Gene expression

A novel strategy to design highly specific PCR primers based on the stability and uniqueness of 3′-end subsequences

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ABSTRACT

Motivation: In contrast with conventional PCR using a pair of specific primers, some applications utilize a single unique primer in combination with a common primer, thereby relying solely on the former for specificity. These applications include rapid amplification of cDNA ends (RACE), adaptor-tagged competitive PCR (ATAC-PCR), PCR-mediated genome walking and so forth. Since the primers designed by conventional methods often fail to work in these applications, an improved strategy is required, particularly, for a large-scale analysis.

Results: Based on the structure of ‘off-target’ products in the ATAC-PCR, we reasoned that the practical determinant of the specificity of primers may not be the uniqueness of entire sequence but that of the shortest 3’-end subsequence that exceeds a threshold of duplex stability. We termed such a subsequence as a ‘specificity-determining subsequence’ (SDSS) and developed a simple algorithm to predict the performance of the primer: the algorithm identifies the SDSS of each primer and examines its uniqueness in the target genome. The primers designed using this algorithm worked much better than those designed using a conventional method in both ATAC-PCR and 5′-RACE experiments. Thus, the algorithm will be generally useful for improving various PCR-based applications.

Availability: The source code of the program is available upon request from the authors or can be obtained from http://itolab.cb.k.u-tokyo.ac.jp/ GATC/

Supplementary information: Supplementary data are available at Bioinformatics online.

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1 INTRODUCTION

Conventional PCR uses a pair of unique primers to selectively amplify the target sequence. The specificity of amplification is thus achieved by a synergistic effect between the specificities of the two primers. In contrast, some PCR applications use only a single unique primer in combination with a common primer. These include rapid amplification of cDNA ends (RACE) (Frohman et al., 1988), PCR-mediated genome walking (Shyamala and Ames, 1989; Riley et al., 1990), adaptor-tagged competitive PCR (ATAC-PCR) (Kato, 1997) and so forth. The specificity of the PCR in these applications depends solely on that of the gene-specific primer. Accordingly, they are often plagued with non-specific amplification products derived from so-called ‘off-targets.’

These problems may be solved by optimizing the PCR conditions, including the annealing temperature and the concentrations of Mg\(^{2+}\), dNTPs, primers and the template. While methods have been developed for the efficient optimization of PCR conditions (Cobb and Clarkson, 1994), it is impractical to perform such adjustments on each of the thousands of primers used in a large-scale experiment. The most reliable solution that would require no preliminary experiments is to employ a two-step amplification that uses an additional nested gene-specific primer (Gibbons et al., 1991). However, this would inevitably double the cost and time. Thus, a reliable method is required to design more specific primers, particularly, for use in a large-scale analysis based on these PCR applications. In addition, the primers should work efficiently under a broad range of experimental conditions so that a single unique condition can be used throughout the large-scale experiments. Such primers would make it possible to optimize the PCR conditions using only a small number of representative primers.

We are developing a genome-wide ATAC-PCR system for the budding yeast Saccharomyces cerevisiae. For these experiments, we had designed each gene-specific primer such that the 12mer including its 3′-end (3′-end 12mer) is unique in the yeast genome. Even with these primers, we often encountered off-target products. To determine the etiology of these off-target products, they were cloned and sequenced. As was expected, we found that they were generated by the misannealing of gene-specific primers and, more importantly, that the length of the homologous sequence shared by the primer and the off-target differed from one case to another.

Based on these observations, we assumed that, at least in some instances, the practical specificity may be better determined by the uniqueness of the minimal 3′-end subsequence that exceeds a threshold of duplex stability rather than the uniqueness of entire primer or that of a 3′-end subsequence with a fixed length. We termed such a subsequence as a ‘specificity-determining subsequence’ (SDSS). Increasing the uniqueness of the SDSS is generally equivalent to its elongation. The longer the SDSS, the more likely it is that the primer functions as a unique one. The shorter the SDSS, the more likely it is that the primer would find off-targets. Primers with longer SDSS tend to be more AT rich in their 3′-end portions, and the duplex formed by this region is rather unstable,
leading to accurate but inefficient priming. Therefore, a balance should be found between the yield and specificity.

To quantitatively evaluate these issues, we have introduced an intuitive parameter termed as ‘association rate’ for duplex stability. We have developed an algorithm that calculates the association rate for every 3′-end subsequence of a given primer, identifies its SDSS as the shortest 3′-end subsequence that exceeds a given threshold and examines the uniqueness of the identified SDSS in the target genome, thereby predicting the performance of the primer. We have proved the principle of this strategy in both ATAC-PCR and 5′-RACE experiments using the primers designed based on this algorithm.

2 METHODS

2.1 Preparation of PCR templates

Yeast genomic DNA was prepared from the S288C strain using a Genomic Tip 500 column (QIAGEN) according to the manufacturer’s instructions. One microgram of its DNA was digested in 150 μl of 1× K buffer (TAKARA) with 10 units of MboI (TAKARA) at 37°C for 1 h. Following the heat inactivation of the enzyme at 70°C for 10 min, the reaction was supplemented with 50 μl of ligation buffer [100 mM Tris–HCl (pH 6.9), 10 mM MgCl₂, 4 mM ATP and 4 mM DTT] and 100 pmol of adapter, which was prepared by annealing equimolar amounts of two oligodeoxynucleotides: 5′-(PO₃)CAT CCA TGC ACA ATA CTC ACA GAA ACA GCT ATG ACT GCG CTC ACA TCG-3′ (the sequence of the adaptor-specific primer is underlined). Adaptor ligation reaction was started by adding 10 Weiss units of T4 DNA ligase (TAKARA) to the mixture and it proceeded overnight (i.e. >10 h) at 16°C. The reaction was stopped by adding 50 μl of 100 mM Na₂EDTA (pH 8.0) to the mixture. The DNAs were precipitated by isopropanol, dissolved in 100 μl of TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM Na₂EDTA] and further purified using MinElute PCR Purification Kit (QIAGEN) according to the manufacturer’s recommendation.

The template for RACE was prepared based on a ligation-anchored PCR (Edwards et al., 1991; Troutt et al., 1992). Total RNAs were extracted from the S288C cells using the hot phenol method described by Iyer and Struhl (1996). Five microgram of total RNAs was mixed with 0.5 μl of MboI (TAKARA) cells using the hot phenol method described by Iyer and Struhl for 3′-end subsequence of a given primer, identifies its SDSS as the shortest 3′-end subsequence that exceeds a given threshold and examines the uniqueness of the identified SDSS in the target genome, thereby predicting the performance of the primer. We have proved the principle of this strategy in both ATAC-PCR and 5′-RACE experiments using the primers designed based on this algorithm.

2.2 PCR amplification

Each PCR was performed in a 10 μl reaction volume that was composed of 1× PCR buffer (Invitrogen) with 4 mM of MgCl₂, 20 μM of each dNTP, 0.2 μM of fluorescence-labeled (at the 5′ terminal) adaptor-specific primer (M13-RV primer 5′-CAG GAA ACA GCT ATG AC-3′ for adaptor-tagged genomic DNA templates, and the SP6 promoter primer for RACE templates), gene-specific primers (Tables 1 and 3), 1 unit of Platinum Taq DNA polymerase (Invitrogen) or TaqHS (TAKARA) and the template DNA prepared as described above. The thermal cycling parameters were as follows: preheating at 94°C for 5 min, 40 (for ATAC-PCR) or 45 (for RACE) cycles of a three-step incubation at 95°C for 20 s, 60°C for 30 s and 72°C for 30 s, followed by a 5 min incubation at 72°C. Amplified PCR products were analyzed by conventional agarose or polyacrylamide gel electrophoresis or by an ABI 3730 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions.

2.3 Cloning and sequencing of RACE products

For the analysis of primer specificity in the 5′-RACE, the PCR products were cloned and sequenced: i.e. 5 μl of the PCR products was purified with AMPure reagent (Agencourt) and cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer’s instructions. For each PCR, 12 positive colonies were picked and their inserts were amplified by PCR with a pair of vector primers, purified with AMPure reagent and sequenced using BigDye version 1.1 cycle sequencing kit (Applied Biosystems).

2.4 Program

To calculate the uniqueness of the SDSS of each primer sequence, we prepared a simple program. This program has two running modes: it evaluates the specificity of a given primer in one mode, whereas in the other mode it extracts candidates for highly specific primers from a given sequence using the SDSS concept. The stability of hybridization was calculated as described in the Results section, using the association rate calculated with the parameters described by SantaLucia (1998), but not ΔG. The parameters for one base-mismatched annealing were taken from Allawi and SantaLucia (1997, 1998a,b,c) and Peyret et al. (1999). The program is written in C++ language using the standard template library and the Boost C++ library. The source code for this program is available upon request or can be downloaded from http://itolab.cb.k.u-tokyo.ac.jp/GATC/.

3 RESULTS

3.1 Rationale for designing PCR primers by SDSS algorithm

Let C₀ and C₀ be the initial concentrations of the template and primer, respectively. Let f be the fraction of the template associated with the gene-specific primer. Then, the concentrations of the template (Cₜ), primer (Cₚ), and primer–template complex (CₜCₚ) at equilibrium state are defined as follows:

\[ Cₜ = (1 - f) \times C₀ \]
\[ Cₚ = C₀ - f \times C₀ \]
\[ CₜCₚ = f \times C₀. \]

Since the association constant Kₐₛ is expressed either in terms of ΔG or Cₜ, Cₚ and CₜCₚ, the following equation is obtained:

\[ Kₐₛ = \frac{CₜCₚ}{CₜCₚCₚ} \]

Note that ΔG can be calculated as described by SantaLucia (1998) and in associated reports (Allawi and SantaLucia, 1997, 1998a,b,c, Peyret et al., 1999), R and T are the gas constant (1.987 cal/K·mol)
and absolute temperature (333.15 K in our conditions), respectively. $C_p$ in our case, is $2.0 \times 10^{-7}$ M. Although $C_t$ is usually unknown, it is obviously negligible compared with $C_p$ and can be omitted from the denominator for simplification. Thus, $f$ can be defined as follows:

$$f = \frac{C_p \times K_{as}}{1 + C_p \times K_{as}}.$$

By its definition, the bona fide target is fully matched to the gene-specific primer. Hence, the duplex stability is high and the $f$ value is close to 1. Alternatively, an off-target is matched only to the 3'-end subsequences of the gene-specific primer, and the $f$ value lies in the 0–1 range. The higher the $f$ value, more likely it is that the sequence serves as an off-target. Once the gene-specific primer anneals and primes using a partial homology in the earlier cycles of the PCR, the product serves as an ideal template in the following cycles, leading to prominent off-target products. We had observed this in the off-target products of the ATAC-PCR experiments. 

We thus assume that what is practically important is the uniqueness of the shortest subsequence including the 3'-end of the primer that can prime with a substantial efficiency to exceed a threshold in $f$, but not that of the entire primer sequence. Using the above equation to calculate $f$ for every 3'-end subsequence of a given primer, we can identify the shortest subsequence that exceeds a predetermined threshold value in $f$. We termed such a sequence as an SDSS.

For instance, the two primers Pdr3-1 and Pdr3-20 shown in Figure 1 differ in the length of SDSS—16mer for the former and 10mer for the latter, when the threshold is set at 0.01. The SDSS of the former primer occurs only once in the budding yeast genome and thus is unique. By contrast, the SDSS of the latter primer occurs five times in the genome. Therefore, the former and the latter are expected to behave as an excellent primer and a poor primer, respectively; we observed that this was the case (Fig. 2). Intriguingly, when judging the two primers based on the uniqueness of their 3'-end 12mers, the former appears worse than

\begin{table}
\centering
\caption{Oligonucleotide primers used in Figure 2}
\begin{tabular}{lllll}
\hline
Name & Sequence & $T_m$ & Frequency of SDSS in genomic sequence & Judgment \\
\hline
Pdr3-1 & GACGCATGCTGATACCTTCAATAATT & 76.0 & 1 & Specific \\
Pdr3-2 & GACGCAGCTCTGATACCTTCAATA & 75.8 & 1 & Specific \\
Pdr3-3 & GACGCATGCTGATACCTTCAATA & 75.6 & 1 & Specific \\
Pdr3-4 & GACGCATGCTGATACCTTCAATA & 76.0 & 1 & Specific \\
Pdr3-5 & GACGCATGCTGATACCTTCAATA & 75.8 & 1 & Specific \\
Pdr3-6 & GACGCATGCTGATACCTTCAATA & 75.5 & 2 & Not-specific \\
Pdr3-7 & GACGCATGCTGATACCTTCAATA & 76.5 & 2 & Not-specific \\
Pdr3-8 & GACGCATGCTGATACCTTCAATA & 77.0 & 1 & Specific \\
Pdr3-9 & GACGCATGCTGATACCTTCAATA & 76.1 & 1 & Specific \\
Pdr3-10 & GACGCATGCTGATACCTTCAATA & 75.8 & 1 & Specific \\
Pdr3-11 & GACGCATGCTGATACCTTCAATA & 76.5 & 2 & Not-specific \\
Pdr3-12 & GACGCATGCTGATACCTTCAATA & 76.0 & 3 & Not-specific \\
Pdr3-13 & GACGCATGCTGATACCTTCAATA & 76.5 & 1 & Specific \\
Pdr3-14 & GACGCATGCTGATACCTTCAATA & 76.4 & 2 & Not-specific \\
Pdr3-15 & GACGCATGCTGATACCTTCAATA & 75.4 & 4 & Not-specific \\
Pdr3-16 & GACGCATGCTGATACCTTCAATA & 76.2 & 14 & Not-specific \\
Pdr3-17 & GACGCATGCTGATACCTTCAATA & 77.6 & 7 & Not-specific \\
Pdr3-18 & GACGCATGCTGATACCTTCAATA & 75.2 & 9 & Not-specific \\
Pdr3-19 & GACGCATGCTGATACCTTCAATA & 76.6 & 8 & Not-specific \\
Pdr3-20 & GACGCATGCTGATACCTTCAATA & 77.1 & 5 & Not-specific \\
Pdr3-r & GACGCATGCTGATACCTTCAATA & 70.9 & 2 & Not-specific \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}Each SDSS determined as described in the text is underlined.
\textsuperscript{b}Each $T_m$ was calculated at 1 M NaCl and 0.2 $m$M primer using the equations described in SantaLucia (1998).
\textsuperscript{c}A primer is judged as specific if its SDSS occurs only once in the genome and is judged as not-specific if it does more than twice.
An antisense primer \((Pdr3-r)\) was also designed downstream of an 4366 whereas that of the latter occurs only once in the genome. The latter, because the 12mer of the former occurs eight times, 4367 of each primer is adjusted so that all of them have approximately the same 4368 designed to tile a segment of the yeast gene for evaluation of the 4369 designed another primer set using the SDSS algorithm with the 4370 uniquely in the yeast genome (Supplementary Table 1). We also 4371 were obtained in low yield and were plagued with many off-target pro- 4372 products are substantially different between the primers. Some PCR 4373 genomic DNA template, the yield and purity of the bona fide target 4374 specificity in amplification when used in combination with a reverse 4375 MboI site (\(5'\ GATC 3'\)) and ligated an adaptor to the cohesive ends. 4376 the yeast genome (Table 1). An inverse correlation was observed 4377 efficiency and specificity in PCR. Note that the length of each primer is adjusted so that all of them have approximately the same \(T_m\). 4378 An antisense primer \((Pdr3-r)\) was also designed downstream of an \(MboI\) site. 4379 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4380 We have developed a simple program which identifies the SDSS 4381 of each primer for systematic evaluation of the SDSS algorithm as follows. We designed 20 primers such that their 3’-ends tile a 20 bp segment with a single nucleotide resolution on the second \(MboI\) restriction fragment of \(PDR3\) gene in budding yeast. The length of each primer is adjusted such that all of them have approximately the same \(T_m\) (Table 1, Fig. 2A). On the other hand, we digested the yeast genome with \(MboI\) (\(5'\ GATC 3'\)) and ligated an adaptor to the cohesive ends. Since an \(MboI\) site is located \(\sim 250\) bp downstream of the primer sites, we can perform PCR using an adaptor-specific primer and one of the gene-specific primers to obtain products of \(\sim 280\) bp including the length of the tagged adaptor.

3.2 Experimental validation of the SDSS algorithm

We designed an experiment using ‘tiling’ primers for a systematic 4391 \(\sim 140\)-fold larger effect than \(\text{Na}^+\). Since the stability of the duplex under these conditions is lower than that predicted under an assumption of the presence of \(1\ \text{M Na}^+\), the calculation provides an underestimated \(f\) value. Accordingly, the primers designed using these values would have sufficient stringency.

We examined the frequency of the SDSS of each primer in the 4392 We have developed a simple program which identifies the SDSS 4393 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4394 We have developed a simple program which identifies the SDSS 4395 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4396 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4397 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4398 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4399 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4400 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would 4401 of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would

3.3 Application of the SDSS algorithm to a large-scale 4409 PCR primer design for adaptor-tagged templates

We examined the performance of the SDSS algorithm in the design 4410 PCR primer design for adaptor-tagged templates

We examined the performance of the SDSS algorithm in the design 4411 PCR primer design for adaptor-tagged templates

We examined the performance of the SDSS algorithm in the design 4412 PCR primer design for adaptor-tagged templates

We examined the performance of the SDSS algorithm in the design 4413 PCR primer design for adaptor-tagged templates

We examined the performance of the SDSS algorithm in the design 4414 PCR primer design for adaptor-tagged templates

We examined the performance of the SDSS algorithm in the design 4415 of the SDSS and primer specificity. (A) A set of primers (\(Pdr3-1–Pdr3-20\), see Table 1 for details) were designed to tile a segment of the yeast \(PDR3\) gene for evaluation of the relationship between the SDSS and specificity in PCR. Note that the length of each primer is adjusted so that all of them have approximately the same \(T_m\). An antisense primer \((Pdr3-r)\) was also designed downstream of an \(MboI\) site. (B) PCR amplification from an adaptor-tagged genomic DNA template with each of the primers \(Pdr3-1–Pdr3-20\) in combination with a primer specific to the adaptor ligated to the \(MboI\) sites. Each lane is numbered according to the primer ID in Table 1. Arrows indicate the approximate position of expected target bands. ‘S’ and ‘N’ indicate that the primer was judged ‘Specific’ and ‘Not-specific,’ respectively, by the SDSS algorithm. Asterisks indicate unexpected byproducts, whose identities were determined by sequencing as those including complex mispriming events (Supplementary Fig. 1). (C) Control amplification from the genomic DNA template with the same primers as those used in B in combination with the reverse primer \(Pdr3-r\). Electrophoretic patterns of PCR products are also shown (upper panel). Real-time monitoring of PCR amplification with ABI 7000 sequence detection system (Applied Biosystems) indicates that all the primers share comparable amplification efficiency (lower panel). This is presumably because all of the amplicons have a similar length and because all of the 21 primers including \(Pdr3-r\) share similar \(f\) values close to 1 under the condition used. The intensity of each amplicon did increase by a factor of \(\sim 2\) at each cycle of the logarithmic phase of the reaction.

One should note that the basic parameters used here to calculate \(\Delta G\) by the nearest-neighbor model are determined in the presence of 1 M \(\text{NaCl}\), which is much higher than the concentration in the PCR solution. Furthermore, the PCR usually involves the use of KCl rather than \(\text{NaCl}\) and also \(\text{MgCl}_2\). While \(\text{K}^+\) has been shown to have almost the same effect as \(\text{Na}^+\), \(\text{Mg}^{2+}\) is assumed to have an \(\sim 140\)-fold larger effect than \(\text{Na}^+\) (Nakano et al., 1999). We set our PCR condition to include 50 mM KCl and 4 mM MgCl2, which can be approximated to be equivalent to a 610 mM concentration of the \(\text{Na}^+\) ion. Since the stability of the duplex under these conditions is lower than that predicted under an assumption of the presence of 1 M Na+, the calculation provides an overestimated \(f\) value. Accordingly, the primers designed using these values would have sufficient stringency.

We have developed a simple program which identifies the SDSS of each primer by calculating the \(f\) for every 3’-end subsequence and counts its frequency in the target genome sequence, which would serve as a predictor for the performance of the primer.

We examined the performance of the SDSS algorithm in the design of gene-specific primers for ATAC-PCR of 96 ORFs from the budding yeast genome. We designed a primer set covering these 96 ORFs based solely on the uniqueness of the 12 nt subsequence including the 3’ end (i.e. the 3′-end 12mer), since most 12mers occur uniquely in the yeast genome (Supplementary Table 1). We also designed another primer set using the SDSS algorithm with the
threshold of $f$ set at 0.01 (Supplementary Table 2). For some ORFs, we could not design any ‘unique’ primer fulfilling the requirements. In these cases, we allowed primers that may generate off-target products longer than 350 bp excluding the length of the tagged adaptor. All of these primers were designed to have a similar $T_m$ and were tested using the adaptor-tagged genomic DNA as the template under a single defined condition without any ad hoc adjustment of the reaction condition.

Target bands were detected in >90% of the cases, regardless of the primer design algorithm. However, in >80% of the cases using the primers based on the 3’-end 12mer specificity algorithm, non-specific or off-target bands were detected; the amplified products contained bands derived from both bona fide targets and off-targets (Table 2). Note that we judged a case as non-specific if the intensity of the off-targets exceeded 5% of that of the bona fide target. In contrast, the use of the SDSS algorithm substantially reduced the occurrence of such cases; off-targets were observed in 43% of the cases (Table 2).

Furthermore, visual inspection of individual electropherograms suggested that the products obtained by the 3’-end 12mer specificity algorithm tend to have more off-targets than those obtained by the SDSS algorithm (Fig. 3A and B). For the quantitative evaluation of these data, we calculated the ratio of the signal intensities between the bona fide target and all other off-targets appearing in a window ranging from 60 to 350 bp. The signal-to-noise ratio (S/N) of each PCR was plotted against the signal intensity of the bona fide product (Fig. 3C). The results clearly demonstrated the superior performance of the SDSS algorithm in designing specific assays; the SDSS primers gave higher bona fide signals and S/Ns than those designed based on the 3’-end 12mer specificity algorithm.

### 3.4 Application of the SDSS algorithm to RACE primer design

Next, we applied the SDSS algorithm to the design of primers for 5’-RACE, because it is one of the most frequently used techniques in cDNA cloning. Even with the complete genome sequence data, it is currently impossible to predict the transcriptional start sites. Thus, these sites have to be determined by various experimental approaches, among which 5’-RACE represents one of the most popular and reliable ones. In RACE, the specificity of the primer per se is not the sole determinant of the ‘practical’ specificity, because the abundance of the targets differs drastically from one case to another. Consequently, the less abundantly the target is expressed, the more stringently the primer should be designed. Even though the $f$ between a primer and an off-target is 0.01, if the off-target is expressed 100-fold more abundantly than the bona fide target, the primer should not be used for RACE. In other words, we have to take the relative expression level between the bona fide target and the off-targets into account. Thus, one should ideally use the following expression instead of just $f$:

$$f' = f \times \text{(relative expression)}.$$
However, it is usually impossible to know the relative expression level between two different genes. Here we used a tentative relative expression value of 10 to design gene-specific primers for the 5′-RACE of 10 ORFs in the yeast chromosome 1. We also designed another set of primers for these 10 ORFs such that their 3′-end 12mer sequences are unique in the budding yeast genome (Table 3). These two sets of primers were used for the 5′-RACE, and the products were resolved by gel electrophoresis (Fig. 4A). Since it is difficult to judge whether the 5′-RACE has worked solely from the electrophoretic patterns, we cloned the products, randomly picked 12 clones and sequenced them for identification. As summarized in Figure 4B, the primers designed using the SDSS algorithm worked strikingly better than those designed using the 3′-end 12mer specificity algorithm. More than 90% of the products obtained using the SDSS primers were derived from the bona fide targets (Fig. 4B). Even in the cases where no distinct bands were observed on the gel, bona fide targets with different ends were detected. These were presumably due to truncated reverse transcription. In contrast, the purity of the products obtained by using the other primer set was rather poor (∼10%) (Fig. 4B). These results clearly demonstrate the efficiency of the SDSS algorithm in designing primers for RACE, particularly for less abundant transcripts such as the 10 ORFs examined above.

### 4 DISCUSSION

A number of programs and web-based services are available for designing PCR primers. Some of them use primer specificity as a parameter to select gene-specific primers from many candidate primers. The specificity of a primer may be defined as ‘the ability of a primer to hybridize to no sequences other than a bona fide target,’ and some objective parameters were employed for its

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**Table 3. Oligonucleotide primers used for the 5′-RACE in Figure 4**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target ORF</th>
<th>Primer sequence (SDSS underlined)</th>
<th>Frequency of SDSS in genomic sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CapSp_01</td>
<td>YAL001C</td>
<td>TCCTTTTTTTGTGTATCCCGTAAATATGT</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_02</td>
<td>YAL007C</td>
<td>CCCCACTCCAAAAGATTTTAATAAAAG</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_03</td>
<td>YAL008W</td>
<td>GCATGCTTGTAATCCGACATACTTATG</td>
<td>1</td>
</tr>
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<td>CapSp_04</td>
<td>YAL013W</td>
<td>CTCGCTCACTGGATATACAGTA</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_05</td>
<td>YAL014C</td>
<td>ACAACTGTGTTGCTGGTAAATAAATA</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_06</td>
<td>YAL018C</td>
<td>CTAGCACACCTCCACTAATAGAT</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_07</td>
<td>YAL023C</td>
<td>GCTGTAACAGTCCCTTTTACATATAA</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_08</td>
<td>YAL024C</td>
<td>CCTCCTAACACCATCGAATCTATAG</td>
<td>1</td>
</tr>
<tr>
<td>CapSp_09</td>
<td>YAL025C</td>
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</tr>
<tr>
<td>CapSp_10</td>
<td>YAL026C</td>
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<tr>
<td>Cap12_01</td>
<td>YAL001C</td>
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<td>YAL007C</td>
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<td>Cap12_03</td>
<td>YAL008W</td>
<td>GCCAGCACGCTCCAAACC</td>
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<td>Cap12_04</td>
<td>YAL013W</td>
<td>GCTCTGTCTGGCTGGG</td>
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<td>YAL014C</td>
<td>GCAAGCGGGCGACTCCCTG</td>
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<td>YAL018C</td>
<td>GCAATAGGGCCCGCA</td>
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<td>Cap12_07</td>
<td>YAL023C</td>
<td>TGGCAGACAGAGCGCGGA</td>
<td>263</td>
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<td>Cap12_08</td>
<td>YAL024C</td>
<td>CGGACGGAGGTCTGCCTC</td>
<td>41</td>
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<tr>
<td>Cap12_09</td>
<td>YAL025C</td>
<td>GGCGAGAGGTGCTCGG</td>
<td>158</td>
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<tr>
<td>Cap12_10</td>
<td>YAL026C</td>
<td>CGCGAGGCGCTTGGCTGCT</td>
<td>26</td>
</tr>
</tbody>
</table>

The primers named as ‘CapSp~’ were designed to tolerate 10-fold abundance of off-targets against the bona fide targets or were designed using the threshold value at 0.001 instead of 0.01 to define the SDSS. The primers named as ‘Cap12~’ were designed so that each 3′-end 12mer is unique in budding yeast genomic sequence.
description. For instance, ‘secondary binding sites’ including mismatched hybridization (Haas et al., 1998) or higher similarity (Rozen and Skaltsky, 2000) are considered simply using the entire primer sequence. An implicit assumption underlying these may be that stable hybridization of a primer with the template is a prerequisite for priming by DNA polymerase. However, this is not always the case; DNA polymerases are known to prime from an incomplete duplex formed between the primer and the template. Accordingly, some other programs paid attention to the 3′-end portion of the primer. These calculate the frequency of 6mers (Oligo program based on Rychlik and Rhoads, 1989; Rychlik et al., 1990; Hyndman and Mitsuhashi, 2003) or the stabilities of the 8 bp (Haas et al., 1998), 6 bp (Chen et al., 2003), or 5 bp segments (Rozen and Skaltsky, 2000) of the 3′-terminal subsequences. Note that these algorithms use either the entire primer sequence or 3′-subsequence of a fixed length for the calculation.

Although the use of a fixed length makes the calculation much simpler, the length to be considered should be, in principle, different from one primer to another, depending on the properties of the sequences. A primer whose 3′-end portion can form a duplex more stable than −11 kcal/mol with the template was shown to support the priming by DNA polymerases (Rychlik, 1995). The minimum length of 3′-end sequences that matches to the template varies from 8 bp for G-C rich one to 13 bp for A-T rich one, under the conditions used by Rychlik (1995). In addition, our analysis of the off-target products of the ATAC-PCR revealed that the length of homology between the off-target sequence and the 3′-end subsequence of the primer is not fixed but quite variable (data not shown). These observations not only reinforce the importance of 3′-end subsequence but also indicate that the length of the subsequence to be considered should be adjusted depending on the primer sequence. How can we determine the length to be considered?

For this purpose, we introduced the concept of the SDSS or the shortest 3′-end subsequence of a primer that exceeds the threshold in association rate. We use the frequency of the SDSS in the target genome as a predictor of the primer specificity. To the best of our knowledge, this is the first algorithm to rationally define the length of the 3′-end subsequence to be considered. Indeed, the efficiency of the SDSS algorithm was demonstrated in both ATAC-PCR and 5′-RACE, compared with an algorithm based on the uniqueness of a 3′-end subsequence with a fixed length. Hence, the SDSS algorithm will improve the performance of various PCR applications, particularly, those using a single unique primer with a common primer.

While the SDSS at f = 0.01 can be as short as 7 nt under the conditions that we used, the DNA polymerase requires at least 8 bp duplex to start extending from the primer (Rychlik, 1995). Accordingly, if the SDSS of a primer is 8 nt or longer, every sequence identical to the SDSS would form only 7 bp duplexes with the primer and hence fail to support the priming by the enzyme. One may thus relax the criteria by considering only the sites that can form duplexes longer than 8 bp with the primer. Such adjustment would not be necessary for the yeast or larger genomes, because even the 3′-end 8mer, which is one-base extended to the 5′-end from the 7 nt SDSS, likely occurs at such a high frequency that the primer cannot be accepted. However, the adjustment may be useful in some instance when applying the algorithm to much smaller genomes such as those of viruses.

Based on its nature, our approach is most suitable to organisms with fully determined genome sequences. However, this approach would be useful even in organisms with partial genome sequence data, because it can at least exclude poor primers.

Even with the primers designed using the SDSS algorithm, we occasionally encounter prominent off-target products in ATAC-PCR (Fig. 2B, asterisks); the generation of these products was found to involve complex patterns of GT and GA mismatches and gapped annealing in the SDSSs (Supplementary Fig. 1). This is also the case for most of the off-targets of 5′-RACE (Fig. 4B). These complex off-targets may be eliminated by optimizing the PCR conditions using the modified Taguchi method (Cobb and Clarkson, 1994). We successfully applied this method to find a condition to reduce some off-targets in ATAC-PCR, but this led to the enhancement of other off-targets (data not shown). Thus, it appears that, in addition to experimental optimization efforts, the integration of a more sophisticated algorithm for off-target search with the SDSS algorithm is necessary to prevent the formation of complex off-target products. Such improvements may provide a more robust SDSS strategy to design highly specific primers that can be used for genomes with a much higher complexity.

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**REFERENCES**


