Solid phase *in vitro* mutagenesis using plasmid DNA template

Thomas Hultman, Maria Murby, Stefan Ståhl, Erik Hornes[1] and Mathias Uhlén*
Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden and [1]Apothekarnes Laboratorium A.S., Harbitzalléen 3, 0275 Oslo 2, Norway

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ABSTRACT

Site-specific mutagenesis was accomplished using a solid support to generate single stranded vector and insert fragments which can be used to form gap-duplex plasmids through flanking, complementary double stranded regions. More than 80% mutants were obtained in both a single and a double primer approach. No special vectors or strains are needed and mismatch repair is avoided as the mutagenesis region is in a single stranded form when transformed into the *Escherichia coli* host cell. The fragments to be immobilized can be produced either by a polymerase chain reaction using general primers or by a site-specific restriction followed by a fill-in reaction. This novel method is rapid, simple and flexible and well suited for both manual and semi-automated *in vitro* mutagenesis protocols.

INTRODUCTION

Oligonucleotide-mediated mutagenesis provides a precise and versatile method to introduce specific changes into cloned DNA sequences. A large number of different approaches have been described to obtain single stranded template needed for the extension reaction (1,2). The aim for all these methods has been to obtain a rapid and reproducible scheme with high yield of mutants.

The original protocols, based on phage M13 vectors, have the disadvantage that the stability of larger DNA inserts is somewhat unpredictable and that additional subcloning steps often are needed to obtain expression vectors, promoter probe vectors etc (2). This has led to the development of several systems, where the plasmid can be converted to a single stranded form *in vivo* (3,4). These methods have often been combined with approaches to increase the yield of mutants, e.g. protocols involving uracil-containing template (5), thio-containing template (6) or gap-duplex mutagenesis (7). These approaches often give high yields of mutants (>50%) with rather general plasmid vectors and thus eliminate the need for additional subcloning steps. However, special strains of *Escherichia coli* are often required and the template preparations are often rather cumbersome. Recently, a large number of new methods for *in vitro* mutagenesis have been described (8,9,10,11) based on the polymerase chain reaction (PCR). Site-specific mutants are created by introducing mismatches into the oligonucleotides used to prime the *in vitro* amplification. Such methods include splicing by overlap extension (8,9,10), which involves a two step PCR procedure and subsequent cloning using blunt ended products. A variant of this technique is the use of a restriction enzyme that cuts at the same distance from the target sequence (12). Both these methods require restriction enzyme digestion of the PCR product and a ligation step is always necessary.

A different strategy is to use the PCR to create recombinant plasmid circles with discrete, cohesive single stranded ends (11). These circles can be formed without the use of restriction enzyme digestion and ligation, and give high yield of mutants. However, due to the necessity to amplify *in vitro* the whole vector, the method might be limited to special small vectors. In addition, the non-specific mutations frequently introduced during the PCR by the *Taq* polymerase (13) make it desirable to sequence the whole fragment produced by PCR, which is difficult for protocols involving *in vitro* amplification of the whole vector.

Here, a new method for *in vitro* mutagenesis is described based on solid phase methodology using magnetic beads, which originally were developed for DNA sequencing (14). Using the biotin-streptavidin system, double stranded plasmid DNA can be immobilized to the solid support and one of the strands can be selectively eluted. Both the bound and the eluted strands can subsequently be used for cloning and/or mutagenesis. A simple protocol involving no special strains or vectors yields more than 80% mutants. The single stranded plasmid template can be obtained either by restriction of purified plasmids or by PCR. The use of the method for large scale automated procedures is discussed.

MATERIALS AND METHODS

Strains, enzymes and oligonucleotides

E.coli strain RRIΔM15 (15) was used as plasmid host. Restriction endonucleases, T4 DNA polymerase, Klenow polymerase, T7 polymerase, T4 polynucleotide kinase and T4 DNA ligase were obtained from Pharmacia LKB Biotechnology (Sweden).

* To whom correspondence should be addressed

Biotin-16-dUTP and *Taq* polymerase were purchased from Boehringer Mannheim (W.Germany). Oligonucleotide primers were synthesized by phosphoramidite chemistry on a Gene Assembler Plus (Pharmacia LKB Biotechnology, Sweden). One primer (RIT 29) was biotinylated in the 5′ end as described by the manufacturer. Plasmid purification and transformation of *E.coli* were performed as described by Hultman *et al* (14).

Preparation of single stranded vector DNA

10 μg of plasmid pRIT28 (16) was restricted with *Hin*dIII and the 5′ extension were filled in using Klenow polymerase, biotin-16-dUTP and appropriate dNTP's (17) The material was purified using a Sephadex G-50 column (Pharmacia LKB Biotechnology, Sweden), followed by etanol precipitation. After redissolving in TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) the plasmid was digested with *Eco*RI. This reaction mixture containing biotinylated double stranded DNA was mixed with 600 mg streptavidin- coated magnetic beads (Dynabeads M280-streptavidin, Dynal AS, Norway), previously washed with TE containing 1 M NaCl, and incubated 15 minutes at room temperature. Immobilization efficiency was checked by agarose gel electrophoresis. About 10 pmole of double stranded DNA can be immobilized per mg magnetic beads. After supernatant removal, using a neodymium-iron-boron permanent magnet (Dynal AS, Norway), the single stranded vector DNA was eluted by incubation in 20 μl 0.15 M NaOH for 10 min at room temperature. The supernatant, containing the single stranded vector, is then neutralized by adding 2.2 μl 10×TE, pH 7.5 and 1.3 μl 1.25 M acetic acid.

When the immobilized single stranded vector was used as template for the synthesis of single stranded vector, the beads with immobilized material were previously washed once with 0.15 M NaOH and three times with TE. To enable buffer removal between each step, sedimentation of the magnetic beads was performed using the permanent magnet. 10 ml of an annealing buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl and 100 μg BSA/ml) supplemented with 15 pmole of an extension primer (RIT66 5′-GGCACTGGCCGTCGTTTT-ACAAC GTCGTGA-3′) was then added to the beads, giving about 3 fold molar excess of primer to theoretically immobilized vector. The reaction mixture was heated to 65°C and allowed to cool to 0°C. After buffer removal, a polymerase buffer (20 mM Tris-HCl pH 8.8, 2 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 100 μg BSA/ml, 0,5 mM of the four dNTP's) and 3.5 u T4 DNA polymerase were added to a total volume of 30 μl. Incubation at 0°C for 10 minutes was followed by a 2 h extension at 37°C. The beads were then washed in TE and a melting and neutralization procedure was performed as described above. The magnetic beads with immobilized single stranded vector can be stored in TE for several weeks at 4°C.

Immobilization of PCR amplified template for *in vitro* mutagenesis

A single colony of *E.coli*, harbouring plasmid pRIT28 with an insert to be mutated, was picked from an agar plate with a sterilized Pasteur pipette and suspended in 10 μl PCR-buffer (25 mM TAPS-HCl, pH 9.3 at 20°C, 50 mM KCl, 0.1% Tween 20). For bacteria lysis, the sample was incubated for 5 minutes at 99°C (14). The PCR was performed with two oligonucleotide primers, RIT28 (5′-AAAGGGGGATGTGCTGCAAGG-CGA-3′) and RIT29 (5′-Biotin-TGCTTCCGGCTCGTATGT-TGTGTG-3′), complementary to regions downstream and upstream of the multilinker region of pRIT28, respectively. The upstream linker was biotinylated in the 5′ end. The reaction mixture (100 μl) consisted of the above described PCR-buffer supplemented with 2.0 mM $MgCl_2$, 10 pmol of each primer, 200 μM of the dNTP's and 2 μl of the above described lysed sample. Two units of *Taq* polymerase were added and temperature cycle reactions (96°C for 0.5 min, 60°C for 1 min, 72°C for 1 min) were carried out using a Techne Dri-Block PHC-1 (Techne, UK). The reaction mixture was covered with a layer of mineral oil. After 20 cycles, the mixture was added to 600 μg Dynabeads M280-streptavidin, previously washed with TE containing 1 M NaCl. The immobilized biotinylated double stranded DNA was converted to single stranded form by incubation at room temperature with 0.15 M NaOH for 10 minutes.

Solid phase *in vitro* mutagenesis using the double primer system

The immobilized single stranded plasmid DNA, containing an insert to be mutated, was washed in TE. 10 ml annealing buffer with 15 pmole of both a general extension primer (RIT67: 5′-AGCACTCCATTGTCATGGTTCAGGCT GCGC-3′) and a phosphorylated mutagenesis primer, RIT109 or RIT110 (Fig. 3A and C), were heated to 65°C and allowed to cool to 0°C. An extension/mutagenesis step was performed as described for the preparation of single stranded vector with the difference that 1 u T4 DNA ligase was added. The synthesized single stranded DNA was eluted by incubation in 20 μl 0.15M NaOH and neutralized by the addition of 2.2 μl 10×TE, pH 7.5 and 1.3 μl 1.25 M HAc.

Solid phase *in vitro* mutagenesis using the single primer system

A combined mutagenesis/extension primer was used for annealing and the following synthesis of a single stranded insert was performed under the same conditions as in the double primer approach, but with the T4 DNA ligase excluded. Elution and neutralization were carried out as for the double primer approach.

Transformation of gap-duplex plasmids

10 μl of the eluted and neutralized single stranded vector DNA was mixed with the same volume of synthesized single stranded DNA containing a mutated insert. The mixture was incubated at 70°C for ten minutes and allowed to cool to room temperature. Competent *E.coli* RRIDM15 cells (14) were directly transformed with the reaction mixtures and the cells were spread on IPTG/XGal plates for blue and white selection (18).

Direct solid-phase DNA sequencing of positive clones

Colonies to be sequenced were picked and lysed as described by Hultman *et al* (14). The PCR amplification, of the plasmid DNA containing the insert, was performed using primers RIT28 and RIT29 (biotinylated). The amplification of fragments, the immobilization on magnetic beads and the subsequent alkali melting to generate a single stranded template were carried out using the scheme described (14). A modified Sanger sequencing with T7 polymerase (19) and a universal fluorescent sequencing primer 5′-CGTTGTAAAACGACGGCCAGT-3′ (Pharmacia LKB Biotechnology, Sweden) was performed. The sequencing reactions were analyzed on a 6% polyacrylamide sequencing gel run on an automatic sequencing apparatus with on line detection of fluorescent bands during electrophoresis (A.L.F., Pharmacia LKB Biotechnology, Sweden).

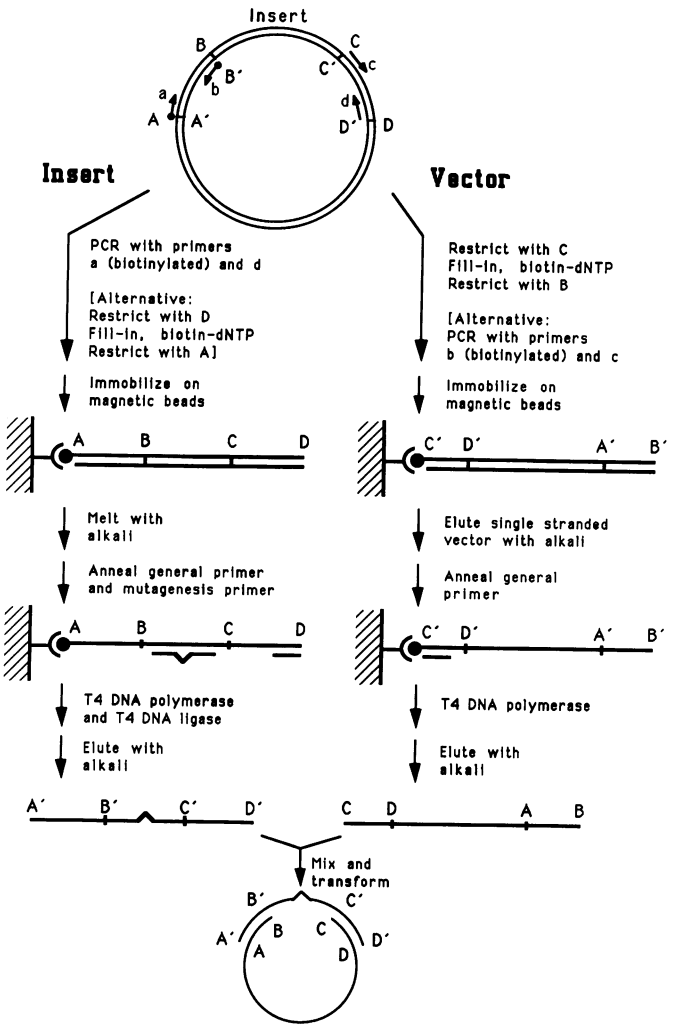

Figure 1. A schematic drawing of the basic concept of the solid phase *in vitro* mutagenesis. Note, that the alkali elution, directly after immobilization to the solid support, generates single stranded vector which can be used to form gap-duplex plasmids. For the experiments described in this paper, C is a *Hin*dIII site, while B is a *Eco*RI site. See text for details.

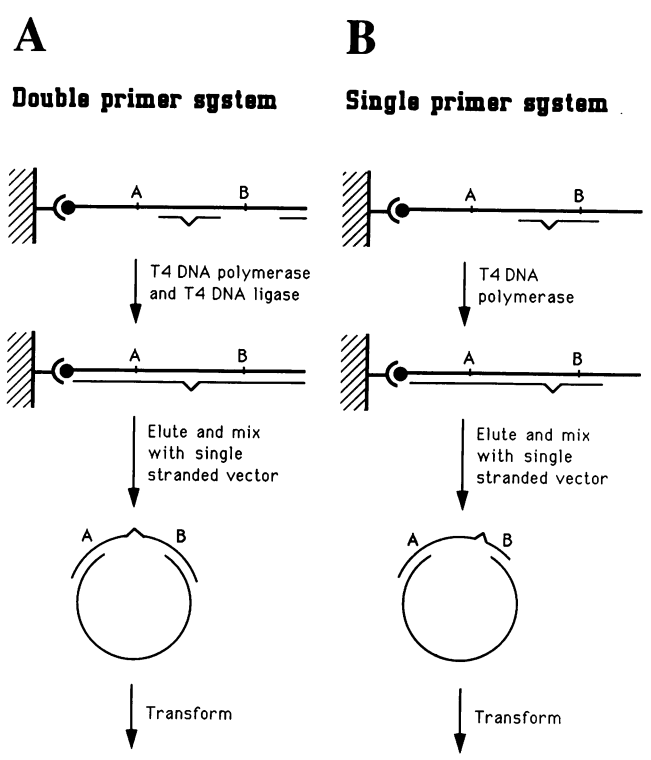

Figure 2. A schematic drawing of the two systems for solid phase *in vitro* mutagenesis using one or two primers. The immobilized strand is bound to the solid support using the biotin-streptavidin system as descrined schematicaaly in figure 1 (Insert).

RESULTS

The basic concept for solid phase *in vitro* mutagenesis

The principle of the solid phase approach is outlined in figure 1. The vector and the insert fragment to be mutated are immobilized separately on magnetic beads through a biotin incorporated into one of the strands. The biotin can be introduced by site-specific restriction followed by a fill-in reaction using DNA polymerase or alternatively by PCR using general primers (denoted a,b,c and d) complementary to regions flanking the multi-cloning site of the vector. Note, that the PCR yields a single strand immobilized in the 5′ end, while the restriction/fill-in route yields a 3′ immobilized single strand (14). This must be taken into account when designing an experiment to ensure that complementary strands are obtained for the vector and the insert (see below).

For the vector, a polymerase extension is not needed as the initial alkali elution generates single stranded vector. However, repeated polymerase extensions followed by elution gives material for many mutagenesis experiments. Both the initial elution as well as the elution after the *in vitro* extention gives an identical, clean, well-defined, linear, single stranded vector fragment. For the insert fragment, a single stranded template suitable for primer directed polymerase reactions is obtained by elution of the non-immobilized fragment with alkali. A double primer system in which a general primer, complementary to the vector part of the immobilized fragment, is normally used together with the specific mutagenesis primer with a mismatch in the region to be changed (Fig. 2A). An extension reaction using T4 DNA polymerase and T4 DNA ligase is subsequently performed to yield a mutated strand containing the desired mismatch (Fig. 1). Other polymerases, such as Klenow and *Taq*, give high yield of displacement of the mutagenesis primer by extension from the general primer (Hultman and Uhlen, data not shown) while T4 DNA polymerase has been reported to have a low displacement tendency (20,21). The synthesized strand is finally eluted by alkali and mixed with the single stranded vector fragment. This yields a gap-duplex plasmid containing small double stranded regions or overlap with large single stranded regions. Note, that the mutated region is single stranded to avoid mismatch repair. The gap-duplex plasmid is finally transformed directly to *E.coli* and clones are screened by conventional methods, ideally by DNA sequencing, to find mutated plasmids.

If the desired mutation in the insert is in close proximity to the vector part, it is possible to use a scheme (Fig. 2B), which is somewhat simpler than the general scheme (Fig. 1 and 2A). A combined mutation/extension primer, complementary to both the mismatch region and part of the vector sequence, is synthesized and used for the extension (Fig. 2B). This strategy gives a rather short complementary region, such as the 18 base

pairs used below, but the gap-duplex plasmid can still be used for successful transformations.

To test these two strategies, different mutation experiments were carried out involving both site-specific substitutions (number 1 and 3) and an insertion (number 2). An experiment allowing for mismatch repair was also tested (number 3) in order to evaluate the importance of having the site of mutation in a single stranded region.

Substitution mutagenesis using the double primer system

In the initial experiment, a mismatch primer was used to introduce a stop codon into the multilinker region of plasmid pRIT28 (16) using the double primer approach (Fig. 2A). A **T** to **A** substitution was carried out using a 25 nucleotide RIT109 primer (Fig. 3A). This mutation allows simple initial screening by blue/white selection on IPTG/Xgal plates. Positive clones (white) were further checked by lack of the *Hin*dIII restriction site.

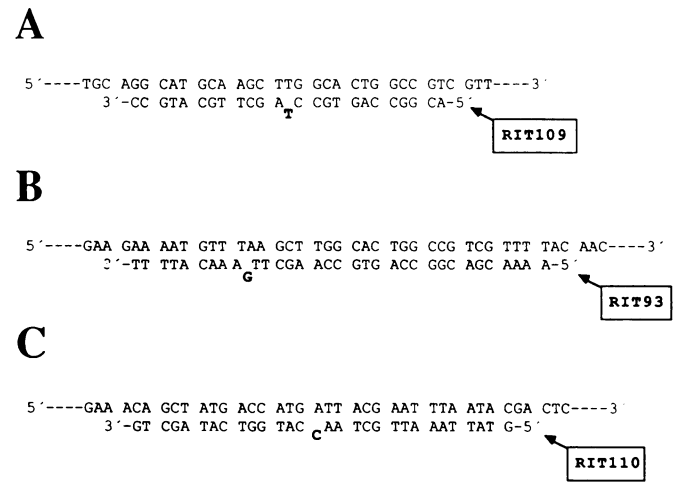

Figure 3. The sequences of the primers and the templates used for the different solid phase *in vitro* mutagenesis experiments. See text for details.

Table 1. Number of colonies obtained after transformation of *E.coli* with different single stranded DNA fragments.

Experiment	Concept	Vector	Insert	Vector+Insert
1	A	7	2	350
2	B	4	4	233

The eluted and neutralized fragments were transformed to competent *E.coli* cells. Vector+Insert, represents the material obtained after mixing the produced single stranded fragments, which are allowed to form the gap-duplex plasmids. See text for details.

The general concept (Fig. 1 and 2A) was followed, in which the immobilized insert template was obtained through PCR and the single stranded vector was produced via the restriction/fill-in route. A general primer (RIT67) and the mutagenesis primer (RIT109) were annealed to the single stranded insert template. After a solid phase polymerase reaction, the extended material was eluted. The single stranded vector was produced by simply eluting the non-bound strand after immobilization of the double stranded vector fragment to the beads. The double stranded overlap regions of the two strands were 105 and 87 base pairs, respectively.

The transformation frequencies for single stranded vector and insert alone is presented in Table 1 (experiment no 1). A low number of transformants (< 10) is obtained in each case. This background most likely represents non-specifically bound vector and can be further reduced by precautions during binding and washing (data not shown). In contrast, when the two single stranded fragments are mixed and transformed into *E.coli*, a large number of colonies are obtained (Table 1). These colonies were screened by the blue/white selection, which showed that 83% have a white phenotype (Table 2). Of these potentially positive mutants, 18 were purified and checked by restriction cleavage (lack of the *Hin*dIII site) and solid phase sequencing (14). Out of these 18 clones 17 were found to have the expected substitution in the mutated region (Table 2). This demonstrates that the general two primer concept, outlined in figures 1 and 2A, can be used for efficient solid phase mutagenesis giving approximately 80% mutants without any special selection procedures.

Insertion mutagenesis using the single primer system

In the second experiment, a mismatch primer was used to introduce an insertion into the multilinker region of plasmid pRIT28EM3 (22) using the single primer approach (Fig. 2B). A **G** insertion was performed using a combined mutagenesis/extension RIT93 primer (34 nucleotides) (Fig. 3B), giving an 18 base pair overlap with the single stranded vector. The insertion introduces a frame-shift mutation in the multilinker region of pRIT28EM3 and this could simply be assayed by white to blue screening on IPTG/Xgal plates.

The concept described for the substitution experiment was followed, although the single primer extension (Fig. 2B) was used for the insert fragment. The transformation frequencies for the vector and the insert alone were also in this case low (Table 1, experiment no 2). In contrast, several hundred colonies were obtained when the two single stranded fragments were mixed before transformation (Table 1). This shows that the rather short overlap (18 base pairs) on one side of the insert allows a functional gap-duplex plasmid to form, which survives a standard transformation protocol.

Table 2. Frequency of site-specific mutations obtained using the various concepts.

Experiment	Concept	Mutation	Selection	Frequency (%)	Verified by sequencing
1	A	Substitution	Blue to white	83	17(18)
2	B	Insertion	White to blue	85	8 (8)[1]
3	A[2]	Substitution	*Nco*I-site	15	4(4)

The frequency is based on the ratio of blue/white or restriction site analysis (selection). The sequencing was performed using the solid phase method (14). [1]Two additional clones were found to contain a deletion in the insert and were therefore not sequenced. [2]Experiment no. 3 involves a variation of concept A with the possibility of mismatch repair in the mutagenesis region.

The results of the mutagenesis is presented in Table 2 (experiment no 2). The initial screening for blue colonies showed 85% positive clones and 10 of these positive clones were further analyzed by restriction mapping (data not shown) and solid phase sequencing (Table 2). Two of the colonies were found to contain a secondary deletion, which might be explained by the extremely repetitive sequence of the insert (22), while the other eight clones showed the correct size and the expected sequence in the mutated region (Table 2). This shows that an approximately 80% mutation frequency can also be obtained for an insertion mutagenesis using the single primer approach outlined in figure 2B.

Substitution mutagenesis involving a mismatch duplex

A third experiment was carried out to investigate the effect of having a mismatch duplex during the gap-duplex plasmid transformation. The two primer approach (Fig. 2A) was applied to introduce a *Nco*I site upstream of the *Eco*RI site of plasmid pRIT28 (16). An **A** to **G** substitution was performed using a 30 base pair RIT110 primer (Fig. 3C). The mutagenesis was performed with a general extension primer (RIT67) and a mismatch primer (RIT110) as in experiment no 1, but differ in that the mismatch primer anneals upstream of the multilinker region and thus gives rise to a mismatch in the double stranded region of the gap-duplex plasmid obtained after vector-insert mixing.

No blue/white selection could be used in this case and therefore 40 of the colonies obtained after transformation were checked by restriction analysis for presence of a new *Nco*I site (data not shown). About 15% of the plasmids were found to have the expected *Nco*I site (Table 2, experiment no 3). Four of the positive clones were further analyzed by solid phase sequencing, which verified that all had the expected sequence (Table 2).

DISCUSSION

Solid phase approaches have proved to be useful for separation and detection of DNA (23), in particular in areas where a clean and reuseable material is needed. Here, we show that magnetic separation of DNA can be used to specifically produce single stranded DNA fragments that can be used for cloning of site-specifically mutated gene fragments. Vector and mutated insert fragments can be produced separately and these fragments can subsequently be mixed and transformed to *E.coli* to give high yields of the specific mutations. The protocol provides the site-specific mutation in a single stranded form, thus eliminating the effect of the mismatch repair system of the host cell. No special strains or template preparations are therefore needed. Note, that the number of transformants per μg of gap-duplex plasmid DNA is relatively low, but the protocol still yields several hundred colonies containing site-specific mutants (Table 2).

The mutagenesis protocol outlined in Figure 1 has the advantage that the same result can be accomplished both with or without the use of PCR. This is important since accumulated polymerase errors are a major concern whenever PCR products are cloned (13). This makes it strongly desirable to sequence all PCR produced fragments when they are used for cloning. In particular for large fragments, such as most cloning and expression vectors, this task is difficult and time consuming. Therefore, protocols such as the solid phase method (Fig. 1) are attractive, where the vector can be produced using a non-PCR approach.

In this paper, we have actually used a combination of the two alternative methods to immobilize the DNA fragments. The single stranded vector was produced by the restriction/fill-in procedure to avoid PCR amplification of large sized fragments. Although this relatively cumbersome method was employed, the same immobilized vector fragment can be used several times to generate single stranded vector as run-off transcripts. Thus, the risk for polymerase induced errors is minimized without sacrificing convenience and speed.

The insert fragments were in all cases produced by the PCR approach. As these fragments were relatively small in size, analysis of the mutated fragments could be performed by sequencing the complete insert regions. Secondary mutations introduced by the PCR can then be found and discarded. However, for the 30 clones sequenced in this paper (Table 2), none had random substitutions, although it cannot be ruled out that the two clones containing deletion mutants (experiment no. 2) were produced during the PCR amplification.

Once the vector and the insert are immobilized on the solid support, the protocol for extension, elution and mixing of the two strands is relatively straightforward and well adapted for automation using a robotic work station. For large scale mutagenesis protocols, it might therefore be convenient to use semi-automated methods, similar to the methods developed for automated solid phase DNA sequencing (23, T. Hultman and M. Uhlen, unpublished).

The insert fragment can be cloned directly into different vectors provided that complementary overlap regions exist between the vector and the insert. This allows for the use of a battery of specifically prepared single stranded vectors, into which the insert can be directly cloned simply by mixing and transforming. Note, that the cloning is achieved without the use of restriction enzymes or ligase. This is similar to the recombinant circle approach (11), although the overlap region in this latter case was several kilobases. Here, an 18 base pair overlap was found to be functional (experiment no 2) which makes the solid phase method very flexible compared to the recombinant circle approach.

The solid phase method can also be used to produce recombinants directly with the help of the general primers exclusively. Recently, a part of the human apolipoprotein E gene was cloned in this manner. A 20 base pair overlap to the cloning vector were introduced to both side of the amplified, chromosomal gene fragment via the PCR (E. Hornes, T. Hultman and M. UhlÅn, unpublished). More than 80% recombinant plasmids were obtained without the use of restriction enzymes, ligase and specific selection methods. These results further accentuate the efficiency and selectively of solid phase approaches for DNA handling.

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