Serious Overestimation in Quantitative PCR by Circular (Supercoiled) Plasmid Standard: Microalgal pcna as the Model Gene

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Abstract

Quantitative real-time PCR (qPCR) has become a gold standard for the quantification of nucleic acids and microorganism abundances, in which plasmid DNA carrying the target genes are most commonly used as the standard. A recent study showed that supercoiled circular confirmation of DNA appeared to suppress PCR amplification. However, to what extent to which different structural types of DNA (circular versus linear) used as the standard may affect the quantification accuracy has not been evaluated. In this study, we quantitatively compared qPCR accuracies based on circular plasmid (mostly in supercoiled form) and linear DNA standards (linearized plasmid DNA or PCR amplicons), using proliferating cell nuclear gene (pcna), the ubiquitous eukaryotic gene, in five marine microalgae as a model gene. We observed that PCR using circular plasmids as template gave 2.65-4.38 more of the threshold cycle number than did equimolar linear standards. While the documented genome sequence of the diatom Thalassiosira pseudonana shows a single copy of pcna, qPCR using the circular plasmid as standard yielded an estimate of 7.77 copies of pcna per genome whereas that using the linear standard gave 1.02 copies per genome. We conclude that circular plasmid DNA is unsuitable as a standard, and linear DNA should be used instead, in absolute qPCR. The serious overestimation by the circular plasmid standard is likely due to the undetected lower efficiency of its amplification in the early stage of PCR when the supercoiled plasmid is the dominant template.

Introduction

Quantitative real-time polymerase chain reaction (qPCR) is a powerful technique that allows accurate and sensitive quantification of starting amounts of DNA without post-PCR manipulation [1]. QPCR in combination with reverse transcription (qRT-PCR) is rapidly becoming the method of choice for mRNA (converted to cDNA) quantification, and is often recommended for the validation of microarray data [2,3,4]. It is also an essential technique for quantifying gene (or noncoding DNA) copy number in a cell [5,6]. Real-time PCR quantification methods are broadly classified as “relative” or “absolute” [7]. Relative qPCR measures the differences in abundances of the target DNA or RNA (reverse-transcribed to cDNA) between samples without showing their actual abundances, and the comparison can only be done for samples run within the same qPCR reaction. Absolute qPCR allows the precise quantification of the target DNA/cDNA based on a standard curve constructed in the same quantification assay as the question samples. The standard curve in an absolute qPCR is generated by amplifying a dilution series of a standard DNA, which can be a plasmid (including plasmid DNA carrying the target DNA, a PCR amplicon, a synthesized oligonucleotide, a genomic DNA, or a cDNA. Among the various types of standard DNA, plasmid DNA, especially the uncut circular one, is the most common choice due to its high stability and reproducibility. It has been shown that uncut circular plasmid DNA is mostly in supercoiled form [8], and that the supercoiled structure of the untreated template plasmid DNA can suppress real-time PCR compared to other relaxed templates [9]. It has also been suggested that careful discrimination of quantitative changes due to either copy number change or structural disruption is needed [9], and linearization may need to be considered for a plasmid to be used as a standard in qPCR [http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf]. However, the magnitude of error a circular plasmid standard may cause and what other conformational types of DNA can be a better choice of standard remain obscure.

In this study, we evaluated three most common forms of standard DNA: circular plasmid, linearized plasmid (digested by restriction enzyme), and linear PCR amplicon. Proliferating cell nuclear antigen gene (pcna), a ubiquitous gene in eukaryotes, from four dinoflagellates and a diatom was used as the model gene for the study. Quantification accuracies of real-time PCR assays based on different standards were compared. Consistently, significant differences were observed in the threshold cycle number (Ct) between the circular plasmid and linear (linearized plasmid or
linear PCR amplicon) DNA. We further used these different conformational types of DNA as standard in qPCR to quantify the pcna copy number in the fully sequenced T. pseudonana genome. Our results demonstrated that the linear DNA standards including linearized plasmids, but not the circular plasmid standard, were reliable for absolute qPCR.

Methods

Microalgal Cultures

The monoclonal cultures of four harmful bloom-forming dinoflagellates and one fully sequenced diatom were used in this study. The dinoflagellate Alexandrium fundyense CA28 was provided by D. M. Anderson at Woods Hole Oceanographic Institution. The dinoflagellates Karlodinium veneficum CCMP1975, Procentrum micans CCMP1589, and Prorocentrum minimum CCMP696, and the diatom T. pseudonana CCMP1335 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, Maine). A. fundyense was grown in F/2-Si seawater medium at 15°C under a 14:10 h light:dark cycle. K. veneficum, P. micans, and P. minimum were grown in F/2-Si seawater medium at 20°C under a 12:12 h light:dark cycle. T. pseudonana was grown in F/2 seawater medium at 15°C under a 14:10 h light:dark cycle. All algal cultures were grown under a photon flux density of 100 μE m−2 s−1. Cell concentrations were measured in triplicate using a Sedgwick-Rafter counting chambers.

DNA and RNA Extraction and cDNA Library Construction

Microalgal cell samples were harvested by centrifugation at 4°C under 3000 xg for 20 min. The A. fundyense and P. micans cell pellets were homogenized using a micropestle to break the theca on the cell surface before nucleic acid extractions as reported [10]. Other species used in this study had weak theca and hence the homogenization step was omitted. For DNA extraction, the cell pellet of each species was resuspended and incubated overnight in 500 μL of DNA extraction buffer (10 mM Tris-HCl, 100 mM EDTA, 0.5% w/v sodium dodecyl sulfate, pH = 8.0) with 200 μg mL−1 proteinase K. Genomic DNA (gDNA) was extracted using a CTAB (cetyltrimethylammonium bromide) protocol [11]. After extraction with chloroform, gDNA was further purified using the Zymo DNA Clean and Concentrator kit (Zymo Research, Orange, California) to remove any remaining impurities. GDNA was finally dissolved in 10 mM Tris-HCl buffer (pH=8) and stored at -20°C. GDNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). For RNA extraction, the cell pellet was resuspended in 1 mL of Trizol Reagent (Invitrogen, Carlsbad, California) and stored in -80°C if not processed immediately. Total RNA was isolated as reported [12]. Alternatively, RNA was extracted using RNAeasy Mini kit (Qiagen, Valencia, California). RNA was dissolved in Diethylpyrocarbonate (DEPC)-treated water and stored at -80°C. The full-length cDNA of K. veneficum was obtained previously [13]. The first-strand cDNA of other algae was synthesized using GeneRacer kit following manufacturer instruction (Invitrogen, Carlsbad, California).

PCR-Based Cloning and Sequencing of pcna cDNA

Proliferating cell nuclear antigen gene (pcna) was chosen as the model in this study because it is a common gene in all eukaryotes and it is a target of our research as a potential cell cycle marker for algal growth rate studies [14]. For T. pseudonana, a pcna fragment was amplified from its gDNA using the specific primer set TpspcnaFl-TpspcnaR1 designed based on its pcna sequence shown in the recently released genome sequence (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html). For A. fundyense, P. micans and P. minimum, pcna fragments were amplified using the first strand cDNA as the template and the spliced leader-based primer (DinoSL) paired with DinoPCNA3d as the primer set (Table 1) under the condition previously reported [14]. For K. veneficum, a pcna fragment was amplified from its full-length cDNA using DinoPCNA5c-RACER3. PCR amplicon was purified using the Zymo DNA Clean and Concentrator kit and cloned into pBluescript II KS vectors (2963 bp, Stratagene, La Jolla, California) using Takara DNA Ligation kit v.1 (TakaraBioUSA, Madison, Wisconsin). Clones were randomly picked and plasmid DNA was isolated from 2 μl of bacterial culture using the Qiagen Miniprep kit to avoid the contamination by bacterial RNA that may occur during a non-column-based plasmid isolation method. pcna insert was sequenced using the M13 Primer Cycle Sequencing kit (Applied Biosystems, Foster City, California). Plasmid DNA was dissolved, measured, and stored in the same way as gDNA described above.

Construction of Circular Plasmid and Linear DNA Standards

Circular plasmid and linear standards were compared to examine the effect of DNA structural confirmation on PCR result and amplification efficiency. Linearized plasmid DNA (3592–3816 bp) and PCR amplicon (436–866 bp) were compared to examine the effects of length and source of DNA (bacterial or PCR amplified). In the A. fundyense qPCR, the pcna recombinant plasmid DNA prepared as mentioned above was used as the circular plasmid standard, named AfuC1 (3816 bp) (Table 2). In order to minimize the experimental error and test the plasmid purity, a second circular plasmid standard (AfuC2) was prepared by further purifying AfuC1 using the Zymo DNA Clean and Concentrator kit. Two linearized plasmid standards for A. fundyense, AfuL1 and AfuL2, were were prepared by digesting AfuC1 with restriction endonuclease EcoRI (4 bp away from the pcna insert) and SalI (26 bp away from the pcna insert), respectively. In parallel, a linear PCR amplicon standard for A. fundyense, AfuL3 (853 bp), was prepared by amplifying the pcna fragment using AfuL1 as the template and DinoSL-DinoPCNA3d as the primer set. Similarly, the circular plasmid standards for P. micans (PmicC), P. minimum (PminC), K. veneficum (KveC), and T. pseudonana (TpsC) (3392–3829 bp) were prepared as for AfuC1. The linearized plasmid standards for K. veneficum (KveL) were similarly prepared as for AfuL1. The linear PCR amplicon standards for P. micans and T. pseudonana (PmicL and TpsL, respectively) were generated from the respective gDNA. The linear PCR amplicon standard of P. minimum (PminL) was amplified from PminC. The complete linearization of the circular plasmid was confirmed by checking the band pattern in the agarose gel. All PCR amplicon standards were purified using the Zymo DNA Clean and Concentrator kit. The optical absorbance at OD260 was measured in triplicates using a NanoDrop ND-1000 spectrophotometer. Based on the OD260 value and the DNA sequence, the molar concentration of the standard DNA was calculated using the OligoCalc oligonucleotide properties calculator [15], and then converted into copy number of DNA molecules per unit volume (in the order of magnitudes of 109–1010 copies μL−1). The standard was finally prepared in dilution series (1×102 to 1×107–1012 copies μL−1) for qPCR. Standard DNA was freshly prepared before use to avoid degradation that may occur during storage.
Microalgal *pcna* qPCR Assays

Five algal *pcna* qPCR were carried out on iCycler iQ Real-Time PCR detection system with SYBR Green supermix (Bio-Rad, Hercules, California). *Pcna*-specific qPCR primers were designed for each species using the program Beacon Designer (Table 1). The specificity of the primers was verified by analyzing the qPCR melt curve and sequencing the PCR amplicon. The standard DNA was diluted in 5–6 serial steps and applied in duplicate (2 \( \times \) \( 10^2 \) to 2 \( \times \) \( 10^6 \)–10\(^7\) copies per reaction). In the case of *T. pseudonana*, three gDNA samples were used as the target DNA, each in six dilutions (100 pg, 200 pg, 500 pg, 1 ng, 2 ng, and 4 ng per reaction) and each dilution was applied in triplicate, which allowed comprehensive evaluation of PCR efficiency and quantification accuracy across a broad range of target DNA quantities. The qPCR condition included a single denaturation cycle of 95°C for 3 min, 40 cycles of 95°C for 20 s, annealing at primer-specific temperature for 30 s (Table 1), and elongation at 72°C for 15 sec.

**Analyses of Threshold Cycle, Amplification Efficiency, and Genomic *pcna* Copy Number**

The threshold cycle number (Ct) was reported by the iCycler iQ program under the “PCR baseline subtracted” option. The standard curve was generated as linear regression between Ct and \( \log_{10} \) starting copy number of standard DNA. The iQ program automatically calculated the amplification efficiency (E) of the standard DNA from the slope of the standard curve: 

\[
E = \frac{10^{(\frac{1}{2})/\text{slope}} - 1
\]

Based on a statistical model in a previous study (Equation 5 in [16]), a multiple regression model was built using SPSS 15 to test the slope and Ct differences between equimolar circular and linear DNA in each qPCR. The model was

\[
y = a + b_1x_1 + b_2x_2 + b_3 x_1 \times x_2 + e
\]

where the dependent variable (y) is threshold cycle (Ct), the covariate (\( x_1 \)) is the logarithmic-transformed known *pcna* copy number in the standard DNA, the fix factor (\( x_2 \)) is the DNA type (circular or linear, coded as 0 or 1 by SPSS 15), and e is the error. If the coefficient of the interaction term (\( b_3 \)) is significant (\( p < 0.05 \)), the slopes of the standard curves (and hence amplification efficiencies) for the circular and the linear standards are significantly different from each other. If the coefficient of the fix factor (\( b_2 \)) is significant (\( p < 0.05 \)), Ct values for the two types of standards are significantly different. The differences in Ct values (\( \Delta \text{Ct} \)) were calculated as the average Ct difference across serial dilutions. When the slopes of the two standard curves (or efficiencies) are significantly different, Ct difference was adjusted (\( \Delta \text{Ct}' \)) as the average of (Ct1 \( \times \) E1 \( - \) Ct2 \( \times \) E2 \( ) \) across all dilution levels, where E’ is another form of amplification efficiency commonly used and also calculated from the slope:

\[
E' = \log_210^{(\frac{1}{2})/\text{slope'}} - 1
\]

Table 1. PCR primers used in this study.

| Primer name  | Primer sequence (5’→3’) | PCR annealing temperature (°C) |
|--------------|-------------------------|-------------------------------|
| **Regular PCR** |                          |                               |
| DinoSL\(^\text{a}\) | TCC GTA GCC ATT TTG GCT CAA G | 55               |
| DinoPCNA3db | TCG TCG ATC TTS GGN GGN AGR TAR AA | 55               |
| DinoPCNA5cb | ATC GCC GGA CTT YGA RCT NAA RCT NAT G | 55               |
| RACER3\(^\text{b}\) | GCT GTC AAC GAT ACG CTA CGT AAG G | 60               |
| PmicpcnaF2 | GCG TTC TCT GAG TCT AAG TGT GAC | 60               |
| PmicpcnaR | GCT GGT GGA CTG TGA GGG TC | 60               |
| TpspcnaF1 | GCA AGC ACC CCT CAC CCA AG | 60               |
| TpspcnaR1 | CTC ATC CTT CTC CGC AGC ACT ATT C |                  |
| **QPCR** |                          |                               |
| *Alexandrium fundyense* |                        |                               |
| AfupcnaF | CAG GTG AAG GCA AGC AAG GA | 57               |
| AfupcnaR | GTT GTC AGT CTT CTC AAG GTC YTA C |                  |
| **Karlodinium veneficum** |                        |                               |
| KvepcnaF | GGA GAT GTY GGH ACW GGN AAT GT | 56.5             |
| KvepcnaR | TAG AAY TGC ATG TAD CCR TTG TC |                  |
| **Prorocentrum micans** |                        |                               |
| PmipcnaF1 | GAG CAG CAV TAC AAG GTG GTG G | 60               |
| PmipcnaR | GCT GGT GGA CTG TGA GGG TC | 60               |
| **Prorocentrum minimum** |                        |                               |
| PminpcnaF | ATH GAG AGC GAG CAC ATG GAG | 65               |
| PminpcnaR | GCT CCA CSG TKC CGC ACA G |                  |
| **Thalassiosira pseudonana** |                        |                               |
| TpspcnaF2 | GAC CTA GTC CAA GAA GCC AAC ATA G | 66-60 touch-down |
| TpspcnaR2 | AAC ACC AAC GCC AAC GAA TCC |                  |

\(^\text{a}\)Zhang et al. 2006, 2007.  
\(^\text{b}\)Zhang et al. 2006.  
\(^\text{c}\)GeneRacer kit, Invitrogen, Carlsbad, California.

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In the case of *T. pseudonana* qPCR, the starting *pcna* copy number in each dilution of *T. pseudonana* gDNA sample (i.e., qPCR-estimated *pcna* copy number) was calculated based on TpsC and TpsL, respectively. The qPCR-estimated copy number was compared with the expected number calculated according to 1 *pcna* per haploid genome (34 Mbp or 0.035 pg of gDNA) [18] (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html). We also did the linear regression analysis between Ct and log_{10} amount of *T. pseudonana* gDNA. From the slope of the regression line, the amplification efficiency of *T. pseudonana* gDNA was calculated as E = 10^{-1/slope}-1.

**Results**

**Remarkably Different Ct Values for Circular Plasmid and Linear Standards**

The threshold cycle numbers (Ct) of circular plasmid standards ranged from 16.79 to 36.72, and Ct of the linearized plasmid and PCR amplicon standards (collectively named linear standards) ranged from 12.89 to 33.59. In all cases, the circular plasmid and the equimolar linear DNA had significant different Ct values (p<0.001). In the *A. fundyense* qPCR, the circular plasmid prepared by the Qiaprep Spin Miniprep kit (AfuC1) and the other further purified by the DNA Clean and Concentrator kit (AfuC2) yielded similar Ct values without significant difference (ΔCt = 0.53). For the linearized plasmid standards (AfuL1-2, 3816 bp) and the PCR amplicon (AfuL3, 853 bp), despite the differences in length, very small ΔCt (0.33) was observed. In contrast, remarkable Ct differences were observed between circular plasmid and linear standards (p<0.001, ΔCt = 2.63–4.29) (Fig. 1a–c). As shown in Fig. 1a, we found that the mean Ct of AfuC1-2 were markedly higher than those of AfuL1-3 (p<0.001, ΔCt = 3.76). Consistently, in all other *pcna* qPCR experiments, the Ct values of the circular plasmid standards were higher than the linear ones: ΔCt = 4.29 in *K. veneficum*, ΔCt = 2.65 in *P. micans*, ΔCt = 4.00 in *P. minimum*, and ΔCt = 3.54 in *T. pseudonana* (p<0.001 in all cases). The conformational state of qPCR standard DNA appeared to exert strong influence on their Ct, with substantially higher values from the circular plasmid than linear standards.

**Comparison of Amplification Efficiencies between Circular Plasmid and Linear DNA Standards**

All standard curves were generated with high coefficients of determination (R^2 = 0.998–1.000) (Table 2). In each qPCR, different standard curves appeared parallel (Fig. 1a–e) and thus the efficiencies derived from their slopes were similar. In the *A. fundyense* qPCR, the overall coefficient of variation (or CV, equal to the standard deviation divided by the mean) of the efficiencies for all five *A. fundyense* standards was 4.45%. The efficiencies between circular plasmids (AfuC1-2) and linear DNA (AfuL1-3) were not significantly different (p = 0.97), neither for the two circular (p = 0.724) or linearized (p = 0.231) plasmids. However, among AfuL1-3, the CV of efficiencies was slightly increased (6.13%), and the efficiency for AfuL3 was significantly different from the other two (p<0.001). In the *K. veneficum*, *P. micans*, and *T. pseudonana* qPCR, efficiencies for the circular plasmid and linear DNA were highly similar (p = 0.994, 0.197, and 0.307 and CV = 0.18%, 2.93%, and 2.52%, respectively). In *P. minimum*, the efficiencies for PminC and PminL were significantly different although the difference was fairly small (p = 0.011 and CV = 3.93%). Incorporating the efficiency difference between PminC and PminL, the Ct difference after adjustment (ΔCt = 4.30) was slightly larger than that without adjustment (ΔCt = 4.00). In summary, no consistent difference in amplification efficiencies was observed between circular and linear standard DNA.

**Pcna Copy Number in *T. pseudonana* gDNA**

Significantly different copies of *pcna* were estimated based on TpsC and TpsL (p<0.001) for each *T. pseudonana* gDNA dilution.

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**Table 2. Types and performance of standard DNA in qPCR in various algal species examined.**

| Algal species | Standard name | Standard type | Length (bp) | Standard curve (R^2) | E (%) |
|---------------|---------------|---------------|-------------|----------------------|-------|
| *Alexandrium fundyense* | AfuC1 | Circular plasmid bearing EF133957 | 3816 | y = –3.642x+40.152 (1.000) | 88.2 |
| | AfuC2 | Circular plasmid bearing EF133957 | 3816 | y = –3.673x+39.770 (0.997) | 87.2 |
| | AfuL1 | Linearized plasmid bearing EF133957 | 3816 | y = –3.799x+36.790 (1.000) | 83.3 |
| | AfuL2 | Linearized plasmid bearing EF133957 | 3816 | y = –3.707x+36.376 (0.998) | 86.1 |
| | AfuL3 | PCR amplicon based on DinoSL-DinoPCNA3d primer set | 853 | y = –3.477x+35.471 (1.000) | 93.9 |
| *Karldinium veneficum* | KveC | Circular plasmid bearing partial EF134029 | 3592 | y = –3.888x+44.484 (0.999) | 90.8 |
| | Kvel | Linearized plasmid bearing partial EF134029 | 3592 | y = –3.897x+40.237 (0.999) | 90.6 |
| *Prorocentrum micans* | PmicC | Circular plasmid bearing EF133939 | 3820 | y = –3.420x+40.272 (1.000) | 96.1 |
| | PmicL | PCR amplicon based on PmipcnaF2-PmipcnaR primer set | 436 | y = –3.523x+38.059 (1.000) | 92.2 |
| *Prorocentrum minimum* | PminC | Circular plasmid bearing EF134019 | 3829 | y = –3.679x+40.874 (1.000) | 87.0 |
| | PminL | PCR amplicon based on DinoSL-DinoPCNA3d primer set | 866 | y = –3.834x+37.560 (1.000) | 82.3 |
| *Thalassiosira pseudonana* | TpsC | Circular plasmid bearing gene fragment | 3631 | y = –3.921x+45.462 (0.999) | 79.9 |
| | TpsL | PCR amplicon based on TspcnaF1-TspcnaR1 primer set | 668 | y = –4.029x+42.437 (0.997) | 77.1 |

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a. The plasmid vector is pBluescript II KS (2963 bp).

b. The linear regression equation between Ct (y) and log_{10} starting copy number (x).

c. Efficiency calculated as E = 10^{-1/slope}-1.

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Figure 1. Standard curves with similar slopes and significant threshold cycle differences (ΔCt) between circular and linear standards in the *pcna* qPCR for (a) *Alexandrium fundyense*, (b) *Karlodinium veneficum*, (c) *Prorocentrum micans*, (d) *P. minimum*, and (e) *Thalassiosira pseudonana*. Standard curves were linear regression lines between Ct and Log_{10} starting *pcna* copy number (calculated from standard DNA concentration), each based on a type of standard DNA. Note that similar slopes of the standard curves indicate similar amplification efficiencies. All ΔCt were calculated as the average Ct difference across serial dilutions and statistically significant (p<0.001). The error bars denote the standard deviations of Ct values among replicates.

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sample (100 pg to 4000 pg per reaction) (Fig. 2). The copy numbers measured from TpsL were very similar to the expected numbers calculated according to 1 copy of pcna per the 0.035 pg DNA genome of T. pseudonana, whereas the estimates from the circular TpsC were 7.77 ± 1.28 times higher (n = 6). Using the results from the TpsL standard, a T. pseudonana haploid genome (0.032 pg of DNA) was estimated to contain 1.02 ± 0.14 copies of pcna (n = 3), in close agreement with the actual 1 copy per genome value. In stark contrast, using supercoiled circular standard resulted in an estimate of 7.77 ± 1.28 copies per genome (n = 3), indicative of serious overestimation by this standard (Table 3).

Discussion

Plasmid DNA containing the target sequence has been commonly used as the standards in quantitative real-time PCR due to its high stability (i.e. little degradation during storage) and ease in preparation [7]. In most applications, circular (i.e. undigested) plasmid gene clones are used directly without linearization, and little attention has been paid to the possible effect of conformational state on quantification accuracy. Other types of standard DNA may be utilized in rare cases, but an explanation for selecting the standard DNA is usually not provided (e.g., PCR amplicon standard used in [19]). In this study, we have conducted a systematic evaluation on the most common types of standards and demonstrated that the linearized plasmid or linear PCR amplicon is the type of choice for a qPCR standard. Our result showed that at any concentration applied (2×10^−8–2×10^10 copies per reaction), the circular plasmid DNA increased Ct by 2.5 more cycles compared to the linear DNA, and in accordance the standard prepared from the circular plasmid DNA led to 8-fold overestimation of T. pseudonana pcna copy number whereas the linear standards gave highly accurate estimates.

Differences observed between the different types of standards were not due to variations in our experiment operation or quality of template DNAs. In this study, the standard DNA and genomic DNA were carefully and freshly prepared to minimize artifacts and each DNA was used in replicated dilution series to assess the intra-

![Figure 2. Comparison of qPCR-estimated and expected pcna copy numbers in Thalassiosira pseudonana gDNA samples.](http://www1.qiagen.com/Plasmid/AgaroseGelAnalysis.aspx). If nicks are introduced at opposite positions on both plasmid DNA strands, e.g., by restriction enzyme digestion, a plasmid is linearized and the supercoiling is relaxed. There is evidence that PCR is suppressed by supercoiling of the template DNA, and that the relaxing of DNA supercoil structure could increase the efficiency for primer binding and elongation in a PCR reaction [9]. This explains well the higher Ct values for circular plasmid than that for linearized plasmid. However, by multiple linear regression analyses, we did not find efficiency differences between the circular and linear DNA in all qPCR that can account for the differential Ct values. Only in one case did we observe a small difference in efficiency, which however contradicted rather than accounted for the Ct difference. It seems likely that the difference in Ct values and quantification accuracies lie in the first several cycles of qPCR when the supercoiled plasmid is the dominant template. Previous research has shown that the efficiency difference in the first few cycles would result in dramatic different qPCR results [21], such as ΔCt measured in this case. However, the efficiencies calculated from the standard curves do not reflect the differences in the early amplification stage, because the standard curves were constructed based on the Ct values identified in the exponential amplification stage (varied from 12.89 to 36.72 in this study) when linear PCR amplicon has become dominant and quantitatively outcompetes the supercoiled plasmid for amplification. Even if the amplification efficiency were calculated using such other methods as one using fluorescent data collected during PCR [22,23], the initial lower efficiency of the supercoiled plasmid DNA still may not be easily detected.

While qPCR results from undigested plasmid DNA standard are strikingly different from those based on linear standards, linearized plasmid and linear PCR amplicon provide similar quantifications. This suggests that the length and source of the DNA template does not have significant effect on PCR efficiency. Practically each of these linear standards has its own advantage and the choice depends on convenience. Plasmid is stable for long-term storage and linearization can be carried out easily at time of standard preparation. PCR amplicon standard comes with the flexibility
that it can be amplified from the stored plasmid if already available or directly from the genomic DNA of the target organism bypassing the tedious gene cloning procedure. Although our observations were based on penta in marine microalgae, the findings likely apply to other genes and other organisms, because all the qPCR reactions are run under in vitro conditions. The only possible exception would be when the target DNA itself is circular (especially if it is in supercoiled state), such as uncut mitochondrial, viral, bacterial, or plasmid DNA, in which whether linear standard still gives more accurate result needs to be individually investigated. In light of our findings in this study, previous results of qPCR based on circular plasmid standards need to be revisited.

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Table 3. QPCR-estimated and expected pcna gene copy numbers (mean ± standard deviation) in Thalassiosira pseudonana genomic DNA samples based on the circular (TpsC) and linear (TpsL) qPCR standards.

| gDNA (n = 3) | 100 pg | 200 pg | 500 pg | 1000 pg | 2000 pg | 4000 pg | Estimated/expected ratio (n = 6) |
|-------------|--------|--------|--------|--------|--------|--------|-------------------------------|
| Based on TpsC (circular) | 2857 ± 10926 | 5714 ± 14286 | 14286 | 28571 | 57143 | 114286 | 7.77 ± 1.28 |
| Based on TpsL (linear) | 2633 ± 19098 | 4707 ± 2400 | 14286 | 29867 | 63200 | 139267 | 7.18 ± 0.14 |

a. Calculated based on 1 pcna per 0.035 pg of gDNA.
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Author Contributions
Conceived and designed the experiments: YH SL. Performed the experiments: YH HZ LNM. Analyzed the data: YH SL. Wrote the paper: YH HZ SL.

Table 3. QPCR-estimated and expected pcna gene copy numbers (mean ± standard deviation) in Thalassiosira pseudonana genomic DNA samples based on the circular (TpsC) and linear (TpsL) qPCR standards.