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Identification of stable reference genes for quantitative PCR in cells derived from chicken lymphoid organs.

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Abstract

Quantitative polymerase chain reaction (qPCR) is a powerful technique for quantification of gene expression, especially genes involved in immune responses. Although qPCR is a very efficient and sensitive tool, variations in the enzymatic efficiency, quality of RNA and the presence of inhibitors can lead to errors. Therefore, qPCR needs to be normalised to obtain reliable results and allow comparison. The most common approach is to use reference genes as internal controls in qPCR analyses. In this study, expression of seven genes, including β-actin (ACTB), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase (GUSB), TATA box binding protein (TBP), α-tubulin (TUBAT) and 28S ribosomal RNA (r28S), was determined in cells isolated from chicken lymphoid tissues and stimulated with three different mitogens. The stability of the genes was measured using geNorm, NormFinder and BestKeeper software. The results from both geNorm and NormFinder were that the three most stably expressed genes in this panel were TBP, GAPDH and r28S. BestKeeper did not generate clear answers because of the highly heterogeneous sample set. Based on these data we will include TBP in future qPCR normalisation. The study shows the importance of appropriate reference gene normalisation in other tissues before qPCR analysis.
Keywords: Reference gene; Chicken; Normalisation; Lymphoid tissues; qPCR

Introduction

Transcriptional regulation in response to infections has been studied using different techniques, for example northern blotting, cDNA microarrays, *in situ* hybridisation and quantitative PCR (qPCR) (Matulova *et al.,* 2013; Sandford *et al.,* 2012; Bojesen *et al.,* 2004). The last technique become a very popular tool in host-pathogen interaction studies because of its high sensitivity and potential for high throughout and enhanced specificity. These characteristics are important in immunological research where genes of interest frequently have many splice variants and very low expression levels (Huggett *et al.,* 2005). It is therefore a very useful technique, especially in chicken immunology, where species-specific antibodies are generally not yet commercially available. 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

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

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

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

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

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

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.

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.

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399

400

Figure 1. The NormFinder analysis of reference genes showing stability values. Lower stability value indicates gene that is more stable.

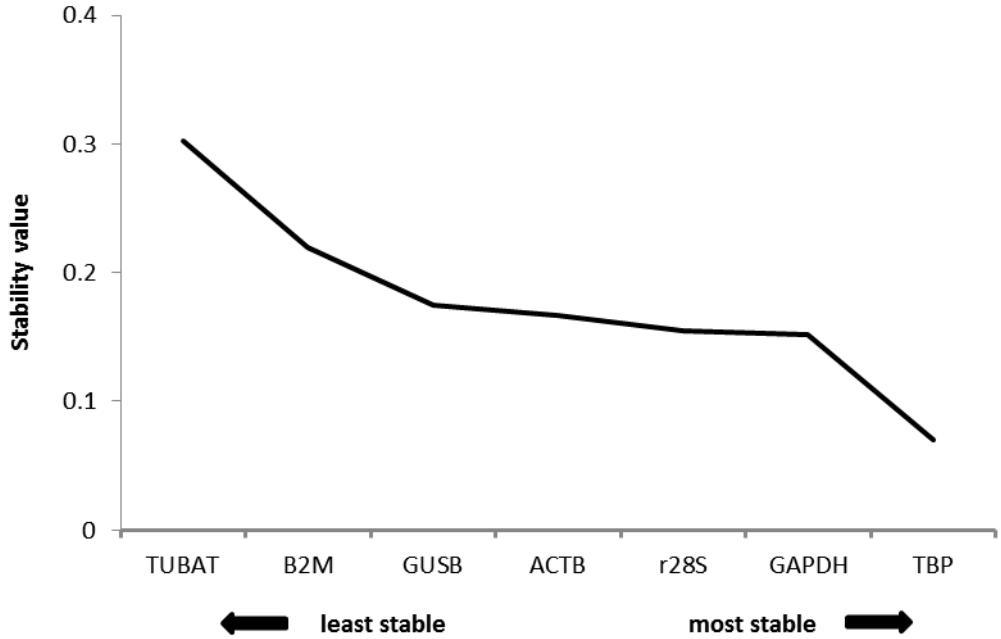
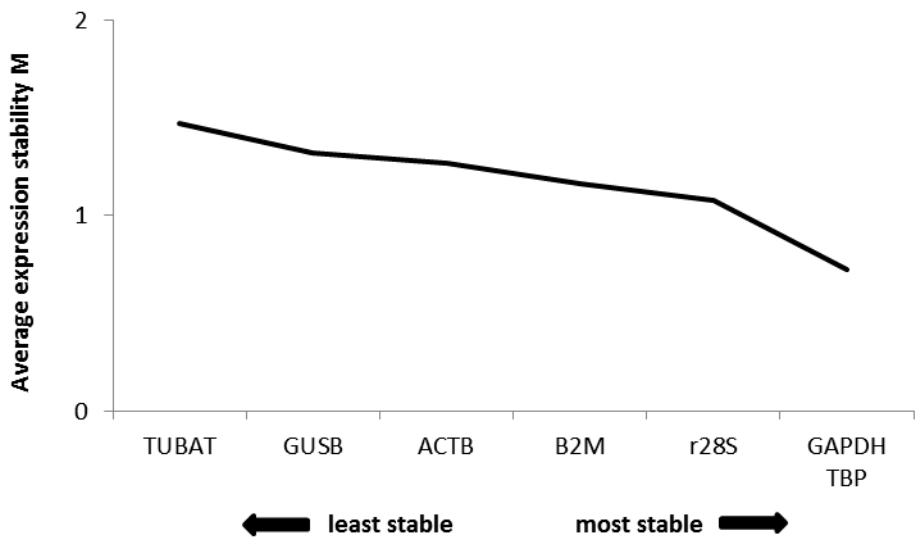


Figure 2. geNorm analysis. A) Average expression stability M of all seven reference genes. The most stably expressed genes have lower M values. B) Optimal number of reference genes required for reliable normalisation calculated by pair-wise variation analysis between normalisation factors NFn and NFn+1.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

A



B

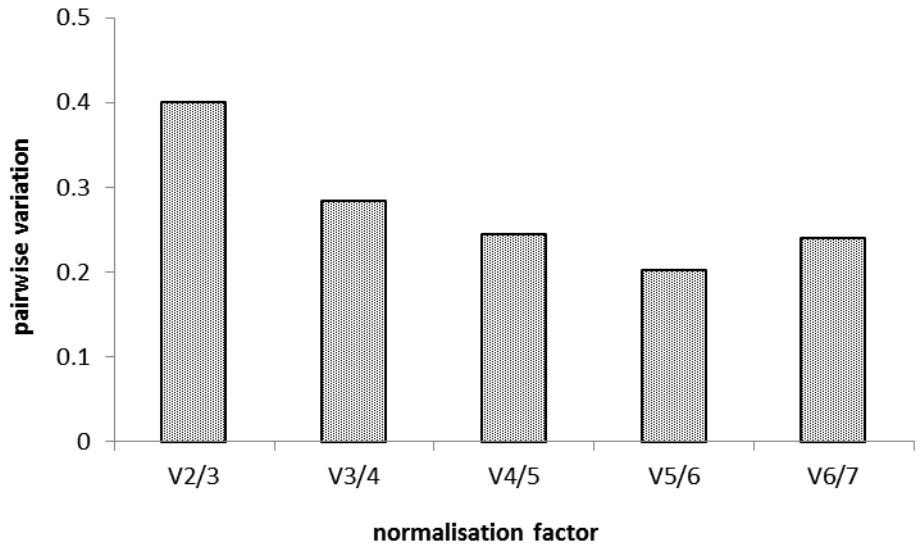
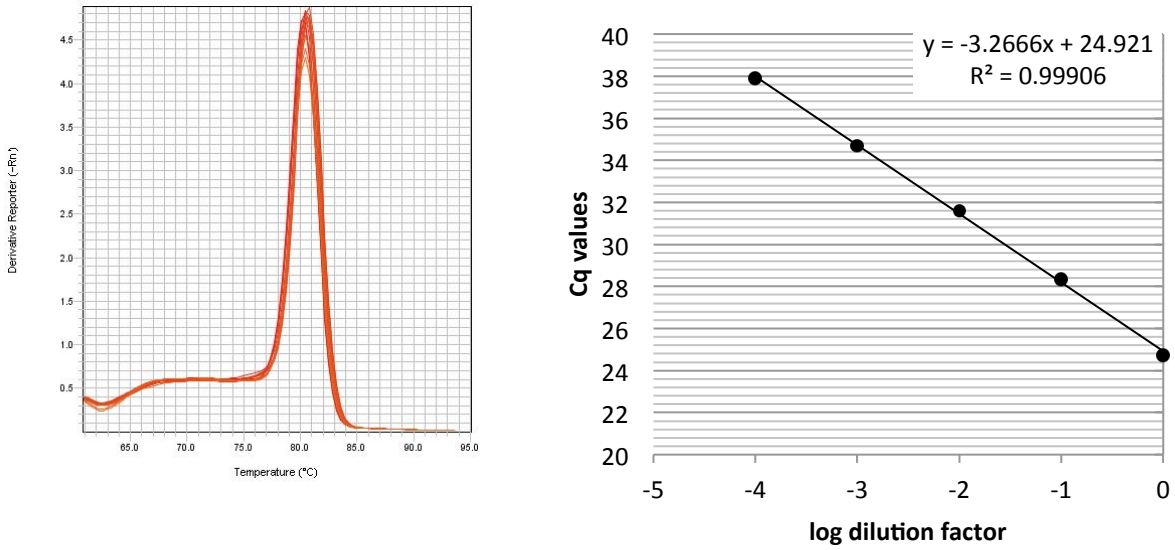


Table 1 Reference genes primer pair sequences and amplicon lengths

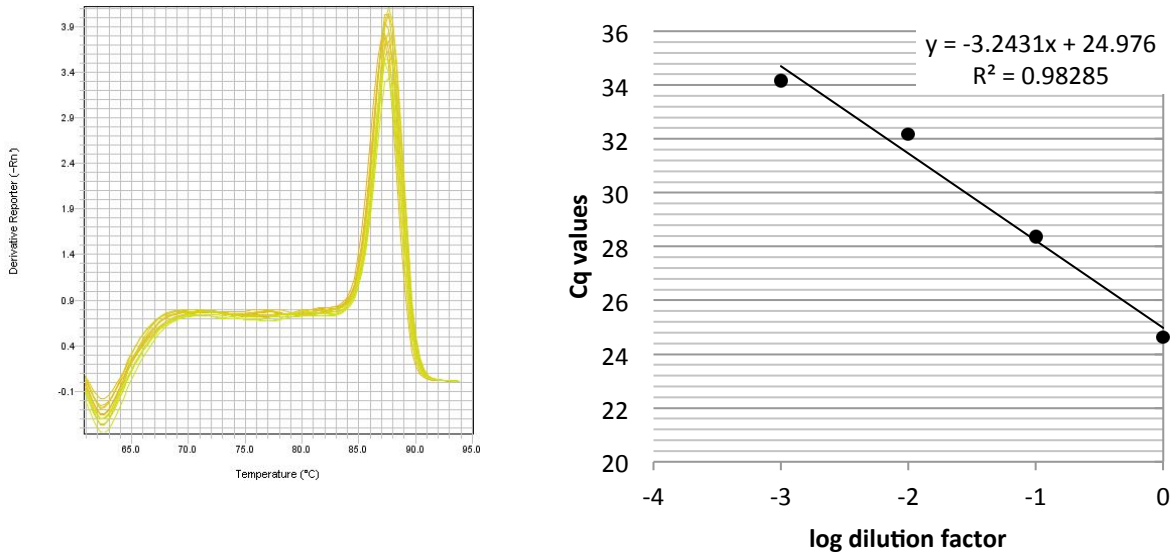
Gene	Sequence	Amplicon length (bp)	Accession number
ACTB	F: CCAGACATCAGGGTGTGATGG R: CTCCATATCATCCCAGTTGGTGA	137	AJ719605
B2M	F: TACTCCGACATGTCCTTCAACG R: TCAGAACTCGGGATCCCACTT	150	AB162661
GAPDH	F: GAAGGCTGGGGCTCATCTG R: CAGTTGGTGGTGCACGATG	150	AF047874
GUSB	F: GGCAGACTGGTCCTGTTGTTG R: GGGTCCTGAGTGATGTCATTGA	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).

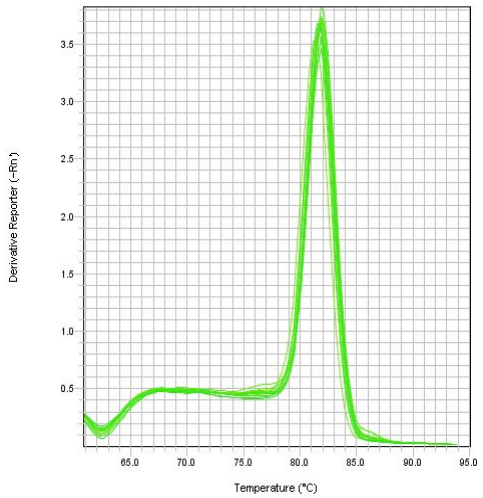
a)

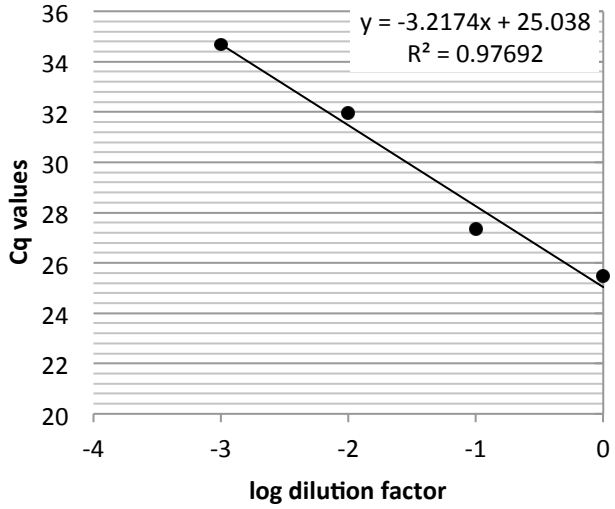


b)

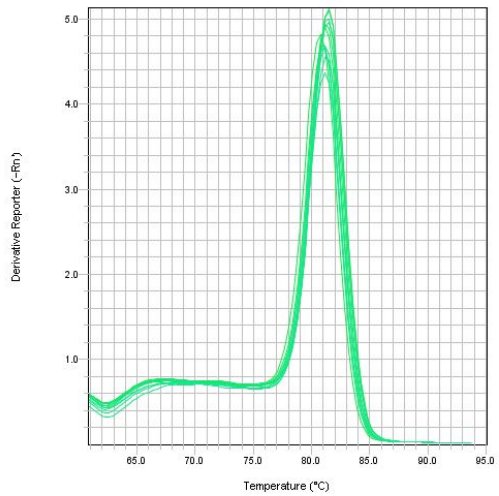


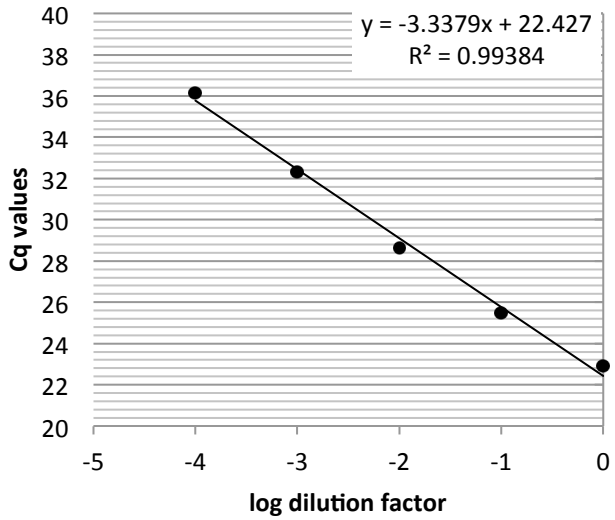
c)


Derivative Reporter (-Rn')
Temperature (°C)

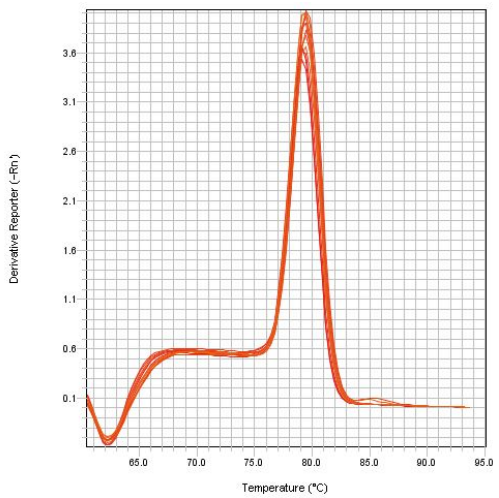

y = -3.2174x + 25.038
R² = 0.97692
Cq values
log dilution factor

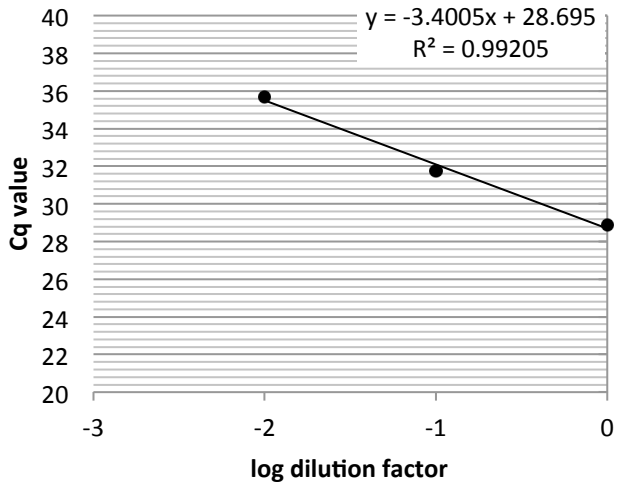
d)


Derivative Reporter (-Rn')
Temperature (°C)

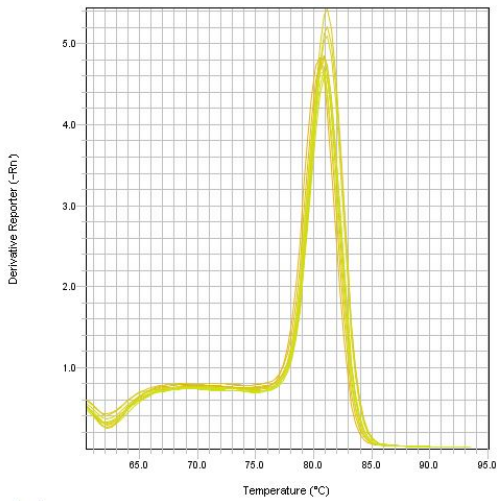

y = -3.3379x + 22.427
R² = 0.99384
Cq values
log dilution factor

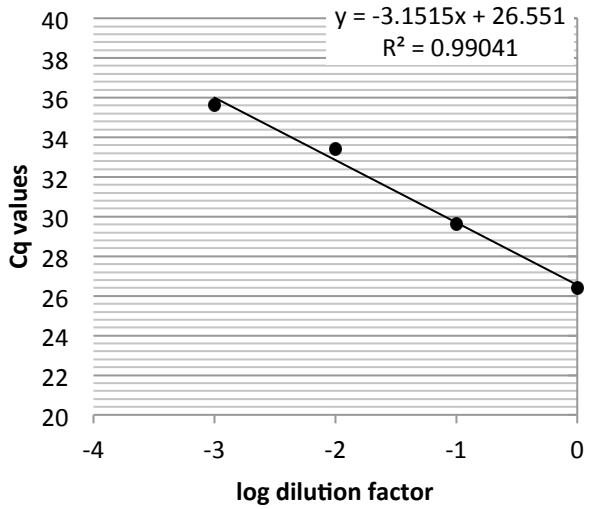
e)


Derivative Reporter (-Rn')
Temperature (°C)

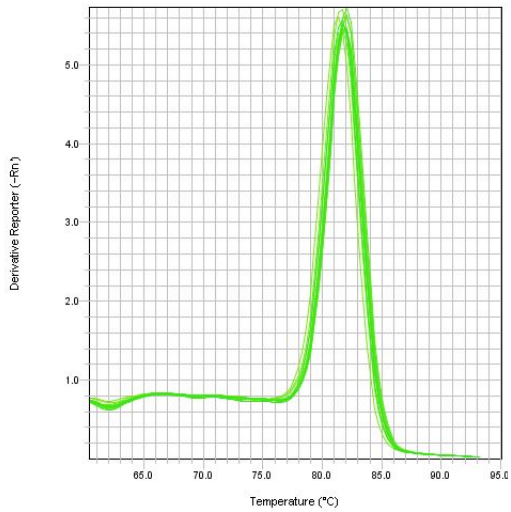

y = -3.4005x + 28.695
R² = 0.99205
Cq value
log dilution factor

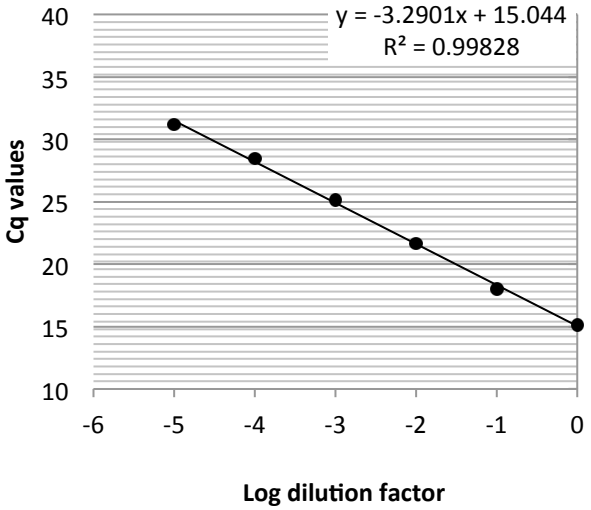
f)


Derivative Reporter (−Rn')
5.0
4.0
3.0
2.0
1.0
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Temperature (°C)


y = -3.1515x + 26.551
R² = 0.99041
Cq values
40
38
36
34
32
30
28
26
24
22
20
-4
-3
-2
-1
0
log dilution factor

g)


Derivative Reporter (−Rn')
5.0
4.0
3.0
2.0
1.0
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Temperature (°C)


y = -3.2901x + 15.044
R² = 0.99828
Cq values
40
35
30
25
20
15
10
-6
-5
-4
-3
-2
-1
0
Log dilution factor