Biomolecular Detection and Quantification 2 (2014) 11–29

Contents lists available at ScienceDirect

Biomolecular Detection and Quantification

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

Review Article

Multi-template polymerase chain reaction

Elena Kalle[a,1], Mikael Kubista[b,c], Christopher Rensing[d,*]

[a] Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Allmas alle 5, 75007 Uppsala, Sweden
[b] TATAA Biocenter, Odinsgatan 28, 41103 Göteborg, Sweden
[c] Institute of Biotechnology, Czech Academy of Sciences
[d] Department of Plant and Environmental Sciences, Faculty of Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 25 April 2014
Received in revised form
25 November 2014
Accepted 26 November 2014
Available online 4 December 2014

Keywords:
Multi-template PCR
Chimera

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.

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.

* Corresponding author. Tel.: +45 29356765.
E-mail address: chres@plen.ku.dk (C. Rensing).
[1] Current address: High-Tech Hub, National Research Institute of Agricultural Biotechnology, Timiryazevskaya 42, 127550 Moscow, Russia.

http://dx.doi.org/10.1016/j.bdq.2014.11.002

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