

Communication

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Communication

Communication on Methodological Considerations Regarding the Quantification of DNA Impurities in the COVID-19 mRNA Vaccine Comirnaty®. Methods Protocols 7, 41 (May 2024)

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Abstract: The purpose of this communication is to clarify criticisms that have arisen in the context of our publication "Methodological Considerations Regarding the Quantification of DNA Impurities in the COVID-19 mRNA Vaccine Comirnaty®" (Methods and Protocols 2024, 7, 41, May 2024 [1]). After an exchange of opinions, a comment was submitted within a Comment and Reply Procedure started by Methods and Protocols by Kaiser and Marschalek [2], in which they assailed our publication. But after reading our reply which we submitted by 24 September 2024, Kaiser and Marschalek decided to submit the data contained in their comment in parallel together with further authors as a regular article to another journal, titled "Quantification of objective concentrations of DNA impurities in mRNA vaccines" (Kaiser et al 2025 [3]), of which an earlier version has been published as a preprint already in November 2024 (Kaiser et al 2024 [4]). This publication and preprint attempted to refute our findings obviously aiming to be published without our response and undermining the Comment and Reply Procedure. However, it does not succeed in doing so with the necessary persuasiveness. First of all, it is particularly important that Kaiser and Marschalek [2] and Kaiser et al [3, 4] have confirmed that our results are reproducible when the quantification is carried out using the Qubit® technology in accordance with the manufacturer's instructions, which is exactly what we did. However, it is then claimed by Kaiser and Marschalek [2] as well as by Kaiser et al [3, 4] that the magnitude of the DNA impurities we have shown would be an effect of high amounts of RNA present in the samples. As a proof they quantified a defined concentration of DNA in the presence of very high concentrations of RNA with the Qubit® methodology. However, even the presence of 250 ng/µL RNA resulted only in a comparatively small increase in the DNA value of 0.655 ng/µL and this is far from explaining the DNA concentrations of 12 to 17.8 ng/µL that we have measured in several batches of Comirnaty®. Further, Kaiser and Marschalek [2] and Kaiser et al [3, 4] mentioned that experiments with DNA extracted from the vaccine would show that the very low legal limits for DNA in Comirnaty® are met. However, the authors of this critique have failed to demonstrate that the extractions they performed are indeed quantitative, i.e. reflect the actual DNA contamination. But, based on the related published literature, this must be denied. With this in mind, we can finally confirm that both our methodology and our data, as published in our above-mentioned article [1], imply that DNA impurities as measured with Qubit® by us in Comirnaty® are reliable according to the manufacturer's premises for this DNA quantification technology. In this sense, the DNA values presented by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] after extraction procedures are obviously artificial effects of the extractions performed and therefore do not represent the true DNA contamination of the concerned Comirnaty® batches. Finally, our data for DNA contaminations in Comirnaty have been confirmed in December 2024 explicitly by research utilizing different Methods which has been conducted in a Laboratory of the US Food and Drug

Administration FDA [5]. However, this publication has been ignored by Kaiser et al. in their own publication of March 2025 [3] which appear almost 3 months later and thus should have cited and discussed the particular findings which are confirming our data.

Keywords: mRNA vaccines; Comirnaty®; DNA impurities; fluorescence spectroscopy; Qubit® fluorometry

Introduction

In our commentary "*Methodological Considerations Regarding the Quantification of DNA Impurities in the COVID-19 mRNA Vaccine Comirnaty®*" published in Methods and Protocols [1], we discussed the methodological aspects of quantifying significant amounts of DNA impurities in Comirnaty®, the mRNA vaccine marketed by Pfizer and BioNTech. Recently, our results and methods as presented in this publication were publicly challenged by Kaiser et al. [3, 4].

In the meantime, however, a new publication from an FDA laboratory confirmed our data with explicit reference to our publication from May 2024: "*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*" [5], where "*another group*" is explicitly referenced to our original publication [1]. Furthermore, the new publication provides evidence that the DNA contaminants in Comirnaty can be mutagenic [5] and is therefore highly worrying in terms of public health.

This did not come as a surprise to us, as the DNA contamination in Comirnaty® had been the subject of heated political debates prior to the publication of our peer-reviewed analysis. In the debate that followed between Rolf Marschalek, one of the authors of Kaiser et al [3, 4] and us, Methods and Protocols proposed a process of clarification by publishing a comment from Marschalek together with a response from us. Both sides agreed, and after some time we received the first version of the comment from Kaiser and Marschalek [2], with an invitation from Methods and Protocols to respond on that. We quickly realised that we could successfully defend our publication on the basis of our own data and, surprisingly, also on the basis of data provided by the comment from Kaiser and Marschalek [2]. Accordingly, we were able to refute all of the critics to our results and interpretations in our first version of this reply submitted to Methods and Protocols already on 24 September 2024 [unpublished]. In this reply we have once again demonstrated that our published data on DNA contamination in Comirnaty® [1] are scientifically sound. Both, our data and our methodology, are robust and the conclusion of our original publication is valid: DNA contaminations in the Comirnaty® batches we analysed were several hundred times higher than the permitted limit, providing evidence that Comirnaty® is seriously challenged in terms of drug safety.

After submitting our response, we expected that both documents, the comment by Kaiser and Marschalek [2] and our response to it, would be reviewed by the editors and peer-reviewed according to the applicable rules [7]. However, this was not the case. Although Methods and Protocols acknowledged receipt of our response, nothing has happened since and our enquiries about this have gone unanswered. But then, about 6 weeks after we submitted our reply, Marschalek, Kaiser and further authors submitted a preprint (Kaiser et al. [4]) which provided some of the criticisms as already submitted with the comment on our original publication (Kaiser and Marschalek [2]). Finally we also published a Preprint [6] on which this publication is based on. However, on 5 February 2025 Methods and Protocols contacted us by sending review reports, what we considered a restart of the comment and reply procedure. But finally this procedure failed with the publication by Kaiser et al. [3] because of the fact that data once published in a different journal can not be published a second time in Methods and Protocols according to the applicable publication rules of Methods and Protocols. This way, Kaiser et al. [3] avoided that their comment would be published together with our reply, aiming that the readers can compare both sides and come to their own conclusion. So, this approach was undermined by Kaiser et al. [3] finally.

Considerations

Comirnaty® is an mRNA vaccine targeting the SARS-CoV2 virus. It consists of a buffer solution with lipid nanoparticles containing the active ingredient, an mRNA encoding a SARS-CoV2 spike protein. A ready-to-use diluted dose of Comirnaty® for adults consists of 300 μ L containing 30 μ g of mRNA [8], which means that the concentration of mRNA is 100 ng/ μ L. Thus, any consideration of an RNA effect on DNA quantification with Qubit® concerning Comirnaty® has to focus on the effect of exactly this RNA concentration of 100 ng/ μ L, for which particularly the manufacturer of the Qubit® system provides concrete data [9] regarding the RNA effect on a Qubit® DNA quantification as considered below. Further, the Australian Therapeutic Goods Administration (TGA) published that as a part of the quality control after production of Comirnaty the Qubit methodology is used to quantify the mRNA contained in the final product [10] and this shows that the authorities don't see lipid nanoparticles as potentially interfering the Qubit® technology as we do according to our spike-in experiments [1].

Since the mRNA active ingredient of Comirnaty® is produced by transcription of specific DNA templates, the Comirnaty® production processes require large amounts of DNA as templates for in vitro synthesis of mRNA. The DNA templates used for commercial Comirnaty® are linearised plasmids from *E. coli* bacteria which serve as the host. After in vitro production of the mRNA for Comirnaty®, the DNA templates have to be removed for drug safety reasons [3]. For the commercial Comirnaty®, this was done by DNase digestion followed by filtration [8]. This means that the commercial Comirnaty® runs the risk of being contaminated with a heterogeneous mixture of DNA fragments, from small to large, as a result of the DNase digestion if the filtration doesn't remove the DNA sufficiently. That this assumption is actually true could be demonstrated by experimental exploration of DNA lengths with the Agilent 2100 Bioanalyzer® System and the Agilent® DNA 12000 Kit as shown in Figure 1.

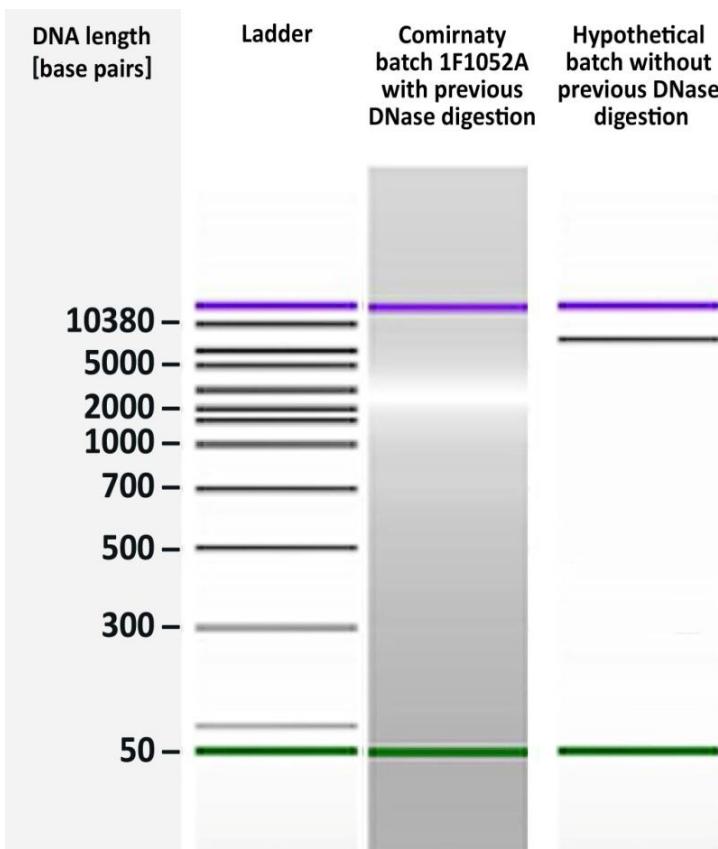


Figure 1: Determination of the length of DNA species contained in Comirnaty Batch 1F1052A.

The Agilent 2100 Bioanalyzer® System in combination with the Agilent® DNA 12000 Kit is a common standard for DNA size determination. It is based on fully automated electrophoresis and uses a standard set of DNA species of defined lengths that provide a so-called ladder with bands

representing the set of DNA lengths contained in the standard and expressed in base pairs. The experiment has been conducted according to the manufacturer's instructions [11]. The figure shows the result for Comirnaty® batch 1F1052, one of several batches tested with similar results. All the batches don't show sharp DNA bands, but a wide range of DNA lengths from small to large, with the largest DNA species likely to be derived from genomic host bacterial DNA. A hypothetical batch model demonstrates what would be expected without a previous DNase digestion: The plasmid would remain intact and thus form one single band at 7824 bp [12] instead of the countless bands which appear after DNase digestion and form a grey smear instead clearly separated bands.

Official documents from the competent authorities indicate that the quantification of residual DNA is performed by quantitative PCR (qPCR) [12]. But this technique only measures DNA fragments containing a specific sequence of bases, and assumes that all fragments containing that sequence are of the same size [13] (→Figure 1: "*Hypothetical batch without previous DNase digestion*"). Since the resulting heterogeneous mixture of DNA fragments can only partially contain the qPCR target sequence. DNA fragments that do not contain this sequence completely escape measurement and this results in values that run the risk of reflecting only a small proportion of the actual DNA fragments, i.e. those that contain the intact qPCR target sequence. This means, that qPCR is not suitable to quantify total DNA in samples that originally contained one single uniform DNA but have been broken down by DNase digestion into a large number of DNA fragments from very short to relatively long, as it is the case with the commercialized version of Comirnaty® [12].

From this circumstance we identified the need to investigate how completely DNA fragments are actually removed from commercial Comirnaty® and how much DNA remains in marketed batches of Comirnaty®. As the DNA limit for Comirnaty® has been set by the authorities at 10 ng/dose [14], this value must be adhered to. Since one dose consists of 300 µL of ready-to-use diluted Comirnaty®, the final concentration of DNA contamination must be below 0.033 ng/µL to be in line with the required limit.

At this point it is important to note that Comirnaty® is a pharmaceutical drug and therefore it is important to consider whether a so called "*recognized pharmaceutical rule*" (this is a legal term as used e.g. in Section 55 of the German Medicines Act [15]) applies to the methods because of being mentioned in the European Pharmacopoeia. The answer can be found in the general chapter "*5.14. Gene transfer medicinal products for human use*" of the European Pharmacopoeia [16], which defines "*gene transfer medicinal products*" to include mRNA pharmaceuticals such as Comirnaty®. This chapter of the European Pharmacopoeia mentions two methods for quantifying plasmid DNA in pharmaceuticals, one based on light absorbance measurement, as used for example by the NanoDrop® device, and the other using a DNA-specific fluorescent dye and measurement of DNA-dependent fluorescence, for which several devices and methods compete, including the Qubit® device, which provides sophisticated features. This is particularly a smartly automated assay process with algorithms which take account the measurements of several light sources, filters and detectors, whereby the composition of the working solution is perfectly co-ordinated with the technical hardware and the result processing algorithms [17]. Thus, we found it suitable for quantifying DNA in Comirnaty®.

As mentioned above, due to the dosage of the active ingredient the RNA concentration in Comirnaty® is 100 ng/µL. It is therefore necessary to investigate whether this high RNA content interferes with DNA quantification. This question has been addressed by ThermoFisher/Invitrogen, the manufacturer of Qubit® and Nano-Drop®. They have provided a technical note which supports these considerations by comparing Nanodrop® (specific absorption) and Qubit® (specific fluorescence) by measuring a DNA standard of 10 ng/µL together with increasing concentrations of RNA [9]. The results are presented in a figure which shows that DNA absorption with Nanodrop® is strongly disturbed by RNA and therefore this method is disqualified under these conditions. But regarding Qubit® the manufacturer's technical note states: "*In a sample containing a 10-fold excess of RNA over DNA, the concentration determined in the DNA assay was only 7% higher than the actual concentration.*" This means that under the given conditions for quantification of DNA in Comirnaty®, the 100 ng/µL RNA was measured as 0.7 ng/µL DNA (7% of 10 ng/µL DNA) and that this effect is

fairly below the dimension of accuracy of 15% which has been defined by the manufacturer for DNA quantification with Qubit® [18].

Figure 2 shows a simplified version of the Figure published by the manufacturers ThermoFisher / Invitrogen [9] which makes it obvious that in case of the presence of high amounts of RNA, DNA quantification with Qubit® is suitable due to a low RNA effect on DNA quantification with Qubit® but absorbance measurement (Nano-Drop®) is not because the last cannot sufficiently distinguish DNA from RNA.

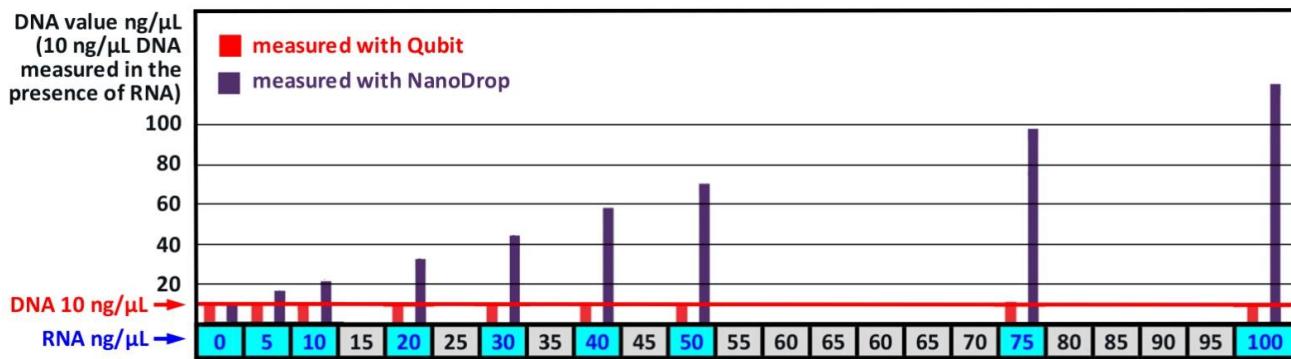


Figure 2. Quantification of 10 ng/µL DNA in the presence of RNA with Qubit® and NanoDrop®. Figure adapted from figure 1 of a technical note provided by the manufacturer [9].

In general, the instructions and technical notes for Qubit® provided by the manufacturer are very clear regarding the specificity of the Qubit® DNA HS Kit in combination with the Qubit® device and show that the specificity is very high, even in the presence of contaminants such as lipids, proteins or even RNA [9, 17, 18]. Nevertheless, we have tested the robustness of these statements. The most appropriate way to test the influence of contaminants in a sample in which one particular substance shall be quantified is a standard addition, means the addition of known amounts of the substance to be quantified and to compare with samples without this additions. This methodology is established since decades [19] and is considered to be one of the simplest methods for detecting the influence of interferences on the result, using minimal resources and fully complies with the rules of Good Laboratory Practice (GLP) [20]. In this sense the standard addition of DNA has been applied by us regarding DNA quantification in Comirnaty® with Qubit®. According to the results of these experiments as provided in the Supplement of our original publication [1] show that DNA has been quantified accurately in our setting [21].

However, Kaiser and Marschalek [2] and Kaiser et al. [3, 4] claim falsely that our results would be wrong and that they actually would show an RNA effect. For this purpose, they presented data that were dimensioned in an unusual way, in that the RNA and DNA values were based on the RNA- or DNA-content of 20 or 10 µL and not expressed as concentrations, i.e. ng/µL, as given in our publication because of the fact that the Qubit® device always provides the measured values this way (→ Fig. 5). Furthermore, the x-axis of the corresponding figure was compressed so that the curve drawn by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] is scientifically misleading. However, this can be easily corrected by converting the values provided by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] to ng/µL and stretching the x-axis to the actual proportions. The result of this procedure is shown in Table 1 and Figure 3, where the value provided by the manufacturer's Technical Note [9] is also included.

Quantity data (ng) provided by Kaiser et al. [2, 3]	Column number	1	2	3	4	5	6	*Add-on	7
ng DNA in 20 µL		5	5	5	5	5	5		5
ng RNA in 20 µL		0	25	50	250	500	1000		5000
Proportion DNA : RNA	n.a.		1:5	1:10	1:50	1:100	1:200	* 1:10	1:1000
ng DNA measured in 10 µL out of 20 µL	2.78		3.1	3.28	4.3	5.16	7.96		9.33
Concentration values (ng/µL) calculated from the data provided by Kaiser et al. [2, 3]	ng DNA in 1 µL (ng/µL)	0.25	0.25	0.25	0.25	0.25	0.25	* 10	0.25
	ng RNA in 1 µL (ng/µL)	0	1.25	2.5	12.5	25	50	* 100	250
	ng DNA measured for 1 µL (ng/µL)	0.278	0.310	0.328	0.430	0.516	0.796	* 10.7	0.933
	RNA effect in ng/µL DNA	*0.000	*0.032	*0.050	*0.152	*0.238	*0.518	* 0.7	* 0.655

* These values quantify the RNA effect in ng/µL DNA and are obtained by subtracting the DNA value measured without RNA in column one (0.278) from the values with accompanying RNA. Example: 0.310 (column two) - 0.278 (column one) = 0.032 (RNA effect in ng/µL DNA)

* Values provided by Invitrogen/ThermoFisher: 100 ng/µL RNA combined with 10 ng/µL DNA (1:10) causes an RNA effect of 0.7 ng/µL DNA

Table 1. RNA effect on the quantification of 0.25 ng/µL DNA with Qubit® based on the data of Kaiser and Marschalek [2] and Kaiser et al. [3, 4] together with a value provided by ThermoFisher [9] standardized to ng/µL (comp. → Figure 3).

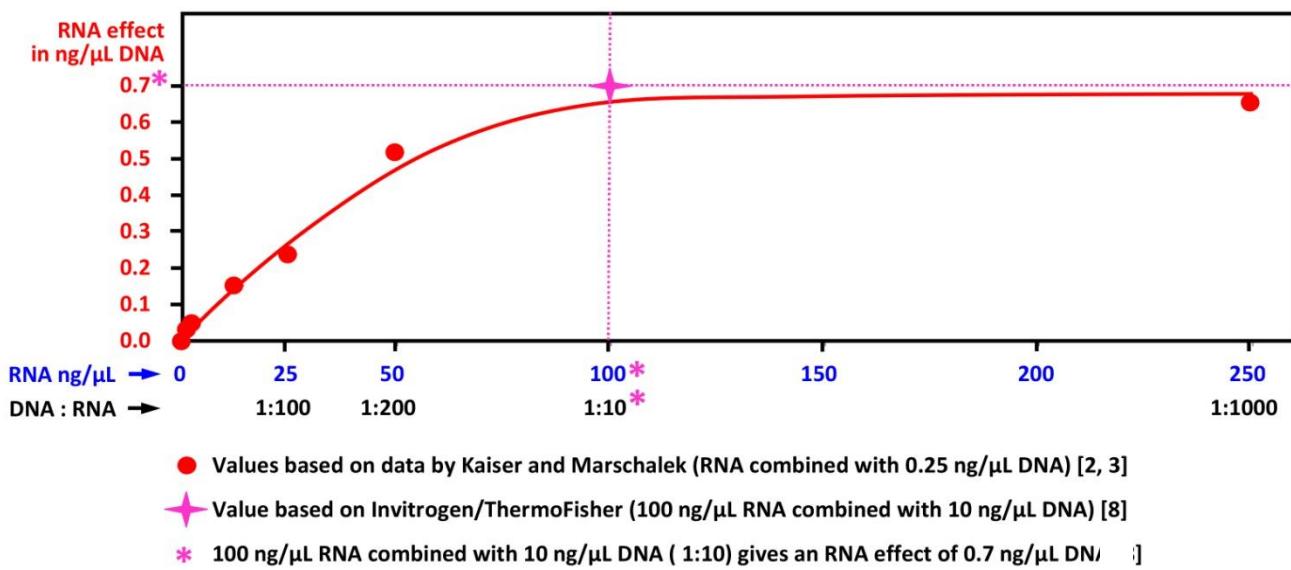


Figure 3. RNA effect on the quantification of 0.25 ng/µL DNA with Qubit® based on the data of Kaiser and Marschalek [2] and Kaiser et al. [3, 4] together with a value provided by ThermoFisher [9] standardized to ng/µL (comp. → Table 1).

Finally the Data of Table 1 and Figure 3 demonstrate, that the RNA effect on DNA quantification with Qubit® is very small, even 250 ng/µL RNA, the 1000fold of the DNA contained in that sample (0.25 ng/µL DNA) is not higher as the RNA effect published by the manufacturer for 100 ng/µL with 0.7 ng/µL DNA if combined with 10 ng/µL DNA (10fold). Therefore, the RNA content of Comirnaty® with a dosing of 100 ng/µL RNA cannot explain the very high amounts of residual DNA as found and published by us and confirmed by Kaiser and Marschalek [2] and Kaiser et al. [3, 4]. In particular, after dissolution of the lipid nanoparticles with Triton-X-100, the DNA values for the Comirnaty® batches investigated by us were 12 to 17.8 ng/µL and thus, even an assumed RNA effect of 1 ng/µL would be far below the 15 % accuracy dimension which is claimed by the manufacturer for Qubit® DNA quantification [18]. Therefore, the assertion by Kaiser and Marschalek [2] and Kaiser et al. [3, 4], the values for DNA impurities in Comirnaty® batches published by us would be explainable by an RNA effect is simply false. Kaiser and Marschalek [2] and Kaiser et al. [3, 4]

themselves provided the data for this conclusion, as clearly shown in Table 1 and Figure 3 above: Surprisingly, they multiplied the original DNA value provided by the Qubit® device in ng/µL by ten to present it in their Figure 2 A [2, 3, 4] in a blown up way as "DNA contained in 10 µL" instead of showing the original value as expressed by the Qubit® device in ng per one µL (the other unit options ng/mL, µg/µL, µg/mL and mg/mL are not applicable, . Kaiser and Marschalek [2] and Kaiser et al. [3, 4] did not provide, nor could we find, any scientific rationale for presenting the data at this 10-fold magnification. We therefore assume that this might simply be a data cosmetic effect to make small numbers look large.

On the other hand, when quantifying DNA in Comirnaty® using Qubit®, for samples with a low DNA concentration, e.g. 0.25 ng/µL as done by Kaiser and Marschalek [2] and Kaiser et al. [3, 4], and a simultaneously RNA concentration of 100 ng/µL or above, the RNA-dependent effect can exceed the accuracy limit as specified by the manufacturer with 15% deviation. This means that for a sample with such a low DNA and high RNA concentration, the RNA content of the sample would have to be reduced relative to the DNA content in order to obtain an accurate DNA value. While this sounds simple, it is challenging. In principle, a quantitative DNA extraction or a selective reduction of the RNA content would be conceivable. However, both are hampered by the fact that meaningful validation experiments would be required for such a method, which in particular can exclude an influence of the components of the Comirnaty® lipid nanoparticles. This applies in particular to the extraction of DNA, but also the reduction of RNA content using the enzyme RNase with subsequent DNA quantification using Qubit® requires a demonstration of a quantitative mode of action since the effects of the components contained in Comirnaty® on the enzymatic efficiency of RNase or the resulting RNA fragments on Qubit® measurements are unknown. A method which probably doesn't introduce interfering factors could possibly be the removal of RNA by using RNA-specific magnetic beads, a method described in the Comirnaty® EMA public assessment report [8] for the production of trail medication. However, also this method would need validation in terms of quantitatitvity.

In addition to the criticism of our methodology, Kaiser and Marschalek [2] and Kaiser et al. [3, 4] suggest in their comments and used in their experiments a method for DNA quantification in Comirnaty® based on Phenol/Chloroform extraction, which has not been published previously in terms of quantitative extraction of DNA from pharmaceutical drugs. The same is given for another extraction used by them, the precipitation of DNA with ethanol after RNase digestion. However, Kaiser and Marschalek [2] and Kaiser et al. [3, 4] did that without presenting the required validation and standardization experiments. This is highly unusual for a publication of new methods since a new method requires extensive validation and standardization before its publication. This is provided in international guidelines such as guidelines 7 and 11 of the Code of Conduct of the German Research Foundation [22] which applies to Kaiser and Marschalek as Members of the German Goethe-University.

With regard to the RNase digestion performed by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] it is stated in Kaiser et al 2024 [4] that RNase H was used. However, RNase H is commonly known as a family of endonuclease enzymes with a common substrate specificity for the RNA strand of RNA-DNA duplexes [23] After the mRNA agent of Comirnaty is not an RNA-DNA duplex the experiments performed by Kaiser et al. with RNase H require a precise justification why this enzyme was used, which is not given by Kaiser and Marschalek [2] or Kaiser et al. [3, 4]. Interestingly, in the publication by Kaiser et al. from March 2025 [3] "RNaseH" was simply replaced by "RNase" without changing the figures. This indicates that Kaiser et al. themselves recognized that RNase H was not appropriate for the experiment they did but instead of an concluding explanation they simply removed the "H" and left the RNase without any specification [3]. That such an approach is scientifically not acceptable is very obvious and thus it has to be asked why the reviewers have not recognized this manipulation.

The method suggested by Kaiser et al. for an extraction of DNA from Comirnaty® is based on phenol/chloroform respectively ethanol after RNase digestion, but both approaches are known as usually not being quantitative [24, 25] and thus not suitable for DNA quantification in a pharmaceutical drug for human use if no proof of quantitatitvity for the particular experiments is provided and neither Kaiser and Marschalek [2] nor Kaiser et al. [3, 4] comply with this precondition.

In terms of literature we found the comparison of six DNA extraction methods and Phenol/chloroform yielded comparatively low 3.92 µg DNA of a standardized sample where Qiagen and Salting out provided above 10 µg [26]. Due to their unambiguous nature, these findings must be regarded as experimental proof that the phenol/chloroform extraction of DNA is fundamentally non-quantitative and thus not suitable for experiments as conducted by Kaiser and Marschalek [2] and Kaiser et al. [3, 4]. Therefore, the values presented by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] for DNA quantification in Comirnaty after phenol/chloroform extraction must be considered scientifically unsustainable. And so it is not surprising that Kaiser and Marschalek [2] and Kaiser et al. [3, 4] did not provide either experimental or literature-based evidence for a quantitative extraction of DNA using phenol/chloroform or ethanol in pharmaceuticals with such a broad variety of DNA lengths from very small to medium. Further, there is no explanation why even after RNase digestion an ethanol extraction is done by them. So, it looks like that Kaiser and Marschalek [2] and Kaiser et al. [3, 4] did the extraction procedure with the intention to eliminate the high share of smaller DNA species as found in Comirnaty (see Figure 1) in order to manipulate the result. Kaiser et al. [3] seem to be aware of this impression, because they make the highly disconcerting attempt to justify the transfer of the particular method of DNA extraction from the level of genomic DNA from cells to a genetically engineered drug with the following sentence: "*The technologies we applied are well-known for over 50 years for isolation of DNA and RNA from natural lipid nanoparticles, namely cells*". However, this attempt cannot succeed because cells are simply not "*natural lipid nanoparticles*" because of their degree of organization, their double membrane containing functional proteins and usually much bigger size far above "nano", to name just a few aspects which demonstrate how bizarre any attempt to define cells as "*natural lipid nanoparticles*" is. It must therefore be considered completely incomprehensible that editors and reviewers waved through such a grotesque comparison.

An appropriate proof of quantitativity of the methods used by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] would require, in particular, that neither the Triton-X-100 added by them before the extraction nor the cationic lipids released from the lipid nanoparticles of Comirnaty® by this treatment impair the extraction procedures, which are also otherwise described only in extremely rudimentary terms by Kaiser and Marschalek [2] and Kaiser et al. [3, 4]. Particularly Triton-X-100 is known to interfere with phenol and ethanol, for example by formation of micelles [27, 28]. Further, the cationic lipids released from the nanoparticles by the Triton extraction could interfere with the DNA in such a way that this DNA accumulates in the phenol phase or the ethanol phase and thus large DNA fractions might be discarded instead of being included in the DNA quantification. Thus, any subsequent DNA quantification to determine the DNA content of the original sample will then be compromised.

Consequently, it has to be assumed that the very low amounts of DNA found in the extraction samples of Kaiser and Marschalek [2] and Kaiser et al. [3, 4] are an artificial result of the insufficiency of the extraction procedure applied by them. This has to be recognized as given until Kaiser and Marschalek [2] and Kaiser et al. [3, 4] provide the required proof that their extraction really works quantitatively. As long as these validation data are not provided, the extraction methodology used by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] doesn't allow to draw any scientific conclusions from their DNA extract - neither quantitatively nor qualitatively and completely independent from the method used for the quantification of the extracted DNA: If the quality of the sample itself is questionable, the quantification of its DNA content is consequently questionable as well.

Thus, we have to conclude that the extraction-based methods of DNA quantification proposed by Kaiser and Marschalek [2] and Kaiser et al. [3, 4] is invalid for the given purpose and cannot replace the recognized pharmaceutical rule based on DNA-specific fluorescent dyes as defined by the European Pharmacopoeia [13] and the German Medicines Act [15] that we followed in our published [1] experiments.

A new method for the quantification of nucleic acids in mRNA vaccines was recently published by the United States National Institute of Standards and Technology (NIST) based on the quantification of RNA bases after hydrolysis using mass spectrometry (MS) [29]. This method is very promising as it does not require any extraction steps, since hydrolysis also dissolves the lipid

nanoparticles of an mRNA vaccine. This is very important, as any separation steps in a purification procedure would lead to DNA losses to an undetermined extent, making any DNA measurement questionable after these steps. According to the authors, DNA impurities can be calculated from the thymine peak of the hydrolysed sample, as obtained from the same MS measurement. However, such a determination of DNA using this method is not yet ready for practical application (personal communication from NIST / Dr. Marc S. Lowenthal). Interestingly, in terms of a validation standard, NIST used a fluorescence dye-based quantification of nucleic acids after Triton treatment of the sample without any extraction of nucleic acids, as was carried out in our original publication [1] as well.

In addition to the aspects discussed above, our use of Qubit® for DNA quantification in Comirnaty® was also challenged in terms of practicality. However, from our perspective these critiques are widely based on some misunderstandings regarding the principles of the method as such. Therefore it is required to answer those questions with an explanation of essential properties of the Qubit® system which elaborates what we have already stated above. In our original Publication [1] we mentioned that technically we followed the instructions of the manufacturer, which is the standard expression for the description of well established methods, particularly if those are suggested by the European Pharmacopoeia for the particular purpose as it is here the case.

In this light the Qubit® device is a highly automated instrument that is perfectly synchronized with DNA-specific test kits. The composition of the working solution and the algorithms for measurement and result calculation have been continuously improved over many years, as has the Qubit® device. Further, the kits contain validated DNA standards that are used to generate a validated reference curve by measuring the original standards in the Qubit® device, which automatically calculates the reference curve and checks its accuracy using special algorithms. Only when the curve has been validated in this way, the Qubit® device accepts samples for DNA quantification. In addition, the Qubit® DNA kits provide a two component working solution that contains all the components necessary for accuracy, including the fluorescent dye [30]. For a standard measurement with the Qubit® DNA HS Kit, 180 µL of the working solution is pipetted together with a sample for which the DNA content is to be quantified whereby the sample volume is 1 to 20 µL and if a volume below 20 µL is chosen, additional buffer has to be added for getting a final volume of 200 µL in the assay tube [31], as demonstrated in Figure 4.

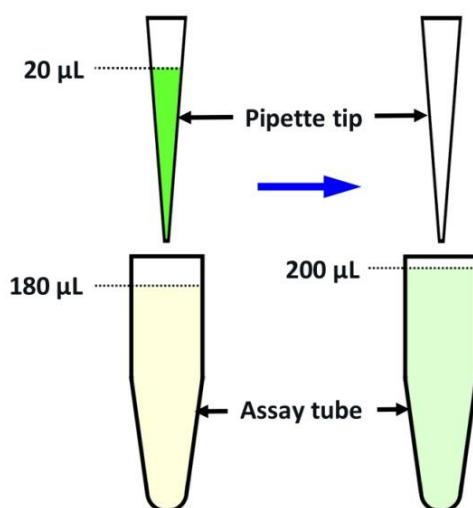


Figure 4. Pipetting of samples for the Qubit® quantification of DNA. Due to the fixed volume of 20 µL sample and 180 µL working solution which result in a final assay volume of 200 µL the sample is always diluted 1:10 before measurement. This might be an advantage in terms of effects caused by concomitant substances as these will be diluted too. Figure adapted from the manufacturer's instructions [30].

Further, for practical application according to the manufacturer's instructions the following sentence is of utmost importance: "The Qubit® dsDNA HS (High Sensitivity) Assay Kit, when used with the Qubit® Fluorometer, provides an accurate and selective method for the quantitation of sensitive DNA

samples. Depending on sample volume, the assay kit is designed to be accurate for initial DNA sample concentrations of 0.005 to 120 ng/μL, providing a detection range of 0.1–120 ng.” [31]

This statement differentiates between the “initial DNA sample concentration” and the “detection range”. The former thus refers to the DNA concentration in the sample which can be measured with the particular assay, while the detection range indicates how much total DNA can be measured in one assay tube. Thus, if a sample with an unknown DNA concentration is analyzed with Qubit®, it is important to ensure that the test kit used is suitable in terms of its detection range. Therefore, it is generally standard practice in professional laboratories to prepare dilutions at high DNA concentrations in order to then measure at a dilution stage that lies within the intended range. If more than one dilution fulfills this requirement, all of those can be used for measurement with Qubit®. Due to the specific algorithms used automatically by the Qubit® device, the various dilutions or sample volumes lead to the same result. This is possible because the data entry mask requires to enter the volume of the original sample contained in the assay sample in μL as shown in Figure 5. Finally the output of all measurements with Qubit® is provided in the chosen unit, e.g. ng/μL DNA, as also shown in Figure 5.



Figure 5. Data entry mask of the Qubit® device for DNA quantification.

The volume of the original sample contained in the pipetted sample has to be entered and also the unit to be used for the output of the Qubit® device has to be chosen, whereby the following output units are possible: ng/μL, ng/mL, μg/μL, μg/mL, mg/mL [18]. Figure adapted from the manufacturer's instructions [18].

For a more detailed explanation of the Qubit® assay process, a sample with a DNA concentration of 10 ng/μL to be quantified with the Qubit® dsDNA HS Kit [30] shall serve as an example. At this value, the initial dilution series comes into play or the sample volume used must be reduced. At an initial DNA sample concentration of 10 ng/μL, an undiluted sample of 20 μL would contain 200 ng of DNA, which is considerably higher than the maximum range of 120 ng. Therefore, the sample must be diluted. This dilution can be, for example, 1:2 with then 5 ng/μL DNA, so that 20 μL of sample with then 100 ng DNA in total can be pipetted into the assay tube. However, since this sample contains only 10 μL of the original sample, this volume has to be entered into the data entry mask of the Qubit® device. Qubit's evaluation algorithm automatically includes this in the calculation of the output value, which then corresponds to the actual DNA of 10 ng/μL of the original sample, since the dilution is taken into account by the algorithm automatically.

For quality assurance purposes, it is recommended to measure several dilutions of the same original sample, which, if set up correctly, will then lead to the same result. This also applies in the case that less than 20 μL of the original sample is pipetted into the assay tube and the difference is compensated for with buffer. This is particularly important when only small sample volumes are available that are too small for a dilution series. So, as is common standard, the values given in our

publication were determined with different dilutions and reproduced twice. This applies to both, DNA and RNA quantification, for which the same procedures were chosen.

Conclusions

Due to the high process quality of DNA quantification with Qubit® and the fact that even the RNA effect of 100 ng/µL RNA would lead to a DNA value of below 1 ng/µL DNA, the values we measured in several batches of Comirnaty® in the range of 12 to 17.8 ng/µL DNA are within the manufacturer's defined accuracy of 15% [18]. Therefore there is no room for a different conclusion than the following: The DNA contaminations in the Comirnaty® batches analyzed by König and Kirchner (2024) [1] are several hundred times higher than the permissible limit of 10 ng of DNA per dose or 0.033 ng/µL concentration in the ready-to-use diluted vaccine.

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