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# Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction

Van Luu-The<sup>1</sup>, Nathalie Paquet<sup>1</sup>, Ezequiel Calvo<sup>1</sup>, and Jean Cumps<sup>2</sup>

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*Quantification of mRNA expression levels using real-time reverse transcription PCR (RT-PCR) is increasingly used to validate results of DNA microarrays or GeneChips®. It requires an improved method that is more robust and more suitable for high-throughput measurements. In this report, we compare a user non-influent, second derivative method with that of a user influent, fit point method that is widely used in the literature. We also describe the advantage of using a double correction: one correction using the expression levels of a housekeeping gene of an experiment as an internal standard and a second using reference expression levels of the same housekeeping gene in the tissue or cells. The first correction permits one to decrease errors due to sample preparation and handling, while the second correction permits one to avoid the variation of the results with the variability of housekeeping in each tissue, especially in experiments using various treatments. The data indicate that the real-time PCR method is highly efficient with an efficiency coefficient close to the theoretical value of two. The results also show that the second derivative method is more accurate than the fit point method in quantifying low gene expression levels. Using triplicate experiments, we show that measurement variations using our method are low with a mean of variation coefficients of <1%.*

## INTRODUCTION

Real-time reverse transcription PCR (RT-PCR), a recently developed fluorescent method of mRNA quantification (1–3), has improved greatly the mRNA quantification performed with PCR. Real-time RT-PCR permits the rigorous control of PCR conditions, and quantification takes place within an exponential phase of the amplification curve (4,5). In fact, the efficiency coefficient is close to the theoretical value of 2, thus indicating that errors due to the PCR amplification process are very low. The most important causes of errors are the RNA preparation and handling (5) because they will be amplified exponentially in the amplification process. The important improvement of real-time RT-PCR compared to classical PCR is that real-time RT-PCR permits one to follow the kinetics of DNA production in real time and to quantify the initial amount of mRNA using a standard curve,

resulting in much lower variability (6). In contrast, with classical PCR quantification, end-product signal that included the exponential amplified errors is measured (7), thus giving rise to high variability. In addition, real-time RT-PCR allows for the estimation of the absolute expression levels as copies of mRNA/microgram of total RNA and thus permits a quantitative appreciation of gene expression levels. This will help to better understand the role and function of the genes under investigation.

Two types of quantification can be performed using real-time PCR: a relative quantification based on the relative expression of a target versus a reference gene (8) and an absolute quantification (9) based either on an internal or an external calibration curve. The most frequently used method to investigate the physiological changes in gene expression is based on a relative expression ratio in order to avoid errors due to RNA and cDNA

preparation. Absolute quantification, on the other hand, yields a quantitative estimate of the concentration of a target mRNA, which allows for a more precise assessment of the importance of its functional role in target tissues or cells.

Two modes of detection are generally used, one employing a gene-specific fluorescent hybridization probe in which fluorescent signal is increased (10,11) or decreased (12) by transfer of energy from one fluorescent dye to another [e.g., fluorescence resonance energy transfer (FRET)] and a second one using a common SYBR® Green I fluorescent dye that binds to a minor groove of DNA (13). With a proper choice of primers and amplification conditions assisted by informatics, it has been shown that real-time RT-PCR using SYBR Green I is a rapid, sensitive, and accurate method to quantify mRNA (4,13).

There are also two methods to determine a crossing point (Cp) value,

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<sup>1</sup>Laval University, Quebec, Canada and <sup>2</sup>University of Louvain, Bruxelles, Belgium

which is a cycle number in a log-linear region (Figure 1) that is used to calculate the quantitative value of real-time RT-PCR. One method, namely fit point (Figure 1B; Reference 4), is performed by drawing a line parallel to the x-axis in the log-linear region of the real-time fluorescence intensity curve; a somewhat variable user-dependent value can be obtained by this method. The second, namely second derivative, calculates a second derivative (2,4,6) value of the real-time fluorescence

intensity curve (Figure 1A), and only one value is obtained. The fit point method is the most currently used method, and the calculation is user-dependent. The second derivative calculation, on the other hand, does not involve any decision by the user because a positive peak corresponds to the beginning of the log-linear phase of the original data.

GeneChips® (Affymetrix, Santa Clara, CA, USA) have been successfully used to monitor gene expression

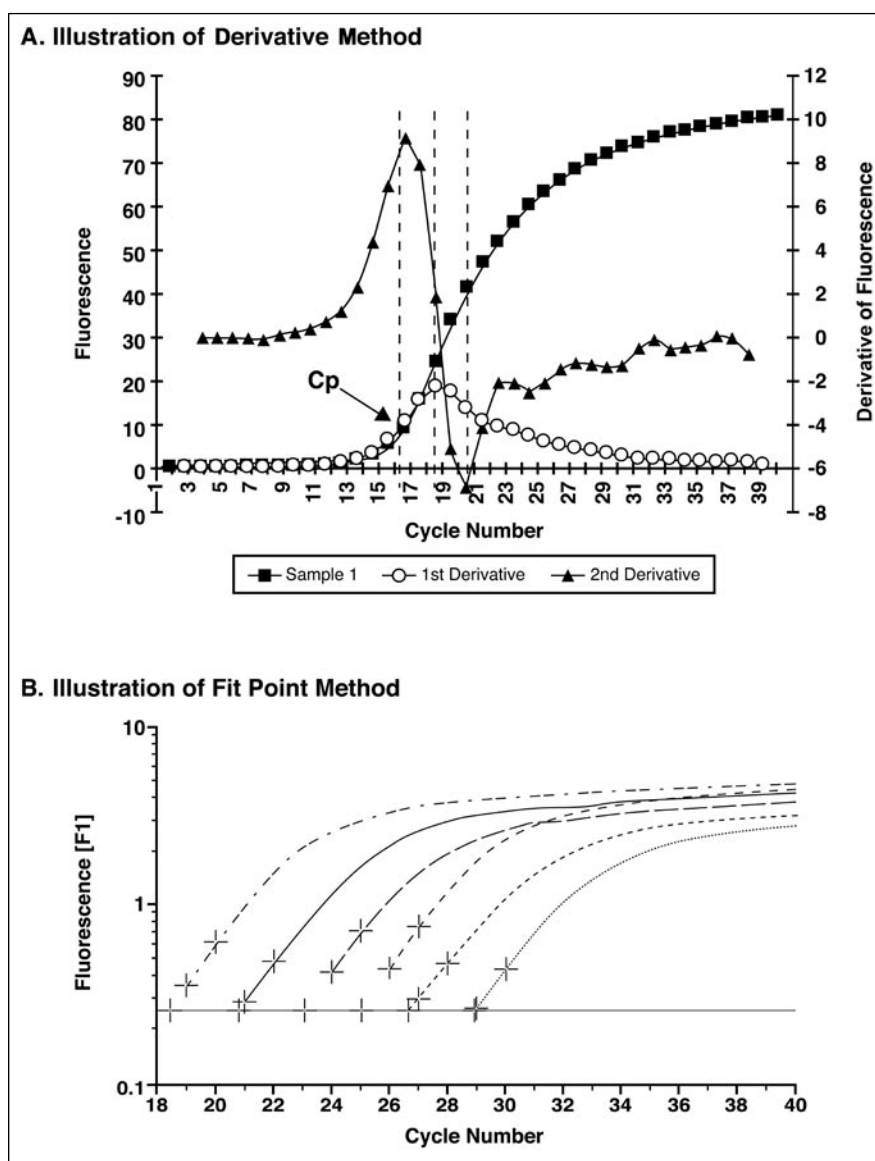
in unicellular model organisms and cell lines (14,15). However, when profiling more complex samples, such as human and murine tissue samples, more variability and false-positive/negative values are more likely to occur. This higher variability is probably related to the cellular heterogeneity of the tissues of mammals. The reliability of GeneChips to detect differences in expression levels following various treatments is also affected by several factors, including the quality of the probe sets, RNA extraction, and quality of the probe preparations and hybridization conditions (14). Even if GeneChip and DNA microarray data are quite reliable, they are not very precise, and thus genes identified as modulated must be validated and quantified. Northern blot hybridization or RNase protection assays are effective methods of validation, however, they often require large amounts of RNA. Classical RT-PCR requires much less RNA but lacks precision due to exponential amplification of potential errors during the PCR amplification process. The most appropriate method of validation is therefore real-time RT-PCR.

In this report, we describe the advantage of the real-time RT-PCR detection method using second derivative by comparing the data obtained with that of the widely used fit point method. We also describe the improvement of the quantification method by using a double correction, and we show an example of an application using this improved method of real-time RT-PCR to validate results obtained from GeneChips. Our results clearly indicate that there are many advantages of using the second derivative method for RT-PCR quantification to validate expression levels of multiple regulated genes from GeneChip analysis.

## MATERIALS AND METHODS

### Animals and Treatment

Ten week-old C57BL6 mice were received from Charles River (St-Constant, Quebec, Canada) and were allowed to acclimate for 3 weeks. The



**Figure 1. Illustration of the second derivative (A) and fit point methods (B).** Illustrations taken from a LightCycler manual (Hoffman-La Roche) showing the variation of fluorescence signal versus number of cycles and the methods to determine the crossing point (Cp) using second derivative peak (A) and fit point (B). In the second derivative method, a Cp corresponds to the first peak of a second derivative curve. This peak corresponds to the beginning of a log-linear phase (A). In the fit point method (B), a Cp is determined by the intersection of a parallel to the threshold line in the log-linear region.

animals were housed individually in an environmentally controlled room (temperature,  $22^{\circ} \pm 3^{\circ}\text{C}$ ; humidity,  $50\% \pm 20\%$ ; 12-h light/dark cycles, lights on at 7:15 h). The mice had free access to tap water and a certified rodent feed [Lab Diet 5002 (pellet); Ralston Purina, St. Louis, MO, USA]. The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC; Ottawa, ON, Canada) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC; Rockville, MD, USA). The study was performed in accordance with the CCAC Guide for the Care and Use of Experimental Animals.

The animals, weighing between 20 and 24 g, were randomized according to their body weight and assigned to 7 groups of 14 animals each as followed: group 1, gonadectomized (GDX) control; groups 2–7, GDX +  $17\beta$ -estradiol ( $\text{E}_2$ ;  $0.05 \mu\text{g}/\text{mouse}$ ). On day 1 of the study, animals were bilaterally ovariectomized under isoflurane anesthesia. Prior to the necropsy performed on day 8 of the study, mice received a single subcutaneous injection ( $0.2 \text{ mL}/\text{mouse}$ ) of the vehicle alone (group 1, 5% ethanol/0.4% methylcellulose) or  $\text{E}_2$  (groups 2–7). The injection of the vehicle was performed 24 h prior to the necropsy for animals in group 1 while  $\text{E}_2$  was injected 1 h (group 2), 3 h (group 3), 6 h (group 4), 12 h (group 5), 18 h (group 6), or 24 h (group 7) prior to the necropsy.

### Tissue Collection and RNA Preparation

On day 8 of the study, mice under isoflurane anesthesia were exsanguinated at the abdominal aorta followed by cervical dislocation. Sixty-four tissues including the uterus were collected and rapidly frozen in liquid nitrogen. Tissues were kept at  $-80^{\circ}\text{C}$  until RNA extraction. These treatments were used routinely in our laboratory and they do not influence the induction due to  $\text{E}_2$  treatment (16).

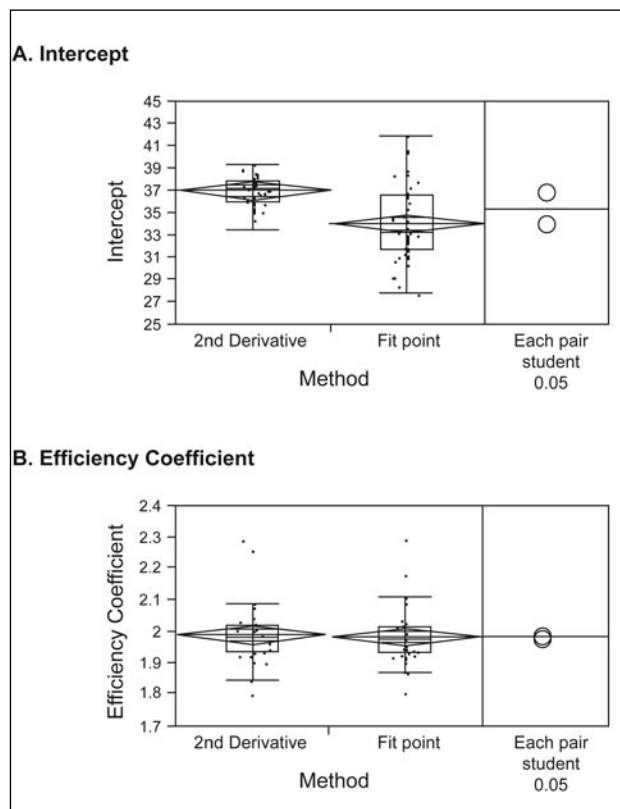
Total RNA was isolated by TRIZOL<sup>®</sup> (Invitrogen, Burlington, ON, Canada) from 14 mouse uteri ranging from 350

(gonadectomized) to 630 mg ( $\text{E}_2$ -treated animals). Twenty micrograms of total RNA were converted to cDNA by incubation at  $42^{\circ}\text{C}$  for 1 h with 400 U SUPERSCRIPT<sup>®</sup> II reverse transcriptase (Invitrogen), oligo(dT)<sub>24</sub> as primer in a reaction buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol (DTT), and 0.5 mM dNTPs.

### Real-Time RT-PCR

cDNA corresponding to 20 ng of the initial total RNA was used to perform fluorescence-based real-time PCR quantification using the LightCycler<sup>®</sup> Realtime PCR apparatus (Hoffman-La Roche, Nutley, NJ, USA). The FastStart DNA Master<sup>PLUS</sup> SYBR Green Kit (Roche Diagnostics, Laval, QC, Canada) was used as described by the manufacturer. The conditions for PCR were denaturation at  $94^{\circ}\text{C}$  for 15 s, annealing at  $50^{\circ}\text{--}65^{\circ}\text{C}$  for 10 s, and elongation at  $72^{\circ}\text{C}$  for 15 s. The reaction was then heated for 3 s at  $2^{\circ}\text{C}$  lower than the melting temperature of the DNA fragment. Reading of the fluorescence signal was taken at the end of the heating to avoid nonspecific signal, and a melting curve was performed to assess nonspecific signal. Annealing temperature was selected based on contamination levels and melting curve results. Oligonucleotide primer pairs that allow for the amplification of approximately 200 bp were design by GeneTools software (BioTools, Edmonton, AB, Canada), and their specificity was verified by blasting in the GenBank<sup>®</sup> database. Prior to mRNA quantification, RNA samples

were verified for genomic DNA contamination by the amplification of two DNA sequences in the intron 3 of mouse type 1  $3\beta$ -hydroxysteroid dehydrogenase gene using 2 oligonucleotide pairs,  $5'$ -TTGGTCTTTGTCTGCAGCTCTGT- $3'$  and  $5'$ -GGGATGTGGTGGATATTGGTG- $3'$  and  $5'$ -CACCCCTTAAGAGACCCATGTT- $3'$  and  $5'$ -CCCTGCAGAGACCTTAGAAAAC- $3'$ . Only RNA having no contamination with genomic DNA was used for quantification. To avoid errors due to RNA and cDNA preparation and handling, we performed a first correction with a housekeeping gene, subunit O of ATPase (*Atp5o*), at each assay. *Atp5o* has shown to be a gene having stable expression levels from embryonic life through adulthood in various tissues (17). Taking advantage of the large-scale quantification of multiple genes in each tissue, which requires more than 30 quantifications of *Atp5o* gene transcripts in each investigated tissue, we have



**Figure 2. Comparison of the second derivative and fit point methods using data of standard curves.** Thirty-nine standard curves have been established using the second derivative and fit point methods. Statistical analysis has been performed to compare both methods. (A) Analysis of the dispersion of intercept values. (B) Analysis of the dispersion of the efficiency coefficient.

determined the mean value of Cp house-keeping (CphK) of multiple assays in the tissue. This Cphk will thus constitute a reference value for the tissue (Cphk\_ref). It will serve to make a correction of the Cp of target genes to minimize errors due to inter-assay variability.

The calculation is as follows:

$$\text{Cpcor\_gene} = \text{Cpgene} / \text{Cphk} \times \text{Cphk\_ref} \quad [\text{Eq. 1}]$$

Where the Cpcor\_gene is the corrected Cp value used for the quantification of the expression levels of the target gene; Cphk is the Cp value of the house-keeping gene measured in the same assay (used to correct technical errors), and Cphk\_ref is the mean value of multiple Cphk determined at different assays using the same tissue (used to minimize inter-assay variation).

The Cpcor\_gene was converted to copies/20 ng total RNA (or any amount corresponding the amount of RNA used in the assay) using a standard curve established by the quantification of cDNA plasmid corresponding to 0, 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, and 10<sup>6</sup> copies of each mRNA species and a LightCycler 3.5 program provided by the manufacturer (Hoffman-La Roche). The resulting values were multiplied by 50 to convert the mRNA expression levels in copies/microgram total RNA. Such a double correction method prevents variability related to the variation of housekeeping mRNA expression levels in various tissues.

### Second Derivative and Fit Point Calculation

PCR amplification in which the number of DNA fragments is doubled at each cycle follows a typical exponential equation:  $N = N_0 \times E^n$ , where  $N_0$  is an initial number of copies,  $E$  is the efficiency coefficient, and  $n$  is the cycle number. The optimum value of  $E$  is 2. Due to experimental condition limitations, the DNA fragments are not synthesized exponentially but

their synthesis saturates after 30–35 cycles. Fluorescence-based real-time PCR permits the visualization of the product and thus allows one to choose a range that corresponds to a log-linear phase. Fit point and second derivative methods allow for the determination of a Cp that is used to calculate the number of copies of initial mRNA species in the sample using a standard curve as described above. Statistical analyses are performed as indicated using the JMP statistical software (SAS Institute, Cary, NC, USA).

## RESULTS AND DISCUSSION

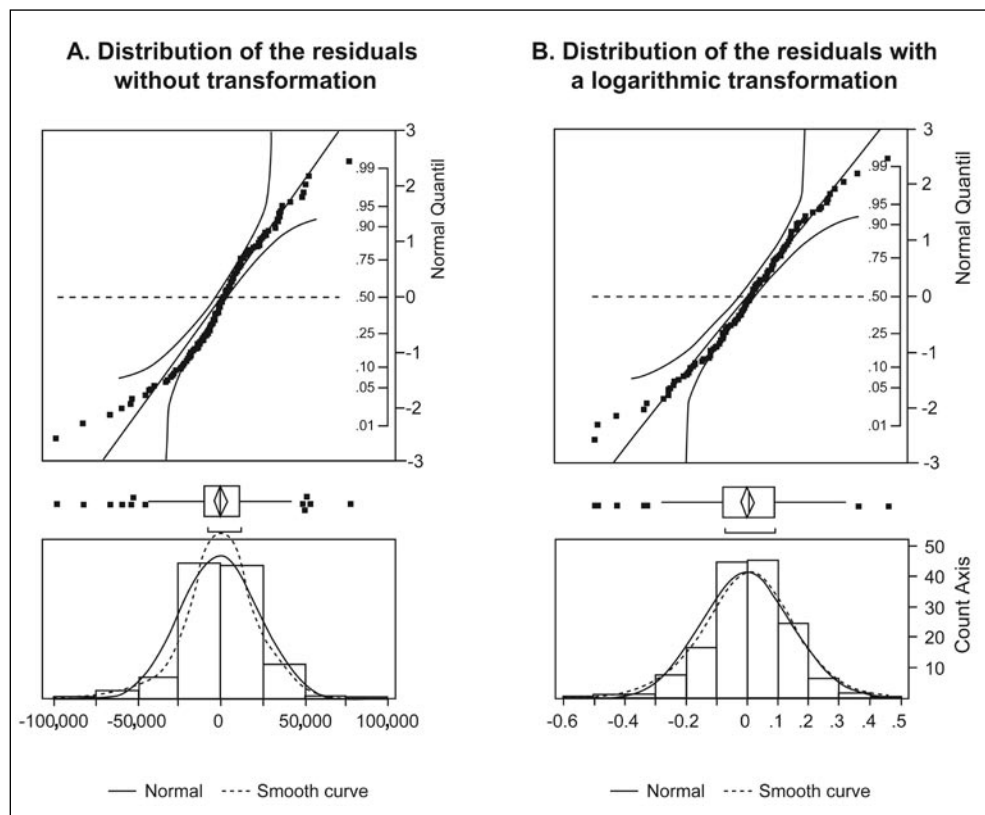
### Comparison of Real-Time RT-PCR Quantification Using Second Derivative and Fit Point Methods

To determine the characteristics of the second derivative and fit point methods, we compared the efficiency coefficients, slopes, and intercepts of

39 different standard curves. As illustrated in Figure 2A, the mean intercept value that represents the Cp corresponding to 1 copy/microgram total RNA is significantly higher [Welch analysis of variance (ANOVA) test,  $P$  value < 0.0001] in the second derivative method (mean Cp value = 37.0) than in the fit point method (mean Cp value = 34.1). We also observed (Figure 2B) a higher dispersion with the fit point method (F test,  $P$  value < 0.0001). On the other hand, there are no significant differences between both methods concerning the mean and the variances of efficiency coefficients.

### Measurements Using a Double Correction Method

The objective of the double correction is to first control errors due to RNA and cDNA preparations as well as to reagent and operator handlings, and second, to control errors due to inter-assay variability. The first



**Figure 3. Shapiro normality test of data of triplicate experiments.** To determine an appropriate method of calculation, the Shapiro normality test has been performed on data of triplicate experiments. (A) Distribution of the residuals without transformation ( $P$  value = 0.0018). (B) Distribution of the residuals with a logarithmic transformation ( $P$  value = 0.21).

correction is performed by dividing the  $C_p$  value of the target gene with that of a  $C_p$  value of the internal housekeeping standard; the second correction is performed by multiplying the result of the first correction with a reference  $C_p$  value of the same housekeeping standard. The reference  $C_p$  value corresponds to the mean of more than 30 measurements using the same tissue or RNA source. The second correction allows for the conversion of the data obtained back to copies/microgram of total RNA. Because mRNA represents only 3–5 percent of the total RNA, calculating the number of copies per total RNA is an established and reliable method to denote mRNA expression levels (9).

To estimate the accuracy of our method, we performed in triplicate the amplification of 50 genes from the same RNA sample but three different batches of cDNA from three different reverse transcriptions. The data are shown in Supplementary Table S1, which is available at the *BioTechniques*' web site at <http://www.BioTechniques.com/February2005/Luu-TheSupplementary.html>. The data are further analyzed using statistical tests. As shown in Figure 3A using the Shapiro normality test (18), the data show a normal distribution when they are expressed in logarithm form ( $P$  value = 0.21), whereas in a non-logarithm form (Figure 3B), the  $P$  value is 0.0018, thus rejecting normality. Analysis using the Grubb discordance test (19) indicates that there are no outlier data. Because the logarithm of the data satisfy the homoscedasticity assumption, we have used these values to perform ANOVA. The results indicate that the mean of variance is 0.003, and the mean of the coefficient of variation is 1%, indicating our method is highly accurate and reproducible.

### Validation of GeneChips Using Real-Time RT-PCR

Although GeneChips are highly reliable, they are not really quantitative; therefore genes identified as modulated must be validated and quantified by another technique. We have previously used the real-time RT-PCR method to validate the expression levels of 60 genes found

to be differentially expressed in the uterus of gonadectomized mouse treated with  $E_2$  for 0, 1, 3, 6, 12, 18, and 24 h. A more than 85% concordance in expression profiles between the GeneChips and the real-time RT-PCR methods has been observed. The concordance value is determined using the Spearman nonparametric test ( $P < 0.05$ ). Figure 4 illustrates an example of a concordant profile between data obtained from GeneChips and real-time RT-PCR. Many genes have the highest expression at 24 h of exposure to  $E_2$  (Figure 4, A and B) while others have an earlier peak of activation. For example, *c-Fos* oncogene is maximally activated at 3 h (Figure 4, C and D).

In this report, we used two sets of data obtained from the project "Atlas of Genomic Profile of Steroid Action," in which we characterized genes that are modulated by steroid hormones in 64 mouse tissues to illustrate the advantage of using the second derivative calculation method and a double correction in real-time RT-PCR to validate the data of the GeneChips. One set of data is the standard curves of 39 genes. These data allow us to assess the quality of two methods of calculation; namely, the second derivative and fit point methods. The results show that the second derivative method is more suitable for the detection of low expression levels because the standard curve shows a higher intercept value (Figure 2A). The intercept value represents, indeed, a  $C_p$  value for one copy. Intercept values in the second derivative method are less disperse than those obtained from the fit point method (Figure 2A). It is noteworthy that the fit point method is the method that is widely described in the literature. The reason why the fit point method is more widely used is probably because it is more straightforward and more visual. It is also easier for users to set a  $C_p$  value where the efficiency coefficient of a standard curve is optimum by moving a straight line up and down in the log-linear region. The drawback of this user influence setting is that the variability between assays is higher. Because of a possible variability of  $C_p$  values in the same tissue sample, this technique is more suitable for a small number of assays. On the other hand,

if we want to avoid user influences on the determination of a  $C_p$ , the second derivative method is a more appropriate the method. In this method, the  $C_p$  is always determined in the same way, a calculation of the first second derivative (4) peak of the curve of fluorescence signal versus the number of cycles (Figure 1A). In addition, theoretically, the parameter related to the nature of the gene is not present in the PCR equation ( $N = N_0 \times E^n$ ); it is likely that this parameter might not have any influence on the standard curve. Accordingly, the dispersion of the efficiency coefficient for different standard curves is very low. It is much less than the variability of other experimental conditions; our results suggest that the use of a standard curve for each gene is not necessary. A standard curve of a reference housekeeping gene is thus sufficient for the quantification of multiple genes in a tissue. This is especially advantageous for the validation of GeneChip results because multiple genes are quantified in one tissue sample. It is noteworthy that Tichopad et al. (20) have also successfully used the second derivative method to quantify tissue-specific expression patterns of the bovine prion gene. They have also proposed a computing method for the estimation of real-time PCR amplification efficiency based on a statistic delimitation of the beginning of exponentially behaving observations in real-time PCR kinetics (21), and a descriptive mathematical model of PCR amplification (22) in which the authors show that the first and second derivative maximum computed from the four parametric sigmoid model is a quite reliable and promising method.

The second set of data is the expression levels of 60 genes that have been found to be modulated in the uterus of gonadectomized mouse treated by  $E_2$  or 0, 1, 3, 6, 12, 18 and 24 h. Some genes are maximally up-regulated after 1 or 3 h of treatment, such as the early gene *FOS*, while others are maximally stimulated only at 24 h. A comparison of the data from GeneChips and real-time RT-PCR shows that 85% of modulated gene profiles are concordant. An example of an expression profile of two genes is illustrated in Figure 4. Although

the modulation profile is concordant, the absolute expression values are not always proportional. This is most probably due to the variability of the signal of the Affymetrix GeneChip probes, which depends on the nucleotide composition and localization of probe sets on the gene. Validation of the GeneChip data required the quantification of multiple genes in one tissue sample. Accordingly, we have repeated many times the quantification of a housekeeping gene standard in the same tissue. This allows us to determine a reference value of the mRNA expression levels of a housekeeping gene in this tissue. This reference value is used in a second correction to convert the expression levels to copies/microgram of total RNA. It also permits one to obtain data that are independent of the variation of housekeeping expression caused by treatment. Indeed, although the expression of housekeeping genes is assumed to not be influenced by experimental treatments, there is evidence that they are somewhat regulated. Our double correction method allows for the correction of the variation in the expression level of the housekeeping gene during treatments. In addition, this variation was also reduced by using ATP5o, which has been shown to have minimal variation during life (17), as a reference gene.

In conclusion, the determination of a Cp using the second derivative method is not user influenced, and there is less variability between

assays. This method is more suitable for high-throughput measurements, such as the validation of multiple genes of interest identified by microarrays such as GeneChips. In addition, the double correction method, as suggested in this manuscript, allows for the prevention of variability of housekeeping gene expression during treatments, and the data expressed as copies/microgram of total RNA allow for a quantitative

assessment of the role and function of individual gene transcripts.

## ACKNOWLEDGMENTS

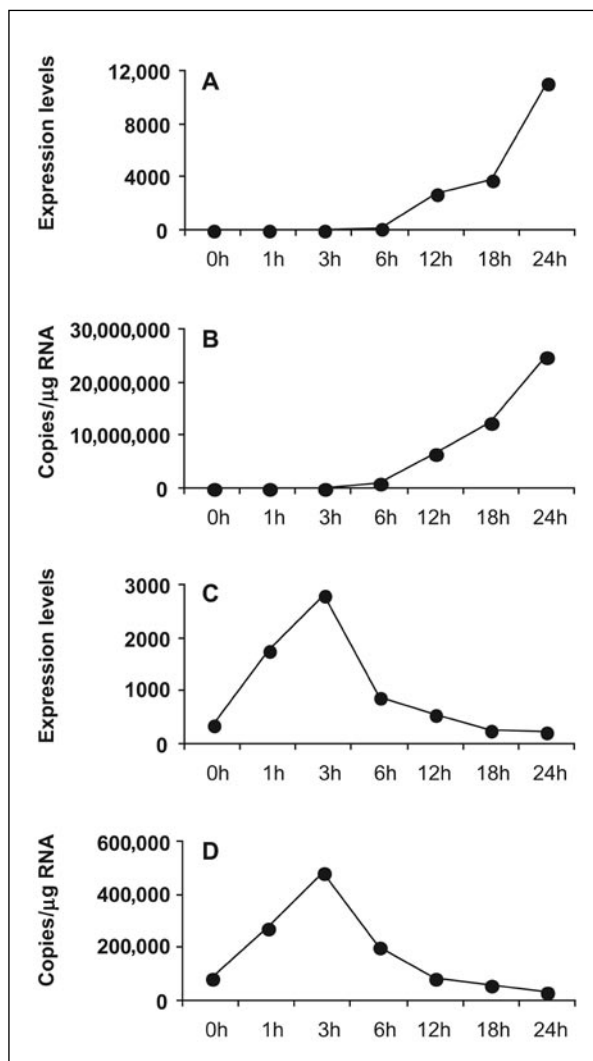
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## COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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**Figure 4.** Comparison of mRNA expression profiles using real-time reverse transcription PCR (RT-PCR) and GeneChips. (A and B) Examples of concordant expression profiles of keratin 16 and (C and D) c-Fos oncogene (C and D), two genes modulated by 17 $\beta$ -estradiol ( $E_2$ ) in the uterus of mouse treated with  $E_2$  for 1, 3, 6, 12, 18, and 24 h and quantified by (A and C) GeneChips and by (B and D) real-time RT-PCR. The accuracy of the method was assessed using a Spearman rank test to compare the profiles obtained using real-time RT-PCR and GeneChips; a  $\rho = 0.964$  and a  $P$  value = 0.003 have been obtained for both genes. Real-time RT-PCR was performed as described in the Materials and Methods section while GeneChips data were calculated using Microarray Suite 5.0 (Affymetrix).

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**Address correspondence to:**

Van Luu-The  
Molecular Endocrinology and  
Oncology Research Center  
Laval University Medical Center (CHUL)  
2705 Laurier Boulevard  
Quebec, QC, G1V 4G2, Canada  
e-mail: Van.Luu-The@crchul.ulaval.ca