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RT-qPCR based quantitative analysis of gene expression in single bacterial cells

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ABSTRACT

Recent evidence suggests that cell-to-cell difference at the gene expression level is an order of magnitude greater than previously thought even for isogenic bacterial populations. Such gene expression heterogeneity determines the fate of individual bacterial cells in populations and could also affect the ultimate fate of populations themselves. To quantify the heterogeneity and its biological significance, quantitative methods to measure gene expression in single bacterial cells are needed. In this work, we developed two SYBR Green-based RT-qPCR methods to determine gene expression directly in single bacterial cells. The first method involves a single-tube operation that can analyze one gene from each bacterial cell. The second method is featured by a two-stage protocol that consists of RNA isolation from a single bacterial cell and cDNA synthesis in the first stage, and qPCR in the second stage, which allows determination of expression level of multiple genes simultaneously for single bacterial cells of both gram-positive and negative. We applied the methods to stress-treated (*i.e.* low pH and high temperature) *Escherichia coli* populations. The reproducible results demonstrated that the method is sensitive enough not only for measuring cellular responses at the single-cell level, but also for revealing gene expression heterogeneity among the bacterial cells. Furthermore, our results showed that the two-stage method can reproducibly measure multiple highly expressed genes from a single *E. coli* cell, which exhibits important foundation for future development of a high throughput and lab-on-chips whole-genome RT-qPCR methodology for single bacterial cells.

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1. Introduction

Microbial cells grown under the same conditions and environments are often considered to be uniform populations that can be adequately described by average values (Brehm-Stecher and Johnson, 2004). However, evidence is emerging that isogenic populations of exponentially growing microorganisms have substantial cell-to-cell heterogeneity at both the gene expression and growth rate levels (Kelly and Rahn, 1932; Maloney and Rotman, 1973; Siegele and Hu, 1997; Becskei et al., 2005; Kuang et al., 2004; Colman-Lerner et al., 2005; Golding et al., 2005; Le et al., 2005; Kaern et al., 2005; Pedraza and van Oudenaarden, 2005; Rosenfeld et al., 2005; Strovas et al., 2007; Strovas and Lidstrom, 2009). It has been suggested that the gene expression heterogeneity could arise from stochasticity, or noise, in the gene expression of each individual. The amplitude of such noise in gene expression is controlled by many factors, including transcription rate, regulatory dynamics, and genetic factors of the cells (Banerjee et al., 2004; Colman-Lerner et al., 2005; Pedraza and van Oudenaarden, 2005; Rosenfeld et al., 2005; Newman et al., 2006; Strovas et al., 2007). As a result of these factors, individual cells in genetically homogeneous populations contain different copy numbers of messenger RNA (mRNA) molecules, which eventually may lead to different numbers of functioning protein molecules. Those noises, once amplified, could offer the opportunity to generate long-term heterogeneity at the cellular level in a clonal microbial population. In addition, within natural ecosystems, microbial cells with diverse genotypes and phenotypes that express distinct metabolic pathways, stress responses and other specific biological activities are juxtaposed (Macfarlane and Dillon, 2007). The mechanisms that contribute to this genetic and physiological heterogeneity include microscale chemical gradients, adaptation to local environmental conditions, stochastic gene expression and the genotypic variation that occurs through mutation and selection (Stewart and Franklin, 2008). The gene expression heterogeneity of a microbial community suggests that by simply harvesting mRNA or proteins from whole populations, the unique patterns of gene expression related to specific regions of the consortia or distinct functional subpopulations in the community might be lost. Furthermore, it is estimated that only less than 1% of microbial species in natural environments can be cultured and accessed by traditional gene expression analysis methods that typically requires a large number of cells. There has been great interest in obtaining individual bacterial cells using methods like fluorescence-activated cell sorting and then analyzing gene expression directly in single bacterial cells.

Several approaches have been proposed to measure gene expression in a single bacterial cell, such as a reporter gene/protein approach that utilizes green fluorescent protein or luciferase (Golding et al., 2005; Le et al., 2005, 2006; Cai et al., 2006; Yu et al.,

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2006; Strovas et al., 2007; Guet et al., 2008; Stewart and Franklin, 2008; Strovas and Lidstrom, 2009), fluorescent probes in fluorescence *in situ* hybridization (FISH) experiments (Levsky et al., 2002; Capodieci et al., 2005), and *in situ* PCR combined with *in situ* reverse transcription (*in situ* RT-PCR) (Aoi, 2002). However, these methods either require genetically engineered strains or very time- and labor-consuming molecular biology protocols to obtain measurement, and it is therefore very hard to improve their measurement throughput. Another method for gene expression analysis is confocal single-molecule detection (SMD) technique to detect single fluorescent molecules with high signal-to-noise ratio (SNR). However, these analyses typically have higher requirements for instruments and are also very time- and labor-consuming (Lu et al., 1998; Korn et al., 2003; Raj et al, 2008; Raj and van Oudenaarden, 2009). An alternative, probably more straightforward and scalable approach, is to perform reverse-transcript (RT) polymerase chain reaction (PCR) directly in single bacterial cells (Kubista et al., 2006; Nolan et al., 2006). Coupled with various cell sorting and collecting methods, several protocols have been published for gene expression analysis by RT-qPCR for single-cell mammalian cells (Lindqvist et al., 2002; Hartshorn et al., 2007; Wacker et al., 2008; Taniguchi et al., 2009; Li et al., 2010). The most advanced protocol was published by Taniguchi et al. (2009) who used a quantitative PCR method featuring a reusable single-cell cDNA library immobilized on beads for measuring the expression of multiple cDNA targets (from several copies to several hundred thousand copies) in a single mammalian cell, and the results showed that an experimental error is less than 15.9%, suggesting that the method is sufficiently accurate to investigate the heterogeneity of single cells.

So far no method has been published for gene expression measurement in single bacterial cells using the RT-qPCR based method, probably due to the fact that most bacterial cells are difficult to lyse efficiently, the half lives of the bacterial mRNA is short and its stability is low when compared with those from eukaryotic cells, and the bacterial cells are much smaller than mammalian cells (2–3 μm *vs.* 10–20 μm) and consequently the concentration for any given mRNA molecule may be relatively low. Many different real-time qPCR methods have been developed, including sequence-unspecific DNA labeling dyes (SYBR Green), primer-based technologies (Ampli-Fluor, Plexor, Lux primers), and techniques involving double-labeled probes, comprising hybridization (molecular beacon) and hydrolysis (TaqMan, CPT, LNA, and MGB) probes (Buh Gašparič et al., 2010). Among them, two most popular methods are probe-based TaqMan PCR which requires a pair of PCR primers as regular PCR does, an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached, and intercalator-based SYBR Green method which requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and emits fluorescence. In general, both the TaqMan and SYBR Green method are considered equally accurate and reliable, but the former is much more expensive (Wong and Bai, 2006). Since our goal is to develop methods that can potentially be used for high throughput analysis, we chose the SYBR Green method as an inexpensive platform to start with. Attempts were made in our laboratory to overcome these issues and develop RT-qPCR based methods to determine the gene expression level directly at the single bacterial level. In this study, we report two SYBR Green-based RT-qPCR approaches to determine gene expression in single bacterial cells. The first method involves a single-tube operation that can analyze one gene from each bacterial cell. The second method features a two-stage operation for RNA isolation/cDNA synthesis and qPCR analysis that allows measurement of multiple genes simultaneously for each bacterial cell. We used the methods to determine the gene expression heterogeneity among the exponentially growing *Escherichia coli* populations and their responses to low pH and high temperature stress. Technical variation (*i.e.* variation when the same samples were analyzed multiple times) of the methods and gene expression heterogeneity (*i.e.* expression difference of the same gene in different single cells) were determined.

2. Materials and methods

2.1. Primer design and selection of target sequence for qPCR

Four genes were chosen for single bacterial cell RT-qPCR in this study. The involvement of both *dnaK* and *groES* genes in heat shock response (Bardwell and Craig, 1987; Tao et al., 1999) and *gadA* in low pH (Tucker and Conway, 2002) in *E. coli* has been well documented before. The choice of 16S rRNA gene was due to the highly expression level and could be used as an initial effort to conduct single bacterial RT-qPCR. Also, 16S rRNA could be used as a housekeeping gene for the purpose of expression calibration of other genes when multiple gene expression analysis was performed within single bacterial cells. DNA sequences of target genes of *E. coli* and *Bacillus subtilis* were retrieved from GeneBank. Primer 3 program available online was used for the primer design (http://frodo.wi.mit.edu/primer3/). For each target gene, several primer pairs capable of amplifying a DNA fragment of about 200 bp were chosen and ordered from Invitrogen (San Diego, CA). The primer effectiveness and efficiency were evaluated first in bulk cells and the primer pairs showing the highest effectiveness and efficiency in qPCR analysis were selected for use in single-cell analysis. PCR primers for four target genes of *E. coli* (16S rRNA, *dnaK*, *groES* and *gadA*) and one target gene of *B. subtilis* (16S rRNA) used in single cell RT-qPCR are as shown in Table 1.

2.2. Cell culturing and single cell picking

Both *E. coli* DH5α and *B. subtilis* 168 were used in this experiment and cultivated in LB liquid broth overnight at 37 °C (Sabina et al., 2003). *E. coli* was also cultivated in minimal M9 medium (glucose as carbon source) overnight at 37 °C (Sabina et al., 2003). Cell picking was done using a robotic single cell manipulation system developed in our research center (Anis et al., 2008), which can aspirate a single bacterial cell in a total volume of 50 nl. Under a light microscopy (10× objective) mounted with a computer monitor, a bacterial cell was selected and positioned with the micropipette tip (20 μm in diameter) using a closed-loop vision-based feedback controller (Anis et al., 2008). When the selected cell was aligned with the micropipette orifice, aspiration could be performed by applying a negative pressure to the micropipette capillary, which generated a drag force on the cell and pulled it inside. To make sure one and only one bacterial cell was pull inside, cell dispense was then performed. This aspiration-dispense process could be repeated several times and visually monitored under microscopy to secure only one bacterial cell was aspirated into the micropipette capillary.

2.3. Single-cell RT-qPCR analysis

For single-tube RT-qPCR, the single cell aspirated was delivered onto the lid of a regular 0.1 ml PCR tube (Applied Biosystems Inc, Foster City, CA), containing 2 μl DEPC-treated water (Ambion, Austin, TX). After closing the lid, the tube was centrifuged briefly in a "Quick-Spin" Minifuge (ISC BioExpress, Kaysville, UT) to let the cell settle to the bottom of the tube. Immediately the tube was then subjected to heating on a thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad Laboratories, Hercules, CA) at 95 °C for 4 min, and then used in single-tube RT-qPCR thereafter, or stored at −80 °C for further gene expression analysis. Alternatively, cell suspension was subject to series dilution with DEPC-treated water to achieve a theoretical given cell number in a final total volume of 2 μl. The solution with cells (2 μl) was then subjected to heating on a thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad Laboratories, Hercules, CA) at 95 °C for 4 min, and then used in single-tube RT-qPCR thereafter, or stored at −80 °C for further

Table 1
Primer pairs for single cell qRT-PCR.

Species	Gene names	GeneBank accession no.	Product size (bp)	Forward primer	Reverse primer
E. coli	16S rRNA	J01859	330	GTTAATACCTTTGCTCATTGA	ACCAGGGTATCTAATCCTGTT
	dnaK	D10765	245	GGCTTCTTCTGGTCTGAACG	TAGCGGCTTTGTCTTCACCT
	groES	EU890979	191	CGTGATCGTCAAGCGTAAAG	CCGTAGCCATCGTTGAAAAC
	gadA	EF547379	162	ACGCAGACGTTCAGAGAGGT	TTACCAGGTTGCCGCTTATC
B. subtilis	16S rRNA	AB042061	177	TCGCGGTTTCGCTGCCCTTT	AAGTCCCGCAACGAGCGCAA

RT-qPCR kit (Invitrogen) was used in the one-tube RT-qPCR analysis. In a 0.1 ml PCR tube (Applied Biosystems, Foster City, CA), a RT-qPCR reaction in 10 μl of total volume was set up as follows: 5 μl of SuperScript III RT/Platinum Taq Mix (including RNaseOUT), 1 μl of each PCR primer (4 μM), 0.1 μl of ROX Reference Dye (25 μM), 2 μl of diluted purified RNA template or cell lysate, as well as 0.9 μl of DEPC-treated water (Ambion, Austin, TX). The thermal cycling program at ABI StepOne (Applied Biosystems, Foster City, CA) was: 48 °C for 30 min, 95 °C for 10 min, 40 cycles of 95 °C for 10 s, and 60 °C for 1 min, followed by melting curve analysis using the defaulted program of the ABI StepOne or StepOne Plus qPCR machine.

For two-step RT-qPCR protocol, the single cell aspirated was delivered into a 100 μl PCR tube (Applied Biosystems, Foster City, CA) containing 100 μl of RNA Lysis Buffer from ZR RNA MicroPrep Kit (Zymo Research, Orange, CA). If a cell was subjected to heat shock treatment, the single cell aspirated was delivered into 100 μl of LB broth of the same type of tube, immediately followed by heat shock treatment in a water bath of 50 °C. After heat shock treatment for 15 min, all LB broth in the 100 μl PCR tube was transferred to a 1.5 ml Eppendorf tube containing 400 μl RNA Lysis Buffer. To make sure the single cell was transferred, the 100 μl PCR tube was rinsed several time with the LB broth–lysis buffer mixture. RNA extraction was carried out using ZR RNA MicroPrep Kit (Zymo Research, Orange, CA) following the instructions provided by the manufacturer. A total of 5 μl of RNA was eluted from the column matrix and immediately used in RT reactions. cDNA synthesis in 10 μl of total volume was as follows: 2 μl of 5× VILO Reaction Mix, 1 μl of 10× SuperScript Enzyme Mix, 5 μl of total RNA from a single cell, as well as 2 μl of DEPC-treated water (Ambion, Austin, TX). After gently mixing tube contents and incubating at 25 °C for 10 min, the cDNA synthesis was performed at 42 °C for 60 min followed by 85 °C for 5 min for inactivation of reverse-transcriptase. Diluted or undiluted cDNA was used in qPCR immediately, or stored at −20 °C until use. During stage two, EXPRESS SYBR GreenER qPCR SuperMixs Kit (Invitrogen, San Diego, CA) was used for qPCR analysis. In a 0.1 ml PCR tube (Applied Biosystems, Foster City, CA), qPCR reaction in 10 μl of total volume was as follows: 5 μl of EXPRESS SYBR GreenER qPCR SuperMix Universal, 1 μl of each primers (4 μM), 0.1 μl of ROX Reference Dye (25 μM), 1 μl of diluted or undiluted cDNA, as well as 2.9 μl of DEPC-treated water. The thermal cycling program at ABI StepOne was: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 80 °C for 10 s (for signal detection), followed by melting curve analysis using the defaulted program of ABI StepOne or StepOne Plus machine. Data analysis was carried out using the software provided by Applied Biosystem Inc.

Total RNA from bulk bacterial cells was extracted and purified according to a method combining Trizol (Invitrogen, San Diego, CA) and RNeasy method (QIAGen, Valencia, CA). Briefly, 1.0 ml recovered cells were transferred to a pre-chilled microcentrifuge tube and centrifuged at 12,000 × *g* for 1 min at 4 °C. After centrifugation, the supernatant was decanted and the cell pellet was re-suspended in preheated 200 μl Max Bacterial Enhancement Reagent and incubated at 95 °C for 4 min. 1.0 ml TRIzol® Reagent was added to the lysate, mixed well and incubated at room temperature for 5 min. For phase separation, 0.2 ml cold chloroform was added to the mixture and mixed by shaking the tube vigorously by hand for 15 s. After being incubated at room temperature for 2–3 min, the sample was centrifuged at 12,000 × *g* for 15 min at 4 °C and the mixture in the tube separated into a lower red, phenol–chloroform phase, an interphase, and a colorless aqueous phase containing RNA. This aqueous phase was transferred into a new Eppendorf tube and proceeded to RNeasy Kit. Equal volume (700 μl) of 70% ethanol was added to the supernatant and mixed well. Then the mixture along with the precipitate was applied to RNeasy mini spin column sitting in a 2 ml collection tube and centrifuged at 12,000 × *g* for 15 s. The flow through was discarded. 350 μl of Buffer RW1 was pipetted on to the RNeasy column and the column was centrifuged at 12,000 × *g* for 15 s. After discarding the flow through and changing the collection tube, DNase treatment on the column using Qiagen RNase-free DNase Set was performed. Then another 350 μl of Buffer RW1 was added onto the spin column, and centrifuged at 12,000 × *g* for 15 s followed by the flow through discarding and the collection tube change. 500 μl of Buffer RPE was applied twice onto the RNeasy column and centrifuged at 12,000 × *g* for 15 s and 2 min respectively. Before this step, RNA was extracted and filtered on the silica-gel membrane of the column. At last, 30 μl of RNase-free water was directly applied onto the membrane and centrifuged at 12,000 × *g* for 1 min.

3. Results and discussion

3.1. Single-tube RT-qPCR of single bacterial cells

DNA amplification and detection have been achieved in single bacterial cells previously (Wong and Bai, 2006; Hutchison and Venter, 2006; Zhang et al., 2006; Marcy et al., 2007). However, it is still an open question whether gene expression analysis in single bacterial cells is achievable. A typical mammalian cell contains 10–30 pg total RNA (http://www.sabiosciences.com/newsletter/RNA.html). However, since no literature or resource reported the total RNA amount within a single bacterial cell, we conducted experiments to estimate this value using *E. coli* cells. The results showed that we obtained 1.92×10^4 ng of total RNA from 5×10^8 *E. coli* cells ($OD_{600} = 1.0$ culture). Assuming 100% RNA recovery, total RNA from one single *E. coli* cell is estimated as approximate 3.84×10^{-5} ng. This total RNA amount is approximately equal to one thousandth of that of a typical mammalian cell. Based on this estimation, total RNA from bulk cells was diluted to the level that equals that of a single cell, and used it for testing in RT-qPCR analysis. We used one-step RT-qPCR procedure to conduct this experiment. The result showed that the bacterial 16S rRNA gene can be amplified and detected at this level (Supplementary Fig. 1A), suggesting that gene expression analysis at a single bacterial level is possible using the current instrumentation. When the same protocol was applied to cells directly from series-dilution, we could amplify the 16S rRNA gene linearly until about 20 cells (Supplementary Fig. 1B). Continuing dilution of cells less than 20 could not generate amplification curves linearly corresponding to the cell dilution level, indicating that we may reach the technical limit of series-dilutions.

We made several modifications to optimize the single-tube RT-qPCR method for direct single-cell analysis. First, it is notable that decreasing the PCR template, the chance of forming primer dimer increased significantly as observed in both qPCR melt curve and agarose gel analysis of the PCR products (Supplementary Fig. 2), which may interfere with the signal detection for the expected PCR

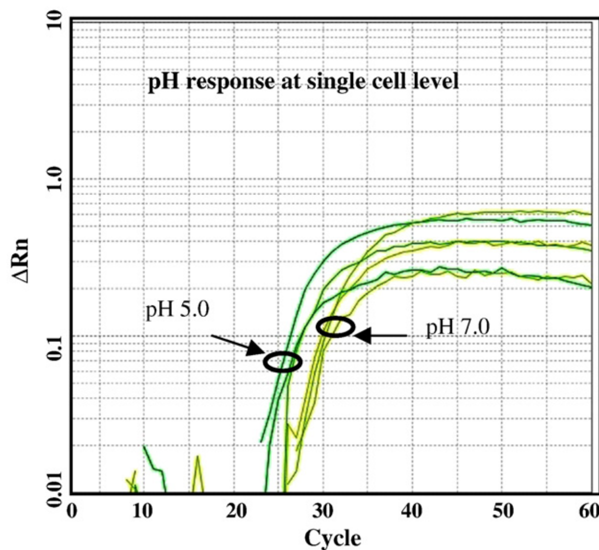

Fig. 1. Expression of *gadA* gene in single *E. coli* cells with or without low pH treatment. Three cells were analyzed for each condition. Each curve is from measurement of one single *E. coli* cell.

products. To avoid this problem, we initially tried to low the primer concentration for single-cell analysis, but very little improvement was observed. We then redesigned primers capable of amplifying a relatively large DNA fragment of about 200 bp, which could be well differentiated from primer dimers that are typically about 100 bp. Second, we redesigned the thermal cycling program by including one step (at about 80 °C for 10 s) immediately following the annealing/amplification step for dsDNA signal detection. At 80 °C, primer dimer will disappear since its melting temperature was less than 75 °C under our RT-qPCR conditions, and the target DNA fragment will remain intact since its melting temperature is more than 80 °C. Although eliminating primer dimers may be possible through continually optimizing PCR conditions including primer design and buffer components, this process is very time-consuming. Alternatively, our approach aimed at avoiding collect fluorescence from primer dimers and believed that even though we could not eliminate primer dimer problem in some cases, we could still collect fluorescence signal purely from PCR products. In addition, based on the data in Supplementary Fig. 2, the primer dimers become a significant issue only when 10^3–10^4 dilution of the single-cell cDNA was used as template in this experiment. Our results demonstrated that under the newly optimized conditions, we could determine gene expression for the *gadA* gene, which encodes a glutamate decarboxylase isozyme and its mRNA copy number is much lower than that of 16S rRNA, directly using single *E. coli* cells. As shown in Fig. 1, three cells from an *E. coli* culture on minimal M9 medium (pH 7.0) and three cells from culture treated by low pH (5.0) were picked and analyzed. Obvious increased expression of the *gadA* was observed for all treated cells, consistent with the expected response of the gene reported in the bulk-cell study (Tucker and Conway, 2002). In addition, a clear difference in terms of gene expression between cells was also observed, although by this single-tube (*i.e.* one gene per cell) analysis we were not able to distinguish technical variation from biological heterogeneity. To further confirm the qPCR results, we also run agarose gel analysis of the PCR products. Single band was observed for the single-cell qPCR and the qPCR products were further confirmed by sequencing.

3.2. Two-stage RT-qPCR of single bacterial cells

The limitation of the single-tube method compelled us to develop an approach to determine gene expression levels per bacterial cell so that biological heterogeneity can be assessed accurately. In order to achieve this goal, we divided the RNA isolation/cDNA synthesis and the qPCR into two stages and optimized each stage separately. During stage one, we first aimed at RNA isolation/purification of bacteria toward single cell level. Since no commercial kits are available for single bacterial cell RNA isolation/purification, we adapted several kits designed for RNA isolation/purification from mammal/eukaryotic cells. We found that ZR RNA MicroPrep Kit (Zymo Research, Orange, CA) was capable of RNA isolation/purification of both gram positive and negative bacterial cells without modifications. When bulk cells were applied, the efficiency and recovery rate of total RNA using this kit was comparable to that of a method combining Trizol (Invitrogen) and RNeasy Kits (Qiagene) (Unpublished data). At a bulk-cell level, we also evaluated the performance of ZR RNA MicroPrep Kit in RNA extraction/purification and found its performance was consistent from time to time and the maximum variation among different extraction was less than 15% of total RNA (Supplementary Table 1). Among kits used in RT reactions, SuperScript VILO cDNA Synthesis Kit (Invitrogen) was used in cDNA synthesis and its performance in RT reactions as evaluated at bulk-cell level was superior to other kits that we have evaluated (data not shown). We divided single-cell RNA

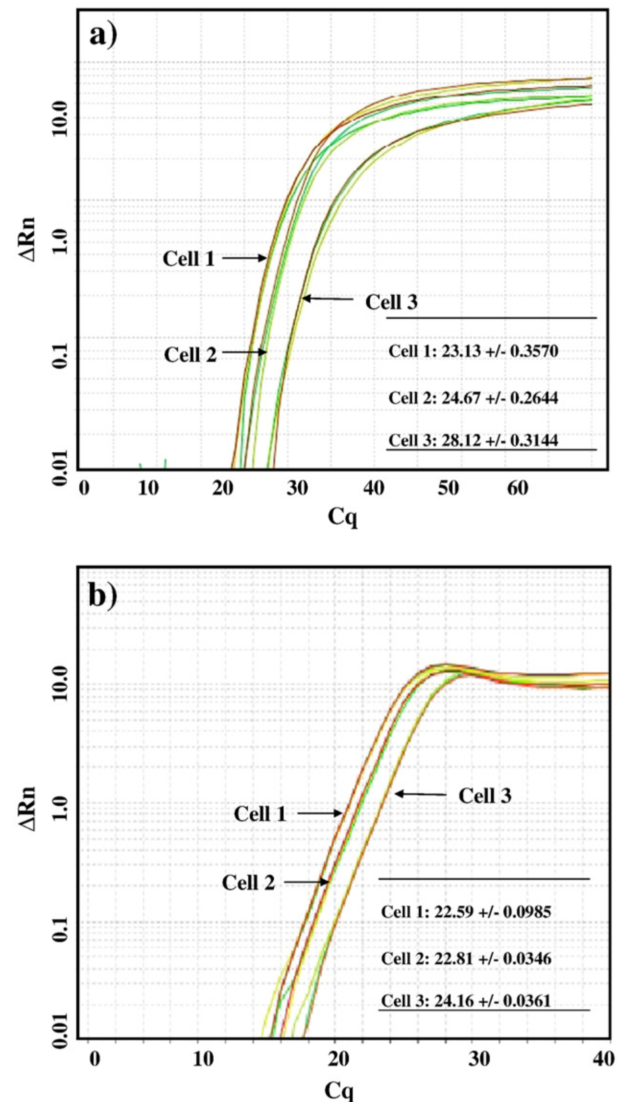

Fig. 2. Single-cell gene expression analysis by the two-step method. Amplification plots for three individual cells from the exponential growing population of *E. coli* (a) and *B. subtilis* (b). 16S rRNA gene is the amplification target. Each reaction used 1/20th of the cDNA, and three technical replicates are run. Average Cq values and standard deviations among three technical replicates are tabled inside the figure.

sample equally into several tubes for RT reaction using SuperScript VILO cDNA Synthesis Kit. The qPCR results showed that Cq value is indistinguishable between cDNA templates generated from different RT reactions, indicating that RT reaction is highly repeatable and variation generated from each RT reaction was minimal in our experiments (Supplementary Table 2).

Using a serial dilution approach, we found that ZR RNA MicroPrep Kit was capable of total RNA isolation/purification from single digit number of bacterial cells. Furthermore, single *E. coli* cells were picked and loaded into Eppendorf tubes containing 100 μl of RNA Lysis Buffer from ZR RNA MicroPrep Kit (Zymo Research, Orange, CA). RNA extraction was carried out using ZR RNA MicroPrep Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. A total of 5 μl RNA was eluted from a column matrix for immediate use in RT reaction using SuperScript VILO cDNA Synthesis Kit (Invitrogen).

We first applied the two-stage protocol to analyze highly expressed 16S rRNA genes from individual *E. coli* cells. The cDNA from each *E. coli* cell was divided into twenty portions and each portion was used in one qPCR analysis. A total of three qPCR analyses were performed for each gene. The results in Fig. 2 demonstrated that 16S rRNA gene expression can be detected with good reproducibility at this sub-single-cell level. To further confirm the qPCR results, we also run agarose gel analysis of the PCR products. Single band was observed for the single-cell qPCR and the qPCR products were further confirmed by sequencing. The standard deviation of qPCR Cq value is less than 0.350 among technical replicates for all cells. In addition, the results also indicated obvious gene-expression heterogeneity for 16S rRNA with standard deviation of qPCR Cq value greater than 2.70 among three cells tested (Fig. 2a). The same protocol was also successfully applied to gram-positive bacterium *B. subtilis* (Fig. 2b). The standard deviation of qPCR Cq value is less than 0.10 among technical replicates for all cells, while cell–cell difference for 16S rRNA expression could be more than 1.5 Cq value among three cells tested.

3.3. Comparative single cell qRT-PCR

To evaluate whether the two-stage method can determine multiple gene expression changes in response to environmental conditions, we then applied it to heat-shock treated *E. coli* cells. Individual *E. coli* cells were picked from the control culture and treated cultures respectively, and each cell was used for analysis of three genes: 16S rRNA, *dnaK* and *groES* genes. In addition, qPCR for each gene was run in triplicates. In this case, we expect the 16S rRNA gene to stay relatively stable after heat-hock treatment, which could serve as an internal control, while *dnaK* and *groES* genes should be up-regulated upon stress (Bardwell and Craig, 1987; Tao et al., 1999). Single-cell gene expression analysis revealed an obvious increase of *dnaK* and *groES* expression levels in heat-shocked cells, in contrast to a minor decrease of 16S rRNA gene expression (Fig. 3). The target genes (*i.e.* 16S rRNA, *dnaK* and *groES* genes) were amplified to detectable levels under both control and treatment conditions. In this case, although negative controls were showing up in several PCR runs, they were well differentiated from the positive samples in terms of Cq number. Standard deviations among technical replicates and among biological replicates (*i.e.* single cells) were summarized in Table 2. The standard deviation among technical replicates is typically 1.0–1.5% of the average Cq value, while standard deviation among biological replicates (cell–cell heterogeneity) is mostly greater than 4.0–7.0% of the average Cq value. The results demonstrated conclusively that the two-step RT-qPCR approach not only allowed reliable measurement of gene expression at the single bacterial cell level, but also provided the resolution needed to distinguish cell

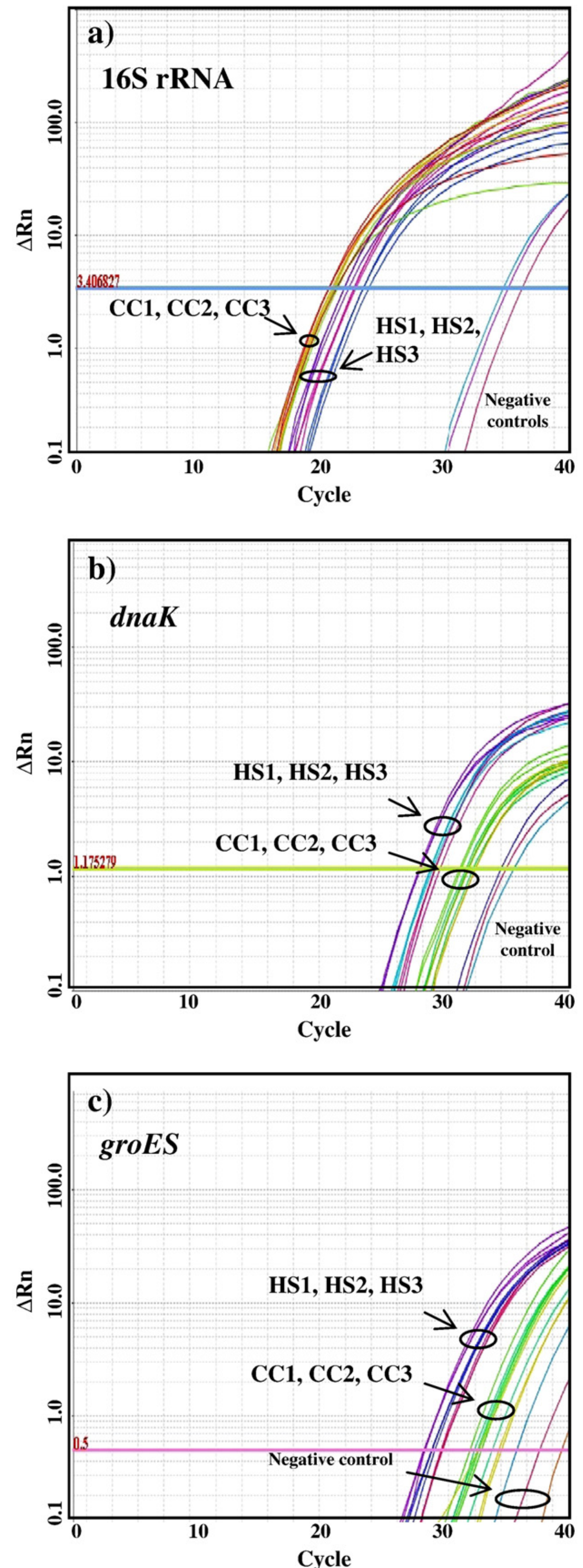

Fig. 3. Single-cell gene expression of *E. coli* for cellular responses to high temperature. Three cells (biological replicates) for each condition (controls *vs.* heat-shock) were individually isolated and each cell was used for analysis of three genes: 16S rRNA, *dnaK* and *groES*. CC: control cell; HS: heat-shock cell. Three technical replicates (same amount of cDNA obtained from the same cell) are used for all qPCR analysis. Each reaction used 1/20th of the cDNA. The standard deviations between biological replicates and among technical replicates are indicated in Table 1. a) Amplification plot for 16S rRNA gene. b) Amplification plot for *dnaK* gene. c) Amplification plot for *groES* gene. Standard deviations among various replicates are listed in Table 1.

Table 2
Average qPCR Cq values and standard deviation among all technical and biological replicates.

	Control CC (Avg Cq ± StDv)		Heat shock HS (Avg Cq ± StDv)	
16S rRNA	Cell no. 1	20.6777 ± 0.3125	Cell no. 1	21.7777 ± 0.1864
	Cell no. 2	20.7948 ± 0.0689	Cell no. 2	23.2901 ± 0.2512
	Cell no. 3	21.0096 ± 0.1281	Cell no. 3	22.4832 ± 0.0818
dnaK	Cell no. 1	30.2822 ± 0.1763	Cell no. 1	28.6768 ± 0.1008
	Cell no. 2	31.7915 ± 0.3143	Cell no. 2	27.7821 ± 0.0468
	Cell no. 3	31.0435 ± 0.3126	Cell no. 3	28.7926 ± 0.2161
groES	Cell no. 1	31.4224 ± 0.4704	Cell no. 1	28.7846 ± 0.1268
	Cell no. 2	32.1555 ± 0.4673	Cell no. 2	28.1949 ± 0.0606
	Cell no. 3	32.5109 ± 0.7372	Cell no. 3	29.5052 ± 0.0537

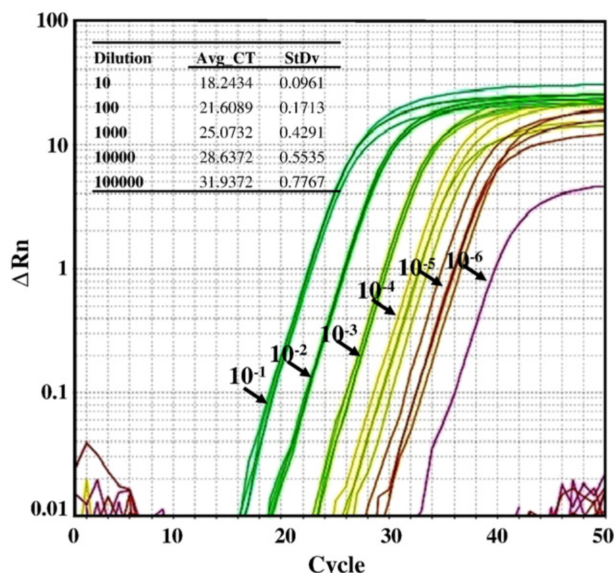

Fig. 4. Gene expression analysis using diluted cDNA from a single bacterial cell. Amplification plots for the 16S rRNA gene are shown. The dilution levels from total cDNA obtained from a single *E. coli* cell are indicated inside the plots. For each dilution level, four technical replicates are used. The standard deviations among technical replicates for various dilution levels are indicated. Average Cq values and standard deviations among four technical replicates are tabulated inside the figure.

responses to environmental factors at the single cell level and gene-expression heterogeneity among single cells.

In some of the experiments, we found that NTC (No Template Control) is also amplified, although typically they are at least 3–10 PCR cycles later than the samples. One possible source of DNA/RNA contamination is the qPCR enzymes and reagents that we purchased directly from the commercial vendors, suggesting in the future the higher QC standard may be needed for commercial enzyme/reagents used for various single-cell based studies.

3.4. How many genes can be analyzed simultaneously from a single bacterial cell?

Our ultimate goal is to establish a method for quantitatively measuring gene expression levels for all the genes in a single bacterial cell. However, the qRT-PCR approach is generally considered a low throughput technique which can analyze only a few dozen genes at the most each time when compared with other gene expression analysis tools, such as the DNA microarray. To address the issues, several attempts have been made to develop chip-based qPCR methods for large-scale gene expression analysis (Stedtfeld et al., 2008). In one recent study, Spurgeon et al. (2008) described a high throughput gene expression platform that allows 2304 simultaneous real time PCR gene expression measurements of 18 different human tissues in a single chip with good reproducibility. The data obtained have excellent concordance with conventional real time PCR and the microfluidic dynamic arrays show better reproducibility than commercial DNA microarrays (Spurgeon et al., 2008). To evaluate whether the two-stage method can be integrated into a similar high throughput platform for whole-genome analysis, we performed experiments to determine whether the method is sensitive enough to amplify low-copy number genes or whether RNA isolated from single bacterial cells is enough for up to several hundred or thousand qRT-PCR reactions (*i.e.* typical microbial genomes contain 2000–7000 genes). To do so, total RNA was isolated from a single *E. coli* cell and was then converted to cDNA as described previously. The cDNA obtained in 10 μl was diluted using ddH_2O up to several thousand folds. One μl of cDNA samples out of 10, 10^2, 10^3, 10^4, 10^5 and 10^6-fold dilutions was used to perform qPCR in a total volume of 10 μl under the identical conditions established above. The 16S rRNA gene was the qPCR target, and each dilution level was run in four replicates. The result in Fig. 4 showed that detectable amplification was achieved even when the cDNA was diluted 10^3 folds with the variation among four technical replicates of the same dilution level still very minimal (mostly with standard deviation less than 1.0%). No amplification was found for negative control. However, when cDNA was diluted by 10^4-fold, the variation among three technical replicates became very significant (greater than 5.0%). Even taking into consideration that expression level of mRNA molecules is much lower than 16S rRNA, the results still suggested that the current two-stage qRT-PCR method can possibly achieve gene expression measurements for multiple highly expressed genes.The method thus has significant advantages over various existing single-cell gene expression technologies (*i.e.* fluorescence microscopy based methods), and can be amenable for whole-genome transcriptomics technology for single bacterial cells in the future.

4. Conclusion

In this work, we developed two RT-qPCR based methods for gene expression at the single bacterial level. Both methods are based on inexpensive SYBR Green chemistry. The first method features a single-tube RT-qPCR with a modified RNA isolation protocol for a single bacterial cell in a low total volume of RT-qPCR reaction (5–10 μl), while the second method features separated RNA isolation/cDNA synthesis and qPCR steps which allow multiple gene expression analyses for single bacterial cells. We tested the methods in single cells isolated from exponential growth and stress-treated *E. coli* populations, and the results demonstrated conclusively that quantitative analysis of gene expression can be achieved in a single bacterial cell with good reproducibility. In addition, significant gene-expression heterogeneity was also observed among the isogenic population. Given the facts that the methods can be easily established in most RT-PCR instrumentation and relatively inexpensive to work, we anticipate the methods should be amenable to many applications involved in gene expression analysis in single bacterial cells. Currently we run the qPCR in a 5–10 μl volume under the standard instrument settings and predict that if further effort is made to combine this qPCR method with microfluidics, it will decrease the templates needed for each qPCR reaction and achieve whole-genome gene expression analysis for single bacterial cells (Kelly and Woolley, 2005). This eventually will enhance our understanding of physiology and biochemistry of microbial cells in many ways.

Supplementary materials related to this article can be found online at doi: 10.1016/j.mimet.2011.03.008.

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