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ORIGINAL ARTICLE

RELATIVE GENE EXPRESSION STUDY USING REAL-TIME QUANTITATIVE PCR FOR *GROEL* GENE OF *ESCHERICHIA COLI (XL1 BLUE)* AND *STAPHYLOCOCCUS EPIDERMIDISISOLATE*

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Abstract:

Real-Time Polymerase Chain Reaction (PCR) is highly sensitive techniques enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time. Quantitative Real-Time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample. A relative quantification assay is used to analyze changes in gene expression in a given sample relative to another reference sample. Real-Time PCR is the favored method for measuring gene expression. Gene expression profiling is a common type of real-time PCR assay in which the relative abundance of a transcript is assessed to determine gene regulation patterns between samples. The relative quantification of the GroEL gene varied according to the bacterial culture Species. Here, we compare GroEL gene of Staphylococcus epidermidisand Escherichia coli,used as a reference gene for gene expression measurement.The SYBR® Green-based Real-Time PCR approach used for the detection of GroEL gene expression.

KEYWORDS:

Escherichia coli XL1 Blue, Gene expression, *GroEL* gene, Heat Shock Protein, Relative Quantification, *Staphylococcus epidermidis*

INTRODUCTION

Culture dependent methods for enumerating bacterial numbers are known to be biased since bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced in vitro. These techniques may take several days to yield a result and therefore are inappropriate in situations where rapid diagnostic decisions are required (Dymock *et al*., 1996; Kroes *et al*., 1999). Fluorescence-based methods can also be used to enumerate bacteria (Veal *et al*., 2000). Today Relative expression is increasingly used, where the expression of target gene is standardized. Quantitative Real-time PCR (RT-qPCR) is a technique to quantify gene transcription levels. Furthermore, it is easy to use, allows high throughput production of data and eliminates the need for radioactive isotopes (Jian et al.2008).As RNA cannot serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction (Bustin et al. 2000).

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has always been central to any research into gene function (Zamorano *et al*. 1996). Here, we analyze *GroEL* gene of *S. epidermidis* and *E.coli (XL1 Blue)* for enumeration. Analysis of the *S. epidermidis* and *(XL1 Blue)* genome indicated that those species are well equipped with genes that are predicted to provide protection from the harsh conditions encountered in its natural habitat. For example, to cope with Temperature condition, they are provided with *GroEL* gene which encodes hsp (Heat Shock Protein). Heat Shock Proteins are belongs to

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Chaperonin family,a more ideal universal DNA target for microbial identification to the species level would be one which has well-conserved DNA sequences within a given species, but with sufficient sequence variation to allow for species-specific identification.

Beginning of work requires successful extraction of RNA. The most important aspect is to prevent degradation of RNA during isolation. The standard protocol that allows high purification of RNA using TRIzol® reagent is mentioned here by. TRIzol®(by Life Technologies, Ambion), a monophasic solution of phenol, red dye and guanidine isothiocyanate minimizes the RNA isolation procedure. RNA isolation procedure was followed by Complementary DNA (cDNA) synthesis process. In this processcDNA products reverse transcribed from Messenger RNA (mRNA). Reverse Transcription followed by the PCR, is the technique of choice to analyze mRNA expression derived from source (Pfaffl et al.2001). The advent of Real-Time PCR methods has further improved the significant benefits of RT-PCR. Compared to conventional gel-based PCR assays, Real-Time PCR has many advantages including rapidity, higher sensitivity, specificity and throughput, lower hand-on-time, minimal contamination rate, quantitative measurement, automation, and easy standardization. The simplest and most cost-effective method involves the use of DNA intercalating dye such as SYBR® Green I as a fluorescent reporter. However, the major disadvantage is that the dye molecules may bind to any double stranded DNA in the reaction including non-specific products and primer-dimmers, which requires melt curve analysis to control the specificity of the reaction (Amer et al. 2010).

Gene expression research is a rapidly evolving field with recent advances in technologies aimed at multi-gene expression profiling and high throughput screening. SYBR® Green real-time PCR, common technique utilized by half of all Real-Time PCR users for gene expression measurement (Arikawa et al. 2008). Gene expression analysis is an important tool in contemporary research, with Real-Time PCR as the method of choice for quantifying transcription levels Co-analysis of suitable reference genes is crucial for accurate expression normalization. Reference gene expression may vary, e.g., among species or tissues (Kessler et al. 2009). Evaluating copy numbers of *GroEL* gene in *S.epidermidis* and *E.coli (XL1 Blue)* is of major importance for this study. SYBR® Green I based reverse transcription Real-Time PCR assay was developed for detection and quantification of *GroEL* gene. The assay was carried out using two sets of primers designed to amplify highly conserved sequences GroEL gene of *S.epidermidisand E.coli (XL1 Blue)*. Data processing can seriously affect interpretation of Real-Time PCR results. The basic choice in relative Real-Time PCR calculations is between standard curve and PCR-efficiency based methods. Widely used in many laboratory techniques this approach is simple and reliable.

MATERIALAND METHODS

All the methods were done simultaneously for both *E.coli (XL1 Blue)* and *S.epidermidis* cells.

Bacterial strain and Culture condition:

E.coli strain XL1 Blue and *S.epidermidisisolate* were grown in Luria-Bertani (LB) broth (Miller, 1972) at 37ºC for in incubator until the growth reaches to the exponential phase (OD600, 0.2; approximately 2 × 108 cells/ mL).

Preparation of serial dilution:

To ensure a thorough and even coverage of your quantification range, enough dilutions should be prepared to cover the expected range of expression within your samples. To study relative gene expression, Dilution of culture *E.coli XL1 Blue* and *S.epidermidisisolate* was made. RNA extraction protocol for these diluted cultures was followed by TRIzol® reagent protocol. Serial dilution of the culture is as followed; Place 9.0 mL of LB Broth media in each dilution tube. Use the Pipetter to add 1.0 mL of *E.coli XL1 Blue* and *S.epidermidis* isolate stock culture to the first dilution tube, to get 10-1 dilution and label it as #1. From the #1 add 1.0 ml from the first tube to the second tube #2, to get 10-2 dilution. Each step results in a further 10-fold change in the concentration from the previous concentration. Continue until the desired dilution of 10-6. Discard the last 1.0 mL from tube #6. Note the decreasing intensity and color change with increasing dilution.

RNA extraction:

RNA extraction of diluted culture was done simultaneously for both *E.coli XL1 Blue* and

S.epidermidis isolate.TRIzol® reagent used for extraction of total RNA, and Chloroform, Isopropyl alcohol, 75% ethanol, RNase-free water, was ready prior to use. Harvest 1.5 mL of *E.coli XL1 Blue* and *S.epidermidis isolate* culture and centrifuged at 12,000×g for 10 minutes. Supernatant was discarded and 0.75 mL TRIzol® reagent was added per 0.25 mL of sample for cell lysis. Cells were lyzed in sample by pippeting up and down several times. Sample was incubated for 5 minutes at room temperature (RT) to permit complete dissociation of the nucleoprotein complex. 0.15 mL of chloroform was added per 0.75 mL of TRIzol® Reagent. Tube was shaken vigorously by hand for 15 seconds. Incubated at RT for 2-3 minutes. Sample was centrifuged at 12,000×g for 15 minutes at 4°C. Aqueous phase of the sample was removed by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase. Aqueous phase was placed into a new tube. 0.38 mL of 99.9% isopropanol was added to the aqueous phase, per 0.75 mL of TRIzol® Reagent and incubated for 10 minutes at RT. Centrifuged at 12,000×g for 10 minutes at 4°C. Supernatant was removed from the tube, leave only the RNA pellet. Pellet was washed with 0.75 mL of 75% ethanol per 0.75 mL of TRIzol® Reagent. Sample was vortexed briefly, and then centrifuged at 7500×g for 5 minutes at 4°C. Wash was discarded, air dried. RNA pellet was resuspended in 20 µL RNase-free water by passing the solution up and down several times through a pipette tip. Sample was incubated in a water bath at 55-60°C for 10-15 minutes and stored at 4°C.

First strand cDNA synthesis:

Complementary DNA(cDNA) synthesis was carried out at the same time for all the RNA samples. RNA sample contaminating DNA was not analyzed further. The presence of target mRNA, *GroEL* gene was analyzed by Reverse Transcriptase Real-Time PCR. M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) was used synthesize cDNA from RNA. For master mix preparation; 1.0µL of Oligo dt primer (500µg/mL), 1.0µL of dNTPs (10mM), 1.6µL of SMQ (sterile milli Q) water, 9.0µL of RNA sample (1ng-5nsg) was added into the PCR tube. Mixture was heated at 65°C for 5 minutes and quickly chilled on ice and 4µL of 5X First strand buffer (FS buffer) and 2µL of 0.1 M DTT (Dithiothreitol) was added. Content was mixed and incubated at 37°C for 2 minutes. 1µL (200 units) of M-MLV RT was added, mixing was done and cDNA was synthesized at; incubated for 50 minutes at 37°C, Inactivate the reaction by heating at 70°C for 15 minutes and sample was stored at 4°C. The cDNA was used as a template for amplification in nested PCR.

Creating a serial dilution of cDNA:

For preparation of StandardcDNA sample which was not diluted during serial dilution of culture was taken and copy no. of the cDNA sample was calculated as described in Table 1.After calculating the copy no. sample was serially diluted till. 5.0 µL of cDNA sample was taken in eppendorf tube and to this 45.0 µL of SMQ was added for dilution. cDNA sample was diluted up to 10-4. Short spin was given to the samples and kept at room temperature. For our relative quantification study we need the dilution 10-4 to 10-7. For Unknown, take the cDNA sample which was diluted during serial dilution of culture. For Unknown, the dilutions of 10-1, 10-2 and 10-3 of the culture was taken.

Table 1 cDNA Copy number calculation:

Organism	Gene	OD	Conc. (µg/mL)	Size in bp	DNA/µL	Copy no.	Copy no. in 5 µL
E.coli XL1 Blue	*GroEL* gene	0.028	1.4	653 bp	0.7 µg	$9.93\ 10^{-11}$ x	49.65×10^{-11}
S.epidermidis isolate	*GroEL* gene	0.009	0.45	154 bp	0.225 µg	$1.35\ 10^{-12}$ x	6.75×10^{-12}

Real-Time PCR amplification:

The primers used to amplify the selected genes using RT-qPCR were described in table 2. Reactions were set up in a total volume of 20 µL using 5 µL of cDNA, 10 µL SYBR® Green I master mix

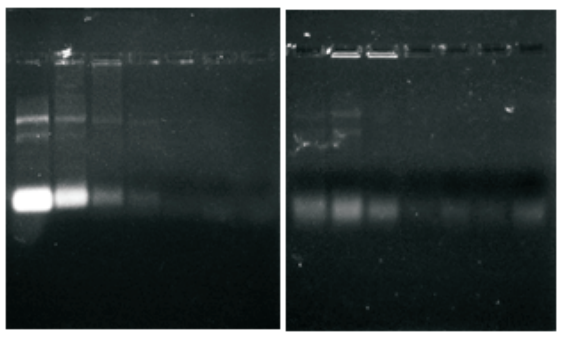
(Qiagen) and 1 μM each of gene-specific primers (Table 2). The cycling conditions; was carried out for *E.coli* was 95°C for 2 minutes, 40 cycles of 95°C for 45 seconds, 50°C for 1 minute, and 72°C for 1 minute; a final elongation step was carried out at 72°C for 10 minutes. For *S.epidermidis* was 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; a final elongation step was carried out at 72°C for 10 minutes. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The melting curve program consisted of temperatures between 60 and 95°C with a heating rate of 0.1°C /s and a continuous fluorescence measurement. After amplification process Data analysis of the Real-Time output was carried out.

Table 2 Primer pairs for individual copies of the *GroEL* gene of *E.coli XL1 Blue)* and *S.epidermidis isolate*:

Organism	Primer name	Target Gene	Sequence (5'-3')	Size of product
E.coli XL1 Blue	IMGROEF* IMGROER	*GroEL*gene	CCGTGGCTACCTGTCTCCTTACTT CCAGCAACCACGCCTTCTTCTACC	653
S.epidermidis isolate	hspsauF* hspsauR	*GroEL* gene	GGTTTACGACAAGGTATCGAC CCATAGCTTCTGAAATATAGCG	154

RESULTS AND DISCUSSION:

RNA extraction :



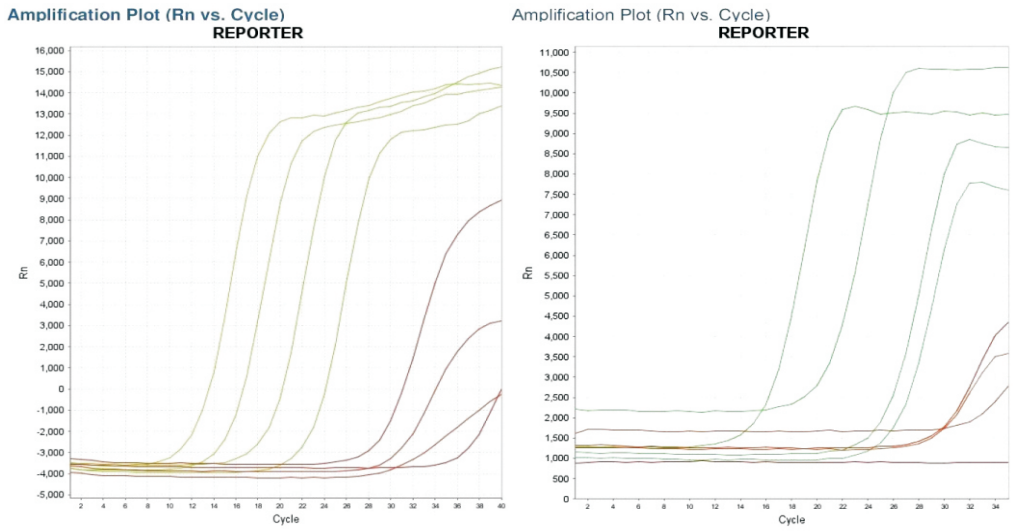
(a) (b)

Figure 1 Electrophoresis of RNA extraction of Diluted sample by TRIzol® reagent was loaded on 1% agarose gel (a) RNA extractionfrom *E.coli XL1 Blue.Lane* 1 contains undiluted sample of RNA, Lane 2 to Lane 7 contains diluted samples of RNA; 10^{-1} to 10^{-6} and (b) RNA extraction from *S.epidermidis isolate* culture. Lane 1 contains undiluted sample of RNA, Lane 2 to Lane 7 contains diluted samples of RNA; 10^{-1} to 10^{-6}.

The choice of TRIzol® reagent was investigated for the RNA isolation from the bacterial sample. Moreover, the time and speed at which the tubes were shaken were critical, since low speeds and short shaking times favored the isolation of DNA while high speeds and long times favored the isolation of RNA. Results for total nucleic acid (RNA) extracted using TRIzol® using the optimized steps directly from the *E.coli XL1 Blue* and *S.epidermidis isolateculture* with serial dilutions of order 10^{-1} to 10^{-6} is shown here. The band intensity justifies the accuracy of dilution. There is serial decrease in the density of band with the increase in serial dilution (Figure 1). The efficiency of mRNA can be predicted indirectly from the band pattern and also the amplification probability can be judged from the result. Contamination is not into consideration as we are interested in checking the concentration and expression of RNA. The result even signifies the minimum limit of concentration required for isolation, indirectly its expression. Also the main perspective i.e. the intensity of the band along the dilution, justifies the result itself. Expression up to folds of dilution can be checked.

Analysis of relative gene expression data using Real-Time quantitative PCR:

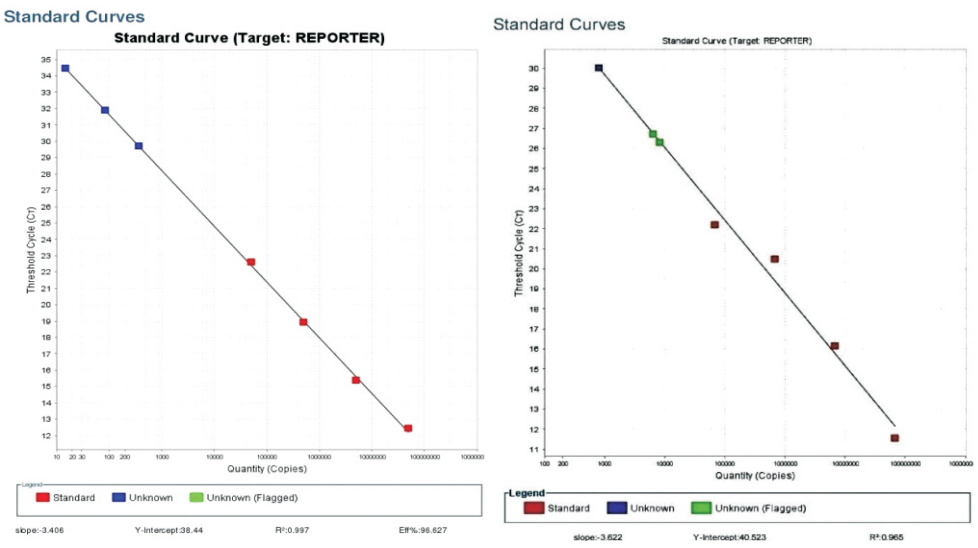
Relative quantification, while still technically challenging, is not quite as rigorous as absolute quantification. In this technique, this applies to most gene expression studies; the expression level of a gene of interest is assayed for up- or down-regulation in a calibrator (normal) sample and one or more experimental samples. Precise copy number determination is not necessary with this technique, which instead focuses on fold change compared to the calibrator sample.



(a) **(b)**

Figure 2 Amplificationplot showing the threshold value of the samples (Rn vs. Cycle) and phases of PCR amplification plot (a) *GroEL* gene Amplification plot of *E.coli XL1 Bluedilution* samples (b) *GroEL* gene Amplification plot of *S.epidermidis isolatedilution* samples.

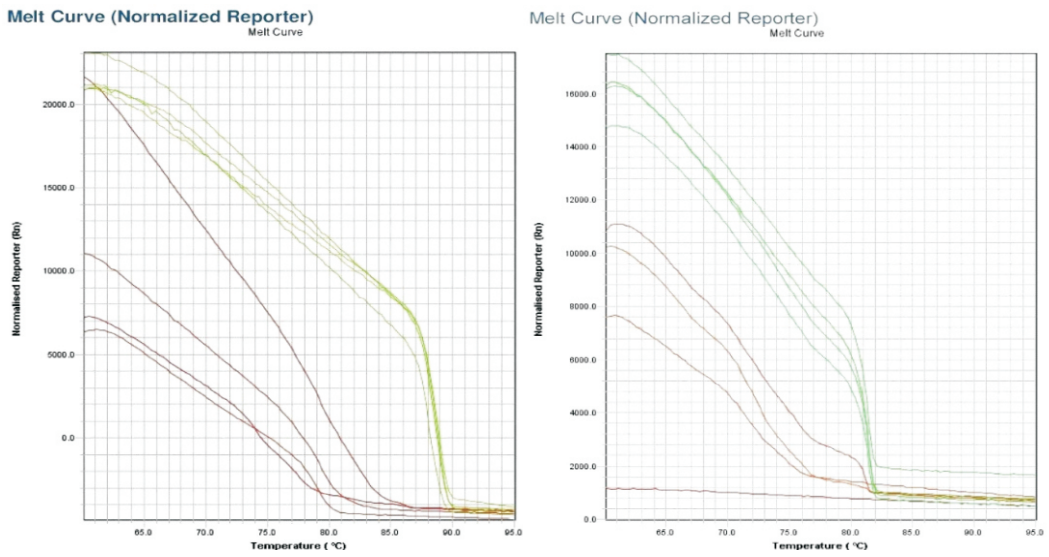
Figure 2 shows the amplification of *GroEL* gene using the Applied Biosystems StepOne™ Instrument Real-Time RT-PCR System. The multiplex strategy was efficient, enabling high throughput and fast results. In addition, this approach limits the complexity of the assay, which can ultimately reduce costs and complexity of the validation process. The difference in the threshold value observed is due to the differences in Bacterial species. When the fluorescence crosses the threshold log phase will start. Amplification of template started at this phase. Log phase is followed by exponential phase in which dNTPs activity is high, product is less, no competition with primers, high activity and high fluorescence. After this phase plateau phase is started where product start accumulating and activity is low. In figure 2 we can see that the threshold value of *S.epidermidis isolate* is higher than the *E.coli XL1 Blue.* During the early cycles of PCR there is little change in the Fluorescence signal. As the reaction progresses the level of signals begin to increase with each cycle. The base line shows the CT value of each reaction. CT values of each reaction used to generate a standard curve.



(a) **(b)**

Figure 3 Standard curve used to calculate genome copies (a) *GroEL* gene standard curve showing the copy numberof *E.coli XL1 Blue* dilution samples (b) *GroEL* gene standard curve showing the copy number of *S.epidermidis isolate* dilution samples.

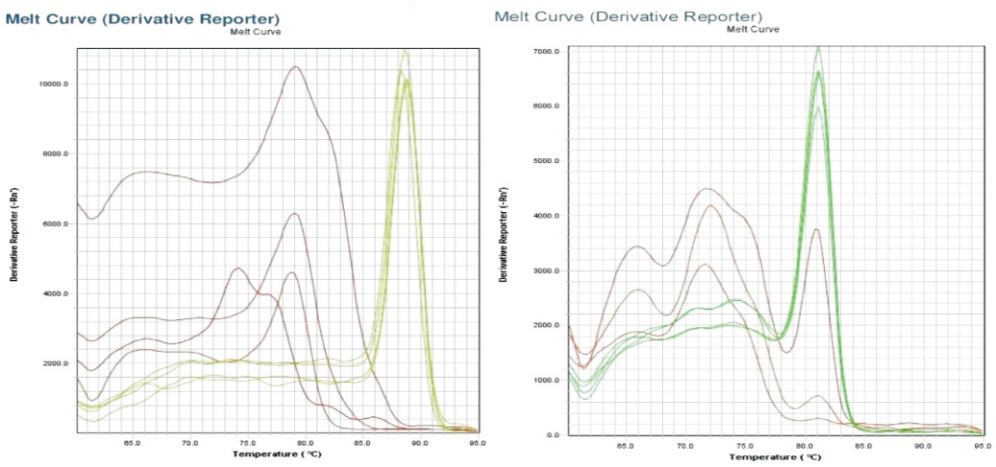
A standard curve was generated by performing serial dilutions of standard of cDNA (10-4 through to 10-7 dilution) and unknown samples whose copy number was not calculated. The standard curve was used to measure calculate the genome copies (Figure 3). The amount of test sample DNA per reaction was adjusted so that the cycle threshold (CT) values were within the linear range of the standard curve then unknown samples were relate to the standard samples for calculate copy number.By using Standard curve, we can calculate the efficiency or performance of amplification of template at each cycle. Slope, Y-intercept, R2 value can be calculated using the Standard curve as shown in figure 3.



(a) **(b)**

Figure 4 Meltcurve (Dissociation curve) for normalized reporter (a) *GroEL* gene dissociation curve of *E.coli XL1 Blue* dilution samples (b) *GroEL* gene dissociation curve of *S.epidermidis* isolate dilution samples.

A melting curve charts the change in fluorescence observed when double stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts", into single-stranded DNA (ssDNA) as the temperature of the reaction is raised. For example, when double-stranded DNA bound with SYBR® Green I dye is heated, a sudden decrease in fluorescence is detected when the melting point (Tm) is reached, due to dissociation of the DNA strands and subsequent release of the dye. The fluorescence is plotted against temperature (Figure 4), and then the –Delta F/ Delta T (change in fluorescence/change in temperature) is plotted against temperature to obtain a clear view of the melting dynamics (Figure 5).



(a) **(b)**

Figure 5 Meltcurve (Dissociation curve) for derivative reporter (a) *GroEL* gene dissociation curve of *E.coli XL1 Blue* dilution samples (b) *GroEL* gene dissociation curve of *S.epidermidis isolate* dilution samples.

Figure 5 showing the melting dynamics which is change in fluorescence vs. change in temperature. The Post-amplification melting-curve analysis is a simple, straightforward way to check real-time PCR reactions for primer-dimer artifacts and contamination and to ensure reaction specificity. Because the melting temperature of nucleic acids is affected by length, GC content, and the presence of base mismatches, among other factors, different PCR products can often be distinguished by their melting characteristics. The characterization of reaction products (e.g., primer-dimers vs. amplicons) via melting curve analysis reduces the need for time-consuming gel electrophoresis. Melting temperatureof *E.coli XL1* Blue is 78.9571 ºC and S.epidermidis isolate is 71.8720ºC.

CONCLUSION:

Real-Time, fluorescence RT-PCR significantly simplifies and accelerates the process of producing reproducible quantification of mRNAs. With the use of appropriate standard curves, absolute copy numbers of mRNA can easily be calculated. Therefore, Real-Time RT-PCR must be the method of choice for any experiments requiring sensitive, specific and reproducible quantification of mRNA.Real-Time PCR using SYBR® Green I fluorescence dye is a rapid and sensitive method to detect low amount of mRNA molecules and therefore offers important physiological insights on mRNA expression level. Our data showed that expression of *GroEL* gene of *E.coli XL1 Blue* and *S.epidermidis isolate* varied.

CONFLICT OF INTEREST

All authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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