Cooperative Oligonucleotides in Purification of Cycle Sequencing Products

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ABSTRACT

Nucleic acid hybridization is an essential component in many of today's standard molecular biology techniques. In a recent study, we investigated whether nucleic acid capture could be improved by taking advantage of stacking hybridization, which refers to the stabilizing effect that exists between oligonucleotides when they hybridize in a contiguous tandem fashion. Here, we describe a specific approach for purification of sequencing products using cooperative probes that hybridize to single-strand targets where one of the probes has been coupled to a magnetic bead. This approach has been developed for standard sequencing primers and has been applied to shotgun plasmid libraries. The cooperative probes have been designed to anneal within the common vector sequence and to avoid co-purification of nonextended sequencing primers and misprimed sequencing products. The reuse of magnetic beads, together with salt independent elution, makes the approach suitable for high-capacity capillary electrophoresis instruments.

INTRODUCTION

The interaction between oligonucleotides and their complementary nucleic acids is used in a wide variety of applications in molecular biology. Oligonucleotides can act as probes in nucleic acid blotting experiments and DNA array technology, for example, or as primers during PCR amplification and cycle sequencing reactions. Important factors in such applications are the stability of the formed duplex and the presence of secondary or tertiary structure in the target or probe.

It has been shown (9,12) that two or more oligonucleotide modules that are annealed adjacently on a template cooperatively increase their affinity for the target. Therefore, a complex with two modular probes and a target molecule can have a higher stability than if a single probe (covering the same region as the two modular probes) is used. This is probably because the modules interact in the opening of the secondary structure and contribute to a base stacking effect, even as a single-nucleotide gap between the probes decreases the stability significantly (12). This increase in affinity can be advantageous in array technology (1–3,6,14), antisense applications and nucleic acid sample preparation (4,8,13,16).

Many of the large-scale genome sequencing projects rely on semiautomated protocols to prepare and analyze the genetic material. However, some of the steps—such as ethanol precipitation of the sequencing reactions that concentrate the sequencing products and remove excess dye-labeled primers and terminators before loading on slab gels or capillaries—are cumbersome to automate.

In this study, we investigated the use of a solid-phase capture method for the

purification of cycle sequencing products. Capture probes complementary to the vectors were covalently coupled to the magnetic beads, and a second modular probe that anneals adjacent to the capture probe site was used to increase the efficiency of capturing sequencing products, as described earlier, for the direct capture of virus genomes (13).

MATERIALS AND METHODS

Preparation of PCR Products

pUC18 and pBluescript[®] plasmids containing various inserts (from bacterial genome shotgun libraries) were used as template in a PCR amplification with RIT27 (5'-GCTTCCGGCTCGTATGTTGTGTG-3') and RIT28 (5'-AAAGGGGGATGTGCTGCAAGGCG-3') primers. Amplification was performed in a 50- μ L reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.1% Tween[®] 20, 0.2 mM each dNTP, 0.5 U AmpliTaq[®] DNA polymerase (PE Biosystems, Warrington, UK) and 75 nM each primer using a model 9600 thermocycler (PE Biosystems). The temperature profile was 94°C for 5 min, followed by 30 cycles of 94°C for 30 s and 72°C for 2 min, ending with 72°C for 10 min.

Cycle Sequencing with Dye Primers

The dye primer cycle sequencing reactions for the MegaBACE 1000 DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) were performed as described in the ET primer protocol. For analysis on the ABI Prism[™] 377 XL DNA sequencer (PE Biosystems, Foster City, CA, USA), the cycle sequencing reactions were carried out in volumes of 10 μ L for the A and

Table 1. Capture and Modular Probes

Vector	Direction	Capture Probe	Modular probe
pUC18	Forward	5'-TCTAGAGTCGACCTGCAG-3'	5'-GCATGCAAGCTT-3'
pUC18	Reverse	5'-CGAGCTCGAATTCGTAATC-3'	5'-ATGGTCTAGCTGTTTCC-3'
pBluescript SK and pBluescript II SK	Forward	5'-ACCCAATTCGCCCTATAG-3'	5'-TGAGTCGTATTAC-3'

C reactions and 20 μ L for the G and T reactions. The following reagents were mixed together for the A and C mixture (final concentrations): 26 mM Tris-HCl, pH 9.0, 6.5 mM $MgCl_2$, 150 μ M dNTP, 0.5 μ M ddNTP (A or C); 1.5 U ThermoSequenase™ (Amersham Pharmacia Biotech); 20 nM M13 forward or reverse (M13-21, USP 5'-CGTTG-TAAAACGACGGCCAGT-3' or RSP 5'-CAGGAAACAGCTATGACC-3') primer with respective rhodamine dye (Genpak, Brighton, UK); 1 μ L PCR product and distilled water for a final volume of 10 μ L per reaction. For the G and T mixture, final concentrations were 26 mM Tris-HCl, pH 9.0, 6.5 mM $MgCl_2$, 150 μ M dNTP, 0.5 μ M ddNTP (G or T), 1.5 U ThermoSequenase; 20 nM M13 forward or reverse primer with respective rhodamine dye; 2 μ L PCR product and distilled water for a final volume of 20 μ L per reaction. Cycle sequencing was carried out using a temperature profile of 95°C for 1 min followed by 28 cycles of 96°C for 30 s and 56°C for 1 min. The cycle sequencing products were pooled before ethanol precipitation or solid phase capture.

Preparation and Regeneration of Beads

Three sets of beads covalently coupled to different capture probes were used (kindly supplied by Erling Finne, Dynal AS, Oslo, Norway). The synthesized thiol-capture oligonucleotides were coupled to super paramagnetic-maleimide beads according to previously published protocols (5). Briefly, the trityl group of a trityl-thiol-linked oligonucleotide was removed by incubating the oligonucleotide with 0.1 M $AgNO_3$ solution for 30 min and then 0.15 M dithiothreitol for 30 min. The

activated oligonucleotides were desalted on a NAP5 column (Amersham Pharmacia Biotech) before incubation with super paramagnetic maleimide beads for direct coupling.

Regeneration (reuse) of beads after elution of sequencing products, using either loading buffer or heated water (see below), was performed by washing the beads twice in 50 μ L binding/washing buffer before resuspension in 15 μ L 6 $\times$ SSPE.

Capture and Elution of Cycle Sequencing Products

The pooled cycle sequencing products were incubated with 30 pmol modular probe (Table 1) and the prewashed beads (150 μ g) in a total volume of 115 μ L 6 $\times$ SSPE at 54°C for 15 min. The beads were then washed once in 100 μ L binding/washing buffer and once in 100 μ L 1 $\times$ TE buffer. The captured cycle sequencing products were then eluted from the beads at room temperature with 2.5 μ L loading buffer (PE Biosystems) before loading onto the model 377 ABI PRISM™ DNA sequencer (PE Biosystems) or with 10 μ L loading buffer (provided with the kit) before injection on the MegaBACE DNA sequencer. Elution by heat and distilled water was also investigated by resuspending the captured sequencing products in 10 μ L distilled water and heating the solution to 95°C for 3 min. The supernatant was transferred to a new tube while still hot. Loading buffer (2.5 μ L) was added to the eluted sequencing products, and the water was evaporated by heating the tubes to 95°C for 5 min before loading.

The assay was optimized by using a bead designed to capture sequencing products that were obtained using the universal sequencing primer (forward)

and inserts cloned in the pUC18 plasmid vector (Table 1). The reproducibility of the assay was investigated by amplifying, sequencing and capturing 15 random pUC18 plasmid clones (obtained from a shotgun library) using vector-specific primers as outlined earlier. As a reference, the same sequencing products were ethanol precipitated by incubating the cycle sequencing product with 138 μ L 96% ethanol and 5.5 μ L 3 M sodium acetate. The resulting pellet was washed in 700 μ L 80% ethanol, dried and resuspended in 2.5 μ L loading buffer (PE Biosystems).

RESULTS

The Basic Concept

We investigated whether a preparative capture method for cycle sequencing products could be developed using stacking modular probes and solid-phase technology. The solid phase used was super-paramagnetic beads, which facilitates the easy removal and exchange of buffers. In these experiments, an in-house bacterial genome project (*Mycoplasma mycoides* spp *mycoides*) involving pUC18 and pBluescript SK II shotgun libraries, with insert sizes in the range of 800–2000 bp, were used as a model system. PCR products were used as DNA templates in cycle sequencing reactions generating single-stranded Sanger fragments. Oligonucleotide probes complementary to common vector sequences of the (universal or reverse sequencing primer) generated Sanger fragments were designed. For each vector and primer combination, two oligonucleotides were used; one was a covalently coupled capture probe and the second was an adjacently positioned

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Figure 1. Schematic representation of the capture protocol. (A) Design of capture and modular sequence. (B) Flowchart.

modular probe (Figure 1A). The obtained Sanger fragments were captured on the beads in a one-step hybridization reaction by mixing the cycle sequencing products with the beads (coupled to the capture probe) and a modular probe as outlined in Figure 1B. The beads with the captured fragments were washed and resuspended in formamide, which eluted the fragments from the bead surface. The supernatant containing the Sanger fragments was loaded directly on an automated DNA sequencer.

Initial tests evaluating the approach on five random pUC18 clones that were amplified by PCR, sequenced by the universal sequencing primer and captured with the pUC18 forward capture bead (Table 1) showed the advantage of using the modular probe for capture efficiency. The gel file, obtained after capture and electrophoresis as shown in Figure 2, gives a quantitative estimate of the capture enhancement effect with the stacking modular probe. In lanes 1–5, the capture was performed in the presence of the modular probe, and in lanes 6–10, it was performed without the modular probe.

Figure 2. The ABI 377 gel-file image of captured sequencing products generated using dye-labeled universal sequencing primer.

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Optimization of Capture Efficiency

A number of parameters—the amount of beads and modular probe, hybridization temperature and duration of incubation at this temperature—were investigated to optimize the capture protocol. The model system consisted of a 1200-bp insert in the pUC18 vector that was amplified by PCR with vector primers and then sequenced using the universal sequencing primer. Initially, the amount of beads necessary for efficient capture was determined. Triplicate samples (in all optimizations) using a bead concentration in the range of 40–250 μg were analyzed, and the capture efficiency was determined by analysis of the peak signals for different fluorophores after gel electrophoresis. As shown in Figure 3A, a decline in efficiency is observed for less than 150 μg beads.

A variation of the modular probe amount in the range of 0–50 pmol (0–0.44 mM) shows that a decline in

efficiency was observed when less than 30 pmol (0.26 mM) probe were used (Figure 3B). In the next experiment, the bead and modular probe concentration remained constant at 150 μg and 0.26 mM, respectively, while the capture temperature was varied from 20°C to 70°C. As Figure 3C demonstrates, the hybridization temperature clearly influences capture efficiency with maximum capture observed at 54°C (close to the melting temperature of the immobilized probe, 56°C) with a sharp decline in capture efficiency at both higher and lower temperatures. Finally, the duration of the incubation at 54°C was investigated. The data presented in Figure 3D show that at least a 15-min incubation at the capture temperature is necessary to achieve maximum capture. A further incubation step at room temperature after capture was found to be unnecessary (data not shown).

To verify these results for other templates, 15 random pUC18 inserts were sequenced. The sequencing products

were either ethanol precipitated or captured with or without modular probe. These samples were then compared for accuracy over the first 500 bp using evaluation software (ABI Prism Sequencing Analysis 3.0; PE Biosystems). As shown in Figure 4, the capture protocol improves the sequence quality during the first 100 bases compared to ethanol precipitation. This is probably because of interference from excess sequencing primer or misannealed and extended sequencing primer that will co-precipitate with the correct sequencing products. However, capture without the modular probe is less accurate in the last interval (400–500 bases) because of the loss of signal intensity, as Figure 2 indicates. Capture with the modular probe has a slightly higher accuracy during the last interval compared with the ethanol-precipitated material. Similar experiments with inserts cloned into the pBluescript vector (and corresponding specific capture beads) also show improved accuracy, which

Figure 3. Optimization of sequence product capture. (A) Amount of beads; (B) amount of modular probe; (C) capture temperature; (D) duration of capture.

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Figure 4. Accuracy on ABI 377 for 15 random clones. Purification of sequencing products by ethanol precipitation, capture with modular probes and capture without modular probe is indicated.

indicates that the modular probes have a cooperative effect in vectors other than pUC18 (data not shown).

Reuse of Beads

Because the capture purification protocol relies on a hybridization event involving a paramagnetic particle, we investigated whether the beads could be reused in an iterative fashion. Two elution formats were analyzed with similar results. The first protocol was based on formamide elution after capture with direct loading on the gel; the second protocol involved heat release at 95°C in a low-salt buffer, transfer of the supernatant to a new tube, addition of formamide and evaporation of the remaining water by heat (data not shown). After elution using both formats, the beads were reconditioned by washing

Figure 5. Iterative reuse of beads. (A) ABI 377 gel image of 12 iterative capture cycles on random clones using pUC18 forward and pUC18 reverse capture beads. (B) Chromatogram of the 12th capture with the pUC18 reverse capture bead.

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with a high-salt buffer (binding/washing buffer, see Materials and Methods); 12 random pUC18 clones were used for this purpose.

Both forward and reverse sequencing reactions were performed on these clones and the corresponding specific capture bead with accompanying modular probe (Table 1). Twelve cycles of iterative use of the beads were performed. The results using the formamide elution protocol are shown both as a gel image (Figure 5A) and as a chromatogram corresponding to the final use of the pUC18 reverse bead (Figure 5B). The gel image displays the expected variability between individual clones for both the forward and reverse specific beads, but with no apparent loss of signal intensity throughout the iterative cycles. The fact that the probe and bead can still participate in capture (without a loss in specificity) is further supported by the chromatogram in Figure 5B, which shows a low background and a high signal-to-noise ratio even after 12 cycles of reuse.

Specificity of the Capture

To test the specificity of the capture protocol, an experiment was designed in which the products of two sequencing reactions were mixed, followed by purification of the individual products.

Thus, two separate sequencing reactions using the forward sequencing primer were performed on PCR products obtained from a pBluescript insert and a pUC18 insert. The sequencing products were then mixed and incubated with the pUC18 (forward) beads, and the remaining supernatant was subsequently mixed with pBluescript (forward) beads. In addition, a control experiment was performed using non-complementary sequencing products. The captured material was eluted and loaded onto an ABI 377 polyacrylamide gel. Figure 6 shows the results as a comparison between signal strength and accuracy over the first 500 bases. The first two bars show the capture of pUC18 and pBluescript forward reactions, respectively, with the two following bars showing the control experiment. A specific purification is achieved with a high degree of accuracy, while the control experiments show no background binding.

Capture of Sequencing Products for Capillary Electrophoresis Analysis

A feature of the solid-phase approach developed here is the possibility of removing salts and template material (i.e., dsDNA) by a simple magnetic separation step before performing capillary electrophoresis. To investigate

Figure 6. Evaluation of the specificity of the capture protocol.

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this possibility, the capture approach was evaluated on 15 random pBlue-script clones that were amplified by PCR and sequenced with the forward sequencing primer on the MegaBACE platform. Elution of captured sequencing products was achieved with 10 μ L formamide loading buffer and the Sanger fragments were then directly electroinjected. Figure 7A shows a summary of the results, which depict a

comparison of the capture protocol (with modular probe) and ethanol precipitation over the first 600 bases. Again, the capture protocol achieves a higher accuracy over the first 100 bases, with the remaining 500 bases showing comparable accuracy for both methods. An example of the sequence quality obtained with the capture protocol is shown in Figure 7B. The chromatogram shows some overlapping

peaks during the first 10 bases that seem to be specific to the capillary electrophoresis platform because these problems were not observed with slab gel electrophoresis using the identical samples (data not shown).

DISCUSSION

We investigated whether the purification of cycle sequencing products by ethanol precipitation could be replaced with a capture assay using modular probes and magnetic beads. Previous reports using magnetic beads to capture single-stranded targets (M13) for sequencing purposes have either relied on long capture probes (50 nucleotides) (4) or a triple-helix capture principle (8). Here, the purification is performed with a capture probe covalently linked to paramagnetic beads and an adjacently annealing modular probe used to stabilize the complex. The improvement in capture efficiency using this modular probe has been documented in this report and in previous studies (12,13). Contributing factors to the improvement of capture include van der Waal's forces that appear between two adjacent positioned primers (i.e., capture and modular probes) and opening the secondary structure of the target sequence. The modular probe has a kinetic advantage compared with the immobilized capture probe that probably aids in hybridization to the bead. As shown in this study, temperature is an important parameter to improve hybridization efficiency and is shown empirically to be a rather narrow temperature interval for optimal performance.

This capture system for purification of sequencing products is based on the presence of common vector sequences accessible for hybridization. The strategy has three major advantages. First, any standard sequencing primer (forward or reverse) and sequencing chemistry (dye primer or dye terminator) (unpublished results) can be used in cycle sequencing to generate sequencing products that are amenable to capture with vector-specific beads. Second, this purification assay does not capture non-incorporated primers or misprimed sequencing products. Thus, only correctly extended Sanger fragments will be

Figure 7. Results using the capture protocol and MegaBACE 1000 capillary electrophoresis instrument with the pBlue-script forward capture bead. (A) Accuracy on the MegaBACE 1000 for 15 random clones. Purification of sequencing products by ethanol precipitation or capture with modular probe is indicated. (B) An example of the obtained MegaBACE 1000 sequence data using the capture protocol.

captured. Obviously, this reduces the background and improves the accuracy as indicated in this report. Third, the sequencing templates can be plasmid, PCR products or M13 phage when using standard sequencing primers. An advantage here is that double-stranded templates that could interfere with the electroinjection step of capillary electrophoresis will not co-purify with the single-stranded sequencing products.

In this study, we used pUC18 and pBluescript SK vector-specific capture beads. In the pUC18 vector that has a rather short stretch between the forward sequencing primer and the multiple cloning site (MCS), the capture probe hybridizes to part of the first restriction sites in the MCS. This pUC18 system is therefore somewhat specialized for shotgun libraries using the *Sma*I restriction site. However, pBluescript and pGEM[®] vectors have longer vector sequences between the sequencing primer and the MCS, which allows for cloning in any of the restriction sites (in the MCS) without having to change the capture probes.

The results described above indicate that we can obtain a high degree of specificity and accuracy in the capture of sequencing products. Future modifications can be envisioned such as designing new probes for capture and adapting the protocol for automated systems. Improvements on probe design could involve varying the capture sequences or the use of nucleotide derivatives (such as PNA) or spacer sequences (10). For such purposes, it would be possible to take advantage of biosensor technology that has been used earlier for real-time, quantitative estimation of hybridization events (10–13). We have previously shown that magnetic separation can successfully be automated using both commercial and in-house instrumentation for similar purposes (7, 15, 17). However, it is important that the chosen instrumentation can facilitate incubation at elevated temperatures (54°C with the current set of probes) that are required to achieve optimal capture efficiency in the present system.

We also show that the beads can successfully be reused at least 12 times, which reduces the cost per reaction by the same magnitude. It appears that the limiting factor regarding reuse is the ef-

iciency of magnetic separation (i.e., the risk of losing beads during the iterative cycles) and not the quality of the bead and its probe.

In addition, the low background binding of noncomplementary sequences onto the beads (Figure 6) suggests that multiplex sequencing reactions can be purified with this method, that is, performing multiple sequencing reactions simultaneously (forward and reverse reactions or multiple vectors) and then iteratively isolating the respective sequencing products with vector-specific capture beads and accompanying modular probes. This will reduce the reagent cost per sample and lead to a system that gives improved sequence data at a lower cost, suitable for high-throughput capillary sequencers.

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