

Evaluation of eight reference genes for quantitative polymerase chain reaction analysis in human T lymphocytes co-cultured with mesenchymal stem cells

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Abstract. Accurate gene expression analysis relies on the selection of a stable reference gene, as unstable reference genes can alter experimental results and conclusions. It is widely-accepted that reference genes exhibit different expression levels in different types of tissues and cells. Therefore, it is essential to screen for stably-expressed reference genes in the cells and tissues used for experimental analysis prior to performing reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In the present study, eight reference genes were screened for their suitability for RT-qPCR in five T lymphocytes co-cultured with mesenchymal stem cells from different sources. Using NormFinder, geNorm, and BestKeeper algorithms consistently demonstrated that RPL13A and B2M were the optimal reference genes for the normalization of RT-qPCR data obtained from T lymphocytes, whereas glyceraldehyde 3-phosphate dehydrogenase was not a suitable reference gene due to its extensive variability in expression. These findings highlight the importance of evaluating reference genes for RT-qPCR.

Introduction

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is considered the most reliable technique for the detection and quantification of mRNA expression due to its high levels of accuracy and sensitivity (1). However, several lines of evidence have suggested that the expression of reference genes vary between cell types and experimental

conditions (2-4). The use of unstable internal controls can lead to incorrect results and erroneous conclusions. Therefore, it is essential to use suitable reference genes as a standard internal control to normalize levels of gene expression (5). An ideal reference gene is that which is unaffected by external or internal factors, including cell type or experimental conditions (6), and they are stably expressed in different samples (7). However, no single reference gene has been reported to exhibit constant expression levels, and there is increasing evidence suggesting that the expression levels of commonly used reference genes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin (ACTB) vary substantially depending on experimental conditions (3,8). Therefore, it is essential to compare and evaluate the stability of each reference gene prior to its use in experiments.

Human mesenchymal stem cells (MSCs) are highly proliferative, plastic adherent, fibroblast-like cells, which are capable of osteogenic, chondrogenic and adipogenic differentiation (9). MSCs are considered to assist healing, in part via modulation and downregulation of the immune response, including decreasing cytokine-associated acute inflammation and increasing blood flow to promote normal healing, rather than scarring (10). Although the mechanisms underlying the behavior of MSCs during an immune response and their immunomodulatory effects remain to be elucidated, it is widely-accepted that tissue-derived MSCs exhibit potent immunomodulatory properties, including T lymphocyte, B lymphocyte and natural killer cell suppression (11-13). To further investigate the molecular mechanisms underlying MSC suppression of T lymphocytes, careful selection of an appropriate reference gene is required. To the best of our knowledge, no previous report has described the experimental identification and validation of suitable endogenous controls for the normalization of T lymphocytes co-cultured with MSCs. The present study aimed to identify the most stable endogenous controls for the normalization of the gene expression of T lymphocytes co-cultured with different MSCs. In total, eight common reference genes were selected and used in the present study, and their expression stability was analyzed using the geNorm (14), NormFinder (15) and BestKeeper (16) algorithms.

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Table I. Summary of the reference genes and primers used in the present study.

Symbol	Gene name	Accession no.	Primer efficiency (%)	Primer sequence	Product size (bp)
18S	18S ribosomal RNA	NM10098.1	F: 5'-GTGGAGCGATTGTCTGGTT-3' R: 5'-AACGCCACTTGTCCTCTAA-3'	92.31	115
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	F: 5'-ATGGGGAAGGTGAAGGTCG-3' R: 5'-GGGGTCATTGATGGCAACAATA-3'	99.04	108
ACTB	β -actin	NM_001101	F: 5'-GAAGATCAAGATCATTGCTCCT-3' R: 5'-TACTCCTGCTTGCTGATCCA-3'	89.78	111
PPIA	Peptidyl-prolylisomerase A	NM_021130.3	F: 5'-TCCTGGCATCTTGTCAT-3' R: 5'-TGCTGGTCTTGCCATTCT-3'	103.09	179
B2M	β -2-microglobulin	NM_004048.2	F: 5'-CTATCCAGCGTACTCCAAAG-3' R: 5'-GAAAGACCAGTCCTTGCTGA-3'	98.89	188
RPL13A	Ribosomal protein L13a	NM_012423.2	F: 5'-CGAGGTTGGCTGGAAGTACC-3' R: 5'-CTTCTCGGCCTGTTCCGTAG-3'	99.79	121
HPRT1	Hypoxanthinephosphoribosyl transferase 1	NM_000194	F: 5'-CCTGGCGTCGTGATTAGTGAT-3' R: 5'-AGACGTTTCAGTCCTGTCCATAA-3'	86.56	131
TBP	TATA box-binding protein	NM_003194	F: 5'-GCACAGGAGCCAAGAGTGA-3' R: 5'-GTTGGTGGGTGAGCACAAG-3'	98.46	174

F, forward; R, reverse.

Materials and methods

Isolation and expansion of MSCs. MSCs were isolated from four human tissue harvest sites: Bone marrow (BM), adipose tissue (AT), umbilical cord Wharton's jelly (WJ) and placenta (PL). Eight BM and AT tissue samples were obtained from healthy donors (age, 18-43 and 23-50 years, respectively), and eight WJ and PL tissue samples were obtained from female patients (age, 23-38 years) following normal birth via caesarean section at the China-Japan Union Hospital, Jilin University (Changchun, China). All patients provided written informed consent, and the present study was approved by the Ethical Committee of the China-Japan Union Hospital, Jilin University. MSCs from the BM, AT, WJ and PL were isolated, using an enzymatic digestion method as previously described (17). Briefly, collagenase and hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) were used to digest the umbilical cord tissue samples following removal of the outer skin layer. The PL and AT were digested by collagenase only. BM-MSCs were obtained by BM adherence culture. Briefly, the cells were maintained in a humidified incubator containing 5% CO₂ at 37°C for 48 h, and non-adherent cells were removed when the media was changed (18). Following processing, the MSCs were plated in a culture flask with α -minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (GE Healthcare Life Sciences, Logan, UT, USA) (17). The culture was maintained at 37°C with saturated humidity in an atmosphere containing 5% CO₂.

Co-culture of MSCs and T lymphocytes. Human T lymphocytes were purchased from Guangzhou Jennio Biological Technology Co., Ltd. (Guangdong, China). Co-culture of the MSCs and T lymphocytes was performed using 6-well

plates. Briefly, the BM, AT, WJ and PL MSCs were seeded at 5x10⁵ cells/well in regular 6-well plates containing α -MEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. After 24 h, 10 μ g/ml mitomycin C (Sigma-Aldrich) was added to inhibit MSC proliferation, and the cells were incubated for 2 h at 37°C, followed by five extensive washes with α -MEM. A total of 5x10⁵ T lymphocytes/well were added and stimulated with 10 ng/ml phytohemagglutinin (PHA; Sigma-Aldrich) and 10 ng/ml interleukin (IL)-2 (Sigma-Aldrich). The IL-2/PHA-activated T lymphocytes were subsequently cultured in presence of the MSCs and the T lymphocytes were obtained following incubation for 4 days.

Reference gene selection and primer design. A total of eight commonly-used stable reference genes were selected, including 18S ribosomal RNA (18S), GAPDH, ACTB, peptidyl-prolylisomerase A (PPIA), β -2-microglobulin (B2M), ribosomal protein L13a (RPL13A), hypoxanthinephosphoribosyl transferase 1 (HPRT1), and TATA box-binding protein (TBP), based on previous studies (19,20). The gene sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The full name of the reference genes, primer sequences, accession number, and corresponding amplicon sizes are listed in Table I. Primers were designed using Primer3 (http://flypush.imgen.bcm.tmc.edu/primer/primer3_www.cgi/). All PCR primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), with melting temperatures of 60°C. All primers were purified using ultrapage (Research Scientific Instruments Co., Ltd., Xiamen, China).

RNA extraction and RT-qPCR. Total RNA from the co-cultured T lymphocytes was extracted and RT-qPCR was performed, as previously described (17). Total cellular RNA was extracted using TRIzol® reagent (Invitrogen Life

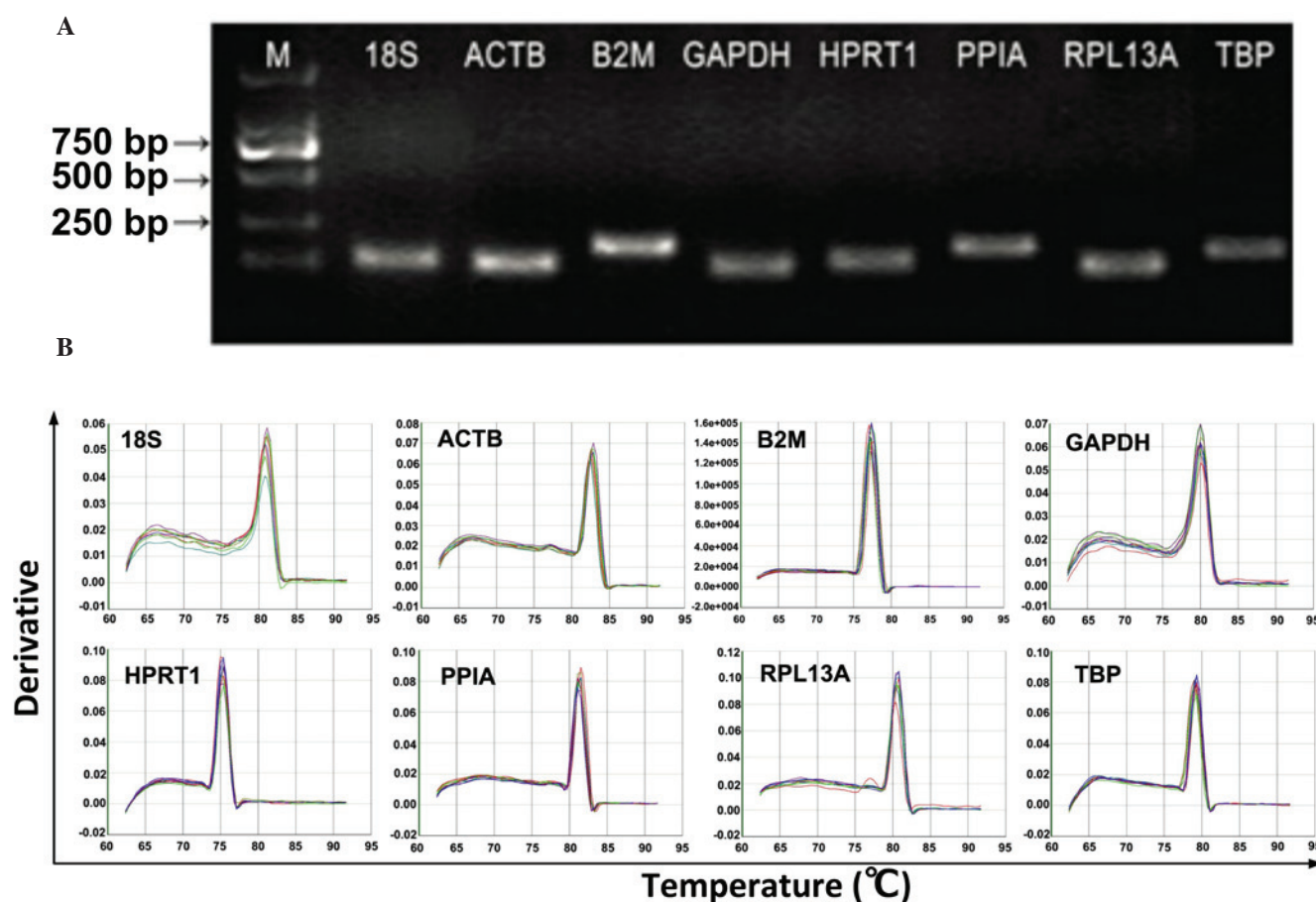


Figure 1. Primer specificity and amplicon length. The PCR amplification products were analyzed using agarose gel electrophoresis and dissociation curves. (A) PCR products were run on a 2% agarose gel. The presence of a single band with anticipate size indicated the PCR product was specific (B) Dissociation curves for the PCR products. The single peak indicates a specific PCR product. PCR, polymerase chain reaction; M, marker; 18S, 18S ribosomal RNA; PPIA, peptidyl-prolylisomerase A; RPL13A, ribosomal protein L13a; HPRT1, hypoxanthinephosphoribosyl transferase 1; ACTB, β -actin; B2M, β -2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box-binding protein.

Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was electrophoretically verified by ethidium bromide (Sigma-Aldrich) staining and an optical density (OD)260/OD280 nm absorption ratio >1.9. Total RNA (500 ng) was reverse transcribed into cDNA using AWW reverse transcriptase, oligo dT (~20 mer, Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. A non-amplification control was included without adding reverse transcriptase. The following reaction conditions were used: 42°C for 30 min, then 95°C for 5 min, and 5°C for 5 min. RT-qPCR was carried out using the ABI PRISM 7900 Sequence Detection system (Applied Biosystems Life Technologies, Foster City, CA, USA). A total of 25 ng cDNA was used in the qRT-PCR reactions with SYBR® Green PCR Master Mix (Applied Biosystems Life Technologies), as well as 5 μ M of gene-specific forward and reverse primers (Sangon Biotech Co., Ltd.). All PCR products demonstrated a single band by a dissociation curve and gel electrophoresis. The thermocycler (Prism 7900; Applied Biosystems Life Technologies) parameters for the amplification of these genes were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 15 s and 72°C for 30 s. To evaluate the efficiency of the PCR, a standard curve was generated using linear regression based

on the cycle threshold (CT) values. The PCR amplification products were analyzed using 2% agarose gel electrophoresis (Sigma-Aldrich) and dissociation curves. A single band with anticipate size indicated the PCR product was specific. Each sample, with 10-fold serial dilutions, was plotted against the logarithm of the cDNA dilution factor. An estimation of PCR efficiency was calculated from the slope of the calibration curve using the following equation: Efficiency = $[10^{1/-\text{slop}} - 1] \times 100\%$, where slop represents the slope of the linear regression (16). All reactions were performed in triplicate and the data were analyzed using the $2^{-\Delta\Delta C_t}$ method (5).

Reference gene evaluation using the GeNorm, NormFinder and BestKeeper algorithms. The expression stability of the eight reference genes were measured using three commonly used algorithms: geNorm (<https://genorm.cmgg.be/>), NormFinder (<http://moma.dk/normfinder-software/normfinder-faq>) and BestKeeper (<http://www.gene-quantification.de/bestkeeper.html>). These three programs are based on Microsoft Excel and use different algorithms to evaluate the expression stability of reference genes. For geNorm and NormFinder, the Ct values were converted into relative quantities using the $2^{-(C_t - \text{lowest } C_t)}$ formula. For BestKeeper, the Ct values were used directly.

Table II. Stability of the eight reference genes, determined using the BestKeeper algorithm.

Parameter	18S	PPIA	RPL13A	HPRT1	ACTB	B2M	GAPDH	TBP
Samples (n)	10	10	10	10	10	10	10	10
CT geo mean	16.556	19.212	18.268	17.964	16.567	20.085	16.204	21.783
CT ar mean	16.556	19.229	18.278	17.988	16.571	20.095	16.219	21.807
CT min	16.529	18.132	17.674	16.452	16.164	19.061	15.279	20.086
CT max	16.593	20.476	19.428	19.664	17.427	21.046	17.382	22.943
CT \pm SD	0.020	0.735	0.509	0.741	0.297	0.548	0.652	0.944
CV (%CT)	0.120	3.820	2.786	4.118	1.792	2.725	4.022	4.327
R-value	0.474	0.865	0.794	0.78	0.578	0.915	-0.04	0.946

CT, threshold cycle value; CT geo mean, geometric mean of the CT; CT ar mean, arithmetic mean of the CT; CT min and max, extreme values of the CT; CT \pm SD, standard deviation of the CT; CV (%CT), coefficient of variance expressed as a percentage of the CT; R, correlation coefficient; 18S, 18S ribosomal RNA; PPIA, peptidyl-prolyl isomerase A; RPL13A, ribosomal protein L13a; HPRT1, hypoxanthinephosphoribosyl transferase 1; ACTB, β -actin; B2M, β -2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box-binding protein.

Table III. Ranking of reference gene stability using the geNorm, NormFinder and BestKeeper algorithms.

Rank	geNorm	M-value	NormFinder	Stability	BestKeeper	r-value
1	B2M/RPL13A	0.364	B2M	0.251	TBP	0.946
2	^a		RPL13A	0.272	B2M	0.915
3	ACTB	0.426	PPIA	0.308	PPIA	0.865
4	18S	0.487	ACTB	0.332	RPL13A	0.794
5	PPIA	0.559	18S	0.341	HPRT1	0.780
6	HPRT1	0.646	TBP	0.465	ACTB	0.578
7	TBP	0.697	HPRT1	0.473	18S	0.474
8	GAPDH	0.803	GAPDH	0.594	GAPDH	0.040

^aNo genes are ranked second as the geNorm identified B2M and RPL13A as the two most stable genes (both rank 1). The M-values indicate expression stability measures determined by geNorm and the r-values indicate the correlation coefficients of the reference genes determined by BestKeeper. 18S, 18S ribosomal RNA; PPIA, peptidyl-prolyl isomerase A; RPL13A, ribosomal protein L13a; HPRT1, hypoxanthinephosphoribosyl transferase 1; ACTB, β -actin; B2M, β -2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box-binding protein.

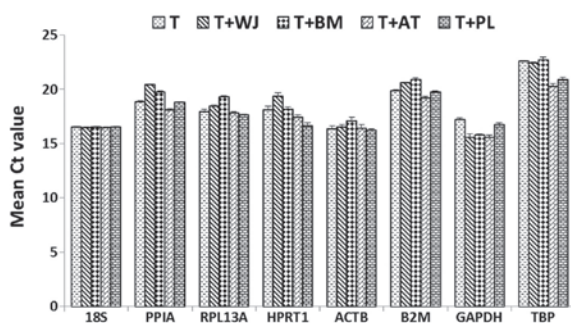


Figure 2. Expression levels of the reference genes in the five cell groups. The values are presented in the form of Ct values as the mean \pm standard deviation (n=5). MSCs, mesenchymal stem cells; WJ, Wharton's jelly; BM, bone marrow; AT, adipose tissue; PL, placenta; WJ+T, T lymphocytes co-cultured with WJ MSCs; BM+T, T lymphocytes co-cultured with BM MSCs; AT+T, T lymphocytes co-cultured with AT MSCs; PL+T, T lymphocytes co-cultured with PL MSCs; Ct, threshold cycle.

GeNorm analyzes the gene expression stability (M-value) and pair-wise variation (V), with the lowest M-value representing the highest stability. V is calculated to determine the

minimal number of reference genes required. If $V < 0.15$, the number of reference genes is sufficient for valid normalization.

NormFinder is based on a variance estimation approach. Higher values indicate lower stabilities. NormFinder is also able to compare inter- and intra-group variations in gene stability.

BestKeeper calculates the expression level variation for reference gene stability based on the standard deviation (SD) and correlation coefficient (r). Genes with $SD > 1.00$ are considered unreliable as reference genes, and the remaining genes are ranked according to their r-values, with the highest r-value indicating the highest stability.

Results

Amplification specificity and primer efficiency. The A260/280 ratio for the isolated RNA was 1.85-2.0. The amplification performance of each primer pair was analyzed using RT-qPCR. The specificity of the PCR products were analyzed using a dissociation curve and 2% agarose gel. No primer/dimers or multibands/peaks were detected, confirming

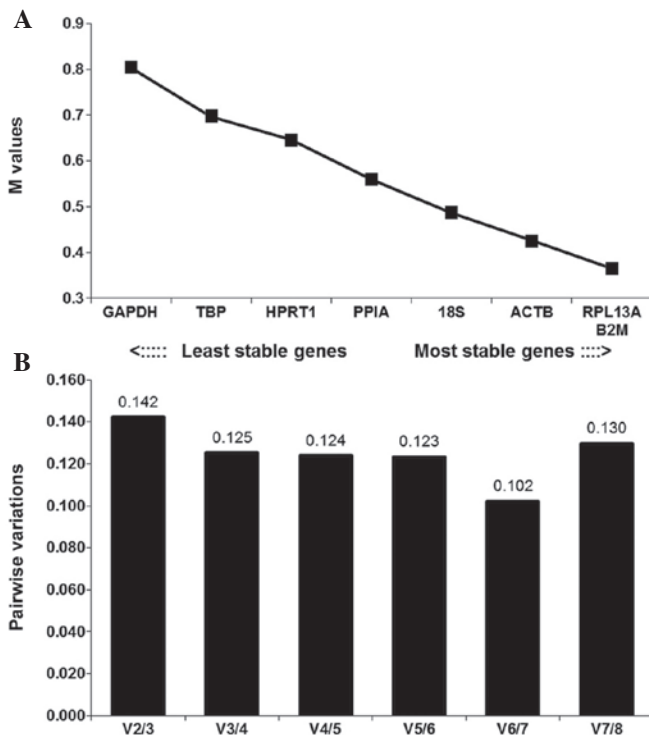


Figure 3. Selection of the most suitable reference genes among the samples, determined by geNorm. (A) M-values of the eight reference genes. Ranking of the genes according to their expression stability is indicated on the x-axis. Low M-values indicate high expression stability. (B) Optimal number of reference genes for normalization based on their V-values. The V-value defines the pair-wise variation between two sequential normalization factors, determined by geNorm. M-value, expression stability measure; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box-binding protein; HPRT1, hypoxanthinephosphoribosyl transferase 1; PPIA, peptidyl-prolylisomerase A; 18S, 18S ribosomal RNA; ACTB, β -actin; RPL13A, ribosomal protein L13a; B2M, β -2-microglobulin.

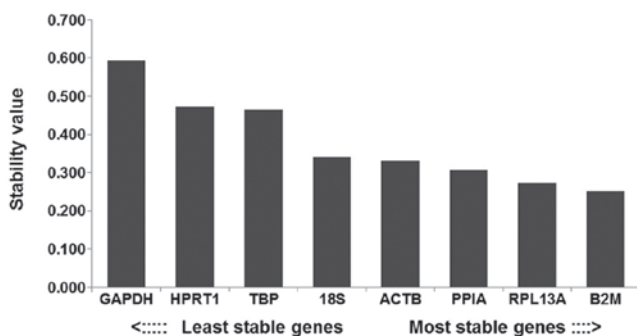


Figure 4. Expression stability values of the reference genes, determined by NormFinder. The genes were ranked according to their expression stability, with the lowest stability values indicating stable expression. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthinephosphoribosyl transferase 1; TBP, TATA box-binding protein; 18S, 18S ribosomal RNA; ACTB, β -actin; PPIA, peptidyl-prolylisomerase A; RPL13A, ribosomal protein L13a; B2M, β -2-microglobulin.

a single amplified band of a predicted size (Fig. 1). All primer pairs exhibited efficiency values ranging between 86.56 and 103.09% (Table I), with correlation coefficients of $R^2 > 0.97$.

Expression levels of the reference genes. An ideal reference gene is expressed at relatively high and stable levels (21).

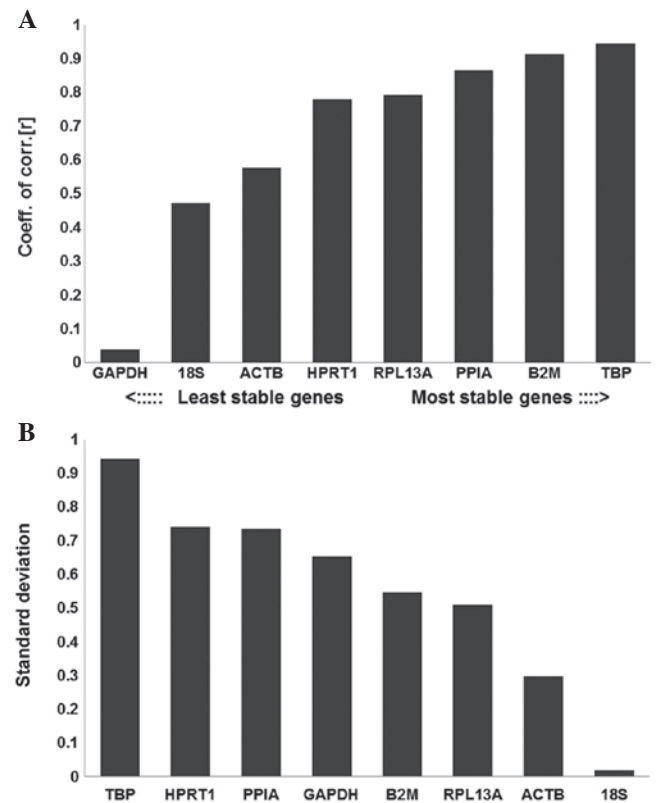


Figure 5. Evaluation of reference gene stability, determined by BestKeeper. (A) Correlation coefficient (r) values of the reference genes, determined by BestKeeper. Ranking of the genes according to their expression stability, with low stability values indicating stable expression. (B) Standard deviation values of the reference genes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 18S, 18S ribosomal RNA; ACTB, β -actin; HPRT1, hypoxanthinephosphoribosyl transferase 1; RPL13A, ribosomal protein L13a; PPIA, peptidyl-prolylisomerase A; B2M, β -2-microglobulin; TBP, TATA box-binding protein.

Figure 2 shows the mean Ct-values for each reference gene in the five samples, indicating the expression levels in the different experimental groups. The eight experimental reference genes exhibited a wide range of expression, with Ct-values ranging between 15.58 for GAPDH and 22.94 for TBP. Among these genes, GAPDH (Ct, 15.58-17.25) and 18S (Ct, 16.54-16.60) exhibited the highest expression levels, and TBP (Ct, 20.32-22.75) exhibited the lowest expression levels in the T lymphocytes. The expression levels of each reference gene was significantly different in all experimental cells, with the smallest difference observed for 18S ($\Delta Ct = 0.06$) and the most marked difference observed for hypoxanthinephosphoribosyl transferase 1 ($\Delta Ct = 2.69$).

RPL13A are the most stably expressed reference genes in T lymphocytes co-cultured with MSCs. The average stability M-values of the eight reference genes in the experimental tissue samples are shown in Fig. 3A. According the principles of geNorm, B2M and RPL13A were the most stably expressed genes ($M < 0.4$). GAPDH and TBP were the least stably expressed genes, although their M-values remained < 0.9 . The V2/3 value, which indicates the pair-wise variation when the number of normalization factors increases between two and three, was 0.142, which was below the cut-off value of 0.15. Therefore, the B2M and RPL13A reference genes

were considered sufficiently stable, and addition of the third gene is optional (Fig. 3B).

According to NormFinder, reference genes that are more stably expressed are indicated by lower stability values. The most stable genes identified in the present study were B2M, with a stability value of 0.251. The most unstable genes were GAPDH, HPRT1 and TBP, which exhibited stability values of 0.594, 0.473 and 0.465, respectively (Fig. 4). The ranking of the selected reference genes used in the present study, according to stability was as follows: B2M>RPL13A>PPIA>ACTB>18S>TBP>hypoxanthinephosphoribosyl transferase 1 (HPRT1)>GAPDH. GAPDH was found to be the most unstable reference gene.

The results of the BestKeeper analysis are presented in Table II. GAPDH was the most unstable reference gene and TBP was the most stable reference gene determined by BestKeeper (Fig. 5). These results suggested that TBP, B2M and PPIA were the most stable reference genes, with the highest *r*-values, whereas GAPDH had the lowest *r*-value (0.04), reflecting the least stable expression.

Overall, following stability analysis of the eight reference genes using the geNorm, NormFinder and BestKeeper algorithms, geNorm and NormFinder indicated that B2M and RPL13A were the most stable reference genes, and that GAPDH was the least stable reference gene. However BestKeeper demonstrated that TBP and B2M were the most stable reference genes. A summary of the rankings produced by the three algorithms is shown in Table III.

Discussion

It is well established that a reference gene requires validation prior to a specific experiment in order to confirm that gene expression is not affected by the experimental conditions. However, increasing evidence suggests that the expression levels of widely-used reference genes vary significantly in independent investigations (3,22). Therefore, it is essential to normalize the expression levels of reference genes, and determine their reliability prior to RT-qPCR analysis. To the best of our knowledge, the present study is the first to investigate the stability of reference genes in T lymphocytes co-cultured with different MSCs.

The results of the present study suggested that the expression of reference genes varied significantly between different cells. Therefore, investigations of gene expression alterations between different cell types requires careful selection of reference genes, which are expressed at similar relative levels between the cell types.

In the present study, the results obtained from the three algorithms, geNorm, NormFinder and BestKeeper, demonstrated discrepancies in the stability ranking of the reference genes. These differences were likely to be caused by the different calculation algorithms used in these software programs (8). According to geNorm, the reference genes with an average *M*-value expression <1.5 are considered reliable. Therefore, RPL13A and B2M were the most stable reference genes determined by geNorm in the present study. Similarly, the results of NormFinder suggested that B2M was the most stable reference gene, followed by RPL13A. When intergroup variation was taken into consideration, 18S and TBP were

revealed as the optimal combination. In addition, the stability value of this combination (0.163) was lower than that of B2M (0.251), indicating that the combination of 18S and TBP as reference genes is sufficiently reliable for relative quantification. Therefore, the number of reference genes also depends on the experimental conditions. BestKeeper identified TBP and B2M as the most stable reference genes. All three algorithms determined GAPDH as the least stable reference gene.

In the present study, GAPDH was confirmed as the least stable reference gene. Another reference gene, ACTB, is frequently used to normalize RT-qPCR data without any prior validation (23). However, ACTB was ranked with low stability in the present study, indicating that it was unsuitable for use in T lymphocytes. The results of the present study demonstrated that the evaluation of reference genes for normalization of RT-qPCR data is essential, and the use of common reference genes, including ACTB and GAPDH, without prior validation may lead to false results.

PPIA has a versatile role as a reference gene in immunosuppression, being regulated by CD4⁺ T cells and inducing leukocyte subsets (24-26). It has been suggested that the expression of PPIA is lower in unstimulated T lymphocytes, but may be higher than normal in T lymphocytes stimulated with allergens or MSCs (19). Although the results of the present study did not indicate high expression levels of PPIA in the MSC co-cultured groups, its readily altered characteristics makes PPIA unreliable as a reference gene. Therefore, when the experimental conditions or experimental treatments may lead to a change in the expression levels of the reference gene, this reference gene cannot be used to normalize the results of RT-qPCR.

In conclusion, among the eight genes analyzed in the present study, RPL13A and B2M were identified as the most suitable for analysis of gene expression levels in T cells co-cultured with MSCs, whereas GAPDH is the least stable gene, and is unsuitable for use as an internal control. These findings may also be useful when validating other genes using selected reference genes from T lymphocytes.

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