Methods 59 (2013) 147–153

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Improving biological relevancy of transcriptional biomarkers experiments by applying the MIQE guidelines to pre-clinical and clinical trials

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ARTICLE INFO

Article history:
Available online 14 August 2012
Communicated by Michael W. Pfaffl

Keywords:
Clinical trial
MIQE qPCR
Transcriptional biomarkers

ABSTRACT

The "Minimum Information for the Publication of qPCR Experiments" (MIQE [3]) guidelines are very much targeted at basic research experiments and have to our knowledge not been applied to qPCR assays carried out in the context of clinical trials. This report details the use of the MIQE qPCR app for iPhone (App Store, Apple) to assess the MIQE compliance of one clinical and five pre-clinical trials. This resulted in the need to include 14 modifications that make the guidelines more relevant for the assessment of this special type of application. We also discuss the need for flexibility, since while some parameters increase experimental quality, they also require more reagents and more time, which is not always feasible in a clinical setting.

1. Introduction

The clinical and pre-clinical trials contain a large volume of processed data. Guidelines may be used to ensure the biological relevancy of each datum and each analysis through coherent checklists [1]. The frequency of the use of qPCR is increasing exponentially, since it permits an easy analysis of several biomarkers, by quantification of their transcriptional precursors [2]; accordingly, the MIQE is expected to be the most relevant guideline for clinical and pre-clinical trials. However, to our knowledge, no clinical or pre-clinical study did use such a guideline before. It is decided in this work to investigate the reasons of this avoidance of its use, to analyze the problem, and to propose some solutions.

Guidelines are not norms. It could be seen rather as blueprints that are strongly linked to the every day's reality and every experiment's setting. Thus, the guidelines objective is to improve the reliability of the data, and should evolve according to the evolution of sciences and practices. The MIQE qPCR app [4], known as a user friendly tool, has a potential of an increasing application in future clinical and pre-clinical setting. This improvement of its application will be investigated in this research.

2. Material and methods

2.1. General information

All trials, adopting the MIQE guideline, have been accomplished over the years in the core facilities of the Institut Polytechnique LaSalle Beauvais (LaSalle Beauvais, France). This institute is one of the leaders of the adoption of the MIQE guideline [3]. The on campus-trials involved molecular biology, and consequently the qPCR. The selected trials used qPCR to quantify the transcriptional precursors of defined biomarkers, such as the expression ratio of immunity related genes.

2.2. Analysis process

All data of the six dietary trials were processed similarly. This available data of each trial is entered into the MIQE qPCR app (LaSalle Beauvais, App Store, Apple), in order to determine the global percentage of MIQE compliance. A global analysis, comparing all studied trials, is performed to diagnose the MIQE items that remain problematic.

The used data include all available documents and records, which will remain as proprietary information of LaSalle Beauvais. The exclusion of certain items from the MIQE is due to unavailable information. The highlighting is restricted to real scientific problems, and not due to constraints of publishing policy at LaSalle Beauvais.

2.3. Analyzed trials

2.3.1. Setting of the trials

All data of the trials used in this research have been performed at LaSalle Beauvais, as indicated in Section 2.1. Some collaboration with industrial partners or others may have existed, but for confidentiality reasons, the affiliations are excluded.

All trials elaborate on the impact of different dietary fibers on organisms, especially on their inflammatory response. Fibers are

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http://dx.doi.org/10.1016/j.ymeth.2012.07.024

expected to up regulate *T helper2* (Th2) and down regulate *T helper 1*(Th1) pathways.

Most of the six trials rely on an *ex vivo* model, with details that are previously published [5,6]. The main milestones are given in the first example, namely the rabbit's pre-clinical assay. This assay was accomplished by the same analysis team, leading to a constant setting for applicability of the MIQE guidelines.

2.3.2. Description of each trial

Different fibers or vegetal extracts have been tried in the four pre-clinical trials on four types of animals, namely: chicken, turkey, rabbit and pig. A brief description of each pre-clinical trial, concerning the transcriptional precursors of biomarkers, and the clinical context is shown below.

2.3.2.1. Rabbit (Mid 2008). Six different groups of a total of 22 rabbits were fed for 56 days different diets, including different compounds and at different doses. One group received an iso-energetic placebo. Markers from Th1 pathway were included namely, Tumor Necrosis factor α (TNF-α), with Accession#: NM_001082263.1, and Interleukine 2 (IL2) with Accession#: NM_001171099.1. The only two included markers from Th2 pathway were Interleukine 10 (IL10), with Accession#: NM_0010 82045.1, and Interleukine 4 (IL4), with Accession#: NM_00116 3177.1.

Sampled blood from each rabbit was put in contact with bacterial lipopolysaccharide (LPS), produced by *Listeria monocytogenes* or *Escherichia coli,* and the contacted materials were continuously shaked under a temperature of around 37 °C. This contact aimed at simulating an *ex vivo* immune reaction against LPS. This led to an inflammatory reaction, modulating the Th1 related early expression of cytokines, followed by the Th2 related cytokines expression. The quantitative expression of the immune markers of the two respective pathways were followed in time, by sampling the reactants at different intervals, followed by RNA extractions from the Th1 and Th2 cells for a qPCR application.

Results were transformed from raw Cq data into expression ratios. Means of treatments were compared at different times of the trial. The hypothesis expected a respective decrease and an increase in the cytokines of Th1 and Th2. The cytokines quantitation in the reacting blood of rabbits was determined at 56 and 70 days following the initiation of administration of the different diets.

2.3.2.2. Chicken (Early 2009). Two studies were applied, the first was *in vitro*, and the second was *in vivo*. The number of samples in the *in vitro* study was high and the procedure was similar to the other cited trials.

The *in vitro* study used several chickens for blood sampling. The blood samples were incubated *in vitro* with various levels of three different vegetal extracts. Following this incubation, the blood was put in contact with LPS of *E. coli,* to simulate a bacterial infection, following the procedure used in the rabbit's trial. The levels of LPS-induced TNF Factor (LITAF) (Accession#: NM_204267.1) and IL-4 (Accession#: GU119892.1) were quantified using qPCR.

The *in vivo* trial included eight groups of chickens, administered in feed or drinking water, fed different diets, for a period of 42 days.

The birds were subjected to humane sacrification at 42 days of age, and their individual blood was incubated in two batches. The first part of the individual blood was incubated with LPS for 6 h (Th1 response), and the second part was left in contact with a different antigen for 20 h to detect the Th2 response. The RNA was extracted from sensitized T cells, and analyzed quantitatively for RNA by qPCR.

2.3.2.3. Turkey (Early 2009). The turkeys were fed for 92 days, and sampled for blood at different times. There were 6 groups with 6 turkeys in each. The blood samples were reacted with a specific antigen, as described before. The analyzed cytokine was the IL-1β (Accession#: DQ393271.1).

2.3.2.4. Pig (Mid 2009). The pig model is appropriate for studying human nutrition, due to the close similarity in the digestive metabolisms of these two organisms. The experimental design included 24 pigs, divided into 4 groups. The protocol of the molecular biology part was similar to the clinical experimental design applied on humans. The pigs were sacrificed at the end of the study, and blood was collected.

The duration of feeding in the pig and in the clinical human trials was the same. In addition, the same protocol of blood T-cells stimulation by LPS, and measurement of the RNA ratios of targeted genes, were used. The targeted quantitated transcription by qPCR was of the following two genes namely, TNF-α with Accession#: NM_214022.1, and IL-1β with Accession#: NM_214029.1.

2.3.2.5. Human (Mid2011). This trial is the only clinical one included in this analysis. The clinical trial was accomplished after all the pre-clinical trials were concluded, expecting a high acceptability of the MIQE compliance.

Four groups of humans drank every day, and for a period of 4 weeks, a preparation containing either a placebo or different levels of a dietary fiber. Blood was collected from each individual on two different occasions, at the beginning and the end of the administration of the specific preparations. The collected blood samples were each incubated with LPS, extracted from Salmonella organism. The RNA was extracted at different times of incubation (6 h and 24 h), and analyzed by qPCR for 18 different transcriptional biomarkers.

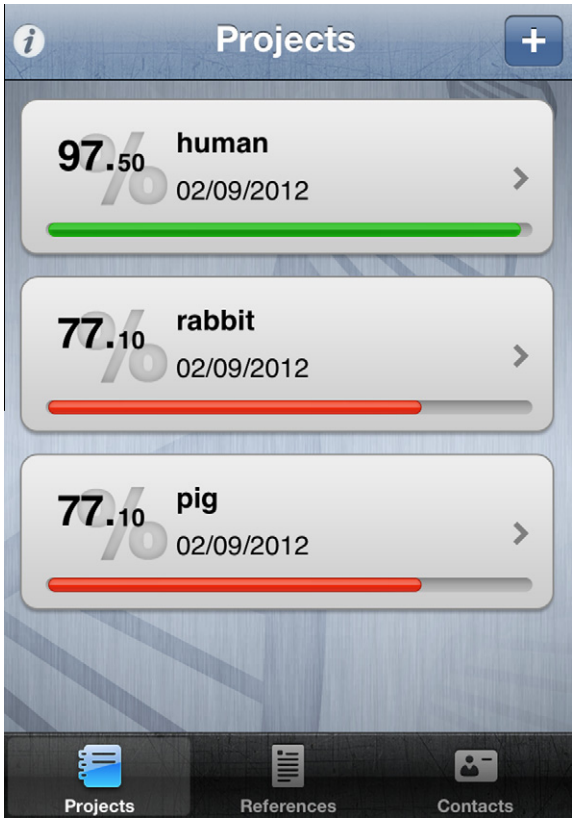

Fig. 1. Screenshot of the MIQE qPCR app.

Table 1
MIQE compliance of six different projects (1 – information available; 0 – information unavailable; n/a – not applicable; bold lines are for the percentage of MIQE compliance for each section of the MIQE guideline, the green color indicates the appropriateness).

Items	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit	Items	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit
% of MIQE compliance	97	81	81	81	81	81	% Reverse Transcription	86	83	86	86	86	86
% Nomenclature	100	40	40	40	40	40	Complete reaction conditions	1	1	1	1	1	1
Reference gene(s) not Housekeeping gene	1	0	0	0	0	0	Amount of RNA and reaction volume	1	1	1	1	1	1
Quantification not quantitation	1	1	1	1	1	1	Priming oligonucleotide (if using GSP) and concentration	n/a	n/a	n/a	n/a	n/a	n/a
Quantify preferable to quantitate	1	1	1	1	1	1	Reverse transcriptase and concentration	1	1	1	1	1	1
Hydrolysis probes not TaqMan®	1	0	0	0	0	0	Temperature and time	1	1	1	1	1	1
Dual Hydrolysis Probes not FRET	n/a	n/a	n/a	n/a	n/a	n/a	Manufacturer of reagents and catalog numbers	1	1	1	1	1	1
Quantification cycle Cq not Ct, Cp, TOP	1	0	0	0	0	0	Cqs with and without RT	0	0	0	0	0	0
% Experimental Design	100	100	100	100	100	100	Storage conditions of cDNA	1	n/a	1	1	1	1
Definition of experimental and control groups	1	1	1	1	1	1	% qPCR Target Information	100	89	89	89	89	89
Number within each group	1	1	1	1	1	1	If multiplex, efficiency and LOD of each assay	n/a	n/a	n/a	n/a	n/a	n/a
Assay carried out by core lab or investigator's lab	1	1	1	1	1	1	Sequence accession number	1	1	1	1	1	1
Acknowledgement of authors' contributions	1	1	1	1	1	1	Location of amplicon	1	1	1	1	1	1
% Sample	100	100	100	100	100	100	Amplicon length	1	1	1	1	1	1
Description	1	1	1	1	1	1	*In silico* specificity screen (BLAST, etc.)	1	1	1	1	1	1
Volume/mass of sample processed	1	1	1	1	1	1	Pseudogenes, retropseudogenes or other homologues	1	1	1	1	1	1
Microdissection or macrodissection	n/a	n/a	n/a	n/a	n/a	n/a	Sequence alignment	1	1	1	1	1	1
Processing procedure	1	1	1	1	1	1	Secondary structure analysis of amplicon	1	1	1	1	1	1
If frozen - how and how quickly	1	1	1	1	1	1	Location of each primer by exon or intron (if applicable)	1	1	1	1	1	1
If fixed - with what, how quickly	n/a	n/a	n/a	n/a	n/a	n/a	What splice variants are targeted	1	0	0	0	0	0
Sample storage conditions and duration (especially for FFPE samples)	1	1	1	1	1	1	% qPCR Oligonucleotides	100	100	100	100	100	100
% Nucleic Acid Extraction	92	69	69	69	69	69	Primer sequences	1	1	1	1	1	1
Procedure and/or instrumentation	1	1	1	1	1	1	RTPrimerDB Identification Number	1	1	1	1	1	1
Name of kit and details of any modifications	1	1	1	1	1	1	Probe sequences	1	1	1	1	1	1
Source of additional reagents used	1	1	1	1	1	1	Location and identity of any modifications	n/a	n/a	n/a	n/a	n/a	n/a
Details of DNase or RNAse treatment	1	1	1	1	1	1	Manufacturer of oligonucleotides	1	1	1	1	1	1
Contamination assessment (DNA or RNA)	0	0	0	0	0	0	Purification method	1	1	1	1	1	1
Nucleic acid quantification	1	1	1	1	1	1							
Instrument and method	1	1	1	1	1	1							
Purity (A260/A280)	1	1	1	1	1	1							
Yield	1	1	1	1	1	1							
RNA integrity method/	1	0	0	0	0	0							

(*continued on next page*)

Table 1 (*continued*)

Items	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit	Items	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit
instrument													
RIN/RQI or Cq of 3′ and 5′ transcripts	1	0	0	0	0	0							
Electrophoresis traces	1	0	0	0	0	0							
Inhibition testing (Cq dilutions, spike or other)	1	1	1	1	1	1							

Table 2
MIQE compliance of six different projects (1 - information available; 0 - information unavailable; n/a - not applicable; bold lines are for the percentage of MIQE compliance for each section of the MIQE guideline, the green colour indicates the appropriateness).

Items	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit	Items	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit
% qPCR Protocol	100	100	100	100	100	100	% Data Analysis	93	86	86	86	86	93
Complete reaction conditions	1	1	1	1	1	1	qPCR analysis program (source, version)	1	1	1	1	1	1
Reaction volume and amount of cDNA/ DNA	1	1	1	1	1	1	Cq method determination	1	1	1	1	1	1
Primer, (probe), Mg++ and dNTP concentrations	1	1	1	1	1	1	Outlier identification and disposition	1	1	1	1	1	1
Polymerase identity and concentration	1	1	1	1	1	1	Results of NTCs	1	1	1	1	1	1
Buffer/kit identity and manufacturer	1	1	1	1	1	1	Justification of number and choice of reference genes	1	0	0	0	0	1
Exact chemical constitution of the buffer	1	1	1	1	1	1	Description of normalization method	1	1	1	1	1	1
Additives (SYBR Green I, DMSO, etc.)	n/a	n/a	n/a	n/a	n/a	n/a	Number and concordance of biological replicates	1	1	1	1	1	1
Manufacturer of plates/ tubes and catalog number	1	1	1	1	1	1	Number and stage (RT or qPCR) of technical replicates	1	1	1	1	1	1
Complete thermocycling parameters	1	1	1	1	1	1	Repeatability (intra-assay variation)	1	1	1	1	1	1
Reaction setup (manual/ robotic)	1	1	1	1	1	1	Reproducibility (inter-assay variation, %CV)	1	1	1	1	1	1
Manufacturer of qPCR instrument	1	1	1	1	1	1	Power analysis	1	1	1	1	1	1
% qPCR Validation	100	20	20	20	20	20	Statistical methods for result significance	1	1	1	1	1	1
Evidence of optimization (from gradients)	1	1	1	1	1	1	Software (source, version)	1	1	1	1	1	1
Specificity (gel, sequence, melt, or digest)	1	0	0	0	0	0	Cq or raw data submission using RDML	0	0	0	0	0	0
For SYBR Green I, Cq of the NTC	n/a	n/a	n/a	n/a	n/a	n/a							
Standard curves with slope and y-intercept	1	0	0	0	0	0							
PCR efficiency calculated from slope	1	0	0	0	0	0							
Confidence interval for PCR efficiency or standard error	1	0	0	0	0	0							
R^2 of standard curve	1	0	0	0	0	0							
Linear dynamic range	1	0	0	0	0	0							
Cq variation at lower limit	1	0	0	0	0	0							
Confidence intervals throughout range	1	0	0	0	0	0							
Evidence for limit of detection	1	1	1	1	1	1							
If multiplex, efficiency and LOD of each assay	n/a	n/a	n/a	n/a	n/a	n/a							

Table 3
Bench related quality indicators of the six different studies. The bench related quality indicator improves with lower values.

	Human	Chicken 1	Chicken 2	Pig	Turkey	Rabbit
Mean	0,27	0,23	0,21	0,32	0,31	0,46
Max	1,09	0,95	1,07	1	1,07	1,45
SD (norm)	1,18	0,91	1,17	0,89	0,78	0,79

3. Results

The results of this research are not related to the effect of fibers on transcription of specific markers, but rather about biological relevancy of produced data. The MIQE compliance results are completed with parameters of the consistency of the handling on the bench.

These obtained data are expected to improve the impact of a published paper, since the used methodology is detailed along with the care of the handling, and may therefore be evaluated in any review or contradictory analysis.

Fig. 1 is a screenshot of the MIQE qPCR app, showing the global percentage of three of the analyzed trials (human, rabbit, and pig). The red bars indicate that not all of the essential items are filled, whereas the green bar indicates that all essential items have been described[1].

The 6 studies are displayed with all items in Tables 1 and 2. Although readability is lower than in app, this presentation allows the pinpointing at unchecked items in each trial. In this analysis, some trends are clearly present, which will be discussed later in the manuscript.

Table 3 presents the bench side of quality management through measurements of consistency in handling. These are statistical parameters of standard deviations. This information presented in Table 3, in addition to the MIQE compliance percentage shown in Tables 1 and 2, show the real biological relevancy of the results obtained in the six trials.

4. Discussion

The section of the manuscript will aim at discussing the entire MIQE guidelines with regard to problems of inclusion of the six trials.

4.1. Nomenclature

The adoption by LaSalle Beauvais of the MIQE guidelines is motivated by the fact that our mother's language is not the English.

4.2. Experimental design

The MIQE compliance percentage has been for years at the 100% level; however, modifications may be suggested for improvement. Compared to other researches, Pre-clinical and clinical trials include variable population size, due to exclusion of experimental subjects during the accomplishment of the experimental designs. This exclusion in experimental subjects might be due to animal death or exclusion of human subjects due to many reasons (5).

On the other hand, even for monocentric studies, several locations may be included in the experimental design, such as animal houses or hospitals. These locations may be different from the place where analysis is carried. Accordingly, the item "Assay carried out by core lab or investigator's lab" does not seem appropriate.

[1] Concerning the human trial, some of the items have been estimated from subpopulation. This approach will be handled later in the text.

These issues are linked to the clinical or pre-clinical setting. The suggestion made is to change the following items in the guideline to better fit these particular settings:

Change from	Change to
Number within each group	Number within each group at the beginning and the end of the trial
Assay carried out by core lab or investigator's lab	Locations of every part of the trial (core facilities or partner's ones)

4.3. Sampling

There is clearly a problem concerning the item "Microdissection or macrodissection" (Tables 1 and 2). Our trials are mainly focused on nutrition, and on analysis of transcriptional biomarkers of inflammation, Thus, only blood is sampled, and the concept of dissection becomes invalid. We suggest making the following replacement:

Change from	Change to
Microdissection or macrodissection	Sampling procedure (especially microdisection or macrodissection if applicable)

This change allows the inclusion of relevant information about the sampling procedure. Moreover, these trials often occur in several locations, even for monocentric studies. Accordingly, the transport conditions should be detailed, since temperature might deteriorate the sample. As a matter of fact, transporting white blood cells at −20 °C could interfere with the RNA extraction procedure due to differential cell lysis. The suggested replacement is:

Change from	Change to
Microdissection or macrodissection	Sampling procedure (especially microdissection or microdissection if applicable) Transport procedure (duration, temperature)

4.4. Nucleic acid extraction

Improvement has occurred, in this section, relying on capillary electrophoresis (Agilent Bioanalyser). In this single experiment, electrophoresis traces may be obtained as well as RIN. This helps in reaching a high MIQE compliance with a low cost per sample, equivalent to about $2.

Table 4
Time-consuming items in clinical context with associated experiment.

Item	Relevant experiment
Contamination assessment (DNA or RNA)	
RNA integrity method/instrument	Capillary electrophoresis
RIN/RQI or Cq of 3′ and 5′ transcripts	Capillary electrophoresis/qPCR
Electrophoresis traces	Capillary electrophoresis
Cqs with and without RT	qPCR
Specificity (gel, sequence, melt, or digest)	Digestion, capillary electrophoresis or melt

The large number of samples collected in clinical trials requires an inclusion of time consuming items (Table 4).

This decision has been made due to the long time needed to check all samples, resulting in significant financial impact. It was decided to process a portion of all collected samples, targeting to save on cost. The sampling procedure is documented, selecting randomly a 10% of every batch of samples, after their RNA is extracted. A confidence interval is determined in this procedure, in order to comply with statistical reliability. Moreover the size of the subpopulation compared to the initial population should be presented. In general, the aim is to have not less than 30 samples to analyze, which simplifies the statistical analysis of the results. The allocation of sample size should relate to the initial size of the population.

The analysis of a representative portion of samples helps in application of the MIQE guidelines to clinical and pre-clinical trials, providing enough information while saving on cost of reagents and technician's time.

4.5. Reverse transcription

The only remaining issue in this section is "Cq with and without RT". This item has a bench-based problem; it requires running a qPCR for each sample, before the procedure of reverse transcription. With huge number of samples, this may require several qPCR runs to satisfy this item.

The estimation approach relies on reduction of qPCR analysis, before reverse transcription, to include only 10% of the sample size.

4.6. The three sections of qPCR target information, oligonucleotides, and protocol

The number of samples has no importance in these three sections. The clinical setting does not interfere with the percentage of MIQE compliance. Some selectivity adopted at LaSalle Beauvais targeted an improvement. In the clinical trial, no time was spent to design primers and probes. A supplier was rather chosen to provide ready to use oligonucleotides. The selection of the supplier is important with regard to the MIQE guidelines. For instance, the implementation of the Solaris probes (ThermoFischer Scientific), known to have MIQE friendly protocols, saved time, while keeping a high MIQE compliance.

4.7. qPCR Validation

The MIQE guidelines at LaSalle Institute took into consideration some parameters that were previously ignored, or assumed to conform to research standards.

The first parameter is the efficiency curve. With one single run per assay, the MIQE compliance percentage is increased by 10%. In addition, the biological relevancy is also increased, since qPCR efficiency determines all expression ratios. Moreover, this run provided information related to expected accuracy at each level of the curve (confidence intervals, Cq variations...).

The other parameter is the specificity. Though specificity is checked *in silico*, the bench verification remains necessary. The problem of checking specificity at each run is time demanding. Again, estimation helps also in reduction of needed time. The core of the issue is the specificity of the assay in sorted samples. The clinical trials have large groups of biologically similar samples; thus, the specificity can be checked by analysis of a part of the grouped samples for each analysis. A simplified approach is to include electrophoresis in every day's analysis. This applies for all assays, targeting also the saving of time. Concerning the Sybr Green assays, another simplified procedure can be adopted by adding a melt phase, helping to check the specificity of the last assay of the day. This procedure requires no additional time, since the melt occurs after the working hours.

4.8. Data analysis

The items under the 'Data Analysis' are not linked to the large number of samples present in clinical or preclinical studies. The submission of the raw data, using RDML is not respected. This is not due to the amount of data, but the internal policy of data management.

There is apparently an evolution of practices in the academic institution. This evolution occurred due to the planned decision to improve quality and compliance with the MIQE guideline, thus improving the biological relevancy. Accordingly, some items have been estimated to reach such an objective. Actually, more than 97% of the essential items have been verified and can be communicated. Such items are filled by data and standard deviations to reflect both the methodology and the quality of handling.

However, and in spite of the fact that this analysis is relevant and allows for improving the applicability of the MIQE guidelines in clinical or pre-clinical settings, another important point must be explained. Available information does not lead systematically to publish information. The constraints of publishing policies remain. The authors, reviewers and editors are responsible to abide by such policies.

4.9. Bench side quality indicators

Some quality indicators may be added to the MIQE information (Table 3). The three added indicators are: 1- the mean of the standard deviations for replicates, giving a rough idea about the global quality of the manipulations on which the results are based, 2- the maximal standard deviation between two replicates giving the error acceptance level, and 3- the normalized standard deviation (nSD) of standard deviations, giving information about the consistency of the manipulations.

5. Conclusion

In conclusion, the MIQE guidelines help in improving the biological relevancy of transcriptional biomarkers experiments. Although very helpful, clinical and pre-clinical trials are specific settings and would therefore require some adjustments in these guidelines. These adjustments are mainly in vocabulary, and statements describing the items.

The large number of samples collected in such trials requires a bench-based control of some critical points by using estimations. The publications of the research should indicate whether controls have been included in all samples, or only estimated. If estimated, the control values must be referred to the analyzed sub-population, sampling procedure, and the confidence interval. It is worth noting that these estimates are of a lower quality than procedures that control all samples. However, they represent a simplified and a cost effective solution compared to the current alternative control protocols.

In addition to these modifications, data related to the standard deviations of qPCR replicates (same assessed sample) should be presented. Variation is more likely to occur with large rather than a few samples. Although the analysis of standard deviations is an end point analysis of the qPCR process, it still gives a good idea of the consistency of the bench side part of the biological analysis. The suggested inclusions of data are:

- The mean of the standard deviations of all Cq, representing the global intra-sample variation for each assay, and

therefore the global level of quality of the bench side process.
- The accepted maximum standard deviation, indicating the level of tolerance assigned to the analysis.
- The standard deviation of standard deviations, divided by the mean of the standard deviations, in order to deduce whether manipulations are consistent, or have been subjected to changes along the qPCR process. The aim of reporting the mean of standard deviation is having a percentage that is easily comprehended, and not relying on the level of quality given by an individual standard deviation.

Acknowledgments

We would like to thank Flore Depeint from LaSalle Beauvais, and the Institut Pasteur de Lille (Pasteur Institute from Lille, France) for their collaboration in the analysed trials.

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