Trends in Biotechnology

Review

The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time

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Quantitative PCR (qPCR) is one of the most common techniques for quantification of nucleic acid molecules in biological and environmental samples. Although the methodology is perceived to be relatively simple, there are a number of steps and reagents that require optimization and validation to ensure reproducible data that accurately reflect the biological question(s) being posed. This review article describes and illustrates the critical pitfalls and sources of error in qPCR experiments, along with a rigorous, stepwise process to minimize variability, time, and cost in generating reproducible, publication quality data every time. Finally, an approach to make an informed choice between qPCR and digital PCR technologies is described.

Highlights

qPCR is more complex than perceived by many scientists.

The production of an amplification curve and an associated quantitative cycle value does not necessarily mean interpretable data.

The MIQE guidelines and associated methodology articles published thereafter, underline the ongoing drive to help scientists produce reproducible data from qPCR, culminating in a simple, stepwise methodology to ensure high-quality, reproducible data from qPCR experiments.

The concept of data normalization has led to the ongoing publication of articles solely focused on this subject for various sample types and experimental parameters.

The analysis of qPCR data can be challenging, especially as experiments grow in sample number and complexity of biological groups. A defined approach to qPCR data analysis is necessary to clarify gene expression analysis.

qPCR Technique: The Perception and Reality

qPCR (see Glossary) is generally viewed by researchers as a powerful technique that can provide precise and quantitative data reflecting the biology of the tested experimental parameters. However, without following strict guidelines, validation and data analysis procedures, the results can be far from valid [1,2]. Unfortunately, the adoption and transfer of inadequate and varied protocols between individual laboratory members and laboratories throughout the scientific community have led to frustration in reproducing data [3–5]. This has driven the production of the minimum information for publication of quantitative real-time PCR experiments (**MIQE**) guidelines and related methodology articles to help the scientific community in augmenting experimental rigor and uniformity to produce more reliable and consistent data [6–8]. Nevertheless, there remain concerns regarding the quality of qPCR results in the published literature [1,2].

When designing experiments for qPCR, all protocols, such as sample handling, harvesting, nucleic acid extraction, reverse transcription, and qPCR should be described and vetted in detail. Mistakes or assumptions can be made in the planning process, resulting in a flawed experimental design with results and conclusions based on artefacts of pre and/or post sample handling procedures as opposed to the true effect of the tested experimental parameters [7]. Poorly optimized reactions can result in data that are consequent to a combination of sample contaminants and/or poor annealing temperature, leading to misinterpreted results and conclusions that are difficult or even impossible to reproduce [9,10].

Despite the MIQE guidelines and other methodology articles, the variability and reproducibility pitfalls associated with qPCR remain elusive for many laboratories [7,11]. This review article describes the major sources of error associated with a qPCR experiment and strategies for their

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minimization, along with a rigorous, stepwise approach to producing accurate and precise results. Finally, a simple decision tree is proposed to choose between digital PCR (**dPCR**) and qPCR technologies to minimize cost and time to publication for any type of sample and target abundance.

Predominant Sources of Error Associated with a qPCR Experiment

There are two major sources of error in any life science experiment that can lead to a high level of variability between test conditions: random and systemic error [12]. Random error is associated with flawed experimental design parameters, which are primarily manifested in biological variability and subsampling error. Systemic error arises from improper use or calibration of equipment and computational software, leading to technical and calculation errors. Each can contribute significantly to the total error derived from an experiment, producing nonstatistically significant and/or artefactual data that is unrepresentative of the tested parameters. By isolating error sources and understanding how to minimize their impact through careful experimental design and good technique, solid, reproducible, and statistically significant results can be achieved that will stand the test of time in the literature.

Random Error Sources

Biological Variability

The most challenging and least considered aspect of many experiments is the appropriate selection of a randomized set of individual samples (i.e., biological replicates) per biological group (i.e., treatment/experimental conditions) while minimizing their inherent variability [13]. Both the transcriptome and proteome are highly sensitive to the inherent biological differences between samples. In fact, gross differences in transcription have been demonstrated between individual cells plated from the same passage in a single petri dish [14,15]. Therefore, careful thought and planning must go into the sourcing, randomized selection, and number of samples per biological group to ensure statistically significant and reproducible results that give the precision and accuracy for publication (Figure 1A).

Apart from the inappropriate selection of a randomized set of samples that represent the population, nonreproducible error can also be introduced from: (i) lot-to-lot differences in growth medium or animal feed [16]; (ii) variation in temperature, time, incubation conditions, and circadian regulation between samples (Figure 1B) [17–19]; (iii) tissue sections or cell subpopulations extracted from a specimen (Figure 1C) [17,18]; (iv) particularly for female animal models, the synchronization of hormonal cycles [20,21]; and (v) for environment and plant material, time of day, amount of light, temperature, soil microenvironment, and *in situ* sampling and storage techniques [22,23]. Each of these experimental design factors can significantly increase biological error and variability, which can ultimately lead to false conclusions.

Subsampling Error

In most molecular biology experiments, a portion of the sample (a subsample) is tested. The error associated with quantification of the number of molecules from a portion of the sample and extrapolating the result to the total is termed subsampling error (Figure 2) [24]. For example, if a 1 μl subsample of cDNA is taken from a total volume of 25 μl with a concentration of six target molecules per μl, the expected number of copies in the subsample would be six. However, the result would likely range between four and eight copies. Assuming no technical error in pipetting, the deviation in the result from the predicted six copies is the subsampling error, which is much more pronounced in sample concentrations below about two copies per microliter (Figure 2).

Glossary

Absolute quantification: the quantification of the absolute quantity of a target gene in a nucleic acid sample. This requires the application of a standard curve using a known concentration of starting sample.
Cq: the quantitative cycle, where the amplification curve from a given target/sample combination crosses the threshold or is fit to a second order derivative to equate the cycle at which the amplification curves are detectable and quantified.
dPCR: digital PCR technology for precise quantification of the absolute concentration of a given target in a nucleic acid sample.
MIQE: minimum information for publication of quantitative real-time PCR experiments. Pronounced ‘MIKEE’, these guidelines provide a framework to conduct qPCR experiments and the information and terminology required for submission of qPCR data to any journal.
Mutation abundance: the relative abundance of a mutant allele with respect to the wild type in a given sample.
Normalized relative expression: the relative expression of the target gene normalized to that of multiple reference genes within the same biological sample (Figures 4D and 5).
qPCR: quantitative polymerase chain reaction. A blanket term that covers any type of qPCR experiment for both absolute and relative quantification.
Relative quantification: the fold difference between a particular reference (typically control) sample relative to all other samples in the experiment. Typically, the calculations result in the control biological group giving a relative expression of 1 and the treatment groups are either fold increase or decrease of control (Figure 5).
RT-qPCR: reverse transcription qPCR, which implies that the starting sample was from messenger RNA, which in turn required reverse transcription to cDNA prior to performing qPCR.

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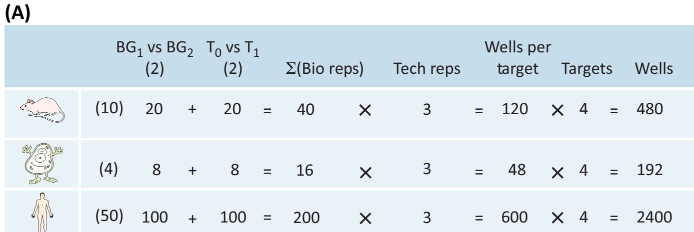
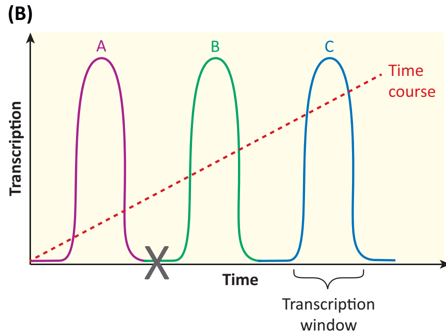
(A)



		BG_1 vs BG_2 (2)		T_0 vs T_1 (2)		Σ(Bio reps)		Tech reps		Wells per target		Targets		Wells
	(10)	20	+	20	=	40	×	3	=	120	×	4	=	480
	(4)	8	+	8	=	16	×	3	=	48	×	4	=	192
	(50)	100	+	100	=	200	×	3	=	600	×	4	=	2400

(B)



(C)

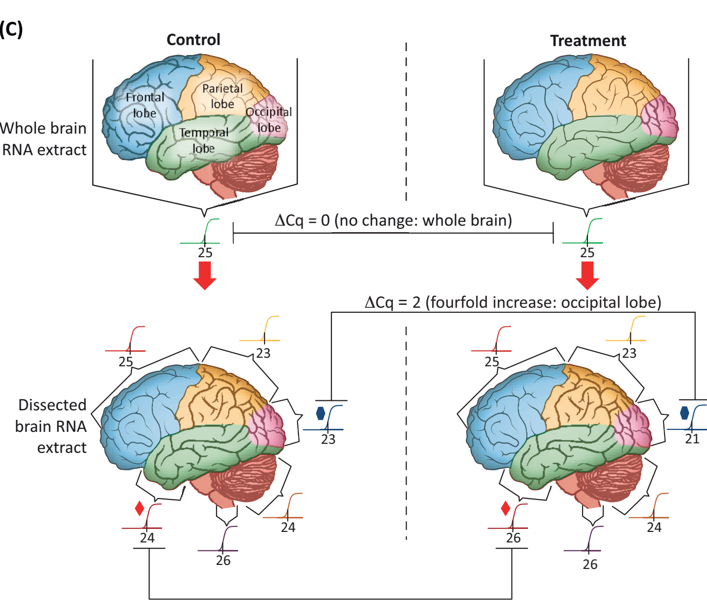


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Figure 1. Planning and Scoping a Quantitative PCR (qPCR) Experiment. (A) Biological group (BG): tested experimental conditions (i.e., control (BG_1) vs treatment (BG_2). Time point (T) (i.e., 0 hour (T_0) and 1 hour (T_1) post-treatment). Biological replicate (Bio reps): number of independent biological specimens from which cDNA/gDNA is produced (i.e., individual samples). Technical replicate (Tech reps): number of repeats per Bio rep (i.e., same cDNA/gDNA sample pipetted into multiple wells). Targets: genes interrogated for differential expression or abundance including chosen reference genes. Wells: total number of microtiter plate wells required for qPCR exclusive of optimization experiments for primer validation (see Figure 4). (B) Time dependence on transcription of targets A, B, and C post-treatment. Choosing a random time point (X) post-treatment may result in no data and/or artefactual data that do not reflect the true response of the tested targets. Performing a preliminary time course study (dotted line) ensures the selection of optimal time points for each target. (C) Influence of tissue sectioning on data quality. Whole tissue RNA extract may show no change, whereas dissected tissue may produce expression differences between biological groups (i.e., fourfold change when comparing expression of occipital lobe or temporal lobe) consequent to target dilution in whole tissue and enrichment in sections.

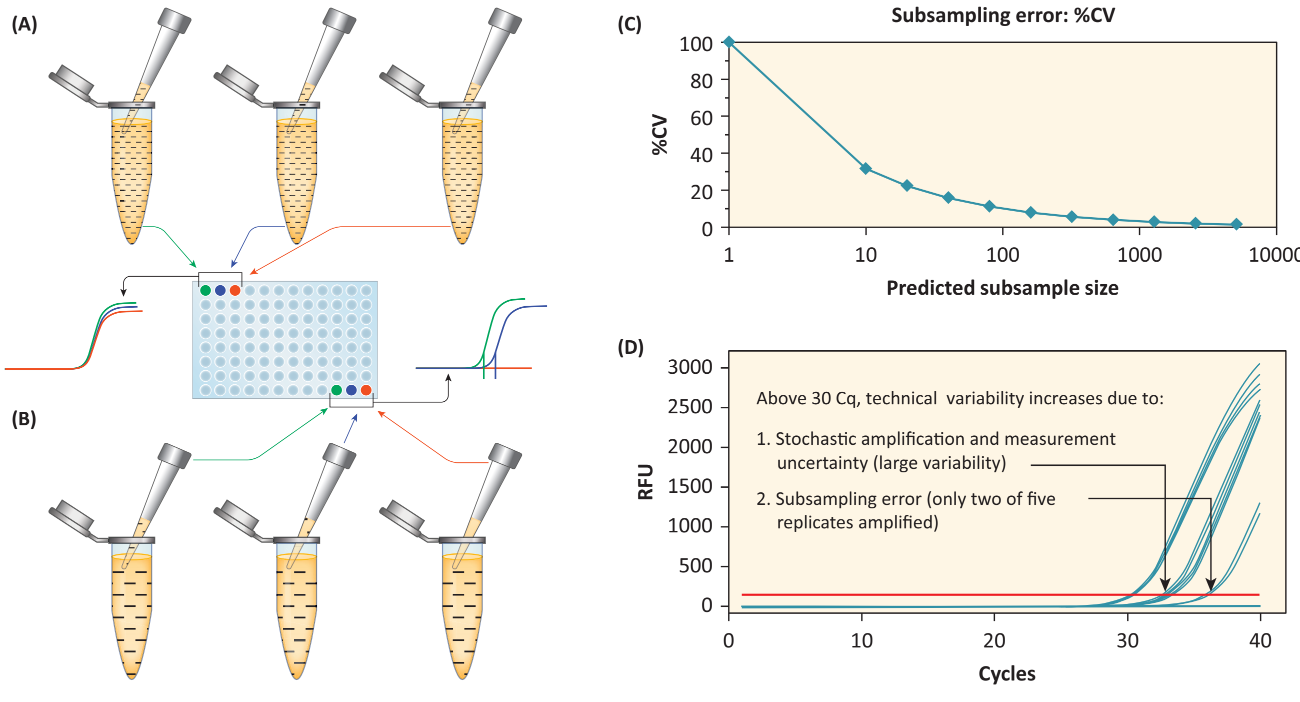

Figure 2. Subsampling Error on Quantitative PCR (qPCR) Data between Triplicate Technical Replicates for High and Low DNA Levels. (A) Minimal error and tight quantitative cycle (Cq) values are observed at higher concentrations of DNA. (B) High error and variability in Cq values between technical replicates occurs for 'sub' samples with low DNA levels. (C) Predicted subsampling error correlated to sample size. (D) Demonstrated effect of stochastic amplification/measurement uncertainty and subsampling error on quintuplicate replicates of three dilutions of DNA. Near 30 Cq the replicates are tight, but above 30 Cq variability increases (stochastic amplification/measurement) until a critical dilution is reached where only two of the five replicates give amplification curves (subsampling error). Abbreviations: CV, coefficient of variation; RFU, relative fluorescence unit.

The coefficient of variation (CV) measures the variation around the mean, and is a useful tool to assess the degree of data inconsistency (Figure 2C). The standard deviation (SD) and CV associated with subsampling error can be calculated from the expected number of copies in a given sample as follows:

M = expected number of target molecules under binomial approximation

$$SD = \sqrt{M}$$

$$CV = \frac{\sqrt{M}}{M}$$

From the previous example, the average number of copies for several 1 μl subsamples will be six but the SD of this measurement is ($\sqrt{6} = 2.45$) and the %CV is (2.45/6*100 = 40.8%). Subsampling error contributes to more than 10% of the variance when the subsample is below 100 copies, and above 30% when the subsample is below ten copies (Figure 2C,D) [24]. This necessitates running more technical replicates (i.e., subsamples) for samples of very low concentration.

Systemic Error Sources

Pipetting Error, Nucleic Acid Contaminants, and Controls

One of the most common and problematic sources of technical variability in molecular biology stems from pipetting and the use of inappropriate pipettes and sample volumes. Many

laboratories inadvertently use poorly calibrated pipettes coupled with tips and technique that may be inaccurate for small volumes [25,26]. This can produce variability ranging between 5% and 37% CV [27]. It becomes a major problem when samples are serially diluted because the pipetting errors between each dilution are propagated, resulting in gross variability between data points [28,29]. For qPCR, a good methodology to minimize pipetting error is to apply a 60:40 ratio of master mix to cDNA/gDNA sample (Figure 3A), which permits: (i) the use of the same pipette and tips, (ii) efficient mixing of sample in the reaction mix, and (iii) pipetting volumes that correspond to the midrange of the pipette for high accuracy.

Nucleic acid contaminants represent another major source of error in qPCR. This necessitates the inclusion of no template controls (NTCs) on each plate to assess the level of cDNA or gDNA contamination that may stem from the individual reaction components (Figure 3A). No reverse transcription controls (NRTs) are also important to assess the levels of genomic DNA contaminants from the initial RNA extracts of a given project [30].

Sample Treatment, Handling, Harvesting, Nucleic Acid Extraction, and Reverse Transcription

Given the sensitivity of the transcriptome to even minor changes in the surrounding environment, care and rigor must be applied to ensure uniformity in: (i) sample handling throughout the experiment; (ii) activity/potency between lots of the same compound or reagent(s) used for

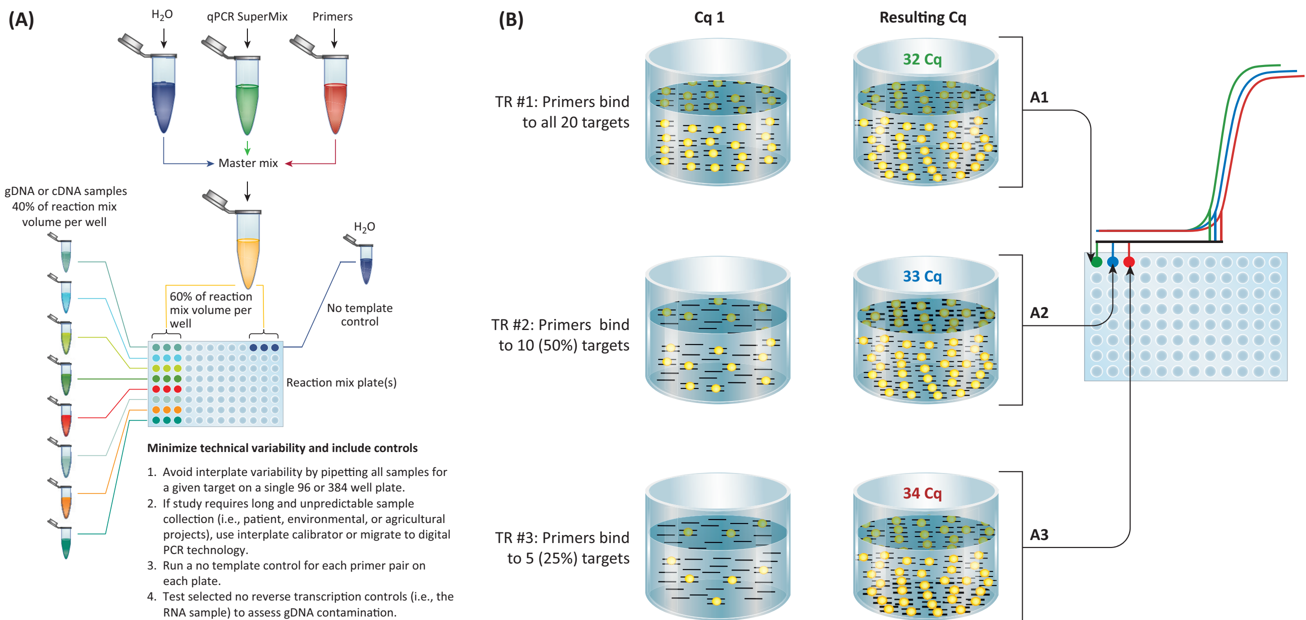

Figure 3. Master Mix Preparation, Plate Loading and Measurement Uncertainty. (A) To minimize error linked to pipetting, the volume of cDNA/gDNA in the qPCR reaction mix should account for approximately 40% of the total reaction volume. Include a no template control for each master mix (target) loaded on a given plate. Test no reverse transcription controls for the first RNA extracts to ensure minimal gDNA contamination. To avoid interplate variability, all samples for a given target should be pipetted on the same plate. If this is not feasible, interplate calibration should be performed, or digital PCR (dPCR) should be considered. (B) Measurement uncertainty clarified. Each well represents a technical replicate (TR) from the same sample, where the amount of target template transferred to each reaction is consistent but low (<100 copies). Measurement uncertainty stems from the amplification of fractional quantities of template in the initial PCR cycles, resulting in variable quantitative cycle (Cq) values between technical replicates (i.e., if only a fraction of the available template amplifies in cycle one, and the remainder begin amplification in the following cycles, the amplification curve will shift higher as shown in TR #2 and TR #3).

treatment [16]; (iii) tissue or cell harvesting and homogenization techniques; and (iv) sample storage and freeze/thaw conditions [31].

RNA/DNA extraction procedures and storage can also play a significant role in data quality, and good kits are available to produce excellent RNA, DNA, or protein extracts to ensure consistency in the quantity, quality, and purity of extracted nucleic acids [31–33]. However, the appropriate choice of extraction kit is critical to minimize background contaminants that can alter the Cq values. This is highly dependent on the sample and no single kit serves all applications [32].

For **RT-qPCR** specifically, significant variability can arise from the reverse transcription (RT) of mRNA to cDNA where the results for the same sample can vary by two to threefold depending on the amount and quality of mRNA used in the RT reaction [34]. Furthermore, the amount of target mRNA with respect to background can have a major impact on the downstream results, which can also be RT kit dependent [35]. Finally, the components of the RT reaction can be inhibitory to the downstream qPCR reaction [35]. Hence, care must be taken in adequately diluting the RNA and the resulting cDNA, to ensure efficient RT and qPCR reactions that reflect the true proportion of the mRNA target(s) in the original samples.

Stochastic Amplification and Measurement Uncertainty

When the target DNA is very dilute (less than ten copies per microliter), the primers may not land on all the template molecules in the first cycle. Thus, different fractions of the original starting template are amplified from the first and second cycles before complete amplification of all template has been initiated (Figure 3B). This can lead to large variability between the technical replicates of a given sample that can easily range between 10% to 200% CV, particularly when template is below 100 copies per reaction [i.e., the quantitative cycle (**Cq**) values are greater than 29 cycles]; (Figure 2D) [6,36,37]. This variability can only be minimized by interrogating a larger proportion of the sample from more technical replicates and using the average Cq for downstream calculations.

Multiplexed Experiments and Reaction Efficiency

One of the advantages of qPCR is the ability to perform multiplexed detection and quantification of several targets within the same sample using probe-based chemistry. This is particularly useful when working with very precious samples containing low quantities of nucleic acids. However, qPCR is entirely dependent on reaction efficiency where a Cq value is only representative of the template amount when the reaction efficiency is near 100% (Figure 4A–C). This necessitates a rigorous validation of individual targets in a representative sample (Figure 4B,C) to ensure their reaction efficiencies and annealing temperatures are similar prior to multiplexing, coupled with limited crosstalk between primers and probes when combined in one reaction [38]. Multiplexed preamplification offers the advantage of increasing the starting quantity of a selected panel of targets to permit their detection and quantification from individual SYBR Green containing qPCR reactions without the constraints of probe-based multiplexing [39,40]. Furthermore, qPCR offers flexibility in running small projects with hand pipetting, and large-scale projects requiring high-throughput sample processing using robotics liquid handlers and automated plate loading and reading.

qPCR Calculations and Error Propagation

The data analysis and associated calculations for relative quantification involve multiple steps (Figure 5): (i) extract the Cq values for each sample after inter-run calibration and calculate the mean Cq from the associated technical replicates; (ii) determine the relative quantity by raising

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Figure 4. Validating a Quantitative PCR (qPCR) Experiment to Minimize Error and Maximize Data Quality. (A) Nonvalidated primers could give variable and/or artefactual data that do not reflect the true target abundance in each sample. (B) Primer validation using thermal gradient and gel electrophoresis. Step #1A: Thermal gradient and agarose gel validation. To validate primers, an equalized pool of samples from each biological group is diluted 1:20 and initially tested using a thermal gradient to determine the optimal annealing temperature, average level of expression, and unique product for each target from melt curve and gel analysis. Step #1B: The quantitative cycle (Cq) value from the optimal annealing temperature range can be used as a guide to establish the standard curve dilution factor for each target (i.e., if the Cq value for optimized temperature range is between 10 and 16, use a 1:8 serial dilution series of the pooled cDNA sample in water). (C) Standard curve validation. An eight-point standard curve is tested for each primer pair using the same pooled sample and the appropriate dilution factor as determined from the thermal gradient data (Step #1B) to cover the widest dynamic range possible. Amplification efficiency, as determined from the slope, should range between 90% and 110%. Deleting the highest and/or lowest concentration points from each primer validation standard curve may be necessary to achieve the best efficiency. The dilution factor from the midpoint is then used to dilute the individual experimental samples per target, assuming that the pooled DNA sample represents the average abundance of each target for the experiment (i.e., equalized pool of the same number of DNA samples from each biological group). This ensures minimal presence of contaminants affecting primer efficiency and accurate quantitative data. (D) Consequence of using poorly validated reference genes. qPCR data can change dramatically when normalization is performed using a stable versus unstable reference gene.

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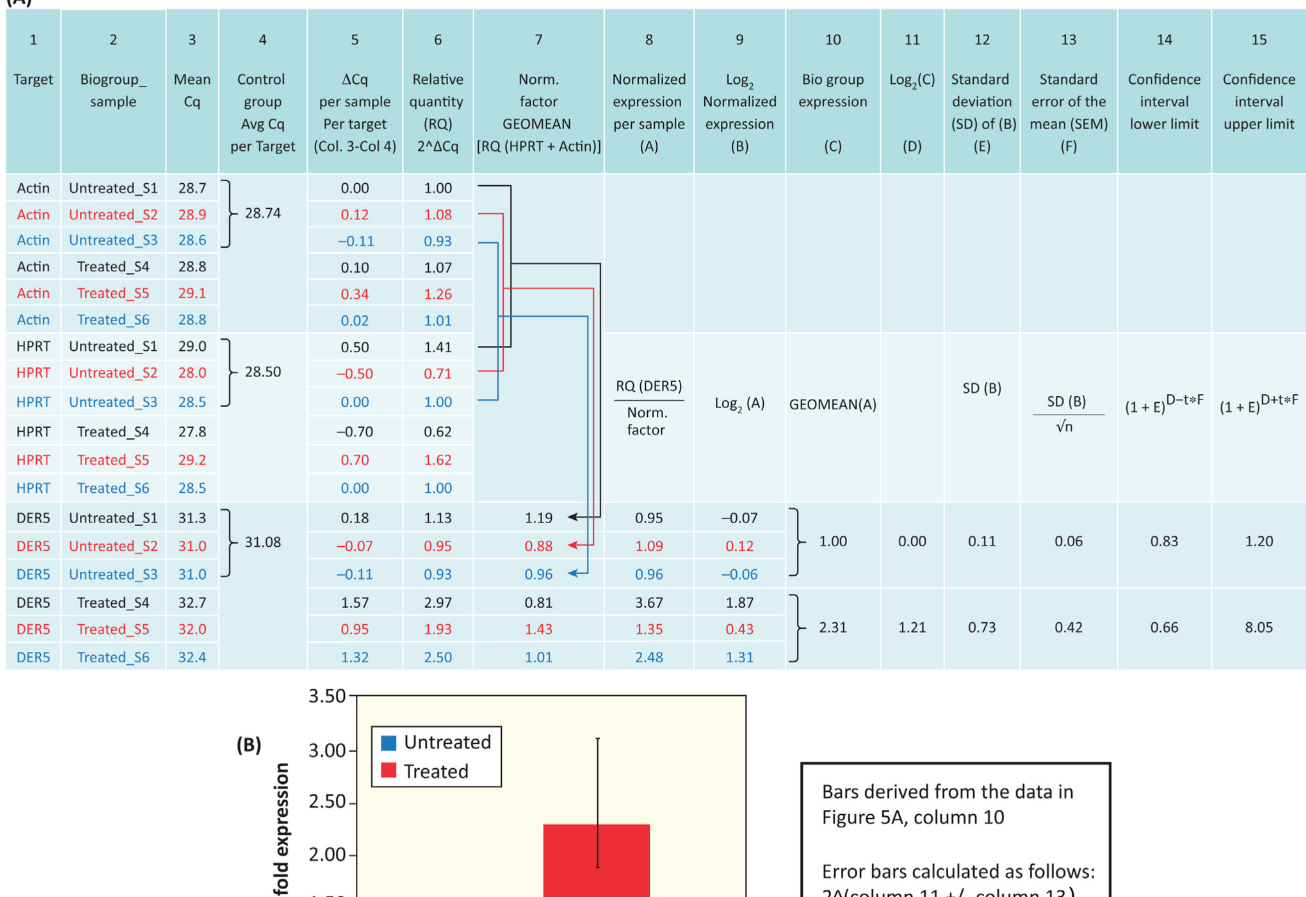
(A)



1 Target	2 Biogroup_ sample	3 Mean Cq	4 Control group Avg Cq per Target	5 ΔCq per sample Per target (Col. 3-Col 4)	6 Relative quantity (RQ) 2^ΔCq	7 Norm. factor GEOMEAN [RQ (HPRT + Actin)]	8 Normalized expression per sample (A)	9 Log_2 Normalized expression (B)	10 Bio group expression (C)	11 Log_2(C) (D)	12 Standard deviation (SD) of (B) (E)	13 Standard error of the mean (SEM) (F)	14 Confidence interval lower limit	15 Confidence interval upper limit
Actin	Untreated_S1	28.7		0.00	1.00									
Actin	Untreated_S2	28.9	28.74	0.12	1.08									
Actin	Untreated_S3	28.6		−0.11	0.93									
Actin	Treated_S4	28.8		0.10	1.07									
Actin	Treated_S5	29.1		0.34	1.26									
Actin	Treated_S6	28.8		0.02	1.01									
HPRT	Untreated_S1	29.0		0.50	1.41									
HPRT	Untreated_S2	28.0	28.50	−0.50	0.71									
HPRT	Untreated_S3	28.5		0.00	1.00		RQ (DER5) / Norm. factor	Log_2 (A)	GEOMEAN(A)		SD (B)	SD (B) / √n	$(1 + E)^{D-t*F}$	$(1 + E)^{D+t*F}$
HPRT	Treated_S4	27.8		−0.70	0.62									
HPRT	Treated_S5	29.2		0.70	1.62									
HPRT	Treated_S6	28.5		0.00	1.00									
DER5	Untreated_S1	31.3		0.18	1.13	1.19	0.95	−0.07						
DER5	Untreated_S2	31.0	31.08	−0.07	0.95	0.88	1.09	0.12	1.00	0.00	0.11	0.06	0.83	1.20
DER5	Untreated_S3	31.0		−0.11	0.93	0.96	0.96	−0.06						
DER5	Treated_S4	32.7		1.57	2.97	0.81	3.67	1.87						
DER5	Treated_S5	32.0		0.95	1.93	1.43	1.35	0.43	2.31	1.21	0.73	0.42	0.66	8.05
DER5	Treated_S6	32.4		1.32	2.50	1.01	2.48	1.31						

Figure 5. Data Analysis for a Typical Relative Quantification Experiment. (A) Rigorous data workup. In this example, DER5 expression is measured between treated and untreated biological groups, each containing three biological replicates and two technical replicates (data for technical replicates is not shown). Actin and HPRT are used as reference genes. First, the mean quantitative cycle (mean Cq; column 3) of the technical replicates for each sample/target combination is calculated [i. e., for actin from the untreated sample 1 (Untreated_S1) the mean Cq is 28.7]. The average Cq of all samples in the control group (i.e., untreated) for each target is then determined (column 4). The relative difference (ΔCq) between the average Cq for the control group (column 4) and the mean Cq (column 3) per individual sample within each target is assessed (column 5). The relative quantities are calculated from the ΔCq (i.e., $2^{\Delta Cq}$) where in this case, it is assumed that reaction efficiency is 100%, and hence a base of two is applied (column 6). Otherwise the base should be one + PCR efficiency (E) [as determined from the standard curve (Figure 4C)]. In effect, the relative quantity represents the fold change between the biological groups for each sample/target combination prior to reference gene normalization. For each biological group/sample combination, a normalization factor is determined from the geometric mean of the associated reference gene relative quantities (column 7). The relative normalized expression for each target gene (DER5 in this example) is then calculated per sample by dividing the relative quantity by the normalization factor (column 8) followed by log transformation (column 9). The average relative normalized and log transformed expression for each biological group is then calculated using the geometric mean (columns 10 and 11). The standard deviation (SD), standard error of the mean (SEM), and 95% confidence interval [in this case a t statistic of 4.3 was used based on three samples per biological group (i.e., 2 degrees of freedom)] of each group are then calculated from the log transformed normalized expression (columns 12–15). (B) Average relative normalized expression graphical representation with appropriate error bars (in this case SEM).

one + the PCR efficiency (E) [determined from the standard curve (Figure 4C)] to the ΔCq [i.e., $(1 + E)^{\Delta Cq}$]; (calculated by subtracting the mean Cq of all the samples within the control group from the mean Cq of the technical replicates from each sample); (iii) enumerate the **normalized relative expression** (equivalent to ΔΔCq after log transformation) per sample by dividing the relative quantity of a given target/sample (step 2) by the geometric mean of the relative quantities of two or more reference targets; (iv) compute the average normalized expression of the samples in each biological group; and (v) perform statistical analysis based on the log transformed normalized expression per sample (Figure 5: column 9) [41].These calculations are complex because qPCR data are relative by nature between samples or groups of samples, with normalization to reference genes, and calibration between plates. Additionally, qPCR measurements are made on the log scale (Cq value) with statistical analysis performed in Cq space (i.e., ΔΔCq values or using log-transformed relative normalized expression), while expression levels are reported after linear transformation of the ΔΔCq results.

The methodology by which normalized expression calculations are performed, coupled with the amalgamation of data from multiple experiments that typically span multiple plates, can be daunting for any researcher. Many laboratories create Microsoft Excel spreadsheets with premade formulas to automate the analysis process. This requires copy/paste manipulations from the raw data files, necessitating the careful triage of results to fit the confines of the formulas and data flow. Multi-plate experiments can introduce another challenge in the manual fashion by which the Cq values from each experiment are entered and potentially reconfigured and rearranged in Excel to accommodate different plate configurations between individual experiments. In the hands of even the most experienced and methodical scientist, the physical management of large datasets in Excel can result in calculation errors that may go unnoticed, resulting in erroneous summarized results and conclusions [42].

The common sources of error in a given experiment cannot be entirely avoided, but by clearly understanding where and how they can arise, measures can be taken for their minimization. Given that scientists continue to push the limits of sensitivity for detection of biomolecules in smaller samples, more care must be taken to reduce the sources of error, improve precision, and achieve reproducible, statistically significant data particularly with low target abundance.

The Design of a qPCR Experiment to Minimize Error and Maximize Data Quality

In order to achieve reproducible data that truly reflect the experimental parameters, a sequence of key steps must be strictly followed to circumvent and minimize both random and systemic sources of error (Table 1), one of the most important of which is primer validation to assess annealing temperature, melt curve, and amplicon size (Figure 4A–B) [43]. Subsequent standard curve analysis is critical, because only when reaction efficiency is close to 100% are the Cq values representative of the target concentration in each sample (Figure 4C) [44]. Furthermore, accurate normalization of qPCR data from the geometric mean stability of multiple reference genes is important because an unstable target can produce artefactual data that do not represent the true expression differences between samples (Figure 4D) [45,46]. For projects that are not conducive to the selection of endogenous reference genes (i.e., developmental studies), an exogenous internal positive control may be required [47]. Finally, to avoid the ramifications of interplate variability, it is best to pipette all samples and associated replicates for a given target on a single plate (Figure 3A).

Table 1. Stepwise Approach to Performing a Valid qPCR Experiment[a]

Step	Description	Sub steps	Instruction
1	Experimental design	Procedure	List the best targets, samples, and treatments based on previously acquired, vetted, and validated data or literature (Figure 1A).
		Biological groups	Define the appropriate biological groups (i.e., treatments, knockout, time points, etc.) and various combinations thereof (Figure 1).
		Replicates	Biological (samples): number of animals or number of cell culture plates per biological group, which is determined with the aid of a biostatistician and/or a statistical power analysis (highly dependent on the complexity of the organism). Technical: number of wells pipetted per cDNA sample from each biological replicate (typically two or three); (Figure 1).
		Experimental conditions	Carefully note all controllable factors, such as: lot consistency in cell culture media, FBS, BSA, animal feed and drugs or compounds, sex, and phenotype. Take pictures throughout the experiment and carefully note any unusual changes in specific samples or specimens that may become outliers.
2	Tissue and cell harvesting	Sample extraction (complex organisms)	Sacrifice animals, extract and dissect tissue reproducibly and sequentially. Flash freeze in liquid nitrogen immediately upon dissection (Figure 1C).
		Sample extraction (cells)	For adherent cells, quickly remove medium and then add lysis buffer from RNA extraction kit directly to the plate. Generate a stable homogenate by scraping cells and pipetting up and down, then freeze.
		RNA extraction procedure	Research, explore, and test appropriate homogenization methods, which can vary between tissue and cell types to ensure quality, reproducible data. Ensure extraction reagents are nuclease-free and use spin columns to purify RNA from protein and chemical contaminants. DNAse treat the RNA extracts using appropriate procedures [61].
3	Test RNA samples	Minimize protein and chemical contaminants	Nanodrop the RNA samples to ensure an OD 260/280 of 1.8 or higher, and an OD 260/230 of 2.0 or higher.
		Ensure RNA is intact	Run about 400 ng per RNA sample on a bleach gel [62] (28S:18S ribosomal RNA bands should give a ratio of at least one or higher). If samples are precious and limited, use an automated electrophoresis instrument like the Bioanalyzer (RIN number of at least 7.0).
4	Reverse transcription	Dilute RNA samples	Normalize all extracted RNA samples to the same approximate concentration. For tighter data, normalize using RiboGreen fluorescence [63]. Use same amount of RNA from each sample for reverse transcription.
		Use a good kit	Use a good reverse transcription kit containing: (i) a mix oligo(dT) and random hexamers for complete coverage of the mRNA; (ii) RNAse H+ for one-to-one conversion of mRNA to cDNA; (iii) RNAse inhibitor and robust enzyme mix to reverse transcribe mRNA over a broad dynamic range of concentrations. Test different amounts of total RNA for a given sample type to produce optimal quality cDNA – using less total RNA may yield better qPCR data.
5	Primer validation	Thermal gradient	The unique, copurified protein and chemical contaminants in nucleic acid extracts can affect primer annealing. Prepare a 1:20 diluted cDNA sample from a pool of equivalent quantities of each treatment condition, and run a thermal gradient (typically between 51 °C and 63 °C) of annealing temperatures (Figure 4B).
		Visualize the amplicon	Run an agarose gel and sequence the amplicon to ensure primer specificity, purity, and identity (Figure 4B).
		Assess reaction efficiency, linear dynamic range, and include controls	For each primer pair, perform an eight-point standard curve from the pooled cDNA sample used for the thermal gradient. Serially dilute the cDNA based on expression level per primer: High (Cq: 10–16), 1:8; Medium (Cq: 16–23), 1:4; low (Cq: $\geq$23), 1:2 (Figure 4B). Dilute individual samples to the midpoint of the efficient range (90% to 110%) of the standard curve (Figure 4C). Prepare and plate reactions appropriately with required controls (Figure 3A).
6	Reference gene validation	Survey literature for potential reference genes	Use the search term 'qPCR reference gene [GeNorm or NormFinder or BestKeeper] [Organism and Tissue of interest]' using Google Scholar. Pick seven to ten targets from the articles, and validate the primers (Figure 4B,C). A spike-in RNA or DNA sample into all test samples can help assess reference gene stability and may also be a useful normalization target [47].
		Confirm target stability	Test the validated reference gene primers against three cDNA samples from each biological/treatment group. Test stability using GeNorm, NormFinder and BestKeeper software. Normalize to multiple reference genes (Figure 4D).

[a]Adapted and updated under a Creative Commons license from [8].

Making the Choice between qPCR and dPCR

dPCR technology permits the quantification of absolute target copy numbers between individual samples at reaction endpoint. This is accomplished through the segregation of the qPCR reaction mix into thousands of partitions which are then interrogated after thermocycling [48]. Thus, the dependence on reaction efficiency (Figure 4C) and interdependence between samples (ΔCq; Figure 5) associated with qPCR, is virtually eliminated [49]. Although qPCR can produce excellent data from a well-designed and executed experiment (Table 1), there are parameters, sample types, and target nucleic acid levels that are more challenging to assess with this technology [49,50]. The consequence of inappropriate use of qPCR can result in dramatically increased cost, time, and most importantly, loss of precious samples with no relevant data. There are some specific applications for which care should be taken in pursuing sample analysis with qPCR and where dPCR could be more appropriate.

Relative and Absolute Quantification in Contaminated and/or Low Target Abundant Samples

qPCR has been the gold standard application with cDNA for gene expression analysis [applying relative quantification (Figure 5)] and for **absolute quantification** using gDNA extracted from environmental (e.g., lake, ocean and runoff water, soil, sludge, and air), human and veterinarian clinical samples (e.g., paraffin embedded tissue or cells), feces, plant material, or food samples. These sample types can be challenging due to the propensity and variability of residual contaminants that could partially inhibit Taq activity and primer annealing [49,50]. If the samples are not adequately diluted with the aid of a primer validation standard curve (Figure 4C), the Cq values may not reflect the actual nucleic acid levels, but rather a combination of target concentration and contaminant levels [49]. However, dilution is only possible if the target nucleic acid levels are high enough to withstand attenuation without compromising the data (Figures 2D and 3B) [49]. The use of externally spiked nucleic acids has become a common tool to assess both differential levels of contaminants and data normalization if required [47,51]. Inhibitor tolerant polymerases have also been developed to help address variability between differentially contaminated samples [52]. In the case of samples derived from widely different sources (i.e., environmental samples), consistency in their dilution may not be possible, and the resulting Cq values would not reflect the cDNA levels, leading to highly variable results [53]. Since dPCR is virtually efficiency independent, consequent to endpoint data acquisition, the effect of contaminants between samples is minimized [49,50]. This also makes the plexing of multiple targets in a single dPCR reaction straightforward, thus permitting the quantification of multiple reference gene targets in each sample for normalization [54,55].

Mutation Abundance Assays

Single nucleotide polymorphisms (SNPs) have been well characterized as biomarkers for a number of cancers and viral infections [56]. qPCR is currently used to detect these targets in circulating DNA purified from extracted body fluids to follow the effect of treatment [57]. However, the limit of quantification is approximately 5% **mutation abundance**, which could represent high disease levels as opposed to early detection [58]. Therefore, many clinical laboratories use standard PCR with preamplification and endpoint detection on agarose gels to detect these biomarkers at much lower levels. However, the tradeoff is an inability to accurately quantify the amount of target, or even to compare the effect of treatment over time with any degree of precision [59].

Under these two circumstances, dPCR technology provides more accurate and precise results because it is much less affected by sample contaminants consequent to data acquisition at reaction endpoint. There is also no requirement for standard curves or interplate calibration. Furthermore, the effect of partitioning permits a much higher level of sensitivity specifically for mutation abundance assays [57]. A simple experimental workflow has previously been described to choose between qPCR and dPCR for any genomics experiment, and is summarized in Figure 6 [49].

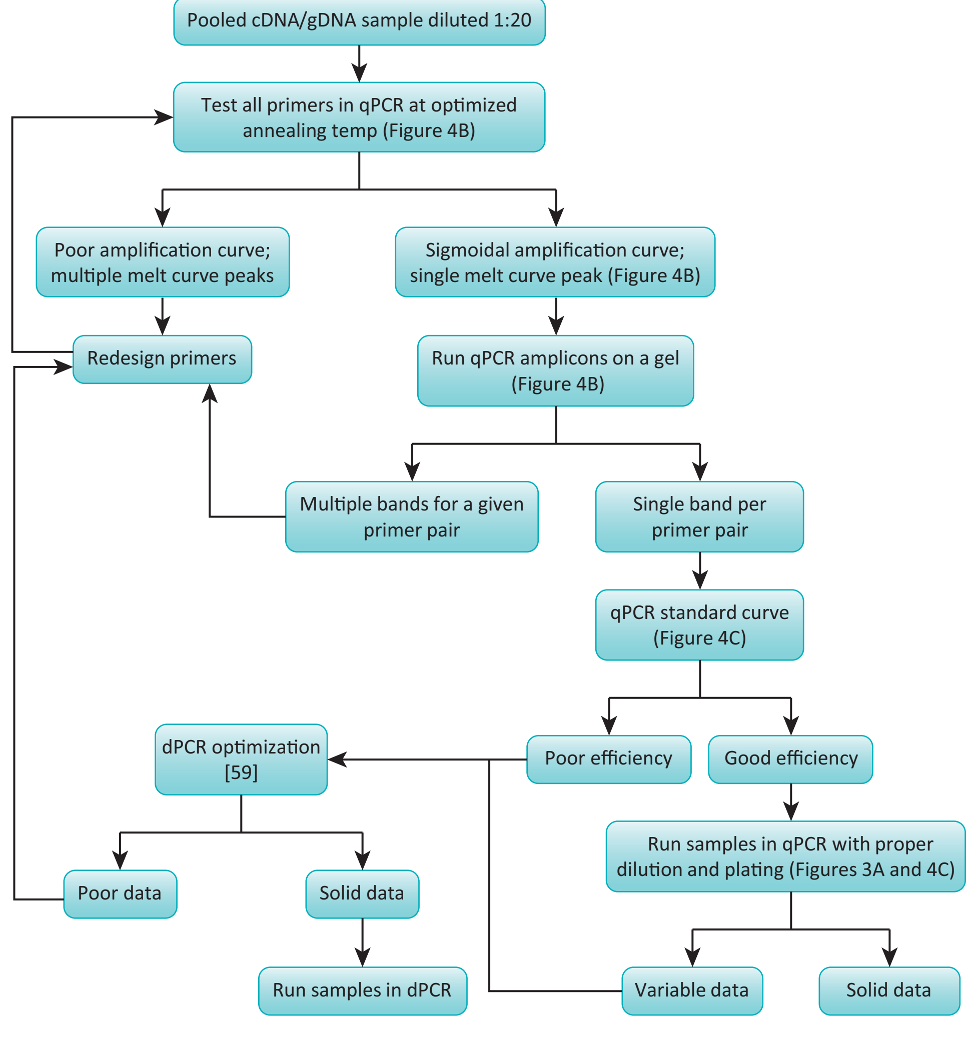


Figure 6. Criteria for Selecting between Quantitative PCR (qPCR) and Digital PCR (dPCR). Primer validation should be performed at optimal annealing temperature to ensure a single amplicon is produced with acceptable reaction efficiency. Primers that result in multiple products should be redesigned. Highly efficient primers (i.e., between 90% and 110%) with broad standard curve dynamic range are good candidates for qPCR. dPCR should be explored when using primers with poor amplification efficiency. Alternatively, targets that are efficient but result in variable, artefactual data from qPCR consequent to a combination of sample dilution requirements and low target abundance, should also be tested in dPCR [59].

Concluding Remarks

qPCR is an excellent tool for nucleic acid detection and quantification with precision down to about twofold differences between biological groups and lower limit of quantification to approximately 100 copies (≤30 Cqs) for gene expression analysis and absolute quantitation (Figures 2D and 3B) [36,37]. However, given the effect of sample contaminants on both the polymerase efficiency and primer annealing, a rigorous, stepwise procedure must be followed to plan the experiment, verify the samples, and validate the primers (Table 1). The consequences of not pursuing this workflow are either the generation of reproducible but artefactual data that are unrepresentative of the samples and experimental conditions, or highly variable, nonstatistically significant results. This underlines several outstanding questions around how best practices for qPCR experiments can be applied, implemented, and enforced globally to ensure the integrity and reproducibility of published data and accelerate advances in the associated fields (see Outstanding Questions).

For those projects where qPCR is deemed inadequate to achieve high-quality data, dPCR could offer a good alternative to minimize time to publication (Figure 6). Since dPCR is much less dependent on reaction efficiency, multiplexed detection and quantification of a larger number of targets per sample is straightforward [54]. Thus, the next generation of dPCR technologies will likely include more excitation wavelengths to increase the number of detectable targets in a single sample. For laboratories who wish to quantify tens to hundreds of targets, next generation sequencing (NGS) technologies may become the methodology of choice. However, the current NGS methodology to achieve high sensitivity and precision requires a large amount of sample (about 250 ng) and several orders of magnitude more cost [60].

Disclaimer Statement

The authors are employed with Bio-Rad Laboratories Inc. and have no financial interest in the subject matter or information discussed in this manuscript.

Outstanding Questions

Although efforts have been made to assess the quality of published qPCR data in the literature, will there ultimately be agreed metrics supported by the scientific community for determining the quality of articles employing this technology?

Can journals establish a standardized set of accepted global guidelines for submission of qPCR data?

Will artificial intelligence software evolve for adaptation by journals to enable a rigorous and automated pre-assessment of the data quality (based on accepted guidelines) from submitted manuscripts prior to peer review?

Will literature search engines incorporate artificial intelligence to permit the selection and perhaps the ranking of articles for data quality?

All of the above will likely require the submission of the raw data with manuscripts to journals. Therefore, will journal databases and archives permit the submission, storage, and public query of raw data to mine the quality with appropriate security to protect the results?

Presuming the raw data used to produce a given article are sensitive and required for future publications in competitive fields or for commercial use, what will be the legal ramifications of requiring the submission of such data with manuscripts?

What steps can be taken (besides departmental seminars and workshops) to educate the global scientific community to seek out guidelines to help produce solid, reproducible data?

References

1. Bustin, S. and Nolan, T. (2017) Talking the talk, but not walking the walk: RT-qPCR as a paradigm for the lack of reproducibility in molecular research. *Eur. J. Clin. Invest.* 47, 756–774
2. Bustin, S.A. and Huggett, J.F. (2017) Reproducibility of biomedical research — the importance of editorial vigilance. *Biomol. Detect. Quantif.* 11, 1–3
3. Hayden, R.T. *et al.* (2008) Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein–Barr virus. *J. Clin. Microbiol.* 46, 157–163
4. Zhang, T. *et al.* (2007) Inter-laboratory comparison of chronic myeloid leukemia minimal residual disease monitoring. *J. Mol. Diagn.* 9, 421–430
5. Jaworski, J.P. *et al.* (2018) Interlaboratory comparison of six real-time PCR assays for detection of bovine leukemia virus proviral DNA. *J. Clin. Microbiol.* 56, 617–628
6. Bustin, S.A. *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622
7. Sanders, R. *et al.* (2018) Improving the standardization of mRNA measurement by RT-qPCR. *Biomol. Detect. Quantif.* 15, 13–17
8. Taylor, S.C. and Mrkusich, E.M. (2014) The state of RT-quantitative PCR: firsthand observations of implementation of minimum information for the publication of quantitative real-time PCR experiments (MIQE). *J. Mol. Microbiol. Biotechnol.* 24, 46–52
9. Dijkstra, J.R. *et al.* (2014) Critical appraisal of quantitative PCR results in colorectal cancer research: can we rely on published qPCR results? *Mol. Oncol.* 8, 813–818
10. Bustin, S.A. *et al.* (2013) The need for transparency and good practices in the qPCR literature. *Nat. Method* 10, 1063–1067
11. Sanders, R. *et al.* (2014) Considerations for accurate gene expression measurement by reverse transcription quantitative PCR when analysing clinical samples. *Anal. Bioanal. Chem.* 406, 6471–6483
12. Althubaiti, A. (2016) Information bias in health research: definition, pitfalls, and adjustment methods. *J. Multidiscip. Healthc.* 9, 211–217
13. Suresh, K.P. (2011) An overview of randomization techniques: an unbiased assessment of outcome in clinical research. *J. Hum. Reprod. Sci.* 4, 8–11
14. Moignard, V. and Gottgens, B. (2014) Transcriptional mechanisms of cell fate decisions revealed by single cell expression profiling. *Bioessays* 36, 419–426
15. Battich, N. *et al.* (2015) Control of transcript variability in single mammalian cells. *Cell* 163, 1596–1610
16. Lanoix, D. and Vaillancourt, C. (2010) Cell culture media formulation and supplementation affect villous trophoblast HCG release. *Placenta* 31, 558–559
17. Kang, H.J. *et al.* (2011) Spatio-temporal transcriptome of the human brain. *Nature* 478, 483–489
18. Gao, S. *et al.* (2018) Tracing the temporal-spatial transcriptome landscapes of the human fetal digestive tract using single-cell RNA-sequencing. *Nat. Cell Biol.* 20, 721–734
19. Panda, S. *et al.* (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109, 307–320
20. Byers, S.L. *et al.* (2012) Mouse estrous cycle identification tool and images. *PLoS One* 7, e35538
21. Mitko, K. *et al.* (2008) Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. *Reproduction* 135, 225–240

22. Woo, H.R. *et al.* (2018) Plant senescence: how plants know when and how to die. *J. Exp. Bot.* 69, 715–718
23. Filipecki, M. and Malepszy, S. (2006) Unintended consequences of plant transformation: a molecular insight. *J. Appl. Genet.* 47, 277–286
24. Gerlach, R.W. and Nocerino, J.M. (2003) *Guidance for Obtaining Representative Laboratory Analytical Subsamples from Particulate Laboratory Samples,* US Environmental Protection Agency
25. Vaccaro, W. (2007) Minimizing liquid delivery risk: operators as sources of error. *Am. Lab.* 39, 16–17
26. Carle, A.B. (2013) Five good reasons: the argument for pipetting technique training. *Med. Lab. Obs.* 45, 58
27. Albert, K.J. and Bradshaw, J.T. (2007) Importance of integrating a volume verification method for liquid handlers: applications in learning performance behavior. *J. Assoc. Lab. Autom.* 12, 172–180
28. Higgins, K.M. *et al.* (1998) The effect of serial dilution error on calibration inference in immunoassay. *Biometrics* 54, 19–32
29. Walling, L.A. (2011) An inline QC method for determining serial dilution performance of DMSO-based systems. *J. Assoc. Lab. Autom.* 16, 235–240
30. Hashemipetroudi, S.H. *et al.* (2018) Assessment of DNA contamination in RNA samples based on ribosomal DNA. *J. Visualized Exp.* 131, e55451
31. Kim, S.J. *et al.* (2007) Effects of storage, RNA extraction, genechip type, and donor sex on gene expression profiling of human whole blood. *Clin. Chem.* 53, 1038–1045
32. Tan, S.C. and Yiap, B.C. (2009) DNA, RNA, and protein extraction: the past and the present. *J. Biomed. Biotechnol.* 2009, 574398
33. Cankar, K. *et al.* (2006) Critical points of DNA quantification by real-time PCR – effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnol.* 6, 37–52
34. Bustin, S. *et al.* (2014) Variability of the reverse transcription step: practical implications. *Clin. Chem.* 61, 201–212
35. Levesque-Sergerie, J.-P. *et al.* (2007) Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR. *BMC Mol. Biol.* 8, 93–111
36. Peccoud, J. and Jacob, C. (1996) Theoretical uncertainty of measurements using quantitative polymerase chain reaction. *Biophys. J.* 71, 101–108
37. Forootan, A. *et al.* (2017) Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol. Detect. Quantif.* 12, 1–6
38. Elnifro, E.M. *et al.* (2000) Multiplex PCR: optimization and application in diagnostic virology. *Clin. Microbiol. Rev.* 13, 559–570
39. Andersson, D. *et al.* (2015) Properties of targeted preamplification in DNA and cDNA quantification. *Expert Rev. Mol. Diagn.* 15, 1085–1100
40. Okino, S.T. *et al.* (2016) Evaluation of bias associated with high-multiplex, target-specific pre-amplification. *Biomol. Detect. Quantif.* 6, 13–21
41. Kitchen, R.R. *et al.* (2010) Statistical aspects of quantitative real-time PCR experiment design. *Methods* 50, 231–236
42. Panko, R.R. (1998) What we know about spreadsheet errors. *J. Organ. End User Comput.* 10, 15–21
43. Opel, K.L. *et al.* (2010) A study of PCR inhibition mechanisms using real time PCR. *J. Forensic Sci.* 55, 25–33
44. Svec, D. *et al.* (2015) How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. *Biomol. Detect. Quantif.* 3, 9–16
45. Robledo, D. *et al.* (2014) Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (*Scophthalmus maximus*) gonad dataset. *BMC Genomics* 15, 648–663
46. Vandesompele, J. *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, research0034
47. Kavlick, M.F. (2018) Development of a universal internal positive control. *Biotechniques* 65, 275–280
48. Hindson, B.J. *et al.* (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* 83, 8604–8610
49. Taylor, S.C. *et al.* (2017) Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. *Sci. Rep.* 7, 2409–2417
50. Rački, N. *et al.* (2014) Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. *Plant Method* 10, 42–52
51. Taruttis, F. *et al.* (2017) External calibration with *Drosophila* whole-cell spike-ins delivers absolute mRNA fold changes from human RNA-Seq and qPCR data. *Biotechniques* 62, 53–61
52. Kermekchiev, M.B. *et al.* (2009) Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucl. Acids Res.* 37, e40
53. Gibson, K.E. *et al.* (2012) Measuring and mitigating inhibition during quantitative real time PCR analysis of viral nucleic acid extracts from large-volume environmental water samples. *Water Res.* 46, 4281–4291
54. Whale, A.S. *et al.* (2016) Fundamentals of multiplexing with digital PCR. *Biomol. Detect. Quantif.* 10, 15–23
55. Coulter, S.J. (2018) Mitigation of the effect of variability in digital PCR assays through use of duplexed reference assays for normalization. *Biotechniques* 65, 86–91
56. Leslie, R. *et al.* (2014) GRASP: analysis of genotype-phenotype results from 1390 genome-wide association studies and corresponding open access database. *Bioinformatics* 30, i185–194
57. Hudecova, I. (2015) Digital PCR analysis of circulating nucleic acids. *Clin. Biochem.* 48, 948–956
58. Whale, A.S. *et al.* (2016) Detection of rare drug resistance mutations by digital PCR in a human influenza A virus model system and clinical samples. *J. Clin. Microbiol.* 54, 392–400
59. Taylor, S.C. *et al.* (2015) Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: clinical implications for quantification of Oseltamivir-resistant subpopulations. *J. Virol. Methods* 224, 58–66
60. Zhang, T.-H. *et al.* (2016) A benchmark study on error-correction by read-pairing and tag-clustering in amplicon-based deep sequencing. *BMC Genomics* 17, 108–117
61. Huang, Z. *et al.* (1996) Optimization of DNase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. *Biotechniques* 20, 1012–1020
62. Aranda, P.S. *et al.* (2012) Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis* 33, 366–369
63. Jones, L.J. *et al.* (1998) RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. *Anal. Biochem.* 265, 368–374