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Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support

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ABSTRACT

Approaches to direct solid phase sequencing of genomic and plasmid DNA have been developed using magnetic beads, coated with streptavidin, as solid support. The DNA is immobilized through selective incorporation of biotin into one of the strands. A single stranded template, suitable for sequencing, is obtained through strand-specific elution. Using this concept, *in vitro* amplified plasmid DNA and chromosomal DNA were sequenced directly from single colonies. The solid phase approach ensures that the amplification and the sequencing reactions can be performed under optimal conditions. The system was found to be suitable for sequencing using both isotope- and fluorescent-labelled primers.

INTRODUCTION

Solid phase methods have proven to be very useful in such areas as peptide synthesis, peptide sequencing and DNA synthesis. The advantage with a solid phase approach is usually a combination of good yields, reproducible reactions and easy automation due to ease of separation of the solid phase from the reaction solution.

In the area of DNA sequencing, there exist a great need for technical improvements to facilitate large scale sequencing projects. Automated systems for on-line sequencing, using fluorescent labelled primers (1–2) or dideoxy chain terminators, (3) have been introduced that are capable of direct detection and storage of the DNA sequence. In addition, several systems have been described for automation of the Sanger dideoxy-nucleotide sequencing method based on robotic workstations (4–6) However, few attempts to adapt solid phase approaches to DNA sequencing have been reported.

Recently, a method for solid phase DNA sequencing using the avidin-biotin system was described (7–8). The method was based on the immobilization of double stranded plasmid DNA to avidin agarose and subsequent use of the immobilized DNA as template for sequencing reactions. The DNA was immobilized through selective introduction of a biotinylated nucleotide into one of the strands of the plasmid vector. The affinity gel containing the immobilized template was consecutively used for the four different dideoxy-nucleotide reactions.

Here, we show that solid phase sequencing can be used for direct genomic sequencing and plasmid sequencing, using DNA template obtained by the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Strains and plasmids.

Bacterial strains *Escherichia coli* RR1 and *Staphylococcus aureus* SA113 were used (9).

The plasmid pRIT27 (7) was used containing a synthetic human proinsulin gene fragment (M.Murby and M.Uhlén, unpublished) as insert.

Enzymes and oligonucleotides.

Restriction endonucleases, T7 DNA polymerase and T4 polynucleotidekinase were purchased from Pharmacia, Sweden. *Taq*I polymerase was obtained from Amersham, England. DNA manipulations and purifications were performed according to standard procedures (9) and biotin-16-dUTP was obtained from Boehringer Mannheim, W.Germany. Four oligonucleotide primers were synthesized by phosphoramidite chemistry on an automated DNA synthesis machine (Gene Assembler, Pharmacia, Sweden): RIT 1 and RIT 2, complementary to the staphylococcal protein A gene and RIT 6 and RIT 13 complementary to sequences upstream and downstream of the multilinker region in pRIT27. Two of the primers were biotinylated in the 5′ end (RIT 1 and RIT 6) as described by the manufacturer (Pharmacia, Sweden).

The use of magnetic beads

Magnetic beads containing covalently coupled streptavidin, Dynabeads M280-Streptavidin, were obtained from Dynal AS, Norway. A neodymium-iron-boron permanent magnet (Dynal AS, Norway) was used to sediment the beads in the tubes during supernatant removal and washing procedures.

Immobilization of template from purified plasmid DNA

Plasmid DNA was digested with *Bst*EII and the 5′ protrusions were filled in using Klenow polymerase, biotin-16-dUTP and appropriate dNTP's (7). The material was purified using a Sephadex G-50 column (Pharmacia, Sweden), followed by ethanol precipitation. After redissolving in TE (10 mM Tris pH 7.5, 1 mM EDTA) the plasmid was digested with *Bgl*II. This reaction mixture containing the biotinylated double stranded DNA was mixed with Dynabeads M280-streptavidin previously washed with 1 M NaCl and TE. Approximately 2 pmole of the plasmid immobilized on 0.150 mg of the magnetic beads were used for each set of the four different dideoxy-nucleotide reactions.

Immobilization of PCR amplified templates

A single colony of *E.coli*, containing the plasmid to be sequenced, was picked from an agar plate with a sterilized Pasteur pipette and suspended in 0.01 ml PCR buffer (67mM Tris, 16.6 mM $(NH_4)SO_4$, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.170 mg/ml BSA), adjusted to pH 10.0. The sample was incubated for 5 minutes at 95°C and after cooling to room temperature, neutralized by the addition of 0.001 ml of 10× concentrated PCR buffer, pH 7.0.

The PCR was performed with two oligonucleotide primers, RIT 6, (biotin-CCATGATTACGAATTTAATAC-3′) and RIT 13 (5′-TTCGATATCGGTAACCA-GCACTCCATGTCATGG-3′) complementary to regions upstream and downstream of the multilinker region of pRIT27, respectively. The upstream primer was biotinylated in the 5′ end. The reaction mixture (0.100 ml) consisted of the above described PCR buffer, pH 8.8, 0.001 mM of each primer, 0.200 mM each of dATP, dCTP, dGTP and dTTP and 0.010 ml of the above described lysed sample. Two units of *Taq*I polymerase were added and temperature cycle reactions were carried out using a Techne programmable Dri-Block PHC-1 (Techne, UK). Each cycle included a heat denaturation step at 92°C for 1 minute, followed by annealing of primers to the DNA for 2 minutes at 50°C, and DNA chain extension with *Taq*I polymerase for 1 minute at 72°C. The reaction mixture was covered with a layer of paraffin oil. After 20 cycles, the mixture were added to 0.3mg of Dynabeads M280 streptavidin, previously washed with 1M NaCl and TE.

Amplification and immobilization of genomic DNA

Staphylococcus aureus SA113 were grown as single colonies on agar plates. A single colony was picked with a sterilized Pasteur pipette and suspended in 0.010 ml PCR buffer, pH 10.0. The sample was incubated for 5 minutes at 95°C and, after cooling to room temperature, neutralized by the addition of 0.001 ml of 10× PCR buffer, pH 7.0.

The PCR was performed with two oligonucleotide primers complementary to the staphylococcal protein A gene. One primer, RIT 1, (biotin-AATAGCGTGATTTTGCGGT-3′) was biotinylated in the 5′ end, the second primer (5′-GTAAAACGACGGCCAGTGCAGGTGTTACGCCA CCAG-3′), RIT 2, contains in the 5′ end a specific 'handle sequence' not complementary to the DNA template. This handle sequence generates an annealing sequence for the general sequencing primer. The PCR amplification followed by immobilization on solid support was performed as described above.

Sequencing reactions using immobilized template DNA

The immobilized biotinylated double stranded DNA was converted into single stranded form by incubation at room temperture with 0.15 M NaOH for 5 minutes. The magnetic beads, with immobilized template DNA was subsequently washed with 0.15 M NaOH and H_2O. Sequencing reactions were performed using both a ^{32}P end labelled primer (5′-GTAAAACGACGGCCAGT-3′), RIT 7, and a fluorescent end labelled primer (5′-CGTTGTAAAACGACGGCCAGT-3′) (2). In both cases the magnetic beads with immobilized template DNA were mixed with 2 pmole of the sequencing primer in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and 0.1 mg/ml BSA in a total volume of 0.01 ml. The annealing mixture was heated to 65°C and allowed to cool to room temperature. A volume of 0.001 ml DTT/NaCl mixture (0.1 M DTT/0.8 M NaCl) and 4 units of T7 DNA polymerase were added before the volume was adjusted to 0.015 ml. 0.0035 ml of the mixture were mixed with 0.0025 ml of the respective nucleotidemixture and incubated for 10 minutes at 37°C. The following nucleotide mixture was used; 0.080 mM of each dNTP, 0.0063 mM of the respective ddNTP, 50 mM NaCl and 40 mM Tris-HCl (pH 7.5). After completed extension the supernatant was removed and the magnetic beads were washed with H_2O. The newly synthesized oligonucleotides were eluted using 0.003 ml of a formamide/sequencing dye mixture consisting of deionized formamide containing 10 mM EDTA, pH 7.5. 0.3% (w/v) xylen cyanol ff and 0.3% (w/v) Bromophenol Blue. In the fluorescent end labelled primer protocol, the melting mixture consisted of deionized formamide containing 10 mM EDTA. After 15 minutes incubation at 37°C the supernatant was removed and diluted with 0.003 ml H_2O. Approx. 0.002 ml were loaded on a 4% standard electrophoresis polyacrylamide gel, or on a 7% sequencing gel run on an automatic sequencing apparatus with detection of fluorescent bands during electrophoresis (2).

RESULTS

Solid phase sequencing of purified plasmid DNA.

The principle of solid phase sequencing of purified plasmids has been described earlier (7–8). General sequencing vectors, pRIT27 and pRIT28 were constructed, into which the DNA to be sequenced is inserted in a multilinker and the plasmid is linearized and biotinylated using flanking restriction sites. To improve the solid phase sequencing approach, the protocol schematically outlined in Fig.1A was developed, in which magnetic beads with covalently coupled streptavidin are used instead of avidin agarose. In addition, the

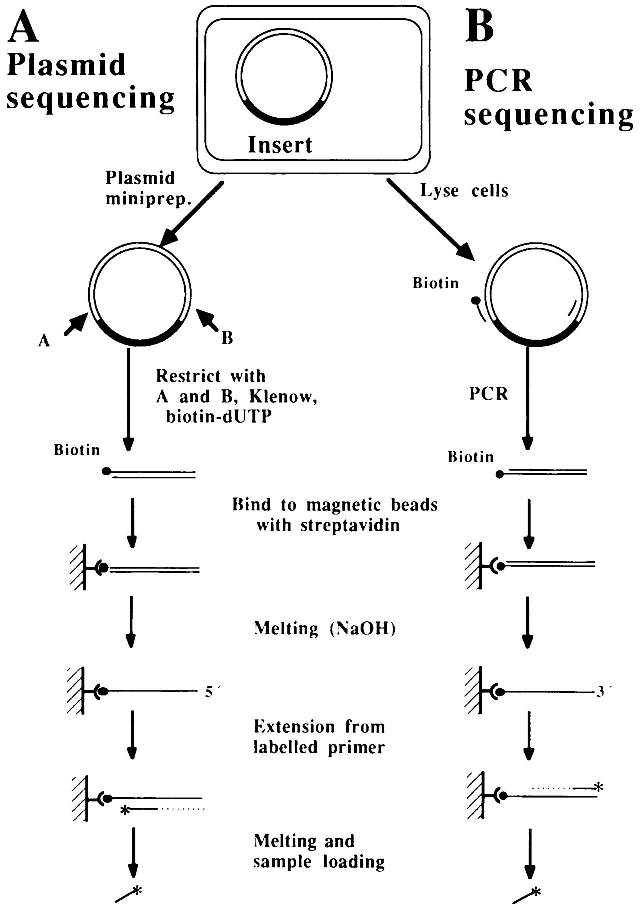

Figure 1. A schematic drawing of the basic concept of the solid phase sequencing using a purified plasmid vector (A) and PCR amplified templates (B). Note that depending on the choice of enzymes A and B, both fragments will or will not be immobilized. See text for details.

primer extension was performed using T7 DNA polymerase to obtain more uniform signal intensities (10). Note, that the extended material is eluted with a formamide/dye mix to enable direct sample loading on sequencing gels. The results from the sequencing of plasmid pRIT27 with a synthetic proinsulin gene fragment as insert is shown in Fig.2A. A ^{32}P labelled 'universal' primer was used and the sample was separated on a 4% field-strength gradient gel (11). A clear and readable sequence is obtained, which corresponds with the expected sequence. A strong band is obtained corresponding to run-off transcripts when the polymerase have extended through the insert and reaches the 5′ end of the immobilized template (site B in Fig.1A). As shown in Fig.2A, a 291 nucleotide fragment is obtained corresponding to a run-off at the *Bgl*II site of plasmid vector pRIT27.

Solid phase sequencing of PCR amplified plasmid DNA.

PCR can be used to amplify specific DNA sequences (12). To obtain a simple protocol

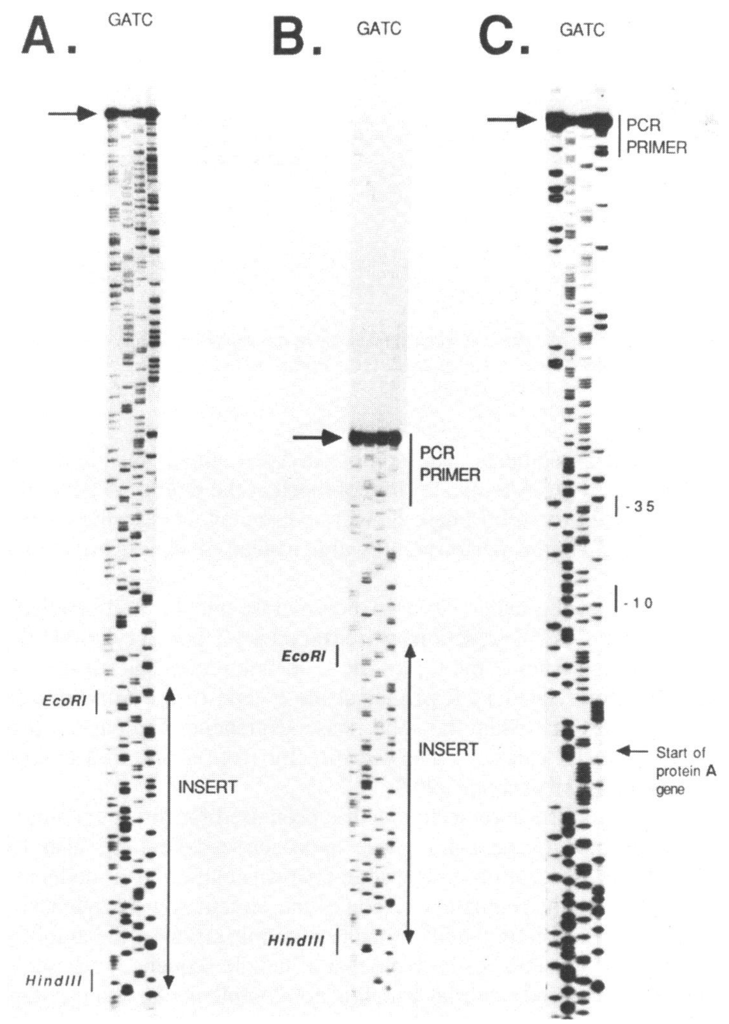

Figure 2. Autoradiograms of sequencing gels with samples obtained by solid phase sequencing. A; purified plasmid vector as tempate; B; PCR amplified templates and C; PCR amplified genomic templates.

to sequence plasmid inserts without having to purify the plasmid, a novel sequencing protocol was developed using the strategy outlined in Fig.1B. A single colony harbouring the pRIT27 plasmid vector with the proinsulin insert was lysed in PCR buffer, pH 10.0, at 95°C for 5 minutes. After neutralization, an *in vitro* amplification was performed using *Taq*I polymerase (see Material and Methods for details). Two general oligonucleotide primers, complementary to upstream and downstream sequences of the multilinker region of the plasmid vector, were used. The upstream primer was biotinylated in the 5′ end. After amplification for 20 temperature cycles, agarose gel electrophoresis reveled a single band corresponding to the DNA insert flanked by vector sequences (data not shown). The amplified material, without purification, was subsequently mixed with magnetic beads,

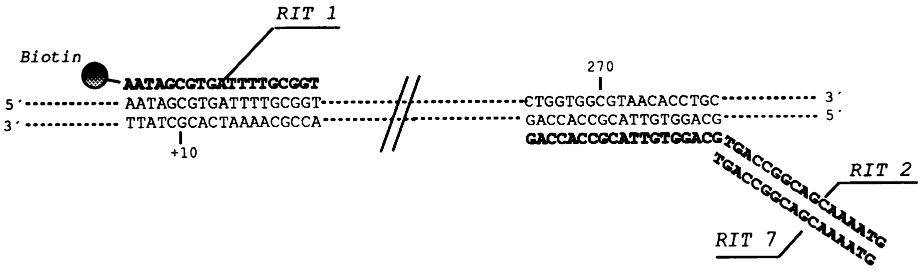

Figure 3. The primers used for solid phase genomic sequencing of the staphylococcal protein A gene. The numbers refer to the nucleotides as described by Uhlén *et al.* (16). Primer RIT 1 was labelled with biotin and RIT 7, the sequencing primer, with ^{32}P in the 5′ end.

to bind the biotinylated fragments. The non-biotinylated strand was eluted with alkali to yield the single stranded DNA template, immobilized at the 5′ end. A general sequencing primer can then be used for solid phase Sanger sequencing as described above, with the difference that the extension is performed towards instead of away from the solid support (Fig.1).

The result of sequencing using T7 DNA polymerase and ^{32}P end labelled 'universal' primer is shown in Fig.2B. The reactions were run on a 4% polyacrylamide field-strength gradient gel. The strong band at the top of the sequence represents run-off transcripts at the position of the biotinylated PCR-primer at the 5′ end of the immobilized template. The result demonstrates that when the solid phase sequencing approach is used for PCR amplified plasmids, DNA can be sequenced directly from a bacterial colony.

Solid phase sequencing of genomic DNA.

Recently, the *in vitro* amplification technique has been used for direct genomic sequencing (13–15). To investigate if the solid phase approach can be used also for genomic sequencing, the staphylococcal protein A gene (16) was chosen as a model system. Two specific primers flanking the regulatory region of the protein A were synthesized (Fig.3). The upstream primer was biotinylated to enable immobilization of the amplified material to streptavidin coated magnetic beads. Note that a 'handle sequence' was introduced into the other end of the amplified material which is not complementary to the staphylococcal genome (Fig.3). In this way, a general sequencing primer (RIT 7) can be used for the sequencing reactions.

A single colony of *S. aureus* SA113 (17) was picked and lysed as described above. After 20 temperature cycles using *Taq*I polymerase, the *in vitro* amplified material was allowed to bind to magnetic beads. After extensive washing, the solid phase sequencing was performed using T7 DNA polymerase and ^{32}P end labelled RIT 7 primer. The results shown in Fig.2C demonstrate that the solid phase approach can be used for direct genomic sequencing. The strong band at the top of the sequence represents run-off transcripts at the position of the biotinylated PCR primer at the 5′ end of the immobilized genomic staphylococcal DNA. The sequence of the promoter region of the protein A gene of *S. aureus* SA113 has not been previously sequenced. Interestingly, the sequence is identical to the *S. aureus* strain 8325-4 sequence, but differs from the sequence of *S. aureus* strain Cowan I (16).

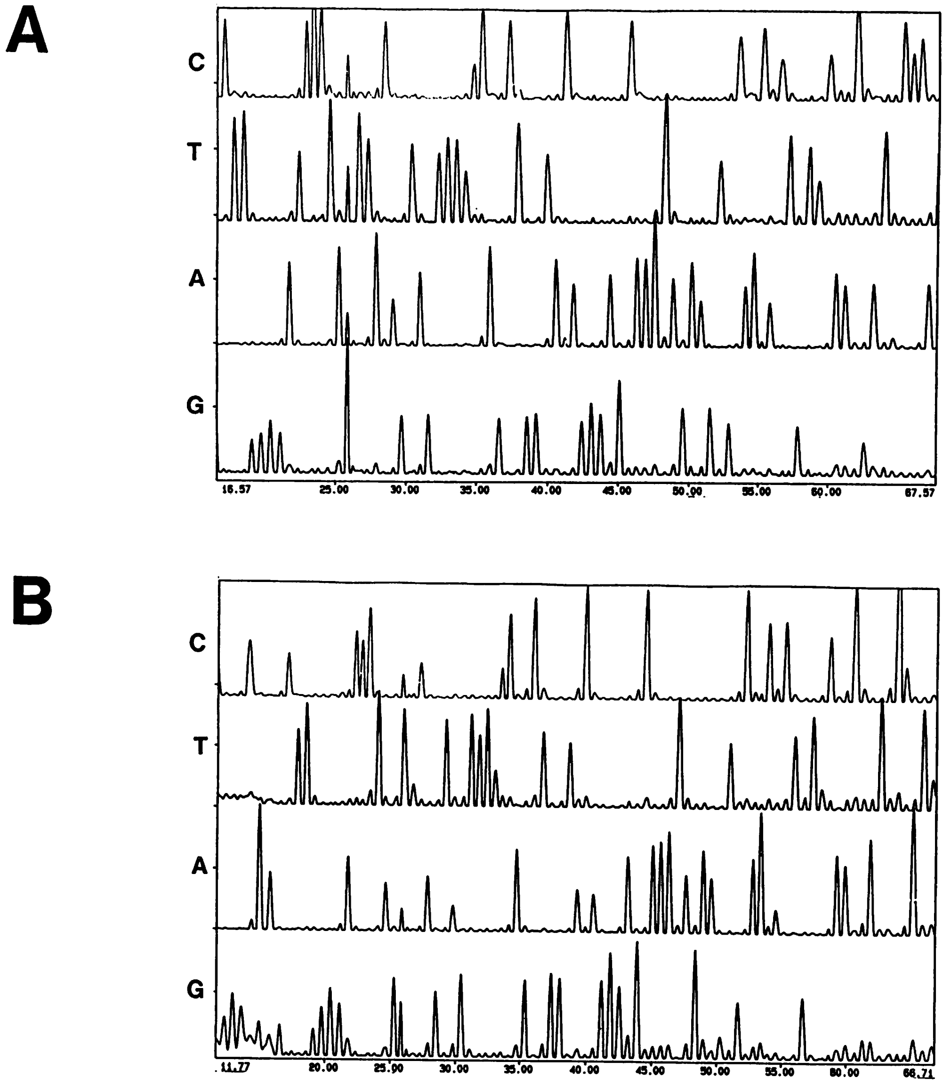

Figure 4. Solid phase sequencing using a fluorescent primer. Data output, from a sequencing run on a 7% polyacrylamide gel with a 20 cm separating length, is shown. A; purified plasmid as template; B; PCR amplified plasmid as template.

Solid phase sequencing using a fluorescent primer.

Recently, systems have been designed enabling on-line detection of DNA during electrophoresis using various fluorescent dyes (2). To test if the solid phase approach could be used also with a fluorescent sequencing primer, the scheme described above for purified and PCR amplified plasmid DNA (Fig.1A and1B) was followed and the eluted fragments were loaded on a 7% sequencing gel with a 20 cm separating length (2). The results using the two strategies are shown in Fig.4, in which the data output corresponding to the proinsulin insert is shown. The results demonstrate that the solid phase approach can be used for fluorescence sequencing using both purified plasmid (Fig.4A) and PCR amplified DNA (Fig.4B) as template.

DISCUSSION

In this paper, we have shown that solid phase approaches can be used for direct DNA sequencing of both plasmid and genomic DNA. The remarkable stability and strength of the biotin-streptavidin complex allows DNA manipulations, such as melting with alkali and elution with formamide, without interfering with the complex binding. The biotin can be introduced either by endonuclease restriction of the purified plasmid followed by a fill-in reaction of biotin-dUTP with polymerase (Fig.1A) or by using a biotinylated primer during an *in vitro* amplification operation (Fig.1B). The successful use of T7 DNA polymerase and fluorescent primers (Fig.4) demonstrates that the concept can be used also for fluorescence sequencing strategies.

The magnetic beads have several characteristics that make them well suited for solid phase sequencing. First, the use of a magnet facilitates liquid handling and the need for centrifugal steps are avoided. Second, the low density of the beads makes it unnecessary to mix the beads during sequencing. Third, the beads are of equal size (monodisperse), which gives uniform kinetics in the magnetic fields. Finally, low or little interference with the enzymes used for sequencing is observed.

The template immobilized by the two alternative methods have different orientation after the elution of the non-bound strand (Fig.1). This has the consequence that the primer extention reaction with the dideoxy-nucleotides is proceeding either away or towards the solid support. Interestingly the PCR amplified DNA template gives a broader run-off fragment (Fig.2B and 2C) compared to the purified plasmid DNA (Fig.2A). Detailed analysis of the sequences reveal that the T7 DNA polymerase terminates at all the four dideoxy reactions 4–5 nucleotides away from the 5′ end of the immobilized template, while the purified plasmid DNA gives a relatively high amount of full length run-off fragments. This suggests that the polymerase is sterically hindered when progressing within 4–5 nucleotides from the solid support. However, a large part of the fragments are indeed of full-length even for the PCR amplified template (Fig.2B and 2C).

The method to sequence plasmid inserts using the solid phase PCR-approach (Fig.1B) has clear advantages as compared to standard sequencing protocols. A single colony from after a transformation can be directly sequenced within a day without the need for plasmid purification and with no or little manual operations. The lysing procedure involves a single temperature cycle in an alkali solution and does not require reagents that might later inhibit the polymerase reaction. General sequencing primers can be used and sequencing from both ends of the insert is easily achieved as either of the two primers used during the *in vitro* amplification step can be biotinylated. In addition, buffers and/or enzymes can be changed between the PCR and sequencing steps. This ensures a flexible system where both the amplification and sequencing reactions can be performed under optimal conditions. The template can be extensively purified after the immobilization, which makes it likely that reproducible results can be obtained. A combined method for automated template preparation and solid phase sequencing can thus be envisioned, which might have potential for large scale sequencing efforts.

Recently, several authors have reported direct genomic sequencing using PCR technology. Using genomic amplification with transcript sequencing (18) a phage promoter was inserted during the *in vitro* amplification step and a RNA template for reverse transcriptase sequencing was obtained using RNA polymerase. Scharf *et al.* (19) used the enzymatically amplified segments for direct cloning into M13 vectors followed by standard Sanger sequencing. An alternative method has been described in which suboptimal quantities of one of the two primers has been used during the *in vitro* amplification (20). In this way, substantial amount of single stranded template is obtained, which can be used for sequencing.

The method described here instead involves *in vitro* amplification of a genomic sequence, with one of the two primers biotinylated, immobilization of the fragment to a solid support followed by solid phase sequencing. This approach has the advantages that a direct sequencing protocol is obtained without *in vitro* amplifications at suboptimal concentration of primers and without the need for additional ethanol precipitation or RNA polymerase reactions.

We have shown that the solid phase DNA sequencing approach can be used for Sanger sequencing of plasmid and genomic DNA. The method to immobilize template in a direct manner can also provide the means for new sequencing strategies. On-line sequencing might be developed to avoid the electrophoresis step (21) and new strategies for the sequencing of large inserts might be developed. Recently, we have successfully performed solid phase sequencing using microtiter wells, with covalently bound streptavidin, as solid support (Hultman and Uhlén, unpublished). The aim of such efforts is to develop completely automated methods for template preparation and solid phase sequencing using laboratory robots.

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