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\cite{25-27}. Before choosing a suitable HKG, a comprehensive evaluation is required to ensure the appropriateness and adequacy of choice\cite{28-30}. Since expression of GOI is normalized to HKG\cite{31-33}, its improper selection may lead to an invalid interpretation\cite{34}. Therefore, choosing the most relevant and more than one HKG in a weighted expression index is expected to address this problem\cite{35}. Incorrect data normalisation and hence misinterpretation remain the most serious issues in RT-qPCR\cite{36-38}. Various methods have been used to assure the validity and reliability of gene expression data\cite{39-42} and one of the most important factors in RT-qPCR is the selection of the right normalisation approach\cite{43}. The synchronised assessment of endogenous HKG is often regarded as the most helpful and relevant method for normalising target genes\cite{44-46}. 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\cite{47}. This also resulted in attempts to adjust for the instability by employing sets of control genes and statistical techniques to calculate normalisation factors\cite{48}. 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 (\(\beta\)-actin)\cite{49}. Literature shows that many studies have used a single HKG for normalisation\cite{50-52}. 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\cite{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\cite{53}. 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 (\(\Delta C_t\)) algorithms are among many that have been integrated and made publicly available in Excel-based applications\cite{54,55,56,57}. Moreover, it also comes in recently built R-based software packages, which has resulted in an increase in HKG-related research globally\cite{58}. These commonly used methods were combined into a single freely accessible web-based software, the Reifinder\cite{59,60}, allowing for a comprehensive review of the most stable HKG. So, the BestKeeper, GeNorm, NormFinder, and comparative \(\Delta C_t\) 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\cite{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 \(C_q\) values as input rather than the specific PCR efficiency of different assays\cite{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\cite{59-61}. 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\cite{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 \(\beta\)-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\cite{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 8–12 wk (200–250 g)\cite{62} 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\cite{48-50}. Blood glucose level was monitored weekly during the experimental period. After 12 wk, animals were sacrificed with an intraperitoneal (IP) injection of sodium pentobarbital (0.14 mg/kg body weight)\cite{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.
(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 stabilization 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 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 prepare 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.

Prime 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[23], 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>NCBI reference ID</th>
<th>Primer length</th>
<th>Sequence</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s ribosomal unit 5 (18s)</td>
<td>100861533</td>
<td>21</td>
<td>GCCATGCAAGTCTAAATGACGC CGGTCGGCAGTTGATGCAC</td>
<td>57.1</td>
</tr>
<tr>
<td>Beta-actin (ActB)</td>
<td>81822</td>
<td>21</td>
<td>ATGGCAGACTGAGTGTTTAC CTCTGCTGCTGCTGAGA</td>
<td>60.0</td>
</tr>
<tr>
<td>Cyclophilin A (CycA)</td>
<td>255158</td>
<td>20</td>
<td>GGATTTGCGTAATAGCGGTCC GTGGTCACCGGGGAGA</td>
<td>60.0</td>
</tr>
<tr>
<td>Glyceraldheyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>24383</td>
<td>18</td>
<td>CCATGGAAGGAGCTGCGG GTAAGGTCAGTGGGAGC</td>
<td>59.4</td>
</tr>
<tr>
<td>Hypoxantherine-guanine phosphoribosyl transferase (Hprt)</td>
<td>24465</td>
<td>19</td>
<td>GACGGGTTCGTCATGTCG ACCTGATTCAATCAATCAC</td>
<td>60.0</td>
</tr>
<tr>
<td>Ribosomal protein, large, P0 (3684)</td>
<td>11837</td>
<td>20</td>
<td>GATCATCCAGACGGTGTGG CCAGTGGGAAAGTTGATGCA</td>
<td>60.3</td>
</tr>
<tr>
<td>Terminal uridyyl transferase 1 (U6)</td>
<td>499314</td>
<td>17</td>
<td>CTCGCTTCGGCAGCAACA AAGGCTTCAAGATTGTGTTG</td>
<td>60.0</td>
</tr>
</tbody>
</table>
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 (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
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

### Table 3 CP data of housekeeping genes by BestKeeper

<table>
<thead>
<tr>
<th>No.</th>
<th>18s</th>
<th>CycA</th>
<th>36B4</th>
<th>GAPDH</th>
<th>HPRT</th>
<th>ActB</th>
<th>U6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cn</td>
<td>SIDR</td>
<td>Cn</td>
<td>SIDR</td>
<td>Cn</td>
<td>SIDR</td>
<td>Cn</td>
</tr>
<tr>
<td>Arithmetic mean (CP)</td>
<td>16.65</td>
<td>16.54</td>
<td>29.99</td>
<td>30.28</td>
<td>27.45</td>
<td>27.51</td>
<td>31.05</td>
</tr>
<tr>
<td>Min (CP)</td>
<td>15.80</td>
<td>16.00</td>
<td>29.30</td>
<td>28.50</td>
<td>27.10</td>
<td>26.40</td>
<td>29.30</td>
</tr>
<tr>
<td>Max (CP)</td>
<td>17.10</td>
<td>17.00</td>
<td>30.90</td>
<td>32.30</td>
<td>27.90</td>
<td>28.80</td>
<td>31.80</td>
</tr>
<tr>
<td>SD (±CP)</td>
<td>0.26</td>
<td>0.29</td>
<td>0.41</td>
<td>0.97</td>
<td>0.25</td>
<td>0.54</td>
<td>0.57</td>
</tr>
<tr>
<td>CV (% CP)</td>
<td>1.58</td>
<td>1.74</td>
<td>1.38</td>
<td>3.20</td>
<td>0.91</td>
<td>1.98</td>
<td>1.85</td>
</tr>
<tr>
<td>Min (x-fold)</td>
<td>-1.80</td>
<td>-1.45</td>
<td>-1.61</td>
<td>-3.37</td>
<td>-1.27</td>
<td>-2.15</td>
<td>-3.34</td>
</tr>
<tr>
<td>Max (x-fold)</td>
<td>1.37</td>
<td>1.38</td>
<td>1.89</td>
<td>4.14</td>
<td>1.37</td>
<td>2.46</td>
<td>1.69</td>
</tr>
<tr>
<td>SD (±x-fold)</td>
<td>1.20</td>
<td>1.22</td>
<td>1.33</td>
<td>1.96</td>
<td>1.19</td>
<td>1.46</td>
<td>1.49</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>18s</th>
<th>CycA</th>
<th>36B4</th>
<th>GAPDH</th>
<th>HPRT</th>
<th>ActB</th>
<th>U6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cn</td>
<td>SIDR</td>
<td>Cn</td>
<td>SIDR</td>
<td>Cn</td>
<td>SIDR</td>
<td>Cn</td>
</tr>
<tr>
<td>CycA</td>
<td>-0.472</td>
<td>0.084</td>
<td>-0.074</td>
<td>0.837</td>
<td>0.789</td>
<td>0.020</td>
<td>0.176</td>
</tr>
<tr>
<td>P</td>
<td>0.238</td>
<td>0.844</td>
<td>0.074</td>
<td>0.949</td>
<td>0.956</td>
<td>0.929</td>
<td>0.656</td>
</tr>
<tr>
<td>36B4</td>
<td>-0.074</td>
<td>-0.023</td>
<td>-0.047</td>
<td>0.949</td>
<td>0.010</td>
<td>0.011</td>
<td>0.144</td>
</tr>
<tr>
<td>P</td>
<td>0.862</td>
<td>0.956</td>
<td>0.074</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.789</td>
<td>0.038</td>
<td>-0.074</td>
<td>0.949</td>
<td>0.010</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>P</td>
<td>0.020</td>
<td>0.929</td>
<td>0.518</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>HPRT</td>
<td>0.176</td>
<td>0.656</td>
<td>0.518</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>P</td>
<td>0.676</td>
<td>0.078</td>
<td>0.176</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>ActB</td>
<td>-0.071</td>
<td>-0.155</td>
<td>-0.176</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>P</td>
<td>0.867</td>
<td>0.283</td>
<td>0.176</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>U6</td>
<td>0.518</td>
<td>0.122</td>
<td>0.518</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
<tr>
<td>P</td>
<td>0.188</td>
<td>0.874</td>
<td>0.518</td>
<td>-0.651</td>
<td>0.001</td>
<td>0.001</td>
<td>0.330</td>
</tr>
</tbody>
</table>

*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.
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 rats, CycA, HPRT, 18s and 36B4 are the four genes that were found to have average to most stable expressions.

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.
Article: Housekeeping genes and diabetes

Table 5: Ranking order of gene expression stability

<table>
<thead>
<tr>
<th>Method</th>
<th>Control 1st</th>
<th>Control 2nd</th>
<th>Control 3rd</th>
<th>Control 4th</th>
<th>Control 5th</th>
<th>Control 6th</th>
<th>Control 7th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta-Ct</td>
<td>18s</td>
<td>18s</td>
<td>HPRT</td>
<td>GAPDH</td>
<td>CyCA</td>
<td>ActB</td>
<td>GAPDH</td>
</tr>
<tr>
<td>BestKeeper</td>
<td>36B4</td>
<td>18s</td>
<td>ActB</td>
<td>U6</td>
<td>U6</td>
<td>ActB</td>
<td>CyCA</td>
</tr>
<tr>
<td>NormFinder</td>
<td>HPRT</td>
<td>18s</td>
<td>GAPDH</td>
<td>CyCA</td>
<td>ActB</td>
<td>GAPDH</td>
<td>U6</td>
</tr>
<tr>
<td>geNorm</td>
<td>CyCA/36B4</td>
<td>18s/HPRT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Recommended ranking</td>
<td>36B4</td>
<td>18s</td>
<td>HPRT</td>
<td>GAPDH</td>
<td>CyCA</td>
<td>ActB</td>
<td>U6</td>
</tr>
</tbody>
</table>

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.

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[41], 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[77-78].

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[80]. 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[81]. 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 Elsherby 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[89]. Given this knowledge, it is reasonable to predict discrepancies and substantial errors in the stability and degree of gene expression under different experimental settings[89], 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[83-85], and high glucose-induced cells[96-98]. However, Ly et al[90] 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


Housekeeping genes and diabetes


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-


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.
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


