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Selection and Verification of Reference Genes for Gene Expression Studies in Different Cell Lines of Golden Pompano (*Trachinotus ovatus*)

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Abstract: The golden pompano snout (GPS) and head kidney (GPHK) cell lines have been proven to be meaningful tools for the study on pathogenic infections in vitro. In this study, we aimed to select the most stable reference genes from seven housekeeping genes (*Actin*, *B2M*, *GAPDH*, *RPL13*, *EF1A*, *18S* and *UBCE*) applied to two cell lines of golden pompano (GPS and GPHK) under both normal physiological conditions and stimulated conditions of the lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (Poly I:C) relying on quantitative real-time PCR (qRT-PCR). Additionally, the raw C_t value resulting from the qRT-PCR was analyzed by the geNorm, NormFinder and BestKeeper algorithm, and the results indicated that expression for all candidate genes exhibited some discrepancy under different experimental conditions or cell types. As for the non-stimulated group, *18S* and *RPL13* were the most appropriate reference genes in GPS and GPHK cells, respectively. Nevertheless, the most suitable reference genes in GPS and GPHK cells, under the stimulation of LPS, were *RPL13* and *18S*, respectively, whereas after being stimulated with Poly I:C, *UBCE* and *EF1A* were recommended as the optimal candidates for GPS and GPHK cells, respectively. To be sure of the reliability of the selected reference genes, immune-related genes (*ISG15*, *BCL2*, *IRF1* and *IRF7*) were chosen as target genes to normalize. The study will provide a direction for various golden pompano cell lines to screen appropriate reference genes, and will set the stage for the application of these cell lines in relevant research areas.

Keywords: golden pompano; cell line; reference genes; LPS; Poly I:C

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1. Introduction

Owing to sensitivity, flexibility, accuracy, dynamic range and high throughput characteristics, quantitative real-time reverse transcription PCR (qRT-PCR), which is a vital powerful technique in target gene expression analysis, has gradually prevailed in the scientific research domain [1–4]. Moreover, in comparison with other conventional means for gene expression analysis, including northern hybridization, semi-quantitative PCR and RNA-seq, qRT-PCR has the advantages of short detection time, high sensitivity, well repeatability and specificity and simplicity of operator [5,6]. Nevertheless, the results generated by qRT-PCR may be influenced by some diverse elements, such as amplification efficiency, quality and quantity of RNA, enzymatic efficiency for reverse transcription proceedings, various sample amounts and so forth [7,8]. Furthermore, it is a pre-requisite for relative quantification of target gene expression to choose stable reference genes so as to standardize the data [9–11]. Despite the stable expression, it has been reported over the recent years that the reference genes will show different expression levels not merely in different types of cells or tissues but also in various physiological stages or diverse stimulating environments [12–16]. It is generally recognized that there has been no single

reference gene that is suitable for expression analysis of any target genes under any conditions [17,18]. Thereby, the reliable gene expression analysis results mainly hinge upon selection for appropriate reference genes.

Some conventional reference genes that possess stable expression are usually adopted as internal control. For instance, beta actin (*Actin*), a highly conserved protein, is related to cell movement, structure and integrality, featuring high transcript abundance and stable expression [19–22]. β-2-Microglobulin (*B2M*) is a subunit of major histocompatibility complex (MHC) class I, whose functions are closely linked to the cell immune activation and regulation and tumor immune process [23–26]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a key enzyme that participates in the glycolytic cycle and plays a vital role in adjusting cellular survival, apoptosis and death [27–29]. Ribosomal protein L13 (*RPL13*), one of the segments of constituted ribosomes, takes part in cell proliferation, differentiation, apoptosis, tumorigenesis and progression, immune response and virus replication [30]. Elongation factor-1-α (*EF1A*) is a conserved and indispensable protein that mainly associates with the extension stage of mRNA translation [31,32]. Moreover, *18S* ribosomal RNA (*18S*) engages in cell growing and breed regulation [33–35], and ubiquitin-conjugating enzyme E2 (*UBCE*) is able to promote apoptosis and regulate the signaling pathway [36–38].

Golden pompano (*Trachinotus ovatus*), a common tropical and subtropical fish with considerable commercial values, is principally cultured in China, Australia, Japan and their vicinity [39–41]. The breeding industry of golden pompano has developed rapidly in recent years, but it is grievously threatened by viruses, bacteria and parasites, resulting in heavy economic losses annually [42,43]. Thereby, it is essential that the mechanism of pathogenic infection is elucidated as quickly as possible.

LPS, as a part of most Gram-negative bacteria tunica externa layers, is a sort of pathogen-associated molecule that can be recognized by immune cells, which plays a pivotal role in pathogenesis [44,45]. Moreover, due to the possession of a stimulating function similar to viral dsRNA, Poly I:C, a viral analogue, is frequently used in investigations on virus infection experiments of immune responses [46,47]. Fish cell lines, as a significant research tool in vitro, have been applied for studies on pathogenic mechanisms of infection, immunology, endocrinology and biotechnology [40]. Nonetheless, to date, there are no published studies on the stability of reference genes in cell lines of golden pompano.

In this study, we aimed to sort out the most stable reference genes from seven candidate reference genes (*Actin*, *B2M*, *GAPDH*, *RPL13*, *EF1A*, *18S* and *UBCE*) in golden pompano snout (GPS) and head kidney (GPHK) cells under normal physiological and stimulating conditions of the lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (Poly I:C). The data produced by qRT-PCR was analyzed using three typical algorithms, including geNorm, NormFinder and BestKeeper [7,48,49]. In order to verify the dependability of the appropriate reference genes we selected, the immune-related target genes (*ISG15*, *BCL2*, *IRF1* and *IRF7*) were chosen to normalize. The results for this study will set the stage for further qRT-PCR research on cell lines of golden pompano.

2. Materials and Methods

2.1. Cell Culture and Stimulation

Golden pompano snout (GPS) cell lines and head kidney (GPHK) cell lines were a generous gift from the South China Sea Institute of Oceanology [40,50]. GPS and GPHK cells were cultivated at 28 °C in 25-cm^2 cell culture flasks, whose composition of culture solution was Leibovitz's L-15 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 0.5% of 1M N-2-Hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES, Gibco, Waltham, MA, USA), 4% penicillin/streptomycin (Gibco, Waltham, MA, USA) and 1% sodium chloride. At 90% cell confluence, 1 mL 0.25% trypsin-EDTA solution (Gibco, Waltham, MA, USA) was used for digestion, and then the cells were inoculated into 6-well plates. Subsequently, when the cells reached the confluence of 90%, the cells were washed three times with PBS whose medium was

replaced. Then, prior to experiments, all cells were divided into three groups (A, B and C group) for treatment. Every experimental group had three biological repetitions. Then, group A and group B were stimulated with LPS or Poly I:C at concentration of 10 μg/mL, respectively, whereas group C incubated with the same volume of PBS was set as the control group. Subsequently, all cells were collected at 2, 4, 8 and 12 h post treatment for follow-up RNA extraction.

2.2. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the cell samples with the FastPure® Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. The purity and integrity of RNA were measured by 260/280 nm UV absorbance ratio with NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA) and confirmed by 1% agarose gel electrophoresis, separately. Purified RNA was reverse transcribed cDNA immediately with Eastep® RT Master Mix Kit (Promega, Madison, WI, USA) [51].

2.3. Reference Gene Primers Design and Amplification Efficiency

Seven candidate reference genes (*Actin*, *B2M*, *GAPDH*, *RPL13*, *EF1A*, *18S* and *UBCE*) were selected and Primer Premier 5.0 software (Premier, Charlotte, NC, USA) was used for designing primers for the qRT-PCR experiments (Table 1). The correlation coefficient (R^2) and PCR efficiency (E) were analyzed by the standard curve. The reliable E value should be between 90–110% with a computational formula of: E (%) = $(10^{-1/\text{slope}} - 1) \times 100$ [52].

Table 1. Information for primers of housekeeping genes and their amplification efficiency in this research.

Gene	Function	GenBank Accession Number	Primer Sequence (5′→3′)	Product Size (bp)	PCR Efficiency (%)	Determination Coefficient (R^2)
Actin	Cytoskeletal protein	KX987228.1	F:CGTGCGTGACATCAAGGAGAA R:AAGGAAGGAAGGCTGGAAGAGG	178	99%	0.992
B2M	Major histocompatibility complex	KX987233.1	F:CCCTGATGCCAAACAGACAGA R:TGGTTGACCCATGAGTGACCTT	125	100%	0.991
GAPDH	Glycolysis enzyme	KY006114.1	F:AGTCCGTCTGGAGAAACCTGC R:GACACGGTTGCTGTAGCCGAACTCA	235	106%	0.993
RPL13	Ribosome Protein	KX987230.1	F:TGAAGGAGTACCGCTCCAAACT R:GCACGGATGCCAAATAGACG	238	104%	0.990
EF1A	Translation	KX987227.1	F:GTCCGTCAAGGAAATCCGTCG R:TTGAACTTGCAGGCAATGTGAG	174	100%	0.996
18S	Ribosome subunit	KY014076.1	F:GCATTCGTATTGTGCCGCTA R:AGTTGGCATCGTTTATGGTCG	160	98%	0.990
UBCE	Protein degradation	KX987232.1	F:CACGATGTCCAGCGAAGTACA R:GACCTCCACTCGTAGATGTTGTC	270	96%	0.991

2.4. qRT-PCR and Data Processing

qRT-PCR was executed via the QuantStudio™ 6 Real-Time PCR System (Thermo Fisher, Waltham, MA, USA) using 2×ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China).

2.5. Stability Verification of Internal Reference Genes

As previously reported, *ISG15*, *BCL2*, *IRF1* and *IRF7* play central roles in the innate immune defense system of fish, protecting them from bacterial or viral infection [53–56]. These four immune-related genes were used to verify the reliability of selected candidate reference genes in GPS and GPHK cells under stimulation of LPS or Poly I:C. Target genes were normalized using combinations in accordance with the following: (1) the most stable expressed reference gene, (2) the second most stable expressed reference genes, (3) the combination of the two most stable expressed reference genes, and (4) the least stable expressed gene. Amplification primers for immune-related genes were listed as Table S1.

3. Results

3.1. qRT-PCR Efficiency and Quality

As shown in Figure S1, total RNA for samples displayed two clear and discernible bands of 28S rRNA and 18S rRNA in agarose gel electrophoresis, whose A260/A280 value was within a reasonable range (2.0 to 2.2), suggesting that the quality and purity of total RNA were in conformity with the follow-up experimental request. In the light of the fact that all amplification products had a sole specific DNA band without obvious non-specific amplification or dimers (Figure S2), the amplification primers for seven candidate reference genes were in accordance with experimental requirements. The *E* values of seven candidate reference genes were within the range of 96% to 106% and their R^2 values ranged from 0.990 to 0.996 (Table 1), manifesting that both the product specificity for each reference gene and amplification efficiency were consistent with the qRT-PCR conditions.

3.2. Threshold Cycle (C_t) Value Analysis

3.2.1. Expression Abundance of Reference Gene Expression among GPS and GPHK Cells under Normal Physiological Conditions

As displayed in Table 2, the C_t values of seven candidate reference genes were from 6.4 to 31.8 in GPS cells under normal physiological conditions. What was noteworthy was that *B2M* had the highest C_t value (31.8), whereas the minimum C_t value was observed in *18S* (6.4). The C_t range of *18S* was 6.4 to 6.6, which suggested that *18S* had the least changed range in comparison with other reference genes. However, the *RPL13* whose C_t range was between 21.3 and 24.3 had the maximum variation. In GPS cells, *B2M* exhibited the lowest expression level with an average C_t value of 31.2, while the expression level of *18S* was the highest with an average C_t value of 6.5.

Table 2. Expression abundance of seven housekeeping genes among GPS and GPHK cells under normal conditions for 2, 4, 8 and 12 h. Values are shown as means ± SD (n = 3).

Cell Lines	Time	*Actin*	*B2M*	*GAPDH*	*RPL13*	*EF1A*	*18S*	*UBCE*
GPS	2 h	12.2 ± 0.3	30.7 ± 0.5	26.8 ± 0.3	24.3 ± 0.3	12.3 ± 0.2	6.4 ± 0.1	21.7 ± 0.3
	4 h	12.4 ± 0.2	30.6 ± 0.6	27.0 ± 0.2	23.9 ± 0.2	12.8 ± 0.4	6.4 ± 0.0	21.9 ± 0.0
	8 h	12.9 ± 0.1	31.8 ± 0.8	27.1 ± 0.0	21.3 ± 0.3	13.1 ± 0.4	6.5 ± 0.1	21.8 ± 0.7
	12 h	12.5 ± 0.3	31.7 ± 0.7	27.1 ± 0.1	22.5 ± 0.3	12.4 ± 0.4	6.6 ± 0.2	22.0 ± 0.7
GPHK	2 h	14.8 ± 0.1	20.3 ± 0.2	33.2 ± 0.1	17.2 ± 0.1	14.9 ± 0.2	7.9 ± 0.1	23.0 ± 0.2
	4 h	15.2 ± 0.1	20.6 ± 0.1	33.5 ± 0.3	17.1 ± 0.1	15.0 ± 0.0	7.7 ± 0.5	22.9 ± 0.3
	8 h	15.7 ± 0.6	21.0 ± 0.2	34.4 ± 0.2	17.4 ± 0.3	15.3 ± 0.2	7.9 ± 0.2	23.6 ± 0.4
	12 h	15.4 ± 0.4	20.3 ± 0.2	33.8 ± 0.5	17.2 ± 0.1	15.4 ± 0.4	7.9 ± 0.0	23.4 ± 0.3

With regard to GPHK cells, the C_t value of all candidate genes was within the range of 7.7 to 34.4. *18S* and *GAPDH* had the minimum C_t value (7.7) and maximum C_t value (34.4), respectively. It was evident that *18S* had the least variation of C_t value while *GAPDH* displayed the largest C_t value changes of 1.2.

3.2.2. Expression Abundance of Reference Gene Expression among GPS and GPHK Cells under LPS Stimulating Condition

According to the data of the C_t value presented in Table 3, in comparison with the control group, there was clearly different variation in the expression level of the seven candidate reference genes in GPS and GPHK cells at different time points under stimuli of LPS. At 2 h and 4 h after being stimulated with LPS, in GPS cells, *Actin* showed the least C_t value variation, whereas *GAPDH* had an obvious variation of expression level. At 8 h, the smallest change of C_t value was *B2M* (0.7); however, C_t variation in the remaining six candidates ranged from 1.1 to 2, among which *GAPDH* presented the highest variation of C_t value (2). At 12 h, *B2M* displayed an inconspicuous variation of the C_t value (0.1), and *Actin* and *18S* were the most unstable candidates with significant change of C_t value (0.8).

Table 3. Expression abundance of seven housekeeping genes among GPS and GPHK cells under stimulation of LPS or Poly I:C for 2, 4, 8 and 12 h. Values are shown as means ± SD (*n* = 3).

Reference Gene	Treatments	GPS				GPHK			
		2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h
Actin	PBS	14.3 ± 0.5	15.7 ± 0.3	15.3 ± 0.2	14.4 ± 0.4	14.3 ± 0.3	14.1 ± 0.2	15.0 ± 0.2	13.9 ± 0.4
	LPS	14.6 ± 0.6	15.8 ± 0.5	14.2 ± 1.0	15.2 ± 0.3	14.1 ± 0.1	13.8 ± 0.4	14.6 ± 0.3	13.6 ± 0.5
	Poly I:C	14.4 ± 0.9	15.5 ± 0.4	15.5 ± 0.6	16.3 ± 0.7	12.9 ± 1.0	15.2 ± 0.6	15.5 ± 0.4	14.0 ± 0.5
B2M	PBS	26.9 ± 1.1	27.3 ± 0.8	28.2 ± 0.5	27.0 ± 0.7	19.6 ± 0.3	19.4 ± 0.1	20.7 ± 0.5	18.9 ± 0.3
	LPS	27.7 ± 0.6	28.4 ± 0.2	27.5 ± 0.3	27.1 ± 0.8	19.1 ± 0.5	18.7 ± 0.4	21.3 ± 0.2	18.5 ± 0.4
	Poly I:C	26.1 ± 0.6	26.0 ± 0.5	26.4 ± 0.1	25.7 ± 0.5	18.1 ± 0.5	18.4 ± 0.5	18.4 ± 0.1	18.0 ± 0.4
GAPDH	PBS	25.0 ± 0.3	26.0 ± 0.4	28.4 ± 0.7	26.4 ± 0.7	30.4 ± 0.5	30.6 ± 0.6	32.2 ± 0.9	31.8 ± 0.6
	LPS	23.7 ± 0.3	27.3 ± 0.3	26.4 ± 0.4	25.7 ± 0.7	31.5 ± 0.3	32.3 ± 0.3	32.7 ± 0.1	32.5 ± 0.9
	Poly I:C	26.6 ± 0.0	27.1 ± 0.2	27.5 ± 0.4	25.8 ± 0.8	29.6 ± 1.5	32.1 ± 0.1	33.0 ± 0.3	30.7 ± 0.2
RPL13	PBS	23.7 ± 0.4	24.2 ± 0.2	25.9 ± 0.8	24.3 ± 0.1	16.8 ± 0.1	15.9 ± 0.3	17.1 ± 0.2	16.3 ± 0.4
	LPS	24.0 ± 0.5	24.4 ± 0.4	24.8 ± 0.2	23.9 ± 0.1	16.5 ± 0.4	15.1 ± 0.1	16.4 ± 0.4	16.5 ± 0.2
	Poly I:C	24.2 ± 1.1	24.9 ± 0.7	26.5 ± 0.5	23.2 ± 0.4	15.1 ± 1.3	16.8 ± 0.7	17.5 ± 0.8	16.4 ± 0.6
EF1A	PBS	13.2 ± 0.5	15.3 ± 0.1	14.7 ± 0.3	12.3 ± 0.1	15.0 ± 0.8	13.9 ± 0.3	14.4 ± 0.1	14.2 ± 0.3
	LPS	12.5 ± 0.4	15.5 ± 0.5	13.4 ± 0.4	12.9 ± 0.8	15.4 ± 0.4	14.2 ± 0.7	14.5 ± 0.9	14.5 ± 0.3
	Poly I:C	13.0 ± 0.7	14.6 ± 0.4	15.2 ± 0.4	13.9 ± 0.7	13.7 ± 0.1	14.8 ± 0.5	14.8 ± 0.3	14.3 ± 0.9
18S	PBS	8.3 ± 0.3	8.1 ± 0.0	8.1 ± 0.0	8.8 ± 0.9	9.5 ± 0.3	7.5 ± 0.1	7.8 ± 0.1	7.8 ± 0.2
	LPS	8.8 ± 1.0	8.3 ± 0.7	9.5 ± 0.9	8.0 ± 0.2	9.3 ± 0.1	7.2 ± 0.1	7.5 ± 0.4	8.1 ± 0.1
	Poly I:C	7.6 ± 0.5	8.5 ± 0.0	8.5 ± 0.5	8.7 ± 0.2	7.4 ± 0.0	7.4 ± 0.0	7.8 ± 0.1	8.1 ± 1.1
UBCE	PBS	24.0 ± 0.8	27.4 ± 0.2	26.1 ± 0.4	24.6 ± 0.5	22.7 ± 0.2	21.7 ± 0.1	22.4 ± 0.3	23.4 ± 0.6
	LPS	24.7 ± 0.3	28.5 ± 0.2	24.6 ± 0.4	25.3 ± 0.3	21.9 ± 0.6	21.1 ± 0.5	22.1 ± 0.6	23.7 ± 0.7
	Poly I:C	24.2 ± 0.3	27.2 ± 0.3	26.5 ± 0.0	26.7 ± 0.6	21.6 ± 0.2	23.2 ± 0.6	23.2 ± 0.1	23.8 ± 1.0

In GPHK cells, at 2 h and 4 h after being stimulated with LPS, the expression level of *GAPDH* possessed maximum fluctuation with C_t value variation while C_t value for both *Actin* and *18S* all showed comparatively small changes. At 8 h and 12 h, the changes for C_t value of all candidates were less than 1.

3.2.3. Expression Abundance of Reference Gene Expression among GPS and GPHK Cells under Poly I:C Stimulating Condition

Likewise, it was noticeable that under stimulation of Poly I:C at diverse time points, expression abundance for all candidate reference genes changed to a certain extent in contrast with the control group. At 2 h, 4 h and 8 h after stimulation with Poly I:C in GPS cells, *Actin* presented the smallest variation of C_t value while *B2M* had the most distinct alteration for expression levels at 4 h and 8 h post stimulation of Poly I:C.

With regard to GPHK cells at 2 h after being stimulated with Poly I:C—except for *GAPDH*, whose C_t value change was 0.8—the C_t value variations of the rest of the six genes were greater than 1, among of which the highest variation (2.1) arose in *18S*. Additionally, in 12 h, *Actin*, *RPL13* and *EF1A* all had the smallest C_t value variation (0.1), whereas *GAPDH* showed the highest variation in expression level (1.1).

3.3. geNorm, NormFinder and BestKeeper Analysis

3.3.1. Stability of Reference Gene Expression in GPS and GPHK Cells under Normal Physiological Conditions

For the purpose of selecting the most stable reference genes in GPS and GPHK cells under normal physiological conditions, the expression stability for candidate reference genes was analyzed via three software, namely geNorm, NormFinder and BestKeeper [7,48,49]. geNorm is a Visual Basic application tool for Microsoft Excel and is able to assess the stability of reference genes based on the principle of keeping the expression ratio of two candidate reference genes in a constant state throughout the different experimental conditions [57]. When the M value is lowest, the candidate is considered the steadiest reference gene and vice versa. Moreover, geNorm is capable of screening the most suitable number of candidate references necessitating the normalization of target gene expression levels through calculation of pairwise variations between one examined gene and the rest of candidate genes. Unlike geNorm, NormFinder can generate the stability value (SV) of reference gene expression relying on the experimental data, producing the most reliable reference gene. Invariably, the candidate reference gene is the most reliable when the SV is the lowest. Apart from that, BestKeeper is able to single out the most reliable reference genes in light of standard deviation (SD) and

the coefficient of variation (CV) of C_t values, and the reference gene is more reliable when the SD and CV values are smaller. Consequently, the most stable candidate reference gene is obtained from the combination of the results generated from the three different software above, as previously reported [58].

According to analysis of geNorm, *18S* and *GAPDH* were the most stable reference genes the in GPS cells (Figure 1). As shown in Figure 2, $V_{2/3}$ values for GPS cells under normal conditions were less than 0.15 in light of the pairwise variations analysis, revealing that accurate normalization necessitate two pairs of the most stable reference genes. The rankings of expression stability from high to low, on the basis of results handled by NormFinder, was *18S* = *GAPDH* (0.026) > *UBCE* (0.039) > *Actin* (0.154) > *EF1A* (0.228) > *B2M* (0.492) > *RPL13* (1.095) (Table 4). Moreover, the results of BestKeeper shown in Table 5 manifested that the most reliable gene was *18S*, with a minimum standard deviation (SD) of 0.11, followed by *GAPDH*, *Actin*, *UBCE*, *EF1A*, *B2M* and *RPL13*. To summarize, analysis results based on three softwares suggested that the rankings of candidates from the most stable to the most unstable were as follow: *18S* > *GAPDH* > *UBCE* > *Actin* > *EF1A* > *B2M* > *RPL13* (Table 6).

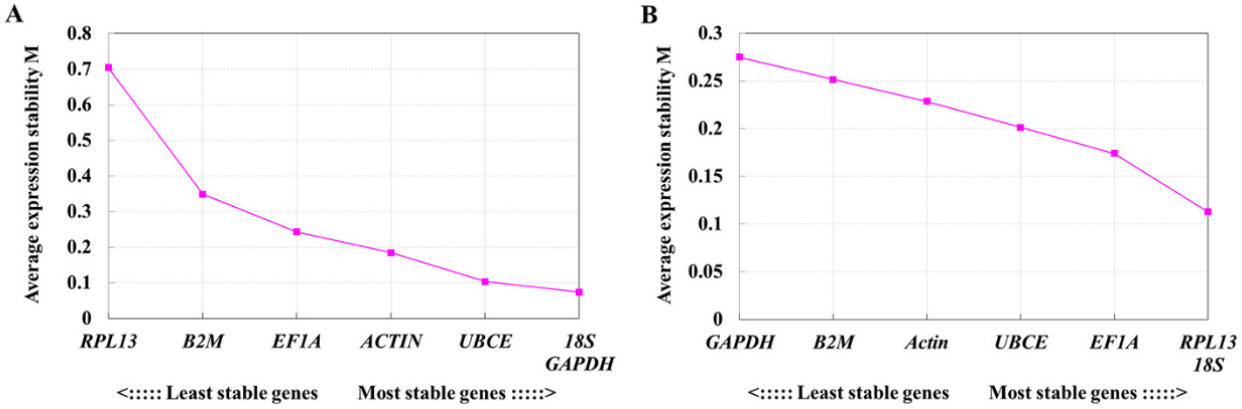

Figure 1. The expression stability of housekeeping gene candidates in GPS (**A**) and GPHK cells (**B**) under normal conditions assessed by geNorm. Based on the C_t value from qRT-PCR, geNorm software was used to analyze the stability of reference genes (M). The most stable reference genes are those who possessed the smallest M value.

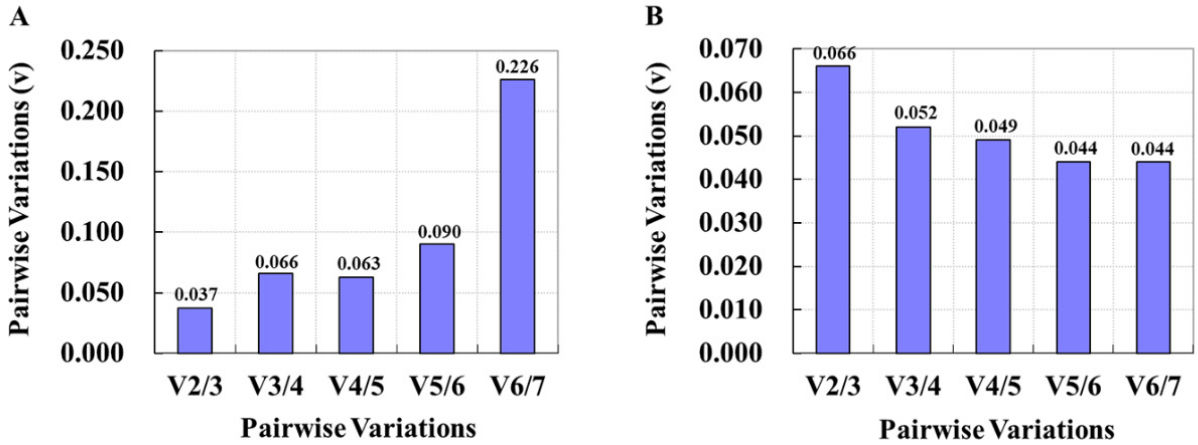

Figure 2. Optimum pairs of housekeeping gene candidates necessitated for normalization in GPS (**A**) and GPHK cells (**B**) under normal conditions according to geNorm analysis. Pairwise variations (v) between normalization factors of the internal reference gene based on geNorm analysis were evaluated.

Table 4. The expression stability of housekeeping gene candidates in GPS and GPHK cells under normal conditions assessed by NormFinder.

Cell Lines	Ranking Order	1	2	3	4	5	6	7
GPS	Gene	*18S*	*GAPDH*	*UBCE*	*Actin*	*EF1A*	*B2M*	*RPL13*
	Stability	0.026	0.026	0.039	0.154	0.228	0.492	1.095
GPHK	Gene	*UBCE*	*Actin*	*RPL13*	*EF1A*	*B2M*	*18S*	*GAPDH*
	Stability	0.075	0.101	0.103	0.114	0.154	0.188	0.206

Table 5. The expression stability of housekeeping gene candidates in GPS and GPHK cells under normal conditions assessed by BestKeeper analysis.

Cell Lines	Genes	Standard Deviation (SD)	Correlation Coefficient (r)	Coefficient of Variation (CV)	*p*-Value	Ranking Order
GPS	*18S*	0.11	0.674	1.64	0.016	1
	GAPDH	0.16	0.280	0.59	0.379	2
	Actin	0.28	0.369	2.25	0.236	3
	UBCE	0.31	0.610	1.44	0.035	4
	EF1A	0.36	0.626	2.86	0.029	5
	B2M	0.61	0.174	1.95	0.588	6
	RPL13	1.09	−0.227	4.76	0.476	7
GPHK	*RPL13*	0.12	0.811	0.71	0.001	1
	18S	0.16	0.386	2.08	0.216	2
	EF1A	0.24	0.524	1.59	0.080	3
	B2M	0.25	0.691	1.21	0.013	4
	Actin	0.30	0.858	1.98	0.001	5
	UBCE	0.32	0.772	1.36	0.003	6
	GAPDH	0.45	0.742	1.33	0.006	7

Table 6. The recommended comprehensive ranking of housekeeping genes based on three algorithm analyses in GPS and GPHK cells under normal conditions.

Cell Lines	Ranking Order	geNorm	NormFinder	BestKeeper	Recommended Comprehensive Ranking
GPS	1	*18S/GAPDH*	*18S/GAPDH*	*18S*	*18S*
	2			*GAPDH*	*GAPDH*
	3	*UBCE*	*UBCE*	*Actin*	*UBCE*
	4	*Actin*	*Actin*	*UBCE*	*Actin*
	5	*EF1A*	*EF1A*	*EF1A*	*EF1A*
	6	*B2M*	*B2M*	*B2M*	*B2M*
	7	*RPL13*	*RPL13*	*RPL13*	*RPL13*
GPHK	1	*RPL13/18S*	*UBCE*	*RPL13*	*RPL13*
	2		*Actin*	*18S*	*18S*
	3	*EF1A*	*RPL13*	*EF1A*	*EF1A*
	4	*UBCE*	*EF1A*	*B2M*	*UBCE*
	5	*Actin*	*B2M*	*Actin*	*Actin*
	6	*B2M*	*18S*	*UBCE*	*B2M*
	7	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*

Similarly, based on geNorm (version 1.0), NormFinder (version 1.0) and BestKeeper (version 1.0) softwares, the stable reference genes in GPHK cells under normal physiological conditions are presented in Figures 1 and 2 and Tables 4 and 5. Through comprehensive analysis, the ranking of optimal candidate reference genes was: *RPL13* > *18S* > *EF1A* > *UBCE* > *Actin* > *B2M* > *GAPDH* (Table 6).

3.3.2. Stability of Reference Gene Expression in GPS and GPHK Cells under LPS Stimulating Condition

Through geNorm analysis on all the C_t values of candidates in GPS cells under the stimulating condition of LPS, *RPL13/Actin*, *RPL13/18S*, *RPL13/Actin* and *RPL13/B2M* were screened as the most suitable reference genes for GPS cells at 2, 4, 8 and 12 h after being stimulated with LPS, respectively (Figure 3). As shown in Figure 4, considering that all the $V_{2/3}$ value were less than 0.15, two pairs of reference genes sufficed to normalize accurately. In view of the analysis of NormFinder, the most appropriate candidates were *RPL13/Actin*, *RPL13*, *RPL13/Actin* and *GAPDH* at 2, 4, 8 and 12 h post stimulation of LPS in GPS cells, respectively (Table 7). Additionally, the results of BestKeeper indicated that *RPL13* (SD = 0.36), *EF1A* (SD = 0.20), *B2M* (SD = 0.41) and *RPL13* (SD = 0.19) were the most appropriate reference genes in GPS cells stimulated with LPS for 2, 4, 8 and 12 h, respectively (Table 8). In sumary, the most reliable reference genes in GPS cells after being stimulated for 2, 4, 8 and 12 h, in view of comprehensive analysis, were all *RPL13* (Table 9).

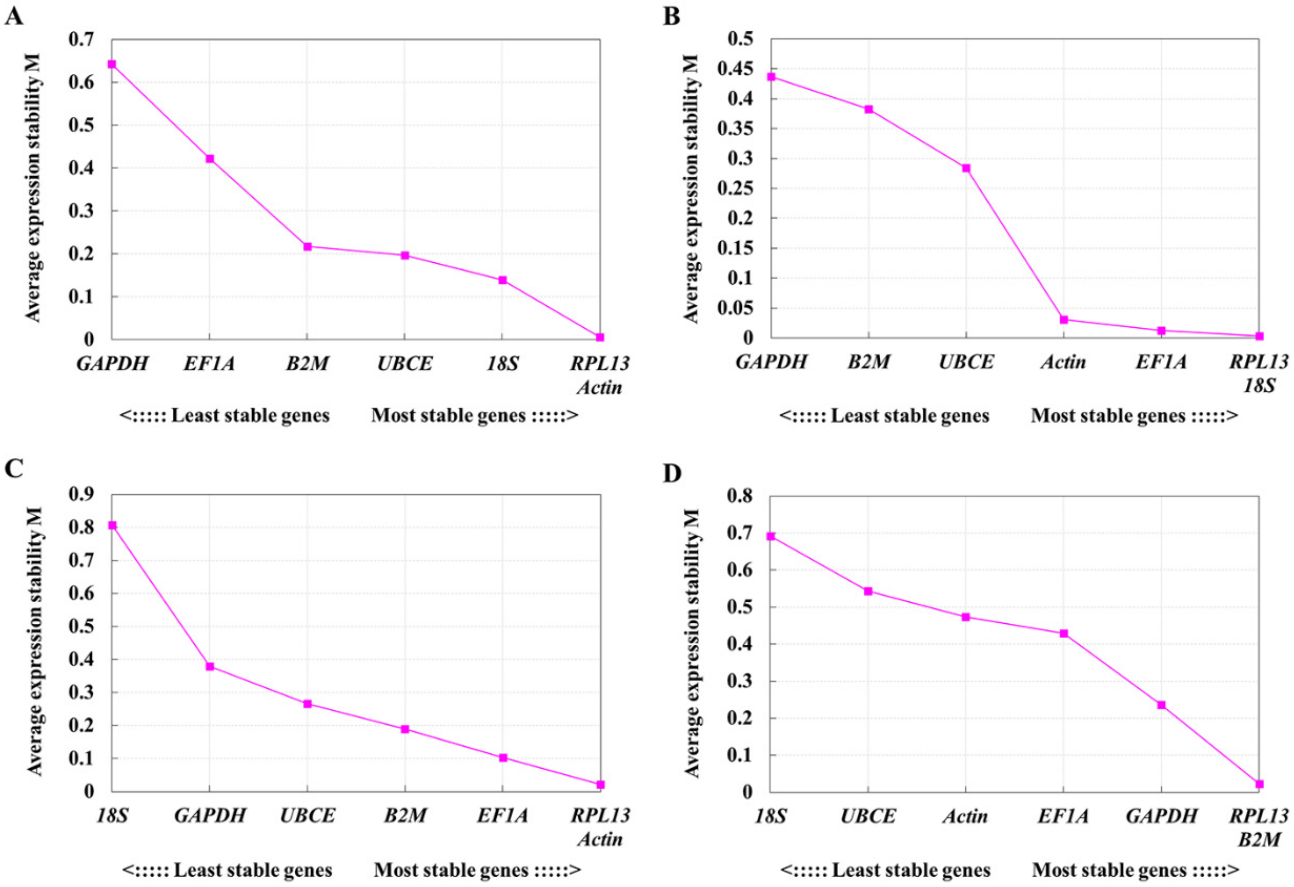

Figure 3. The expression stability of housekeeping gene candidates in GPS cells under stimulation with LPS for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) assessed by geNorm. According to the C_t values presented by seven housekeeping gene candidate stimulations with LPS or PBS, the average expression stability of housekeeping gene candidates can be evaluated as M by geNorm; not only can the M value not be more than 1.5, but the most reliable housekeeping genes have the most minimal M value as well.

In GPHK cells, after being stimulated with LPS, the optimal candidate gene was determined by virtue of the analysis of geNorm (Figures 5 and 6), NormFinder (Table 7) and BestKeeper (Table 8). To summarize, it was conspicuous that *18S* was the most appropriate reference gene in GPHK cells stimulated with LPS for 2, 4, 8 and 12 h, respectively (Table 9).

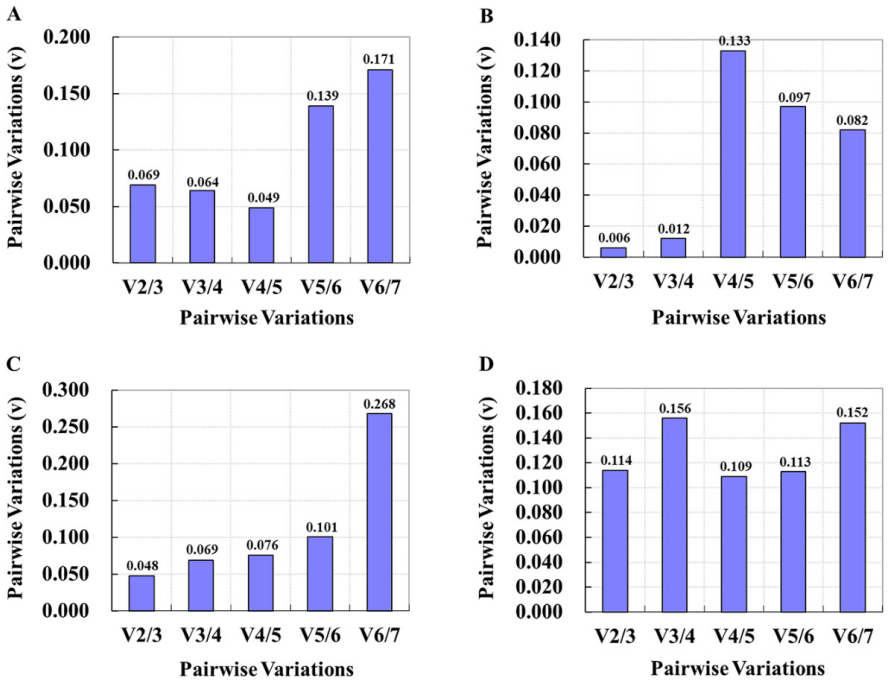

Figure 4. Optimum pairs of housekeeping gene candidates necessitated for normalization in GPS cells under stimulation with LPS for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) according to geNorm analysis. The optimal number of the reference gene depends on $V_{n/n+1}$ value via geNorm analysis. Moreover, the value is popularly less than 0.15.

Table 7. The expression stability of housekeeping gene candidates in GPS and GPHK cells under stimulation with LPS or Poly I:C for 2, 4, 8 and 12 h, as assessed by NormFinder.

Cell Lines	Stimuli	Ranking Order	1		2		3		4		5		6		7	
GPS	LPS	2 h	*RPL13/Actin*	(0.002)			*18S*	(0.211)	*UBCE*	(0.327)	*B2M*	(0.377)	*EF1A*	(0.393)	*GAPDH*	(0.820)
		4 h	*RPL13*	(0.207)	*18S*	(0.209)	*EF1A*	(0.224)	*Actin*	(0.259)	*UBCE*	(0.264)	*B2M*	(0.306)	*GAPDH*	(0.384)
		8 h	*RPL13/Actin*	(0.007)			*EF1A*	(0.047)	*B2M*	(0.075)	*UBCE*	(0.307)	*GAPDH*	(0.588)	*18S*	(1.299)
		12 h	*GAPDH*	(0.114)	*B2M*	(0.203)	*RPL13*	(0.228)	*EF1A*	(0.273)	*Actin*	(0.345)	*UBCE*	(0.562)	*18S*	(0.725)
	Poly I:C	2 h	*UBCE/Actin*	(0.026)			*EF1A*	(0.066)	*RPL13*	(0.130)	*18S*	(0.385)	*B2M*	(0.508)	*GAPDH*	(0.848)
		4 h	*UBCE/Actiin*	(0.010)			*18S*	(0.164)	*EF1A*	(0.387)	*RPL13*	(0.412)	*GAPDH*	(0.605)	*B2M*	(0.662)
		8 h	*Actin*	(0.055)	*18S*	(0.207)	*UBCE*	(0.213)	*EF1A*	(0.299)	*RPL13*	(0.324)	*GAPDH*	(0.435)	*B2M*	(0.985)
		12 h	*UBCE/EF1A*	(0.362)			*18S*	(0.413)	*Actin*	(0.443)	*RPL13*	(0.508)	*GAPDH*	(0.535)	*B2M*	(0.634)
GPHK	LPS	2 h	*18S/Actin*	(0.003)			*RPL13*	(0.029)	*EF1A*	(0.210)	*B2M*	(0.228)	*UBCE*	(0.417)	*GAPDH*	(0.673)
		4 h	*18S/Actin*	(0.002)			*EF1A*	(0.137)	*UBCE*	(0.223)	*B2M*	(0.256)	*RPL13*	(0.360)	*GAPDH*	(1.001)
		8 h	*EF1A*	(0.030)	*UBCE*	(0.140)	*18S*	(0.142)	*RPL13*	(0.196)	*Actin*	(0.198)	*GAPDH*	(0.305)	*B2M*	(0.348)
		12 h	*EF1A*	(0.001)	*18S*	(0.007)	*RPL13*	(0.020)	*UBCE*	(0.055)	*Actin*	(0.254)	*GAPDH*	(0.295)	*B2M*	(0.339)
	Poly I:C	2 h	*EF1A/Actin*	(0.001)			*B2M*	(0.040)	*UBCE*	(0.153)	*RPL13*	(0.154)	*GAPDH*	(0.354)	*18S*	(0.392)
		4 h	*EF1A/RPL13*	(0.013)			*Actin*	(0.135)	*UBCE*	(0.374)	*18S*	(0.384)	*GAPDH*	(0.430)	*B2M*	(0.963)
		8 h	*EF1A*	(0.010)	*RPL13*	(0.016)	*Actin*	(0.031)	*18S*	(0.114)	*UBCE*	(0.285)	*GAPDH*	(0.399)	*B2M*	(1.383)
		12 h	*RPL13*	(0.052)	*EF1A*	(0.062)	*Actin*	(0.109)	*18S*	(0.199)	*UBCE*	(0.281)	*B2M*	(0.426)	*GAPDH*	(0.552)

Table 8. The expression stability of housekeeping gene candidates in GPS and GPHK cells under stimulation with LPS or Poly I:C for 2, 4, 8 and 12 h, as assessed by BestKeeper analysis.

Cell Lines	Stimuli	Ranking Order	Genes				Standard Deviation (SD)				Correlation Coefficient (r)				Coefficient of Variation (CV)				*p*-Value			
			2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h
GPS	LPS	1	*RPL13*	*EF1A*	*B2M*	*RPL13*	0.36	0.20	0.41	0.19	0.768	0.607	0.767	0.476	1.49	1.33	1.48	0.77	0.074	0.201	0.075	0.341
		2	*Actin*	*RPL13*	*EF1A*	*B2M*	0.40	0.23	0.63	0.43	0.968	0.454	0.757	0.738	2.76	0.93	4.49	1.60	0.002	0.365	0.081	0.094
		3	*EF1A*	*18S*	*RPL13*	*18S*	0.48	0.26	0.64	0.48	0.594	0.754	0.674	0.021	3.77	3.19	2.52	5.60	0.213	0.083	0.143	0.970
		4	*18S*	*Actin*	*Actin*	*GAPDH*	0.52	0.29	0.67	0.55	0.923	0.765	0.914	0.883	6.14	1.81	4.53	2.05	0.009	0.076	0.011	0.020
		5	*UBCE*	*UBCE*	*UBCE*	*EF1A*	0.54	0.53	0.78	0.58	0.544	0.628	0.807	0.719	2.21	1.88	3.09	4.52	0.263	0.183	0.052	0.107
		6	*GAPDH*	*B2M*	*18S*	*Actin*	0.69	0.57	0.81	0.67	−0.022	0.619	−0.166	0.712	2.83	2.05	9.17	4.46	0.970	0.189	0.751	0.112
		7	*B2M*	*GAPDH*	*GAPDH*	*UBCE*	0.76	0.62	1.00	0.81	−0.059	0.705	0.779	0.662	2.80	2.33	3.63	3.18	0.910	0.117	0.067	0.151
	Poly I:C	1	*18S*	*UBCE*	*UBCE*	*18S*	0.39	0.17	0.26	0.29	0.768	0.737	0.497	0.061	4.93	0.64	0.99	3.43	0.074	0.095	0.314	0.910
		2	*UBCE*	*18S*	*Actin*	*UBCE*	0.46	0.18	0.27	0.32	0.576	0.012	0.756	0.368	1.92	2.21	1.73	1.19	0.231	0.985	0.082	0.474
		3	*EF1A*	*Actin*	*18S*	*EF1A*	0.48	0.25	0.30	0.35	0.927	0.841	0.774	0.488	3.69	1.57	3.64	2.59	0.008	0.036	0.071	0.325
		4	*Actin*	*EF1A*	*EF1A*	*Actin*	0.50	0.43	0.35	0.45	0.972	0.325	0.586	0.571	3.44	2.85	2.31	2.80	0.001	0.528	0.221	0.237
		5	*B2M*	*RPL13*	*GAPDH*	*RPL13*	0.58	0.43	0.46	0.59	0.005	0.318	0.348	0.338	2.20	1.74	1.64	2.46	0.993	0.540	0.500	0.511
		6	*RPL13*	*GAPDH*	*RPL13*	*B2M*	0.59	0.52	0.56	0.69	0.798	0.365	0.824	0.414	2.47	1.95	2.13	2.60	0.057	0.479	0.044	0.414
		7	*GAPDH*	*B2M*	*B2M*	*GAPDH*	0.79	0.69	0.91	0.70	0.003	0.235	−0.079	0.665	3.07	2.60	3.32	2.63	0.993	0.656	0.881	0.150

Table 8. *Cont.*

Cell Lines	Stimuli	Ranking Order	Genes				Standard Deviation (SD)				Correlation Coefficient (r)				Coefficient of Variation (CV)				*p*-Value			
GPHK	LPS	1	*18S*	*18S*	*18S*	*18S*	0.14	0.16	0.22	0.16	0.496	0.781	0.838	0.367	1.50	2.14	2.82	1.97	0.318	0.067	0.037	0.474
		2	*Actin*	*Actin*	*Actin*	*EF1A*	0.15	0.21	0.26	0.22	0.795	0.793	0.835	0.490	1.07	1.49	1.73	1.54	0.059	0.060	0.039	0.325
		3	*RPL13*	*B2M*	*UBCE*	*RPL13*	0.20	0.33	0.33	0.25	0.285	0.567	−0.147	0.322	1.20	1.72	1.46	1.55	0.587	0.240	0.779	0.534
		4	*B2M*	*EF1A*	*EF1A*	*Actin*	0.37	0.34	0.36	0.34	0.626	0.129	0.663	0.510	1.91	2.39	2.48	2.45	0.183	0.808	0.151	0.300
		5	*UBCE*	*UBCE*	*RPL13*	*B2M*	0.48	0.40	0.38	0.35	0.309	0.562	0.649	0.011	2.14	1.87	2.29	1.88	0.551	0.245	0.162	0.985
		6	*EF1A*	*RPL13*	*B2M*	*UBCE*	0.48	0.40	0.39	0.50	0.439	0.907	0.069	0.337	3.15	2.60	1.85	2.11	0.383	0.013	0.895	0.511
		7	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*	0.56	0.82	0.49	0.66	−0.083	−0.575	0.207	0.784	1.83	2.62	1.51	2.05	0.873	0.231	0.696	0.065
	Poly I:C	1	*UBCE*	*18S*	*18S*	*Actin*	0.55	0.04	0.08	0.39	0.883	−0.611	−0.106	0.341	2.47	0.47	1.07	2.76	0.020	0.198	0.844	0.506
		2	*EF1A*	*EF1A*	*EF1A*	*RPL13*	0.69	0.48	0.25	0.40	0.853	0.978	0.402	0.729	4.80	3.33	1.70	2.45	0.031	0.001	0.429	0.100
		3	*Actin*	*B2M*	*Actin*	*EF1A*	0.69	0.53	0.29	0.43	0.913	−0.341	0.609	0.904	5.06	2.79	1.94	3.01	0.011	0.506	0.198	0.013
		4	*GAPDH*	*Actin*	*UBCE*	*18S*	0.70	0.55	0.36	0.44	0.657	0.991	0.426	0.949	2.35	3.76	1.57	5.57	0.157	0.001	0.400	0.004
		5	*B2M*	*RPL13*	*RPL13*	*B2M*	0.75	0.57	0.37	0.46	0.987	0.953	0.482	0.257	3.95	3.48	2.16	2.48	0.001	0.003	0.333	0.624
		6	*RPL13*	*UBCE*	*GAPDH*	*GAPDH*	0.99	0.71	0.61	0.59	0.921	0.959	0.672	−0.138	6.22	3.16	1.87	1.87	0.009	0.002	0.143	0.793
		7	*18S*	*GAPDH*	*B2M*	*UBCE*	1.05	0.75	1.18	0.60	0.929	0.722	0.055	−0.563	12.49	2.40	6.01	2.53	0.007	0.105	0.918	0.245

Table 9. The recommended comprehensive ranking of housekeeping genes based on three algorithm analyses in GPS and GPHK cells under stimulation with LPS and Poly I:C for 2, 4, 8 and 12 h.

Cell Lines	Stimuli	Ranking Order	geNorm				NormFinder				BestKeeper				Recommended Comprehensive Ranking			
			2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h	2 h	4 h	8 h	12 h
GPS	LPS	1	*RPL13/ Actin*	*RPL13/ 18S*	*RPL13/ Actin*	*RPL13/ B2M*	*RPL13/ Actin*	*RPL13*	*RPL13/ Actin*	*GAPDH*	*RPL13*	*EF1A*	*B2M*	*RPL13*	*RPL13*	*RPL13*	*RPL13*	*RPL13*
		2						*18S*		*B2M*	*Actin*	*RPL13*	*EF1A*	*B2M*	*Actin*	*18S*	*Actin*	*B2M*
		3	*18s*	*EF1A*	*EF1A*	*GAPDH*	*18S*	*EF1A*	*EF1A*	*RPL13*	*EF1A*	*18S*	*RPL13*	*18S*	*18S*	*EF1A*	*EF1A*	*GAPDH*
		4	*UBCE*	*Actin*	*B2M*	*EF1A*	*UBCE*	*Actin*	*B2M*	*EF1A*	*18S*	*Actin*	*Actin*	*GAPDH*	*UBCE*	*Actin*	*B2M*	*EF1A*
		5	*B2M*	*UBCE*	*UBCE*	*Actin*	*B2M*	*UBCE*	*UBCE*	*Actin*	*UBCE*	*UBCE*	*UBCE*	*EF1A*	*EF1A*	*UBCE*	*UBCE*	*Actin*
		6	*EF1A*	*B2M*	*GAPDH*	*UBCE*	*EF1A*	*B2M*	*GAPDH*	*UBCE*	*GAPDH*	*B2M*	*18S*	*Actin*	*B2M*	*B2M*	*GAPDH*	*UBCE*
		7	*GAPDH*	*GAPDH*	*18S*	*18S*	*GAPDH*	*GAPDH*	*18S*	*18S*	*B2M*	*GAPDH*	*GAPDH*	*UBCE*	*GAPDH*	*GAPDH*	*18S*	*18S*
	Poly I:C	1	*UBCE/ Actin*	*UBCE/ Actin*	*UBCE/ 18S*	*UBCE/ EF1A*	*UBCE/ Actin*	*UBCE/ Actin*	*Actin*	*UBCE/ EF1A*	*18S*	*UBCE*	*UBCE*	*18S*	*UBCE*	*UBCE*	*UBCE*	*UBCE*
		2						*18S*			*UBCE*	*18S*	*Actin*	*UBCE*	*Actin*	*Actin*	*18S*	*EF1A*
		3	*EF1A*	*18S*	*EF1A*	*18S*	*EF1A*	*UBCE*	*18S*	*EF1A*	*EF1A*	*Actin*	*18S*	*EF1A*	*EF1A*	*18S*	*Actin*	*18S*
		4	*RPL13*	*RPL13*	*RPL13*	*Actin*	*RPL13*	*EF1A*	*EF1A*	*Actin*	*Actin*	*EF1A*	*EF1A*	*Actin*	*18S*	*EF1A*	*EF1A*	*Actin*
		5	*18S*	*GAPDH*	*Actin*	*RPL13*	*18S*	*RPL13*	*RPL13*	*RPL13*	*B2M*	*RPL13*	*GAPDH*	*RPL13*	*RPL13*	*RPL13*	*RPL13*	*RPL13*
		6	*B2M*	*EF1A*	*GAPDH*	*GAPDH*	*B2M*	*GAPDH*	*GAPDH*	*GAPDH*	*RPL13*	*GAPDH*	*RPL13*	*B2M*	*B2M*	*GAPDH*	*GAPDH*	*GAPDH*
		7	*GAPDH*	*B2M*	*B2M*	*B2M*	*GAPDH*	*B2M*	*B2M*	*B2M*	*GAPDH*	*B2M*	*B2M*	*GAPDH*	*GAPDH*	*B2M*	*B2M*	*B2M*
GPHK	LPS	1	*18S/ Actin*	*18S/ Actin*	*18S/ UBCE*	*18S/ EF1A*	*18S/ Actin*	*18S/ Actin*	*EF1A*	*EF1A*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*
		2						*UBCE*	*18S*	*Actin*	*Actin*	*Actin*	*EF1A*	*Actin*	*Actin*	*Actin*	*UBCE*	*EF1A*
		3	*RPL13*	*UBCE*	*RPL13*	*UBCE*	*RPL13*	*EF1A*	*18S*	*RPL13*	*RPL13*	*B2M*	*UBCE*	*RPL13*	*RPL13*	*UBCE*	*EF1A*	*RPL13*
		4	*B2M*	*B2M*	*Actin*	*RPL13*	*EF1A*	*UBCE*	*RPL13*	*UBCE*	*B2M*	*EF1A*	*EF1A*	*Actin*	*B2M*	*B2M*	*Actin*	*UBCE*
		5	*UBCE*	*RPL13*	*EF1A*	*GAPDH*	*B2M*	*B2M*	*Actin*	*Actin*	*UBCE*	*UBCE*	*RPL13*	*B2M*	*UBCE*	*EF1A*	*RPL13*	*Actin*
		6	*EF1A*	*EF1A*	*GAPDH*	*Actin*	*UBCE*	*RPL13*	*GAPDH*	*GAPDH*	*EF1A*	*RPL13*	*B2M*	*UBCE*	*EF1A*	*RPL13*	*GAPDH*	*GAPDH*
		7	*GAPDH*	*GAPDH*	*B2M*	*B2M*	*GAPDH*	*GAPDH*	*B2M*	*B2M*	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*	*B2M*	*B2M*
	Poly I:C	1	*EF1A/ Actin*	*EF1A/ RPL13*	*EF1A/ Actin*	*EF1A/ RPL13*	*EF1A/ Actin*	*EF1A/ RPL13*	*EF1A*	*RPL13*	*UBCE*	*18S*	*18S*	*Actin*	*EF1A*	*EF1A*	*EF1A*	*RPL13*
		2						*RPL13*	*EF1A*	*EF1A*	*EF1A*	*EF1A*	*EF1A*	*RPL13*	*Actin*	*RPL13*	*Actin*	*EF1A*
		3	*B2M*	*Actin*	*RPL13*	*Actin*	*B2M*	*Actin*	*Actin*	*Actin*	*Actin*	*B2M*	*Actin*	*EF1A*	*UBCE*	*Actin*	*RPL13*	*Actin*
		4	*UBCE*	*UBCE*	*UBCE*	*18S*	*UBCE*	*UBCE*	*18S*	*18S*	*GAPDH*	*Actin*	*UBCE*	*18S*	*B2M*	*18S*	*18S*	*18S*
		5	*RPL13*	*GAPDH*	*GAPDH*	*UBCE*	*RPL13*	*18S*	*UBCE*	*UBCE*	*B2M*	*RPL13*	*RPL13*	*B2M*	*RPL13*	*UBCE*	*UBCE*	*UBCE*
		6	*GAPDH*	*18S*	*18S*	*B2M*	*GAPDH*	*GAPDH*	*GAPDH*	*B2M*	*RPL13*	*UBCE*	*GAPDH*	*GAPDH*	*GAPDH*	*B2M*	*GAPDH*	*B2M*
		7	*18S*	*B2M*	*B2M*	*GAPDH*	*18S*	*B2M*	*B2M*	*GAPDH*	*18S*	*GAPDH*	*B2M*	*UBCE*	*18S*	*GAPDH*	*B2M*	*GAPDH*

3.3.3. Stability of Reference Gene Expression in GPS and GPHK Cells under Poly I:C Stimulating Condition

At 2, 4, 8 and 12 h after being stimulated with Poly I:C, seven reference gene candidates in GPS cells presented relatively stable expression level on account of their M and $V_{2/3}$ values all being less than 1.5 and 0.15, respectively. Moreover, in light of the analysis by geNorm, *UBCE/Actin*, *UBCE/Actin*, *UBCE/18S* and *UBCE/EF1A* were considered as the most appropriate reference genes for GPS cells, respectively (Figures 7 and 8). On the basis of NormFinder, the most stable reference genes in GPS cells stimulated with Poly I:C for 2, 4, 8 and 12 h were *UBCE/Actin*, *UBCE/Actin*, *Actin* and *UBCE/EF1A*, respectively (Table 7), which was consistent with the results analyzed by geNorm. Moreover, with the aid of the BestKeeper algorithm, *18S*, *UBCE*, *UBCE* and *18S*, all of which had the lowest SD value, were deemed the most suitable reference genes for GPS cells at 2, 4, 8 and 12 h post stimulation of Poly I:C (Table 8).

Comprehensive analysis indicated that at 2, 4, 8 and 12 h after being stimulated with Poly I:C, the most reliable candidates in GPS cells were *UBCE* (Table 9). Combined with three softwares, geNorm (Figures 9 and 10), NormFinder (Table 7) and BestKeeper (Table 8), the most suitable reference genes were *EF1A*, *EF1A*, *EF1A* and *RPL13* after being stimulated with Poly I:C in GPHK cells at 2, 4, 8 and 12 h, respectively (Table 9).

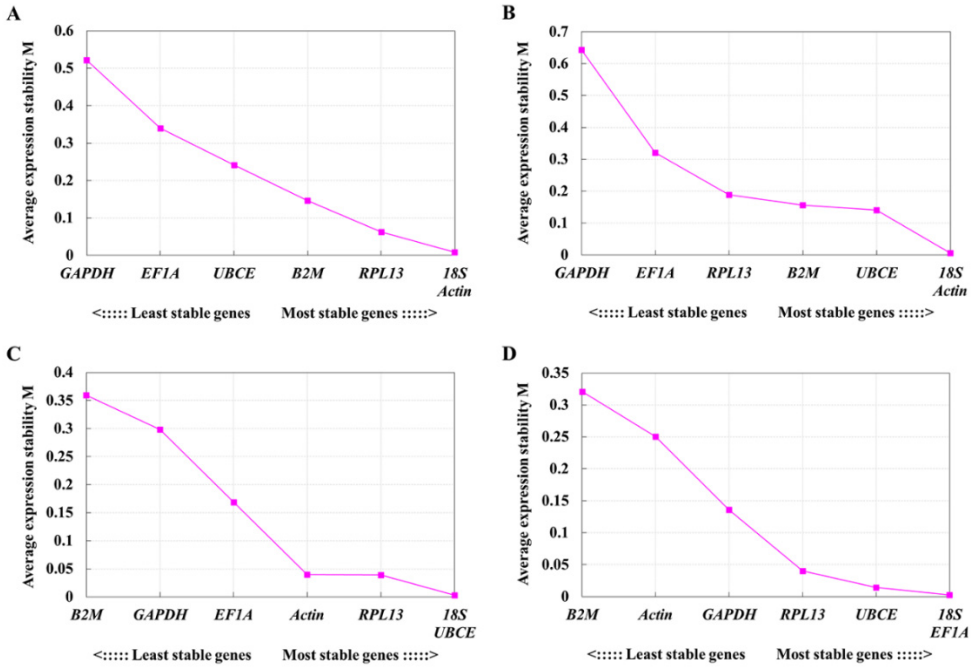

Figure 5. The expression stability of housekeeping gene candidates in GPHK cells under stimulation with LPS for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**), assessed by geNorm. According to the C_t values presented by the seven housekeeping gene candidate stimulations with LPS or PBS, the average expression of stability for housekeeping gene candidates can be evaluated as M by geNorm; not only can the M value not be more than 1.5, but the most reliable housekeeping genes have the most minimal M value as well.

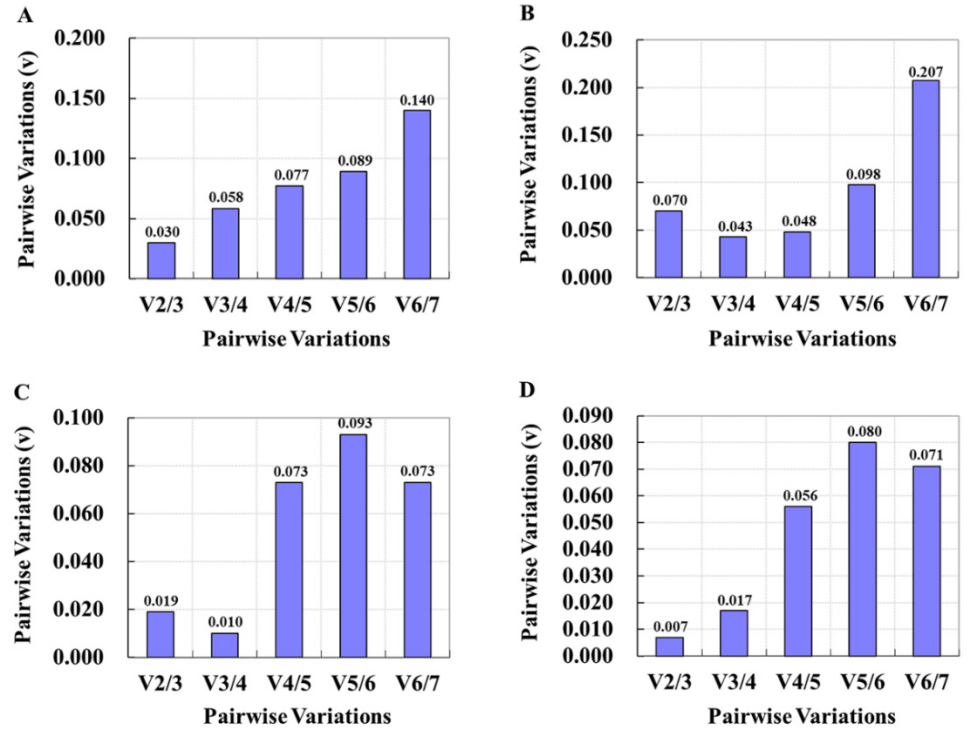

Figure 6. Optimum pairs of housekeeping gene candidates necessitated for normalization in GPHK cells under stimulation with LPS for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) according to geNorm analysis. The optimal number of reference genes depends on the $V_{n/n+1}$ value via geNorm analysis; moreover, the value is popularly less than 0.15.

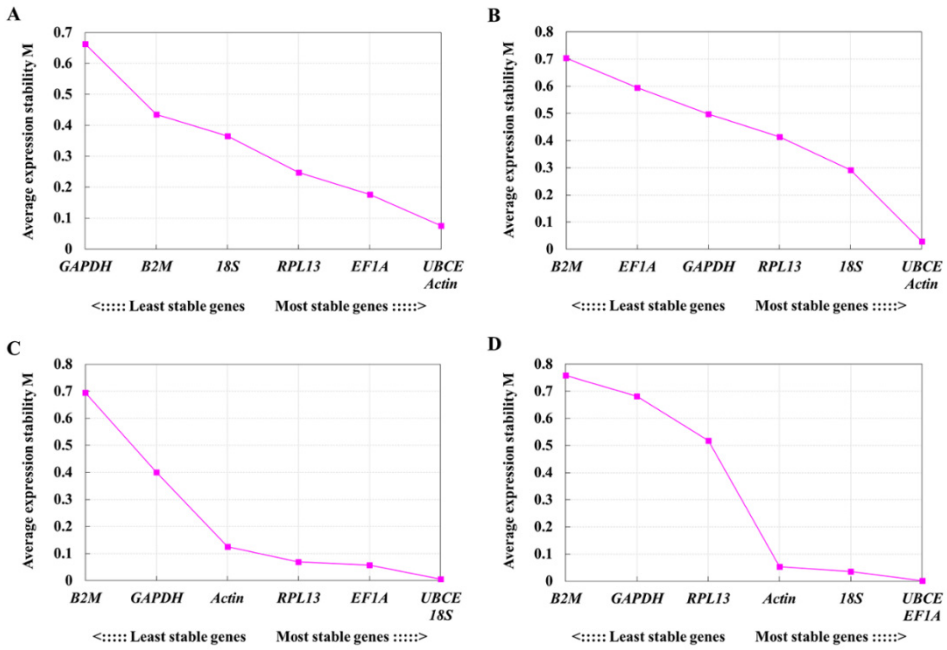

Figure 7. The expression stability of housekeeping gene candidates in GPS cells under stimulation with Poly I:C for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) assessed by geNorm. According to the C_t values presented by seven housekeeping gene candidate stimulations with Poly I:C or PBS, the average expression stability of housekeeping gene candidates can be evaluated M by geNorm, and not only can the M value not be more than 1.5, but the most reliable housekeeping genes have the most minimal M value as well.

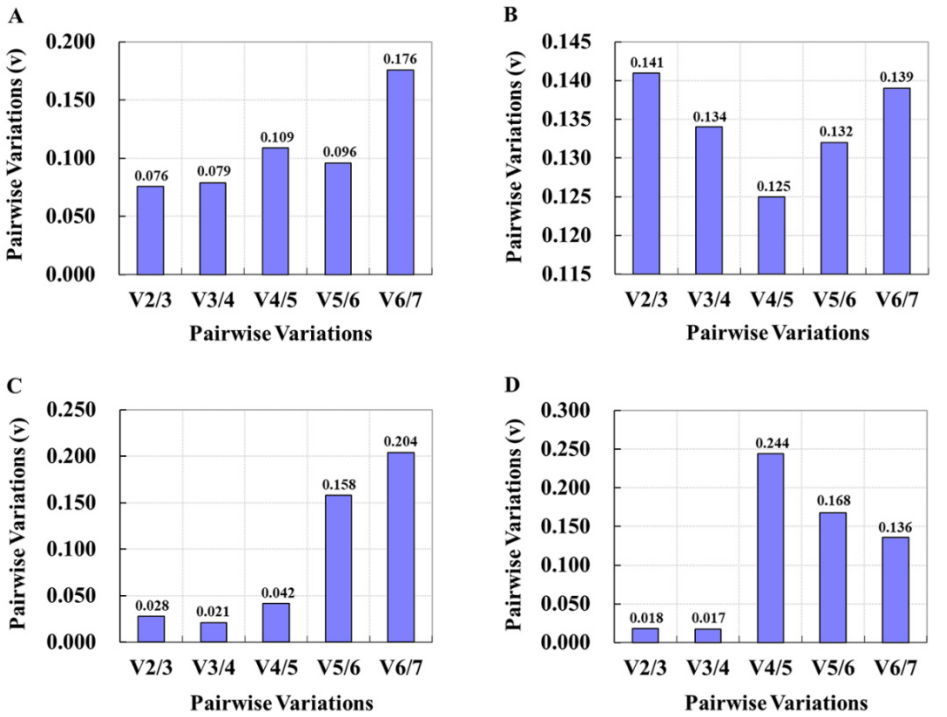

Figure 8. Optimum pairs of housekeeping gene candidates necessitated for normalization in GPS cells under stimulation with Poly I:C for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) according to geNorm analysis. The optimal number of reference genes depends on the $V_{n/n+1}$ value via geNorm analysis; moreover, the value is popularly less than 0.15.

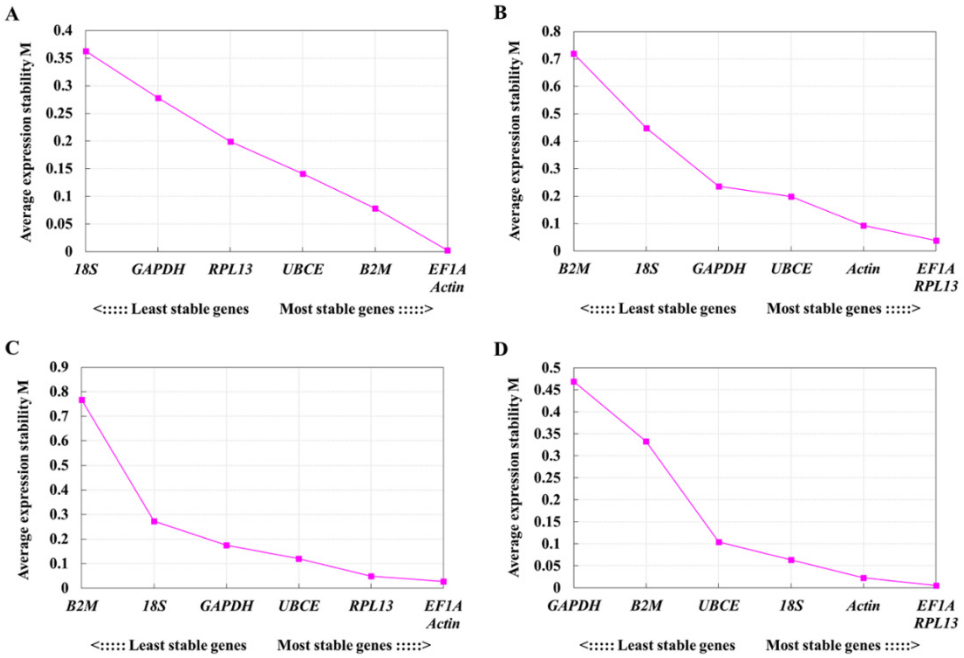

Figure 9. The expression stability of housekeeping gene candidates in GPHK cells under stimulation with Poly I:C for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) assessed by geNorm. According to the C_t values presented by seven housekeeping gene candidate stimulations with Poly I:C or PBS, the average expression stability of housekeeping gene candidates can be evaluated M by geNorm, and not only can the M value not be more than 1.5, but the most reliable housekeeping genes have the most minimal M value as well.

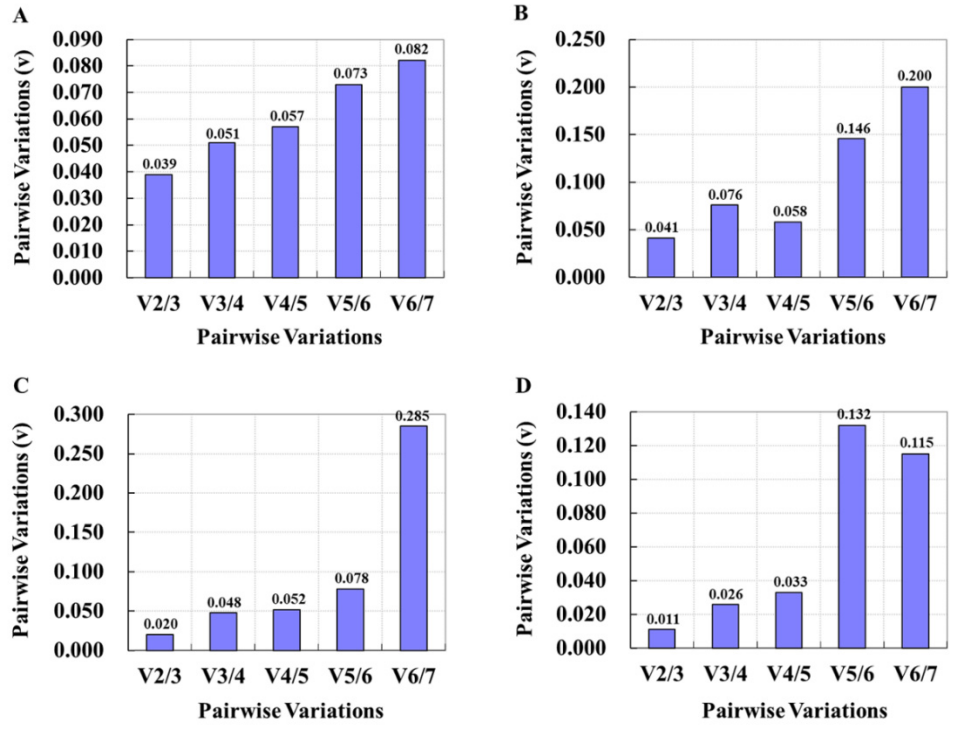

Figure 10. Optimum pairs of housekeeping gene candidates necessitated for normalization in GPHK cells under stimulation with Poly I:C for 2 (**A**), 4 (**B**), 8 (**C**) and 12 h (**D**) according to geNorm analysis. The optimal number of reference genes depends on the $V_{n/n+1}$ value via geNorm analysis; moreover, the value is popularly less than 0.15.

3.4. Verification of Screened Reference Genes

In the light of the pairwise variation results analyzed by geNorm, two pairs of the most stable reference genes sufficed to normalize accurately for gene expression analysis because all $V_{2/3}$ values were less than 0.15. Thereby, in a bid to further verify the reliability of screened suitable reference genes, two pairs of the most stable genes and one pair of the most unstable gene were selected to standardize the expression of target genes.

As shown in Figure 11, there was considerable discrepancy between the expression profile of target genes when the difference for expression stability of selected reference genes was relatively great. Under stimulation with LPS, *RPL13*/*Actin* and *18S*/*Actin* were the most stable genes in GPS and GPHK cells, respectively, whereas *GAPDH* was the least stable gene in two cell lines. After being stimulated with Poly I:C, the most appropriate reference genes in GPS and GPHK cells were *UBCE*/*Actin* and *EF1A*/*Actin*, respectively, while *B2M* and *GAPDH* were identified as the most unstable candidates in GPS and GPHK cells, respectively.

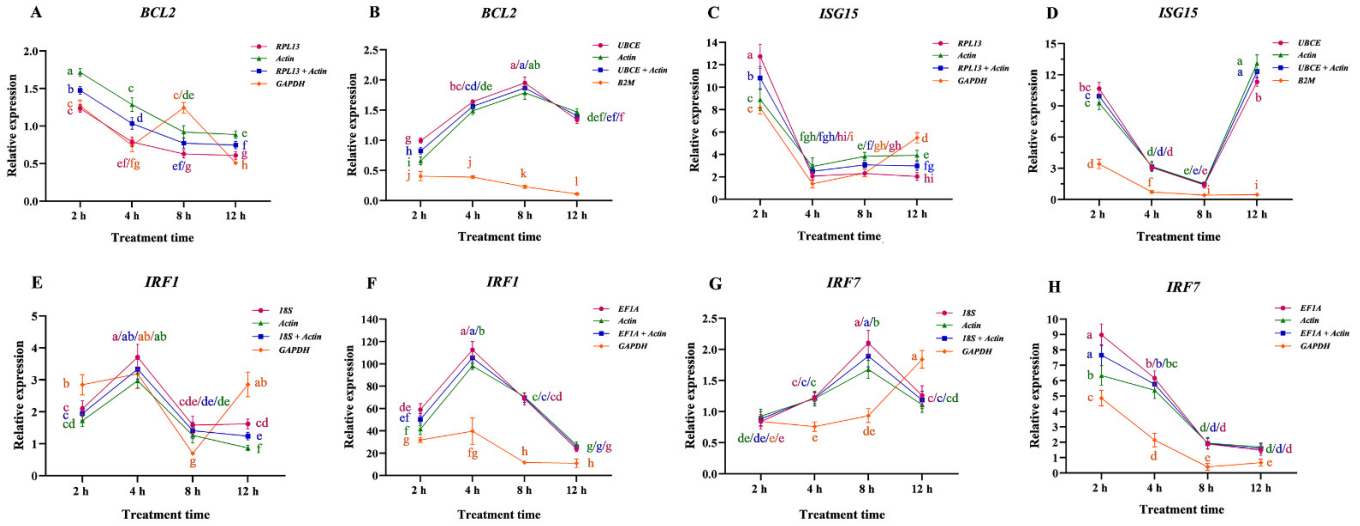

Figure 11. Expression profile for target genes in GPS and GPHK cells under stimulation with LPS or Poly I:C by virtue of screened reference genes. (**A**,**C**) show the relative expression levels of *BCL2* and *ISG15* in GPS cells after being stimulated with LPS. (**B**,**D**) show the relative expression levels of *BCL2* and *ISG15* in GPS cells after being stimulated with Poly I:C. Relative expression levels of *IRF1* and *IRF7* in GPHK cells after being stimulated with LPS were shown in (**E**,**G**), respectively. Relative expression levels of *IRF1* and *IRF7* in GPHK cells after being stimulated with Poly I:C were shown in (**F**,**H**), respectively. The bars represent standard error (n = 3). Different letters manifest statistically significant differences in each condition ($p < 0.05$).

For instance, in GPS cells stimulated with Poly I:C, when *UBCE* or *Actin* or a combination of them was selected as reference genes, the expression level of *BCL2* was up-regulated gradually along with the increase of stimulation time and peaked at 8 h; however, when using the least stable reference gene *B2M*, the expression pattern for *BCL2* was fundamentally different and was down-regulated at all four time points (Figure 11B). After being stimulated with LPS, in GPS cells, using the stable candidates or their combination as internal controls, the expression profile for *ISG15* was that its expression was elevated drastically at 2 h and then maintained a lower level with small fluctuation at 4, 8 and 12 h; when adopting *GAPDH* as the reference gene, the change for target gene expression at 2, 4 and 8 h was similar to the above results, but at 12 h the expression level was remarkably increased to 5.5-fold (Figure 11C). As expected, under stimulating condition of LPS or Poly I:C, expression levels of immune-related genes in GPHK cells changed abnormally when adopting the unstable candidate, which was similar to the results in GPS cells (Figure 11E–H).

To summarize, if the stable reference genes or a combination of them were adopted, the expression pattern of target genes was normal and had a striking similarity to each other; in contrast, if the unstable candidates were selected for standardization of gene expression, target gene expression profiles would be greatly distinct from the counterpart that was using stable candidates or their combination as internal controls, and its expression would manifest great fluctuation.

4. Discussion

An ideal reference gene should display excellent expression stability among most tissues or cell types that have not been appreciably affected by endogenous or exogenous factors [59,60]. It has been reported in many studies that in teleost, several common reference genes exhibited preferable expression stability either across different tissues or under various conditions [61–64]. As for goldfish, *EF1A* and *ACTB* were recommended as the optimal reference genes both in healthy and CyHV-2 infected fish, whereas *18S* presented great expression stability under healthy conditions but was the least stable candidate under infection with CyHV-2 [64]. Moreover, in the half-smooth tongue sole, the most appropriate genes in samples for eighteen developmental phases was *B2M* and *GAPDH* [62]; for Japanese flounder, *UBCE* and *ACTB* with minimum expression variation were deemed as the most stable candidates across eight tissues under healthy states [61]. Regarding humpback grouper, *RPL13* was evaluated as the most suitable reference gene across five immune tissues under healthy states [63]. However, when it comes to the selection of reference genes for cell lines in fish, its relevant research was relatively rare and not systemic. Thereby, in this study, we evaluated and detected expression stability of seven candidate reference genes (*Actin*, *B2M*, *GAPDH*, *RPL13*, *EF1A*, *18S* and *UBCE*) in GPS and GPHK cells under normal physiological conditions or stimulated conditions of LPS or Poly I:C in order to screen appropriate reference genes applied to different cell lines of golden pompano.

Conventional methods that are used to evaluate expression stability for reference genes include: geNorm [7], REST [65], BestKeeper [49], NormFinder [48] and the comparative delta-Ct method [66]. As previously reported, the rankings of the most stable reference genes assessed via different software manifested similarity to a certain extent, but slight discrepancies due to adopting of various algorithms also occurred [62]. For example, in the pituitary of turbot, the results of geNorm demonstrated that *actb* and *ctsd* were the most suitable reference genes, and the optimal candidates assessed by Normfinder were *actb* and *b2m*, whereas in accordance BestKeeper analysis, *18S* was the most appropriate reference gene [67]. Consistently, in this study, the three software all proposed *18S* as the most suitable candidate in GPS cells under normal physiological conditions; regarding GPHK cells, in the light of results generated by BestKeeper or geNorm, the optimal reference genes were *RPL13* and *RPL13/18S*, respectively, whereas *UBCE* was evaluated as the most stable reference gene according to NormFinder. Hence, in this research, the final results were produced by the combination of ranking order for the most stable candidates evaluated by the three software. In light of comprehensive analysis, *18S* and *RPL13* separately were the most appropriate reference genes in GPS and GPHK cells under normal physiological conditions. Similarly, it has been reported in previous research that after being infected with ISAV, *18S* was recommended as the most appropriate reference gene in the kidney cells of Atlantic salmon [68].

Previous studies have demonstrated that reference gene expression levels fluctuated with the variation of experimental conditions, developmental phase or tissue or cell types [69–71]. For instance, in the peripheral blood mononuclear cells of porcine, geNorm results revealed that in the non-stimulated group, the most appropriate reference genes were *PPIA*, *BLM* and *GAPDH* while *PPIA*, *B2M* and *RPL4* were identified as the most suitable candidates in the LPS-stimulated group [72]. In this study, under stimulation of LPS or Poly I:C at four time points, the most reliable candidates were *RPL13* and *UBCE* in GPS cells, respectively. Similarly, it has been reported that *RPL4*, belonging to the ribosomal

protein family, was evaluated as the most stable candidate in porcine peripheral blood mononuclear cells after being stimulated with LTA [72]. Apart from that, *UBE2D2*, in light of expression stability and suitability, was assessed as the optimal candidate reference gene in human T-cells as well as in peripheral blood mononuclear cells [73].

Moreover, in GPHK cells after being stimulated with LPS or Poly I:C, *18S* and *EF1A* were recommended as the most appropriate reference genes based on comprehensive analysis, respectively. In line with our results, for grass carp, *EF1a* ranked as the most stable reference gene in kidney cells stimulated with Poly I:C [74]; moreover, under stimulation of west nile virus (WNV) antigen, the optimal candidate reference gene was identified as *18S* in peripheral blood mononuclear cells [75].

Four immune-related genes, *ISG15*, *BCL2*, *IRF1* and *IRF7*, were selected to verify the reliability of selected stable reference genew in GPHK and GPS cells under stimulation with LPS or Poly I:C. In two cell lines under different stimulating conditions, when adopting the unstable candidates, the expression pattern for all target genes would be abnormal and their expression would fluctuate to some degree, whereas the results were contrary when using the stable candidates or combination as internal control. The above results were consistent with similar observations in other studies, revealing that mistaken and inaccurate conclusions would occur if unreliable reference genes were screened for normalization [76,77]. Thereby, our results will provide a firm basis for gene expression analysis in golden pompano cell lines in vitro.

5. Conclusions

In this study, using geNorm, Normfinder and BestKeeper, we analyzed and evaluated the expression stability of seven candidate reference genes in GPS and GPHK cells under different conditions. For GPS and GPHK cells under normal physiological conditions, the most stable reference genes were *18S* and *RPL13*, respectively. Contrarily, *RPL13* and *18S* were proposed as the optimal candidates in GPS and GPHK cells under stimuli with LPS. After being stimulated with Poly I:C, *UBCE* and *EF1A* were the most reliable reference genes in GPS and GPHK cells, respectively. The results were further validated by normalization analysis on four immune genes (*ISG15*, *BCL2*, *IRF1* and *IRF7*). In conclusion, expression stability for all candidate genes exhibited some discrepancies under different experimental conditions or cell types, and our results will provide a firm basis for gene expression analysis in golden pompano cell lines in vitro.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8010008/s1, Figure S1: Analysis of agarose gel of total RNA extracted from different cell lines of golden pompano under normal physiological condition (A) and stimulation of LPS (B) or Poly I:C (C); Figure S2: The amplification of qRT-PCR of the seven housekeeping genes of golden pompano. Table S1: Primers for immune genes used for validation experiment.

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