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Research Article

Comparing two conventional methods of emulsion PCR and optimizing of Tegosoft-based emulsion PCR

The selection of aptamers represents a promising route in the development of high affinity ligands. In these processes the formation of by-products is a common problem during the PCR-based amplification of complex oligonucleotide libraries. One approach to overcome this drawback is to separate each template oligonucleotide into an individual reaction compartment provided by a droplet. This method, termed emulsion PCR (ePCR), has already emerged to a standard method in sample preparation for 2nd generation sequencing. In this work, we compare different literature protocols that have been developed to generate stable emulsions for ePCR. We investigate different emulsification methods and evaluate the importance of the initial template concentration. We demonstrate that emulsion stability is of utmost importance for the successful inhibition of by-product formation and give an optimized protocol for generation of an emulsified PCR.

Keywords: Aptamer / By-product formation / Emulsion PCR / Polymerase chain reaction / SELEX



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1 Introduction

Modern biology and medicine rely heavily on the use of specific ligands for molecular recognition of biomarkers or other molecules. Historically, this field is dominated by the use of antibodies that are produced by either immunization of animals and subsequent harvesting of antibodies or by large-scale cell culture. However, in the past 20 years the generation of artificial ligands has made substantial progress. One type of artificial ligands is represented by single-stranded oligonucleotides, termed aptamers. Depending on their sequence and applied conditions (e.g. buffer composition) these aptamers fold into specific 3D structures. In their folded state aptamers are able to bind to a variety of targets such as ions, small molecules, and peptides as well as to proteins or cells [1]. Aptamers are usually described as high affinity ligands with a distinct specificity for their corresponding target, which makes them excellent ligands for affinity

purification [2–4] and offers great potential in sensing applications [5–7]. The aptamer-target binding is based on electrostatic interactions, van der Waals interactions, and hydrogen bonding that are nonspecific by nature, but the synergistic combination of these interactions may result in a high affinity binding. Consequently, aptamers do display high specificities for their target, due to an excellent structural match with the target.

Aptamers are generated via an iterative selection process called systematic evolution of ligands by exponential enrichment (SELEX). SELEX typically is initiated by incubation of a target molecule with an oligonucleotide library consisting of 10^{14} – 10^{15} different oligonucleotides. All oligonucleotides are composed of a central randomized region that is flanked by defined priming regions. After incubation of library and target, unbound oligonucleotides are washed away, while bound oligonucleotides are later recovered by elution. Subsequently, these recovered aptamer-candidates are amplified using PCR. Following a strand separation, the enriched library of aptamer candidates is subjected to the next round of selection and amplification. Typically, it takes 8–15 rounds of selection to enrich high affinity aptamers from a randomized library [8]. After sufficient enrichment the aptamer-candidates are commonly identified by cloning and sequencing. Once identified, affinity and specificity can be assessed for each aptamer candidate individually. Despite employing only

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Abbreviation: SELEX, systematic evolution of ligands by exponential enrichment

standard methods the success rate of conventional SELEX is only about 30% [9].

Since the beginning of aptamer development many technologies have been applied to overcome inherent barriers and limitations of SELEX. Here, we only give a short overview of major improvements, while in-depth reviews on these topics can be found elsewhere [1, 9, 10]. A core step in SELEX is the separation of bound and unbound aptamer-candidates after incubation with the target. Today, highly efficient microfluidic, bead-based, or electrophoretic partitioning techniques are most utilized in aptamer selection, while traditional SELEX relied on nitrocellulose filter binding [9]. Another key factor for SELEX success is the starting library, as the structure of an aptamer and its possible interactions with the respective target directly depends on the aptamer's sequence. Therefore, an increasing effort has been put on modifying starting libraries. These modifications include optimized motif distribution, degenerated libraries of a known binding motif or secondary structure and the use of artificial nucleotides with an extended nucleotide alphabet [11, 12]. Furthermore, downstream identification and characterization of aptamer-candidates is greatly facilitated by 2nd generation sequencing and high-throughput characterization [10]. Altogether these improvements have raised the overall success rate of SELEX to $\approx 85\%$.

Although, this suggests an overall improvement of SELEX processes, little attention has been given to the particular field of aptamer-candidate amplification. Researches frequently report the formation of by-products in conventional PCR, but only a few publications address this issue. In recent years, it has been discovered that different PCR mechanisms apply to complex libraries in comparison to PCR of a single amplicon. Nonspecific primer annealing and the formation of primer dimers, which are the main source of by-products in conventional PCR, seem to be outpaced by product–product hybridization (transpriming) of partially homologous sequences that occurs during amplification of complex libraries once a critical template concentration is reached [13–15]. In further investigations Tolle et al. discovered mechanisms that lead to the formation of “ladder-type” and “nonladder-type” by-products by sequence analysis [16]. Beside the formation of by-products, the product formation in PCR is biased toward shorter or structurally less stable sequences and sequences that match the polymerase sequence preference (PCR bias) [10, 17]. Overall, studies demonstrate that conventional PCR results in a library diversity loss of $\approx 50\%$ within each PCR [18].

To overcome these drawbacks, conventional PCR has been adapted to SELEX conditions by careful optimization of reaction parameters [13, 19]. Furthermore, limitation of PCR cycles and real-time PCR also has been applied to avoid overamplification [20]. Other approaches include multiple partitioning steps between each amplification step (non-SELEX [21]) or reduction of selection cycles by using powerful partitioning methods, such as CE, ultimately leading to a one-step selection [22, 23]. Although these optimizations are able to reduce the amount of by-products, the formation of by-products and the PCR bias still are a major problem in SELEX processes.

However, in past years another PCR variation has emerged in the preparation of 2nd generation sequencing libraries, which is also prone to by-product formation and PCR bias. In order

to prevent transpriming each member of the initial library is encapsulated in a separate PCR droplet that is surrounded by a hydrophobic organic phase. This method is called emulsion PCR (ePCR). Due to this separation each target is amplified individually as each droplet only contains a single species. Consequently, ePCR has been found to significantly reduce the PCR bias and the formation of by-products to a nondetectable level while preserving library diversity at the same time [17, 18]. Thus ePCR should be considered the standard method for amplification of aptamer-candidates during SELEX.

The current literature features two different protocols for emulsification of a PCR mixture: While Williams et al. describe the formulation of an emulsion PCR based on a mixture composed of mineral oil supplemented with the detergents Triton-X-100 and Tween 80 [14], another organic phase for ePCR was developed by Diehl et al. using the emollient Tegosoft DEC mixed with mineral oil and the emulsifier ABIL WE 09 [24]. Both methods aim to create a stable emulsion that is a challenging task considering the high temperature applied during PCR. Based on the protocol of Williams, Shao et al. evaluated and optimized different parameters for ePCR, e.g. the starting template concentration, the annealing temperature, the primer concentration, and the polymerase concentration [15]. They demonstrated that the initial target concentration is the most important parameter of an emulsion PCR besides the emulsion stability. Following the Poisson distribution, the starting concentration defines the number of different oligonucleotides that will be encapsulated in the same droplet. Reaching a critical number, templates will start to form by-products within the droplet again [15]. Additionally, PCR bias will be inevitable at high target concentration. Another important parameter is the concentration of BSA that is supplemented to the PCR mixture. Williams stated that BSA is essential in ePCR to saturate the aqueous/organic interface with a “bulk protein” to protect the polymerase from getting inactivated at that interface [14]. Shao et al. required a high amount of DNA polymerase as they did not add BSA to the aqueous PCR phase [15]. However, BSA and DNA polymerase concentration should be optimized for each PCR and emulsification protocol individually [25].

In this study, we compare the most common protocols for PCR emulsification with focus on emulsion stability, by-product formation, and ease of preparation.

2 Materials and methods

2.1 Chemicals and materials

Mineral oil, ethyl acetate, Tween 80, and Span 80 were purchased from Sigma-Aldrich (Munich, Germany). Platinum[®] Pfx DNA Polymerase, and dNTPs were purchased from Thermo Scientific (Rockford, USA). Tegosoft DEC and ABIL WE 09 were kindly supplied by Evonik (Essen, Germany). Diethyl ether, agarose, and Roti[®]-GelStain were purchased from Carl Roth (Karlsruhe, Germany). Tween 20 was purchased from AppliChem PanReac (Darmstadt, Germany). Isobutanol was purchased from Honeywell Riedel-de Haën (Seelze, Germany). DNA purifications were performed with QIAquick[®] PCR Purification Kit from Qiagen (Hilden, Germany) according to the manufacturer's protocol.

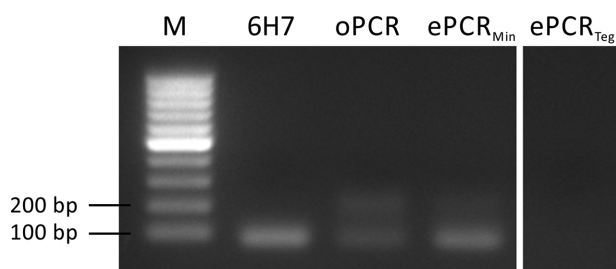


Figure 2. Comparison of organic phases for the emulsification of PCR (mineral oil mixture; ePCR_{Min}/Tegosoft DEC mixture; ePCR_{Teg}). 6H7 was amplified as a length standard. For clarification, only selected samples of the whole gel are shown.

in the open PCR control. On the other hand, the emulsion generated with Tegosoft remained white/creamy after thermocycling, indicating a stable emulsion. Surprisingly, no PCR product was produced in the Tegosoft emulsion PCR. However, both Williams et al. [14] and Schütze et al. [25] stated that the polymerase may become trapped at the emulsion interface. Therefore, they advise the addition of BSA and the optimization of its concentration. Considering the poor emulsion stability using the mineral oil mixture we focused on optimizing the Tegosoft protocol for ePCR. The effect of BSA was investigated by applying different BSA concentrations of 0 mg/mL, 0.5 mg/mL, or 1 mg/mL during PCR in a Tegosoft-based emulsion. Again the PCR products were degreased with diethyl ether/ethyl acetate, purified using the Qiaquick PCR purification kit (Qiagen) and analyzed via agarose gel electrophoresis (Fig. 3).

As observed before, no PCR product was formed using BSA concentrations of 0 mg/mL. Also a BSA concentration of 0.5 mg/mL was not sufficient to successfully perform ePCR. However, increasing the BSA concentration to 1 mg/mL led to the formation of PCR products without any detectable amounts of by-products. Therefore, all further emulsification of PCR mixtures was performed using the Tegosoft-based organic phase and BSA at the concentration of 1 mg/mL.

3.2 Preparation of emulsions-methods and important parameters

Besides the formulation of the organic phase, the emulsification technique is a key factor for the generation of a stable emulsion. Comparing the literature protocols, many emulsions have been

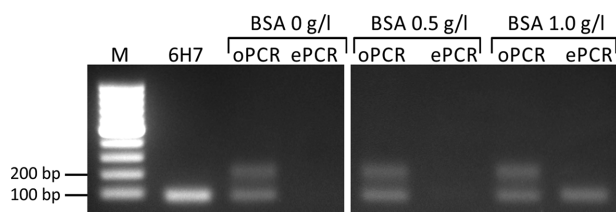


Figure 3. Effect of BSA concentration on amplification results of Tegosoft DEC-based emulsion PCR. For clarification, only selected samples of the gel are shown.

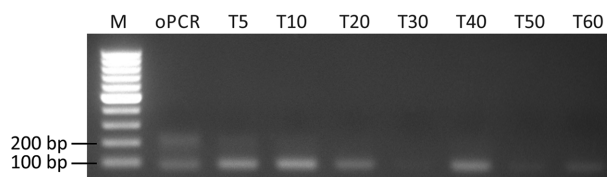


Figure 4. Formation of products and by-products in emulsion PCR, depending on time of emulsification. Emulsification times of 5, 10, 20, 30, 40, 50, and 60 s were tested and analyzed on 1.5% agarose gel with Roti®-GelStain.

generated using magnetic stirrers. In general, during this method the organic phase is continuously stirred while the PCR mixture is successively added in small portions. The resulting emulsion is then usually stirred for additional 5 min before performing PCR. Despite the emulsification of PCR with a magnetic stirrer seems to be the standard procedure, we instead focused on using a mixer mill (tissue lyser) as it is not only time efficient but also could be used for high parallel processing of many reactions. To assay the effect of mixing time on the emulsion stability, different Tegosoft DEC-based PCR emulsions were prepared by variation of the emulsification time in a ball mill at 30 Hz. The generated emulsions were subjected to 20 cycles of PCR, and PCR products were subsequently purified and analyzed by agarose gel electrophoresis (Fig. 4). Phase contrast microscopy was used to determine the mean droplet diameter for the emulsions prepared by mixing for 10, 30, and 60 s (see Supporting Information Fig. 1).

After thermocycling the emulsions of 5–20 s have partially separated into the designated phases indicating that these mixing times are insufficient for the generation of stable emulsions. The other investigated emulsions have been stable. Generally, mixing times between 30 and 40 s yielded in the highest product concentration without any detectable by-products. Here, droplet size is about 6 μm resulting in about 10^9 compartments. However, in this particular experiment emulsification of PCR for 30 s resulted in minimal amplification. Elongating the mixing time to 60 s lead to a decreased droplet diameter of 5 μm and increased the numbers of droplets by 75%. As the droplet diameter decreased, the amount of formed product is decreasing as well. This is consistent with theory considering that smaller droplets carry less PCR reagents, which will deplete earlier. This is not balanced out by the higher droplet amount, since many droplets remain empty with regard to target sequence. These experiments demonstrated that emulsions are best prepared in a mixer mill shaking for 40 s.

3.3 Overloading droplets-Impact of initial template concentration

The initial template concentration is another important parameter to consider during ePCR. Emulsion PCR aims to circumvent product–product hybridization by separation of the templates into individual droplets. However, the number of compartments is limited. Therefore, high target concentrations can lead to droplets that initially contain more than one single template.

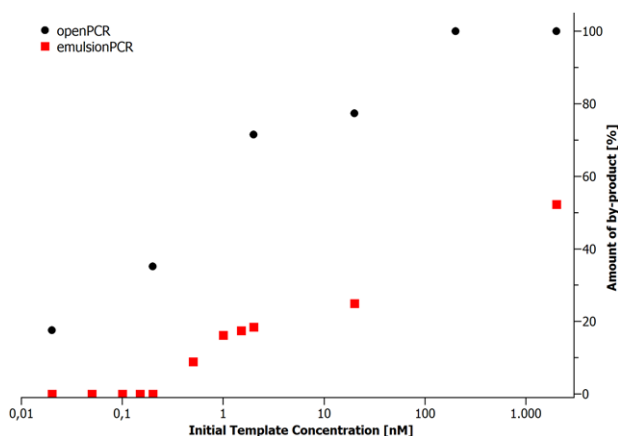


Figure 5. Concentration-dependent formation of by-products in open PCR and emulsion PCR. Amounts were determined by agarose gel electrophoresis and subsequent densitometric analysis of the corresponding band-pattern.

Increasing the initial template number per droplet raises the likelihood of product–product hybridization drastically and facilitates by-product formation. In contrast, too low template concentrations might result in low PCR efficiency due to loss of PCR reagents in droplets not containing a template molecule.

Here, we demonstrate that overloading of droplets with templates lead to by-product formation and therefore limit the ePCR's effectiveness. For this purpose, we performed ePCR and open PCR with an initial target concentration range between 0.02 and 2000 nM and compared the amounts of formed by-products (Fig. 5).

In the open PCR by-products are formed, even at low template concentrations. The amount of by-products steadily increases in the concentration range between 0.2 and 20 nM. Above the initial concentration of 20 nM nearly all oPCR product is transformed into by-product. For the ePCR, by-product formation starts at the concentration above 0.2 nM with a reduced degree of by-product formation in comparison to the open PCR control. At the highest investigated concentration of 2000 nM the amount of by-product reaches $\approx 50\%$. However, by-product formation is totally inhibited for starting concentrations up to 0.2 nM. This demonstrates the advantages of ePCR for the amplification of highly diverse oligonucleotide libraries.

4 Concluding remarks

PCR bias and the formation of by-products are common problems during the amplification of complex oligonucleotide libraries. To overcome these drawbacks, ePCR, which relies on the separation of templates into individual droplets, has been applied. We compared different literature protocols that have been developed to generate stable emulsions for PCR. During our experiments we found that emulsions based on Tegosoft were far more stable than emulsions based on mineral oil. However, initial ePCR attempts with Tegosoft-based emulsions failed. Successful amplification was achieved by increasing BSA concentration to 1 mg/mL. Therefore, we strongly advise the optimization

of BSA concentration. In addition to oil-surfactant comparison, different emulsification methods have been evaluated. In most literature protocols emulsions were generated by stirring with a magnetic rod. To allow parallel reaction setup and shorten the emulsification times we employed a bead mill instead. Notably, incubation time is a key parameter as short emulsification lead to instable emulsions but longer emulsification times decreased the obtained product amount. This is due to an increased droplet number, which do not contain a target sequence to be amplified. Beside these parameters being key factors for the emulsion stability, the initial template concentration is critical to circumvent the formation of by-products. High template concentrations result in the entrapment of multiple templates per droplet which then may form by-products. Therefore, ePCR's effectiveness is significantly reduced at high template concentrations. Our findings suggest that the tolerable template concentration can be determined by titration.

Overall, in this work we demonstrated the advantages of ePCR over open PCR for the amplification of complex libraries in regard to by-product formation and PCR bias. Moreover, we present key parameters for successful ePCR along with corresponding methods to optimize each parameter.

Practical Application

Amplification of complex oligonucleotide libraries via PCR is essential, e.g. for the selection of aptamers. This amplification is hampered by the formation of by-products and PCR-bias. To overcome these limitations, we have compared different protocols for emulsion PCR and optimized conditions for emulsion PCR. Besides providing optimized protocols, this study can also be used as a guideline for systematic optimization of emulsion PCR.

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