Novel methods in molecular cloning

Ph.D. thesis

Candidate: József Gál

Environmental Biochemistry and Biotechnology Doctoral Program

Supervisor: Miklós Kálmán, Ph.D.

University of Szeged

2000.

Table of Contents

1. Introduction and Aims

During molecular cloning, the DNA fragment to be multiplied is attached to a cloning vector, a DNA molecule which is capable of self-replication within the host cell. The present thesis describes the elaboration of two new methods in molecular cloning: the development of a *gltS*-based cloning system enabling positive selection of recombinant clones and a method for the directional cloning of native products of the polymerase chain reaction (PCR).

The development of techniques for the cutting of DNA molecules into discrete fragments, linking of different DNA fragments together and introduction of the resulting chimaeric molecules into host cells were prerequisites for molecular cloning. The methods offering solutions for these basic steps became available by the early 1970's.

The discovery of site-specific endodeoxyribonucleases offered a solution for the generation of discrete DNA fragments. An endodeoxyribonuclease (or simply endonuclease) used during genetic manipulation should both recognize and cut DNA molecules at specific sequences. The discovery of restriction endonucleases came with the research of host-controlled restriction-modification systems. In the 1950's it was recognized that phage grown on a certain bacterial strain sometimes infect cells of another strain of the same species with very low efficiency (Luria, 1953; Lederberg, 1957; Arber and Dussoix, 1962; Dussoix and Arber, 1962), so the phage were "restricted" by the second strain. Later it turned out that the DNA of the infecting bacteriophage is degraded by an

endodeoxyribonuclease, called "restriction endonuclease" or "restriction enzyme" in the non-accepting cells (Lederberg and Meselson, 1964), whereas the DNA of the host cell is protected from digestion by methylation (Arber, 1965). The recognition sequence of the restriction endonuclease and the methylase is the same.

The first purified restriction endonuclease was *Eco*K from *Escherichia coli* (Meselson and Yuan, 1968). Unfortunately, although DNA recognition by the *Eco*K enzyme was specific, mapping of the cleavage site failed. As it turned out, the *Eco*K and the related *Eco*B restriction endonucleases cleave at least 1 kb away from the recognition sequence at a random position, so these enzymes cannot be used for the cutting of the DNA at a certain given site.

The first purified restriction enzyme whose recognition site coincided with its cleavage site was *Hin*dII from *Hemophilus influenzae* (Smith and Wilcox, 1970; Kelly and Smith, 1970). This was the first discovered TypeII restriction endonuclease (see below).

Most of restriction endonucleases can be classified into three groups (for a review on the biochemical and evolutionary relationships of restriction endonucleases, see Wilson and Murray, 1991). Type I restriction endonucleases recognize specific nucleotide sequences, but they are not particularly useful for gene manipulation since their cleavage sites are generally at least 1 kb apart from the recognition site, at an essentially random position. Their activity requires S-adenosyl-methionine (SAM). Although these restriction enzymes are not used during gene manipulation, they are important from another point of view. The most important host used during molecular cloning is *Escherichia coli*, and the *Eco*K and *Eco*B restriction endonucleases are Type I enzymes. Elimination of the

latter systems is sometimes vital to prevent restriction of incoming DNA after introduction into host cells.

Type III restriction endonucleases cut the DNA at a measured distance (usually 24-26 bps) to one side of the recognition sequence, and two recognition sites positioned in opposite directions are needed for the cleavage to occur. Their activity is stimulated by SAM.

TypeII enzymes recognize a particular target sequence in a duplex DNA molecule and break the polynucleotide chains within that sequence to give rise to discrete DNA fragments of defined lenght and sequence. The restriction endonucleses used during molecular cloning are usually TypeII enzymes. These endonucleases consist of a single polypeptide, usually as a homodimer. They require no cofactor other than Mg^{2+}. The cognate modification methylases act independently of the endonucleases, unlike in the case of TypeI and III systems. They recognition sequences are usually palindromic, and cleavage occurs symmetrically within the sites. Some enzymes cut in a staggered manner, resulting in either 3' or 5' single-stranded protruding ends, called "cohesive" or "sticky ends", while others produce ends without protrusions, called "blunt" or "flush" ends. It is important to note that not all site specific endonucleases purified from bacteria were actually proven to be part of a host-controlled restriction-modification system.

TypeIIs ("shifted") restriction endonucleases are similar to the TypeII enzymes in their subunit organization and cofactor requirement. However, their recognition sequence is not palindromic, and cut a few base pairs away from the recognition site to one side of that, usually generating sticky ends. Some of these enzymes are applied during DNA manipulation.

DNA fragments can be joined by the enzyme DNA ligase (Gellert, 1967; Olivera and Lehman, 1967; Olivera et al., 1968; Gumport and Lehman, 1971). The ligase encoded by phage T4 joins fragments with blunt ends or base-pairing sticky ends (Sgaramella, 1972). However, the *E. coli* enzyme joins blunt ends only among conditions of macromolecular crowding (Zimmerman and Pheiffer, 1983), so the T4 enzyme is the most widely used DNA ligase.

Another method for the joining of DNA molecules makes use of the annealing of complementary homopolymer sequences formed by terminal deoxynucleotidyl transferase, or terminal transferase (Kato et al., 1967). The method is called homopolymer tailing (Chang and Bollum, 1971). Terminal transferase acts as a template-independent DNA polymerase, inserting nucleotides to the 3' termini of DNA fragments. If only a single deoxyribonucleotide triphosphate is added to the reaction mixture then a single-stranded stretch composed of that nucleotide is formed. The DNA fragments to be attached are "tailed" by complementary nucleotides. Typically 10-40 homopolymeric residues are added to each end. Originally, after the annealing of the DNA fragments to be joined, the site of the attachment was repaired *in vitro* by DNA polymerase and DNA ligase (Jackson et al., 1972). However, the annealed fragments are stably kept together by the homopolymeric tails even without ligation, so commonly the annealed circles are used directly for transformation with repair of the gaps occuring *in vivo*. A disadvantage of the method is that the homopolymeric sequences are contained in the resulting chimaera.

Usually different strains of *Escherichia coli* are used as host during molecular cloning. The process for the introduction of foreign genetic material into host cells is called transformation, and cells harboring the introduced nucleic

acid molecule are referred to as transformants. Originally, *E. coli* was thought to be unable to take up DNA from its environment. Later it was found that $CaCl_2$ treatment allowed *E. coli* cells to take up bacteriophage λ DNA (Mandel and Higa, 1970), and also plasmid DNA (Cohen et al., 1972).

Among the first *in vitro* DNA manipulation experiments, a circular dimer of the genome of simian virus 40 (SV40) (Jackson et al., 1972) and a similar dimer of phage P22 DNA (Lobban and Kaiser, 1973) were constructed. The first reported construction of a chimaeric DNA molecule was the attachment of the genome of SV40 to that of a defective λ phage (Jackson et al., 1972), but this chimaera was not transformed into any cell because of safety precautions. Later, engineered bacterial plasmids were created and transformed into *E. coli* cells (Cohen et al., 1973).

The first interspecific plasmid chimaera transformed into *E. coli* was a pSC101 derivative carrying a fragment of the *Staphylococcus aureus* plasmid p1258 (Chang and Cohen, 1974). The p1258 fragment conferred penicillin resistance on the harboring *E. coli* cells, so this experiment can be concerned as the first functional expression of a cloned gene in a heterologous host.

The cloning of fragments of the rDNA from *Xenopus laevis* in *E. coli* (Morrow et al., 1974) was the first experiment when eucaryotic DNA was replicated in a procaryotic host. The expression of the human somatostatin hormone in *E. coli* (Itakura et al., 1977) was the first time when a functional gene product was obtained from a chemically synthesized gene.

The most frequently used cloning vectors are plasmids. Plasmids are dispensable, genetically homogeneous extrachromosomal nucleic acid molecules

with a constant monomeric unit size. Plasmids are most frequently double-stranded, circular DNA molecules. During the cloning process, the plasmid vector is linearized, and the DNA fragment to be cloned (the "insert") is usually attached to it by DNA ligase. After ligation, cells competent for DNA uptake are transformed with the ligation mixture. The cells are then spread onto selective medium which enables the growth of transformants only. The vector-borne marker gene enabling this selection is kept intact during the cloning process. This marker gene usually renders cells resistant to an antibiotic or complements a metabolic deficiency of the host cell. After the appearance of the transformant colonies, it is necessary to verify the presence of the insert sequence in the clones. It is a relatively easy task when there is positive selection for the presence of the insert, but in most cases the cloned DNA fragment does not endow the harboring host cell with any easily detectable phenotype.

Although the DNA fragment to be cloned itself might not cause an easily recognizable change in the general shape or appearance of transformant colonies, its insertion can result in marked changes in a vector function, enabling the identification of insert-harboring clones on the level of the appearing colonies. The earliest methods to distinguish insert-harboring and insert-free clones were based on an approach called "negative selection". The insertion of the cloned fragment into the vector inactivated a vector-borne function, e. g. tetracycline resistance (Hamer and Thomas, 1976). The transformation mixture was plated onto tetracycline-free medium, then the tetracycline sensitivity of the appearing colonies was tested either by replica plating or inoculating individual colonies onto tetracycline-containing medium. This negative selection method is rather

laborious, because a two-step procedure is required for the identification of positive clones.

The most widespread cloning vectors, e. g. the commercially available Bluescript vectors (Stratagene) utilize the blue/white color screening system based on the *lacZ* gene of *Escherichia coli* encoding β-galactosidase. β-galactosidase hydrolyzes the glycosidic bond of β-galactosides, e. g. that of lactose. The enzyme also accepts XGal, a chromogenic, precipitate-forming compound as substrate (Miller, 1972). Cells harboring wild-type *lacZ* form blue colonies in the presence of XGal. In β-galactosidase deficient cells, a vector-borne *lacZ* gene can be exploited for the discrimination between insert-harboring and insert-free cells. Inactivation of the gene by the DNA fragment to be cloned results in the lack of β-galactosidase activity of transformants, which is indicated by the white color of appearing colonies on XGal indicator plates. The size of the intact *lacZ* structural gene is over 3 kb, so usually only its short promoter-proximal *lacZα* fragment is included in the vectors. The great fragment of β-galactosidase expressed *in trans* complements the LacZα peptide (Ullmann et al., 1967; Landy et al., 1974; Langley et al., 1975; Messing et al., 1977). Several polycloning sites were inserted into the N-terminal part of *lacZα* without the loss of β-galactosidase activity, providing numerous restriction sites for cloning (see e. g. Messing et al., 1981; Vieira and Messing, 1982). The system is powerful, but in-frame insertions and transcriptional or translational reinitiation within the insert often causes the formation of false negative (blue) colonies.

Positive selection (also called "direct selection") cloning vectors offer a further possibility. Among selective conditions, cells harboring the unaltered

vector are counterselected, and only the insert-harboring derivative of the vector support colony formation of the transformants.

Most of positive selection cloning vectors utilize a conditionally lethal gene as the marker for the selection of insert-harboring clones. Insertional inactivation of the gene enables transformants to grow, but cells transformed by the recircularized vector are counterselected among selective conditions. The conditionally lethal genes utilized by positive selection cloning systems are summarized in Table 1.

Marker gene	Reference
Mu phage killing function	Schumann, 1979
colE1	Ozaki et al., 1980
rpsL (*strA*)	Dean, 1981
pheS	Hennecke et al., 1982
ush	Burns and Beacham, 1984
colE3	Vernet et al., 1985
Gene E of ϕX174	Henrich and Plapp, 1986
xylA	Stevis and Ho, 1987
xylB	Stevis and Ho, 1988
rglB	Noyer-Weidner and Reiners-Schramm, 1988
ccdB	Bernard et al., 1994
GATA-1	Trudel et al., 1996
barn	Yazynin et al., 1996
amy	Barros et al., 1996
crp	Schlieper et al., 1998

Table 1. Counterselectable marker genes exploited in positive selection cloning vectors.

Even the β-galactosidase activity of transformants can be counterselected in *galE* mutant strains (Gossen et al., 1992). The *galE* gene of *E. coli* encodes UDP-glucose 4-epimerase (also known as UDP-galactose 4-epimerase). *galE* mutant cells are highly sensitive to galactose, one of the products of the reaction

mediated by β-galactosidase. Although the selection scheme is promising, it did not become widely applied.

Some of the positive selection cloning vectors contain a gene rendering cells resistant to an antibiotic, and another gene encoding a transcriptional repressor controlling the expression of the antibiotic resistance gene (Roberts et al., 1980; Nikolnikov et al., 1984). Insertional inactivation of the repressor gene by the cloned DNA fragment derepresses the expression of the antibiotic resistance gene, resulting in the appearance of antibiotic resistance. Cells harboring the recircularized vector are counterselected by a medium containing the respective antibiotic.

Another selection principle is based on the inviabililty of long uninterrupted palindromes in bacteria (Hagan and Warren, 1982). The DNA fragment to be cloned is inserted into the middle of the palindromic sequence. The insert serves as a spacer between the halves of the palindrome, making possible the replication of the plasmid.

A peculiar system is based on the *Eco*K restriction activity of *Escherichia coli* K-12 cells (De Backer et al., 1994). Insertion of the DNA fragment to be cloned destructs the single *Eco*K recognition sequence of the vector, thus enabling the positive selection of recombinant clones in restriction-proficient *Escherichia coli* K-derived strains.

Identification of the correct recombinant clones is often laborious despite the availability of the blue/white screening and positive selection cloning systems. Most of these systems are not free from particular problems which limit their general applicability (see Messing, 1991; Bernard et al., 1994; Bernard, 1996;

Hengen, 1997; Kast, 1994 and Schlieper et al., 1998 for overview). The most frequent problems are the need for a special host strain, the low number of available restriction sites for cloning, and the expensive selection medium. Sometimes the selection system does not completely exclude the growth of insert-free cells, just causes a difference in the sizes of the appearing colonies. We decided to construct a set of positive selection cloning vectors free from the above mentioned problems. The new vectors utilize a novel selection principle, namely the conditional inhibitory effect of the *gltS* gene from *E. coli* K-12 on the growth of *E. coli* cells.

The *gltS* gene product GltS is a glutamate-specific permease (Deguchi et al., 1990; Kálmán et al., 1991) which is also responsible for the transport of the toxic glutamate analog α-methyl glutamate (αMG) (Halpern and Umbarger, 1961). This analog interferes with the conversion of glutamate to glutamine and inhibits the growth of *E. coli* on glucose minimal medium (Halpern and Umbarger, 1961). Inactivation of the *gltS* gene by the insertion of foreign DNA abolishes the αMG sensitivity allowing recombinant clones to grow.

The wild type *gltS* gene offers a limited set of unique restriction sites for cloning. To overcome this problem, the KS and SK polycloning sites of the pBC vectors (Stratagene) were inserted into the *gltS*, leaving the original reading frame unaltered. The position of insertion of the polycloning sites corresponds to a hydrophylic loop located cytoplasmatically (Gál et al., manuscript in preparation). The function of the permease was left intact by this in-frame insertion. The resulting *gltS*-derivative marker genes offer a wide set of restriction sites for cloning, enable *in vitro* transcription of the insert sequence and the expression of

protein fusions to the N-terminal part of the GltS, even that of proteins incapable of secretion from *E. coli* cells.

The polymerase chain reaction (Mullis and Faloona, 1987; Saiki et al., 1988) is a method of central importance in molecular biology. Difficulties are frequently encountered during the cloning of PCR products, although numerous methods have been developed for the purpose to date.

The simple blunt ligation of the PCR product to a vector is not directional, and rather inefficient. *Pfu* polymerase generates PCR products with blunt ends (Hu, 1993), but *Taq* polymerase possesses 3' terminal extendase activity (Clark, 1988; Hu 1993), so end-polishing of *Taq* products with Klenow (Hemsley et al., 1989), T4 or *Pfu* polymerase (Costa and Weiner, 1994) is advisable.

The extendase activity of *Taq* polymerase is exploited by the method called T-cloning (Holton and Graham, 1991; Marchuk et al., 1991; Mead et al., 1991). Since almost exclusively a dAMP residue is added to the 3' end (Clark, 1988; Hu, 1993), native PCR products can be ligated to vectors displaying single 3' TMP overhangs. The T-cloning is fairly efficient, but not directional.

Original restriction sites of the template rarely support cloning. The directional cloning of PCR products is frequently accomplished by the inclusion of extra restriction sites in the 5' end of amplification primers and restriction enzyme digestion of the product before ligation to a suitably cleaved vector (Scharf et al., 1986). Since many restriction enzymes cut rather inefficiently close to DNA termini (Jung et al., 1990; Kaufman and Evans, 1990), often it is necessary to include several further overhanging nucleotides in the primer, elevating the cost of oligonucleotide synthesis.

Limited degradation of the PCR product by uracil DNA glycosylase (Nisson et al., 1991; Smith et al., 1993) to form 3' overhangs was reported, but the method requires special primers, enzymatic modification and alkaline treatment of the product. dUTP can be incorporated into the primer during oligonucleotide synthesis. During PCR, a template-strand dUMP residue behaves as a TMP residue. The dUMP-containing PCR product is treated by uracil DNA glycosylase resulting in the removal of the uracil base and the formation of an abasic site. The sugar-phosphate backbone can be cleaved at the abasic site by alkaline treatment, resulting in 3' overhangs. The method can be applied using *Taq* polymerase, but archaeal DNA polymerases, e. g. *Pfu* or Vent polymerase are seriously inhibited by uracil-containing primers (Greagg et al., 1999). Archaeal DNA polymerases possess a so-called "read-ahead" function detecting promutagenic template-strand dUMP residues, and remain tightly blocked by that.

The 3'-5' exonuclease activity of T4 DNA polymerase is rather strong (Hershfield and Nossal, 1972). In the absence of certain dNTPs, limited degradation of PCR products amplified by suitably designed primers results in the formation of 5' single-stranded overhangs on the ends of the PCR product (Aslanidis and de Jong, 1990). Unfortunately, enzymatic modification of the product is necessary, and the controlling of the limited degradation by T4 DNA polymerase is rather difficult.

The fusion of insert and vector sequences is also possible by PCR (Jones and Howard, 1990; Shuldiner et al., 1990), but the methods are dependent on more than one primer pair and more than a single amplification reaction.

A staggered reannealing method to form single-stranded overhangs on PCR product ends was published (Ailenberg and Silverman, 1996), but it is also

based on two primer pairs. The DNA fragment to be cloned is amplified by two primer pairs in separate reactions. The two PCR products are mixed, denatured and reannealed. Some of the reannealed duplexes contain strands from the same reaction, whereas others are heteroduplexes formed by strands from different reactions. The latter bear single-stranded overhangs on their termini.

Recombination of the PCR product and the cloning vector can be accomplished *in vivo* (Oliner et al. 1993), but a special host strain and more than one primer pair (or rather long primers) are needed.

We aimed to develop a novel method for the directional cloning of PCR products using a single primer pair without the need of any modification of the product after PCR. Abasic sites in a DNA template are non-instructional for a DNA polymerase, so it is stalled at the site during synthesis of the complementary strand (Schaaper et al., 1983; Paabo et al., 1990). The 5' ends of PCR product strands contain built-in amplification primers. Abasic sites within the primers result in the formation of 5' single-stranded overhangs at the ends of the PCR product, enabling its direct ligation to a suitably cleaved cloning vector without the need of any further modification.

The original AS-PCR method was described with *Taq* polymerase, using primers with a single tetrahydrofuran abasic site and a 5 nucleotides long overhang comprising an *Eco*RI or *Bam*HI sticky end and a site-regenerating nucleotide (Gál et al., 1999). Products amplified by *Pfu* or Vent polymerase using the above primers were cloned with very low efficiency, although tetrahydrofuran abasic sites were reported to block a variety of DNA polymerases (Takeshita et al.,

1987; Ng et al., 1989; Takeshita and Eisenberg, 1994; Paz-Elizur et al., 1996; Moran et al., 1996; Paz-Elizur et al., 1997; Efrati et al., 1997; Shibutani et al., 1997; and Shimizu et al., 1997; Gál et al., 1999), including *Pfu* (Greagg et al., 1999). A set of primers was synthesized to test the effect of various overhang structures on the efficiency of cloning. Our results show that depending on the 3' non-templated extra nucleotide addition activity of the used polymerase, products with different overhang structures are cloned efficiently.

2. Materials and Methods

Chemicals

The Chemical Phosphorylation Reagent (Horn and Urdea, 1986) and dSpacer (Takeshita et al., 1987) were obtained from Glen Research, Sterling, VA, USA, all other protected nucleotides were from Millipore. Other chemicals used during oligonucleotide synthesis were from Sigma, Aldrich or Merck. *Taq* DNA polymerase was obtained from Zenon Biotechnology Ltd., Szeged, Hungary, *Pfu* DNA polymerase from Promega, Vent DNA polymerase from New England Biolabs. Alkaline phosphatase was from Boehringer, all other modification enzymes and the restriction endonucleases were purchased from MBI-Fermentas or New England Biolabs. α-methyl-DL-glutamic acid was obtained from Sigma. Media components were from Difco. All other chemicals were obtained from Reanal, Budapest, Hungary or Sigma.

Cloning experiments

All enzymatic manipulations were performed according to the manufacturers' recommendations, and cloning steps were done following conventional methods (Sambrook et al., 1989).

Media used

During our experiments, M9 glucose minimal or LB media were used (Sambrook et al., 1989). The final concentrations of the supplements were: 100 μg/ml for αMG (Sigma), 1 μg/ml for thiamine, 100 μg/ml for ampicillin and

carbenicillin, 25 μg/ml for chloramphenicol, XGal and IPTG, unless otherwise indicated.

Amplification primers

The primers used during the construction of the *gltS*-based positive selection cloning vectors were synthesized by custom synthesis. The sequences of the primers:

AT473 5'-TTAATGTAAGTTAGCTCACTCAT-3'

AT478 5'-TGCTGCAAGGCGATTAAGTTG-3'

AT481 5'-TCTTCTTCATATGAAGGAGTAACTATGTTTCATCT-3'

AT482 5'-TTCTTGTAGATCTGGAGATTCGCGGC-3'

GJ6 5'-GCCAAGCGCGCCAATTAACCCTCAC-3'

All primers used during the Autosticky PCR experiments were synthesized on a Cruachem model PS250 DNA synthesizer using phosphoramidite chemistry and purified by polyacrylamide gel electrophoresis on 10% 19:1 acrylamide:bisacrylamide gel containing 7M urea. The structure of the primers is shown in Figure 3 and Table 5.

Amplification and purification of AS-PCR products

*Eco*RI-linearized pMK1 (Kálmán et al., 1991) was used as template DNA for amplification. A 262 bp fragment of the *E. coli* K-12 genome was amplified during our experiments. In a 100 μl reaction volume, 100 pmol of each primers were used. The amplification reactions were performed following the manufacturers' recommendations. PCR reactions were performed on a LEP Scientific PREM III thermal cycler using the program 94°C, 2.5 minutes initial

denaturation followed by 30 cycles of denaturation at 94°C, 1 minute, annealing at 55°C, 1 minute, extension at 72°C, 50 seconds, and after the cycles a final extension step at 72°C, 5 minutes. PCR mixtures were analyzed by agarose gel electrophoresis.

Taq and *Pfu* polymerases amplified a single fragment with the expected length. However, in the Vent mixtures, products with unexpected mobilities also appeared. *Taq* and *Pfu* products were purified using the QIAquick PCR Purification Kit (QIAGEN), unless otherwise indicated. The Vent products with the proper length were purified by preparative agarose gel electrophoresis, and DNA was recovered using the QIAquick Gel Extraction Kit (QIAGEN).

Analysis of AS-PCR clones

The *Eco*RI-*Bam*HI great fragment of pBC, SK(–) (Stratagene) was purified by preparative agarose gel electrophoresis using the QIAquick Gel Extraction Kit. Similarly, an already cloned AS-PCR fragment was also isolated as an *Eco*RI-*Bam*HI fragment.

During our first experiments (Gál et al., 1999), about 200 ng of the linearized vector and 100 ng of the purified PCR fragment or the isolated *Eco*RI-*Bam*HI restriction fragment were ligated in a 15 µl reaction volume using 1.5 units of T4 DNA ligase, and were incubated overnight at 16°C. Later, the cloning efficiency was optimized, and about 50 ng of the linearized vector and 20 ng of the purified PCR product or the isolated *Eco*RI-*Bam*HI restriction fragment were ligated (Gál et al., 2000, accepted for publication). Ligation mixtures were transformed into competent DH5αF' (Life Technologies) *E. coli* cells (with about 10^7 colonies/µg pBR322 transformation efficiency), and plated onto XGal/IPTG

indicator plates containing chloramphenicol. DNA was isolated from white and pale blue colonies. Plasmid clones were analyzed by *Pvu*II and *Kpn*I digestion and agarose gel electrophoresis.

All sequencings were performed in the DNA Sequencing Laboratory of the Biological Research Center of the Hungarian Academy of Sciences using Ampli*Taq* DNA Polymerase, FS (Perkin-Elmer). Plasmid clones were sequenced using Reverse, T3 and T7 general sequencing primers.

Dephosphorylation of AS-PCR products

The *Taq*-5N/1S, the *Taq*-4N/1S and the *Pfu*-3N/1S products were treated by alkaline phosphatase according to the manufacturer's recommendations. After dephosphorylation, the products were cleaned up by the QIAquick Gel Extraction Kit.

In silico analysis of sequence data

During our work the GCG Program Package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, 53711, USA) and a public BLAST (Altschul et al., 1990) homology searching server (http://www.ncbi.nlm.nih.gov/BLAST) of the National Center for Biotechnology Information was used extensively.

3. Results

3.1. Development of *gltS*-Based Positive Selection Cloning Vectors

Abstract

Positive selection cloning vectors based on the *gltS* glutamate permease gene of *E. coli* K-12 were constructed. Expression of the cloned *gltS* gene from a weak, constitutive promoter does not interfere with cell growth. Under selective conditions, however, the GltS-dependent uptake of α-methyl glutamate causes severely retarded growth. Insertion of foreign DNA into the *gltS* gene restores cell growth, therefore positive selection of the recombinant clones is possible. Several naturally occurring restriction sites of the *gltS* gene as well as polycloning regions engineered into the *gltS* gene can serve as cloning sites.

Key words: Cloning vector, Positive selection, *gltS*, MCS

Testing of the positive selection principle

Inactivation of the *gltS* gene was observed when a translational frameshift was introduced at the unique *Nhe*I site of pMK242 within the *gltS* structural gene (Kálmán et al., 1991).

The promoter of the *gltS* gene is rather weak. In pMK242, expression of the gene is under transcriptional control of its own promoter and tandem P_{tac} and P_{lac} promoters. The *gltS* gene can be overexpressed by the addition of IPTG, but high-level expression of the gene is toxic to the cells (Kálmán et al., 1991). pMK242 renders cells highly sensitive to αMG even without the addition of the inducer, primarily due to leaky expression from the P_{tac} and P_{lac} promoters.

To test whether insertion of a foreign DNA fragment into the gene also abolishes its activity, a 2 kb *Nhe*I-*Nhe*I *Pichia pastoris* genomic fragment was inserted into the *Nhe*I site of pMK242. After ligating the fragment and *Nhe*I-linearized pMK242, XL1-Blue (Stratagene) cells were transformed with the ligation mixture. Equal amounts of the transformed cells were spread onto αMG-containing and toxin-free glucose M9 minimal medium supplemented with ampicillin and thiamine. It was found that the number of arising colonies was about ten times higher in the absence of αMG. Plasmid DNA was isolated from ten colonies picked from the plate with αMG, and all clones were found to be insert-harboring.

Translational frameshifts introduced at the *Hin*dIII or at the *Cla*I site also abolished the αMG sensitivity, showing that a substantial part of the coding region could be used for insertional inactivation (see Figure 1 for restriction map).

Construction of the basic vectors

pMK242 has several disadvantages as a positive selection cloning vector. Its size is relatively great, it offers a limited set of restriction sites for cloning, and the promoter structure of the *gltS* gene is rather complicated, so we decided to construct a new set of vectors.

The *gltS* gene was fused to a weak, constitutive derivative of the *E. coli lac* promoter. The promoter region lacks both the CAP binding site and the *lac* operator. It was derived from the pRIZ'O(–) vector (Simoncsits et al., 1997). First, the single *Nde*I site of pTC01 (Collet et al., 1992) was destructed by filling-in with Klenow polymerase, resulting in pTC01, *Nde*I(–). The *lac* promoter of pRIZ'O(–) was amplified by *Taq* polymerase using AT473 and AT478, treated by Klenow polymerase and T4 polynucleotide kinase, and blunt-ligated to the Klenow-blunted and dephosphorylated *Hin*dIII-*Sac*I great fragment of pTC01, *Nde*I(–), resulting in pTC-P_{lac}-$O^{(-)}$-*lacZ'*. The orientation of the promoter was checked by *Nde*I-*Xho*I digestion.

The *gltS* structural gene was amplified by PCR along with its own Shine-Dalgarno sequence and terminator region using primers AT481 and AT482. The PCR product was cut by *Nde*I and *Bgl*II, and ligated to the *Nde*I-*Bam*HI great fragment of pTC-P_{lac}-$O^{(-)}$-*lacZ'*, resulting in pCP1(–) (Figure1). pCP1(–) contains the p15A origin of replication and a chloramphenicol resistance gene. The resulting *lac* promoter-*gltS* cassette is applied in all *gltS*-based positive selection cloning vectors. The arrangement of the cassette is the following:

TAATGTAAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATG

CTTCCGGCTCGTATGTTGCATATG**AAGGAG**-*gltS* structural gene-terminator

region, where the -35 and the -10 regions are underlined and the Shine-Dalgarno sequence is in bold.

The promoter-*gltS* cassette was amplified by *Taq* polymerase using primers AT473 and AT482, treated by Klenow polymerase and T4 polynucleotide kinase, and ligated to the *Pvu*II large fragment of the pBS(–) phagemid (Stratagene) dephosphorylated by alkaline phosphatase. Since the latter cloning step was not directional, the *gltS* cassette was ligated to the vector in both orientations, resulting in pGA(+) and pGA(–) (Figure 1). The orientation of the cassette was checked by *Nde*I digestion.

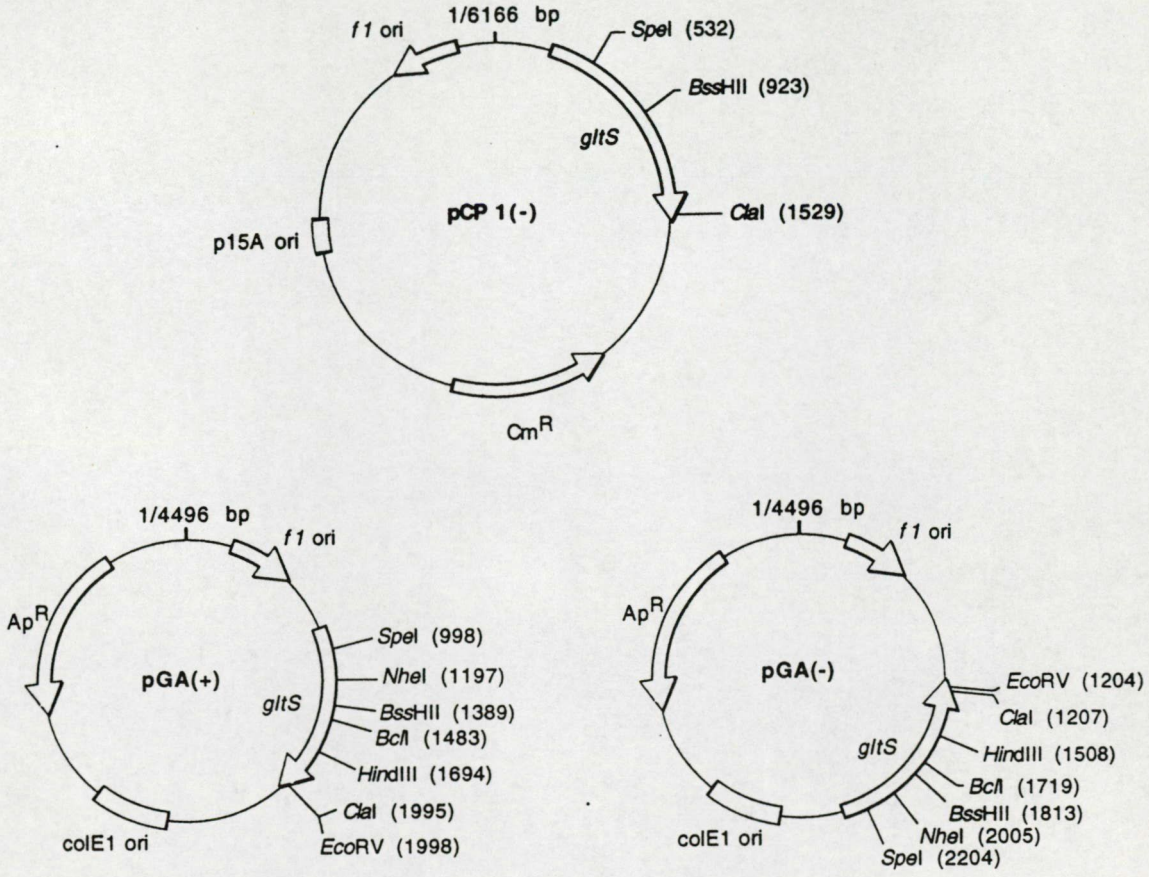


Figure 1. The stucture of the basic *gltS*-based positive selection cloning vectors pCP1(–), pGA(+) and pGA(–).

(+) and (–) refer to the direction of the promoter-*gltS* fusion with respect to that of the f1 origin of replication: (+) means identical whereas (–) divergent orientations. In the (+) vectors the sense, whereas in the (–) vectors the antisense strand of *gltS* is recovered after infection with helper phage (Dotto et al., 1981; Zinder and Boeke, 1982).

These vectors offer a limited set of restriction sites for positive selection cloning (*Spe*I, *Bss*HII and *Cla*I in pCP1(–); *Spe*I, *Nhe*I, *Bss*HII, *Bcl*I, *Hin*dIII, *Cla*I and *Eco*RV in the pGA vectors) (Figure 1).

Design and construction of the polycloning site-containing vectors

In order to increase the cloning flexibility, we introduced the polycloning regions of the pBC, KS and SK vectors (Stratagene) into the *gltS* coding region so that the permease function was maintained. The polycloning sites were inserted into the unique *Bss*HII site of the *gltS* gene, after the codon of arginine at position 181 (R181). Although the polycloning regions could be isolated as *Bss*HII-*Bss*HII fragments, their direct insertion into the *gltS* at the *Bss*HII site would shift the reading frame of the gene.

The upstream primer for the amplification of the polycloning regions was primer GJ6: 5'-GCCAAGCGCGCCAATTAACCCTCAC-3', where the upstream *Bss*HII site is underlined and an extra dCMP residue used to maintain the reading frame of the *gltS* is shown in bold. The chosen nucleotide for maintaining the reading frame of the gene was dCMP, because TMP would have generated a stop codon (TAA), dAMP lysine (AAA), dGMP glutamic acid (GAA), and dCMP glutamine (CAA). The amino acid following R181 in wild-type GltS is tirosine. The chemical character of glutamine is the closest to the original tirosine among

the possibilities, and lysine and glutamic acid were excluded because amino acids with charged side chains can perturb the topology of membrane proteins (von Heijne and Gavel, 1988). The site of insertion of the polycloning sites corresponds to the beginning of a hydrophylic loop following the predicted sixth transmembrane region of the GltS (Deguchi et al., 1990). As it turned out later, the first five predicted transmembrane regions really traverse the membrane, but the sixth hydrophobic region is located cytoplasmatically in its whole length, and the polycloning sites were inserted into the longest cytoplasmatic loop of the GltS (Gál et al., manuscript in preparation).

The KS and SK polycloning regions were amplified by PCR using GJ6 and the generic primer *M13* (-20), the products were cleaved by *Bss*HII and cloned into the *Bss*HII site of the *gltS* gene of pGA(–) to obtain pGAII(–) KS and SK. The orientation of the polycloning sites was checked by *Cla*I digestion. pGAII(+) KS and SK were constructed by replacing the *Nhe*I-*Mlu*I small fragment of pGA(+) by that of pGAII(–) KS and SK, respectively. pCP1(–) KS and SK were constructed by ligating the *gltS*-carrying *Nde*I-*Bgl*II fragments of pGAII(+) KS and SK to the *Nde*I-*Bam*HI great fragment of pTC-P_{lac}-$O^{(-)}$-*lacZ'*. The structures of pCP1(–) KS and pGAII(–) KS are shown in Figure 2.

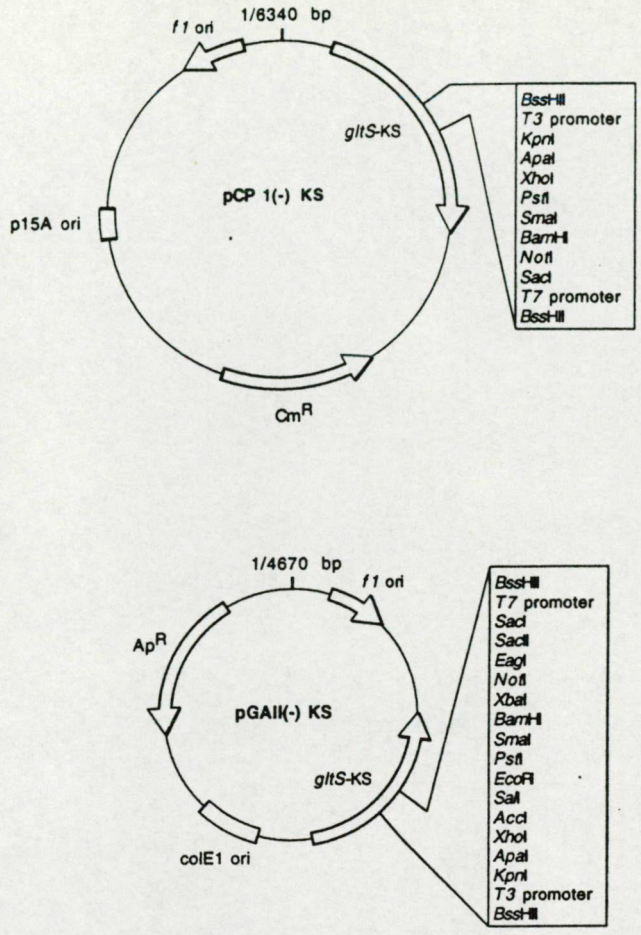


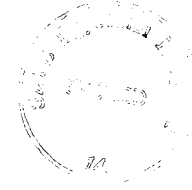
Figure 2. The structure of vectors with polycloning sites. *gltS*-KS: *gltS* gene containing the KS polycloning region. All indicated restriction sites, except for the *Bss*HII sites are unique. In the SK derivatives, the order of restriction sites within the polycloning site is reversed.

XL1-Blue cells transformed with these *gltS*-harboring vectors did not grow on plates of M9 glucose minimal medium (Sambrook et al., 1989) supplemented with thiamine, the appropriate antibiotic and 40 μg/ml αMG (up to six days) at 37 °C. Colonies were obtained, however, when αMG was omitted (two days) or when LB-based plates were used (12-16 hr, with or without αMG). The sequences

of the vectors were deposited in the EMBL Nucleotide Sequence Database under accession numbers AJ005323-AJ005330 and AJ005339.

Testing of the gltS-based positive selection cloning vectors

The applicability of the positive selection cloning system is demonstrated here by several examples in which different restriction fragments of the HDHII/Ku autoantigen subunits were used (Tuteja et al., 1994). In the ligation reactions, the cleaved vectors were used without phosphatase treatment and gel purification. XL1-Blue cells transformed with the ligation mixtures were washed with 150 mM NaCl and plated on M9 glucose minimal plates containing thiamine, αMG (Sigma), carbenicillin (for pGA vectors) or chloramphenicol (for pCP1(–) KS) and incubated at 37 °C for 2-3 days. The results (see Table 2) show that the selection is highly efficient. Even when the efficiency of the cloning (colony number on selective *versus* nonselective plate) was very low, all or nearly all selected and tested clones contained the expected insert. The only false positive detected in six different experiments contained vector-size DNA with probably a short deletion causing frameshift in *gltS*. Such cloning background is common in other systems and could be minimized by carefully performed enzymatic manipulations (vector cleavage and ligation).



Vector	Cloning sites used	Insert[a] size (kb)	Colony number on Nonselective[b] plate	Colony number on Selective plate	Recombinant clone[c] per tested clone
pGA(+)	*Hin*dIII	1.43	1480	154	10/10
pGAII(-) KS	*Pst*I	1.08	2090	111	9/10
pGAII(-) SK	*Pst*I	1.08	1425	67	10/10
pGAII(-) KS	*Eco*RI-*Bam*HI[d]	1.5	765	723	9/9
pGAII(-) SK	*Eco*RI-*Bam*HI[d]	1.5	795	730	10/10
pCP1(-) KS	*Bam*HI	0.24-1.42	1650	176	9/9

Table 2. Examples of cloning and selection experiments using the positive selection vectors.

[a] The inserts were obtained by the same cleavage as indicated for the corresponding cloning vectors, except for in the pCP1(-) KS cloning. In this example a mixture of fragments ranging from 0.24-1.42 kbp was obtained by partial *Sau*3AI cleavage.

[b] Estimated by plating aliquots of the transformation mixture onto LB-Ap or LB-Cm plates.

[c] Plasmid preparations were analyzed by appropriate restriction cleavage(s) and agarose gel electrophoresis.

[d] The double-cleaved vectors were purified by using the QIAquick Gel Extraction Kit (QIAGEN) so that the cleavage mixture was not subjected to gel purification but mixed directly with solution QX1 of the Kit.

3.2. Development of the Autosticky PCR* Cloning Method

* Patent pending Nº P9801320, Hungarian Patent Office

Abstract

A novel method for the directional cloning of native PCR products was developed. Abasic sites in DNA templates make DNA polymerases stall at the site during synthesis of the complementary strand. Since the 5' ends of PCR product strands contain built-in amplification primers, abasic sites within the primers result in the formation of 5' single-stranded overhangs at the ends of the PCR product, enabling its direct ligation to a suitably cleaved cloning vector without any further modification. This "Autosticky PCR" (AS-PCR) overcomes the problems caused by end sensitivity of restriction enzymes, internal restriction sites within the amplified sequences and enables the generation of apparently any desired 5' overhang.

Key words: PCR, Cloning, Sticky end, Abasic primer

Primer design

A 262 bp *E. coli* K-12 genomic fragment was chosen as a model fragment for amplification. Primers GJ8 and GJ10 hybridize with the template in their whole length, whereas primers GJ11 and GJ17 are extended with 6 nucleotides on their 5' end comprising 4 nucleotides *Eco*RI or *Bam*HI restriction overhangs, a dCMP residue for the regeneration of the restriction sites upon ligation to the vector and *in vivo* repair and an abasic site for stopping the polymerase before completion of the synthesis of the complementary strand (Figure 3).

```
GJ8     5′-CGGCACAAGACACGCAACAC-3′
GJ10    5′-ATGCAGAACGCGGGAGATCA-3′
GJ11    5′-P-GATCC-S-GJ8-3′
GJ17    5′-P-AATTC-S-GJ10-3′
```

Figure 3. The structure of the used oligonucleotides. P, phosphate group; S, tetrahydrofuran abasic site.

A tetrahydrofuran derivative, a stable structural analog of 2'-deoxyribose was chosen to mimic naturally occurring abasic sites. It differs from a 2'-deoxyribose abasic site by having a hydrogen instead of a hydroxyl group on 1' carbon of the deoxyribose ring (Takeshita et al., 1987). The sugar-phosphate backbone is cleaved at 2'-deoxyribose abasic sites among alkaline conditions, causing problems during deprotection after oligonucleotide synthesis. However, tetrahydrofuran abasic sites are highly resistant to alkaline treatment.

GJ11 and GJ17 were chemically phosphorylated during primer synthesis on their 5' end to enable ligation of the AS-PCR product to an *Eco*RI-*Bam*HI cleaved vector.

Analysis of the PCR mixtures on agarose gel showed that both primer pairs amplified the 262 bp genomic fragment without aspecific products and no visible trace of primers could be detected.

Polymerase action at abasic sites

The behavior of the polymerase at abasic sites was tested by high temperature sequencing reactions using the GJ11-GJ17 PCR product as template, GJ11 or GJ17 as sequencing primer and Ampli*Taq* DNA polymerase, FS (Perkin-Elmer). The results showed that the polymerase inserts predominantly dAMP opposite tetrahydrofuran (1',2'-dideoxyribose) abasic sites but is unable to complete the synthesis of the complementary strand, resulting in a 5 nucleotides long 5' overhang. According to these findings, the structure of the vector-PCR product ligation joint is reminiscent of proposed double-strand lesions caused by bleomycin (Steighner and Povirk, 1990) and neocarzinostatin (Povirk et al., 1988), where an abasic site is accompanied by a closely located break in the complementary strand. The structure of a ligation joint suggested by our results is shown in Figure 4.

NNNNG**GATCCSNNNN**
NNNNCCTAG **aNNNN**

Figure 4. The suggested structure of the ligation joint between the vector and the AS-PCR product at the *Bam*HI side. The PCR product is shown in bold, the vector in normal letters. **S**, tetrahydrofuran abasic site. Letter "**a**" in lowercase indicates the extra 3' dAMP residue added by *Taq* polymerase.

Cloning of the AS-PCR products

The PCR mixtures were phenol-chloroform extracted and precipitated by ethanol. The pellets were dissolved in sterile distilled water, and directly used in ligation reactions. The PCR products and an *Eco*RI-*Bam*HI restriction fragment of the same length were ligated to *Eco*RI-*Bam*HI-cut pBC, SK(–) and *Xcm*I-cut pMON38201 (Borovkov and Rivkin, 1997), a T-cloning vector. In both vectors, insertional inactivation of the *lacZa* fragment enables blue-white color selection of positive clones. DH5αF' competent cells (with about 10^7 transformants/μg pBR322 cloning efficiency) were transformed with the ligation mixtures and transformed cells were plated on LB medium containing IPTG, XGal and the appropriate antibiotic. Plasmid DNA was isolated from white and pale blue colonies and analyzed by *Pvu*II digestion and agarose gel electrophoresis. The results of cloning experiments are summarized in Table 3. As shown in Table 3, the cloning of the GJ11-GJ17 PCR product and the *Eco*RI-*Bam*HI restriction fragment gave similar results. When the GJ11-GJ17 product was cloned into *Eco*RI-*Bam*HI-cut pBC, SK(–), positive clones were obtained with $4\text{-}8\times10^3$ positives/μg insert efficiency, about a magnitude lower than in the case of the fragment generated by restriction digestion. With the GJ11-GJ17 PCR product, we were unable to isolate insert-containing clones using *Xcm*I-cut pMON38201. These results also suggest that the polymerase is stalled at the abasic site, the further synthesis of the complementary strand is severely inhibited, and no 3' overhanging dAMP residue is present at the end of the PCR product.

Fragment cloned	Vector used	
	*Eco*RI-*Bam*HI-cut pBC, SK(–)	*Xcm*I-cut pMON38201
GJ8-GJ10 PCR product	–	$1\text{-}2\times10^3$ pos./μg
GJ11-GJ17 AS-PCR product	$4\text{-}8\times10^3$ pos./μg	–
*Eco*RI-*Bam*HI restriction fragment	6×10^4 pos./μg	–

Table 3. Summary of the cloning efficiencies. "pos./μg": the number of positive clones per μg insert; "–": no positive clones found.

From a pBC, SK(–)/GJ11-GJ17 PCR product cloning experiment, 12 white or pale blue colonies were tested. 11 of 12 clones were found to be insert-containing and sequenced. In all cases, the PCR product was inserted in the expected orientation. The results of the sequencings are summarized in Table 4.

Primer structure	P-GATCC-S-GJ8 (GJ11)	P-AATTC-S-GJ10 (GJ17)
Structure of the sequenced clones in the terminal regions of the insert	GGATCC-T-GJ8 (3 clones) GGATCC-GJ8 (7 clones) GGATCt/c-GJ8 (1 clone)	GAATTC-T-GJ10 (11 clones)

Table 4. Structure of the insert-vector junctions in the sequenced clones. P, phosphate group; S, tetrahydrofuran abasic site.

As shown in Table 4, in all cases the *Eco*RI site was regenerated, and the abasic site was repaired as TMP. Strikingly, next to the *Bam*HI site, the abasic site was deleted 8 times, and was repaired as TMP only 3 times. The repair of abasic sites as TMP corresponds to the addition of dAMP opposite the site.

In the case of one of our clones, sequencing was rather ambiguous at the *Bam*HI site. Retransformation and reisolation of plasmid DNA from several colonies proved that the clone was a mixture of plasmids harboring normal (GGATCC) or mutant (GGATCT) *Bam*HI site, probably due to the action of the repair system of host cells.

When the unpurified AS-PCR mixture was used in a ligation reaction with *Eco*RI-*Bam*HI-cut pBC, SK(–), numerous white colonies were observed upon transformation and plating onto IPTG/XGal-containing medium, but these clones did not prove to be insert-harboring. Sequencing of a clone showed that the polymerase filled in the 5' restriction overhangs of the vector and self-circularization of the plasmid occured by blunt-ligation. Based on this finding, it is advised to purify the AS-PCR mixture before ligation to a suitable vector.

3.3. Polymerase Dependence of Autosticky PCR*

* Patent pending Nº P9801320, Hungarian Patent Office

Abstract

During Autosticky PCR (AS-PCR), primers containing abasic sites are incorporated into the amplification product. DNA polymerases are stalled at abasic sites, resulting in the formation of 5' single-stranded overhangs at the ends of the product, enabling its directional ligation to a vector without any further modification. A set of primers was synthesized to test the effect of various overhang structures on the efficiency of cloning. It was found that the cloning efficiencies of products with different overhang structures strongly depend on the 3' non-templated extra nucleotide addition activity of the applied polymerase. According to our results, the properties of the amplifying polymerase should be respected during Autosticky PCR. The optimal primer structures for *Taq*, Vent and *Pfu* polymerases are suggested.

Key words: Autosticky PCR, Cloning, Abasic primer, Sticky end

Primer design

The nomenclature of AS-PCR primers and products follows the structure of the overhang. E. g., "the *Pfu*-5N/1S PCR product" refers to a product amplified by *Pfu* polymerase using primers having a 5 nucleotides long overhang and a single abasic site. The structure of the primers is shown in Table 5.

Primer Structure	GJ8 side	GJ10 side
5N/1S	5′-P-GATCC-S-GJ8-3′	5′-P-AATTC-S-GJ10-3′
4N/1S	5′-P-GATC-S-GJ8-3′	5′-P-AATT-S-GJ10-3′
3N/1S	5′-P-GAT-S-GJ8-3′	5′-P-AAT-S-GJ10-3′
5N/2S	5′-P-GATCC-SS-GJ8-3′	5′-P-AATTC-SS-GJ10-3′
4N/2S	5′-P-GATC-SS-GJ8-3′	5′-P-AATT-SS-GJ10-3′
3N/2S	5′-P-GAT-SS-GJ8-3′	5′-P-AAT-SS-GJ10-3′

Table 5. The structure of the used amplification primers. P, phosphate group; S, tetrahydrofuran abasic site.

The original AS-PCR method was described using *Taq* polymerase and 5N/1S primers (Gál et al., 1999). The 5 nucleotides long overhangs comprise an *Eco*RI or a *Bam*HI sticky end and a site-regenerating nucleotide. Without the site-regenerating nucleotide, a 4N/1S primer pair was also synthesized. A 3N/1S primer pair was designed so as the abasic site of the AS-PCR product could be opposed by the distal residue of the vector-borne sticky end. It was reported that templates containing two non-instructional sites in tandem block DNA polymerases more efficiently than a single site (Newton et al., 1993), so all three structures were also sythesized with double abasic sites.

Cloning results with Taq polymerase

The cloning efficiencies are summarized in Table 6. With a single abasic site, only the *Taq*-5N/1S and *Taq*-4N/1S PCR products were cloned. After several experiments with the 3N/1S product, no positive clones were found. The *Taq*-5N/1S product had the highest cloning efficiency among all AS-PCR products, however, even it was a magnitude lower than that of the isolated *Eco*RI-*Bam*HI restriction fragment (data not shown). Surpisingly, the cloning efficiency of the *Taq*-4N/1S product was more than 10-fold lower than that of the *Taq*-5N/1S product.

Primer Structure	*Taq* polymerase	Vent polymerase	*Pfu* polymerase
5N/1S	1.3×10^4 pos./μg	Sporadic	Sporadic
4N/1S	1.0×10^3 pos./μg	–	–
3N/1S	–	2.2×10^3 pos./μg	1.8×10^3 pos./μg
5N/2S	Sporadic	–	–
4N/2S	–	Sporadic	–
3N/2S	–	Sporadic	–

Table 6. Summary of the cloning efficiencies. "pos./μg": the number of positive clones per μg AS-PCR product; "–": no positive clones found; "Sporadic": positive clones were found only sporadically.

The behavior of *Taq* polymerase at the abasic positions of different primer structures was tested in high temperature sequencing reactions using Ampli*Taq* DNA polymerase, FS (Perkin-Elmer) and different AS-PCR products as template. The results showed that the polymerase inserts predominantly dAMP opposite tetrahydrofuran abasic sites. It explains the cloning failure of the *Taq*-3N/1S product, because the space opposite the abasic site was occupied, making ligation to the vector impossible.

Generally, Ampli*Taq* DNA polymerase was unable to complete the synthesis of the complementary strand after the abasic site. However, on the GJ10 side, a 1-2 nucleotides readthrough was detected on templates with a single abasic site, so the addition of a nucleotide opposite the abasic site was sometimes followed by the incorporation of one or two further nucleotides. The addition of the first nucleotide after the abasic site is much stronger than that of the second one. This readthrough is very similar to that seen with exo(-) *Pfu* polymerase (Greagg et al., 1999), suggesting that it is due to the lack of the 3'-5' exonuclease activity of *Taq* polymerase (Chien et al., 1976; Tindall and Kunkel, 1988). This effect does not seriously hamper the cloning of the *Taq*-5N/1S product, because the site-regenerating nucleotide provides a 1-nucleotide gap which is not occupied by the vector overhang (see Figure 5), but it explains the relatively low cloning efficiency of the *Taq*-4N/1S product.

a. NNNNG**GATCCSNNNN**
NNNNCCTAG **aNNNN**

b. NNNNG**GATCSNNNN**
NNNNCCTAG**aNNNN**

c. NNNNG**GATSNNNN**
NNNNCCTAG**NNNN**

Figure 5. The suggested structures of the ligation joints on the *Bam*HI side between the vector and AS-PCR products which gave the best cloning results. a., the *Taq*-5N/1S product; b., the *Taq*-4N/1S product and c., the *Pfu*- or the Vent-3N/1S product. PCR products are shown in bold, the vector in normal letters. **S**, tetrahydrofuran abasic site. Letter "**a**" in lowercase indicates the extra 3' dAMP residue added by *Taq* polymerase.

Cloning results with Pfu and Vent polymerases

The *Pfu*-5N/1S and the Vent-5N/1S products were hardly clonable, and no positive clones were found with the 4N/1S products (Table 6). However, the *Pfu*- and Vent-3N/1S products were cloned with much better results, showing that these polymerases are stalled at 1', 2'-dideoxyribose (tetrahydrofuran) abasic sites without extra nucleotide addition. Similar behavior was reported for T4 DNA polymerase (Sagher and Strauss, 1983), which has strong 3'-5' exonuclease activity (Hershfield and Nossal, 1972). *Pfu* and Vent polymerases also possess 3'-5' exonuclease activity (Lundberg et al., 1991; Mattila et al., 1991), unlike *Taq* polymerase (Chien et al., 1976; Tindall and Kunkel, 1988). *Pfu* polymerase is known to be completely devoid of the 3' non-templated extra nucleotide addition activity, and even that of Vent polymerase is rather weak (Hu, 1993). It was previously reported that the behavior of DNA polymerases at abasic sites is affected by the 3'-5' proofreading exonuclease activity (Kunkel et al., 1983; Sagher and Strauss, 1983; Hu, 1993; Paz-Elizur et al., 1996; Paz-Elizur et al., 1997; Greagg et al., 1999). The failure with the 5N/1S and 4N/1S products might have been caused by the lack of a nucleotide opposite the abasic site.

Results with double abasic sites

In high-temperature sequencing reactions, Ampli*Taq* DNA polymerase was stalled on both sides of the *Taq*-5N/2S template after the addition of a single dAMP residue opposite the first abasic position. It is obvious from this observation and previously reported results (Newton et al., 1993) that polymerase stalling is more efficient at tandem non-instructional sites. Paradoxically, with double abasic sites, only sporadic or no cloning was found. It suggests that the low

cloning efficiency of these products might have been caused by the difficult repair situation.

The structure of the insert-vector junctions in the positive clones

The structures of the insert-vector junctions of the sequenced AS-PCR clones are summarized in Tables 7 and 8. The sequencing results of the *Taq*-5N/1S clones were adapted from Gál et al., 1999. In the case of products with at least 10^3 positive clones/μg insert cloning efficiency, 8-8 clones were sequenced. Under 10^3 positives/μg insert cloning efficiency, all found positive clones were sequenced.

Primer structure	GJ8 side		GJ10 side	
5N/1S	GATCC-GJ8	Taq:7	AATTC-T-GJ10	Taq:11
	GATCC-T-GJ8	Taq:3	AATTC-GJ10	Pfu:1
	GATC-GJ8	Vent:2	AATT-T-GJ10	Vent:1
		Pfu:2	AATT-GJ10	Pfu:1
	GATCt/c-GJ8	Taq:1	Δ(AATTC)-Δ_4	Vent:1
4N/1S	GATC-T-GJ8	Taq:7	AATT-T-GJ10	Taq:7
	GATC-C-GJ8	Taq:1	AATT-Δ_1	Taq:1
3N/1S	GAT-GJ8	Vent:7	AAT-T-GJ10	Vent:8
		Pfu:5		Pfu:7
	GAT-C-GJ8	Vent:1	AAT-Δ_1	Pfu:1
		Pfu:1		
	Δ_1-Δ(GAT)-GJ8	Pfu:1		
	Δ_1-Δ(GAT)-Δ_1	Pfu:1		

Table 7. The structure of the insert-vector junctions in the sequenced clones generated by cloning of AS-PCR products with a single abasic site. Δ: deletion; either the deleted sequence or its length is shown.

On the GJ10 side, substitution of the abasic position by a TMP residue was the predominant result of the repair. It corresponds to the addition of a dAMP residue opposite the abasic site. This result is in accordance with the tendency of *Taq* polymerase (Paabo et al., 1990; Gál et al., 1999) and *E. coli* DNA

polymerases (Kunkel et al., 1983; Sagher and Strauss, 1983; Schaaper et al., 1983; Kunkel, 1984; Takeshita et al., 1987; Hevroni and Livneh, 1988; Lawrence et al., 1990; Takeshita and Eisenberg, 1994; Paz-Elizur et al., 1997; and Shibutani et al., 1997) to insert a dAMP residue opposite abasic sites, following the A-rule (Strauss, 1991). On the GJ8 side, however, the repair pattern is not so homogeneous; deletion of the abasic position and its substitution by a TMP or rarely a dCMP residue all occured. Unfortunately, at present, the prediction of the repair pattern is rather difficult, because both the addition of nucleotides opposite abasic sites (Randall et al., 1987; Lawrence et al., 1990) and the deletion frequency of the site (Kunkel, 1990; Lawrence et al., 1990; Shibutani and Grollman, 1993; and Shimizu et al., 1997) are sequence context dependent.

Primer structure	GJ8 side		GJ10 side	
5N/2S	GATCC-GJ8 GATCC-T-GJ8 GATC-T-GJ8	Taq:3 Taq:2 Taq:1	AATTC-T-GJ10 AATTC-GJ10	Taq:5 Taq:1
4N/2S	GATC-Δ_2	Vent:1	AATT-GJ10	Vent:1
3N/2S	Δ_1-Δ(GAT)-GJ8	Vent:1	AAT-T-GJ10	Vent:1

Table 8. The structure of the insert-vector junctions in the sequenced clones generated by cloning of AS-PCR products with double abasic sites. See Table 7 for explanations.

In a few cases, the deletion of the abasic position was accompanied by minor deletions in its vicinity. Probably misalignment during bypass synthesis is responsible for this effect (Kunkel, 1990; Shibutani and Grollman, 1993).

The sequences of the *Pfu*- and Vent-3N/1S clones on the GJ8 side suggest that although the repair of the abasic position is primarily determined by the abasic site, the nucleotide opposite the site might have some influence on that.

With a single abasic site, this is the only situation where the abasic position is opposed by a vector overhang dGMP, not by a dAMP residue (either *Taq* polymerase-added or vector-borne) in the ligation junction (see Figure 5). In this case, the predominant result of the repair was the deletion of the abasic position, but in two clones it was substituted by dCMP, and no clones were found with TMP substitution.

Among the clones which originated from low efficiency clonings, the frequency of deletions in the vicinity of abasic positions was elevated. It suggests that the difficult repair process of these structures constitutes a bottleneck during their cloning.

The importance of primer phosphorylation

Treatment of AS-PCR products with alkaline phosphatase severely reduced the efficiency of the cloning. In the case of the *Taq*-5N/1S product, only the primer-borne phosphate group can be ligated to the vector (see Figure 5). However, in the case of the *Taq*-4N/1S and the *Pfu*-3N/1S products, the 5' terminal residue of the vector overhang might be ligated to the polymerase-added 3' extra nucleotide or to the last 3' nucleotide of the product strand, respectively (Figure 5). In the latter cases, the strong drop in the cloning efficiency after dephosphorylation could be attributed to the low ligation efficiency at mismatched sites (Alves and Carr, 1988; Landegren et al., 1988; Wu and Wallace, 1989a and 1989b). It is also supported by the observation that, unlike the isolated *Eco*RI-*Bam*HI restriction fragment, the *Taq*-4N/1S PCR product was not multimerized by T4 DNA ligase (data not shown), although the structure of its termini would theoretically allow that.

4. Discussion

The vectors described here combine the versatility of the Bluescript vectors with the possibility of positive selection. A slight disadvantage of our system is the use of the minimal medium which causes generally slow cell growth (2-3 days). However, special additives in the medium or special host strains are not required. The αMG is commercially available and inexpensive (about 3 times cheaper than the carbenicillin). Since the conditionally toxic *gltS* gene is expressed from a weak, constitutive promoter, there is no need to use an inducer in combination with a host strain overproducing the corresponding repressor (e. g. IPTG and $lacI^q$ host). No special strain is required with respect to the chromosomal *gltS* allele either, since the latter does not interfere with the selection. There are no limitations as to the propagation of the empty cloning vectors. Although overproduction of the GltS is toxic to the cells even without added αMG (Kálmán et al., 1991), the low-level synthesis in our system allows stable propagation of the vectors in commonly used laboratory strains. These features, together with the highly efficient selection mechanism, should make our system an attractive alternative to other positive selection cloning systems.

Stalling of *Taq* polymerase at abasic sites and its tendency to insert a dAMP residue opposite the site was already reported (Paabo et al., 1990). The ability of a tetrahydrofuran derivative abasic site to block several DNA polymerases was demonstrated (Takeshita et al., 1987). *Taq* polymerase was stalled at tetrahydrofuran abasic sites in high temperature sequencing reactions,

and a dAMP residue was added opposite them. The preferred addition of dAMP by several DNA polymerases opposite natural (Kunkel et al., 1983; Sagher and Strauss, 1983; Schaaper et al., 1983; Kunkel, 1984; Hevroni and Livneh, 1988; Lawrence et al., 1990; Shibutani et al., 1997) and synthetic (Randall et al., 1987; Takeshita et al., 1987; Takeshita and Eisenberg, 1994; Shibutani et al., 1997) abasic sites, either *in vivo* or *in vitro*, was reported. Based on these findings, the A-rule of mutagen specificity was postulated (Strauss, 1991).

Cloning experiments of the Autosticky PCR product showed that the behavior of the product is similar to a DNA fragment generated by restriction enzyme digestion, but not to that of a conventional PCR product. The abasic site was either deleted or repaired as a TMP, rarely as a dCMP residue. Deletion events were already reported at abasic sites (Kunkel, 1984; Lawrence et al., 1990). In a previous study, a tetrahydrofuran abasic site was inserted into the single-stranded part of a gapped duplex plasmid (Takeshita and Eisenberg, 1994). In that case, the repair pattern was predominated by insertion of dAMP opposite the abasic site or deletion of the site. The reason for the differential action of the repair system on the GJ11 and GJ17 sides is not completely clear, although the sequence context-dependence of deletion frequency at template lesions was reported (Shibutani and Grollman, 1993). It is yet uncertain to what extent the extra dAMP added by *Taq* DNA polymerase affected the repair of abasic sites as TMP. The possible deletion of the abasic position has to be taken into consideration during the planning of cloning experiments.

In one of the clones, the *Bam*HI site was mutated in a part of the plasmids. Rare mutation events at flanking positions of abasic sites were also encountered previously (Lawrence et al., 1990).

The repair events observed are unlikely to be SOS-dependent (for a review, see Walker, 1995), since the used *E. coli* strain DH5αF' is RecA deficient. *E. coli* DNA polymerases I, II and III core each can bypass a tetrahydrofuran abasic site unassisted by other proteins (Paz-Elizur et al., 1996), although bypass at abasic sites is less efficient under non-SOS circumstances than after SOS-induction (Lawrence et al., 1990). In a previous study, no differences were found in the mutagenic spectra of synthetic abasic sites in RecA deficient and proficient strains (Takeshita and Eisenberg, 1994).

Our AS-PCR method has several advantages. It allows directional cloning of PCR products. There is no need to modify the amplification product before ligation, so the end sensitivity of restriction enzymes does not disturb cloning. Theoretically any desired 5' overhang can be generated, including overhangs that correspond to restriction sites occurring within the amplified sequences.

The tested polymerases behave similarly at abasic sites and at the ends of a linear template. *Taq* polymerase possesses 3' non-templated extra nucleotide addition activity, whereas *Pfu* and Vent polymerases are stalled before the abasic position. In the ligation junction, the abasic site should be opposed either by a polymerase-added extra nucleotide, or the 5' terminal residue of the vector-borne overhang. From these results we conclude that during AS-PCR primer design, the non-templated 3' extra nucleotide addition activity of the desired polymerase should be taken into consideration. 5N/1S primers are suggested for *Taq*, whereas 3N/1S primers should be used with Vent and *Pfu* polymerases.

5. Summary

We have developed a set of positive selection cloning vectors based on a novel principle exploiting the conditionally toxic nature of the *gltS* gene. The product of the *gltS* is a Na^+/glutamate permease which also transports the toxic amino acid analog α-methyl-glutamate (αMG). Strong overexpression of the *gltS* gene is itself toxic to *E. coli* cells, but intermediate level expression of the gene is tolerated, causing high sensitivity to αMG. Insertional inactivation of the *gltS* by the DNA fragment to be cloned rescues transformants from the inhibitory effect of the gene.

Inactivation of the *gltS* gene was observed when a *Pichia pastoris* genomic fragment was inserted into the unique *Nhe*I site of pMK242, within the *gltS* structural gene. Translational frameshifts introduced at the *Hin*dIII or at the *Cla*I site of the *gltS* also abolished the αMG sensitivity, showing that a substantial part of the coding region could be used for insertional inactivation.

Vectors with and without polycloning sites inserted into the *gltS* gene were also constructed. The polycloning sites were inserted into the unique *Bss*HII site of the *gltS* gene, after the codon of arginine at position 181 (R181). The site of insertion of the polycloning sites corresponds to a hydrophylic loop located cytoplasmatically.

The vectors combine the versatility of the Bluescript vectors with the possibility of positive selection. Several naturally occurring restriction sites of the *gltS* gene as well as polycloning regions engineered into the *gltS* gene can serve as cloning sites. The T3 and T7 phage promoters enable *in vitro* transcription of

insert sequences. The f1 origin of replication enables the recovery of single-stranded plasmid DNA after infection with helper phage.

No special host strain is required for the selection, and there are no limitations as to the propagation of the empty cloning vectors. The only necessary extra additive in the medium is the commercially available and inexpensive αMG. These features, together with the highly efficient selection mechanism, should make our system an attractive alternative to other positive selection cloning systems.

The described Autosticky PCR (AS-PCR) method is based on the polymerase stalling effect caused by abasic sites in the template strand. *Taq*, Vent and *Pfu* polymerases are all stalled at abasic sites located in amplification primers, resulting in the formation of 5' single-stranded overhangs at the ends of the PCR product.

Cloning experiments with the Autosticky PCR products showed that the behavior of the products is similar to that of a DNA fragment generated by restriction enzyme digestion, not to that of a conventional PCR product. In the resulting clones, the abasic position was either deleted or repaired as a TMP, or much less frequently, as a dCMP residue. Sometimes minor deletions in the vicinity of the abasic site were also seen. The possible deletion of the abasic position has to be taken into consideration during the planning of cloning experiments.

The tested polymerases behave similarly at abasic sites and at the ends of a linear template. *Taq* polymerase possesses 3' non-templated extra nucleotide addition activity, whereas *Pfu* and Vent polymerases are stalled before the abasic

position. In the ligation junction, the abasic site should be opposed either by a polymerase-added extra nucleotide, or the 5' terminal residue of the vector-borne overhang. From these results we conclude that during AS-PCR primer design, the non-templated 3' extra nucleotide addition activity of the desired polymerase should be taken into consideration. 5N/1S primers are suggested for *Taq*, whereas 3N/1S primers should be used with Vent and *Pfu* polymerases.

Polymerase stalling is more efficient at tandem non-instructional sites. Paradoxically, with double abasic sites, only sporadic or no cloning was found. It suggests that the low cloning efficiency of these products might have been caused by the difficult repair situation.

Treatment of AS-PCR products with alkaline phosphatase severely reduced the efficiency of the cloning, so phosphorylation of the AS-PCR primers should not be omitted.

The AS-PCR method has several advantages. It allows directional cloning of PCR products. There is no need to modify the amplification product before ligation, so the end sensitivity of restriction enzymes does not disturb cloning. Theoretically any desired 5' overhang can be generated, including overhangs that correspond to restriction sites occurring within the amplified sequences.

5. Összefoglalás

Kifejlesztettünk egy új klónozó vektor-családot, amely egy új elven, a *gltS* gén feltételes növekedést gátló hatásán alapul. A *gltS* terméke egy Na^+/glutamát permeáz, amely a toxikus aminosav analógot, az α-metil-glutamátot (αMG) is szállítja. A *gltS* gén erős túltermeltetése önmagában is toxikus az *E. coli* sejtek számára, azonban annak közepes szintű expresszióját a sejtek még elviselik, ami magas αMG érzékenységet okoz. A *gltS* inszerciós inaktivációja a klónozandó DNS szakasz által menekíti a transzformáns sejteket a gén gátló hatásától.

A *gltS* gén inaktiválódott, amikor egy *Pichia pastoris* genomi szakaszt építettünk be a pMK242 plazmid egyedi *Nhe*I helyére, a *gltS* struktúrgén területére. A *gltS* *Hin*dIII és *Cla*I helyénél létrehozott leolvasási keret-eltolódás szintén megszűntette az αMG-érzékenységet, ami azt mutatja, hogy a kódoló régió legnagyobb része felhasználható az inszerciós inaktiváció szelekciójára.

Mind poliklónozó hely nélküli, mind a *gltS* gén területén poliklónozó helyet tartalmazó vektorokat létrehoztunk. A poliklónozó helyeket a gén funkciójának megőrzésével egy citoplazmatikus elhelyezkedésű hurkot kódoló szakasz területére, a *gltS* egyedi *Bss*HII helyére építettük be a 181. pozícióban lévő arginin (R181) kodonja után.

A kifejlesztett vektorok a Bluescript vektorok sokoldalúságát egyesítik a pozitív szelekció lehetőségével. A *gltS* gén számos természetes restrikciós hasítóhelye, valamint a *gltS* génbe beépített poliklónozó szakaszok egyaránt felhasználhatóak a klónozás során. A T3 és T7 fág promoterek lehetővé teszik az

inszert szekvencia *in vitro* transzkripcióját, az f1 replikációs origó pedig egyes szálú plazmid DNS előállítását helper fág segítségével.

A szelekcióhoz nincs szükség speciális törzsre, és nincsenek megkötések a kiindulási vektorok felszaporításával kapcsolatban sem. Az egyedül szükséges kiegészítés a táptalajban a kereskedelmileg kapható és viszonylag olcsó αMG.

A kifejlesztett Autosticky PCR (AS-PCR) módszer azon alapszik, hogy a templátban elhelyezkedő abázikus helyek gátolják a másolást végző DNS polimeráz enzim működését. A *Taq*, Vent és *Pfu* polimerázok egyaránt elakadnak a beépült láncindító oligonukleotidokban elhelyezkedő abázikus helyeknél, ami 5' egyes szálú túllógó végek keletkezéséhez vezet a PCR termék végén.

Az Autosticky PCR termékek klónozási kísérletekben úgy viselkednek, mint egy restrikciós emésztéssel előállított DNS szakasz, nem pedig úgy, mint egy hagyományos PCR termék. A létrejött klónokban az abázikus pozíció vagy deletálódott, vagy TMP-re, ritkábban dCMP-re cserélődött. Néha az abázikus hely delécióját annak környezetében kisebb deléciók kísérték. Az abázikus pozíció lehetséges delécióját figyelembe kell venni a klónozási kísérletek tervezése során.

A vizsgált polimerázok lineáris templátok végénél és abázikus helyeknél tapasztalt viselkedése hasonlónak bizonyult. A *Taq* polimeráz rendelkezik 3' templáttól független nukleotid-adó képességgel, míg a *Pfu* és a Vent polimerázok megállásra kényszerülnek az abázikus pozíció előtt. A ligálási pontban az abázikus hellyel szemben vagy egy polimeráz által adott nukleotidnak, vagy a vektor túllógó vége 5' nukleotidjának kell elhelyezkednie. A fentieknek megfelelően az AS-PCR primerek tervezése során a felhasználandó polimeráz templáttól független 3' nukleotid-adó képességét figyelembe kell venni. 5N/1S

primereket javaslunk a *Taq*, míg 3N/1S primereket a Vent és *Pfu* polimerázokkal való munkához.

A polimerázok működése hatékonyabban gátolható több egymás utáni, mint egy egyedüli abázikus hellyel. Ennek ellenére dupla abázikus helyeket tartalmazó primerek felhasználásával amplifikált AS-PCR termékekkel csak szórványos klónozódást tapasztaltunk. Valószínűleg az alacsony hatékonyság oka a sejtek által nehezen javítható szerkezet a ligálási pontban.

Az AS-PCR termékek kezelése alkalikus foszfatázzal jelentősen csökkentette a klónozás hatékonyságát, emiatt az AS-PCR primerek foszforilációja nem elhagyható.

Az AS-PCR módszernek számos előnye van. Lehetővé teszi a PCR termékek irányított klónozását. Nincs szükség a termék enzimatikus módosítására, emiatt a restrikciós enzimek végérzékenysége nem zavarja a klónozást. Elméletileg bármilyen 5' túllógó vég létrehozható, olyan is, amely megfelel egy, az amplifikált szekvenciában megtalálható restrikciós hasítóhelynek.

6. List of abbreviations

SAM: S-adenosyl-methionine

p15A ori: p15A plasmid origin of replication

ColE1 ori: ColE1 plasmid origin of replication

f1 ori: f1 phage origin of replication

Cm: chloramphenicol

Cm^R: Chloramphenicol acetyltransferase gene, conferring chloramphenicol resistance on host cells

Ap: ampicillin

Ap^R: β-lactamase gene, conferring ampicillin and carbenicillin resistance on host cells

hr: hour

PCR: Polymerase Chain Reaction

AS-PCR: Autosticky PCR

bp: base pair

kb: kilobase

XGal: 5-bromo-4-chloro-3-indolyl-β,D-galactoside

IPTG: isopropyl-β,D-thiogalactoside

αMG: alpha-methyl glutamic acid

7. Acknowledgments

First of all, I would like to thank my supervisor, Dr. Miklós Kálmán for training me, the controlling of my work and the much patience and encouragement I got from him.

I would like to thank the Szeged University of Sciences and the Bay Zoltán Foundation for Applied Research, especially Professor Ernő Pungor for the Ph.D. fellowship between 1996 and 2000.

I would like to thank the Institute of Genetics of the Biological Research Center of the Hungarian Academy of Sciences, especially Professor István Raskó for the research possibility.

I would like to thank Dr. Sándor Pongor and Dr. András Simoncsits for the supervision of my work during my one-year ICGEB fellowship in 1997.

I would like to thank my colleagues for the helpful discussions, especially Professor Lajos Alföldi, Dr. László Dorgai, Dr. Péter Putnoky, Professor István Raskó and Dr. Gábor Rákhely.

I would like to thank Andrea Vörös and Aranka Bálint for the excellent technical assistance.

I would like to thank all of my teachers for training and educating me.

And, of course, I would like to thank my loving wife, Emese for her encouragement and my family, without whom nothing would have been possible.

7. Köszönetnyilvánítás

Először is, szeretném megköszönni témavezetőmnek, Dr. Kálmán Miklósnak tanításomat, munkám irányítását és a sok türelmet és bíztatást, amit kaptam Tőle.

Szeretném megköszönni a Szegedi Tudományegyetemnek és a Bay Zoltán Alkalmazott Kutatási Alapítványnak, különösen Pungor Ernő Professzor Úrnak a Ph.D. ösztöndíjat 1996 és 2000 között.

Szeretném megköszönni a Magyar Tudományos Akadémia Szegedi Biológiai Központja Genetikai Intézetének, különösképpen Raskó István Professzor Úrnak a kutatási lehetőséget.

Szeretném megköszönni Dr. Pongor Sándornak és Dr. Simoncsits Andrásnak munkám irányítását egyéves ICGEB ösztöndíjam alatt 1997-ben.

Szeretnék köszönetet mondani kollégáimnak a hasznos eszmecserékért, különösképpen Alföldi Lajos Professzor Úrnak, Dr. Dorgai Lászlónak, Dr. Putnoky Péternek, Raskó István Professzor Úrnak és Dr. Rákhely Gábornak.

Szeretném megköszönni Bálint Arankának és Vörös Andreának a kiváló technikai segítséget.

Szeretném megköszönni valamennyi tanáromnak, hogy oktattak és neveltek.

Végül, de nem utolsósorban, köszönetet mondok feleségemnek, Emesének szeretetéért és buzdításáért, szüleimnek pedig azért, hogy felneveltek, és lehetővé tették számomra mindazt, amit eddig elértem.

8. List of Publications

Gál, J., Szekeres, S., Schnell, R., Pongor, S., Simoncsits, A., and Kálmán, M. (1999) A positive selection cloning system based on the *gltS* gene of *Escherichia coli*. *Anal. Biochem.* **266,** 235-238.

Gál, J., Schnell, R., Szekeres, S., and Kálmán, M. (1999) Directional cloning of native PCR products with preformed sticky ends (Autosticky PCR). *Mol. Gen. Genet.* **260,** 569-573.

Gál, J., Schnell, R., and Kálmán, M. (2000) Polymerase dependence of Autosticky PCR. *Anal. Biochem.*, accepted for publication.

9. References

Ailenberg, M., and Silverman, M. (1996) Description of a one step staggered reannealing method for directional cloning of PCR-generated DNA using sticky-end ligation without employing restriction enzymes. *Biochem. Mol. Biol. Int.* **39,** 771-779.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403-410.

Alves, A. M., and Carr, F. J. (1988) Dot blot detection of point mutations with adjacently hybridising synthetic oligonucleotide probes. *Nucleic Acids Res.* **16,** 8723.

Arber, W. (1965) Host specificity of DNA produced by *Escherichia coli*. V. The role of methionine in the production of host specificity. *J. Mol. Biol.* **11,** 247-256.

Arber, W., and Dussoix, D. (1962) Host specificity of DNA produced by *Escherichia coli*. I. Host controlled modification of bacteriophage λ. *J. Mol. Biol.* **5,** 18-36.

Aslanidis, C., and de Jong, P. J. (1990) Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res.* **18,** 6069-6074.

De Backer, O., Chomez, P., and De Plaen, E. (1994) Positive selection of recombinant plasmids based on the *Eco*K restriction activity of *Escherichia coli* K-12. *Gene* **150,** 197-198.

Barros, E. V., Bataus, L. A., Valencia, F. F., Maranhao, A. Q., and Astolfi-Filho, S. (1996) A novel cloning system for direct screening using a suicidal strategy. *Gene* **179,** 287-289.

Bernard, P. (1996) Positive selection of recombinant DNA by CcdB. *BioTechniques* **21,** 320-323.

Bernard, P., Gabant, P., Bahassi, E. M., and Couturier, M. (1994) Positive-selection vectors using the F plasmid *ccdB* killer gene. *Gene* **148,** 71-74.

Borovkov, A. Y., and Rivkin, M. I. (1997) *Xcm*I-containing vector for direct cloning of PCR products. *Biotechniques* **22,** 812-814.

Burns, D. M., and Beacham, I.R. (1984) Positive selection vectors: a small plasmid vector useful for the direct selection of *Sau*3A-generated overlapping DNA fragments. *Gene* **27,** 323-325.

Chang, L. M. S., and Bollum, F. J. (1971) Enzymatic synthesis of oligodeoxynucleotides. *Biochemistry* **10,** 536-542.

Chang, A. C., and Cohen, S. N. (1974) Genome construction between bacterial species *in vitro*: replication and expression of *Staphylococcus* plasmid genes in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **71,** 1030-1034.

Chien, A., Edgar, D. B., and Trela, J. M. (1976) Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.* **127,** 1550-1557.

Clark, J. M. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* **16,** 9677-9686.

Cohen, S. N., Chang, A. C., Boyer, H. W., and Helling, R. B. (1973) Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci. USA* **70,** 3240-3244.

Cohen, S. N., Chang, A. C., and Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69,** 2110-2114.

Collet, T. A., Roben, P., O'Kennedy, R., Barbas, C. F. III, Burton, D. R., and Lerner, R. A. (1992) A binary plasmid system for shuffling combinatorial antibody libraries. *Proc. Natl. Acad. Sci. USA* **89,** 10026-10030.

Costa, G. L., Weiner, M. P. (1994) Polishing with T4 or *Pfu* polymerase increases the efficiency of cloning of PCR fragments. *Nucleic Acids Res.* **22,** 2423.

Dean, D. (1981) A plasmid cloning vector for the direct selection of strains carrying recombinant plasmids. *Gene* **15,** 99-102.

Deguchi, Y., Yamato, I., and Anraku, Y. (1990) Nucleotide sequence of *gltS*, the Na^+/glutamate symport carrier gene of *Escherichia coli* B. *J. Biol. Chem.* **265,** 21704-21708.

Dotto, G. P., Enea, V., and Zinder, N. D. (1981) Functional analysis of bacteriophage f1 intergenic region. *Virology* **114,** 463-473.

Dussoix, D., and Arber, W. (1962) Host specificity of DNA produced by *Escherichia coli*. II. Control over acceptance of DNA from infecting phage λ. *J. Mol. Biol.* **5,** 37-49.

Efrati, E., Tocco, G., Eritja, R., Wilson, S. H., and Goodman, M. F. (1997) Abasic translesion synthesis by DNA polymerase β violates the "A-rule". *J. Biol. Chem.* **272,** 2559-2569.

Gál, J., Schnell, R., Szekeres, S., and Kálmán, M. (1999) Directional cloning of native PCR products with preformed sticky ends (Autosticky PCR). *Mol. Gen. Genet.* **260,** 569-573.

Gál, J., Schnell, R., and Kálmán, M. (2000) Polymerase dependence of Autosticky PCR. *Anal. Biochem.*, accepted for publication.

Gellert, M. (1967) Formation of covalent circles of lambda DNA by *E. coli* extracts. *Proc. Natl. Acad. Sci. USA* **57,** 148-155.

Gossen, J. A., Molijn, A. C., Douglas, G. R., and Vijg, J. (1992) Application of galactose-sensitive *E. coli* strains as selective hosts for LacZ$^-$ plasmids. *Nucleic Acids Res* **20,** 3254.

Greagg, M. A., Fogg, M. J., Panayotou, G., Evans, S. J., Connolly, B. A., and Pearl, L. H. (1999) A read-ahead function in archaeal DNA polymerases detects promutagenic template-strand uracil. *Proc. Natl. Acad. Sci. USA* **96,** 9045-9050.

Gumport, R. I., and Lehman, I. R. (1971) Structure of the DNA ligase-adenylate intermediate: lysine (epsilon-amino)-linked adenosine monophosphoramidate. *Proc. Natl. Acad. Sci. USA* **68,** 2559-2563.

Hagan, C. E., and Warren, G. J. (1982) Lethality of palindromic DNA and its use in selection of recombinant plasmids. *Gene* **19,** 147-151.

Halpern, Y. S., and Umbarger, H. E. (1961) Utilization of L-glutamic and 2-oxoglutaric acid as sole sources of carbon by *Escherichia coli. J. Gen. Microbiol.* **26,** 175-183.

Hamer, D. H., and Thomas, C. A., Jr. (1976) Molecular cloning of DNA fragments produced by restriction endonucleases *Sal*I and *Bam*I. *Proc. Natl. Acad. Sci. USA* **73,** 1537-1541.

von Heijne, G., and Gavel, Y. (1988) Topogenic signals in integral membrane proteins. *Eur. J. Biochem.* **174,** 671-678.

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J. (1989) A simple method for site-directed mutagenesis using the polymerase chain reaction. *Nucleic Acids Res.* **17,** 6545-6551.

Hengen, P. N. (1997) Methods and reagents. Reducing background colonies with positive selection vectors. *Trends Biochem. Sci.* **22,** 105-106.

Hennecke, H., Günther, I., and Binder, F. (1982) A novel cloning vector for the direct selection of recombinant DNA in *E. coli*. *Gene* **19,** 231-234.

Henrich, B., and Plapp, R. (1986) Use of the lysis gene of bacteriophage ϕX174 for the construction of a positive selection vector. *Gene* **42,** 345-349.

Hershfield, M. S., and Nossal, N. G. (1972) Hydrolysis of template and newly synthesized deoxyribonucleic acid by the 3' to 5' exonuclease activity of the T4 deoxyribonucleic acid polymerase. *J. Biol. Chem.* **247,** 3393-3404.

Hevroni, D., and Livneh, Z. (1988) Bypass and termination at apurinic sites during replication of single-stranded DNA *in vitro*: a model for apurinic site mutagenesis. *Proc. Natl. Acad. Sci. USA* **85,** 5046-5050.

Holton, T. A., and Graham, M. W. (1991) A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. *Nucleic Acids Res.* **19,** 1156.

Horn, T, and Urdea, M. S. (1986) A chemical 5'-phosphorylation of oligodeoxyribonucleotides that can be monitored by trityl cation release. *Tetrahedron Lett.* **27,** 4705-4708.

Hu, G. (1993) DNA polymerase-catalyzed addition of nontemplated extra nucleotides to the 3' end of a DNA fragment. *DNA and Cell Biol.* **12,** 763-770.

Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., and Boyer, H. W. (1977) Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* **198,** 1056-1063.

Jackson, D. A., Symons, R. H., and Berg, P. (1972) Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **69,** 2904-2909.

Jones, D. H., and Howard, B. H. (1990) A rapid method for site-specific mutagenesis and directional subcloning by using the polymerase chain reaction to generate recombinant circles. *Biotechniques* **8,** 178-183.

Jung, V., Pestka, S. B., and Pestka, S. (1990) Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites. *Nucleic Acids Res.* **18,** 6156.

Kálmán, M., Gentry, D. R., and Cashel, M. (1991) Characterization of the *Escherichia coli* K12 *gltS* glutamate permease gene. *Mol. Gen. Genet.* **225,** 379-386.

Kast, P. (1994) pKSS - a second-generation general purpose cloning vector for efficient positive selection of recombinant clones. *Gene* **138,** 109-114.

Kato, K. I., Goncalves, J. M., Houts, G. E., and Bollum, F. J. (1967) Deoxynucleotide-polymerizing enzymes of calf thymus gland. II. Properties of the terminal deoxynucleotidyltransferase. *J. Biol. Chem.* **242,** 2780-2789.

Kaufman, D. L., and Evans, G. A. (1990) Restriction endonuclease cleavage at the termini of PCR products. *Biotechniques* **9,** 304-306.

Kelly, T. J., Jr., and Smith, H. O. (1970) A restriction enzyme from *Hemophilus influenzae*. II. Base sequence of the recognition site. *J. Mol. Biol.* **51,** 393-409.

Kunkel, T. A., Schaaper, R. M., and Loeb, L. A. (1983) Depurination-induced infidelity of deoxyribonucleic acid synthesis with purified deoxyribonucleic acid replication proteins *in vitro*. *Biochemistry* **22,** 2378-2384.

Kunkel, T. A. (1984) Mutational specificity of depurination. *Proc. Natl. Acad. Sci. USA* **81,** 1494-1498.

Kunkel, T. A. (1990) Misalignment-mediated DNA synthesis errors. *Biochemistry* **29,** 8003-8011.

Landegren, U., Kaiser, R., Sanders, J., and Hood, L. (1988) A ligase-mediated gene detection technique. *Science* **241,** 1077-1080.

Landy, A., Olchowski, E., Ross, W., and Reiness, G. (1974) Isolation of a functional *lac* regulatory region. *Mol. Gen. Genet.* **133,** 273-281.

Langley, K. E., Villarejo, M. R., Fowler, A. V., Zamenhof, P. J., and Zabin, I. (1975) Molecular basis of beta-galactosidase alpha-complementation. *Proc. Natl. Acad. Sci. USA* **72,** 1254-1257.

Lawrence, C. W., Borden, A., Banerjee, S. K., and LeClerc, J. E. (1990) Mutation frequency and spectrum resulting from a single abasic site in a single-stranded vector. *Nucleic Acids Res.* **18,** 2153-2157.

Lederberg, S. (1957) Supression of the multiplication of heterologous bacteriophages in lysogenic bacteria. *Virology* **3,** 496-513.

Lederberg, S., and Meselson, M. (1964) Degradation of non-replicating bacteriophage DNA in non-accepting cells. *J. Mol. Biol.* **8,** 623-628.

Lobban, P. E., and Kaiser, A. D. (1973) Enzymatic end-to-end joining of DNA molecules. *J. Mol. Biol.* **78,** 453-471.

Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A., and Mathur, E. J. (1991) High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* **108,** 1-6.

Luria, S. E. (1953) Host-induced modifications of viruses. *Cold Spr. Harb. Symp. Quant. Biol.* **18,** 237-244.

Mandel, M., and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* **53,** 159-162.

Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* **19,** 1154.

Mattila, P., Korpela, J., Tenkanen, T., and Pitkanen, K. (1991) Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase - an extremely heat stable enzyme with proofreading activity. *Nucleic Acids Res.* **19,** 4967-4973.

Mead, D. A., Pey, N. K., Herrnstadt, C., Marcil, R. A., and Smith, L. M. (1991) A universal method for the direct cloning of PCR amplified nucleic acid. *Biotechnology* **9,** 657-663.

Meselson, M., and Yuan, R. (1968) DNA restriction enzyme from *E. coli. Nature* **217,** 1110-1114.

Messing, J. (1991) Cloning in M13 phage or how to use biology at its best. *Gene* **100,** 3-12.

Messing, J., Crea, R., and Seeburg, P. H. (1981) A system for shotgun DNA sequencing. *Nucleic Acids Res.* **9,** 309-321.

Messing, J., Gronenborn, B., Müller-Hill, B., and Hofschneider, P. H. (1977) Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*. *Proc. Natl. Acad. Sci. USA* **74,** 3642-3646.

Miller, J. H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Moran, S., Ren, R. X.-F., Sheils, C. J., Rumney, S. 4th, Kool, E. T. (1996) Non-hydrogen bonding 'terminator' nucleosides increase the 3'-end homogeneity of enzymatic RNA and DNA synthesis. *Nucleic Acids Res.* **24,** 2044-2052.

Morrow, J. F., Cohen, S. N., Chang, A. C., Boyer, H. W., Goodman, H. M., and Helling, R. B. (1974) Replication and transcription of eukaryotic DNA in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **71,** 1743-1747.

Mullis, K. B., and Faloona, F. A. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155,** 335-350.

Newton, C. R., Holland, D., Heptinstall, L. E., Hodgson, I., Edge, M. D., Markham, A. F., and McLean, M. J. (1993) The production of PCR products with 5' single-stranded tails using primers that incorporate novel phosphoramidite intermediates. *Nucleic Acids Res.* **21,** 1155-1162.

Ng, L., Weiss, S. J., and Fisher, P. A. (1989) Recognition and binding of template-primers containing defined abasic sites by *Drosophila* DNA polymerase α holoenzyme. *J. Biol. Chem.* **264,** 13018-13023.

Nikolnikov, S., Pósfai, G., and Sain, B. (1984) The construction of a versatile plasmid vector that allows direct selection of fragments cloned into six unique sites of the cI gene of coliphage 434. *Gene* **30,** 261-265.

Nisson, P. E., Rashtchian, A., and Watkins, P. C. (1991) Rapid and efficient cloning of Alu-PCR products using uracil DNA glycosylase. *PCR Methods and Appl.* **1,** 120-123.

Noyer-Weidner, M., and Reiners-Schramm, L. (1988) Highly efficient positive selection of recombinant plasmids using a novel *rglB*-based *Escherichia coli* K-12 vector system. *Gene* **66,** 269-278.

Oliner, J. D., Kinzler, K. W., and Vogelstein, B. (1993) *In vivo* cloning of PCR products in *E. coli*. *Nucleic Acids Res.* **21,** 5192-5197.

Olivera, B. M., Hall, Z. W., and Lehman, I. R. (1968) Enzymatic joining of polynucleotides, V. A DNA-adenylate intermediate in the polynucleotide-joining reaction. *Proc. Natl. Acad. Sci. USA* **61,** 237-244.

Olivera, B. M., and Lehman, I. R. (1967) Linkage of polynucleotides through phosphodiester bonds by an enzyme from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **57,** 1426-1433.

Ozaki, L. S., Maeda, S., Shimada, K., and Takagi, Y. (1980) A novel ColE1::Tn3 plasmid vector that allows direct selection of hybrid clones in *E. coli*. *Gene* **8,** 301-314.

Paabo, S., Irwin, D. M., and Wilson, A. C. (1990) DNA damage promotes jumping between templates during enzymatic amplification. *J. Biol. Chem.* **265,** 4718-4721.

Paz-Elizur, T., Takeshita, M., Goodman, M., O'Donnell, M., and Livneh, Z. (1996) Mechanism of translesion DNA synthesis by DNA polymerase II. *J. Biol. Chem.* **271,** 24662-24669.

Paz-Elizur, T., Takeshita, M., and Livneh, Z. (1997) Mechanism of bypass synthesis through an abasic site analog by DNA polymerase I. *Biochemistry* **36,** 1766-1773.

Povirk, L. F., Houlgrave, C. W., and Han, Y.-H. (1988) Neocarzinostatin-induced DNA base release accompanied by staggered oxidative cleavage of the complementary strand. *J. Biol. Chem.* **263,** 19263-19266.

Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J., and Goodman, M. F. (1987) Nucleotide insertion kinetics opposite abasic lesions in DNA. *J. Biol. Chem.* **262,** 6864-6870.

Roberts, T. M., Swanberg, S. L., Poteete, A., Riedel, G., and Backman, K. (1980) A plasmid cloning vehicle allowing a positive selection for inserted fragments. *Gene* **12,** 123-127.

Sagher, D., and Strauss, B. (1983) Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during *in vitro* synthesis: uniqueness of adenine nucleotides. *Biochemistry* **22,** 4518-4526.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487-491.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA

Schaaper, R. M., Kunkel, T. A., and Loeb, L. A. (1983) Infidelity of DNA synthesis associated with bypass of apurinic sites. *Proc. Natl. Acad. Sci. USA* **80,** 487-491.

Scharf, S. J., Horn, G. T., and Erlich, H. A. (1986) Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* **233,** 1076-1078.

Schlieper, D., von Wilcken-Bergmann, B., Schmidt, M., Sobek, H., and Müller-Hill, B. (1998) A positive selection vector for cloning of long polymerase chain reaction fragments based on a lethal mutant of the *crp* gene of *Escherichia coli. Anal. Biochem.* **257,** 203-209.

Schumann, W. (1979) Construction of an *Hpa*I and *Hin*dII plasmid vector allowing direct selection of transformants harboring recombinant plasmids. *Mol Gen Genet* **174,** 221-224.

Sgaramella, V. (1972) Enzymatic oligomerization of bacteriophage P22 DNA and of linear Simian virus 40 DNA. *Proc. Natl. Acad. Sci. USA* **69,** 3389-3393.

Shibutani, S., and Grollman, A. P. (1993) On the mechanism of frameshift (deletion) mutagenesis *in vitro*. *J. Biol. Chem.* **268,** 11703-11710.

Shibutani, S., Takeshita, M., and Grollman, A. P. (1997) Translesional synthesis on DNA templates containing a single abasic site. *J. Biol. Chem.* **272,** 13916-13922.

Shimizu, H., Yagi, R., Kimura, Y., Makino, K., Terato, H., Ohyama, Y., and Ide, H. (1997) Replication bypass and mutagenic effect of α-deoxyadenosine site-specifically incorporated into single-stranded vectors. *Nucleic Acids Res.* **25,** 597-603.

Shuldiner, A. R., Scott, L. A., and Roth, J. (1990) PCR-induced (ligase-free) subcloning: a rapid reliable method to subclone polymerase chain reaction (PCR) products. *Nucleic Acids Res.* **18,** 1920.

Simoncsits, A., Chen, J., Percipalle, P., Wang, S., Törö, I., and Pongor, S. (1997) Single-chain repressors containing engineered DNA-binding domains of the phage

434 repressor recognize symmetric or asymmetric DNA operators. *J. Mol. Biol.* **267,** 118-131.

Smith, C., Day, P. J., and Walker, M. R. (1993) Generation of cohesive ends on PCR products by UDG-mediated excision of dU, and application for cloning into restriction digest-linearized vectors. *PCR Methods and Appl.* **2,** 328-332.

Smith, H. O., and Wilcox, K. W. (1970) A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. *J. Mol. Biol.* **51,** 379-391.

Steighner, R. J., and Povirk, L. F. (1990) Bleomycin-induced DNA lesions at mutational hot spots: implications for the mechanism of double-stand cleavage. *Proc. Natl. Acad. Sci. USA* **87,** 8350-8354.

Stevis, P. E., and Ho, N. W. (1987) Positive selection vectors based on xylose utilization suppression. *Gene* **55,** 67-74.

Stevis, P. E., and Ho, N. W. (1988) A novel *xylB*-based positive selection vector. *Plasmid* **20,** 92-95.

Strauss, B. S. (1991) The 'A rule' of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? *Bioessays* **13,** 79-84.

Takeshita, M., Chang, C.-N., Johnson, F., Will, S., and Grollman, A. P. (1987) Oligodeoxynucleotides containing synthetic abasic sites. *J. Biol. Chem.* **262,** 10171-10179.

Takeshita, M., and Eisenberg, W. (1994) Mechanism of mutation on DNA templates containing synthetic abasic sites: study with a double strand vector. *Nucleic Acids Res.* **22,** 1897-1902.

Tindall, K. R., and Kunkel, T. A. (1988) Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* **27,** 6008-6013.

Trudel, P., Provost, S., Massie, B., Chartrand, P., and Wall, L. (1996) pGATA: a positive selection vector based on the toxicity of the transcription factor GATA-1 to bacteria. *Biotechniques* **20,** 684-693.

Tuteja, N., Tuteja, R., Ochem, A., Taneja, P., Huang, N. W., Simoncsits, A., Susic, S., Rahman, K., Marusic, L., Chen, J., Zhang, J., Wang, S., Pongor, S., and Falaschi, A. (1994) Human DNA helicase II: a novel DNA unwinding enzyme identified as the Ku autoantigen. *EMBO J.* **13,** 4991-5001.

Ullmann, A., Jacob, F., and Monod, J. (1967) Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol.* **24,** 339-343.

Vernet, T., Lau, P. C., Narang, S. A., and Visentin, L. P. (1985) A direct-selection vector derived from pColE3-CA38 and adapted for foreign gene expression. *Gene* **34,** 87-93.

Vieira, J., and Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19,** 259-268.

Walker, G. C. (1995) SOS-regulated proteins in translesion DNA synthesis and mutagenesis. *Trends Biochem. Sci.* **20,** 416-420.

Wilson, G. G., and Murray, N. E. (1991) Restriction and modification systems. *Annu. Rev. Genet.* **25,** 585-627.

Wu, D. Y., and Wallace, R. B. (1989a) Specificity of the nick-closing activity of bacteriophage T4 DNA ligase. *Gene* **76,** 245-254.

Wu, D. Y., and Wallace, R. B. (1989b) The ligation amplification reaction (LAR) - amplification of specific DNA sequences using sequential rounds of template-dependent ligation. *Genomics* **4,** 560-569.

Yazynin, S. A., Deyev, S. M., Jucovic, M., and Hartley, R. W. (1996) A plasmid vector with positive selection and directional cloning based on a conditionally lethal gene. *Gene* **169,** 131-132.

Zimmerman, S. B., and Pheiffer, B. H. (1983) Macromolecular crowding allows blunt-end ligation by DNA ligases from rat liver or *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **80,** 5852-5856.

Zinder, N. D., and Boeke, J. D. (1982) The filamentous phage (Ff) as vectors for recombinant DNA - a review. *Gene* **19,** 1-10.