Optimization and design of oligonucleotide setup for strand displacement amplification☆

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Abstract

Several advantages of strand displacement amplification (SDA) as an all-purpose DNA amplification reaction are due to it isothermal mechanism. The major problem of isothermal amplification mechanism is the accumulation of non-predictable byproduct especially for longer incubation time and low concentrations of initial template DNA. New theoretical strategies to tackle the difficulties regarding the specificity of the reaction are experimentally verified. Besides improving the reaction conditions, the stringency of primer hybridization can be distinctly improved by computer based sequence prediction algorithms based on the thermodynamic stability of DNA hybrid a described by the partition function of the hybridization reaction. An alternative SDA mechanism, with sequences developed by this means is also investigated.
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1. Introduction

Strand displacement amplification (SDA) is based on the primer-directed nicking activity of a restriction enzyme and an exonuclease-deficient polymerase which is capable of initiating synthesis at a nick and displacing the downstream strand. The method exploits the ability of several restriction enzymes to create a nick on one DNA strand within a hemiphosphothioate form of its recognition site. Because of repeated nicking, strand displacement and priming of displaced strands, DNA will be exponentially amplified (see Fig. 3b). Since the development of the strand displacement amplification method by Walker et. al [1], many enhancements, particularly the use of thermophilic enzymes which allows the reaction to take place at incubation temperatures of 50–60 °C [2], have decreased non-specific background amplification and thereby turned the method into a sensitive and universal tool for amplification of nucleic acids.

In addition, isothermal amplifications systems like SDA, a general system which allows the amplification of a chosen target by the flanking primers, become increasingly important with the widespread use of microstructured systems, where thermal convection and expansion has to be circumvented. Another potential advantage of SDA is the heightened production of single-stranded DNA by asymmetric SDA, i.e. using two different primer concentrations that is important especially with respect to the use as a fluorescent probe for microarrays, single-nucleotide polymorphism, etc.

Several techniques to analyze the products by end-point detection using sandwich based assays [3] or primer extension [2] and real-time detection by fluorescence polarization [4] or fluorescence energy transfer [5] are described in the literature. These techniques allow the specific detection of a known and distinct DNA subsequence even if only few copies are present, and signal can be further amplified by enzymatic reaction. This makes SDA valuable in diagnostics [6]. However, byproducts and exhaustion of resources due to the synthesis of byproducts were not necessarily detected by these detection methods. A variety of rules for primer design in PCR are described in detail in the literature, see for example Ref. [7], and a wide choice of tools for oligonucleotide design in view of optimizing probe-target binding is publicly available. The applications range from primer search and analysis for PCR [8–11] to the calculation of probes for the development of large-scale projects in microarray analysis [12–16]. Most of these tools are based on several general criteria for oligonucleotide design like melting temperature, oligonucleotide length, product length and GC content, although some include secondary structure prediction by energy minimization using nearest neighbor energy parameters.

Since isothermal amplification systems are more susceptible to the occurrence and accumulation of non-specific products, none of these tools fulfill the needs of primer design satisfactorily for strand displacement amplification. In contrast to polymerase chain reaction, the temperature induced steps and thereby synchronization are missing. This non-specificity presents a problem, especially in its application to long amplification reactions like in vitro evolution [17,18], where the product is unknown and must be analyzed afterwards. As a result special care has to be taken in the optimization process of reaction conditions and oligonucleotide design. For this reason we searched for a way to improve the design strategy using the partition function method to calculate the base pairing

probabilities in thermodynamic equilibrium [19] and defining a criterion for elongation after binding by the initial primer and target sequences as well as all new build products. This allows the design of DNA oligonucleotides which are specific to their respective targets.

2. Materials and methods

2.1. Design of an oligonucleotide set

All calculations are based on the algorithms of the Vienna Folding Package (http://www.tbi.univie.ac.at/ivo/RNA/[20]).

For computation of the oligonucleotide design of standard SDA and nicking SDA (Table 1) the primer and template sequences were assembled from 6 DNA-words with 16 bases each [21]. The template is composed of 4 words, 2 words in the middle flanked by two primer binding sites. The primers in turn are made up of the recognition sequence of the restriction enzyme and 1 word forming the template binding sequence.

The discrimination of specific and nonspecific hybridization is based on the thermodynamic stability of the DNA hybrids formed, whereby the thermodynamic stability is described by the free energy of the hybridization reaction. The calculation of the probability distribution of alternative DNA/DNA duplex structures was done by computation of the partition function [19] and the free energy for the ensemble of structures for a sequence, which consists of the two binding partners connected via a spacer of 15 artificial nucleotides which are defined to have no binding properties. The length of the words was set to 16 nt as mentioned above, the GC content to 50% and the melting temperature to 55 °C calculated by nearest neighbor thermodynamics [22,23], where $T_m = \frac{\Delta H^0}{\Delta S^0 + Rln\frac{C_T}{4}}$, with the total nucleic acid concentration C.

Table 1
Primer and target sequences (recognition site in upper case and bold types)

System	Description	Sequence (5′ → 3′)
Standard SDA	Template tSDA	cgt tca tct cag tag caa gga cgt acc att ggg cgc aat ttg gta acc aca ctg tgc tga tct c
	Primer P(SDA)1V	cga ttc cgc tcc aga ctt CTC GGG cgt tca tct cag tag c
	Primer P(SDA)2R	acc gca tcg aat gca tgt CTC GGG gag atc agc aca gtg t
	Internal probe (R6G-5/6)	gcg caa ttt ggt aac c
Nicking SDA	Template tSDAnick	is equivalent to tSDA
	Primer P(SDA)7V	cga ttc cgc tcc aga ctt GAG TCa aaa cgt tca tct cag tag c
	Primer P(SDA)8R	acc gca tcg aat gca tgt GAG TCa aaa gag atc agc aca gtg t
Minimal SDA	Template SDAmin	tgc act ctg gaa ttt taa agg gaa cac tgg
	Primer SDAminR	ctc gat cat ctc acc CTC GGG cca gtg ttc cct tta
	Primer SDAmin5V	agg act gac gca taa CTC GGG tgc act ctg gaa ttt

In standard SDA, the restriction enzyme *Bso*BI, in the nicking SDA the nicking endonuclease N.*Bst*NBI is used (see Materials and methods). Starting from the oligonucleotide design of the standard SDA and nicking SDA, an improved design strategy is evolved and tested, resulting in the minimal SDA system (see Fig. 1).

For details on this design strategy we refer to [24]. To improve the design strategy, the algorithms used for computation of the minimal SDA system (Table 1) were adapted to the SDA reaction and the calculated oligonucleotides were tested by simulation of the mechanism. We took into consideration secondary structures as well as the elongation of the 3′ end because of mis-priming. In this calculation, even less probable intermediate products were regarded.

The test system contains only the essential components for SDA to take place. That means, the primer consists of a 5′ overlapping end following the recognition site of the restriction enzyme and a template-binding region on its 3′ end. The template only consists of the primer binding target sequence, so there is no sequence inside the two primer flanking regions (Fig. 1). The system is also built up of DNA building blocks, each 15 nt long with a melting temperature from 50 to 60 °C and a GC content from 40% to 60%.

To check possible and non-specific binding of a free 3′ end of any product, we estimated the elongation probability. The *elongation probability* represents the maximum product of the last base pairing probability and the three base pairing probability, which is
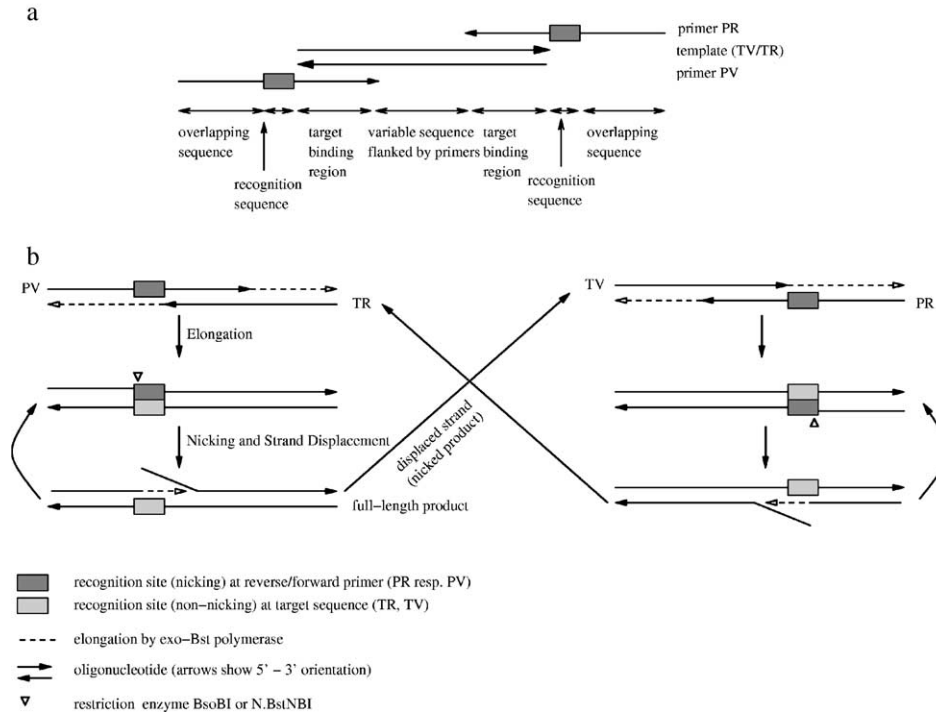

Fig. 1. Setup and mechanism of strand displacement amplification. (a) Oligonucleotide setup. All primers (see Table 1) consist of a 5′ overlapping sequence, the recognition site for the restriction enzyme located 5′ to the target binding region and a template/target binding region at their 3′ end. The two primer bind to opposite strands of the target sequence. For the minimal SDA system, they bind without flanking the variable region inside. (b) Underlying mechanism of exponential amplification in SDA. Besides amplification by repeated elongation, nicking and strand displacement, the displaced strand can also serve as a template for the opposing strand.

allowed. The three base pairing probabilities means the sum of the base pairing probabilities of any base of a DNA sequence at position N to the last base and at position N+1 to the last but one base and at position N+2 to the last but two base at the 3′ end.

These probabilities are calculated either for one or two sequences linked by a spacer of 15 artificial nucleotides with no binding properties, to take inter- as well as intramolecular binding into account. Every combination is tested: that means every primer, template and intermediate product with itself and with any other sequence from the reaction (see Fig. 2).

As long as this maximum product is greater than the elongation probability one randomly selected base will be exchanged by another base chosen randomly as well (point mutation). Fixing the elongation probability is therefore a criterion for the specificity of the computed oligonucleotides.

2.1.1. Materials

*Bso*BI (10 U/μl), N.*Bst*NBI (10 U/μl), *Bst* DNA Polymerase Large Fragment (exo-*Bst*, 8 U/μl)were purchased from New England Biolabs. Denaturing ladder was a 10
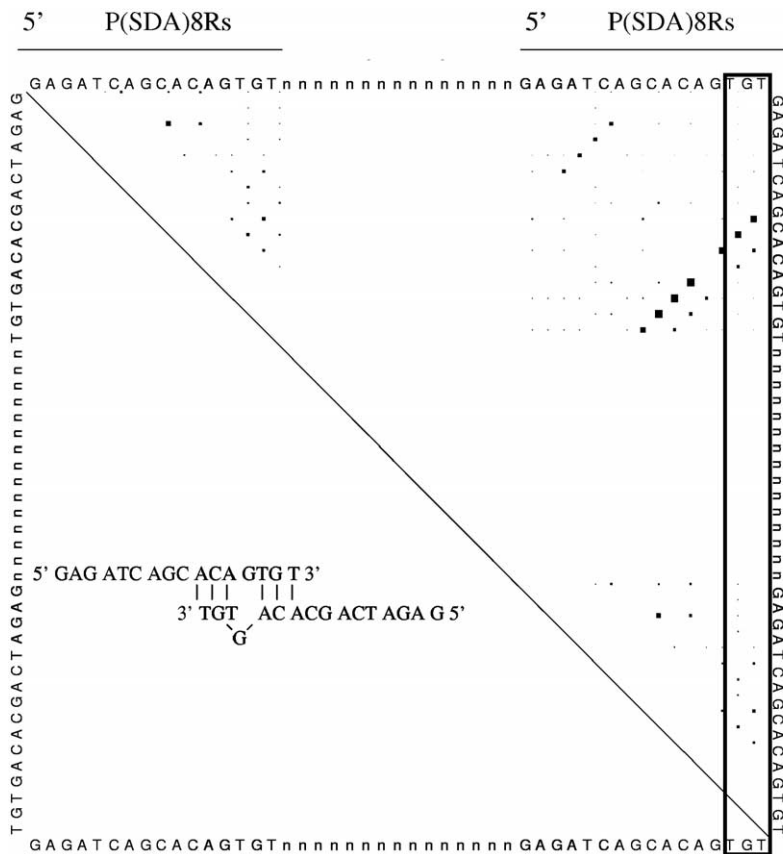

Fig. 2. Partition function as a tool for the search of non-specific hybridization reaction.

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