Selection of housekeeping genes for gene expression normalization in chicken embryo fibroblasts infected with Newcastle disease virus

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Abstract

In this study, we described the first systematic selection and evaluation of suitable housekeeping genes (HKGs) for genes expression studies in chicken embryo fibroblasts (CEF) infected with Newcastle disease virus (NDV) which adopt the criterion of $\Delta Ct \leq +/-0.5$. Results from our experimental conditions indicated that ACTB, HPRT1 and HMBS were the stable HKGs, while 18S RNA, GAPDH and SHDA were precluded for normalization. Relative expression levels of B2M and IFN-a were normalized to these HKGs suggested that inappropriate HKGs selection can have profound influence on experimental outcome ranging from divergent statistical results to inaccurate data interpretation of significant magnitude.

Keywords: Newcastle disease virus, housekeeping gene, qRT-PCR, chicken embryo fibroblasts

With the current rise in NDV-caused economic losses [1] and as an oncolytic agent [2], real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has become a widely used method for assessment of target genes transcriptional profiles in NDV-related studies. However, a widely used method of correcting for intersample variability using qRT-PCR involves normalizing to one or more HKGs whose expression should not change by the treatment or between study conditions [3; 4]. Therefore, it is import to distinguish technical variability from true biological changes in gene expression. Meanwhile, to our knowledge, there are no studies regarding the choice of HKGs for gene expression studies in CEF infected with NDV. For this purpose, we chose to use guidelines previously described by Gorzelniak and Ferguson [4; 5], which the $\Delta Ct \leq +/-0.5$, that means relative expression levels of HKG fell in between 0.7 and 1.4, are considered fluctuation in gene expression that is largely due to technical variance that should be reflected similarly between both housekeeping and target genes; while $\Delta Ct > +/-0.5$ (relative expression beyond out between 0.7 and 1.4) are suggestive of biological variability resulting from treatment or experimental conditions, precluding the use of such HKG for target gene normalization.

In the present study, Newcastle disease virus strain NA-1 was purified directly from the

allantonic fluid and primary CEF was prepared from 10-day-old specific pathogen-free (SPF) chicken eggs have been described previously [6]. 0.4×10^6 CEF cells per well were seeded into 24-well plates 1 day before viral treatment. When the cells reached 70-80%, the cells were washed and overlaid with 100 µl serum-free E-MEM medium containing virus suspensions at a multiplicity of infection (MOI) of 10. Supernatant were completely removed at 12, 24, 48 and 72h after viral infection and 800 µl of ZR RNA Buffer (ZYMO, Beijing, China) were directly added into each well for total RNA isolation. First strand cDNA synthesis, qRT-PCR performance with SYBR green and statistical data analysis have been described in our previous studies [6; 7]. Sample reactions, including negative controls, were performed in triplicate in a 96 well plate. All experiments were performed by twice time. All validation data were converted to fold-changes using the $2^{-\Delta Ct}$ method for the raw data or $2^{-\Delta\Delta Ct}$ for normalized data. All experimental protocols were reviewed and approved by the Experimental Animal Council of Jilin University, China.

The transcriptional profiles of 6 commonly used HKGs (for full gene information see table 1 in supplementary material) in NDV infected with CEF over time up to 72h were examined using absolute Ct values. All HKGs showed moderate to high expression with a mean Ct values below 25 for each gene (Figure 1A). As shown in figure 1A, expression levels of 18S RNA was highest, with median Ct values at 13 cycles, followed by the HKGs GAPDH and SHDA, with median Ct values between 15 and 16 whereas ACT, HMBS and HPRT displayed the lowest levels with median Ct values between 21 and 22 cycles. Among the 6 HKGs, the maximum and the minimum variation of the expression range were 5.1 cycles for GAPDH and 1.7 cycles for HMBS, respectively. In a next step, these HKGs as a delimiter of HKG suitability were evaluated using the criterion of $\Delta Ct \leq +/-0.5$. As illustrated in figure 1B, relative expression of 50% HKGs tested (ACTB, HPRT1 and HMBS) fluctuated within the $\Delta Ct \leq +/-0.5$ limits in a manner that would be consistent with intersample variability and thus, considered suitable for use in target gene normalization in CEF following NDV treatment (Figure 1B). However, 18S RNA and SHDA were increased steadily above $\Delta Ct \leq +/- 0.5$ ranges at more than contiguous time point (Figure 1C); in contrast, GAPDH was the only one HKG that steadily and consistently dropped below the

88 ΔCt ≤ +/-0.5 at time points following 24 hours post-NDV infection (Figure 1C). The relative
89 fold changes for GAPDH, 18S RNA or SHDA were likely due to biological variability, thus
90 precluding these commonly used HKGs for suitability under this experimental condition.
91 To assess the impact of using GAPDH as a HKG, we examined relative expression of
92 B2M without and with normalization to either ACTB, the geometric mean of three stable
93 HKGs (ACTB, HMBS and HPRT1) or GAPDH in CEF treated with NDV over time up to
94 72h. B2M was chosen for this experiment as a target gene that is not responsive to NDV
95 infection [8]. As illustrated in figure 2B, fold changes in B2M gene expression when
96 normalized to ACTB or the geometric mean of three stable HKGs closely approximated fold
97 changes as shown for raw B2M without normalization (Figure 2A), where data points
98 following treatment were not significantly different from untreated control. In contrast, when
99 B2M was normalized to GAPDH, each data point where GAPDH drop down the ΔCt ≤ +/-
100 0.5 delimiter range was significantly different from untreated control (Figure 2B). Thus,
101 normalizing B2M to GAPDH resulted in an inaccurate gain of significance that did not occur
102 with ACTB or the geometric mean of stable HKGs.
103 To examine the impact of the HKGs which increase above the ΔCt ≤ +/- 0.5 limits on
104 target gene expression, relative changes of IFN-a gene expression, which is known for its
105 expression significantly increase upon NDV infection [9; 10], was validated without and with
106 normalization to either ACTB, the geometric mean of three stable HKGs (ACTB, HMBS and
107 HPRT1), SHDA or 18S RNA under the same experimental condition. Normalization of IFN-a
108 to ACTB or the geometric mean of three stable HKGs resulted in fold changes in gene
109 expression (Figure 3B) that were nearly identical to fold changes in raw expression without
110 normalization (Figure 3A) where 24h post-infection yielded 7.6-fold induction in target gene
111 expression that increased to 13-fold induction by 48h where it decreased to 5-fold induction
112 for 72h. These results were very similar to the well-documented expression profile of IFN-a
113 using conventional methodology during NDV infection [10; 11]. In contrast, however, the
114 increase in gene expression at 24h and 72h post-infection were not significance when IFN-a
115 was normalized to SHDA or 18S RNA, where the 7.6-fold and 5-fold induction of the HKG
116 ablated the 4.6-fold and 2.5-fold induction of the target gene (Figure 3B). Although INF-a

gene was significantly increased at 48h post-infection by 7-fold, remarkably enough there is a decrease of 5-fold induction as compared with normalization to ACTB or the geometric mean of three stable HKGs. Thus, normalizing IFN-a to SHDA or 18S RNA upon NDV infection over time up to 72h resulted in an inaccurate loss of significance that did not occur with ACTB or the geometric mean of three stable HKGs.

Data presented here and elsewhere clearly demonstrate that whether statistically significant differences between experiment groups are present or absent can depend on which HKG is used for normalization even when variability in HKG expression is marginal [4; 12]. Comparing the outcome of normalizing to among ACTB, 18S RNA or GAPDH, we present data from two genes; B2M whose raw profile did not vary over time following NDV treatment and IFN-a which increased under same experimental conditions with statistical significance. Neither the expression profile nor significance in gene expression of either gene was markedly influenced by normalized to ACTB or the geometric mean of three stable HKGs. In contrast, normalizing B2M to GAPDH resulted in an inaccurate gain of significance as well as IFN-a to either 18S RNA or SHDA resulted in an inappropriate loss of significance, respectively, demonstrating even small variation in HKG can have a significance influence on statistical outcome.

In summary, we validated six commonly used HKGs for the purpose of interpreting the impact of HKG selection on normalization of two target genes in chicken embryo fibroblast following NDV treatment over time up to 72h. Data are presented here demonstrating that using of unvalidated controls has led to flawed outcomes where reported changes in target gene expression were actually due to changes in HKG expression. Until HKGs are evaluated on an individual condition, the erroneous impact of inappropriate HKG selection on data interpretation and biological outcome will undoubtedly continue to contribute to inaccurate experimental conclusions and inconsistencies between reports.

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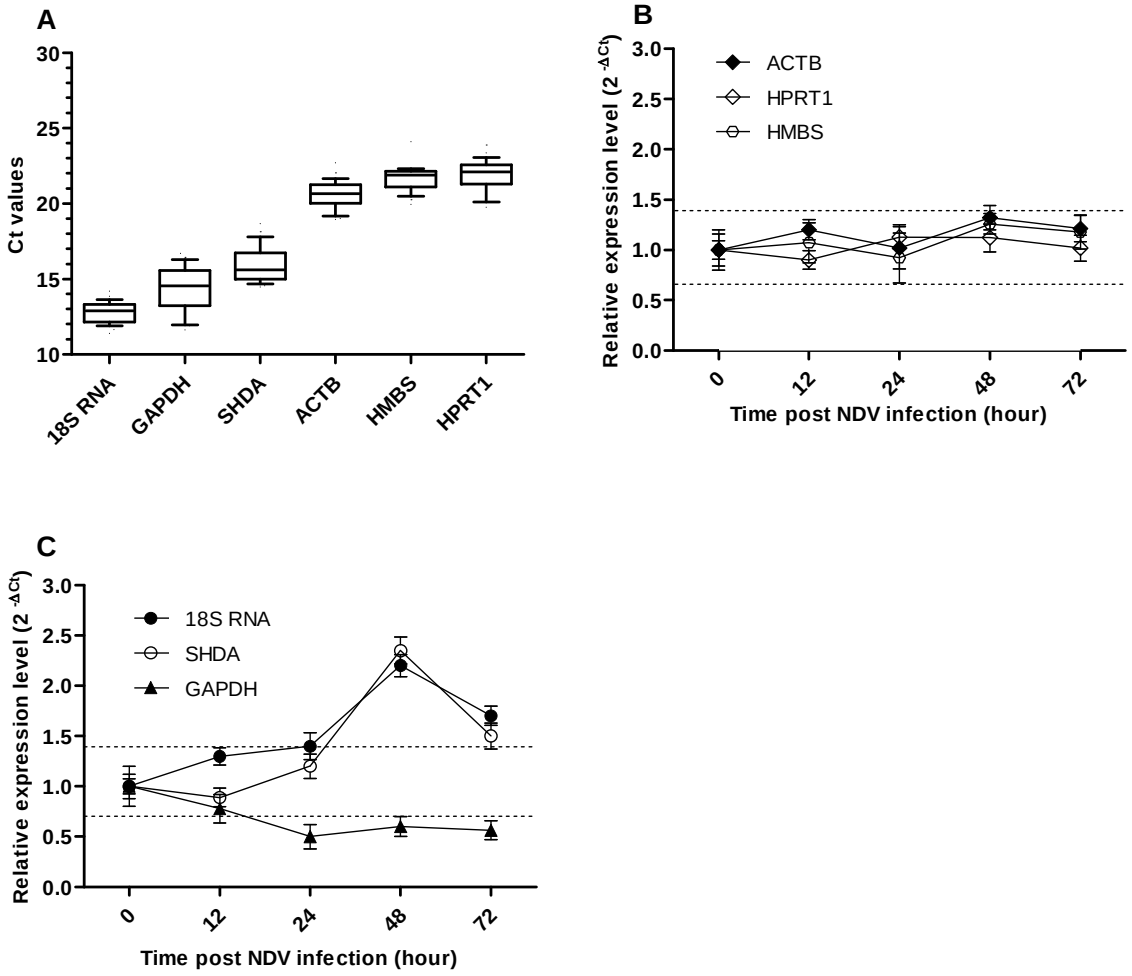
Fig.1. Transcriptional profiles of 6 housekeeping genes in chicken embryo fibroblasts over time up to 72 hours infected with Newcastle disease virus strain NA-1 at an MOI of 10. The raw cycle threshold (Ct) values of each housekeeping gene in all samples (n=27) are plotted in a box-and-whisker diagram (A). Boxes represent the interquartile interval (25-75%) with median value (50%); whiskers represent the 90^{th} and 10^{th} percentiles, respectively. Fold changes in housekeeping genes that fell within (B) and outside (C) the △Ct≤+/-0.5 limits of suitability (n=5-6).

Fig.2. Gain or loss of statistical significance of target genes with inappropriate housekeeping gene selection in CEF infected with NDV. Fold changes in both B2M gene expression without (A) or with normalization (B) to the geometric mean of three housekeeping genes (ACTB, SHDA and HMBS), ACTB, or GAPDH and IFN-a gene expression without (C) or with normalization (D) to the geometric mean of three housekeeping genes (ACTB, SHDA and HMBS), ACTB, SHDA, or 18s rRNA. Asterisks indicates significant differences between treated and untreated samples (n=5-6; *, $p<0.05$; **, $p<0.01$).

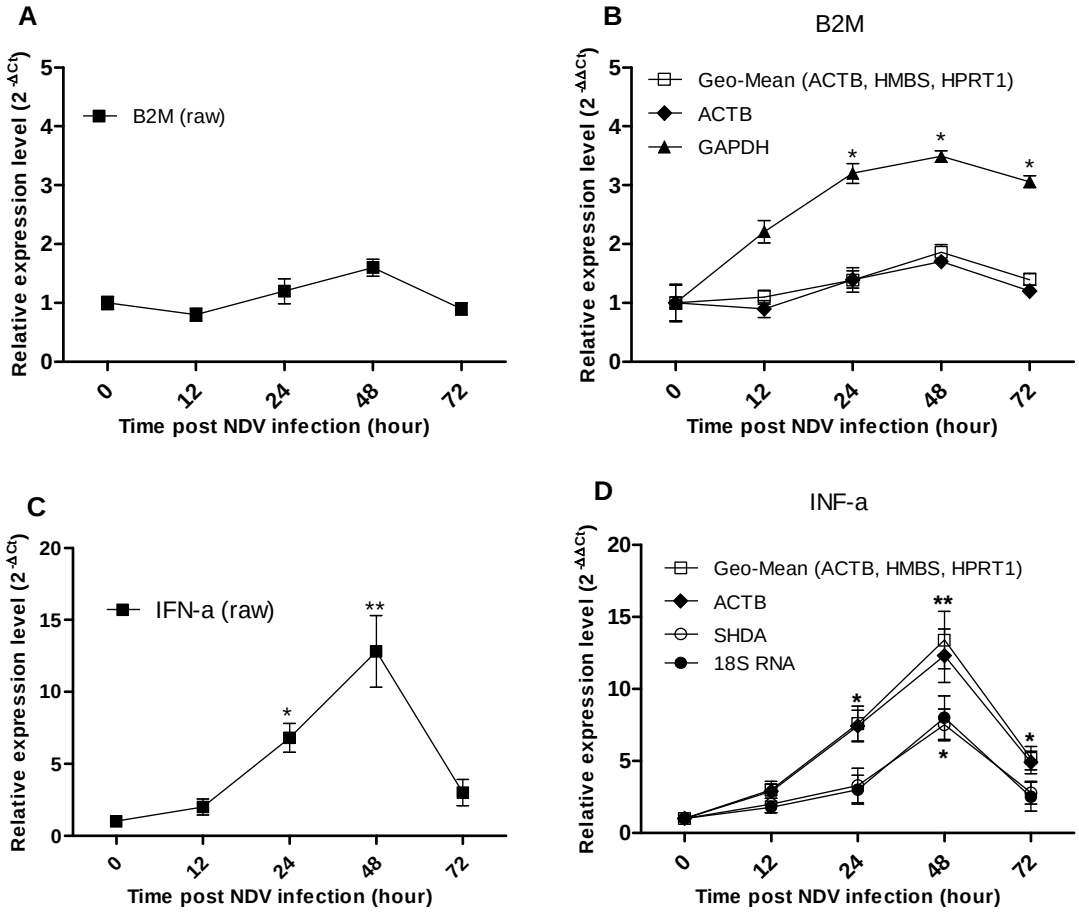
Table 1

Primer sequences and amplicon characteristics of housekeeping genes and target genes in this study

Symbol	Gene name	sequence	ampicon (bp)	Accession NO.
ACTB	beta-actin	F：cagacatcagggtgtgatgg R：tcaggggctactctcagctc	183	L08165.1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	F：tgggcagatgcaggtgctga R：tggtgcacgatgcattgctgaga	201	X01578.1
18S RNA	18S ribosomal RNA	F：ggcggctttggtgactctag R：atcgaaccctgattccccgt	148	AF173612.1
HMBS	hydroxymethylbilane synthase	F：ggctgggagaatcgcatagg R：tcctgcagggcagataccat	131	XM_417846.2
HPRT1	hypoxanthine phosphorribosyltransferase 1	F：tggtggggatgacctctcaa R：ggccgatatcccacacttcg	177	NM_204848.1
SDHA	succinate dehydrogenase complex, subunit A	F：ttcccgttttgcctacggtg R：ctgcctcgccacaagcatat	126	XM_419054.2
IFN-a	interferon alpha	F：agcaatgcttggacagcag R：aggcgctgtaatcgttgtct	123	GU119896.1
B2M	beta-2-microglobulin	F：aaggagccgcaggtctac R：cttgctctttgccgtcatac	151	NM_001001750.1


A
Ct values
30
25
20
15
10
18S RNA
GAPDH
SHDA
ACTB
HMBS
HPRT1
B
Relative expression level (2 -ΔCt)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ACTB
HPRT1
HMBS
0
12
24
48
72
Time post NDV infection (hour)
C
Relative expression level (2 -ΔCt)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
18S RNA
SHDA
GAPDH
0
12
24
48
72
Time post NDV infection (hour)

202
203 Fig.1


A
B2M (raw)
Relative expression level (2-ΔCt)
Time post NDV infection (hour)
B
B2M
Geo-Mean (ACTB, HMBS, HPRT1)
ACTB
GAPDH
Relative expression level (2-ΔΔCt)
Time post NDV infection (hour)
C
IFN-a (raw)
Relative expression level (2-ΔCt)
Time post NDV infection (hour)
D
INF-a
Geo-Mean (ACTB, HMBS, HPRT1)
ACTB
SHDA
18S RNA
Relative expression level (2-ΔΔCt)
Time post NDV infection (hour)

204

205 Fig.2