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1   Identification of stable reference genes for quantitative PCR in cells

2   derived from chicken lymphoid organs.

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4   Borowska D.[1], Rothwell L.[1], Bailey R. A.[2], Watson K.[2], Kaiser P.[1]

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7   [1]The Roslin Institute and R(D)SVS, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG,

8   United Kingdom

9

10   [2]Aviagen Ltd, Edinburgh EH28 8SZ, United Kingdom

11

12

13   Corresponding author: Dominika Borowska

14   Phone: (+44) 0131 651 9213

15   Fax: +44 (0) 131 651 9105

16   E-mail: dominika.borowska@roslin.ed.ac.uk

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21   **Abstract**

22   Quantitative polymerase chain reaction (qPCR) is a powerful technique for quantification of gene

23   expression, especially genes involved in immune responses. Although qPCR is a very efficient and

24   sensitive tool, variations in the enzymatic efficiency, quality of RNA and the presence of inhibitors

25   can lead to errors. Therefore, qPCR needs to be normalised to obtain reliable results and allow

26   comparison. The most common approach is to use reference genes as internal controls in qPCR

27   analyses. In this study, expression of seven genes, including β-actin (ACTB), β-2-microglobulin

28   (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase (GUSB), TATA box

29   binding protein (TBP), α-tubulin (TUBAT) and 28S ribosomal RNA (r28S), was determined in cells

30   isolated from chicken lymphoid tissues and stimulated with three different mitogens. The stability of

31   the genes was measured using geNorm, NormFinder and BestKeeper software. The results from both

32   geNorm and NormFinder were that the three most stably expressed genes in this panel were TBP,

33   GAPDH and r28S. BestKeeper did not generate clear answers because of the highly heterogeneous

34   sample set. Based on these data we will include TBP in future qPCR normalisation. The study shows

35   the importance of appropriate reference gene normalisation in other tissues before qPCR analysis.

36   Keywords: Reference gene; Chicken; Normalisation; Lymphoid tissues; qPCR

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38   **Introduction**

39   Transcriptional regulation in response to infections has been studied using different

40   techniques, for example northern blotting, cDNA microarrays, *in situ* hybridisation and quantitative

41   PCR (qPCR) (Matulova *et al.,* 2013; Sandford *et al.,* 2012; Bojesen *et al.,* 2004). The last technique

42   become a very popular tool in host-pathogen interaction studies because of its high sensitivity and

43   potential for high throughout and enhanced specificity. These characteristics are important in

44   immunological research where genes of interest frequently have many splice variants and very low

45   expression levels (Huggett *et al.,* 2005). It is therefore a very useful technique, especially in chicken

46   immunology, where species-specific antibodies are generally not yet commercially available.

47   Although qPCR is the most relevant technique, there are still many problems associated with its use,

48   mainly inherent variability of RNA, and differences in efficiencies of reverse transcription (RT) and

49   PCR (Bustin, 2002). To make analysis of qPCR reliable, the data need to be normalised using

50   reference genes (also known as internal controls or housekeeping genes). The process of selecting

51   internal control genes needs to be cogent to avoid errors in interpreting the mRNA quantification

52   results (Gantasala *et al.*, 2013). Reference genes usually have well-characterised and permanent

53   functions and, in theory, their expression is stable. Ideal reference genes have consistent expression in

54   varying experimental and environmental conditions. Expression of target genes can be normalised

55   with internal control genes in samples that vary in qualities and quantities of starting RNA. It also

56   compensates for differences in enzymatic efficiencies in individual templates because the reference

57   genes undergo the same preparation steps and are exposed to the same treatments as the gene of

58   interest. An ideal reference gene is yet to be identified (Bär *et al.*, 2009). Many authors suggest that a

59   definite or universal internal control gene for every condition in different tissues and cells does not

60   exist (Maltseva *et al.*, 2013; Coulson *et al.*, 2008; Vandesompele *et al.*, 2002). There are increasing

61   numbers of studies on widely used reference genes that prove many of them are not resistant to

62   changes in the experimental environment (Yang *et al.*, 2013; Yin *et al.*, 2011; Sugden *et al.*, 2010;

63   Yue *et al.*, 2010; He *et al.*, 2008; Bas *et al.*, 2004; Dheda *et al.*, 2004; Lupberger *et al.*, 2002;

64   Schmittgen and Zakrajsek 2000). It has therefore been suggested that determination of appropriate

65   reference genes should be performed for experiments involving a specific cell type or tissue with

66   different experimental settings before qPCR (Riemer *et al.*, 2012).

67   The use of reference genes as internal controls in qPCR normalisation studies is now a

68   standard procedure. Researchers have used many methods to identify reference genes. The most

69   popular strategies are the use of software and algorithms such as GeNorm (Vandesompele *et al.*,

70   2002), BestKeeper (Pfaffl *et al.*, 2004) and NormFinder (Andersen *et al.*, 2004). Many studies use

71   more than one of these programs as they differ in their underlying assumptions (Chang *et al.*, 2012;

72   Ledderose *et al.*, 2011; Perez *et al.*, 2008).

73   In this study, the stability of seven reference genes was measured with the aim of creating a

74   set of genes that could be used as internal controls in mRNA expression studies in chicken lymphoid

75   organs, and to confirm ribosomal 28S (r28S), which we have used as a reference gene in these studies

3

76   for fifteen years, was appropriate. A group of standard reference genes (ACTB, B2M, GAPDH,

77   GUSB, TBP, TUBAT, r28S) was chosen for evaluation of their mRNA expression in cells isolated

78   from the spleen, bursa and thymus and stimulated with different mitogens (see Materials and

79   Methods). The three softwares described above were used to calculate gene stability in an effort to

80   select the least variable genes as appropriate controls in future expression studies.

81

82   **Materials and methods**

83   *Tissue-cell collection and stimulation*

84   J-line layer chickens were bred and hatched at The Roslin Institute. Birds were reared in floor pens

85   and water and feed was provided *ad libitum*. Bursa of Fabricius, spleen and thymus were collected

86   from each bird and single-cell suspensions prepared by gently squeezing the tissues through a 40 μm

87   nylon strainer. Leukocytes were isolated with density gradient centrifugation for 20 min at 300 x g

88   using Histopaque 1.077 (Sigma-Aldrich, Poole, UK). Isolated cell numbers were adjusted to 5 x 10^6

89   cells/ml with pre-warmed RPMI media containing 10% CS. Cells were cultured in 25 mm^2 flasks for

90   4 h with the addition of 500 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin for bursal

91   cells, 1 μg/ml Concanavalin A (ConA) for splenocytes and 25 μg/ml phytohaemagglutinin (PHA) for

92   thymocytes.

93

94   *RNA extraction and cDNA synthesis*

95   Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Crawley, UK) according to the

96   manufacturer's protocol. The quality and quantity of the extracted total RNA was evaluated by

97   spectrophotometry using NanoDrop™ 1000. First-strand cDNA synthesis was performed using a

98   SuperScript III reverse transcription kit (Paisley, UK) containing random primers (Sigma-Aldrich)

99   and according to the manufacturer's instructions. The cDNA was stored at -20°C until further use.

100

101   *Gene selection and quantitative PCR*

102   Seven genes commonly used as reference genes in quantitative PCR (qPCR) gene expression

103   experiments were selected: beta-actin (ACTB, structural framework inside cells) (Gunning *et al.,*

104   1983); beta-2-microglobulin (B2M, part of the major histocompatibility complex) (Gűssow *et al.,*

105   1987); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, related to carbohydrate metabolism)

106   (Sirover, 1997); beta-glucuronidase (GUSB, involved in the breakdown of glycosaminoglycans)

107   (Shipley *et al.,* 1993); TATA box binding protein (TBP, indicates transcription start sites) (White *et*

108   *al.,* 1992); alpha-tubulin (TUBAT, forms and organizes microtubules) (Ludueña, 1997); 28S

109   ribosomal RNA (28S rRNA, structural RNA for the large component of cytoplasmic ribosomes)

110   (Wool, 1979). All qPCR primers were designed using Primer Express Software 3.0 (Life

111   Technologies) and synthesised by Sigma Aldrich. Primer sequences and amplicon lengths are shown

112   in Table 1.

113   Reaction mixes were prepared using the following components for each of the samples: 5 μl ABI

114   TaqMan Gene Expression Master Mix (Applied Biosystems, Paisley, UK, 0.5 μl 20X EvaGreen

115   (Biotum, VWR-Bie & Berntsen), 2.5 μl 20 μM specific primer (forward and reverse) and 10 μl of

116   water. Each reaction contained 2 μl of cDNA diluted 1:3 in low EDTA TE buffer. Quantitative PCR

117   was performed with an Applied Biosystems 7500 Fast Real-Time PCR System with the following

118   cycle profile: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles with denaturing for 15 s at 95°C,

119   and annealing/elongation for 1 min at 60°C. Melting curves were generated to confirm a single-PCR

120   product for each reaction (from 60°C to 95°C, increasing 1°C every 3 s). All reactions were

121   performed in duplicate and in each run internal standard curves (serial dilutions of a pooled cDNA

122   sample for each tissue-type, mock and antigen-stimulated) were used to assign relative concentrations

123   to the samples.

124   *Statistical analyses*

125   To select suitable internal controls, the stability of each gene was statistically analysed with three

126   software packages: GeNorm (Vandesompele *et al.,* 2002); NormFinder (Andersen *et al.,* 2004) and

127   BestKeeper (Pfaffl *et al.,* 2004). All three packages were used according to the supplied instructions.

128   The BestKeeper input format requires untransformed quantification cycle (Cq) values. The GeNorm

129   and NormFinder input formats require gene expression data to be transformed to relative quantities

130   (Q) where Cq values for each particular gene are normalised to the sample with the highest expression

131   level (the lowest Cq) by a delta Cq formula (Equation 1).

132    $$Delta\ Cq = \min Cq\ (reference\ sample) - Cq\ sample \qquad (1)$$

133   Relative quantities (Equation 2) are calculated based on PCR amplification efficiency (E) (Equation

134   3) that is derived based on the slope of the standard curve, by graphing the log of the DNA

135   concentration used versus Cq value for the sample (Supplementary file).

136    $$Q = E^{delta\ Cq} \qquad (2)$$

137    $$E = 10^{(-1/slope)} \qquad (3)$$

138   The three software programs generate measures of reference gene stability. geNorm, using Q values,

139   calculates the M value of a given gene based on the arithmetic mean of all pair-wise variations

140   between a particular gene and all other genes examined (Equation 4).

141    $$Mj = \frac{\sum_{k=1}^{n} Vjk}{n-1} \qquad (4)$$

142   where:

143   *Mj* – gene stability measure,

144   *Vjk* – pairwise variation of gene j relative to gene k,

145   *n* – total number of examined genes

146   geNorm calculates the optimal number of reference genes required for the analysis. Based on the

147   geometric mean of the expression levels, normalisation factors are calculated by stepwise inclusion of

148   an additional reference gene. If the subsequent gene causes a decrease in variation ($V_{n/(n+1)}$), it should

149   be included in the analysis as it has a significant effect. Vandesompele *et al.* (2002) suggest the M

150   value not to be greater than 1.5, where lower values indicate an increase in gene stability and decrease

151   in gene expression variability. The pair-wise comparison approach selects genes based on their degree

152   of similarity. Therefore, candidates with lower M values do not necessarily become top ranked. The

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153   pair-wise stability measure indicates if the addition of extra genes to the normalisation process is

154   beneficial and changes the accuracy of the analysis. NormFinder also relies on Q values to estimate

155   the stability of each gene. Quantities are first log-transformed and then used in an ANOVA model-

156   based approach to calculate expression variation where intra- and inter-group variations are estimated.

157   The two sources of variation represent systematic error that will occur when the given gene will be

158   used (Andersen *et al.,* 2004). BestKeeper analyses the expression stability using descriptive statistics:

159   geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) value, standard

160   deviation (SD) and coefficient of variance (CV). CV and SD values are used to determine the stability

161   of the reference gene expression, where the most stably expressed genes have the lowest CV and the

162   SD value is below one. Internal controls with SD higher than one can be regarded as unreliable. The

163   genes that are considered to be stably expressed are used to calculate a BestKeeper Index (BKI) as the

164   geometric mean. BestKeeper also analyses inter-housekeeping gene (HKG) relations using the

165   Pearson correlation coefficient (r) and the probability (p) value.

166   **Results and Discussion**

167   Quantitative PCR is now a standard technique to study RNA expression levels. To precisely

168   determine amplification of transcript fragments, normalisation strategies are necessary (Bustin, 2000).

169   There are several guidelines that can be followed to minimise inaccuracies in gene expression studies.

170   For example, uniform sample size, RNA extraction methods, reduction of gDNA contamination and

171   internal controls. These methods are not mutually exclusive and can all be included in the protocol

172   (Huggett *et al.,* 2005). Using a reference gene as an internal control for amplification of the mRNA is

173   the most commonly used and suitable technique (Radonić *et al.,* 2004). In this study, expression levels

174   of seven reference genes (Table 1) were measured in cells isolated from chicken lymphoid organs

175   (bursa, spleen and thymus) and then stimulated with different mitogens. To identify the most suitable

176   genes for normalisation of qPCR, the geNorm, NormFinder and BestKeeper algorithms were used.

177   All three software programs recognise control genes by determining their expression stability.

7

178   NormFinder, using calculated relative quantities, identified TBP as the most stably expressed

179 reference gene with a stability value of 0.070 followed by GAPDH (0.151) and 28S rRNA (0.155)

180 (Figure 1). For the best combination of two reference genes, the program suggested GAPDH and TBP

181 with a stability value of 0.083. Ribosomal RNA 28S performed equally well as GAPDH, whereas

182 B2M and TUBAT were the worst scoring genes in the panel, with stability values of 0.22 and 0.30

183 respectively.

184   Relative quantities as input data were also used in the geNorm algorithm (Figure 2A).

185 Analogous to NormFinder, geNorm identified TBP and GAPDH as the two most stable genes with an

186 average gene stability M score of 0.72, which is characteristic for heterogeneous samples, where the

187 acceptable M value should be lower than 1 (Hellemans *et al.,* 2007). The M value for r28S was

188 second lowest after the TBP M value, yet the program chose GAPDH as one of two ideal internal

189 controls in the tissue panel tested. The frequently used housekeeping gene ACTB performed poorly

190 and, according to the geNorm algorithm, should not be used as a reference gene in the samples tested.

191 The pair-wise variation $V_{n/(n+1)}$ for seven reference genes is shown in Figure 2B. The results suggested

192 that three reference genes were sufficient, but the inclusion of a fourth gene did not cause an increase

193 in the variation. Although the pair-wise variation cut-off value (0.15) has not been achieved, using at

194 least three of the most stable reference genes is in agreement with the recommendation from the

195 geNorm software developers.

196 Opposite to the previously described algorithms, BestKeeper uses a raw qPCR Cq as input data to

197 calculate descriptive statistics. Standard deviation values for all reference genes tested in this

198 experiment were higher than one. Therefore, all genes were disregarded from further analysis. In

199 contrast to geNorm or NormFinder, BestKeeper does not allow ranking of the reference genes using

200 the stability value and it does not suggest an optimal number of reference genes. The studied sample

201 set was very heterogeneous which theoretically invalidated the use of the Pearson correlation

202 coefficient. However, Pfaffl *et al.* (2004) mentioned very high correlation between lower Cq values

203 (UBQ, GAPDH, ACTB) and higher (18S) Cq values, which was the reason not to exclude 18S from

204 their index. BestKeeper and geNorm are based on the same principle. However, the two algorithms do

205   not always display overlapping reference genes (Cinar *et al.,* 2012). BestKeeper is robust against

206   sampling errors but it requires in-depth knowledge of the co-regulation of the candidate genes (Tong

207   *et al.,* 2009). Considering the BestKeeper statistical methods, modification of candidate gene

208   expression levels by any other gene in the panel could bias the results and any interpretation would be

209   false. Similar problems may be encountered when using the geNorm algorithm, as its pair-wise

210   comparison does not correct for co-regulation. NormFinder calculations, on the other hand, are not

211   influenced by co-regulation (Andersen *et al.,* 2004). Thus, inclusion of genes that represent a cross-

212   section of independent cellular functions should correct for putative co-regulation effects in the same

213   experimental settings (Riedel *et al.,* 2014). NormFinder and geNorm results in this study agree that

214   TBP is the most stably expressed gene among all seven candidates tested. Both softwares indicate

215   GAPDH as the best gene when combined with TBP.

216   There are few published studies on reference gene normalisation in chicken cells or tissues

217   and all of the existing results differ in their ranking of the genes. Most of the studies have focused on

218   chicken embryo fibroblasts (CEFs) as a virus infection model. The results of Yin *et al.* (2011)

219   indicated ACTB as the most stably expressed gene in CEFs infected with NDV and GAPDH along

220   with 18S as the least stable genes, based on their transcriptional profiles only. Yue *et al.* (2010) used

221   CEFs infected with AIV and in cell response studies YWHAZ was the most stable gene, whereas in

222   virus replication studies ACTB and RPL4 were the most reliable controls according to geNorm. The

223   same software was used to determine the best reference gene in CEFs infected with ALV-J. geNorm

224   ranked RPL30 and SDHA as the best candidates and ACTB and GAPDH as the least stable genes

225   (Yang *et al.,* 2013). de Boever *et al.* (2008) identified GAPDH and UBC together as the best pair of

226   internal controls in cells of chickens stimulated with LPS. In duck and chicken primary lung cells,

227   infected with LPAIV and HPAIV, GAPDH was ranked as the second best reference gene after 18S

228   (Kuchipudi *et al.,* 2012).

229   In this study, GAPDH and r28S were ranked as the second-most stable reference genes in

230   chicken lymphoid tissues. There have been many reports of GAPDH expression being unstable in

231   other experiments (Barber *et al.,* 2005; Lin *et al.,* 2012; Sudgen *et al.,* 2010). The use of ribosomal

232 RNA as a normaliser can be controversial, based on its technical limitations and can lead to its

233 exclusion from analyses (Lu *et al.*, 2013). The ubiquitous abundance of ribosomal RNA and lower

234 rate of degradation, compared to mRNA, may influence the results of qPCR (Vandesompele *et al.,*

235 2002). This is very important for studies on genes characterised with general low abundance where

236 smaller changes in relative expression cannot be detected. The cDNA require dilutions prior to qPCR

237 analysis using a ribosomal reference gene, which may introduce dilution errors. Nevertheless,

238 ribosomal RNAs, including r28S and r18S, has been shown to be stably expressed reference genes

239 (Wang *et al.,* 2011; Røge *et al.,* 2007). Li *et al.* (2005) reported that r28S was among few genes with

240 stable expression in CEFs infected with IBDV but in the same experiment B2M and TBP were the

241 least stable. In *in vitro* stimulation of human blood cells, TBP was a good reference gene in studies on

242 T lymphocytes, neutrophils and total blood leukocytes (Ledderose *et al.,* 2011).

243 Our own laboratory has used r28S as a reference gene for nearly two decades, as published in

244 more than 50 papers (e.g. Rothwell *et al.,* 2012; Wu *et al.,* 2009; Kogut *et al.,* 2003). This decision

245 was based on early studies in the laboratory, which were never published, comparing expression of

246 r28S, GAPDH, β-actin and ovotransferrin in splenocytes and thymocytes stimulated with a variety of

247 mitogens for various times (Kaspers, Rothwell, Kaiser, unpublished). Ribosomal 28S was by far the

248 most stably expressed of the four genes, and has thus been the laboratory standard housekeeping gene

249 since, until it was decided to revisit the subject with modern analyses.

250 The current study is the first published report of reference gene normalisation in stimulated

251 chicken lymphoid organ-derived cells. These results demonstrate the need to carefully select reference

252 genes for immune genes expression studies. Although this study showed that TBP, GAPDH and r28S

253 are suitable gene expression normalisers for chicken lymphoid cells, we strongly recommend testing

254 internal control genes before gene expression studies in other chicken tissues or cells.

255 **Acknowledgments**

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400

Figure 1. The NormFinder analysis of reference genes showing stability values. Lower stability value indicates gene that is more stable.
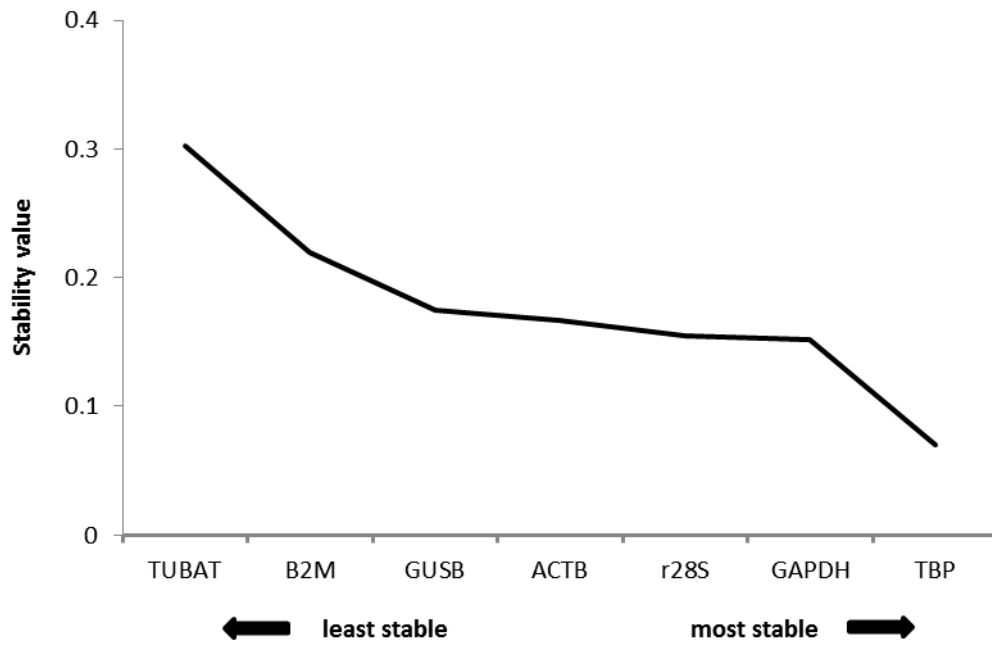
Figure 2. geNorm analysis. A) Average expression stability M of all seven reference genes. The most stably expressed genes have lower M values. B) Optimal number of reference genes required for reliable normalisation calculated by pair-wise variation analysis between normalisation factors NFn and NFn+1. According to geNorm, addition of a fourth gene has significant effect.

A
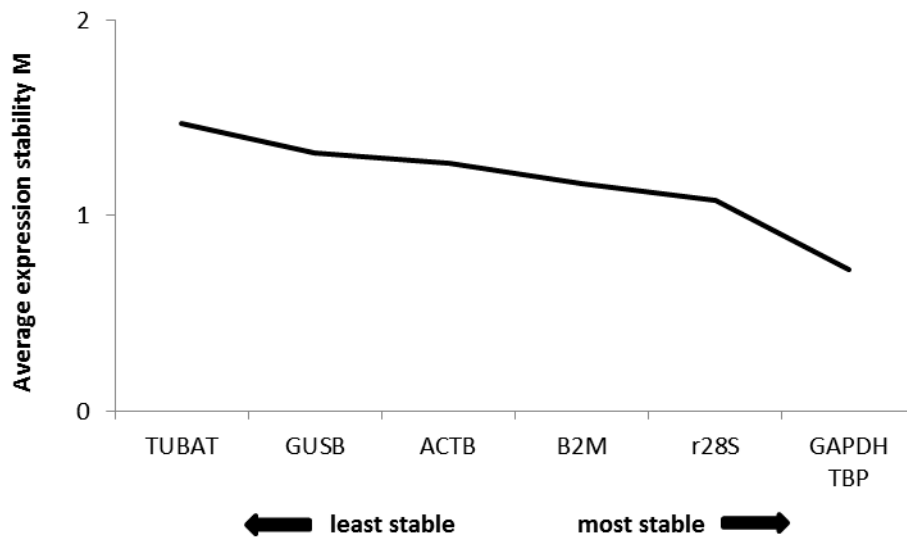
B
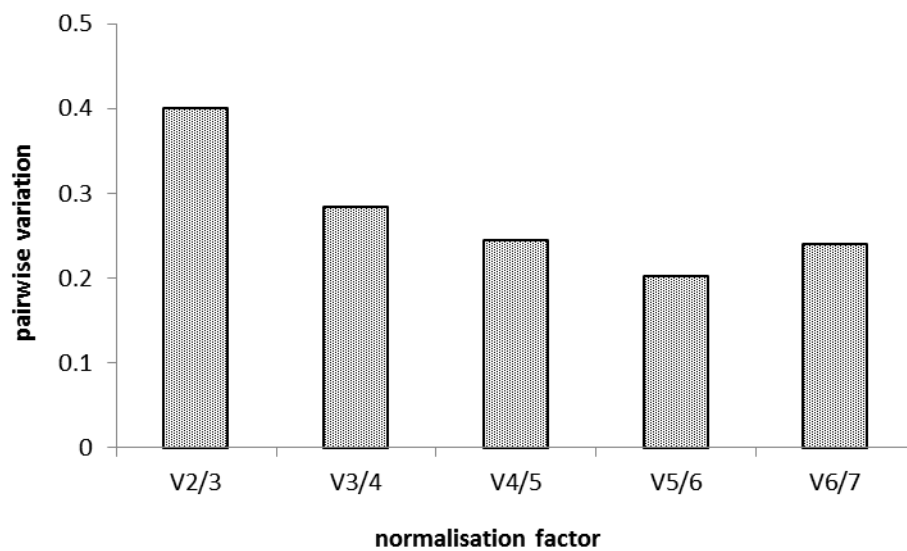
Table 1 Reference genes primer pair sequences and amplicon lengths

Gene	Sequence	Amplicon length (bp)	Accession number
ACTB	F: CCAGACATCAGGGTGTGATGG R: CTCCATATCATCCCAGTTGGTGA	137	AJ719605
B2M	F: TACTCCGACATGTCCTTCAACG R: TCAGAACTCGGGATCCCACTT	150	AB162661
GAPDH	F: GAAGGCTGGGGCTCATCTG R: CAGTTGGTGGTGCACGATG	150	AF047874
GUSB	F: GGCAGACTGGTCCTGTTGTTG R: GGGTCCTGAGTGATGTCATTGA	64	AJ720880
TBP	F: AGCTCTGGGATAGTGCCACAG R: ATAATAACAGCAGCAAAACGCTTG	134	AF221563
TUBAT	F: CAGCTCTCAGTGGCTGAAATCA R: CCTTGTTGCGGGTCACACTT	77	M16030
r28S	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	62	FM165415

Figure 1. Confirmation of specificity of primer pair products for seven reference genes using melting curve analysis (left panel). For analysis of reaction efficiency, standard curves were generated using log-fold serial dilutions of pooled cDNA from spleen cells, thymus cells and bursa cells (control and stimulated); Data for ACTB (a); B2M (b); GAPDH (c); GUSB (d); TBP (e); TUBAT (f); r28S (g).
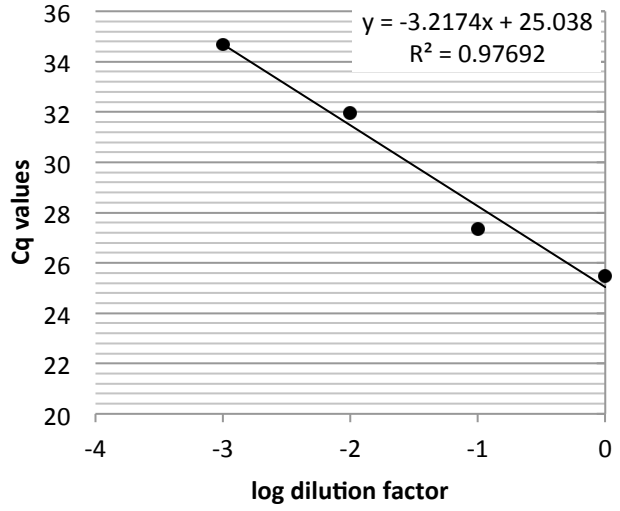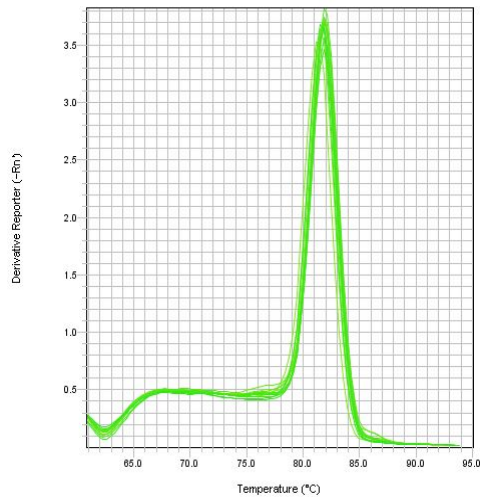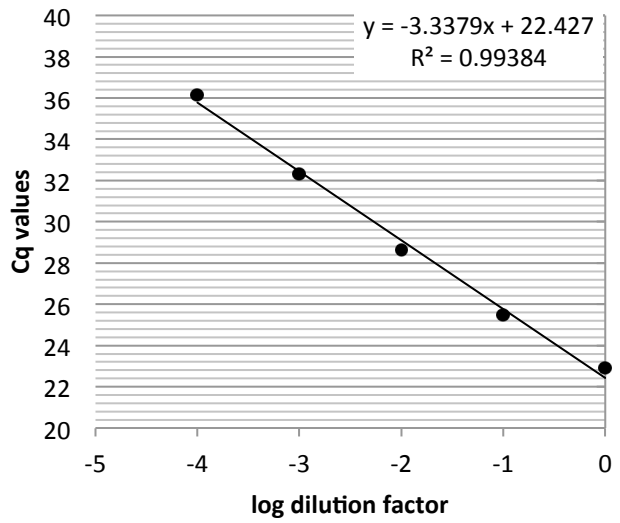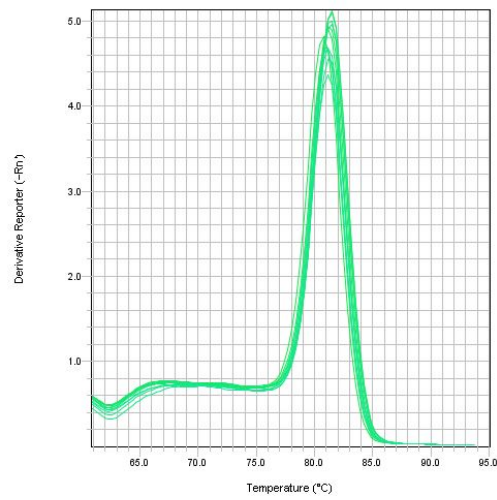
a)

b)

c)

y = -3.2174x + 25.038
R² = 0.97692

d)

y = -3.3379x + 22.427
R² = 0.99384

e)

y = -3.4005x + 28.695
R² = 0.99205

f)

$y = -3.1515x + 26.551$
$R^2 = 0.99041$

g)

$y = -3.2901x + 15.044$
$R^2 = 0.99828$