• Basic Research •

Identifying the stability of housekeeping genes to be used for the quantitative real-time PCR normalization in retinal tissue of streptozotocin-induced diabetic rats

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Abstract

● **AIM:** To investigate the stability of the seven housekeeping genes: beta-actin *(ActB)*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18s ribosomal unit 5 (*18s*), cyclophilin A (*CycA*), hypoxanthine-guanine phosphoribosyl transferase (*HPRT*), ribosomal protein large P0 (*36B4*) and terminal uridylyl transferase 1 (*U6*) in the diabetic retinal tissue of rat model.

● **METHODS:** The expression of these seven genes in rat retinal tissues was determined using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) in two groups; normal control rats and streptozotocin-induced diabetic rats. The stability analysis of gene expression was investigated using geNorm, NormFinder, BestKeeper, and comparative delta-Ct (ΔCt) algorithms.

● **RESULTS:** The *36B4* gene was stably expressed in the retinal tissues of normal control animals; however, it was less stable in diabetic retinas. The *18s* gene was expressed consistently in both normal control and diabetic rats' retinal tissue. That this gene was the best reference for data normalisation in RT-qPCR studies that used the retinal tissue of streptozotocin-induced diabetic rats. Furthermore, there was no ideal gene stably expressed for use in all experimental settings.

● **CONCLUSION:** Identifying relevant genes is a need for achieving RT-qPCR validity and reliability and must be appropriately achieved based on a specific experimental setting.

● **KEYWORDS:** housekeeping genes stability; real-time reverse transcription polymerase chain reaction; retinal tissue; streptozotocin-induced diabetic rats

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INTRODUCTION

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) measurement of transcript abundance has become the method of choice for high-throughput and accurate expression monitoring of chosen genes due to its high sensitivity, specificity, and broad quantification range[1-3]. This approach is most often employed for molecular diagnostics, verifying microarray data of a narrower collection of genes and is especially beneficial when just a limited number of cells or tissue samples are available[2,4]. The expression of the gene of interest (GOI) is compared to that of an internal control gene known as a housekeeping gene (HKG) to obtain quantification. The use of this HKG to normalise the mRNA fraction is the gold standard since it is not expected to

vary under the test conditions[2,5-6]. Before choosing a suitable HKG, a comprehensive evaluation is required to ensure the appropriateness and adequacy of choice[7-9]. Since expression of GOI is normalized to HKG[10-11], its improper selection may lead to an invalid interpretation[12]. Therefore, choosing the most relevant and more than one HKG in a weighted expression index is expected to address this problem[13].

Incorrect data normalisation and hence misinterpretation remain the most serious issues in RT-qPCR[14-15]. Various methods have been used to assure the validity and reliability of gene expression data[16-18] and one of the most important factors in RT-qPCR is the selection of the right normalisation approach[19]. The synchronised assessment of endogenous HKG is often regarded as the most helpful and relevant method for normalising target genes[20-22]. However, it is known that there is significant variation in gene expression of regularly used HKG and this introduces baseline noise to the corresponding data resulting in inaccurate measurement of the expression of GOI[23]. This also resulted in attempts to adjust for the instability by employing sets of control genes and statistical techniques to calculate normalisation factors[24]. The most widely employed HKGs utilized to measure the expression of various GOI in diabetic rat retinas are glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-actin (*β-actin*)[25]. Literature shows that many studies have used a single HKG for normalisation[26-27]. To some degree, the use of a single HKG is acceptable provided it has already been evaluated in identical experimental conditions and its expression has been accurately confirmed[28], yet, it has been recorded that this may result in a significant bias, as high as a 3- to 6-fold bias in 10%–25% of the examined data[29].

To support the usage of a single or multiple HKG, some algorithms were developed to recognise the difference in HKG stability. BestKeeper, NormFinder, geNorm and comparative delta-Ct (ΔCt) algorithms are among many that have been integrated and made publicly available in Excel-based applications[19,30-33]. Moreover, it also comes in recently built R-based software packages, which has resulted in an increase in HKG-related research globally[34]. These commonly used methods were combined into a single freely accessible web-based software, the RefFinder[35-38], allowing for a comprehensive review of the most stable HKG. So, the BestKeeper, GeNorm, NormFinder, and comparative ΔCt algorithms may be used to identify the most stable internal control genes, which use a measure of gene stability that is unaffected by gene abundance and does not need normalisation[39-41]. These software were developed and tested on a wide range of biological materials in order to discover the most stably expressed HKG that uses raw Cq values as input rather than the specific PCR efficiency of different assays[42].

Despite the growing importance of RT-qPCR technology in recent years, few studies have been conducted to validate the stability of HKG for use as endogenous controls in ocular disorders[43-45], none of them, however, is unique to retinal or diabetic retinal tissue. As a result, identifying a stable expressed HKG has become a significant problem for precise normalisation procedures, particularly in the case of precious biopsies such as retinal tissue from the samples[45]. In this regard, molecular genetic analysis of a particular gene or biochemical marker is critical for discovering innovative therapeutics in ocular disorders like diabetic retinopathy (DR). Hence, we performed real-time RT-qPCR on RNA extracted from the retina of control and diabetic Sprague Dawley rats to quantify the expression of *β-actin*, *GAPDH*, 18s ribosomal unit 5 (*18s*), ribosomal protein large P0 (*36B4*), terminal uridylyl transferase 1 (*U6*), cyclophilin A (*CycA*), and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*). These genes have been used as an endogenous control previously in retinal and non-retinal tissue of animals with a wide range of metabolic disorders[25,46-47].

MATERIALS AND METHODS

Ethical Approval The study was carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals for ophthalmic and vision research and animal ethics guidelines of Universiti Teknologi MARA (UiTM). The institutional committee for animal research granted ethics permission for this work, with approval number UiTM CARE 3/2019/ (286/2019).

Animals Male Sprague Dawley rats aged 8–12wk (200–250 g)[48] were obtained from UiTM. The animals were individually caged and housed in Laboratory Animal Care Unit (LACU), UiTM under standard laboratory conditions of 12-hour light-dark cycle. The food and water were available *ad libitum*. All animals were subjected to general and ophthalmic examination. Those found to have no abnormalities were included in the study.

Animals were divided into two groups: healthy (normal control) rats (n=8) and streptozotocin-induced diabetic retinopathy (SIDR) rats (n=10). The number of rats included in the SIDR group was higher due to mortality associated with this model[48-50]. Blood glucose level was monitored weekly during the experimental period. After 12wk, animals were sacrificed with an intraperitoneal (IP) injection of sodium pentobarbital (0.14 mg/kg body weight)[51]. Eyeballs were enucleated, and retinas were preserved for subsequent RT-qPCR analysis.

Induction of Diabetes To induce diabetes, rats (n=10) were fasted overnight and then were administered with IP injection of 2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose (Santa Cruz Biotechnology Inc., Texas, USA) streptozotocin

Table 1 Primer design

Primer source	NCBI reference ID	Primer length	Sequence	Temperature (°C)
18s ribosomal unit 5 *(18s)*	100861533	21 20	GCCATGCATGTCTAAGTACGC CCGTCGGCATGTATTAGCTC	57.1
Beta-actin (*ActB*)	81822	21 22	ATTGGCAATGAGCGGTTCCGC CTCCTGCTTGCTGATCCACATC	60.0
Cyclophilin A (*CycA*)	25518	20 18	GGATTTGGCTATAAGGGTTC GTTGTCCACAGTCGGAGA	60.0
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	24383	18 20	CCATGGAGAAGGCTGGGG CAAAGTTGTCATGGATGACC	59.4
Hypoxanthine-guanine phosphoribosyl transferase (*HPRT*)	24465	19 24	GACCGGTTCTGTCATGTCG ACCTGGTTCATCATCACTAATCAC	60.0
Ribosomal protein, large, P0 (*36B4*)	11837	20 20	GATCATCCAGCAGGTGTTTG CCAGTGGGAAGGTGTAGTCA	60.3
Terminal uridylyl transferase 1 (*U6)*	499314	17 20	CTCGCTTCGGCAGCACA AACGCTTCACGAATTTGCGT	60.0

NCBI: National Center for Biotechnology Information.

(STZ), dissolved in ice-cold sodium citrate buffer (10 mmol/L, pH 4.5) at a single dose of 55 mg/kg body weight[52]. Blood was collected from the tail vein, 48-hour post-STZ injection for blood glucose estimation using an Accu Chek Performa glucometer (Roche Diagnostic, Basel, CH). Rats with a blood glucose level of more than 20 mmol/L were included for further study[53]. The control group similarly received an IP injection of sodium citrate buffer.

RNA Isolation Two retinas from the same rat were pooled together as one sample. Collected retinas were rinsed with ice-cold phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4) prior to being immersed in RNA stabilisation solution (RNAlater®) to prevent cellular RNA degradation. The extraction and purification of RNA were performed using the commercially available spin-column nucleic acid purification kit. RNA concentration was quantified using a micro-volume ultraviolet (UV)-Vis spectrophotometer. Samples with RNA concentrations of more than 40 ng/μL were considered suitable for DNA conversion. The machine measured the absorbance ratio at 260 nm and 280 nm (A260/A280), which provides an estimate of the purity of RNA for contaminants absorbed by the UV spectrum, such as proteins, ethanol or phenol contamination. Good quality RNA has an A260/A280 ratio of 1.8 to 2.2.

cDNA Synthesis The cDNA synthesis was performed using OneScript® Plus cDNA Synthesis Kit. The 1 μL of 10 mmol/L dNTP mix and 1 μL of 10 μmol/L random primers were added to extracted RNA samples. Nuclease-free water was added to the dNTP-primer-RNA mixture to a total volume of 14.5 μL. The mixture was incubated at 65°C for 5min, followed by incubation on ice for 1min. A master mix containing 5×RT buffer, RNaseOFF Ribonuclease Inhibitor and OneScript RTase were then added. The mixture was then incubated at 25°C for 10min, followed by a second incubation at 42°C for 15min. The reaction was stopped by incubating the mixture at 85°C for 5min. The cDNA was then stored at -20°C until further use.

Primer pair specificity was verified with the Nucleotide Basic Local Alignment Search Tool (BLASTN), an online software tool used by the National Centre for Biotechnology Information (NCBI, USA), against the genome of rats (Table 1). All primers were supplied by Macrogen Inc., Gangnam-gu, Seoul, Republic of Korea. Stock concentrations were diluted to 10 μmol/L before use.

RT-qPCR The RT-qPCR was performed according to the manufacturer's protocol[54]. cDNA templates and all the reaction mixture were prepared on the ice at 10 μL volume according to the manufacturer's instructions (Cat. No.G270, abm, Richmond, Canada). The PCR cycling condition used was enzyme activation at 95°C for 20s, denaturation at 95°C for 3s with 40 cycles of amplification and annealing at 60°C for 30s with 40 cycles and a dissociation curve. PCR products were analysed for melting curves to verify their sensitivity and amplification specificity. Three independent biological replicates and two technical replicates were used for each HKG and GOI. A standard curve was obtained with a 4-fold dilution series (1:9 to 1:6561) for each gene to calculate PCR energy efficiency ranging from 90% to 110%. The cycle threshold (Ct) values were measured using Quantstudio 12K Real-Time System.

Statistical Analysis Blood glucose level of control and diabetic rats were expressed as mean±standard deviation (SD). The statistical significance of differences between means was determined by the unpaired *t*-test and the distribution of *t* in a two-tailed test. *P* values less than 0.05 were considered significant.

For the stability analysis of the seven HKGs, geNorm[22], NormFinder[55], and BestKeeper[13] software were utilized. The comparative ΔCt method was also used to assess the stability of each HKG candidate. In concise, the algorithm

system by geNorm gives its calculation according to pairwise deviation (V-value) among all the analysed HKG to assess the stability estimate (M-Value), and greater stability represented by smaller M-value[22]. Whereas the NormFinde uses the Excel-based platform that measure the stability of gene's Cq values by assessing their inter- and intra-group difference in expression values[55]. The algorithm of BestKeeper appraises stability measures with reference to an index numeracy derived from the SD and coefficient of variance (CV) among the expression[13]. Conclusively, a comparative evaluation platform, the RefFinder (https://www.heartcure.com.au/reffinder/#) was applied to assimilate the findings of each distinct algorithm system by providing the rank of the HKG stability according to their geometric mean (GM)[34].

RESULTS

Blood Glucose Level In general, diabetic rats seemed weaker and thinner than normal control rats. During the experiment, SIDR group has mortality rate of 20% where two rats died, whereas there was no mortality in the normal control group. Hence, at the end of the experiment, there were 8 rats in the control group and 8 rats in the SIDR group (each n=8). The blood glucose level in diabetic rats remained significantly higher compared to control rats starting from 48h post-STZ injection until the end of the experimental period (P<0.001; Figure 1).

Sample RNA Quantity and Quality RNA concentration was quantified after RNA extraction. RNA was quantified using micro-volume UV-Vis. This step is to ensure sufficient RNA was obtained to proceed with RT-qPCR. All samples in the current experiment contained sufficient RNA (higher than 40 ng/μL) to proceed with cDNA conversion[50].

RNA quality was also assessed to confirm the purity of RNA prior to cDNA conversion. The sample absorbance at 260 nm and 280 nm was assessed by a similar device. A 260/280 ratio of approximately 2.0 is generally accepted as good-quality RNA. All samples showed an acceptable range of 260/280 ratio (Table 2).

Expression Profile of the Candidate Reference Genes The stability of mRNA expression for each of the 7 candidate reference genes in STZ-induced rat retinal tissues was analyzed through Ct values. Regarding expression level, only *18s* and *ACTB* were highly abundant with average Ct values range 15.98–17.04 and 16.80–18.31, respectively (Figure 2). Other candidate reference genes (*U6*, *HPRT,* and *36B4*) were expressed at a medium level, with average Ct values ranging from 24.78–28.76, whilst *GAPDH* and *CycA* showed considerably low levels of expression (mean Ct range: 30.36–32.23 and 29.27–32.33 respectively).

BestKeeper For each HKG, the following descriptive statistics were computed: GM, arithmetic mean (AM), minimal

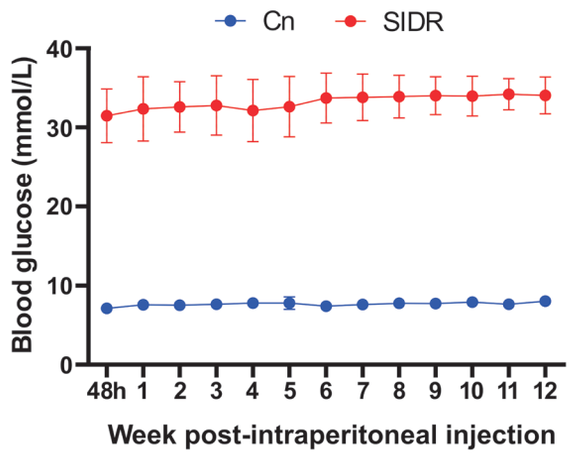

Figure 1 Blood glucose level in control and SIDR rats (mean±SD, n=8) Cn: Control; SIDR: Streptozotocin-induced diabetic rats.

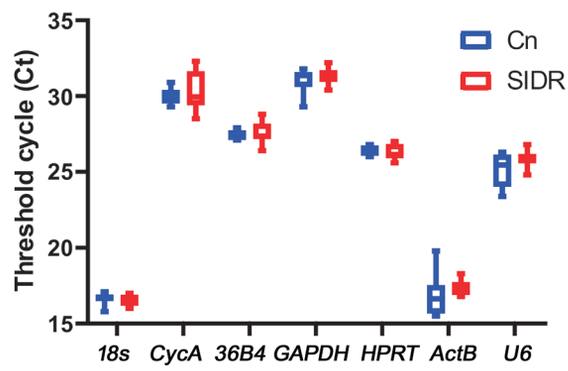

Figure 2 Expression of reference gene candidates determined by raw threshold cycle (Ct) values in retinal samples from control and SIDR rats The bars indicate the maximum and minimum values of the Cts (n=8). Cn: Rats received vehicle intraperitoneally; SIDR: Streptozotocin-induced diabetic rats.

Table 2 Nucleic acid concentration and A260/A280 of each sample

Sample No.	Nucleic acid conc. (ng/μL)		A260/A280	
	Cn	SIDR	Cn	SIDR
1	120.5	111.3	2.1	2.1
2	193.9	227.6	2.1	2.1
3	172.4	164.7	2.1	2.1
4	215.9	174.0	2.0	2.1
5	62.4	151.8	2.0	2.1
6	185.8	199.6	2.1	2.1
7	102.6	315.2	2.1	2.1
8	168.6	289.5	2.1	2.1

Cn: Rats received vehicle intraperitoneally; SIDR: Streptozotocin-induced diabetic rats.

(Min) and maximal (Max) value, SD, and CV (Table 3). All crossing point (CP) data from both groups were compared. Seven genes, each with n=8, were studied. Individual samples' x-fold over- or under-expression towards the GM CP is calculated, and the multiple factors of their minimal and maximal values are expressed as the x-fold ratio and its SD.

After calculating the descriptive statistics for each HKG expression level, the first estimation of HKG expression

Table 3 CP data of housekeeping genes by BestKeeper *n*=8

No.	*18s*		*CycA*		*36B4*		*GAPDH*		*HPRT*		*ActB*		*U6*	
	Cn	SIDR	Cn	SIDR	Cn	SIDR	Cn	SIDR	Cn	SIDR	Cn	SIDR	Cn	SIDR
Geometric mean (CP)	16.65	16.63	29.98	30.25	27.45	27.50	31.04	31.36	26.41	26.30	16.83	17.42	25.15	25.82
Arithmetic mean (CP)	16.65	16.54	29.99	30.28	27.45	27.51	31.05	31.36	26.41	26.30	16.88	17.43	25.18	25.83
Min (CP)	15.80	16.00	29.30	28.50	27.10	26.40	29.30	30.40	26.00	24.60	15.50	16.80	23.40	24.80
Max (CP)	17.10	17.00	30.90	32.30	27.90	28.80	31.80	32.20	26.80	27.00	19.80	18.30	26.30	26.80
SD (±CP)	0.26	0.29	0.41	0.97	0.25	0.54	0.57	0.40	0.29	0.43	0.99	0.36	0.88	0.38
CV (% CP)	1.58	1.74	1.38	3.20	0.91	1.98	1.85	1.27	1.09	1.62	5.89	2.04	3.50	1.48
Min (x-fold)	-1.80	-1.45	-1.61	-3.37	-1.27	-2.15	-3.34	-1.94	-1.33	-1.62	-2.51	-1.54	-3.37	-2.03
Max (x-fold)	1.37	1.38	1.89	4.14	1.37	2.46	1.69	1.79	1.31	1.63	7.85	1.84	2.21	1.97
SD (±x-fold)	1.20	1.22	1.33	1.96	1.19	1.46	1.49	1.32	1.22	1.34	1.99	1.28	1.84	1.30

Descriptive statistics of seven candidate housekeeping genes based on their CP scores. *n*: Number of samples; CP: Crossing point; SD (±CP): Standard deviation of the CP; CV (%CP): Coefficient of variance expressed as a percentage on the CP level; Min (x-fold) and Max (x-fold): Extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD (±x-fold): Standard deviation of the absolute regulation coefficients; Cn: Control; SIDR: Streptozotocin-induced diabetic rats; *18s*: 18s ribosomal unit 5; *CycA*: Cyclophilin A; *36B4*: Ribosomal protein large P0; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; HPRT: Hypoxanthine-guanine phosphoribosyl transferase; *ActB*: Beta-actin; *U6*: Terminal uridylyl transferase 1.

Table 4 Pair-wise correlation

Parameters	*18s*		*CycA*		*36B4*		*GAPDH*		*HPRT*		*ActB*		*U6*	
	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR
CycA	-0.472	0.084	-	-	-	-	-	-	-	-	-	-	-	-
P	0.238	0.844	-	-	-	-	-	-	-	-	-	-	-	-
36B4	-0.074	-0.023	0.837	0.949	-	-	-	-	-	-	-	-	-	-
P	0.862	0.956	0.010	0.001	-	-	-	-	-	-	-	-	-	-
GAPDH	0.789	0.038	-0.651	0.409	-0.226	0.457	-	-	-	-	-	-	-	-
P	0.020	0.929	0.081	0.314	0.590	0.255	-	-	-	-	-	-	-	-
HPRT	0.176	0.656	0.330	0.114	0.335	-0.049	-0.195	0.533	-	-	-	-	-	-
P	0.676	0.078	0.425	0.788	0.418	0.908	0.643	0.174	-	-	-	-	-	-
ActB	-0.071	0.434	-0.155	0.245	-0.334	0.268	-0.425	-0.161	0.414	-0.046	-	-	-	-
P	0.867	0.283	0.715	0.558	0.419	0.521	0.294	0.703	0.309	0.914	-	-	-	-
U6	0.518	0.122	-0.487	-0.707	-0.031	-0.655	0.781	0.161	-0.209	0.427	-0.315	-0.195	-	-
P	0.188	0.774	0.221	0.050	0.943	0.078	0.022	0.704	0.620	0.291	0.447	0.644	-	-
BestKeeper *vs*														
[a]*r*	0.602	0.553	0.001	0.751	0.001	0.715	0.323	0.604	0.499	0.569	0.577	0.497	0.468	0.001
P	0.114	0.155	0.400	0.032	0.893	0.046	0.435	0.113	0.208	0.141	0.134	0.210	0.243	0.655

[a]Pearson correlation coefficient (*r*) by BestKeeper. SIDR: Streptozotocin-induced diabetic rats; *18s*: 18s ribosomal unit 5; *CycA*: Cyclophilin A; *36B4*: Ribosomal protein large P0; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *HPRT*: Hypoxanthine-guanine phosphoribosyl transferase; *ActB*: Beta-actin; *U6*: Terminal uridylyl transferase 1.

stability could be made based on the inspection of calculated variations (SD and CV values). The BestKeeper Index specific to the sample is calculated as the GM of its candidate HKGs CP values from the genes considered stably expressed. Multiple pair-wise correlation analyses are performed to estimate the inter-gene relationships of all possible HKG pairs. The Pearson correlation coefficient (*r*) and the probability *P*-value are calculated for each such correlation (Table 4).

All the highly correlated HKGs are aggregated into an index. The correlation between each candidate HKG and the index is then calculated, with the Pearson correlation coefficient (*r*) and *P*-value describing the relationship between the index and the contributing candidate HKG (Table 4).

HKGs were placed in order from the most stably expressed, with the lowest variation, to the least stable, with the highest variation, based on the variability observed. Any studied gene with an SD greater than one (=starting template variation by factor 2) can be considered inconsistent. In this evaluation by BestKeeper, *36B4* demonstrated the highest stability in normal control whereas *18s* had the highest stability in SIDR among all seven candidate genes. All seven genes did not exceed SD threshold value across both experimental groups and thus

they were regarded as suitable for subsequent gene expression normalization (Figure 3). *ActB* and *CycA* were the least stable candidates in the control and SIDR groups, respectively, however, none crossed the 1 SD threshold value. Overall, using this analytical approach, *18s*, *CycA*, *36B4*, *GAPDH*, *HPRT*, *ActB,* and *U6* remained stable in all sample sets.

geNorm The geNorm algorithm evaluated the stability of reference genes based on expression stability value (M), as shown in Figure 3. All evaluated HKG had an M value below 1.5 which is the recommended geNorm cutoff value for stable gene selection through RT-qPCR analysis. This result confirms that the candidate HKG was stable across retinal tissues from both normal control and SIDR rats. With the lowest M values (both 0.320), *CycA* and *36B4* were the most stably expressed genes among the normal control samples whereas *18s* and *HPRT* (both 0.381) were the most stable genes in SIDR rats. Although overall gene candidates showed stability for subsequent gene analysis, *ActB* and *CycA* were the least stable genes with the highest M value for normal control and SIDR groups, respectively.

NormFinder Based on the evaluation by NormFinder, the top five ranking stable HKG for normal control rats were *HPRT>18s>36B4>CycA>GAPDH*. For SIDR rats, the order was *18s>GAPDH>HPRT>ActB>36B4*. As shown in Figure 3, NormFinder evaluation suggested that *ActB* was the least stable for control and *CycA* for SIDR. Moreover, *U6*, the second least stable gene in normal control rats, was in line with the geNorm, BestKeeper, and ΔCt output. Interestingly, in all these four algorithm results, *18s* remains the best candidate gene and could be considered a stable gene in SIDR rats. Whereas *U6* and *ActB* were the candidate genes that crossed 1 stability value in the normal control group similar to *CycA* in the SIDR group and they could be considered the least stable gene in this analysis.

Comparative Delta-Ct According to the comparative ΔCt method, again, *18s* was found to be the most stable and *HPRT* was the second most stable gene in both experimental groups. These results were very similar to SIDR group analysis by GeNorm but contrasted with results generated by the NormFinder and BestKeeper algorithms (Figure 3). Nevertheless, *CycA* was the most unstable gene across all tested algorithms in this experimental DR model.

Comprehensive Ranking Values The stably expressing genes were ranked differently by the four distinct systems. Previous research has also shown this variation. As shown in Table 5 and Figure 3, comprehensive analyses utilising four algorithms rated the tested genes from the most stable (lowest value) to least stable (highest value) based on the derived HKG stability measure. All geNorm, BestKeeper, NormFinder and ΔCt stability values were summarized together. For normal control rats, *CycA*, *HPRT*, *18s* and *36B4* are the four genes that were found to have average to most stable expressions. For SIDR

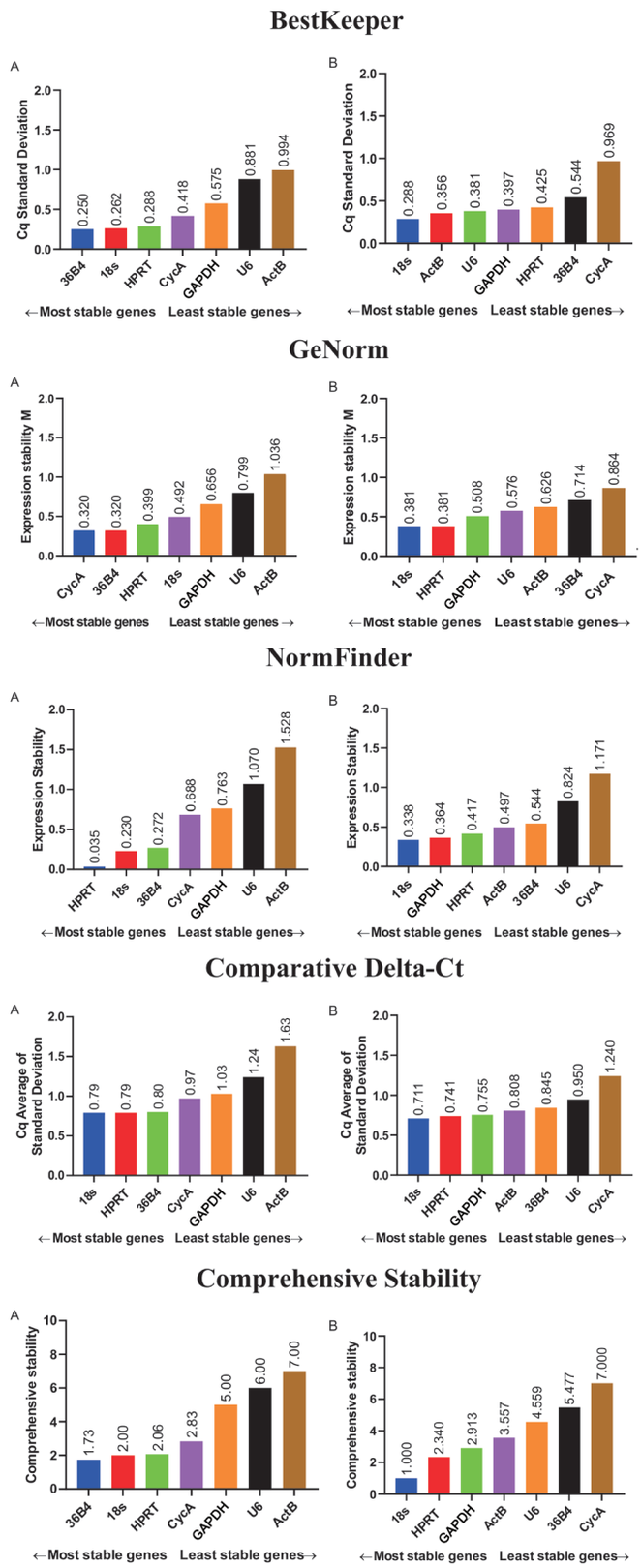

Figure 3 Gene stability as shown by BestKeeper, Genorm, NormFinder, and Delta-Ct *18s*: 18s ribosomal unit 5; *CycA*: Cyclophilin A; *36B4*: Ribosomal protein large P0; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *HPRT*: Hypoxanthine-guanine phosphoribosyl transferase; *ActB*: Beta-actin; *U6*: Terminal uridylyl transferase 1.

Table 5 Ranking order of gene expression stability

Method	1st		2nd		3rd		4th		5th		6th		7th	
	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR	Control	SIDR
Delta-Ct	*18s*	*18s*	*HPRT*	*HPRT*	*36B4*	*GAPDH*	*CycA*	*ActB*	*GAPDH*	*36B4*	*U6*	*U6*	*ActB*	*CycA*
BestKeeper	*36B4*	*18s*	*18s*	*ActB*	*HPRT*	*U6*	*CycA*	*GAPDH*	*GAPDH*	*HPRT*	*U6*	*36B4*	*ActB*	*CycA*
NormFinder	*HPRT*	*18s*	*18s*	*GAPDH*	*36B4*	*HPRT*	*CycA*	*ActB*	*GAPDH*	*36B4*	*U6*	*U6*	*ActB*	*CycA*
geNorm	*CycA/36B4*	*18s/HPRT*	-	-	*HPRT*	*GAPDH*	*18s*	*U6*	*GAPDH*	*ActB*	*U6*	*36B4*	*ActB*	*CycA*
Recommended comprehensive ranking	*36B4*	*18s*	*18s*	*HPRT*	*HPRT*	*GAPDH*	*CycA*	*ActB*	*GAPDH*	*U6*	*U6*	*36B4*	*ActB*	*CycA*

Ranking order (better-good-average). SIDR: Streptozotocin-induced diabetic rats; *18s*: 18s ribosomal unit 5; *CycA*: Cyclophilin A; *36B4*: Ribosomal protein large P0; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *HPRT*: Hypoxanthine-guanine phosphoribosyl transferase; *ActB*: Beta-actin; *U6*: Terminal uridylyl transferase 1.

rats, *ActB*, *GAPDH*, *HPRT*, and *18s* showed the most stable expression. Overall, *36B4* (control) and *18s* (SIDR) were the most stable genes in all the samples by RefFinder because they expressed the lowest Geomean of the ranking values.

DISCUSSION

DR is a metabolic disorder associated with high blood glucose[56-57], thus it would be logical to expect that the expression of several genes would be altered in diabetic animals[58]. To determine the appropriateness of the selection of specific HKG while determining the expression of GOI particularly in the diabetic rat retinas, we examined the expression of seven HKG widely described in the literature: *GAPDH*, *β-actin*, *HPRT*, *18s*, *36B4*, *ActB* and *U6*. We showed that *36B4* and *18s* were stably expressed in the rat retina of the normal control and SIDR groups, respectively and were considered suitable for selection as HKG. *U6* and *ActB* expressions were less stable. Expression of *CycA* incorporates a substantial percentage of processed pseudogenes, and pseudogene amplification may remain even when all steps were taken to reduce genomic DNA contamination[59], as was evident in the current study. Although *18s* and *28s* ribosomal RNA are known to have considerable variance in terms of the expression ratios of rRNA and mRNA[14], this study proved that this gene could be used for subsequent RT-qPCR to determine the expression of GOI. Moreover, *18s* was not expressed to a far greater extent than are most other genes, making it relevant as HKG for various applications. Hyperglycaemia has a profound influence on gene expression[60-63]. With the exception of a relatively small number of hyperglycaemia-induced genes, transcription is greatly altered under conditions of hyperglycaemia. Interestingly, many studies examining the effects of diabetes on gene expression have utilized *GAPDH* as a reference gene, even though its expression is upregulated by hyperglycaemia-induced oxidative stress[64-66]. Our findings in the SIDR group align with other recent research that revealed that *18s* is extensively utilised for the standardisation of RT-qPCR responses in a rat diabetes experimental paradigm[67-68]. Recent studies in diabetic rats' foot lesions and muscle tissue have used *18s* as a reference gene without its validation as the appropriate HKG. However, the appropriateness of the selection of HKG for each experimental set-up should be independently confirmed. Ahmad *et al*[69] employed both *18s* and *GAPDH* as the reporter genes for measuring the anti-inflammatory effects of a natural product in an STZ-induced mice model of DR. There is also another study that used *18s* as the HKG, especially in STZ-induced DR rats[70-72], mice[73-76], and cells[76-78].

In any application of RT-qPCR experimentation, it is recommended that data should be normalised with at least two HKG which should be verified before choosing them[79]. This is important to avoid any misinterpretation of the expression analysis, regardless of the application[29]. Constitutively expressed genes associated with housekeeping or structural activities are typically employed as HKG because they are expressed across numerous cell and tissue types and exhibit minimal variation among samples or experimental circumstances[80]. Previous studies have employed *GAPDH* for normalisation of responses in type 1 or type 2 diabetic mice without mentioning the previous validation methods, which may under- or overestimate target gene expression. Since DR pathology can results in hypoxia due to abnormal vascularization, HKG selected in this study can be related to its stability against a low oxygen tension environment. However, the stability expression of HKG among retinopathy or hypoxic experimental models is very limited. For the cardiac tissue of rats exposed to intermittent hypoxia, *GAPDH* was the most stable gene, followed by *ActB*. According to Moein *et al*[81], these genes can be stable when fibrotic lesions occur. This could be due to its ability to generate cross-reactions of the expression of the different actin isoforms, influencing its gene expression. Despite the fact that the *ActB* is also frequently employed as an HKG for RT-qPCR in rodent models, its stability varies[82]. Previously, Zhong and Kowluru[83] have used both *ActB* and *18s* as HKG for metabolic memory research, whereas Elsherbiny *et al*[84] used *GAPDH* and *18s*. However, as compared to the other HKGs, this *ActB* candidate was not

found to be the most stable gene in the current study and its' ranking varied depending on the algorithms utilised and the treatment to which the animal was subjected.

Two HKG, *HPRT* and *36B4*, were examined for RT-qPCR in a study of light-induced clock gene expression and dopamine levels in the DR mouse retina. The *HPRT* gene was utilised for internal standardisation of target gene expression since it demonstrates constitutively non-regulated expression in both groups regardless of physiologic state or experimental circumstances[85]. In contrast, when the steady-state transcript levels of four commonly used HKG, *β-actin*, *GAPDH*, *CycA*, and *36B4*, were examined in various rat tissues, the *36B4* gene appeared to be the most suitable as a standard for comparing gene expression levels across tissues. According to the findings of this study, the *36B4* gene has a moderate level of stability in the SIDR samples analysed. This relevancy of *36B4* in diabetes research was also shown previously in STZ-induced diabetic rats[86]. Based on previous literature authors found that the utilization of this candidate gene for RT-qPCR normalization is limited, and they used northern blotting for the control probe[87-89]. The stability values fluctuate depending on the method utilised, and the candidate gene reference differs depending on the tissue and experimental conditions imposed[90]. Given this knowledge, it is reasonable to predict discrepancies and substantial errors in the stability and degree of gene expression under different experimental settings[91], even if they are conducted on animals of the same species.

The finding of *HPRT* in the current study was in line with previous research on STZ-induced DR rats[92], STZ-induced mice[93-95], and high glucose-induced cells[96-98]. However, Ly *et al*[99] used both *HPRT* and *GAPDH* for the detection of early inner retinal astrocyte dysfunction in STZ-induced DR rats. To compare with the *U6* gene, Kovacs *et al*[71] identified that this candidate gene provided the best stability for investigating microRNAs in early DR in STZ-induced rats. When multiple HKG candidates are employed, it is critical to evaluate all of them together to create findings that are more reliable and accurate in assessing gene expression[100]. Normalization of, say, seven reference genes becomes impractical when just two GOI need to be investigated. An ideal HKG demonstrates continuous expression in that experimental environment[101], rather than throughout the animal's life.

In a systematic review by Dheda *et al*[102] where 1700 publications were assessed, it was discovered that the majority of them used poorly standardised techniques or did not have them at all. Some published research currently does not even incorporate the process of gene validation for using a single HKG[103]. One probable explanation for this is a lack of awareness about the significance of validating HKG for each experimental set-up[104]. The optimum selection of genes for normalization reduces variability[105-106], ensuring improved repeatability of the results[107]. It has been discovered that reducing variability by including many reference genes does not compensate for some disadvantages, such as the time and additional cost associated with such inclusions.

Given that genes participate in various cellular functions[108-110], there is no universal HKG that can be used with the greatest efficacy in any metabolic disorder especially in diabetic diseases[111]. Furthermore, experimental manipulation of the tissue alters the stability and validity of the same HKG[112-113]. The addition of more HKG does not reduce the variability of gene expression[114], but it increases the cost of research[115]. This emphasises the importance of determining the optimal number of HKG appropriate for a specific setting[116]. The current study indicates that the use of at least two validated stable HKG is the most appropriate.

In summary, it is not possible to find an ideal HKG for a specific species. The experimental situation will have an impact on gene stability because it alters cellular metabolism. By using at least two most appropriate HKG, it is possible to maintain the accuracy and reliability of the research while saving the cost. As conclusion, this experiment demonstrated that the most stable HKG for RT-qPCR in retinal tissue of normal control and diabetic rats are *36B4* and *18s*, respectively. However, *36B4* is the most stable gene for the control group, *18s* provides competitive stability and thus could be considered the most consistent across the retinal tissues of normal control and diabetic rats. It is recommended that a practice of using commonly used HKG in literature must be complemented with optimization for appropriateness for specified experimental settings.

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REFERENCES

1 Torres S, Lama C, Mantecón L, Flemetakis E, Infante C. Selection and validation of reference genes for quantitative real-time PCR in the green microalgae Tetraselmis Chui. *PLoS One* 2021;16(1):e0245495.

2 Grätz C, Bui MLU, Thaqi G, Kirchner B, Loewe RP, Pfaffl MW. Obtaining reliable RT-qPCR results in molecular diagnostics-MIQE goals and pitfalls for transcriptional biomarker discovery. *Life* (*Basel*) 2022;12(3):386.

3 Pagacz K, Kucharski P, Smyczynska U, Grabia S, Chowdhury D, Fendler W. A systemic approach to screening high-throughput RT-qPCR data for a suitable set of reference circulating miRNAs. *BMC Genomics* 2020;21(1):111.

4 Letovanec I, Finn S, Zygoura P, *et al*, European Thoracic Oncology Platform Lungscape Consortium. Evaluation of NGS and RT-PCR methods for ALK rearrangement in European NSCLC patients: results from the European thoracic oncology platform lungscape project. *J Thorac Oncol* 2018;13(3):413-425.

5 Tu CY, Du TS, Shao CC, Liu ZJ, Li LL, Shen YW. Evaluating the potential of housekeeping genes, rRNAs, snRNAs, microRNAs and circRNAs as reference genes for the estimation of PMI. *Forensic Sci Med Pathol* 2018;14(2):194-201.

6 Li YK, Han JB, Wu JY, *et al*. Transcriptome-based evaluation and validation of suitable housekeeping gene for quantification real-time PCR under specific experiment condition in teleost fishes. *Fish Shellfish Immunol* 2020;98:218-223.

7 Zhang JP, Deng CC, Li JL, Zhao YJ. Transcriptome-based selection and validation of optimal house-keeping genes for skin research in goats (Capra hircus). *BMC Genomics* 2020;21(1):493.

8 Sarwar MB, Ahmad Z, Anicet BA, Sajid M, Rashid B, Hassan S, Ahmed M, Husnain T. Identification and validation of superior housekeeping gene(s) for qRT-PCR data normalization in *Agave sisalana* (a CAM-plant) under abiotic stresses. *Physiol Mol Biol Plants* 2020;26(3):567-584.

9 Miao JX, Wang JY, Robl N, Guo HR, Song SH, Peng Y, Wang YC, Huang SN, Li XM. Validation of stable housekeeping genes for quantitative real time PCR in golden Syrian hamster. *Indian J Anim Res* 2020:10.18805/IJAR.B-1237.

10 Chen ZW, Halford NG, Liu CH. Real-time quantitative PCR: primer design, reference gene selection, calculations and statistics. *Metabolites* 2023;13(7):806.

11 Soltanian S, Sheikhbahaei M. Identification of suitable housekeeping genes for quantitative gene expression analysis during retinoic acid-induced differentiation of embryonal carcinoma NCCIT cells. *Journal of Cell and Molecular Research* 2021;12(2):104-10.

12 Kloubert V, Rink L. Selection of an inadequate housekeeping gene leads to misinterpretation of target gene expression in zinc deficiency and zinc supplementation models. *J Trace Elem Med Biol* 2019;56:192-197.

13 Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnol Lett* 2004;26(6):509-515.

14 de Jonge HJ, Fehrmann RS, de Bont ES, *et al*. Evidence based selection of housekeeping genes. *PLoS One* 2007;2(9):e898.

15 Want A, Staniak K, Grabowska-Pyrzewicz W, *et al*. Optimized RT-qPCR and a novel normalization method for validating circulating miRNA biomarkers in ageing-related diseases. *Sci Rep* 2023;13(1):20869.

16 Hu CM, Zhou CL, Wan JN, *et al*. Selection and validation of internal control genes for quantitative real-time RT–qPCR normalization of Phlebopus portentosus gene expression under different conditions. *PLoS One* 2023;18(9):e0288982.

17 Dong HQ, Hu XY, Liang SJ, Wang RS, Cheng P. Selection of reference genes in liproxstatin-1-treated K562 Leukemia cells via RT-qPCR and RNA sequencing. *Mol Biol Rep* 2024;51(1):55.

18 Feria-Romero IA, Bribiesca-Cruz I, Coyoy-Salgado A, Segura-Uribe JJ, Bautista-Poblet G, Granados-Cervantes A, Guerra-Araiza C. Validation of housekeeping genes as an internal control for gene expression studies in the brain of ovariectomized rats treated with tibolone. *Gene* 2021;769:145255.

19 De Spiegelaere W, Dern-Wieloch J, Weigel R, *et al*. Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One* 2015;10(3):e0122515.

20 Wang X, Gardiner EJ, Cairns MJ. Optimal consistency in microRNA expression analysis using reference-gene-based normalization. *Mol Biosyst* 2015;11(5):1235-1240.

21 Vandesompele J, Kubista M, Pfaffl MW. Reference gene validation software for improved normalization. *Real-time PCR: current technology and applications* 2009;4:47-64.

22 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3(7):RESEARCH0034.

23 Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* 2005;21(3):389-395.

24 Bonham-Carter O, Thu YM. Systematic normalization with multiple housekeeping genes for the discovery of genetic dependencies in cancer. In Niagara Falls, New York. ACM, New York, NY, USA, 2019:10 pages.

25 Biederman J, Yee J, Cortes P. Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis. *Kidney Int* 2004;66(6):2308-2314.

26 Bustin SA, Benes V, Garson J, *et al*. The need for transparency and good practices in the qPCR literature. *Nat Methods* 2013;10(11):1063-1067.

27 Ceelen L, De Craene J, De Spiegelaere W. Evaluation of normalization strategies used in real-time quantitative PCR experiments in HepaRG cell line studies. *Clin Chem* 2014;60(3):451-454.

28 Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. Housekeeping genes as internal standards: use and limits. *J Biotechnol* 1999;75(2-3):291-295.

29 Kozera B, Rapacz M. Reference genes in real-time PCR. *J Appl Genet* 2013;54(4):391-406.

30 Xie FL, Wang JY, Zhang BH. RefFinder: a web-based tool for comprehensively analyzing and identifying reference genes. *Funct Integr Genomics* 2023;23(2):125.

31 Petersen PHD, Lopacinska-Jørgensen J, Høgdall CK, Høgdall EV. Expression stability analysis of candidate references for normalization of RT-qPCR data using RefSeeker R package. *Bio Protoc* 2023;13(17):e4801.

32 Jiang CF, Zhou L, Zhao QC, Wang MK, Shen SR, Zhao T, Cui KD, He LM. Selection and validation of reference genes for reverse-

transcription quantitative PCR analysis in *Sclerotium rolfsii*. *Int J Mol Sci* 2023;24(20):15198.

33 Wang H, Cai QZ, Liu L, Yang Q, Zhou LY. Reference gene screening for Real-time quantitative PCR in Polygonum multiflorum. *Zhongguo Zhong Yao Za Zhi* 2021;46(1):80-85.

34 Xie FL, Xiao P, Chen DL, Xu L, Zhang BH. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol* 2012;80:75-84.

35 Sarker N, Fabijan J, Emes RD, *et al*. Identification of stable reference genes for quantitative PCR in koalas. *Sci Rep* 2018;8(1):3364.

36 Yang J, Yang XZ, Kuang Z, Li B, Lu XY, Cao XY, Kang JF. Selection of suitable reference genes for qRT-PCR expression analysis of Codonopsis pilosula under different experimental conditions. *Mol Biol Rep* 2020;47(6):4169-4181.

37 Oto J, Plana E, Fernández-Pardo Á, Cana F, Martínez-Sarmiento M, Vera-Donoso CD, España F, Medina P. Identification of miR-29c-3p as a robust normalizer for urine microRNA studies in bladder cancer. *Biomedicines* 2020;8(11):447.

38 da Conceição Braga L, Gonçalves BÔP, Coelho PL, da Silva Filho AL, Silva LM. Identification of best housekeeping genes for the normalization of RT-qPCR in human cell lines. *Acta Histochem* 2022;124(1):151821.

39 Vasu M, Ahlawat S, Choudhary V, *et al*. Identification and validation of stable reference genes for expression profiling of target genes in diverse ovine tissues. *Gene* 2024;897:148067.

40 Jin G, Zhang XH, Yu SY, Du YP, Wang MX, Zhao CL, Zhang MF. Screening and validation of optimal miRNA reference genes in different developing stages and tissues of Lilium henryi Baker. *Sci Rep* 2024;14(1):1545.

41 Zhu XX, Shen AR, Li N, *et al*. Identification of stable reference genes for relative quantification of long RNA expression in urinary extracellular vesicles. *J Extracellular Bio* 2024;3(1).

42 Lee SY, Park CJ, Nam YK. Assessment of Suitable Reference Genes for RT-qPCR Normalization with Developmental Samples in Pacific Abalone *Haliotis discus hannai*. *J Anim Reprod Biotechnol* 2019;34(4):280-291.

43 Kulkarni B, Mohammed I, Hopkinson A, Dua HS. Validation of endogenous control genes for gene expression studies on human ocular surface epithelium. *PLoS One* 2011;6(8):e22301.

44 Wei RX, Stewart EA, Amoaku WM. Suitability of endogenous reference genes for gene expression studies with human intraocular endothelial cells. *BMC Res Notes* 2013;6:46.

45 Cinici E, Caglar O, Arslan ME, Dilekmen N, Utlu B, Mardinoglu A, Turkez H. Targeted gene candidates for treatment and early diagnosis of age-related macular degeneration. *Biomed Res Int* 2021;2021:6620900.

46 Zhang WX, Fan J, Ma J, Rao YS, Zhang L, Yan YE. Selection of suitable reference genes for quantitative real-time PCR normalization in three types of rat adipose tissue. *Int J Mol Sci* 2016;17(6):968.

47 van Wijngaarden P, Brereton HM, Coster DJ, Williams KA. Stability of housekeeping gene expression in the rat retina during exposure to cyclic hyperoxia. *Mol Vis* 2007;13:1508-1515.

48 Sadikan MZ, Nasir NAA, Iezhitsa I, Agarwal R. Antioxidant and anti-apoptotic effects of tocotrienol-rich fraction against streptozotocin-induced diabetic retinopathy in rats. *Biomedecine Pharmacother* 2022;153:113533.

49 Ghasemi A, Jeddi S. Streptozotocin as a tool for induction of rat models of diabetes: a practical guide. *EXCLI J* 2023;22:274-294.

50 Sadikan MZ, Abdul Nasir NA, Bakar NS, Iezhitsa I, Agarwal R. Tocotrienol-rich fraction reduces retinal inflammation and angiogenesis in rats with streptozotocin-induced diabetes. *BMC Complement Med Ther* 2023;23(1):179.

51 Sadikan MZ, Nasir NAA, Agarwal R, Ismail NM. Protective effect of palm oil-derived tocotrienol-rich fraction against retinal neurodegenerative changes in rats with streptozotocin-induced diabetic retinopathy. *Biomolecules* 2020;10(4):556.

52 Sadikan MZ, Abdul Nasir NA, Iezhitsa I, Agarwal R. Open field mirror test as a tool for the assessment of visual functions in rats with streptozotocin-induced diabetes. *Neurosci Res Notes* 2021;4(3):11-20.

53 Sadikan MZ, Abdul Nasir NA, Abdul Ghani NA, Lambuk L, Iezhitsa IN, Agarwal AR. The use of Fiji image J as an image analysis tool for measuring retinal vessel diameter in rodent model of diabetic retinopathy. *Asian J Med Biomed* 2021;5(1):61-66.

54 Abdul Ghani NA, Abdul Nasir NA, Lambuk L, Sadikan MZ, Agarwal R, Ramli N. The effect of palm oil-derived tocotrienol-rich fraction in preserving normal retinal vascular diameter in streptozotocin-induced diabetic rats. *Graefes Arch Clin Exp Ophthalmol* 2023;261(6):1587-1596.

55 Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004;64(15):5245-5250.

56 Sadikan MZ, Abdul Nasir NA, *et al*. Diabetic retinopathy: a comprehensive update on *in vivo*, *in vitro* and *ex vivo* experimental models. *BMC Ophthalmol* 2023;23(1):421.

57 Sadikan MZ, Abdul Nasir NA. Diabetic retinopathy: emerging concepts of current and potential therapy. *Naunyn Schmiedebergs Arch Pharmacol* 2023;396(12):3395-3406.

58 Kowluru RA, Kowluru A, Mishra M, Kumar B. Oxidative stress and epigenetic modifications in the pathogenesis of diabetic retinopathy. *Prog Retin Eye Res* 2015;48:40-61.

59 Pink RC, Wicks K, Caley DP, Punch EK, Jacobs L, Carter DRF. Pseudogenes: pseudo-functional or key regulators in health and disease? *RNA* 2011;17(5):792-798.

60 Li BX, Wang Y, Liu Y, Ma JX, Li YK. Altered gene expression involved in insulin signaling pathway in type II diabetic osteoporosis rats model. *Endocrine* 2013;43(1):136-146.

61 Ali Beg MM, Verma AK, Saleem M, Saud Alreshidi F, Alenazi F, Ahmad H, Joshi PC. Role and significance of circulating biomarkers:

miRNA and E2F1 mRNA expression and their association with type-2 diabetic complications. *Int J Endocrinol* 2020;2020:6279168.

62 Bheda P. Metabolic transcriptional memory. *Mol Metab* 2020;38:100955.

63 Lother A, Bondareva O, Saadatmand AR, *et al*. Diabetes changes gene expression but not DNA methylation in cardiac cells. *J Mol Cell Cardiol* 2021;151:74-87.

64 Mrudula T, Suryanarayana P, Srinivas PNBS, Reddy GB. Effect of curcumin on hyperglycemia-induced vascular endothelial growth factor expression in streptozotocin-induced diabetic rat retina. *Biochem Biophys Res Commun* 2007;361(2):528-532.

65 Li EH, Huang QZ, Li GC, Xiang ZY, Zhang X. Effects of miRNA-200b on the development of diabetic retinopathy by targeting *VEGFA* gene. *Biosci Rep* 2017;37(2):BSR20160572.

66 Gong CY, Lu B, Hu QW, Ji LL. Streptozotocin induced diabetic retinopathy in rat and the expression of vascular endothelial growth factor and its receptor. *Int J Ophthalmol* 2013;6(5):573-577.

67 Obrosova IG, Minchenko AG, Marinescu V, Fathallah L, Kennedy A, Stockert CM, Frank RN, Stevens MJ. Antioxidants attenuate early up regulation of retinal vascular endothelial growth factor in streptozotocin-diabetic rats. *Diabetologia* 2001;44(9):1102-1110.

68 Zhou ZK, Wang F, Ren XC, Wang YY, Blanchard C. Resistant starch manipulated hyperglycemia/hyperlipidemia and related genes expression in diabetic rats. *Int J Biol Macromol* 2015;75:316-321.

69 Ahmad S, ElSherbiny NM, Jamal MS, *et al*. Anti-inflammatory role of sesamin in STZ induced mice model of diabetic retinopathy. *J Neuroimmunol* 2016;295-296:47-53.

70 Krady JK, Basu A, Allen CM, Xu YP, LaNoue KF, Gardner TW, Levison SW. Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. *Diabetes* 2005;54(5):1559-1565.

71 Kovacs B, Lumayag S, Cowan C, Xu SB. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. *Invest Ophthalmol Vis Sci* 2011;52(7):4402-4409.

72 Hu J, Dziumbla S, Lin JH, *et al*. Inhibition of soluble epoxide hydrolase prevents diabetic retinopathy. *Nature* 2017;552(7684):248-252.

73 Li JM, Wang JJ, Yu Q, Wang M, Zhang SX. Endoplasmic reticulum stress is implicated in retinal inflammation and diabetic retinopathy. *FEBS Lett* 2009;583(9):1521-1527.

74 Mishra M, Duraisamy AJ, Kowluru RA. Sirt1:a guardian of the development of diabetic retinopathy. *Diabetes* 2018;67(4):745-754.

75 Kowluru RA, Mishra M, Kumar B. Diabetic retinopathy and transcriptional regulation of a small molecular weight G-Protein, Rac1. *Exp Eye Res* 2016;147:72-77.

76 Portillo JA C, Lopez Corcino Y, *et al*. CD40 in retinal Müller cells induces P2X7-dependent cytokine expression in macrophages/microglia in diabetic mice and development of early experimental diabetic retinopathy. *Diabetes* 2017;66(2):483-493.

77 Mortuza R, Feng B, Chakrabarti S. MiR-195 regulates SIRT1-mediated changes in diabetic retinopathy. *Diabetologia* 2014;57(5):1037-1046.

78 Santos JM, Tewari S, Goldberg AFX, Kowluru RA. Mitochondrial biogenesis and the development of diabetic retinopathy. *Free Radic Biol Med* 2011;51(10):1849-1860.

79 Ling DJ, Salvaterra PM. Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. *PLoS One* 2011;6(3):e17762.

80 Davies KA, Welch SR, Sorvillo TE, *et al*. Optimal reference genes for RNA tissue analysis in small animal models of hemorrhagic fever viruses. *Sci Rep* 2023;13(1):19384.

81 Moein S, Javanmard SH, Abedi M, Izadpanahi MH, Gheisari Y. Identification of appropriate housekeeping genes for gene expression analysis in long-term hypoxia-treated kidney cells. *Adv Biomed Res* 2017;6:15.

82 Chapman JR, Waldenström J. With reference to reference genes: a systematic review of endogenous controls in gene expression studies. *PLoS One* 2015;10(11):e0141853.

83 Zhong Q, Kowluru RA. Role of histone acetylation in the development of diabetic retinopathy and the metabolic memory phenomenon. *J Cell Biochem* 2010;110(6):1306-1313.

84 Elsherbiny NM, Ahmad S, Naime M, *et al*. ABT-702, an adenosine kinase inhibitor, attenuates inflammation in diabetic retinopathy. *Life Sci* 2013;93(2-3):78-88.

85 Lahouaoui H, Coutanson C, Cooper HM, Bennis M, Dkhissi-Benyahya O. Diabetic retinopathy alters light-induced clock gene expression and dopamine levels in the mouse retina. *Mol Vis* 2016;22:959-969.

86 McVicar CM, Hamilton R, Colhoun LM, Gardiner TA, Brines M, Cerami A, Stitt AW. Intervention with an erythropoietin-derived peptide protects against neuroglial and vascular degeneration during diabetic retinopathy. *Diabetes* 2011;60(11):2995-3005.

87 Otani A, Takagi H, Oh H, Koyama S, Honda Y. Angiotensin II induces expression of the Tie2 receptor ligand, angiopoietin-2, in bovine retinal endothelial cells. *Diabetes* 2001;50(4):867-875.

88 Yokota T, Ma RC, Park JY, *et al*. Role of protein kinase C on the expression of platelet-derived growth factor and endothelin-1 in the retina of diabetic rats and cultured retinal capillary pericytes. *Diabetes* 2003;52(3):838-845.

89 Takagi H, King GL, Aiello LP. Hypoxia upregulates glucose transport activity through an adenosine-mediated increase of GLUT1 expression in retinal capillary endothelial cells. *Diabetes* 1998;47(9):1480-1488.

90 Fedoseeva LA, Shevelev OB, Kolosova NG, Dymshits GM. MS2 phage ribonucleoproteins as exogenous internal control for RT-qPCR data normalization in gene expression study of developing rat brain. *Biochemistry* 2014;79(7):706-716.

91 Ho KH, Patrizi A. Assessment of common housekeeping genes as reference for gene expression studies using RT-qPCR in mouse choroid plexus. *Sci Rep* 2021;11(1):3278.

92 Cammalleri M, Dal Monte M, Locri F, Marsili S, Lista L, De Rosa M, Pavone V, Rusciano D, Bagnoli P. Diabetic retinopathy in the spontaneously diabetic torii rat: pathogenetic mechanisms and

preventive efficacy of inhibiting the urokinase-type plasminogen activator receptor system. *J Diabetes Res* 2017;2017:2904150.

93 Lahouaoui H, Coutanson C, Cooper HM, Bennis M, Dkhissi-Benyahya O. Clock genes and behavioral responses to light are altered in a mouse model of diabetic retinopathy. *PLoS One* 2014;9(7):e101584.

94 Kandpal RP, Rajasimha HK, Brooks MJ, Nellissery J, Wan J, Qian J, Kern TS, Swaroop A. Transcriptome analysis using next generation sequencing reveals molecular signatures of diabetic retinopathy and efficacy of candidate drugs. *Mol Vis* 2012;18:1123-1146.

95 Shalaby L, Thounaojam M, Tawfik A, *et al*. Role of endothelial ADAM17 in early vascular changes associated with diabetic retinopathy. *J Clin Med* 2020;9(2):400.

96 Gong QY, Xie JN, Li Y, Liu Y, Su GF. Enhanced ROBO4 is mediated by up-regulation of HIF-1α/SP1 or reduction in miR-125b-5p/miR-146a-5p in diabetic retinopathy. *J Cell Mol Med* 2019;23(7):4723-4737.

97 Manasson J, Tien T, Moore C, Kumar NM, Roy S. High glucose-induced downregulation of connexin 30.2 promotes retinal vascular lesions: implications for diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2013;54(3):2361-2366.

98 Liu R, Liu H, Ha Y, Tilton RG, Zhang WB. Oxidative stress induces endothelial cell senescence via downregulation of Sirt6. *Biomed Res Int* 2014;2014:902842.

99 Ly A, Yee P, Vessey KA, Phipps JA, Jobling AI, Fletcher EL. Early inner retinal astrocyte dysfunction during diabetes and development of hypoxia, retinal stress, and neuronal functional loss. *Invest Ophthalmol Vis Sci* 2011;52(13):9316-9326.

100 Wang Z, Meng QQ, Zhu X, Sun SW, Liu AQ, Gao SF, Gou YF. Identification and evaluation of reference genes for normalization of gene expression in developmental stages, sexes, and tissues of diaphania caesalis (lepidoptera, Pyralidae). *J Insect Sci* 2020;20(1):6.

101 Hounkpe BW, Chenou F, de Lima F, De Paula EV. HRT Atlas v1.0 database: redefining human and mouse housekeeping genes and candidate reference transcripts by mining massive RNA-seq datasets. *Nucleic Acids Res* 2021;49(D1):D947-D955.

102 Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 2004;37(1):112-119.

103 Balogh A, Paragh G Jr, Juhász A, *et al*. Reference genes for quantitative real time PCR in UVB irradiated keratinocytes. *J Photochem Photobiol B* 2008;93(3):133-139.

104 Chen C, Li SY, Zhu H, Fan BQ, Wang Y, Hao DJ. Identification and evaluation of reference genes for gene expression analysis in the weevil pest Pagiophloeus tsushimanus using RT-qPCR. *J Asia Pac Entomol* 2020;23(2):336-344.

105 Bhattacharya A, Hamilton AM, Furberg H, *et al*. An approach for normalization and quality control for NanoString RNA expression data. *Brief Bioinform* 2021;22(3):bbaa163.

106 Arora S, Pattwell SS, Holland EC, Bolouri H. Variability in estimated gene expression among commonly used RNA-seq pipelines. *Sci Rep* 2020;10(1):2734.

107 Jain N, Nitisa D, Pirsko V, Cakstina I. Selecting suitable reference genes for qPCR normalization: a comprehensive analysis in MCF-7 breast cancer cell line. *BMC Mol Cell Biol* 2020;21(1):68.

108 Chen LL. The expanding regulatory mechanisms and cellular functions of circular RNAs. *Nat Rev Mol Cell Biol* 2020;21(8):475-490.

109 Statello L, Guo CJ, Chen LL, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. *Nat Rev Mol Cell Biol* 2021;22(2):96-118.

110 Schjoldager KT, Narimatsu Y, Joshi HJ, Clausen H. Global view of human protein glycosylation pathways and functions. *Nat Rev Mol Cell Biol* 2020;21(12):729-749.

111 Pérez-Gómez JM, Porcel-Pastrana F, De La Luz-Borrero M, *et al*. LRP10, PGK1 and RPLP0:best reference genes in periprostatic adipose tissue under obesity and prostate cancer conditions. *Int J Mol Sci* 2023;24(20):15140.

112 Song J, Cho J, Park J, Hwang JH. Identification and validation of stable reference genes for quantitative real time PCR in different minipig tissues at developmental stages. *BMC Genomics* 2022;23(1):585.

113 Liu XY, Liu SF, Zhang J, *et al*. Optimization of reference genes for qRT-PCR analysis of microRNA expression under abiotic stress conditions in sweetpotato. *Plant Physiol Biochem* 2020;154:379-386.

114 Everaert BR, Boulet GA, Timmermans JP, Vrints CJ. Importance of suitable reference gene selection for quantitative real-time PCR: special reference to mouse myocardial infarction studies. *PLoS One* 2011;6(8):e23793.

115 Köhsler M, Leitsch D, Müller N, Walochnik J. Validation of reference genes for the normalization of RT-qPCR gene expression in Acanthamoeba spp. *Sci Rep* 2020;10(1):10362.

116 Linardić M, Braybrook SA. Identification and selection of optimal reference genes for qPCR-based gene expression analysis in Fucus distichus under various abiotic stresses. *PLoS One* 2021;16(4):e0233249.