

Transcriptome-based Selection and Validation of Optimal House-keeping Genes for Skin Research in Goats (*Capra hircus*)

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Abstract

Background: In Quantitative real-time polymerase chain reaction (qRT-PCR) experiments, accurate and reliable target gene expression data is dependent on optimal amplification of house-keeping genes (HKGs). RNA-seq technology offers a novel approach to detect new HKGs with improved stability. Goat (*Capra hircus*) is an economically important livestock species and plays an indispensable role in the world animal fiber and meat industry. Unfortunately, uniform and reliable HKGs for use in goat skin research have not been identified. Therefore, this study seeks to identify a set of stable HKGs for the skin tissue of *C. hircus* using high-throughput sequencing technology.

Results: Based on the transcriptome dataset of 39 goat skin tissue samples, 8 genes (*SRP68*, *NCBP3*, *RRAGA*, *EIF4H*, *CTBP2*, *PTPRA*, *CNBP*, and *EEF2*) with relatively stable expression levels were identified and selected as new candidate HKGs. Commonly used HKGs including *SDHA* and *YWHAZ* from a previous study, and 2 conventional genes (*ACTB* and *GAPDH*) were also examined. Four different experimental variables: (1) different development stages, (2) hair follicle cycle stages, (3) breeds and (4) sampling sites, were used for determination and validation. Four algorithms (geNorm, NormFinder, BestKeeper, and Δ Ct method) and a comprehensive algorithm (ComprFinder, developed in-house) were used to assess the stability of each HKG. It was shown that *NCBP3+SDHA+PTPRA* were more stably expressed than previously used genes in all conditions analysed, and that this combination was effective at normalizing target gene expression. Moreover, a new algorithm for comprehensive analysis, ComprFinder, was developed and released.

Conclusion: This study presents the first data of candidate HKGs for *C. hircus* skin tissues based on an RNA-seq dataset. We propose that the *NCBP3+SDHA+PTPRA* combination be used as a triplet set of HKGs in skin molecular biology experiments in *C. hircus* and other closely related species to standardize analyses across studies. In addition, we also encourage researchers who perform candidate HKG evaluations and who have the need for comprehensive analysis to adopt our new algorithm, ComprFinder.

Background

In molecular biology research, determining the relative changes in target gene expression at the transcriptional level requires precise quantitative analysis. The emergence and development of quantitative real-time polymerase chain reaction (qRT-PCR) has enabled comprehensive mRNA quantification. Furthermore, qRT-PCR is a commonly used technique due to its accuracy, sensitivity, reproducibility, and cost-effectiveness in analyzing gene expression [1, 2] and detects real-time fluorescence generated by a dye bound to, and proportional to, the total amount of nucleic acid. This value is typically reported as a cycle threshold value (Ct) in the comparative Ct method [3]. The qRT-PCR assay is reliant on house-keeping genes (HKGs) to obtain relative expression level data [4, 5] and choosing HKGs has become a major source of error and bottlenecks in qRT-PCR experiments.

In qRT-PCR experiments, inadequate HKG selection may lead to an inappropriate interpretation of target gene expression [6]. There are two commonly made mistakes when selecting HKGs: **(I)** HKGs are selected based on experience without reviewing HKG research papers, and **(II)** a single HKG with poor stability is used. In recent years, it has been reported with increasing frequency [7, 8] that the instability of commonly used HKGs, such as *ACTB*, *GAPDH*, and *18sRNA*, means that they have critical limitations. Ideal endogenous HKGs should exhibit consistent expression levels across all experimental conditions (e.g. cell types, physiological states, and growth conditions) [9, 10]. Unfortunately, no HKGs are stable across all experimental conditions, which means that each experimental system may need to use unique HKG(s) to accurately explore the specific research question being investigated.

Goat (*Capra hircus*) is an economically important livestock species as a source of meat, hair, and dairy products [11]. Skin tissue, as the largest biological organ, serves many functions including physical protection from injury and infection, thermal insulation, and is a substrate for growing hair. To reveal the molecular regulatory mechanism of hair follicle activity, it is necessary to clarify the pattern of target gene expression under different conditions, such as different stages of the hair follicle cycle or in different breeds. Unfortunately, most molecular studies examining goat skin have only included a single HKG such as *ACTB* [12-14] or *GAPDH* [15, 16]. In 2014, Bai et al. [17] selected 10 commonly used HKGs based on a literature review to explore their stability in different hair follicle cycles of Liaoning cashmere goats. However, due to lack of experiments used and testing only of commonly used HKGs, that study resulted in a limited impact. The development of high-throughput RNA-seq technology provides a means of determining spatio-temporal expression at the transcriptome level, and provides a novel approach for the identification of HKGs [18, 19]. This strategy was successfully used to identify candidate HKGs for *Artemisia sphaerocephala* [7], *Pyropia yezoensis* [22], *Euscaphis* [23], *Arabidopsis pumila* [24], fish [20], tomato leaves [21], and holstein cows [25], all from transcriptome datasets. Therefore, we hypothesized that novel, credible HKGs can be predicted and validated via transcriptome sequencing data from goat skin tissue.

In this study, the transcriptome dataset of 39 goat skin tissue samples was analysed. Potential HKGs were predicted, of which 8 genes (*SRP68*, *NCBP3*, *RRAGA*, *EIF4H*, *CTBP2*, *PTPRA*, *CNBP*, and *EEF2*) were selected based on their relatively stable expression levels. An additional 4 commonly used HKGs (*SDHA*, *YWHAZ*, *ACTB*, and *GAPDH*) were selected for comparison and validation. These 12 genes were amplified using qRT-PCR in 4 groups with different experimental treatments. Four different algorithms (geNorm [26], Δ Ct method [27], NormFinder [28], and BestKeeper [29]) and a comprehensive method (developed the ComprFinder method) were used to evaluate the stability of each HKG. Finally, the reliability of the recommended optimal HKGs were validated and confirmed.

Results

Selection of novel candidate HKGs based on RNA-seq data

From a complete transcriptome dataset, the fragments per kilobase of exon model per million mapped reads (FPKM) of all transcripts from each individual animal were obtained. We first removed some transcripts which did not have a credible function annotation, or exhibited low levels of expression (FPKM=0). This resulted in 15853 unigenes being found for further selection. Next, genes with a relatively high expression level as determined by the average of the FPKM value, and genes with low variability as determined by the coefficients of variation (CV, %), maximum fold change (MFC), and dispersion measure (DPM), were evaluated (see Methods section). As shown in **Figure 1**, the probability density curve of all 15853 unigenes was evaluated based on the following 4 indicators:

(1) FPKM. Potential HKGs were relatively highly expressed genes (FPKM ≥ 10) [8]. In this study, 5623 genes had FPKM values ≥ 10 (35.5% of 15853 genes, the green area in **Figure 1A**).

(2) CV (%). The most promising HKGs would have the lowest CV values. A total of 2266 genes with a CV $\leq 20\%$ (14.3% of 15853 genes, the red area in **Figure 1B**) were retained in this step with CVs ranging from 7.7% to 20.0%.

(3) DPM. Most stable genes exhibited lower DPM values. The default parameter of DPM <0.3 returned an excessive 7025 unigenes, and so a more stringent DPM < 0.2 was used. Following this, 2026 genes (12.8% of 15853 genes, the yellow area in **Figure 1C**) were retained in this step with DPM values ranging from 0.09 to 0.2.

(4) MFC. This parameter reflects the range of extremum value, and the lowest MFC values are preferable. In this study, MFC < 2.5 was used which produced 2508 genes (15.8% of 15853 genes, the blue area in **Figure 1D**), all within the range of 1.35 to 2.5.

A Venn diagram was constructed for the 4-color blocks (green, red, yellow, and blue corresponding to those used in **Figure 1A-D**, respectively). This showed that 1325 genes (**Figure 1E**) met all 4 of the above requirements, and are significantly enriched in 11 signalling pathways ($q < 0.05$) as shown in **additional file 1: Figure S1**. These genes were considered as candidate HKGs and of these, 8 genes (*RRAGA*, *PTPRA*, *SRP68*, *EIF4H*, *NCBP3*, *CTBP2*, *CNBP*, and *EEF2*) that with lower CV value, higher FPKM value and easier primers design were selected as novel candidate HKGs for further qualification. Each gene was ranked based on its CV value with a lower CV receiving a higher-ranking order (**Table 1**). In addition, 4 genes outside of the initial 1325 were considered, including *SDHA* and *YWHAZ*, as they had previously been proposed by other researchers [17], and *ACTB* and *GAPDH* genes were included as the most commonly used endogenous HKGs for exploring target gene expression in goats. In total, 12 candidate HKGs were analyzed in subsequent steps.

Amplification specificity and efficiency of the candidate HKGs and target genes

A total of 15 primer pairs targeting 12 candidate HKGs and 3 target genes were designed for qRT-PCR. The gene, primer and amplicon specifications are shown in **Additional file 1: Table S1**. All primers were designed to anneal at around 60°C. Amplification efficiency for all 15 genes ranged from 96.4% for *DKK1* to 103.9% for *PTPRA*, and the coefficient of determination (R^2) varied from 0.9986 to 0.9999. The specificity for each paired primer was validated by the melting curve analysis, which showed a single amplification peak (**Additional file 1: Figure S2**).

Expression profiles of the candidate HKGs

The mean Ct (the average of 3 technical replicates in the same sample) values were used to calculate gene expression levels among samples with distinct experimental factors. As shown in **Figure 2** and **Additional file 1: Table S2**, the expression level of the 12 candidate HKGs varied widely from 20.74 to 31.60. The most highly expressed gene was *ACTB* (mean Ct value: 23.25 cycles), and the lowest was *SPR68* (mean Ct value: 29.07 cycles). The top 3 genes with low standard deviations were *SRP68* (0.875), *NCBP3* (0.970), and *PTPRA* (0.972). The 3 most deviantly expressed genes were *ACTB* (1.483), *CNBP* (1.277), and *GAPDH* (1.258). The narrower standard deviation range of a gene means it has a higher expression stability in different samples. Although some genes had a lower standard deviation than others, experimental errors are always possible. Therefore, to obtain reliable HKGs to normalize target gene expression levels, further analysis with more scientific algorithms and evaluation for candidate HKGs is needed.

Analysis of HKG expression stability

In this study, 4 publically available algorithms were used to evaluate HKGs for higher-accuracy stability rankings: geNorm, NormFinder, BestKeeper, and the Δ Ct method.

geNorm analysis

Gene expression stability was determined by the M-value in geNorm analysis; the lower M value suggests a higher gene expression stability. For group 1, the two most stable genes were *EIF4H* and *EEF2* with the lowest M value, and *GAPDH* was the most unstable gene (**Figure 3A**). For group 2, the two most stable genes were *EIF4H* and *PTPRA*, and *ACTB* was the most unstable gene (**Figure 3B**). For group 3, the two most stable genes were *EIF4H* and *PTPRA*, whereas *ACTB* was the most unstable gene (**Figure 3C**). For group 4, the two most stable genes were *NCBP3* and *PTPRA*, and *GAPDH* was the most unstable gene (**Figure 3D**). For all samples, geNorm analysis was conducted on 39 samples and 12 HKGs. It was determined that the 3 most stable genes were *PTPRA*, *EIF4H*, and *NCBP3*. Conversely, *ACTB*, *CNBP*, and *GAPDH* were the most unstable genes (**Figure 3E**).

geNorm can be used to determine the minimum optimal number of HKGs needed for accurate normalization under different experimental treatments by analysing pairwise variation (V_n/V_{n+1}). This

method recognizes $V_n/V_{n+1} < 0.15$ as a threshold value, and “n” as an appropriate number of HKG needed. The V2/3 values for all the experimental variables were below the cut-off value of 0.15 (0.067, 0.078, 0.099, 0.091 and 0.081 for group 1, 2, 3, 4, and all samples, respectively), which indicate that using double HKGs (first two genes in each group) is sufficiently accurate for use in normalizing qRT-PCR derived gene expression data (**Additional file 1: Figure S3**). The triplet or more gene combinations can also be used as $V_n/V_{n+1} < 0.15$.

NormFinder analysis

Expression stability values, as determined by NormFinder, are shown in **Table 2**. For group 1, *SDHA* and *EIF4H* were the most stable HKGs, and *ACTB* was the least stable gene, which was the same as was determined by geNorm. In group 2, *SDHA* and *NCBP3* were the most stable HKGs while *ACTB* was the least stable gene. In group 3, *SDHA* and *YWHAZ* got the top rank, while *ACTB* ranked at the lowest. In group 4, *PTPRA* and *NCBP3* were the most stable, while *GAPDH* ranked at the lowest. In all samples, *SDHA* and *NCBP3* were the most stable, while *ACTB* was the least.

BestKeeper analysis

The BestKeeper algorithm used std-values to assess HKG stability with the lower the std-value, the more stable HKG expression was. As shown in **Table 3**, in group 1, *SDHA* and *PTPRA* were the most stable HKGs, whereas *RRAGA* was the least stable. The same was observed with the geNorm analysis. In group 2, *SDHA* and *EEF2* were the most stable HKGs, while *ACTB* was the least stable. In group 3, *SDHA* and *YWHAZ* got the top rank, while *SPR68* ranked at the lowest. In group 4, *EEF2* and *NCBP3* were most stable, while *GAPDH* was the least. In all samples, *SDHA* and *EEF2* were most stable, while *ACTB* was the least.

Δ Ct analysis

The 12 candidate HKGs were analyzed using the Delta Ct method, the data of which is presented in **Table 4**. The stability of the gene is inversely related to the std-value, thus a lower value indicates greater stability. In group 1, the two most stably expressed genes were *PTPRA* and *SDHA*, and the lowest were *GAPDH* and *ACTB*. In group 2, the two most stable genes were *EEF2* and *SDHA*, and the least were *ACTB* and *CNBP*. In group 3, *SDHA* and *PTPRA* were the most stably expressed, whereas *ACTB* and *CNBP* were the least. In group 4, the top two stably expressed genes were *NCBP3* and *EEF2*, whereas *CNBP* and *GAPDH* were the least. In all samples, the 3 most stable genes were *PTPRA*, *EEF2*, and *SDHA*, while *GAPDH*, *CNBP*, and *ACTB* were the least stable genes.

A comprehensive ranking of the four methods examined

Next, the ComprFinder algorithm was employed to obtain a comprehensive score which was used to rank the potential HKGs (**Table 5**). In group 1, the 3 most stable HKGs were *EIF4H*, *PTPRA*, and *SDHA*. In group 2, *SDHA*, *NCBP3*, and *EEF2* were the most stable HKGs analyzed. In group 3, *SDHA*, *PTPRA*, and *EIF4H* were the three most stable HKGs analysed. In group 4, *NCBP3*, *PTPRA*, and *EEF2* were the most stable

genes. The overall rankings, from the highest to the lowest stability, were *NCBP3* > *SDHA* > *PTPRA* > *EEF2* > *EIF4H* > *SRP68* > *CTBP2* > *YWHAZ* > *RRAGA* > *GAPDH* > *CNBP* > *ACTB*. It is interesting to note that the top 3 genes in different group rankings have at least 2 of *NCBP3*, *SDHA* and *PTPRA*. In contrast, the commonly used HKGs, *ACTB* and *GAPDH*, were relegated to the bottom 2 and 4 positions, respectively.

NCBP3, *SDHA*, *PTPRA* were the 3 most stable HKGs across all samples with scores within a tight range, calculated at 0.096, 0.099, and 0.108, respectively. They were also preferably ranked in groups 1-4 relative to other genes and were therefore considered to be the 3 most promising candidate HKGs, and were advanced for further validation.

Validation of HKGs by *DKK1*, *SHH*, and *FGF5* genes

Based on the above analyses, 3 target genes (*DKK1*, *SHH*, and *FGF5*) were further characterized based on their changes in expression levels during the secondary hair follicle cycle (T1, T2, T3) with normalizations using different single HKG and multi-gene combinations. It was observed that *NCBP3*, *SDHA*, and *EEF2* were the top 3 HKGs in group 2 (factor: hair follicle cycle) based on expression stability. Therefore, it can be concluded that the combination of *NCBP3+SDHA+EEF2* was the best-normalized gene set for group 2. Since these 3 genes (*NCBP3*, *SDHA*, and *PTPRA*) are possibly the most important candidate HKGs, they were further characterized to determine optimal combinations for normalization of gene expression studies. Four multi-gene combinations, including *NCBP3+SDHA+PTPRA*, *NCBP3+SDHA*, *NCBP3+PTPRA*, and *SDHA+PTPRA*, in addition to 3 single-genes (*NCBP3*, *SDHA*, and *PTPRA*) were used for the analysis. Conversely, *ACTB* and *GAPDH* were used for comparison and were also examined as the multi-gene combination *ACTB+GAPDH*. In total, 11 multi-gene combinations or single genes were used as normalization factors. For multiple gene combinations, the geometric average of their Ct value was calculated. The relative gene expression level was calculated as $2^{-\Delta Ct}$, $\Delta Ct = \Delta (Ct_{\text{target gene}} - Ct_{\text{HKGs}})$.

As is shown in **Figure 4A**, the expression profiles of *DKK1* were similarly obtained using the 8 stable single-gene and multi-gene combinations. Furthermore, it was observed that *DKK1* was more highly expressed in T2 compared to T1, and it was most highly expressed during the T3. Among the unstable single- and multi-gene combinations, only *ACTB* and *ACTB+GAPDH* performed similarly to the stable genes. However, the gene expression profile as normalized by *GAPDH* was different from the other conditions, and no significant difference has been identified among T1, T2, and T3. Expression of the *SHH* gene was even during the T1 and T2, but there was a significant decrease in T3 (**Figure 4B**). The 5 multi-gene combinations and *NCBP3*, *SDHA* identified this trend, but *PTPRA* did not. Though *GAPDH*-normalized gene expression profile had similar trends to stable multi-gene combinations, but *ACTB* was different. The combination of *ACTB + GAPDH* identified this expression change as a trend, but was not able to detect significant changes in expression. The expression profile of the *FGF5* gene, when normalized by the most stable candidate HKGs used individually or in combination here, were very similar. High expression levels were observed in T2, but no statistical significance was identified relative to T1 and T3 (**Figure 4C**). The combination of *ACTB + GAPDH* showed a similar pattern to the stable

HKGs, but when *ACTB* and *GAPDH* were used individually, the expression patterns were completely different. Furthermore, significant differences in *ACTB* were also identified in T2 relative to T1.

The above-mentioned results derived from **Figure 4** reflect the differences of expression profiles of a single target gene normalized by 11 types of single or multiple-gene combinations. To further understand the relationship of those single or multi-HKG combinations, a correlation analysis on these relative expression data ($2^{-\Delta Ct}$) of 3 target genes was performed. As shown in **Figure 5**, the normalized results using *NCBP3+SDHA+EEF2* and *NCBP3+SDHA+PTPRA* had a high correlation coefficient ($R=0.990$, $P < 0.001$), suggesting that they have extremely similar normalization capabilities. Other double-gene combinations including *NCBP3+SDHA*, *NCBP3+PTPRA*, and *SDHA+PTPRA* had high correlation coefficients, ranging from 0.969-0.997 with *NCBP3+SDHA+EEF2*. Also, these double-gene combinations had high correlation coefficients of 0.989-0.994 with *NCBP3+SDHA+PTPRA*. This indicated that these 3 types of double-gene combinations exhibited similar normalization capabilities to *NCBP3+SDHA+EEF2* and *NCBP3+SDHA+PTPRA*. For single stable HKGs, *NCBP3*, *SDHA*, and *PTPRA* also exhibited high correlation coefficients with *NCBP3+SDHA+EEF2* (0.942-0.973) and *NCBP3+SDHA+PTPRA* (0.952-0.977). The *ACTB*, *GAPDH*, and *ACTB+GAPDH* combinations had relatively low correlation coefficients with any of the stable single- (0.513-0.780) and multi-gene combinations (0.548-0.738).

Discussion

Standard criteria for HKG screening for skin tissue research in goats

Which candidate HKGs should we choose?

Four original algorithms were used to determine the expression stability values of 12 candidate HKGs and other final values were determined using a comprehensive algorithm. However, even for the final ComprFinder value, the results varied between different groups. If the top 3 genes were used, groups 1-4 should theoretically be *EIF4H+PTPRA+SDHA*, *SDHA+NCBP3+EEF2*, *SDHA+PTPRA+EIF4H*, and *NCBP3+PTPRA+EEF2*, and a total of 5 HKGs (*EIF4H*, *PTPRA*, *SDHA*, *NCBP3*, *EEF2*) would be needed. In theory, it is preferable to use multiple high-performing HKGs as a normalization factor, however, in practice, the additional cost and excessive number of HKGs, limits the number of samples that can be tested. Therefore, the fewest number of HKGs possible should be used to meet the relevant statistical needs, in addition to reducing experimental costs [10, 30]. In this study, *NCBP3*, *SDHA*, and *PTPRA* were the top 3 most stable HKGs for all samples, and 2 of those ranked in the top 3 of each group. They were therefore considered to be the 3 most powerful candidate HKGs, and were considered for further validation.

How many candidate HKGs should be used?

There is still no specific theory prescribing a specific number of HKGs to be used. In the above discussion, *NCBP3*, *SDHA*, and *PTPRA* were proposed for their excellent stability, however, which single or multiple gene combinations (*NCBP3+SDHA+PTPRA*, *NCBP3+SDHA*, *NCBP3+PTPRA*, *SDHA+PTPRA*, *NCBP3*, *SDHA*, *PTPRA*) should be used? In other words, is the efficacy sufficient to use a single or double HKG? Compared with *NCBP3+SDHA+EEF2* or *NCBP3+SDHA+PTPRA*, the detection efficacy of *NCBP3* (**Figure 4A**), *SDHA* (**Figure 4B**), and *PTPRA* (**Figure 4B**, **Figure 4C**) were not consistent. Considering that the single gene performances were not good, it is recommended that single HKGs should be avoided, even if they were the top-ranked HKGs.

For the double gene combinations *NCBP3+SDHA*, *NCBP3+PTPRA* and *SDHA+PTPRA* (**Figure 4A-C**), similar expression patterns and detection efficacy were seen, consistent with the *NCBP3+SDHA+EEF2* or *NCBP3+SDHA+PTPRA* combinations. It was shown that *NCBP3+SDHA* behaved more similarly to the *NCBP3+SDHA+EEF2* or *NCBP3+SDHA+PTPRA* combinations compared to *NCBP3+PTPRA* and *SDHA+PTPRA*, possibly because *NCBP3* and *SDHA* were among the top in the final stability ranking in group 2. This also implies that *SDHA+PTPRA*, *SDHA+PTPRA*, and *NCBP3+PTPRA* may be the optimal double gene combinations for groups 1, 3, and 4, respectively. There are still 3 genes, *NCBP3*, *SDHA*, and *PTPRA*, and there is no type of double gene combination able to cope with multiple factors (groups 1-4).

Considering that the 3-gene combination of *NCBP3+SDHA+PTPRA* exhibited better stability performance, it can be applied to various factors in goat dermatologic research, and 3 HKGs is still an acceptable number for qRT-PCR experiments. Therefore, it is recommended that *NCBP3+SDHA+PTPRA* be adopted as the HKG combination for dermatologic research in goats.

The HKGs of skin tissue in goats and other species

As noted previously, common HKGs used in traditional skin research of goats were either *ACTB* [12-14], or *GAPDH* [15, 16]. Almost all of these studies used a single HKG, so it can be speculated that some of these previous studies may have obtained erroneous data and it is therefore crucial that scientists appreciate the advantage of a set of appropriate HKGs. Regarding target genes that have undergone significant changes, these can be identified by less stable HKGs, but for target genes that show slight changes, these can only be identified by optimal HKGs [30]. As far as we know, only one previous HKGs study reported on goat skin tissue, Bai et al. [17] selected ten commonly used HKGs by consulting the literature to select optimal HKGs to span 3 stages of hair follicle cycle in Liaoning cashmere goats (referred to here as T1, T2, and T3 of IMCG), the authors recommended *SDHA+YWHAZ+UBC* as the HKGs be used in combination, but their geNorm values ($V_2/V_3=0.159$ and $V_3/V_4=0.144$) imply that the combination of 3 genes was not ideal. In the present study, an RNA-seq dataset was used for prediction and selection, and a large number of biological samples were provided for determination and validation, and multiple algorithms were used for evaluation. Therefore, in terms of both the number and quality of HKGs, this study is a significant step forward from previous studies.

When studying the expression of target genes in skin tissue from other species, such as Angora rabbits [31], mink [32], mice [33], and even humans [34], *ACTB* or *GAPDH* are generally used as the HKGs. The

selection of HKGs from neighbouring species has been widely recognized and accepted and thus the data presented here can describe HKGs for skin tissue research, not only for goats, but also for other species.

Selection and validation of HKGs based on RNA-seq data

Selection and validation of HKGs using RNA-seq produced results that were more reproducible, had greater sensitivity, and yielded better correlation with protein expression levels, in addition to having more accurate detection and higher coverage [35]. To the best of our knowledge, this study is the first to report on the selection and validation of suitable HKGs for qRT-PCR analysis in goats. Two novel HKGs (*NCBP3* and *PTPRA*) and a known HKG (*SDHA*) belonging to the *NCBP3+SDHA+PTPRA* combination were recommended. Using a similar approach as other studies [19, 22, 23], new and improved HKGs were identified through analysing an RNA-seq dataset. In total, 1325 candidate genes were identified that met the HKG criteria (**Figure 1E**) and eight of them were selected for additional determination and validation analyses. While this study demonstrated the advantages of using RNA-seq datasets in the discovery of new HKGs, it is also possible that the prediction of HKGs by RNA-seq datasets may be lacking in some respects. For example, the ranking order of these candidate HKGs (**Table 1**) and the determined final score (**Table 5**) did not match (compared in **Additional file 1: Table S3**). Specifically, the CV values of *RRAGA* and *SRP68* were in the top 3, although in the final ranking they did not appear in the top 3 positions of any group (groups 1-4 and all samples). This may be the reason why RNA-seq samples in the selection stage and the qRT-PCR samples used in the determination stage did not completely overlap. This phenomenon is consistent with those reported by Gao et al. [22] and implies that the HKGs predicted by RNA-seq screening were not completely reliable, and need further confirmation and validation by qRT-PCR experiments.

As said before, *ACTB* and *GAPDH* are currently the most popular HKG in the literature, but their limited normalizing capacity was verified here. This suggests that scientists must be cautious when selecting traditional HKGs, especially when identifying target genes that have slight changes in expression. Therefore, it is recommended that common HKGs be included as a comparison, to provide direct evidence. Of course, it must also be acknowledged that mining reliable HKGs requires scientific experimental design, complete experimental materials, more algorithmic tools, and a certain amount of scientific research time and funding [30], though these are not available to every research laboratory. Therefore, those experimental systems that do not meet the above conditions were recommended to search for HKGs in close species using the ICG platform [36], then use as many HKGs as possible and calculate their arithmetic mean as the normalization factor to increase experimental stability, instead of simply using a single HKG such as *ACTB* or *GAPDH*.

Comprehensive analysis

The requirement of comprehensive analysis, and the shortcomings of the previous algorithms

After evaluating candidate HKGs with the above-mentioned algorithms (geNorm [26], NormFinder [28], BestKeeper [29], and the Δ Ct method [27]), it is not surprising that the rankings of candidate genes may vary depending on the algorithm used [30] and thus another algorithm is needed for comprehensive ranking. After reviewing the literature on HKGs, it was determined that 2 types of comprehensive algorithms were mainly used: (1) the primary ranking order was used to calculate the arithmetic average to get the final ranking [21, 37]; (2) using the primary ranking order, the geometric average is then calculated to get the final ranking. RefFinder [38] is a typical representative (<https://www.heartcure.com.au/reffinder/?type=reference>) and many studies [7, 19, 22, 24, 39-41] have used it (Times Cited: 352, on Web of Science, 2020/2/3), which illustrates its vast impact. Both of the above comprehensive algorithm types depend on the ranking number of the original algorithms. Due to this, it is possible that the use of ranking numbers may cause some errors.

These ranking numbers reflect the true size of stability values of these candidate HKGs, but they should not be used as the input numerical value for the next calculation. Doing so would excessively reduce or enlarge the real differences among them. Taking our experimental data as an example, these candidate HKGs were evaluated and unevenly distributed (**Figure 6A**) on the axis of the four algorithms. The RefFinder algorithm provides them with the uniform rank of 1-12 (**Figure 6B**), and then calculates the geometric average for each candidate HKG. The RefFinder algorithm increases the gap between *PTPRA* and *EIF4H*, and reduces the gap between *CNBP* and *ACTB* (the axis of the geNorm algorithm in **Figure 6A** and **Figure 6B**). We consider this unreasonable for comprehensive evaluation and therefore, in this study, a new algorithm for comprehensive analysis was developed.

The ComprFinder algorithm

ComprFinder performs the next step of the integration analysis on the results obtained through the algorithms including, but not limited to, the above mentioned geNorm, BestKeeper, NormFinder, and Δ Ct method. The schematic diagram of the ComprFinder algorithm is presented in **Figure 7**. Specifically, **STEP 1**, according to the original algorithm results ordered the values from small to large to find the minimum and the maximum. From this, the range was calculated (1) where A is one of the original algorithms, and is also used to calculate Δ_B , Δ_C and Δ_D . In **STEP 2**, the values from the original algorithm were standardized in the interval [0,1], where the minimum value=0 and the maximum value=1. The normalized value was calculated (2) where *i* is one of these candidate HKGs, and is also used to calculate B_i , C_i and D_i . All the other data were assigned normalized values between 0 and 1. This step makes these stability values that belong to the same HKG but from different original algorithms abide by the additive property. In **STEP 3**, arithmetic averages of the standardized values for each candidate HKG were calculated, and then these final scores (FS) were sorted to obtain their final rankings (**Figure 7**). The FS_i (3) was determined with *n* being the number of original algorithms we used.

$$\Delta_A = A_{max} - A_{min} \quad (1)$$

$$A_i = (A_i - A_{min}) / \Delta_A \quad (2)$$

$$FS_i = (A_i + B_i + C_i + D_i) / n \quad (3)$$

ComprFinder was intentionally developed to replace RefFinder and the comparison of the two algorithm's results are shown in **Additional file 1: Table S4**. It can be seen that the relative final scores and ranking order of the 12 candidate HKGs calculated using the two algorithms are different. For example, *NCBP3*(0.096), *SDHA*(0.099), and *PTPRA*(0.108) were the top 3 genes for all samples calculated by ComprFinder, whereas *PTPRA*(2.14), *SDHA*(2.45), and *EEF2*(3.13) were the top three calculated by RefFinder. The ComprFinder algorithm directly standardizes the results of the original algorithms, unlike RefFinder, which uses the ranking numbers of original algorithms. Using the ranking numbers, the real differences among the candidate HKGs will excessively reduce or increase, therefore, the new comprehensive algorithm can overcome the intrinsic errors caused by artificial assignment. Standardized processing allows the results of different algorithms to have the same dimension, and makes them essentially comparable. Finally, the standardized results can be integrated to get a series of scores and a final evaluation. Therefore, ComprFinder is a more reasonable algorithm than RefFinder for the comprehensive evaluation of HKGs.

We provide a ComprFinder algorithm tool (**Additional file 2**) for researchers who have comprehensive evaluation needs for candidate HKGs. This tool is based in Microsoft Excel and can be downloaded from the supplementary materials of this article. Briefly, after inputting the original algorithm results into the input area, the ComprFinder algorithm automatically processes the data and all candidate HKGs will be scored and presented in the output area. Although the use of ComprFinder in this study was based on geNorm, BestKeeper, NormFinder, and the ΔCt method, analysis is not limited to these 4 algorithms.

Conclusion

In this study, we present the first data of candidate HKG selection for goat skin tissue based on transcriptome data. The *NCBP3+SDHA+PTPRA* combination was identified and finally recommended as the triplet HKGs for skin molecular biology studies in goats and other closely related species. In addition, a comprehensive algorithm tool, ComprFinder, was developed for the comprehensive evaluation of candidate HKGs.

Methods

Animals and skin tissue samples

All animals and sampling procedures in this study were supervised and approved by the Institutional Animal Care and Use Committee of Southwest University. Each 1 cm² skin tissue section was sampled from the Inner Mongolia cashmere goat (IMCG), Dazu black goat (DBG), Hechuan white goat (HCWG), or

the first filial generation (F_1 , DBG × IMCG). Information regarding animal source, sampling procedure, and sample preservation is found in **Additional file 1: Table S5** and **S6**. All samples were stored at $-80\text{ }^\circ\text{C}$ until required.

A total of 48 skin tissue samples were collected to determine and validate potential HKGs. In the determination stage of this study (**Figure 8C, Additional file 1: Table S5**), all samples were collected from does. Four groups (factors) were used, including age (4 development stages, group 1), sampling time (3-stages of hair follicle cycle, group 2), breed (4 different breeds, group 3), and sampling site (5 different sampling sites on the body of the goat, group 4). As shown in **Figure 8C**, **group 1** included F_1_P0 , F_1_P60 , F_1_P240 and F_1_Adult , which were sampled at 0-day, 2 months, 8 months and 2 years after birth from F_1 . **Group 2** included IMCG_T1, IMCG_T2, and IMCG_T3, which were sampled during the anagen (September), catagen (December) and telogen (March) from IMCG. **Group 3** consisted of animals from 4 breeds and included IMCG, DBG, F_1 , and HCWG. Skin samples from each of the breeds were sampled in the anagen phase of the hair follicle cycle. Skin biopsies collected from **group 4** (#4, #5, #6, #12, and #14 from IMCG), were taken from the forearm, dorsal chest, lateral chest, thigh, and the inner side of the forearm, respectively. Except for samples #4, #5, #12 and #14, all samples analyzed here were collected from the lateral chest of the goat body. Except for samples belonging to F_1_P0 , F_1_P60 and F_1_P240 , all samples were collected from adult goats. In the validation stage of this study (**Figure 8E, Additional file 1: Table S6**), 3 bucks of IMCG were added to group 2, to enhance validation accuracy.

RNA isolation and cDNA synthesis

Total RNA was extracted using the RNAiso Plus kit (#9109, TaKaRa, China) according to the manufacturer's instructions. The concentration and purity were determined using the Nanodrop2000 (Thermo, USA) with the 260/280 ratios being between 1.8 and 2.0, and the 260/230 ratios were greater than 1.6 in all analyzed RNA samples. First-strand cDNA was synthesized using the 5X All-In-One RT MasterMix (with AccuRT Genomic DNA Removal Kit) (#G492, ABM, Canada) and 1:4 volumes of DEPC water was added to dilute the samples.

Selection of candidate HKGs

Transcriptome sequencing data of 39 goat skin tissues (unpublished data, **Figure 8A**) was performed using the Illumina paired-end sequencing technology on an Illumina Hi-Seq™ 2000 platform. After assembly and annotation, the gene expression profiles and read counts of unique transcripts were converted into FPKM values. Based on the FPKM value of every gene in each transcriptome, the CV, MFC and DPM [42] were calculated. The CV is defined as the ratio of the SD_{FPKM} to the mean of the FPKM of all samples for one gene. The DPM parameter was introduced for the identification of the HKGs on pattern gene finder (PaGeFinder) [42], and their jar package was downloaded and run from the PaGeFinder website. The MFC, which is defined as the fold change between the largest and smallest

FPKM values within 39 RNA-seq were calculated. The standard criteria for HKGs are relatively high expression level, and low expression variation, therefore, a candidate HKG should have a relatively high FPKM value, and low CV, DPM and MFC values.

Genes with RPKM, CV, MFC, and DPM fulfilling the criteria of HKGs were retained for further analysis (**Figure 8B**). Moreover, two HKGs (*SDHA* and *YWHAZ*) from a previous study by Bai [17], and two commonly used HKGs (*ACTB* and *GAPDH*) were also considered as HKGs. All candidate HKGs were amplified using qRT-PCR for subsequent determination and validation. The probability density curves were drawn using in house MATLAB scripts (**Additional file 3**) (<https://ww2.mathworks.cn/products/matlab.html>). Venn diagram analysis was performed using the OmicShare online platform tools (<http://www.omicshare.com/tools>).

Primer design and amplification efficiency analysis

Specific primers were designed using the Primer-BLAST [43] web tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) based on the sequences of the unigenes. The criteria for primer design were as follows: primer lengths of 17–24 bp, GC content of 50%– 66%, melting temperature of 58–61°C, and amplicon lengths of 100–200 bp. All primers were synthesized by the Beijing Genomics Institute (Beijing, China).

qRT-PCR analysis

Sample reactions were performed in a 10 µL reaction volume with 5 µL of 2× qPCR MasterMix (#MasterMix-S, ABM, Canada), 1 µL cDNA template, 0.3 µL each primer, and 3.4 µL DNase/RNase-free water and run on the Bio-Rad CFX96 Real-Time PCR Detection System. The thermal cycling conditions were conducted according to the reagent kit instructions as follows: enzyme activation at 95 °C for 10 mins, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 60 s. The specificity of the SYBR green PCR signal was confirmed by melting curve analysis. All samples were analyzed in 3 replicates. Serial tenfold dilutions of cDNA template (note: in primer amplification test, cDNA template is PCR product with the same primer) were used to generate a slope of the standard curve to calculate the amplification efficiency and R^2 of each paired primer. All qRT-PCR experiments and data analyses in the present study were performed in accordance with the MIQE guidelines [44, 45].

Determination of expression stability of HKGs

The cycle threshold (Ct) data of all the candidate HKGs obtained from the qRT-PCR experiments were evaluated by 4 algorithms, geNorm [26], NormFinder [28], BestKeeper [29], and the Δ Ct method [27](**Figure 8D**). After using the above-mentioned traditional evaluation algorithms, another algorithm was developed

for comprehensive ranking. The new algorithm, ComprFinder, standardizes the output values from the above 4 algorithms then arithmetically averages them to get a final score and final ranking order.

Experimental validation of the HKGs

To verify the results, the 3 best candidate HKGs were selected (*NCBP3*, *SDHA*, and *PTPRA*), as well as the most unstable and most commonly used HKGs (*ACTB* and *GAPDH*). Next, the HKGs were verified and evaluated using 3 target genes, which are the most important genes in hair follicle research of goats (*DKK1*, *SHH*, and *FGF5*) (Figure 8E). Considering the need to accurately evaluate gene expression, 3 new bucks were included to the original sample size of 3 does, (6 adult IMCG, 3♂ and 3♀) and were sampled over 3 time-points (T1, T2, and T3). The qRT-PCR was conducted as described above. The paired sample t-test was performed using Microsoft Excel, and the graph was plotted using GraphPad Prism 6. The results are presented as Mean ± SEM, * $P < 0.05$, ** $P < 0.01$. For multiple gene combinations, the geometric average of their Ct value was calculated [26]. The relative gene expression level was calculated as $2^{-\Delta Ct}$, $\Delta Ct = \Delta (Ct_{\text{target gene}} - Ct_{\text{HKGs}})$.

To further evaluate the internal relationship of these candidate HKGs, a correlation analysis was performed. First, the target genes were normalized by different HKGs or HKG combinations. Their copy numbers were converted to relative expression levels with $2^{-\Delta Ct}$, and then their normalized-based expression levels were examined by the correlation analysis.

Declarations

Ethics approval and consent to participate

All animals and sampling procedures in this experiment were supervised and approved by the Institutional Animal Care and Use Committee of Southwest University.

Consent for publication

Not applicable.

Availability of data and materials

The RNA-seq dataset analyzed during the current study is available in the Sequence Read Archive (SRA) database, accessible through NCBI Bioproject ID PRJNA630571.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

ZJP and DCC designed the experiments. ZJP and LJL fed animals, collected experimental tissues, and extracted the RNA. ZJP performed all qRT-PCR experiments, analyzed and interpreted the data, drafted the manuscript. ZYJ revised the manuscript. All authors read and approved the final manuscript.

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Abbreviations

ACTB: Actin Beta; CNBP: CCHC-Type Zinc Finger Nucleic Acid Binding Protein; Ct: Cycle threshold value; CTBP2: C-Terminal Binding Protein 2; CV: Coefficient of variation; DBG: Dazu black goat; DKK1: Dickkopf WNT Signaling Pathway Inhibitor 1; DPM: Dispersion measure; EEF2: Eukaryotic Translation Elongation Factor 2; EIF4H: Eukaryotic Translation Initiation Factor 4H; F1: First filial generation; FGF5: Fibroblast Growth Factor 5; FPKM: Fragments per Kilobase per Million; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HCWG: Hechuan white goat; HKGs: House-keeping genes; IMCG: Inner Mongolia cashmere goat; MFC: Maximum fold change; NCBP3: Nuclear Cap Binding Subunit 3; PTPRA: Protein Tyrosine Phosphatase Receptor Type A; qRT-PCR: Quantitative real-time polymerase chain reaction; R²: coefficient of determination; RNA-seq: RNA Sequencing; RRAGA: Ras Related GTP Binding A; SD: Standard deviation; SDHA: Succinate Dehydrogenase Complex Flavoprotein Subunit A; SHH: Sonic Hedgehog Signaling Molecule; SRP68: Signal Recognition Particle 68; YWHAZ: Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta.

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Supplementary Files Legend

Additional file 1: Supplementary Figure and Tables (PDF 1047 kb)

Figure S1 The top 14 enriched signaling pathways of the 1325 candidate HKGs based on KEGG analysis

Figure S2 Melting curves for the 12 candidate HKGs and 3 target genes

Figure S3 Optimal number of HKGs in different experimental groups calculated by geNorm

Table S1 Primer sequences and amplicon information of candidate HKGs and target genes for qRT-PCR

Table S2 Ct values of the 12 candidate HKGs in all samples

Table S3 The comparison of experimental results and RNA-seq data

Table S4 The comparison of final results using ComprFinder and RefFinder algorithms

Table S5 The sample information in the determination stage

Table S6 The sample information in the validation stage

Additional file 2: Downloadable Excel file with the ComprFinder algorithm (Excel 260 kb)

Additional file 3: Matlab_script (Matlab file 1 kb)

Figures

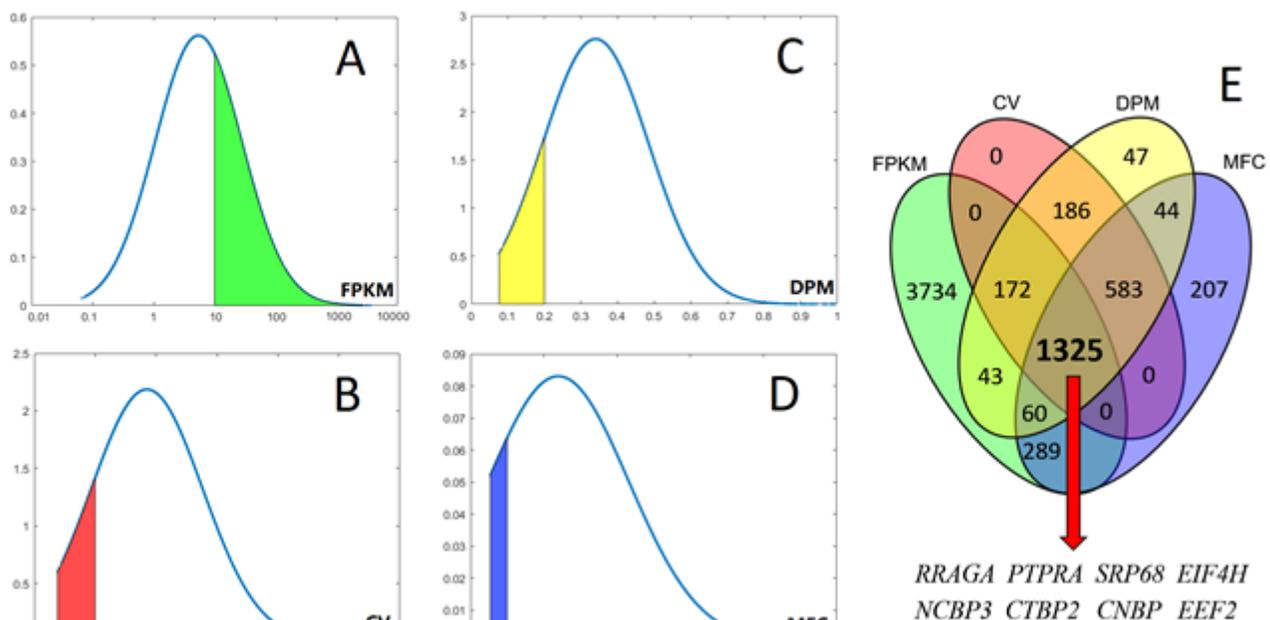


Figure 1

Probability density curve of FPKM, CV, DPM and MFC of 15853 unigenes

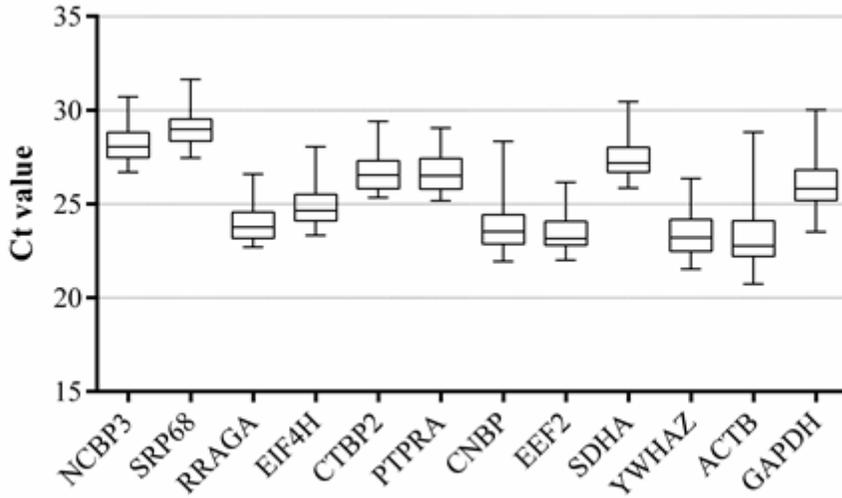


Figure 2

Boxplot of absolute Cq value of the 12 candidate genes in all skin tissue samples

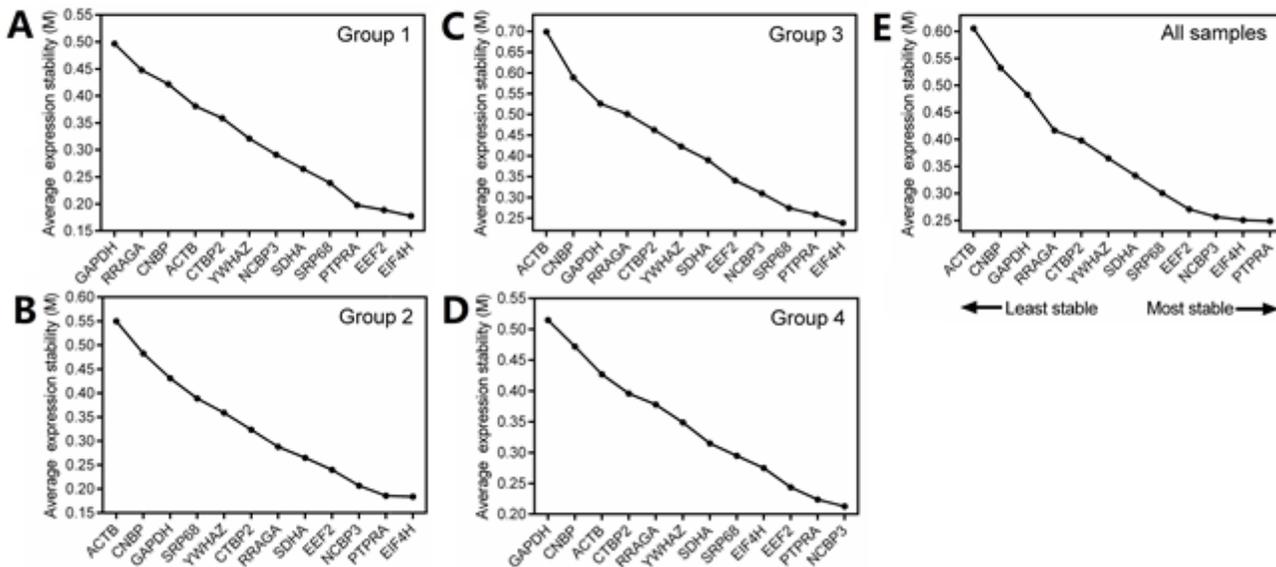


Figure 3

Average expression stability (M-value) calculated by geNorm

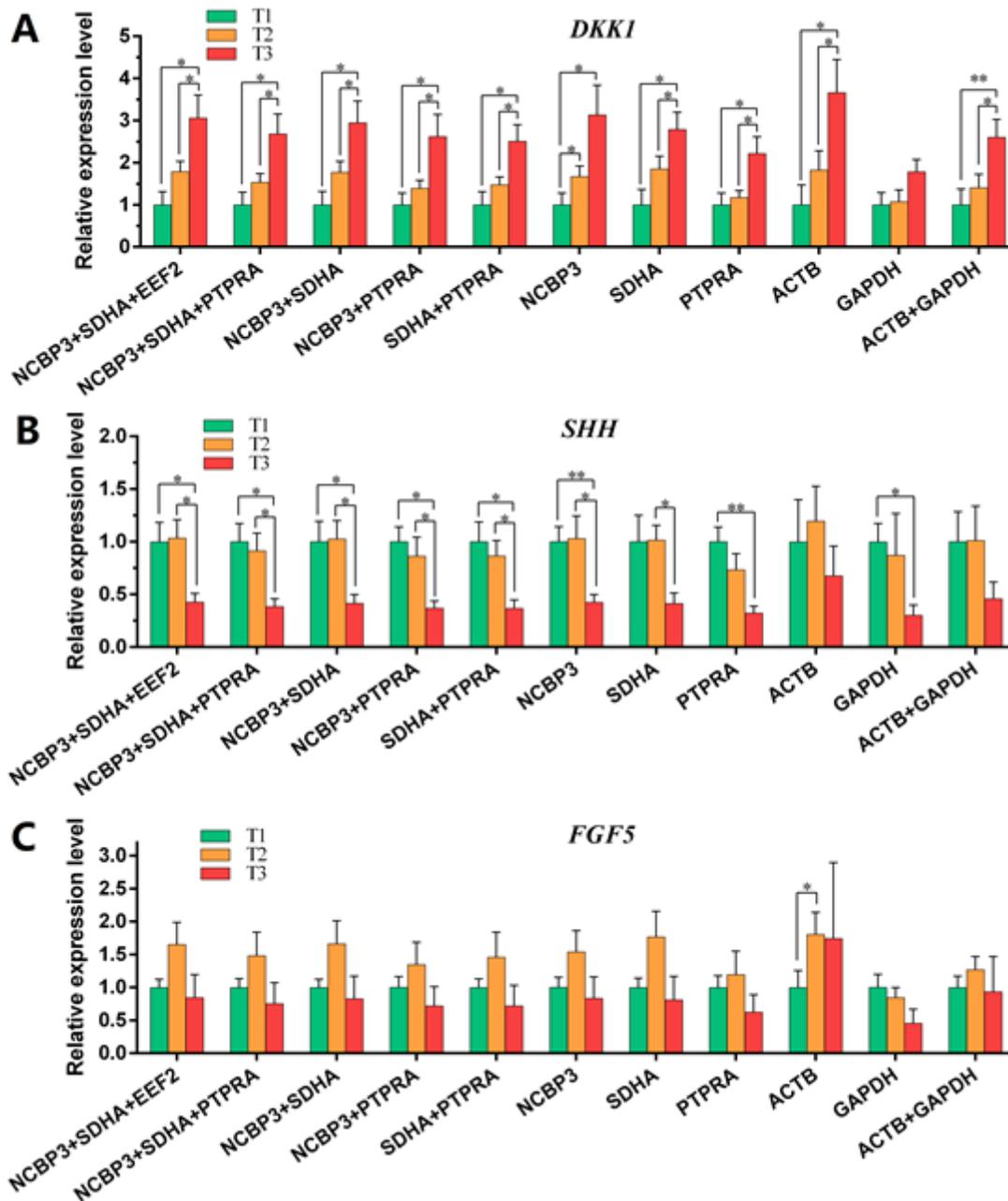


Figure 4

Relative expression levels normalized by 11 types of single or multiple gene combinations of HKGs

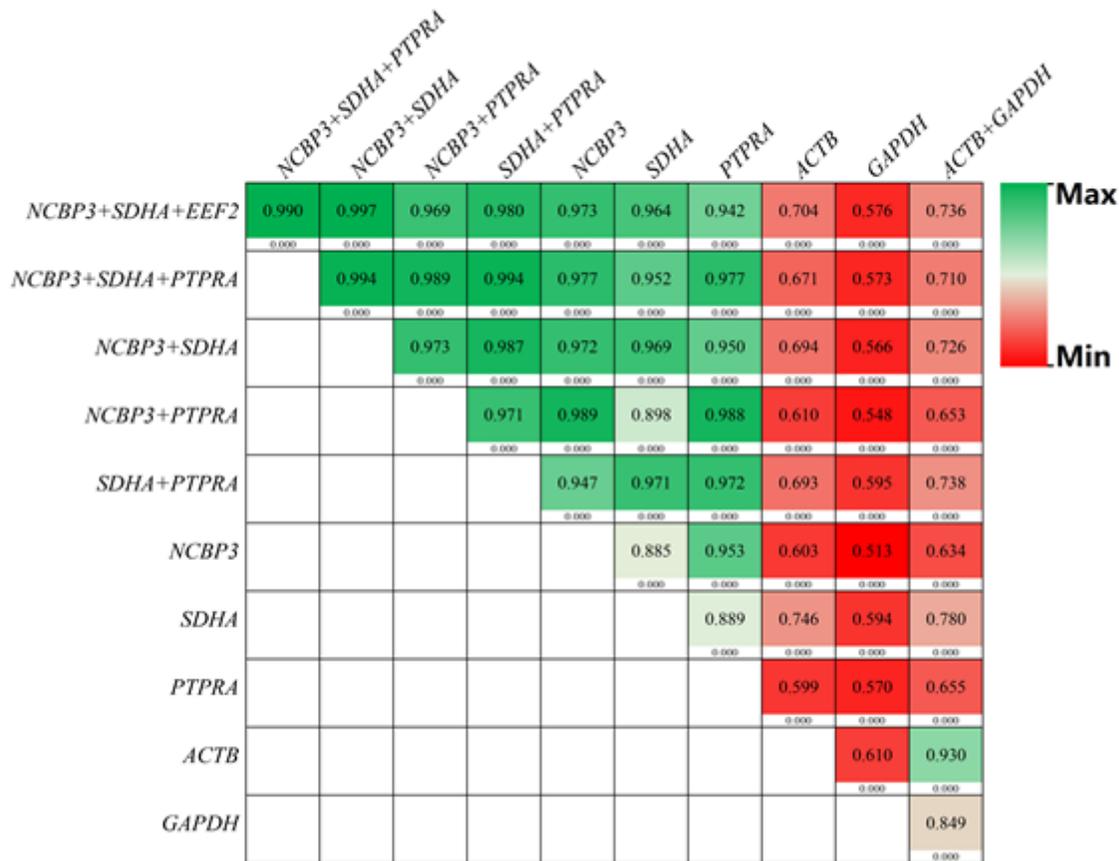


Figure 5

Heat map of correlation coefficients of relative expression levels based on different normalized HKGs

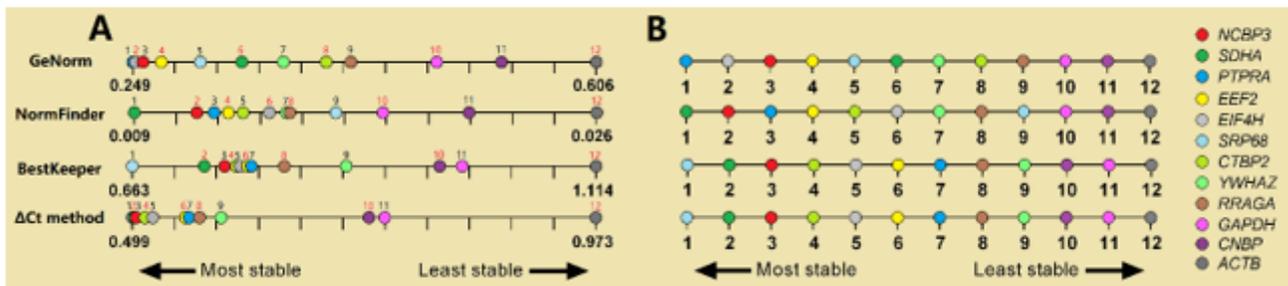


Figure 6

Gene stability values and rank order

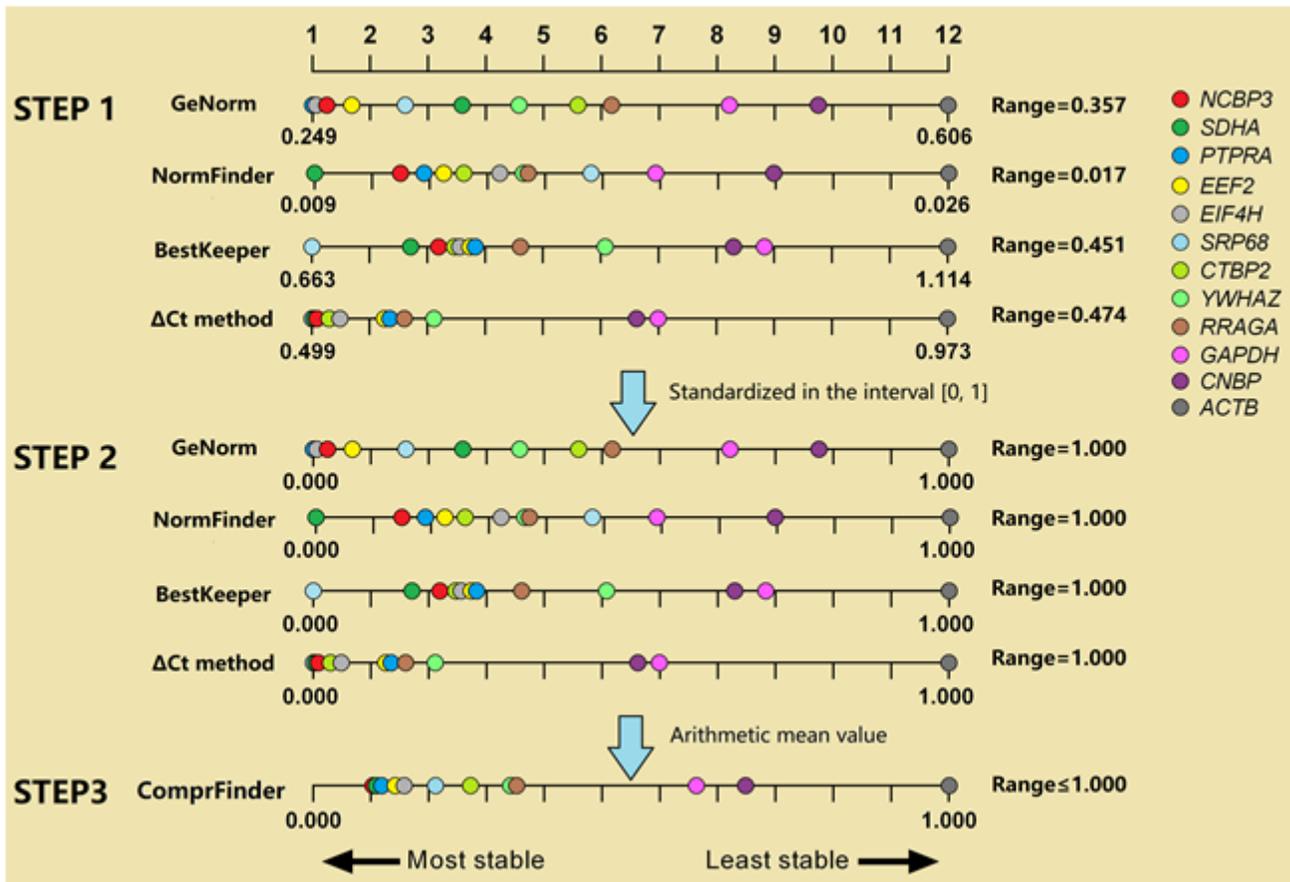


Figure 7

Schematic diagram of the ComprFinder algorithm

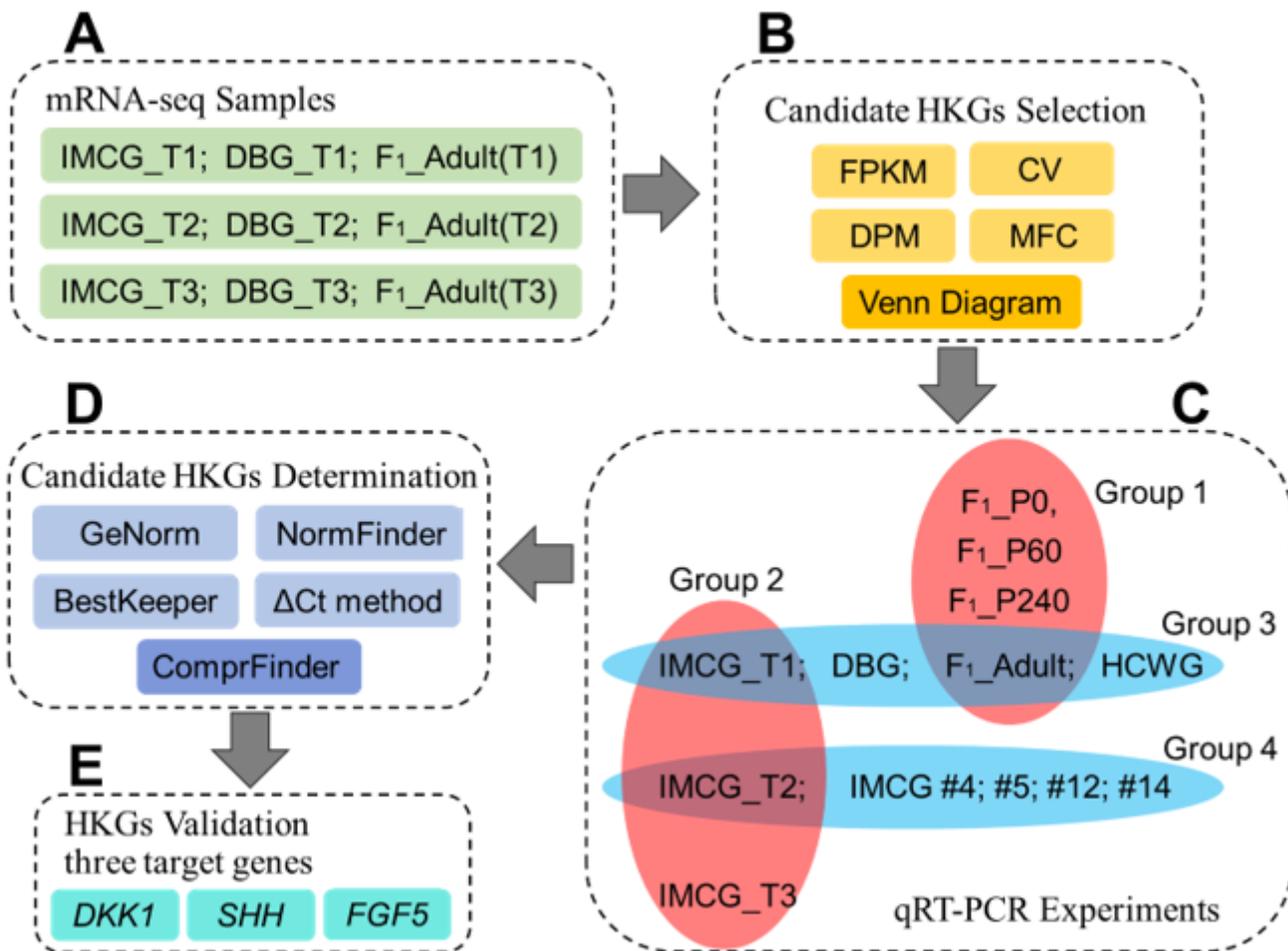


Figure 8

The workflow of this study

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Matlabscript.m](#)
- [DownloadableExcelfilewiththeComprFinderalgorithm.xlsx](#)
- [renamed9cbdf.pdf](#)