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Review Article

Multi-template polymerase chain reaction

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ABSTRACT

PCR is a formidable and potent technology that serves as an indispensable tool in a wide range of biological disciplines. However, due to the ease of use and often lack of rigorous standards many PCR applications can lead to highly variable, inaccurate, and ultimately meaningless results. Thus, rigorous method validation must precede its broad adoption to any new application. Multi-template samples possess particular features, which make their PCR analysis prone to artifacts and biases: multiple homologous templates present in copy numbers that vary within several orders of magnitude. Such conditions are a breeding ground for chimeras and heteroduplexes. Differences in template amplification efficiencies and template competition for reaction compounds undermine correct preservation of the original template ratio. In addition, the presence of inhibitors aggravates all of the above-mentioned problems. Inhibitors might also have ambivalent effects on the different templates within the same sample. Yet, no standard approaches exist for monitoring inhibitory effects in multitemplate PCR, which is crucial for establishing compatibility between samples.

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Contents

Abbreviations: CDCE, constant denaturing capillary electrophoresis; DGGE, denaturing gradient gel electrophoresis; DHPLC, denaturing high-performance liquid chromatography; HPLC, high-performance liquid chromatography; PAAG, polyacrylamide gel; SSCA, single strand conformation analysis; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism.

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1. What makes multi-template PCR so different?

Polymerase chain reaction (PCR) amplifies the target segment of DNA by several orders of magnitude via repetitive cycles. In experiments where DNA served as an indicator molecule, PCR produced sufficient DNA material for the analysis, starting from a sample in which the sequence of interest may have been present in just a single copy. Increased detection sensitivity is both the result of the production of high quantities of the sequence and also of the increase of the target to non-target DNA ratio. This simplicity and universality make PCR probably the most widespread technique in molecular biology nowadays. However, due to its simplicity a PCR assay may erroneously be perceived as undemanding. Practical elegance and minimalism mask the complicated molecular processes that occur during the reaction and give the false impression of a clear, well-trodden path that, without special effort, always would lead to success. Nevertheless, it is essential to remember that every new application of PCR requires appropriate validation. The validation procedures should appropriately address all difficult passages of that particular application and this requires a high level of background knowledge. This review will discuss the use of PCR for simultaneous amplification of homologous sequences in a mixed template and the possible pitfalls for an unaware user. Previous studies already alerted the scientific community about the numerous problems of applying PCR technology to genetically and chemically complex samples, as in e.g. [1,2]. Unfortunately, we still turn a blind eye to the majority of the difficulties identified by these authors since they are tricky to address properly. This review focuses on yet another set of problems, which arise exclusively during the course of polymerase chain reactions in multi-template samples and leaves out all other weaknesses of this approach as this would be outside the scope of this review. We put together pieces of knowledge acquired by researchers from different fields, added our own results and experiences and then attempted to put together a coherent picture to better understand the nature of multi-template PCR. We also made an attempt to identify the challenges impeding a further development of this PCR technology.

PCR techniques can be divided into three groups based on the type of target (Fig. 1). The first group encompasses techniques where a single target sequence is amplified from single type template molecules using a single primer set. The template can be present in the test tube in multiple copies but all these copies have the same sequence. This assay is referred to as single-template PCR (Fig. 1a) and is what is typically referred to as PCR. The product of such an assay is analyzed *en masse* and on an agarose gel where it appears as a single band of a specific size. The second group of PCR techniques encompasses assays where several non-homologous target sequences are amplified simultaneously in the same reaction tube. Each target sequence is amplified with its own primer set. This type of PCR is referred to as multiplex PCR and is widely used in diagnostics (Fig. 1b). In such an assay, the precise sequence of each target gene is known. Products of multiplex PCR differ in size and can be fractionated. An agarose gel is usually used for separation of amplicons as each type of amplicon can be visualized as a distinct band. The third group of PCR techniques encompasses reactions where a set of similar target sequences is amplified from a mixture of homologous DNA sequences with just a single set of primers. This is called multi-template or mixed template PCR (Fig. 1c). In a multi-template assay the exact target sequences are unknown and a single set of primers is designed for the conserved part of a gene with the aim of amplifying all alleles in a mixed sample. After the PCR, amplicons of such an assay are fractionated so that the product from each template in the original sample can be distinguished from the other products and, if possible, quantified. Unfortunately, agarose gels fail to provide adequate separation (all products appear as a single band) since amplicons are almost of identical size, and more sensitive methods have to be used for fractionation (methods of fractionation and detection are discussed in Section 5.1).

A special case of the multi-template assay is applying it to measure microbial load. In this case, PCR is also performed using mixed templates but the final product escapes fractionation and is instead analyzed *en masse*. However, the demanded outcome for this type of assay should be a quantitative measure. When multi-template PCR is used for quantifying microbial load, biases and artifacts characteristic for the mixed template assay might occur. Yet, since the product is analyzed without fractionating, the effect of the PCR-induced artifacts could become ambiguous: some artifacts distort the quantification while others have no such effect. The compulsory need for quantitative results imposes additional troublesome requirements for this type of multi-template assay (to be discussed in more detail in Section 5.3).

Multi-template PCR is intensively employed in studies of molecular evolution and phylogeny [3–6], forensic investigations [7], medical research and diagnostics [8–10], and environmental research [11–13]. While no more than two different homologous templates are usually amplified together in forensic or medical applications, environmental studies perform PCR on high-order mixtures with up to hundreds of different types of templates, each present in a different copy number. Usually environmental and not laboratory-generated samples are used for multi-template assays; thereby adding further complicating chemical factors to the reaction. These chemical factors can often be co-purified with the extracted nucleic acids thereby exacerbating other PCR-generated problems [1]. If not specifically mentioned, this review uses the terms multi- or mixed template PCR for those assays where samples with more than two homologous templates are employed.

2. Artifacts and bias in multi-template PCR

The high complexity of the samples predisposes multi-template PCR for artifacts and biases. Whereas some PCR-induced errors are common in all types of PCR assays, other artifacts such as heteroduplexes and chimeras are the exclusive attributes of multi-template reactions (Fig. 2).

2.1. Artifacts exclusive for mixed template reactions: heteroduplexes and chimeras

Genomic DNA exists in the form of homoduplexes with all corresponding base pairs being complementary. Double-stranded DNA molecules form a heteroduplex once they contain any

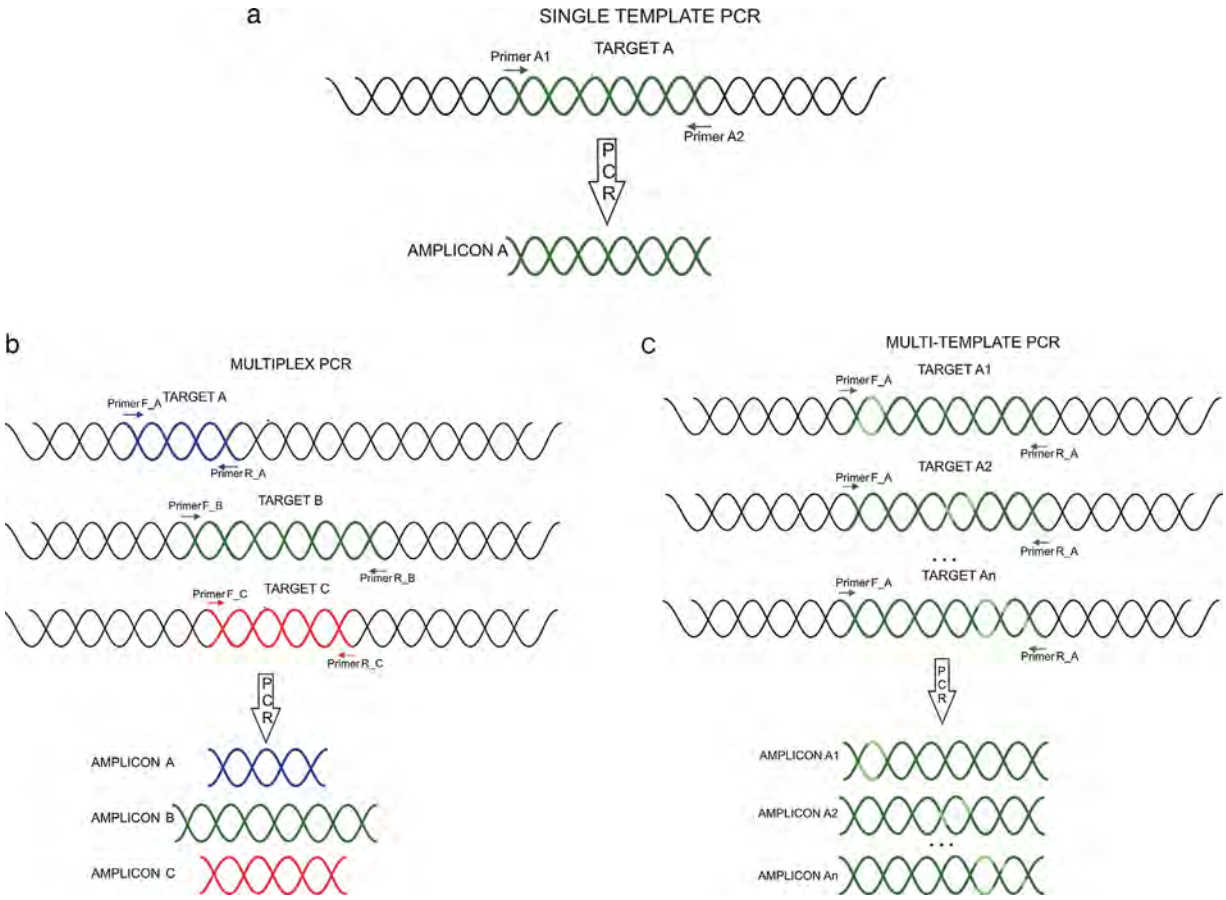

Fig. 1. Types of polymerase chain reactions (explanations are provided in the text).

non-complementary base pair. In a PCR seeded with a single template the only source for heteroduplex formation are amplification errors produced by polymerases. In contrast, the phenomenon of heteroduplex formation in the presence of more than one template is well known and has even been exploited for evaluating the fidelity of enzymes [14]. Since the products of single-template PCRs are usually analyzed together, random and rare insertions of mismatch base pairs do not cause methodological problems in routine PCRs. However, in a multi-template assay the original sample is a mixture of homologous DNA sequences. Therefore, the DNA strands amplified from different homologous templates in the course of a PCR could cross-hybridize under annealing conditions and form heteroduplex DNA molecules [15,16]. Such cross-hybridized products formed during the last amplification cycle would not be denatured and could form a significant proportion of the final product: the potential number of heteroduplexes arising from n distinct allelic sequences is $n(n-1)$ [17]. Heteroduplex amplicons form separate clusters during fractionation, which are distinct from the fractions formed by the parental DNA molecules. These separate fractions create false additional signals visible in the detection step and their presence could lead to an overestimation of the sample complexity. In a mixed template PCR with two homologous sequences, the heteroduplex amplicons are easy to identify. For a quantitative analysis, the results can be corrected by quantifying the heteroduplex [18]. However, this problem becomes increasingly severe when more complex templates are used. Numerous, and unfortunately, often contradicting strategies were proposed to decrease the presence of heteroduplexes in the final PCR products [16,19–21]. One of the most efficient ways to reduce the number of heteroduplex DNA molecules is a post-PCR treatment of amplicons with exonucleases that specifically cut single-stranded DNA [16,22]. However, such practice can eliminate products originating from rare templates because rare amplicons only have a small chance to form homoduplex molecules but instead usually end up in heteroduplex molecules with more abundant amplicons.

To circumvent the problem of heteroduplex formation, Uejima and co-workers [23] proposed a hot-stop PCR approach. Rather than eliminate heteroduplexes, their method ignores them by adding an end-labeled oligonucleotide just before the last PCR step. Heteroduplexes escape detection because they do not contain the labeled primer.

Chimeric amplicons are formed when a single DNA strand is amplified from more than one template. Chimeras never occur in a single-template reaction. However, during simultaneous amplification of homologous sequences the generation of chimeric DNA molecules is a common artifact resulting in the most severe outcomes. Several mechanisms can lead to the appearance of chimeric DNA molecules. For instance, incomplete amplification can be the cause of chimeras. When many homologous sequences are amplified, prematurely terminated partial-length DNA molecules have a low probability to anneal with molecules that exactly complement them instead the probability of recombination events that could result in chimeric molecules is higher [24–26]. In addition, template switching to pre-existing templates or to the complimentary nascent strand has been shown to create recombinant sequences even after only a single round of PCR. For details see [27]. When an enzyme with proofreading activity is used for amplification it may shorten primers and degrade amplicons, creating random DNA fragments, which might became incorporated into chimeric sequences during the subsequent PCR cycles [28]. The phenomenon of DNA recombination during PCR has been known for a long time and has even been exploited by molecular biologists to generate

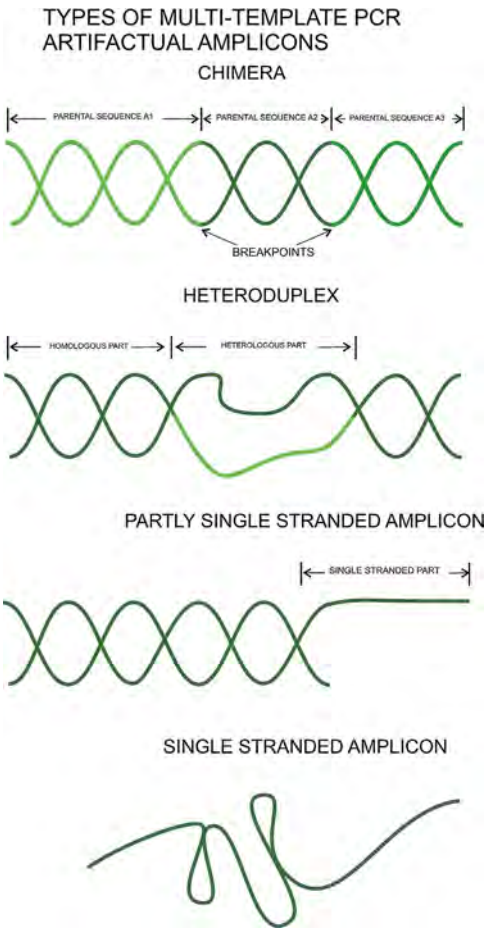

Fig. 2. Multi-template polymerase chain reaction artifacts (detail explanations are provided in the text).

new DNA sequences in vitro [25,29]. Nevertheless, the appearance of chimeric DNA sequences in PCR where several homologous templates are amplified in the same reaction still remains a surprising discovery for researchers from other scientific fields as they continue to verify this old finding by providing new evidence, although now employing modern methods of chimera detection.

Larh and Katz [30] demonstrated that the frequency with which a specific DNA sequence from the template pool was involved in recombination events corresponded to the frequency that this sequence was recovered in total in the entire PCR; the more frequently available sequences were more likely to become a part of chimeras. In their experiments, PCRs were seeded with a template consisting of eight partly amplified homologous sequences of genes encoding actin and generated varying chimeras that ranged from having a single breakpoint with two clearly identifiable parental sequences to having eight breakpoints and six parental sequences alternating in participation. The majority of chimeras (65%) had more than one breakpoint and more than two parental sequences for each chimera.

Even in the simplest mix of having two distinct genomes and relatively short target sequences (240 bp), artificial chimeras were formed at a frequency ranging from 1.5% to 7.4% depending on the total amount of input DNA. In these types of reaction mixes between 1 and 3 breakpoints were found in chimeric molecules [31]. Between 15% and 60% chimeric sequences (target sequence 200b) were detected in the denoised 454 pyrosequencing data set originated from mock communities of different nematode species [32].

The negative effect of chimeras is manifold. Chimeric sequences artificially increased the apparent diversity of an investigated community by creating false-positive signals. Furthermore, chimeric amplicons are created randomly and that is why each type of chimeric sequence behaves as a rare template. Since rare templates have low probability to hybridize with the homologous sequence they tend to form heteroduplexes. Thus, chimeras might increase false-positive signals not only directly, but also indirectly by increasing the numbers of heteroduplexes. So, chimeric sequences might significantly inflate the apparent diversity of a community being investigated or the pool of alleles in a population. What is even more precarious, when these data sets are deposited in public databases, new sequence variants can pollute these repositories with references to non-existing microorganisms or alleles. Sampling ten published and two unpublished studies of 16S rDNA databases Hudenholtz and Huber [33] revealed 21 inter-phylum and 18 intra-phylum chimeras and numerous smaller local topological rearrangements of sequences in the partial trees. After screening 1399 sequences from 19 phyla deposited at the Ribosomal Database Project, Ashelford and co-workers [34] found 5% of the records to be corrupted; most of these (78.6%) were chimeras or other, similarly insidious errors. Many chimeras (43.1%) were formed from parental sequences belonging to different phyla. While most contained two fragments, 13.7% were composed of at least three fragments, often from three different sources. The same research group, when analyzing most of the large libraries of cloned bacterial 16S rRNA gene sequences submitted to the public repository during 2005, found that the average anomaly content per clone library was 9%, that is 4% higher than had previously been estimated for the public repository overall; 90.8% of the anomalies had characteristic chimeric patterns [35]. The authors stressed that anomalous sequences continue to be added to the public databases with an increasing rate and urged quick action to be taken. The problematic presence of chimeric sequences in the public databases is not only restricted to 16S rDNA libraries as chimeras from other experiments have also been found in GenBank [36,37].

Since chimeras can contain more than one breakpoint it creates a problem for chimera-detecting software, which bases their search criteria on finding one breakpoint per sequence. For example, Bellerophon software was able to detect only an average of $65 \pm 18\%$ of the chimeras in a data set experiment performed on an artificial mix template with eight sequence variants with a false-positive rate of $40 \pm 31\%$ [30]. Authors warned that once a chimeric sequence is added to the public databases it becomes invisible to CHIMERA_CHECK and other software that use analogous algorithms, because it is simply compared against itself in the analysis.

2.2. Artifacts that occur in all PCR but especially affect mixed template assays: product-template ratio bias, single- and partly single-stranded amplicons

PCR efficiency is defined as the fraction of double-stranded DNA molecules that is copied at a given cycle [38]. Variability in the efficiency of single-template reactions is a common phenomenon and although low amplification efficiency might pose a problem in some PCR applications, it does not cause a bias in single template PCRs. In multi-template samples the situation is different as targets vary both in the primary DNA structures and in the frequencies with which they occur in a mixture. Templates which have different starting concentrations will have an unequal probability of being amplified [3,39]. This is coupled to variations in the amplification efficiencies of each individual template due to the slight differences in the DNA primary structure. The phenomenon of different amplification efficiency among homologous sequences in a mixed template has been noticed by many research groups

[3,30,40–44]. This problem is complicated even further by the fact that different targets can be given preferences under only slightly altered PCR conditions [45]. In practice this means that minor differences in composition or concentration of inhibitors as well as slight variations in other PCR components can drastically change the amplification efficiency of the same template. Thus, dissimilar amplification efficiencies of templates within a mixed sample may lead to a biased (and even false) outcome of the original sample composition; for a review see [46].

Single-stranded and partially single-stranded amplicons are not the exclusive attributes of mixed template PCR as they can be generated in any PCR assay. Nevertheless, single stranded and partially single-stranded amplicons formed during PCR amplification are a potential source of bias in multi-template PCR assays [47], because these artifactual products give false-positive signals, which do not correspond to any template in the original sample. In denaturing gradient gel electrophoresis (DGGE) or denaturing high-performance liquid chromatography (DHPLC) analysis, single-stranded and partially single-stranded amplicons form smeary, poorly stainable extra bands/picks due to altered electrophoretic mobility and stainability compared to the relevant fully synthesized amplicons. In case of the T-RFLP assay, single-stranded DNA molecules escape analysis and amplicons which are single-stranded at their terminal restriction site favor the formation of restriction fragments longer than the true, expected terminal restriction fragments because type II restriction enzymes cut double-stranded DNA only [22]. Presence of single-stranded termini also impairs cloning and sequencing of these types of artifactual PCR products.

3. Factors that can diminish artifacts formation in multi-template assays

As discussed earlier, multi-template PCR is predisposed to artifacts and biases due to the complex nature of its template. Factors, like primers, polymerases alongside their supplemental buffers, template status, PCR additives, and PCR conditions do not cause the artifacts. These factors, if chosen correctly, can only alleviate negative tendency. Some of these factors are more potent, some are less; and their effects are specific for each individual assay and even for each template in a particular sample. Below we are going to discuss the potency of these factors to mitigate the negative attributes of multi-template PCR.

3.1. Primers

In a single-template and a multiplex PCR the failure of the primers can be recognized by (i) the absence of the target sequence; (ii) presence of non-specific product(s); (iii) presence of ssDNA due to the non-efficient amplification of one of the primers. In addition, it is common practice to include both positive and negative controls to monitor PCR conditions under which the primer efficiency can be estimated. In assays with mixed templates, it is difficult to implement the same principles due to the presence of multiple homologous targets, which have unknown identities in most cases. Therefore, the failure will not be noticed if some targets within a mix escape amplification.

The problem with primers that tend to amplify only a particular part of the target in a mixed sample had been noticed before [44,48,49]. However, this effect was thought to be due to the selectivity of the primers on their own and not dependent on the particular polymerase used, the PCR conditions and template status. Common recommendations for overcoming this bias were to optimize primers, to perform several assays using different primer sets or even the very questionable practice of using a mix of primers simultaneously. Clearly, the problem is obviously much more complicated since primer specificity should only be seen in a context that takes the interaction of the polymerase with its substrate (primer-template duplex and dNTP) under the particular conditions of the cyclic enzymatic reaction into account.

The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors, including: (i) the kinetics of association and dissociation of the primer-template duplex at the annealing and extension temperature, (ii) the presence of mismatches between primer and template, (iii) the effect on duplex stability of mismatched bases and their location, and (iv) the efficiency with which a DNA polymerase can recognize and extend a mismatched duplex [50]. Primer binding energy differs among different templates. If the mismatch occurs elsewhere in the primer binding site, then the annealing temperature of the primer would be affected, resulting in a reduced amplification efficiency. The reduction in efficiency is individually different for each template under particular conditions. If the mismatch occurred at the 3′termini of a primer, the outcomes become even more complicated. Polymerases are known to have different abilities to extend mismatched primer-template duplexes. In editing DNA polymerases, the arrest of strand synthesis following the incorporation of a mismatched base allows the 3′ to 5′ exonuclease activity to remove the incorrect nucleotide. This makes proofreading polymerases insensitive to 3′ mismatches of the primer-template duplex. Polymerases which lack 3′ to 5′ exonuclease activity cannot correct mismatches at the 3′terminus and as a result strongly discriminate correct from incorrect templates for PCR. Extensive studies have shown that the presence of a newly incorporated mismatch reduced the efficiency of subsequent nucleotide insertion and extension by a hundred- to a million-fold [50–53] and the magnitude of this effect depends on the properties of both the mismatch and the polymerase. Mismatch-induced stalling is not limited to the point of incorporation. Some polymerases retain a “short-memory” of replication errors, responding to mismatches of up to four base pairs distant from the primer terminus [53].

Difference in primer binding efficiency and effects of primer-template mismatches may play a significant role during the first rounds of PCR but have less of an effect in later cycles. Once a mismatched primer has been extended and its extension product became a perfectly matched template, it will be faithfully amplified during the subsequent cycles.

Another important issue is the considerable discrepancy between in silico analysis of primer specificity and efficacy and the results of empirical testing [54]. In an exemplary study, it could be shown that specific care must be taken when interpreting new or previously published results obtained with PCR primers that have not been fully validated. Since it is impossible to design primers that perfectly match all sequences in a mixed sample, the amplification of some templates will be always affected. These differences would have the strongest effect during the first rounds of PCR, and the evolving bias would be amplified further during the following cycles. Thus, the amplicon ratio in a final PCR product would fail to correctly reflect the initial composition of templates in the sample. An additional complication occurs if amplification by forward and reverse primers is affected by a different magnitude. Under these conditions, accumulation of single-stranded amplicons would occur which in turn could lead to an increasing number of both heteroduplexes and single-stranded DNA in the final product.

Primers with modified properties can be tested for their potency to improve the performance of multi-template PCR. For example, primers containing the temperature-sensitive 4-oxo-tetra-decyl (OXT) phosphotriester modification demonstrated superior performance compared to unmodified PCR primers in their ability to amplify four targets from human total RNA both separately and

simultaneously in a one-step reverse-transcription PCR [55]. Still, the probability to find an optimal set of primers able to target all templates in a mixed sample is very low. In addition, other components of PCR can affect primer selectivity.

3.2. Polymerases

The real PCR machinery is the polymerase despite the fact that a PCR device is the first thing that catches the eyes due to its visibility. Therefore, a profound understanding of the qualities of every individual polymerase is crucial to PCR users. The environment encountered by individual polymerases in multiplex and multi-template PCR differs considerably when compared to the single-template assay. In a single-template reaction a polymerase only encounters one type of target sequence and one possible combination of a primer-template duplex. In contrast, a PCR polymerase has more opportunities to manifest preferences among slightly different primer-template combinations in multiplex and mixed templates [56] and different characteristics of the particular enzyme can lead to a different spectrum of PCR artifacts.

Amplification of mismatched 3′ termini discussed above is only a special case for a DNA polymerase characteristic known as fidelity – the frequency of polymerase-induced errors. Polymerase fidelity is influenced by multiple factors, including the tendency of an enzyme to insert an incorrect nucleotide [14,57] the presence of a proofreading 3′ to 5′ exonuclease activity which can remove mismatches [57–59], and the ease with which particular mismatches can be extended [53,57]. The type and rate of error depend on the specific DNA polymerase [57–61] the template properties [26,61] and PCR conditions [14,57–59]. The varieties of changes in DNA sequences that can occur during amplification vary from single base substitution to deletions and insertions [57,62–64]. Despite the very high fidelity that are claimed for some commercial DNA polymerases, the actual error statistics in final PCR products often greatly exceeds the predicted level [20,28,65–68]. Each error, once it has occurred, is amplified along with the original sequences thereby increasing the fraction of polymerase-induced mutant sequences.

In a single template reaction, the PCR product, as a rule, is analyzed in an aggregate. Thus, low-level, random mutagenesis during the amplification should not have a significant impact because the great majority of DNA molecules have an unaltered nucleotide at any given position. However, when PCR is used to disclose the molecular structure of a complex sample and when amplicons are analyzed by fractions, PCR-induced mutagenesis may create a serious background problem [14,69]. In early observations, Clayton and colleagues [70] reported unexpectedly high levels of intraspecific variation (within and between strains) of bacterial SSU rRNA sequences deposited in GenBank. Among possible causes for the variability, the authors mentioned PCR-based sequencing and other laboratory errors. Whatever the underlying cause, the authors believed that undetected 16S rRNA sequence variability can render any phylogenetic, ecological, or clinical conclusions unreliable. Pompanon and colleagues [10], analyzing the causes and consequences of genotyping errors, concluded that all studies reported a non-negligible error rate from 0.2% to more than 15% when errors were checked. Taking in account that error rates as low as 0.5–1.0% have the potential to obscure medically important findings, the authors saw the need to confront this issue and proposed a strategy for estimating error rates.

In addition to the potential ability of polymerases to incorporate wrong nucleotides, another intrinsic property of these enzymes can be a cause of PCR artifacts in a mixed template – their relatively low processivity. The average number of nucleotides added by a DNA polymerase in a single binding event is described as processivity and depends on the components of the reaction medium and on the DNA template sequence [61,68]. Some DNA polymerases dissociate from the DNA template after the attachment of an average of about 40 nucleotides, whereas others have an even lower processivity of 4–30 nucleotides (see Supplementary information for [68]). Since the number of nucleotides that the respective polymerase is able to incorporate during one binding event is much less than the length of amplified template, uncompleted DNA sequences can dissociate from a primary template and serve as primer in the next cycle. In single-template PCR this does not cause a serious problem (except diminishing the reaction efficiency) since only one type of target sequence is present in the reaction tube and the incomplete sequence only has the possibility to hybridize with the identical template during the next cycle. In a multiplex PCR, target sequences are heterologous and do not hybridize with each other. However, in a multi-template PCR uncompleted DNA sequence can bind to any available complimentary sequence and its subsequent amplification can create a chimeric DNA molecule. Since longer DNA sequences dissociate from complementary strands at a higher temperature than shorter sequences [42], the efficiency of annealing of partly amplified DNA molecules to a template can be higher than with of primers. Thus, incomplete amplicons are very efficient in competing for a template.

Polymerases commonly used for PCR might possess some extra activities, which are not essential for the routine assays but ought to be considered in multi-template reactions. One of these additional enzymatic capacities is the reverse transcriptase activity described for some polymerases [71–73]. The presence of high amount of rRNA in a sample (if treatment with RNAse was omitted) coupled with the reverse transcriptase activity of a polymerase might significantly affect the results especially given that the most popular target sequence used in multi-template assays is the ribosomal DNA. Mn^{2+} cations, necessary for this reaction, might be present in the sample among the co-extracted substances. Another additional enzymatic activity observed for some polymerases is the structure-specific 5′ nuclease activity that cleaves single-stranded DNA or RNA at the bifurcated end of a base-paired duplex [74–76]. The ribosomal DNA sequence extensively used as a marker in multi-template assays has the intrinsic ability to form loop-stem structures which can serve as substrate for a polymerase switched into 5′ nuclease mode. The outcome of structure-specific cleavage of amplicons by a polymerase would be an increased amount of incomplete DNA sequences. These incomplete sequences, if amplified during subsequent cycles, might become chimeras. Otherwise they can also form partly single-stranded products.

Nowadays, many new polymerases have become available on the market. Of particular interest are new thermostable DNA polymerases that can potentially bring significant changes into the current technologies as enzymes with enhanced processivity and strand displacement ability. Significantly, these enzymes can synthesize DNA fragments of more than 70 kb without dissociating from the template [68]. Many of these new polymerases have attractive properties to facilitate experimental procedures, as they can, for example, provide a simplified protocol for the direct amplification from whole blood and crude soil samples [77]. New polymerases that are able to utilize shorter oligomers open the possibility of decreasing the length of the primers and thereby broadening the scope of sequences detectable by multi-template PCR [78]. If these enzymes can also demonstrate low artifact induction into mixed templates, they could become a useful tool in this type of PCR assay.

3.3. PCR facilitators

In multi-template samples the goal is to amplify all target sequences in the proportion that directly correlates to the proportions in the original mix. To overcome some of the problems linked to amplification of recalcitrant templates, different

PCR-enhancing compounds can be used. Usually the difficulties with amplification are connected to GC-rich sequences [79,80]. However, AT-rich templates also can also be challenging to amplify [81]. The influence of PCR-enhancers on amplification of recalcitrant sequences was thoroughly studied in single-template reactions, and there were very few, systemic attempts to evaluate the influence of these compounds in PCR with complex templates [45,82]. Many co-solvents were shown to depress the melting temperature of the DNA molecule; however, at the same time they decrease enzymatic activity and thermostability of the Taq polymerase [79]. Even in a single template PCR the task to find the optimal concentration for a suitable enhancing compound is challenging [83]. Given that targets in a mixed template have very diverse properties, it becomes even more difficult to optimize the multi-template reaction in such way that all templates get an equal chance to be amplified. On one hand, low concentrations of PCR enhancers might prove to be ineffective. On the other hand, at high concentration of PCR enhancers the decline in polymerase activity and thermodegradation decrease product yield by increasing the minimum extension time necessary to complete the replication of a template. In addition, the same enhancing compounds can have an ambiguous influence on the amplification of AT- and GC-rich templates. In summary, the presence of multiple targets with different properties leaves a very narrow window for optimizing the concentration of PCR additives.

4. Factors that can increase artifacts formation in multi-template assay

4.1. Template structure and conditions

Template quality is one of the most important issues among many factors that determine sensitivity, accuracy and reliability of a PCR assay. Template quality is a combination of many parameters, including complexity of the target and neighboring sequences, the level of DNA damage and the presence of inhibitors. Benita and co-workers [84] in an extensive study of factors influencing PCR success demonstrated that the template rather than the primer was most often the cause of PCR failure. These conclusions cast more doubts on our ability to provide PCR conditions equally favorable for amplification of all templates in complex samples.

Amplification efficiency can vary across a genome [84,85]. Genomic regions resistant to amplification by PCR correlate with regions having a high GC content as these do not denature efficiently under routinely used conditions [64,86]. Veal and co-workers [85] proposed that regions of extreme GC content remain duplexed during standard DNA denaturation procedures, and in so doing also prevented their flanking regions from separating. As such, these neighboring strands are able to quickly reanneal as soon as non-denaturing conditions are re-established. They concluded that DNA samples of varying quality carry different numbers of nicks and breaks and so are differentially affected by this amplification suppression mechanism. The same assumption can be applied to the different templates within the mixed sample, so templates with DNA strand(s) breaks might be given advantages during first amplification cycles.

Since resistance to denaturation can selectively prevent some templates from being amplified it is tempting to solve this problem by more stringent denaturing conditions. However, when double-stranded DNA molecules separate into single-stranded DNA under denaturing conditions, they become more susceptible to hydrolytic attack, oxidation and depurination and thus, increase the potential for polymerase-induced errors [26,46,57]. A second common type of DNA damage is spontaneous base release. The resulting abasic sites in the DNA can inhibit synthesis by DNA polymerases [26,87]. In some cases, DNA polymerases are capable to replicate through abasic lesions, however, as such sites are noncoding, such bypass replication is error-prone. The errors caused by template damage happen at random and that is why they are difficult to monitor. It is therefore reasonable to keep the conditions of the denaturing step as relaxed as possible. Thus, a dilemma arises: on one hand, harsh denaturing conditions increase the probability for GC-rich templates to be amplified; on the other hand, the same conditions increase the probability of PCR-induced artifacts.

Applications of PCR assays in microbial ecology, probably from the very beginning, suffer from the consequences of having to choose non-optimal target sequences. In culture-independent studies, DNA has become the dominant signature molecule and the predominant targets for the assessment of microbial diversity are DNA sequences encoding 16S rRNA or 18S rRNA. The comparative analysis of small subunit rRNA sequences was introduced into bacterial systematics by Fox and colleagues [88]. 16S rRNA sequences almost perfectly meet the basic requirements for a general phylogenetic marker, i.e., ubiquitous distribution, functional constancy, low frequency of lateral gene transfer and recombination combined with a comprehensive database of rDNA sequences [2]. However, estimation of diversity and identification based on 16S rRNA sequences has often been criticized for overestimating the relevance of a single genomic marker with respect to the evolutionary history of the whole organism and for potentially misleading conclusions due to the intragenomic heterogeneity between multiple 16S rRNA operons [2,20,70,89,90]. In addition to this problem, the single-stranded ribosomal DNA sequence has an intrinsic tendency to form stem-loop structures. Such secondary structures formed during the extension step may cause the polymerase to stall and fall off [90,91] or can serve as substrate for the 5′ nuclease activity that some polymerases possess. Non-functional 16S rDNA sequences in general, have a diminished ability to form stem-loop structures. Thus, it would be useful to test whether such nonfunctional sequences would be preferentially amplified in a mix with functional 16S rDNA sequences.

4.2. Inhibitors

PCR is an enzymatic reaction and therefore sensitive to inhibitors. Inhibiting compounds are very diverse and can originate from the sample itself or be introduced during sample processing (for reviews see [92–94]). Inhibitors can interfere practically with every step of the PCR analysis. Their effects are very diverse, usually concentration-dependent [92–95], and can vary during the course of the PCR [96]. The presence of inhibitors is already problematic for a single-template PCR but becomes an even more severe problem for multi-template assays and particularly for environmental samples [1,45,64].

Inhibiting compounds can intensify the negative tendency of erroneous amplifications to which multi-template PCR is already predisposed. First, inhibitors might ambivalently affect the amplification of different targets in the mixed sample. The effect of differential susceptibility has been observed in single template assays [97–100]. Such differential susceptibility of templates to the inhibitors can considerably aggravate template-to-product bias in multi-template PCR. Secondly, some inhibitors were shown to block the DNA template [100–102]. Template blocking may increase the amount of partially amplified products [103], which can then serve as primers in the next cycles and thereby increasing the number of chimeric amplicons in the final product. Thirdly, interfering with the availability or activity of essential reaction components [92,93,100] or by direct modification of the DNA molecule, inhibitors can increase the rate of polymerase-induced errors [104].

Apart from increasing the tendency for erroneous amplification in a multi-template PCR, inhibitors can alter the sensitivity of the assay. Wernars and co-workers [105] found that the sensitivity of detection was at least 10-fold less than the theoretical minimum, varying between 10^3 and $>10^8$ CFU 0.5 g^{-1} due to the presence of inhibitors in different brands of soft cheeses. Sensitivity was reduced 1000-fold in milk powder, where 10^5 CFU ml^{-1} was required for detection of *Staphylococcus aureus* despite the ability to detect fewer than 10 cells in poor culture [106]. The sensitivity of a real-time PCR assay to quantify *S. aureus* cells in artificially contaminated cheeses depended on the cheese matrix [107]. Although these results were obtained using simplex PCR techniques, these examples indicate that inhibitors can reduce the assay sensitivity in unpredictable ways. This fact introduces additional obstacles for a correct comparison between samples. Considering the severity of the problem, it is very surprising that, to the best of our knowledge, no systematic studies of PCR inhibition in a mixed template have been reported so far.

Many different strategies were developed to reduce the amount of inhibitors in samples (for reviews see [92–95]). Again, all of these approaches were designed for single-template PCRs. For example, the first approach to minimize the presence of inhibitors during the DNA extraction would entail increasing the intensity and number of cleaning steps. In samples containing only one type of template, the aim of the DNA extraction procedure is usually limited to obtaining PCR compatible samples. In multi-template assays, an additional task is to preserve the initial ratio of templates in the sample. This task demands special protocols for DNA extraction. On one hand, these protocols must be as gentle as possible to maintain the template ratio and integrity within a mixture. On the other hand, they must provide the highest possible level of purification to enable stable performance of the PCRs on concentrated samples.

Dilution of the original sample is yet another commonly used approach to escape PCR inhibition in single-template assays. However, in mixed samples the dilution might cause a loss of low- and medium-concentrated target sequences [108]. The dilution of the template also markedly increases the risk of contamination, because contaminating molecules have a higher probability of being amplified when the number of template molecules is low. Thus, the elimination of inhibitors or reducing their effect on multi-template PCR is a very complex task.

Despite efforts to eliminate inhibitors, they still can persist in samples and interfere with the course of the PCR. There are no tools for the direct identification and quantification of inhibitory compounds in the samples. In single-template PCR, an internal amplification control is carried out to determine the inhibitory effect of all the substances present in the sample [99,109,110]. Huggett and co-authors [99] stated that if two PCRs are to be compared, it is important that both reactions are affected by potential inhibitors to the same extent. They called it inhibition compatibility and called for devoting proper attention to its measurement. However, the application of internal controls in multi-template assays is impeded by the complexity of the samples. When the samples themselves represent mixes of homologous targets, the products of their amplification will fail to be distinguished from the internal control. Moreover, the signal obtained cannot be corrected for heteroduplexes, which are inevitably formed between the control and target sequences. To estimate inhibition compatibility of samples with mixed templates, we recently proposed a new approach that is a compromise between external and internal amplification controls. We called this approach a semi-internal control [111] and the idea was to amplify the mixed template with known identity together with an aliquot of a DNAse-treated sample. In a semi-internal control, the native sample DNA is destroyed and thus only DNA from an added model mix can serve as the template. This makes the amplification profile predictable. On the other hand the sample aliquot provides a cocktail of potentially PCR-inhibiting compounds. If inhibitors present in two different test samples differently influence the amplification of the model mix, then these samples are incompatible. If the inhibitors present do not alter amplification of the model mix or influence them in a similar pattern, e.g. ceasing amplification of GC-rich templates, then these samples can be compared. However, in the latter case the researcher should be aware that amplification is biased and therefore any differences between test samples which occur in the omitted template fractions cannot be accounted for.

If multi-template PCR assays are to be continued in the future, more attention should be paid to all aspects of the problems linked to inhibitors. The most urgent challenges that need to be addressed are (i) the development of DNA extraction protocols which effectively eliminate inhibiting compounds from the mixed samples and at the same time preserve template ratio; (ii) the screening of PCR-enhancing compounds for their potency to mitigate inhibitory effects in complex templates without altering amplification efficiencies of templates with different GC content; (iii) developing procedures for estimation of inhibition compatibility of the mixed samples.

4.3. Erroneous methodology

While classic PCR approaches operate with concepts of accuracy, precision, sensitivity, limits of detection and other precise notions. In contrast, multi-template PCR uses a variety of superstitious omens to judge the PCR aptness. A broadly used indication of “reliable” multi-template PCR is when there is little variability between replicates; e.g. [112–115]. Yet, biased profiles can be highly reproducible depending on the polymerase used for the reaction [111]. Although unsupported by experimental data, criteria such as the maximum yield of a PCR product [116] or the highest variety of amplicon types [117,118] are often used for multi-template PCR optimization. Representation of a sequence by more than one clone, preferably obtained in independent PCRs, is used as a threshold to assign new alleles of the gene coding for the major histocompatibility complex (MHC) in vertebrates [119]. However, Lenz and Becker [5] showed that when these criteria were applied to the results of standard PCR protocols, it more than doubled the initial pool of real alleles. The use of the abovementioned criteria for multi-template PCR optimization has neither been supported by mathematical models nor by empirical studies.

The most severe fault in current multi-template PCR assays is the lack of appropriate amplification controls. Two kinds of controls, external and internal, are usually employed to monitor single-template PCR. An external control is a template amplified in parallel with test samples to verify the integrity of one or more reagent(s) in the cocktail; an internal control is a second target molecule that can be jointly amplified but distinguished from other products in the same tube [120]. However, multi-template assays possess distinct characteristics and therefore demand adequately designed amplification controls. A single template cannot serve as a control in such an assay because such control is unable to monitor for characteristic artifacts and biases. Instead, a mixed sample with known identity can be used as an external control in this case. The same control amplified with an aliquot of the DNAse-treated test sample can be a rational compromise for the internal control [111].

Model mixtures used for amplification control can be of different complexity. The complexity of the control sample must reflect the expected diversity of the sample and the detection capacity of the post-PCR analysis. The performance of amplification controls must be clearly described in publications to evaluate compatibility of samples and the level of PCR-induced bias of the particular assay. Unless appropriate methodological sections in research papers

have been provided, any data generated with a control-free protocol will stay questionable.

Another error-inducing practice is a relaxed procedure of assay installation. Most of the samples will already contain significant amounts of single-stranded DNA even before they have been heated deliberately in the thermocycler to induce strand separation. Such partial DNA denaturation can occur during DNA extraction ([65] and reference within) and might be target-specific. For instance, if DNA is extracted from a population consisting of many different cell types, DNA from easily digested cells undergo more harsh treatment compared to recalcitrant cells and these DNA molecules might then be mainly present in single-stranded or partly denatured form. The same process can also occur with less stable AT-rich sequences. The single-stranded targets and primers can anneal at room temperature during installation of the reaction and since polymerases are sufficiently active at room temperature, they can subsequently extend the primer-DNA duplex. Under low temperature the reaction will be more prone to errors due to an increase of non-specific priming [121] combined with high initial concentration of dNTP and a polymerase that can extend from a mispaired termini more easily [57]. During this single amplification event under low-stringent conditions (i) some templates can get a head start and (ii) an erroneous template can be created.

It is almost impossible to give ready-to-use protocols or recommendations on improving the performance of multi-template PCR. First, due to the mixed nature of the template any modifications in reaction solution (e.g. Mg^{2+}/dNTP and primer concentrations, type and concentration of additives) or in cycling conditions have a different influence on different template fractions. Secondly, polymerases and as a consequence their supplemental buffers used in different labs have diverse properties; what is good for one enzyme is bad for another. Regretfully, the role of polymerases is very often overlooked. Finally, manufactures of PCR supplies made much progress in optimizing PCR buffer composition. As a result, such buffers work well under a wide range of temperatures and also became less sensitive to suboptimal concentrations of Mg^{2+}/dNTP. In addition, the exact composition of commercially available buffers is often unknown. Thus (i) the effects of certain changes in PCR conditions are poorly predictable and (ii) the optimal conditions established for one particular polymerase/buffer conjunction might manifest dissimilar effect when another combination is tried.

A reliable indicator of the optimal performance of multi-template PCR could be the behavior of the appropriate control under defined conditions.

4.4. Contamination

The downside of PCR's high sensitivity is its susceptibility to contamination. Again, the multi-template assay has some features that ought to be taken into account when planning countermeasures. One of such features is (as a rule) the large number of targets in the samples. Together with the commonly encountered adherence to lax protocols during the preparation of the environmental samples these two factors can lead to a high rate of cross-contamination. Plasmid clones carrying the templates from previously analyzed samples may be present in large numbers in the laboratory environment and might pose another problem. Nevertheless, the most important, multi-template assay (as a rule) applies nested or semi-nested approaches plus post-amplification analysis and might consist of several steps. Thus, the close-tube format is not applicable for the multi-template assay. These unsafe, open-tube procedures are further burdened by the need for the same sets of primers used for the assay. Repeated amplification of the same target sequences with the same set of primers leads to a massive accumulation of the amplification product in the laboratory environment. The only bonus that multi-template PCR has, compared with other types of PCR assays is its reduced (to a certain point) sensitivity to contamination. In order to preserve native patterns, samples for the multi-template PCR ought to be maintained as concentrated as possible. All in all, the multi-template assay although less susceptible to contamination, produces quite a bit of contaminant, making the risk of cross-contamination almost inescapable.

Several pre- and post-amplification techniques have been developed for the simplex and multiplex PCR to deal with contamination. Some of these techniques are applicable for the multi-template assay while others are not. One set of measures aims to prevent ingression of non-native templates into the PCR tubes; e.g. a strict separation of the areas of the laboratories where post-PCR manipulations are performed, regular cleaning of the work surfaces in the lab, careful disposal of PCR products and used plastic ware. These are equally applicable for every PCR-based assay, including multi-template one. Another group of measures aim to neutralize previously generated amplicons by either converting those amplicons into ineligible targets for further amplification (e.g. ultra-violet light irradiation, hydroxylamine or isopsoralen treatment) or by destroying them prior the next amplification step (e.g. enzymatic inactivation with uracil-N-glycosylase). These methods might be not appropriate for the multi-template PCR because they interfere with a nested protocol or with the downstream analysis. Due to the high risk of contamination, the multi-template assay requires a mandatory application of negative control(s). The simplest example of a negative control is the use of dH_2O as a PCR template. Negative result obtained in no-template controls indicates the absence of contamination or spurious amplification arising from the basic PCR "master mix." A more informative negative control would be a true blank sample, processed through the extraction procedure, in parallel with the test samples. An expected negative result here would confirm that contamination or spurious amplification is not arising from something in the sample matrix or generated during the extraction process. This latter option would be considered as a "negative process control." Optimally, any PCR-based assay should employ both a negative amplification control and a negative process control; because such practice allows almost immediate identification of extraction step contaminations.

5. Multi-template PCR measurements

5.1. Multi-template PCR product detection and identification

In multi-template assays the modes of product detection differ in many ways from those of single template assays. Firstly, in single template assays the product is visualized *en masse* while the multi-template assay demands post-PCR product fractionation. Secondly, in single-template PCR, real-time detection is feasible while in the multi-template assay it is not. This is due to the fact that in mixed template assays, product fractionation must anticipate the detection. At present, there are no methods to combine these two processes. The only exception is measuring total microbial load since here product fractionation is not performed. This case will be discussed in more detail in Section 5.2.

Products of multi-template PCR are of almost the same size and cannot be separated by an agarose gel. The exception here is rDNA internal spacer analysis (RISA). This method is frequently used in microbial ecology, and relies on long amplicons that vary both in sequence and in length (for review see [122]). For other applications of multi-template PCR, separation capacities higher than the conventional agarose gels are required. Looking at amplicon fractionation, all detection methods employed for multi-template assays can be divided in three groups. The first group of detection

methods uses minor differences in amplicon structure to distinguish them from each other: DGGE, double gradient DGGE, TGGE, SSCA or SSCP, CDCE, DHPLC, and T-RLFP all belong to that group [123,124]. The second group employs PCR product sequencing, followed either by a cloning step or by direct massive parallel sequencing. Finally the third group includes methods that use a DNA–DNA hybridization approach (dot-blot, microarray). Every detection method has its own level of sensitivity which can be higher, lower or equal to the detection limit of PCR. In addition, some detection methods can add new artifacts to the pool of already existing PCR-generated errors. In this review, only the sensitivity of these detection methods to artifacts and biases induced by multi-template PCR is discussed. The artifacts introduced by the detection method itself are not considered in this discussion. First, pitfalls of the detection methods deserve their own scrupulous review. Secondly, the differences between the errors occurring at different steps of the analysis ought to be distinguished.

All detection techniques without exception are sensitive to PCR-induced template-to-product bias. This means when the template-to-product bias occurs in the course of a PCR, none of the methods can neither correct nor ignore it. The ability to detect other types of PCR-generated artifacts is conditional. For example, techniques using single stranded DNA for analysis are insensitive to heteroduplexes and, undoubtedly, to the presence of single-stranded amplicons. Methods, which employ DNA–DNA hybridization or restriction analysis, can be insensitive to chimeras if the breakpoint does not interfere with the site of recognition. There is a need to emphasis that all types of artifacts and biases described in this review originated in the course of a PCR and therefore none of the described detection methods can be held responsible for these types of errors.

5.2. Qualitative measurements: forensic approach vs chiromancy

In multi-template assays, amplicons are sorted by fractionation and each amplicon type is treated as a direct reference to a particular template in the original sample. In microbial ecology studies, distinct types of amplicons are called operational taxonomic units (OTUs) and are treated as directly corresponding to a particular microorganism in the studied community. Based on this assumption, diversity indices for a particular community are estimated [125]. How justified is this approach? The particular traits and the quality of multi-template PCR should be considered before treating the results of an assay like this as a direct inventory of the original sample. The first intrinsic attribute of the multi-template PCR is the formation of artifactual products. As described above, PCR amplification of such complex samples almost inevitably leads to formation of artifacts and with current protocols, false signals cannot be distinguished from correct ones. The second obstacle is the unknown detection limit of multi-template PCR. This question has not been properly analyzed so far. The limit of detection is the minimum amount of target DNA sequence that can be detected in a sample with a given level of confidence [126]. Imagine a sample where homologous templates A, B, C, D and F are present in 10,000, 1000, 100, 10 and 1 copies respectively. Will templates D and F be amplified in a mix with the more abundant A, B and C homologs? Where does the low detection limit lie? Are the detection limits equal for all targets in the mixture or are they individual (Fig. 3)? How can detection limits be estimated for each particular assay?

As a consequence of the unknown limit of detection in multi-template assays, the fate of rare templates in a mixed sample is unknown. Can they be amplified at all? There is evidence that rare templates when amplified in a mixture have only a low chance to form related double-stranded products. Instead, they most often end up as part of chimeras and heteroduplexes. For example, Wang and Wang [127] showed that when two 16S rDNA fragments with 99.3% similarity were mixed 1:10, nearly all the molecules of the less abundant species recombined because the partial DNA strands of this species only had a 10% chance to reanneal with their own species. What happens in mixtures that are more complex?

Issues concerning the detection limit are aggravated by the well-known fact that homologous templates compete for resources during amplification. This phenomenon is used in competitive PCR for precise quantification of the product [128,129] (competitive PCR will be discussed in more details in Section 5.2). The results of titration experiment for competitive PCR have shown that a 2-log domination in either of the templates eventually leads to cessation of amplification of the less abundant homolog (qPCR manual, Ambion Inc, CA, USA). To the best of our knowledge, no studies have been done that convincingly demonstrate that low-copy templates can be amplified in a mix together with their counterparts present in a several-log higher concentration. Therefore, compelling reasons exist to expect that only dominant templates are amplified in multi-templates assay. This means that only the most abundant members of the sample can be detected by this assay irrespective of the detection method. Although massive parallel sequencing is the most sensitive detection method, this potential is limited by the detection limit of the previous step – the multi-template PCR itself [130].

So, both theoretical models and experimental data argue against the assumptions that in a multi-template assay (i) each original template is amplified, and (ii) each amplicon type corresponds to the particular template in an original sample. On the more positive side is the fact that patterns of the mixed template PCR might be highly reproducible [111]. It might therefore be reasonable to abandon the palmistry approach and stop assigning significance to each separate band or phylotype. Instead, the results of multi-template PCR assays should be perceived and analyzed as a whole – as a pattern characteristic of a particular sample and compared to each other as criminologists compare real fingerprints and footsteps.

5.3. Quantitative measurements: mission currently impossible

There is an urgent need to discuss whether mixed template PCR can provide any quantitative measurements at all. The goal of quantification by PCR is to determine the initial copy number of the template at the start of the reaction. Exponential accumulation of the product is the basis for the high sensitivity of the PCR-based assay. At the same time, exponential accumulation is the main drawback for a quantitative measurement because small differences in amplification efficiencies can lead to high differences in the ratio and in the concentrations of individual amplicons in the final product. As a result the high variability can preclude reliable quantification. For this reason, the ability of PCR to provide a quantitative analysis was initially viewed with skepticism by many researchers (discussed by [131,132]). Two approaches have been developed to help solve the problem of quantification: kinetic methods based on the determination or comparison of the amplification factor; and co-amplification methods comparing the amount of the product to a simultaneously amplified standard template (for reviews see [133,134]).

Constant amplification efficiency and subsequent accurate estimation constitute the basis of the kinetic quantification method. However, in a mixed-template PCR target sequences vary both in copy number and amplification efficiency. Targets are amplified discordantly, spending unequal time at each amplification phase. It is practically impossible to establish the range when products from all templates in a mixture accumulate exponentially. Furthermore, the signal of the product of a multi-template PCR cannot be corrected for the presence of artifactual amplicons because it is impossible to determine which particular template participated in their formation, and the proportion of DNA strands that are

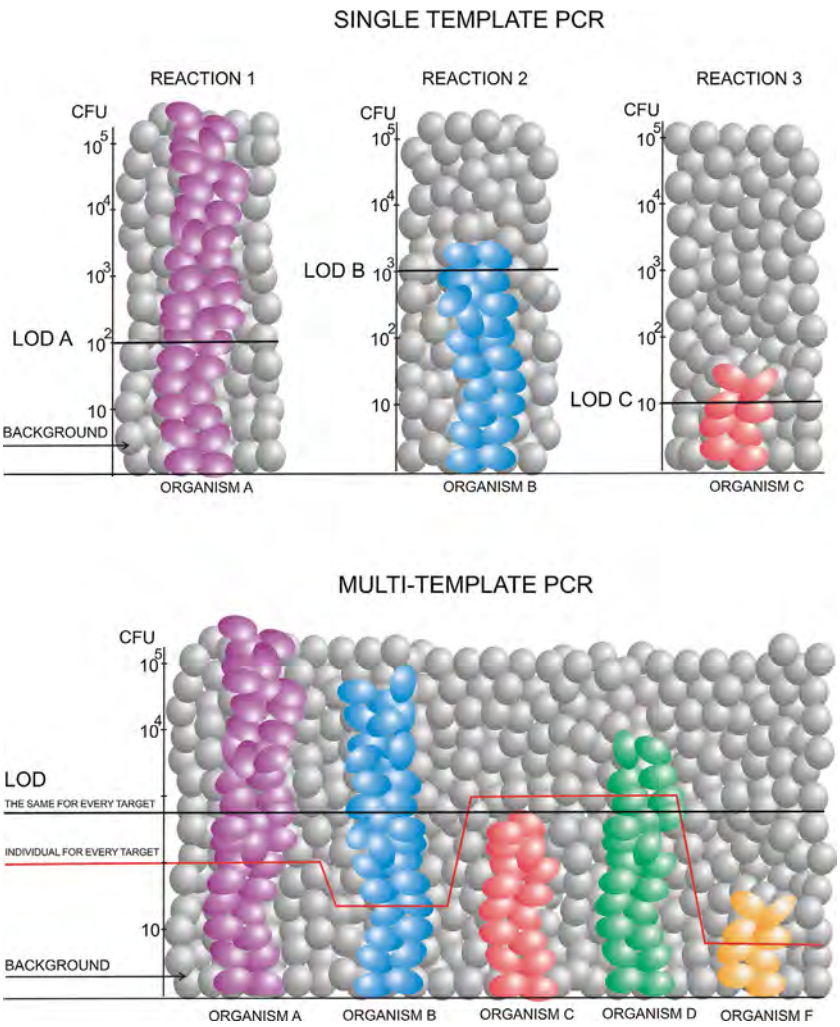

Fig. 3. Limit of detection in single and multi-template PCR. In the single-template PCR limit of detection (LOD) varies depending on the type of microorganism, background DNA, level of PCR inhibition and other factors. At present there is a lack of experimental evidences confirming that all types of the homologous targets have the same LOD in the multi-template PCR. On the contrary, there are experimental data supporting the idea that LODs of the different targets within the mixed sample differ (for discussion see the text).

involved in formation of artifacts. In addition, the kinetic method demands real-time detection, which is technically impossible to apply in the multi-template assay. These are the major reasons why the kinetic approach is not applicable for multi-template PCR.

The co-amplification method quantifies the sequence of interest relative to a second control sequence by co-amplification in the same PCR tube. The control template can be a related DNA sequence with a known concentration and amplified with the same set of primers; or it can be an unrelated DNA sequence amplified with separate primer set. The first case is called competitive PCR and represents the simplest example for a multi-template PCR as the mix consists of only two homologous sequences. Co-amplification methods rest on the assumption that the amplification efficiencies are equal for target and standard sequences [133]. Reliable quantification requires several reactions in parallel, each containing the same number of target sequences but different concentrations of the standard template added. The range of the dilution series should encompass the copy number of the target template [135].

When the co-amplification method was adopted for multiplex PCR, a thorough validation of the concept and methodology was undertaken [136]. In these exemplary trials, competitive templates of the internal standard were prepared for each target gene and relative abundances of internal standards in a final mix were thoroughly adjusted. Furthermore, a strategy of correction for the presence of heteroduplexes in the final PCR product has been developed and validated.

There was an attempt to adopt a co-amplification method for the quantitative analysis of the multi-template assay [137]. The authors reasonably argued for the need of an internal amplification control for the multi-template assay. However, in the final analysis this work was unconvincing because some important methodological issues had not been properly addressed. First, information about the PCR assay condition was missing. It was not even mentioned whether a one-step or a nested PCR approach had been used. Secondly, the authors failed to demonstrate that the basic requirement for the co-amplification quantitative analysis was satisfied, specifically that the amplification efficiencies of standard and template (all templates in case of the study in discussion) were the same. It is questionable that the AT-rich sequence used in this work as a standard has an equal amplification efficiency with each of the templates in a mix. On the contrary, theoretical and empirical evidence exists that templates with different primary sequences are amplified with different efficiencies [43,138–142]. An extreme example was presented in a study by Ogino and Wilson [18] where a single nucleotide polymorphism, not in a primer-binding site, caused a reproducible 20% disparity in amplification efficiencies of two homologous sequences. Thirdly, in the work of Bruggemann and co-workers [137], PCR products were visualized with ethidium

bromide. The fundamental concept of quantitative PCR is the principle that accumulation of the signal is proportional to the accumulation of amplification product. It ought to be questioned, however, whether intercalating dye produces a linear signal when they bind templates with diverse primary sequences; especially after these sequences undergo partial melting while passing through the denaturing gradient gel. To ensure that the bands being analyzed were all within a dynamic range of the image analytical system, it ought to be shown that ethidium bromide staining was proportional to the total number of base pairs present; and that the total number of base pairs was related to both the size and the number of amplicons present in each band [136]. In addition, a band densitometry method was shown to have a high coefficient of variation (44.9%) [143] and therefore demanded a considerable number of replicates.

Finally, the copy number of the internal control must be close to the copy number of the tested template, otherwise qualitative measurements give erroneous results [136,144]. The amplification efficiencies of homologous templates depend on their relative concentration. Early in 1990s Dostal and co-workers [144] showed that the first source of error results from changes in the relative efficiencies between two homologous sequences during amplification when validating multiplex competitive PCR. The more abundant component had a higher efficiency then the less abundant component. Moreover, once there was a large difference between competing sequences, the PCR product from the less abundant template had a low signal that was close to background. When Souaze and co-workers [145] aimed to determine the limits and accuracy of competitive PCR, they discovered that once the ratio between two competing homologous templates was between 0.66 and 1.5 (i.e., the difference between templates was 1.5-fold), the final results had an error of approximately 10%. However, a twofold divergence between competing templates resulted in errors approaching 60%. It became obvious that with a single internal control it is impossible to find an appropriate concentration which would corresponds to all templates in a mix. Thus, methodological shortcomings of an otherwise very needed study made the results inconclusive. These difficulties demonstrate how challenging the task of converting multi-template PCR into a quantitative assay is.

There is one interesting feature of the multi-template assay which poses an additional difficulty for an application of the competitive PCR methodology. In the classic single template PCR, the advantage of the competitive approach was the ability to run the reaction to the plateau stage and still obtain quantitative results. In the multi-template assay a particular phenomenon has been noticed, the rate of amplification for abundant products generally declines faster than for less abundant products in the same reaction tube in the late cycles of PCR [146]. As a consequence, differences in product abundance diminish as the number of cycles increase. This observation is in conflict with findings of other studies stating that templates with low abundance have lowed chances to be amplified. However, this discrepancy between different studies just reflects the complicated and unpredictable nature of the multi-template PCR and manifests the current existing chaos in methodology.

Another important methodological problem for quantitative analysis of multi-template PCR is the multiplicity of the types of replicated molecules (native DNA, long product and short product) and its impact on the reaction kinetics. Long product is the DNA strand synthesized during the first cycle with the 5′-terminus restricted by the previous cycle primer and the 3′-terminus extended beyond the site of the complementary of the second primer. Extension, using the long product as a template, generates a strand restricted by the primer on one side and by sequence complementarity to the site of the other primer on the other side. This DNA strand is called a short product [147,148]. In a single-template PCR, the input of the native DNA and the formation of the long products can be lowered by dilution of the original sample. In multi-template PCR, however, the goal is to disclose the structure of the original sample as accurate as possible. Thus dilution of the sample is undesirable because it can lead to loss of essential fractions of templates. In the multi-template assay, the input of native DNA and long products can be considerable and might interfere in unpredictable ways with PCR kinetics due to the high concentration of templates.

We already mentioned the special case of using the multi-template assay for quantification of microbial load. This approach also aims to amplify all bacterial or fungal marker sequences in a sample but here the product is analyzed without fractionation unlike common multi-template assays. Quantitative real-time PCR with DNA binding dyes or with the more specific 5′-nuclease assay (TaqMan technology) are exploited in this case. The absence of need for post-PCR fractionation canceled some technical obstacles for quantitative analysis. Still, the intrinsic features of multi-template samples demand special consideration.

Since the aim of measuring the microbial load is to determine the total number of bacteria or fungi in a sample, it is rational to avoid dilution of a sample or, at least, keep the dilution rate low. This task, however, conflicts with basic requirements of real-time PCR detection methods. Real-time technology determines the PCR cycle at which the increase in fluorescence of the reporter dye reaches a quantification cycle – C_q is proportional to the log of target DNA amount and hence the log of the number of microorganism in a sample, provided that there is only one copy of the target sequence per genome. At extremely high or low C_q values (synonym C_t, for the “threshold cycle”, is used in the referred source) a twofold error in estimating the relative amount of DNA can occur [149]. The baseline fluorescence is due to the fluorescence of unbound fluorochrome and to fluorochrome bound to double stranded DNA, primers annealing to DNA and primers annealing to themselves [150]. Other sources of fluorescence also contribute to the baseline fluorescence. The presence of external fluorescence sources as well as high concentrations of template in some environmental samples increase the level of background fluorescence. High levels of initial fluorescence interfere with correct C_q measurements. The erroneous treatment of raw real-time PCR data might further aggravate the problem [97,150]. Although for single template PCR, statistical methods to identify samples that were erroneously quantified have been developed [97]. To the best of our knowledge, similar methods have not been available for the multi-template assay.

Environmental samples are frequently diluted to the point where the concentration of inhibitors in the extract is no longer inhibitory to PCR. In the single template assay the target sequence stays the same throughout the dilution panel. In the multi-template assay the situation is different. First, the dilution of a complex template might be biased. Quantifying mitochondrial DNA relative to genomic DNA, Malic and co-workers [151] observed that sample dilution can introduce significant errors as mitochondrial and nuclear DNA did not dilute equally. They termed this effect as “dilution bias”. Researchers explained this observation by the different sizes and viscosity of mitochondrial and nuclear genomes and advised shearing of template DNA as a method to remove dilution bias. Whether or not similar phenomena take place in all types of complex templates has not been investigated yet.

Secondly, sample dilution may reduce the number of genomes sampled during PCR and via this mechanism might alter the composition of templates. The inability to reproducibly amplify DNA at low template concentrations has been noticed even in mixed samples with a low complexity. Genomic template concentration of 5 ng or more (≈78 diploid cell equivalents) were necessary to generate consistent signal intensities when amplifying androgen receptor alleles [139]. As allelic concentration dropped to 0.5 ng, quantification of allelic PCR products deteriorated. A similar

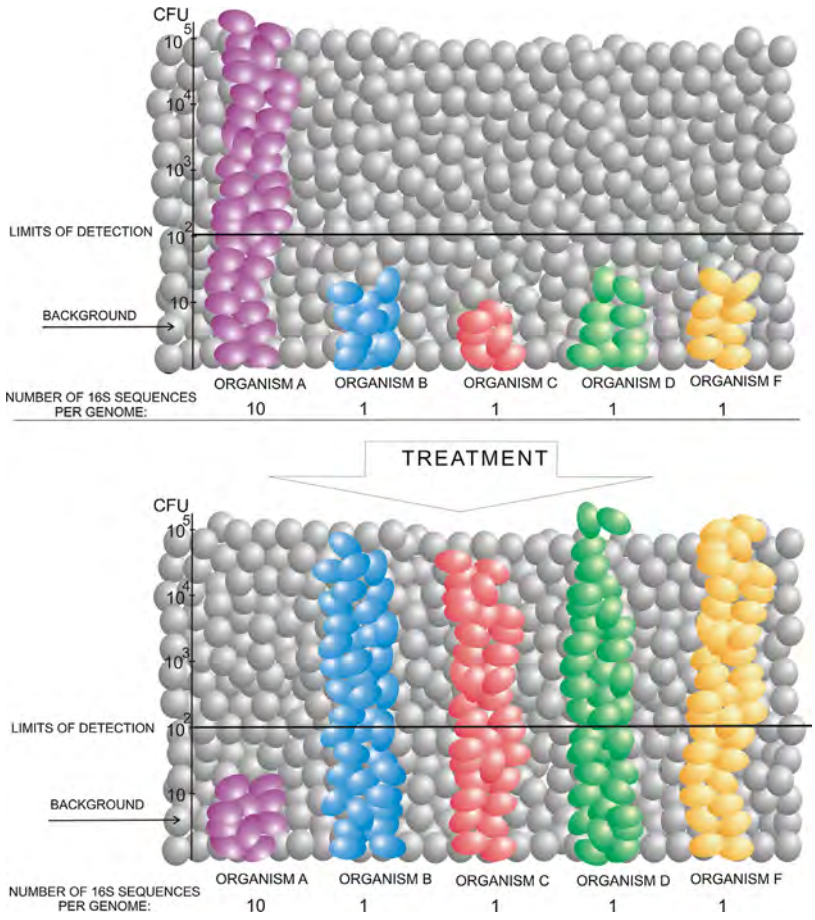

Fig. 4. A simulation of the fluctuations in the microbial community structure induced by the treatment in the course of an experiment and the effect of such change on the quantification of microbial load. Apart from the multi-template PCR burden of artifacts and biases this assay often uses untrustworthy target sequences, e.g. 16S or 18S rDNA. The numbers of these sequences vary among microbial species. Imagine a situation when single specie with 10 copies of 16S rDNA grew above the LOD at the beginning of the experiment. A treatment stimulates a shift within microbial community and four other bacterial species grew above the LOD, while the former one was suppressed under the new conditions.
Load before treatment: 1 microbial strain grew above LOD $\times 10^5$ CFU $\times$ 10 copies of 16SrDNA/cell = 10^6 copies of 16S rDNA ought to be detected (although really there are only).
Load after treatment: 4 microbial strains grew above LOD $\times 10^5$ CFU $\times$ 1 copy of 16SrDNA/cell = 4×10^5 copies of 16S rDNA ought to be detected under new conditions.
So, the analysis based on the 16S rDNA would show one order of magnitude decrease in bacterial load after treatment. While in fact, the load of microbes before treatment was 10^5 and after treatment – 4×10^5. The rate of the possible error is much higher than the twofold difference in number of DNA molecules that real-time PCR usually aims to detect.

situation was observed in the amplification of HLA-DQα alleles when the total copy number decreased to less than 20 copies [138]. Chandler and co-workers [152] reported that template dilution prior to PCR can seriously affect the composition of 16S rDNA libraries derived from low-biomass environments. Although the DNA concentrations in their experiments were lower than those required for quantitative amplification of relatively simple allelic systems, the number of 16S rDNA sequences present in the PCRs was higher than the number of allelic targets required for a reproducible assay. Nevertheless, only 15–24% of the total RFLP types recovered from a sample were present both in the undiluted and in the 1:10 diluted extracts. Their results indicate that at least with low template concentrations, proportional representation of specific products was not reproducible upon template dilution, confirming that PCR amplification of 16S rDNA cannot be used directly to infer microbial abundance.

Apart from the problem surrounding sample dilution, another technical difficulty might arise from the complex nature of the samples used in multi-template assays. Treatment might not only change the concentration of particular microorganisms but also the community structure as a whole. As a result, not only the quantities but also the qualities of the original templates might vary between treatments. The prevailing groups of microorganisms after different treatments might differ in the number of target sequences on their genomes. There is evidence that the number of 16S rDNA loci correlates with the rate with which bacteria respond to the availability of resources and that the species responding to stimuli faster will have a higher copy number [153]. Thus, the measurement of 16S rDNA quantities might lead to wrong conclusions about the total bacterial number if species with low copy number dominate in one treatment while those with higher copy number in another (Fig. 4). A warning was given that the failure to compare DNA from similar groups of bacteria and possessing similar growth rates, readily leads to an under- or over-estimation of the amount of DNA by one order of magnitude [149]. The rate of the possible error is much higher than the twofold difference in number of DNA molecules that real-time PCR usually aims to detect [97]. Nadkarni and co-workers [149] recommended that a DNA standard representing those bacteria most likely to predominate in a given habitat should be used for a more accurate determination of total bacterial load. However, this sensible recommendation is difficult to follow in most of the environmental studies. On the other hand, ignoring it often makes the results questionable.

The lack of attention to the specific properties of multi-template PCR leads to even meticulous and extensive studies losing their full value. Suzuki and co-workers [154] developed and tested quantitative reliability of primers and probe sets for quantification of prokaryotic 16S rDNA. The assay has been validated on mixtures composed of two bacterial templates or one bacterial and one archaeal template. This simplest case of a multi template PCR has already been thoroughly studied under the term of 'competitive PCR'. However, methods for quantitatively measuring microbial load ought to be validated on mixtures with complexities that reflect real environmental samples. In 2012, two quantitative assays for measuring bacterial and fungal load were developed [155,156]. Authors claimed that using the qPCR platform they can design an assay capable of concurrently detecting and quantifying all unique bacteria that constitute a complex community. However, the validation of these techniques was performed only in the single template format. It has been demonstrated that new primers can amplify a broad range of fungal or bacterial species. Yet, primers were tested in *single* template reactions. It was also shown that the assay can efficiently detect microbial template in a mixture with human DNA. Yet again, all tests were performed on mixtures of human DNA and a *single* microbial template. There were no results showing how well the same primers would amplify a mixture of bacterial or fungal templates. In addition, no attempts were made to clarify how all microbial species in complex mixtures would be amplified and how the experimentally obtained microbial load would correspond to the number of microorganisms in the original sample. In other words, the issues of multi-template PCR were not addressed.

Another group of researchers analyzing potential PCR biases and utility of fungi-specific primers, stated that on one hand quantitative real-time PCR is a robust method to determine total fungal biomass in a mixed-template sample, while on the other hand they reported a significant PCR bias and differential amplification of templates [157]. The reasonable question that thus has to be asked is how can the sum be correct if at least one of the summands is faulty? In other words, if at least one of the templates in a mixed sample is amplified with an efficiency considerably different from the amplification efficiencies of the other templates, then it follows, the total amount of PCR products has to be disproportionate to the original template concentration.

There can be no doubt that PCR techniques have several advantages over previously used methods for measuring microbial load in environmental samples [149]. However, a further development of this PCR application must take into account the specific characteristics of multi-template PCR.

In summary, we can conclude that feasibility of quantitative measurements using multi-template PCR has neither been confirmed by theoretical models nor by empirical evidence. Several inherent features of the multi-template assay constitute obstacles for reliable quantification. First, the heterogeneity of template, both in the sense of primary sequences and copy number, does not allow for selection of reaction conditions that guarantee equal amplification efficiency for each template in a mix. One of the consequences therefore is that the exponential phase for each particular template cannot be determined accurately. This technological limitation ruins the basis of the quantitative analysis. The possibility of estimating some kind of 'average' exponential phase for all targets in multi-template PCR has not been validated and appears very doubtful. Secondly, many artifactual amplicons are generated during the course of multi-template PCR with the result that the signal from a particular template can be split among several different PCR products. The identity and proportions of such products are undetectable. Therefore, the results of an assay cannot be freed from these biases. Thirdly, due to the need of post-PCR fractionation a multi-template assay usually has two steps. If cloning is used for detection of the product, then PCR is followed by a cloning step. PAAG fractionation (in the form of TGGE or DGGE) usually demands a nested PCR. Most of the massive parallel sequencing approaches also employ additional PCRs. In all these cases, the quantitative measures are made at the second step (post-fractioning). Is it methodologically sound to make a quantification of PCR (first step) by detecting and counting the products of the second step? When a two-step analysis is employed to quantify RNA by RT-PCR, at least the first step of this assay is a linear reaction. In addition, rigorous procedures to monitor bias have been developed [158]. In case of multi-template assays, approaches for the correct quantification of these many-stage procedures have not been discussed at all. Fourth, some quantitative approaches employ serial dilutions in amplification of the original template. However, a dilution of a mixed template would change the ratio of the templates to each other and even worse, eliminate some templates from the mix. As a result, other types of chimeric and heteroduplex amplicons would be formed and the profiles obtained with different dilutions would become incomparable. This phenomenon can be aggravated by the presence of inhibitors, which can in unpredictable ways affect amplification of different samples and different dilutions of the same sample.

In conflict with existing theoretical models and experimental evidences, a quantitative power has been assigned to the multi-template PCR assay as an uncontested attribute. To correct such a conclusion a serious discussion about the suitability of the mixed template assay for quantitative measurements must be renewed. In the meantime and based on current methodologies, multi-template PCR cannot be used for quantitative analysis.

6. Future of multi-template PCR assay

In the 1990s two approaches for enumerating microorganisms in environmental samples were competing: the most-probable-number PCR and the quantitative PCR [1,159 and references within,160]. At that time, the quantitative PCR procedure became the prevalent method due to its technical simplicity. Nowadays it appears as if the pendulum has swung back and the basic idea of the most-probable-number PCR is now being increasingly used in modern methods, particularly in the so called digital PCR. The basic idea behind this approach is to convert multi-template PCR into separate single-template reactions. Unlike quantitative PCR techniques, there are relatively few assumptions that can be violated in the most-probable-number PCR method. Quantification by the latter method does not require the use of an internal standard and the endpoint is a simple all-or-none determination [1,160,161]. The novelty of modern approaches lies in methods in which a multi-template sample is split into a set of single-template reactions. In the most-probable-number PCR, a dilution to extinction method was used for this purpose. However, apart from the disadvantage of being very cumbersome, the dilution to extinction method also has the shortcoming of detecting only the most abundant templates in mixed samples. Fortunately, the approach used in a digital PCR overcomes these drawbacks.

6.1. Emulsion PCR

In emulsion PCR, the multi-template sample is transformed into a batch of simplex PCRs by dividing it into small droplets. The emulsion PCR matrix contains cell-like compartments (10^8–10^9 droplets/ml) with a single or at most a few DNA molecules in each droplet. These are then separated from each other without exchange of macromolecules [162,163]. The segregation of template DNA molecules prevents recombination between homologous gene fragments, thus eliminating chimeras, as well as preventing heteroduplex formation. The compartmentalization of

templates also reduces the competition between DNA sequences [163]. Nevertheless, emulsion PCR may be inefficient in reducing PCR generated biases due to either the secondary structures formed by template DNA molecules or the reduction in annealing efficiency of primers to particular templates [164]. Emulsion PCR might also be ineffective in reducing errors if the individual DNA molecule contains several target sequences (e.g. several rRNA operons per genome). Nowadays, emulsion PCR is employed in several massively parallel sequencing technologies. However its advantages are abolished if "conventional" PCR is used in a previous step to multiply target sequences *en masse*.

6.2. Digital PCR

The same idea of "divide and conquer" is exploited in another elegant PCR technology called digital PCR. There is some confusion about the term 'digital', since emulsion PCR is based on similar principals but performed in a droplet format whereas the 'classic' digital PCR is carried out in a microchamber format. The concept behind digital PCR was described in 1992 and is based on a combination of limiting dilution, end-point PCR and Poisson statistics [159,160]. A few years later it was used for the identification of predefined mutations expected to be present in a minor fraction of a cell population [165]. Ottesen and co-workers [166] were the first, to the best of our knowledge, who applied digital PCR to examine a complex microbial community. However, the advantage of the new PCR technique was not fully revealed in their study for at least two reasons. First, there was no method validation performed preceding application of the technique to the new field. In addition, there was no assay control(s) reported in the study. Secondly, the downstream methods of handling the results almost canceled the advantages of the digital PCR format since only 28 individual reaction chambers were further analyzed by standard methods (re-amplification, cloning, and sequencing). Until recently sequencing was the only method for analysis of digital PCR products. However, Fraley and co-workers [167] recently proposed an alternative method. They applied post-PCR high resolution melting analysis for profiling of mixed samples. Amplicons from each individual DNA sequence were represented by a single and specific melting profile. Creation of a database of known sequence-specific melting curves might provide additional benefits by enabling direct sequence identification.

Digital PCR is considered by experts as an especially powerful technique in experiments requiring the quantitative investigation of individual alleles in DNA samples isolated from a mixed cell population [168]. Furthermore, a step toward standardization of the digital PCR protocols was already undertaken [169]. At the moment, there are still factors such as high costs, limited throughput, and complicated work flow that have hampered the broad adoption of digital PCR.

6.3. Quality-insurance policy

If assays based on multi-template PCR continue to be employed in the future, the proof of concept followed by the development of a quality-insurance policy is imperative. In every scientific discipline the reliability of the conclusions strongly depends on the quality of the data. The trend toward implementation of quality standards is notable in many scientific communities. The need for quality control has been recognized early on during the development of forensic DNA profiling and has led to the formation of the European DNA profiling Group (EDNAP) in 1989 [170]. The European Molecular Genetics Quality Network, established in 1996, is aiming to spread quality assurance policy on genotyping assays across Europe [171]. Microarray assays are known to be error-prone, so the scientific community reacted by designing strict standards presented in the 'Minimum Information About a Microarray Experiment' (MIAME) document [172]. After a crisis of confidence, caused by publishing a series of erroneous papers in leading journals, the scientific community dealing with ancient DNA and gene expression analysis set up strict standards to ensure data quality [10]. A set of recommendations summarized in 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) lists the minimum information required for potential reproduction as well as for unambiguous quality assessment of qPCR-based experiments [173,174]. A batch of requirements for digital PCR has already been identified during the early stage of its development and commercial implementation and the guidelines in "Minimum Information for Publication of Quantitative Digital PCR Experiments" have already been developed [169].

Although an awareness that amplification in a mixed template is prone to different biases exists in the scientific community [19,34,36,108] there have been no attempts yet to develop a policy for quality insurance. The central part of this policy should include appropriate controls for each step of an experiment, starting from the extraction of nucleic acids.

To the best of our knowledge, the first attempt to evaluate the implementation of a standardized operating procedure in microbial ecology studies was undertaken as a part of the Human Microbiome Project [175]. The authors demonstrated the value of model communities as a quality control in microbial ecology studies and urged for inclusion of mock community samples on each sequencing run to calculate the level of chimeras, sequencing error rate, and drift in the representation of a community structure. They also emphasized that any microbial analysis is only as good as the underlying biological question, study design, DNA extraction method, PCR conditions, sequencing, and bioinformatics analysis, thus proper controls must be implemented on each step and the entire procedure must be clearly presented in the published data.

The other important issue is the clear and concise communication of the procedure that was used in the method section of the publication. The guidelines developed by Apfalter and colleagues [176], Bustin and colleagues [173], Huggett and co-workers [169] for the minimum information that should be presented in publications based on results of PCR assays should be recommended reading to all researchers as well as reviewers and editors of the biological journals.

7. Conclusions

PCR is a formidable and potent technology that serves as an indispensable tool in wide range of biological disciplines. However, due to the ease of use and often lacking rigorous standards, many PCR applications can lead to highly variable, inaccurate, and ultimately meaningless results. Thus, rigorous method validation must precede broad adoption of PCR to any new application. Multi-template samples possess particular features, which make their PCR analysis prone to artifacts and biases such as multiple homologous templates present in copy numbers that vary within several orders of magnitude. Such conditions are a breeding ground for chimeras and heteroduplexes. Differences in template amplification efficiencies and template competition for reaction compounds undermine the correct preservation of the original template ratio. In addition, the presence of inhibitors further aggravates all of these problems. Moreover, inhibitors might have varying effects on the different templates within the same sample. Yet, no standard approach exists for monitoring inhibitory effects in multi-template PCR, which would be crucial for establishing sample compatibility.

Apart from being prone to errors, multi-template PCR also represents a challenge for product detection since amplicons are of almost equal size and must be fractionated before detection. The need of fractionation makes multi-template PCR analysis a

multistage process. Necessity of several steps (i) makes real-time detection by currently available methods technically impossible and (ii) together with PCR-induced errors, imposes insurmountable difficulties for quantitative analysis.

Concepts such as amplification control, sensitivity, specificity and limits of detection have not been properly established in multi-template assays. Meanwhile, using definitions based on concepts from single-template assays can lead to misunderstandings or confusion.

The sensitivity of product detection methods must correspond to the sensitivity of the PCR itself. If the sensitivity of the detection method is lower than the sensitivity of the polymerase chain reaction, then some amplicon groups will not be detected. On the contrary, if the polymerase chain reaction is able to amplify only the major templates from the mix, then employment of high-resolution methods (e.g. massive parallel sequencing) is worthless. Thus, use of such sensitive (and therefore expensive) detection method must first be justified. Finding the balance between PCR sensitivity and the resolution capacity of the detection method should become one of the central questions in further methodology development for the multi-template assay.

Further application of multi-template PCR requires systematic method validation and the development of a quality-insurance policy. A stringent policy would increase the relevance of published data, and, allow a fair competition between research groups that practice different levels of accuracy in their experimental execution.

In conclusion, the multi-template assay brings along intractable difficulties for its validation and standardization at this time. This fact might stimulate the research community to abandon the practice of amplifying multiple homologous sequences in the same PCR tube. At the same time, most-probable-number PCR reanimated in a new format of digital PCR represents an attractive alternative for the multi-template assay.

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