Placenta (2005), **26**, 601–607
doi:10.1016/j.placenta.2004.09.009

# Evaluation of Housekeeping Genes in Placental Comparative Expression Studies

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Paper accepted 20 September 2004

Preeclampsia and diabetes are complications of pregnancy that contribute to maternal and perinatal mortality worldwide. Results emerging from molecular studies of placentae may elucidate etiologically important genomic alterations. Appropriate application of real time reverse transcription (RT) PCR in comparative gene expression studies requires endogenous housekeeping genes to normalize between sample variations. Ideal housekeeping genes must have stable tissue expression, but few have been specifically studied in the placenta. We sought to identify candidate control genes by analyzing seven functionally distinct housekeeping genes (B2M, GAPDH, HMBS, HPRT, SDHA, TBP, YWHAZ) for their expression stability and level in the placenta. mRNA isolated from 20 placentae was analyzed for gene expression using RT-PCR. Expression stability ($M$) was assessed using normalization strategies previously used for other tissues. TBP and SDHA were the most stable, with an average expression stability of $M = 0.43$, followed by YWHAZ ($M = 0.44$) > HPRT ($M = 0.53$) > HMBS ($M = 0.57$) > GAPDH ($M = 0.61$) > B2M ($M = 0.69$). The genes tested ranged in abundance, with an approximately 300-fold increase from the lowest (HMBS) to the highest (B2M). By using TBP, SDHA and YWHAZ, with greater expression stability than those housekeeping genes commonly used in placenta studies, gene expression profile comparisons will have more sensitivity and specificity.
Placenta (2005), **26,** 601–607 

**Keywords:** Real time PCR; mRNA expression; Endogenous housekeeping genes; Internal standards; Placenta

## INTRODUCTION

Recent studies on the placenta establish that regulation of specific genes in this tissue may be related to gestational disorders such as preeclampsia and gestational diabetes [1,2]. These changes in gene expression may enable investigators to advance the knowledge of the pathophysiology of disorders in the placenta. Additionally, monitoring of gene expression changes may identify new candidate biomarkers, which might expedite disease prediction earlier in the pregnancy than current methods allow. By measuring RNA expression in the placenta, we can determine whether certain genes are specifically transcribed in that tissue and how their regulation relates to abnormal gestational effects.

Comparison of RNA samples by controlling the amount of total RNA put into each reaction is time consuming to validate, requires accurate quantification of the RNA sample and is complicated by many physiologically irrelevant sources of variation [3]. Endogenous controls, usually housekeeping genes, are measured to better normalize between samples. Reporting the amount of a particular gene of interest relative to a housekeeping gene helps to avoid sample-to-sample variation [4].

RNA has traditionally been analyzed by assays such as Northern blots or RNase protection assays which can determine relative amounts of RNA between samples [5]. However, RNA measurement by real time PCR and microarray analysis can show subtle changes in relative quantities for larger numbers of genes and consume smaller amounts of each sample [6]. In order to take advantage of this sensitivity, variation between samples must be quantified, accounted for and minimized whenever possible. If the choice for controls is not based on the most stable genes in the placenta, but genes that have been traditionally used in other tissues, then results may be spurious. For this purpose, endogenous control genes must be empirically evaluated for stability between many types of placenta samples, including samples from complicated pregnancies.

In this study we compared seven housekeeping genes in the placenta by real time PCR. The seven genes evaluated were: β-2 microglobulin (B2M), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hydroxymethyl-bilane synthase (HMBS), Hypoxanthine phosphoribosyl-transferase I (HPRT), Succinate dehydrogenase complex, subunit A (SDHA), TATA

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box binding protein (TBP) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ). To account for possible differences in tissues, samples were taken from uncomplicated and complicated pregnancies as well as from the maternal and fetal sides of the organ. Comparisons of gene expression stability were made by analyzing results using procedures previously described for neuroblastoma, fibroblast, leukocyte, bone marrow and normal pool tissues [7]. Finally, to identify which endogenous control genes express at levels in the dynamic range of potential genes of interest, we assessed the relative overall levels of expression of these seven genes in the placenta.

## MATERIALS AND METHODS

### Collection of placentae

The procedures used in this study were in agreement with the protocol approved by the Institutional Review Board of Swedish Medical Center. All participants provided written informed consent. Unselected placentae from 20 uncomplicated and complicated pregnancies that included diabetes and hypertension were collected at delivery. Gestational age was determined by last menstrual period and confirmed by first trimester ultrasound. The population included women with insulin-dependent diabetes mellitus (IDDM) and gestational diabetes mellitus (GDM) diagnosed according to criteria advocated by the American Diabetes Association (ADA) [8]. Pregnancy induced hypertension (PIH) was defined according to criteria set forth by the American College of Obstetricians and Gynecologists (ACOG) in 1996 [9].

The placental tissue was collected immediately after delivery. The chorionic plate, including overlying membranes, was removed and tissue biopsies were taken from the villous tissue, which consists of the intervillous tissues and chorionic villi on the fetal side, and decidual tissue, which consists of the decidua basalis and the utero placental arteries on the maternal side. Both villous tissue and decidual tissue biopsies were taken from each of the 20 placentae collected. Biopsies of approximately 0.5 $cm^3$ were taken from a lateral position, approximately one-third the distance from the placental edge. Biopsies were placed in cryotubes containing RNAlater (Qiagen Inc., Valencia, CA) at 10 µl per 1 mg of tissue and stored at −80 °C.

### RNA extraction

Thirty milligrams of tissue from stored biopsies was cut and weighed. The weighed samples were homogenized using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Total RNA was extracted by using the RNeasy Fibrous Mini Kit (Qiagen Inc.), following the standard protocol including DNase and with increased incubations at the RW1 and RPE buffer steps to 5 min to decrease protein contamination. Total RNA was eluted from the columns in 50 µl of sterile water. Total RNA concentration was calculated by determining absorbance at 260 nm (Spectramax Plus 384 spectrophotometer, Molecular Devices, Sunnyvale, CA). Protein contamination was monitored by $A_{260}/A_{280}$ ratio. All samples ranged in concentration from 0.33 µg/µl to 1.10 µg/µl and had $A_{260}/A_{280}$ ratio of $>1.8$. They were diluted to 0.25 µg/µl in sterile water and aliquoted for storage at −80 °C.

### Reverse transcription and real time PCR

The seven housekeeping genes tested in this study were chosen based on their distinct gene functions (for a summary of gene function, see Vandesompele et al. [7]). The seven genes are: B2M, GAPDH, HMBS, HPRT, SDHA, TBP and YWHAZ. Leptin, an adipocytokine known to express at high levels in the placenta and to show up to 3-fold changes in mRNA expression in diabetic placentas compared to controls [10], was also tested to compare gene expression analysis. First strand cDNA was synthesized by using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Amounts of 2.0 µg or less of input total RNA were run in 25 µl reactions and diluted in sterile water to 5.0 ng/µl cDNA. Real time PCR was performed in duplicate on 25 µl mixtures, containing 50 ng of template cDNA, 12.5 µl of 2× Taqman Universal Master Mix (Applied Biosystems), and 1.25 µl of Taqman Gene Expression Assays (Applied Biosystems), see Table 1. These assays use the

**Table 1**. Gene expression assays used in this manuscript

| Gene symbol | Gene name | ABI catalog number | Accession number | Approximate probe nucleotide location |
|---|---|---|---|---|
| B2M | β-2 Microglobulin | Hs00187842 | NM_004048 | 128 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | Hs99999905 | NM_002046 | 130 |
| HMBS | Hydroxymethyl-bilane synthase | Hs00609297 | NM_000190 | 183 |
| HPRT | Hypoxanthine phosphoribosyl-transferase I | Hs99999909 | NM_000194 | 568 |
| SDHA | Succinate dehydrogenase complex, subunit A | Hs00188166 | NM_004168 | 796 |
| TBP | TATA box binding protein | Hs99999910 | M34960 | 738 |
| YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | Hs00237047 | NM_003406 | 75 |
| LEP | Leptin | Hs00174877 | NM_000230 | 201 |

All assays used were designed such that their probe spans an exon–exon junction and therefore will not detect genomic DNA. ABI = Applied Biosystems (Foster City, California).

5′ nuclease ability of Taq polymerase to disrupt the bound probe and release the dye, which can be quantified for gene expression. Since probe binding is an added level of specificity, this improves real time PCR detection over detection with SYBR green dye by eliminating false positives. Each assay is supplied as a 20× mix of forward primer, reverse primer and minor groove binding FAM probe with a dark quencher. Each assay is functionally tested on pooled human cDNA and on human genomic DNA to exclude genomic DNA detection.

Reactions were run on an ABI PRISM 7000 Real Time PCR machine (Applied Biosystems) using the default cycling conditions of 50 °C, 2 min; 95 °C, 10 min; followed by 40 cycles of 95 °C, 15 s and 60 °C, 1 min. Four point standard curves of Human Universal Total RNA Standard (BD Biosciences/Clontech) were used for primer efficiency comparison of all Taqman Gene Expression Assays based on the slope of each standard curve calculated by the ABI PRISM 7000 SDS Software, version 1.1. Briefly, primer efficiency was determined by the equation:

$$\text{Primer efficiency} = 10^{(-1/\text{slope})} - 1$$

Data were then transformed into amplification efficiency by adding 1 to the primer efficiency. For example: when primer efficiency is 100%, then amplification efficiency is 2 ($2 = 1 + 1$), but if primer efficiency is 95%, then amplification efficiency is 1.95 ($1.95 = 1 + 0.95$). All raw expression data presented in this paper are corrected by the amplification efficiency.

A control of Human Universal total RNA for assay-to-assay variability was run for every cDNA reaction and run on every real time PCR plate. ΔCt values were determined for each gene by subtracting the Ct value from the geometric mean of the Ct values for SDHA, TBP and YWHAZ run on the same plate. Inter-assay plate variation was $<1.0$ΔCt for every gene tested.

### Data analysis

Results from the ABI PRISM 7000 SDS Software, using the Absolute Quantitation method with auto thresholds and baselines, were exported into Excel (Microsoft Corporation, Redmond, WA) for data analysis. Only one of the Ct values of the duplicates differed by a $>0.5$ standard deviation. This sample was re-tested. Ct value duplicates differing by $\leq 0.5$ standard deviations were averaged.

For analysis of gene stability we used the geNorm application, a Microsoft Excel program available at http://medgen.ugent.be/~jvdesomp/genorm/. The premise of this program is that ratios between constantly expressed, non-normalized housekeeping genes should remain regular. To show this, $M$, the average pair-wise variation of a single endogenous control gene was calculated from the raw expression data. The lowest $M$ value is the most stable, so the gene with the highest instability (highest $M$ value) is removed at each step. A new $M$ value for each remaining gene is calculated until only two genes remain. Since these calculations are based on ratios, the final two genes cannot be resolved from each other. Normalization factors were calculated using the geometric mean to control for changes in relative gene expression and outlying values. Pair-wise variation ($V_{n/n+1}$) was calculated by geNorm between two sequential normalization factors for all the samples [7].

Pearson's correlation coefficients were calculated to further determine gene stability. Since control genes were tested and there were no internal controls with which to compare them, it was assumed that high pair-wise variability between samples, shown by low correlation, reflects low expression stability. Therefore, the highest significant pairing was reported as the most stable. This approach has been used by other investigators [11].

## RESULTS

The relative gene expression values (Table 2) were calculated using the comparative Ct method according to the geNorm Manual (version 1.4, November 2003) and run on the geNorm program [7]. The seven genes were ordered according to stability rank by using this program (Figure 1). Since the program eliminated the most unstable gene and recalculated new $M$ values for the remaining genes, the top two genes could not be further compared. TBP and SDHA showed the highest stability ($M = 0.43$), followed closely by YWHAZ ($M = 0.44$). The remaining genes were ordered from most stable to least stable: HPRT ($M = 0.57$), HMBS ($M = 0.57$), GAPDH ($M = 0.61$) and B2M ($M = 0.69$). Analysis of pair-wise correlation of the raw relative expression data showed TBP and YWHAZ to be the most significant pair ($r = 0.902$; $P < 0.001$), followed closely by YWHAZ and SDHA ($r = 0.901$; $P < 0.001$) and SDHA and TBP ($r = 0.868$; $P < 0.001$).

We sought to evaluate the extent to which results differed according to the diagnosis of the placenta or the site of tissue biopsy. When hypertensive and diabetic placentas were excluded from the analysis, SDHA, TBP and YWHAZ remained the most stable (Table 3, control alone). Pair-wise coefficient analysis shows similar results, with the highest coefficients for TBP and YWHAZ ($r = 0.96$; $P < 0.001$), SDHA and YWHAZ ($r = 0.95$; $P < 0.001$), and SDHA and TBP ($r = 0.93$; $P < 0.001$). When tissue from the maternal side—decidual tissue—or from the fetal surface—villous tissue—were compared, similar results were seen, regardless of mRNA source (Table 3, Maternal and fetal). However, we did notice a minor shuffling of the rank order of these three genes according to stability. Pair-wise coefficient comparisons of separate villous and decidual tissues showed similar results. RNA expression from decidual tissue samples had the highest coefficients for TBP and YWHAZ ($r = 0.91$; $P < 0.001$), SDHA and YWHAZ ($r = 0.89$; $P < 0.001$), and SDHA and TBP ($r = 0.84$; $P < 0.001$). Similarly, corresponding RNA expression for villous tissue had the highest coefficients for SDHA and YWHAZ ($r = 0.92$; $P > 0.001$), SDHA and TBP

**Table 2**. Raw relative expression levels of seven genes from 40 placental RNA samples by real time PCR

| Sample | Tissue | GAPDH | B2M | HPRT | YWHAZ | TBP | HMBS | SDHA |
|---|---|---|---|---|---|---|---|---|
| 1 | Decidual | 0.71 | 0.14 | 0.50 | 0.79 | 0.62 | 0.38 | 0.88 |
| 2 | Villous | 0.80 | 0.23 | 0.91 | 1.00 | 1.00 | 0.55 | 1.00 |
| 3 | Decidual | 0.80 | 0.20 | 0.94 | 0.98 | 0.91 | 1.00 | 0.79 |
| 4 | Villous | 0.64 | 0.27 | 0.90 | 0.53 | 0.56 | 0.76 | 0.59 |
| 5 | Decidual | 0.13 | 0.11 | 0.73 | 0.22 | 0.37 | 0.34 | 0.34 |
| 6 | Villous | 0.14 | 0.07 | 0.75 | 0.18 | 0.33 | 0.43 | 0.23 |
| 7 | Decidual | 0.43 | 0.46 | 1.00 | 0.28 | 0.51 | 0.39 | 0.31 |
| 8 | Villous | 0.34 | 0.16 | 0.61 | 0.24 | 0.48 | 0.30 | 0.32 |
| 9 | Decidual | 0.26 | 0.43 | 0.65 | 0.23 | 0.32 | 0.14 | 0.50 |
| 10 | Villous | 0.19 | 0.10 | 0.39 | 0.24 | 0.32 | 0.11 | 0.36 |
| 11 | Decidual | 0.15 | 0.09 | 0.45 | 0.13 | 0.20 | 0.12 | 0.23 |
| 12 | Villous | 0.27 | 0.12 | 0.51 | 0.21 | 0.34 | 0.12 | 0.35 |
| 13 | Decidual | 0.16 | 0.09 | 0.35 | 0.13 | 0.13 | 0.11 | 0.22 |
| 14 | Villous | 0.39 | 0.14 | 0.47 | 0.26 | 0.25 | 0.14 | 0.30 |
| 15 | Decidual | 0.21 | 0.11 | 0.38 | 0.21 | 0.31 | 0.11 | 0.29 |
| 16 | Villous | 0.14 | 0.10 | 0.32 | 0.12 | 0.19 | 0.08 | 0.22 |
| 17 | Decidual | 0.51 | 0.72 | 0.93 | 0.20 | 0.17 | 0.15 | 0.38 |
| 18 | Villous | 0.66 | 0.13 | 0.34 | 0.25 | 0.21 | 0.09 | 0.44 |
| 19 | Decidual | 0.24 | 0.08 | 0.40 | 0.26 | 0.29 | 0.24 | 0.34 |
| 20 | Villous | 0.30 | 0.11 | 0.44 | 0.22 | 0.26 | 0.14 | 0.31 |
| 21 | Decidual | 0.22 | 0.17 | 0.44 | 0.25 | 0.18 | 0.14 | 0.23 |
| 22 | Villous | 0.32 | 0.18 | 0.45 | 0.36 | 0.24 | 0.14 | 0.30 |
| 23 | Decidual | 0.18 | 0.09 | 0.33 | 0.41 | 0.38 | 0.20 | 0.35 |
| 24 | Villous | 0.22 | 0.12 | 0.34 | 0.38 | 0.37 | 0.19 | 0.33 |
| 25 | Decidual | 0.24 | 0.11 | 0.38 | 0.38 | 0.31 | 0.18 | 0.35 |
| 26 | Villous | 0.18 | 0.13 | 0.34 | 0.33 | 0.24 | 0.17 | 0.25 |
| 27 | Decidual | 0.30 | 0.45 | 0.52 | 0.31 | 0.31 | 0.11 | 0.35 |
| 28 | Villous | 0.50 | 1.00 | 0.84 | 0.33 | 0.39 | 0.14 | 0.45 |
| 29 | Decidual | 0.16 | 0.11 | 0.25 | 0.17 | 0.12 | 0.07 | 0.21 |
| 30 | Villous | 0.15 | 0.10 | 0.27 | 0.20 | 0.19 | 0.08 | 0.24 |
| 31 | Decidual | 0.48 | 0.18 | 0.46 | 0.27 | 0.21 | 0.20 | 0.26 |
| 32 | Villous | 0.34 | 0.09 | 0.33 | 0.24 | 0.21 | 0.15 | 0.19 |
| 33 | Decidual | 0.23 | 0.10 | 0.33 | 0.22 | 0.19 | 0.16 | 0.19 |
| 34 | Villous | 0.14 | 0.05 | 0.22 | 0.20 | 0.22 | 0.08 | 0.37 |
| 35 | Decidual | 0.28 | 0.10 | 0.35 | 0.13 | 0.18 | 0.13 | 0.22 |
| 36 | Villous | 0.25 | 0.12 | 0.31 | 0.14 | 0.18 | 0.10 | 0.23 |
| 37 | Decidual | 0.37 | 0.18 | 0.59 | 0.15 | 0.12 | 0.18 | 0.31 |
| 38 | Villous | 1.00 | 0.25 | 0.96 | 0.45 | 0.34 | 0.28 | 0.44 |
| 39 | Decidual | 0.32 | 0.12 | 0.64 | 0.21 | 0.27 | 0.25 | 0.37 |
| 40 | Villous | 0.30 | 0.09 | 0.52 | 0.14 | 0.21 | 0.19 | 0.27 |

Quantities were calculated according to the geNorm manual (version 1.4) by the comparative Ct method. Decidual = sampled from the maternal side of the placenta, Villous = sampled from the fetal side of the placenta.

($r = 0.90$; $P > 0.001$), and TBP and YWHAZ ($r = 0.89$; $P < 0.001$).

Next we evaluated the number of genes required for normalization to determine accurate expression levels (Figure 2). To determine if the benefit of using additional $(n + 1)$ control genes outweighed the cost, pair-wise variation ($V_{n/n+1}$) was calculated between consecutively ranked normalization factors [7]. Since there are no significant changes between $V_{2/3}$ (0.13) and $V_{3/4}$ (0.14) in our samples, two genes could potentially be used. However, use of a third gene is suggested by Vandesompele et al. [7] for increased accuracy and reliability of normalization.

Next we compared the gene expression profiles of a gene of interest when it was normalized to the three most stable housekeeping genes, SDHA, TBP and YWHAZ, and the most commonly used housekeeping gene in expression studies, GAPDH. To do this, we evaluated the relative expression of the leptin gene. Normalization of leptin with the geometric mean of the three housekeeping genes (SDHA, TBP and YWHAZ) was compared to leptin normalization with GAPDH. Difference in normalized gene expression of up to 500% was seen in the 40 samples tested (standard deviation (SD) = 1.20). Normalization of the same samples with one of the three genes (SDHA, TBP or YWHAZ) alone compared to the geometric mean of all three resulted in change of up to 50% (SDHA SD = 0.14, TBP SD = 0.15, YWHAZ SD = 0.16). When the geometric mean of two genes were compared to three, the difference was reduced to 30% (SDHA and TBP

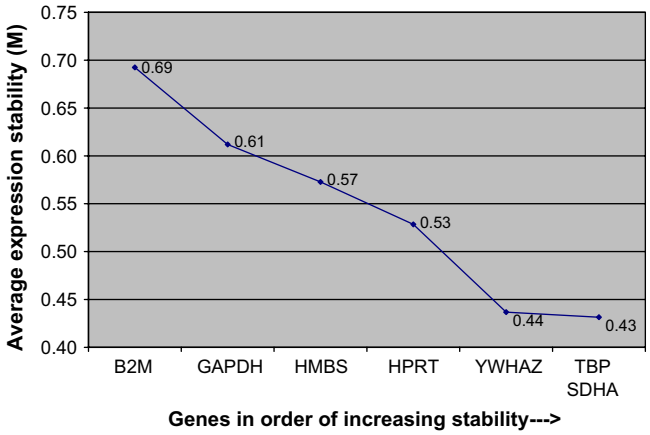


**Figure 1.** Stability ranking of control genes tested. The expression stability value (*M*) was determined using the geNorm program. After the removal of the least stable control gene, the *M* value for the remaining housekeeping genes was computed during six total rounds of calculations. Genes are ranked from left to right in order of increasing expression stability, which is indicated by lower *M* values.

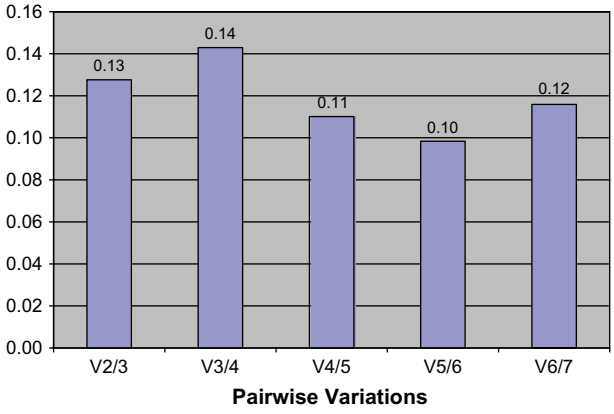


**Figure 2.** Graph of optimal control gene number. Pair-wise variation ($V_{n/n+1}$) was calculated (see Methods) to determine the least number of control genes necessary for accurate normalization.

SD = 0.08, SDHA and YWHAZ SD = 0.06, TBP and YWHAZ SD = 0.09).

Finally, we measured the relative gene expression of all seven housekeeping genes in the placenta (Figure 3). The abundance of these genes ranged from approximately 300-fold difference from the highest expressing gene (B2M) to the lowest expressing gene (HMBS). The three most stable genes in the placenta, YWHAZ, SDHA and TBP, all expressed at the same level, approximately 4-fold that of HMBS, the lowest expressing gene in the placenta of the seven genes tested.

## DISCUSSION

Selection of control genes for quantitative real time PCR is based on the ability of the gene to maintain a constant level of expression between samples, regardless of the conditions. However, control genes, particularly for placental studies, are often selected on the basis of observations from research on other tissues. Testing of commonly used endogenous control genes such as GAPDH and β-actin show that their level of gene expression is affected by a number of conditions [12].

**Table 3**. Comparison of average expression stability (*M*) for maternal, fetal and combined tissues

| | B2M | GAPDH | HMBS | HPRT | YWHAZ | TBP | SDHA |
|---|---|---|---|---|---|---|---|
| All samples | 0.69 | 0.61 | 0.57 | 0.53 | 0.44 | 0.43 | 0.43 |
| Decidual alone | 0.72 | 0.58 | 0.52 | 0.61 | 0.43 | 0.43 | 0.46 |
| Villous alone | 0.66 | 0.61 | 0.56 | 0.48 | 0.42 | 0.38 | 0.38 |
| Controls alone | 0.64 | 0.67 | 0.56 | 0.60 | 0.44 | 0.42 | 0.42 |

As in Figure 1, the M value was recalculated at every step after the stepwise exclusion of the least stable gene.

Notably, hypoxia [13,14] and dysregulation of growth factors [15,16] are of etiologic and pathophysiologic importance in preeclampsia and GDM, two relatively common complications of pregnancy with placental involvement. 5-Aminolevulinate synthase (ALAS) is a heme biosynthetic enzyme used commonly as a housekeeping gene. However, it is up-regulated by insulin [17], hence, its use as a control gene in studies of GDM and preeclampsia is contraindicated. This effect is also seen with GAPDH [18]. Additionally, GAPDH has been shown to be involved in other functions aside from basal cell metabolism [19], which may affect its level of expression in stressed tissues. During pregnancy, the placenta is a developing tissue that undergoes many different endocrine and immune system changes, which vary depending on the health of the mother, the fetus and the implantation of the placenta itself. Therefore, it is likely that the effects on these genes in the placenta may be influenced during pregnancy. For these reasons we tested GAPDH and B2M, another commonly used housekeeping gene, in this study. In addition, we assessed five other control genes used in studies of comparative expression

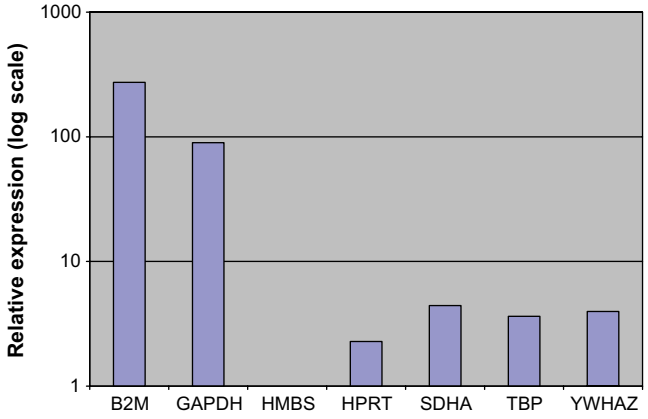


**Figure 3.** Graph of relative expression of control genes in the placenta. Geometric means of all samples were normalized to the geometric mean of SDHA, TBP and YWHAZ, and then relative expression was calculated using the comparative Ct method.

from those evaluated in a variety of tissues including neuroblastoma, fibroblast, leukocyte and bone marrow [7] to have additional levels of stability comparison. They were selected based on functional class, so that they were not co-regulated and therefore do not show a false level of stability compared to each other. Overall, we found that B2M was the least stable of the seven genes tested in the placenta, followed by GAPDH. Our data suggest that three other genes, SDHA, TBP and YWHAZ, may be good candidates for endogenous control genes in placental studies. The steep drop in instability as measured by *M* value (Figure 2), as the less stable genes were removed in sequential rounds of unstable gene elimination, shows the increased stability of these three genes over the excluded genes.

Since both the villous and decidual sides of the placenta may be exposed to different factors during pregnancy, such as different fluids, growth factors and perfusion, we chose to sample both sides of all the placentae we analyzed. Overall, the stability profiles of the housekeeping genes are consistent in tissue sampled from both sides of the organ. SDHA, TBP and YWHAZ had very close expression stability scores, so there is a shuffling of rank order for the first three genes when comparing the villous to decidual tissue. Similarly, these three genes have the highest pair-wise correlation relative to each other, regardless of whether maternal or fetal side was sampled. Additionally, when diabetes and hypertensive placentas were excluded from the analysis, SDHA, TBP and YWHAZ remained the most stable genes. On the basis of these observations, we suggest using all three of these genes as endogenous controls for normalization of assays in the placenta.

To verify the suggestion that all three genes be used for greatest reliability of expression reporting, we looked at variability of gene expression profiles based on changes in leptin expression. In placental tissue, we show that the choice of housekeeping genes determines the sensitivity at which relative quantitation data can be reported. Assuming that the geometric mean of the three most stable housekeeping genes represents the most accurate measurement for endogenous control, comparison to GAPDH or use of only one or two of the three most stable housekeeping genes was analyzed. GAPDH normalized leptin fold change varied up to 5-fold (500%) from the geometric mean of SDHA, TBP and YWHAZ. Such large variability is unacceptable for comparison of gene expression by relative quantitation. When leptin fold change normalized to any one of the three genes alone was compared to normalization with the geometric mean of the three, change of up to 50% was seen. When results are reported as a 2-fold change in expression, an error of 50% can affect the significance of the results. Use of two genes showed only a 30% change, which is more acceptable for comparison of small fold changes, though use of three genes strengthens the results of this assay even further. Therefore, due to the small variability seen between these three genes within the placenta, use of only one or two of them is acceptable. However, use of all three for normalization is recommended for increasing the sensitivity in reporting gene expression changes.

Despite this conclusion, note that comparison of placental tissue to any other tissue type would not only require further housekeeping gene analysis, but it may demand the use of three or more housekeeping genes. It is likely that the regulation of these genes between tissue types may vary more extremely than they do within a particular tissue.

Finally, real time PCR shows gene expression profiles over a large dynamic range. However, for calculation of relative quantitation, it is still best to select for a control gene that has a comparable number of copies per cell as the gene of interest, allowing for measurement of the genes to be performed on the same linear scale. This will vary depending on the gene of interest, but we chose five genes, HPRT, HMBS, SDHA, TBP and YWHAZ because they express at low- to mid-range levels in the placenta. Other genes, such as 18s rRNA were not tested due to their extremely high expression levels in the placenta [20], which is out of the sensitive range of many less abundant genes. The expression data show that the genes tested express at levels in the range of many of the genes used in our laboratory (Figure 3). SDHA, TBP and YWHAZ all expressed at the mid-range compared to the other genes in this study.

In conclusion, we recommend using the normalization strategy developed by Vandesompele et al. [7] for comparison of genes in placental tissue samples. This can be done by geometrically averaging SDHA, TBP and YWHAZ to determine a normalization factor for each sample. Normalization of the gene of interest to this factor increases the accuracy and reliability of the relative quantitation data reported.

## ACKNOWLEDGEMENTS

We thank Dr. Martin Muy for his help in critically discussing the data presented in this manuscript and Dr. Chunfang Qiu for her help with correlation analysis.

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