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External and semi-internal controls for PCR amplification of homologous sequences in mixed templates

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ABSTRACT

In a mixed template, the presence of homologous target DNA sequences creates environments that almost inevitably give rise to artifacts and biases during PCR. Heteroduplexes, chimeras, and skewed template-to-product ratios are the exclusive attributes of mixed template PCR and never occur in a single template assay. Yet, multi-template PCR has been used without appropriate attention to quality control and assay validation, in spite of the fact that such practice diminishes the reliability of results. External and internal amplification controls became obligatory elements of good laboratory practice in different PCR assays. We propose the inclusion of an analogous approach as a quality control system for multi-template PCR applications. The amplification controls must take into account the characteristics of multi-template PCR and be able to effectively monitor particular assay performance. This study demonstrated the efficiency of a model mixed template as an adequate external amplification control for a particular PCR application. The conditions of multi-template PCR do not allow implementation of a classic internal control; therefore we developed a convenient semi-internal control as an acceptable alternative. In order to evaluate the effects of inhibitors, a model multi-template mix was amplified in a mixture with DNAse-treated sample. Semi-internal control allowed establishment of intervals for robust PCR performance for different samples, thus enabling correct comparison of the samples. The complexity of the external and semi-internal amplification controls must be comparable with the assumed complexity of the samples. We also emphasize that amplification controls should be applied in multi-template PCR regardless of the post-assay method used to analyze products.

1. Introduction

Polymerase chain reaction is a method encompassing a number of techniques. Good laboratory practice dictates that method validation must precede its employment to a new application. However, the technical ease of PCR and its extensive ingrowths into every day laboratory routine can lead to relaxed practices. The transition of PCR techniques to a new field might be so natural that the need of method validation stays unrecognized. This happened when the PCR assay, initially developed for single-template analysis, was applied to amplify complex mixed samples.

PCR techniques can be divided in to three categories based on the complexity of the template. The first group of methods analyzes samples containing only one type of target template and employs one set of primers. The product of such reaction is analyzed en masse. This type of technique is called single-template or simplex PCR. The second group of PCR methods amplify several heterologous targets from the same sample using specific set of primers for each type of template. Amplicons originated from different templates are separated by fractions due to the difference in length. This approach is referred as multiplex PCR (Henegariu et al., 1997). Finally, some PCR assays aim to amplify all homologous DNA sequences from the mix sample using single primer set. Amplicons ought to be fractioning during post-PCR analysis. Products of such PCR have almost the same size, but slightly vary in primary DNA structure. Agarose gels possess inadequate separating capacity for such PCR products and therefore other fractioning techniques are employed. This approach is called a multi-template or a mixed template PCR, although the term might need a revision, since multiplex PCR can also be considered as a mixed template assay. In this paper we will refer to it as multi-template or mixed template PCR if the sample contains more than two homologous sequences. The simplest case of multi-template PCR is excluded because it is extensively used as one of

Abbreviations: DGGE, Denaturing Gradient Gel Electrophoresis; DG-DGG, double-gradient denaturing gradient gel; DG-DGGE, Double-Gradient Denaturing Gradient Gel Electrophoresis; DHPLC, denaturing high-performance liquid chromatography; OTU, operational taxonomic unit; PAAG, polyacrylamide gel; CTAB, cetyltrimethylammonium bromide; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; TMA, tetramethylammonium chloride.

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the quantitative PCR approaches, called co-amplification method, and therefore has been thoroughly discussed (for review see Raeymaekers, 2000; Wong and Medrano, 2005).

Multi-template PCR assay is employed in many fields, e.g. forensic investigations (Jobling and Gill, 2004), medical research and diagnostics (Pompanon et al., 2005), molecular evolution and systematic studies (Wagner et al., 1994; Nikolaev et al., 2004; Yoon et al., 2008), and environmental research (Pace, 1997; Dawson and Pace, 2002; Doherty et al., 2007).

Even in a single template PCR the specificity, efficiency and fidelity of the reaction might be a challenge (Cha and Thilly, 1993; Booth et al., 2010). When the complexity of the template increases so does the challenge to keep PCR on an appropriate level of specificity and fidelity, since the presence of multiple homologous sequences *per se* creates the environment for biases and artifacts. Heteroduplexes, chimeras and skewed template-to-product ratios are the exclusive attributes of mixed template PCR and can never occur in a single-template reaction. In addition, formation of single-stranded and partially single-stranded amplicons, and polymerase-induced errors although not being the exclusive attributed of multi-template PCR, are a potential source of bias. This occurs because the aim of such assays is to estimate the composition of the original sample; therefore amplicons are analyzed by fractions. Each type of artifactual product originating in the course of multi-template PCR produces a false-positive signal, which does not correspond to any target in the original sample.

A high potential risk of artifacts in multi-template PCR stimulated the scientific community to develop strategies to monitor and minimize such biases. Two different approaches were taken. The first was to thoroughly investigate all characteristics of the system in use; to apply controls in each experimental stage and to determine the limitations of the system (Schneider, 1997; Clayton et al., 1998; Gill et al., 2006). This strategy is routinely used in forensic and diagnostic PCR assays. Another approach was to develop ready-to-use protocols with recommendations to apply them in other experiments. This last tendency is especially noticeable in environmental studies. In microbial ecology the most complex templates are used comprising of hundreds of target sequences. In addition, environmental samples contain inhibitors, which vary both in composition and concentration and increase the risk of PCR failure, biases and sensitivity reduction (for reviews see Wilson, 1997; Schrader et al., 2012; Allaeddini, 2012). Built-in predisposition of multi-template assay to artifacts and biases aggravated by the negative effect of inhibitors calls for especially strict quality insurance policy. One of the basic elements of such policy should be the development and obligatory employment of amplification controls. Such amplification controls ought to comply with the specificity of multi-template assays and therefore should differ from the amplification controls employed for single-template or multiplex PCR techniques.

There were a few attempts to adopt amplification control in environmental studies. However, the aim of these particular attempts was developing a standard for quantitative analysis. A competitive internal standard was included in the PCR assay of environmental samples by Bruggemann et al. (2000). A single template representing the most AT-rich 16S rDNA sequence described to date was used for a quantitative estimation of the most common components of the environmental samples. Petersen and Dahllöf (2005) argued for using internal standards for both DNA extraction and PCR. The PCR internal standard they developed originated from the *Drosophila melanogaster* genome and was a 140-basepair long PCR product, which was amplified by non-competitive primers in the same PCR reaction as the target DNA and analyzed together with the target PCR product on the same DGGE gel. Although an internal standard with non-competitive primers will ot allow for estimating PCR-induced artifacts in the target community, it helps to monitor and estimate the variations between replicate samples. Thereby the relative changes can be used to assess the respective impact of treatments.

However, the amplification of a single sequence cannot serve as an appropriate control for an amplification of a mixture of homologous targets, because different types of PCR-induced errors take place in the multi template PCR compared with a single template. Thus, the nature of artifacts demands an appropriate type of quality control.

The most adequate amplification controls are samples or standards that are similar to unknown samples, in this case, mixed samples composed by templates with known identity. Model communities have been successfully used to evaluate the influence of different factors on the level of PCR-induced artifacts in a mixed template. To investigate the variables relevant to production of artifacts during amplification of a multi-gene family of murine immunoglobulin germ lines V genes, Zylstra et al. (1998) constructed several different model mixtures composed from one up to 11 homologous templates. Jae-Hyung et al. (2006) used a set of 16S rDNA sequences from thirteen soil bacteria cloned into separate plasmids to investigate the effect of primer set and annealing temperature on the level of amplification bias. Frey et al. (2006) attempted to quantify biases attributed to DNA extraction and PCR amplification using a model community of five bacterial species mixed in different combinations. They found that biases attributable to these factors led to deviations from expected PCR product ratios by 6% to 38%. Although the authors thoroughly investigated a range of different factors, the interactions were so complex that a single suitable factor could not be derived. Ahn et al. (2009) used an artificial mix of six 16S rDNA sequences cloned in separate plasmids to investigate the effect of various PCR parameters and denaturing gradient gel electrophoresis (DGGE) conditions on the level of PCR-induced bias aiming to develop a protocol for accurate quantitative estimation of soil bacterial communities. Kunin et al. (2010) smartly used *Escherichia coli* MG1655 bearing five homologous rRNA operon types as a natural mixed template to estimate the rate of pyrosequencing errors. They expected to observe a single operational taxonomic unit (OTU) when clustering at 97% of identity threshold, but instead observed 16 OTU for the V1 and V2 regions and 7 OTU for the V8 region. Their approach can be recommended as one of the possible variants of an external control, which ought to be included in similar experiments. Morgan et al. (2010) constructed a synthetic community of ten microbial species and sequenced its metagenome by both Sanger and 454 pyrosequencing methods. They encountered several sources of observational bias that likely affected most metagenomic experiments to date and present challenges for comparative metagenomic studies. Mock communities consisting of pooled genomic DNA from 12, 24 and 48 individual nematode worms, all belonging to different species, were used by Fonseca et al. (2012) to analyze the effect of species richness and phylogenetic diversity on the formation of chimeras. After clustering sequences into OTUs at a 99% identity threshold they found that the mean OTU numbers were approximately double the number of unique nematode species in each pool.

All researchers, who exploited model communities as a simplified test system to examine the causes of artifacts and to optimize the PCR parameters, demonstrated the efficiency and simplicity of an artificial community for these purposes. The next step would be to advocate for inclusion of this test as an amplification quality control in routine assays. Although an artificial community does not represent the complexity of environmental samples, even a simplified test is better than the complete absence of a quality control that we are currently witnessing in ecology studies. All of the above mentioned considerations and the concern expressed by Stephan Bustin (2010) about the quality of published qPCR assays in relation to “ill-assorted pre-assay conditions, poor assay design and inappropriate data analysis methodologies that have resulted in the recurrent publication of data that are at best inconsistent and at worst irrelevant and even misleading”, stimulated us to put more attention to quality control in PCR assays when complex mixtures of homologous DNA sequences were used as a template.

The aim of this study was to remind users about the complex nature of PCR, thus discouraging the adoption of ready-to-use protocols and instead stimulate the systematic validation of multi-template PCR assays.

The amplification control must be adjusted to the particular features of the PCR assay. Two types of amplification controls are proposed in this study and their efficiency in PCR optimization and monitoring of specific biases was demonstrated. Optimally, standard panels of model communities with different complexity should be available. The results of model template amplification should be reported in publications to ensure the reliability of the data. We propose the inclusion of different types of amplification controls as an obligatory element of quality assurance of multi-template PCR assays.

2. Materials and methods

2.1. Preparation of the model mixed sample and environmental templates

Twenty four bacterial stocks were randomly picked from a microbial collection obtained from the rhizoplane of *Avena fatua* plants. Genomic DNA was extracted according to the protocol described by Frey et al. (2006), except that final DNA preparations were dissolved in PCR-grade mQ water (Sigma, Saint Louis, MO, USA). 16S rDNA sequences of each microbial stock were amplified separately according to the procedure explained below and four samples that produced single bands on double gradient denaturing gradient gel electrophoresis (DG-DGGE) were selected. The main criterion for selection was that sequences with different GC content were represented in the model community. This allowed controlling the amplification of templates across the wide gradient of GC content. DNAs from four selected bacterial stocks were diluted to 5 ng/μl with PCR-grade mQ water and mixed together in equal proportions. This mixture of genomic DNA from four bacterial stocks was used as template representing a model microbial community.

DNA extracts from native microbial communities used in the experiment were obtained at earlier studies and kept at −20 °C before use. Original DNA extracts were diluted in proportion with the starting material. DNA from soil samples were extracted from 5 g of soil and were diluted in 100 μl of PCR-grade distillate water, thus 1 μl of solution contained DNA extracted from 0.05 g of soil. Samples of rhizoplane microbial communities were diluted proportionally to the fresh weight of roots, from which microorganisms were extracted. One μl of each these templates contained DNA obtained from microorganisms extracted from 0.5 g of roots.

The amounts and the purity of the DNA extracts for all templates used in study were estimated using a NanoDrop (Thermo Scientific, Wilmington, DE, USA) spectrophotometer.

2.2. Sample preparation for semi-internal control

The most recalcitrant DNA extracts from the collection of stocks were selected to serve as templates for the test. Original extracts were treated with DNase according to the protocol developed by Invitrogen. Briefly, 10 μl of each DNA solution was incubated with 0.5 U of DNase I (Invitrogen, Carlsbad, CA) in 1× DNase buffer (10 mM Tris pH 7.5; 2.5 mM $MgCl_2$; 0.5 mM $CaCl_2$) for 1 h at 37 °C. DNase was inhibited by heating at 95 °C for 15 min. Control treatment with PCR-grade distilled water instead of DNA solution was included. Final DNA-free solutions were mixed with DNA from model microbial communities in a 1:1 ratio and used as templates for PCR.

2.3. Basic PCR protocol

To avoid DNA contamination during PCR, all reaction mixtures were prepared in a laminar flow cabinet equipped with a bactericidal UV lamp. Water used for dilutions, reactions, and PCR blanks was molecular biology grade. Pipetting was done on ice, and the vials were placed directly from ice into the preheated metal block (94 °C) of the thermocycler. Negative controls, with molecular grade water as template, were included in all sets of PCR reactions to provide for a check on contamination. A re-amplified negative control from the first round PCR plus a fresh negative control were also included. All disposable plastic ware was autoclaved and/or UV treated prior to use. Primers were synthesized by TAG Copenhagen. A list of primers used to amplify bacterial 16S rDNA sequences are shown in Supplementary material 1.

The main characteristics of polymerases and correspondent PCR buffers as well as PCR conditions are summarized in Supplementary material 2.

A list of PCR-enhancing compounds with their supposed mode of actions and concentrations in the final PCR mixes are shown in Supplementary material 3.

Amplification of 16S rDNA sequences from model and native microbial communities was performed using a nested approach. Nested PCR is routinely used when DGGE or TGGE are employed for post-PCR detection. The purpose of this approach is (i) to increase the specificity of the assay; (ii) to add GC-rich clamps on the 5′-end of the amplicons. GC-clamps are used to increase the separation capacity of DGGE. In the primary amplification reactions primer sets A, B, C, D and F were used.

The preliminary denaturation step was adjusted in agreement with the demands of a particular polymerase. During the cycling phase of PCR, the denaturing step was held for 25 s at 94 °C for all polymerases, if not specifically mentioned otherwise. Annealing temperature was set at 5 °C lower than the lowest melting temperature of the two corresponding primers. Times for primer annealing as well as extension temperature and time was according to manufacturers' protocols. For primary amplification 21 cycles were used. In experiments evaluating the effect of PCR-enhancing compounds, the additives were used during the primary reactions. In experiments with semi-internal control, the concentration of Mg^{2+} ions in the primary PCR was increased by 10% to compensate for the inhibitory effect of Ca^{2+} ions introduced alongside DNase-treated solutions.

The secondary (nested) PCR amplification step was carried out with primer set N on 20 fold diluted products obtained from the primary reactions. The preliminary denaturation step was adjusted in agreement with demands of the respective particular polymerase. During the cycling phase of PCR, the denaturing step was held for 15 s at 94 °C for all polymerases. An annealing temperature at 54 °C was used. Time for annealing of primers as well as extension temperature and time were set according to manufacturers' manuals. For secondary amplification 33 cycles were used.

All PCR reactions were performed using the recommended buffer supplied by the manufacturer. All comparisons employed identical concentrations of PCR primers and template; however, DNA polymerase amount, dNTP and Mg^{2+} concentrations were used as a point of departure at the lowest concentration recommended by the manufacturer.

Although PCR products in amounts necessary for a visualization step can be produced within fewer cycles when the model community was used as a template, 21 and 33 cycles were used in this study because a satisfactory yield for downstream processing in the experiments with native microbial communities was only obtained with these cycling conditions.

2.4. Gel analysis of PCR products

2.4.1. Agarose gels

The PCR products were separated by electrophoresis on a 1% agarose gel in 1× TAE (0.04 M Tris-acetate; 0.001 M EDTA; pH 8.0) buffer at room temperature using a voltage gradient of 4.5 V/cm. For any given gel analysis, the same volume of PCR products was loaded into each gel slot. The equivalent of about 5 μl PCR products was loaded in each gel lane, after mixing it with loading buffer.

2.4.2. Polyacrylamide gels with denaturing and porosity gradients

Denaturing gradient gel electrophoresis (DGGE) (Lerman et al., 1984; Myers et al., 1985) was carried out on the nested PCR products. The same volume of PCR products (5 μl) was loaded in each gel slot after mixing in loading buffer. DGGE was performed on a D-Code

Universal Mutation Detection System (BioRad, Hercules, CA, USA). PCR products were loaded onto TAE polyacrylamide gels (PAAG) with a porosity gradient from 6% to 12% coupled with a denaturing gradient from 45% to 65% to separate partly-amplified bacterial rDNA sequences. One hundred per cent denaturing conditions were defined as 7 M urea and 40% (v/v) formamide (Lerman et al., 1984). Empirical observations showed this range of denaturants coupled with acrylamide gradient gave the best resolution of the fragments.

To facilitate handling during the staining procedure, gels were poured onto the hydrophilic side of GelBond® PAG film (Lonza Rockland, ME, USA) that was hydrophobically bonded to the glass plate. Gels were poured with the aid of a gradient mixer (Fisher Scientific, Loughborough, UK) using a peristaltic pump and electrophoresis was carried out at 75 V for 16 h in 1× TAE buffer. During electrophoresis, the gels remained immersed in a tank with recirculated buffer held at a constant 60 °C with a constant temperature circulator. Each percentage unit of the denaturant reference solution is roughly equivalent to 0.3 °C. The equivalent "temperature" was thus 72 °C at the beginning of the gradient and 79.5 °C at the end.

2.4.3. PAAGs staining

Gels were rinsed with deionized water, fixed for 15 min (10% ethanol, 0.5% glacial acetic acid in distilled water) prior to silver staining, then incubated for 20 min with agitation in freshly prepared staining solution (0.2% $AgNO_3$, 10% ethanol, 0.5% glacial acetic acid in distilled water). Gels were washed 3 times for 5 min in deionized water; this was followed by incubation in fresh developing solution (3% KOH, 0.5% formaldehyde in distilled water) under darkness until bands appeared. The gels were then incubated for 2 min with agitation in distilled water and again fixed for 10 min. Silver-stained gels were scanned with high-resolution scanner Epson Perfection V700 Photo.

3. Results

3.1. Significant differences in outcomes of bacterial community analysis between different DNA polymerases and primers

The overall goal of this study was to find reliable criteria to diminish artifacts and biases in multi-template PCR assays. Since most of the publications ranked the choice of the correct primer set as the most significant factor of any PCR assay, we started with evaluation of the existed broad-spectrum primers targeting 16S rDNA. During preliminary screening experiments five primer sets were selected out of 72 combinations of primers commonly used to amplify 16S rDNA sequences. The main criteria for selection were the highest number of QTU produced and the reproducibility of three independent PCRs (data not shown). DNA from a native microbial community extracted from soil and BioTaq polymerase with supplemental buffer were used throughout the screening step.

Unfortunately, all tested primer sets, although claiming to be specific for bacterial 16S rDNA sequences, amplified DNA extracted from sterile plants Supplementary material 4.

A model bacterial community was created by mixing equal amounts of genomic DNA from four bacterial species which differed considerably in GC content.

Five polymerases were used to compare the rate of PCR artifacts produced during amplification of 16S rDNA sequences from these model bacterial communities. Two of the enzymes (DreamTaq and Phusion) were routinely used in the laboratory for studying native microbial communities. Two others (AccuPrime and OneTaq) were chosen based on the manufacturers' claim of being the most accurate-priming polymerase and performing well in multiplex PCR. BioTaq was added to the list because it demonstrated highly reproducible results in previous experiments and demanded very few adjustments of PCR conditions.

The expected profile of the bacterial community comprising of four species would show four bands, with positions on a gel matching the individually amplified 16S rDNA sequences from each bacterial species.

Polymerases differed considerably in their ability to reproduce the correct profile of the model microbial community (Fig. 1). The most reliable enzyme was AccuPrime (Fig. 1a), which generated the correct profile and minimal artifacts with all primer sets, except set A. It was followed by BioTaq (Fig. 1b), which correctly reproduced the profile of the model community when used in combination with primer set D and had tolerable (potentially adjustable) level of artifacts when used in combination with primer set F. Other polymerases failed to correctly replicate the profile of the model community. Patterns of artifacts varied from being highly reproducible (DreamTaq and OneTaq) to inconsistent (Phusion). Primer set B, although able to amplify all templates with low and high G + C content in separate reactions, demonstrated a strong tendency to miss sequences with low G + C content when amplifying a mixture template.

3.2. No significant differences in the amplification profile was found under a broad span of annealing temperatures

In order to increase the specificity of amplification by BioTaq and Phusion polymerases, different annealing temperature (52 °C, 54 °C, 56 °C, and 58 °C) were tested (Supplementary material 5). Primer set D was used for primary reactions under all examined annealing temperatures. No PCR-enhancing additives were used. The results showed no effect of the annealing temperature, within the tested interval, on the outcome of PCRs.

3.3. The post-PCR treatment of amplicons with Klenow exo$^-$ and exonuclease S1 had very little effect

Although BioTaq polymerase in combination with primer set D generated a correct profile of the model bacterial community, the appearance of faint bands in non-specific positions along the gel was noticed. In order to decrease the number of non-specific bands, the amplicon pool was treated with i. Klenow exo$^-$; ii. exonuclease S1 enzymes and iii. both enzymes in combination (Supplementary material 6). Attempt to fill in single stranded amplicons at their ends with Klenow exoenzyme did not reduce the number of non-specific bands. At the same time, digestion of (partly) single-stranded amplicons with exonuclease S1 or sequential treatment with both enzymes slightly reduced the number of non-specific bands. Since BioTaq polymerase under particular PCR conditions generated very few faint non-specific bands, the effect of post-PCR treatment with Klenow exo$^-$ and exonuclease S1 was low.

3.4. The suboptimal PCR conditions rather than the direct effect of inhibitors led to artifactual PCR products

In order to evaluate the effect of inhibitors present in DNA extracts, a semi-internal control was applied to the assay. The most recalcitrant samples were selected from the DNA stock collection. These were the genomic DNA isolated from the rhizosphere microbial communities inhabiting roots of *Tussilago farfara* and *Tarantella officinale* plants (referred to below as rhizosphere samples). The samples were probably so challenging due to co-extraction of high amounts of inhibitors from the milky latex present in the roots of these plants. Native DNA in the rhizosphere samples was digested with DNaseI and DNA from the model microbial community was added in a 1:1 ratio to the resulting solutions. Several rounds of PCR adjustment were performed before the amplification succeeded with the rhizosphere samples. Addition of BSA, betaine and DMSO to the primary PCR was unsuccessful. The adjustment of Mg^{2+}–dNTP balance at the primary reactions gave stable amplification in the rhizosphere samples. Visualization of the results

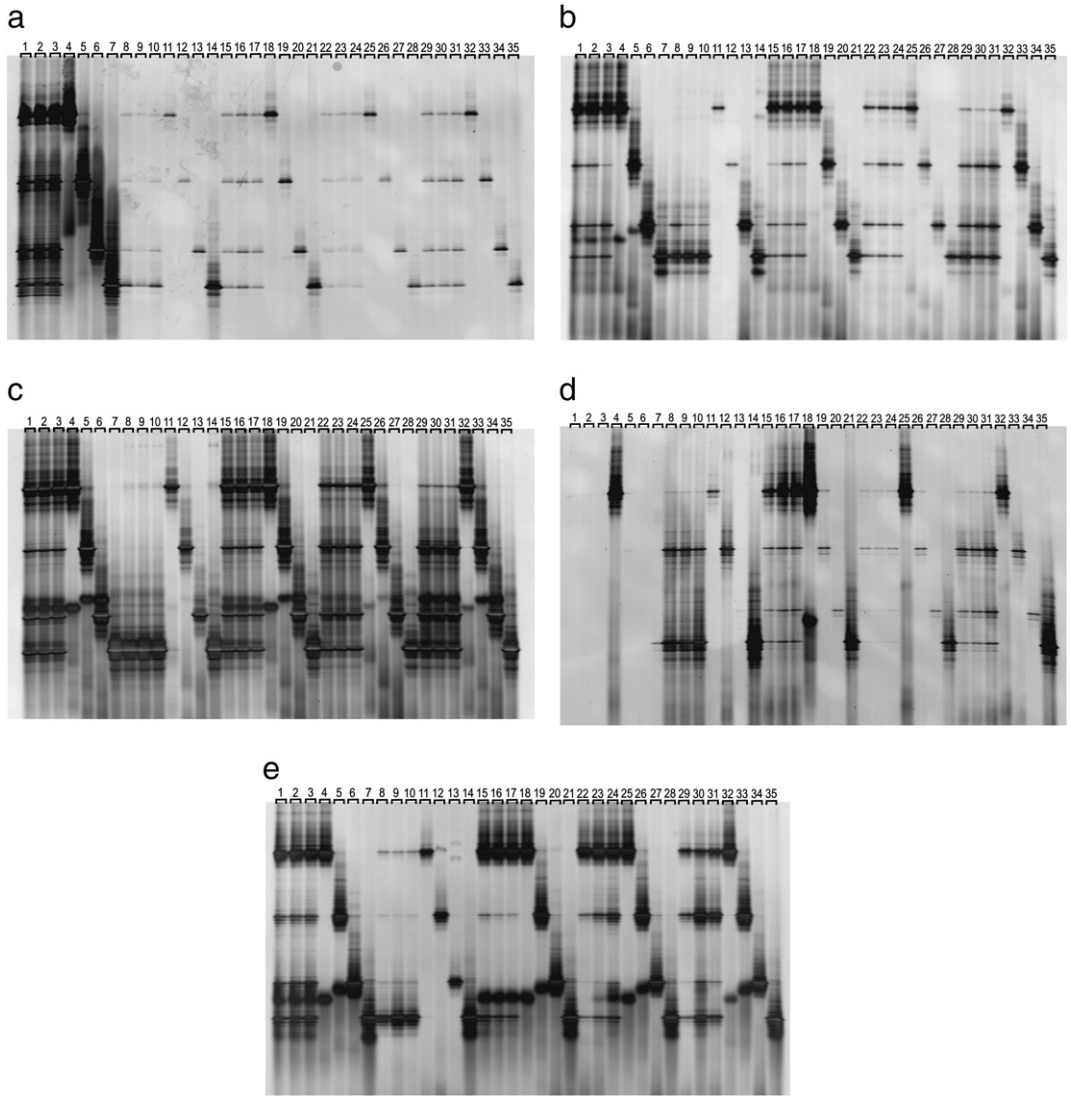

Fig. 1. The effects of different polymerases and primer sets on PCR artefacts during amplification of 16S rDNA sequences from a model bacterial community. Polymerases used for amplification of different regions of 16S rDNA sequences were: (a) AccuePrime Taq; (b) BioLine Taq; (c) DreamTaq; (d) OneTaq; (e) Phusion. Amplicons were obtained by nested PCR with the primer Set N for the second PCR reaction for all samples and with the different primer sets for the first PCR reaction: Set A (lines 1–7); Set B (lines 8–14); Set C (lines 15–21); Set D (lines 22–28); Set F (lines 29–35). PCR products were separated on PAAG gels contained a porosity gradient of 6–12% and a denaturant gradient of 45–65% for 16 h at 75 V.

of PCR amplification on the gel revealed several effects of the inhibitors (Fig. 2): i. development of spurious products; ii. presence of multiple bands, especially for the templates with high G + C content; and iii. appearance of additional non-specific bands. The character of the PCR artifacts was similar in profile to that of the external and semi-internal controls amplified under the same conditions. By deviation of the obtained profile of the model mixed template from the expected results, one can approximate the level of distortion in the samples under study.

3.5. The PCR-enhancing compounds were unable to reduce artifacts when other PCR conditions were suboptimal

Seven PCR-enhancing compounds with different modes of action were tested for their ability to decrease amplification of artifacts (Supplementary material 7). DNA extracts from soil (in three replicates) were amplified alongside with the model bacterial community template (one replicate). Primer set D was used for amplification with all polymerases. Acetamide and betaine reduced the number of faint non-specific bands in a profile of a model bacterial community in reactions with BioTaq polymerase. However, none of the additives helped to amplify templates with DNA extracted from soil. Surprisingly, AccuPrime polymerase produced a range of non-specific products in control reactions when no additives were added. Betaine was the only compound that almost eliminated artifacts. However, similar to BioTaq, no amplification of samples with templates from soil was obtained.

DreamTaq, OneTaq and Phusion polymerases were able to amplify templates with DNA from soil, but the profile of the model bacterial community was so altered that the results of these reactions were unreliable. None of the PCR-enhancers were able to reduce the PCR artifacts in reactions with these polymerases. The aim of this study was to demonstrate the usefulness of the model mixed sample as the amplification control for multi-template PCR. The thorough optimization of all PCR components, including co-solvents was beyond the scope of this study. Thus, only one concentration of PCR-enhancing compounds was tested. The lack of effect of the tested co-solvents on the outcome of the multi-template PCR in this study does not mean the absence of the effect of the same compounds under different conditions.

4. Discussion

The primary goal of this work was to demonstrate the need of firm criteria to control the performance of PCR assays on mixed templates. One of the key criteria for such assays could be the performance of amplification controls under tested conditions — the golden standard for all

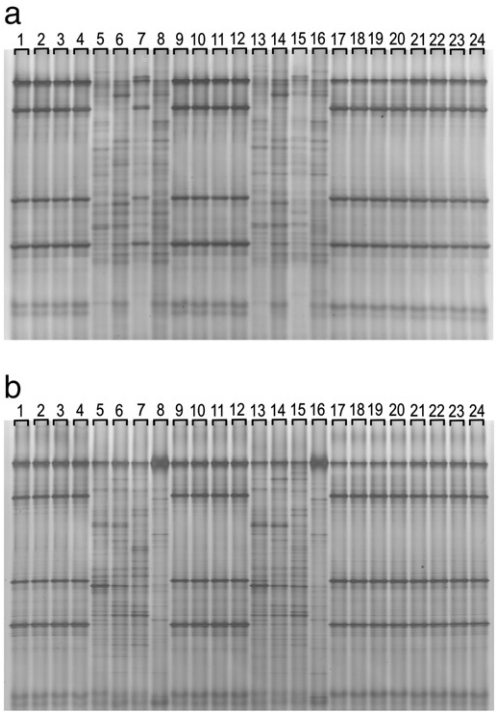

Fig. 2. The performance of the external and internal controls and the result of the corresponding PCR assays. Samples of DNA extracted from the rhizosphere microbial communities inhabiting roots of *Tussilago farfara* (a); *Taraxacum officinale* (b) were used to demonstrate the effect of inhibitors and the performance of the semi-internal control under the corresponding conditions. Amplicons were obtained with BioTaq polymerase by nested PCR with the primer Sets D (lines 1–12) and primer Set J (lines 13–24) for the primary reaction and with the primer Set N for the second PCR reaction. PCR conditions were adjusted until the amplification succeeded in the rhizosphere samples. The profiles of the external control (lines 1–4, and lines 21–24); the profiles of the semi-internal control (lines 9–12, and lines 17–20); the profiles of the rhizosphere bacterial communities extracted from the roots of four individual plants (the same samples were used for the both primer sets) (lines 5–8, and 13–16). PCR products were separated on PAAG gels contained a porosity gradient of 6–12% and a denaturant gradient of 45–65% for 16 h at 75 V.

PCR techniques. However, in order to work successfully, amplification controls should fit the purposes and nature of the particular PCR application. We demonstrated the efficiency of external and semi-internal controls to monitor characteristic biases of the multi-template PCR and have also shown that the wrong conclusions could be drawn practically on each step of the assay in the absence of such controls.

4.1. Mixed templates possess inbuilt properties that unavoidably lead to PCR artifacts and biases

While numerous manuals provide a detailed discussion on conditions influencing the quality of PCR in general, relatively little has been published regarding important experimental factors and common difficulties encountered with mixed template PCR. Amplification of homologous sequences in a mixed template is a special case of PCR application. Users need to recognize the differences in PCR environments when there is a mix of homologous targets instead of a single target that is amplified *en masse* in the same reaction. In fact, the presence of many targets with high levels of homology provided a breeding ground for PCR-induced artifacts in our study. In addition, the window for optimal PCR conditions that exists for a reaction with a single target narrows considerably when the number of homologous targets increases. However, until now few attempts have been made to understand the scale of the problem with PCR-induced artifacts caused by increased complexity of the template.

4.2. Factors that affect the rate of PCR-induced artifacts and biases

4.2.1. The choice of the correct primer set is crucial

In a single template PCR the failure of the primers is recognized by i. the absence of the target sequence; ii. the presence of non-specific product(s); and iii. the 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 is estimated. In PCRs with mixed templates it is difficult to implement the same principles, due to the presence of multiple targets. However, at this stage no criteria have been developed to evaluate the competence of primers for this particular PCR application.

In the present study, 72 different combinations of primers, commonly used for studying microbial communities were examined in the aim to choose 2–3 optimal pairs. However, the practical criteria for choosing an optimal primer set were absent. All combinations of primers were able to amplify complex microbial communities. As was expected, the amplification profiles produced by different primer sets varied greatly (data not shown). That was not a surprising finding. Previous work (Shannon et al., 2008; Hong et al., 2009; Neilson et al., 2013) had already demonstrated that some primers failed to amplify all targets with the same efficiency when a mixed-template is used in the reaction despite being able to amplify a broad spectrum of 16S rDNA sequences in single-template PCR. Still, the practical question remained as to the criteria on which the competent primer sets should be selected for. It is tempting to take the maximum number of phylotypes in the final PCR products as a criterion for selection of the optimal primer set or other PCR conditions (Hongoh et al., 2003; Acinas et al., 2005). Yet, the increasing number of amplicon types might be due to the presence of artifactual DNA molecules. Thus, optimization of the multi-template PCR assays based on achieving higher number of amplicon fractions is a questionable approach. In this study a model community containing genomic DNA from four bacterial species was used to find optimal primer combinations and PCR parameters.

The effect of primer mismatches is poorly detectable when PCR is seeded with single templates, because to some extend all polymerases are able to generate product in this reactions. This effect can also be overlooked when a mixed template with unknown profile is amplified. For example, in this study, the primer set B, among others, was selected in a pilot experiment after screening using a native bacterial community. However, further testing on the model community disclosed a tendency of this particular primer set to discriminate AT-rich templates when amplified in a mixture. The effect was consistently repeated with all polymerases tested.

Thus, a model mixed template is a helpful tool to select the optimal polymerase and primer combinations. Ideally, a commercial kit with polymerase, suitable primers and a model mixed template should be developed. The sequences in such model template should have a complete spectrum of mismatches with primers and the adjustments of PCR conditions can be monitored by the changes in the profile of the model community.

4.2.2. The significance of polymerases has been underestimated

Polymerase behavior in PCR reactions with single template differs from that of mixed template. In a single-template PCR polymerase has unique target sequence and one possible combination of primer–template duplex. In reactions containing multiplex and mixed templates, the respective PCR polymerase has more opportunities to manifest preferences among slightly different primer–template combinations (Henegariu et al., 1997) and different characteristics of the particular enzyme can lead to a different spectrum of PCR artifacts.

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 mixed template reactions — their relatively low processivity. Since the number of nucleotides that the respective polymerase is able to incorporate during one binding event is much

less than the length of the amplified template, uncompleted DNA strands can dissociate from a primary template and serve as primer in the next cycle. In single-template PCR it does not cause any serious problem, since there is only one type of target sequence and in the next cycle the incomplete nucleotide strand can hybridize only with the identical template. However, in a mixed template uncompleted DNA strand can bind to any available complimentary sequence and then its amplification can create a chimeric DNA molecule. Since longer DNA sequences recognize complementary strands at a higher rate than do shorter sequences (Suzuki and Giovannoni, 1996), the efficiency of annealing of partly amplified DNA molecules to template is higher than the rate of primers. Probably, the high level of artifacts induced in PCRs reactions catalyzed by DreamTaq and Phusion DNA polymerases can be explained by their low processivity, since they belong to the B family (judging by patents US5500363 and US7425423 respectively) although the manufacturer of Phusion polymerase claim it to possess enhanced processivity.

In the absence of proper standards, it is difficult to predict the rate and the nature of artifacts that each polymerase can induce. This is especially true for the reactions with mixed templates. All properties of enzymes, as described in the manuals are determined in PCRs with single templates. None of the manufacturers provides information about the behavior of their polymerase in reactions with mixtures of homologous genes. Wu et al. (2010) found that amplification of the same sample with two different polymerases (PfuUltra II Fusion HS, Stratagen and Ex Taq, Takara) showed two different community structures. However, without firm criteria for choosing the right enzyme it is impossible to judge which of the two tested polymerases provided correct results. In this study, DreamTaq or Phusion should be the first choice for the further experiments if the criteria for selecting the optimal polymerase were the yield of the PCR product. Nevertheless, a simple test with the model template demonstrated that these polymerases are not suitable for amplification of homologous sequences in PCRs with mixed templates. Our results demonstrating the high level of artifactual amplicons generated by Phusion polymerase support the findings of Lahr and Katz (2009) regarding the same enzyme. In their experiments, 60% to 65% of amplicons generated by Phusion polymerase were recombinant sequences. In experiments performed by Aird et al. (2011), genomic libraries amplified with AccuPrime polymerase were less skewed then libraries amplified with Phusion. These results should not let readers come to the conclusion that polymerases that produce artifactual DNA molecules in a mixed template are defective enzymes; these polymerases are simply not suitable for this particular purpose while they can be indispensable for other applications. In this study, only BioTaq and AccuPrime polymerases were able to correctly reproduce the structure of the model community when given two particular primer sets. These correct primer–polymerase combinations were only possible to be determined by an experimental approach using a model template as an amplification control.

4.2.3. Templates should be maximally preserved from degradation and damage

Among many factors that determine the sensitivity, accuracy and reliability of PCR assay template quality is one of the most important determinants of reproducibility and biological relevance. The template quality is a combination of many parameters, including complexity of target and neighboring sequences, the level of DNA/RNA damage, and the presence of inhibitors.

Amplification efficiency can vary across a genome (Veal et al., 2012). Genomic regions resistant to amplification by PCR correlate with high GC content (Wintzingerode et al., 1997; McDowell et al., 1998) that do not denature efficiently under routinely used conditions. Another problem is that some sequences, for example single stranded 16S rDNA, are potentially prone to secondary structure formation during extension; which may cause the polymerase to fall off (Gutell et al., 1994).

Since resistance to denaturation can selectively prevent some templates from being amplified, it is tempting to solve this problem by tightening denaturing conditions. However, when double-stranded DNA molecules separate into single-stranded DNA under denaturing conditions, these single strands became more susceptible to hydrolytic attack, oxidation and depurination, thus increasing potential for polymerase-induced errors (Pääbo et al., 1990; Eckert and Kunkel, 1991; Booth et al., 2010). Thus, it is important to monitor the efficiency of denaturing conditions during PCR to ensure that GC-rich templates are available for amplification while at the same time prevents templates from being damaged due to unnecessary stringent settings. The adequacy of denaturing conditions can be adjusted on a model template by monitoring the efficiency of amplification of GC-rich sequences included in a model mixture. In addition, molecular mimics that simulate targets with extreme resistance to denaturation can be included in model mixtures; for example, one of the sequences can be embraced by GC-rich clamps and by the appearance of its amplicons in the final PCR product the efficiency of the denaturation step can be supervised.

4.2.4. Well-balanced supplemental buffers widen the window for non-biased PCR conditions

There is a large database of evidence on how PCR parameters affect the appearance of PCR artifacts in a mixed template (Wagner et al., 1994; Hongoh et al., 2003; Acinas et al., 2005; Jae-Hyung et al., 2006). Acinas et al. (2005) reported considerable differences in sequence diversity between two cloned libraries obtained from the same sample but amplified under the different PCR conditions.

We believe that PCR conditions can be discussed only after the appropriate primer–polymerase combination is determined. In this study polymerase–primers combination was the most significant factor responsible for minimizing PCR artifacts. Inappropriate polymerase or primer sets generated the rate of amplification errors that was beyond mitigation by optimization of PCR protocol. In our study, the amplification of the model community under a range of annealing temperature neither changed the rate of PCR bias caused by Phusion polymerase, nor influenced the outcome of reactions with BioTaq polymerase. The unchanged behavior of PCRs under a wide range of conditions can be explained by the considerable amendments of the modern PCR buffers. Thus, new PCR buffers widen intervals of PCR robustness, particularly for the annealing and extension temperatures.

4.2.5. The inhibition compatibility of the samples should be evaluated

PCR is an enzymatic reaction and therefore sensitive to inhibitors. PCR inhibitors can originate from the sample or be introduced during sample processing (for a review see Wilson, 1997). Inhibition may be total or partial and can manifest itself as a complete reaction failure or as a reduced sensitivity of detection (Nolan et al., 2006; Huggett et al., 2008).

Huggett et al. (2008) stated that if two PCRs are compared it is important that both reactions are affected by potential inhibitors to the same extent. They called it inhibition compatibility and argued for showing proper attention to its measurement.

The presence of inhibitors being a difficulty for a single-template PCR became even more complex problem for reaction performed on a mixed template (Weissensteiner and Lanchbury, 1996; Wintzingerode et al., 1997). First, the amplification of homologous targets can be inhibited differently by the same level of inhibitor, thus inducing template-to-product skew PCR bias. Second, non-specific blocking of DNA template may increase the amount of partially amplified products which could then serve as primers in the next cycles increasing the number of chimeric amplicons in the final product (Waterhouse and Glover, 1993). Third, inhibitors can increase the rate of polymerase-induced errors by interfering with the availability or activity of essential reaction components. Considering the severity of this problem it is very surprising that, to our knowledge, no systemic studies of PCR inhibition in a mixed template have been reported.

We propose to implement a semi-internal control to estimate inhibition compatibility of samples with mixed templates. The inclusion of this control into PCR assays allows (i.) comparing activity of inhibitors between different samples; (ii.) locating intervals of PCR robustness

for different samples and thus enable their correct comparison; and (iii.) estimating and monitoring the level of PCR-induced errors.

4.2.6. PCR additives possess restricted potential to decrease the level of artifacts in mixed template PCR

Different PCR-enhancing compounds can be used to overcome some problems with amplification of recalcitrant templates. Usually the difficulties with amplification are connected to GC-rich sequences (Chakrabarti, 2003; Kang et al., 2005), however, AT-rich templates can also be challenging to amplify (Chevet et al., 1995).

The effects of PCR-enhancers on amplification of recalcitrant sequences was thoroughly studied in a single-template reactions, and there were very few, systematic attempts to evaluate the effects of these compounds in PCR with complex templates (Baskaran et al., 1996; Weissensteiner and Lanchbury, 1996). All co-solvents, selected for our experiment, were shown previously to depress the melting temperature of DNA molecule. However, they were shown to decrease enzymatic activity and the thermostability of the Taq polymerase at the same time (Chakrabarti, 2003). It is a challenge to find the optimal concentration of enhancing compound even in PCR with a single template (Kovarova and Draber, 2000). Targets in a mixed template have very diverse properties so it became even more difficult to optimize PCR conditions in order for all templates to have an equal chance to be amplified. Low concentrations of PCR enhancers might be ineffective, while at high additive concentration the decline in polymerase activity and thermodegradation decreased product yield by increasing the minimum extension time necessary to complete the replication of a template. In addition, the same enhancing compounds can cause ambivalent effects on the amplification of AT- and GC-rich templates. Altogether the presence of multiple targets with different properties left a very narrow window for the optimization of PCR additive concentrations. In our study, only betaine slightly decreased the number of faint non-specific bands in amplification with BioTaq polymerase under the tested concentrations. The absence of any effect of PCR-enhancing compounds can be partly explained by both the well-balanced conditions provided by BioTaq and AccuPrime supplemental buffers and by the presence of unspecified additives in commercial buffers which interact with added PCR enhancers.

4.2.7. The efficiency of the post-PCR treatment of artifactual amplicons depends on the types of PCR-induced errors

It is possible to reduce a number of particular artifacts by post-PCR treatment of the amplicons. First, the number of heteroduplex molecules can be reduced by digestion with exonuclease (Jensen and Straus, 1993; Qiu et al., 2001; Egert and Friedrich, 2003). However, the stretch of ssDNA must be long enough to allow the recognition and cleavage by the enzyme. The second type of artifacts that can be corrected, at least partly, by post-PCR treatment is the presence of amplicons, which are single-stranded at the terminal sites. Incubation of PCR products with Klenow fragment converts partially single stretches of amplicons to the relevant double-stranded molecules (Egert and Friedrich, 2005). Our attempt to reduce artifactual amplicons by the treatment with Klenow fragment was not successful, in contrast to the very noticeable effect demonstrated by Egert and Friedrich (2005). This may be explained by the differences in PCR conditions (primer set, polymerase, supplemental buffer, composition and condition of the template, presence of inhibitors), that lead to a different spectrum of artifacts. Probably, the conditions that occurred in our PCR tubes were less favorable for generation of amplicons with single-stranded termini. In agreement with results by Egert and Friedrich (2005), treatment with exonuclease also reduced (though slightly) the number of non-specific bands in the profile of the model bacterial community in our experiment. However, one should take into account that the rare sequences almost completely form heteroduplexes with other, more abundant sequences (Ruano and Kidd, 1992; Ruano et al., 1994). So, treatment with exonuclease might lead to the complete loss of the rare products.

4.3. Post-PCR visualization method cannot alleviate errors occurred during PCR

For reasons that are not fully understandable, the PCR bias and artifacts were sometimes attempted to be explained by defects of post-PCR analysis; and DGGE was the most frequent method to be blamed (e.g. Neilson et al., 2013). This would be equivalent to accusing agarose gel electrophoresis for a wrong profile obtained by cutting a plasmid with restriction enzymes. The sensitivity and adequacy of DGGE and related methods (non-denaturing PAAG, double gradient denaturing gel electrophoresis (DG-DGGE), temperature gradient gel electrophoresis (TGGE), denaturing gradient high performance liquid chromatography (DG-HPLC)) were confirmed by a long history of use in many research fields, even in of polymerase fidelity assays (Keohavong and Thilly, 1989). However, we agree with the idea that these methods should be systematically tested for their suitability and the interval of robust performance when employed for a new application, particularly, for detection of multiple products.

There is a superstition that modern technologies, like massively parallel sequencing, can solve the problem of PCR bias in a mixed template. Yet, sequencing of PCR products is just another way of visualization of results, and none of the post-PCR visualization methods can reduce artifacts, which occurred in a previous step. PCR is widely employed to amplify DNA fragments before they are processed for sequencing. In addition, the majority of current high-throughput parallel sequencing methods involve a step of PCR amplification. Those researchers who examined modern visualization techniques for the presence of artifacts found the same spectrum of PCR-induced errors (Gorzer et al., 2010; Kunin et al., 2010; Morgan et al., 2010; Wu et al., 2010; Aird et al., 2011; Schloss et al., 2011; Fonseca et al., 2012).

4.4. Strategy for controlling and limiting the production of errors during assays

Many recipes for decreasing or mitigating PCR-induced artifacts have been proposed (Wagner et al., 1994; Qiu et al., 2001; Acinas et al., 2005; Ahn et al., 2009). However, the microconditions in each PCR tube and at each particular cycle of reaction are unique and settings that can be optimal for one reaction can became error-generating for the other (Bastien et al., 2008). There are too many factors and interactions that can drive the particular PCR system into a biased state, so a singular reason could not be derived (for review see Bustin, 2012).

4.4.1. External control for mixed template PCR should incorporate multiple targets

All PCR applications that require confidence should be designed to control for false results. Two kinds of controls can be employed to monitor PCR: an external and an internal control. An external control is an amplicons added to the PCR and allowed to amplify so as to verify the integrity of one or more reagent(s) in the cocktail (Lee et al., 2004). For most molecular tests, the external control is the only control that can be implemented. However, PCR has an added advantage that it can be readily multiplexed to include internal controls. An internal control is a second target molecule that can be amplified with, but distinguished from other products in the same tube (Lee et al., 2004). Implementation of external and internal controls is the golden standard of PCR-based tests for forensic and diagnostic applications, where demand for reliability is high. So this standard should also become the norm for other applications of PCR methods.

Model communities have been successfully used to evaluate the influence of different factors on the level of PCR-induced artifacts in a mixed template (Zylstra et al., 1998; Jae-Hyung et al., 2006; Frey et al., 2006; Ahn et al., 2009; Kunin et al., 2010; Morgan et al., 2010; Schloss et al., 2011) The next logical step would be the inclusion of model mixed templates as an amplification quality control into routine PCR assays.

We consider that an external control for amplification of homologous sequences from mixed templates should represent a mixture of targets that can be amplified with the same primer sets. We emphasize the need for a mixture of targets rather than a single template, because a single target does not allow controlling the generation of PCR artifacts due to the presence of homologous sequences (chimeras, heteroduplexes, and ratio screw). Sequences combined in a model template should ideally have a broad range of GC content to cover the whole spectrum of variation in native targets. Differently designed molecular mimics can be included into a mix to control particular PCR artifacts or difficulties. For example, sequences embraced by GC-rich flanking regions can be included to monitor the efficiency of the denaturing step. The behavior of this template can help to adjust the conditions in such way that they allow melting of resistant templates but at the same time maintaining the denaturation settings as relaxed as possible to prevent ssDNA degradation and damage.

4.4.2. Semi-internal control aims to measure inhibition compatibility of different samples

An external control only monitors the reliability of the PCR composition and cycling conditions. It does not allow identifying and testing samples for inhibition of the PCR reaction, or using the term coined by Huggett and co-workers as 'the inhibition compatibility' of the samples. Ideally the internal control should be used for these purposes. However, when samples themselves represent mixes of homologous targets the products of their amplification cannot be distinguished from amplicons of an internal control. Still, it is very important to estimate the presence of inhibitors in samples and adjust the PCR mix and cycling conditions to obtain the lowest possible level of artifacts. We proposed to employ a hybrid between the two approaches — a semi-internal control, when a model mixed template is amplified in the same tube with an aliquot of a DNAse-treated sample. After treatment with DNAse, the sample represents a DNA-free solution with the original cocktail of inhibitors. In addition, the outcomes of the amplification of the model template in the presence of the inhibitors can to some extent predict the behavior of the native targets under similar conditions. Ideally, amplification on a dilution series of the model template with the same concentration of a DNA-free sample solution should be performed because at a high concentration the control might not detect a weak inhibition that could cause drop-off of the targets present in a low copy numbers of the original sample (Hoorfar et al., 2004). When applied together, external and semi-internal controls can monitor and detect a considerable part of PCR-induced artifacts.

The encouraging results from this study were that under the same PCR conditions the profiles of external and semi-internal controls were almost identical. The possible explanation for this phenomenon in these particular cases could lie in the suboptimal PCR conditions rather than in inhibitors present in the samples that increased the development of PCR artifacts. In the tested samples inhibitors could probably act indirectly, by sequestering of Mg^{2+} cations. However, under other circumstances, the behavior of the semi-internal control would constitute the better indicator of the performance of the multi-template PCR then the external one.

Ideally, both controls should be available as commercial products. If possible, it should contain a panel of mixes with differing complexity, e.g. ranging from ten up to hundreds of homologous sequences. Users should be able to choose the appropriate control based on the expected complexity of the studied community and detectable capacity of the visualization method. For example, if massively parallel sequencing is chosen for post-PCR analysis, and researchers report detection of several thousand different OTUs, then a control mix that includes at least a hundred templates must be used to support an unbiased procedure. The performance of the amplification control must be reported as in Kunin et al. (2010). Using uniform controls would facilitate comparison of experimental quality and results produced by different research groups.

4.5. Accounting for errors during data analysis

Efforts should be undertaken to minimize both the production of artifacts and their impact on final results because PCR-induced artifacts can be generated even in the presence of quality controls.

Pompanon et al. (2005) argued that the best way to deal with errors in genetic studies is to set up a strategy that is appropriate for the particular situation and apply a strict error-detection policy on every step of the experiment.

To minimize the effect of errors, attention should be paid on choosing statistic methods that are robust to errors (Akey et al., 2001). For example, when comparing different treatments based on PCR-assays in mixed templates, the choice should be in favor of qualitative rather than quantitative approaches (e.g. Neilson et al., 2013). In the absence of quantitative amplification control and appropriate number of replicates, quantitative results remain questionable regardless of the method (PAAG, cloning, DHPLC, or massively parallel sequencing) used for post-PCR analysis.

It is difficult and sometimes impossible to distinguish between PCR-induced errors and rare alleles in population studies. Thus, whatever technology is used to visualize PCR results, caution should be taken to draw conclusions when difference between treatments might be due to variations in rare alleles. Ideally, the tolerable error rate should be clearly communicated in the method section of the publication.

5. Conclusions

Amplification of homologous sequences in a mixed template should be recognized as a special application of PCR technology which is extremely prone to PCR-induced artifacts and has a narrow window for optimal conditions. Following ready-to-use protocols is dangerous because they do not include error-detection strategies and therefore leave artifacts unnoticed. To prevent potentially misleading results, systematic investigations should be undertaken to evaluate the level of PCR-induced bias during the amplification of homologues sequences in a mixed template. Furthermore, multi-template PCR assay must be validated for the different levels of template complexity. External and semi-internal controls presented in this study aim to monitor the major types of the amplification biases characteristic to multi-template PCR. Ideally model mixtures should be available as commercial products to reduce the time for their preparation and to facilitate comparison of results between research groups. Model mixtures can be of different complexity, fitting the detection capacity of the post-PCR analysis. The performance of amplification controls must be clearly shown in the publication. Unless appropriate methodological section in research papers is ensured, any data generated on rampant PCR assay will be questionable. In the absence of amplification controls PCR assay can be nothing more than a voodoo ritual.

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