Selection of reference genes for gene expression studies in rats

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Running title: reference genes in postprandial status

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Abstract

Selection of the most stable reference gene is critical for a reliable interpretation of gene expression data using RT-PCR. In order so, 17 commonly used genes were analyzed in Wistar rat duodenum, jejunum, ileum and liver following a fat gavage and at two time periods. These reference genes were also tested in liver from Zucker (fa/fa) on a long-term dietary trial. Four strategies were used to select the most suitable reference gene for each tissue: ranking according biological coefficient of variation and further validation by statistical comparison among groups, geNorm, NormFinder and BestKeeper programs. No agreement was observed among these approaches for a particular gene, nor a common gene for all tissues. Furthermore we demonstrated that normalizing using an inadequate reference conveyed into false negative and positive results. The selection of genes provided by BestKeeper resulted in more reliable results than the other statistical packages. According to this program, *Tbp, Ubc, Hprt* and *Rn18s* were the best reference genes for duodenum, jejunum, ileum and liver, respectively following a fat gavage in Wistar rats and *Rn18s* for liver in another rat strain on a long-term dietary intervention. Therefore, BestKeeper is highly recommendable to select the most stable gene to be used as internal standard and the selection of a specific reference expression gene requires a validation for each tissue and experimental design.

Keywords: dietary trial, duodenum, jejunum, ileum, liver, rat, reference gene, postprandial,

Introduction

Several studies have found significant associations between impaired elimination of postprandial lipoproteins and cardiovascular diseases (Kannel and Vasan, 2009; Redgrave, 2008). Triglyceride rich lipoproteins observed in the postprandial state are from intestinal and hepatic origins and referred to, considering the source of lipids, as exogenous and endogenous, respectively (Iqbal and Hussain, 2009). These organs are gatekeepers (Cianflone et al., 2008) that regulate postprandial lipemia and potential targets for regulation in response to a great variety of stimuli such as hormones, feeding schedules, composition of foods, etc (Bergeron and Havel, 1997; Cianflone et al., 2008; Lairon, 2008; Perez-Martinez et al., 2008; Xu et al., 2009). In order to answer these regulatory questions, the rat has been chosen as model, an animal model with a new interest due to its recent incorporation of transgenic and knock-out technology (Cozzi et al., 2009; Geurts et al., 2009).

Quantitative real-time fluorescence-based reverse transcription polymerase chain reaction (RT-qPCR) has been the method of choice for quantification of steady-state mRNA levels due to its sensitivity, specificity, wide dynamic range and high-throughput possibilities. Despite these obvious advantages, several drawbacks referred to sample manipulation, RT and PCR procedures may introduce analytical errors that compromise the gathered biological information. To minimize the influence of the latter sources of error, accuracy of RT-qPCR relies on normalisation to an internal control, often referred to as a reference or housekeeping gene (Dheda et al., 2004; Thellin et al., 1999). An ideal housekeeping gene should be present at constant levels in all tissues and have an essential role in the maintenance of the cellular function (Butte et al., 2001). Therefore, it should show minimal variability in expression among samples and under different experimental

conditions used and its steady-state expression levels should be similar to those of the target gene (Herrera et al., 2005).

Commonly used reference genes in RT-qPCR such as β-actin (*Actb*), glyceraldeyde-3-phosphate dehydrogenase (*Gapdh*), ribosome small subunit (18s) ribosomal RNA, ubiquitin C (*Ubc*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Ywhaz*) have exhibited large variability in expression in function of tissues, experimental conditions or pathological states (Bahr et al., 2009; Banda et al., 2008; Bar et al., 2009; Byun et al., 2009; Cleal et al., 2009; Derks et al., 2008; Fajardy et al., 2009; Foldager et al., 2009; Gubern et al., 2009; Hurtado del Pozo et al., 2010; Lu et al., 2009; Schroder et al., 2009; She et al., 2009; Valente et al., 2009). Therefore, the identification of a valid reference for data normalisation to achieve accurate, reproducible, and biologically relevant mRNA quantification remains a serious problem and should be determined for each experimental condition. Assuming this perspective, the aim of this study was to find the most suitable reference genes for mRNA expression studies in different tissues and two experimental designs: following an acute intake of fat at two time-periods and after a long-term dietary intervention.

Materials and Methods

Rats

Male Wistar rats, weighing 250- 300 g aged 2 months (purchased from Charles River, Barcelona, Spain), were used for postprandial experiments. Rats, housed in sterile filter-top cages (3-4 per cage), were acclimatized in a room maintained at 20ºC with a 12-h light-dark cycle for 10 days, allowed *ad libitum* access to water and standard chow diet (Pascual S.A., Barcelona, Spain), and fasted for 18 h before experiments. As an experimental model of type-2 diabetes and obesity, male Zucker (fa/fa) rats, weighing 220-225 g aged 7 weeks (supplied by Charles River), were used for a nine-week dietary intervention after 2 weeks of quarantine. Animals were handled and killed, always according to criteria from the European Union for care and use of animal laboratory in research, and the protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza.

Study designs

In the postprandial experiment, rats were randomly allocated into 3 groups of 5 rats each. The control group did not receive any fat. The other two groups were fed 5 ml of extra virgin olive oil (Aceites Toledo, Spain) as a bolus and sacrificed 4 and 8 hours after the feeding, respectively. This amount represents the use of a dose of 16 mL olive oil/ kg, sufficient to induce a plasma postprandial response in mice (Maeda et al., 1994). Olive oil was directly administered to stomach using a 1.1 mm diameter and 50 mm long flexible Abbocath connected to a sterile polypropylene syringe and delivered in 4 seconds. At the moment of sacrifice, rats were anesthetized with 1 ml of 8% Avertine (Aldrich Chemical Co., Madrid, Spain) in 0.1 M phosphate, pH 7.2, and blood drawn from hearts. Blood was

collected in tubes containing 1 g/l sodium EDTA. Duodenum, jejunum, ileum and liver were removed and quickly frozen in liquid N_2 until total RNA was extracted.

In the dietary experiment, 12 rats were randomly allocated into 2 groups of 4 (control) and 8 rats (dietary restriction), respectively. The control group received normal chow rat (Purina 5008 diet, Charles River) ad libitum. The dietary restricted group was fed on a hypocaloric diet (30% less than the control group) during 9 weeks. The distribution of calories in both groups was similar (27% proteins, 57 % carbohydrates and 16% fat). At the moment of sacrifice, rats were anesthetized and their livers removed and quickly frozen in liquid N_2 until total RNA was extracted.

RNA isolation.

RNA from each liver was isolated using Tri reagent (Sigma). DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from AMBION (Austin, TX, USA). RNA was quantified by absorbance at 260 and 280 nm. As shown in supplementary Table 1, the $A_{260/280}$ ratio did not vary significantly among groups. The integrity of the 28 S and 18 S ribosomal RNAs was verified by 1% agarose gel electrophoresis of 500 ng of total RNA. Ethidium-bromide stained gels were exposed to UV light and images were captured (BioRad, Madrid, Spain). Intensity of bands for each condition was calculated using Quantity One® software version 4.5.0 (BioRad). The 28S/18S ratio did not differ among groups (supplementary Table 1). RNA integrity number (RIN) from samples was obtained by RNA nano kit using an Agilent 2100 Bioanalyzer. RIN values were not significantly different among tested groups (supplementary Table 1).

Quantification of mRNA.

The mRNA expression was analyzed by reverse transcriptase and quantitative real-time polymerase chain reaction (RT-qPCR). Equal amounts of DNA-free RNA from each sample of each animal were used. First-strand cDNA synthesis and the PCR reactions were performed using the SuperScript II Platinum Two-Step RT-qPCR Kit with SYBR Green (Invitrogen, Madrid, Spain), according to the manufacturer's instructions and as previously described (Arbones-Mainar et al., 2006). Primers were designed by Primer Express® (Applied Biosystems, Foster City, CA) and checked by BLAST analysis (NCBI) to verify specificity and selective amplification of the target gene as well as to get amplification of the cDNA and not of genomic DNA. The sequences are shown in Table 1. Real time PCR reactions were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) following the standard procedure. The specificity of the PCR reaction was confirmed by observing a single dissociation curve and no template controls were carried out to reject unintended amplification. Each sample was analyzed in duplicate obtaining an average *Cq* for sample and the coefficient of variation (CV) for groups was obtained. This CV, expressed as percentage and calculated as the standard deviation divided by the mean *Cq*, was used to compare the variation degree among the 17 control genes. To confirm results, geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) applications were also used. These bioinformatics packages calculate a stability value, whereas a lower value means a higher stability in gene expression. The genes were ranked according to these obtained gene stability values.

Statistical analysis

The results are expressed as means ± SD. Comparisons were made using one-way ANOVA and the Tukey-Kramer multiple comparison test (*post hoc*) when the distribution of the variables was normal. When the variables did not show such a distribution (according to the Shapiro-Wilk's test), or failed to show homology of variance, comparisons were made using the Mann-Whitney U test. All calculations were performed using SPSS version 15.0 software (SPSS Inc, Chicago, IL). Significance was set at $P \leq 0.05$.

Results

Ranking of candidate genes by RT-qPCR.

Based on the global value biological coefficient of variation in the three conditions – control (fasted animals), 4 and 8 hours after a fat gavage- of the first experiment, the 17 analyzed genes were ranked and the results obtained for duodenum are shown in Table 2. According to this data, *Rn18s* showed the lowest inter-experiment variation while *Rplp2* represented the quite opposite situation. Jejunal ranking for the selected reference genes, depicted in Table 3, indicates that *Cck* was the gene with the lowest variation and that *Ywhag* showed the highest among studied genes in this tissue. In ileum and shown in Table 4, *Rn18s* expression displayed the lowest variation and the opposite was true for *B2m* in the present experimental setting. When the ranking was used to classify the liver reference gene expression (Table 5), *Ubc* expression showed the lowest biological variability in clear contrast with *Ppib* which was ranked in the last position. In order to verify that no variation in the expression of any of these genes existed among experimental groups, analysis of variance of the top five gene expressions for each tissue was carried out. As shown in Table 6 and with the exception of the significant change of *Ubc* expression in liver, no other significant change was observed. Thereby, these two approaches are proposing the use of *Rn18s* as reference gene for duodenum, ileum and liver and *Cck* for jejunum in this specific experimental design.

To test the reliability of the former hepatic candidate gene, a second experimental design was carried out in a different strain of rats (Zucker variant fa/fa) and with a long-term dietary intervention. As shown in Table 7, the ranking used to classify the liver reference gene expression in this setting showed that *Tbp* had the lowest biological variability, while *Gapdh* the highest. Surprisingly in this experimental approach, *Rn18s* as reference gene for

liver was not obtained. The top five gene expressions of Table 7 were also tested to test that no variation in their expression took place. As shown in Table 8, no significant change was observed.

Stability of reference genes

In the postprandial experiment design, stability of the 17 reference genes was also evaluated by the most commonly used software based methods: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004). The geNorm uses a gene-stability measure M, which is defined as the average pair wise variation between a particular gene and all other control genes and calculates the optimal number of genes necessary for normalisation. Using this method, the values obtained are shown in Figure 1. Based on the M value, the best reference pairs to be used were: *Arbp* + *Tfrc* in duodenum, *Hprt* + *Ppia* in jejunum, *Pgk1*+ *Ywhag* in ileum and *Hprt* + *Ppia* in liver. The NormFinder software uses a model-based approach and gives a ranking order. According to this program (Figure 1), *Arbp*, *Pgk1*, *Tbp* and *Cck* were identified as the most stable genes for duodenum, jejunum, ileum and liver, respectively. BestKeeper program, using geometric means, provides a stability ranking order and a single best gene for normalisation. As shown in Figure 2, *Tbp, Ubc, Hprt* and *Rn18s* were the optimal reference genes for duodenum, jejunum, ileum and liver.

Analysis of stability in the long-term dietary intervention by different software methods for the different reference genes is reflected in Figure 3. According to geNorm (Figure 3A), the best reference pairs would be *Hprt* + *Actb*. As shown in Figure 3 (panels B and C), NormFinder and BestKeeper showed that the best option were *Tbp* and *Rn18s*, respectively.

Relevance of selecting a particular candidate reference gene

To address this issue, the expression of hepatic gene *Slc34a2* was analyzed and referred to several reference genes in the postprandial experiment in Wistar rats. As shown in Figure 4A, the use of *Ppib* as reference, the worst one for liver, resulted in high biological variation and absence of significant changes in the experimental conditions. Normalisation of *Slc34a2* hepatic expression either using the best combination of genes (*Hprt* + *Ppia)* suggested by geNorm (Figure 4B) or the best single gene *Cck* calculated by NormFinder software (Figure 4C) resulted in lower variability and significant decreases in animals receiving the fat gavage. When the *Slc34a2* expression was normalized taking as reference *Rn18s,* as proposed from coefficient of variation (Tables 5 and 6) and from BestKeeper program, the lowest variation and a dramatic decrease in the animals receiving the fat gavage were observed (Figure 4D).

As a second example, the expression of hepatic gene *Apoa1* was analyzed and referred to several reference genes in the long-term dietary intervention using Zucker (fa/fa) rats. When *Gapdh* expression (Figure 4E) was used as reference gene, a significant decrease in the *Apoa1* gene was observed. This change was lost when the best choices of geNorm (*Hprt* + *Actb),* NormFinder (*Tbp*) and BestKeeper *(Rn18s*) were used (Figure 4 panels F, G and H). Overall, these results indicate that searching for appropriate reference gene or genes in any experimental design is crucial since false negative and positive results may be obtained when an inappropriate reference gene is used. Furthermore, since a uniform outcome from the different software methods was not obtained, the use of at least two of them is required to reach a certain confidence in the observed changes.

Discussion

Our study provides a search for finding the most suitable reference gene or genes for mRNA gene expression studies in two experimental designs: first, in hepatic and intestinal tissues following a postprandial approach in adult Wistar rats and in liver after long-term dietary trial in Zucker (fa/fa) rats. To this end, 17 reference genes by quantitative real-time PCR were investigated in duodenum, jejunum, ileum and liver of Wistar rats after a fat gavage at two time-periods. The same genes were also studied in the other strain of rats consuming a hypocaloric diet. Four strategies of selection such as biological coefficient of variation and statistical comparison among groups, geNorm, NormFinder and BestKeeper applets were also employed to select a single gene or a combination of genes that better suits as a control.

Gene expression data in RT-qPCR based gene expression studies are normalised relative to an internal control. Thus, the choice of an appropriate control is crucial to prevent biased results. Furthermore, a universal control gene does not exist (Thellin et al., 1999) what motivates new validations for different tissues and experimental conditions. Several studies have addressed the influence of reference genes in liver (Butte et al., 2001; Chen et al., 2006; Frericks and Esser, 2008; Kouadjo et al., 2007; Pohjanvirta et al., 2006; Rhoads et al., 2003; Verma and Shapiro, 2006; Warrington et al., 2000; Waxman and Wurmbach, 2007) or hepatocytes (Nishimura et al., 2006). Few have done in intestine (Hoque et al., 2007) and, to our knowledge; this is the first to address the influence of fat intake on reference gene variations in these tissues.

In this work, four different strategies have been adopted to validate the results. The first one was based on Cq and the calculation of biological coefficient of variation, as recently proposed (Hurtado del Pozo et al., 2010), and ranking according to this value. Using this

approach, the 17 genes were ranked in the different tissues (Tables 2-5). To further reinforce this approach, the top five gene expressions were statistically compared to verify that no significant change existed (Table 6). With the remarkable exception of hepatic *Ubc* expression, the other genes showed small variation and could be considered good candidate reference genes. In fact, if the best and the worst candidates were used to normalize *Slc34a2* expression, a dramatic influence would be obtained (Figure 4 A and D). To compare the reliability of the approach, other common programs were also used. In this way, the M values of the geNorm program (Vandesompele et al., 2002) for the 17 candidate reference genes in the four tissues were calculated. In all cases, the values were under the arbitrary threshold of 0.5 (Figure 1) what in practical terms indicated that any of the reference genes would be feasible to be used. The geometric average of the two highest score reference genes suggested by the geNorm program has been proposed by Silver et al. (Silver et al., 2008) as a procedure to normalize. Following this advice, the *Slc34a2* expression was normalized to the geometric mean of *Hprt* and *Ppia* expressions. Despite the notable improvement compared to the normalisation using *Ppib* (Figure 3 A and B), higher variability was still observed in the groups of animals receiving the fat gavage. Equally, stability analysis by NormFinder was carried out and proposed different top-ranking genes that those selected by geNorm. The reference gene suggested by this program, *Cck*, was employed to normalize *Slc34a2* expression and this resulted in a similar outcome (Figure 3 C) to that obtained from geNorm. The use of BestKeeper (Figure 2) to calculate the stability of reference genes in the different tissues resulted in a ranking that suggested *Rn18s* as the best for rat liver following a fat gavage in agreement with results obtained by the combination of biological coefficient of variation and statistical comparison. This double agreement and the simplicity of BestKeeper make the latter a

reasonable choice to be used in selecting reference genes. In order to gain more insight into the relevance of this finding, a second experimental design was used. In this regard, Zucker fa/fa rats were fed a hypocaloric diet for nine weeks and the search for best reference gene carried out. No such agreement on the selected gene between biological coefficient of variation (Tables 7 and 8) and BestKeeper (Figure 3) existed. While the former suggested *Tbp*, the latter proposed *Rn18s.* From results of Figure 4, it is clear that a combination of both procedures provides a reasonable certainty regarding to which gene expression change better reflects the tissue situation.

In agreement with previous studies (Andersen et al., 2004; Feng et al., 2010; Hurtado del Pozo et al., 2010; Pfaffl et al., 2004), we have observed that the different theoretical methods (NormFinder, geNorm and BestKeeper) provide different outcomes. These discrepancies have been attributed to the different mathematical models used. In this regard, geNorm uses the average pair wise variation between a particular gene and all other control genes and calculates the optimal number of genes necessary for normalisation. NormFinder software, using all candidate genes, gives a ranking order based on the estimated intra- and intergroup variation. All candidate genes are also considered in BestKeeper but a geometric mean is calculated to provide its stability ranking order. These two methods suggest a single best gene for normalisation. Recently, Feng et al (Feng et al., 2010) have described that the worst genes in the rankings of geNorm and NormFinder were the same. Something corroborated by data shown in Figures 1 and 3. These authors preferred geNorm data. Unfortunately, they did not use BestKeeper in their analysis. However, when geNorm and NormFinder software methods in long-term dietary intervention were used and the best and worst candidate genes taken into consideration, results were similar (Figure 4 F, G and H). This would indicate an insensitivity of to detect

a false positive in contrast with the results of the worst gene selected by BestKeeper that clearly did (Figure 4 E). A new and interesting feature of BestKeeper is the calculation of the intrinsic variance (InVar) of expression for a single sample that allows the researchers to detect outliers, due to inefficient sample preparation, incomplete reverse transcription or sample degradation. When this index was calculated (data not shown), no change was detected among samples in agreement with other RNA quality values obtained and shown in supplementary Table 1. A potential explanation of high biological standard deviation, reflected in Figure 4, may be the presence of a particular animal with higher value for a certain gene and different for the studied genes. This may take place when no inbred animals are used, and its relevance is more important when sample size is small.

Conclusions

To study gene expression changes it is important to verify the reference gene that shows the highest stability for each specific experimental design and tissue. Among the 17 genes used in four different tissues following a fat gavage, a specific gene expression was found optimal for each tissue. Based on the outcome of the different strategies adopted to validate reference genes, the use of BestKeeper is highly recommended and according to this, *Tbp, Ubc, Hprt* and *Rn18s* were found to be the optimal reference genes for duodenum, jejunum, ileum and liver to study gene expression. When another experimental condition was tested in liver, it has been observed that this software also discriminates better than the others but rejection of a false positive may require an additional use of other software tools.

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List of Abbreviations:

Actb, β-actin; *Arbp*, acidic ribosomal phosphoprotein; *B2m*, β-2-microglobulin; *Cck*, cholecystokinin; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Gusb*, β-glucuronidase; *Hmbs*, hidroxym-ethylbilane synthase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Pgk1*, phosphoglycerate kinase 1; *Ppia*, peptidylprolyl isomerase A; *Ppib*, peptidylprolyl isomerase B; *Rn18s*, 18S ribosomal RNA; *Rplp2*, ribosomal large P2; RT-PCR, reverse transcription polymerase chain reaction; *Tbp*, TATA box binding protein; *Tfrc*, transferrin receptor; *Ubc*, ubiquitin; *Ywhag*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma polypeptide.

Authors' contribution

RMB and MS carried the molecular and statistical analyses, participated in drafted the manuscript. MAN and MAG carried the postprandial experiment and obtained samples. AL, PI carried the long-term dietary experiment and obtained samples. SA and CB prepared the RNA samples and participated in drafted the manuscript. JO conceived the

study, coordinated the work and helped to draft the manuscript. All authors read and approved the final manuscript.

References

Andersen, C.L., Jensen, J.L., Orntoft, T.F., (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.

Arbones-Mainar, J.M., Navarro, M.A., Acin, S., Guzman, M.A., Arnal, C., Surra, J.C., Carnicer, R., Roche, H.M., Osada, J., (2006) Trans-10, cis-12- and cis-9, trans-11-Conjugated Linoleic Acid Isomers Selectively Modify HDL-Apolipoprotein Composition in Apolipoprotein E Knockout Mice. J Nutr 136, 353-359.

Bahr, S.M., Borgschulte, T., Kayser, K.J., Lin, N., (2009) Using microarray technology to select housekeeping genes in Chinese hamster ovary cells. Biotechnol Bioeng 104, 1041-1046.

Banda, M., Bommineni, A., Thomas, R.A., Luckinbill, L.S., Tucker, J.D., (2008) Evaluation and validation of housekeeping genes in response to ionizing radiation and chemical exposure for normalizing RNA expression in real-time PCR. Mutat Res 649, 126-134.

Bar, M., Bar, D., Lehmann, B., (2009) Selection and validation of candidate housekeeping genes for studies of human keratinocytes--review and recommendations. J Invest Dermatol 129, 535-537.

Bergeron, N., Havel, R.J., (1997) Assessment of postprandial lipemia: nutritional influences. Curr Opin Lipidol 8, 43-52.

Butte, A.J., Dzau, V.J., Glueck, S.B., (2001) Further defining housekeeping, or "maintenance," genes Focus on "A compendium of gene expression in normal human tissues". Physiol Genomics 7, 95-96.

Byun, J., Logothetis, C.J., Gorlov, I.P., (2009) Housekeeping genes in prostate tumorigenesis. Int J Cancer 125, 2603-2608.

Cianflone, K., Paglialunga, S., Roy, C., (2008) Intestinally derived lipids: metabolic regulation and consequences--an overview. Atheroscler Suppl 9, 63-68.

Cleal, J.K., Day, P., Hanson, M.A., Lewis, R.M., (2009) Measurement of housekeeping genes in human placenta. Placenta 30, 1002-1003.

Cozzi, J., Anegon, I., Braun, V., Gross, A.C., Merrouche, C., Cherifi, Y., (2009) Pronuclear DNA injection for the production of transgenic rats. Methods Mol Biol 561, 73-88.

Chen, J., Rider, D.A., Ruan, R., (2006) Identification of valid housekeeping genes and antioxidant enzyme gene expression change in the aging rat liver. J Gerontol A Biol Sci Med Sci 61, 20-27.

Derks, N.M., Muller, M., Gaszner, B., Tilburg-Ouwens, D.T., Roubos, E.W., Kozicz, L.T., (2008) Housekeeping genes revisited: different expressions depending on gender, brain area and stressor. Neuroscience 156, 305-309.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G., Zumla, A., (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 37, 112-114, 116, 118-119.

Fajardy, I., Moitrot, E., Vambergue, A., Vandersippe-Millot, M., Deruelle, P., Rousseaux, J., (2009) Time course analysis of RNA stability in human placenta. BMC Mol Biol 10, 21.

Feng, X., Xiong, Y., Qian, H., Lei, M., Xu, D., Ren, Z., (2010) Selection of reference genes for gene expression studies in porcine skeletal muscle using SYBR green qPCR. J Biotechnol 150, 288-293.

Foldager, C.B., Munir, S., Ulrik-Vinther, M., Soballe, K., Bunger, C., Lind, M., (2009) Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. BMC Mol Biol 10, 94.

Frericks, M., Esser, C., (2008) A toolbox of novel murine house-keeping genes identified by meta-analysis of large scale gene expression profiles. Biochim Biophys Acta 1779, 830-837.

Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., Jenkins, S.S., Wood, A., Cui, X., Meng, X., Vincent, A., Lam, S., Michalkiewicz, M., Schilling, R., Foeckler, J., Kalloway, S., Weiler, H., Menoret, S., Anegon, I., Davis, G.D., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., Jacob, H.J., Buelow, R., (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325, 433.

Gubern, C., Hurtado, O., Rodriguez, R., Morales, J.R., Romera, V.G., Moro, M.A., Lizasoain, I., Serena, J., Mallolas, J., (2009) Validation of housekeeping genes for quantitative real-time PCR in in-vivo and in-vitro models of cerebral ischaemia. BMC Mol Biol 10, 57.

Herrera, F., Martin, V., Antolin, I., Garcia-Santos, G., Rodriguez-Blanco, J., Rodriguez, C., (2005) Standard curve for housekeeping and target genes: specific criteria for selection of loading control in Northern blot analysis. J Biotechnol 117, 337-341.

Hoque, T., Bhogal, M., Webb, R.A., (2007) Validation of internal controls for gene expression analysis in the intestine of rats infected with Hymenolepis diminuta. Parasitol Int 56, 325-329.

Hurtado del Pozo, C., Calvo, R.M., Vesperinas-Garcia, G., Gomez-Ambrosi, J., Fruhbeck, G., Corripio-Sanchez, R., Rubio, M.A., Obregon, M.J., (2010) IPO8 and FBXL10: New Reference Genes for Gene Expression Studies in Human Adipose Tissue. Obesity (Silver Spring) 18, 897-903.

Iqbal, J., Hussain, M.M., (2009) Intestinal lipid absorption. Am J Physiol Endocrinol Metab 296, E1183- E1194.

Kannel, W.B., Vasan, R.S., (2009) Triglycerides as vascular risk factors: new epidemiologic insights. Curr Opin Cardiol 24, 345-350.

Kouadjo, K.E., Nishida, Y., Cadrin-Girard, J.F., Yoshioka, M., St-Amand, J., (2007) Housekeeping and tissue-specific genes in mouse tissues. BMC Genomics 8, 127.

Lairon, D., (2008) Macronutrient intake and modulation on chylomicron production and clearance. Atheroscler Suppl 9, 45-48.

Lu, B., Yu, J., Xu, J., Chen, J., Lai, M., (2009) A novel approach to detect differentially expressed genes from count-based digital databases by normalizing with housekeeping genes. Genomics 94, 211-216.

Maeda, N., Li, H., Lee, D., Oliver, P., Quarfordt, S.H., Osada, J., (1994) Targeted disruption of the apolipoprotein C-III gene in mice results in hypertriglyceridemia and protection from postprandial hypertriglyceridemia. J Biol Chem 269, 23610-23616.

Nishimura, M., Koeda, A., Suzuki, E., Shimizu, T., Kawano, Y., Nakayama, M., Satoh, T., Narimatsu, S., Naito, S., (2006) Effects of prototypical drug-metabolizing enzyme inducers on mRNA expression of housekeeping genes in primary cultures of human and rat hepatocytes. Biochem Biophys Res Commun 346, 1033-1039.

Perez-Martinez, P., Lopez-Miranda, J., Perez-Jimenez, F., Ordovas, J.M., (2008) Influence of genetic factors in the modulation of postprandial lipemia. Atheroscler Suppl 9, 49-55.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., (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.

Pohjanvirta, R., Niittynen, M., Linden, J., Boutros, P.C., Moffat, I.D., Okey, A.B., (2006) Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin-treated rats. Chem Biol Interact 160, 134-149.

Redgrave, T.G., (2008) Chylomicrons in disease-future challenges Invited keynote address. Atheroscler Suppl 9, 3-6.

Rhoads, R.P., McManaman, C., Ingvartsen, K.L., Boisclair, Y.R., (2003) The housekeeping genes GAPDH and cyclophilin are regulated by metabolic state in the liver of dairy cows. J Dairy Sci 86, 3423-3429.

Schroder, A.L., Pelch, K.E., Nagel, S.C., (2009) Estrogen modulates expression of putative housekeeping genes in the mouse uterus. Endocrine 35, 211-219.

She, X., Rohl, C.A., Castle, J.C., Kulkarni, A.V., Johnson, J.M., Chen, R., (2009) Definition, conservation and epigenetics of housekeeping and tissue-enriched genes. BMC Genomics 10, 269.

Silver, N., Cotroneo, E., Proctor, G., Osailan, S., Paterson, K.L., Carpenter, G.H., (2008) Selection of housekeeping genes for gene expression studies in the adult rat submandibular gland under normal, inflamed, atrophic and regenerative states. BMC Mol Biol 9, 64.

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

Valente, V., Teixeira, S.A., Neder, L., Okamoto, O.K., Oba-Shinjo, S.M., Marie, S.K., Scrideli, C.A., Paco-Larson, M.L., Carlotti, C.G., Jr., (2009) Selection of suitable housekeeping genes for expression analysis in glioblastoma using quantitative RT-PCR. BMC Mol Biol 10, 17.

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

Verma, A.S., Shapiro, B.H., (2006) Sex-dependent expression of seven housekeeping genes in rat liver. J Gastroenterol Hepatol 21, 1004-1008.

Warrington, J.A., Nair, A., Mahadevappa, M., Tsyganskaya, M., (2000) Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiol Genomics 2, 143-147.

Waxman, S., Wurmbach, E., (2007) De-regulation of common housekeeping genes in hepatocellular carcinoma. BMC Genomics 8, 243.

Xu, T., Li, X., Ma, X., Zhang, Z., Zhang, T., Li, D., (2009) Effect of diacylglycerol on postprandial serum triacylglycerol concentration: a meta-analysis. Lipids 44, 161-168.

Table 1. Nucleotide sequence of primers used for analysis by RT-qPCR of 17 candidate reference genes.

Gene	Accession #		Sequence	junction	[primer]	Efficiency	Amplicon length
Actb	NM_031144.2	sense	CTGACTGACTACCTCATGAAGATCCT	exon 4	100 nM	92%	86
		antisense	CTTAATGTCACGCACGATTTCC				
Arbp	NM_022402.1	sense	CCCTTCTCCTTCGGGCTGAT	exon 4/5	100 nM	94%	165
		antisense	TGAGGCAACAGTCGGGTAGC				
B2m	NM_012512.1	sense	CGAGACCGATGTATATGCTTGC	exon 2/4	100 nM	96%	114
		antisense	GTCCAGATGATTCAGAGCTCCA				
Cck	NM_012829.1	sense	GGCTATTTAAGAGGAGTCGCCC	exon 1/2	100 nM	98%	111
		antisense	CACGCCGCACTTCATATCTTC				
Gapdh	NM_017008.3	sense	CTCCCTCAAGATTGTCAGCAA	exon 4/6	100 nM	96%	312
		antisense	GTCAGATCCACAACGGATACATT				
Gusb	NM_017015.2	sense	CCTTTCTACTTCCAAGGCGTCA	exon 6/7	100 nM	95%	101
		antisense	CAACGGAGGAGGTTGAAATCC				
Hmbs	NM_013168.2	sense	TGAAACTCTGCTTCGCTGCA	exon 11/13	100 nM	90%	113
		antisense	TCAGGTACAGTTGCCCATCCTT				
Hprt	NM_012583.2	sense	TCCCAGCGTCGTGATTAGTGA	exon 1/3	100 nM	97%	152
		antisense	CCTTCATGACATCTCGAGCAAG				
Rn18s	X01117.1	sense	ACTCAACACGGGAAACCTCA	exon 5	100 nM	99%	114
		antisense	TCTTAGTTGGTGGAGCGATT				
Pgk1	NM_053291.3	sense	GCAGATTGTTTGGAACGGTCC	exon 8/9	100 nM	98%	113
		antisense	TAGTGATGCAGCCCCTAGACGT				
Ppia	NM_017101.1	sense	CCAAACACAAATGGTTCCCAGT	exon 3/4	100 nM	95%	135
		antisense	ATTCCTGGACCCAAAACGCT				
Ppib	NM_022536.1	sense	TCGGAGCGCAATATGAAGGT	exon 1/2	100 nM	97%	102
		antisense	CTTCTTCTTATCGTTGGCCACG				
Tbp	NM_001004198.1	sense	TAATCCCAAGCGGTTTGCTG	exon 4/6	100 nM	92%	111
		antisense	TTCTTCACTCTTGGCTCCTGTG				
Tfrc	XM_340999.3	sense	ATCATCAAGCAGCTGAGCCAG	exon 4/5	100 nM	93%	124

		antisense	CTCGCCAGACTTTGCTGAATTT				
Rplp2	NM_001030021.1	sense	ATGCGCTACGTTGCCTCTTATC	exon 2/3	100 nM	91%	126
		antisense	GACCTTGTTGAGTCGTTCATCG				
Ubc	NM_017314.1	sense	ATCTAGAAAGAGCCCTTCTTGTGC	exon 3	100 nM	98%	51
		antisense	ACACCTCCCCATCAAACCC				
Ywhag	NM_019376.2	sense	TTCCTAAAGCCCTTCAAGGCA	exon 1	100 nM	96%	101
		antisense	GGCTTTCTGCACTAGTTGCTCG				

Table 2. Duodenal gene expression of 17 commonly used reference genes in rats following a postprandial regimen organized according to their biological coefficient of variation obtained by RT-qPCR.

	Global				Control		4h		8h	
Gene symbol	Mean Cq	SD	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank
Rn18s	24.8	0.5	2.1	1	1.2	2	2.6	1	2.1	7
Ubc	24.9	0.6	2.3	2	2.3	11	3.3	2	1.0	1
Tfrc	28.5	0.7	2.6	3	1.6	5	3.9	3	1.1	3
Ppia	23.1	0.6	2.8	4	1.3	3	4.0	4	2.5	10
Ppib	24.9	0.7	2.9	5	2.4	12	4.1	6	2.3	9
Cck	27.8	0.9	3.3	6	3.6	16	4.2	7	1.8	6
Hmbs	25.5	0.9	3.4	7	1.3	4	4.4	8	1.7	4
Arbp	23.6	0.8	3.5	8	2.2	10	5.3	10	2.2	8
Ywhag	23.8	0.9	3.9	9	1.1	1	4.6	9	2.5	11
Gusb	22.4	0.9	4.0	10	2.0	9	5.3	11	3.3	13
Gapdh	22.8	1.0	4.5	11	2.9	15	7.4	14	3.7	14
Pgk1	21.8	1.0	4.5	12	1.8	7	6.9	12	3.7	16
Actb	20.4	0.9	4.5	13	2.6	13	7.4	13	2.5	12
Hprt	27.2	1.4	5.0	14	2.8	14	4.1	5	5.2	17
B2m	24.2	1.2	5.0	15	4.5	17	7.4	15	3.7	15
Tbp	27.8	2.3	8.2	16	1.7	6	8.6	16	1.7	5
Rplp2	22.1	2.5	11.4	17	1.9	8	12.5	17	1.0	2

Table 3. Jejunal gene expression of 17 commonly used reference genes in rats following a postprandial regimen organized according to their biological coefficient of variation obtained by RT-qPCR.

	Global				Control		4h		8h	
Gene symbol	Mean Cq	SD	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank
Cck	33.3	1.2	3.6	1	2.0	8	4.1	8	4.6	2
Gusb	27.5	1.1	4.0	2	2.5	12	1.4	1	6.0	3
Ppia	24.6	1.0	4.1	3	1.4	4	3.8	5	6.2	4
Hprt	30.9	1.3	4.3	4	1.4	5	3.8	6	6.4	5
Tfrc	24.9	1.1	4.3	5	3.0	15	3.3	4	6.5	6
Ubc	21.0	0.9	4.4	6	4.2	17	3.9	7	4.4	1
Pgk1	29.4	1.3	4.5	7	0.7	1	4.1	9	6.8	7
Rplp2	26.5	1.2	4.6	8	2.0	10	4.2	10	7.0	8
Hmbs	25.2	1.3	5.1	9	2.9	13	3.0	2	8.2	10
Ppib	27.5	1.5	5.5	10	0.8	2	4.4	11	9.4	14
Actb	23.4	1.3	5.7	11	1.4	6	6.1	14	7.1	9
Arbp	26.0	1.5	5.7	12	2.0	9	5.6	12	9.2	13
B2m	25.0	1.4	5.8	13	3.1	16	6.1	15	8.8	12
Tbp	25.7	1.6	6.0	14	3.0	14	3.3	3	10.3	15
Rn18s	25.1	1.7	6.9	15	2.1	11	9.5	17	8.5	11
Gapdh	24.5	1.8	7.4	16	1.9	7	6.2	16	11.0	17
Ywhag	26.1	1.9	7.4	17	1.2	3	5.9	13	10.9	16

Table 4. Ileal gene expression of 17 commonly used reference genes in rats following a postprandial regimen organized according to their biological coefficient of variation obtained by RT-qPCR.

	Global				Control		4h		8h	
Gene symbol	Mean Cq	SD	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank
Rn18s	27.7	1.2	4.3	1	3.1	3	4.9	1	2.8	1
Ubc	24.0	1.1	4.5	2	2.9	2	5.6	4	4.3	8
Tbp	25.9	1.4	5.2	3	5.9	7	5.1	2	6.4	16
Hprt	29.8	1.6	5.4	4	3.7	5	7.9	11	3.8	4
Ppib	28.7	1.6	5.5	5	3.5	4	8.4	13	4.0	7
Tfrc	27.9	1.7	6.0	6	4.9	6	7.7	10	4.7	11
Gusb	27.3	1.7	6.0	7	6.4	10	7.0	8	3.9	5
Ywhag	32.0	2.0	6.3	8	6.1	8	6.9	7	5.5	14
Ppia	26.1	1.7	6.3	9	2.0	1	10.8	17	2.9	2
Hmbs	30.8	2.0	6.3	10	7.3	12	6.4	6	5.0	12
Pgk1	25.8	1.8	7.0	11	7.5	13	8.9	16	4.0	6
Rplp2	26.9	1.9	7.2	12	7.1	11	6.1	5	5.4	13
Cck	33.5	2.5	7.4	13	6.3	9	5.2	3	3.7	3
Actb	23.6	1.9	8.1	14	10.2	16	8.1	12	5.5	15
Arbp	26.4	2.2	8.3	15	7.6	14	8.5	15	4.5	10
Gapdh	26.8	2.3	8.6	16	10.3	17	8.4	14	4.3	9
B2m	25.3	2.8	11.2	17	8.5	15	7.1	9	10.6	17

Table 5. Hepatic gene expression of 17 commonly used reference genes in rats following a postprandial regimen organized according to their biological coefficient of variation obtained by RT-qPCR.

	Global				Control		4h		8h	
Gene symbol	Mean Cq	SD	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank
Ubc	20.8	0.5	2.4	1	1.9	1	1.1	2	2.1	1
Rn18s	21.5	0.6	2.7	2	3.6	4	0.7	1	1.1	2
Ywhag	24.8	0.8	3.2	3	3.4	3	3.4	4	2.7	3
Tfrc	27.3	1.0	3.6	4	4.1	7	1.6	3	4.4	8
Cck	32.8	1.5	4.4	5	4.1	8	5.1	6	3.2	5
Gusb	27.9	1.4	5.0	6	4.8	9	6.1	7	5.4	11
Rplp2	27.2	1.4	5.2	7	4.0	6	7.7	11	3.4	6
Ppia	28.4	1.6	5.7	8	6.7	12	6.3	8	2.7	4
Pgk1	29.4	1.8	6.0	9	5.7	10	5.1	5	5.5	13
B2m	25.0	1.6	6.5	10	8.5	15	7.1	10	6.0	15
Hprt	29.2	1.9	6.5	11	7.3	13	6.7	9	5.4	12
Hmbs	25.7	1.9	7.2	12	7.5	14	9.7	15	4.9	10
Arbp	27.1	2.0	7.3	13	4.0	5	7.9	12	4.1	7
Actb	24.4	2.1	8.4	14	6.3	11	10.6	17	4.7	9
Gapdh	27.4	2.3	8.5	15	3.0	2	10.2	16	6.0	14
Tbp	25.4	2.2	8.6	16	8.5	16	9.0	13	9.6	16
Ppib	27.7	2.4	8.8	17	9.7	17	9.5	14	12.0	17

Table 6. Gene expression of top five reference genes in different tissues selected according to their biological coefficient of variation obtained by RT-qPCR in Wistar rats following a fat gavage.

Gene symbol	Control	4 h	8 h
Liver			
Ubc	20.3 ± 0.4	20.9 ± 0.2	21.2 ± 0.4[a]
Rn18s	21.5 ± 0.6	21.9 ± 0.8	21.1 ± 0.1
Ywhag	24.8 ± 0.8	24.4 ± 0.8	24.8 ± 0.8
Tfrc	27.2 ± 0.9	27.8 ± 1.1	26.8 ± 0.4
Cck	32.8 ± 1.4	32.0 ± 1.3	33.3 ± 1.7
Duodenum			
Rn18s	24.7 ± 0.5	24.5 ± 0.3	25.0 ± 0.7
Ubc	24.9 ± 0.5	24.8 ± 0.6	25.1 ± 0.8
Tfrc	28.5 ± 0.7	28.5 ± 0.5	28.9 ± 1.1
Ppia	23.1 ± 0.6	23.1 ± 0.3	23.3 ± 0.9
Ppib	24.9 ± 0.7	24.9 ± 0.6	25.0 ± 1.0
Jejunum			
Cck	33.3 ± 1.2	33.3 ± 0.6	33.7 ± 1.4
Gusb	27.5 ± 1.1	28.3 ± 0.7	27.1 ± 0.4
Ppia	24.5 ± 1.0	25.1 ± 0.3	24.5 ± 0.9
Hprt	30.9 ± 1.3	31.7 ± 0.4	30.8 ± 1.2
Tfrc	24.9 ± 1.1	24.7 ± 0.7	25.2 ± 0.8
Ileum			
Rn18s	27.7 ± 1.2	27.3 ± 0.9	27.0 ± 1.3
Ubc	23.9 ± 1.1	23.4 ± 0.7	24.4 ± 1.4
Tbp	25.9 ± 1.4	25.6 ± 1.5	25.8 ± 1.3
Hprt	29.8 ± 1.6	29.1 ± 1.1	29.9 ± 2.4
Ppib	28.7 ± 1.6	28.1 ± 1.0	28.9 ± 2.4

Data expressed as Cq are means ± SD for each group. Statistical analysis to evaluate dietary response was done using one-way ANOVA and the Mann Whitney's U as post hoc test. [a], $P < 0.05$ vs control.

Table 7. Hepatic gene expression of 17 commonly used reference genes in Zucker (fa/fa) rats fed a hypocaloric diet organized according to their biological coefficient of variation obtained by RT-qPCR.

	Global				Control		Diet	
Gene symbol	Mean Cq	SD	CV (%)	Rank	CV (%)	Rank	CV (%)	Rank
Tbp	26.5	0.6	2.4	1	3.8	7	5.2	8
Ywhag	24.2	0.6	2.6	2	3.4	3	5.8	9
Tfrc	25.4	0.7	2.7	3	3.9	9	5.9	10
Cck	32.5	0.9	2.7	4	3.0	2	3.0	2
Hmbs	28.1	0.8	2.8	5	3.9	10	2.7	1
Ubc	20.8	0.6	2.9	6	2.1	1	4.6	6
Gusb	21.4	0.6	3.0	7	3.8	8	9.9	16
Hprt	27.6	0.8	3.0	8	4.4	15	4.6	5
Actb	23.5	0.7	3.1	9	4.3	14	5.9	11
Ppib	26.3	0.8	3.2	10	3.6	6	4.3	4
Arbp	26.1	0.8	3.2	11	3.9	11	7.1	14
Rplp2	23.4	0.8	3.3	12	3.4	4	8.4	15
Ppia	23.4	0.8	3.4	13	4.3	13	6.1	12
Pgk1	26.2	0.9	3.6	14	3.5	5	4.7	7
B2m	23.6	1.0	4.1	15	5.3	16	6.8	13
Rn18s	23.9	1.0	4.1	16	4.3	12	3.3	3
Gapdh	23.2	1.0	4.4	17	5.8	17	11.3	17

Table 8. Hepatic gene expression of top five reference genes selected according to their biological coefficient of variation obtained by RT-qPCR in Zucker (fa/fa) rats fed a hypocaloric diet

Gene symbol	Control	Diet
Tbp	26.4 ± 1.0	27.1 ± 1.4
Ywhag	24.1 ± 0.8	24.7 ± 1.4
Tfrc	25.4 ± 1.0	25.9 ± 1.5
Cck	32.5 ± 1.0	32.4 ± 1.0
Hmbs	28.1 ± 1.1	28.3 ± 0.8

Data expressed as Cq are means ± SD for each group. Statistical analysis to evaluate dietary response was done using one-way ANOVA and the Mann Whitney's U as post hoc test.

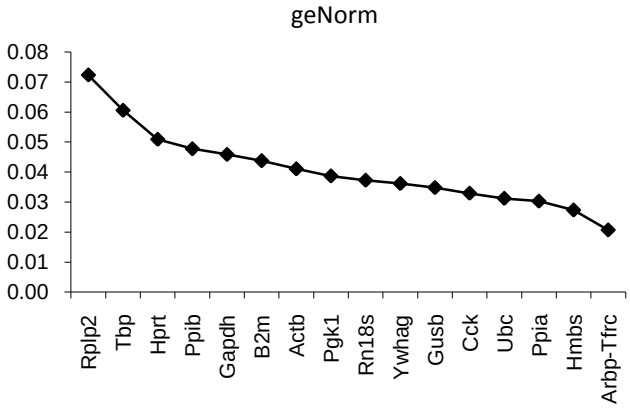
Figure 1. Gene stability according to different procedures. The geNorm and NormFinder softwares were used to calculate the most stable genes among the 17 genes in four different organs of Wistar rats after a fat gavage. The most stable genes are those with the lowest stability values.

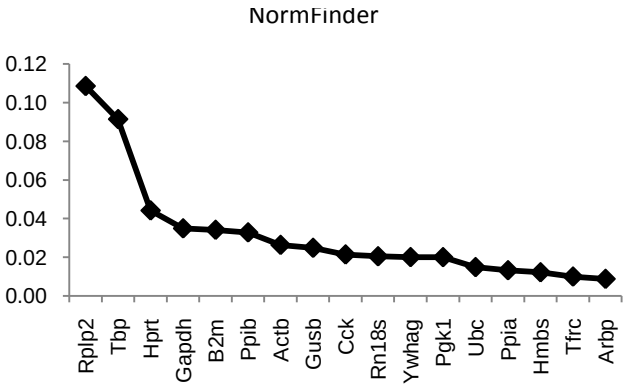
Figure 2. Gene stability ranking order for selected reference genes using BestKeeper. The 17 genes in four different organs of Wistar rats given a fat gavage were ranked according to data obtained with this software. The most stable genes are those with the lowest stability values.

Figure 3. Gene stability according to different procedures. The geNorm (A), NormFinder (B) and BestKeeper (C) softwares were used to calculate the most stable genes among the 17 genes in livers of control and following a hypocaloric diet Zucker rats. The most stable genes are those with the lowest stability values.

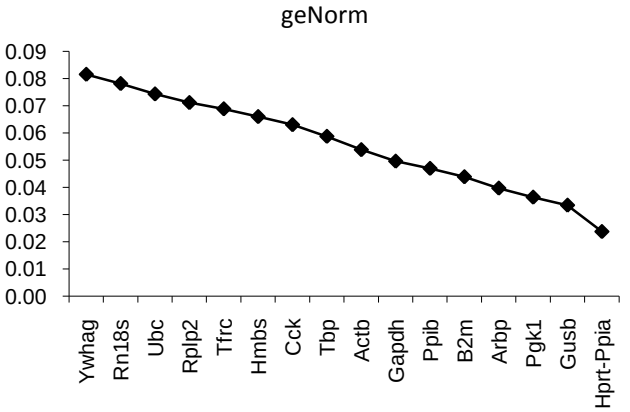
Figure 4. Influence of normalisation with different reference genes in rat. Hepatic *Slc34a2* mRNA expression referred to that of *Ppib* (A), *Hprt* + *Ppia* (B), *Cck* (C) and *Rn18s* (D) in control and 4 and 8 hours after a fat gavage in Wistar rats. Hepatic *Apoa1* mRNA expression referred to that of *Gapdh* (E), *Hprt* + *Actb* (F), *Tbp* (G) and *Rn18s* (H) in control and following a hypocaloric diet Zucker (fa/fa) rats. Data are means ± SD. Statistical analysis to evaluate dietary response was done using one-way ANOVA and the Mann Whitney's U as post hoc test. [a], $P < 0.05$ vs control.

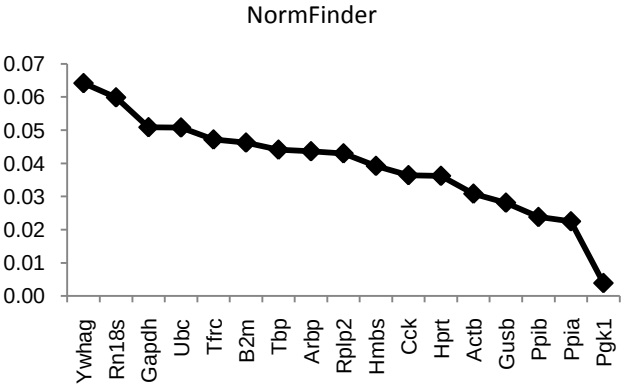
Duodenum





Jejunum





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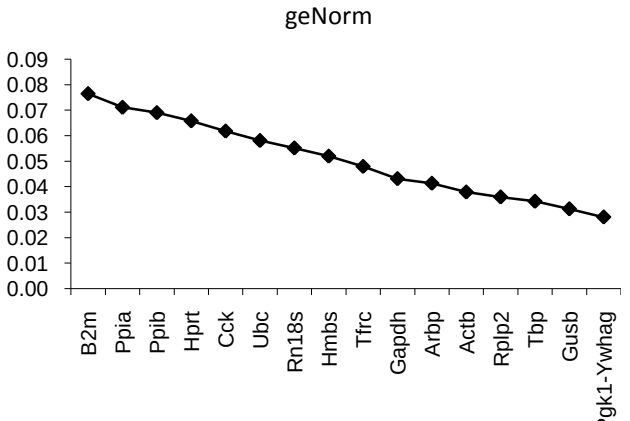
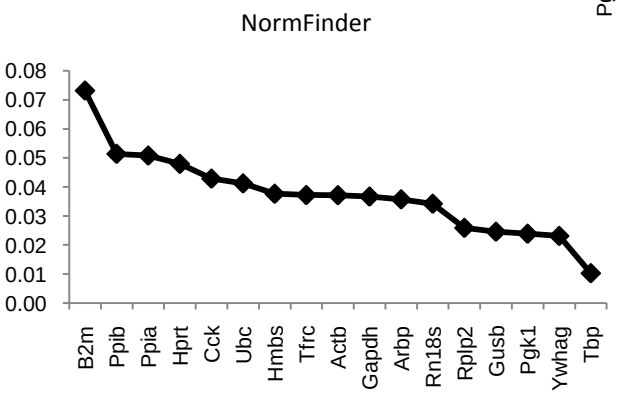
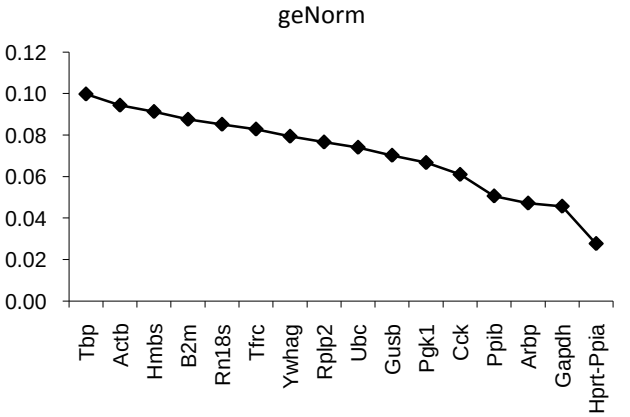
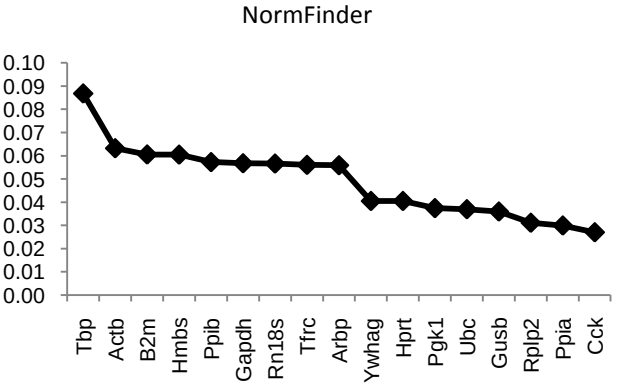


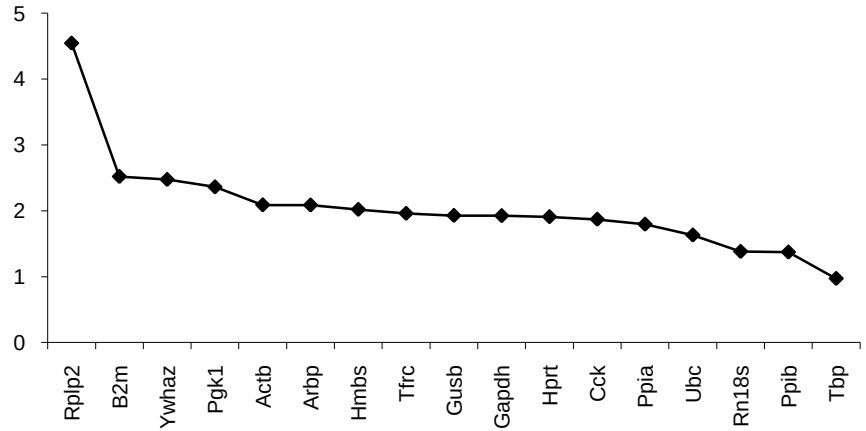


Liver

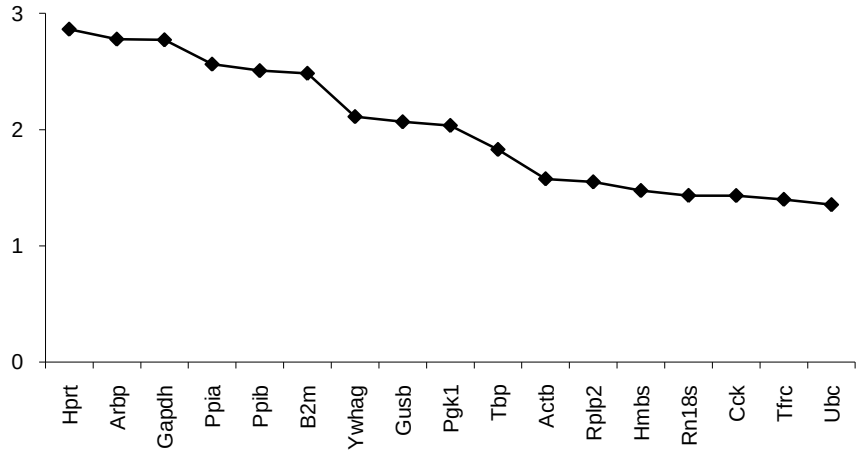




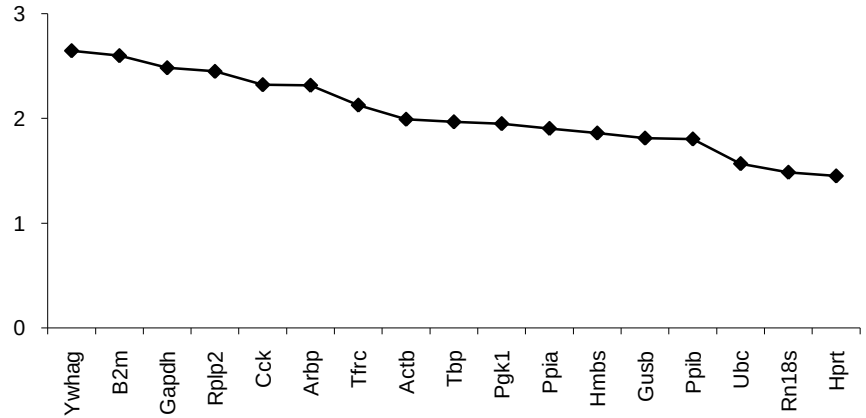
Duodenum



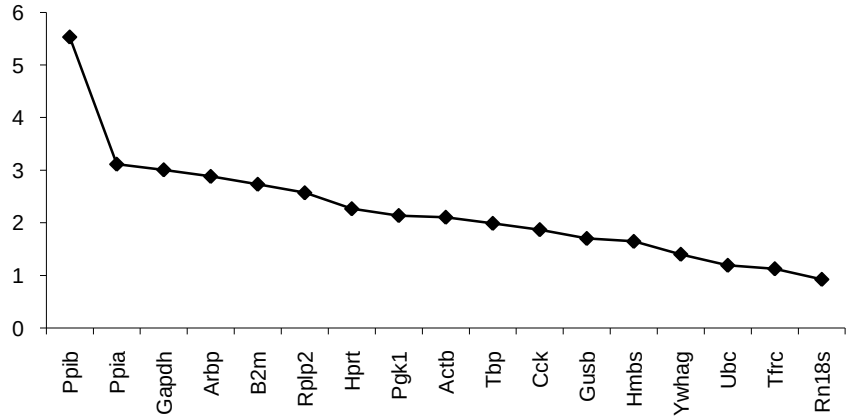
Jejunum



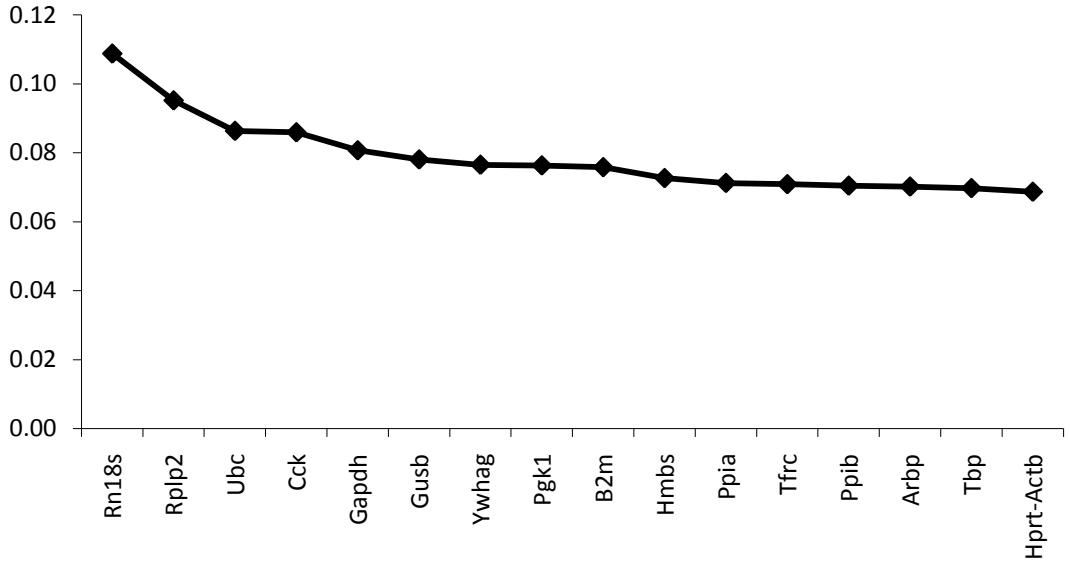
Ileum



Liver



A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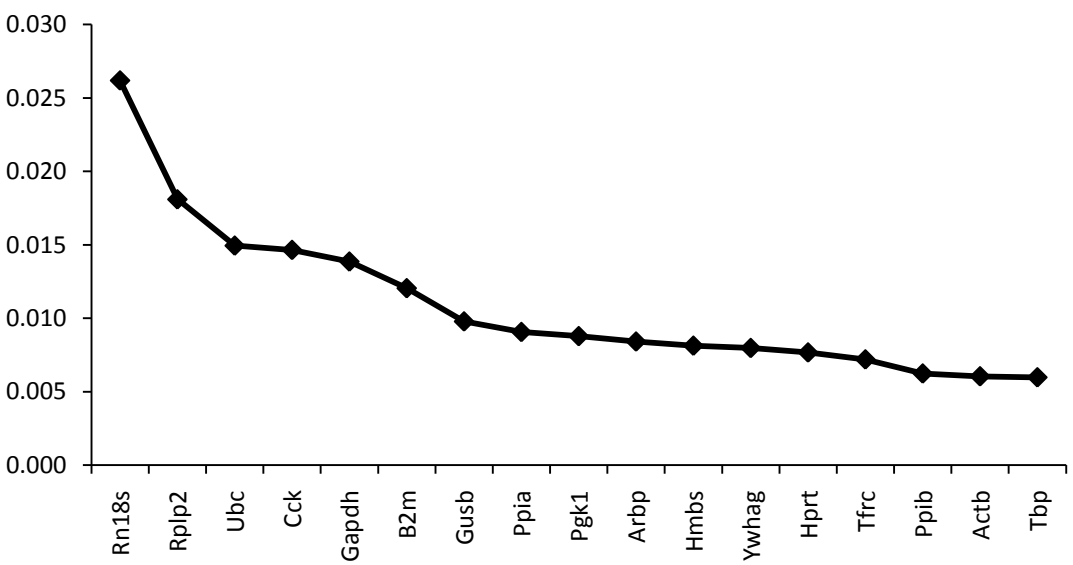
0.10
0.08
0.06
0.04
0.02
0.00
Rn18s
Rplp2
Ubc
Cck
Gapdh
Gusb
Ywhag
Pgk1
B2m
Hmbs
Ppia
Tfrc
Ppib
Arbp
Tbp
Hprt-Actb

B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0.025
0.020
0.015
0.010
0.005
0.000
Rn18s
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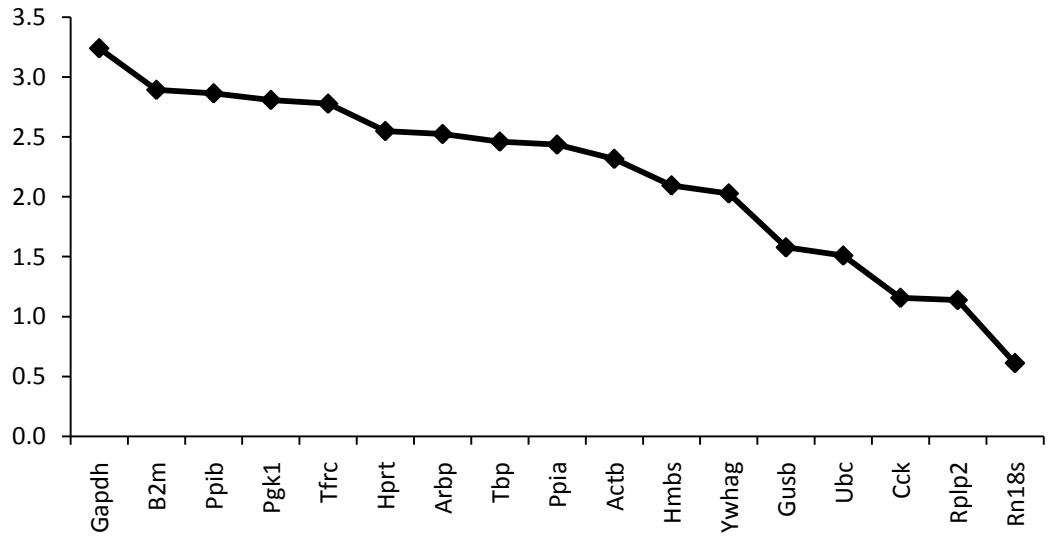
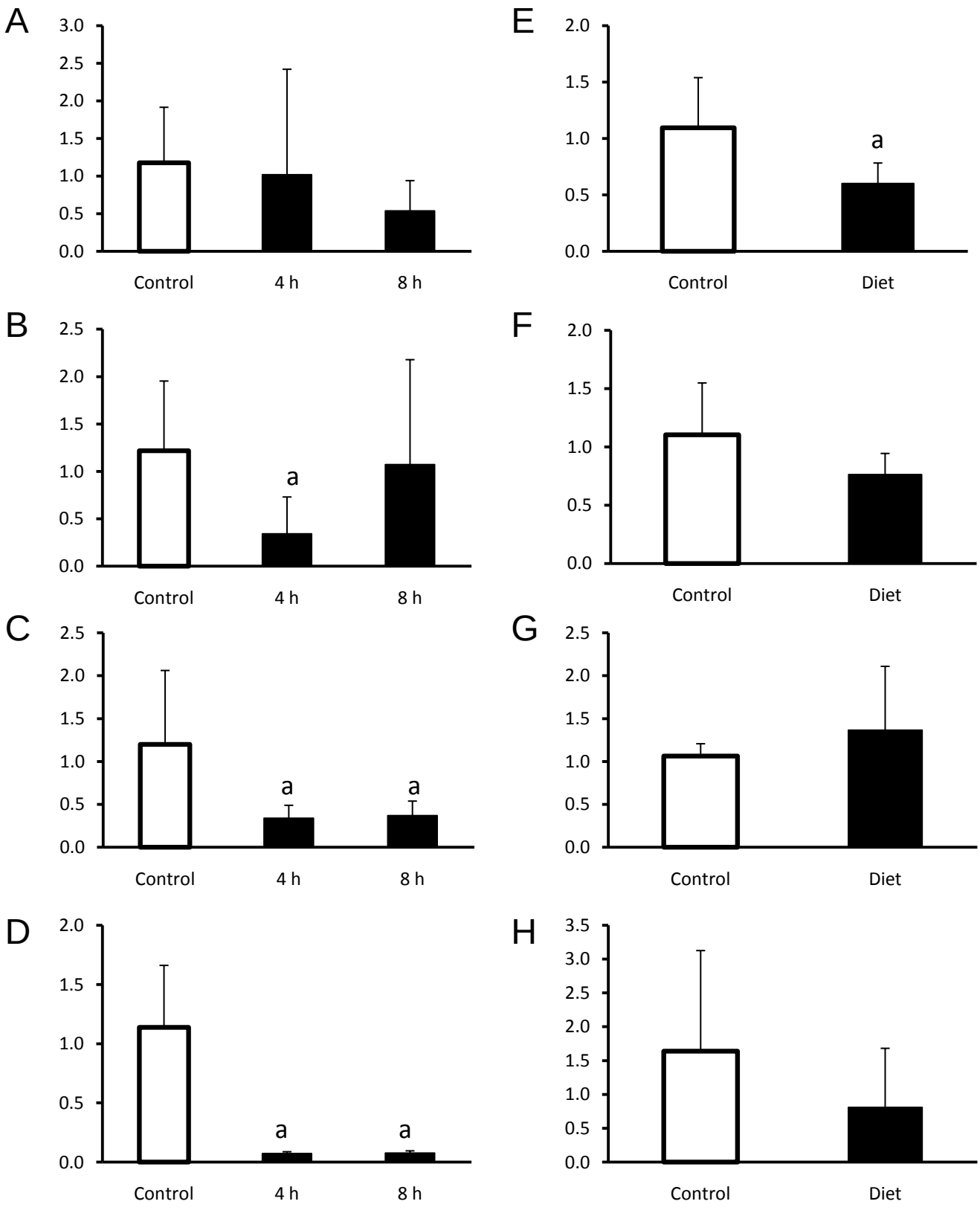
Ubc
Cck
Gapdh
B2m
Gusb
Ppia
Pgk1
Arbp
Hmbs
Ywhag
Hprt
Tfrc
Ppib
Actb
Tbp

C


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Gapdh
B2m
Ppib
Pgk1
Tfrc
Hprt
Arbp
Tbp
Ppia
Actb
Hmbs
Ywhag
Gusb
Ubc
Cck
Rplp2
Rn18s


A
3.0
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2.0
1.5
1.0
0.5
0.0
Control
4 h
8 h
B
2.5
2.0
1.5
1.0
0.5
0.0
a
Control
4 h
8 h
C
2.5
2.0
1.5
1.0
0.5
0.0
a
a
Control
4 h
8 h
D
2.0
1.5
1.0
0.5
0.0
a
a
Control
4 h
8 h
E
2.0
1.5
1.0
0.5
0.0
a
Control
Diet
F
2.0
1.5
1.0
0.5
0.0
Control
Diet
G
2.5
2.0
1.5
1.0
0.5
0.0
Control
Diet
H
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Diet