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Assessing recovery rates of distinct exogenous controls for gDNA extraction efficiency using phenol-chloroform or silica-column based extractions

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Abstract

Assessment of genomic DNA (gDNA) extraction efficiency is required for accurate bacterial quantification by qPCR. Exogenous DNA molecules are often added after bacterial cultures are lysed, but before DNA purification steps, to determine extraction efficiency. Herein we found that different exogenous DNA controls have different recovery rates, suggesting distinct DNA extraction efficiencies. Recovery rates are also affected by the gDNA extraction method being more affected in silica-based columns than in phenol-chloroform extraction. Overall, we determined that the use of long DNA fragments, such as gDNA, as exogenous controls have a higher recovery rate than use of smaller size DNA molecules.

Main text

Quantitative PCR (qPCR) is a well-established method to quantify bacteria and is used in multiple types of biological samples (Edslev et al., 2021; Pacha-Herrera et al., 2020;

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Yin et al., 2021). Overall, bacterial quantification by qPCR requires nucleic acid extraction followed by the thermal amplification process. Variability in nucleic acid extraction efficiency can lead to significantly different concentrations of RNA (Magalhães et al., 2019) or DNA extracted from similar samples (Greathouse et al., 2019). To quantify this inherent technical variability, the utilization of exogenous controls has been proposed as a method to normalize total nucleic acid extraction (Johnston et al., 2012; Revilla-Fernández et al., 2005). This is of utmost importance when quantifying bacterial species by DNA extraction, as the lack of adequate controls hinders comparison of results between studies (Ducarmon et al., 2020; Schwager et al., 2018).

We previously observed that, for lower bacterial concentrations, a luciferase cDNA exogenous control was not sufficient to accurately quantify bacterial populations in three species consortia, when using a silica-column based extraction commercial kit (Cerca et al., 2022). We hypothesized that the low DNA mass of such small exogenous controls could result in a very low recovery rate in the DNA optimized silica columns commonly used for genomic DNA (gDNA) extraction, which could bias quantification.

To test this hypothesis, *Gardnerella vaginalis* ATCC 14018T was grown in culture, as previously described (Rosca et al., 2020). Bacterial suspensions with culture densities ranging from 3 × 10^8 cells.mL-1 to 1 × 10^7 cells.mL-1 were prepared in PBS 1x. Each culture was divided in equal aliquots and gDNA was extracted with either the DNeasy Ultraclean Microbial Kit (QIAGEN), using the manufacturer's instructions with minor modifications (Cerca et al., 2022), or with the classical phenol/chloroform method (Kumar and Mugunthan, 2018). A mixture containing three different exogenous controls, namely, commercially available luciferase cDNA (Promega) (10 µL of 10^8 copies of luciferase cDNA, with 67 bp), 10 µL of 1 ng.µL-1 *Staphylococcus epidermidis* gDNA (2.6 Mb, (NCBI, 2005), extracted with DNeasy Ultraclean), and 10 µL of 1 ng.µL-1 of a plasmid (piMAY, 5473 bp (Monk et al., 2012)), was spiked before DNA isolation to determine the % of DNA recovered, in both methods. The recovery rate was calculated as the difference between the qPCR cycle threshold of the target gene in the DNA samples versus the cycle threshold of the pure exogenous DNA controls, at equivalent concentrations. DNA concentration and purity were measured using a Nanodrop one (Thermo-Scientific). Three extractions per independent condition were performed. The qPCR runs were performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad) with the following cycle parameters: 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. The qPCR amplifications were conducted in 10 µL reaction mixtures, containing 5 µL of Xpert Fast SYBR MasterMix (Grisp, Porto, Portugal), 1 µL of primer mixture (10 µM), 2 µL of DNase free water, and 2 µL of a 1:40 dilution of extracted gDNA.

Three control reactions (one for each exogenous control) were performed in triplicate. To assess the efficiency of gDNA extraction and to calibrate fluorescence intensity between qPCR runs, a control was used by adding 2 µL of 1:200 of each exogenous control tested to all independent qPCR runs. Melt-curve analysis was performed to ensure the absence of unspecific products and primer-dimers. The primers used to quantify the target genes were previously developed: Fw CAACGGTATCCTGACCGTCT; Rv CCTTGCAAAGGCAGTTAAGC for *G. vaginalis* detection (Sousa et al., 2021); Fv TACAACACCCCAACATCTTCGA and Rv GGAAGTTCACCGGCGTCAT for luciferase detection (Magalhães et al., 2019), Fw TACATGTCAAGAATAAACTGCCAAAGC and Rv AATACCTGTGACGGAA GATCACTTCG for piMAY detection (Monk et al., 2012), and Fv TCAGACG ACATCATTGCGCT; Rv ACGTTGTCCCCTTATCTCCTC for *S. epidermidis* gDNA detection (Brás, 2020). Primer specificity at 60ºC was experimentally determined. qPCR amplification efficiency was determined through the slope of a standard curve, constructed with serial dilutions of DNA. Efficiencies were 94% for *G. vaginalis*, 100% for luciferase, 89% for piMAY, and 90% for *S. epidermidis* primers. Statistical analysis was performed using an unpaired-samples t-test ($p < 0.05$).

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As previously shown (Greathouse et al., 2019), for the same initial bacterial loads, different gDNA yields were observed, from a minimum of 1.5x fold variation, in samples extracted with Ultraclean at 1 x 10^8 cells.mL-1, to a maximum of 6.6x fold variation, in samples extracted with phenol/chloroform at 3 x 10^8 cells.mL-1. On average we found a 3.3x fold variation in gDNA extraction yields for the same initial bacterial load (**Table 1**). Without normalization of the different gDNA extraction efficiencies, performing calibration curves for qPCR quantification would result in significant quantification errors, as we have previously shown (Cerca et al., 2022). Not surprisingly, gDNA extraction with the phenol/chloroform method yielded more gDNA than the silica-column based approach (Ibrahim and Nassar, 2018; Schiebelhut et al., 2017), except at very low bacterial concentrations, where both extraction methods yielded similar results.

Table 1. Variability in gDNA extraction efficiency of pure *G. vaginalis* cultures adjusted to specific concentrations, using two different extraction methods. DNA concentration and purity was assessed by Nanodrop (Thermo-Scientific).

	Ultraclean kit	Phenol/ chloroform method

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Assay	Bacterial concentration (cells.mL-1)	DNA yield (ng.µL-1)	A260/280	A260/A230	DNA yield (ng.µL-1)	A260/280	A260/A230
1.	3×10^8	41.8	1.84	0.28	228.3	1.91	2.85
2.		25.7	1.82	0.15	41.9	1.85	2.44
3.		48.9	1.90	2.22	235.9	1.92	2.76
1.	1×10^8	4.4	1.72	0.14	53.9	1.84	2.69
2.		6.6	1.83	1.29	16.4	1.83	2.71
3.		6.8	1.85	1.22	59.9	1.79	2.59
1.	3×10^7	1.8	1.32	0.23	12.7	1.78	2.59
2.		3.8	1.31	0.51	43.0	1.70	2.38
3.		2.5	1.56	0.67	20.7	1.52	1.85
1.	1×10^7	0.2	1.09	0.00	1.6	1.20	1.56
2.		0.7	1.33	0.13	1.2	1.58	1.82
3.		0.9	0.92	0.14	4.2	1.38	1.93

When analysing the recovery rate of the 3 exogenous controls by both methods, it became aparent that the variability observed in the gDNA yield was further aggravated. Generally, a lower recovery rate was observed in samples with a lower total gDNA (**Figure 1**). Interestingly, experiments performed with DNA obtained by the phenol-chroroform extraction method presented similar extraction efficiencies, suggesting that the size and conformation of the exogenous controls had little effect on the exogenous control recovery rate. However, for the silica column-based extraction method, recovery rates were strongly affected by the nature of the exogenous controls, at 3×10^8 cells.mL-1 to 1×10^7 cells.mL-1. For these culture densities, piMAY presented a significantly lower recovery rate than *S. epidermidis* gDNA, while luciferase showed a significantly lower recovery rate than the gDNA exogenous control at 3×10^8 cells.mL-1. The main differences between extraction methods were observed when using piMAY as an exogenous control, wherein, in 2 out of the 4 bacterial densities tested, the recovery rates by the two methods were statistically different (**Figure 2**).

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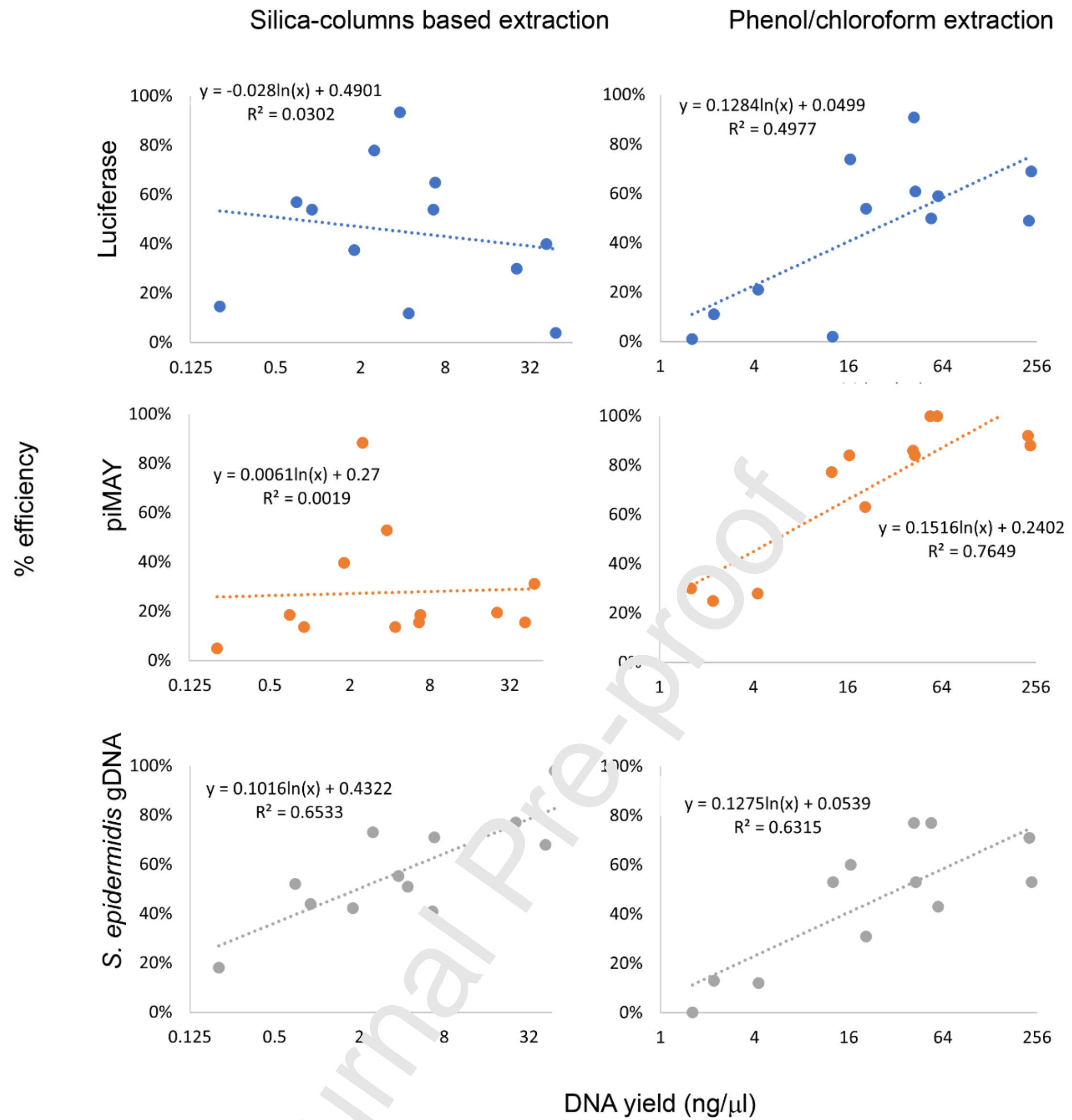

Figure 1. Variability of gDNA extraction efficiency of *G. vaginalis* cultures with different bacterial concentrations for each exogenous control and for the two different extraction methods.

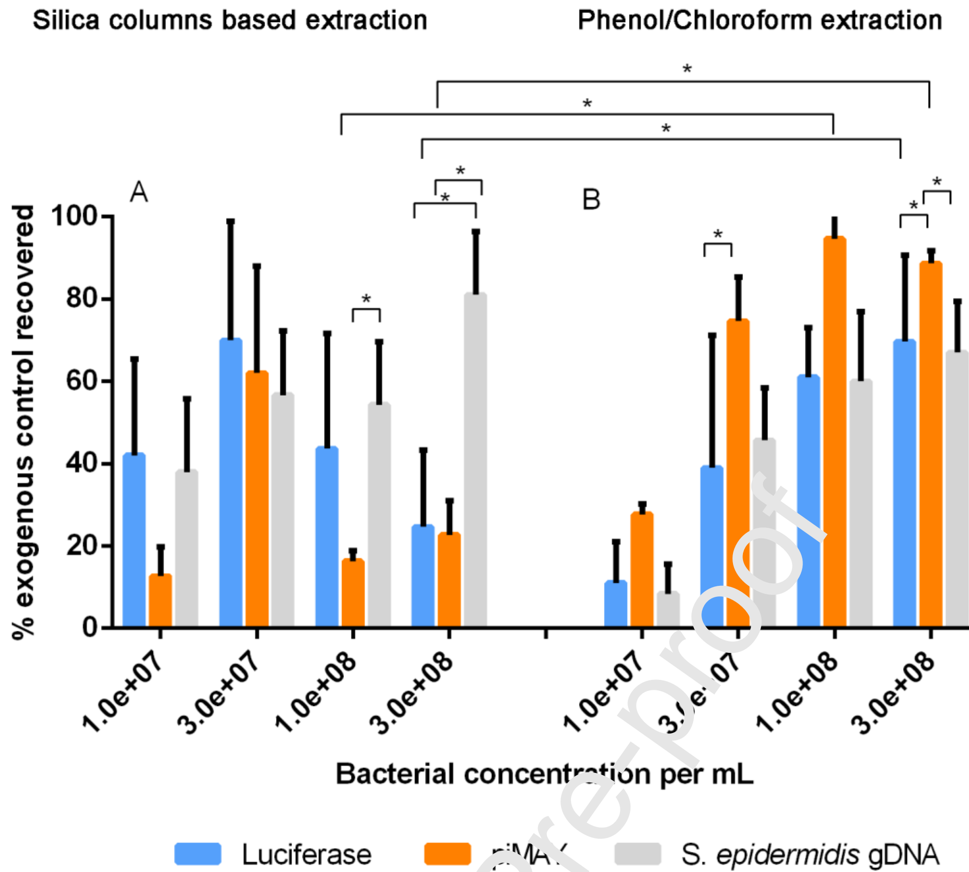

Figure 2. Quantitative differences between DNA extraction methods and the recovery rate of the exogenous controls. The bars represent the mean and the standard deviation of 3 independent assays. * represents a statistical significance between each extraction method (Two-way ANOVA, Turkey's multiple comparisons test, $p < 0.05$).

Overall, these data support our initial hypothesis that differential affinity for the silica-columns can impact bacterial quantification by qPCR. Despite the better performance of the phenol/chloroform extraction method, researchers often use silica-based protocols, due to greater time constraints and the utilization of toxic chemicals in the classical phenol//chloroform method (Ali et al., 2017). Importantly, we have shown that when using an exogenous control composed of gDNA molecules unrelated to the target genome, the recovery rates were similar in both DNA extraction processes (**Figure 2**). This leads us to conclude that, for silica-based columns that are optimized for gDNA extraction, exogenous controls should also be composed of unrelated gDNA molecules. Alternatively, as shown recently, commercially available bacterial community standards can also be used as exogenous controls (Scarsella et al., 2021).

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Author contributions

NC and CAM designed the experiments. LS and FM performed the DNA extractions. ÂL performed the qPCR experiments. ÂL and NC drafted the manuscript. All authors critically reviewed and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the contents of this manuscript. the contents of this manuscript.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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- gDNA isolation process has inherent variability
- Addition of exogenous DNA controls are often used to normalize extraction efficiency.
- The size of the exogenous DNA can impact normalization efficiency.

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