

Multiplex amplification enabled by selective circularization of large sets of genomic DNA fragments

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ABSTRACT

We present a method to specifically select large sets of DNA sequences for parallel amplification by PCR using target-specific oligonucleotide constructs, so-called selectors. The selectors are oligonucleotide duplexes with single-stranded target-complementary end-sequences that are linked by a general sequence motif. In the selection process, a pool of selectors is combined with denatured restriction digested DNA. Each selector hybridizes to its respective target, forming individual circular complexes that are covalently closed by enzymatic ligation. Non-circularized fragments are removed by exonucleolysis, enriching for the selected fragments. The general sequence that is introduced into the circularized fragments allows them to be amplified in parallel using a universal primer pair. The procedure avoids amplification artifacts associated with conventional multiplex PCR where two primers are used for each target, thereby reducing the number of amplification reactions needed for investigating large sets of DNA sequences. We demonstrate the specificity, reproducibility and flexibility of this process by performing a 96-plex amplification of an arbitrary set of specific DNA sequences, followed by hybridization to a cDNA microarray. Eighty-nine percent of the selectors generated PCR products that hybridized to the expected positions on the array, while little or no amplification artifacts were observed.

INTRODUCTION

The PCR (1) along with Sanger sequencing (2) are the two molecular techniques that have contributed the most to our understanding of genomes. Today, the vast majority of methods for identifying sequences in the human genome involve target sequence amplification through PCR.

PCR is a bimolecular mechanism to probe and copy DNA, which performs optimally with single target sequences. Increasingly, however, large numbers of sequences are under investigation and procedures have therefore been developed for parallel amplification. Non-specific amplification of whole genomes is possible using methods such as PEP- and DOP-PCR (3,4) or by multiple-displacement amplification (5). A few methods exist that permit the user to amplify subsets of genomes using variants of so-called adaptor PCR (6–8). For example, Kennedy *et al.* (6) present a technique for fragment selection and complexity reduction through adaptor ligation of digested genomic DNA samples, followed by PCR. The method is optimized to amplify fragments in particular size ranges, but it is difficult to control the composition of the amplified sets of fragments. Adaptor PCR methods are also used to *in vitro* clone individual amplicons on microbeads or in microwells (9,10). Currently, the only way to amplify many defined sequences in the same reaction is by combining several different PCR primer pairs. A crucial problem with this approach is the appearance of amplification artifacts, such as primer-dimers, truncated fragments or amplification products of irrelevant target sequences. The risk of generating such artifacts in a multiplex PCR tends to increase roughly as the square of the number of added primer pairs (11). Even with careful attention paid to the design of the primers, PCR is usually limited to about 10 simultaneous amplification reactions before the analysis of the resulting amplification product is significantly compromised (12,13). Impressive results have been obtained with simultaneous amplification of sets of 10 long-range PCR products (14), but it has proven difficult to greatly increase the number of simultaneously amplified fragments. Therefore, large numbers of separate PCRs are typically performed whenever many genomic sequences need to be analyzed.

Here, we present an approach to enable robust amplification of large sets of selected DNA sequences. The procedure is based on the hybridization of oligonucleotide constructs, called selectors, to defined target nucleic acid sequences. The selectors contain target-complementary end-sequences, joined by a general linking sequence, and they act as ligation

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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templates to direct circularization of target DNA fragments. These circularized targets are then amplified in multiplex using one universal PCR primer pair specific for the general linking sequence in the selectors. We demonstrate the method by performing a 96-plex amplification reaction and we argue that this method will perform well at still greater levels of multiplexing.

MATERIALS AND METHODS

Oligonucleotides and design

Ninety-six cDNA clone sequences from a cDNA microarray were chosen in a UU pattern. Corresponding genomic target sequences for selector design were found by sequence similarity search using BLAST (15). For each cDNA sequence, the genomic sequence yielding the highest-scoring hit was used as a target sequence. The target sequences and an additional 700 nt of sequence information on both sides were downloaded and *in silico* restriction digested. Restriction fragments were considered suitable for selection if they contained at least 70 consecutive nucleotides complementary to the cDNA and they were between 140 and 750 nt in length. Selector probes were designed against one suitable genomic fragment for each target. The selectors, 5'-phosphorylated vector, and primers were from DNA Technology (Aarhus, Denmark). Sequences are shown in the Supplementary Table S1.

Circularization and amplification of 96 fragments

Genomic DNA was extracted from human blood samples (Flexigene, Qiagen). Two combinations of restriction enzymes, **FH**: 10 U of Fsp I (Fermentas) and 10 U of HpyCH4 V (New England Biolabs); **AC**: 10 U of Acu I (New England Biolabs) and 10 U of CviA II (New England Biolabs), were added to two different aliquots of 10 µg genomic DNA and 0.5 µg BSA, in a total volume of 50 µl NEBuffer 4. The restriction digestion was performed at 37°C for 1 h. Two different circularization reactions containing 1.6 pM of each of 87 and 9 different selectors were combined with 1 µg of DNA from the restriction digestion reactions **FH** and **AC**, respectively. The circularization reactions were performed in PCR buffer (Invitrogen) supplemented with 10 mM MgCl₂, 1 mM NAD and 3.2 nM of vector oligonucleotide, using 2.5 U Platinum *Taq* DNA polymerase (Invitrogen) and 5 U Ampligase (Epicentre) in a volume of 25 µl. The circularization reactions were incubated at 95°C for 15 min, 60°C for 20 min, followed by 50°C overnight. To enrich for circularized DNA by degrading linear strands including selectors, 10 µl of the circularization mixtures (0.4 µg DNA) were then added to a 10 µl mixture of 5 U Exonuclease I (New England Biolabs), 110 mM Tris-HCl, pH 9.0, 3 mM MgCl₂ and 0.2 µg BSA and incubated for 2 h at 37°C, followed by 95°C for 10 min. Amplification was performed using 4 µl of each exonuclease-treated circularization reaction (80 ng DNA each) added to 17 µl mixture of 1× PCR buffer (Invitrogen), supplemented with 0.5 U Platinum *Taq* DNA polymerase (Invitrogen), 0.25 mM dNTP, 0.4 µM Cy-3-labeled forward and reverse primer, respectively, and 2 mM MgCl₂. Cycling was performed as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 20 s.

Array hybridization

cDNA arrays were obtained from the microarray core facility at Uppsala University. Arrays were prepared according to the manufacturer's recommendations. In brief, 7776 cDNA clones were obtained from the Sequence Verified Known Genes Collection (Research Genetics). Clone inserts were prepared using the standard protocol and printed in duplicate on UltraGAPS slides (Corning Life Sciences) using a Cartesian Prosys 5510A (Cartesian Technologies) printer. Slides were cross-linked with 450 mJ ultraviolet light using UV-Stratalinker 1800 (Stratagen). To verify the quality of the array, a Cy-3-labeled random 9mer (Operon) was hybridized to one of the arrays. An aliquot of 25 µl of amplification reaction was hybridized to a cDNA array together with 25 µl MICROMAX hybridization buffer (NEN) at 55°C overnight. The array was washed in 0.02× SSC and 0.1% Triton X-100 for 2 min, transferred to 0.1 mM NaCl₂ for 5 s, and then scanned using a GenePix 4000B (Axon Instruments). Images were analyzed using GenePix Pro 5.0 (Axon Instruments). Signals were defined as positive if the ratio of fluorescence to local background exceeded a threshold value for both duplicate spots. This threshold was set so that <1% of the 7680 spots outside the UU pattern were scored as positive.

In silico restriction digestion

Sequence data for the first 2 kb of every 100 kb of the first 100 Mb of each of the human chromosomes 1–6 were extracted from the NCBI genome database. These sequences were randomly allocated into five sets of 1000 sequences each. The sequences were *in silico* digested with the PieceMaker program (16), using a set of 20 commercially available restriction enzymes and all combinations of two of these enzymes. Enzymes are presented in Supplementary Table S2.

RESULTS

Selectors provide a means to choose a large number of defined target sequences for amplification in parallel. Each selector contains two oligonucleotides: one selector probe with two end sequences complementary to the target sequence to be selected for amplification, separated by a general primer-pair motif, and one vector oligonucleotide complementary to the general primer-pair motif (Figure 1A). As a first step in the circularization reaction, a DNA sample is digested by restriction enzymes to generate target fragments with defined ends. The digested DNA sample is then denatured to allow the selector to hybridize to the restriction fragments and template ligation to the vector oligonucleotide, forming single-stranded circular DNA molecules. This step can be performed in two different ways (Figure 1B). (i) When the ends of a targeted restriction fragment hybridize to the appropriate selector probe, the ends become juxtaposed to the vector oligonucleotide guided by the selector probe. Next, a ligase joins the restriction fragment to the vector oligonucleotide generating a circular DNA strand. (ii) Alternatively, the 3' end segment of the selector probe is designed to hybridize to the 3' end of a targeted restriction fragment as above, but the 5' end segment of the selector probe is designed to hybridize to an internal sequence in the target fragment, forming a branched structure. This structure serves as substrate for the

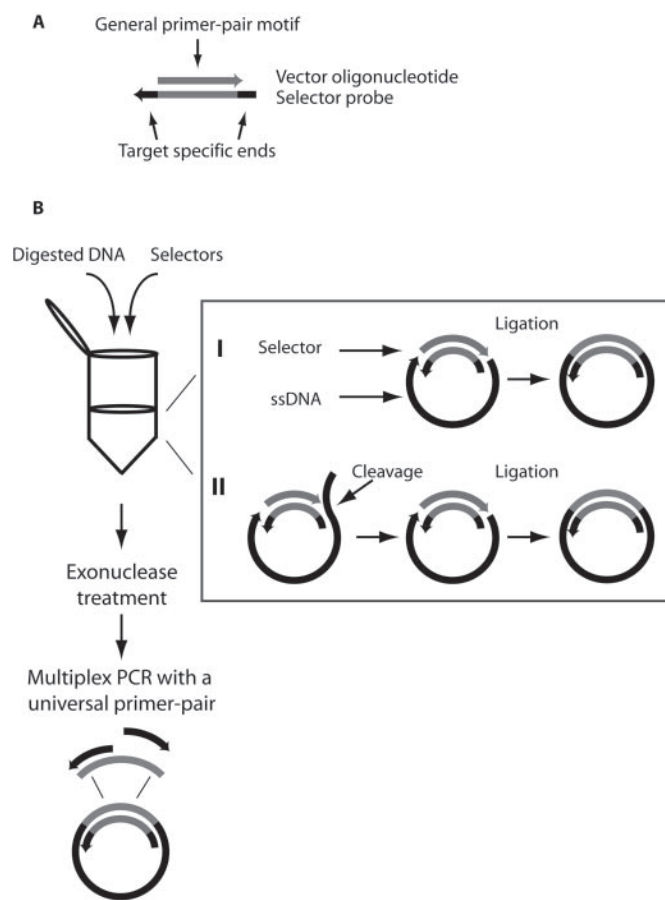


Figure 1. Selector construct and circularization reaction principles. (A) The selector consists of two oligonucleotides, one selector probe of ~70 nt and one general vector oligonucleotide of 34 nt. The selector probe has two target-specific ends (black) of ~18 nt complementary to the selected target and a primer-pair motif (gray) of 34 nt. The vector oligonucleotide is complementary to the primer-pair motif (gray). (B) The circularization procedure starts with digestion of the DNA to generate targets with defined ends. The digested DNA is then combined with selectors and the mixture is denatured, allowing the selectors to hybridize to their respective targets. This can be performed in two different ways: (i) a selector probe hybridizes to both ends of the selected target and the ends are connected to the vector by ligation, or (ii) a selector probe hybridizes with one end to the 3' end of the selected target and the other end to an internal sequence of the target, forming a branched structure that can be cleaved by the endonucleolytic activity of *Taq* DNA polymerase. Both ends of the selected single-stranded target are now ready for ligation to the vector oligonucleotide, forming a closed circular molecule. The circularization mixture is then exonuclease-treated and finally PCR-amplified using a common primer pair.

endonucleolytic activity of *Taq* polymerase, resulting in an invasive cleavage (17). The reaction product is then converted to a circular molecule as under (i) above. In the second procedure, the 5' end of the target, and also the size of the amplified sequence, can be defined without being limited by the presence of restriction sites. Both procedures require two hybridization and ligation events in order to circularize a target sequence, while procedure (ii) also requires an invasive cleavage. These strict requirements should provide sufficient specificity to allow analysis of unique sequences in human genomic DNA. After the circularization reaction, linear sample DNA is degraded by exonuclease. Thereby, the sample is enriched for circularized DNA fragments, having

the general vector oligonucleotide inserted. These fragments are then amplified in a PCR using a universal primer pair specific for the vector oligonucleotide (Figure 1B).

We assessed the performance of the method by selecting an unbiased set of 96 genomic fragments for amplification, and then analyzing the product by hybridization to an array of ~7500 spotted human cDNA sequences. To demonstrate that we can amplify a defined set of targets, we choose targets in the genome that corresponded to cDNA sequences at 96 positions on the array in the pattern of UU (as in Uppsala University). The first step in the design of the 96 selectors was to perform an *in silico* restriction digestions of the human genomic DNA sequence to find a combination of restriction enzymes that generated suitable fragments for all targets. We chose two different restriction enzyme combinations, each with two different enzymes (FH: Fsp I/HpyCH4 V; AC: Acu I/CviA II), which formed suitable fragments for 87 and 9 targets, respectively. The selector probes were designed for strategy (i) if the fragments were of the desired size (140–160 bp), and for strategy (ii) if they were larger, to generate amplification products of ~190 bp (Figure 2A). The selector probes were purchased as a standard 96-plate synthesis and mixed in two pools, one containing 87 selector probes (FH) and the other containing 9 selector probes (AC). Each pool was then combined with DNA from the appropriate digested genomic DNA sample in two separate circularization reactions. These were then subjected to exonuclease treatment, combined and amplified in a single PCR with a universal Cy3-labeled primer pair. The PCR product was analyzed on a 1.5% agarose gel (Figure 2A). The gel analysis showed one sharp band of ~190 bp, demonstrating the specificity of the amplification. The PCR product was then hybridized to the 7.5 k cDNA microarray (Figure 2B).

To evaluate the reproducibility of the method, the experiment was repeated using five different DNA samples. First, to verify the quality of the array, a Cy-3-labeled random 9mer was hybridized to an array from the same spotting session. This analysis showed that 7 of the 96 selected spots lacked cDNA (data not shown), limiting the number of positions that we could analyze to 89. Next, the samples were hybridized to five different slides and a threshold value was defined for each slide, such that 76 of 7684 (1%) spots were scored positive outside the UU pattern. These spots could represent false amplification products or be due to misprinting or cross hybridizations. Seventy-nine (89%) of the selected fragments generated positive signals in at least three of five experiments, and 71 (80%) were scored positive in all five experiments. The average signal intensities of the 79 positive fragments are shown in Figure 3. The signal intensities were reproducible with an average variation (CV) of 24%. We further verified the inter- versus intra-experimental reproducibility in dual-color co-hybridization experiments, comparing a pair of reactions using the same genomic sample, and a pair of reactions using two different samples. We found that the correlation coefficients in the two comparisons were very similar, indicating that the intra- and inter-experimental variation is similar (Supplementary Figure S3).

Applying the selector method requires finding restriction enzymes, or combinations of enzymes, that generate DNA fragments suitable for the particular application. To investigate whether this process is scalable, we performed *in silico*

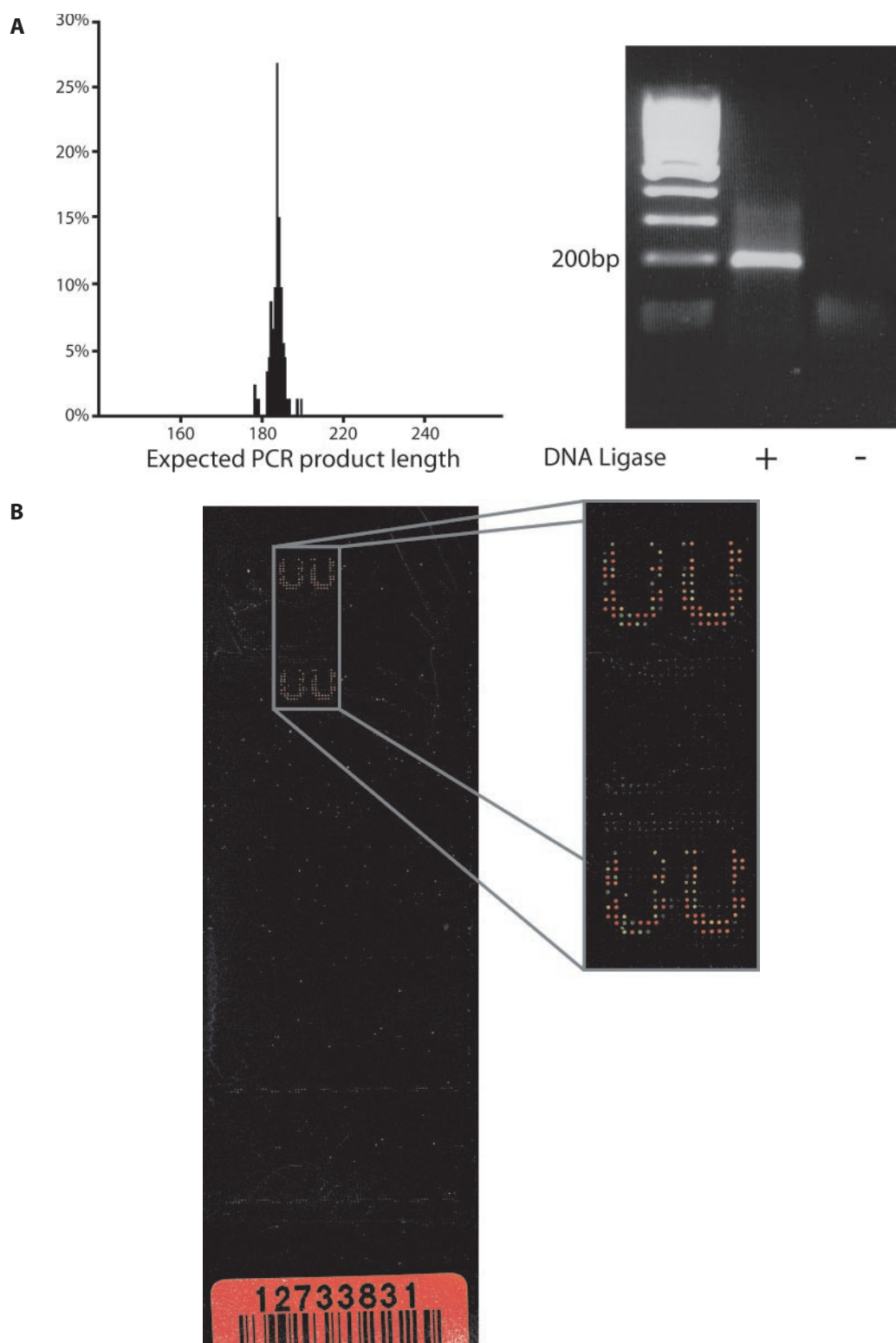


Figure 2. Multiplex selector PCR. A total of 96 sequences were selected from a 7.5 k cDNA array in a pattern of a UU. Ninety-six selectors were then designed to select and amplify the corresponding targets from the human genome. The circularization reaction was performed as described in Figure 1B, where three fragments were circularized using procedure (i) and 93 using procedure (ii). A multiplexed PCR with a Cy3-labeled primer pair was then performed. **(A)** The PCR product from the 96-plex PCR was analyzed on a 1.5% agarose gel. The gel was loaded with a 100 bp ladder (left lane), PCR product from circularization reaction with ligase (middle lane) and PCR product from a circularization reaction without ligase. A histogram showing the expected length distribution of the 96 PCR products plotted against relative frequency (%). **(B)** The PCR product was also hybridized to the cDNA array. Fluorescence intensity is represented as a pseudo-color gradient ranging from green to red.

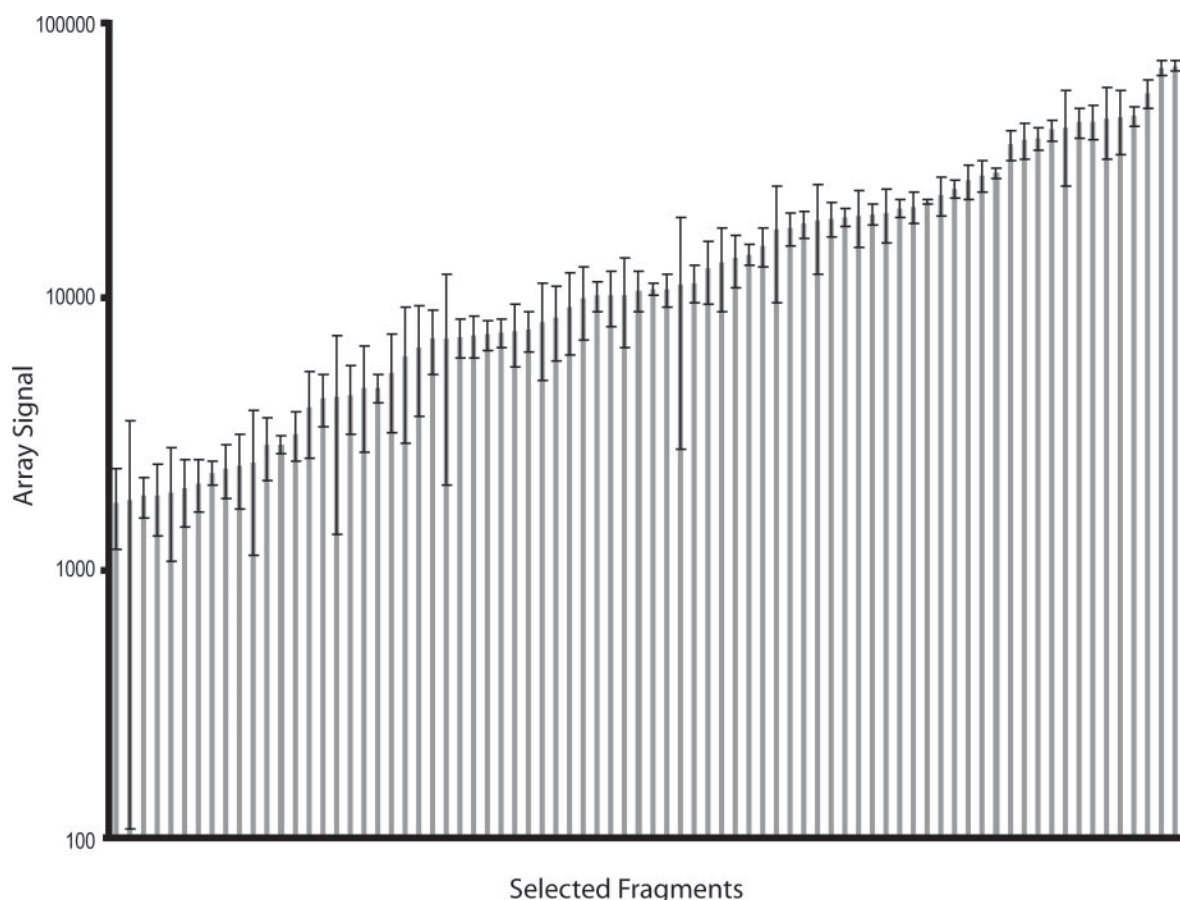


Figure 3. Reproducibility of selector PCR. The selection reaction described in Figure 2 was performed using DNA from five different individuals to investigate the reproducibility of the method. The graph demonstrates the variation in signal for each of the 78 selected fragments that were defined as positive. Signals were normalized relative to the total intensity of the spots in the UU pattern. Fragments are ordered with increasing average signal with bars representing 1 SD.

restriction digestions on increasing the number of sequences from five different sets of arbitrarily chosen target sequences from the human genome. In this simulation, 20 commercially available restriction enzymes were used in either one or two reactions, each with one or two restriction enzymes. Design parameters were selected to be similar to those used in the 96-plex experiment presented. The design success rates of these different restriction digestions are presented in Figure 4. The results show that the rate of success does not tend to decrease with increasing number of targets.

DISCUSSION

We present a strategy for multiplex PCR amplification of large sets of specifically selected DNA fragments from total genomic DNA using oligonucleotide selectors. Current investigations where many gene sequences must be amplified by PCR require multiple separate amplification reactions, and therefore tend to be time-consuming, expensive and require large amounts of target DNA. In contrast, selector-based amplification permits amplification of multiple specific DNA sequences in individual reactions, requiring fewer amplification reactions and less sample material.

Selector design requires that combinations of restriction enzymes are found that generate suitable fragments for

as many target sequences as possible, while minimizing the number of separate digestion reactions. For the demonstrated 96-plex PCR, two digestion reactions using two different restriction enzymes each were required to achieve a 100% design success rate. In an accompanying paper, design criteria are discussed in more detail and a computer program is described that guides the choice of selectors and restriction enzymes for given sets of amplicons (16).

The selector-based amplification method requires a single ~70mer oligonucleotide for each amplicon, compared with the two oligonucleotides of 20 or so nucleotides each used in conventional PCR. It may be possible to reduce the cost of generating a multitude of selectors by using microarray-based oligonucleotide synthesis methods (18).

A single sharp band of ~190 bp was seen upon gel analysis. No evidence of non-specific amplification was observed, as this would be expected to generate differently sized fragments. The array-based analysis demonstrated that 89% of the selected targets were amplified. By analyzing the intensity of the array signals, we further found that the amplification efficiency of each fragment was highly reproducible with an average signal variation of 24% (CV). However, we cannot determine to what extent the amplification efficiency differs between the selected fragments since the variation in signal intensities between different spots on the array does not directly reflect

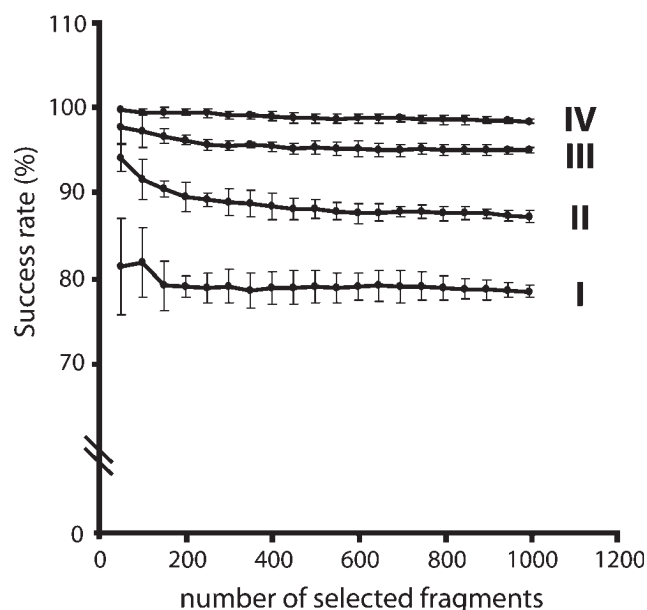


Figure 4. Design simulation demonstrating scalability of the method. Selector design was performed on five arbitrary sets of human genomic sequences. The figure shows the average design success rate for increasing numbers of fragments. Bars represent 1 SD. Line I represents one digestion reaction with one restriction enzyme, line II represents one digestion reaction with two restriction enzymes, line III represents two digestion reactions with one restriction enzyme in each reaction and line IV represents two digestion reactions with two restriction enzymes in each reaction. Arbitrarily selected 2 kb fragments containing 250 bp long sequences of interest were chosen as targets. After *in silico* digestion with different enzymes and combinations thereof, those target-enzyme combinations resulting in at least one fragment fulfilling the design parameters were considered successful. The parameters for the restriction fragments were set to a content of at least 50 bp of the desired sequence, a maximum flap length of 500 bp, and size of the selected fragment of 100–200 bp.

the concentration of the selected fragments, due to the semi-quantitative nature of cDNA array analyses. The failure to demonstrate hybridization to 10 of the targets on the array could potentially be due to poor oligonucleotide quality. To investigate this, we repeated the experiment using high-performance liquid chromatography purified selector probes for the failed fragments, but none of them was rescued (data not shown). Another potential cause of failure is the presence of polymorphic sequences influencing restriction digestion, but we found no known polymorphisms in SNP databases that could explain these failures. However, we did find a correlation between the failure rate and the GC content of the targets. The success rate for targets of <60% GC content was 95%, while for targets with >60% GC content the success rate was only 71%. We are currently investigating ways to improve the selection and amplification protocol to further increase the efficiency of amplification of GC-rich targets.

It has previously been shown that thousands of oligonucleotide probes can selectively be circularized and then amplified in parallel using a universal PCR primer pair (19–21). Unlike this probe amplification method, which distinguishes single-nucleotide variation among DNA samples, the selectors guide amplification of the target DNA, enabling analysis of longer sequences. On the other hand, since the selector method is based on selective circularization of DNA molecules and a universal PCR amplification, similar to the probe amplification

method, we expect that the selector technique can be applied for higher levels of multiplexing than the 96 shown in this paper. We have further demonstrated that the selector design can be extended to far larger numbers of amplicons. As an alternative to PCR, circle-to-circle amplification (C2CA) can be used to amplify the selected DNA circles (22). The C2CA procedure has been shown to yield greater amounts of individual amplicons than PCR, potentially enabling even higher levels of multiplexing. The C2CA process has also been shown to amplify different amplicons more evenly than PCR (22), and should furthermore introduce fewer polymerase errors in the amplified sequences (23).

As discussed, the selector-based amplification procedure is suitable for highly parallel DNA analysis and can be applied for, for example, parallel large-scale resequencing. Currently, considerable efforts are invested in developing the so-called ultra low cost sequencing (ULCS) methods, with the goal of sequencing the entire human genome for US \$1000, a 4–5-log improvement over current methods (24). A more realistic goal in the shorter term is to sequence only parts of the genome of particular interest, e.g. all exons and promoters. The use of selectors for parallel amplification could prove of value for several of the ULCS methods, whether based on sequencing by hybridization (14,25) or sequencing by synthesis (9,10,26,27). Selectors could also reduce time and cost required for analyses in PCR-based genotyping (13) and in other mutation detection methods (28).

In conclusion, we present a new method for multiplexed amplification of selected DNA fragments by PCR. The method provides a robust mechanism and a simple protocol that should be of great value for a wide range of PCR-based applications.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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Conflict of interest statement. F. Dahl, M. Gullberg, U. Landegren and M. Nilsson have licensed the commercial rights to the technology to Olink AB (Uppsala, Sweden), a company in which M. Gullberg, U. Landegren and M. Nilsson also hold stock. J. Stenberg declares no conflict of interest.

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