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ORIGINAL ARTICLE

qPCR Analysis of Quorum Sensing Genes of *Pseudomonas aeruginosa*: Primer Design and qPCR Efficiency

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ABSTRACT – A well-designed and properly validated primer sequences should produce a specific and efficient real-time quantification polymerase chain reaction (qPCR) assay. The primer's melting temperature, primer's content of guanine-cytosine (G-C) and its size, as well as the amplicon's size were meticulously considered in designing the primers for this study, in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Designed primers were evaluated prior to the qPCR analysis to verify its efficiency and precision for the relative quantification of the gene transcription analysis. The validity of a primer is reliant on three elements: the qPCR efficiency (*E*) and the linear regression (R^2) as well as the slope of the standard curve. The resulted *E* for the genes *lasI, lasR, rhlI, rhlR* and *rplS* are 92%, 93%, 96%, 92% and 94%, respectively. While the R^2 and the slope value for these genes are 0.9991 and -3.523 for the *lasI*, 0.9991 and -3.501 for the *lasR*, 0.9989 and -3.434 for the *rhlI*, 0.9999 and -3.535 for the *rhlR*, and 0.9935 and -3.487 for the *rplS*. Melt curve analysis verified a single product amplification for tested genes. Resulted *E*, R^2 and slope for all studied genes fell between the acceptable range, validating the use of designed primer in the analysis of changes in transcription level of quorum sensing genes in treated *Pseudomonas aeruginosa*.

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INTRODUCTION

A Nobel Prize-worth development of the polymerase chain reaction (PCR) by Kary Mullis in 1992 revolutionized the field of molecular biology into a whole new era. PCR is a technique used to amplify a segment of DNA containing the gene of interest with a wide range of application, from mapping the human genome to the detection of bacteria and viruses, as well as diagnosis of hereditary disorders and investigation of gene transcription profile [1]. In PCR, the terms PCR, qPCR, RT-PCR, and RT-qPCR are often mistakenly used interchangeably with lack of attention to what the terms described. In traditional PCR, the amplified DNA segment is qualitatively assessed by gel-aided visualization. qPCR on the other hand, denotes real-time quantification of DNA amplification and measures the amplified DNA segment via fluorescent probe or intercalating dye. The acronym RT-PCR denotes reverse transcription PCR where generation of single-stranded complementary DNA (cDNA) using reverse transcriptase enzyme is coupled to a PCR while RT-qPCR combined previously described techniques in just one reaction tube for rapid quantitative detection of gene expression level [2].

A precise, sensitive, and robust qPCR assay relies on several key components, namely, the primer, a purely intact sample, the PCR reagent, and the thermal cycler [3]. With the qPCR assay condition remains mostly constant, together with a wide selection of PCR reagents, the latter two components are conveniently worked out. On the other hand, careful and precise consideration should be put into the primer design and the isolation process of the sensitive genetic material. Between these two components, the primer essentially dictates the success of a qPCR assay [4]. An efficient primer would specifically binds to the target molecule at its annealing temperature and remains hybridized at higher temperature to allow the extension by DNA polymerase to take place [5]. Primer's melting temperature (T_m), the G-C content and its size, as well as the targeted amplicon's size are among the parameters that should be considered when designing an optimal primer [4]–[6]. Further in silico analysis of the primer for possible secondary structures either from self- or hetero dimerization as well as the hairpin structure are worthy doing to avoid discrepancies in the qPCR results, especially when intercalated dye is opted as the detection method [7]. Unlike the fluorescent probe, the dye would intercalate into any double stranded nucleotide sequence present in the reaction and could falsely emit nonspecific fluorescence signal into the quantification. Nonetheless a proper execution of a qPCR protocol together with a well-designed primer, the intercalating dye detection method could achieve a high precision qPCR assay with a straightforward approach at a lower cost [8], [9].

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The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) recommends the qPCR assay to be validated prior to the actual analysis for an accurate and reliable assay [10]. A well-designed primer must perform accordingly during the assessment of the qPCR assay to produce a linear standard curve with a negative slope and a qPCR efficiency in the range of 90 – 110% [11]. qPCR efficiency (*E*) is defined as the capacity of one PCR cycle to copy and double the number of the target molecule, regardless of the target molecule's concentration with an assumption of perfect doubling at 100% efficiency [3]. The standard curve is constructed by plotting the qPCR-measured quantification cycle (Cq) values against logarithmic scale of serial dilution of target molecule. [4]. The determination of qPCR *E* value is particularly recommended for genetic expression profiling that employs an endogenous control as reference to relatively quantify the amplified target molecule [3].

We adapted qPCR analysis in our study to investigate the changes in the transcription level of quorum sensing (QS) genes in bacterial pathogen under given treatment. QS system is an intricate, multifactorial network of cell-to-cell communication employed by several bacteria, including *Pseudomonas aeruginosa*, to regulate its pathogenesis and biofilm formation [12]. *P. aeruginosa* is an opportunistic pathogen that often causes healthcare-associated infections (HAIs), due to surgery or the usage of medical devices [13]. A healthy individual is also at risk of contracting *P. aeruginosa* infection when the normal physiological function is disturbed, resulting in infections of either in the eye, ear, skin, soft tissue, or urinary tract [14]. The communication network used by *P. aeruginosa* is regulated by means of density of signaling molecules that would activate cascades of signaling pathway of virulence importance once it reached a threshold level [15]. The treatment given in this study is expected to interfere with the QS system without adversely affect the bacterial cell, with the aim to identify bioactive compound(s) possessing therapeutic effect without inducing the resistance mechanism in the pathogen. This could be accomplished by comparing the expression level of the QS genes between the control and the treated group of *P. aeruginosa*. To obtain a highly reliable result of gene expression profile, it is crucial to first and foremost, determine the efficiency of the designed primer via standard curve generated from qPCR assay. In this study, we designed four sets of primer of QS genes as well as the primer for the endogenous gene. Designed primers were subjected to qPCR efficiency analysis to ensure primer's specificity and efficiency in subsequent analysis of QS gene expression profile.

MATERIALS AND METHODS

Designing primer

Primer sequences were designed according to the recommended parameters [7]. Primer was designed using an online tool, PrimerQuest™, by Integrated DNA Technologies (IDT). Briefly, a complete sequence of gene of interest obtained from the National Center for Biotechnology Information (NCBI) was submitted to PrimerQuest™ for qPCR 2 Primers Intercalating Dyes type of analysis. Three primer criteria were set according to the recommended values: melting temperature (T_m) of 62 ± 3°C, a G-C content of 40 – 60%, and a primer size of 20 – 24 nucleotides with amplicon size between 75 – 150 base pair (bp). Returned primer sequences with best fit criteria was selected for further in silico analysis of secondary structure using OligoAnalyzer™, an online tool by IDT as well. The primer sequences were submitted for prediction of primer dimerization and hairpin formation. Primer with likeliness to form a stable secondary structure at assay condition would be redesigned accordingly.

qPCR assay optimization

The qPCR assay for every gene of interest was subjected to optimization step via standard curve analysis with a 5 point of 5-fold serial dilution of the template [3]. Resulted quantification cycle (Cq) values were plotted against the logarithm scale of the template's concentration to generate the standard curve. The assay was performed on StepOnePlusTM Real-Time PCR Systems (Applied Biosystem) with SYBR Green qPCR Master Mix (Applied Biosystem) as the assay reagent. Briefly, a master mix was prepared; separately for each gene; with each reaction contained 1X SYBR Green qPCR Master Mix and primer's concentration of 200 nM. The master mix was transferred to the reaction plate followed by the serial diluted cDNA template. An internal negative control of no template control (NTC) was included in the reaction plate as well. Thermal cycling program was initiated with initial denaturation at 95°C for 60 s and followed by the data acquisition cycle at 95°C for 5 s and 65°C for 15 s for each cycle. A post-run melt curve analysis was carried out at temperature range of 65°C to 95°C at 0.5°C/s increment for verification of a single product amplification. The slope and the R^2 value were derived from the generated standard curve while the qPCR efficiency (*E*) value was calculated from the slope value using following formula:

$$E = 10^{-(1/\text{slope})} - 1$$

RESULTS AND DISCUSSION

Primer sequences and their criteria

The primer sequences used in this study were designed according to the principal recommendations in primer design and are summarized in Table 1. In brief, the primers used in this study met all the principal criteria for a specific and

sensitive qPCR assay. The recommended optimal T_m for a primer is 62°C ± 3°C, with a maximum difference of 3°C between the forward and the reverse primers. T_m is the temperature at which 50% of the primer is bound to the template [4]. While it is almost impossible to have a same T_m for both forward and reverse primers, a closer T_m would allow the primer pair to anneal to the target site at approximately the same temperature, thus permitting an efficient reaction of the assay [4]. T_m for all the genes of interest is within the recommended value with a narrow T_m difference between the forward and the reverse primer.

Table 1. Primers sequences and its criteria.

Primers	Sequence (5'-3')	Size (nucleotides)	Melting temperature, T_m (°C)	% GC content	Amplicon (bp)
lasI-F	TACATGCTGAAGAACACCTTCC	22	62.39	45.5	99
lasI-R	TCCAGAGTTGATGGCGAAAC	20	62.36	50.0	
lasR-F	CTGTGGATGCTCAAGGACTAC	21	61.96	52.4	111
lasR-R	CCACTGCAACACTTCCTTCT	20	62.33	50.0	
rhlI-F	GCTACCGGCATCAAGTCTTC	20	63.00	55.0	100
rhlI-R	GTTTGCGGATGGTCGAACTG	20	63.00	55.0	
rhlR-F	GGCTTCGATTACTACGCCTATG	22	62.28	50.0	119
rhlR-R	CCGTAGTTCTGCATCTGGTATC	22	62.01	50.0	
rplS-F	CAGGTCAAGGTGAAGGAAGG	20	62.00	55.0	149
rplS-R	CTGTAGGTCTGGAAGGTACG	20	61.00	55.0	

**F: forward; R: reverse.*

The second criteria are the G-C content of a primer, which is recommended to be between 40 – 60%, and the G-C content of the primers used in this study fell between this range. G-C content promotes a strong binding of primer to the template but too rich of it would produce stronger binding and might not denature well during the thermal cycling step [17]. It is also recommended for the primer to be at the length of 20 – 24 nucleotide and it was kept between 20 – 22 nucleotides for this study. This size is sufficient for a specific binding of primer to the targeted site on the cDNA. Any size smaller than 20 nucleotides might resulted in unspecific binding while extended primer size might prolong the amplification cycle and thus reducing the qPCR assay efficiency [6]. Meanwhile, the size for the amplicon is recommended to be in the range of 50 to 150 bp for an efficient amplification and the amplicons of genes of interest in this study have a size ranging from 99 – 149 bp. Longer amplicon size reduces the efficiency of the qPCR assay as larger amount of intercalating dye would be incorporated into the amplicon [6].

Additionally, primers of genes under investigation in this study were also made sure to follow the recommendations for the GC clamp at 5' end of the primer, and consecutive runs of nucleotides in the primer sequence. GC clamp referred to the G and C nucleotides within the five bases at the 5' end where a maximum of two G and/or C is recommended for a firm binding of the primer to the template [6]. It is recommended too, to avoid consecutive run of more than four of the same nucleotides in the primer sequence to prevent non-specific interaction of the primer [6].

Possible secondary structure of designed primer

Any well-designed primer bears the possibility of forming secondary structure(s) either by dimerization with itself or with its pair, known as primer dimer. The primer could also fold back into a loop and hybridize with a portion of itself creating a hairpin structure. In silico analysis of possible secondary structure of designed primers were carried out to ensure least interference of the secondary structure to the qPCR assay which could compromise the specificity and efficiency of the assay. Since this study used fluorescence as the detection method, it is important to verify that the signal was detected solely from the targeted double stranded DNA. The criteria of concern in secondary structure of a primer are the presence of matched bp(s) at the 3' end, with a maximum of two matched bp is allowed. A matched of more than two bp promotes the formation of primer dimer and hairpin structure better than the hybridization of primer with the template [6].

Generally, when there is more than two matched bp at the 3' end of the primer dimer, the Gibbs free energy (ΔG) of the dimer should be verified to not exceed -9 kcal/mol. When the ΔG value is more negative than -9, there would be a higher chance for the dimer to be amplified, and therefore such primer should be disregarded [6]. Predicted secondary structure of primer dimer in this study showed absence of matched bp(s) at the 3' end, as shown in Table 2. Even though the ΔG value for the self-dimerization of forward primer of *rhlI* exceeded by 0.75 of the recommended value, this structure is free from matched bp(s) at the 3' end and therefore was accepted for the study.

Table 2: Possible primer dimer formation of designed primers

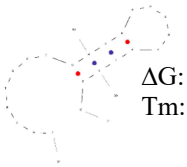
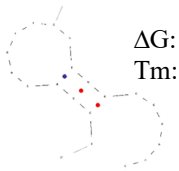
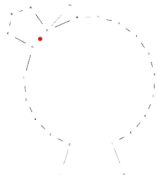
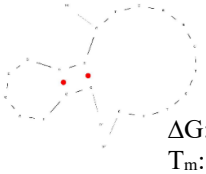
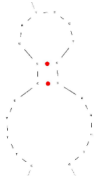
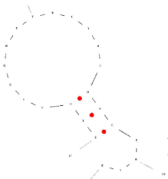
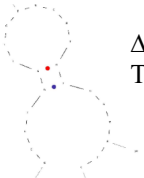
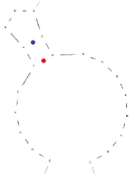
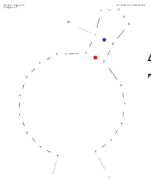
Gene	Secondary structure	Predicted secondary structure	ΔG (kcal/mol)
lasI	Self-dimer (F-F) Max ΔG: -38.91 kcal/mol	5' TACATGCTGAAGAACACCTTCC 3' CCTTCCACAAGAAGTCGTACAT	-5.38
	Self-dimer (R-R) Max ΔG: -38.24 kcal/mol	5' TCCAGAGTTGATGGCGAAAC 3' CAAAGCGGTAGTTGAGACCT	-5.02
	Hetero dimer (F-R) Max ΔG: -38.91 kcal/mol	5' TACATGCTGAAGAACACCTTCC 3' CAAAGCGGTAGTTGAGACCT	-3.55
lasR	Self-dimer (F-F) Max ΔG: -36.62 kcal/mol	5' CTGTGGATGCTCAAGGACTAC 3' CATCAGGAACTCGTAGGTGTC	-3.14
	Self-dimer (R-R) Max ΔG: -36.50 kcal/mol	5' CCACTGCAACACTTCCTTCT 3' TCTTCCTTCACAACGTCACC	-7.05
	Hetero dimer (F-R) Max ΔG: -36.62 kcal/mol	5' CTGTGGATGCTCAAGGACTAC 3' TCTTCCTTCACAACGTCACC	-8.19
rhlI	Self-dimer (F-F) Max ΔG: -38.47 kcal/mol	5' GCTACCGGCATCAAGTCTTC 3' CTTCTGAACTACGGCCATCG	-9.75
	Self-dimer (R-R) Max ΔG: -40.02 kcal/mol	5' GTTTGCGGATGGTCGAACTG 3' GTCAAGCTGGTAGGCGTTTG	-6.76
	Hetero dimer (F-R) Max ΔG: -40.02 kcal/mol	5' GCTACCGGCATCAAGTCTTC 3' GTCAAGCTGGTAGGCGTTTG	-6.68
rhlR	Self-dimer (F-F) Max ΔG: -41.94 kcal/mol	5' GGCTTCGATTACTACGCCTATG 3' GTATCCGCATCATTAGCTTCGG	-6.76
	Self-dimer (R-R) Max ΔG: -39.11 kcal/mol	5' CCGTAGTTCTGCATCTGGTATC 3' CTATGGTCTACGTCTTGATGCC	-7.05
	Hetero dimer (F-R) Max ΔG: -41.94 kcal/mol	5' GGCTTCGATTACTACGCCTATG 3' CTATGGTCTACGTCTTGATGCC	-8.86

Table 2: Possible primer dimer formation of designed primer; continued

Gene	Secondary structure	Predicted secondary structure	ΔG (kcal/mol)
rplS	Self-dimer (F-F) Max ΔG: -37.77 kcal/mol	5' CAGGTCAAGGTGAAGGAAGG ||| ::: 3' GGAAGGAAGTGGAACTGGAC	-3.53
	Self-dimer (R-R) Max ΔG: -36.51 kcal/mol	5' CTGTAGGTCTGGAAGGTACG |||| 3' GCATGGAAGGTCTGGATGTC	-3.65
	Hetero dimer (F-R) Max ΔG: -37.77 kcal/mol	5' CAGGTCAAGGTGAAGGAAGG ||| 3' GCATGGAAGGTCTGGATGTC	-3.55

Furthermore, the in-silico prediction of hairpin structure formation by designed primers showed no formation of hairpin structure with matched bp(s) at the 3' end and therefore, were accepted to be used in subsequent qPCR assay. It is crucial to make sure the matched bp(s), if presence, is less than two, with a T_m of the hairpin structure that is significantly low than the temperature used in the thermal cycling protocol [6]. Hairpin structure with a low Tm would not interfere with the annealing phase in the PCR assay and therefore ensure the efficiency of the assay.

Table 3: Possible hairpin structure of designed primer

Gene	Hairpin structure, F-primer	Hairpin structure, R-primer
lasI	 ΔG: -2.55 kcal/mol Tm: 48.2°C	 ΔG: -0.81 kcal/mol Tm: 35.7°C
lasR	 ΔG: 0.38 kcal/mol T_m: 16.5°C	 ΔG: 1.73 kcal/mol T_m: -16.2°C
rhlI	 ΔG: -1.23 kcal/mol T_m: 43.0°C	 ΔG: -1.10 kcal/mol T_m: 41.0°C
rhlR	 ΔG: -1.28 kcal/mol T_m: 40.4°C	 ΔG: 0.46 kcal/mol T_m: 18.3°C
rplS	 ΔG: 0.42 kcal/mol T_m: 15.3°C	 ΔG: 0.00 kcal/mol T_m: 25.0°C

qPCR efficiency (*E*)

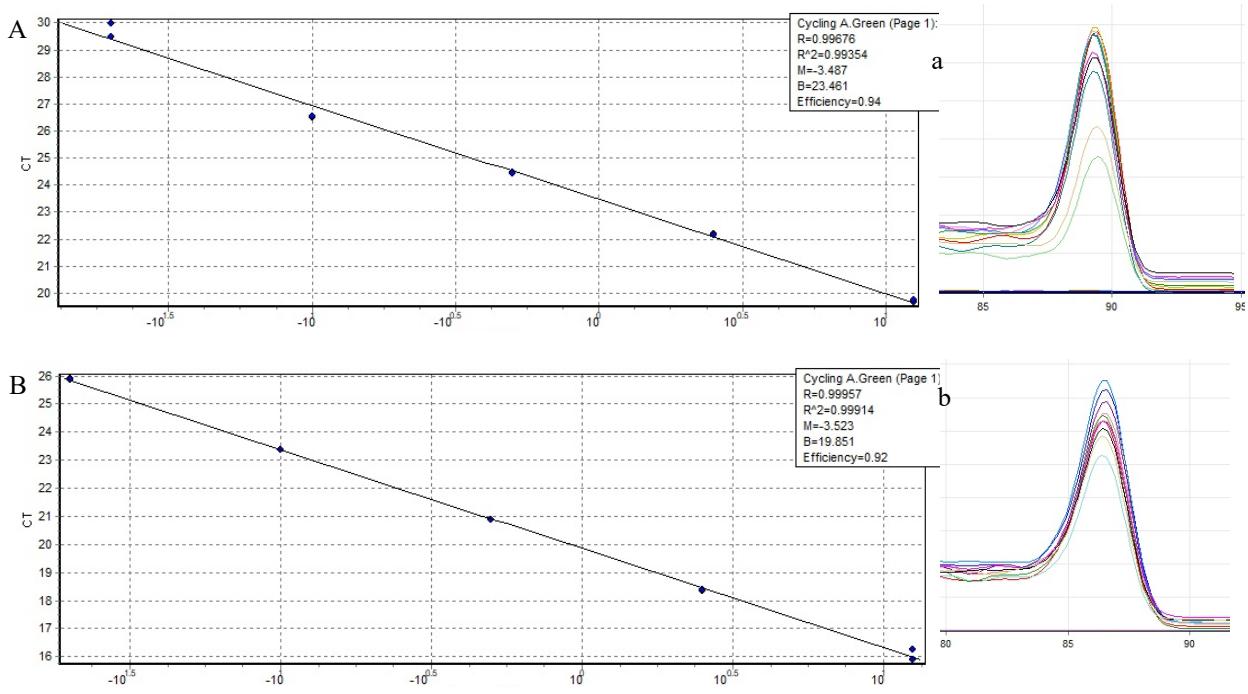
qPCR *E* defines the specificity and the sensitivity of a qPCR assay, that would allow accurate data analysis and interpretation. The qPCR *E* is practically depending on the performance of the primer in the assay condition, and it describes the capacity of the primer to drive the thermal reaction to double the number of target molecule in one complete PCR cycle, irrespective of the target molecule's concentration [3]. An acceptable range for qPCR *E* is from 90 – 110% with an assumption of perfect doubling at 100% efficiency. In a condition where the polymerase inhibitor is presence in the sample, the qPCR *E* could exceed 100% efficiency due to the delayed Cq in the concentrated sample [18]. Table 4 summarized the R^2 and the slope values that were derived from the standard curve, as well as the calculated value of qPCR *E* for each of genes of interest. The determined values met the criteria to pass the qPCR assay evaluation.

Table 4: Validation of designed primers.

Genes	qPCR efficiency, *E* (90 – 110%)	R^2 (> 0.95)	Slope (- 3.10 – - 3.59)	Pass/ Fail
rplS	94	0.9935	- 3.487	Pass
lasI	92	0.9991	- 3.523	Pass
lasR	93	0.9991	- 3.501	Pass
rhlI	96	0.9989	- 3.434	Pass
rhlR	92	0.9999	- 3.525	Pass

Generated standard curves and the resulted melt curves for all five genes of interest are shown in Figure 1. As expected, the Cq for the concentrated template was observed at earlier cycle and as the concentration of the template was decreased, the Cq value appeared at later cycle. As a result, a linear graph with negative slope was obtained for tested genes indicating a linear relationship between the template's concentration and the Cq value. As shown further in Figure 1a – 1e, multiple peaks were observed at the same temperature in the temperature gradient melt-curve analysis, indicating a single amplification of target molecule during the PCR cycle.

All the primers designed for this study produced acceptable values for all the required criteria to validate the accuracy and precision of the qPCR assay. Primers' sequences followed and obeyed the principal recommendations in designing the primers, while the standard curves established a degree of confidence for quantification of gene expression using intercalating dye. Thus, it could safely be concluded that the primers were at their optimum condition, and therefore could be applied in the subsequent qPCR analysis of the QS gene expression in treated *P. aeruginosa*.



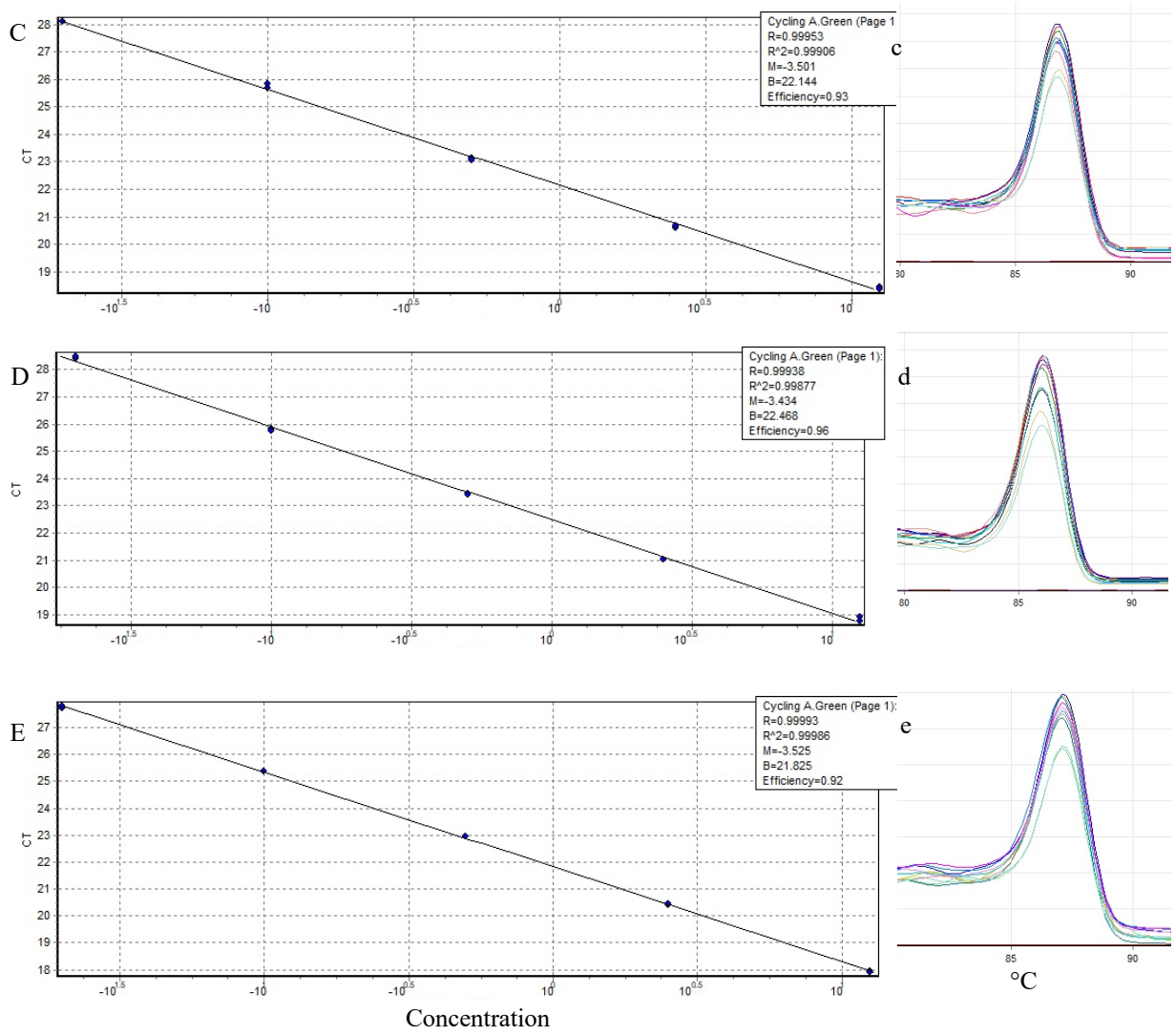


Figure 1: Standard curve (uppercase letter) and melt curve (lowercase letter) generated from the qPCR assay optimization. A/a) *lasI* gene, B/b) *lasR* gene, C/c) *rhlI* gene, D/d) *rhlR* gene and E/e) *rplS* gene.

CONCLUSION

qPCR is a convenient method to investigate variations in QS genes expression by *P. aeruginosa* under given treatment in this study. Primers for the qPCR assay were carefully designed based on principal guidelines in primer design and were evaluated for their specificity and efficiency in the qPCR assay. We successfully designed primer sequences that passed the qPCR assay optimization with a single amplification of target molecule in the post-qPCR run melt curve analysis. It is crucial for this study to accomplish the optimization step since we aimed to demonstrate the ability of a plant extract to attenuate the expression of the QS genes in treated *P. aeruginosa*, in our next study. It is predicted that QS genes inhibition would offer us an alternative approach in treating bacterial infection as opposed to antibiotics. Further elucidation in QS genes expression profile is needed to support appropriate drug development. Since this anti-infective drug manipulates the QS system of *P. aeruginosa*, rather than the bacterial cells, it is hoped that this drug would be able to decelerate the acquisition of antibiotic resistance while delivering successful treatment for the bacterial infection.

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