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COMPARISON OF METHODS FOR RELATIVE QUANTIFICATION OF GENE EXPRESSION USING REAL-TIME PCR

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Comparison of methods for relative quantification of gene expression using real-time PCR

Quantitative real-time PCR (qPCR) has become a widely used tool for quantifying gene expression. Several methods for relative quantification have been developed, enabling rapid and reliable detection and quantification of specific nucleic acids. These methods, based on qPCR include: the standard curve method, the efficiency calibrated method and the $2^{-\Delta\Delta Cq}$ method. Here we analyzed if these three methods generate comparable results. To evaluate their performance, we analyzed the expression of the nuclease gene MS53_0284 from *Mycoplasma synoviae* type strain WVU 1853 during *in vitro* infection of CEC-32 cells, using qPCR. As determined, all three methods generated comparable and reliable results when all necessary conditions were fulfiled. Also, the efficiency calibrated and the standard curve methods were more suitable for quantifying small differences in relative gene expression than the $2^{-\Delta\Delta Cq}$ method.

Key words: molecular genetics / genes / gene expression / quantitative real-time PCR / methods

Primerjava metod za relativno kvantifikacijo genskega izražanja s PCR v realnem času

Metoda kvantitativne verižne reakcije s polimerazo v realnem času (qPCR) je postala najpogosteje uporabljen način analize izražanja genov. Razvitih je bilo več metod za relativno kvantifikacijo genske ekspresije, ki omogočajo hitro in zanesljivo detekcijo ter kvantifikacijo specifičnih nukleinskih kislin. Mednje spadajo: metoda z umeritveno krivuljo, metoda z upoštevanjem učinkovitosti pomnoževanja in metoda po enačbi $2^{-\Delta\Delta Cq}$. V tej študiji smo z analizirajem izražanja gena MS53_0284, ki kodira nukleazo pri bakteriji *Mycoplasma synoviae* WVU 1853, po okužbi celic CEC-32 *in vitro* s qPCR preverili primerljivost rezultatov, dobljenih z uporabo omenjenih metod. Pokazali smo, da z upoštevanjem potrebnih pogojev z omenjenimi metodami pridobimo primerljive rezultate ter da sta metoda z umeritveno krivuljo in metoda z upoštevanjem učinkovitosti pomnoževanja primernejši za ugotavljanje majhnih razlik v izražanju genov kot metoda po enačbi $2^{-\Delta\Delta Cq}$.

Ključne besede: molekularna genetika / geni / gensko izražanje / kvantitativni PCR v realnem času / metode

1 INTRODUCTION

Quantitative real-time PCR (qPCR) is an extensively used method for gene expression and quantification analysis in molecular biology. The principle of the method is based on classical PCR, where employment of fluorescent dyes or probes and fluorescent signal measurement enables quantification of starting DNA material in the sample during amplification (Valasek and Repa, 2005). The main advantages of qPCR are its high sensitivity, accuracy and the ability to quantify rare transcripts and small changes in gene expression, producing reliable and rapid quantification results (Pfaffl, 2001; Yuan *et al.*, 2006). Because of the lacking consensus on how to best perform qPCR, MIQE guidelines have been developed to uniform qPCR experiment setup, optimization and data analysis, making the protocols comparable between different research groups and organizations and ensuring the relevance, accuracy, correct interpretation and repeatability of the results (Bustin *et al.*, 2009).

When analyzing gene expression, qPCR data can be subjected to absolute or relative quantification (Livak and Schmittgen, 2001; Pfaffl, 2001; Yuan *et al.*, 2006). Absolute quantification employs internal or external cali-

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bration curves to derive the input template copy number of the standard material to which unknown samples are compared. This type of analysis is suitable when the actual transcript copy number in a given sample needs to be determined. Generally, obtaining, quantifying and validating an independent reliable standard for each gene that is to be analyzed is time consuming and represents the main limitation of this type of quantification (Kuhne and Oschmann, 2002; Pfaffl, 2001; Yuan *et al.*, 2006). Relative quantification, however, enables determination of relative changes in gene expression, irrespective of the internal standard absolute quantity. This approach is useful for most studies that investigate physiological differences in gene expression. Relative quantification is based on normalizing the expression of a target gene to the expression of a stabile reference gene, which serves as an internal standard, and on comparing the expression of these normalized gene expressions in target versus control samples (calibrators). To obtain accurate results with this type of quantification, it is necessary to include one or more stable internal standards (housekeeping genes), that are not expected to change their expressions under selected experimental conditions (Livak and Schmittgen, 2001; Pfaffl, 2001; Pfaffl *et al.*, 2004; Valasek and Repa, 2005; Vandesompele *et al.*, 2002; Yuan *et al.*, 2006).

Several data analysis procedures for relative quantification have been developed. These include: i) the standard curve method, also known as the Roche Applied Science E-method (Soong *et al.*, 2000; Pfaffl, 2001; Pfaffl *et al.*, 2002; Tellman, 2006) or the assumption-free method (Ramakers *et al.*, 2003); ii) the efficiency calibrated method (Pfaffl, 2001; Pfaffl *et al.* 2002); and iii) the $2^{-\Delta\Delta Cq}$ method (Livak, 1997; Livak and Schmittgen, 2001). Data generation in the first two methods is based on determination of qPCR efficiency for the target and reference genes, whereas the latter method is based on the assumption that efficiencies of target and reference genes are the same. Thus, the $2^{-\Delta\Delta Cq}$ method can be used only if the suitability of the designed primers is confirmed by a validation experiment (Livak and Schmittgen, 2001; Pfaffl, 2001; Yuan *et al.*, 2006).

The focus of this paper was to compare the standard curve method, the efficiency calibrated method and the $2^{-\Delta\Delta Cq}$ method, since all three methods are frequently employed in relative gene quantification. We wanted to evaluate if and to what extent, when all necessary conditions are taken into consideration, generated data varies between the three methods and if they are to generate comparable results. To examine that, we analyzed the expression of the nuclease gene MS53_0284 from *Mycoplasma synoviae* type strain WVU 1853 during *in vitro* infection of CEC-32 cells, which was compared to the expression of the same gene in *M. synoviae* WVU 1853 grown in mycoplasma broth medium.

2 MATERIALS AND METHODS

2.1 CHICKEN CELLS AND *MYCOPLASMA SYNOVIAE* WVU 1853 BROTH CULTURE

Transfected chicken embryonic fibroblast cells (CEC-32) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 2% chicken serum (all Sigma-Aldrich, Germany) and 0.1% gentamicin (Krka, Slovenia) and kept in a CO_2 incubator at 37 °C in 5% CO_2 atmosphere. Cells from the 3rd to 6th passage were used in the experiment.

Mycoplasma synoviae type strain WVU 1853 was cultured as described before (Benčina *et al.*, 2001; Dušanić *et al.*, 2009) and the number of colony forming units (CFU) determined by standard procedures (Rodwell and Whitcomb, 1983).

2.2 EXPERIMENTAL DESIGN

CEC-32 cells were grown in 75 cm^2 culture flasks, to reach 5×10^6 cells per flask and 99% cell viability was determined using trypan blue staining (Sigma-Aldrich, Germany). Before the experiment began, supplemented DMEM containing gentamicin was removed. CEC-32 cells were washed with Hank's Balanced Salt Solution (HBSS) and 10 ml of supplemented DMEM without gentamicin was added. *M. synoviae* cells were cultivated until they reached the late log phase of growth. Then, 5 ml of mycoplasma broth medium with approximately 5×10^8 CFU was used to inoculate each flask. The multiplicity of infection (MOI) ranged from 10 to 100 *M. synoviae* WVU 1853 cells per CEC-32 cell. Cells were incubated in a CO_2 incubator at 37 °C in a 5% CO_2 atmosphere and collected 6, 12, 24 and 48 h after infection (samples 1 to 4), using a cell scraper and centrifuged at 500×g for 10 min. To remove mycoplasmas that were not attached to host cells, CEC-32 were washed with 1×PBS (pH 7.5) twice. *M. synoviae* cells grown separately in Frey's medium were diluted in a 1:2 ratio with supplemented DMEM without gentamicin, pelleted (20,000×g for 10 min) at the same incubation time-points and used as a control (the calibrator). Experiments for every incubation time-point were performed in triplicates.

Table 1: *M. synoviae qPCR primers*
Preglednica 1: *Oligonukleotidni začetniki uporabljeni za qPCR pomnoževanje genov pri bakteriji M. synoviae*

Gene	Gene name	Primer sequence	Amplicon size	Accession number[e]
gap[a]	Glyceraldehyde-3-P dehydrogenase	F[c]: 5' AAGTAGTGATACCGTGATT 3' R[d]: 5' TTATGTGGAGCATCTTGA 3'	156 bp	YP_278330.1
rsuA[a]	16S rRNA uridine-516 pseudouridylate synthase family protein	F: 5' AGGAATTCTTCTCGAGGCCAGA 3' R: 5' CCTTCGGTATCGATATCTAGTCTTCCG 3'	177 bp	YP_278540.1
gidB[a]	glucose-inhibited division protein B	F: 5' CCTAACAGGATTTAGTGGCGAGTC 3' R: 5' AGCCAACGCCAGAACCGATATCTA 3'	125 bp	YP_278282.1
MS53_0284[b]	hypothetical protein MS53_0284 (hypotetical nuclease)	F: 5' GCAACAACAGCATCAAGTATTCCAC 3' R: 5' TGGTGAAGCCACTATTGAATCGCT 3'	120 bp	YP_278410.1

[a] Reference gene; [b] Target gene; [c] Forward, [d] Revese; [e] GenBank sequence accession numbers

2.3 RNA ISOLATION AND REVERSE TRANSCRIPTION

In order to analyze gene expression of *M. synoviae* MS53_0284 nuclease gene, total RNA was isolated from samples of collected CEC-32 and attached or intracellular *M. synoviae* cells using PureLink™ RNA Mini Kit (Invitrogen, US) according to the manufacturer's instructions. RNA concentration was measured at 260 nm and purity was assessed using the 260:280 nm ratio with NanoVue (GE Healthcare, UK). RNA samples were treated with DNaseI (EN0523, Fermentas, Canada) to remove contaminating genomic DNA and analyzed on 1.5% agarose gels. Reverse transcription was performed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1632, Fermentas, Canada) with random hexamer

Table 2: *qPCR data for generating standard curves for target (MS53_0284 nuclease) gene and the endogenous reference used for the standard curve method and the efficiency calibrated method and for validation of the $2^{-\Delta\Delta Cq}$ method*
Preglednica 2: *qPCR podatki za pridobitev standardnih krivulj tarčnega gena (MS53_0284) in endogene reference pri metodi z umeritveno krivuljo in metodi z upoštevanjem učinkovitosti pomnoževanja ter podatki za validacijo metode po enačbi $2^{-\Delta\Delta Cq}$*

Dilution of cDNA template[a]	cDNA copy number[b]	Log copy number	Cq (Target)[c]	Cq (Endogenous reference)[d]	ΔCq ($Cq_{Target} - Cq_{End.\ ref.}$)[e]
0	10000	4.0	18.13	20.37	−2.19 ± 0.04
0	10000	4.0	18.19	20.32	
1:2	5000	3.7	19.05	20.89	−1.68 ± 0.16
1:2	5000	3.7	19.37	20.89	
1:10	1000	3.0	21.65	23.29	−1.56 ± 0.13
1:10	1000	3.0	21.89	23.36	
1:50	200	2.3	24.24	26.03	−1.76 ± 0.05
1:50	200	2.3	24.33	26.07	
1:100	100	2.0	25.19	27.07	−1.84 ± 0.13
1:100	100	2.0	25.41	27.22	
1:500	20	1.3	27.41	29.38	−1.79 ± 0.12
1:500	20	1.3	27.57	29.19	
NTC			No Cq	No Cq	
NTC			No Cq	No Cq	
		k	−0.2855	−0.2897	
		n	9.1973	9.8167	

[a] Starting concentration of cDNA template was 5 ng/µl (0); [b] Relative cDNA copy number; [c] Nuclease gene MS53_0284; [d] Endogenous reference Cq values were calculated by geometric averaging of gap, rsuA and gidB reference gene Cq values. Cq data for individual reference genes are not shown; [e] The data were generated by subtracting geometric averages of Cq values (± S.D.)

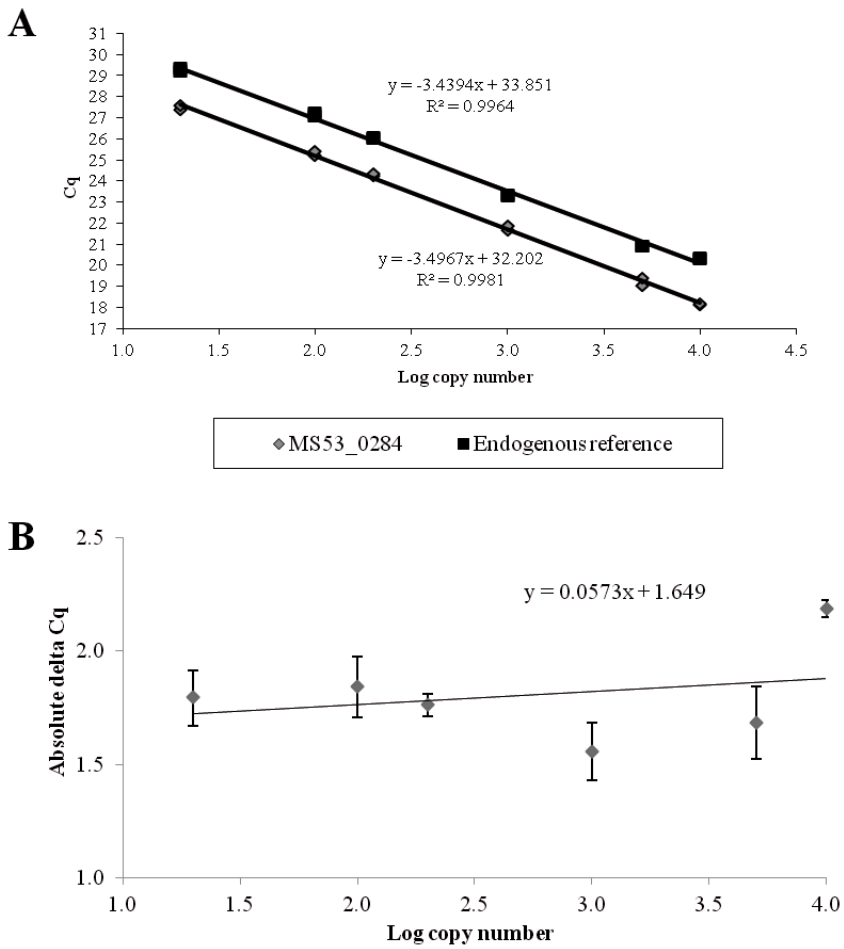

Figure 1: A) *Standard curves for qPCR efficiency calculation of target MS53_0284 nuclease gene and endogenous reference used for standard curve method and the efficiency calibrated method data generation. B) Validation curve of the* $2^{-\Delta\Delta Cq}$ *method.*
Slika 1: *A) Standardni krivulji za izračun učinkovitosti qPCR pomnoževanja tarčnega gena MS53_0284, ki kodira nukleazo in endogene reference pri metodi z umeritveno krivuljo in metodi z upoštevanjem učinkovitosti pomnoževanja. B) Validacijska krivulja za metodo po enačbi* $2^{-\Delta\Delta Cq}$.

primers, according to manufacturer's instructions. The average amount of RNA used in reverse transcription reaction was 100 ng and its purity was above 2.1 (260:280 nm ratio). cDNA was stored at −20 °C until qPCR assays were performed.

2.4 PRIMERS AND QUANTITATIVE REAL-TIME PCR

Real-time PCR experiment was performed according to the MIQE guidelines (Bustin *et al.*, 2009). Specific *M. synoviae* primers were designed using IDT PrimerQuest℠ Software and synthesized by IDT (Integrated DNA Technologies, Belgium). Specifity of the designed primers and amplicons was confirmed by BLAST analysis. *M. synoviae* primer specifications are listed in Table 1. For relative quantification of gene expression, target gene quantification cycle (Cq) values were compared to Cq geometric means of the reference genes (gap, rsuA and gidB), representing the endogenous reference which was used for normalization of qPCR data.

SYBR Green I chemistry, containing a ROX passive reference dye was used for all qPCR assays which were performed in Mx3000P strip tubes and optical caps (401428 and 401425, Agilent Technologies, UK) on Mx3000P QPCR System (Stratagene, US) in a 20 µl reaction mixture volumes. Each reaction contained 10 µl of FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Germany), 250 nM of each primer, PCR grade water and 2 µl of cDNA template (diluted in a 1:2 or 1:10 ratio). All samples were assayed in duplicates and a no template control was included with each run.

PCR cycling program for MS53_0284, gidB and

Table 3: *Calculation of MS53_0284 nuclease gene fold change with the standard curve method*
Preglednica 3: *Izračun podatkov o spremembi izražanja gena MS53_0284 pri metodi z umeritveno krivuljo*

Sample	Dilution [a]	Cq	Rel. copy number [b]	Log [c]	Inhibition control [d]	Log sample copy number	Copy number	Corrected [e]	Average copy number	Normalization to end. reference	Fold change	S.E.
Target												
Sample 1	1:2	20.60	5000	3.7	−3.3406	3.32	2074.92	2074.92	2039.49	0.90	1.16	0.02
Sample 1	1:2	20.78	5000	3.7		3.27	1843.40	1843.40				
Sample 1	1:10	22.86	1000	3.0		2.67	469.76	2348.82				
Sample 1	1:10	23.19	1000	3.0		2.58	378.16	1890.82				
Control	1:2	20.97	5000	3.7	−3.1332	3.21	1626.99	1626.99	1575.81	0.77	1.00	0.04
Control	1:2	21.35	5000	3.7		3.10	1267.40	1267.40				
Control	1:10	23.27	1000	3.0		2.55	358.79	1793.97				
Control	1:10	23.43	1000	3.0		2.51	322.98	1614.89				
End. ref.												
Sample 1	1:2	22.26	5000	3.7	−3.3196	3.37	2327.36	2327.36	2274.89	/	/	/
Sample 1	1:2	22.43	5000	3.7		3.32	2076.99	2076.99				
Sample 1	1:10	24.54	1000	3.0		2.71	511.12	2555.60				
Sample 1	1:10	24.80	1000	3.0		2.63	427.92	2139.61				
Control	1:2	22.55	5000	3.7	−3.2107	3.28	1926.63	1926.63	2040.08	/	/	/
Control	1:2	22.55	5000	3.7		3.28	1917.08	1917.08				
Control	1:10	24.67	1000	3.0		2.67	468.42	2342.10				
Control	1:10	24.92	1000	3.0		2.60	394.91	1974.54				

[a] Dilution of cDNA template; [b] Relative copy number was designated with regard to the template dilution in Table 2; [c] Log of relative copy number; [d] Amplification efficiency of an individual sample that was appropriate when between −3.1 and −3.6 (90% < Esample < 120%).; [e] Corrected sample copy number with regard to sample dilution

rsuA genes consisted of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 58 °C. PCR program for gap gene consisted of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. An additional step was used (95 °C for 15 s, 58 °C or 55 °C for 30 s and 95 °C for 15 s) for dissociation curve analysis. Data were analyzed by MxPro-Mx3000P software (Stratagene, US), with automatically set baseline and manually adjusted fluorescence threshold used for determination of Cq values. Statistical comparison between samples was performed using unpaired Student's *t*-test. A p value of < 0.05 was considered statistically significant.

2.5 GENERATION OF STANDARD CURVES

To determine qPCR amplification efficiency (E) and the dynamic range of the standard curve method and the efficiency calibrated method, relative standard curves were generated. Dilution series of the reference material representing the cDNA mixture of all analyzed samples was performed. Serial dilutions were expressed in relative units corresponding to the logarithmic values of the number of copies of the analyzed genes (Table 2). Standard curves were generated by plotting Cq values against the logarithmic values of the estimated cDNA relative copy numbers in the reference sample (Livak, 1997; Pfaffl *et al.*, 2001; Ramakers *et al.*, 2003; Tellmann, 2006) (Fig. 1A).

To determine the suitability of the $2^{-\Delta\Delta Cq}$ method, a validation curve was generated by plotting geometric averages of Cq values against the logarithmic values of the estimated cDNA relative copy numbers in the reference sample (Livak, 1997) (Fig. 1B).

2.6 DATA ANALYSIS BY THE STANDARD CURVE METHOD

We calculated the gene expression fold change data using the standard curve method as described below. Logarithmic input amounts of target gene and endogenous reference relative copy numbers for each individual sample were calculated from parameters k and n of standard curves (Table 2) by using the equation ($Cq \times k_{(Target\ or\ End.\ ref.)}$) + $n_{(Target\ or\ End.\ ref.)}$) or by using the equations of the standard curves (Fig. 1 A). Input amounts (relative copy numbers) of the target and reference genes were then calculated as $10^{(log\ input\ amount\ of\ analyzed\ gene)}$ and corrected according to the starting dilution of the cDNA template. To normalize target gene expression to the endogenous reference, average values of target gene input amounts were divided with average values of reference gene input amounts for every sample separately. Normalized sample amounts were then divided by normalized amounts of the calibrator, representing the relative fold change ratio of the target gene in the sample, compared to the expression in the calibrator sample (Livak, 1997; Ramakers *et al.*, 2003; Tellman, 2006) (Table 3). Standard error (S.E.) was calculated to illustrate deviations from the mean from the target gene/endogenous reference ratio.

Table 4: *Calculation of MS53_0284 nuclease gene fold change with the efficiency calibrated and the $2^{-\Delta\Delta Cq}$ method*
Preglednica 4: *Izračun podatkov o spremembi izražanja gena pri metodi z upoštevanjem učinkovitosti pomnoževanja in metodi po enačbi $2^{-\Delta\Delta Cq}$*

				The efficiency calibrated method				The $2^{-\Delta\Delta Cq}$ method		
Sample	Dilution	Cq	Average Cq	ΔCq (target)	ΔCq (reference)	Fold change	S.E.	ΔΔCq	Fold change	S.E.
Target gene										
Sample 1	1:2	20.60	21.32	0.47	/	1.19	0.005	0.27	1.20	+0.11; −010
Sample 1	1:2	20.78								
Control	1:2	20.97	21.16	0.00	/	1.00	0.19	0.00	1.00	+0.14; −0.12
Control	1:2	21.35								
Endogenous reference										
Sample 1	1:2	22.26	22.89	/	0.20	/	/	/	/	/
Sample 1	1:2	22.43								
Control	1:2	22.55	22.55	/	0.00	/	/	/	/	/
Control	1:2	22.55								

2.7 DATA ANALYSIS BY THE EFFICIENCY CALIBRATED METHOD

Analysis of the data was performed as follows. The relative expression ratio was calculated based on the E of target gene and geometric average of all endogenous references. These were calculated from the slopes of the standard curves shown in Fig. 1 by using the equation $E = 10^{(-1/\text{slope of the standard curve})}$ (Rasmussen, 2001). Deviations of Cq values (ΔCq) for the target gene and endogenous reference were obtained by subtracting averaged Cq values of the sample from averaged Cq values of the calibrator: $(Cq_{Calibrator}) - (Cq_{Sample})$. Relative fold change ratio was then calculated by using the following formula: $((E_{Target})^{\Delta Cq\ (Target)}) / ((E_{End.\ ref.})^{\Delta Cq\ (End.\ ref.)})$. S.E. was calculated from subtracted target-reference Cq values (Table 4).

2.8 DATA ANALYSIS BY THE $2^{-\Delta\Delta CQ}$ METHOD

In order to analyze the gene expression by using the $2^{-\Delta\Delta Cq}$ method, a validation curve was generated as described above (Fig. 1B), and the slope was used to determine suitability of the designed primers for the analysis. Then, differences between Cq values (ΔΔCq) were calculated from the average Cqs from all experimental repetitions of target and reference genes in the calibrator and treated samples as $((Cq_{Target} - Cq_{End.\ ref.})_{Sample} - (Cq_{Target} - Cq_{End.\ ref})_{Calibrator})$. Fold change calculation was then performed by $2^{(-\Delta\Delta Cq)}$. S.E. of ΔΔCq was calculated as $SQRT((S.E._{Target})^2 + (S.E._{End.\ ref.})^2)$ and positive/negative values of S.E. determined by $2^{(\text{Absolute } \Delta\Delta Cq \pm S.E.\ (\Delta\Delta Cq))}$. The range of absolute fold change values was then calculated as (Fold change $-S.E._{positive\ value}$) and (Fold change $-S.E._{negative\ value}$) (Table 4).

3 RESULTS

3.1 STANDARD CURVES

The slopes of the standard curves for the standard curve and efficiency calibrated methods were used to determine primer E and linear dynamic range, which was between 20 and 10000 relative copies of the reference cDNA material. The limit of detection (LOD) was determined at 10 and the limit of quantification (LOQ) at 20 relative cDNA copies. The coefficient of determination (R^2) was higher than 0.98 and Pearson correlation coefficient (r^2) higher than 0.99. Standard curves are shown in Fig. 1A and their specifications listed in Table 5.

The slope of the curve for the $2^{-\Delta\Delta Cq}$ method validation was < 0.1, indicating that the assumption of equal target gene and endogenous reference E was correct. Hence, the designed primers were suitable for relative quantification of gene expression using the $2^{-\Delta\Delta Cq}$ method (Fig. 1B).

3.2 COMPARISON OF FOLD CHANGES OBTAINED BY DIFFERENT METHODS

To compare and evaluate the standard curve method, the efficiency calibrated method and the $2^{-\Delta\Delta Cq}$ method, we analyzed the expression of the *M. synoviae* WVU 1853 nuclease gene MS53_0284 during *in vitro* infection of CEC-32 cells. Gene expression was analyzed in four samples, representing four incubation time-points after *M. synoviae* inoculation, which were compared to the control (*M. synoviae* broth culture that was used for the *in vitro* infection, diluted in a 1:2 ratio with supplemented DMEM without gentamicin) as described in Materials and methods.

Table 5: *Characteristics of target and reference standard curves used for relative quantification of gene expression by the standard curve method and the efficiency calibrated method*
Preglednica 5: *Značilnosti tarčne standardne krivulje in referenčnih standardnih krivulj, uporabljenih za relativno kvantifikacijo genske ekspresije pri metodi z umeritveno krivuljo in metodi z upoštevanjem učinkovitosti pomnoževanja*

Target/reference gene	Slope of the standard curve[b]	E[c]	E [%][d]	(R^2)[e]	(r^2)[f]
MS53_0284	−3.4967	1.93	93	0.9981	−0.9991
gap	−3.2544	2.03	103	0.9889	−0.9944
rsuA	−3.5269	1.92	92	0.9959	−0.9980
gidB	−3.5411	1.92	92	0.9979	−0.9989
Endogenous reference [a]	−3.4394	1.95	95	0.9964	−0.9982

[a] Endogenous reference was calculated by geometric averaging of gap, rsuA and gidB reference genes and used for normalization of qPCR data; [b] Standard curves were generated by plotting quantification cycle values (Cq) against the logaritmic values of the relative cDNA copy numbers in the reference sample (cDNA mixture of all samples analyzed); [c] PCR amplification efficiency calculated from the slope of the standard curve by using $10^{(-1/\text{slope of the standard curve})}$; [d] PCR amplification efficiency expressed in%; [e] Coefficient of determination ($R^2 > 0.98$); [f] Pearson correlation coefficient ($r^2 > 0.99$). Negative value represents the orientation of the standard curve.

The results of comparing relative fold change data, generated by the three methods, are shown in Fig. 2 in a $\log_2$ scale. The results were highly similar between the evaluated methods with minor deviations. Although only calculation steps for sample 1 are shown, it is clear that all three methods generated comparable relative fold change values, with no regard to the template dilution in the dynamic range (data not shown). They were between 1.16 and 1.20 for sample 1, 2.33 and 2.52 for sample 2, 0.92 and 0.96 for sample 3 and 0.99 and 1.12 for sample 4. The expression of gene MS53_0284 was significantly upregulated in samples 2 ($P < 0.001$) and 3 ($P < 0.01$), as determined by the standard curve method, whereas the efficiency calibrated and $2^{-\Delta\Delta Cq}$ method showed significant upregulation of MS53_0284 gene expression only in sample 2 ($P < 0.05$), when compared to the control.

4 DISCUSSION

Analysis of gene expression by qPCR has become one of the most common and useful modes for investigation of physiological processes in various biological systems (Yuan *et al.*, 2006), where many methods for relative quantification have been developed and improved during the last decade (Livak, 1997; Livak and Schmittgen, 2001; Pfaffl, 2001; Pfaffl *et al.*, 2002; Pfaffl *et al.*, 2004; Ramakers *et al.*, 2003; Soong *et al.*, 2000; Tellman, 2006; Vandesompele *et al.*, 2002; Yuan *et al.*, 2006). Because many research groups use different methods for gene quantification (Bustin *et al.*, 2009), we wanted to examine if three most commonly used methods for relative gene quantification generate comparable results. We performed a relatively simple qPCR experiment, obtained the Cq values for the target and reference genes and compared and evaluated the results generated by interpreting the Cq values using the three methods: the standard curve method, the efficiency calibrated method and the $2^{-\Delta\Delta Cq}$ method.

As shown in Fig. 2, mean values of generated data were comparable, whereas, S.E. values differed between the methods. The lowest S.E. were present with the standard curve method, meaning the average values were closest to the actual mean of the generated data. Standard errors in the other two methods were higher, mostly due to the manner data for fold change calculation was derived. Fold change calculations using the standard curve method were performed from relative target gene copy numbers in test samples, gained from raw Cq values, k and n from the standard curves, which were then normalized to the endogenous reference and the control sample. By doing this, normal data distribution was generated. Similar normalization of the data was performed in the efficiency

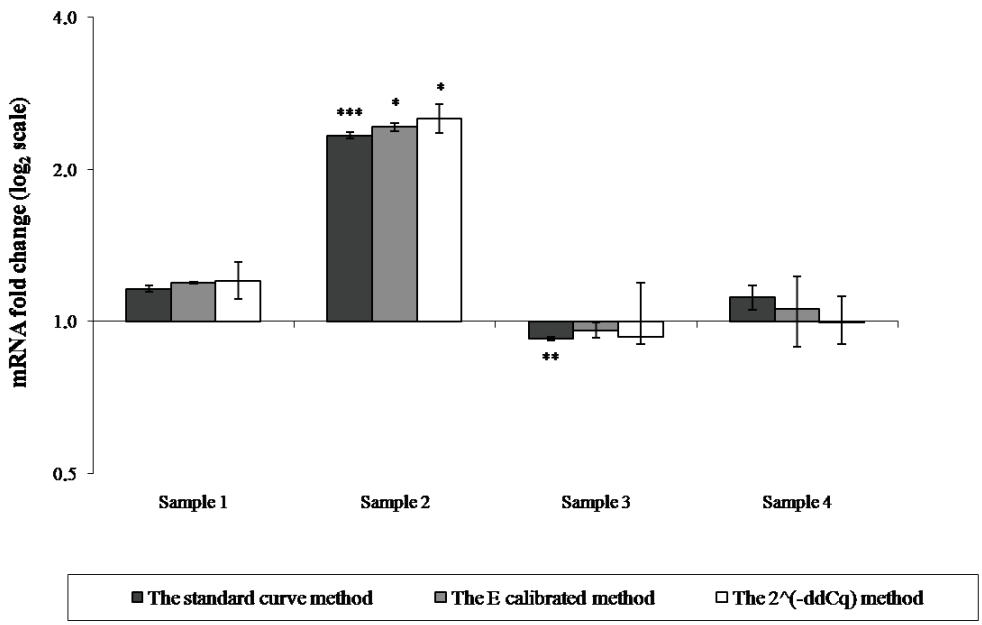

Figure 2: *M. synoviae MS53_0284 nuclease gene mRNA fold change, generated by the standard curve method, the efficiency calibrated method and $2^{-\Delta\Delta Cq}$ method (2^(−ddCq)) from four samples and compared to the control sample (fold change 1). Bars represent the means ± S.E. of the mean, n = 2–4. *P < 0.05, **P < 0.01, ***P < 0.001.*

Slika 2: *A) Standardni krivulji za izračun učinkovitosti qPCR pomnoževanja tarčnega gena MS53_0284, ki kodira nukleazo in endogene reference pri metodi z umeritveno krivuljo in metodi z upoštevanjem učinkovitosti pomnoževanja. B) Validacijska krivulja za metodo po enačbi $2^{-\Delta\Delta Cq}$.*

calibrated method, where logarithmic transformations of relative gene expression were used for data generation. Thus, the standard curve and the efficiency calibrated method generate equal S.E. for positive and negative deviations. This is an advantage, because S.E. calculation is relatively easy, once the data is normally distributed (Ramakers *et al.*, 2003; Yuan *et al.*, 2006). In the $2^{-\Delta\Delta Cq}$ method, however, the data distribution was not normal, so positive and negative S.E. values differ and need to be calculated separately for positive and negative deviations from the mean with some additional calculation steps (Livak, 1997; Pfaffl, 2001; Pfaffl *et al.*, 2002; Yuan *et al.*, 2006). Therefore, if we take S.E. as a criterion, the standard curve method generated most accurate results with greatest precision, which was confirmed by the statistical significance of gene expression fold change in sample 3, excluded by the other two methods.

To gain the desired accuracy and precision, inhibition controls should be taken in consideration in the standard curve method. Two dilutions of cDNA were used to generate the range of slope for every analyzed sample which was appropriate when between −3.1 and −3.6 (90% < E < 110%). If the range of the slope deviated from these values, causing intensification of the error and gaining of erroneous results, analysis of the sample was repeated (Buh Gašparič *et al.*, 2008). The use of inhibition controls is, generally, purely informative and has no direct role in fold change calculations. With it, we gain information about E, possible presence of inhibitors and primer specificity for every sample analyzed. Thus, the weakness of the efficiency calibrated method is that fold change calculations are performed only from the slopes of standard curves, generated from a mixture of cDNA from all analyzed samples, whereas amplification efficiency of an individual sample is neglected.

In contrast to the standard curve method, the $2^{-\Delta\Delta Cq}$ method uses arithmetic formulas to achieve results for relative quantification. For this method to be valid, efficiencies of target and reference gene amplifications must be approximately equal. To test this, validation experiments by generating validation curves need to be performed, as described in Materials and methods. If the slope of the validation curve is < 0.1, the assumption that target and reference efficiencies are approximately equal is correct, therefore, relative quantification using the $2^{-\Delta\Delta Cq}$ method can be performed. If the slope is > 0.1, new primers for target and reference genes should be designed or other methods for relative quantification used. Generally, this method is relatively robust and suitable for determinating larger differences in gene expression (Livak, 1997; Livak and Schmittgen, 2001). On the other hand, the efficiency calibrated method is the improved version of the $2^{-\Delta\Delta Cq}$ method, where target and reference primer efficiencies are not assumed to be approximately equal, but are determined by the use of standard curves (Pfaffl, 2001). With precise primer efficiencies taken in consideration, data generation with this method is more accurate and suitable for determinating smaller differences in gene expression.

5 CONCLUSIONS

Considering the results gained in this study, we can conclude that the standard curve method, the efficiency calibrated method and the $2^{-\Delta\Delta Cq}$ method generate comparable and reliable results when all necessary conditions for each method are fulfilled. However, when quantifying small differences in gene expression, $2^{-\Delta\Delta Cq}$ method should be avoided. The efficiency calibrated and the standard curve method are both suitable for determinating very small differences in relative gene quantification, where the latter was shown to be the most accurate method.

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