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SURVEY AND SUMMARY

Validation of kinetics similarity in qPCR

Tzachi Bar[1,*], Mikael Kubista[2,3] and Ales Tichopad[3,4]

[1]Labonnet Ltd., 2 Hamelacha St., Ramat-Hasharon, 47445, Israel, [2]TATAA Biocenter, Odinsgatan 28, 411 03 Göteborg, Sweden, [3]Biotechnology Institute, Academy of Science of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic and [4]Charles University, Medical Faculty Hospital in Pilsen, Dr. E. Beneše 13, 305 99 Pilsen – Bory, the Czech Republic

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ABSTRACT

Quantitative real-time PCR (qPCR) is the method of choice for specific and sensitive quantification of nucleic acids. However, data validation is still a major issue, partially due to the complex effect of PCR inhibition on the results. If undetected PCR inhibition may severely impair the accuracy and sensitivity of results. PCR inhibition is addressed by prevention, detection and correction of PCR results. Recently, a new family of computational methods for the detection of PCR inhibition called kinetics outlier detection (KOD) emerged. KOD methods are based on comparison of one or a few kinetic parameters describing a test reaction to those describing a set of reference reactions. Modern KOD can detect PCR inhibition reflected by shift of the amplification curve by merely half a cycle with specificity and sensitivity >90%. Based solely on data analysis, these tools complement measures to improve and control pre-analytics. KOD methods do not require labor and materials, do not affect the reaction accuracy and sensitivity and they can be automated for fast and reliable quantification. This review describes the background of KOD methods, their principles, assumptions, strengths and limitations. Finally, the review provides recommendations how to use KOD and how to evaluate its performance.

INTRODUCTION

Real-time polymerase chain reaction (PCR) is the method of choice for sensitive detection and precise quantification of minute amounts of targeted DNA sequence. When combined with reverse transcription (RT) real-time PCR is the preferred method also for the detection and quantification of RNA. It is widely used in bio-medical research and is at present the reference method for molecular diagnostics, water, food and feed testing, forensic and most other testing of nucleic acids. Search in PubMed generates >250 000 hits. At present, 25 years after the breakthrough invention of PCR and 15 years after the invention of real-time PCR, validation and standardization of real-time PCR are hot topics (1) attracting the attention of regulatory bodies such as the FDA (www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm126957.pdf), EPA (http://www.epa.gov/microbes/qa_qc_pcr10_04.pdf), ISO (2), and CLSI (http://www.clsi.org/source/orders/free/mm16a.pdf) as well as multinational projects (www.spidia.eu).

PCR kinetics and efficiency

PCR has been studied extensively (3–6); here a brief description follows. There is some confusion between PCR efficiency and PCR kinetics in the literature. Here, we follow the IUPAC definition of chemical yield, the fraction of the amount of an element or chemical compound following a specified chemical reaction (http://goldbook.iupac.org/C01041.html) and define PCR efficiency (E) as the fraction of double-stranded DNA molecules that is copied at a given cycle. E is expressed in percentage (%). For example, $E = 100\%$ represents perfect doubling of all DNA molecules, and 0% represents no change in reaction product. Similarly, we follow the IUPAC definition of macroscopic kinetics being the behavior of bulk systems, e.g. in changes in concentrations of reactants and products (http://goldbook.iupac.org/M03677.html) and define PCR kinetics as the change in concentration of DNA along several consecutive cycles, often during the entire reaction. Since PCR efficiency changes as the reaction develops, PCR kinetics is best described by a function (7–10) (Figure 1).

The confusion between the two terms stems from the common assumption that efficiency is constant until the threshold level is reached. However, recently, Rutledge and Stewart showed that this is not the case (11). At the time the amplification signal is distinguishable from

*To whom correspondence should be addressed. Tel: +972 50 8424208; Fax: +972 77 4424640; Email: tzachi.bar@labonnet.com

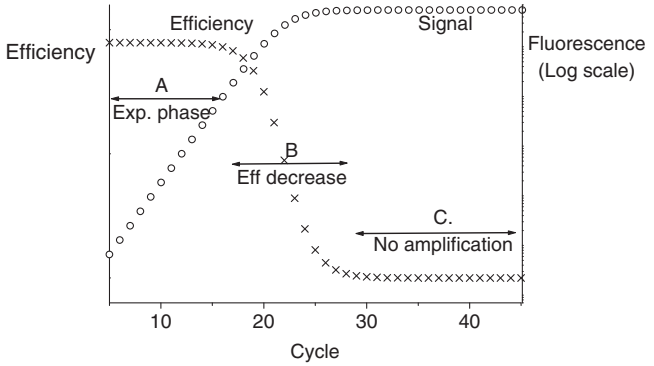

Figure 1. Schematic representation of PCR product accumulation (open circle) and PCR efficiency (cross mark) during the reaction. As long as all reagents are in excess and little amount of double-stranded DNA is in the tube, the PCR progresses with seemingly constant high-efficiency, segment A. As reagents are depleted and product is accumulated, the efficiency decreases at increasing rate, segment B (12). When too little reagents are available, or too much double-stranded DNA is in the tube, the efficiency of the reaction gets practically to zero, segment C.

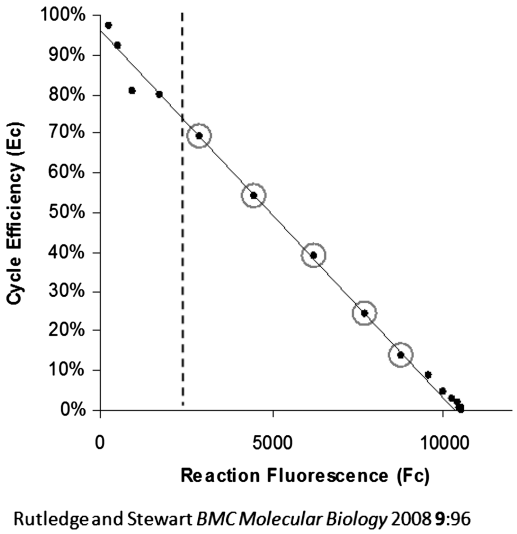

Figure 2. PCR kinetics from (11). The efficiency estimated at the lower part of the amplification curve, i.e. the left side of the *x* axis, is imprecise due to the low signal-to-noise ratio. Circles indicate precise estimation of efficiency. For the same reason, the threshold for quantification is set above the reaction fluorescence level (vertical dashed line), where efficiency already decreased by ~25%. These data are from SYBR Green reactions, given the lower signal-to-noise ratio of probes the dashed line is expected to be shifted to the right when probe is used.

background, PCR efficiency is already 10–25% below its maximum value (Figure 2).

The term PCR efficiency is widely used in the context of standard curve made of a dilution series. The term relates to the value calculated from the slope of a standard curve and relies on the assumption that the amplification curves in the dilution series are parallel at least until reaching the threshold. Mathematically this value reflects the average efficiency in the first cycles during which the reaction moves from one DNA concentration to the next in the dilution series, i.e., this average efficiency value reflects the constant kinetics during the very early cycles.

The assumption of similar kinetics

The strength of PCR as diagnostic method stems from its exponential amplification of a targeted sequence, which enables the investigator to detect even a single DNA molecule in less than 1 h. However, the exponential amplification is also the technology's Achilles Heel; almost all quantitative approaches based on real-time PCR (qPCR) compare the number of amplification cycles required to reach a threshold signal level when a target sequence is amplified in two reactions, or two groups of reactions. This is equivalent to measuring the distance between the two amplification curves. Hence, for proper quantification these methods assume similar amplification kinetics up to the threshold among compared reactions of the *same sequence*. If the assumption is not valid, substantial error into quantification can be introduced. Even a difference of only 5% in PCR efficiency will produce more than 100% error, i.e. 2-fold difference in amplicon amount, after 30 cycles. Larger difference of 15% will produce an order of magnitude error (>1000%) after the same number of cycles (13).

Note that relative quantification of gene expression with the $\Delta\Delta C_T$ method (14) assumes similarity of amplification kinetics between *different sequences*, the target and reference genes, as well as similar amplification kinetics among the same sequence reactions.

The error introduced by violating the requirement of similar kinetics among reactions of the same sequence is much larger than the error introduced by violating the requirement for equal kinetics of target and reference genes. While the second error propagates only during the cycles between the curves, i.e. the error is not affected by the absolute values of Cq but by the distance between the curves, the first error propagates during the entire reaction, from cycle 0 to Cq. Hence, the higher the Cq, the larger the error introduced by dissimilar kinetics among reactions of the same sequence. A typical 10% difference in kinetics will result in an error of few tens percents in the second case and an error of hundreds percents in the first case. Violation of the requirement for similar kinetics between compared sequences is easily solved by analyzing the data with Pfaffl's equation (15). For detailed example of the differences in error due to violation of the two assumptions, see the Supplementary Data.

While quantification errors of tens to several hundred percent may be of little practical importance when changes in quantities are measured by orders of magnitude and primary trends are studied, it may have serious implication in all other cases. Such error may make the difference between up- or down-regulation, affect medical and veterinary decisions, determine approval or disapproval of food and water for use, change qualification or disqualification of genetically modified products, distort allocation of rare forensic sample to different forensic tests and harm many other important decisions, which are based on the measured levels. In these cases, it is critical

to verify that the assumption of similar kinetics among reactions of the same sequence indeed fulfilled. The most common cause that this assumption is not fulfilled is PCR inhibition.

PCR inhibition

PCR inhibition is a well-recognized problem that can be caused by diverse substances via several mechanisms (16–19). For example, PCR mixtures based on the widely used *Taq* DNA polymerase are totally inhibited in the presence of 0.004% (v/v) human blood (20). The PCR inhibitors originate either from the test sample matrix or from sample preparation prior to PCR or from both (17,21). Examples of inhibiting substances present in original samples include bile salts and complex polysaccharides in feces; collagen in food samples; heme, immunoglobulin G (IgG) and lactoferrin in blood; humic substances in soil; melanin and myoglobin in tissue; polysaccharides in plants; proteinases and calcium ions in milk; indigo dye in denim and urea in urine (17). Fatty tissues are in general very problematic.

Components from sampling and extraction that inhibit PCR include chelators such as EDTA, which complexes Mg^{2+} rendering it unavailable for the polymerase. Interestingly, trace amounts of phenol inhibit *Taq* polymerase, while *Tth* polymerase maintains both DNA- and RNA-dependent DNA polymerase activity in the presence of 5% (v/v) phenol (20). Excess of KCl, NaCl and other salts, ionic detergents such as sodium deoxycholate, sarkosyl and sodium dodecyl sulfate (SDS) also inhibit PCR (22), as well as alcohols such as ethanol and isopropanol (23).

For expression analysis, it has been shown that active reverse transcriptase brought over from the RT reaction can have inhibitory effect on the PCR (24) and stimulate primer–dimer formation (25). The effect is profound during the first cycles and declines as the reverse transcriptase denatures by the applied heat and as the DNA accumulates. Apparent PCR efficiencies of >100% occasionally reported (25,26) and may be obtained when standard curves are constructed based on serial dilution of cDNA, which also dilutes the contaminating reverse transcriptase (27).

Wilson (17) classifies PCR inhibitors into three categories: failure of lysis of biological material, poor lysis of cell and capture of nucleic acids, and inhibitors. *Taq* inhibitors may be classified into three sub-categories based on mechanism: (i) binding of the inhibitor to the polymerase; (ii) interaction of the inhibitor with the DNA and (iii) interaction with the polymerase during primer extension (19). Some inhibitors may interfere via more than one mechanism (17,19).

No matter the cause inhibition should be considered. There is a common saying that a wise person avoids problems that a clever person knows how to solve. Near the PCR bench the wise person carefully removes inhibitors from the sample before quantification. When a large set of similar samples is analyzed, extraction and purification methods may be customized and optimized for reproducibility. However, when samples vary in their composition as often is the case with food, soil, seed, feces, fatty samples, bacterial samples, forensic samples and samples containing mixtures of tissues and cells types reproducibly removing all inhibitors from the samples may be a challenging task.

PCR inhibition and the errors it may introduce into nucleic acid quantification are approached by three complementary ways. First, the degree of inhibition is reduced, optimally prevented experimentally. This includes extensive purification, adding agents that reduce inhibition and interference such as T4 gene 32 protein (28,29) and dsDNA-specific nuclease, using DNA polymerases that are less prone to inhibition (30,31) and polymerases with higher capacity of synthesizing through GC-rich sequences (32) and diluting the sample (33). Second, detecting PCR inhibition includes experimental approaches based on internal amplification controls (IAC) (34–37) and computational methods based on kinetics outlier detection (KOD). Thirdly, computational methods can be used to reduce the errors imposed by the inhibition, among them 'assumption-free' quantification methods that do not assume similar kinetics of the compared reactions (8,9,38,39) and methods that correct the measured results (40,41).

First in this article, we discuss what would be the ideal solution for PCR inhibition: assumption-free methods. Then, we treat the most common approach at present for detection of PCR inhibition, the IAC. Then, the error correction methods are discussed and finally we describe the KOD methods. Each method is examined and evaluated both by evidence from the literature and according to the strength of the assumptions upon which it relies.

'ASSUMPTION FREE' QUANTIFICATION METHODS

If inhibition is not eliminated in advance during sample preparation, the ideal solution for PCR inhibition would be to use a quantification method that does not rely on similar reaction kinetics, but determines reaction-specific kinetics that are used to calculate the initial number of target molecules. Such methods have been developed. They are based on nonlinear regression (NLR) to fit parameters in an equation assumed to describe the reaction-specific kinetics, to the measured fluorescence signal. The equation is then used to calculate the number of molecules at cycle 0 by extrapolation. For details of the different NLR methods, the reader is referred to recent reviews on the topic (7,42–44). Although intensive efforts have been invested in the development of assumption-free methods in the last decade (5,6,8–11,13,38,45), independent studies show that for quantification of non-inhibited reactions the NLR methods do not outperform conventional cycle of quantification (Cq) method, i.e. methods that do assume kinetics similarity among reactions of the same sequence.

Goll *et al.* evaluated the quantification error of 11 NLR methods in a comprehensive study and compared it to the quantification error obtained when using the conventional Cq method (7). They report that the best NLR model yield quantitative results with an intra-assay variation of 58%

versus 24% for the Cq-derived molecule numbers. Similar result was obtained by Guescini *et al.* (40) who tested Rutledge's new method (9). These findings support the conclusions of Yann *et al.* (43), Nordgard *et al.* (42) and Peirson *et al.* (46) who present evidence that reaction-specific kinetics and efficiency estimated from individual amplification curves primarily increase the random error of qPCR and should not be used instead of classical methods.

Several factors may contribute to the imprecision of NLR methods. Major limitation is our poor understanding of the complex mechanism of real-time PCR. The changing ratio between DNA and the dye or probe during the reaction affects their binding (47,48). The higher the ratio between the dye or probe and the DNA, the higher the binding rate and stronger the emitted signal. Unfortunately, the exact behavior depends on various factors, which are difficult to model and predict precisely in practice. Another source of error is the modeling of background (49). When background is not well modeled by the fitted equation, it may introduce error to the fit and to all resulting calculations (13,49) (Figure 3).

The precision that can be achieved with NLR methods depends on the qPCR instrument. Some of the early platforms (e.g. AB7700 and Roche Lightcycler 1.0–2.0) had more sensitive optics and measured signal changes over a 3–4 log units, which is not the case for some of the newer and cheaper instruments. Using linear scale data may seem to fit the model very well, but the sum of squares of the regression is biased by the higher fluorescent readings. It is, therefore, better to fit data in the logarithmic scale. This creates ambiguity in the reporting of the estimated error. It would be good for the field to define a precision measure similar to the standard deviation of Cq. Such an error measurement will need to combine both the quality of the fit and the Cq, as efficiency exponentially affects the measured quantity.

Perhaps the most conspicuous obstacle on the way to panacea NLR is the poor reproducibility of PCR at the detectable region. Although the signal-to-noise ratio of fluorescence emitted from SYBR Green in real-time PCR conditions is relatively high, about two to three orders of magnitude, when DNA amount in the tube reaches the detectable region, PCR efficiency rapidly decreases with every cycle (phase B, in Figure 1). At this stage, the reproducibility of the reaction and our understanding of its are too low for precise modeling of the process.

Since qPCR is mainly about quantification of minute amounts of nucleic acids, in most cases the detectable signal arrives above cycle 20, which leads to a long extrapolation down to cycle 0. Thus, currently, reasonably precise estimation of initial quantities using NLR methods seems possible only when several replicates for each reaction are performed. Chemistries and instrumentation that will produce and support signal acquisition with higher signal-to-noise ratio will enable detection of DNA at lower amounts of DNA, when the reaction is more reproducible and would be modeled more accurately and precisely.

If 'assumption-free' quantification methods can hardly be used then conventional methods that do assume kinetics similarity should be used and kinetics similarity should be verified. The rest of this paper describes the most common tools for kinetics validation.

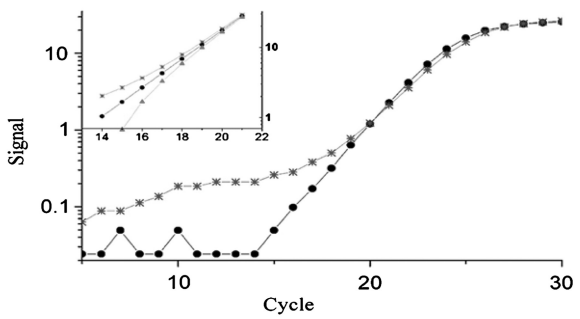

Figure 3. Effect of improper background subtraction on the shape of the amplification curve (49). Data points from a sample with properly modelled and subtracted background (filled circles) fall on what looks like a straight line in the exponential phase of the amplification curve, while under background subtracted (stars) and over background subtracted amplification curves (triangles, inset only) form concave and convex shapes, respectively. This not only affects the results of quantification due to Cq shift at the lower part of the curve, but also directly affects the efficiency estimated from the amplification curve.

IAC

Validation of PCR kinetics in RNA quantification

RNA quantification with RT–qPCR is a multi-step process that each proceeds with limited efficiency and introduces variability, which makes absolute quantification virtually impossible unless known amount of RNA is spiked to the sample and used to validate and normalize the result. Therefore, the common use of RT–qPCR is for relative quantification, where fold of change in RNA amount between samples is calculated. Relative quantification by qPCR is based on normalizing the measured expression of genes of interest with the expression of one or several reference genes that are minimally affected by treatment.

In this process, it is assumed that the measured amounts of the target and reference genes' transcripts are influenced equally by changes in the steps involved in the quantification, including extraction, purification, transportation, storage, RT, etc. The yields in these steps do not have to be the same for the gene of interest and the reference gene, because their expression ratio is typically compared among samples, but their relative yields in the experimental steps should stay the same.

However, despite all their advantages, reference genes are not suitable for correction of errors due to PCR inhibition in relative quantification. PCR inhibition is not only often sample specific, but also sequence dependent. That is, two sequences quantified from the same sample may be inhibited to different levels (19,50–53) and the error in quantification of one sequence will not compensate for the error in the other sequence. Several possible explanations have been proposed for the differential inhibition. During denaturation, where DNA is in

single-stranded form it may form secondary structures that interact with inhibitors (19), or even directly interact with the primer region to decrease the reaction kinetics (54). Since secondary structure of DNA depends both on the sequence and the ionic composition, PCR is also sensitive to ionic composition including the amounts of K^+ and Mg^{2+} ions. Substances present in the sample that bind any of these ions, such as genomic DNA, may differentially affect the kinetics of PCRs.

Regardless of the inhibition source and mechanism, it should be detected, and not by reference genes. One way to estimate PCR efficiency is by serial dilution of a sample and calculating the efficiency from the slope of the dilutions curve (50). This has been adapted by Roche under the name E-method (http://www.roche-applied-science.com/PROD_INF/BIOCHEMI/no4_06/pdf/16.pdf). Drawback is that it is laborious and costly, since a sample must be analyzed multiple times, and is not applicable on samples with small number of molecules, since they cannot be diluted. Also, interfering substances will be diluted as well and PCR efficiency will change.

Identification of PCR inhibition with IAC

IAC is a non-target DNA or RNA spiked to a test sample at known amount and co-amplified with the target sequence. Following the amplification, the Cq of the IAC is compared to the Cq of the same amount of IAC quantified without the test sample. A difference between the Cqs indicates inhibition. It was adopted 7 years ago for detection of PCR inhibition in DNA quantification by the European Standardization Committee (CEN) and the International Organization for Standardization (ISO) (2).

Various approaches to the design and use of IAC have been presented (16,36,37,55). Hoorfar (36) classifies IACs into two categories based on their competitiveness with the target sequence. Competitive IAC shares primer set with the target sequence. The concentration of the IAC has to be carefully adjusted to the (unknown) quantity of the target sequence in the samples; too many IAC molecules and the IAC will consume the primers of the target and will itself inhibit the target sequence amplification; too little of IAC and the target sequence will consume the primers and inhibit the IAC amplification. If added at sub-optimal concentration, IAC may severely affect the accuracy and sensitivity of the quantification (16,34,56) and the control will not correctly reflect the presence of inhibitors. If properly optimized, the advantage of competitive IAC is that it will have very similar kinetics as the target sequence and the sensitivity to inhibitors of the two PCRs will also be similar. Competitive IAC is recommended by the United States Environmental Protection Agency in its quality control guidance for PCR (http://www.epa.gov/microbes/qa_qc_pcr10_04.pdf) and required by ISO 22174(2).

Non-competitive IAC is amplified with a different set of primers from the target. The final amount of non-competitive IAC amplicon produced is limited by primers' concentrations and does not deplete resources needed by the other reaction. Hence, the amount of IAC added is not critical for proper amplification of the target. But the same dissimilarity of sequences between the target and the non-competitive IAC that makes it so easy to use and popular (55) might cause it not to properly reflect sequence-dependent inhibition. This could be the reason why the United States Environmental Protection Agency recommends in its quality control guidance for PCR (49), and ISO 22174 requires the IAC to be amplified by the same primers set. Dedicated qPCR data analysis software such as GenEx include an option to correct Cqs of targets based on the differential Cq measured on an IAC (www.multid.se).

Whatever type of IAC is used, spiking the sample with IAC earlier than the PCR might not indicate PCR inhibition, but rather the loss of material during the steps the IAC passed, e.g. during extraction and purification of nucleic acids. It is then not possible to separate effects from losses and degradation during pre-analytics and PCR inhibition on a shift of Cq. Since the correction for loss of material is linear, while the effect of PCR inhibition accumulates exponentially and is Cq dependent, the shift in Cq of an IAC added during pre-analytics cannot be used to correct the result of quantification. To control for loss of material during pre-analytics as well as for PCR inhibition two IACs are needed: one added during pre-analytics and one added for the qPCR. Still, in spite of the obvious value of IACs for QC of qPCR, they are still not used in most qPCR publications at present.

COMPENSATION FOR PCR INHIBITION

The search for accurate and precise quantification led to the development of methods to compensate for errors in quantification due to PCR inhibition. The first one, dedicated to real-time PCR, was published by Meijerink *et al.* (41). It is based on multiplex amplification of two genes present in different copy numbers in the human genome (beta-actin and albumin). Normally, the two PCR systems are amplified with the same kinetics. Hence, in the absence of inhibition a constant distance between the amplification curves of the two genes that corresponds to the difference in copy numbers is expected. A larger distance between the two amplification curves indicates PCR inhibition. Using a mathematical model, the Cq values can be corrected based on the measured difference. This method assumes that PCR inhibition is not sequence dependent. While this assumption may be correct for some cases, it does not always hold (19,50–53). In addition, the method is only applicable for DNA quantification and it is species specific.

'Cy0' is another method for error correction in cases of slight inhibition recently published by Guescini *et al.* (40). Here, a reaction-specific parameter named 'Cy0' replaces the Cq of each reaction. Cy0 is the intersection between the abscissa and the tangent of the inflection point of the curve obtained by the nonlinear regression of raw data (Figure 4). Although Cy0 is a single parameter, as is the regular Cq, it does account for some of the reaction kinetic because it is calculated on the basis of the slope of the inflection point of fluorescence data. The authors compared the Cy0 method with three other quantification

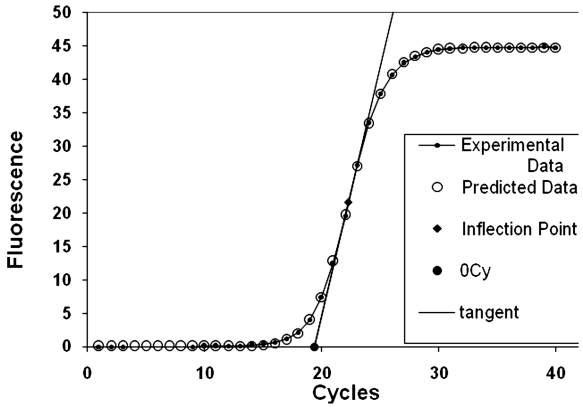

Figure 4. Example of the Cy0 method (64). Curve fitting of Richards function to amplification data generates values for the kinetic parameters from which the inflection point (solid black rhombus) and the slope of the curve can be derived. The quantitative entity Cy0 (solid black dot) shows the cross point between the *x* axis and the tangent crossing the inflection point of amplification curve.

methods: maximum second derivative, fixed threshold and NLR and found that quantification based on Cy0 on SYBR data is the most accurate when the assays are slightly inhibited. This holds promise and it will be interesting to learn how the method performs on probe data, where the inflection point is lower, how it performs with different kinds of inhibitors, and its robustness for sub-optimal background subtraction. The Cy0 method is freely available at www.cy0method.org.

Another approach to avoid quantification errors due to PCR inhibition was proposed by Gallup and Ackermann (33). First, a small amount of template is collected from all test samples and pooled. Then, the pooled sample is serially diluted and Cq is plotted versus the dilution factor, similarly to a standard curve. Since the slope of this curve describes PCR efficiency at the very early cycles, the optimal dilution for inhibition removal can be identified from the shape of the curve and applied to all test samples.

While the concept is highly robust since virtually any inhibitor can be diluted out so its effect vanishes, in practice this requires large amounts of DNA. The authors recommend dilutions by two to six orders of magnitude, which drastically reduce the sensitivity of quantification. In addition, the method assumes that all samples are inhibited to the same degree reflected by the pooled sample, which is not practical for analysis of samples where variable inhibition is expected, such as with food, water, forensic, clinical, soil and many other types of samples. Gallup's method is implemented in Prexcel software available at http://www.gene-quantification.de/download.html#gallup.

KOD

Pre KOD methods

The ability to identify inhibited PCRs by analyzing their reaction kinetics attracted already the attention of the developers of the first NLR method (39). However, the authors didn't elaborate on the statistical details and only suggested a general approach for quality check based on a single estimated efficiency value.

The idea of using an 'efficiency threshold' below which a reaction is disqualified was introduced by then Corbett Research, a real-time PCR instrument manufacturer that recently was acquired by Qiagen. Although attractive because of its simplicity, it is difficult to justify an efficiency threshold value, at least if an outlier concept shall be used since the selection criterion should be based on similarity in kinetics rather than just having high efficiency. Reactions from the same sequence that are equally inhibited can readily be accurately quantified. Further, using a single parameter, such as an estimated value of the efficiency, the method is sensitive to noise and only strongly inhibited reactions could be confidentially detected. After some years, Corbett Research deleted this functionality from their software.

Contrary to the 'efficiency threshold' concept, Massart *et al*. (57) directly test the assumption of similar kinetics, i.e. the similarity of the slopes of two sets of compared amplification curves. For this purpose, they use six replicates in each set and fit linear equation to five log-transformed data points. It is based on well-established method for comparison of slopes often used in analytical chemistry, e.g. when testing similarity of calibration curves (58). Although the authors indicate the optimal number of fitted data points and the minimal number of replicates required for reliable testing, they don't measure the quantification error detected with these settings. While the logic of the method is sound, the need for at least six replicates may be laborious and expensive for many users.

The KOD concept

KOD is a family of statistical methods designed to identify real-time PCRs with dissimilar kinetics. KOD methods are based on comparison of one, or more, parameters describing the kinetics of a test reaction to kinetic parameters describing a set of reference reactions that preferably has been validated by independent means. The first paper implementing this concept was published by Peirson *et al*. (46) and the term KOD was coined 3 months later in an independent study (49).

Univariate KOD

The first KOD methods (46,49,59,60) assumed that the exponential phase, with its constant efficiency continues at least up to the threshold, where Cq is determined. Thus, a single parameter, the efficiency at the early cycles, could be used to validate similarity of the kinetics of compared reactions.

In the first KOD method, deviant amplification efficiency was identified by comparing the variance of the reference set with that of each individual reaction essentially identifying outliers based on leave-one-out validation (46). In this method, all reactions make the reference set, regardless of their quality. Hence, reactions with the most divergent kinetics are identified and disqualified independent of the degree of divergent. Thus, quantification error and the method's performance

cannot be characterized and quantified. This method was implemented in a Microsoft XL application called DART and is available at http://www.gene-quantification.de/download.html#dart.

In the second KOD paper, the authors presented a somewhat different approach (49). The estimated efficiency of a reaction that deviated by more than ± 1.96 standard deviations (SD) (the approximate value of the 97.5 percentile point of the normal distribution) from the mean efficiency of all reactions in the reference set was classified kinetic outlier. While the mean efficiency is calculated from a reference set from the same run, the SD is calculated from a large set of independent reference runs. Using an external estimate for the SD behind the exclusion criteria makes the method more robust since SD calculated from a single reference set varies with every slight change in settings of the efficiency estimation procedure. The authors discuss the reference set composition and consider the possibility of basing the reference set on only reactions used in the standard curve and not on all the test reactions. A variant of this method was thoroughly tested by Elias *et al.* (61) who used Liu and Saint's method to estimate PCR efficiency (8). The authors concluded that the KOD method provides a sensitive and statistically powerful strategy to identify inhibited reactions.

Later work by Chervoneva *et al.* (59) pointed out that the second KOD method, which is intended to retain 95% of samples with efficiencies comparable to those in the reference set, the acceptance interval included the desired 95% coverage only if the mean and standard deviation used in the computations are true parameters describing the efficiency distribution in the underlying reference population. However, the employed estimates of the mean and standard deviation are calculated from the reference set without any adjustment for the error of estimation and, consequently, the second KOD method is not a valid statistical procedure. In particular, if the reference set is small, the second KOD method may erroneously reject reactions with efficiencies that would be consistent with those of the infinite reference setpopulation (59).

Chervoneva *et al.* suggested an improved, third, KOD method (59). Here, reaction-specific efficiency is estimated by fitting an exponential growth model to the fluorescence data in the early detectable cycles of the reaction. Next, reactions within the reference set having outlying efficiency are eliminated using the box plot outlier detection rule (62). Estimates of amplification efficiencies of the trimmed outlier-free reference set are then employed to define tolerance intervals that are subsequently used to eliminate kinetic outliers from test reactions. The authors demonstrate superior performance of their method relative to the second KOD method.

The fourth KOD method published by Bar and Muszta (60) is designed for relative quantification, and is particularly suitable for trend studies where many reactions are compared with each other, such as time series and dose–response experiments. In such experiments, the kinetics of all compared reactions should be similar. Here, the variance of efficiency, estimated from the set of compared reactions, is tested for similarity to the variance of a reference set. If the variance of the test reactions is similar to that of the reference set, the set of test reactions are comparable. If the variances differ reactions with extreme kinetics are designated outliers using a corrected version of the second KOD method that considers the size of the reference set.

Multivariate KOD

The early KOD methods (first to fourth) are based on a single parameter estimate of the reaction kinetics to describe reaction kinetics and compare between a test reaction and a reference set. It is further assumed that PCR kinetics is constant at least up to a threshold level. Rutledge and Stewart (11) pointed out that this assumption is rarely tested and probably often it is not fulfilled, which makes these methods useful mainly for the detection of severe inhibition (Fig. 2).

A later version of KOD called, Multivariate KOD (MKOD), was introduced by Tichopad *et al.* and Sisti *et al.* (63,64). MKOD is based on pattern recognition principles. The amplification curve is described with $N \geq 2$ discrete geometrical measures (e.g. derivative maxima, inflexion point, height, etc.) describing features of the amplification curve regardless of the formal mode of the amplification. This set of geometrical measures defines a unique 'kinetic finger print' to each amplification curve. Next, kinetically outlying reactions are excluded from reference set, establishing exact exclusion margins for kinetics parameters of retained references. Then, test reactions with kinetics parameters outside the exclusion margins are identified by multivariate statistics such as the Mahalanobis distance or the Z-score for the geometric measures (65).

The two MKOD methods of Tichopad *et al.* and Sisti *et al.* have common principles but they differ in several aspects. Tichopad *et al.* characterize an amplification curve by the y coordinates of its first and second derivative maxima and then identify outliers, while Sisti *et al.* use the y coordinate of the inflection point, the tangent to the inflection point and the maximal signal. Although low maximal signal is a strong indicator of PCR inhibition, it may also be prone to variation that is not necessarily associated with quantification error. In fact, this is the one reason instrument manufacturers recommend selecting threshold for Cq readout above the noise, but still close to the baseline where the signal is low and reproducibility higher (14). Sisti *et al.* considered this, but found that the maximal signal has significant impact on the variance-covariance matrix and improves the performance of the method.

Further, Tichopad *et al.* model the response curve with four-parameters symmetric equation, while Sisti *et al.* fit five-parameters asymmetric equation. The difference may be relevant for probe-based amplification curves, which often are asymmetric. However, since Tichopad *et al.* fit the lower part of the amplification curve only (six points below the inflection point and only two points above it), the asymmetry of the amplification curves has little effect

on the method's performance. The asymmetric fit, however, is highly relevant for Sisti *et al.*'s method, since the entire amplification curve is modeled.

The fact that the two groups after extensive testing end up with similar results using different parameters and number of parameters implies that fine differences in the methods' performance may result from differences in the analyzed data. For certain data, one method may perform better, while the other method may suit more in other cases. To find out which method performs best requires a comprehensive study on data confounded by many different factors that affect the shape of amplification curves. This may include data generated using different detection chemistries, buffer compositions, presence of various inhibitors, cycling conditions, etc.

Not being limited to any particular amplification model and being free from wrong assumption of exponential amplification up to the threshold, the advantage of MKOD over the univariate KODs is superior sensitivity and specificity in the identification of outlier amplifications (63,64). The MKOD method of Tichopad *et al.* was implemented in Kineret software and is available at www.labonnet.com.

Composition of the reference set

Being based on 'test reaction versus reference set' comparison, the performance of all KOD methods is directly derived from the composition of the reference set. The higher the similarity among the reference set reactions, the smaller the differences in kinetics the methods will detect. Therefore, the selection of the reference set should consider several parameters.

(i) *Dynamic range*. PCR replicates are highly reproducible and generate amplification curves that are very similar in shape. Hence, a reference set consisting of replicates based on a single concentration are likely to result in many false positive alerts, i.e. kinetics outliers without relevant quantification error. Hence, the reference set should span and thus reflect the test reactions to account for the random variability in kinetics as well as the systematic variability associated with starting concentration within the expected range.

(ii) *Number of reactions*. Developers of KOD report that a reference set of 10–15 reactions is usually enough to estimate the kinetic parameters with sufficient precision (51,63).

(iii) *Composition*. The composition of the reference set is driven by the requirement for similar kinetics. Optimum composition of the reference set depends on whether a standard curve is used or not.

  (a) If quantification is based on a standard curve, the kinetics of test reactions shall be similar to that of the standard curve reactions (66). The reference set shall then be based on the standard curve reactions. Noteworthy, when using a diluted sample to construct the standard curve, the investigator should take care of not using an inhibited sample since the dilution of the sample will necessarily dilute the inhibitor and will result in amplification curves with different kinetics, which will reduce the sensitivity of the method. A standard curve made of an inhibited sample is identified by its nonlinear shape, expressed in low R^2.

  (b) When quantifying without a standard curve (15), the test reactions are compared to each other and make up an internal reference set.

The material from which the standard curve is prepared shall be relevant to the quantified samples. The exact protocol depends on the quantification target, DNA or RNA, but except for this fact the composition of the standard curve has been the subject of a long dispute in the qPCR community. Some investigators pick one representative sample (67), and some use linearized plasmid DNA carrying the cloned target sequence, or purified PCR products. Others use, for RT-PCR, synthetic, usually *in vitro* transcribed, RNA or total RNA/mRNA containing the target sequence (55,66). Others pool small amounts of all test samples and use the resulting mixture to construct the standard curve (33). Whatever source is used, using KOD (KODing) the test reactions with the standard curve as a reference set, each investigator can easily check the kinetics similarity between the standard curve reactions and the test reactions, and find the best composition of the standard curve for their own requirements.

Taking all the test reactions as reference set will automatically disqualify the reactions with the most extreme kinetics, regardless of quantification error. This implication of KOD emphasizes the need to estimate the quantification error actually detected using KOD.

Evaluating KOD performance

Application of KOD supplies the investigator with probabilistic result about the similarity of kinetics between test and reference reactions, but it says nothing about the magnitude of the quantification error associated with the detected dissimilarity. To overcome this limitation, the developers of KOD methods characterized the methods performance by a simple experiment described below. They demonstrated that proper application of KOD can under certain condition detect quantification errors of less than half amplification cycle, with sensitivity and specificity >90% (63,64). However, since the performance of KOD depends on the relevance and quality of the reference set, the performance of KOD should be determined by each investigator in their own lab conditions.

An investigator who starts 'KODing' their data can obtain a good idea about the method's performance on their data, including the detected error, sensitivity and specificity by repeating the developers' simple characterization experiment. Briefly, a standard curve is constructed based on some 10–15 reactions covering 4–6 log concentrations, which serves as a reference set (Figure 5). The same standard curve is then repeated in the same run in the presence of increasing level of PCR inhibitor, which is expected to shift the Cqs to the

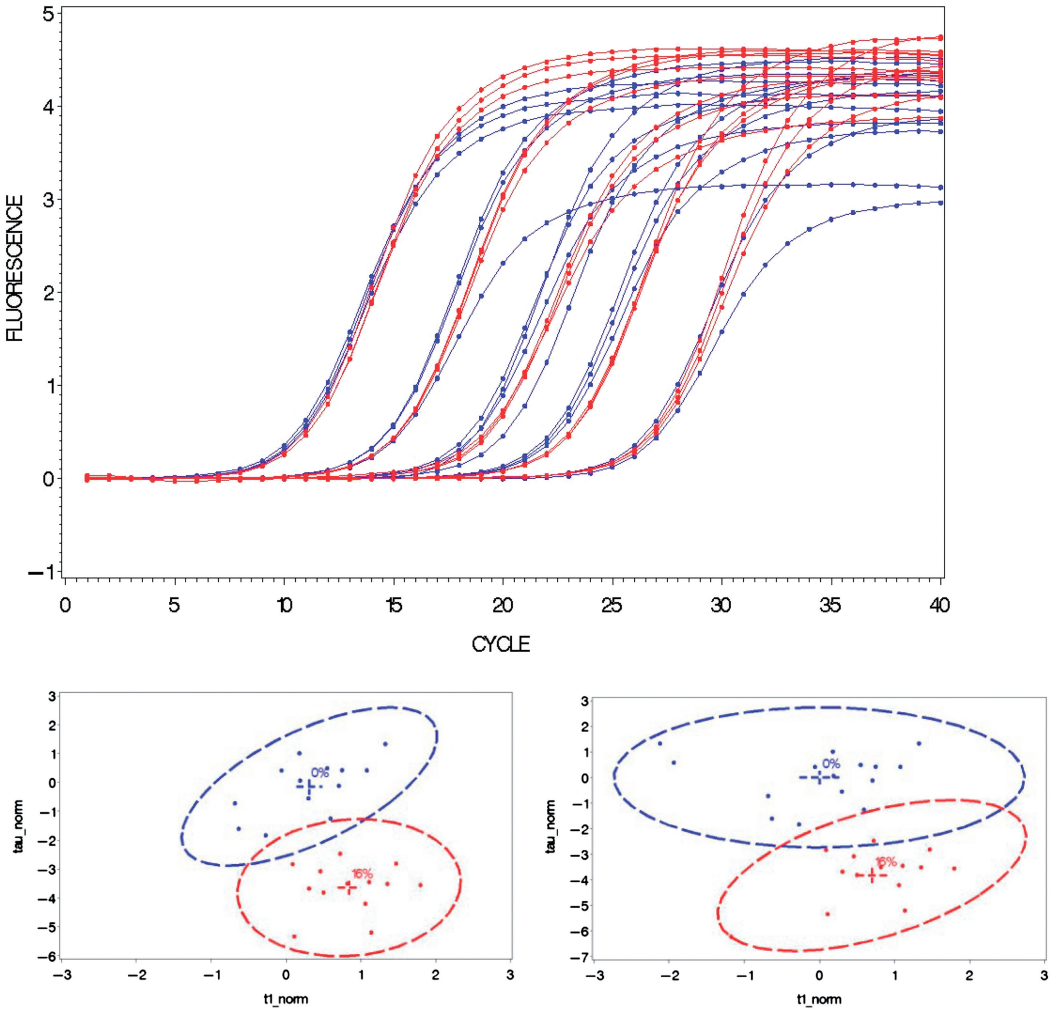

Figure 5. Upper figure from (63): the blue curves are 15 reference reactions and the red curves are 15 reactions produced from the same DNA stocks as the reference with 2.0 ng tannic acid added per 15 µl reaction mix. Lower figure: two-dimensional 95% confidence region produced by the reference set in the upper figure. Both dimensions are normalized to mean = 0 and SD = 1. Left figure: before outliers' exclusion from the reference set, right figure, after exclusion, resulting in stronger outlier detection.

right. This shift is readily translated into fold of quantification error as

$$\text{Error} = E^{\text{shift of Cq}},$$

where $1 \leq E \leq 2$ is the PCR efficiency calculated from the slope of the dilution series. Note, Error = E^0 = 1 means no error.

KOD developers have used several well-characterized PCR inhibitors in their papers. However, PCR inhibition in biological samples may differ by its mechanism and effect on quantification from the clean inhibitor. Using one of their inhibited samples, or pooled sample, as inhibitor for the characterization experiment, the characterization experiments will be more relevant to the investigator's particular data. The inhibitors in the inhibited sample do not have to be chemically characterized, but the sample should contain much less DNA of the detected sequence than the lowest concentration in the standard curve (of the characterization experiment) so it won't affect the Cq of the standard curve. Such sample can be prepared by DNase treatment followed by inactivation of the enzyme.

Error calibrator

The characterization experiment described above gives the investigator general term of the method performance over broad dynamic range, but since the performance of KOD is derived from the reference set, which changes from run to run, the characterization experiment will not tell about the detected error in a particular run. To obtain an empirical estimation of the detected error in a particular run, the investigator should include reactions from one concentration from the characterization experiment, i.e. three to six PCR replicates containing DNA concentration representing the major body of the reactions, with one replicate containing no inhibitor and the rest containing increasing level of inhibitor. The Cq of the inhibited replicates will be shifted differentially, according to their inhibitor concentration. The replicates with

shifted Cq that were detected supply an empirical evidence to the detected error by the particular reference set of the run. We name these inhibited replicates as 'error calibrators'.

CONCLUSION

KOD methods seem to be useful for detection of PCR inhibition in virology (68), bacteriology (69), gene expression analysis (70), forensic testing (61), and essentially all other qPCR applications were quality matters. Under optimal conditions, interference causing as small as a half cycle error in quantification can be detected using KOD with high sensitivity and specificity with an empirical estimation of the detected error. KOD analysis can be fully automated and is recommended in several textbooks (51, 71,72). The ability to objectively disqualify reactions based on aberrant amplification is an important step toward standardizing qPCR, which is a critical step needed for addressing regulatory requirements for evidence-based medicine (www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm126957.pdf). The appearance of MKOD with its superior performance, and the development of commercial MKOD tool is a step toward broader adoption of MKOD. A significant move in this direction was recently taken by the European consortium SPIDIA (www.spidia.eu) that aims to tackle the standardization and improvement of pre-analytical procedures for *in vitro* diagnostics, which used the MKOD implemented in the Kineret software for the detection of PCR interference in a large European ring trial.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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