Evaluation and validation of the suitable control genes for quantitative PCR studies in plasma DNA for non-invasive prenatal diagnosis

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Received March 27, 2014; Accepted September 9, 2014

DOI: 10.3892/ijmm.2014.1944

Abstract. Quantitative polymerase chain reaction (qPCR) is widely used in quantitation of plasma DNA for non-invasive prenatal diagnosis (NIPD). Control genes are indispensable as standard normalizers in qPCR analysis, and there is increasing evidence indicating that the content levels of commonly used control genes vary significantly in different independent experiments. The commonly used control genes for DNA quantitation using qPCR in plasma DNA analysis are frequently chosen without any preliminary evaluation of their suitability. The present study aimed to examine a panel of six common control genes (*HBB*, *TERT*, *GAPDH*, *ALB*, *ACTB* and *TRG*) in order to evaluate and validate the most reliable control genes for qPCR studies in the quantitation of plasma DNA from pregnant and non-pregnant females for NIPD. Plasma DNA was extracted from the peripheral blood of 18 pregnant females and 18 non-pregnant females by the QIAamp DNA mini kit. qPCR followed by geNorm, NormFinder and BestKeeper based analysis was conducted to evaluate the DNA content stabilities of the six candidate control genes. *DSCR3* was used to validate the result. The study recommended *TERT* and the combination of *ACTB* and *TERT* as the optimal control genes for qPCR studies on pregnant/non-pregnant plasma DNA quantitation. Thus, the study reveals that the DNA content stability of widely used control genes varies significantly in pregnant and non-pregnant plasma DNA.

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Key words: quantitative polymerase chain reaction, control gene, pregnant females, plasma DNA, normalization

Introduction

Quantitative polymerase chain reaction (qPCR) is the most fundamental, sensitive and common method used for quantitative analysis of DNA. However, its accuracy is influenced by a number of external and internal factors, including the amount of starting samples, the quality of templates and PCR efficiency (1). At present, using control genes as a standard normalizer is the most common method to minimize the effects (2). Control genes are commonly defined as genes that ubiquitously exist at stable levels in various biological contexts and are used to confirm the presence and content of DNA, as well as quantitatively measure the total DNA in each sample (3,4). However, accumulating evidence indicates that content levels of widely used control genes vary significantly in different independent studies (5,6).

Since the presence of cell-free fetal DNA in maternal plasma and serum was confirmed by the Lo *et al* (7) study in 1997, there are increasing studies focusing on the utilization of plasma DNA for non-invasive prenatal diagnosis (NIPD). Thus far, plasma DNA analysis is widely studied in numerous NIPD, including fetal gender detection, Rhesus blood group, D antigen (RhD) status determination, monogenic diseases and chromosomal aneuploidies prenatal diagnosis. To the best of our knowledge, the commonly used control genes for plasma DNA analysis are frequently chosen empirically and without any preliminary evaluation of their suitability. Thus, it is essential to compare and evaluate the content stability of each control gene prior to use for normalization in quantitative analysis of plasma DNA.

The focus of the present study is on the content stability of six commonly used control genes (*HBB*, *TERT*, *GAPDH*, *ALB*, *ACTB* and *TRG*) in pregnant and non-pregnant plasma DNA using qPCR. The candidate control genes were selected from previous studies on pregnant plasma DNA (8-11). Three common programs, geNorm (12), NormFinder (13) and BestKeeper (14), were used for data analysis. In order to confirm the analysis results, each of the candidate control genes was used as a normalizer to quantitatively measure the *DSCR3* gene. The *DSCR3* region only exists in chromosome 21 and it is supposed to have the same relative quantity in pregnant

Table I. Primer sequences, product sizes and PCR efficiency.

Symbol	Gene name	Primers sequences (5'→3')	Amplicon size (bp)	PCR efficiency
HBB	β-globin	F-GTGCACCTGACTCCTGAGGAGA R-CCTTGATACCAACCTGCCCAG	101	2.58
TERT	Telomerase	F-GGTGAACCTCGTAAGTTTATGCAA R-GGCACACGTGGCTTTTCG	97	2.00
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F-GGACTGAGGCTCCCACCTTT R-GCATGGACTGTGGTCTGCAA	157	1.72
ALB	Albumin	F-TGAAACATACGTTCCCAAAGAGTTT R-CTCTCCTTCTCAGAAAGTGTGCATAT	80	1.79
ACTB	β-actin	F-CCTGTACGCCAACACAGTGC R-ATACTCCTGCTTGCTGATCC	211	2.08
TRG	T cell receptor γ	F-AGGGTTGTGTTGGAATCAGG R-CGTCGACAACAAGTGTTGTTCCAC	160	1.82
DSCR3	Down syndrome critical region-3	F-CAGTGCAATGACAGCAGTAT R-TGGGATCACATCAAGCTAA	141	2.11
SRY	Gender-determining region Y	F-AAAGGCAACGTCCAGGATAGAG R-CCACTGGTATCCCAGCTGCT	137	2.19

bp, basepair; PCR, polymerase chain reaction.

and non-pregnant groups of normal females (15,16). The result may reveal the optimal control gene selections for further quantitative studies on plasma DNA from pregnant females.

Materials and methods

Plasma sample collection and DNA extraction. A total of 2 ml peripheral blood donated from 18 pregnant females (gestational age, 12.87±1.25 weeks) and 18 non-pregnant volunteers was collected. A form of consent was obtained from each volunteer and the experiment was approved by the Ethical Committee of Second Hospital, Jilin University (Jilin, China). The blood samples were anti-coagulated by EDTA. The plasma supernatant was separated from the entire blood by centrifugation at 2,000 x g for 20 min at room temperature, followed by further centrifugation at 12,000 x g for 5 min to remove the residual intact cells. The supernatant was collected carefully. DNA was extracted from 350 μl plasma from each sample by the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The whole process was performed within 4 h of the withdrawal time.

qPCR analysis. qPCR was carried out using Roche LightCycler 480 [Roche Diagnostics (Schweiz) AG, Risch, Switzerland]. The primers of the control (*HBB*, *TERT*, *GAPDH*, *ALB*, *ACTB* and *TRG*) and two target genes (*DSCR3* and *SRY*) were synthesized by Sangon Biotech Shanghai Co., Ltd., (Shanghai, China) (Table I).

The reactions were performed in a 20 μl volume containing 8 ng DNA using the All-in-One qPCR Mix kit (GeneCopodia, Inc., Rockville, MD, USA) following the protocol. The amplification was as follows: An initial step of 95˚C for 10 min, 50 cycles of 95˚C for 15 sec, 58˚C for 15 sec and 72˚C for 30 sec. Each assay was performed in triplicate. qPCR results were subjected to 1% agarose gel electrophoresis. To estimate the efficiencies of amplification, a standard curve was generated for each primer pair based on four points of serial 2-fold DNA dilution. The efficiencies were calculated using the slope of the calibration curve following the equation: $E=2^{-1/slope}$.

Data analysis. Microsoft Excel was used to calculate the mean and standard deviation (SD) values. The content stabilities of the six candidate-control genes were assessed by three commonly used programs: geNorm, NormFinder and BestKeeper, as described in their manuals. geNorm calculates a gene content stability measure (M) and pairwise variation (V) parameter. Lower M values represent higher content stability. V is calculated to determine the minimal number of control genes required. When V<0.15, the number of control genes is enough for valid normalization. NormFinder computes inter- and intra-group content stability values by an analysis of variance-based model. Lower value indicates higher content stabilities. BestKeeper analyses content stability based on SD and coefficient of correlation (r) of all the candidate control genes. The genes with SD >1.00 are suggested to be considered unreliable as a stable control gene and the remaining genes are ranked according to their r values, with a higher r value indicating higher stability. All the analyses were performed separately for the following three groups: Pregnant, non-pregnant and total sample (pregnant and non-pregnant) groups.

Control gene validation. *DSCR3* was used as the target gene in order to validate the control genes for normalization of relative quantity in the pregnant and non-pregnant groups (17). The

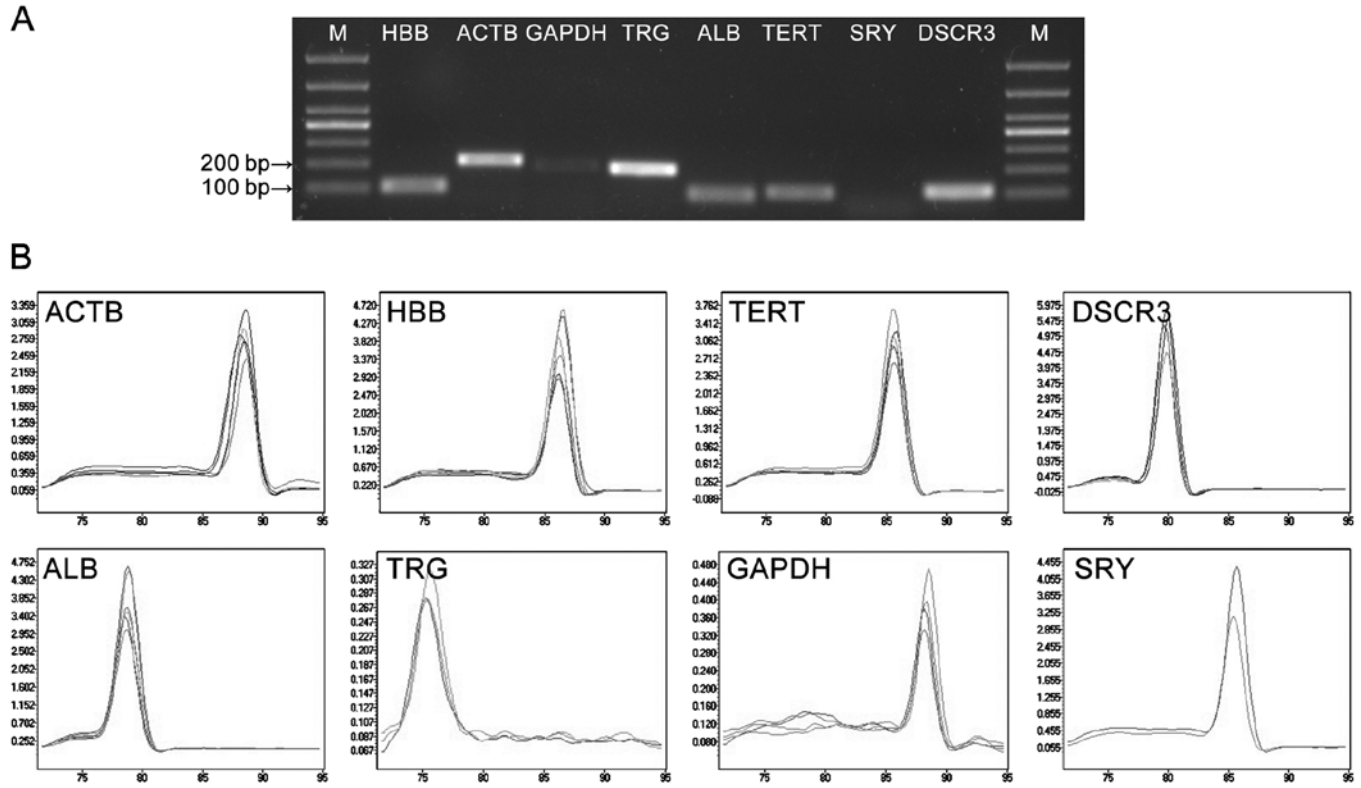

Figure 1. Specificity of primers and amplicon length. (A) 1% agarose gel electrophoresis of amplified fragments. M, DNA marker DL 1000. (B) Melting curve of six control genes and two target genes.

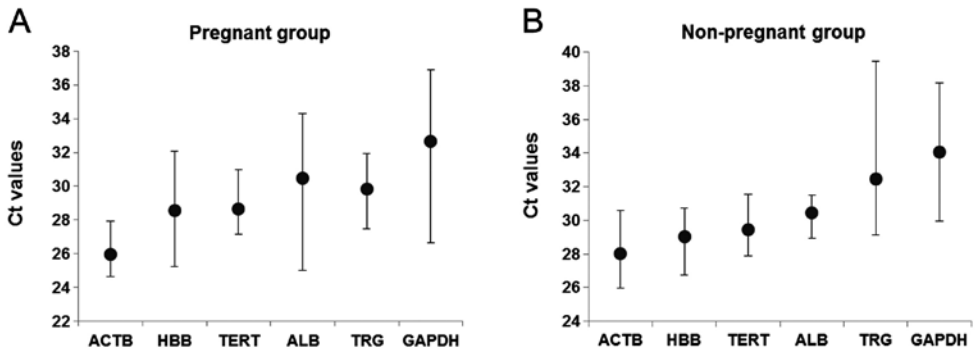

Figure 2. Mean Ct values of the candidate control genes in (A) pregnant (n=18) and (B) non-pregnant (n=18) group samples. The circle represents the arithmetic mean; the bar indicates the minimal to maximal Ct value.

relative quantity in each sample was normalized by each of the six control genes and the most stable combination recommended by geNorm and NormFinder independently, using the $2^{-\Delta\Delta Ct}$ method (18). *SRY* is only presented in pregnant females carrying male fetuses (19), and was used to detect whether or not the extracted DNA was contaminated with exogenous DNA.

Results

Amplification performance of primers. The qPCR amplification product was detected in 1% agarose gel electrophoresis. The results showed clear bands with expected size and no primer dimers (Fig. 1A). One single peak was obtained in each amplification reaction during the analysis of the melting curves; this confirmed the specific amplification of primers (Fig. 1B). The efficiencies of the primers are listed in Table I. *SRY* was only amplified in the pregnant group, indicating that there was no exogenous DNA contamination.

Amplification profile of candidate control genes. The amplification profile of the candidate control genes was estimated as Ct values. Fig. 2 shows the mean Ct values of each gene in the pregnant and non-pregnant groups. The Ct values of the groups varied between 25.99 to 32.66 for the pregnant group (Fig. 2A) and 28.02 to 34.09 for the non-pregnant group (Fig. 2B). *ACTB* exhibited the lowest Ct value (mean ± SD is 25.99±0.99 and 28.02±1.86) and *GAPDH* exhibited the highest Ct value (mean ± SD is 32.66±3.21 and 34.09±2.92) in the two groups respectively. In the pregnant group, *GAPDH* is the most variable with a high SD value (3.21), whereas *ACTB* had the lowest

Table II. Rank of six candidate control genes in order of their quantity stability calculated by NormFinder.

	Total sample		Pregnant group		Non-pregnant group	
Rank	Gene	Stability value	Gene	Stability value	Gene	Stability value
1	*TERT*	0.340	*ACTB*	0.115	*HBB*	0.318
2	*ACTB*	0.418	*TERT*	0.299	*ALB*	0.419
3	*HBB*	0.462	*TRG*	0.439	*TERT*	0.782
4	*GAPDH*	0.552	*ALB*	0.928	*ACTB*	0.820
5	*ALB*	0.577	*HBB*	1.218	*GAPDH*	0.930
6	*TRG*	0.771	*GAPDH*	2.042	*TRG*	2.360

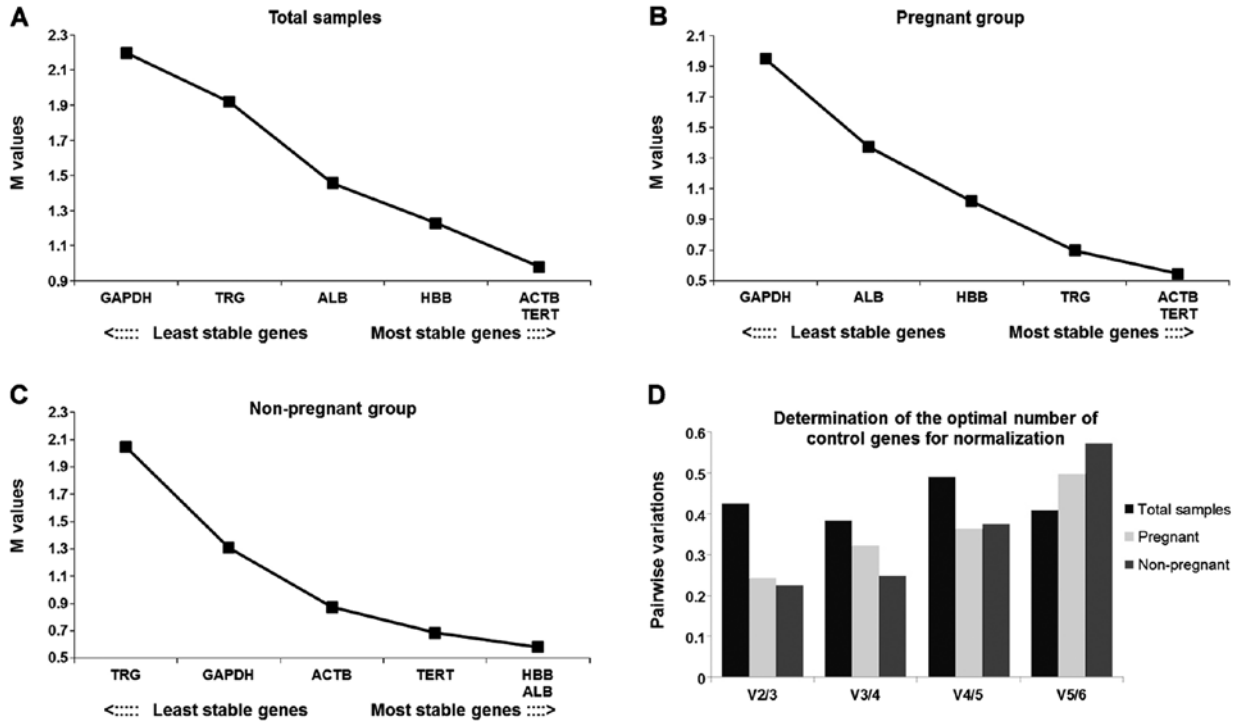

Figure 3. Stability values of the candidate control genes analyzed by geNorm. Expression stability measures (M) of the six control genes analyzed and the quantity stability was plotted in the (A) total samples, (B) pregnant and (C) non-pregnant groups. The x-axis from left to right indicates the ranking of the genes according to their stability; lower M values indicate higher stability. (D) Determination of the optimal number of control genes for normalization was conducted. The program calculates the normalization factor from at least two genes at which the variable, V, defines the pair-wise variation between two sequential normalization factors.

SD values (0.99). In the non-pregnant group, *TRG* was the most variable with a high SD value (4.13), whereas *ALB* had the lowest SD values (1.19). There was no significant difference of the Ct values between maternal- and fetal-derived DNA in each gene.

geNorm analysis. The geNorm analysis result revealed that *ACTB* and *TERT* were the most stable genes and *GAPDH* was the least stable among the total samples (Fig. 3A). Similar results were obtained in the pregnant group (Fig. 3B). In the non-pregnant group, *HBB* and *ALB* were the most stable genes and *TRG* was the least stable (Fig. 3C). None of the V values were below the cut-off value (Fig. 3D) indicating that there was no optimal combination number of control genes for normalization.

NormFinder analysis. The results of the NormFinder analysis showed that *ACTB* and *TERT* were the top two content stable genes in the total and pregnant groups, whereas *HBB* and *ALB* were the top two genes in the non-pregnant group (Table II). *GAPDH* and *TRG* were the least stable genes in the pregnant and non-pregnant groups, respectively, and *TRG* was also considered as the least stable in the total group. The best combination of two genes for total sample analysis was *ACTB* and *TERT*, and the stability value (0.224) was lower than *TERT* (0.340). This indicated that the combination of these two genes provide higher stability than using TERT alone.

BestKeeper analysis. According to BestKeeper analysis, when considering the total samples, *TERT* was found to be the optimal control gene with SD<1.00 and the highest r value (0.870). In

Table III. Descriptive statistics of six candidate control genes based on their cycle threshold values as calculated by BestKeeper.

Group	CP data	*ACTB*	*HBB*	*TERT*	*ALB*	*TRG*	*GAPDH*
Total samples	geo Mean (CP)	26.74994	28.67918	28.94395	30.42784	30.74505	33.09142
	Min (CP)	24.63311	25.21569	27.16459	25.00278	27.47232	26.64072
	Max (CP)	30.58614	32.06806	31.57339	34.2869	39.44698	38.16773
	SD (± CP)	1.310503	1.699849	0.999535	1.369684	1.955511	2.533179
	coeff. of corr. (r)	0.853	0.754	0.870	0.776	0.784	0.774
Pregnant	geo Mean (CP)	25.96939	28.4677	28.61777	30.40847	29.79071	32.51344
	Min (CP)	24.63311	25.21569	27.16459	25.00278	27.47232	26.64072
	Max (CP)	27.91232	32.06806	30.97218	34.28690	31.92536	36.89494
	SD (± CP)	0.76351	1.804439	0.877834	1.575063	1.160367	2.65516
	coeff. of corr. (r)	0.954	0.711	0.887	0.888	0.852	0.644
Non-pregnant	geo Mean (CP)	27.96496	28.99936	29.44021	30.45691	32.23419	33.97771
	Min (CP)	25.98068	26.73664	27.9093	28.95515	29.13656	29.93401
	Max (CP)	30.58614	30.70807	31.57339	31.51242	39.44698	38.16773
	SD (± CP)	1.545731	1.419053	0.983466	1.066481	3.279869	2.360286
	coeff of corr (r)	0.814	0.920	0.815	0.951	0.785	0.948

CP, cycle threshold; geo Mean (CP), geometric mean (CP); min, max (CP), extreme values of (CP); SD (± CP), standard deviation (CP); coeff of corr (r), pairwise correlation coefficient.

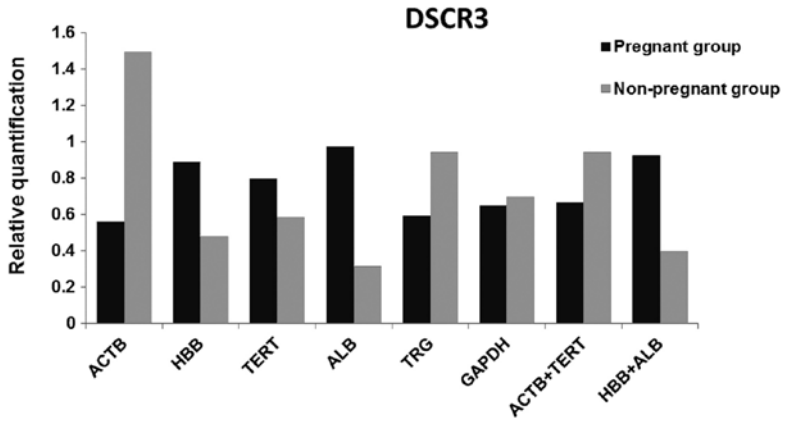

Figure 4. Relative quantities of the target gene *DSCR3* (n=36) in the plasma DNA upon different normalization approaches. Quantitative polymerase chain reaction (qPCR) data were normalized by six single control genes and two different combinations.

the pregnant group, *ACTB* and *TERT* were acceptable with SD<1.00, whereas *ACTB* had a higher r value (0.954) and was considered to be the most optimal control gene. In the non-pregnant group, *TERT* was the only gene with SD<1.00, but *ALB* had the highest r value (0.951). Although the SD of *ALB* was higher than 1.00 (SD=1.07), it was still considered to be a reliable control gene, similar to *TERT* (Table III). *GAPDH* and *TRG* were the least stable genes as shown by the results of geNorm and NormFinder in the pregnant and non-pregnant groups.

Validation of control genes. In order to verify the results of the control genes from geNorm, NormFinder and BestKeeper, the relative quantities of *DSCR3* were determined using six candidate control genes and the combinations recommended by geNorm (*ACTB* + *TERT*) and NormFinder (*HBB* + *ALB*) were the normalizers (Fig. 4). *GAPDH* had the minimum difference between the two groups, followed by *TERT*. *ACTB* had the largest difference, although it was ranked as one of the top two in all three algorithms. *TERT* combined with *ACTB* provided a smaller difference between the two groups compared to *ALB* + *HBB*.

Discussion

The discovery of cell-free fetal DNA in maternal plasma has become a primary target for NIPD (7). In healthy gravidae, fetal DNA can be detected in maternal plasma as early as the seventh week after conception (20), and it increases with the pregnancy progresses (10), reaches the plateau in the ensuing three months, is promptly cleared from maternal plasma and disappears within 2 h of delivery (21). These properties caused

plasma DNA to be the optimal material for NIPD. Thus far, qPCR is the most fundamental, sensitive and accurate method, widely used in studies of maternal plasma DNA. Due to its low cost and ease of use, a number of diseases, including gender determination (22-24), β-thalassemia (25-27), rhesus fetal blood group genotyping (28-30) and aneuploidies diseases (31), have been successfully diagnosed by qPCR. Although it is an extremely useful technique, there are challenges concerned with its use. The most important is normalization with an accurate, reliable control gene. To avoid the incorrect analysis results caused by pipetting errors, inhibitory compounds, quality of starting material or other systematic errors in qPCR (32), control genes should be stably contained in pregnant and non-pregnant female plasma. Ideally, control genes in plasma should not be influenced or regulated by pregnancy conditions, stress response, stimulation or any other physiological or pathological states between different individuals (4,33). However, there is accumulating evidence indicating that content levels of widely used control genes varies significantly in different independent studies, for example, *B2M*, *ACTB* and *SDHA* showed significant variation in expression levels in human epileptogenic brain tissues (34), and the single-copy DNA control gene *HBB*, which is used for representing the cell number, has been proved to not be the most reliable control gene (3). Therefore, it has become indispensable to normalize the control gene quantity levels and determine reliable control genes prior to any qPCR relative quantitative analysis. To the best of our knowledge, this is the first study to evaluate the content stability of control genes commonly used in the plasma DNA from pregnant and non-pregnant females. In the present study, the samples in the second trimester of the gestational age were selected, as in this stage the content of plasma DNA is stable. Six commonly used control genes (*HBB*, *TERT*, *GAPDH*, *ALB*, *ACTB* and *TRG*) were analyzed by qPCR of the plasma DNA from pregnant and non-pregnant females. Three common statistical algorithms (geNorm, NormFinder and BestKeeper) were used for data analysis and *DSCR3* was used to confirm the data analysis results.

On the basis of the results obtained from the three algorithms, the rank of the candidate genes stability was slightly different. These variations were possibly caused by different calculation algorithms (35,36) and indicated different features of the correlations between these control genes. Among the six candidate control genes, *ACTB* and *TERT* in the total samples and pregnant group, and *HBB* and *ALB* in the non-pregnant group showed the highest stability. This conclusion is consistent with the Ct values. *ACTB*, *TERT* and *TRG* had the lowest SD (0.99, 1.16 and 1.43) of the Ct values in the pregnant group; *ALB*, *TERT* and *HBB* had the lowest SD (1.19, 1.25 and 1.64) in the non-pregnant group. By contrast, *GAPDH* was unanimously affirmed as the least stable gene by the three algorithms in the pregnant group, as was *TRG* in the non-pregnant group. This corresponded to their high SD (3.21 and 4.13, respectively) for the Ct values, which means that they clearly vary.

In order to evaluate the exactitude of the control genes recommended by the three algorithms, the candidate control genes were used as a normalizer to detect the relative levels of the *DSCR3* gene. The *DSCR3* region only exists in chromosome 21, which is supposed to have the same relative quantity in the plasma DNA from pregnant and non-pregnant females. The content variance between the pregnant and non-pregnant groups was performed at maximum when using *ACTB* as the control gene, but minimum when using *GAPDH*. There is a slight discrepancy between the *DSCR3* evaluation and algorithm results. When using *TERT* as the normalizer, the content variance is within the tolerable range. When combining more than one control gene as the normalizer, the best combination chosen was *ACTB + TERT,* suggested by geNorm, and *HBB + ALB* from NormFinder. The result reveals that *ACTB + TERT* obtain an improved result compared to using *TERT* alone.

The optimal number of control genes for normalization was indicated by the V value below the cut-off of 0.15, as shown in geNorm (12). However, as the result from geNorm showed, there was no optimal combination of the selected control genes below the cut-off value. It has been suggested that when conditions permit, three of the most stable control genes could be used instead of a single gene (37,38).

Furthermore, it is of note that the concentration of plasma DNA in plasma is extremely low (21) and highly originates from the apoptosis process (39,40). These characteristics influence the amplification efficiency of plasma DNA. For example, firstly, the amplificon sizes should be short enough, as the longer the template of the target gene is, the more opportunities there are to be digested in the apoptosis process, which reduces the effective templates. Secondly, the succesful amplification of every single target gene from plasma DNA cannot be guaranteed. There are increasing studies focusing further on the clinical application of plasma DNA, which is required for NIPD. However, to the best of our knowledge, all the control genes used in plasma DNA analysis are chosen by experience, and no evaluation has been performed to confirm the content stability of these control genes in the plasma DNA from pregnant and non-pregnant females. The present study validated the most content stable control genes at the second trimester of gestational age, which can be used as a criterion in subsequent studies.

In conclusion, the present study indicated that the content stability of control genes used for DNA showed significant variation in pregnant and non-pregnant plasma DNA. Every qPCR DNA study should commence with the selection of an appropriate control gene individually. According to the study, *TERT* and the combination of *ACTB* and *TERT* permit an efficient normalization for DNA quantitation using qPCR in pregnant and non-pregnant plasma, whereas *GAPDH* and *TRG* were proved to be the least reliable control genes.

Acknowledgements

The present study was supported by the Project supported by the Key Foundation of Jilin Provincial Science and Technology Department, China (nos. 20130727038YY and 20100942) and the Jilin Provincial Development and Reform Commission, China (no. 20101928).

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