Selection of Appropriate Reference Genes for RT-qPCR Analysis in a Streptozotocin-Induced Alzheimer’s Disease Model of Cynomolgus Monkeys (Macaca fascicularis)

Sang-Je Park1,2,*, Young-Hyun Kim1,3,*, Youngjeon Lee1,4,*, Kyoung-Min Kim1,3, Heui-SooKim2, Sang-Rae Lee1, Sun-Uk Kim1, Sang-Hyun Kim1, Ji-Su Kim1, Kang-Jin Jeong1, Kyoung-Min Lee5, Jae-Won Huh1,3,*, Kyu-Tae Chang1,3,*

1 National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Chungbuk, Republic of Korea, 2 Department of Biological Sciences, College of Natural Sciences, Pusan National University, Busan, Republic of Korea, 3 University of Science & Technology, National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Chungbuk, Republic of Korea, 4 Department of Rehabilitation Science in Interdisciplinary PhD Program, Graduate School of Inje University, Gimhae, Gyeongnam, Republic of Korea, 5 Department of Neurology, Seoul National University Hospital, Seoul, Republic of Korea

Abstract

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) has been widely used to quantify relative gene expression because of the specificity, sensitivity, and accuracy of this technique. In order to obtain reliable gene expression data from RT-qPCR experiments, it is important to utilize optimal reference genes for the normalization of target gene expression under varied experimental conditions. Previously, we developed and validated a novel icv-STZ cynomolgus monkey model for Alzheimer’s disease (AD) research. However, in order to enhance the reliability of this disease model, appropriate reference genes must be selected to allow meaningful analysis of the gene expression levels in the icv-STZ cynomolgus monkey brain. In this study, we assessed the expression stability of 9 candidate reference genes in 2 matched-pair brain samples (5 regions) of control cynomolgus monkeys and those who had received intracerebroventricular injection of streptozotocin (icv-STZ). Three well-known analytical programs geNorm, NormFinder, and BestKeeper were used to choose the suitable reference genes from the total sample group, control group, and icv-STZ group. Combination analysis of the 3 different programs clearly indicated that the ideal reference genes are RPS19 and YWHAZ in the total sample group, GAPDH and RPS19 in the control group, and ACTB and GAPDH in the icv-STZ group. Additionally, we validated the normalization accuracy of the most appropriate reference genes (RPS19 and YWHAZ) by comparison with the least stable gene (TBP) using quantification of the APP and MAPT genes in the total sample group. To the best of our knowledge, this research is the first study to identify and validate the appropriate reference genes in cynomolgus monkey brains. These findings provide useful information for future studies involving the expression of target genes in the cynomolgus monkey.

Citation: Park S-J, Kim Y-H, Lee Y, Kim K-M, Kim H-S, et al. (2013) Selection of Appropriate Reference Genes for RT-qPCR Analysis in a Streptozotocin-Induced Alzheimer’s Disease Model of Cynomolgus Monkeys (Macaca fascicularis). PLoS ONE 8(2): e56034. doi:10.1371/journal.pone.0056034

Introduction

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is a widely used experimental method for the detection and evaluation of mRNA levels because of the specificity, accuracy, sensitivity, and cost-effectiveness. Despite these advantages, a number of parameters such as differing sample amounts, RNA quality, purity, enzymatic efficiency in reverse transcription, and PCR efficiency can lead to inaccurate quantification of gene expression data by using RT-qPCR experiments [1]. To overcome this problem, normalization strategies are commonly used with constitutively expressed gene, termed the reference gene or the internal control gene [2]. Traditional reference genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) are frequently used to normalize the target gene expression levels. However, these genes have been shown to have variable expression patterns across tissue types and experimental conditions [1,3]. Therefore, selection of suitable reference genes is important to avoid incorrect results obtained from differential expression patterns in specific tissue types and experimental conditions [4].

Previous studies have shown that rodents injected intracerebroventricular streptozotocin (icv-STZ) show pathological features similar to those of Alzheimer’s disease (AD), such as neuronal loss, accumulation of Aβ, hyperphosphorylation of tau, and impairment of spatial learning. In particular, a chronic decrease in cerebral glucose uptake and production has been observed in icv-STZ rats [5,6]. Abnormalities of brain glucose metabolism are major features of early stages of AD [7], and therefore, the icv-STZ rat is a useful animal model for the investigation of AD. However, rodent models are not ideal for the investigation of spatial distribution and regional differences in pathogenic...
vulnerability because the small rodent brains do not allow detailed spatial mapping. To better understand spatial- and regionalspecific differences in the pathological features of AD, we established a primate model of AD using icv-STZ of cynomolgus monkeys [5]. Interestingly, 18F-deoxyglucose uptake monitoring by high-resolution micro-PET of this primate model showed hypometabolism of glucose. However, this study has not been performed molecular characterization of AD-related genes in the detailed region of icv-STZ monkey brain. Therefore, studies are needed to characterize the genes showing altered expression in the icv-STZ primate model brain. In order to further investigate this model, it is important to determine the appropriate reference genes before completing experiments on target genes such as those involved in the insulin signaling pathway and AD-related genes in the brain tissues of normal and icv-STZ primate models. To our knowledge, studies designed to select suitable reference genes have not been performed in cynomolgus monkeys. In order to provide a more suitable model organism for studies of neurodegenerative diseases such as AD and Parkinson’s disease (PD), appropriate reference genes must be identified for RT-qPCR experiments in a range of regions in normal and icv-STZ brains.

The aim of this study was to select and evaluate the stability of 9 candidate reference genes in order to identify reliable genes that may be used for normalization when studying AD-related gene expression. To this end, samples from different regions of control and icv-STZ cynomolgus monkey brains were used. The stability ACTB, B-2-microglobulin (B2M), GAPDH, hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein S5 (RPS5), RPS19, succinate dehydrogenase complex subunit A (SDHA), TATA box binding protein (TPB), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Z polypeptide (YWHAZ) were analyzed using the geNorm [1], NormFinder [9], and BestKeeper [10] software programs. In addition, we validated the reference genes identified in our study by comparison with the mRNA levels of amyloid beta (A4) precursor protein (APP) and microtubule-associated protein tau (MAPT) in the control and icv-STZ brains.

Results

Expression Stability of Candidate Reference Genes

We analyzed the 9 candidate genes to select the most stable and suitable reference genes using 3 software programs (geNorm, NormFinder, and BestKeeper). These are freely available and generally accepted tools.

a) GeNorm analysis. The expression stability of the 9 candidate reference genes was analyzed by the geNorm program. GeNorm selects the most suitable reference genes by calculating the stability values (M values) of the tested samples. The M value is calculated by the average pair-wise variation of a particular gene compared with all other control genes. Thereafter, the gene with the highest M value is excluded, and then new M values are calculated from the remaining genes. The genes with high M values are highly variable and less stably expressed, and the genes with low M values possess low variability and are stably expressed. The average M values of the 9 reference genes in the 3 tested sample groups are displayed in Fig. 1. The reference genes RPS19 and YWHAZ were identified as the 2 most stably expressed genes in the total sample group (Fig. 1A); GAPDH and RPS19 were identified in the control group and icv-STZ group (Fig. 1C and 1E).

The geNorm program was also utilized to calculate the optimal number of required reference genes to obtain reliable results from RT-qPCR studies. This calculation was performed by analysis of the pair-wise variation (V value) of sequential normalization factors (NF) with an increasing number of reference genes (NFn and NFn+1) in all tested sample groups (Fig. 1B, 1D, and 1F). The original paper using the geNorm program proposed 0.15 as the cut-off value; implying that if a value below this is obtained an additional reference gene is not required [1]. The pair-wise variation V2/3 was lower than 0.15 in all sample groups; therefore, additional reference genes were not required to calculate the NF.

b) NormFinder analysis. NormFinder is a tool to identify the optimal stable reference genes using a model-based approach. NormFinder calculated the stability value and standard error according to the expression variation of the candidate reference genes; stably expressed genes, which have less varied expression levels, have lower stability values [9]. Analysis of our data showed that RPL19 (0.090) was the most stable reference gene with the lowest stability value, whereas YWHAZ (0.148), ACTB (0.148), RPS5 (0.153), GAPDH (0.188), SDHA (0.196), HPRT1 (0.205), B2M (0.208), and TBP (0.221) had respectively increasing stability values in the total group (Table 1). GAPDH (0.051) and ACTB (0.022) were the most stable reference genes in the control group and the icv-STZ group, respectively.

c) BestKeeper analysis. The BestKeeper program is also an Excel-based software tool like geNorm and NormFinder. The program determines the coefficient of correlation analysis for all pairs of candidate reference genes (≤10 genes), and calculates the % coefficient of variation (CV) and standard deviation (SD) for each candidate gene’s crossing point (CP) value (the raw quantification cycle value; Cq) [10]. Based on these indices, the most stable reference gene for the accurate normalization of the RT-qPCR data was determined (Table 2). The TBP gene had the lowest CV (1.00) and SD (0.27) values of the candidate reference genes; indicating that it was stably expressed across all tested samples. However, TBP had a very low coefficient of correlation value (0.836) in the total sample group; indicating that its expression did not correlate well with the expression patterns of the other candidate reference genes. On the contrary, RPS3 had a high coefficient of correlation value (0.981) and high CV (3.05) and SD (0.61) values. Therefore, YWHAZ, RPS19, HPRT1, and GAPDH were stably expressed with high coefficient of correlation, and low CV and SD values in the total sample group. Similarly, GAPDH, HPRT1, RPS3, and RPS19 in the control group and GADPH, ACTB, YWHAZ, and SDHA in the icv-STZ group were stable reference genes.

Finally, we selected the most stable reference genes in the total sample group (RPS19 and YWHAZ) control group (GAPDH and RPS19) and icv-STZ group (ACTB and GAPDH) by using the combined data from the geNorm, NormFinder, and BestKeeper programs.

Comparative Analysis of most and Least Stable Reference Genes on the Normalization of APP and MAPT Genes

To demonstrate the importance of using carefully selected normalization genes to estimate the relative expression of the APP and MAPT genes, we tested the 2 most stable genes (NF of RPS19 and YWHAZ) and least stable gene (TBP) for normalization in the total sample group (Fig. 2A and 2B). The relative expression of the APP gene following normalization by NF of the RPS19 and YWHAZ genes showed increased levels across the icv-STZ group compared with the control group, except in the hippocampal tissue (Fig. 2A). In particular, the expression level was more than double in the icv-STZ precuneus sample. However, there was no significant difference in the expression level of the control group and the icv-STZ group in the frontal cortex, hippocampus, and posterior cingulate. When the TBP gene was used for normaliza-
Figure 1. GeNorm analysis of the total sample, control, and icv-STZ groups. Average expression stability (M) of 9 candidate reference genes and the best combination of 2 genes were calculated (A, C, and D). Lower M values indicate more stable expression. Determination of the optimal number of reference genes for normalization was conducted (B, D, and F). The geNorm program calculated the normalization factor (NF) from at least 2 genes and the variable V defines the pair-wise variation between the 2 sequential NF.

doi:10.1371/journal.pone.0056034.g001

Table 1. Gene stability value calculations by NormFinder.

| Total sample group | Control group | Icv-STZ group |
|--------------------|---------------|--------------|
| Gene name          | Stability value | Gene name    | Stability value | Gene name | Stability value |
| RPS19              | 0.090          | GAPDH        | 0.051          | ACTB      | 0.022          |
| YWHAZ              | 0.148          | RPS5         | 0.112          | GAPDH     | 0.073          |
| ACTB               | 0.148          | RPS19        | 0.119          | RPS19     | 0.077          |
| RPS5               | 0.153          | B2M          | 0.153          | SDHA      | 0.083          |
| GAPDH              | 0.188          | YWHAZ        | 0.155          | RPS5      | 0.094          |
| SDHA               | 0.196          | ACTB         | 0.186          | YWHAZ     | 0.161          |
| HPRT1              | 0.205          | TBP          | 0.191          | HPRT1     | 0.193          |
| B2M                | 0.208          | SDHA         | 0.197          | B2M       | 0.260          |
| TBP                | 0.221          | HPRT1        | 0.244          | TBP       | 0.261          |

doi:10.1371/journal.pone.0056034.t001
tion, the APP gene showed increased expression levels in the frontal cortex, posterior cingulate, and precuneus, whereas decreased expression levels were observed in the occipital cortex. These normalization results reveal significant difference in the frontal cortex, precuneus, and occipital cortex. Furthermore, the expression level of the APP gene showed the opposite pattern in the hippocampus and occipital cortex. The MAPT gene demonstrated increased expression patterns in the frontal cortex, hippocampus, precuneus, and occipital cortex; and decreased expression patterns in the posterior cingulate compared to the normalization against the NF of RPS19 and YWHAZ. However, the expression levels of the MAPT gene were not significantly different between the control group and the icv-STZ group. Following normalization using the TBP gene, the MAPT gene showed increased expression patterns in the frontal cortex and hippocampus, and decreased expression patterns in the posterior cingulate, precuneus, and occipital cortex. Among these, the expression of the MAPT gene showed opposing results in the precuneus and occipital cortex.

Discussion

In recent studies, the RT-qPCR method has been widely used for gene expression analysis because of its specificity, sensitivity, and accuracy. To obtain accurate gene expression data from RT-qPCR experiments, stably expressed reference genes are needed to normalize the target gene’s transcript levels. Therefore, studies of suitable reference genes are essential for accurate normalization and data analysis. Indeed, reference genes have been widely analyzed in various species (including animals and plants), tissues, and under different experimental conditions; and these reference genes have been widely applied for the accurate quantification of target gene expression levels.

The rhesus monkey (Macaca mulatta) and cynomolgus monkey (Macaca fascicularis) are widely used animal models for the studying neurodegenerative diseases such as AD, PD, Huntington’s disease, and amyotrophic lateral sclerosis [11–13]. However, only a few studies on the selection of suitable reference genes have been performed on the rhesus monkey brain and organs [16–18]. Furthermore, to our knowledge, studies on the selection of suitable reference genes have not yet been performed on brain and organ tissues of the cynomolgus monkey. Therefore, more accurate and detailed reference gene information is required for the accurate normalization of expression levels of neurodegenerative disease-related genes in cynomolgus monkey brain tissues.

In this study, we identified the most stable reference genes from 9 commonly used candidate reference genes in the brain tissues of cynomolgus monkey using the geNorm, NormFinder, and BestKeeper programs. And also, to provide a more useful and reliable information for studies of various gene expression in the cynomolgus monkey, we carried out selection of appropriate reference genes in the three groups such as total sample group, control group, and icv-STZ group. Although the rankings of the candidate reference genes in the 3 programs showed slightly different patterns between each sample group, there were similarities between each sample group with respect to the composition of the high ranked genes from each program. This difference in the stability ranking of the candidate reference genes may be due to the different algorithms and analytical procedures utilized by the 3 programs. Therefore, the most suitable reference genes for the accurate normalization of target gene expression were selected by combining the data obtained for the top 3 reference genes from each program in each sample group. With respect to the total sample group, RPS19, YWHAZ, and GAPDH were the top 3 genes from the geNorm program, RPS19, YWHAZ, and ACTB from the NormFinder program, and YWHAZ, RPS19, HPRT1, and GAPDH from the Bestkeeper program. Among these genes, RPS19 and YWHAZ were present in all the programs. These 2 genes were ranked in the top 2 in the geNorm and BestKeeper programs; comparatively, HPRT1 and GAPDH showed low stability values in the geNorm program, RPS19, YWHAZ, and ACTB showed low stability values in the geNorm program. Therefore, RPS19 and YWHAZ were selected as the most suitable reference genes in the total sample group. Similarly, we selected GAPDH and RPS19 in the control group, and ACTB and GAPDH in the icv-STZ group. Previous studies have indicated that commonly used reference genes such as ACTB, GAPDH, and B2M are not stably expressed across tissue types and experimental conditions [4,19,20]. However, our results showed that ACTB and GAPDH were the most suitable reference genes in the control group and the icv-STZ group for the cynomolgus monkey. The different results regarding the reference genes may be due to different of species, tissue types, and experimental conditions. Therefore, the selection of appropriate reference genes must be performed before analyzing the target genes. Specifically, it is important to identify appropriate reference genes for the accurate normalization of target genes in a variety of tissues and experimental conditions in the cynomolgus monkey, to ensure this

### Table 2. Expression stability analysis of the reference genes by BestKeeper.

| Genes  | Total sample group | Control group | Icv-STZ group |
|--------|--------------------|---------------|--------------|
|        | R     | CV   | SD   | R     | CV   | SD   | R     | CV   | SD   |
| GAPDH  | 0.939 | 2.96 | 0.55 | 0.975 | 1.28 | 0.23 | 0.986 | 3.02 | 0.58 |
| ACTB   | 0.916 | 1.85 | 0.36 | 0.533 | 0.80 | 0.15 | 0.994 | 2.82 | 0.54 |
| HPRT1  | 0.935 | 2.63 | 0.60 | 0.969 | 1.93 | 0.43 | 0.957 | 3.04 | 0.69 |
| RPS5   | 0.981 | 3.05 | 0.61 | 0.979 | 1.59 | 0.31 | 0.996 | 3.46 | 0.70 |
| RPS19  | 0.983 | 2.80 | 0.53 | 0.930 | 1.44 | 0.27 | 0.995 | 3.42 | 0.66 |
| TBP    | 0.836 | 1.00 | 0.27 | 0.496 | 0.58 | 0.15 | 0.879 | 1.50 | 0.40 |
| YWHAZ  | 0.952 | 2.46 | 0.53 | 0.828 | 1.16 | 0.25 | 0.970 | 2.98 | 0.65 |
| B2M    | 0.857 | 1.67 | 0.39 | 0.687 | 0.70 | 0.16 | 0.889 | 2.56 | 0.60 |
| SDHA   | 0.859 | 1.70 | 0.39 | 0.651 | 1.23 | 0.28 | 0.987 | 2.17 | 0.46 |

doi:10.1371/journal.pone.0056034.t002
animal model is a suitable organism for studies of human neurodegenerative diseases.

In this study, the expression levels of APP and MAPT were measured by normalization with the NF of RPS19 and YWHAZ in the total sample group (Fig. 2A and 2B). The expression levels of the APP and MAPT genes in the control and icv-STZ samples did not differ in most samples; however, the expression levels of the APP gene did differ in the precuneus and occipital cortex tissues. Previous studies have revealed significantly reduced expression levels of the MAPT gene and significantly increased expression levels of the APP gene in frontal cortex, hippocampus, and hypothalamus samples of human AD relative to the control [21,22]. These altered expression patterns may be explained by spontaneous recovery of the cynomolgus monkey against brain damage induced by STZ injection because in our previous study, the icv-STZ cynomolgus monkey had a recovered voxel count of brain parenchyma (gray matter + white matter) [8].

Finally, to evaluate the suitability of the selected reference genes in this study, we performed comparative analysis of the expression levels of the APP and MAPT genes by normalization using the least stable reference gene (TBP, Fig. 2A and 2B). These results indicated that normalization using inappropriate reference genes could lead to misinterpretation of target gene expression data. Indeed, diametrically opposed expression patterns of APP were observed in the hippocampus and occipital cortex and of MAPT in the precuneus and occipital cortex. Moreover, recent studies also demonstrated that target gene expression data following normalization using unstable reference genes showed significantly different results compared with expression data following normalization with suitable reference genes [2,23,24]. Unfortunately, many studies still use traditional reference genes such as GAPDH and ACTB or randomly selected single reference genes for the normalization of gene expression, which is likely to jeopardize the accuracy of the analyzed data [25–29]. Therefore, studies aimed at the selection of appropriate reference genes are essential to ensure the accuracy of quantification data of target gene expression obtained using RT-qPCR experiments.

This study was the first to evaluate suitable reference genes in control and icv-STZ brain tissue of cynomolgus monkeys using the geNorm, NormFinder, and Bestkeeper programs. Our results showed that the RPS19 and YWHAZ genes in the total sample group, GAPDH and RPS19 in the control group, and ACTB and GAPDH in the icv-STZ group were the most appropriate reference genes. Moreover, we validated the normalization accuracy of the
most stable reference genes \((\text{RPS}19 \text{ and } \text{YWHAZ})\) in the total sample group by quantifying the mRNA levels of \(\text{APP}\) and \(\text{MAPT}\). These results provide reliable information for future quantitative analyses of gene expression, particularly related to AD, in the brain tissue of cynomolgus monkeys.

**Materials and Methods**

**Experimental Animals and Sampling**

Four healthy 3-year-old, 3–4 kg female cynomolgus monkeys were used. Their origin is Vietnam. All animals were provided by the National Primate Research Center (NPRC) of Korea. This monkey was kept in an indoor individual cage and fed a commercial monkey chow (Harlan) supplemented daily with various fruits, and supplied water ad libitum. Environmental conditions were controlled to provide a temperature of 24±2°C, a relative humidity of 50±5%, 100% fresh air at a rate of ≥12 room changes per hour, and a 12:12 h light:dark cycle. Their health was monitored by the attending veterinarian consistent with the recommendations of the Weatherall Report.

Four cynomolgus monkeys were divided into 2 groups, namely, the icv-STZ group \((n = 2)\) and the control group \((n = 2)\). In the icv-STZ group, the STZ was injected into the cerebrospinal fluid (CSF) via the cerebellomedullary cistern (CM) using a 25-g spinal needle on day 1, 7, and 14. The monkeys were treated with 2 mg/kg STZ dissolved in 0.3 mL of normal saline. The control monkeys were injected with the same volume of normal saline. The animals were sacrificed at 20 weeks after the STZ or saline treatments following deep anesthesia using ketamine (20 mg/kg) for 10 sec. The amplification specificity of each RT-qPCR assay was confirmed by melting curve analysis. The temperature range for analysis of the melting curves was 55°C to 99°C for 5 sec. As shown in Fig. S2, each primer pair showed a single, sharp peak, thereby indicating that the primers amplified only one specific PCR product. The no-template control (NTC) was detected with any of the tested genes except for SDHA and MAPT. However, the NTC of these 9 genes was amplified over the 32 cycles (Table 3). Therefore, it did not affect the amplification of the target gene. These data generated 3 independent experiments.

**Ethics Statement**

All procedures and use of monkeys were approved by the Korea Research Institute of Bioscience and Biotechnology (KIRBB) Institutional Animal Care and Use Committee (Approval No. KIRBB-AEC-11010).

**Total RNA Isolation and cDNA Preparation**

Total RNA was extracted from the 2 matched-pair brain samples (5 regions) of control cynomolgus monkeys and those who had received intracerebroventricular injection of streptozotocin (icv-STZ) \((n = 20)\) samples using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. The RNase-free DNase set (Qiagen) was used to eradicate DNA contamination from the total RNA preparations. Total RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase was used for the reverse transcription reaction in the presence of the RNase inhibitor (Promega), with an annealing temperature of 42°C. We performed PCR amplification without the reverse transcription reaction using pure RNA samples \((n = 2)\) and determined that the prepared mRNA samples did not contain genomic DNA.

**Primer Design and Standard Curve Analysis**

For the development of specific primers for the 9 candidate reference genes and 2 target genes \((\text{APP} \text{ and } \text{MAPT})\), primer pairs were designed using the Primer3 program \(\text{http://frodo.wi.mit.edu/primer3/}; \text{Table 3})\ [30]. The gene sequences were obtained from our previous study sequencing the transcriptome of the cynomolgus monkey [31]. BLAST searches were performed to confirm the gene specificity of the primer sequences, and the results showed the presence of multi-locus matching at individual primer sites. Most primers spanned at least 2 exons or have a great size of introns sequence between forward and reverse primer in order to avoid false-positive amplification of contaminating genomic DNA in the RNA samples. The nucleotide sequences of the RT-PCR products for the 9 reference genes and 2 target genes were obtained by using standard cloning and sequencing procedures \(\text{Fig. S1})\. Amplification efficiencies and correlation coefficients \((R^2)\) of the 11 genes were generated using the slopes of the standard curves obtained by serial dilutions. Standard curves with a 10-fold dilution series were used to calculate the amplification efficiency \((\text{Table 3})\). The amplification efficiency was calculated according to the formula: efficiency \((\%) = \left(10^{-1/\text{slope}} - 1\right) \times 100\). The efficiency range of the real-time RT-PCR amplifications for all the tested genes was 88%–100%.

**RT-qPCR Amplification**

RT-qPCR using SYBR Green was performed using a Rotor Gene Q thermocycler (Qiagen). In each run, 1 μL of cDNA was used as a template for each reaction. The samples were added to 19 μL of the reaction mixture, containing 7 μL H₂O, 10 μL RotorGene SYBR Green PCR mastermix (Qiagen), and 1 μL each of the forward and reverse primers. RT-qPCR amplification of the 11 genes was performed for 40 cycles of 94°C for 5 sec and 60°C for 10 sec. The amplification specificity of each RT-qPCR assay was confirmed by melting curve analysis. The temperature range for analysis of the melting curves was 55°C to 99°C for 5 sec. As shown in Fig. S2, each primer pair showed a single, sharp peak, thereby indicating that the primers amplified only one specific PCR product. The no-template control \((\text{NTC})\) was detected with any of the tested genes except for SDHA and MAPT. However, the NTC of these 9 genes was amplified over the 32 cycles \((\text{Table 3})\). Therefore, it did not affect the amplification of the target gene. These data generated 3 independent experiments.

**Characterization of the Analysis Programs**

The geNorm program [1] provides a measure of gene expression stability \((M\text{ value})\), which is the mean pair-wise variation between an individual gene and all other tested control genes. This method differs from model-based approaches, as it compares genes based on the similarity of their expression profiles. Cq values were converted to scale expression quantities using the ΔCq method and recorded in the geNorm program, which then ranked the genes based on their M values; the gene with the most stable expression has the lowest value. NormFinder [9] focuses on finding the 2 genes with the least intra- and inter-group expression variation or most stable reference gene in the intra-group expression variation. A BestKeeper [10] index was created using the geometric mean of each candidate gene’s Cq values. This index was then compared to each individual candidate reference gene by pair-wise correlation analyses, with each combination assigned a value for the Pearson correlation coefficient \((r)\), probability \((p)\), SD, and CV.

**MIQE Guidelines**

All the experiments were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [32].
| Abbreviation | Gene name | Primer* | Exon(s) | Amplicon size (bp) | PCR efficiency(%) | R² | NTC** (Cq) |
|--------------|-----------|---------|---------|-------------------|-------------------|----|------------|
| ACTB         | Beta-actin| F: ACAGAGCTCGACCTTTGAC
R: CAGATGGAGGGAAAGAC | 1st 2nd | 160 | 92 | 0.99094 | 32 |
| B2M          | Beta-2-microglobulin | F: GTTCGGCTGAGTGCCTTA
R: GTGGATGCGGCTGAAGTAAACC | 1st 2nd | 101 | 88 | 0.99174 | 32.5 |
| GAPDH        | Glyceraldehyde-3-phosphate dehydrogenase | F: ACAAAGCCCTCAAGATCTCGAG
R: ACCTGTCGATCTGCTCCTCC | 6th 7th/8th | 112 | 90 | 0.99273 | 34.18 |
| HPRT1        | Hypoxanthine phosphoribosyltransferase 1 | F: GACCAGTACAAGGGAAC
R: AAAGTCTGCACTGTTTGGC | 4th 6th | 116 | 92 | 0.99380 | 32.6 |
| RPS5         | Ribosomal protein S5 | F: GTCCTGGTGAAGCCATC
R: TCAGACATTGTCCTTACTGCC | 4th 5th | 182 | 100 | 0.99338 | 33.5 |
| RPS19        | Ribosomal protein S19 | F: AGCTGGCTCCCAGGATAG
R: GACGACCAACCTCTGGGA | 3rd 4th | 174 | 93 | 0.99581 | 36.04 |
| SDHA         | Succinate dehydrogenase complex subunit A | F: AAACCAATGCTGAAAGGAAAT
R: TCGTATAGCTGTGATTT | 11th 12th | 180 | 88 | 0.99386 | N.d. |
| TPB          | TATA box binding protein | F: CCACCCACTGATCTGGCC
R: TATAATGGCTTCTGGG | 3rd 4th | 174 | 97 | 0.99045 | 35.51 |
| YWHAZ        | Tyrosine 3-monoxygenase/tyrosine 5-monoxygenase activation protein, zeta polypeptide | F: AGCGAGATGCGTCGAGAATACA
R: GTATACACCGCCGGCAAC | 2nd 3rd | 185 | 97 | 0.99120 | 38.44 |
| APP          | Amyloid beta (A4) precursor protein | F: TACCAGTGCTTAG/TTGCTGA
R: CTCACTGACGTC/CTCTTTGG | 3rd/4th 4th/5th | 138 | 90 | 0.99179 | 32.3 |
| MAPT         | Microtubule-associated protein tau | F: TAGCAAGTCATCAGTCAAGT
R: TCTGGCTTTGAAAGTCCAGCT | 11th 13th | 196 | 90 | 0.99080 | N.d. |

*If a primer is located on 2 exons, the junctions are shown with a virgule.
**No template control.
N.d: Not detected.

doi:10.1371/journal.pone.0056034.t003
Supporting Information

Figure S1 Nucleotide sequences of the candidate reference genes and target genes from the cynomolgus monkey brain. (TIF)

Figure S2 Melting curve analyses of the candidate reference genes and target genes in the total samples.

References

1. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.

2. Mafra V, Kubo KS, Alves-Ferreira M, Ribeiro-Alves M, Stuart RM, et al. (2012) Reference genes for accurate transcript normalization in citrus genotypes under different environmental conditions. PLoS One 7: e31263.

3. Yperman J, De Vos P, Hooft P, Flameng W (2004) Beta-actin cannot be used as a control for gene expression in ovine interstitial cells derived from heart valves. J Heart Valve Dis 13: 848–853.

4. Park SJ, Huh JW, Kim YH, Lee SR, Kim SH, et al. (2012) Selection of Internal Reference Genes for Normalization of Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis in the Canine Brain and Other Organs. Mol Biotechnol. In press.

5. Lester-Coll N, Rivera EJ, Soucia SJ, Doiron K, Wands JR, et al. (2006) Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer’s disease. J Alzheimer Dis 9: 13–33.

6. Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S (2007) Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem 101: 177–770.

7. Mistur R, Mosconi L, Santi SD, Guzman M, Li Y, et al. (2009) Current Challenges for the Early Detection of Alzheimer’s Disease: Brain Imaging and CSF Studies. J Clin Neurol 5: 153–166.

8. Heo JH, Lee SR, Lee ST, Lee KM, Oh JH, et al. (2011) Spatial distribution of glucose hypometabolism induced by intracerebroventricular streptozotocin in monkeys. J Alzheimer Dis 25: 317–323.

9. Andersen CL, Jensen JL, Orntoft TF (2004) 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 64: 5245–5250.

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

11. Li W, Wu Y, Min F, Li Z, Huang J, et al. (2010) A nonhuman primate model of Alzheimer’s disease generated by intracranial injection of amyloid-beta42 and thiophan. Metab Brain Dis 25: 277–284.

12. Krikis S, Shim JW, Fiao J, Ganad YM, Wakedman DR, et al. (2011) Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson’s disease. PLoS ONE 6: 1–11.

13. Mi Y, Peng S, Sperandio PG, Sossi V, Eidelberg D, et al. (2012) Abnormal metabolic brain networks in a nonhuman primate model of parkinsonism. J Cereb Blood Flow Metab 32: 633–642.

14. McBride JL, Patzer M, Boudreau RL, Dufoir B, Hobbs T, et al. (2011) Preclinical safety of RNAi-mediated HTT suppression in the rhesus macaque as a potential therapy for Huntington’s disease. Mol Ther 19: 2152–2162.

15. Uehida A, Sasaguri H,Kimura N, Fujiri M, Okubo T, et al. (2012) Non-human primate model of amyostrophic lateral sclerosis with cytoplastmic mislocalization of TDP-43. Brain 135: 833–846.

16. Ahn K, Huh JW, Park SJ, Kim DS, Ha HS, et al. (2008) Selection of internal reference genes for SYBR green qRT-PCR studies of rhesus monkey (Macaca mulatta) tissues. BMC Mol Biol 9: 78.

17. Noriega NC, Kohama SG, Urbanski HF (2009) Gene expression profiling in the rhesus macaque: methodology, annotation and data interpretation. Methods 49: 42–49.

18. Noriega NC, Kohama SG, Urbanski HF (2010) Microarray analysis of relative gene expression stability for selection of internal reference genes in the rhesus macaque brain. BMC Mol Biol 11: 47.

19. Furguson BS, Niam H, Hopkins RG, Morrison RF (2010) Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipoocytes. PLoS One 5: e15208.

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

21. Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, et al. (2005) Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer’s disease: link to brain reductions in acetylcholine. J Alzheimers Dis 8: 247–260.

22. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, et al. (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer’s disease—is this type 3 diabetes? J Alzheimers Dis 7: 63–80.

23. Chang Z, Ling C, Yamashita M, Welham NV (2010) Microarray-driven validation of reference genes for quantitative real-time polymerase chain reaction in a rat vocal fold model of mucosal injury. Anal Biochem 406: 214–221.

24. Marum I, Miguel A, Ricardo CP, Miguel C (2012) Reference gene selection for quantitative real-time PCR normalization in Qcrubus suber. PLoS One 7: e50113.

25. Tachartsomdach C, Kantachuesris S, Changsirikulchai S, Wimolheuck S, Prunpad K, et al. (2012) Connective tissue growth factor gene expression and decline in renal function in lupus nephritis. Exp Ther Med 3: 713–718.

26. Shen B, Dong P, Li D, Gao S (2011) Expression and function of ABCG2 in head and neck squamous cell carcinoma and cell lines. Exp Ther Med 2: 509–513.

27. Saigusa S, Inoue Y, Tanaka K, Toiyama Y, Kawamura M, et al. (2012) Significant correlation between LKB1 and LGR5 gene expression and the association with poor recurrence-free survival in rectal cancer after preoperative chemoradiotherapy. J Cancer Res Clin Oncol. In press.

28. Yuasa K, Takeda S, Hikjaka T (2012) A conserved regulatory element located far downstream of the glos locus modulates glos expression through chromatin loop formation during myogenesis. FEBS Lett 586: 3464–3470.

29. Miura C, Sugawara K, Neriya Y, Minato N, Krima T, et al. (2012) Functional characterization and gene expression profiling of superoxide dismutase from plant pathogenic phytoplasma. Gene. In press.

30. Rozen S, Skalitisky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 363–380.

31. Huh JW, Kim YH, Park SJ, Kim DS, Lee SR, et al. (2012) Large-scale transcriptome sequencing and gene analyses in the crab-eating macaque (Macaca fascicularis) for biomedical research. BMC Genomics 13: 163.

32. Bustin SA, Benes V, Garson JA, Hellmans J, Huggett J, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611–622.