eppendorf

APPLICATION NOTE No. 459

# Fake DNA in Your qPCR? – Comparative Evaluation of Leaching Levels in PCR Plates.

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## Abstract

The influence of lab consumables on PCR assays remains largely underestimated. This poses a critical misconception, since laboratory plastics are an integral part of any PCR workflow and may have a direct and profound impact on experimental outcome and data validity. This study provides a comparative analysis of UV-absorbing leachables and qPCR fluorescence signals upon incubation of water samples in PCR plates from several manufacturers. The observed leaching levels and resulting false DNA concentrations were considerably high in the majority of the non-Eppendorf plates tested (up to 3,08 µg/mL). This indicates, that leaching may strongly influence qPCR assays and interfere with both photometric and fluorescence signal quantification leading to poor reproducibility results. Furthermore, a high variability of leaching itself was observed in the non-Eppendorf plates, which may adversely affect intra- and inter-plate assay reproducibility.

## Introduction

Despite its conceptual simplicity, PCR is not a trivial technique: accurate assay preparation, data analysis and interpretation may pose challenges and be influenced by various factors. Typically, only the assay-specific factors are perceived as relevant and tend to be properly controlled: sample material, reagents and laboratory equipment, whereas the influence of lab consumables remains largely underestimated.

This poses a critical misconception, since laboratory plastics are integral part of any PCR workflow and may have a direct and serious impact on experimental outcome and data validity. In particular, an increasing amount of studies indicate, that a large part of processing additives may be released (leach) from the consumable into the samples and pose a source of error in various assay systems including PCR [1, 2, 3].

The leaching effects are particularly relevant for plate-based assays, where a high variability in temperature conditions as well as position-dependent leaching can dramatically influence the data validity and reproducibility of a PCR/qPCR assay [4].

The range of PCR applications is broad including end-point PCR, qPCR or digital PCR as a means for simple quantification, but also ligation or amplification assays, which serve as preparation steps for further downstream analysis workflows such as NGS. Leachables may impact any of those assays in multiple ways – here qPCR and quantification served as a simple means to visualize interference.

This study provides a comparative analysis of UV-absorbing leachables and qPCR fluorescence signal readings upon incubation of water samples in PCR plates from several manufacturers.

## Material and Methods

### UV-absorbing leachables

Two different 96-well PCR plate lots (3 plates per Lot) of the following manufacturers were assessed: Eppendorf (twin.tec® PCR Plates), vendor "4T" and vendor "Ar". 48 wells of each PCR plate were filled with 100 µL of ultra-pure water in a chessboard pattern. The plates were sealed with the Eppendorf Heat Sealing Film and centrifuged for 1 min at 500 x g and then placed in a Mastercycler® X50s for 40 minutes at 96 °C. Further, the plates were mixed (Eppendorf MixMate®, 10 min at RT and 1200 rpm) and centrifuged (Eppendorf Centrifuge 5920 R, 1 min at RT, 500 x g). Subsequently aliquots of 90 µL were transferred from each well to a UV-VIS, 96/F Microplate to measure the absorbance on a Microplate Spectrophotometer (xMark™, Bio-Rad®). An absorbance wavelength spectrum from 220 nm to 400 nm was measured. Non-incubated water was used to set the blank values. Absorbance at 260 nm and the factor 50 µg/mL were used to calculate the false DNA concentration derived from UV-absorbing leachables for each sample.

### qPCR fluorescence signals

Two different 96-well PCR plate lots (1 exemplary plate per LOT) of the following manufacturers were assessed: Eppendorf (twin.tec® PCR Plates), vendor "4T" and vendor "Ar". All 96 wells of each PCR plate were filled with 20 µL of ultra-pure water. The plates were sealed with a self-adhesive Masterclear® real-time PCR Film and centrifuged (Eppendorf Centrifuge 5920 R, 1 min at RT, 500 x g). Subsequently, PCR plates were placed on a real-time PCR cycler (CFX96 Touch™, Bio-Rad) and subjected to the following thermal conditions: 5 minutes at 95 °C, followed by 35 cycles of 95 °C for 30 seconds and 60 °C for 45 seconds. All fluorophore channels of the CFX96 Touch real-time instrument were selected for the scanning: SYBR, HEX, TEXAS, CY5, CY5.5. The amplification data were analyzed using the CFX Maestro™ software (Bio-Rad). Fluorescence values were determined using either the Base Line Subtracted Curve Fit setting or without baseline subtraction. The baseline threshold was calculated automatically by the software.

## Result and Discussion

### UV-absorbing leachables

Figure 1 shows that during the incubation conditions tested (40 minutes at 96 °C) the plates of manufacturer "4T" and "Ar" released considerable amounts (up to 3,08 µg/mL) of UV-absorbing contaminants into the incubated water samples, which closely mimic the spectrum of nucleic acids. While absorbance levels are highest for shorter wave length, measurements at 260 nm and 280 nm still resulted in an absorbance of around 0.04 and 0.01 – 0.02 respectively. Simply put, as depicted in figure 2, those readings incorrectly indicate high levels of DNA with values up to 3,08 µg/mL (manufacturer 4T) in an absolutely DNA-free sample.

These UV-active leachables may thus heavily influence DNA spectrophotometric measurements and lead to false DNA readings. They certainly may pose a source of technical error for downstream applications that would need to be accounted for and corrected – given that the scientist is aware of the bias and that he can quantify the effect of the propagation of errors in his own application.

Significant reduction of such sources of error and applicational risks might be achieved by using high quality plates, such as the Eppendorf PCR plates, which exhibit the by far lowest levels of leachables (fig. 2).

To mitigate the influence of technical background scientists commonly use reference samples on each single plate. The reference signals are then used as a baseline. Those signals of the reference samples however are only meaningful if the variance of background signals across single plates is low and consistent across multiple plates and production lots. Non-consistent variance has a direct impact on assay reproducibility: it will significantly reduce achievable assay sensitivity and will increase risk of false positive and false negative results [4].

Figure 3 shows that consistent background signal across different plates and production batches, due to the leachables, is not a given (compare 4t lot 1 and 4t lot 2). While, due to sampling size, the results displayed here might not be reflective to all plates sold in the market it nevertheless should call attention during the assay development phase.

Assays with longer PCR protocol times and environments with a high variability in temperature conditions may boost the leaching effect. Therefore it is advisable to pay special attention to plate impact here.

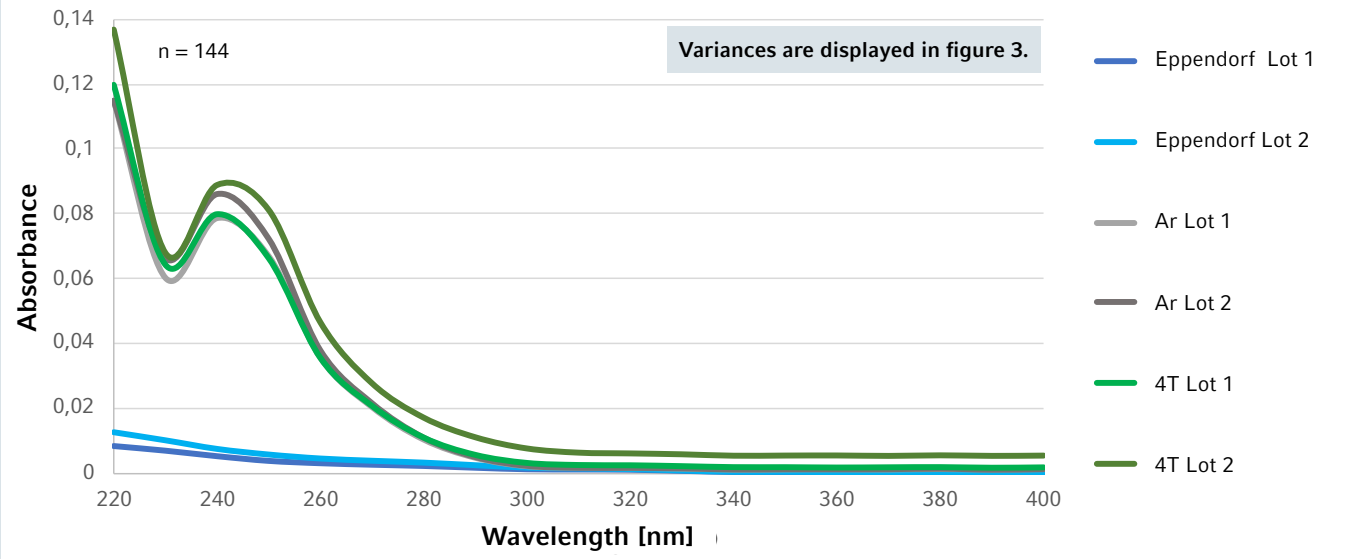


**Figure 1:** Absorption spectra of UV-absorbing (nucleic acid-mimicking) leachables detected after incubation of ultrapure water samples. Samples were incubated at 96 °C for 40 min in various PCR plates. Mean values of three standard 96-well PCR plates (48 wells per plate) from two separate lots are depicted.

**Did you know**
that you can calculate DNA concentration based on your OD signal using the Promega Biomath Calculators?

**https://www.promega.de/resources/tools/biomath/**

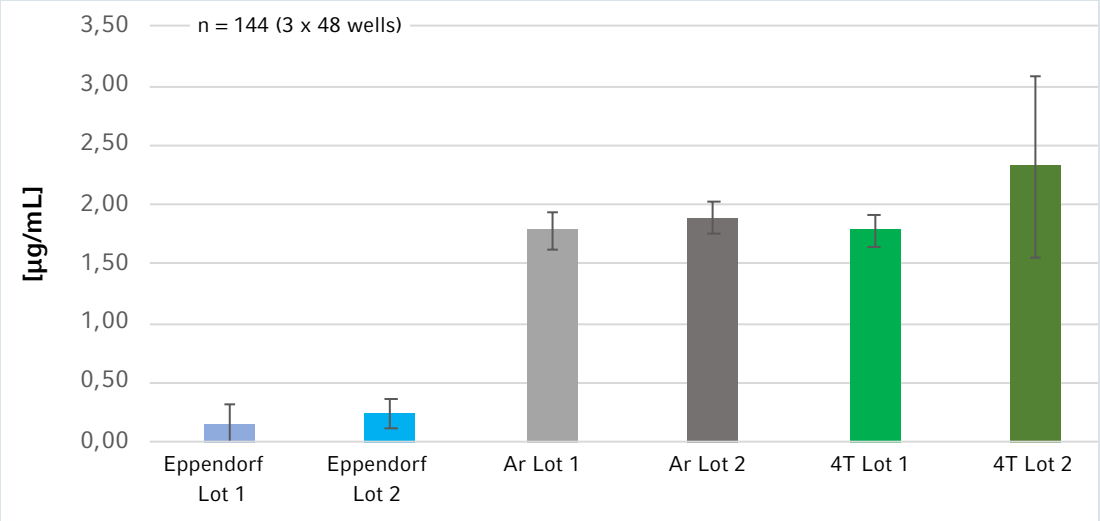


**Figure 2:** False DNA concentration (µg/mL) based on UV-absorbing leachables (A 260 nm). Leachables are released from 96-well PCR plates into ultrapure water samples after incubation at 96 °C for 40 min. Three standard 96-well PCR plates from two separate lots, with 48 wells per plate were analyzed. Mean values of three plates per lot are depicted.

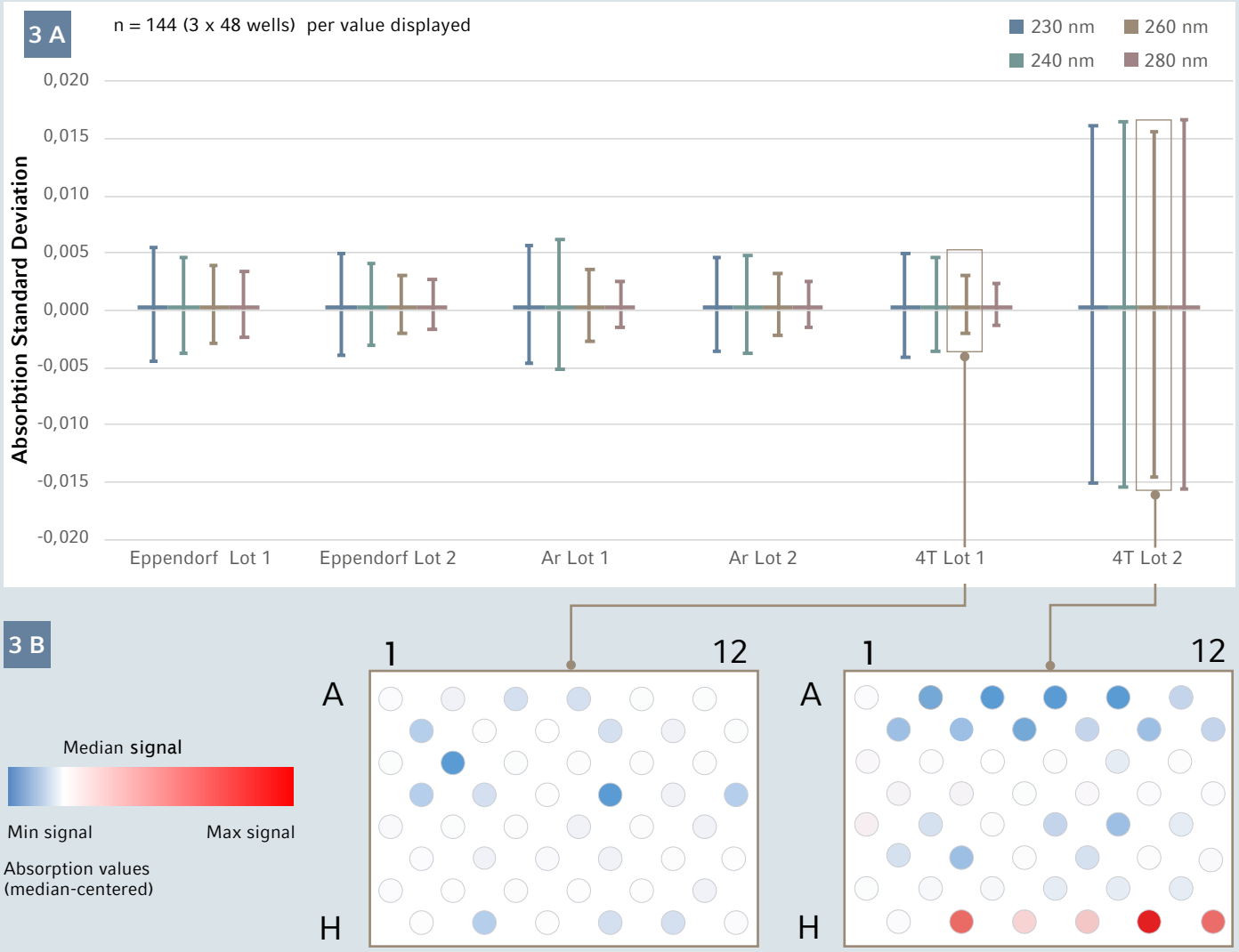


**Figure 3:** Inter-plate reproducibility of leachable levels.
**3A:** Standard deviation of absorption values for four different wavelengths are depicted. Three standard 96-well PCR plates from two separate Lots per manufacturer (six plates in total per manufacturer) were tested. Every second well of each plate (48 wells per plate) was analyzed. Values of three plates per lot are depicted.

**3B**: This image shows the potential real-life impact of the fluctuating "blank signal" variance displayed in figure 3A. It demonstrates how such fluctuation might lead to decreased meaningfulness of reference samples. Displayed are median-centered absorption values measured in the 48 wells of each one plate from lot 1 and from lot 2 of manufacturer 4T. The signals in lot 2 show a much higher variance across the plate with especially high signals in row H. In the laboratory practice, this might lead to false positive or false negative results.

### qPCR fluorescence signals

To further evaluate if leaching may directly influence a qPCR assays, ultrapure water samples were subjected to a standard qPCR thermal cycling protocol and resulting fluorescence signals were assessed. As shown in figure 4, specifically for the readings of the plates from manufacturers Ar and 4T, signals can indeed be impacted by released chemical substances. Here the signal interference was most notable for the commonly used SYBR/FAM wavelength, observable through the spread of the set of curves, while CY5, HEX, Texas RED or CY5.5 were less impacted. Noteworthy, the plates from manufacturer Ar have very transparent wells, which commonly may be perceived as high-quality parameter. Unfortunately, the well clarity often relates to high amounts of clarifying agents used during production process, which have been shown as critical and hamper various assay systems including PCR [4].

**Did you know**

That polypropylene by nature has a milky hue and that some of the most common leachables found in consumables are clarifiers added to the polypropylene to increase vessel transparency?

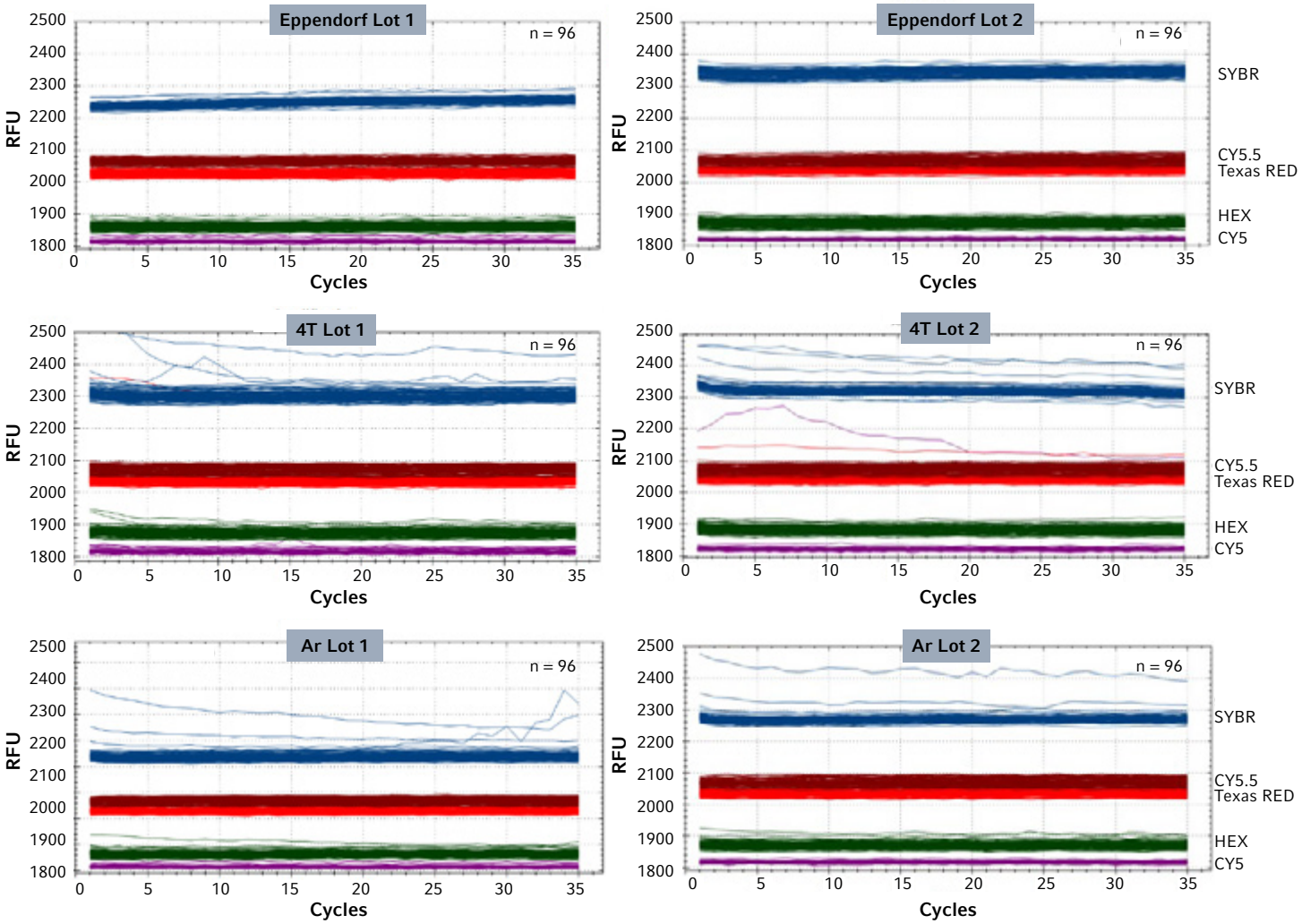


**Figure 4:** Fluorescence signals without baseline subtraction measured for water samples incubated in PCR plates of different manufacturers. Depicted are signals of 96 wells from one plate from each lot over 35 cycles of amplification protocol. The fluorescence signal of the 5 channels is present on each graph: FAM/SYBR (Excitation 450-490 / Detection 515-530): Blue lines, HEX (Excitation 515-535 / Detection 560-580): green lines, TEXAS Red (Excitation 560-590 / Detection 610-650): red lines, CY5 (Excitation 620-650 / Detection 675-690): purple lines, CY5.5 (Excitation 672-684 / Detection 705-730): burgundy lines.

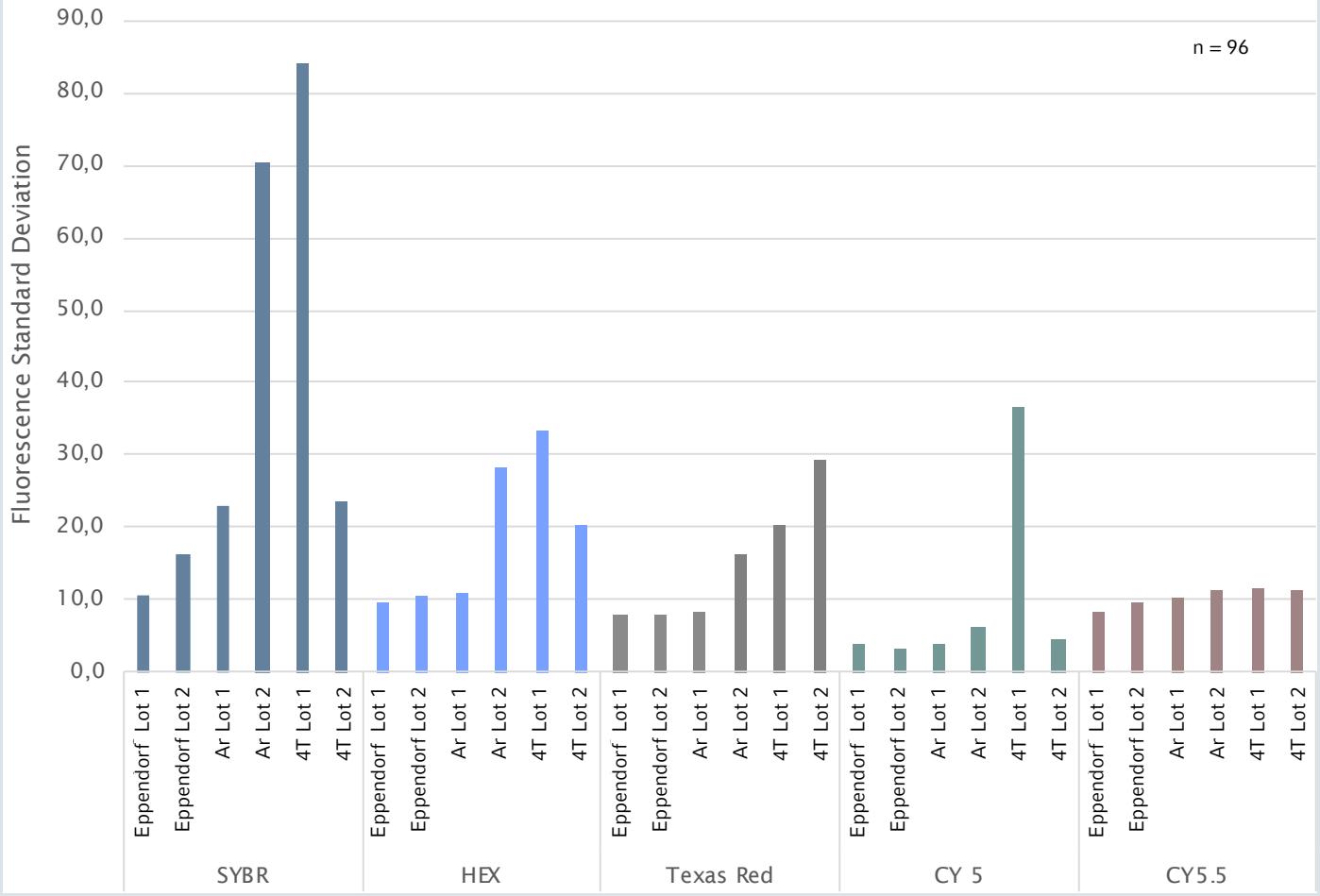


**Figure 5:** Standard deviation of the fluorescence signals measured for water samples incubated in PCR plates of different manufacturers. Depicted are standard deviation values in fluorescence units calculated from 96 signals of one plate from each lot.

Figure 5 displays the inter-plate variation of fluorescence signals of all detection channel used in the experiment. The particularly high interference in the FAM/SYBR channel is recognizable through the clearly higher standard deviation bars for plates of the manufacturers Ar and 4T, indicating that the technical bias affecting the signals detected in the single wells of a single plate is prone to higher variation.

Noteworthy, even though on a lower scale, two things can be observed: First, relatively constant fluorescence signal variability for Eppendorf plates across the single plates and across the lots. Second a rather fluctuating level of variability for the other manufacturers' plates analysed here. This indicates, that leaching may interfere with fluorescence signal quantification in qPCR assays leading to false or unreproducible results. Keep in mind that high variability of the leaching process itself may adversely affect intra- and inter-plate assay reproducibility especially when outlier signals might be clustered in the wells used for reference samples.

## Conclusion

This study provides a comparative analysis of UV-absorbing leachables and qPCR fluorescence signals. Using ultra-pure water samples incubated in PCR plates from several manufacturers the data shows that PCR plates of some manufacturers can release considerable amounts of UV-absorbing contaminants (up to 3.08 µg/mL for manufacturer 4T), which closely mimic the spectrum of nucleic acids and may interfere with quantification of nucleic acids and downstream applications, such as sequencing or cloning reactions.

Furthermore, both Ar and 4T plates released high levels of chemical substances directly interfering with fluoresces signal measurement during standard qPCR protocol. In particular, these leachables interfered with commonly used SYBR and CY5 detection channels and they also exhibited high inter-plate and inter-lot variability levels (CV values up to 3.6 %). This high variation may be particularly relevant, where a position-dependent leaching may dramatically influence both intra- as well as inter plate reproducibility and thus data validity of a qPCR assay [4]. For example: Imagine your lab's reference samples were pipetted into a row of plate wells with specifically high or low background signals. Or imagine you validated your assay with an outlier lot with low background signal only to continue to perform future analysis with lots with higher background signal. Both scenarios can have major impact, not only on a single experiment but the final conclusion drawn from a number of assays.

While it is legitimate to question whether the level of variation observed might be significant for one's own assay or a deviation between different plates could be circumvented via reference measurements, the main intention of this study is to increase awareness that consumables may negatively influence PCR and related or dependent assays (e.g. NGS) and hamper their reproducibility. It also vital to note, that interference of nucleic acid quantification is indeed a prominent, but only one example of negative effect leachables may pose on experiments. Amongst others, leachables may interfere with sample isolation, NGS library preparation, the PCR reaction itself or potentially as an entrained contaminant impacting downstream analysis steps. The exemplary experiment setting (UV-absorbing leachables and qPCR) here has been only chosen in order to quickly visualize and assess the leachable levels.

Ultimately, it is up to the scientist to judge, optimize and validate the single components of the workflow in his own assay. Single assay components such as samples, reagents and consumables work in concert and so the impact of certain components might highly depend on the specific setting. The more aware the scientist is of potential sources of error the more capable he is to reduce the total level of technical bias and ultimately to increase his level of reproducibility.

## Literature

[1] Lewis LK, Robson M, Vecherkina Y, Ji C, Beall G. Interference with spectrophotometric analysis of nucleic acids and proteins by leaching of chemicals from plastic tubes. Biotechniques 2010; 48(4):297-302.
[2] Grzeskowiak R. Extractables and Leachables in Microcentrifuge Tubes – Extensive HPLC/GC/MS Analysis. Eppendorf AG Application Note. 2018: No. 417; www.eppendorf.com
[3] McDonald GR, Hudson AL, Dunn SM, You H, Baker GB, Whittal RM, Martin JW, Jha A, Edmondson DE, Holt A. Bioactive contaminants leach from disposable laboratory plasticware. Science 2008; 322(5903):917
[4] Pfaffl M. Afif M. How to apply the MIQE Guidelines – a visual, interactive and practical qPCR guide! iBook (ISBN-9783000488061)

## Ordering information

**Ordering information**

| Description | Order no. international | Order no. North America |
|---|---|---|
| **Eppendorf twin.tec® Trace PCR Plate 96,** skirted, PCR clean, clear, 25 plates | 0030 129.768 | 0030129768 |
| **Eppendorf twin.tec® PCR Plate 96,** skirted, PCR clean, colorless, 25 plates | 0030 128.648 | 951020401 |
| **Eppendorf twin.tec® PCR Plate 96,** semi-skirted, PCR clean, colorless, 25 plates | 0030 128.575 | 951020303 |

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