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Identification of optimal reference genes for RT-qPCR in the rat hypothalamus and intestine for the study of obesity

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Abstract

BACKGROUND—Obesity has a complicated metabolic pathology, and defining the underlying mechanisms of obesity requires integrative studies with molecular endpoints. Real time quantitative PCR (RT-qPCR) is a powerful tool that has been widely utilized. However, the importance of using carefully validated reference genes in RT-qPCR seems to be overlooked in obesity-related research. The objective of this study was to select a set of reference genes with stable expressions to be used for RT-qPCR normalization in rats under fasted vs. re-fed and chow vs. high fat diet (HFD) conditions.

DESIGN—Male Long-Evans rats were treated under four conditions, chow/fasted, chow/re-fed, HFD/fasted, and HFD/re-fed. Expression stabilities of the13 candidate reference genes were evaluated in the rat hypothalamus, duodenum, jejunum, and ileum using ReFinder software program. The optimal number of reference genes needed for RT-qPCR analyses was determined using geNorm.

RESULTS—Using geNorm analysis, we found that it was sufficient to use the two most stably expressed genes as references in RT-qPCR analyses for each tissue under specific experimental conditions. Unique subsets of reference genes out of the 13 candidate genes were identified, each of which is specific for one type of rat tissue (hypothalamus, duodenum, jejunum, or ileum) under a different combination of diet and feeding condition.

CONCLUSIONS—Our study demonstrates that gene expression levels of reference genes commonly used in obesity-related studies, such as ACTB, or RPS18, are altered by changes in acute or chronic energy status. These findings underline the importance of using reference genes that are stable in expression across experimental conditions when studying the rat hypothalamus and intestine, because these tissues play an integral role in regulation of energy homeostasis. It is our hope that this study will raise awareness among obesity researchers on the essential need for reference gene validation in gene expression studies.

Keywords

Obesity; reference gene; hypothalamus; intestine; RT-qPCR; rat

INTRODUCTION

The worldwide incidence of obesity has led to an increasing need for understanding the molecular mechanisms that drive this epidemic. Obesity is the combined consequence of

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genetic, behavioral, and environmental factors that drive an imbalance between energy intake and energy expenditure towards an increase in adiposity[1]. Hormones and peptides secreted from the adipose tissue and the gastrointestinal tract act as signals representing current energy status to the central nervous system (CNS)[2]. The hypothalamus is a particularly important CNS region that is responsive to these peripheral signals to initiate changes in food intake and/or energy expenditure in order to tightly regulate overall energy balance[3]. Gut to brain signaling is also thought to be important for lipid sensing[4] and glucose sensing[5]. Due to the increase in frequency and success of bariatric surgery to treat obesity, attention has turned to understanding mechanisms underlying the gut-brain axis in regulating energy homeostasis[6].

RT-qPCR has become a valuable method for gene expression analysis and is used extensively in obesity research. In order to avoid measuring the absolute amount of mRNA within a sample, data is analyzed relative to the control group. However, errors are introduced in the technique through the process of RNA isolation, reverse transcription, and real time PCR. To overcome these variables, a reference gene is used for normalizing gene expression. However, this assumes that the expression of the reference gene must remain constant in all cell/tissue types and under specific experimental conditions. Unfortunately, increasing data have shown that no single gene has constant expression across all cell types or under all physiological/pathological conditions[7–9]. Therefore, to obtain accurate gene expression information, it is imperative that stable reference genes are chosen for the specific type of tissue and experimental condition. Several algorithms, including the geNorm[9], NormFinder[10], BestKeep[11], and comparative ΔCt (cycle thresholds) method[12], have been developed for selection of suitable reference genes and are widely used. The use of at least three reference genes for the correct normalization of RT-qPCR data has been proposed by Vandesompele[9] and is a recommended approach for normalizing RT-qPCR data[9,13]. Recently, ReFinder[14], a web based program that integrates four mathematical programs, i.e. geNorm, NormFinder, BestKeeper, and comparative ΔCt method, was developed to provide a convenient and adequate means for reference gene evaluation[12].

Rodents, such as mice and rats, have been commonly used in studying diet induced obesity and diabetes. Surprisingly, evaluation of reference genes in obesity research has received little attention[15]. In most gene expression studies, a single gene, including ACTB and RPS18, is still commonly used without any mentioning of whether these genes are affected by the experimental conditions[16–19]. In other fields, it has been realized that the expression stability of reference genes are influenced by conditions used in studies[20–22]. Therefore, we studied 13 commonly used reference genes in the rat hypothalamus, duodenum, jejunum, and ileum across different diets and feeding schemes typically used in obesity research, to determine which reference genes would be most appropriate for these particular investigations. We found that the expression levels of several commonly used reference genes, such as RPS18 in the hypothalamus or ACTB in the intestine, fluctuated across the varied diet and nutrition conditions. These data underscore the systematic validation of reference genes in obesity research.

MATERIALS AND METHODS

Animals

Male Long-Evans rats were single-housed under controlled conditions (12:12-hour light-dark cycle, 50–60% humidity, 25°C with free access to water and food except where noted) in the Metabolic Diseases Institute at University of Cincinnati. Rats were fed either a high fat diet (HFD) (n=12), (Research Diets, New Brunswick, NJ, D12451; 45% fat; 4.73kcal/g) or a standard chow diet (Harlan-Teklad, Indianapolis, IN) (n=12) for 8 weeks prior to

experiments. All procedures for animal use were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Animal groups and tissue collection

Rats were assigned to one of the four treatment groups (n=6/group): chow/fasted, chow/re-fed, HFD/fasted, HFD/re-fed. All rats were fasted overnight with free access of water and then half of the rats were re-fed for 2 hours. At the end of 2-hr feeding in the dark cycle, all rats were individually placed in a CO_2 chamber and then sacrificed by decapitation. Brain, duodenum, jejunum, and ileum were taken quickly and flash frozen in sub-zero 2-methylbutane and stored at −80°C until RNA isolation.

RNA isolation and cDNA synthesis

The hypothalamus was quickly dissected from a semi-frozen brain. Total RNA was isolated from the hypothalamus, duodenum, jejunum, and ileum using RNeasy mini-columns (Qiagen, Valencia, CA). Genomic DNA was eliminated by DNase I on column treatment with RNase-free DNase set (Qiagen, Valencia, CA). RNA integrity was confirmed by visualizing an approximate 2/1 ratio of 28S to 18S band on the 1% agarose gel stained by ethidium bromide. RNA purity was confirmed with absorbance ratio of >2.0 at 260nm/280nm using NanoVue (GE Healthcare, Piscataway, NJ). 1 μg of total RNA was used to generate cDNA using iScript following manufacturer's protocol (Bio-Rad, Hercules, CA). cDNA was made from all samples for each tissue at the same time to minimize experimental variations.

Primers and qPCR

TaqMan Gene Expression Assays [Applied Biosystems (ABI) (Carlsbad, CA)] were used for qPCR. Pre-optimized (with nearly 100% efficiency) primers/probes of 13 candidate reference genes (Table 1) were purchased from ABI. qPCR reactions were set at 10 μl with 5μl of the TaqMan Gene Expression Master Mix, 0.5μl primer/probe, 2μl of 6× diluted cDNA, and 2.5μl H_2O. Plates were run on the ABI Prism 79000HT Fast Real-Time PCR System. All qPCRs were run in duplicates on the same thermal cycles (95°, 10min, 40 cycles of 0.01sec @95°C, 20sec @60°C). No amplification signal was detected in water or no-RT RNA samples. Each gene was run with all samples for each tissue on the same plate. The threshold value was manually set to 0.2 to guarantee the comparability between the Cts obtained from different genes and different runs.

Data analysis and statistics

The gene expression stabilities of the 13 candidate reference genes from each tissue were determined by ReFinder. Upon the input of Ct values, ReFinder invokes four commonly used computational programs, geNorm, NormFinder, BestKeeper, and comparative ΔCt method, to process those data, respectively. The processed ranking results from each program were aggregated by ReFinder to generate gene expression stability rank orders. Based on their rankings from each program, ReFinder assigns an appropriate weight to each individual reference gene and then calculates the geometric mean of the weights for each gene to reach its overall final ranking, the comprehensive ranking order, among all 13 candidate genes. The details in the calculation procedures have been previously described[23]. We used the comprehensive rank order as our results.

The gene expression stabilities of the 13 candidate genes were analyzed under 4 separated conditions for each tissue: (1) fasted vs. re-fed under chow diet, (2) fasted vs. re-fed under HFD, (3) chow vs. HFD in fasted condition, and (4) chow vs. HFD in fasted then re-fed condition. 12 Cts from each individual gene were analyzed under four conditions described

above, for each tissue. We also did an overall evaluation of gene expression stabilities in pooled conditions, in which 24 Ct values of each candidate gene were analyzed for each tissue from chow/fasted, chow/re-fed, HFD/fasted, and HFD/re-fed animals.

The optimal numbers of reference genes required for accurate normalization were determined by the pairwise variation (V_n/V_{n+1}) using geNorm. A $V_{n/n+1}$ value (n = the number of reference genes desired) represents a pairwise variation between two sets of reference genes with the second set containing an additional gene. A large variation means that the added gene has a significant effect and should be included in normalization analyses. To calculate V values, Cts from all candidate genes were input into geNorm excel based program. An arbitral cut-off value of 0.15 for $V_{n/n+1}$ is adopted[9] to assist evaluation of optimal gene numbers. For example, if a $V_{2/3}$ is 0.22 and a $V_{3/4}$ is 0.12, then three reference genes are recommended for RT-qPCR analyses. Since $V_{3/4}$value is 0.12 (< cutoff value), using four reference genes will not make a significant impact on RT-qPCR analyses.

RESULTS

Transcription profiles of 13 candidate reference genes

The expression level of 13 candidate genes (Table 1) was evaluated as threshold cycle (Ct) in rat hypothalamus, duodenum, jejunum, and ileum that were treated in chow/fasted, chow/re-fed, HFD/fasted, and HFD/re-fed conditions (Fig. 1, for each gene, n=6/group). These genes were selected from ABI rat endogenous control array gene card (Applied Biosystems, Carlsbad, CA). They were chosen because they were routinely used as reference genes for normalization and they are expected to have minimal differential expression across different tissues and experimental conditions.

The Cts across the candidate housekeeping genes ranged from 20.6 to 31.22 in the hypothalamus (Fig. 1a), 19.13 to 32.27 in the duodenum (Fig. 1b), 17.97 to 31.51 in the jejunum (Fig. 1c), and 17.98 to 31.67 in the ileum (Fig. 1d). The wide range of Ct values suggested that these candidate genes had different expression levels in the four tissues examined. Among the 13 candidate genes, ACTB mRNA was the most abundant whereas TBP mRNA was the least abundant in all four tissues.

Gene expression stability analysis of candidate reference genes

Hypothalamus—When comparing re-fed vs. fasted conditions, the most stably expressed reference genes within the hypothalamus were PGK1 and B2M in rats fed chow and PGK1 and HPRT in rats fed HFD. When comparing chow or HFD, the most stable reference genes were B2M and ACTB in fasted rats and RPLP2 and YWHAZ in re-fed rats. When the pooled conditions were considered, the reference genes that expressed consistently were B2M and RPLP0. The expression of TBP and PPIB genes were the most volatile across all conditions (Table 2).

Duodenum—In rat duodenum, when comparing fasting vs. re-feeding, the most stable genes were HPRT and RPLP2 in rats fed chow and HMBS and RPS18 in rats fed HFD. When comparing diets, the most stable genes were RPLP0 and RPS18 in fasted rats and HMBS and RPS18 in refed rats. Under the pooled conditions, RPS18 and HMBS were found to be the most stably expressed genes, ACTB and PGK1 were among the least stably expressed genes across all conditions (Table 3).

Jejunum—In the jejunum, when comparing fasted vs. re-fed conditions, the most stably expressed genes were identified as RPS18 and HPRT in rats fed chow and HMBS and YWHAZ in rats fed HFD. When comparing diets, RPLP0 and HMBS were identified as

most stable genes in fasted rats, and RPLP2 and YWHAZ in fed rats. RPLP2 and RPLP0 were found to be the most stable genes under the pooled conditions. ACTB was among the genes with the least stable expressions across all conditions (Table 4).

Ileum—In rat ileum, when comparing fasted vs. re-fed conditions, HMBS and RPLP2 were identified as the genes with most stable expressions in rats fed chow, and RPS18 and RPLP2 in rats fed HFD. However, when comparing diets, YWHAZ and RPS18 were found to be the most stable genes in fasted rats, and RPS18 and RPLP2 in re-fed rats. When the pooled conditions are considered, RPS18 and YWHAZ were found to be the most stably expressed genes. And again, ACTB was found among the least stable genes across all experimental conditions (Table 5).

Optimal reference genes required for normalization

The optimal numbers of reference genes need for RT-qPCR analyses were determined in pooled conditions for each tissue by pairwise variation method ($V_{n/n+1}$) using geNorm software. Figures 2a–d showed that the $V_{2/3}$ values were 0.042, 0.063, 0.079, and 0.052 for the hypothalamus, duodenum, jejunum, and ileum, respectively. Since they are all smaller than the recommended cut-off value of 0.15, it indicates that, under the pooled conditions used in this study, using two reference genes for normalization would be sufficient to obtain accurate data.

DISCUSSION

The concept that reference genes used for normalization in RT-PCR analyses should be validated prior to use was initially suggested in 2002[24] and has been realized in various scientific research disciplines such as plant sciences[23,25,26], cancer[27–29], stem cell[30–32], and cardiovascular research[33–35]. Some limited data have been published from metabolic research in rodents, which clearly show that different sets of reference genes are found only to be suitable for each experimental condition and for each tissue[15,20–22]. However, no previous research has identified the most stably expressed reference genes within the rat hypothalamus or small intestine under different dietary and feeding conditions for energy homeostasis studies. Therefore, our study is the first attempt in this area to provide first hand evidence on the necessity of reference gene optimization.

This study was designed (1) to evaluate gene expression stability across different dietary conditions among 13 commonly used endogenous control genes, and (2) to identify the reference genes most suitable for obesity studies that use RT-PCR analysis in the rat hypothalamus and intestine. Our data confirmed that expression of many of the 13 commonly used reference genes can be affected at different levels by both tissues and conditions used in experiments (Tables 2–5). As a result, the ranking order in gene expression stability among the 13 candidate genes from each tissue was found to vary across different experimental settings. For example, in the rat hypothalamus and jejunum, there was no single pair of reference genes that were stable across all conditions, suggesting that gene expression in these two tissues were more susceptible to the experimental conditions. Our studies also showed that TBP and PPIB were consistently found to be the least stable reference genes in the hypothalamus, while ACTB was regularly scored among the least stable reference genes in the rat intestine under all but three conditions (Table 3–5).

The lack of stability in expression by reference genes in each tissue across the various laboratory conditions exemplifies the complex physiological responses to changes in feeding conditions. In order to identify accurate gene expression during the course of obesity development, it is critical to carefully select reference genes used in RT-qPCR analyses. Unfortunately, in studies reported by several groups[36–39], ACTB was frequently used as a

reference gene for normalization in RT-qPCR analyses in the hypothalamus and intestine. Based on our findings in this study, the expression of ACTB is one of the least consistent in the intestine and can fluctuate quite widely in the hypothalamus (Tables 3–5). Therefore, depending on the experimental condition, the use of ACTB could lead to either an over- or underestimation of the role of the target gene.

In 2011, Lavin et al. published a study on the effect of HFD on anti-inflammation[40]. In an experiment with conditions similar to what we used in our study, they reported that in mice fed on a HFD for 10–12 weeks, no difference was observed in the expression of inflammation related genes, such as F4/80, CD11b, and IL1α, between fed vs. fasted conditions. However, in mice fed a low fat diet gene expressions of these inflammatory genes were down regulated in fasted vs. fed condition. Since their gene expression analyses were based on a single reference gene, ACTB which we now know can vary under the conditions that change energy homeostasis, their experimental design may be flawed.

It is recommended to use three reference genes in RT-qPCR normalization studies to ensure data quality[9,13]. However, due to the constraints in cost and time, it is not always feasible to include that many reference genes in an analysis. Our data analyses using geNorm (Fig. 2) indicated that two reference genes were adequate for RT-qPCR analyses under the experimental conditions we used. Therefore, in certain conditions, it would be sufficient to use two reference genes in RT-qPCR analyses.

In conclusion, our study demonstrated that expression of 13 candidate reference genes commonly used in obesity research was differentially affected by dietary and feeding conditions, as well as by tissue. From the 13 candidate reference genes, we have identified a subset of reference genes suitable for RT-qPCR normalization in the rat hypothalamus and intestine. The development of obesity is a complex event and subtle changes in gene expression during the development of obesity can impact a cascade of signaling pathways that further contribute to obesity and/or its comorbidities. Thus, in order to detect the small but significant changes in gene expression, normalization using highly stable candidate genes becomes critical. It is our hope that the reference genes identified here can be a resource for future obesity studies.

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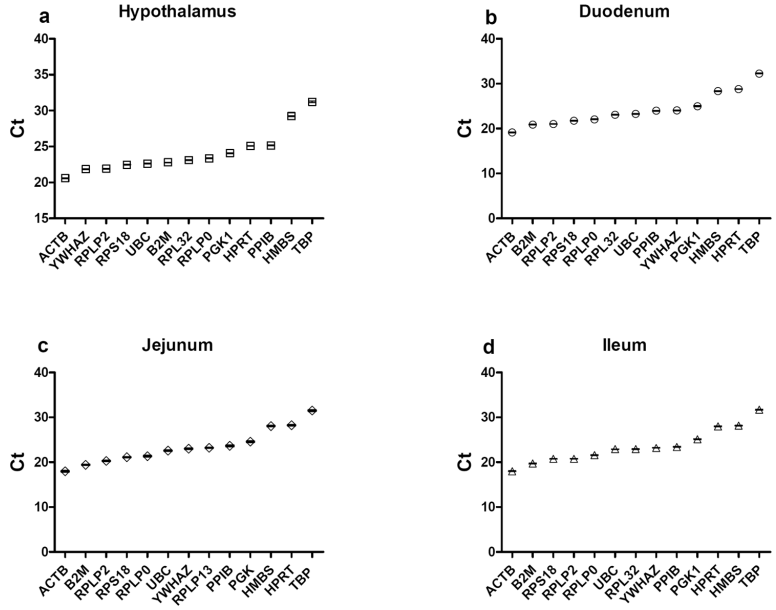

Figure 1. Distribution of threshold cycle (Ct) values of 13 candidate reference genes

The boxes show the Ct of each gene in the hypothalamus (a), duodenum (b), jejunum (c), and ileum (d). Black center line indicates the median Ct. Samples were pooled from all four conditions: chow/fasted, chow/re-fed, HFD/fasted, and HFD/re-fed. Data are presented as mean±SEM (n=24)

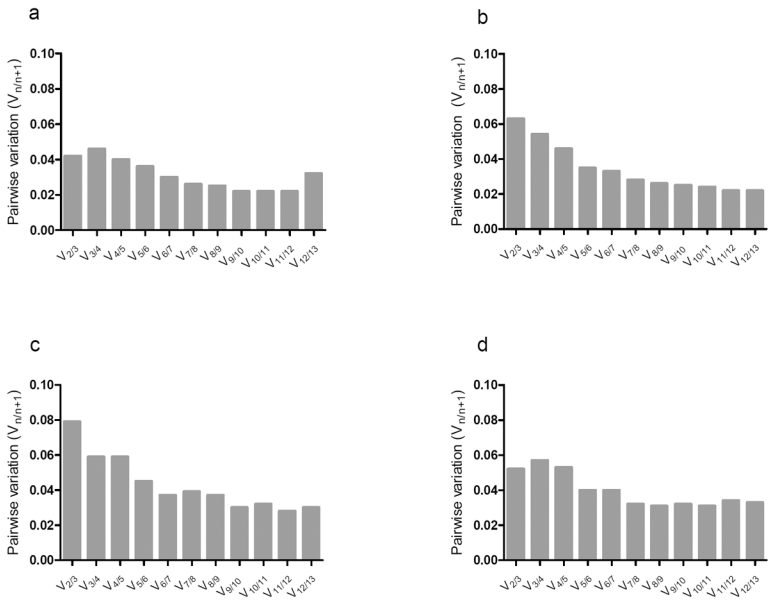

Figure 2. Pairwise variation ($V_{n/n+1}$) of 13 candidate reference genes

To derive the numbers of reference genes needed for accurate RT-qPCR, $V_{n/n+1}$ were calculated by geNorm software by inputting the Ct of each candidate gene from each tissue, hypothalamus (a), duodenum (b), jejunum (c), and ileum (d). $V_{n/n+1}$, n = the number of reference genes desired, represents the pairwise variation between two sets of reference genes with the second set containing an additional gene. The cutoff value of V is 0.15.

Table 1

Candidate reference genes and catalog numbers (ABI)

Symbol	Gene name	Accession number	Function	Cat. No.
ACTB	Actin, beta	NM_031144.2	Cytoskeletal structural protein	4352931E
B2M	beta-2 microglobulin	NM_012512.2	Assembly and surface expression of MHC class I molecules	Rn00560865_m1
HMBS	Hydroxymethylbilane synthase	NM_013168.2	Heme synthesis, porphyrin metabolism	Rn00565886_m1
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_012583.2	Generation of purine nucleotides through the purine salvage pathway	Rn01527840_m1
PGK1	Phosphoglycerate kinase I	NM_053291.3	Phosphoprotein glycolyosis	Rn00821429_g1
PPIB	Peptidylprolyl isomerase B	NM_022536.1	Endoplasmic reticulum cyclosporine-binding protein	Rn00574762_m1
RPLP0	Ribosomal protein large, P0	NM_022402.2	Protein synthesis	Rn00821065_g1
RPLP2	Ribosomal protein large, P2	NM_001030021.1	Protein synthesis	Rn01479927_g1
RPL32	Ribosomal protein L32	NM_013226.2	Protein synthesis	Rn00820748_g1
RPS18	Ribosomal protein S18	NM_213557.1	Protein synthesis	Rn01428915_g1
TBP	TATA box binding protein	NM_001004198.1	General RNA polymerase II transcription factor	Rn01455648_m1
UBC	Ubiquitin C	NM_017314.1	Involved in muscle protein metabolism	Rn01789812_g1
YWHAZ	Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide	NM_013011.3	Belongs to the 14-3-3 family of proteins which mediate signal transduction by binding to phosphoserine-containing proteins	Rn00755072_m1

Table 2

Rank order of reference gene expression stability in the hypothalamus

Ranking[a, b]	Chow fasted vs. re-fed	HFD fasted vs. re-fed	Fasted chow vs. HFD	Re-fed chow vs. HFD	Pooled conditions
1	**PGK1**	**PGK1**	**B2M**	**RPLP2**	**B2M**
2	**B2M**	**HPRT**	**ACTB**	**YWHAZ**	**RPLP0**
3	RPS18	ACTB	UBC	B2M	ACTB
4	RPLP0	RPS18	RPLP0	RPLP0	HMBS
5	ACTB	RPL32	HMBS	RPS18	UBC
6	HMBS	B2M	HPRT	HPRT	YWHAZ
7	YWHAZ	UBC	YWHAZ	HMBS	HPRT
8	HPRT	HMBS	RPS18	UBC	RPS18
9	RPLP2	YWHAZ	RPL32	ACTB	RPLP2
10	UBC	RPLP0	PGK1	PGK1	PGK1
11	RPL32	RPLP2	RPLP2	RPL32	RPL32
12	PPIB	PPIB	PPIB	PPIB	PPIB
13	TBP	TBP	TBP	TBP	TBP

[a] The ranking order is the recommended comprehensive ranking by ReFinder. Gene expression stability decreases as the ranking order number increases.

[b] Top two ranked genes are in bold.

Table 3

Rank order of reference gene expression stability in the duodenum

Ranking[a, b]	Chow fasted vs. re-fed	HFD fasted vs. re-fed	Fasted chow vs. HFD	Re-fed chow vs. HFD	Pooled conditions
1	**HPRT**	**HMBS**	**RPLP0**	**HMBS**	**RPS18**
2	**RPLP2**	**RPS18**	**RPS18**	**RPS18**	**HMBS**
3	HMBS	RPLP2	RPLP2	RPLP2	HPRT
4	RPS18	RPLP0	HMBS	PPIB	RPLP2
5	RPLP0	RPL32	HPRT	RPL32	PPIB
6	PPIB	B2M	YWHAZ	HPRT	YWHAZ
7	YWHAZ	HPRT	RPL32	YWHAZ	RPL32
8	UBC	YWHAZ	PPIB	UBC	RPLP0
9	RPL32	PPIB	UBC	B2M	UBC
10	TBP	UBC	B2M	TBP	B2M
11	PGK1	PGK1	ACTB	RPLP0	ACTB
12	B2M	ACTB	PGK1	ACTB	TBP
13	ACTB	TBP	TBP	PGK1	PGK1

[a] The ranking order is the recommended comprehensive ranking by ReFinder. Gene expression stability decreases as the ranking order number increases.

[b] Top two ranked genes are in bold.

Table 4

Rank order of reference gene expression stability in the jejunum

Ranking[a, b]	Chow fasted vs. re-fed	HFD fasted vs. re-fed	Fasted chow vs. HFD	Re-fed chow vs. HFD	Pooled conditions
1	**RPS18**	**HMBS**	**RPLP0**	**RPLP2**	**RPLP2**
2	**HPRT**	**YWHAZ**	**HMBS**	**YWHAZ**	**RPLP0**
3	RPLP13	RPLP0	YWHAZ	HPRT	HPRT
4	RPLP2	TBP	RPS18	RPS18	HMBS
5	UBC	HPRT	B2M	RPLP13	RPS18
6	HMBS	B2M	RPLP2	HMBS	B2M
7	RPLP0	RPLP2	ACTB	PGK1	YWHAZ
8	PGK1	RPLP13	RPLP13	UBC	PGK1
9	B2M	PGK1	TBP	RPLP0	RPLP13
10	PPIB	RPS18	PGK1	B2M	UBC
11	YWHAZ	ACTB	HPRT	TBP	ACTB
12	TBP	UBC	UBC	ACTB	TBP
13	ACTB	PPIB	PPIB	PPIB	PPIB

[a]The ranking order is the recommended comprehensive ranking by ReFinder. Gene expression stability decreases as the ranking order number increases.

[b]Top two ranked genes are in bold.

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Table 5

Rank order of reference gene expression stability in the ileum

Ranking[a, b]	Chow fasted vs. re-fed	HFD fasted vs. re-fed	Fasted chow vs. HFD	Re-fed chow vs. HFD	Pooled conditions
1	**HMBS**	**RPS18**	**YWHAZ**	**RPS18**	**RPS18**
2	**RPLP2**	**RPLP2**	**RPS18**	**RPLP2**	**YWHAZ**
3	RPL32	RPL32	PPIB	YWHAZ	RPLP2
4	UBC	HMBS	HMBS	RPL32	RPL32
5	RPS18	UBC	RPLP2	PPIB	UBC
6	YWHAZ	YWHAZ	PGK1	UBC	PPIB
7	PGK1	PGK1	UBC	HMBS	HMBS
8	RPLP0	PPIB	RPLP0	HPRT	RPLP0
9	PPIB	HPRT	RPL32	RPLP0	HPRT
10	HPRT	RPLP0	ACTB	TBP	ACTB
11	ACTB	ACTB	HPRT	PGK1	PGK1
12	TBP	B2M	B2M	ACTB	TBP
13	B2M	TBP	TBP	B2M	B2M

[a]The ranking order is the recommended comprehensive ranking by ReFinder. Gene expression stability decreases as the ranking order number increases.

[b]Top two ranked genes are in bold.

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