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Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria

Chai Lian Lee, Dave Siak Wei Ow, Steve Kah Weng Oh *

Microbial Fermentation Group, Bioprocessing Technology Institute, 20 Biopolis Way #06-01 Centros 138668, Singapore

Abstract

A method for determination of plasmid copy number (PCN) in bacteria by real-time quantitative polymerase chain reaction (QPCR) was developed as an alternative to current PCN assays. Conventional methods for PCN estimation are generally not of high throughput, laborious, have low reproducibility, require large amounts of biological samples and are applicable only for a narrow dynamic range. Real-time QPCR, using the ABI Prism® 7000, was able to sensitively detect the quantity of the pUC ori based plasmid, NS3, transformed into *Escherichia coli* host, DH5α, to be 411 ± 6.1. The PCN of pBR322 plasmid DNA in DH5α was estimated to be 40 ± 0.6 which is within its previously reported PCN range of approximately 30 to 70. QPCR was found to show good reproducibility and high sensitivity in detecting a two fold difference in template concentration, and a wide linear dynamic range covering 0.5 pg to 50 ng of DNA. PCNs of DH5α bearing plasmids pBR322 and NS3 computed from real-time QPCR assay were validated by that of agarose gel assay, and a marginal difference of only 13.0% and 10.7% was found for the two plasmids respectively. The QPCR assay was able to detect changes in PCN of plasmid producing DH5α during the course of a 2 l batch fermentation.
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1. Introduction

The plasmid copy number (PCN) is defined as the number of copies of a plasmid present per chromosome in a bacteria (Gerhardt et al., 1994). Most available methods for determining the plasmid copy number are laborious, of low reproducibility and applicable only for a narrow dynamic range (Schmidt et al., 1996). They generally rely on an initial plasmid DNA separation or isolation from the chromosomal DNA, followed by their respective quantification. These methods include CsCl-EtBr centrifugation (Weisblum et al., 1979), gel electrophoresis (Projan et al., 1983), and high performance liquid chromatography (Coppella et al., 1987).

On the other hand, indirect PCN assays such as the one using enzyme activity of reporter proteins (Schendel et al., 1989) are prone to errors due to

enzyme kinetics and protein denaturation. Even though capillary electrophoresis allows rapid quantification of plasmid DNA with high resolution and precision, PCN is computed based on plate count to assess the number of cells present (Schmidt et al., 1996). Although the amount of plasmid DNA can be analyzed by capillary electrophoresis in less than 30 min, plate counting can be time consuming and laborious.

PCR based methods have an important advantage over all other methods mentioned above due to its high sensitivity that allows the study of low abundance or limited amounts of biological samples (Santagati et al., 1997). By virtue of the 96-well format, a large number of samples can be processed during each PCR reaction (Whelan et al., 2003). Nonetheless, conventional endpoint PCR allows only semi quantitative results because of the saturation of amplicons before the completion of PCR.

Recent advances in kinetic PCR have permitted the development of extremely sensitive and truly quantitative real-time PCR methods. This method is well established and widely used for detecting copy number of genes, especially in cases of detection of gene amplification or bacteria identification, however it had not been established for determination of plasmid copy number.

A wide dynamic range of linear quantitative detection have been reported using real-time QPCR (Nonnenmacher et al., 2004; Furet et al., 2004; Herrmann et al., 2004) which allowed the real-time monitoring of fluorescence levels during PCR. A common dye used for real-time detection of PCR amplification is SYBR Green, which gives off enhanced fluorescence by binding with high affinity to double stranded DNA (Wittwer et al., 1997; Ririe et al., 1997; Howell et al., 1999; Hernández et al., 2003). However, it is possible to distinguish a specific PCR product by monitoring the fluorescence continuously during a melting curve analysis (Wittwer et al., 1997). The melting temperature (T_m) of the PCR product is dependent on GC content, length, and sequence (Ririe et al., 1997). This melting temperature is indicated by a rapid fall in fluorescence, or a peak in the negative first derivative of the fluorescence curves (Howell et al., 1999).

Absolute quantification of the initial template concentration is achievable as the dye emits fluorescence proportional to the double stranded DNA or PCR product present upon excitation (Ririe et al., 1997; Howell et al., 1999). Serial ten fold dilutions of pure DNA with known concentrations were used to set up a standard curve to which the threshold cycle of a sample was compared to give its absolute quantity (Kühne and Oschmann, 2002). In this work, the plasmid copy numbers computed from real-time QPCR were verified with those obtained by agarose gel assay (Pushnova et al., 2000), where the intensity of the bands were indicative of the quantity of genomic and plasmid DNA present. We found that this new real-time QPCR assay is sensitive, less laborious, reproducible, of high throughput, and does not require the separation of plasmid and genomic DNA or large amount of biological sample. Finally, we applied the method to monitor PCN in a batch fermentation culture.

2. Materials and methods

2.1. Bacterial strains, plasmids and cultivation

E. coli strain, DH5α, was used as bacterial host for the plasmids. The plasmids used in this study were pBR322 (4.36 kb) and NS3 (7.3 kb), which were obtained from Promega (Madison, WI USA) and Dr. Vincent Chow at the Department of Microbiology, National University of Singapore, respectively. Non Structural 3 (NS3) was derived from pUC ori based pcDNA3.1 vector (Invitrogen, Carlsbad, CA) and a 1.8 kb gene fragment from Dengue virus. These plasmids were transformed into the *E. coli* host cells following the established protocol from Promega (Madison, WI USA).

E. coli host cells were inoculated from freshly streaked overnight Luria Bertani or LB (Becton, Dickinson and Company, Maryland, USA) agar plates into 10 ml LB media. After incubation at 37 °C for 8 h, 0.5 ml of the culture was inoculated into 25 ml of LB broth and allowed to grow in an incubator shaker (220 rpm) at 37 °C overnight (approximately 15 h) to the stationary phase. *E. coli* bearing plasmids pBR322 or NS3 was cultivated in the same media except ampicillin was added for selection at a final concentration of 50 μg/ml.

2.2. Bacterial DNA isolation and purification

Genomic or total DNA were isolated from 1 ml cell pellets spun down at 13,200 rpm for 1 min in Eppendorf Centrifuge 5415D (B. Braun, Melsungen, Germany). DNA extraction method reported in previous publication (Pushnova et al., 2000) was adopted with the following modifications. First, to ensure complete cell lysis, mechanical destruction of cell walls was performed by mixing with a syringe with 0.7 mm needle, after addition of 10% SDS and Proteinase K, followed by incubation at 50 °C. Second, in order to further purify the DNA obtained, one volume of 100% isopropanol was added to precipitate the DNA after chloroform extraction and then washed with one volume of 70% ethanol before rehydration in one volume double deionised water (ddH$_2$O) at 65 °C for 1 h.

Plasmid DNA for standard curve was extracted from the host cells DH5α according to the QIAprep Spin Miniprep Kit protocol (Qiagen, Hilden, Germany).

2.3. Agarose gel assay — scanning of negative film and computation of plasmid copy number

Following the protocol of a previously reported agarose gel assay (Pushnova et al., 2000) for plasmid copy number quantification, serial two fold dilutions of the template was run on 0.8% SeaKem® LE agarose (Cambrex Bio Science, Rockland, ME USA) which contains 0.2 μg/ml ethidium bromide solution (Bio-Rad Laboratories, Hercules, CA). Buffer TAE was used to conduct the agarose gel electrophoresis at 100 V for 40 min.

AlphaEaseFluorChem™ software (Alpha Innotech, San Leandro, CA), version 3.1.2, was used to scan the negative film of the agarose gel pictures and to quantify the peak area of both the genomic and plasmid DNA band. Since the peak area of each band is proportional to the amount of DNA present, accepting a linear range that differed by less than 20% after normalization, the plasmid copy number (PCN) can be computed as follows (Pushnova et al., 2000).

$$\text{PCN per genome} = \frac{\text{Size of chromosomal DNA (bp)} \times \text{Amount of plasmid DNA(pg)}}{\text{Size of plasmid DNA (bp)} \times \text{Amount of genomic DNA(pg)}} \cdots \quad (1)$$

$$= \frac{\text{Size of chromosomal DNA(bp)} \times \text{Peak Area of plasmid DNA band } (Int^*\text{mm}^2)}{\text{Size of plasmid DNA(bp)} \times \text{Peak Area of genomic DNA band } (Int^*\text{mm}^2)} \cdots \quad (2)$$

2.4. Oligonucleotide primers

PCR primers (Table 1) were designed using Primer Express software Version 1.0 (Applied Biosystems, Foster City, CA, USA). Detection of the plasmid DNA was targeted at the bla gene coding for its ampicillin resistance, while the genomic PCR primer pair was targeted at the 16S rDNA gene. As PCN is typically more than 100 per chromosome, it would be an advantage to use a multicopy gene like 16S rDNA as the genomic target. This is so that the threshold cycle values of both the plasmid and genomic targets would be closer, and PCN could be determined from the same template dilution. Since the threshold cycle fluorescence value is proportional to the amount of target DNA and the same E. coli strain (DH5α) was used to establish the standard curves, no further cor-

rection factor was needed for the multicopy nature of the 16S rDNA gene (Song et al., 2004).

2.5. Quantitative real-time PCR assay

The ABI PRISM® 7000 instrument (Applied Biosystems) was used for QPCR amplification and detection. QPCR was prepared in triplicates of 25 μl reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). Each reaction well contains 2.5 μl of template DNA, 2.5 μl of 10 × SYBR® Green PCR Master Mix (Applied Biosystems), and 12.5 pmol each of forward and reverse primers.

Concentrations of plasmid and genomic DNA standards were measured by absorbance at 260 nm with a UV/Vis Spectrophotometer (Beckman Coulter, Full-

Table 1
Real-time QPCR primers used in this study

	Primers	Sequence (5′–3′)	Target Gene	Size	T_m
Plasmid DNA (NS3 or pBR322)	bla1f	GTA TGC GGC GAC CGA GTT	bla	50 bp	82.2 ± 0.2 °C
	bla1r	GCT ATG TGG CGC GGT ATT ATC			
Genomic DNA (DH5α)	Xlf	CCG GAT TGG AGT CTG CAA CT	16S rDNA	50 bp	78.5 ± 0.2 °C
	Xlr	GTG GCA TTC TGA TCC ACG ATT AC			

erton, CA). The ratio of absorbance at 260 and 280 nm, (OD_{260}/OD_{280}), was routinely found to be between 1.8 and 2.0, indicating minimum protein contamination. Serial ten fold dilutions of both plasmid and genomic DNA were conducted in triplicates to establish the standard curves. The standard curve is a plot of the threshold cycle (Ct) versus log concentration (Co). For any unknown total DNA sample, by interpolating its Ct value against the standard curve, the absolute quantity of both plasmid and genomic DNA would be obtained. From Eq. 1, PCN was calculated for each sample.

Negative control and positive control were included in the experimental runs. The negative control was set up by substituting the template with ddH$_2$O and that routinely had a high Ct value which represented the lower detection limit. For the positive control, we used a template of known PCN quantified by the agarose gel method (Pushnova et al., 2000) for comparison with QPCR derived values.

Real-time QPCR was conducted with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1min each. Upon completion of 40 PCR amplification cycles, there was a dissociation step of ramping temperature from 60 to 95 °C steadily for 20 min, while fluorescence signal was continually monitored, for melting curve analysis. At the melting temperature as the template denatures, a rapid fall in fluorescence could be detected resulting in a peak in the dissociation curve when plotting the first derivative of fluorescence signal against temperature. Table 1 shows the sizes of the amplified products and their melting or denaturation temperature (T_m), for determining of specific PCR products amplification.

2.6. Fermentation conditions

Freshly streaked E. coli NS3 cells were inoculated into 40 ml shake flask culture for incubation at 37 °C overnight with shaking at 250 rpm. This served as the inoculum (2%) for the 2 l bioreactor run. Fermentation temperature was set at 37 °C, with automatic pH buffering by addition of 2 M sodium hydroxide (NaOH) and 2 M sulphuric acid (H$_2$SO$_4$) to maintain the pH at 7.0. Stirrer speed and airflow was kept constant at 600 rpm and 1.5 slpm respectively.

The fermentation media comprised of 12 g/l yeast extract, 6 g/l tryptone, 6 g/l dipotassium phosphate, 0.48 mg/l magnesium sulphate, and 5 g/l glucose. A 2 l E. coli NS3 batch fermentation was operated for 13 h, and 1 ml samples were centrifuged at 14,000 rpm for 1 min, and the cell pellets stored in − 20 °C for DNA extraction.

3. Results and discussion

3.1. QPCR specificity and sensitivity

As shown in Fig. 1a, specific PCR products amplified with plasmid primer set bla1f and bla1r are expected to give a single sharp peak at the melting temperature of 82.4 °C. 12 identical peaks with the expected T_m were seen. The single dashed line indicated a product that was non specific, resulting in a lower T_m. This would not be used for analysis.

A two fold difference in template concentrations was used to test the sensitivity of real-time QPCR. 2.1 ng and 1.05 ng of genomic DNA were run in triplicates and the Ct values for these two samples were 18.32 ± 0.045 and 19.41 ± 0.024 respectively. As shown in Fig. 1b, the triplicate fluorescence curves overlapped closely and the two samples were easily distinguishable, with an average of 1.09 Ct between them. This demonstrated the sensitivity and ability of real-time QPCR to distinguish concentrations that would theoretically result in only one Ct difference, with a deviation of only 1%.
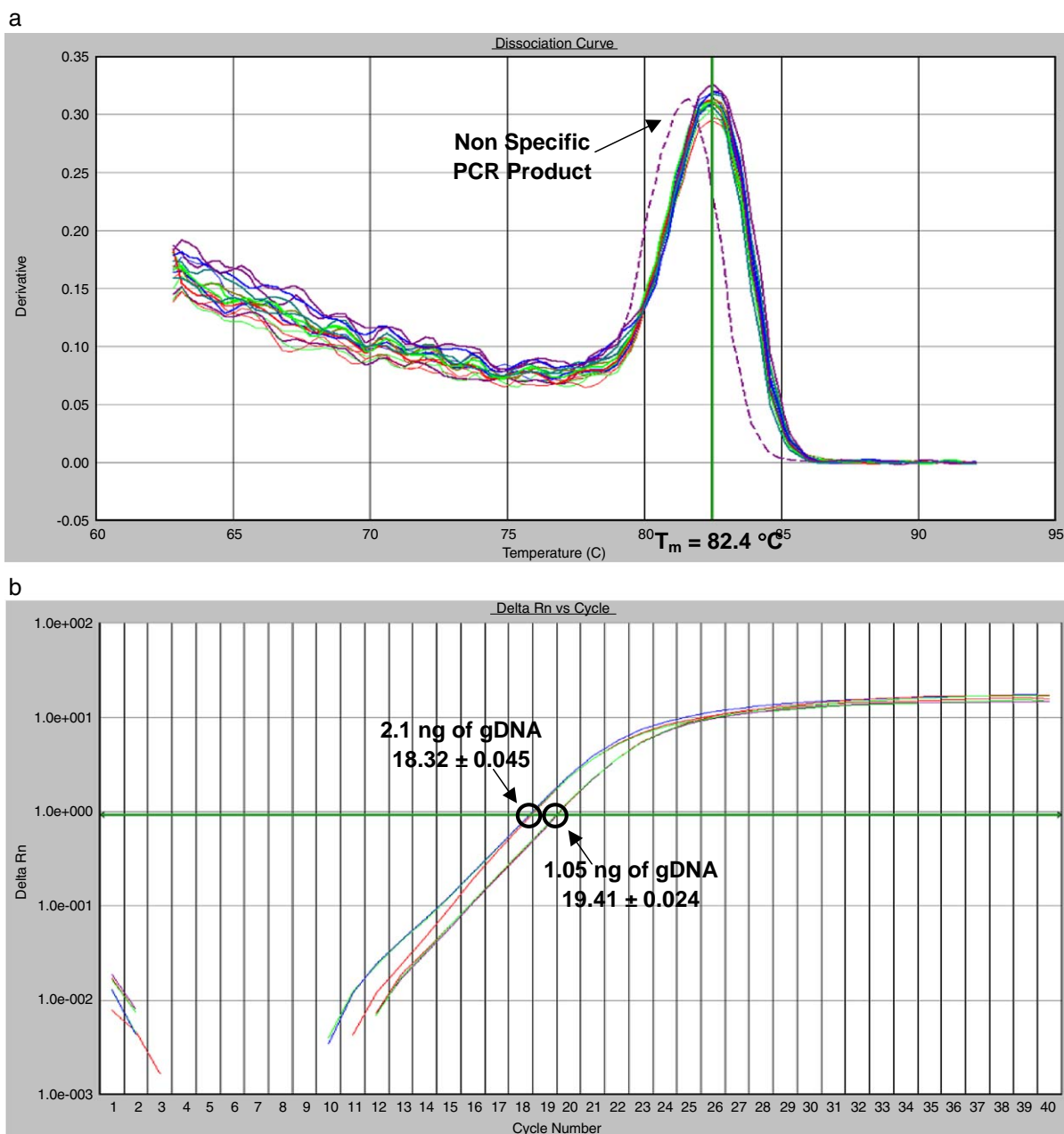
a

b

Fig. 1. Real-time QPCR results (a) Melting curve analysis of plasmids using primer set *bla1f* and *bla1r* by plotting first derivative of fluorescence versus dissociation temperature, dashed line (---) represents a non specific PCR product with a lower melting temperature (81.6 °C) than the others (82.4 °C). (b) Fluorescence versus PCR cycle curve for triplicates of 2.1 ng and 1.05 ng of genomic DNA template amount. This shows the ability of real-time QPCR to detect a two fold difference in amount of template and small deviation between the triplicates. (c) Threshold cycle versus log concentration with standard error bars (which are negligible) for triplicates of each concentration to determine the linear dynamic range of real-time QPCR for plasmid and genomic DNA. Absolute gradient values obtained for plasmid ($\bigcirc$) and genomic (Δ) DNA were 3.3808 and 3.3606 respectively. Theoretical value should be 3.322 with 100% PCR efficiency.
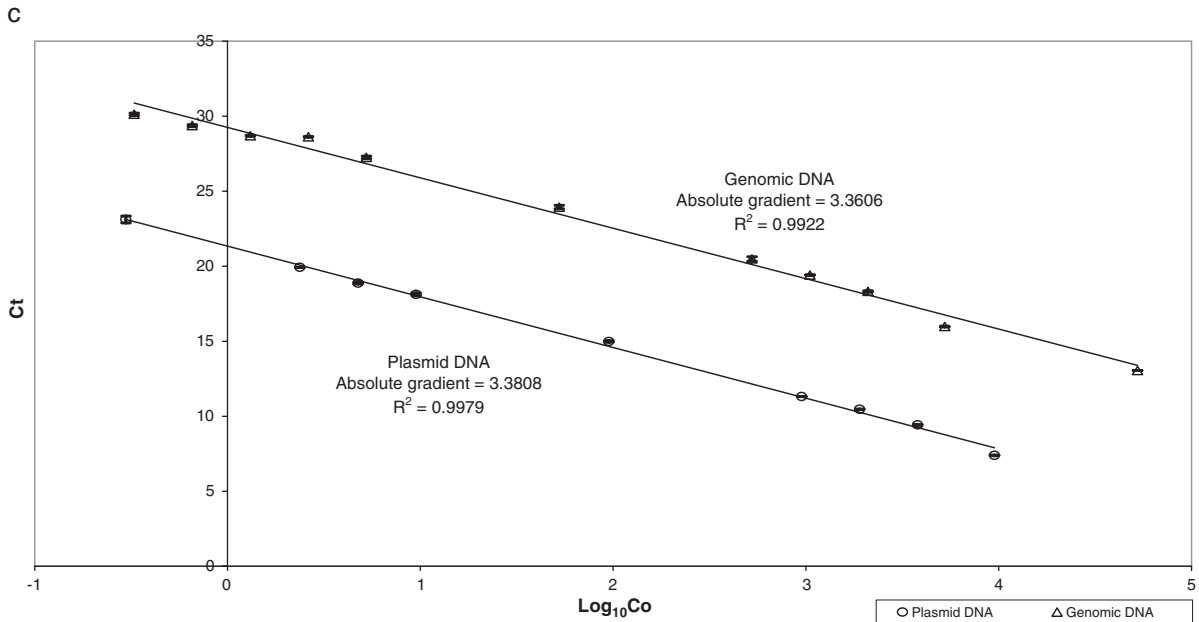
Fig. 1 (*continued*).

3.2. Standard curve and dynamic range

Amounts of DNA template ranging from 0.297 pg to 95 ng for NS3 plasmid DNA and 0.164 pg to 52.5 ng for DH5α genomic DNA were used to find the linear dynamic range of template that can be detected and quantified accurately. Serial two fold dilutions were used for the upper and lower ranges, while serial ten fold dilutions were used for the mid range.

Before determining the linear dynamic range, we evaluated the PCR amplification efficiency from the absolute gradient value of Ct versus log Co curve. In theory, PCR efficiency or the slope of the standard curve should be computed as absolute gradient = $\frac{1}{\log_{10} 2} = 3.322$. In other words, theoretically, for a ten fold difference in template amount, a Ct value of 3.322 cycles should be expected. As shown in Fig. 1c, the absolute gradients of the curves for targeted plasmid and genomic DNA PCR product were 3.38 and 3.36 respectively, resulting in a small percentage difference (less than 2%) from the theoretical value. From the results and analysis, it could be concluded that the linear detection range of the real-time QPCR method ranged from 0.5 pg to 50 ng of DNA template, covering a wide 10^5 fold range (Fig. 1c).

For negative controls of the plasmid and genomic DNA, we obtained high Ct values of 25.43 ± 0.150 and 31.76 ± 0.274 respectively. These values are way above Ct values of 20 where DNA samples are typically detected thus background amplification was negligible.

3.3. Agarose gel assay and real-time QPCR assay

Fig. 2a shows the agarose gel picture for *E. coli* bearing NS3 with serial two fold dilutions from lanes 1 to 7. The two bands obtained per lane were genomic (top) and plasmid (bottom) DNA. Using the AlphaEaseFC software, we were able to quantify the bands intensities by measuring the areas under the peaks (*int**mm^2), as shown in Fig. 2b. These values were then adjusted by their respective dilution factors to obtain an average intensity that would be proportional to the amount of DNA present, with an allowance of 20% deviation between the serial dilutions (Pushnova et al., 2000). On occasions when more than one isoform (supercoiled, open circular and denatured) of plasmid DNA bands were observed, all these intensity values were added together to represent the total amount of plasmid DNA present.
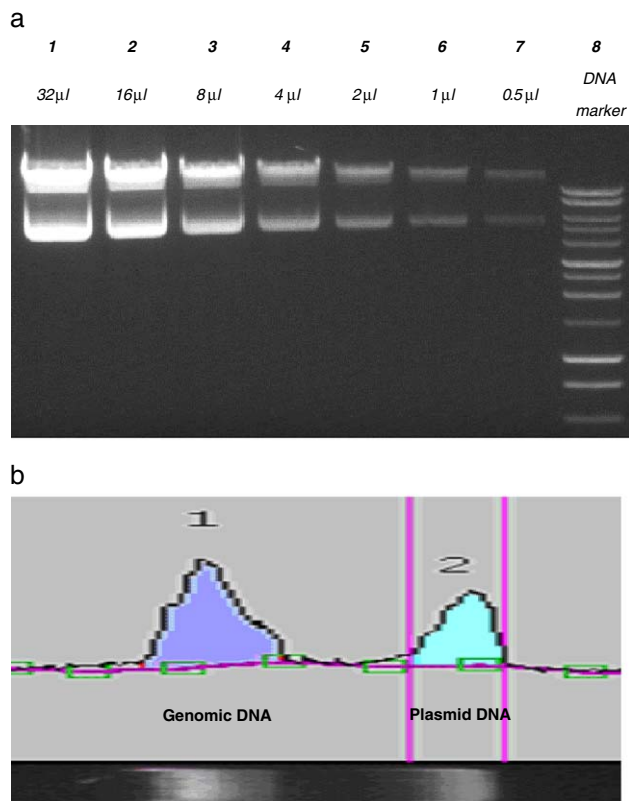
Fig. 2. Agarose gel assay (a) Agarose gel picture of NS3/DH5α for quantitation of plasmid copy number with 32, 16, 8, 4, 2, 1 and 0.5 μl of total DNA in lanes 1 to 7 respectively and 4 μl of 1 kb DNA marker in lane 8. The higher molecular weight genomic DNA and lower molecular weight plasmid DNA constitutes the top and bottom band respectively. (b) Negative scanning of lane 3 in (a) using Alpha Ease FC for analysis of the intensity of the bands where peak 1 and 2 represents intensity areas for genomic and plasmid DNA respectively.

The linear range obtainable for agarose gel assay was only for three consecutive lanes, that is, 2^3 or 8 fold difference. In contrast, a linear range of at least 10^5 fold was easily detectable by real-time QPCR, and standard deviation of less than 0.15 Ct value or 10% deviation in quantity was easily obtainable within triplicates in a 96-well run. Lesser amount of DNA is required for the real-time QPCR assay compared with the agarose gel assay. With the agarose gel method, 63.5 μl of total DNA (typically around 2.5 mg) would be used in each assay. For QPCR, 2.5 μl of template was added into triplicates of plasmid and genomic reactions, resulting in the use of only 15 μl of a 1000 fold dilution. Thus only 0.015 μl of DNA sample (typically only around 0.6 ng) is required.

The PCN results obtained from QPCR were verified using the classical agarose gel electrophoresis method for *E. coli* pBR322 and *E. coli* NS3 (Table 2). For each

sample, three agarose gel assays and real-time QPCR runs were conducted, and PCNs were computed for these three separate trials. Other than the PCN of *E. coli* pBR322 computed by agarose gel method, the relative standard deviation for both methods were less than 1.6%, indicating excellent reproducibility. PCN of *E. coli* pBR322 is low (approximately 40), hence its plasmid DNA band was much fainter than the genomic DNA band. The large difference in band intensities made it difficult to adjust and quantify the intensity of each band, leading to the high standard deviation between the three gel runs. This highlights the advantage of real-time QPCR in computing low PCN with high accuracy.

It was also noted that the average plasmid copy numbers computed by the two different methods were comparable. As shown in Table 2, using agarose gel electrophoresis and real-time QPCR, the average

Table 2
Comparison of plasmid copy numbers obtained for triplicate runs of agarose gel electrophoresis method and real-time QPCR method for one sample each of *E. coli* pBR322 and *E. coli* NS3

Sample	Quantification method	Plasmid copy number					
		Trial 1	Trial 2	Trial 3	Average	Standard deviation (copy number)	Relative standard deviation (%)
E. coli pBR322	Agarose gel electrophoresis	57	45	35	46	11.0	24.1
	Real-time QPCR	39	40	40	40	0.6	1.5
E. coli NS3	Agarose gel electrophoresis	452	464	465	460	7.2	1.6
	Real-time QPCR	406	410	418	411	6.1	1.5

PCN of *E. coli* pBR322 were 46 ± 11.0 and 40 ± 0.6 respectively, and that for *E. coli* NS3 were 460 ± 7.2 and 411 ± 6.1 respectively. This resulted in only 13.0% and 10.7% difference in PCN computed by the two different methods, for *E. coli* pBR322 and *E. coli* NS3 respectively. The similarity in copy numbers to that of the established agarose gel assay validated the PCN results of the QPCR assay. In addition, the PCN of ~40 obtained for pBR322 is within its commonly reported range of 30 to 70 (Atlung et al., 1999).

3.4. Application of assay to batch fermentation run

During the 2 l fermentation run, samples were collected every hour and optical density (OD_{600}) was measured to monitor growth. Total DNA were extracted from cell pellets at each time point and the amount of plasmid and genomic DNA were quantified using real-time QPCR. The plasmid copy number was then computed as described earlier and plotted alongside the OD_{600} measurements, as shown in Fig. 3.

During the lag phase (2nd and 3rd h), the plasmid copy number per chromosome in *E. coli* decreased from that of the overnight shake flask culture to less than 200. Over this period of time, the cells were adjusting to the changes in the environment, and there would be minimal cell growth and DNA replication. During the exponential growing phase (4th to 7th h), cells multiply rapidly as could be seen by the rapid increase in OD_{600}. PCN was maintained at an average
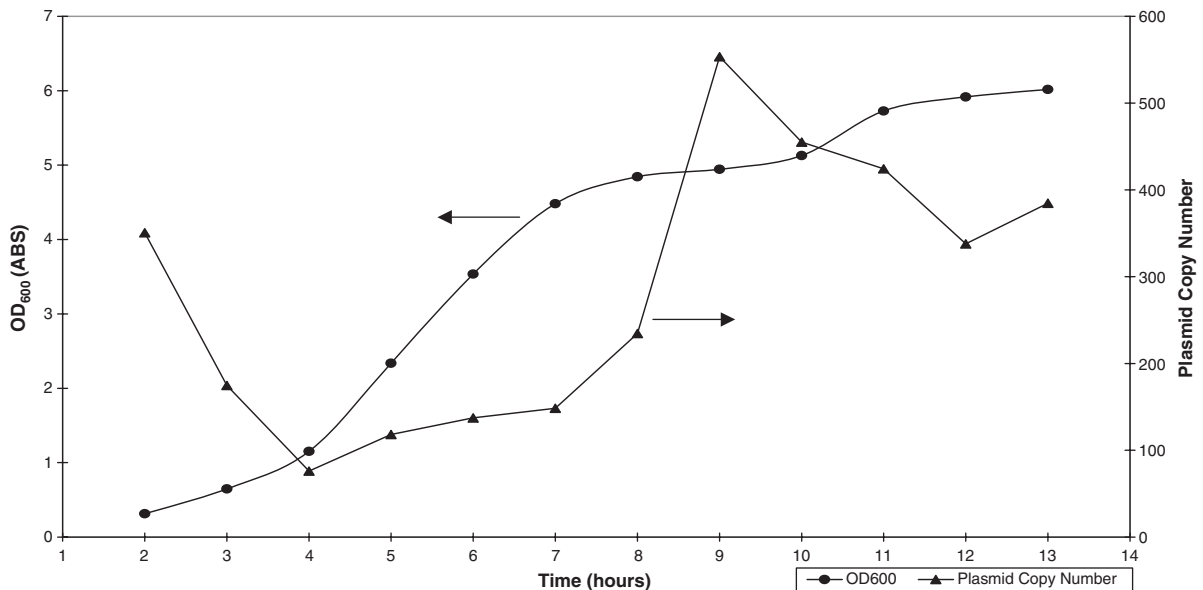

Fig. 3. Growth of DH5α carrying NS3 plasmid measured by optical density (OD) at 600 nm wavelength or OD_{600} (●) and plasmid copy number (▲) computed from real-time QPCR results in a 2 l fermentation run.

of only 120 copies. The low PCN could be due to the presence of multiple chromosomes in the rapidly growing cells. During the late exponential phase (8th to 9th h), cell growth slowed down while plasmid replication continued. Thus, the PCN increased sharply to as high as 550 copies. During the stationary phase (10th to 13th h), there was almost no genomic nor plasmid replication, which could possibly be due to the depletion of limiting substrate, and thus the PCN remained constant at an average of 400 copies. Separately, we have also shown a similar trend in PCN profile when carrying out another 4 l fermentation run with *E. coli* JM109 carrying plasmid NS3 (data not shown).

In this paper, we described a total DNA isolation protocol followed by real-time QPCR amplification using two primer sets targeting the plasmid and genomic DNA specifically for quantitation of PCN, and minimal amount of samples were needed. Current methods for PCN quantitation are tedious and time consuming for multiple samples like those generated during the fermentation processes. Taking the agarose gel assay as an example, at least one agarose gel had to be prepared and bands intensities quantified for samples at each time point. This procedure was tedious, and the amount of DNA required was 10^5 times more than that required for real-time QPCR method. QPCR allowed the quantitation of plasmid and genomic DNA for multiple samples collected throughout the whole fermentation run in one single 96-well plate, thus minimizing batch to batch handling error, while enabling greater sensitivity and precision.

Acknowledgements

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