Mitigation of the effect of variability in digital PCR assays through use of duplexed reference assays for normalization

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Digital PCR has been promoted as a technique for obtaining absolute measures of the amount of nucleic acid target sequence in a sample, but still lacks standardization in data reporting. The initial method of representing data as copies per microliter produced inconsistent results and made inter-assay comparisons difficult. Normalizing copies to amount of nucleic acid gives more uniform results, but factors influencing the effective concentration of nucleic acid in the final digital PCR assay must be considered. Using droplet digital PCR and previously validated reference genes duplexed with target genes, a method of normalization was developed to estimate the amount of input nucleic acid in individual assays, subsequently reporting the number of copies of target gene relative to this amount. Correcting for the actual amount of amplifiable nucleic acid present demonstrated a higher correlation between various dilutions of sample mRNA and allowed more accurate comparisons of digital PCR results.

Digital PCR (dPCR), which has found various uses in DNA quantification (including copy number determination [1–4], quantification, fluorescence-based size characterization [5] and SNP detection [2]) differs from quantitative PCR (qPCR) in that the reaction is divided into numerous partitions, ideally with one or only a few copies of the target in a portion of the partitions [6]. Quantification is considered to be absolute, so much smaller differences in expression can be regarded as significant [4]. Since dPCR uses an end-point measurement for quantification, it is less dependent on PCR efficiency than qPCR [7] and, along with the physical partitioning of the sample effectively enriching the target sequence in positive partitions, is less sensitive to inhibitors and interference from nontarget sequences that may be present in the nucleic acid sample [8–10].

One disadvantage of dPCR is that it has a narrower dynamic range than qPCR, with the upper limit being dependent on the number of partitions per reaction (more partitions equal greater dynamic range) [11]. Droplet dPCR (ddPCR), which partitions the PCR reaction into individual droplets in an oil emulsion rather than individual chambers on a chip, improves the dynamic range somewhat, but reliance on a Poisson correction for accurate quantification necessitates the presence of a population of droplets that are negative for the target, limiting its accuracy at higher template concentrations. This limited range necessitates the use of different amounts of input cDNA for genes with different expression levels to ensure that the template copies will be within that range.

Assay optimization is also essential for adapting qPCR assays to the ddPCR platform, as it affects the determination of which droplets are assigned as negative and which as positive. The default setting for Bio-Rad Laboratories' QuantaSoft analysis program (CA, USA) is to set a threshold just above the negative droplet cluster and define all other droplets as positive. There is some rationale in this approach, since intermediate droplets (commonly referred to as 'rain') may simply represent suboptimal PCR amplification due to partial PCR inhibition or sequence variances in the gene of interest [12]. However, it is also possible that these droplets could be from nonspecific amplification, so some researchers think that they should be excluded from analysis to obtain a more reliable result [13,14]. Witte *et al.* [12] concluded that biased amplification was a major factor contributing to rain in their assay, and that adjusting annealing temperature, ramp rate, and number of cycles allowed minimization of such intermediate droplets. Other researchers have reported the same results [15–18], with varying elongation time being another potential factor [5]. Several groups have introduced independent open source data analysis methods that deal with intermediate droplets differently than QuantaSoft, either excluding them altogether or attempting to determine to which population they should appropriately be assigned, for both single channel and multiplex ddPCR [13,19–23].

METHOD SUMMARY

A method for normalization of digital PCR data was developed using duplexed reference genes to eliminate sources of variability and estimate the actual amount of input RNA or DNA present in individual assays. This value can then be used to standardize reporting of the results for target genes or sequences.

Several crucial factors must also be considered when using dPCR to measure gene expression. The reverse transcription (RT) reaction is notably problematic, with efficiency dependent on mRNA structure, priming strategy, RT enzyme chosen, and concentration of total RNA [24]. One proposed strategy to compensate for gene-specific variation in efficiency is to partition RNA prior to performing an RT reaction with gene-specific primers, referred to as one-step RT-dPCR, so that positive partitions would only represent RNA molecules in the original sample [25]. However, Sanders *et al.* found that this method was both kit- and transcript-specific, especially for lower copy numbers [25]. Also, since components of the RT reaction have been shown to inhibit subsequent PCR, it might be more susceptible to this type of inhibition because the RT components would not be diluted as in two-step reactions where the mRNA is reverse transcribed separately [26].

While these considerations and others, including DNA/RNA isolation, measurement, and the numerous pipetting steps involved, allow considerable opportunity for variability in both dPCR and qPCR, in qPCR data are generally normalized to one or more reference genes, compensating for some of the variability. In addition, comparison between genes and across tissues requires that some form of normalization be used. Synthetic targets are available for some assays for normalization, although Sanders *et al.* found that measurement of endogenous targets was more variable in practice. Therefore, normalization to reference genes is still recommended [27].

In an effort to provide a more standard measure, some researchers have chosen to relate measured copies of target to the amount of nucleic acid added to the assay [28,29] rather than the originally reported copies per microliter (copies/µl). Another potentially promising method that was recently introduced that is specific to gene expression, Selfie-digital PCR, uses the same gene-specific primers for both the RT reaction and the subsequent digital PCR, comparing the number of copies generated with and without RT to determine the level of transcription compared with the number of copies of genomic DNA for the target gene in a cell or tissue lysate [30]. The method presented here uses the more general approach of normalization to an endogenous reference gene, comparing the measured copies of the reference gene template with the starting amount of nucleic acid in the assay, and using this relationship to estimate the actual amount of nucleic acid originally added and calculate the copies of target gene on that basis. While originally developed for gene expression analysis, it could also be applied to other ddPCR analyses, such as copy number variation, where reliable reference genes or sequences are available. Using this method of normalization allows comparison of gene expression levels between genes and tissues.

Materials & methods

Animal treatment & RNA isolation

Liver RNA from vehicle and TBBPA-treated Wistar Han rats (Charles River, NC, USA) from a previous study approved by the Institutional Animal Care and Use Committee of the National Institute of Environmental Health Sciences (NIEHS) and described in detail in Sanders *et al.* [31] was utilized for the current study. Briefly, female Wistar Han rats in the same estrus stage were treated for 5 consecutive days by gavage with either 250 mg/kg of TBBPA (3,3′,5,5′-tetrabromobisphenol A, Sigma-Aldrich 330396, MO, USA) or vehicle (1:3:3 ratio of ethanol, Cremophor EL [Sigma-Aldrich 238470] and water), then euthanized by CO_2 asphyxiation. The central portion of the left lobe of the liver was cubed, flash frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from 50–60 mg of tissue using a RNeasy kit (Qiagen, MD, USA) and QIAcube instrument (Qiagen). RNA was quantified using a Nanodrop 2000c (Thermo Scientific, DE, USA), integrity was measured on a QIAxcel instrument (Qiagen) using a QIAxcel RNA QC Kit V2.0 and RNA was stored at -80°C.

Reverse transcription & ddPCR

cDNA was prepared as described [31] using Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase (Thermo Fisher Scientific N8080018, MA, USA). FAM™ (6-carboxyfluorescein)-labeled TaqMan gene expression assays (Thermo Fisher Scientific) previously used for qPCR were optimized for ddPCR (Supplementary Material) and tested with reference gene Sdha for duplexing suitability. Reference-gene-only assays (Sdha, Elk4) were run singleplex for each sample concentration, while all target genes were run as duplex assays with Sdha-VIC™ (4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein). All assays were run in triplicate. A detailed description of the ddPCR workflow is given in Hindson *et al.* [1]. A brief description follows. QX200 ddPCR instrument and all specific reagents and supplies were purchased from Bio-Rad Laboratories (CA, USA).

To reduce concentration errors due to pipetting, stock dilutions of the needed concentrations of liver cDNA from all samples were made in RNase/DNase-free water so that all assays in a set using the same amount were drawn from a single stock. Resulting stock solutions contained cDNA generated from either 0.1, 1, 5, 10, 20 or 40 ng of input RNA per 8 µl aliquot. Diluted cDNA was then combined with 10 µl of 2X ddPCR Supermix for Probes, 1 µl of target TaqMan gene expression assay, and 1 µl of either water or 20X Sdha-VIC assay and loaded into a DG8 cartridge eight samples at a time, along with 70 µl of droplet generation oil for probes. Cartridges were sealed with a DG8 rubber gasket and placed into the droplet generator where reactions were partitioned into droplets. Droplet emulsions were subsequently transferred to an Eppendorf semi-skirted 96-well PCR plate (Eppendorf North America, NY, USA), sealed with a pierceable foil heat seal in a PX1 Plate Sealer (Bio-Rad), and incubated in a T100 Thermal Cycler (Bio-Rad) using the following parameters: 1 cycle of 10 min at 95°C; 40 cycles of 30 s at 94°C followed by 1 min at either 59°C (Pparg, Slc16a2, Sult2a2) or 56°C (all others) at a ramp rate of 2.0°C/s; 1 cycle of 10 min at 98°C. Samples were then cooled to 4°C and held until the plate was transferred to the QX200 Droplet Reader. Data generated were analyzed with QuantaSoft™ Analysis Pro version 1.7.4.0917 (Bio-Rad) in 2D Amplitude mode. Cutoffs for positive droplets were set to exclude any intermediate droplets ('rain'). Droplet counts were exported into Microsoft Excel for further analysis.

Computation of average reference concentration

ddPCR assays for Sdha and Elk4 were run in triplicate at a range of concentrations. As demonstrated in Figure 1, the copies per 20 µl assay (reference copies per 20 µl well, column f) were divided by the theoretical amount of input RNA (Theoretical Input Amount, column b) as calculated from the original RNA measurement for the sample. RT efficiency was assumed to be consistent across samples for the individual reference genes. The resulting values were

defined as copies per theoretical ng input RNA (Reference Copies per Theoretical ng Input RNA, column g), also referred to as 'theoretical concentration'. These values were averaged to obtain a reference value equal to average copies per ng (average reference value, column h). Copies per theoretical ng input RNA were then divided by the average reference value to determine the percent of the average represented by the individual value (percent of average, column i), and this value was multiplied by the theoretical concentration for the sample to obtain the estimated concentration of the sample (calculated ng input RNA, column j; see also example spreadsheet in Supplementary Material). Estimated sample concentrations (reference values) were calculated with both Sdha and Elk4 and with Sdha alone and gave comparable results. Sdha reference values were calculated both within each concentration and across all concentrations; subsequent concentration estimates were determined for each sample and plotted against the number of copies measured for the samples. The resulting linearity of the values was compared by performing linear regression analysis in SigmaPlot version 12.5 (Systat Software, Inc., CA, USA).

Target gene analysis

For theoretical or standard reference methods, copies of target gene per 20 µl assay (Figure 1B, column d) were divided by either calculated or theoretical ng of input RNA as determined above for the relevant sample and concentration used for each assay to obtain the target copies per ng input RNA (Figure 1B, column k). For the duplex method, a reference value was calculated as above for the Sdha copies per 20 µl in the duplex reactions. This value was used to calculate a ng value for each individual assay that was subsequently used to calculate the copies per ng value for the target gene. Triplicate values for each sample were averaged, exported to SigmaPlot, and graphically represented as box and whisker plots for comparison. P values for vehicle versus TBBPA-treated samples were determined for each calculation method using the unpaired t-test function in SigmaPlot.

Results & discussion

It has been previously determined that protein and chemical contaminants in extracted nucleic acid samples can partially inhibit both the RT and qPCR reactions [27], and that components of the RT reaction also can inhibit the qPCR reaction [26], so endogenous reference genes were used to normalize the sample concentrations. Bustin *et al.* [32] promote the use of at least three reference genes from different pathways; however, it had previously been determined using the geNorm^{PLUS} function of qbase^{PLUS} (Biogazelle, Zwijnaarde, Belgium) that two reference genes, Sdha and Elk4, were most stable and sufficient in this sample set (Figure S1) [31]. No significant difference was observed between treatment groups of the current sample set for these genes in data compiled from previous analyses (Figure S2).

To evaluate different methods of calculating sample concentration from measured reference gene copies, a range of amounts of liver cDNA (0.1–40 ng) from 20 samples was assayed for Sdha expression and the different methods used to compute the amount of cDNA in the sample. The computed values were then plotted against the number of copies and linearity of the resulting line evaluated. For each individual liver sample, the number of measured copies was plotted against the theoretical concentration, the concentration calculated from averaging all the values over all the concentrations, and the concentration calculated from averaging across each individual concentration; linear regression analysis was then performed. Table 1 shows the resulting R^2 values for each line. The best fit was obtained when averaging across all the concentrations, so this method was used for further analyses.

Initially, sample dilutions were assayed for both reference genes individually and sample concentrations were calculated

Ⓐ

Reference copies per 20 µl well (*column f*)

↓

Divide by **Theoretical Input Amount (ng)** (*column b*) to obtain **Reference Copies per Theoretical ng Input RNA** (*column g*)

↓

Average **Reference Copies per Theoretical ng Input RNA** (*column g*) across all samples and concentrations to obtain **Average Reference Value** (*column h*)

↓

Divide **Reference Copies per Theoretical ng Input RNA** (*column g*) by **Average Reference Value** (*column h*) to obtain **Percent of Average** (*column i*)

↓

Multiply **Reference Copies per Theoretical ng Input RNA** (*column g*) by **Percent of Average** (*column i*) to obtain **Calculated ng Input RNA** (*column j*)

↓

Divide **Target Copies Per 20 µl Well** (*column d*) by **Calculated ng Input RNA** (*column j*) to obtain **Target Copies per ng Input RNA** (*column k*)

Ⓑ

	a	b	c	d	e	f	g	h	i	j	k
1	Sample	Theoretical input amount (ng)	Target	Target Copies Per 20 µl Well	Reference	Reference Copies Per 20 µl Well	Reference Theoretical copies per ng	Average Reference Value	Percent of Average	Calculated ng input RNA	Target copies per ng input RNA
2							f(line n)/ b(line n)	AVERAGE of all values in column g	g(line n)/ Average from Column h	b(line n)* i(line n)	d(line n)/ j(line n)
3	L52	0.1	Sult2a1	4360	Sdha	538	5380	1325.05	406.02%	0.41	10738.30
4	L52	0.1	Sult2a1	4540	Sdha	498	4980		375.84%	0.38	12079.74
5	L52	0.1	Sult2a1	4440	Sdha	452	4520		341.12%	0.34	13015.95
6	L53	0.1	Sult2a1	5440	Sdha	442	4420		333.57%	0.33	16308.27
7	L53	0.1	Sult2a1	5500	Sdha	474	4740		357.72%	0.36	15375.01
8	L53	0.1	Sult2a1	5580	Sdha	486	4860		366.78%	0.37	15213.50

Figure 1. Workflow diagram of calculations and excerpt from example spreadsheet. Column letters in diagram **(A)** refer to columns in example spreadsheet excerpt **(B)**. Example spreadsheet is available in Supplementary material.

Table 1. Linearity of concentration versus number of copies for reference gene Sdha using theoretical versus calculated values.

Sample	R^2 (theoretical concentration)	R^2 (using total of all concentrations)	R^2 (using individual concentration)
L52	0.987	1.000	0.990
L53	0.989	0.999	0.990
L54	0.987	1.000	0.988
L55	0.989	0.999	0.988
L56	0.987	1.000	0.993
L57	0.985	1.000	0.995
L58	0.986	1.000	0.992
L59	0.990	1.000	0.992
L60	0.988	1.000	0.990
L61	0.990	1.000	0.992
L62	0.987	0.999	0.989
L63	0.991	0.999	0.990
L64	0.990	1.000	0.993
L65	0.984	1.000	0.993
L66	0.990	1.000	0.990
L67	0.978	0.991	0.977
L68	0.984	1.000	0.992
L69	0.993	1.000	0.990
L70	0.989	1.000	0.991
L71	0.992	1.000	0.991

R^2 values were determined by plotting theoretical concentration or concentration calculated using all concentration values or within individual concentration groups (all in copies per ng) and performing linear regression analysis in SigmaPlot version 12.5 (see Methods).

from the average of both. Best results were obtained when at least three different concentrations were considered, although increased variability was seen in the lowest concentration samples, likely due to the difficulty of obtaining accurate measurements of stock for the lowest concentrations. Serial dilutions were not used, since an inaccuracy in one dilution would be carried through to any further dilutions and affect the accuracy of the resultant concentration values. While this method did compensate for measurement errors in the steps leading up to and including the stock solution preparation, it still allowed variability in the actual assays. To correct for assay variability, it was necessary to measure the reference gene in the actual assay through duplexing the target gene assay with the reference gene assay. For practical reasons, only one reference gene was used for this purpose; Sdha was selected since it was the most stable reference gene according to geNorm^{PLUS} analysis and also had the best expression level in the samples. A subset of 11 assays previously used for qPCR that had shown a range of expression was chosen and ddPCR assays were performed. Both reference genes were run separately, and Sdha was duplexed with the target assays. Gene expression was then calculated using the theoretical concentration, the separately assayed values both for Sdha alone and the combination of the two reference genes, and the duplexed Sdha reference.

Figure 2 demonstrates the impact of the different calculation methods on the statistical significance of a comparison of treated and control samples. It is clear that normalization of the data to account for variables inherent in the assay process affects the distribution of values. For Nr1i2, the median when calculated using the theoretical value was almost equivalent for the two treatment groups. However, when differences in sample concentration were considered, there was a marked difference. The opposite was the case for

Table 2. Comparison of p-values for different methods of computing droplet digital PCR values.

Gene	Theoretical (copies/μl)	Standard reference	Standard reference (SDHA only)	Duplexed reference
Ccnb2	0.0788	0.0669	0.0506	0.0540
Ccnd2	0.0662	0.123	0.106	0.378
Cyp2b1	0.0525	0.0404	0.0306	0.0237
Nr1i2	0.773	0.157	0.204	0.149
Pparg	0.920	0.831	0.691	0.752
Slc16a2	0.0611	0.0923	0.136	0.0370
Sult1e1	0.657	0.690	0.626	0.564
Sult2a2	0.734	0.850	0.791	0.791
Thra	0.00609	0.00374	0.00623	0.00328
Tp53	0.436	0.772	0.859	0.989

Data for Cyp1b1, Sult1e1 and Sult2a2 did not represent normal distributions, so p-values were determined using the Mann–Whitney Rank Sum test. p-values for all other assays were computed using an unpaired t-test. Statistics were performed in SigmaPlot version 12.5. p-values for copies/μl were also computed and were equal to those for theoretical concentration.

Ccnd2. The calculation method can also affect the computed significance within a data set. With Cyp2b1, the difference in treatment groups was not significant using the theoretical values but was significant when the calculation compensated for concentration variability. A similar result was obtained for Slc16a2, where only the duplexed reference showed a significant difference. Nr1i2 and, to a lesser extent, Ccnd2 also demonstrate situations where the calculated concentration of the samples is significantly different from the theoretical concentration, most likely in this case due to a poorly calibrated pipette.

Table 2 compares the p-values for all the gene assays considered. Little difference was noted between the p-values for the standard reference (using both Sdha and Elk4) and the Sdha-only reference, whereas those for the theoretical concentration showed wide variation for some of the assays. Statistics were also run for copies/µl, and the results were equivalent to those for theoretical concentration.

Ultimately, the method that accounted for the most sources of variance and gave the most consistent results was duplexing a reference gene into the assays and using that value to calculate the individual concentration of input RNA that was present in the sample. Placing the reference in the sample assay itself allowed compensation for measurement variation throughout the RNA isolation and assay procedure. While this study utilized probe-based assays for duplexing, dye-binding assays such as EvaGreen can also be duplexed in ddPCR by exploiting the effect of amplicon length, annealing temperature, or dye concentration on the amplitude of the resulting droplets [33,34]. Duplexing of reference and target assays could still be susceptible to amplification bias from inhibitors within the specific samples, but it should allow normalization for both sample concentration variations and assay-specific bias across samples.

When comparing gene expression or DNA copy number across different assays and sample sets, it is necessary to have a common point of reference. The method presented here uses reference genes to normalize RNA or DNA concentration in a sample set and relate digital PCR results to that concentration, reducing the variability seen within experiments and allowing more reliable intra-assay and inter-assay comparisons to be made.

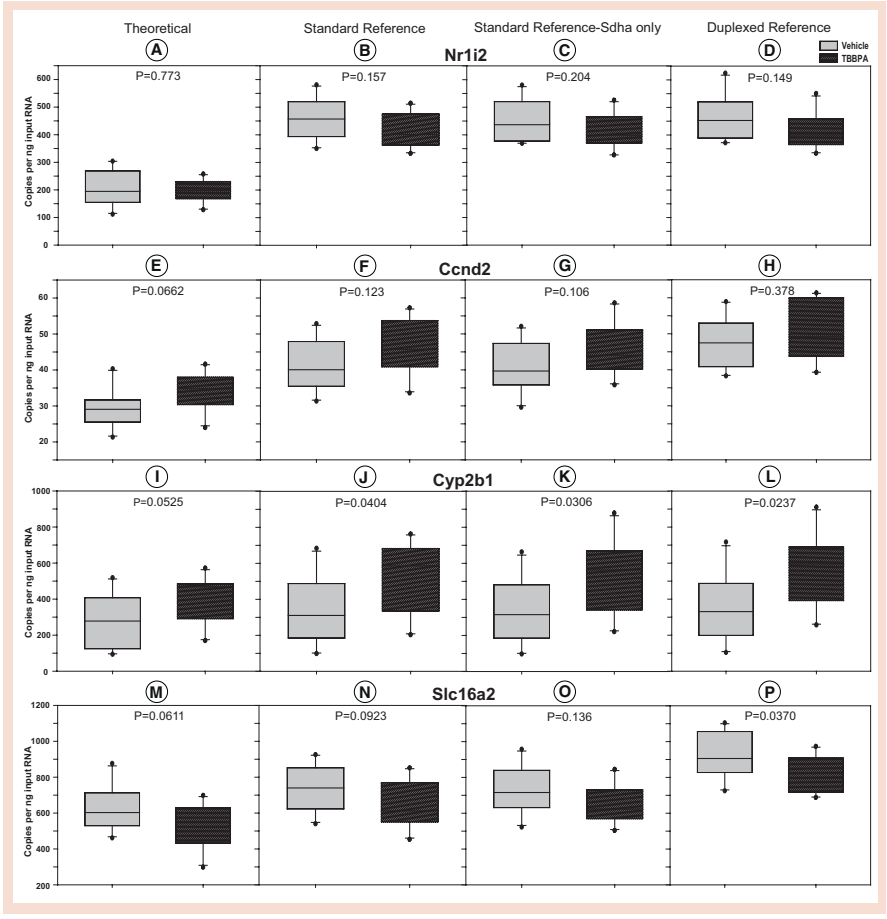

Figure 2. Box and whisker plots of droplet digital PCR results for TaqMan gene expression assays of liver RNA samples from TBBPA-treated versus vehicle-treated Wistar Han rats showing effect of different calculation methods on computed p-values and significance of difference between treatment groups. Copies per ng input RNA were calculated using theoretical concentration (no reference; **A, E, I, M**), concentrations calculated using both reference genes Sdha and Elk4 (standard reference; **B, F, J, N**) or Sdha only (standard reference-Sdha only; **C, G, K, O**) run as separate assays, and concentrations calculated using duplexed Sdha (duplexed reference; **D, H, L, P**), showing median and range of values. p-values were determined using an unpaired t-test in SigmaPlot version 12.5 and are indicated on each graph. **(A–D)** Nr1i2 shows greater difference in treatment groups with normalization of sample concentrations, and also demonstrates a situation where the calculated concentration differs significantly from the theoretical concentration. **(E–H)** Ccnd2 shows less difference in treatment groups with normalization. **(I–L)** Cyp2b1 shows significant difference ($p < 0.05$) after normalization for all methods. **(M–P)** Slc16a2 shows significant difference only with duplexed reference **(P)**.

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Supplementary data

To view the supplementary data that accompany this paper please visit the

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