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NOTE

Anatomy

Identification of reference genes for quantitative PCR analyses in developing mouse gonads

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ABSTRACT. Stable reference genes are important for gene expression analyses such as quantitative PCR. The stability of 15 candidate reference genes that can be used to developing mouse gonads was thoroughly verified using combinations of multiple algorithms. The expression of these genes fluctuated greatly depending on the analysis period and/or gender. Peptidylprolyl isomerase A (*Ppia*) and polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*) were the reference genes that were used stably for a wide analysis period in developing mouse gonads. Furthermore, the stable reference genes corresponding to the analysis period and/or gender were ranked. These results are useful for the selection of the optimal reference gene required for high-precision measurements.

KEY WORDS: developing mouse gonad, gene expression, normalization, quantitative PCR, reference gene

Testes in males (XY) and ovaries in females (XX) are the organs that generate gametes (germ cells). They arise from an undifferentiated bipotential gonad replies on the expression of the sex-determining region of the Y chromosome (*SRY/Sry*). In XY gonads that differentiate into testes, cell differentiation and proliferation occur under the influence of *Sry*-expressing Sertoli cells [15]. In this process, various kinds of genes are expressed in a specific spatiotemporal manner. As a result, the cell types in the gonad increase and their ratio change. Both expressed genes and developmental stages of germ cells differ in the gonads of males and females. Gene transfer and gene knockout methods in mice are important for elucidating these mechanisms. In particular, the C57BL/6 strain, which is referred to as a mouse reference sequence, is frequently used, and the expression of many genes has been measured [15]. In order to elucidate the developmental process of gonads, both histological methods to examine localization of expression and molecular biological methods to examine the expression level are essential.

Stably expressed genes called housekeeping genes, such as actin, beta (*Actb*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and 18S ribosomal RNA (*Rn18s*), are commonly used to normalize mRNA expression levels between different samples in quantitative polymerase chain reaction (qPCR) studies [4, 11]. Nucleic acids used for measurement include experimentally generated errors such as cell number, mRNA extraction degree, and transcription efficiency into complementary DNA (cDNA), and are normalized by dividing by the value of the stably expressed gene (reference gene). The expression levels of these genes may vary depending on cell type, tissue, gender, and developmental stage, and may change further under experimental conditions [11]. Adjusting the cell number or reverse-transcription efficiency using fluctuating genes produces large errors [19]. Therefore, the selection of reference genes is crucial for gene expression studies [4, 11]. However, the information on stable reference genes in mouse gonads has been reported only at the early development stage (11.5–14.5 days post coitum (dpc)) [17] and at the postnatal stage (neonate to 5 months) [8].

Today algorithm-based ranking methods, such as geNorm (https://genorm.cmgg.be/) [21], NormFinder (https://moma.dk/normfinder-software) [2], and BestKeeper (https://www.gene-quantification.de/bestkeeper.html) [13], are commonly used for the selection of reference genes. GeNorm repeats the procedure of eliminating the lowest stability gene, using the correlation of gene

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Table 1. Primer sequences for reference genes

Gene symbol	Accession number	Gene name	Primers (5′-3′)	Size (bp)	References of primer sequences
Actb	NM_007393	Actin, beta	F: CTAAGGCCAACCGTGAAAAG R: ACCAGAGGCATACAGGGACA	104	[17]
B2m	NM_009735	Beta-2 microglobulin	F: TGCTACTCGGCGCTTCAGTC R: AGGCGGGTGGAACTGTGTTAC	200	[9]
Gapdh	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase	F: CGTCCCGTAGACAAAATGGT R: TTGATGGCAACAATCTCCAC	110	[17]
Gusb	NM_010368	Glucuronidase, beta	F: CACGGCGATGGACCCAAGAT R: CCCATTCACCCACACAACTGC	86	-
Hprt	NM_013556	Hypoxanthine guanine phosphoribosyl transferase	F: AGGCCAGACTTTGTTGGATTTG R: CTTAGGCTTTGTATTTGGCTTTTCC	136	-
Pgk1	NM_008828	Phosphoglycerate kinase 1	F: CTGACTTTGGACAAGCTGGACG R: GCAGCCTTGATCCTTTGGTTG	110	[22]
Polr2a	NM_009089	Polymerase (RNA) II (DNA directed) polypeptide A	F: ATCAACAATCAGCTGCGGCG R: GCCAGACTTCTGCATGGCAC	144	-
Ppia	NM_008907	Peptidylprolyl isomerase A	F: CGCGTCTCCTTCGAGCTGTTTG R: TGTAAAGTCACCACCCTGGCACAT	150	[22]
Rn18s	NR_003278	18S ribosomal RNA	F: GATCCATTGGAGGGCAAGTCT R: CCAAGATCCAACTACGAGCTTTTT	103	[17]
Rplp0	NM_007475	Ribosomal protein, large P0	F: AGATTCGGGATATGCTGTTGGC R: TCGGGTCCTAGACCAGTGTTC	109	[25]
Sdha	NM_023281	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	F: TGTTCAGTTCCACCCCACA R: TCTCCACGACACCCTTCTGT	66	[17]
Tbp	NM_013684	TATA box binding protein	F: GCTCTGGAATTGTACCGCAG R: TGACTGCAGCAAATCGCTTG	130	-
Tfrc	NM_011638	Transferrin receptor	F: GGCGCTTCCTAGTACTCCCT R: TCTGCAGCCAGTTTCATCTCCA	162	-
Ubc	NM_019639	Ubiquitin C	F: AGGTCAAACAGGAAGACAGACGTA R: TCACACCCAAGAACAAGCACA	80	[24]
Ywhaz	NM_011740	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	F: TTGATCCCCAATGCTTCGC R: CAGCAACCTCGGCCAAGTAA	88	[22]

expression [21]. NormFinder determines the stability of genes using intragroup and intergroup gene expression variance [2]. The feature of BestKeeper is to use the Ct (threshold cycle) value, and the stability is determined based on the variance of the cycle number [13]. However, because the results of each calculation algorithm are different from each other, it is difficult to decide which to adopt [11]. Several reports have merely described the results for each method [5, 14, 23], while other reports decided the final ranking from the arithmetic mean or geometric mean of ranks on each test [3, 18]. In the present study, we calculated the stability of reference genes from three different algorithms and identified stable reference genes in the gonads from the undifferentiated to adult stages.

C57BL/6NCrSlc mice were purchased from SLC Japan (Hamamatsu, Japan) and maintained as described elsewhere [20]. Male and female mice or their fetuses were used at 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5 dpc, neonate, 5-week-old, and adult (male: 39 weeks; female: 29 weeks). This study was approved by the Institutional Animal Care and Use Committee (Permission #22-8-03) and carried out according to the Kobe University Animal Experimental Regulations.

The gonad-mesonephros complex (only at 10.5 dpc) or gonads were collected from three animals at each time point immediately after euthanasia, which was accomplished under deep anesthesia with isoflurane. Bilateral samples of each individual were collected and used together. Total RNA was extracted with the ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, U.S.A.), including on-column DNaseI treatment. For cDNA synthesis, a PrimeScript RT reagent Kit (Takara Bio, Kusatsu, Japan) with both random hexamer primers and oligo (dT) primers was used according to the manufacturer's procedures. The cDNA samples were diluted 10 times with EASY Dilution (Takara Bio) and divided into small amounts to avoid freeze-thaw cycles. Real-time PCR was performed on a Thermal Cycler Dice TP-860 system (Takara Bio) using SYBR Premix EX TaqII (Takara Bio). The cycling parameters were as follows: thermal activation for 10 sec at 95°C and 50 cycles of PCR (melting for 5 sec at 95°C, annealing for 10 sec at 60°C, and extension for 120 sec at 72°C). The applied primers are listed in Table 1. Melting temperature analysis and electrophoresis of the PCR products were performed for each experiment to verify that only single products were amplified. The second derivative maximum method was used to determine the Cycle quantification. Each run was designed to

Table 2. Ranking of candidate reference genes from each algorithm in the all-period

Algorithm\gene name		*Ppia*	*Polr2a*	*Rplp0*	*Gusb*	*Hprt*	*Ubc*	*Pgk1*	*Tfrc*	*B2m*	*Tbp*	*Actb*	*Rn18s*	*Sdha*	*Gapdh*	*Ywhaz*
BestKeeper		1	2	4	3	8	7	6	5	10	9	11	12	13	14	15
	Std dev	(0.51)	(0.53)	(0.75)	(0.74)	(0.93)	(0.87)	(0.84)	(0.84)	(1.09)	(1.03)	(1.73)	(1.75)	(1.91)	(3)	(4.35)
NormFinder		1	4	3	6	2	7	5	8	9	10	11	12	13	14	15
	Stability value	(0.81)	(0.98)	(0.87)	(1.06)	(0.84)	(1.15)	(1.05)	(1.25)	(1.31)	(1.37)	(1.48)	(1.68)	(1.76)	(3.34)	(4.26)
GeNorm		1.4	1.4	3	5	6	4	8	9	7	10	11	12	13	14	15
	Stability value	(0.48)	(0.48)	(0.59)	(0.83)	(0.92)	(0.69)	(1.05)	(1.11)	(0.99)	(1.16)	(1.29)	(1.45)	(1.58)	(1.89)	(2.23)
Geometric mean of rank		1.1	2.2	3.3	4.5	4.6	5.8	6.2	7.1	8.6	9.7	11.3	11.7	13	14	15

All-period consist of 10.5, 11.5, 12.5, 13.5, 15.5, 17.5 dpc, neonate, 5-week-old and adult.

include all samples to reduce variability between experiments. To evaluate the stability of candidate reference genes, commonly used algorithms geNorm, NormFinder, and BestKeeper were used according to the developer's instructions.

Fifteen genes from different pathways were used as candidate genes (Table 1). We used such a large number because when the number of candidate genes is small, correlation may occur even if the expression of two or three genes simultaneously changes. To avoid this problem, many previous reports have used 8 to 15 primers [7, 10, 12, 14, 17, 25]. If multiple genes in the same pathway are used, correlation occurs between the candidate genes [11]. Therefore, in the selection of candidate genes, genes were selected from different pathways with reference to the Mouse Housekeeping Gene Primer Set (Takara Bio), TaqMan Human Endogenous Control Plate (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and previous reports [9, 14, 17, 22, 24, 25].

The samples were assigned to one of three periods according to developmental stage: a sex determination period (10.5–12.5 dpc), a sex differentiation period (13.5–18.5 dpc), and a postnatal period (neonate, 5-week-olds, and adults) (Tables 2–4). The postnatal period should be divided into before and after puberty, but since there are only three time points, they were combined under "postnatal". In addition, the sex differentiation period included six time points, but the sex determination period and the postnatal period each have only three time points (Table 4). In order to avoid an imbalance toward the sex differentiation period, half of the samples in the sex differentiation period were used for the all-period and fetal-period analyses (Tables 2–4).

The results of the calculation of candidate gene stability in the all-period analysis by the three algorithms are shown in Table 2. The order of reference genes by each algorithm was calculated. We then obtained the geometric mean of those ranks and made it the final rank (Table 2). Simply calculating the mean of the rank despite the existence of different judgment criteria in each algorithm is criticized as not being a scientific method [6]. However, it is impossible to uniformly decide which is better, because each algorithm has advantages and disadvantages [6, 11]. Among the 45 ranks calculated by each algorithm, there were 3 items with rank-order fluctuations of 3, 5 items with fluctuations of 2, and more than half were the same as the mean value (Table 2). This is why we think it is useful to use mean values as rough indicators to select reference genes. Interestingly, genes with a rank of less than 1/3 were common to each algorithm. Even simply excluding these unstable genes from the reference genes will stabilize gene expression analysis experiments.

Several periods commonly used for gonadal sex determination and sexual differentiation studies were analyzed, such as 10.5 to 12.5 dpc and 10.5 to 14.5 dpc. As a result, the expression of peptidylprolyl isomerase A (*Ppia*) and polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*) was stable under many conditions, especially after 12.5 dpc, but the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Ywhaz*) and *Gapdh* was always unstable, and that of *Rn18s*, *Actb*, and succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*Sdha*) was also unstable in many cases (Table 3). The sexually dimorphic expression pattern was confirmed. That is, although ribosomal protein, large P0 (*Rplp0*) and beta-2 microglobulin (*B2m*) were stable in males, TATA box binding protein (*Tbp*), glucuronidase, beta (*Gusb*), and ubiquitin C (*Ubc*) were stable in females.

As described later, in the period of 10.5 to 12.5 dpc, the stably expressed genes were largely different from those in the other periods. The stability of *Gusb* and *Ubc* was high, but that of *Ppia* and *Polr2a* was relatively low. Notably, stable expression of *Sdha* was observed, especially in males. Furthermore, the expression of *Gusb* and *Ubc* after 13.5 dpc became more unstable in males than in females (Table 3). *Rn18s*, ribosomal protein S29 (*Rsp29*), *Tbp*, and *Sdha* are recommended for gene expression analysis ranging from 11.5 to 14.5 dpc [17], but this result is different from ours. This may be due to differences in calculation algorithms, differences in samples, differences in candidate genes, or differences in mouse strain. In the period of 11.5 to 14.5 dpc, the stability of the candidate gene is relatively high, and it can be used even with the low-rank *Rn18s*. Particularly in females, the expression of most genes other than *Gapdh* and *Ywhaz* was stable. However, the use of a more stable reference gene enables high-precision measurement, so it is better to use *Gusb*, *Ppia*, and *Tbp* during this period.

In the postnatal period, hypoxanthine guanine phosphoribosyl transferase (*Hprt*), *Ppia*, and phosphoglycerate kinase 1 (*Pgk1*) were calculated to be stable, but *Hprt* and *Pgk1* were relatively unstable in males compared to females. When we analyzed only males, *Ppia*, *Rplp0*, *B2m*, and *Tfrc* were stable and *Actb*, *Sdha*, *Gapdh*, and *Ywhaz* were unstable (Table 3). In the report that evaluated 11 time points from male mice after birth using six genes, *Ppia*, *Gapdh*, and *Actb* were very stable and *Hprt* and *Tbp* were unstable [8]. Since *Gapdh* and *Actb* are always unstable in our study and are not recommended for studies in adult human testicular cells [16], it is unknown whether these genes are stable. These differences may be primarily caused by differences in the number of candidate genes evaluated.

Table 3. Ranking of candidate reference genes from geometric mean in gonads

Male + Female gonad		Most stable ←														Least stable
	Period (sample) \ Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
All-period	(10.5, 11.5, 12.5, 13.5, 15.5, 17.5, neonate, 5-week-old, adult)	***Ppia****	***Polr2a****	***Rplp0****	*Gusb*	*Hprt*	*Ubc*	*Pgk1*	*Tfrc*	*B2m*	*Tbp*	*Actb*	*Rn18s*	*Sdha*	*Gapdh*	*Ywhaz*
Fetal-period	(10.5, 11.5, 12.5, 13.5, 15.5, 17.5)	***Polr2a****	***Ppia****	*Tbp*	*Ubc*	*Gusb*	*Rplp0*	*Pgk1*	*B2m*	*Hprt*	*Tfrc*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
Postnatal period	(neonate, 5-week-old, adult)	***Hprt****	***Ppia****	***Pgk1****	*Rplp0*	*Polr2a*	*B2m*	*Tfrc*	*Gusb*	*Ubc*	*Rn18s*	*Tbp*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
10.5–18.5 dpc	(10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	***Polr2a****	***Ppia****	***Tbp****	*Rplp0*	*Ubc*	*Gusb*	*B2m*	*Hprt*	*Pgk1*	*Tfrc*	*Actb*	*Rn18s*	*Sdha*	*Gapdh*	*Ywhaz*
10.5–12.5 dpc	(10.5, 11.5, 12.5)	***Gusb****	***Ubc****	***Tbp****	*Sdha*	*Hprt*	*Polr2a*	*B2m*	*Ppia*	*Rplp0*	*Pgk1*	*Tfrc*	*Actb*	*Gapdh*	*Rn18s*	*Ywhaz*
10.5–14.5 dpc	(10.5, 11.5, 12.5, 13.5, 14.5)	***Gusb****	***Tbp****	*Polr2a*	*Ppia*	*Ubc*	*Hprt*	*Rplp0*	*B2m*	*Pgk1*	*Tfrc*	*Sdha*	*Actb*	*Rn18s*	*Gapdh*	*Ywhaz*
12.5–14.5 dpc	(12.5, 13.5, 14.5)	***Polr2a****	***Gusb****	*Ppia*	*Ubc*	*Tbp*	*Pgk1*	*Rplp0*	*Hprt*	*B2m*	*Tfrc*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
13.5–18.5 dpc	(13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	***Polr2a****	***Ppia****	*Tbp*	*Rplp0*	*Ubc*	*Pgk1*	*Gusb*	*B2m*	*Hprt*	*Tfrc*	*Actb*	*Rn18s*	*Sdha*	*Gapdh*	*Ywhaz*
Male gonad		Most stable ←														Least stable
	Period (sample) \ Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
All-period	(10.5, 11.5, 12.5, 13.5, 15.5, 17.5, neonate, 5-week-old, adult)	***Ppia****	***Polr2a****	*Rplp0*	*Gusb*	*Hprt*	*Ubc*	*Pgk1*	*Tfrc*	*B2m*	*Tbp*	*Actb*	*Rn18s*	*Sdha*	*Gapdh*	*Ywhaz*
Fetal-period	(10.5, 11.5, 12.5, 13.5, 15.5, 17.5)	***Polr2a****	***Ppia****	*Rplp0*	*Hprt*	*Tbp*	*Ubc*	*Gusb*	*Pgk1*	*Tfrc*	*B2m*	*Actb*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
Postnatal period	(neonate, 5-week-old, adult)	***Ppia****	***Rplp0****	***B2m****	***Tfrc****	*Polr2a*	*Pgk1*	*Hprt*	*Ubc*	*Gusb*	*Tbp*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
10.5–18.5 dpc	(10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	***Polr2a****	***Ppia****	***Rplp0****	*Tbp*	*Hprt*	*Gusb*	*Ubc*	*B2m*	*Pgk1*	*Tfrc*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
10.5–12.5 dpc	(10.5, 11.5, 12.5)	***Sdha****	***Gusb****	*Ubc*	*Hprt*	*Tbp*	*Polr2a*	*B2m*	*Ppia*	*Rplp0*	*Pgk1*	*Tfrc*	*Actb*	*Rn18s*	*Gapdh*	*Ywhaz*
10.5–14.5 dpc	(10.5, 11.5, 12.5, 13.5, 14.5)	***Polr2a****	*Ppia*	*Rplp0*	*Tbp*	*Hprt*	*Gusb*	*Ubc*	*B2m*	*Pgk1*	*Tfrc*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
12.5–14.5 dpc	(12.5, 13.5, 14.5)	***Polr2a****	***Ppia****	*Ubc*	*Gusb*	*Hprt*	*Tbp*	*Rplp0*	*B2m*	*Pgk1*	*Tfrc*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
13.5–18.5 dpc	(13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	***Polr2a****	***Ppia****	*B2m*	*Rplp0*	*Hprt*	*Pgk1*	*Tbp*	*Ubc*	*Gusb*	*Tfrc*	*Rn18s*	*Actb*	*Sdha*	*Gapdh*	*Ywhaz*
Female gonad		Most stable ←														Least stable
	Period (sample) \ Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
All-period	(10.5, 11.5, 12.5, 13.5, 15.5, 17.5, neonate, 5-week-old, adult)	***Tbp****	***Ppia****	*Polr2a*	*Gusb*	*Rplp0*	*Ubc*	*Sdha*	*Pgk1*	*Actb*	*Hprt*	*Tfrc*	*B2m*	*Rn18s*	*Gapdh*	*Ywhaz*
Fetal-period	(10.5, 11.5, 12.5, 13.5, 15.5, 17.5)	***Gusb****	***Tbp****	*Polr2a*	*Ubc*	*Ppia*	*Rplp0*	*Sdha*	*B2m*	*Actb*	*Pgk1*	*Hprt*	*Tfrc*	*Rn18s*	*Gapdh*	*Ywhaz*
Postnatal period	(neonate, 5-week-old, adult)	***Hprt****	***Ubc****	*Ppia*	*Pgk1*	*Tbp*	*Rplp0*	*Polr2a*	*Gusb*	*Sdha*	*Actb*	*Tfrc*	*B2m*	*Gapdh*	*Rn18s*	*Ywhaz*
10.5–18.5 dpc	(10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	***Polr2a****	***Tbp****	*Gusb*	*Ppia*	*Ubc*	*Rplp0*	*B2m*	*Sdha*	*Actb*	*Pgk1*	*Hprt*	*Tfrc*	*Rn18s*	*Gapdh*	*Ywhaz*
10.5–12.5 dpc	(10.5, 11.5, 12.5)	***Gusb****	***Ubc****	*Ppia*	*Tbp*	*Sdha*	*B2m*	*Hprt*	*Polr2a*	*Rplp0*	*Gapdh*	*Tfrc*	*Actb*	*Pgk1*	*Rn18s*	*Ywhaz*
10.5–14.5 dpc	(10.5, 11.5, 12.5, 13.5, 14.5)	***Gusb****	***Ppia****	***Tbp****	*Ubc*	*Polr2a*	*Rplp0*	*B2m*	*Sdha*	*Hprt*	*Tfrc*	*Actb*	*Pgk1*	*Gapdh*	*Rn18s*	*Ywhaz*
12.5–14.5 dpc	(12.5, 13.5, 14.5)	***Tbp****	***Gusb****	***Ppia****	*Polr2a*	*Rplp0*	*Ubc*	*Pgk1*	*Actb*	*B2m*	*Hprt*	*Sdha*	*Tfrc*	*Gapdh*	*Rn18s*	*Ywhaz*
13.5–18.5 dpc	(13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	***Tbp****	***Polr2a****	*Ppia*	*Gusb*	*Ubc*	*Rplp0*	*Actb*	*Pgk1*	*B2m*	*Hprt*	*Sdha*	*Tfrc*	*Rn18s*	*Gapdh*	*Ywhaz*

Geometric mean ranking points are indicated with underline and astarisc 3 or less, underline 3 to 4 less than, gray and underline 7 or more, gray 10 or more.

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Table 4. Selected reference genes in each stages

All-period (10.5, 11.5, 12.5, 13.5, 15.5, 17.5, neonate, 5-week-old, adult)	
Male+Female	*Ppia*, *Polr2a*, *Rplp0*
Male	*Ppia*, *Polr2a*, *Rplp0*
Female	*Tbp*, *Ppia*, *Polr2a*, *Gusb*
10.5–18.5 dpc (10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	
Male+Female	*Polr2a*, *Ppia*, *Tbp*, *Rplp0*
Male	*Polr2a*, *Ppia*, *Rplp0*
Female	*Polr2a*, *Tbp*, *Gusb*
Sex determination period (10.5, 11.5, 12.5)	
Male+Female	*Gusb*, *Ubc*, *Tbp*
Male	*Sdha*, *Gusb*, *Ubc*
Female	*Gusb*, *Ubc*
Sex differentiation period (13.5, 14.5, 15.5, 16.5, 17.5, 18.5)	
Male+Female	*Polr2a*, *Ppia*, *Tbp*
Male	*Polr2a*, *Ppia*, *B2m*, *Rplp0*
Female	*Tbp*, *Polr2a*, *Ppia*
Postnatal period (neonate, 5-week-old, adult)	
Male+Female	*Hprt*, *Ppia*, *Pgk1*
Male	*Ppia*, *Rplp0*, *B2m*, *Tfrc*, *Polr2a*
Female	*Hprt*, *Ubc*, *Ppia*, *Pgk1*, *Tbp*

Geometric mean ranking points are indicated with underline 3 or less.

In our results, the ranking of recommended reference genes changed greatly depending on gender, developmental stage, and analysis period (Table 3). In particular, when we analyzed only a specific gender in a limited time period, it became clear that highly accurate measurement is possible by using a specific reference gene specialized for experiments (Table 4). In the developmental process of the gonads, the cell type, the number of cells, and the composition ratio contained in the gonad all change with time, and accordingly the expression of many genes, including the reference gene, changes greatly [8, 15, 17]. Because of the high risk of normalization with a single reference gene, it is recommended that multiple reference genes from different pathways be used [1, 11].

In this study, we ranked the stability of reference genes according to developmental stage and/or gender. This is very useful for selecting reference genes in gene expression analyses such as quantitative PCR in developing mouse gonads.

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