

COMPARISON OF DNA STANDARDS FOR REAL-TIME PCR-BASED QUANTIFICATION OF *LACTOBACILLUS ACIDOPHILUS* IN DAIRY PRODUCTS

Monir-Sadat Shakeri *

Address(es):

Department of Food Biotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

*Corresponding author: m.shakeri@rifst.ac.ir

<https://doi.org/10.55251/jmbfs.3738>

ARTICLE INFO

Received 21. 9. 2020
Revised 26. 9. 2021
Accepted 29. 9. 2021
Published 1. 2. 2022

Regular article



ABSTRACT

Probiotic bacteria are an essential part of the healthy gut microbiota. Fermented foods as potential sources of health-promoting bacteria can regulate the intestinal microbial population. However, the exact quantification of these bacteria in such multiple-strain matrixes continues to remain elusive. In this study, we evaluated the reliability of genomic DNAs and cloned recombinant plasmids as standard controls for absolute real-time PCR assay. The associated standard curves were constructed and used for the quantification of *Lactobacillus acidophilus* probiotics. All stages from the design and construction of standards and related curves met the criteria for high-quality products. There were no significant differences between the two enumeration methods. However, plasmid-based standard curves resulted in a lower detection limit than the curves of genomic DNA standards. Our findings showed that the non-linearized recombinant plasmids had long-term stability at high concentrations during storage at -20 °C, which strongly depended on the purification methods. We propose that the recombinant plasmid standards can supersede the traditional genomic DNA standards for accurate quantification of probiotic bacteria.

Keywords: Recombinant DNA; Plasmid standard; DNA calibrator; Standard curve; Bio-yoghurt

INTRODUCTION

Lactobacilli are common probiotics in food owning the specific beneficial health properties (Fijan *et al.* 2019). *Lactobacillus acidophilus* is one of the best-recognized species of the genus *Lactobacillus*. Based on morphological properties, these bacteria are gram-positive and non-spore-forming rods. They are found in different commercial fermented milks because of intestinal probiotic effects and modulation of the host microbiome (Widyastuti *et al.* 2021). Identification of these bacteria is essential to discriminate them from phylogenetically similar strains with different properties. Therefore, reliable procedures are required for qualitative and quantitative detection of probiotic bacteria.

There are different PCR-based quantification techniques such as competitive PCR, real-time PCR, and digital PCR (Zentilin and Giacca 2007; Papic *et al.* 2017) (Fig. 1). Real-time PCR (qPCR) is the most precise method by which to measure genes. It is now a well-known method for identification, quantification, and microbial community analysis that covers a wide range of applications in medicine and food safety. In real-time PCR, DNA amplification is tracked through the monitoring of fluorescence. Although the basic principles of PCR are simple, there are some specific issues in qPCR to achieve reliable quantification (Kralik and Ricchi 2017). Problems such as primer dimers, amplification of non-specific products, and heterogeneous efficiency of amplification may occur that users must have previously thought about them.

Absolute real-time PCR quantification using the standard curve method is the most common technique used in environmental and food microbiology (Brankatschk *et al.* 2012). In fact, to address the issues associated with non-uniform PCR efficiencies in different samples, the standard curve method is uncomplicated and reliable (Larionov *et al.* 2005). A standard curve can be created by amplification of serial dilutions of known concentration of standard template followed by linear regression of the log of DNA concentrations of the standard templates versus threshold cycle (Ct). In this method, it is presumed that the efficiency of both sample and standard are the same (Mackay 2004; Brankatschk *et al.* 2012). However, in practice they may not be similar, which affects the quantification accuracy. Hence the reliability and validity of the standard curve are a matter of interest. It depends on the design and production of standard control, measurement of the exact concentration, and long storage stability (Pfaffl 2004). To overcome the fluctuations of efficiency, it is crucial that both the standard dilutions and the unknown samples amplify in the same protocol and with the same amplification

properties (Taylor *et al.* 2019). Moreover, it is undoubtedly associated with the choice and quality of standard controls. They should have excellent properties such as purity, indivisibility, and stability. Purified PCR product, plasmid DNA construct, genomic DNA, cDNA, or synthetic oligonucleotide spanning the PCR amplicon can be used as a DNA standard control or calibrator. Between them, cloned recombinant plasmid DNA and genomic DNA generate reproducible standard curves due to high stability (Pfaffl 2004; Boulter *et al.* 2016). However, MIQE guidelines have introduced the best practices to facilitate standardization of qPCR assay. Some issues related to finding suitable controls to generate a standard curve for each gene of interest are still remained (Boulter *et al.* 2016).

In the current study, we designed a plasmid DNA construct containing the target gene of *L. acidophilus* bacteria. The constructed plasmid was compared with genomic DNA as standards for absolute real-time PCR assay with the aim of accurate enumeration of these probiotics.

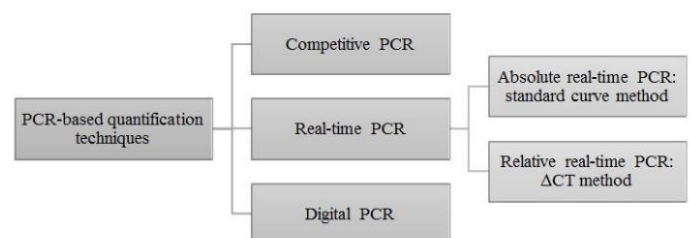


Figure 1 Different types of PCR-based quantification methods

MATERIALS AND METHODS

Bacterial Strain and Food Samples

L. acidophilus bacteria were from American Type Culture Collection (ATCC 4356) and activated in de Man Rogosa Sharpe (MRS) broth (Merck-Darmstadt, Germany) at 37°C. Commercial bio-yoghurts with yoghurt culture organisms, *L. acidophilus* and bifidobacteria, were obtained in IRAN. Surface plating on MRS agar was used for the classical enumeration of the bacteria. The plates were incubated at 37°C for 72 h under anaerobic conditions.

DNA Extraction

Genomic DNAs of bacterial pellets from overnight liquid cultures and bio-yoghurt samples were extracted with an AccuPrep™ Genomic DNA Extraction Kit (Bioneer, Korea). The protocol slightly modified for increased extraction efficiency as previous work by Shakeri *et al.* (Shakeri *et al.* 2018). Briefly, bacteria were harvested from 1 mL of overnight culture based on McFarland turbidity standards (approximately 10^8 cfu/mL) by centrifugation. The pellet was mixed with 500 μ L of TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA) buffer and 15 mg/mL lysozyme followed by incubation at 37 °C for 1 h. All other purification steps were done according to the protocol described in the kit. The purity of DNA was checked on a nanodrop spectrophotometer (Nanodrop Technologies, DE, USA) by absorbance detection at 260, 280, and 230 nm.

Construction of the Plasmid DNA as Standard

The plasmid encoding part of the *L. acidophilus* 16S rRNA gene was constructed through the amplification of a 227-bp fragment within the conserved region of the 16S rRNA gene using the specific primer pairs including Acidfor (5'-AGCGAGCTGAACCAACAGAT-3') and Acidrev (5'-AGGCGTTACCCTACCAACT-3'). This primer set was previously designed by Tabasco *et al.* (Tabasco *et al.* 2007), and has been used before by Shakeri *et al.* (Shakeri *et al.* 2018). The primer sequences and PCR conditions with the genomic DNA of the bacteria as the template were similar to the previous study published by these authors.

The amplified fragments were visualized using agarose gel electrophoresis, and the band size verified. They were purified using a DNA extraction kit K0513 (EN0525; Fermentas, Vilnius, Lithuania), according to the manufacturer's protocol. The purified DNA segment was TA cloned into *Escherichia coli* DH5 alpha using TA Cloning Kit K1214 (EN0525; Fermentas). The plasmid DNA was then extracted using the High Pure Plasmid Isolation Kit (Roche, Germany) based on the kit manual. Freshly extracted plasmids were linearized with the restriction endonuclease that cuts the BamHI site near the inserted PCR target (Fig. 2) and purified. The concentration of linearized plasmids quantified using the UV absorbance (OD_{260}) method. PCR was performed on the purified plasmids using Acidfor and Acidrev primers. Moreover, the presence of the target gene was confirmed via the digestion of BamHI and EcoRI sites. All the enzyme assays were carried with 2 U of each enzyme at 37°C for 2 h. Based on the molecular weight of the designed plasmid DNA (plasmid plus insert) and its concentration, the number of copies per μ L was calculated using the formula described by Kibbe, (Kibbe, 2007).

We also extracted the plasmid DNA by the basic manual alkaline lysis method for stability analysis during storage (Sambrook and Russel, 2001). The stability of the standards (plasmid and genomic DNAs) was checked by measuring the total DNA concentrations after three years of storage at -20 °C. The mean of the percentage of variance from triplicates was calculated using Excel (Microsoft, Unterschleissheim, Germany).

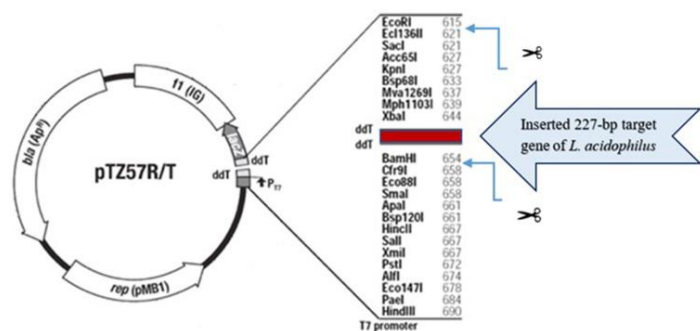


Figure 2 Physical genetic map of recombinant pTZ57R/T plasmid carrying the 16S rRNA-based target gene of *L. acidophilus*

Construction of Standard Curves

To prepare the standard curves for molecular quantification of *L. acidophilus* bacteria, two standard controls, including genomic DNA and cloned recombinant plasmid DNA containing the target sequence, were used.

To construct the standard curve with genomic DNA, bacterial genomic DNA from an overnight culture of *L. acidophilus* bacteria with a cell concentration of 6×10^8 cfu/mL was serially diluted in nuclease-free water (Sigma). In this way, we prepared a set of standard dilutions of genomic DNA that is equivalent with the known concentration of bacterial cells ranging from about 6×10^2 to 6×10^7 cfu/mL. The real-time PCR assay was performed with five serial dilutions of standard DNA samples as a template. The correlation between Ct values and log concentration of *L. acidophilus* bacteria (cfu/mL) in standard samples was used for generating the standard curve by linear regression analysis in Microsoft Excel.

To generate the plasmid DNA-based standard curve, the stock plasmid solution with a concentration of 1.49×10^{10} copy/ μ L was serially diluted in DNA grade water. Subsequently, these serial 10-fold dilutions of the standard plasmid (2.98×10^1 to 2.98×10^8 DNA copies per reaction in 2 μ L) were used to qPCR amplification. The standard curve was constructed by plotting the Ct values against the log copy numbers of the designed plasmid in standard samples using the linear regression, as described above.

To obtain the numbers of cfu, extracted DNA from a bacterial suspension of 6×10^5 cfu/mL was subjected to the qPCR assay using this standard plasmid curve, and the copy number of plasmids equivalent to the number of bacterial cells was calculated. Mean Ct values from duplicates (two repeats) were calculated and used for generating an average standard curve from each standard construct.

Real-time PCR Quantification and Data Analysis

Real-time PCR was carried on CFX96 Touch™ Bio-Rad Cyclor (Bio-Rad, Hercules, CA, USA). The final reaction volume was 25 μ L, including Maxima SYBR Green qPCR Master Mix (Fermentas, Vilnius, Lithuania) and 5 pmol of both Acidfor and Acidrev primers, 1 μ L of DNA template, and DNase free water. The real-time PCR cycling parameters were the following: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 30 s. Reactions containing ddH₂O as no-template controls (NTC) were included in each run. At the end of the amplification, melting curve analysis was run to confirm the presence of the single desired product in each reaction. All reactions were performed in triplicate. According to the constructed standard curves, the number of *L. acidophilus* bacteria in pure cultures (containing a known concentration of *L. acidophilus* bacteria) and commercial bio-yoghurts was calculated and compared. Statistical differences in the quantification observed between two standard curve methods were examined by paired t-test using SigmaStat (SigmaStat Statistical Software, Version 4.0, Jandel Corporation, San Rafael, CA, USA). 95% confidence interval ($P \leq 0.05$) was considered in the test.

RESULTS AND DISCUSSION

Constructed Plasmid DNA

The designed plasmid, including the target region of *L. acidophilus* 16S rRNA gene, was successfully generated, and the structural integrity of the plasmid sample was confirmed (Fig. 3A). The dominant conformation of the isolated plasmid DNA was a supercoiled form. However, linear and nicked-circular plasmid forms were also observed. Non-linearized plasmids result in different efficiencies during the PCR reaction (Dhanasekaran *et al.* 2010). So the extracted plasmid DNA was linearized and then used as templates for the standard curve. Moreover, enzymatic digestion analysis and PCR amplification with the specific target primers verified the accuracy of the plasmid carrying the 227-bp target gene of *L. acidophilus* (Fig. 3B).

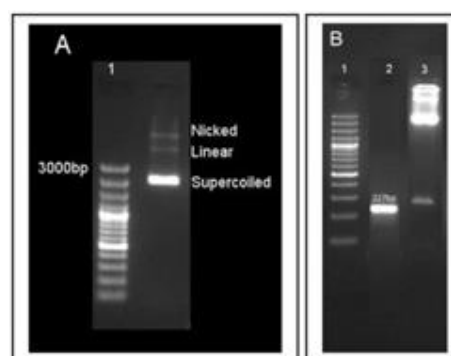


Figure 3 Properties of constructed plasmid DNA: A, Gel electrophoresis of the isolated plasmid DNA (nicked, linear and supercoiled forms). B, verification of inserted target through restriction enzyme digestion and colony PCR. Lane 1: 100 bp DNA ladder (Thermo Scientific, USA). Lane 2: PCR product (227 bp) on the generated plasmid DNA. Lane 3: a band of ~ 266 bp produced from double digestion of BamHI/EcoRI sites on the constructed plasmid

Generated Standard Curves

As shown in Fig. 4A, the standard curve generated from genomic DNA as a calibrator had a linear range between 6×10^2 and 6×10^7 cfu/mL with slopes of -3.424. The amplification efficiency of the genomic DNA used for developing the standard curve was 95.9%, with a regression coefficient (R^2) value of 0.996.

The standard curve of 10-fold dilutions of the recombinant plasmid DNA as a calibrator is shown in Fig. 4B. The amplification efficiency and R^2 were 99.8% and 0.99, respectively. The linear dynamic range was considered between 2.98×10^1 and 2.98×10^8 DNA copies per reaction with slopes of -3.326, which indicated that the amplification was well performed. For both standard curves, the thermal dissociation curves were checked at the end of each PCR, and always had a unique

melting point of 82.5°C, without any additional peak, indicating single PCR products. Amplified fragments of the expected size were also verified by gel electrophoresis (Fig. 5).

In general, a suitable standard curve is characterized by factors including slope, PCR efficiency, and correlation coefficient. Ideally, the slope should be -3.33, which corresponds to 100% efficiency or two-fold (precisely, 2.0092) amplification at each cycle. Also, correlation coefficient should be ≥ 0.99 for gene quantitation analysis (Pfaffl 2004; Dorak 2006). In practice, an optimal standard curve should have efficiency as close to 100% as possible (90-110%), the slope between -3.1 to -3.6 with low variation and a high correlation coefficient. However, slope values between -3.2 and -3.4 indicate that the reaction is well optimized (Dorak 2006; Matijasic et al. 2010; Taylor et al. 2019). According to our results, appropriate efficiency, accuracy, and sensitivity were observed for both standard curve methods. However, the linear range of the plasmid-based standard curve was greater with a lower limit of detection (LOD). According to the lowest number point on the standard curves, the LOD for genomic and plasmid DNAs were 600 and 10 cfu/mL, respectively.

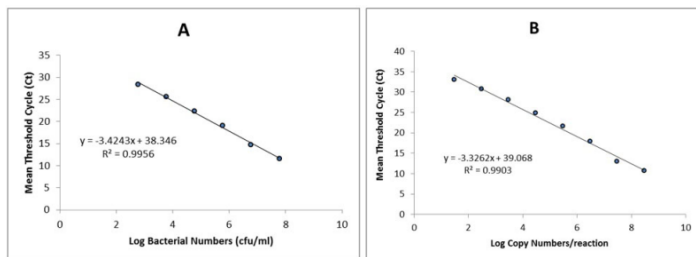


Figure 4 Standard curves for qPCR enumeration of *L. acidophilus* bacteria: A, Standard curve obtained from real-time PCR of 10-fold dilution series of extracted genomic DNA of *L. acidophilus* bacteria. B, Standard curve obtained from real-

time PCR of serial dilutions of the designed plasmid DNA in which the target 16S rRNA gene of *L. acidophilus* bacteria is inserted

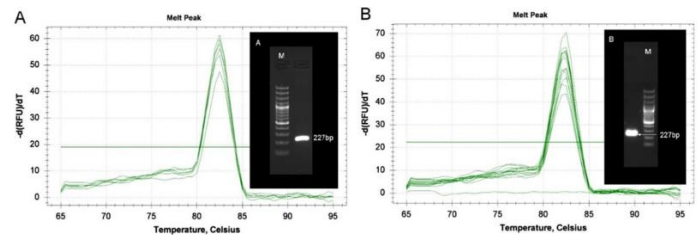


Figure 5 Melting curve analysis and gel electrophoresis visualization for the real-time PCR products with the used primer set and standard DNAs: A, genomic *L. acidophilus* DNA as a template and B, the constructed plasmid DNA as template. M: 100 bp DNA ladder (Thermo Scientific, USA)

Comparative Analysis of Two Standard Curve Methods

For comparison between two generated standard curves, DNA extracted from three suspensions of *L. acidophilus* bacteria was quantified using two methods. The results showed that there were no significant differences between the counts of bacterial cells (Table 1). Both assays represented more than 97% recovery with low variability in repeatability. Similarly, two standard curve methods did not have significant differences on the enumeration of these bacteria in bio-yoghurt samples (Table 2). Most of the previous studies on molecular quantification of probiotic bacteria have used genomic DNAs or PCR products as standards (Furet et al. 2004; Ongol et al. 2009; Sattler et al. 2014). However, plasmid DNAs have presented highly efficient detections of food-borne pathogens, which is similar to our results (Liu et al. 2019). Moreover, the plasmids were suitable calibrators for the quantification of genetically modified content of the food (Burns et al. 2006).

Table 1 qPCR quantification of *L. acidophilus* bacteria in pure cultures based on genomic and recombinant plasmid DNA standard curves.

Standard curve methods	Expected numbers (log cfu/mL)	Observed numbers (log cfu/mL) ^a	Reliability		Paired t- test ($\alpha=0.05$)
			Repeatability	Variability	
			Recovery ^b (%)	RSD ^c (%)	
GDM	7.176	7.077 ± 0.122	98.561	1.727	P = 0.695 (ns) ^d
PDM		7.100 ± 0.126	98.886	1.773	
GDM	5.176	5.127 ± 0.117	98.970	2.274	P = 0.691 (ns)
PDM		5.147 ± 0.131	99.357	2.550	
GDM	3.176	3.107 ± 0.116	97.694	3.739	P = 0.971 (ns)
PDM		3.113 ± 0.115	97.904	3.704	

GDM; genomic DNA-based method, PDM; Plasmid DNA-based method, ns; non-significant.

^a Mean ± SD (n=3).

^b Recovery: observed number/expected number × 100%.

^c RSD: relative standard deviation = SD/Mean × 100%.

^d Two-tailed P-value.

Table 2 Enumeration of *L. acidophilus* bacteria in bio-yoghurt samples based on genomic and recombinant plasmid DNA standard curves.

Standard curve methods	Number of bacteria (log cfu/mL) ^a	Paired t- test ($\alpha=0.05$)
Genomic DNA-based method	7.033 ± 0.051	non-significant p = 0.560
Plasmid DNA-based method	7.006 ± 0.032	

^a Mean ± SD (n=3).

As shown in Fig. 6, the percentage of the variance of DNA concentration for different types of standards was also evaluated after three years of storage at -20 °C. It was clear that the non-linearized recombinant plasmid DNA extracted by the used kit (non-linearized) had a variance of <2%, which is acceptable. However, the manual extracted plasmid DNA and genomic DNA showed a variation of >10%, indicating that the nature of the standard and isolation method could change the standard concentration during the storage time. Moreover, diluted standards were degraded completely (data was not shown). Similarly, Dhanasekaran et al. (Dhanasekaran et al. 2010) demonstrated that PCR products cloned into non-linearized plasmids were more stable than linearized plasmids containing the PCR products during 14 days of storage at -20 °C. In spite of the PCR suppression effect of the supercoiled conformation of plasmids (Lin et al. 2011), our findings showed that this form of plasmids could maintain high stability. In similar, long-term stability (3 years for -20 °C) of the plasmids containing information DNAs have been previously demonstrated by Nguyen et al. (Nguyen et al. 2018). Changes in standard concentrations and copy numbers due to the degradation of the DNA

targets cause the main variation in PCR efficiency of standards without altering the correlation coefficient. Hence, all of the mentioned factors, including PCR efficiency, concentration, and copy number variations as well as R², are equally significant for accurate enumeration of probiotic bacteria.

According to these results, the use of the plasmid could be more precise because of its ease of maintenance and stability under long storage time, which can lead to a more accurate enumeration of probiotic bacteria between various laboratories. Table 3 represents the technical and performance properties of these two standards. Because plasmid DNA is more stable and requires fewer replicates, the plasmid-based standard curve method has a higher throughput than genomic-based assay. The technique is also easy with universal applicability and reliable estimation of the copy number of the standards. The determination of plasmid DNAs is based on the molecular weight of the nucleotides, which is more precise than genomic DNAs. In general, the determination of the concentration and length of the shorter templates is more accurate (Pfaffl 2004). Finally, it is possible to construct a cloned recombinant plasmid containing several target genes of bacteria, which leads to the amplification of more target genes in a single reaction in qPCR assay. In this way, more bacteria can be detected simultaneously (Liu et al. 2019). However, the production of standard material is an expensive and time-consuming process.

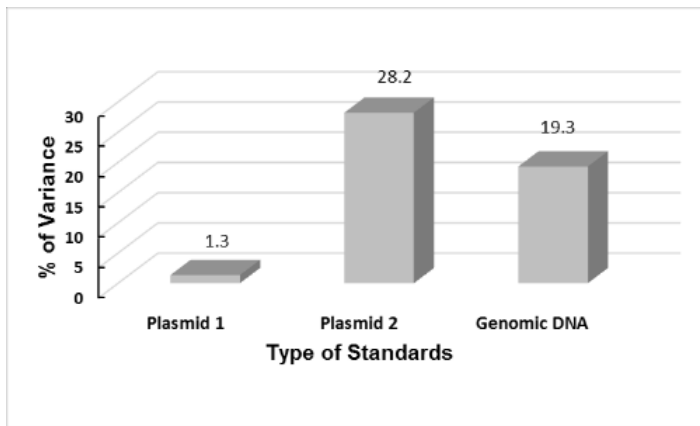


Figure 6 The Average percentage of the variance of DNA concentration for different standards (n=3). Plasmid 1: non-linearized recombinant plasmid DNA extracted by the commercial kit, Plasmid 2: non-linearized recombinant plasmid DNA obtained by the basic manual alkaline lysis method, and Genomic DNA: extracted by the commercial kit

Table 3 Comparison of cloned recombinant plasmid DNA to genomic DNA as a calibrator in qPCR assay.

Properties	Plasmid DNA	Genomic DNA
Relative quantification	✓	✓
Absolute quantification	✓	✓
High accuracy	✓	✓
No specialized equipment	✓	✓
High throughput	✓	
Low replicates	✓	
No special preparation		✓
Applicable for certified reference materials	✓	
High stability	✓	
High precision in copy number estimation	✓	
Multiple and simultaneous quantifications of bacteria	✓	

CONCLUSION

Performance characteristics related to plasmid and genomic calibrators were investigated to find the best practice guide. The results demonstrated that plasmid standard outperformed genomic DNA in the quantification of *L. acidophilus* bacteria. When the related standard curve was generated, the limit of detection was 10 cfu/mL, which was remarkably lower than the genomic DNA standard. The plasmid standard was very stable after three years of storage at -20 °C. It was the first report on the long-term stability of the plasmid standards for real-time PCR quantification of probiotic bacteria. Conclusively, we tend to suggest using the plasmid-based standard curve method as a reliable and efficient alternative for quantification of probiotic bacteria in absolute real-time PCR. It can create a reproducible quantification which helps the standardization of qPCR assay. It should be added that standard curve materials should be routinely assessed for quality and stability.

Acknowledgements: The authors thank the personnel of the genomics laboratories of the Ferdowsi University of Mashhad.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals.

Informed Consent: This article does not contain any individual participants.

REFERENCES

Boulter N, Suarez FG, Schibeci S, Sunderland T, Tolhurst O, Hunter T, Hodge G, Handelsman D, Simanainen U, Hendriks E, Duggan K (2016) A simple, accurate and universal method for quantification of PCR. *BMC Biotechnology* 16, 27. <https://doi.org/10.1186/s12896-016-0256-y>
 Brankatschk R, Bodenhausen N, Zeyer J, Burgmann H (2012) Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. *Applied and Environmental Microbiology*, 78(12), 4481-4489. <https://doi.org/10.1128/AEM.07878-11>
 Burns M, Corbisier P, Wiseman G, Valdivia H, McDonald P, Bowler P, Ohara K, Schimmel H, Charels D, Damant A, Harris N (2006) Comparison of plasmid and

genomic DNA calibrants for the quantification of genetically modified ingredients. *European Food Research and Technology* 224, 249-258. <https://doi.org/10.1007/s00217-006-0376-z>
 Dhanasekaran S, Mark Doherty T, Kenneth J, TB Trials Study Group (2010) Comparison of different standards for real-time PCR-based absolute quantification. *Journal of Immunological Methods*, 354, 34-39. <https://doi.org/10.1016/j.jim.2010.01.004>
 Dorak MT (2006) *Real-time PCR*. New York: Taylor and Francis Group.
 Fijan S, Frauwallner A, Varga L, Langerholc T, Rogelj I, Lorber M, Lewis P, Povalej Brzan P (2019) Health professionals' knowledge of probiotics: an international survey. *International Journal of Environmental Research and Public Health*, 16, 3128. <https://doi.org/10.3390/ijerph16173128>
 Furet J, Quenee P, Tailliez P (2004) Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *International Journal of Food Microbiology*, 97, 197-207. <https://doi.org/10.1016/j.ijfoodmicro.2004.04.020>
 Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research*, 35, Web Server issue W43-W46. <https://doi.org/10.1093/nar/gkm234>
 Kralik P, Ricchi M (2017) A basic guide to real-time PCR in microbial diagnostics: definitions, parameters, and everything. *Frontiers in Microbiology*, 8, 108. <https://doi.org/10.3389/fmicb.2017.00108>
 Larionov A, Krause A, Miller W (2005) A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics*, 6, 62. <https://doi.org/10.1186/1471-2105-6-62>
 Lin C, Chen Y, Pan T (2011). Quantification bias caused by plasmid DNA conformation in quantitative real-time PCR assay. *PLoS ONE*, 6(12), e29101. <https://doi.org/10.1371/journal.pone.0029101>
 Liu Y, Cao Y, Wang T, Dong Q, Li J, Niu C (2019). Detection of 12 common food-borne bacterial pathogens by TaqMan real-time PCR using a single set of reaction conditions. *Frontiers in Microbiology*, 10, 222. <https://doi.org/10.3389/fmicb.2019.00222>
 Mackay IM (2004) Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection*, 10, 190-212. <https://doi.org/10.1111/j.1198-743X.2004.00722.x>
 Matijasic BB, Obermajer T, Rogelj I (2010) Quantification of *Lactobacillus gasserii*, *Enterococcus faecium* and *Bifidobacterium infantis* in a probiotic OTC drug by real-time PCR. *Food Control*, 21, 419-425. <https://doi.org/10.1016/j.foodcont.2009.07.001>
 Nguyen HH, Park J, Park SJ, Lee CS, Hwang S, Shin YB, Ha TH, Kim M (2018). Long-term stability and integrity of plasmid-based DNA data storage. *Polymers*, 10, 28. <https://doi.org/10.3390/polym10010028>
 Ongol MP, Tanaka M, Sone T, Asano K (2009). A real-time PCR method targeting a gene sequence encoding 16S rRNA processing protein, rimM, for detection and enumeration of *Streptococcus thermophilus* in dairy products. *Food Research International*, 42, 893-898. <https://doi.org/10.1016/j.foodres.2009.04.010>
 Papic B, Pate M, Henigman U, Zajc U, Gruntar I, Biasizzo M, Oceppek M, Kusar D (2017) New approaches on quantification of *Campylobacter jejuni* in poultry samples: the use of digital PCR and Real-time PCR against the ISO standard plate count method. *Frontiers in Microbiology* 8, 331. <https://doi.org/10.3389/fmicb.2017.00331>
 Pfaffl MW (2004) *Quantification strategies in real-time PCR*. In: Bustin SA (ed) A-Z of quantitative PCR. La Jolla, CA: International University Line (IUL).
 Sambrook J, Russel D (2001) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
 Sattler VA, Mohnl M, Klose V (2014) Development of a strain-specific real-time PCR assay for enumeration of a probiotic *Lactobacillus reuteri* in chicken feed and intestine. *PLoS ONE*, 9(2), e90208. <https://doi.org/10.1371/journal.pone.0090208>
 Shakeri M, Shahidi F, Mortazavi A, Bahrami AR, Nassiri MR (2018) Combination of competitive PCR and cultivation methods for differential enumeration of viable *Lactobacillus acidophilus* in bio-yoghurts. *International Journal of Dairy Technology* 70, 1-6. <https://doi.org/10.1111/1471-0307.12536>
 Tabasco R, Paarup T, Janer C, Pelaez C, Requena T (2007) Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk. *International Dairy Journal* 17, 1107-1114. <https://doi.org/10.1016/j.idairyj.2007.01.010>
 Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J (2019) The ultimate qPCR experiment: producing publication quality, reproducible data the first time. *Trends in Biotechnology* ,37(7), 761-774. <https://doi.org/10.1016/j.tibtech.2018.12.002>
 Widyastuti Y, Febriantosa A, Tidona F (2021) Health-promoting properties of lactobacilli in fermented dairy products. *Frontiers in Microbiology*, 12: 673890. <https://doi.org/10.3389/fmicb.2021.673890>
 Zentilin L, Giacca M (2007) Competitive PCR for precise nucleic acid quantification. *Nature Protocol*, 2(9), 2092-2104. <https://doi.org/10.1038/nprot.2007.299>