Folia Microbiol (2013) 58:657–662
DOI 10.1007/s12223-013-0255-5

Error estimation in environmental DNA targets quantification due to PCR efficiencies differences between real samples and standards

Leonardo Martín Pérez · Mariana Fittipaldi · Bárbara Adrados · Jordi Morató · Francesc Codony

Received: 25 June 2012 / Accepted: 23 April 2013 / Published online: 7 May 2013

Introduction

Nowadays, as in the past, our knowledge of microbiology is limited because of the capacity of available technology tools. In recent decades, molecular techniques are playing a central role in the understanding of the microbial world. In this sense, a complete scientific evaluation of biological samples is not possible without using molecular biology. The most influential technique has probably been the polymerase chain reaction (PCR), which allows us to work beyond classical microbiology. With PCR, in some cases, the need for culture may be avoided. Nevertheless, until the development of real-time PCR, our evaluation of the microbial complexity has been merely qualitative.

Real-time PCR is an evolution of the conventional endpoint PCR wherein the amplification of a target gene and its fluorescence detection occurs simultaneously during each cycle. Different strategies based on the use of nonspecific DNA intercalating dyes or specific fluorescent probes exist for linking target amplification to fluorescence detection. Regardless of the fluorescence emission mechanisms involved, real-time PCR enables both detection and quantification of a genetic target by a continuous fluorescence monitoring of DNA amplification (Higuchi et al. 1993).

An advantage in real-time PCR is that target quantification sensitivity is independent of the copy number within the sample (Freeman et al. 1999). Moreover, the key aspect that differentiates real-time PCR from previous semi-quantitative methodologies is that target DNA concentration can be determined from the fractional cycle at which a threshold amount of target DNA is produced (i.e., measured signal exceeds a background level), set at a point where amplicon DNA just becomes detectable but is still within the exponential phase of the amplification (Higuchi et al. 1993; Rasmussen 2001). This quantification cycle (*Cq*) (or cycle threshold, *Ct*) are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the *Cq* value, the greater the amount of target nucleic acid in the sample). This approach ensures that interfering factors associated with later stages of the amplification are minimized and considerably improves the measurement precision (Rasmussen 2001).

One of the most important points is that real-time PCR allows quantitative measurements. In order to estimate a target concentration in environmental samples, absolute quantification can be performed using a standard curve constructed by amplifying known amounts of target DNA (Rutledge and Côté 2003). Nowadays, this approach is the most commonly used in environmental microbiology.

Real-time PCR has many advantages over conventional endpoint PCR including speed, broad dynamic range of quantification, reduced risk of contamination, and good sensitivity. However, despite all these objective strengths, real-time PCR sensitivity is not better than conventional PCR if a protocol validation is not performed (Bastien et al. 2008).

L. M. Pérez · M. Fittipaldi · B. Adrados · J. Morató · F. Codony (✉)
Laboratori de Microbiologia Sanitaria i Mediambiental (MSM-Lab) & Aquasost - UNESCO Chair in Sustainability, Universitat Politècnica de Catalunya (UPC),
Edifici Gaia, Rambla Sant Nebridi 22,
08222 Terrassa, Barcelona, Spain
e-mail: codony@oo.upc.edu

L. M. Pérez
Departamento de Química Analítica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR) & Instituto de Química Rosario (IQUIR, UNR-CONICET),
Suipacha 570,
2000 Rosario, Santa Fe, Argentina

The amplification process is driven by the PCR efficiency as stated by the Eq 1 (Rebrikov and Trofimov 2006):

$$N = N_0 E^n \quad (1)$$

N, total copies of the genetic target after PCR
N_0, initial number of copies of the genetic target
E, PCR efficiency
n, number of cycles

Theory indicates that DNA is duplicated in each amplification cycle; as a result, the efficiency (E) should be 2. But different factors related with reagents, primers performance, or DNA quality, frequently have a considerable impact on the amplification process making the efficiency value lower than the theoretical one (Tuomi et al. 2010). Example 1 shows numerically how different PCR efficiencies for standard and sample amplification reaction could impact in target quantification, especially at lower concentrations (i.e., high *Cq* values).

PCR efficiency can be estimated using two different experimental protocols. The first one explores the "*window of linearity*":

$$E = \left(\frac{N}{N_0}\right)^{\frac{1}{n}} \quad (2)$$

window of linearity approach

This approach estimates the slope of the fluorescence detection in individual amplifications during log phase (Ramakers et al. 2003). The second approach uses the curve fitting method, which is based on the construction of standard curves and regression analysis (Rasmussen 2001):

$$E_{Standard} = 10^{\frac{-1}{slope}} \quad (3)$$

curve fitting approach

The latter methodology (curve fitting) is frequently used to report PCR efficiencies in most published scientific works, but it is based only on data obtained from curves performed with good-quality standard DNAs (pure cultures, plasmids, or cDNA) that may be different (generally lower) than that obtained for DNA from real environmental samples (Love et al. 2006). Moreover, once an experimental protocol has been selected and subjected to internal validation and optimization, the PCR efficiency is frequently evaluated only during the standards quantification. Under this point of view, PCR inhibitions can be detected only if they were absolutes.

In the present study, we have analyzed real-time PCR data for *Bacteroides* spp., *Legionella pneumophila*, or *Adenovirus* spp. obtained from a large number of different environmental samples with the aim of elucidating the bias on the microbial target quantification due to its environmental nature. We also hypothesize that this bias is a consequence of the differences between the PCR efficiencies for the standard DNA and the sample DNA. Our proposal can be used to determine the uncertainty and bias in the real sample analysis which would enable environmental microbiologists to make the right decisions.

Materials and methods

Microbiological analysis

Table 1 summarizes the information related to environmental samples (n=92), microbial targets, source for standards DNA, environmental sample treatment, and real-time PCR protocol used throughout this study. Quantitative PCR (qPCR) reactions were carried out in a Lightcycler 1.5 instrument (Roche, Manheim, Germany). Reaction mixtures contained 10 μL of the 2x QuantiTect probe or Sybrgreen PCR Master Mix (Qiagen, Hilden, Germany); 5 μL of DNA extract template; primers and probes according to the manufacturer's instructions in order to achieve the work/method concentration, and molecular grade water. Total final PCR reaction volume was 20 μL. Thermal cycling conditions were used in accordance with the protocols used (Table 1) in previously published works (Jothikumar et al. 2005; Layton et al. 2006; Behets et al. 2007). The method used for *Adenovirus* spp. detection was performed without an internal probe but verifying each positive result by a melting point analysis.

Genomic DNA obtained from pure cultures was used to develop standard curves which were used in bacterial target PCR detection and quantification. For *Adenovirus* spp., standard DNA was obtained from a plasmid containing a fragment of the Hexon gene cloned into a competent cell using the Qiagen PCR cloning kit (Qiagen, Hilden, Germany). The latter method was the only one suitable for our laboratory allowing us to obtain a good quality viral DNA.

PCR reactions were performed in duplicate. In each case, data from standards were obtained from three separate serial dilutions with different target levels (from 10^1 to 10^5 gene copy number for PCR reaction). The *Cq* values were automatically determined by the Lightcycler software.

Target DNA concentration was estimated using two approaches: firstly, according to the "dilution of the signal" strategy (i.e., previous dilution to signal loss is considered as 10^1 target copy number per reaction). Secondly, the DNA amount was quantified by spectroscopy and divided by the genome or plasmid size. The coherence between both approaches was used as a reference to estimate target concentration in our standards.

Quantification error estimation

Efficiencies per sample (E_{Sample}) were estimated using the window of linearity approach described by Ruijter et al.

Table 1 Summary of sample processing, microbial target and real-time PCR protocols used throughout the present study

Sample (*n*)	Sample processing	DNA extraction (kit)	Microbial target (gene)	Source of standard DNA	Reference method
Soil (42)	100-g soil+100-mL sterile NaCl (0.85 %), vortex 30 s, sedimentation 2 min	2-mL supernatant (DNA soil Omega bio-tek)	*Bacteroides* spp. (16S rRNA)	ATCC 51477	Layton et al. 2006
Wastewater (30)	Haramoto et al. 2005	0.2-mL concentrated (EZNA tissue DNA Omega bio-tek)	*Adenovirus* ser. 40–41 (Hexon)	Plasmid (fragment Hexon gene) ATCC VR-930	Jothikumar et al. 2005
Cooling tower waters (20)	100-mL filtration, (nylon 0.22 μm Ø), recovery in 10-mL sterile NaCl (0.85 %), vortex 30 s, ultrasound bath 3 min	2-mL concentrated (EZNA tissue DNA Omega bio-tek[a])	*Legionella pneumophila* (MIP)	NCTC 12821	Behets et al. 2007

[a] This kit was also used for standards DNA purification

(2009). The correction factor (CF) was estimated according to Eq 4 as follows:

$$CF = \left(\frac{E_{Standard}^{Cq}}{E_{Sample}^{Cq}} \right) \quad (4)$$

Where $E_{Standard}$ and E_{Sample} are the PCR efficiencies calculated at the *Cq* cycle for standard and environmental sample DNAs, respectively, and *Cq* is the *Cq* value determined by the thermal cycler software. The use of CF also assumes that a true value is known; in this case, we are assuming that the best possible efficiency is that obtained with good quality DNA.

Statistical analysis

For each microbial target, PCR efficiency obtained for standard (using the mean of each duplicates per dilution) or environmental sample DNAs (using the mean of each replication per sample) was expressed with a confidence interval (CI) of 95 %, using SigmaStat® (Version 3.1, Systat Software Inc., California, USA).

Results

Table 2 shows PCR efficiencies, *Cq* values, and the calculated CF for each environmental sample type and microbial target during the quantification process. For all three microbial targets, the *Cq* value obtained from environmental sample DNA amplification reaction was around 30. According to our knowledge, this value corresponds to a target concentration per reaction ranging from 100 to 1,000 copies. These levels are low but good enough to be well detected and they are inside of the range where quantification is possible and generates reliable results. In the case of results values lower than 25 copies per reaction, they often show a great variability in quantification.

The PCR efficiency achieved by analyzing soil samples and industrial cooling tower water samples was excellent for *Bacteroides* spp. (1.92–1.95) and *L. pneumophila* (1.91–1.95), respectively. In both cases, no statistical differences were detected between PCR efficiencies for standard and environmental sample DNAs. The two analyzed microbial target efficiencies obtained using standard DNAs were included into the 95 % CI of the samples PCR efficiencies.

For *Adenovirus* spp., PCR efficiencies of standards (1.89–1.93) were statistically different ($p < 0.05$) from sample PCR efficiencies (1.81–1.87). As environmental sample DNA amplification proceeds differently from the standard, the detection will be biased affecting sample *Cq* and, consequently, the final quantification. Example 1 illustrates the error magnitude due to differences in the amplification reaction efficiencies for samples and standard DNAs. At the same target concentration (i.e., same *Cq*), a 0.10-amplification E difference between the standard DNA and the sample DNA produces a final quantification error of 3.75. In other words, for the same initial target concentration, the decrease in the sample PCR product amount due to lower amplification reaction efficiency has a proportional variation in the fluorescence signal and, therefore, the target concentration estimation in the environmental sample is 3.75 times lower. Following this reasoning, our results for *Adenovirus* spp. shows that different PCR efficiencies for the amplification reaction of the standard DNA and the environmental sample DNA resulted in a significant error at the final viral load quantification at the environmental sample. The concept is that if two reactions (standard used for calibration and sample) have different behaviors, the quantification will have an error. In this case, CF varies from 4.89 to 48.89 suggesting the need for analytical improvements or modifications in the final quantification by means of a bias correction for the samples (Table 2). As showed in Fig. 1, CF

Table 2 Global result summary

Sample		PCR efficiency for microbial target		
Type (target)	qPCR *Cq* 95 % CI	Sample DNA 95 % CI	Standard DNA 95 % CI	CF 95 % CI
Soil (*Bacteroides* spp.)	(29.47–30.75)	(1.92–1.95)	(1.94–1.97)	(1.37–3.66)
Wastewater (*Adenovirus* spp.)	(31.97–33.03)	(1.81–1.87)	(1.89–1.93)	(4.89–48.89)*
Industrial water (*L. pneumophila*)	(32.81–34.21)	(1.91–1.95)	(1.87–1.95)	(0.77–1.80)

*p <0.05, statistically significant

increases by the contribution of two factors. First, the difference between PCR efficiencies and, second, the *Cq* values. As error increases at higher *Cq*, the quantification bias when environmental samples with low target levels (i.e., high *Cq* values) are analyzed may be considerable, even with small differences in the PCR efficiencies.

Discussion

Although amplification reaction efficiency is key for real-time PCR DNA quantification, most scientific papers in environmental microbiology do not include information about PCR efficiencies of the processed samples. In environmental samples, the hybridization process between primers and target DNA is not always perfect. Multiple primers hybridization sites with slight differences with the target sequence may change PCR efficiency, and it can differ from the efficiency value calculated using pure laboratory standard DNAs during the prior validation of the PCR reaction. However, reaction efficiency must not be the only parameter to be considered when real-time quantification is performed. Any change in sample type, sample treatment, used reagents, PCR materials (e.g., tubes), or thermal cycler can affect the DNA amplification reaction performance and subsequently, the PCR efficiency. Some other factors are also critical for real-time PCR quantification: (1) the *Cq* value, (2) the DNA molar absorbance, and (3) the error associated to the calibration procedure. Both *Cq* and DNA molar absorbance are related with the thermal cycler optical performance and with the software algorithms, and formally, they cannot be improved easily. Linear regression quality, in qPCR quantification with external standards, is frequently associated with errors during the manipulation and preparation of the standard dilutions. Indeed, the abovementioned factors also contribute to the bias in the quantification affecting the accuracy and uncertainty of the results, but not as much as the reaction efficiency (Love et al. 2006). While the aim of the present work is not to elucidate the significance of all the factors related with real-time PCR accuracy and uncertainty, it is important to notice that such factors can be significantly reduced by increasing the workflow quality, performing a proper instrument calibration, or applying more rigorous criteria for the regression line construction and acceptance. On the other hand, Kitchen et al. 2010 reported some strategies that minimize the total confounding in qPCR analysis that could help researchers in optimizing the quantification procedures.

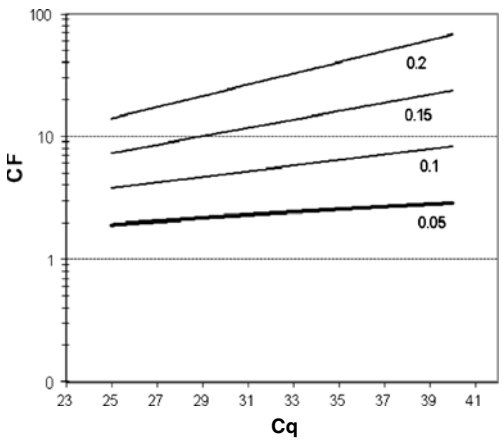

Fig. 1 Graphical representation of correction factor (CF) in real-time PCR absolute quantification model (Eq 4) as a result of the differences in PCR efficiencies between sample and standard DNA for the same microbial target. Each line represents a 0.05-magnitude difference between PCR efficiencies obtained for good quality standard DNA and environmental sample DNA. CF increases exponentially and this increment is larger for environmental samples with low DNA target concentration (i.e., high *Cq* values)

Recently, Brankatschk et al. (2012) have also demonstrated the impact of PCR efficiency in microbial quantification on environmental samples using degenerated primers and calculating the PCR efficiency by the method of multiple dilutions (fitting method). In that work, PCR efficiency differences were detected up to several orders of magnitude. As stated by the authors, the contribution of using degenerate primers was found to be relevant in order to explain those differences. On the other hand, the approach used by Brankatschk et al. (2012) for efficiency estimation could be probably finer because it includes more PCR reactions, but for the same reason, it could also be a practical limitation in common workflows.

Limitations in real-time PCR quantification using standard curves approaches were pointed out several years ago for mRNA quantification (Ramakers et al. 2003). To solve these problems, a new algorithm to estimate PCR efficiencies was developed by Ruijter et al. (2009). This method was adapted by Töwe et al. (2010) in an environmental survey using DNA from soil samples, but DNA quantification was only possible for a limited range. Therefore, in environmental microbiology it must be necessary to apply a practical alternative in order to obtain quantitative values when differences between sample and standard DNAs PCR efficiencies exist. We proposed that as stated in Example 1, in such cases, once the sample reaction efficiency has been estimated, a practical approach could be to calculate the bias and to correct the final value obtained by the thermal cycler software by multiplying it by the CF.

Multiple methods to calculate PCR efficiency exist (Rasmussen 2001; Ramakers et al. 2003; Rebrikov and Trofimov 2006). Therefore, in our opinion, researchers need to know the contribution of the reaction performance in the quantification regardless of the approach used to finally estimate DNA targets concentration. This step is critical in environmental microbiology because, in some environmental samples, specific targets may not be present at high levels and the detection must to be done at high *Cq* values where small differences between standard and samples PCR reaction efficiencies are more important (Fig. 1). In these cases, as we demonstrated for *Adenovirus* spp., the quantification error in the final concentration estimation will be considerable (see Table 2). For accurate target quantification it is important to know the PCR efficiency for each procedure and environmental sample type. This knowledge could lead to evaluate the need for an entire workflow optimization or, at least, be able to correct the results using an adequate factor. Despite of the method used to estimate PCR efficiency, using the approach from Example 1, it is possible to estimate the bias and correct the quantification error in the final result.

We encourage researchers to estimate PCR efficiency in their environmental samples and to know the quantification bias. In addition, editors and reviewers must persuade authors to include this information in their scientific communications.

Acknowledgments We thank the Ministry of Education and Science of Spain and the Comissionat per a Universitats i Recerca del Departament d'Innovacio´ and Universitats i Empresa de la Generalitat de Catalunya i del Fons Social Europeu, for supporting this study. Financial support was provided by Alfa Network TECSPAR (RED ALFA II-0543- FI-FAFCD; Sustainable technologies for potabilization and wastewater treatment), and by grant CTM2008-06676-C05-02/ TECNO from the Ministry of Science and Innovation of Spain to Jordi Morató. The authors also want to thank to the peer review process of Folia Microbiologica for improving the quality of the present work.

Example 1

Using Eq 4 for the same reaction at the same Cq (i.e. 25)

$$E_{Standard} = 1.95 \quad E_{sample} = 1.85$$
$$N = N_0 1.95^{25} \quad N = N_0\ 1.85^{25}$$
$$N = N_0 1.8 \cdot 10^7 \quad N = N_0\ 4.8 \cdot 10^6$$
$$\mathrm{CF} = \left(\frac{E^{Cq}_{Standard}}{E^{Cq}_{Sample}}\right)$$
$$\mathrm{CF} = 3.75$$

References

Bastien P, Procop GW, Reischl U (2008) Quantitative real-time PCR is not more sensitive than "conventional" PCR. J Clin Microbiol 46:1897–1900. doi:10.1128/JCM.02258-07

Behets J, Declerck P, Delaedt Y, Creemers B, Olleviera F (2007) Development and evaluation of a TaqMan duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. J Microbiol Methods 68:137–144. doi:10.1016/j.mimet.2006.07.002

Brankatschk R, Bodenhausen N, Zeyer J, Bürgmann H (2012) Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl Environ Microbiol 78(12):4481–4489. doi:10.1128/AEM.07878-11

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. Biotechniques 26:112–125

Haramoto E, Katayama H, Oguma K, Ohgaki S (2005) Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan. Appl Environ Microbiol 71:2403–2411. doi:10.1128/AEM.71.5.2403-2411.2005

Higuchi R, Fokler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11:1026–1030. doi:10.1038/nbt0993-1026

Jothikumar N, Cromeans TL, Hill VR, Lu X, Sobsey MD, Erdman DD (2005) Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. Appl Environ Microbiol 71:3131–3136. doi:10.1128/AEM.71.6.3131-3136.2005

Kitchen RR, Kubista M, Tichopad A (2010) Statistical aspects of quantitative real-time PCR experiment design. Methods 50(4):231–236. doi:10.1016/j.ymeth.2010.01.025

Layton A, Mckay L, Williams D, Garrett VR, Sayler G (2006) Development of *Bacteroides* 16S rRNA gene Taqman-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol 72:4214–4224. doi:10.1128/AEM.01036-05

Love JL, Scholes P, Gilpin B, Savill M, Lin S, Samuel L (2006) Evaluation of uncertainty in quantitative real-time PCR. J Microbiol Methods 67:349–356. doi:10.1016/j.mimet.2006.04.005

Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 339:62–66. doi:10.1016/S0304-3940(02)01423-4

Rasmussen R (2001) Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K (eds) Rapid cycle real-time PCR—methods and applications. Springer Press, Heidelberg, pp 21–34

Rebrikov DV, Trofimov DY (2006) Real-time PCR: a review of approaches to data analysis. Appl Biochem Microbiol 42(5):455–463. doi:10.1134/S0003683806050024

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB, Moorman AFM (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucl Acids 37:e45. doi:10.1093/nar/gkp045

Rutledge RG, Côté C (2003) Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res 31(16):e93. doi:10.1093/nar/gng093

Töwe S, Kleindeindam K, Schloter M (2010) Differences in amplification efficiency of standard curves in quantitative real-time PCR assays and consequences for gene quantification in environmental samples. J Microbiol Methods 82:338–341. doi:10.1016/j.mimet.2010.07.005

Tuomi JM, Voorbraak F, Jones DL, Ruijter JM (2010) Bias in the Cq value observed with hydrolysis probe based quantitative PCR can be corrected with the estimated PCR efficiency value. Methods 50(4):313–322. doi:10.1016/j.ymeth.2010.02.003