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Reference genes selection for real-time quantitative PCR analysis in mouse germinal vesicle oocytes

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Research Article

Cite this article: Filatov MA *et al*. (2019) Reference genes selection for real-time quantitative PCR analysis in mouse germinal vesicle oocytes. *Zygote* **27**: 392–397. doi: 10.1017/S0967199419000492

Received: 19 September 2018
Revised: 5 August 2019
Accepted: 11 August 2019
First published online: 23 September 2019

Keywords:
Germinal vesicle; Mice; Oocyte; PCR; Reference gene

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Summary

Reference gene selection in mouse oocytes is an important task required to perform further adequate analysis of target gene expression levels. In the current work we have analyzed expression stability of the seven most commonly used reference genes (*Actb, Eef1e1, Gapdh, H2afz, Ppia, Rpl4* and *Ubc*) in mouse oocytes at the germinal vesicle (GV) stage. We have performed analysis of expression stability of the above-mentioned reference genes with the three most commonly used software tools: geNorm, BestKeeper and NormFinder. Taking into account the results obtained from all of these programmes *Gapdh, Rpl4* and *H2afz* seem to be suitable candidate reference genes in GV oocytes of mouse.

Introduction

In recent years, research projects dedicated to the examination of gene expression levels in oocytes have been actively developed (Zhang *et al*., 2013; Zare *et al*., 2017; Gumus *et al*., 2018). Determination of gene expression levels may be important for the quantification of oocyte quality, therefore making possible the evaluation of the assisted reproductive technology (ART) protocol applied, such as oocyte *in vitro* maturation (IVM) or ovarian follicle *in vitro* culture followed by oocyte retrieval for further fertilization. Quantitative real-time PCR is a standard method for gene expression analysis. Nevertheless, investigation of gene expression requires careful selection of reference genes (which are also often called housekeeping genes) for normalization (Kozera and Rapacz, 2013; Chapman and Waldenström, 2015). Despite the large number of studies in the field of reference gene selection, there is evidence that the expression of genes ordinarily used as reliable internal controls often depends on the specific experimental conditions and samples (Zhong and Simons, 1999; Selvey *et al*., 2001). Therefore, it is very important to select appropriate genes that display a stable expression pattern in the experimental probes (Nikishin *et al*., 2018).

Nowadays, there are only a few research works dedicated to reference gene selection for gene expression analysis in mammalian oocytes (Kuijk *et al*., 2007; Mamo *et al*., 2007, 2008; Kumar *et al*., 2012; O'Connor *et al*., 2013; Macabelli *et al*., 2014; Wang *et al*., 2017). The mouse is the most commonly used animal model for research studies. Therefore, the selection of appropriate reference genes for mouse oocytes is one of the most important tasks, it is the first step required to enable further investigation of target gene expression in mouse oocytes. The studies of Macabelli *et al.* (2014), O'Connor *et al.* (2013) and Kumar *et al.* (2012) were performed on bovine and ovine oocytes and the studies of Wang *et al.* (2017), Kuijk *et al.* (2007) and Mamo *et al.* (2008) were performed on swine and rabbit oocytes, respectively. Although, the general regulation of meiosis in oocytes of different mammalian species is the same, there are some distinctions in the processes of meiosis. Therefore, the works of Macabelli *et al.* (2014), O'Connor *et al.* (2013), Kumar *et al.* (2012) Wang *et al.* (2017), Kuijk *et al.* (2007) and Mamo *et al.* (2008) can be a starting point for identifying the most consistently expressed reference genes in mouse oocytes. The survey of Mamo *et al.* (2007) was carried out on mouse oocytes. However, more than 10 years have passed since the publication of this work. Only one mathematical approach called geNorm (Vandesompele *et al*., 2002) for the selection of reference genes was used in that study. New and more accurate mathematical algorithms that allow evaluation of the stability of reference gene expression have appeared. Furthermore, Mamo *et al.* (2007) performed analysis to evaluate the stability of reference gene expression levels in oocytes that resumed first meiotic division in comparison with the further stages of mouse embryo development (zygote, 2-cell embryo, morula and blastocyst). In the current study we used oocytes from the earlier germinal vesicle (GV) stage, when the nuclear envelope of the oocyte is intact.

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Table 1. Reference genes for gene expression normalization

Target gene	GenBank accession no.	Localization and primer sequence	Product size (bp)	Annealing temperature (°C)
Actb	NM_007393.5	Exon 3F: 5′-CTAAGGCCAACCGTGAAAAGAT-3′ Exon 4R: 5′-TGGCGTGAGGGAGAGCATA-3′	189	62.1 61.8
Eef1e1	NM_025380.2	Exon 1F: 5′-GACTGAAGCCGGGGAATAAG-3′ Exon 2R: 5′-ACTAGATGGGTGGCGATGGT-3′	112	60.8 61.1
Gapdh	NM_001289726.1	Exon 5F: 5′-GACGTGCCGCCTGGAGA-3′ Exon 6R: 5′-GAAGAGTGGGAGTTGCTGTTGAA-3′	144	63.0 62.9
H2afz	NM_016750.3	Exon 4–5F: 5′-ATTGCTGGTGGTGGTGTCATC-3′ Exon 5R: 5′-GCCTCCAACTTGCTCAAATAGAAT-3′	212	62.3 62.9
Ppia	NM_008907.1	Exon 4–5F: 5′-CAAGACTGAATGGCTGGATGG-3′ Exon 5R: 5′-GCCCGCAAGTCAAAAGAAAT-3′	164	62.3 61.4
Rpl4	NM_024212.4	Exon 1–2F: 5′-TCCCCGTCATGGCTTGTG-3′ Exon 2R: 5′-ACGGCATAGGGCTGTCTGTT-3′	169	62.7 61.6
Ubc	NM_019639.4	Exon 2F: 5′-TTACCACCAAGAAGGTCAAACAGG-3′ Exon 2R: 5′-CTCCCCCATCACACCCAAGAAC-3′	100	64.1 67.0

In the current study, evaluation of reference gene stability was performed on oocytes retrieved from mature and immature mice. Seven most commonly used reference genes from different functional classes were selected to minimize the risk of their co-regulation: β-actin (*Actb*), eukaryotic translation elongation factor 1 epsilon 1 (*Eef1e1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), H2A histone family, member Z (*H2afz*), peptidyl-prolyl isomerase A (*Ppia*), ribosomal protein L4 (*Rpl4*) and ubiquitin C (*Ubc*).

Materials and Methods

Animals

C57BL/6J mice were purchased from the animal house of the Federal Medical Biological Agency, Branch 'Andreevka'. Animals were maintained under controlled room conditions (22–24°C and a 14 h light:10 h dark photoperiod). Mice were given *ad libitum* access to food and water. All manipulations with animals were performed accordingly to the Moscow State University Bioethical Committee recommendations.

Oocyte collection

In the current work we used oocytes retrieved from two groups of animals. The first group contained oocytes obtained from immature mice (21–24 days old) and oocytes from the second group were retrieved from the adult mice (3 months old) at the proestrus stage of the estrous cycle. The estrous cycle stages were evaluated by vaginal smears as described previously (Zenclussen *et al.*, 2014).

Mice were sacrificed by cervical dislocation and ovaries were removed and placed into pre-equilibrated HEPES-containing medium (Human Tubal Fluid Medium, Irvine Scientific). in a CO_2/air incubator. Then, ovaries were dissected using insulin needles and oocytes were collected and measured. Only denuded oocytes with no attached cumulus cells or oocytes with small numbers of cumulus cells were used in this study. Cumulus cells were removed from the oocytes by gentle pipetting of oocytes three times. Oocytes were divided into groups, 10 oocytes in each group, for further qRT-PCR analysis. For the analysis, eight samples (each sample contained 10 oocytes) were obtained from each group.

cDNA synthesis

Synthesis of cDNA libraries was carried out using the commercial kit SuperScript III cDNA Synthesis CellDirect (ThermoFisher, USA) following the manufacturer's instructions. All obtained cDNA samples were stored at −20°C until qRT-PCR analysis.

Primer design for selected candidate reference genes

Primer design (Table 1) was performed using Lasergene PrimerSelect (DNASTAR) software and the online utility Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using sequences from the NCBI GenBank database.

Quantitative real-time PCR

Quantitative RT-PCR was carried out on a StepOnePlus real-time PCR system (Applied BioSystems, USA) using SYBR® green I with ROX™ as an internal loading standard. The qPCRmix-HS SYBR+ROX kit (Evrogen, Russia) was utilized for all reference gene qRT-PCR assays, according to the manufacturer's protocol. Each reaction contained: 0.5 µl of solution containing forward primers (0.5 µM), 0.5 µl of solution containing reverse primers (0.5 µM), 5 µl of 5× qPCRmix-HS SYBR+LowROX, 0.5 µl of solution containing template cDNA obtained using the commercial kit SuperScript III cDNA Synthesis CellDirect in accordance with the manufacturer's recommendations, and 18.5 µl of diethylpyrocarbonate (DEPC)-treated and autoclaved water. Lyophilized primers were previously diluted with DEPC-treated and autoclaved water.

PCR cycles were run as follows: 2 min at 50°C, then denaturation for 10 min at 95°C followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Fluorescence was measured following each annealing and extension phase. A melt curve was produced to confirm a single gene-specific peak and to detect primer/dimer formation by heating the samples from 70 to 95°C in 0.5°C increments with a dwell time of 10 s at each temperature, while continuously monitoring the fluorescence. The reactions were set up in a 96-well PCR plate (Applied BioSystems, USA) in three technical replicates.

Controls included non-RT controls (total RNA without reverse transcription was used to monitor genomic DNA contamination) and non-template controls (water template). For total RNA

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isolation the same protocol as for cDNA synthesis was used, but water was added to the samples instead of the reverse transcriptase from the kit.

Data analysis

Statistical significance of oocyte diameters differences between samples from different groups was calculated using the Mann–Whitney *U*-test. Also the Mann–Whitney *U*-test was used to find the differences in the C_t levels of the examined reference genes between samples from mature and immature oocytes. To test the hypothesis of normal distribution the Kolmogorov–Smirnov and Lilliefors normality test was used. The Kolmogorov–Smirnov and Lilliefors normality test, Mann–Whitney *U*-test and calculation of standard error of the mean (SEM) were performed using Statistica 7.0 software (Dell, Inc., USA). Differences were considered statistically significant if the *P*-value was < 0.05. The expression stability of seven selected internal control genes was evaluated using three commonly used freeware software tools: geNorm v.3.5 (Vandesompele *et al.*, 2002), BestKeeper (Pfaffl *et al.*, 2004) and NormFinder (Andersen *et al.*, 2004).

The inner diameter of the oocyte excluding the zona pellucida was calculated using freely available ImageJ software (NIH, USA).

Results

We measured oocytes diameters and analyzed the retrieved data. The median value of oocyte diameter in the group of oocytes retrieved from immature mice was approximately 68 µm, and the median value of oocyte diameter in the group of oocytes retrieved from mature mice was approximately 76 µm. Analysis of the data showed that there is a statistically significant difference in oocyte diameters between these two groups (Mann–Whitney *U*-test, $P < 0.05$). These results correlated well with previously obtained data (Otoi *et al.*, 2000). Therefore, based on measurements of diameters we could conclude that oocytes obtained from mature and immature mice belong to different groups. This conclusion is essential for adequate mathematical analysis because the above-mentioned software tool required data on gene expression levels obtained from two different groups of samples (Andersen *et al.*, 2004). Data obtained from both mature and immature mouse oocytes were used for the analysis.

The C_t levels in oocytes from two groups (mature and immature ones) were measured. The C_t value for each gene in sample was calculated as the arithmetic mean of three technical replicates. The C_t values are given in Table 2. Interestingly, the mean C_t levels of all seven examined candidate reference genes were higher in immature oocytes in comparison with mature oocytes, indicating that the number of initial cDNA matrices in immature oocytes may be lower. There are two possible reasons of such differences in expression levels. The first one is potential difference in RNA extraction or cDNA synthesis between two groups of oocytes, however this hypothesis seems to be rather weak as the same protocol was applied for both groups of samples. Another reason of such discrepancies may lie in the different status of retrieved oocytes. The mean volume of mature oocyte was approximately 1.4-fold larger in comparison with immature oocyte volume. Furthermore, it is well known that during prophase I meiotic arrest, mammalian oocytes grow and accumulate various molecules and organelles that will be necessary for early embryo development, thereby oocytes acquire the ability to resume meiosis, i.e. oocyte maturation (Filatov *et al.*, 2018). Therefore, accumulation of key housekeeping gene mRNAs followed by an increase in oocyte volume seems to be logical. However, there are no statistically significant differences ($P > 0.05$) in the C_t levels for all the examined genes across the two groups of oocytes. Therefore, the C_t levels data from mature and immature oocytes can be considered as one population.

Table 2. The mean of C_t values ± standard error of the mean (SEM) of investigated reference genes in mice oocytes (samples $n = 8$ for both groups)

Target gene	Mature oocytes	Immature oocytes
Actb	33.90 ± 0.43	35.42 ± 1.02
Eef1e1	31.76 ± 0.46	34.16 ± 1.26
Gapdh	30.38 ± 0.84	32.49 ± 0.80
H2afz	30.92 ± 1.20	33.12 ± 0.84
Ppia	28.64 ± 1.00	31.37 ± 1.36
Rpl4	31.37 ± 0.95	32.50 ± 0.94
Ubc	26.37 ± 0.50	28.30 ± 0.87

The C_t values of seven reference genes were analyzed using three different software tools (geNorm, BestKeeper, and NormFinder) to identify the reference genes with stable expression. The C_t values of stably expressed reference genes that may be used in further research work dedicated to the examination of target genes expression levels were sought.

As there was no statistically significant differences in the C_t values between the groups of mature and immature oocytes, the C_t levels data from both groups of oocytes were considered as one population for analysis using geNorm and BestKeeper programs.

geNorm is software program that is commonly used to identify the most stably expressed reference genes (Vandesompele *et al.*, 2002). In this software, a complex approach is used to analyze gene expression stability. As a result, the M value (the parameter of gene expression stability) of each gene is calculated. The gene with the lowest M value may be accepted as the most stably expressed gene. In mouse GV oocytes, geNorm ranked *Gapdh* and *Rpl4* as the most stable reference genes (M value = 1.238 and 1.259, respectively), followed by *H2afz* (M value = 1.533) and *Ppia* (M value = 1.539). The other genes were ranked from most to least stable as follows: *Actb* > *Ubc* > *Eef1e1*.

BestKeeper is also a software tool applied to identify the most suitable reference genes (Pfaffl *et al.*, 2004). Analysis of gene expression stability is based on calculation and comparison of the correlation coefficients for all possible pairs of candidate reference genes. In mouse GV oocytes the BestKeeper software tool classified *Rpl4* and *Ppia* as the most stable reference genes. The other genes were ranked from most to least stable as follows: *Gapdh* > *H2afz* > *Eef1el* > *Ubc* > *Actb*.

NormFinder is another software tool that allows selection of the most stable reference genes. NormFinder measures intragroup and intergroup variations in gene expression stability. Then, the examined reference genes are ranked according to their expression stability (Andersen *et al.*, 2004). Immature and mature oocytes were selected as two different groups as mature and immature oocytes have different diameters. In mouse GV oocytes the NormFinder tool determined *Gapdh* and *H2afz* as the most stable pair of genes, followed by *Rpl4* and *Eef1e1*. The other genes were ranked from most to least stable as follows: *Actb* > *Ppia* > *Ubc*.

The summary list of genes ranked by three software tools (geNorm, BestKeeper and NormFinder) is given in the Table 3.

https://doi.org/10.1017/S0967199419000492 Published online by Cambridge University Press

Table 3. Reference genes ranked by their expression stability in mice mature and immature GV oocytes by three most commonly used software tools: geNorm, BestKeeper, NormFinder. The genes considered by the authors as the most stable reference genes are marked by bold letters

Rank	geNorm	BestKeeper	NormFinder
1	***Gapdh***	***Rpl4***	***Gapdh***
2	***Rpl4***	*Ppia*	***H2afz***
3	***H2afz***	***Gapdh***	***Rpl4***
4	*Ppia*	***H2afz***	*Eef1e1*
5	*Actb*	*Eef1e1*	*Actb*
6	*Ubc*	*Ubc*	*Ppia*
7	*Eef1e1*	*Actb*	*Ubc*

It should be noticed that according to the data obtained in the current study, *Actb* is unsuitable as a reference gene in mouse oocytes even though it is one of the most commonly used reference genes mentioned in many research studies. All software applications (geNorm, BestKeeper and NormFinder) classified *Actb* as a gene with unstable expression. Furthermore, *Ubc*, which shows the highest expression levels among all the examined candidate reference genes, also has been classified as one of least stable expressed genes by three software tools: geNorm, BestKeeper and NormFinder.

Discussion

Several research studies dedicated to the evaluation of reference gene expression level stability in mammalian oocytes have been performed previously (Mamo *et al.*, 2007, 2008; Kuijk *et al.*, 2007; Kumar *et al.*, 2012; O'Connor *et al.*, 2013; Macabelli *et al.*, 2014; Wang *et al.*, 2017). Key data obtained in these research studies are listed in the Table 4. Interestingly, genes with the most stable expression patterns differ across different mammalian species. It should be mentioned that selection of the most stable reference genes depends on many factors such as primers used for PCR, protocol of cDNA synthesis, methods of mathematical analysis, and type of probes. In works of Kuijk *et al.* (2007) and Wang *et al.* (2017) different genes were selected as the most stable reference genes for porcine oocytes. The same situation was observed in works of Kumar *et al.* (2012) and Macabelli *et al.* (2014) performed on bovine oocytes. Such differences in results may be explained by using the different sets of candidate reference genes and primer pairs in the above-mentioned works. Furthermore, in the research of Wang *et al.* (2017) it has been shown that the results of gene expression stability depend on the type of protocol applied for cDNA synthesis. Intriguingly, the final results of gene expression stability also depend on the primers used for cDNA synthesis. Therefore, in the work of O'Connor *et al.* (2013) it was been shown that in ovine oocytes the most stable reference genes may alter depending on primers used for cDNA synthesis: *Sdha, Tubb* and *Ppia* in the case of random hexamer primers and *Ywhaz, Tubb* and *Sdha* in the case of oligo(dT) primers.

In the current study we used oocytes at the GV stage because, in GV oocytes, maternal mRNAs are bound by MSY2 protein, which prevents their destruction. Once oocyte meiotic resumption is induced, phosphorylation of MSY2 occurs, resulting in maternal mRNAs degradation (Mehlmann, 2013). Therefore, determination of stable reference genes in GV oocytes is needed for further research works that require evaluation of total maternal mRNA transcript levels in oocytes; the data obtained in the current study will allow the performance of this kind of analysis.

The results of the current work differ from that described previously in the work of Mamo *et al.* (2007) except for one gene (*H2afz*). We have also shown stable expression of *H2afz* in mouse oocytes. Based on this, we concluded that this gene can be used as the reference gene that corresponds with results of Mamo *et al.* (2007). The reason for discrepancies in these two studies may be as follows: different sets of genes selected for analysis were used; different primer pairs and cDNA synthesis protocols were applied; oocytes at different stages of meiotic resumption were used (MII in the work from Mamo *et al.* (2007) versus GV oocytes in the current work). Moreover, in this research, the three most commonly used software tools were applied to analyze gene expression stability: geNorm (Vandesompele *et al.*, 2002), BestKeeper (Pfaffl *et al.*, 2004) and NormFinder (Andersen *et al.*, 2004), whereas in other studies dedicated to identifying the most suitable reference genes in mammalian oocytes only geNorm software was used (Kuijk *et al.*, 2007; Mamo *et al.*, 2007, 2008; Kumar *et al.*, 2012; O'Connor *et al.*, 2013; Macabelli *et al.*, 2014; Wang *et al.*, 2017). Unexpectedly, these tools (geNorm, BestKeeper and NormFinder) found that different reference genes were the most suitable for mouse oocytes because three different mathematical approaches were used in these programs to analyze gene expression levels.

According to the values of reference genes expression stability calculated using three different mathematical approaches (geNorm (Vandesompele *et al.*, 2002), BestKeeper (Pfaffl *et al.*, 2004) and NormFinder (Andersen *et al.*, 2004)) *Gapdh, Rpl4 and H2afz* seem to be suitable candidate reference genes for mouse GV oocytes.

The selection of the most appropriate software for gene expression and reference gene stability analysis is widely discussed in the scientific community, and there is no current consensus. Previously, it has been shown that the geNorm analysis is more affected by fluctuations in PCR efficiencies compared with NormFinder analysis (De Spiegelaere *et al.*, 2015). Another drawback of the geNorm algorithm is the impossibility to conduct target gene analysis (Vandesompele *et al.*, 2002). Also it has been shown that the NormFinder algorithm is less robust for small sample sizes compared with geNorm (Neuvians *et al.*, 2005). Nevertheless, the important advantage of NormFinder is that it can differentiate intragroup variation from intergroup variation and, therefore, is a suitable tool for reference gene evaluation when different sample groups are used (De Spiegelaere *et al.*, 2015).

In the current work, three statistical software tools (NormFinder, BestKeeper and geNorm) were applied to find the most stably expressed reference genes for mouse oocytes. These programs use different calculation algorithms and gave different results (Mallona *et al.*, 2010; Mafra *et al.*, 2012). NormFinder and geNorm use relative quantities calculated from C_t values, while BestKeeper uses C_t values directly (Ali *et al.*, 2015). Therefore, the better approach to select the most stable reference genes is to use several software tools with different mathematical algorithms for analysis, and then compare the data obtained by these programs for the selection. This approach was used in the current work.

It is very important to select the appropriate reference genes that will show a stable level of expression in the specific samples. The use of inappropriate reference genes may distort the results of a study. Moreover, it should be taken into account that the use of only one reference gene for analysis can lead to distortion in research findings, as fluctuations in gene expression levels always

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Table 4. Researches dedicated to the examination of stable expressed reference genes in mammalian oocytes

Animal	Most stably expressed reference genes	Examined reference genes	Study details	Reference
Sheep	*Sdha, Tubb* and *Ppia* for random hexamer primers; *Ywhaz, Tubb* and *Sdha* for oligo(dT) primers	*Actb, Gapdh, H2afz, Hprt1, Ppia, Sdha, Tubb* and *Ywhaz*	geNorm analysis of gene expression patterns in GV oocytes retrieved from mouse and MII oocytes obtained after *in vitro* maturation	O'Connor *et al.*, 2013
Rabbit	*H2afz, Hprt1* and *Ywhaz*	*Actb, Gapdh, Hprt1, H2afz, Ubc, Ppia, Eef1e1, Polr2a, Tbp, G6pdx, B2m, Pgk1* and *Ywhaz*	geNorm analysis of sample groups obtained *in vivo* and *in vitro*: MII oocytes, zygotes, 2-cell embryos, 8-cell embryos, morulae, blastocysts	Mamo *et al.*, 2008
Pig	*Gapdh, Pgk1, S18* and *Ubc*	*B2m, Bact, Gapdh, H2a, Pgk1, Sl8* and *Ubc*	geNorm analysis of GV oocytes retrieved from mouse and obtained after *in vitro* culture: MII oocytes, 2-cell embryos, 4-cell embryos and blastocysts	Kuijk *et al.*, 2007
Pig	*Ywhag* and *Bact*	*Gapdh, 18S, Ywhag, Bact, Rpl4, Hprt1* and *Ppia*	geNorm analysis of gene expression patterns in GV oocytes retrieved from mouse and germinal vesicle breakdown (GVBD) and MII oocytes obtained after *in vitro* maturation. Comparison of two different kits (RNeasy Kit and Lysis Kit) for evaluation of gene expression stability levels in oocytes was carried out in this research	Wang *et al.*, 2017
Buffalo	*Rps15, Rps18* and *Gapdh*	*Ywhaz, Sdha, Gapdh, Rps15, Rps18* and *Rn18s1*	geNorm analysis of gene expression patterns in oocytes matured *in vitro* until MII stage and 2-cell, 4-cell, 8-cell and 16-cell embryos, morulae and blastocysts obtained after IVF.	Kumar *et al.*, 2012
Buffalo	*Ywhaz, Gusb* and *Gapdh*	*Actb, Gapdh, Gusb, Hist1h2ag, Hprt1, Ppia, Rpl15, Sdha, Tbp* and *Ywhaz*	geNorm analysis of reference genes expression patterns in oocytes retrieved from cumulus–oocyte complexes (COCs) in winter and summer	Macabelli *et al.*, 2014
Cattle	*Rpl15, Ppia* and *Gusb*	*Actb, Gapdh, Gusb, Hist1h2ag, Hprt1, Ppia, Rpl15, Sdha, Tbp* and *Ywhaz*	geNorm analysis of reference genes expression patterns in oocytes retrieved from COCs in winter and summer	Macabelli *et al.*, 2014
Mice	*Ppia, H2afz* and *Hprt1*	*Actb, Gapdh, H2afz, Hprt, Ppia, Ubc, Eef1e1, Tubb4, Hist2h2aa1, Tbp, Bmp7* and *Polr2a*	geNorm analysis of gene expression patterns in MII oocytes obtained after ovarian stimulation and embryos at the different stages of development: 2-cell, 8-cell and blastocysts	Mamo *et al.*, 2007

occur. A possible way to avoid such problems is to calculate a specific index based on information on expression levels of several reference genes for further assessment of target gene expression changes. This method of analysis would minimize the effect of fluctuations in the expression level of each individual gene.

The novel data obtained in the current work will allow adequate analysis of target gene expression levels in mouse oocytes to be performed in further studies. Therefore, new publications dedicated to this subject can be expected in the near future.

Acknowledgements. We thank Filatova Polina for language editing and proofreading.

Financial support. This study was funded by the Russian Science Foundation (project no. 14-50-00029).

Statement of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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https://doi.org/10.1017/S0967199419000492 Published online by Cambridge University Press