Published in final edited form as:

Arch Oral Biol. 2016 February ; 62: 33–42. doi:10.1016/j.archoralbio.2015.11.004.

Appropriate Real-Time PCR Reference Genes for Fluoride Treatment Studies Performed *In Vitro* or *In Vivo*

D. Faibish, M. Suzuki, and J.D. Bartlett*

Department of Mineralized Tissue Biology and Harvard School of Dental Medicine, The Forsyth Institute, 245 First Street, Cambridge, MA, USA

Abstract

Objective—Quantitative real-time PCR (qPCR) is routinely performed for experiments designed to identify the molecular mechanisms involved in the pathogenesis of dental fluorosis. Expression of reference gene(s) is expected to remain unchanged in fluoride-treated cells or in rodents relative to the corresponding untreated controls. The aim of this study was to select optimal reference genes for fluoride experiments performed *in vitro* and *in vivo*.

Design—Five candidate genes were evaluated: *B2m*, *Eef1a1*, *Gapdh*, *Hprt* and *Tbp*. For *in vitro* experiments, LS8 cells derived from mouse enamel organ were treated with 0, 1, 3 and/or 5 mM sodium fluoride (NaF) for 6 or 18 hours followed by RNA isolation. For *in vivo* experiments, six-week old rats were treated with 0 or 100 ppm fluoride as NaF for six weeks at which time RNA was isolated from enamel organs. RNA from cells and enamel organs were reverse-transcribed and stability of gene expression for the candidate reference genes was evaluated by qPCR in treated versus non-treated samples.

Results—The most stably expressed genes *in vitro* according to geNorm were *B2m* and *Tbp*, and according to Normfinder were *Hprt* and *Gapdh*. The most stable genes *in vivo* were *Eef1a1* and *Gapdh*. Expression of *Ddit3*, a gene previously shown to be induced by fluoride, was demonstrated to be accurately calculated only when using an optimal reference gene.

Conclusions—This study identifies suitable reference genes for relative quantification of gene expression by qPCR after fluoride treatment both in cultured cells and in the rodent enamel organ.

*Corresponding author at: Department of Mineralized Tissue Biology, The Forsyth Institute, 245 First Street, Cambridge, MA 02142, USA. Tel.: +1 617 892 8288; fax: +1 617 892 8510.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Form

The authors declare no conflicts of interest.

Ethical approval

All animals used in this study were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facilities (animal welfare assurance number: A3051-01) and were treated humanely based on a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Forsyth Institute. Experimental protocols were designed along institutional and National Institutes of Health guidelines for the humane use of animals.

Keywords

Fluoride; Reference genes; Real-time PCR; fluorosis; Gene expression

1. Introduction

Supplementation of drinking water with fluoride is an established method for prevention of caries.¹ The recommended level of water fluoridation in the U.S.A. is 0.7 ppm.² This level of fluoride was determined to present minimal risk for dental fluorosis and reduced the prevalence of caries, which is an important public health concern. In many areas of the world, natural levels of fluoride in drinking water are higher than the levels recommended for prevention of caries. The risk of dental fluorosis increases when the levels of fluoride in drinking water exceed 1.6 ppm.³ In recent years the prevalence of dental fluorosis has increased.⁴ Studies showed that fluoride plasma levels depend on weight, nutrition, growth status, renal function, and genetic background.^{5–8} However, the severity of fluorosis is positively associated with the concentration of fluoride within the plasma.^{9, 10} In the United States, prevalence of dental fluorosis among children aged 12–15 increased from 22.6% in 1986–1987 to 40.7% in 1999–2004.¹¹ Approximately a third of the 6–29 year old age group is affected by fluorosis, due to the expansion of water fluoridation and increased availability of additional sources of ingested fluoride such as processed foods.¹²

The mechanism by which high levels of fluoride induce defects in mineralization is the subject of active research. Dental fluorosis causes sub-optimal enamel mineralization, which can manifest as a chalky-white appearance and loss of transparency,^{13, 14} and this may have a negative impact on a child's quality of life.¹⁵ More severe fluorosis causes brownish enamel discoloration and weakening of the enamel that tends to chip from the teeth. The reduced mechanical properties of fluorosed enamel result in malfunction and increased susceptibility to decay.^{16, 17} Despite its normal thickness, fluorosed enamel is deficient in mineral and contains excessive matrix proteins.^{14, 18–23} Excessive levels of fluoride mainly affect developing unerupted teeth during the maturation stage of enamel development when the fully thick enamel layer begins hardening into its final form.^{18, 24–27} We have previously demonstrated that ameloblasts respond to excess fluoride in part by phosphorylation of eIF2 α ^{28, 29} and we have recently shown that expression of the proteinase kallikrein-related peptidase-4 (KLK4), responsible for cleaving enamel matrix proteins prior to their removal from the hardening enamel, is inhibited by fluoride treatment.³⁰ Serum fluoride levels in rat drinking water supplemented with 50 ppm fluoride are similar to humans ingesting 2–5 ppm fluoride.³¹ Clearance of fluoride from rodents is more efficient than clearance from humans.¹⁰ In addition, development of the rodent incisor takes approximately 35 days to complete whereas in humans tooth development takes at least three years.³¹ Thus, in humans, ameloblasts are exposed to fluoride present in drinking water for a longer time relative to rodent ameloblasts and this may be a contributing factor to the large difference in sensitivity to fluoride between humans and rodents.

Analysis of gene expression is routinely utilized to investigate fluoride toxicity *in vivo* and *in vitro*. Currently, a frequently used technique for gene expression analysis is quantitative

real-time polymerase chain reaction (qPCR). The qPCR workflow is comprised of RNA isolation, synthesis of cDNA, selection of primers, followed by the qPCR reaction and analysis of gene expression. Specific limitations were reported for each of these steps. For example, RNA isolation results in variable co-purification of PCR inhibitors, such as polyphenolics or polysaccharides. In addition, there are differences in primer amplification efficiencies.³² In an effort to standardize this workflow, a series of guidelines for design and analysis of qPCR experiments were published by Bustin et al.³³ A common method of analysis for physiological changes in gene expression by qPCR is relative quantification. Relative quantification experiments are designed to compare the expression of treated samples with non-treated samples from the same group. The basic assumption of this design is that the treatment to which the samples were exposed results in differences in gene expression.

Accepted analyses of relative quantification experiments are the Ct method³⁴ and the primer efficiency method.³⁵ The Ct method assumes an ideal amplification efficiency of 2, meaning that the quantity of amplicon doubles in each cycle of amplification. In reality, experimental conditions determine the efficiency of amplification. The primer efficiency method takes into account differences in amplification, and relies on estimation of the efficiency from a standard curve. An amplification efficiency value greater than 1.8 is regarded as appropriate.³⁶ In both methods, the expression of a chosen target gene is calculated relative to one or more reference genes. The reference gene or genes are required to be unaffected by the experimental treatment.³⁴ The efficiency of amplification of the reference gene should be similar to that of the target gene. When fluorescence incorporated into the qPCR amplification product rises above background level, the quantification cycle (C_q) is recorded for each sample in the exponential phase of the PCR reaction.

Genes involved in recurrent cell processes, such as amino acid synthesis, are termed “housekeeping genes” and these genes are typically used as reference genes for qPCR. The expression level of housekeeping genes, however, may change depending on the experimental conditions.^{37–44} For example, the expression of β -actin (*Actb*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNAs were found to fluctuate upon activation of T-lymphocytes.⁴³ The use of inappropriate reference genes may lead to inaccurate and misleading quantification of target gene expression.^{45, 46} Results from qPCR experiments should accurately reflect gene expression levels and this can be obtained by using a minimum of three biological replicates.⁴⁷

In fluoride experiments, expression levels of reference genes must remain stable regardless of fluoride treatment. The aim of the current study was to identify optimal qPCR reference genes for NaF treatments both *in vivo* and *in vitro*. Four commonly-used reference genes were tested *in vivo*, and five were tested *in vitro*: beta-2 microglobulin (*B2m*), transcription factor TATA binding protein (*Tbp*), hypoxanthine phosphoribosyltransferase (*Hprt*) and eukaryotic translation elongation factor 1 alpha 1 (*Eef1a1*). These genes have a variety of cellular functions: *B2m* is a component of major histocompatibility complex (MHC) class 1, and a cell surface marker for all nucleated cells,⁴⁸ *Tbp* is a member of the family of TATA-box transcription factors,⁴⁹ *Gapdh* is a glycolytic enzyme,⁵⁰ *Hprt* is part of purine synthesis in the salvage pathway,⁵¹ and *Eef1a1* functions in the translational machinery.⁵² The data

presented here will facilitate accurate and reproducible transcript profiling studies in fluoride treated rats and in enamel organ-derived LS8 cells.

2. Materials and Methods

2.1. Animals

All animals were treated humanely. Sprague–Dawley rats (6-week-old) were purchased from Charles River Laboratories (Wilmington, MA). Animals were euthanized by CO₂ inhalation after 6 weeks of fluoride treatment. Three rats were used in each group. Incisor enamel organs in the maturation stage of enamel development, were separated from the secretory stage by appearance and hardness. A micro-scalpel blade that slices into soft enamel dissects the secretory stage enamel organ whereas hard resistant enamel is in the maturation stage. RNA from the maturation stage was isolated for use in qPCR assays.

2.2. Cell Culture

LS8 cells⁵³ were maintained in alpha minimal essential medium with GlutaMAX supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate (Life Technologies, Grand Island, NY) and 3×10^5 were plated for experiments. NaF (Cat. S299-100, Fisher Scientific, Pittsburgh, PA) was used as indicated. Fluoride concentrations of 1, 3, or 5 mM were used for the LS8 experiments. Rats were provided water containing 0 or 100 ppm fluoride as NaF *ad libitum*.

2.3. RNA Isolation and Processing

Total RNA was isolated from LS8 cells or rat enamel organs (Direct-zol™ RNA MiniPrep, Zymo Research Corp., Irvine, CA) according to the manufacturer's instructions. Briefly, cells were lysed using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA). Lysates were transferred to Zymo-Spin IIC Columns. Columns were then washed using Direct-zol™ RNA PreWash buffer and RNA was eluted using RNA Wash Buffer. RNA concentration was assessed by use of a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA). A 260/280 ratio of approximately 2.0 indicated high purity RNA. RNA integrity was assessed by gel electrophoresis (Bioanalyzer, Agilent Technologies, Santa Clara, CA). Total RNA (1 µg) was reverse-transcribed into cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Minneapolis, MN). The reaction mixture was incubated for 10 min at 25°C, 120 min at 37°C and 5 sec at 85°C. Primers (Table 1) were designed using Primer Blast.⁵⁴ Melting temperature was limited to 60°C ± 1°C, primer length 18–25 bases, GC content 40–60%, and product size 60–150 base pairs. Primers were designed for *B2m*, *Eef1a1*, *Gapdh*, *Hprt*, *Tbp* and *Ddit3* (Table 1).

2.4. qPCR

cDNA was subjected to real-time PCR amplification on a Light Cycler 480 System using SYBRGreen I Mastermix (Roche Diagnostics Corporation, Indianapolis, IN). The PCR protocol started with a heat activation step of 95°C for 10 min. Then, 40 cycles of thermocycling were performed with a denaturation step at 95°C for 30 sec, an annealing step at 60°C for 30 sec and an extension step at 72°C for 15 sec. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by

incubating the product at 95°C for 5 sec, 65°C for 1 min, and then slowly heating to 97°C. Fluorescence was measured through the slow heating phase. Melting curves were used to ensure that only one PCR product was amplified. C_q was measured using the baseline-independent second derivative maximum method.⁵⁵ A standard curve was prepared for each primer set from the C_q values of 5-point 5-fold serial cDNA dilutions. Each dilution was assayed in triplicate. The assay amplification efficiency, also termed primer efficiency, is the derivation of the linear regression of the standard curve ($E = 10^{(1/-\text{slope})-1}$). This value represents the increase in the quantity of amplicon in each qPCR cycle. In an ideal reaction the amplification efficiency is 2. No-template controls were negative in all runs. C_q was smaller than 40 for all reactions. C_q was smaller than 0.5 for all replicates.

2.5. qPCR Data Analysis

Mean C_q values for treated and non-treated samples were recorded for each assay. Fold change for each sample and for each target gene was calculated from raw expression values using primer efficiencies from the standard curves.³⁵ The stability of reference genes was calculated from these fold change values using two methods. The first method was based on the geNorm algorithm using the SLqPCR package (version 1.28.0) on R (version 3.0.2).⁵⁶ According to this method, base 2 logarithm for the ratio of fold change values of all pairs of target genes was calculated for all samples. The standard deviation of the result for all treatments for these two target genes was then calculated. An average of the standard deviations for all target gene combinations was calculated and presented as stability (M).^{56, 57} The second method was based on the NormFinder algorithm using a Microsoft Excel Add-In (version 0.953) downloaded at <http://moma.dk/normfinder-software>.⁵⁷ This method ranks the genes with the smallest intra- and intergroup variation.

3. Results

3.1. Analysis of Reference Gene Expression after 6 h or 18 h Fluoride Treatment

LS8 cells were treated with NaF at 0, 1, 3 or 5 mM for 6 hours (Fig. 1) and at 0, 1 or 3 mM for 18 hours (Fig. 2). RNA was isolated at the respective time points and cDNA was prepared. PCR reactions were performed to identify consistency of expression of the candidate genes at multiple doses. The candidate genes tested were: *B2m*, *Eef1a1*, *Gapdh*, *Hprt*, and *Tbp*. Small variations in fold change were observed in the expression of *B2m* and *Tbp*, and a higher variation was observed in the expression of *Eef1a1* at 6 hours (Fig. 1) and at 18 hours (Fig. 2) relative to untreated cells.

3.2. Analysis of Reference Gene Expression Stability

Multiple algorithms have been developed to assess stability of reference genes.^{56–59} In this study, the geNorm and the Normfinder algorithms were used for analysis of gene stability.⁵⁶ In the 6 hour experiment, *B2m* and *Tbp* had the lowest standard deviation (0.03) and therefore the highest stability according to geNorm (Fig. 1F). *Eef1a1* had the highest standard deviation (0.37) and the lowest stability (Fig. 1F). After 18 hour fluoride treatment, *B2m* and *Tbp* had the lowest standard deviation (0.12) according to the geNorm method (Fig. 2F) and *Eef1a1* had the highest standard deviation (0.5). According to Normfinder, in the 6 hour experiment *Hprt* had the lowest standard deviation (0.06), and *Eef1a1* the highest

(0.41) (Figs. 1G). By the same method, in the 18 hour experiment, *Gapdh* had the lowest standard deviation (0.04), and *Eef1a1* the highest (0.5) (Fig. 2G).

3.3. Effect of Reference Gene Choice on Quantification of Experimental Gene Expression

DNA-damage inducible transcript 3 (*Ddit3*, Chop, Gadd153) is a stress response gene that increases its expression with fluoride treatment.²⁸ In order to test whether utilization of different reference genes affects the quantification of target gene expression, qPCR reactions were performed for *Ddit3* at 6 and 18 hours after exposure to NaF (Fig. 3). *Ddit3* expression was calculated using both the $\Delta\Delta C_T$ method³⁴ and the primer efficiency method.³⁵ Using both methods, NaF-treated *Ddit3* expression values were calculated relative to *Ddit3* expression in non-treated cells. As expected, normalization of *Ddit3* using a geometric mean of the three most stable genes resulted in *Ddit3* expression values (Fig. 3A,D) that were very different than when calculated relative to the reference gene *Eef1a1* (Fig. 3B,E).

Normalization of *Ddit3* was also performed relative to *B2m* only, and the result was similar to normalization with three most stable genes (Figs. 3C, F). Results were similar when the efficiency method was used for analysis (Fig. 4). Similar to the $\Delta\Delta C_T$ method, normalization to *Eef1a1* induced an overestimation of *Ddit3* levels in cells that were treated with NaF for 6 or 18 hours.

3.4. Analysis of Reference Gene Expression In Vivo

To test reference gene expression *in vivo*, drinking water for 6-week-old male Sprague Dawley rats was supplemented with 0 or 100 ppm fluoride as NaF. After 6 weeks, rats were sacrificed and RNA was extracted from enamel organs. Maturation-stage rat incisor enamel organs were dissected and RNA was isolated. qPCR analysis of these samples showed the smallest variation in expression of *Gapdh* and *Eef1a1* (0.18), and a higher variation for *Hprt* (0.27) (Fig. 5E) according to the geNorm method. By Normfinder, *Gapdh* had a variation of 0.06 and *Eef1a1* had a variation of 0.13 (Fig. 5F).

4 Discussion

Relative quantification of genes of interest in qPCR experiments depends on selection of appropriate reference genes. Optimal reference gene expression remains unchanged regardless of experimental conditions. Reference gene normalization greatly improves statistical significance, power and may dramatically reduce sample size. In this study, the goal was to evaluate stability of candidate reference gene expression upon treatment with NaF.

Ameloblast-derived LS8 cells were treated for 6 or 18 hours with NaF and the expression of five potential reference genes was tested: *B2m*, *Eef1a1*, *Gapdh*, *Hprt* and *Tbp*. The most stable genes in these experiments were *B2m* and *Tbp* by geNorm and *Gapdh* and *Hprt* by Normfinder. In order to test the effect of choosing an appropriate gene for normalization, the expression of *Ddit3*, an ER stress response gene shown to be upregulated by NaF, was assessed in these experiments relative to the *B2m* and *Eef1a1* reference genes. *Ddit3* expression levels relative to *Eef1a1* were higher than if calculated relative to the more stably expressed *B2m* gene. Similar results were obtained when using the $\Delta\Delta C_T$ or the efficiency

methods of analysis. Data in this *Ddit3* experiment was normalized to the most stable gene⁵⁶ or to the three most stable genes.⁶⁰ The results in both cases were comparable and therefore the benefit of using multiple reference genes must be considered. For example, a small number of targets of interest may only require one or two reference genes.

In a separate experiment, the expression of *B2m*, *Eef1a1*, *Gapdh* and *Hprt* was tested in samples from the enamel organs of 6-week-old male Sprague-Dawley rats treated with 100 ppm fluoride. *Gapdh* and *Eef1a1* were the most stable genes in this experiment using both the geNorm and Normfinder algorithms. The differences in gene expression *in vitro* versus *in vivo* underscore the importance of testing genes under specific experimental conditions. In the above-described experiments the variability in gene expression of the more stable genes was relatively low. The differences in output of the geNorm and Normfinder algorithms reflect the different statistical approaches to data analysis.⁵⁷ Normfinder uses a mathematical model that describes gene expression values, analyzes the sample subgroups separately, estimates both the intra- and inter-group variation of gene expression, and calculates candidate gene stability. geNorm employs a pairwise comparison, and therefore selects genes with the highest degree of similarity in expression across the sample set. geNorm, however, tends to select genes that are correlated. The NormFinder algorithm on the other hand is less robust in experiments with a small sample size.⁵⁷ Nevertheless, according to both methods the least stable gene observed *in vitro* was *Eef1a1*, while the others had a standard deviation lower than the accepted threshold of 0.15.⁵⁶

The differences observed between the *in vitro* and *in vivo* experiments likely reflect the differences in the starting material. The cell population in the *in vitro* experiments was homogenous, and the treatment with fluoride was acute (6 hours or 18 hours). The enamel organ is composed of ameloblasts and connective tissue cells, and the contribution of each subpopulation may explain the differences in gene expression relative to the *in vitro* experiments. In addition, fluoride treatment *in vivo* was chronic (3 weeks of fluoride-supplemented drinking water) providing an additional source of variation by eliciting a more complex cellular response.

Given the risk of inducing reference gene expression instability when altering experimental conditions, it is recommended to validate a stable set of reference genes as an initial and an essential step in all qPCR experiments. A recent review presented the software packages that were developed for qPCR analyses.⁶¹

In conclusion, qPCR experiments must first validate the stability of reference gene expression in context of the specified conditions. This study identified stable reference genes for fluoride experiments using an ameloblasts-derived cell line (LS8) and by using rat enamel organ.

Acknowledgements

The mouse enamel organ derived cell line (LS8) was a generous gift from Malcolm L. Snead. The authors would like to thank Katharine Sodek for stimulating conversations on qPCR and Kate Steinhacker for helping prepare the manuscript.

Funding

Research reported in this publication was supported by the National Institute of Dental and Craniofacial Research of the National Institutes of Health (DF) T32DE007327, (JDB) R01DE018106.

Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
C_q	quantification cycle
IACUC	Institutional Animal Care Use Committee
qPCR	quantitative real-time PCR

References

1. Truman BI, Gooch BF, Sulemana I, Gift HC, Horowitz AM, Evans CA, et al. Reviews of evidence on interventions to prevent dental caries, oral and pharyngeal cancers, and sports-related craniofacial injuries. American journal of preventive medicine. 2002 Jul 23.(1 Suppl):21–54. [PubMed: 12091093]
2. Recommendations for using fluoride to prevent and control dental caries in the United States. Centers for Disease Control and Prevention. MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control. 2001:1–42.
3. Whitford GM. The metabolism and toxicity of fluoride. Monographs in oral science. 1996; 16:1–153. Rev 2. [PubMed: 8813212]
4. Beltran-Aguilar ED, Barker LK, Canto MT, Dye BA, Gooch BF, Griffin SO, et al. Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis--United States, 1988–1994 and 1999–2002. Morbidity and mortality weekly report Surveillance summaries. 2005 Aug 26; 54(3):1–43. [PubMed: 16121123]
5. Ekstrand J, Spak CJ, Ehrnebo M. Renal clearance of fluoride in a steady state condition in man: influence of urinary flow and pH changes by diet. Acta pharmacologica et toxicologica. 1982 May; 50(5):321–325. [PubMed: 7113707]
6. Jarnberg PO, Ekstrand J, Irestedt L, Santesson J. Renal fluoride excretion during and after enflurane anaesthesia: dependency on spontaneous urinary pH-variations. Acta anaesthesiologica Scandinavica. 1980 Apr; 24(2):129–134. [PubMed: 7386145]
7. Spak CJ, Berg U, Ekstrand J. Renal clearance of fluoride in children and adolescents. Pediatrics. 1985 Mar; 75(3):575–579. [PubMed: 3975128]
8. Everett ET, McHenry MA, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, et al. Dental fluorosis: variability among different inbred mouse strains. Journal of dental research. 2002 Nov; 81(11):794–798. [PubMed: 12407097]
9. Angmar-Mansson B, Ericsson Y, Ekberg O. Plasma fluoride and enamel fluorosis. Calcified tissue research. 1976 Nov 24; 22(1):77–84. [PubMed: 1000344]
10. Angmar-Mansson B, Whitford GM. Enamel fluorosis related to plasma F levels in the rat. Caries research. 1984; 18(1):25–32. [PubMed: 6580951]
11. Beltran-Aguilar ED, Barker L, Dye BA. Prevalence and severity of dental fluorosis in the United States, 1999–2004. NCHS data brief. 2010 Nov.(53):1–8. [PubMed: 21211168]
12. Mascarenhas AK. Risk factors for dental fluorosis: a review of the recent literature. Pediatric dentistry. 2000 Jul-Aug;22(4):269–277. [PubMed: 10969430]
13. Robinson C, Connell S, Kirkham J, Brookes SJ, Shore RC, Smith AM. The effect of fluoride on the developing tooth. Caries research. 2004 May-Jun;38(3):268–276. [PubMed: 15153700]
14. Wright JT, Chen SC, Hall KI, Yamauchi M, Bawden JW. Protein characterization of fluorosed human enamel. Journal of dental research. 1996 Dec; 75(12):1936–1941. [PubMed: 9033447]

15. Marshman Z, Gibson B, Robinson PG. The impact of developmental defects of enamel on young people in the UK. *Community dentistry and oral epidemiology*. 2009 Feb; 37(1):45–57. [PubMed: 19046330]
16. Mottled Enamel. *American journal of public health and the nation's health*. 1933 Jan; 23(1):47–48.
17. Mc KF. The study of mottled enamel (dental fluorosis). *Journal of the American Dental Association*. 1952 Feb; 44(2):133–137.
18. Zhou R, Zaki AE, Eisenmann DR. Morphometry and autoradiography of altered rat enamel protein processing due to chronic exposure to fluoride. *Archives of oral biology*. 1996 Aug-Sep;41(8–9): 739–747. [PubMed: 9022911]
19. Triller M. Structural and histochemical observations of fluorotic enamel matrix. *Journal of dental research*. 1979 Mar; 58(Spec Issue B):1028–1029. [PubMed: 84005]
20. Shinoda H. Effect of long-term administration of fluoride on physico-chemical properties of the rat incisor enamel. *Calcified tissue research*. 1975 Jul 25; 18(2):91–100. [PubMed: 1148900]
21. Shinoda H, Ogura H. Scanning electron microscopical study on the fluorosis of enamel in rats. *Calcified tissue research*. 1978 Feb 28; 25(1):75–83. [PubMed: 647440]
22. DenBesten PK, Heffernan LM. Enamel proteases in secretory and maturation enamel of rats ingesting 0 and 100 PPM fluoride in drinking water. *Advances in dental research*. 1989 Sep; 3(2): 199–202. [PubMed: 2701159]
23. Den Besten PK. Effects of fluoride on protein secretion and removal during enamel development in the rat. *Journal of dental research*. 1986 Oct; 65(10):1272–1277. [PubMed: 3463596]
24. Denbesten PK, Crenshaw MA, Wilson MH. Changes in the fluoride-induced modulation of maturation stage ameloblasts of rats. *Journal of dental research*. 1985 Dec; 64(12):1365–1370. [PubMed: 2416791]
25. Lyaruu DM, de Jong M, Bronckers AL, Woltgens JH. Ultrastructure of in-vitro recovery of mineralization capacity of fluorotic enamel matrix in hamster tooth germs pre-exposed to fluoride in organ culture during the secretory phase of amelogenesis. *Archives of oral biology*. 1987; 32(2): 107–115. [PubMed: 3478008]
26. Sharma R, Tsuchiya M, Skobe Z, Tannous BA, Bartlett JD. The acid test of fluoride: how pH modulates toxicity. *PloS one*. 2010; 5(5):e10895. [PubMed: 20531944]
27. Smith CE, Nanci A, Denbesten PK. Effects of chronic fluoride exposure on morphometric parameters defining the stages of amelogenesis and ameloblast modulation in rat incisors. *The Anatomical record*. 1993 Oct; 237(2):243–258. [PubMed: 8238976]
28. Kubota K, Lee DH, Tsuchiya M, Young CS, Everett ET, Martinez-Mier EA, et al. Fluoride induces endoplasmic reticulum stress in ameloblasts responsible for dental enamel formation. *The Journal of biological chemistry*. 2005 Jun 17; 280(24):23194–23202. [PubMed: 15849362]
29. Sharma R, Tsuchiya M, Bartlett JD. Fluoride induces endoplasmic reticulum stress and inhibits protein synthesis and secretion. *Environmental health perspectives*. 2008 Sep; 116(9):1142–1146. [PubMed: 18795154]
30. Suzuki M, Shin M, Simmer JP, Bartlett JD. Fluoride affects enamel protein content via TGF-beta1-mediated KLK4 inhibition. *Journal of dental research*. 2014 Oct; 93(10):1022–1027. [PubMed: 25074495]
31. Lyaruu DM, Bronckers AL, Santos F, Mathias R, DenBesten P. The effect of fluoride on enamel and dentin formation in the uremic rat incisor. *Pediatric nephrology*. 2008 Nov; 23(11):1973–1979. [PubMed: 18563453]
32. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of biomolecular techniques : JBT*. 2004 Sep; 15(3):155–166. [PubMed: 15331581]
33. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*. 2009 Apr; 55(4):611–622. [PubMed: 19246619]
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001 Dec; 25(4):402–408. [PubMed: 11846609]

35. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*. 2001 May 1;29(9):e45. [PubMed: 11328886]
36. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology*. 2007; 8(2):R19. [PubMed: 17291332]
37. Koch I, Weil R, Wolbold R, Brockmoller J, Hustert E, Burk O, et al. Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA. *Drug metabolism and disposition: the biological fate of chemicals*. 2002 Oct; 30(10):1108–1114. [PubMed: 12228187]
38. Ullmannova V, Haskovec C. The use of housekeeping genes (HKG) as an internal control for the detection of gene expression by quantitative real-time RT-PCR. *Folia biologica*. 2003; 49(6):211–216. [PubMed: 14748434]
39. Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, et al. Quantitative realtime reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. *Analytical biochemistry*. 2002 Oct 15; 309(2):293–300. [PubMed: 12413463]
40. Schulz WA, Eickelmann P, Hallbrucker C, Sies H, Haussinger D. Increase of beta-actin mRNA upon hypotonic perfusion of perfused rat liver. *FEBS letters*. 1991 Nov 4; 292(1–2):264–266. [PubMed: 1959616]
41. Schmid H, Cohen CD, Henger A, Irrgang S, Schlondorff D, Kretzler M. Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. *Kidney international*. 2003 Jul; 64(1):356–360. [PubMed: 12787429]
42. Bemeur C, Ste-Marie L, Desjardins P, Hazell AS, Vachon L, Butterworth R, et al. Decreased beta-actin mRNA expression in hyperglycemic focal cerebral ischemia in the rat. *Neuroscience letters*. 2004 Mar 11; 357(3):211–214. [PubMed: 15003287]
43. Bas A, Forsberg G, Hammarstrom S, Hammarstrom ML. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scandinavian journal of immunology*. 2004 Jun; 59(6):566–573. [PubMed: 15182252]
44. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes and immunity*. 2005 Jun; 6(4):279–284. [PubMed: 15815687]
45. Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, et al. The need for transparency and good practices in the qPCR literature. *Nature methods*. 2013 Nov; 10(11):1063–1067. [PubMed: 24173381]
46. Gutierrez L, Mauriat M, Guenin S, Pelloux J, Lefebvre JF, Louvet R, et al. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant biotechnology journal*. 2008 Aug; 6(6):609–618. [PubMed: 18433420]
47. Udvardi MK, Czechowski T, Scheible WR. Eleven golden rules of quantitative RT-PCR. *The Plant cell*. 2008 Jul; 20(7):1736–1737. [PubMed: 18664613]
48. Gussow D, Rein R, Ginjaar I, Hochstenbach F, Seemann G, Kottman A, et al. The human beta 2-microglobulin gene. Primary structure and definition of the transcriptional unit. *Journal of immunology*. 1987 Nov 1; 139(9):3132–3138.
49. Jung M, Ramankulov A, Roigas J, Johannsen M, Ringsdorf M, Kristiansen G, et al. In search of suitable reference genes for gene expression studies of human renal cell carcinoma by real-time PCR. *BMC molecular biology*. 2007; 8:47. [PubMed: 17559644]
50. Tang WW, Qi M, Van GY, Wariner GP, Samal B. Leukemia inhibitory factor ameliorates experimental anti-GBM Ab glomerulonephritis. *Kidney international*. 1996 Dec; 50(6):1922–1927. [PubMed: 8943475]
51. Seegmiller JE, Rosenbloom FM, Kelley WN. Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science*. 1967 Mar 31; 155(3770):1682–1684. [PubMed: 6020292]
52. Mateyak MK, Kinzy TG. eEF1A: thinking outside the ribosome. *The Journal of biological chemistry*. 2010 Jul 9; 285(28):21209–21213. [PubMed: 20444696]

53. Chen LS, Couwenhoven RI, Hsu D, Luo W, Snead ML. Maintenance of amelogenin gene expression by transformed epithelial cells of mouse enamel organ. *Archives of oral biology*. 1992 Oct; 37(10):771–778. [PubMed: 1444889]
54. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics*. 2012; 13:134. [PubMed: 22708584]
55. Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *Journal of computational biology : a journal of computational molecular cell biology*. 2005 Oct; 12(8):1047–1064. [PubMed: 16241897]
56. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*. 2002 Jun 18; 3(7) RESEARCH0034.
57. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research*. 2004 Aug 1; 64(15):5245–5250. [PubMed: 15289330]
58. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology letters*. 2004 Mar; 26(6):509–515. [PubMed: 15127793]
59. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC molecular biology*. 2006; 7:33. [PubMed: 17026756]
60. Nicot N, Hausman JF, Hoffmann L, Evers D. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *Journal of experimental botany*. 2005 Nov; 56(421):2907–2914. [PubMed: 16188960]
61. Pabinger S, Rödiger S, Kriegner A, Vierlinger K, Weinhäusel A. A survey of tools for the analysis of quantitative PCR (qPCR) data. *Biomolecular Detection and Quantification*. 2014; 1(1):23–33.

Highlights

Identification of reference genes for fluoride qPCR experiments is proposed.

RNA was isolated from LS8 ameloblast-derived cells and rat enamel organs.

qPCR experiments must first validate the stability of reference gene expression in context of the specified conditions.

Gapdh and *Eef1a1* were the most stably expressed genes in enamel organs from fluoride-treated rats.

Fig. 1.

Expression of reference genes in LS8 cells after treatment with increasing doses of NaF. Cells were plated and treated with 0, 1, 3, or 5 mM NaF for 6 hours. RNA was isolated and cDNA was prepared. The expression of *B2m* (A), *Tbp* (B), *Eef1a1* (C), *Gapdh* (D), and *Hprt* (E) was evaluated by qPCR. Expression stability was assessed using the geNorm (F) and Normfinder (G) methods, and the most stable genes have a lower M value. Each experiment was performed in triplicate. Fold change for each NaF concentration was calculated relative

to untreated cells and error bars represent standard error of the mean (SEM). *Eef1a1* had the largest variation in gene expression. C_q, quantification cycle.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

Expression of reference genes in LS8 cells after treatment with increasing doses of NaF. Cells were plated and treated with 0, 1 or 3 mM NaF for 18 hours. RNA was isolated and cDNA was prepared. The expression of *B2m* (A), *Tbp* (B), *Eef1a1* (C), *Gapdh* (D), and *Hprt* (E) was evaluated by qPCR. Expression stability was assessed using the geNorm (F) and NormFinder (G) methods, and the most stable genes have a lower M value (F). Each experiment was performed in triplicate. Fold change for each NaF concentration was

calculated relative to the untreated cells and error bars represent standard error of the mean (SEM). *Eef1a1* had the largest variation in gene expression. C_q, quantification cycle.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

The choice of reference gene determines the calculated expression level of *Ddit3* in LS8 cells. Cells were treated with NaF for 6 hours (A–C) or 18 hours (D–F). *Ddit3* was normalized to the mean of *B2m*, *Tbp*, and *Hprt* at 6 hours (A) and 18 hours (D). *Ddit3* was normalized to *Eef1a1* at 6 hours (B) and 18 hours (E). *Ddit3* was normalized to *B2m* at 6 hours (C) and 18 hours (F). Analysis was performed using the CT method. Normalization with *B2m* produced results similar to normalization with a mean of *B2m*, *Tbp*, and *Hprt*, but these results were different from normalization using *Eef1a1*.

Fig. 4.

Expression of *Ddit3* using the primer efficiency method. Cells were treated with NaF for 6 or 18 hours. *Ddit3* expression was calculated relative to *B2m* at 6 hours (A) or 18 hours (C). *Ddit3* expression was calculated relative to *Eef1a1* at 6 hours (B) or 18 hours (D). The increase in expression of *Ddit3* with fluoride treatment is significantly smaller when calculated relative to the more stable gene *B2m* relative to the less stable *Eef1a1* gene.

Fig. 5.

Expression of reference genes in rat enamel organ. The smallest variation with fluoride treatment was observed in the expression of *Eef1a1* and the largest was with *Hprt*. Rat drinking water was supplemented with 0 or 100 ppm fluoride as NaF for 6 weeks. RNA was isolated from maturation stage incisor enamel organs and cDNA was prepared. The expression of *B2m* (A), *Gapdh* (B), *Hprt* (C), and *Eef1a1* (D) was evaluated by qPCR. Expression stability was assessed using the geNorm (E) and the Normfinder (F) methods. The most stable genes have a lower M value. Each experiment was performed in triplicate.

C_q, quantification cycle. Fold change for NaF treatment was calculated relative to untreated rats and error bars represent standard error of the mean (SEM).

Table 1

qPCR Primer Sequences

Target gene / Amplicon length	Accession	Sequence / GC content	Efficiency
Mus musculus Beta-2 microglobulin (<i>B2m</i>) / 119 bp	NM_009735.3	Fwd: 5'-GGT CTT TCT GGT GCT TGT CTC-3' / 52.38 Rev: 5'-CGT AGC AGT TCA GTA TGT TCG G-3' / 50.00	1.963
Mus musculus Hypoxanthine phosphoribosyltransferase (<i>Hprt</i>) / 142 bp	NM_013556.2	Fwd: 5'-TCA GTC AAC GGG GGA CAT AAA-3' / 47.62 Rev: 5'-GGG GCT GTA CTG CTT AAC CAG-3' / 57.14	1.943
Mus musculus TATA box binding protein (<i>Tbp</i>) / 206 bp	NM_013684	Fwd: 5'-CCA ATG ACT CCT ATG ACC CC-3' / 55 Rev: 5'-GTT GTC CGT GGC TCT CTT ATT C-3' / 50	1.936
Mus musculus Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>) / 63 bp	NM_001289726.1	Fwd: 5'-ACT GGC ATG GCC TTC CG-3' / 64.71 Rev: 5'-CAG GCG GCA CGT CAG ATC-3' / 66.67	1.96
Mus musculus Eukaryotic translation elongation factor 1 alpha 1 (<i>Eef1a1</i>) / 98 bp	NM_010106.2	Fwd: 5'-ATT CCG GCA AGT CCA CCA CAA-3' / 52.38 Rev: 5'-CAT CTC AGC AGC CTC CTT CTC AAA C-3' / 52.00	1.895
Mus musculus DNA-damage inducible transcript 3 (<i>Ddit3</i>) / 67 bp	NM_001290183.1	Fwd: 5'-CCA CCA CAC CTG AAA GCA GAA-3' / 52.38 Rev: 5'-AGG TGA AAG GCA GGG ACT CA-3' / 55.00	1.995
Rattus norvegicus beta-2 microglobulin (<i>B2m</i>) / 79 bp	NM_012512.2	Fwd: 5'-CGTCGTGCTTGCCATTCAGAA-3' / 52.38 Rev: 5'-GAAGTTGGGCTTCCCATCTCC-3' / 54.55	1.938
Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>) / 89 bp	NM_017008.4	Fwd: 5'-ATGATTCTACCCACGGCAAG-3' / 50.00 Rev: 5'-CTGGAAGATGGTGATGGGTT-3' / 50.00	1.932
Rattus norvegicus hypoxanthine phosphoribosyltransferase 1 (<i>Hprt1</i>) / 96 bp	NM_012583.2	Fwd: 5'-AATGCAGACTTTTGCTTTCTTGG-3' / 43.48 Rev: 5'-TATCCAACACTTCGAGAGGTCCTTT-3' / 44.00	1.978
Rattus norvegicus eukaryotic translation elongation factor 1 alpha 1 (<i>Eef1a1</i>) / 109 bp	NM_175838.1	Fwd: 5'-CTCCACTTGGTCGTTTTGCTG-3' / 52.38 Rev: 5'-GCAGACTTGGTGACTTTGCC-3' / 55.00	1.876