quality Control" is misleading becasue these methods are already available and used widely in the literature. I would prefer something along the lines of " Quantification of Plasmid DNA in COVID-19 mRNA vaccines".

8.Compared to your finding of average bp of 100 in Pfizer vaccines, this reference used the Oxford nanopore sequencing and found average bp of 214 bp and a maximum size of 3.5 kb (see: https://osf.io/preprints/osf/mjc97). This goes contrary to your claim in point 6. I think it is necessary for you to state that the amount/bp/replicability of residual DNA is strongly dependent on the method employed. Hence, it is all the more imperative - especially since RNA vaccines are probably here to stay - to establish a correlation between the probability of DNA integration into the human genome and the method used to examine that probability.

9.Please also state explicitly what specific work in this study was directly peformed by the student.

We thank the reviewer for the timely review and constructive comments. A point-by-point response to the refree's comments is provided as below (original comments in black and our responses in blue):
Reviewer #1:

1. Assuming that the spec of 10 ng/dose was set by qPCR, this reference, found that the DNA was below this limit for vaccine lots when tested by qPCR (https://osf.io/preprints/osf/mjc97), but that smaller fragments are not detectable by this technique due to the size range of approx 100 bp of the qPCR amplicons. Can the size range of amplicons be decreased 10X without interfering with their ability to amplify DNA? This is an interesting point to discuss and is even more relevant when the same reference found a correlation between DNA per dose and SAE.

   Our response: first of all, we would like to point out that it is a common "misunderstanding" that 10 ng per dose is the limit set by WHO or FDA to be the amount of DNA allowed in a viral vaccine. The specific references cited by the reviewer apply to DNA derived from the cells in which the vaccines are made. In other words, there are no clear guidance on the amount of plasmid DNA in the mRNA vaccine. Unfortunately, this point is largely misunderstood by most people, including the authors of the reference. We learned this from FDA professionals.

   Depending on the design (the location of the primers and probes), quantitative PCR may not be able to linearly amplify fragments smaller than 100 bp. As far as we know, qPCR amplicons are typically between 100-200bp. If the size of the amplicon decreases by 10-fold (i.e., 10-20bp), qPCR will surely fail and miss a significant amount of DNA.

   The relationship between DNA per dose and SAE is interesting, but it is not the topic of investigation of our paper.

2. You state "….After ligated DNA was transformed into E. coli cells, we only detected two colonies from the Pfizer biosimilar vaccine-derived DNA….." Did you perform experiments

where you tried to transform DNA without the ligation step into E.Coli Cells? In other words, does linear DNA transform E.Coli and if not, how do you know that the DNA you detected using Gel electrophoresis and/or the Nanodrop method was not ligated to begin with?

Our response: We thank the reviewer for the question. Please refer to https://blog.addgene.org/plasmids-101-what-is-a-plasmid, "at their most basic level, plasmids are small circular pieces of DNA that replicate independently from the host's chromosomal DNA." Linear plasmid DNA can get in E.coli cells, but cells will gradually lose all linear plasmid DNA as these DNA do not replicate as cells propagate. In order to form colonies, E. coli cells will have to contain a replication-competent plasmid DNA which confers drug resistance. Linearized DNA (without ligation) would yield zero colonies on a LB plate containing antibiotics. This is common knowledge in molecular biology.

3. You state "….These results support a complete removal of replication-competent pDNA from the Pfizer mRNA lots…." Please avoid statistically incorrect generalization based on small sample sizes. Please restate to read " These results support a complete removal of replication-competent pDNA from the analyzed vials of the Pfizer mRNA lot". Please check for these errors throughout the manuscript.

   Our response: The point is well taken. The relevant text has been revised accordingly.

4. You state "….However, the DNA is nearly all composed of small fragments of around 35 bp in length. Once again, this finding is consistent with a fully executed DNase I treatment of the commercial mRNA vaccine…." That may be so but how then do you explain that a Pfizer biosimilar demonstrated growth at a DNA concentration of 16 ng/uL, whereas the Moderna biosimilar and in-house mRNA standard did not, at DNA concentrations of 26 and 95 ng/uL ? Were the bp dis-similar between these samples? Please include an average bp for all these samples in the table as well as the method used to measure the DNA concentrations. Please note that this reference Plasmid DNA vaccines: assay for integration into host genomic DNA. Ledwith BJ 1 , Manam S , Troilo PJ , Barnum AB , Pauley CJ , Griffiths TG 2nd , Harper LB , Schock HB , Zhang H , Faris JE , Way PA , Beare CM , Bagdon WJ , Nichols WW, Developments in Biologicals, 01 Jan 2000, 104:33-43, states that is is possible for bp as low as 7 to integrate into the genome. Please discuss in the manuscript. Also discuss the fact that the plasmid derived ori, the SV40 promoter-ori DNA can be detected in the Pfizer vaccine and its implications on reverse integration into the human genome. This is counter to your stated claim in the manuscript "…As the pDNA template does not contain eukaryotic promoters or replication origins, the potential harm caused by the pDNA fragments may be lower than that from cellular DNA….." Please check and provide references.

   Our response: It is not surprising that a Pfizer biosimilar demonstrated growth at a DNA concentration of 16 ng/uL, whereas the Moderna biosimilar and in-house mRNA standard did not, at DNA concentrations of 26 and 95 ng/uL. The ability to form antibiotics-resistant colony is not necessarily correlated with the concentration of the DNA. One product may contain more DNA, but as long as the DNA is thoroughly digested (linearized), it would not form colonies.

As to the reference that the reviewer cited as well as the statement that "the plasmid derived ori, the SV40 promoter-ori DNA can be detected in the Pfizer vaccine and its implications on reverse integration into the human genome", they are not really the focus on our paper. However, for more information, plasmid derived ori is only bacterial origin of replication, it does not initiate replication in mammalian cells. The SV40 promoter-ori DNA "consists of a few palindromes, a 17-base-pair (bp) A + T-rich sequence, three copies of a G + C-rich 21-bp repeat, and two copies of a 72-bp repeat" (Proc Natl Acad Sci U S A. 1983 Feb;80(3):721). Because we only detected DNA fragments <35 bp in our study, we reason those detected by others are broken pieces of SV40 promoters that are non-functional.

5. You state that "….By contrast, Qubit uses 8fluorometric dye for specific quantification of dsDNA, which is more specific for DNA quantification…." However, some references have stated that there is possibility of intercalation between the modified RNA residues of the mRNA and the dye, that may lead to overestimation. Please discuss in the manuscript.

   Our response: we welcome the comment and have revised the manuscript accordingly.

6. You state "….The plasmid DNA template does not contain oncogene or any eukaryotic promoters/enhancers, it is also not known that these small DNA fragments can integrate into host DNA. Therefore, it is less likely that these DNA fragments will be oncogenic…." Please re-evaluate and rephrase this statement considering the evidence provided in point 4.

   Our response: Please refer to our response to point #4.

7. Your title "….A Rapid Detection Method of Plasmid DNA from COVID-19 mRNA Vaccines for Quality Control" is misleading becasue these methods are already available and used widely in the literature. I would prefer something along the lines of " Quantification of Plasmid DNA in COVID-19 mRNA vaccines".

   Our response: we thank the reviewer for the comment, but we would politely disagree. Typically, a declarative title that summarizes the scope and main finding of the research project is preferred (Nat Hum Behav 7, 465 (2023). https://doi.org/10.1038/s41562-023-01596-8), although this is not always possible.

   In our case, despite DNA ligation and transformation are standard molecular biology techniques, we creatively applied these methods in detecting replication-competent (or replicative) plasmid DNA from the COVID-19 mRNA vaccines. Such a method is different from the standard qPCR method that has been adopted by industry. Because our goal is to monitor vaccine safety and impurity, hence the current title reflects the scope of the research. Quantification of Plasmid DNA in COVID-19 mRNA vaccines would be less specific. To further mitigate the reviewer's concern, we have now revised the title to read "A Rapid

Detection Method of Replication-competent Plasmid DNA from COVID-19 mRNA Vaccines for Quality Control."

8. Compared to your finding of average bp of 100 in Pfizer vaccines, this reference used the Oxford nanopore sequencing and found average bp of 214 bp and a maximum size of 3.5 kb (see: https://osf.io/preprints/osf/mjc97). This goes contrary to your claim in point 6. I think it is necessary for you to state that the amount/bp/replicability of residual DNA is strongly dependent on the method employed. Hence, it is all the more imperative - especially since RNA vaccines are probably here to stay - to establish a correlation between the probability of DNA integration into the human genome and the method used to examine that probability.

   Our response: nanopore sequencing is typically used to sequence fragments longer than multiple kilobases. Nonetheless, we included a short discussion comparing our findings with the one cited by the reviewer.

9. Please also state explicitly what specific work in this study was directly peformed (sic) by the student. Please also submit a word copy of your revised manuscript along with figures submitted as separate JPEG files.

   Our response: the authors performed all the work except the DNA fragment size analysis on the Agilent 2100 Bioanalyzer. This has already been stated in the Materials and Methods section.

   We have included a revised manuscript with track changes and a clean copy as well as separate figures in JPEG files.

Thank you for addressing my comments. In general, however, I would like to see the content of those responses discussed in the body of the manuscript so that readers are made aware of wider context that was taken into account. Hence,
For point 1: please include your response in the manuscript with the pertinent point of DNA per dose and SAE mentioned along with the reference.
For point 4: please include the body of your response in the manuscript along with the reference. You have not adequately addressed "Please note that this reference Plasmid DNA vaccines: assay for integration into host genomic DNA. Ledwith BJ 1 , Manam S , Troilo PJ , Barnum AB , Pauley CJ , Griffiths TG 2nd , Harper LB , Schock HB , Zhang H , Faris JE , Way PA , Beare CM , Bagdon WJ , Nichols WW, Developments in Biologicals, 01 Jan 2000, 104:33-43, states that is is possible for bp as low as 7 to integrate into the genome. Please discuss in the manuscript.". Please address this comment and include your response in the manuscript. Point 6 is hence not adequately addressed in view of the above non-responded comment.

I could not see the tracked changes in the manuscript submitted. Please address the comments above in the manuscript and resubmit using the same guidelines as before.

Point-to-point response:

For point 1: please include your response in the manuscript with the pertinent point of DNA per dose and SAE mentioned along with the reference.
Response: we have now added the discussion to the Discussion section with the reference (Page 8 of the Manuscript with track changes).

For point 4: please include the body of your response in the manuscript along with the reference. You have not adequately addressed "Please note that this reference Plasmid DNA vaccines: assay for integration into host genomic DNA. Ledwith BJ 1 , Manam S , Troilo PJ , Barnum AB , Pauley CJ , Griffiths TG 2nd , Harper LB , Schock HB , Zhang H , Faris JE , Way PA , Beare CM , Bagdon WJ , Nichols WW, Developments in Biologicals, 01 Jan 2000, 104:33-43, states that is is possible for bp as low as 7 to integrate into the genome. Please discuss in the manuscript.". Please address this comment and include your response in the manuscript. Point 6 is hence not adequately addressed in view of the above non-responded comment.

Response: we have now added the discussion to the Discussion section with reference (Pages 9-10 of the Manuscript with track changes). We also indicated in Figure 4 legend that the DNA concentrations were measured by Nanodrop.

I could not see the tracked changes in the manuscript submitted. Please address the comments above in the manuscript and resubmit using the same guidelines as before.
Response: we apologize for the inconvenience. Please find the manuscript copy with track changes (can be opened in Microsoft Word).

Thank you for addressing my comments.
Please rewrite and reformat the references so that they are consistent with each other. Each reference needs to list at least 6 authors (if there are more than 6 authors for that manuscript) followed by et al. Each reference needs to be followed by a live link (please see published manuscripts at the JHSS website for guidance). Please do NOT number the references using the software's automatic numbering system - instead number each reference manually. Lastly, do not link the references in the references section to the reference numbers in the body of the manuscript.
Please also move the Materials and Methods section to immediately below the Introduction section.

Thank you for addressing my comments. Accepted.