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(71) Applicant *(for all designated States except US)*: PLANT BIOSCIENCE LIMITED [GB/GB]; Norwich Research Park, Colney Lane, Norwich, Norfolk NR4 7UH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LOMONOSSOFF, George, Peter [GB/GB]; Magnolia House, Chester Place, Norwich, Norfolk NR2 3DG (GB). LIU, Li [CN/GB]; 15 Suffolk Walk, Norwich, Norfolk NR4 7TJ (GB).

(74) Agents: KREMER, Simon, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

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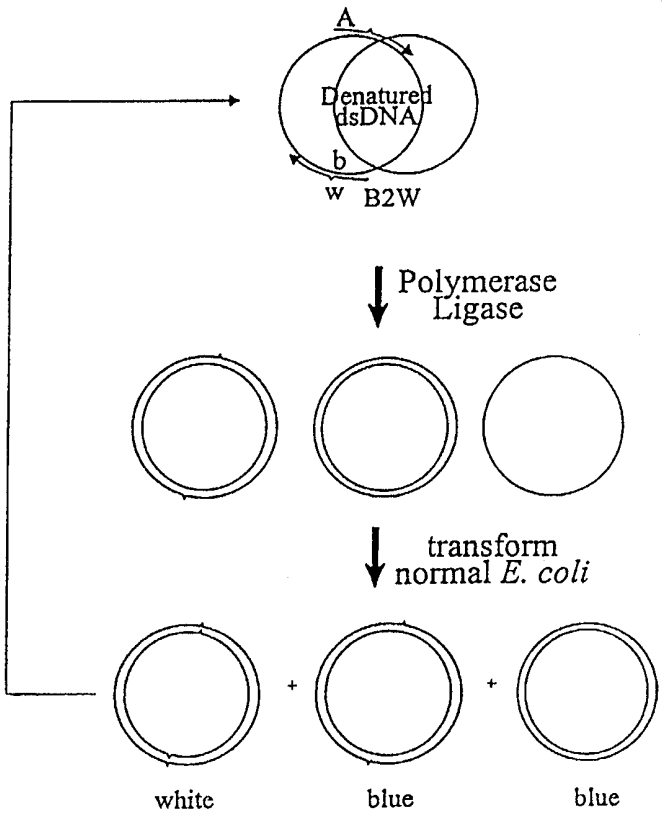
(57) Abstract

Disclosed are methods of introducing a mutation into a target nucleotide sequence. The method facilitates the introduction of more than one mutation sequentially into the target sequence using multiple rounds of mutagenesis, allowing the appropriate mutated sequences to be positively selected after each step. More than one target primer may be used simultaneously to introduce more than one mutation into the target sequence. Preferably hosts carrying the selection sequence and the mutated selection sequence can be simultaneously positively selected and distinguished on the basis of colour e.g. arising from mutations in the alpha–complement factor or an active variant thereof e.g. as encoded by lac Z.



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MUTAGENESIS

TECHNICAL FIELD

The present invention relates to methods and materials and kits for introducing mutations into target nucleotide sequences and recovering mutated material.

PRIOR ART

Site-directed mutagenesis is an important tool for the study of structure and functions of nucleic acids and proteins (see Ling & Robinson, 1997, for a recent review). Different strategies have been developed to perform the reaction and in particular to selectively recover mutated product material following the mutagenesis, which may represent as little as 1% of the reaction material.

By using uracil-containing single-stranded DNA (ssDNA) templates, in which dUMP are inserted randomly at the dTMP position in a *dut*$^-$ and *ung*$^-$ bacteria strain such as CJ236, more than 50% percent of progeny DNA molecules are mutated (Kunkel *et al.*, 1991). Although this method is efficient, only ssDNA can be used as templates, and special host organisms must be used.

Since ssDNA is more time-consuming to obtain than double-stranded plasmid DNA (dsDNA), attempts have been made to develop methods to use dsDNA as templates.

These methods include using phosphorothioate-modified nucleotide to protect from restriction enzyme and exonuclease III digestion (Sugimoto *et al.*, 1989). One restriction

enzyme based kit is disclosed in WO 97/20950 (Stratagene *et al.*)

Further methods employ linearized DNA and a closing oligonucleotide (Silaty *et al.*, 1990), deletion of a unique restriction site to give protection against digestion by restriction enzyme treatment (Deng & Nickoloff, 1992; Wong and Komaromy, 1995), reversion of an RNA binding site mutation to recover DNA replication in normal bacteria strains (Ohmori, 1994), and reversion of a selectable marker, such as ampicillin-resistance (amp^r) gene (Lewis and Thompson, 1990).

Certain of these possibilities are shown in Fig 1, schemes (1)-(3).

For those methods employing selectable markers, it is desirable that they do not require extra enzyme treatments, and that multiple rounds of mutagenesis can be performed without recloning target DNA. One method which avoids recloning is that of Promega (1994) wherein two selectable markers are used. The method is performed by using two extra oligonucleotide sequences, one for repairing an ampicillin sensitive gene, and another one to inactivate the tetracycline resistant gene. Since this method needs two selection markers, the vector size is of necessity relatively large, and two selection steps are needed, which increases the time and reduces the efficiency of the process. Another disadvantage of this method is that after the mutation reaction, it needs two round of transformations for selection of homologous clones.

It will be appreciated that the provision of novel methods

and materials for introducing one or more mutations into a target nucleotide sequence, particularly those which overcame one or more of the problems associated with the prior art, would provide a contribution to the art.

DISCLOSURE OF THE INVENTION

The present inventors have devised a novel, rapid and efficient protocol which avoids the use of antibiotic mutation and repair but that can, if required, be used for multiple rounds of mutagenesis without recloning. Compared with the antibiotic selection method, it only needs one screenable, selection marker which permits the use of relatively small vectors (or allows the use of larger target sequences).

In preferred forms homologous mutated clones can be obtained after only one round of transformation, using double-stranded plasmid DNA as templates, and bacterial strains commonly used in the art, wherein host cells carrying the desired mutation can be distinguished by colour alone.

In the most preferred embodiment the selection is carried out on the basis of mutation and repair of a selection nucleotide sequence encoding the α-complement for β-galactosidase in conjunction with an appropriate chromogenic substrate. The α-complement system in *E. coli* has *per se* been known for a considerable period of time (see e.g. Ullmann *et al.*, 1967; Gronenborn, 1976). Essentially the α-complement is an intra-allelic complement of a defective form of β-galactosidase encoded by a host, with the amino-terminal fragment of β-galactosidase being encoded by a segment of DNA (*LacZ*) inserted in a suitable construct. Bacteria which synthesize

both fragments of the enzyme will produce blue colonies when plated on media containing the chromogenic substrate `X-gal', those which do not will be a 'white' colour.

The α-complement has previously been used in cloning (Messing *et al.*, 1977), including use as a control to check mutagenesis efficiency in many mutagenesis methods (for example, in Kunkel, 1985; Taylor *et al.*, 1985; Lewis an Thompson, 1990; Deng and Nickoloff, 1992). In these methods the aim is generally to check that the components being used in the method are functioning as they should. The mutagenesis of the LacZ gene is not linked to that of the target sequence, and is generally carried out using a separate plasmid.

A different method employing LacZ mutagenesis is disclosed in WO 97/05484 (Beth Israel Hospital Association). Here a LacZ region, based on the pUR288 plasmid, is integrated into the genome of an organism; the organism is subjected to mutagenesis techniques and then the LacZ region is recovered in circular form and mutation is investigated using a positive selection procedure in *E. coli* C (*LacZ*$^-$). The object of the method is to assess the efficiency of the mutagenesis method, with the LacZ itself being the 'target'.

Thus none of these prior art methods employ a plasmid carrying separate target and LacZ sequences as used in the preferred methods of the present invention.

In the most preferred form of the present invention the selection can be commenced on the basis of either repair of a deficient (e.g. frame-shifted) LacZ, or mutation of an active, read-through LacZ, and can proceed accordingly. The

cloning sites (for the target sequence) are positioned elsewhere in the vectors. Two oligonucleotides controlling the *LacZ* expression and allowing the colonies exhibit blue or white colours are used as screenable primers. This property can be used for multiple rounds of mutagenesis by using the two screenable primers alternatively. Figure 1, scheme (4) summarises the method.

Various aspects and embodiments of the present invention will now be discussed in more detail.

Thus in a first aspect of the present invention there is disclosed a method of introducing a mutation into a target nucleotide sequence, the method comprising the steps of:
(i) providing a template nucleic acid construct comprising the target sequence and a selection nucleotide sequence encoding a selection polypeptide,
(ii) providing a first primer capable of hybridising with the target sequence but carrying a mutation with respect to that sequence,
(iii) providing a second primer capable of hybridising with the selection sequence but carrying a mutation with respect to that sequence,
(iv) incubating the template and primers under conditions suitable for template-dependent primed nucleic acid synthesis such as to generate a product nucleic acid construct comprising mutated target and mutated selection sequences,
(v) introducing the product into a suitable host capable of expressing the selection sequence or the mutated selection sequence to a respective polypeptide,
(vi) selecting a host carrying the product on the basis of expression of the selection sequence or the mutated selection sequence,

characterised in that the selection sequence is such as to allow simultaneous positive selection of a host expressing the selection sequence or a host expressing the mutated selection sequence

In this embodiment those hosts carrying a mutated selection sequence are selected since these will also comprise the mutated target sequence.

In one embodiment of this aspect, there is disclosed a method of introducing more than one mutation sequentially into a target sequence by using multiple rounds of mutagenesis, the method comprising the steps of:

(i) providing a product nucleic acid comprising a mutated target sequence and a mutated selection nucleotide sequence as described above,

(ii) repeating the method above using: the product nucleic acid as a template; a first primer capable of introducing a further mutation into the mutated target sequence; a second primer which is a selection repair primer capable of reverting the mutated selection sequence to the original selection sequence.

In this (multiple round mutagenesis) embodiment those hosts carrying an authentic (original) selection sequence are selected since these will also comprise the multiply-mutated target sequence. Subsequent rounds will select the mutant and authentic sequences alternately.

Thus the methods of the present invention differ from the use of antibiotic resistance markers in that the mutated selection sequence is not effectively a lethal mutation under the conditions used to select the non-mutated version. Thus

the need for two separate markers each of which is positively selectable under different conditions is avoided.

As the skilled person will appreciate, site-directed mutagenesis generally uses circular ssDNA as wild-type template for one or more mismatch primers. The primers are extended with a DNA polymerase to produce a double stranded DNA dsDNA product and the nick is ligated with DNA ligase. Thus the mutagenesis step in the methods above will initially yield a single stranded mutated `product' annealed to the template (a heterogenous product). Generally this duplex may be used to generate a homogenous dsDNA construct corresponding to either double stranded `original' material or double stranded mutated `product' material. The double stranded homogenous `product' with the appropriate mutated selection sequence is preferably selected in appropriate host cells.

In preferred embodiments of the invention a host carrying the selection sequence and a host carrying the mutated selection sequence can be simultaneously positively selected, and distinguished, on the basis of colour. However the invention embraces other single (mutated) selection sequences which give non-lethal phenotypes to the hosts carrying them under the conditions used to select them.

Thus in preferred embodiments either the selection sequence or the mutated selection sequence will encode an "active selection polypeptide" which is capable of altering the colour of the host in which it is expressed (or the vicinity thereof, in the case of extracellular enzyme activity). The selection sequence or the mutated selection not encoding the active selection polypeptide may, for instance, comprises a

mutation which either prevents transcription of the sequence, or prevents translation of an active polypeptide from the transcribed sequence. The mutation may optionally be a frame-shift mutation. However in each case the mutation is reversible with the appropriate primer.

Preferably the active selection polypeptide complements the activity of a host-encoded polypeptide, or is entirely new to the host. Preferably the complemented activity of the host-encoded polypeptide is β-galactosidase activity. Most preferably the active selection polypeptide is the α-complement factor or an active variant thereof.

An α-complement "active variant" is one which is encoded by a derivative of the wild-type LacZ sequence which incorporates changes therein. Changes may be desirable for a number of reasons. For instance they may introduce or remove restriction endonuclease sites or alter codon usage. Alternatively changes to a sequence may produce a derivative by way of one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded α-complement polypeptide. Changes may be by way of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Also included are variants having non-conservative substitutions. In all cases, however, the active variant polypeptide is capable of complementing β-galactosidase activity in a suitable host as described above.

It is reiterated that the starting point for the mutagenesis can be a vector encoding functional or non-functional selection polypeptide e.g. in embodiments where the "selection nucleotide sequence" encodes α-complement, this can either be functional or non-functional, the only proviso being that the "mutated selection sequence" encodes the opposite one. The terms "mutated" and "reverted" are therefore relative and should be construed accordingly.

Appropriate host cells for use in the present invention will occur to those skilled in the art by analogy with those used in the prior art above. Example hosts include (but are not limited to) the *E. coli* strains JM109 and the commonly used DH5α (or DH5αF') which are discussed in the Examples below. It may be preferred to use a mis-match repair minus *E. Coli* host (e.g. XLblue mutS) in order to increase mutagenesis efficiency. The use of *dam*$^-$ or *dcm*$^-$ strains may allow an increase in the ratio of desired colours in the whole colonies. Other preferred hosts may be *dut-,ung*-bacteria.

Naturally the methods of the present invention will generally be preceded by the insertion of the target sequence into the template nucleic acid construct. This will generally be by means of a "polylinker" region in the construct i.e. one containing multiple restriction sites which is adapted to accept appropriately cleaved target sequences.

If desired more than one target-mutagenesis primer can be used simultaneously to introduce more than one mutation into the target sequence in a single selection round. This may require the ligation of more than two polymerised nucleic acids.

The construct, or vector, used in the method may be any appropriate one suitable for site-directed-mutagenesis in the selected host. Example vectors include plasmids based on the pBluescriptIISK(+) which are disclosed in the Examples below. As disclosed in the Examples, the mutagenesis may employ a dsDNA template.

Vectors adapted for use in the methods of the present invention form a further aspect of the invention. The vectors will comprise an optionally mutated selection nucleotide sequence encoding a selection polypeptide, and a polylinker region. The selection sequence is such as to allow simultaneous positive selection of a host expressing the selection sequence or a host expressing the mutated selection sequence as described above. Preferably the vectors will be less than 5.8 Kbp in size, more preferably less than 5.0, 4.0, 3.0 Kbp, more preferably approximately 2.9 Kbp. Example vectors include pM81B, pM81W, and pM83B. Vectors in which target sequence has been included (e.g. in the polylinker region) are also included, and naturally these could be much larger (e.g. 13 Kbp or more, as in pBin19).

A primer adapted for use in the methods of the present invention forms a further aspect of the invention. These will be oligonucleotides, preferably of DNA although analogs may be used if preferred. Techniques for the synthesis of oligonucleotides can be found, amongst other places, in "Oligonucleotides and Analogues: A practical Approach" Ed. Eckstein, IRL Press, Oxford, (1992).

Such primers will be either a mutagenesis primer capable of hybridising with the selection sequence such as to allow the introduction of a mutation therein (contains a mismatch), or

a repair primer capable of hybridising with the mutated selection sequence such as to allow reversion of the mutated selection sequence to the original selection sequence, in each case the selection sequences being as described above i.e. they give non-lethal phenotypes to the hosts carrying them under the conditions used to select them.

Pairs of such primers are particularly preferred.

Example primers include W2B and B2W:

W2B (white to blue, 5' CCAGTGAGCGCG**ATC**GCGTAATCATGG 3').

B2W (blue to white, 5' CCAGTGAGCGCG**CTTG**GCGTAATCATGG 3').

In a further aspect of the present invention there is disclosed a kit for performing the methods of the present invention. Such kits may include the primers and vectors discussed above. In particular these is disclosed a kit for introducing a mutation into a target nucleotide sequence, the kit comprising:

(a) a template nucleic acid construct comprising an optionally mutated selection nucleotide sequence encoding a selection polypeptide, and a polylinker region,

(b) a mutagenesis primer capable of hybridising with the selection sequence such as to allow the introduction of a mutation therein,

(c) a repair primer capable of hybridising with the mutated selection sequence such as to allow reversion of the mutated selection sequence to the original selection sequence,

characterised in that the selection sequence is such as to allow simultaneous positive selection of a host expressing the selection sequence or a host expressing the mutated

selection sequence

The primers and vectors may be those described above. The kit may comprise further materials for ligating target sequences, for performing the polymerisation, or for detecting the selection polypeptide. Specific details of kit materials suitable for the polymerisation are found in commercially available kits e.g. as described in current Catalogues or Websites of Amersham, Pharmacia, Stratagene, Clonetech, Bio-Rad, and Promega. See also WO 97/20950 or references disclosed therein.

Examples of possible kit materials are described below. Components and concentrations are cited purely by way of example; those skilled in the art will appreciate that the precise nature of components and concentrations may vary e.g. depending on the number of reactions which the kit provides and the identity of the other materials with which it is to be used:

1. Vectors (as described above).
2. Polymerases (klenow polymerase 4U/ μl; Native T7 (2.5U/μl) or T4 DNA polymerase (5U/μl),
3. Ligase (T4 DNA ligase)
4. dNTP mix (1.25 mM dATP, dCTP, dGTP, dTTP, 2.5 mM ATP and 35 mM $MgCl_2$)
5. Host cells (e.g in freeze dried form, or pre-made competent cells in glycerol)
6. Control DNAs (appropriate for the vector and oligonucleotides)
7. Chromagenic substrates (e.g. X-Gal 50 mg/ml; IPTG)
8. Instruction booklet
9. Buffers (for annealing; synthesising; enzyme dilution)

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

Figure 1 shows a schematic diagram of a method in accordance with the present invention - Scheme (4). The other schemes represent the prior art. Scheme (1) is based on restriction site elimination, (2) is a replication defect protocol, (3) is an antibiotic repair protocol with two antibiotic markers.

Figure 2 schematically shows the result of a typical "blue to white" protocol resulting in double stranded heterogenous and homogenous products. The ^ shows the mutations introduced. A is the target; b is the sceenable selection marker. The mutant product gives white colonies on X-gal and IPTG. In embodiments where extended incubation time is taken, the heterozygous blue double stranded product shown in the Figure can be avoided.

Figure 3 shows a scheme of various vector construction and mutagenesis reactions according to the present invention, as described in the Examples below. Functional LacZ is shown as a block. Deficient LacZ is shown in outline. Also shown are cloning sites and insertions. Mutated sites (other than in Lac Z) are shown by a dark circle.

EXAMPLES

Example 1 - Mutation efficiency by selection based on white

colonies to blue colonies

Referring to Figure 3, to determine if circular dsDNA was a suitable template for site-directed mutagenesis in the present system, we first mutagenized pM0 by using W2B and V4514. At the same time, ssDNA was used as a control. Among 20 blue colonies, 19 samples contained the desired mutations, as checked by *Asc*I restriction analysis (Table 1, Reaction A), which is similar to the reaction using ssDNA as template (92 percent mutation). These results indicate that dsDNA is an effective template for site-directed mutagenesis. After inserting a multiple cloning sequence into pM0W, we mutagenized it by using W2B and a target primer. As a result, 53 percent simultaneous mutations were obtained (Table 1, Reaction B).

When using the blue colour as the screening marker, it was found that the desired colonies often contained heterogenous (mutated and unmutated) plasmids. To eliminate this, plasmid DNA from each blue colony was retransformed into the bacteria strain DH5α. After this second selection, all the blue colonies contained homologous mutated DNA.

In other embodiments, described in the 'General materials and methods' below, *MutS* strains were used for transformation, and the cells were incubated for two to three hours in liquid culture and then transferred to petri dishes. After overnight, single colonies containing the desired screenable marker were picked up. Up to 60% of colonies were homologous and contained the targeted mutations.

Example 2 - Mutation efficiency by selection based on blue colonies to white colonies

To confirm that multiple rounds of mutations could be achieved, pM81B was mutagenized by using the B2W and a target primer. Two mutagenesis reactions were performed and 50 to 63 percent of colonies contained the desired mutations (Table 1, Reaction C and D).

Interestingly heterogenous plasmids were not found by restriction analysis (data not shown) and plasmid re-transformation and sequencing (data not shown). This result is unsurprising since colonies containing unmutated plasmids will show a blue colour and hence will not be selected.

Table 1 Mutagenesis efficiency by using dsDNA as template

Reaction	Vector size (kbp)	Selection marker	size of target primer (nt)[1]	Type of Mutation[1]	Efficiency (%)
A	2.7	W2B	35	2 substitution among 4 nt	95
B	2.9	W2B	37	3 substitution among 4 nt	53
C	2.9	B2W	37	3 substitution among 4 nt	63
D	2.9	B2W	36	3 substitution among 4 nt 3 nt deletion	50
E	3.6	B2W	35	1 nt insertion	100

F	5.3	W2B	40	4 nt substitution	70
G[2]	3.6	B2W	35 33	1 nt insertion 1 nt substitution	75 75
H[3]	5.2	W2B	30 30	2 nt substitution 2 substitution among 4 nt	80 60
I[4]	5.3	W2B	28 40 35 45	1 nt substitution 4 nt substitution 2 nt substitution 7 substitution among 10 nt	71 57 83 67

[1] nt for nucleotide(s).

[2] The simultaneous mutation rate is 75 percent.

[3] The simultaneous mutation rate is 60 percent.

[4] The simultaneous mutation rate is 38 percent.

Example 3 - testing mutation efficiency with inserts

Plasmid DNA of pM81W2.4K and pM83B0.7K was mutagenised by using target primers and W2B or B2W. The mutagenesis rate is 70 to 100 percent analysed by restriction digestion for pM81B2.4K and sequencing for pM83W0.7K (Table 1, Reaction E and F).

Example 4- multiple simultaneous mutations

To test the efficiency of the method in multiple simultaneous

mutations, two mutagenesis oligonucleotides were used to mutagenize pM83B0.7K and pM81W2.3K, and four target primers were used for pM81W2.4K. The simultaneous mutation rate is from 60 to 75 percent for two-site samples, and 38 percent For the four-site sample (Table 1, Reaction G, H, and I).

Example 5 - effect of Bacteria strains

It has been reported that DNA mis-matches are repaired by a methyl-directed DNA mismatch-repair system in *E. coli* (Kramer *et al.*, 1984). Using mis-match repair minus *E. Coli* host is reported to be important for high mutation efficiencies (Kramer *et al.*, 1984; Lewis and Thompson, 1990). A mismatch repair minus bacteria strain XLblue mutS was used for the transformation and compared with `normal' hosts JM109 and DH5α. The results showed that while JM109 did repair the mutations, there is no difference between using DH5α and XLblue mutS. However, 'normal' strains may give unstable results when using large insert plasmids. Therefore it may be preferable to use mutation repair minus strains (such as *mutS* or *mutL* strains) under these circumstances.

Example 6 - effect of enzyme concentration

The effects of T4 and T7 DNA polymerases, together with different concentrations of T4 DNA ligase, were tested in order to find the best combination. Mutated DNA was transformed and directly transferred to Petri dishes to check the blue/white ratio. With template DNA from different *E. coli* hosts, the enzyme effects were not the same. In general, results showed that when T4 DNA polymerase was used, mutated colonies (blue/white ratio) were usually less than 0.1%. When T7 DNA polymerase was used, the enzyme appeared to reduce the

total number of colonies at higher concentrations (Table 2).

Table 2 Effects of T4 and T7 DNA polymerases (b/w) (4 kbp insert)

units of T4 DNA ligase	units of T7 DNA polymerase				
	0.5	1	2	2.5	5
2.5	8/1492	10/936	15/672	24/644	4/124
5	13/1564	22/948	15/764	NT*	2/123

*NT not tested.

Example 7 - effect of denaturing method

Methods of DNA denaturing by use of boiling water (Slilaty *et al.*, 1989; Deng & Nickoloff, 1992) or alkali (Sugimoto et al., 1988) were compared. In nine mutation experiments conducted with heating denatured DNA, nearly half produced no screenable mutants. This was particularly the case where large insertion plasmids were used. When a modified alkaline denaturing method was used, in twenty-four experiments with sodium hydroxide, all produced mutated colonies. The results suggest that alkaline denaturing may be preferable.

Example 8 - effect of ratio of screenable and target oligonucleotides

Oligonucleotide ratio and amount may affect the efficiency of targeted mutagenesis. The amount of screenable and target oligonucleotides and its effect on targeted mutation efficiency were investigated. The results showed that both oligonucleotides at 4 pmol produced best targeted mutations (Table 3).

Table 3 amount of screenable and target oligonucleotides and efficiency of target mutagenesis (4 kbp insertion).

amount of W2B and target oligonucleotides (pmol)	0.5:4	1:4	2:4	4:4
target mutagenesis (%) (mutated colonies/blue colony)	83 (5/6)	66 (4/6)	80 (4/5)	100 (7/7)

Example 9 - effect of size of insert

Plasmid DNA containing insertions from 2 kbp to 6.0 kbp was used to test the target mutation rate over marker mutation. The mutagenesis rate was 25 to 71 percent analysed by restriction digestion analysis (Table 4).

Table 4 mutation rates of a target site over screenable marker mutation

size of insertion (kbp)	mutated /analysed colonies	mutation rate (%)
2.0	2/6	33
4.1	106/149	71
4.2	1/4	25
5.6	3/6	50
6.0	5/12	41

Example 10 - further example of multiple simultaneous mutations

To confirm the efficiency of the method in multiple simultaneous mutations, two or four target mutagenesis oligonucleotides were used in addition to a screenable

oligonucleotide. The simultaneous mutation rate was 58 percent for the two-site mutation sample, and 16 percent for the four-site sample (Table 5).

Table 5 Multiple simultaneous mutation rates

size of insertions	number of target mutation oligonucleotide	mutated /analysed colonies	simultaneous mutation rate (%)
4.0	4	6/12, 4/12, 5/12, 3/7	16
4.4	2	10/12, 7/12	58

Example 11 - effect of incubation time on the segregation of cells containing heterologous DNA

Extension of the time in which transformed cells were incubated in liquid culture permitted improved segregation of cells containing heterologous DNA plasmids with the result that homologous mutated DNA could be achieved in a single transformation (Table 6).

Table 6 Effects of incubation time on the segregation of cells containing heterologous DNA (4 Kbp insert)[1]

Incubated hours	2	3	4
Experiment 1	3/6	2/6	4/6
Experiment 2	3/12	8/12	5/12
Experiment 3	NT[2]	5/12	NT[2]

[1] number of homologous colonies containing marker and target mutations/total colonies analyzed.

[2] not tested.

Conclusions

Thus the invention provides, *inter alia*, an efficient and rapid site-directed mutagenesis method using α-complement for colour selection as a screenable marker. This new method has several advantages over current approaches:

Normal dsDNA may be used as templates compared to Kunkel's method which uses only uracil-containing ssDNA (Kunkel 1985; Kunkel *et al.*, 1991).

Enzyme digestion is not necessary after the reaction, which is a vital step for successful unique site elimination method (Deng & Nickoloff, 1992).

One transformation was sufficient to select homologous mutated DNA when white colonies were the selection target. Commonly used methods need two round of transformations to separate mutated DNA from the heterogenous plasmids (Lewis & Thompson, 1990; Deng & Nickoloff, 1992; Andrews & Lesley, 1998).

When a second transformation is needed, DNA from individual colonies containing a marker gene may be used for the second transformation, thus eliminating the possibility that plasmids selected from the second transformation are multiplied from same colonies.

Mismatch DNA repair deficient strains are not necessary for

the new method.

Multiple rounds of mutations can be performed using same plasmid DNA. Although Promega's Protocol can be used for this purpose (Promega, 1994), the new method needs only one selection marker (switch `on' or switch `off') rather than two, hence saving the selection time, increasing the mutational efficiency and keeping the plasmid small (less than 3 kbp). The Small vector size allows large insertions compared with other methods which are usually used for mutation of small fragments of DNA. The present system can be easily used to mutate at least a 6.1 kbp DNA insert.

Simultaneous multiple mutations are efficiently performed compared to PCR-based mutagenesis. Additionally, the procedure can employ high fidelity T4, or more preferably T7, DNA polymerase which reduce the unwanted secondary mutations that are often encountered in PCR-based mutagenesis. Additionally, T7 polymerase gives fast synthesis and high processivity (Tabor *et al*, 1987) which is useful when using large inserts. T7 was also found to increase the ratio of mutated screenable colonies, by reducing the activity of unmutated DNA.

General materials and methods

Enzymes, bacteria strains and reagents

Restriction enzymes were purchased from Boehringer, GIBCO-BRL or New England BioLabs. T4 DNA polymerase and polynucleotide kinase were from GIBCO-BRL or Pharmacia. T4 DNA ligase was from Boeringer. T7 DNA polymerase was from New England. *E. coli* strain DH5α and DH5αIQ was from GIBCO-BRL, JM109 from

Promega, XLblue *mutS* from Stratagene. X-gal and IPTG from Pharmacia. DNA purification kits and columns were from Promega.

Plasmids Construction

The pM0 was constructed by deleting the *Bss*HII fragment in pBluescriptIISK(+). The mutagenesis method was tested by using W2B (white to blue, 5' CCAGTGAGCGCGATCGCGTAATCATGG 3') and V4514, an oligonucleotide of 35 nucleotides, to mutate dsDNA or ssDNA of pM0 to pM0B, in which a additional *Sca*I was created at the *LacZ* gene by W2B and a *Asc*I site downstream of the *LacZ* gene by V4514. To produce pM0W, ssDNA of pM0 was mutated by using B2W (blue to white, 5' CCAGTGAGCGCGCTTGGCGTAATCATGG 3') and V4514, in which restriction sites *Bgl*II was produced at the *LacZ* gene by B2W, and a *Asc*I site downstream of the *LacZ* gene by V4514. The LacZ gene was reading through in pM0B and was disrupted in pM0W. When these vectors were transformed into LacZ negative bacteria strains, colonies will be show blue or white, respectively.

To introduce a polylinker sequence into pM0B and pM0W, these vectors were treated with *Asc*I and ligated with the *Bss*HII-*Asc*I fragment containing a polylinker sequence from pN81'. The constructed vectors, pM81B and pM81W, were used as mutation vectors for cloning target fragments.

To test the system with insertions, A 2.4 Kbp fragment consisting of CaMV 35S promoter and a cDNA fragment of cowpea mosaic virus RNA1, and a 2.3 Kbp fragment containing a NPTII gene were cloned into pM81W to produce pM81W2.4K and pM81W2.3K, respectively. A 0.7 Kbp fragment containing a cDNA

fragment of red clover mosaic virus was cloned into pM83B (a derivative of pM81B) to produce pM83B0.7K. The dsDNA of constructs were applied as templates. One or more mutagenic primers together with W2B or B2W were used to perform mutagenesis.

Oligonucleotide Synthesis and Phosphorylation

The oligonucleotides were synthesized on the ExpediteTM Nucleic Acid Synthesis System (Millipore). The DNA was treated with 35% NH_4OH, precipitated with 70% ethanol and dissolved in distilled water. The concentration was determined by spectrophotometer.

Phosphorylation were performed at 37°C for 30 minutes and stopped by incubating at 70°C for 10 minutes in a 25 μl of volume containing 4 pmol/μl primer, 50 mM This-HCl, pH7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine HCl, 0.1 mM EDTA, pH8.0, 0.8mM ATP, 10U T4 DNA polynucleotide kinase.

Template DNA Preparation

The dsDNA was purified using the alkaline method (Sambrook *et al.*, 1989) and further treated using a DNA purification kit. Purified DNA was adjusted to 0.05 pmol/μl. Mini-prep DNA (Sambrook *et al.*, 1989) was sometimes used to test the adaption of the system.

Site-Directed Mutagenesis

For Examples 1 to 5, mutagenesis was performed as follows. 0.05 pmol of template DNA was mixed with 4 pmol of screenable W2B or B2W primer, 4 pmol of target primer(s) in a 20 μl of

volume containing 20 mM Tris-HCl, pH7.5, 10 mM $MgCl_2$, 50 mM NaCl. The mixture was incubated in a 100°C water bath for 3 minutes, and immediately placed on ice for another 3 minutes. After adding 10 μl of a solution containing 30 mM Tris-HCl, pH7.5, 1.5 mM dNTPs, 3 mMATP, 6 mM dithiothreitol, 5U T4 DNA polymerase and 5U T4 DNA ligase, the mixture was incubated at 37°C for 180 minutes and placed on ice for transformation. The reactions was incubated at 75°C for 10 minutes and placed on ice before transformation. A volume of 3 to 15 μl of reactions was used in a standard calcium chloride transformation (Sambrook *et al.*, 1989).

For Examples 6 to 10, mutagenesis was performed as follows, if not indicated otherwise. An aliquot (0.05 pmol) of column-cleaned DNA (1 μl) or 15 μl of mini-prep DNA was placed in a 0.5 ml micro-centrifuge tube, to which 4 μl of 2N NaOH, and H_2O to a total volume of 20 μl were added. The mixture was incubated at room temperature for 10 minutes to denature the DNA, and 10 μl of 3M sodium acetate, pH5.2 and 100 μl of ethanol were added. The sample was then placed on dry ice for 15 minutes. The DNA was collected by centrifugation for 10 minutes in a micro-centrifuge, washed with 70% ethanol, and finally redissolved in up to 16 μl of H_2O. Screenable W2B or B2W oligonucleotide (1 μl, 4 pmol), target oligonucleotide(s) (1 μl, 4 pmol), annealing buffer (2 μl, 200 mM Tris-HCl, pH7.5, 100 mM $MgCl_2$, 500 mM NaCl) and H_2O were added to a volume of 20 μl and the mixture was incubated at 75°C for 5 minutes and then room temperature for 10 minutes. After adding 10 μl of a solution containing 30 mM Tris-HCl, pH7.5, 1.5 mM dNTPs, 3 mMATP, 6 mM dithiothreitol, 2.5U T7 DNA polymerase and 2.5U T4 DNA ligase, the mixture was incubated at 37°C for 90 minutes. Before transformation, the reactions was incubated at 75°C for five minutes and placed on ice for 1

minute. A volume of 5 to 15 μl of reactions was mixed with 50 μl of Xlblue *mutS* competent cells (calcium chloride method) in a 0.5 ml tube and placed on ice for 30 minutes, at 42°C for 90 seconds, and on ice for 90 seconds.

For testing the effects of different conditions, the sample was added with 300 μl of LB, incubated at 37°C for 10 to 20 minutes and transfer to Petri dish containing LB, carbenicillin, X-gal and IPTG. The screenable mutating colonies were recorded after 20 hours according to the colour, and targeted mutations were further tested by enzyme digestion of purified DNA.

To obtain homogenous mutants directly, the sample was treated as follows. After 90 seconds on ice, the cells were transferred to 10 ml of LB and the mixture incubated at 37°C for one hour. Antibiotic (carbenicillin) was the added and the cells incubated for a further tow hours under the same conditions before transfer to a Petri dish. Blue or white colonies were selected according to screenable oligonucleotide for confirmation of target mutations.

References

Andrews, C. A., and Lesley, S. A. (1998). Selection strategy for site-directed mutagenesis based on altered β-lactamase specificity. *BioTechniques* **24**(6), 972-980.

Bebenek, K., and Kunkel, T. (1989). The use of native T7 DNA polymerase for site-directed mutagenesis. *Nucleic Acids Research* **17** (13), 5408.

Gronenborn, B. (1976). Overproduction of phage lambda repressor under control of the *lac* promoter of *Escherichia coli*. *Molecular and General Genetics* **148**,

243-250.

Deng, W. P., and Nickoloff, J. A. (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Analytical Biochemistry* **200,** 81-88.

Hultman, T., Murby, M., Stahl, S., E., H., and Uhlen, M. (1990). Solid phase in vitro mutagenesis using plasmid DNA template. *Nucleic Acids Research* **18**(17), 5107-5112.

Kunkel, T. A., Bebenek, K., and McClary, J. (1991). Efficient site-directed mutagenesis using uracil-containing DNA. *In* "Methods in Enzymology", Vol. 204, pp. 125-139. Academic Press, Inc.

Lewis, M. K., and Thompson, D. V. (1990). Efficient site directed in vitro mutagenesis using ampicillin selection. *Nucleic Acids Research* **18**(12), 3439-3443.

Ling, M. M., and Robinson, R. H. (1997). Approaches to DNA mutagenesis: An overview. *Analytical Biochemistry* **254,** 157-178.

Messing, J., Gronenborn, R., Mueller-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 from *in vitro*. *Proceedings of the National Academy of Sciences of The United States of America* **74**(9), 3642-3646.

Moor, J. T., Maley, G. F., and Mayley, F. (1993). A modified unique site elimination plasmid mutagenesis procedure utilizing standard calcium chloride transformations. *Analytical Biochemistry* **208,** 402-403.

Ohmori, H. (1994). A new method for strand discrimination in sequence-directed mutagenesis. *Nucleic Acids Research* **22**(5), 884-885.

Osei-Frimpong, J., and Lebovitz, R. (1993). Improving the efficiency of mutagenesis during oligonucleotide-directed *in vitro* mutagenesis. *BioTechniques* **14**(4), 554-

556.

Promega (1994) Instruction of Site-directed Mutagenesis Kit: "Altered Sites II - *in vitro* Mutagenesis Systems - Instructions for products Q6210, Q6080 and Q6090" Rev. 8/94 Part# TM001.

Ray, F. A., and Nickoloff, J. A. (1992). Site-specific mutagenesis of almost any plasmid using a PCR-based version of unique site elimination. *BioTechniques* **13**(3), 342-347.

Sayers, J. R., Krekel, C., and Eckstein, F. (1992). Rapid high-efficiency site-directed mutagenesis by the phosphorothioate approach. *BioTechniques* **13**(4), 592-596.

Silaty, S. N., Fung, M., Shen, S. H., and Lebel, S. (1990). Site-directed mutagenesis by complementary-strand synthesis using a closing oligonucleotide and double-stranded DNA templates. *Analytical Biochemistry* **185**, 194-200.

Sugimoto, M., Esaki, N., Tanaka, H., and Soda, K. (1989). A simple and efficient method for the oligonucleotide-directed mutagenesis using plasmid DNA template and phosphorothioate-modified nucleotide. *Analytical Biochemistry* **179**, 309-311.

Tabor et al, (1987). *The Journal of Biological Chemistry* **262**: 16212-16223

Taylor, J. W., Ott, J., and Eckstein, F. (1985). The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acids Research* **13**(24), 8765-8785.

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

Wong, F., and Komaromy, M. (1995). Site-directed mutagenesis using thermostable enzymes. *BioTechniques* **18,** 1995.

Claims

1. A method of introducing a mutation into a target nucleotide sequence, the method comprising the steps of:
(i) providing a template nucleic acid construct comprising the target sequence and a selection nucleotide sequence encoding a selection polypeptide,
(ii) providing a first primer capable of hybridising with the target sequence but carrying a mutation with respect to that sequence,
(iii) providing a second primer capable of hybridising with the selection sequence but carrying a mutation with respect to that sequence,
(iv) incubating the template and primers under conditions suitable for template-dependent primed nucleic acid synthesis such as to generate a product nucleic acid construct comprising mutated target and mutated selection sequences,
(v) introducing the product nucleic acid into a suitable host capable of expressing the selection sequence or the mutated selection sequence to a respective polypeptide,
(vi) selecting a host carrying the product nucleic acid on the basis of the presence of the selection sequence or the mutated selection sequence, characterised in that the selection sequence is such as to allow simultaneous positive selection of a host expressing the selection sequence or a host expressing the mutated selection sequence

2. A method of introducing more than one mutation sequentially into a target sequence by using multiple rounds of mutagenesis, the method comprising the steps of:
(a) performing a method as claimed in claim 1 to provide a product nucleic acid comprising mutated target and mutated selection sequences,

(b) providing a further first primer capable of introducing a further mutation into the target sequence, and a further second primer which is a selection repair primer capable of reverting the mutated selection sequence,

(c) performing a method as claimed in claim 1 using the product nucleic acid as a template and using said further primers to generate a further product nucleic acid construct which comprises a further mutated target sequence and a reverted selection sequence, and

(d) optionally repeating steps (a) to (c) using said further product nucleic acid as a template.

3. A method as claimed in claim 1 or claim 2 wherein more than one primer capable of hybridising with the target sequence but carrying a mutation with respect to that sequence is used simultaneously to introduce more than one mutation into the target sequence.

4. A method as claimed in any one of the preceding claims wherein the target sequence is larger than 6 kbp.

5. A method as claimed in any one of the preceding claims wherein step (iv) employs T7 polymerase.

6. A method as claimed in any one of the preceding claims wherein the selection sequence is such that a host carrying the selection sequence and a host carrying the mutated selection sequence can be simultaneously positively selected and distinguished on the basis of colour.

7. A method as claimed in claim 6 wherein either the selection sequence or the mutated selection sequence encodes

an active selection polypeptide which is capable of altering the colour of a host cell in which it is expressed.

8. A method as claimed in claim 7 wherein the mutation created or repaired by the second primer prevents transcription of the selection sequence or prevents translation of an active selection polypeptide from the transcribed sequence.

9. A method as claimed in claim 7 or claim 8 wherein the active selection polypeptide complements the activity of a host-encoded polypeptide.

10. A method as claimed in claim 9 wherein the complemented activity is β-galactosidase activity.

11. A method as claimed in claim 10 wherein the active selection polypeptide is the α-complement factor or an active variant thereof.

12. A method as claimed in claim 10 or claim 11 wherein the either the selection sequence or the mutated selection sequence comprises lac Z.

13. A method as claimed in any one of the preceding claims wherein the host cell is a mis-match repair minus *E. Coli* strain.

14. A method as claimed in any one of the preceding claims wherein the host cell is a selected from: *E. coli* strain XLblue mutS; JM109; DH5α; DH5αF'.

15. A method as claimed in any one of the preceding claims

wherein the template nucleic acid of step (i) is obtained by denaturing double stranded nucleic acid using an alkali.

16. A method as claimed in any one of the preceding claims wherein the selection of the host cell of step (vi) is carried out by incubating host cells in a liquid culture for at least about 30 mins, 1 hour, 2 hours or 3 hours prior to transfer to a solid medium and selecting host cells directly therefrom without the intermediate step of retransforming the product nucleic acid into a second host.

17. A process for producing a host cell carrying a nucleic acid comprising a target nucleotide sequence into which at least one mutation has been introduced, the process comprising selecting a host cell in accordance with a method as claimed in any one of the preceding claims and optionally culturing said host cell.

18. A process for producing a nucleic acid comprising a target nucleotide sequence into which at least one mutation has been introduced, the method comprising producing a host cell in accordance with a method as claimed in claim 17 and isolating the nucleic acid therefrom.

19. A kit for performing a method or process of any of the preceding claims, wherein said methods employ a template nucleic acid construct comprising an optionally mutated selection nucleotide sequence encoding a selection polypeptide, and a polylinker region, which kit comprises:
(a) a mutagenesis primer capable of hybridising with the selection sequence such as to allow the introduction of a mutation therein,
(b) a repair primer capable of hybridising with the mutated

selection sequence such as to allow reversion of the mutated selection sequence to the original selection sequence, characterised in that the selection sequence is such as to allow simultaneous positive selection of a host expressing the selection sequence or a host expressing the mutated selection sequence

20. A kit as claimed in claim 17 further comprising a template nucleic acid adapted which comprises (i) an optionally mutated selection nucleotide sequence encoding a selection polypeptide, and (ii) a polylinker region, whereby the selection sequence is such as to allow simultaneous positive selection of a host expressing the selection sequence or a host expressing the mutated selection sequence

21. A kit as claimed in claim 20 wherein the template nucleic acid construct is less than 5.8, 5.0, 4.0, or 3.0 Kbp in size.

22. A kit as claimed in claim 21 wherein the template nucleic acid construct is derived from pBluescriptIISK(+).

23. A kit as claimed in claim 22 wherein the template nucleic acid construct is pM81B, pM81W or pM83B as described herein in Fig 3.

24. A kit as claimed in any one of claims 19 to 23, which kit further comprises one or more of the following materials: a polymerase; a ligase; a dNTP; a host cell; control nucleic acids; a chromagenic substrates; instructions for use; buffer.

25. A nucleic acid primer selected from:

W2B (white to blue, 5' CCAGTGAGCGCGATCGCGTAATCATGG 3'), or
B2W (blue to white, 5' CCAGTGAGCGCGCTTGGCGTAATCATGG 3')

26. A composition of matter comprising primer W2B and B2W of claim 24.

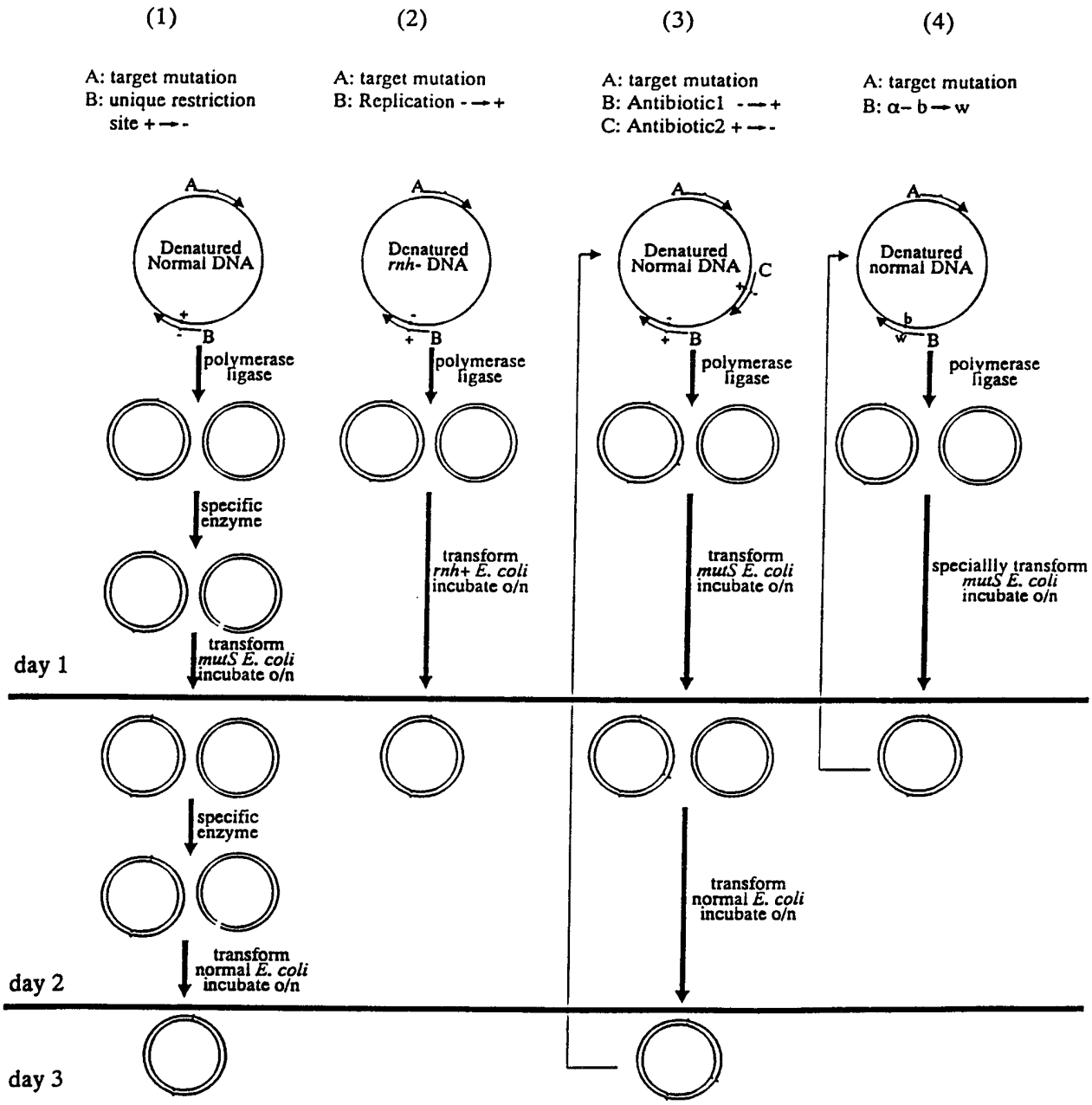
(1)
A: target mutation
B: unique restriction site + → -
Denatured Normal DNA
polymerase ligase
specific enzyme
transform muts E. coli incubate o/n
(2)
A: target mutation
B: Replication - → +
Denatured rnh- DNA
polymerase ligase
transform rnh+ E. coli incubate o/n
(3)
A: target mutation
B: Antibiotic1 - → +
C: Antibiotic2 + → -
Denatured Normal DNA
polymerase ligase
transform muts E. coli incubate o/n
transform normal E. coli incubate o/n
(4)
A: target mutation
B: α- b → w
Denatured normal DNA
polymerase ligase
specially transform muts E. coli incubate o/n
specific enzyme
transform normal E. coli incubate o/n
day 1
day 2
day 3

FIG. 1

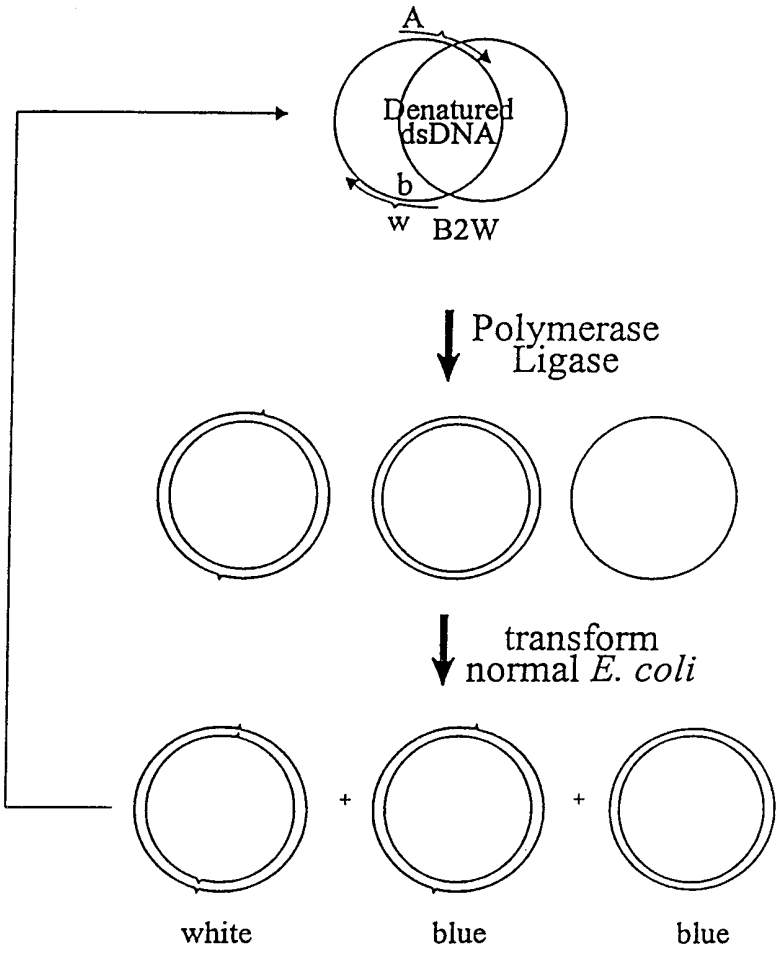
A
Denatured
dsDNA
b
w
B2W
Polymerase
Ligase
transform
normal E. coli
+
+
white
blue
blue

FIG. 2

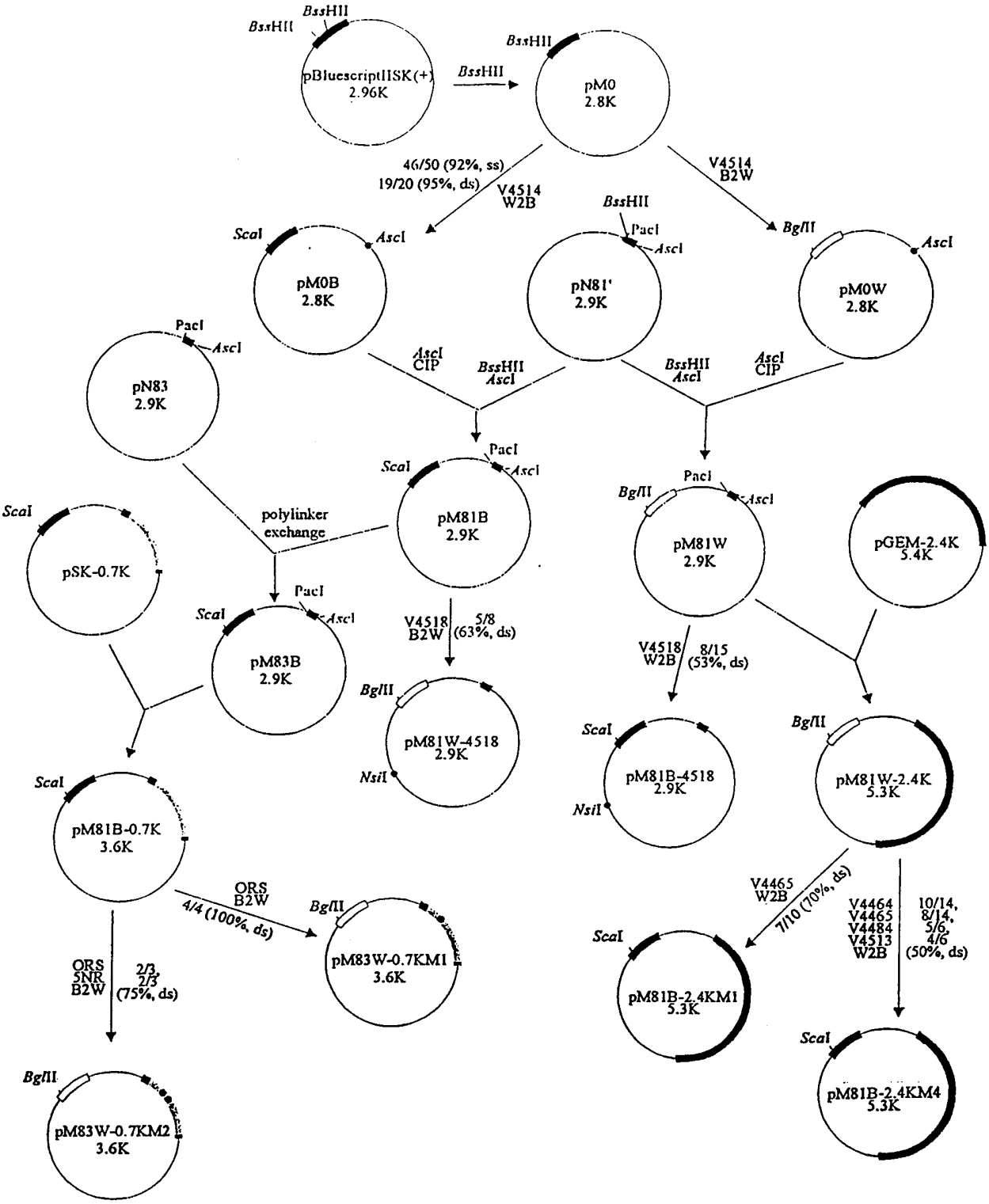
BssHII
pBluescriptIISK(+)
2.96K
pM0
2.8K
46/50 (92%, ss)
19/20 (95%, ds)
V4514
W2B
V4514
B2W
ScaI
AscI
pM0B
2.8K
PacI
pN81'
2.9K
BglII
pM0W
2.8K
pN83
2.9K
AscI
CIP
pM81B
2.9K
pM81W
2.9K
pGEM-2.4K
5.4K
pSK-0.7K
polylinker
exchange
pM83B
2.9K
V4518
B2W
5/8
(63%, ds)
pM81W-4518
2.9K
NsiI
V4518
W2B
8/15
(53%, ds)
pM81B-4518
2.9K
pM81W-2.4K
5.3K
pM81B-0.7K
3.6K
ORS
B2W
4/4 (100%, ds)
pM83W-0.7KM1
3.6K
ORS
5NR
B2W
2/3
2/3
(75%, ds)
pM83W-0.7KM2
3.6K
V4465
W2B
7/10 (70%, ds)
V4464
V4465
V4484
V4513
W2B
10/14,
8/14,
5/6,
4/6
(50%, ds)
pM81B-2.4KM1
5.3K
pM81B-2.4KM4
5.3K

FIG. 3

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/GB 00/01075

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, STRAND, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEWIS M K ET AL: "EFFICIENT SITE DIRECTED IN VITRO MUTAGENESIS USING AMPICILLIN SELECTION" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 18, no. 12, 1 January 1990 (1990-01-01), pages 3439-3443, XP002049079 ISSN: 0305-1048 cited in the application	1-4, 13-21
Y	the whole document	6-12,24

X	"Sequencing and Mutagenesis" PROMOGA PRODUCT CATALOG 1990/1991, pages 44-45, XP002142035	1-4, 13-21,24
Y	Altered sites in vitro Mutagenesis system, pSELECT-1;	6-12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 July 2000	24/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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PCT/GB 00/01075

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	"ALTERED SITES II IN VITRO MUTAGENESIS SYSTEMS" PROMEGA PRODUCT CATALOG, 1 January 1994 (1994-01-01), XP002049080 cited in the application	1-4, 13-21
Y	the whole document	6-12,24
X	SHEN T -J ET AL: "A MARKER-COUPLED METHOD FOR SITE-DIRECTED MUTAGENESIS" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 103, 15 July 1991 (1991-07-15), pages 73-77, XP002049081 ISSN: 0378-1119	1-4, 13-21
Y	the whole document	6-12,24
X	WO 98 02537 A (PROMEGA CORP) 22 January 1998 (1998-01-22) cited in the application	1-4, 13-21
Y	the whole document	6-12,24
X	DENG W P & NIKOLOFF J A: "Site-directed mutagenesis of virtually any plasmid by eliminating a unique site" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 200, 1 January 1992 (1992-01-01), pages 81-88, XP002096494 ISSN: 0003-2697 cited in the application the whole document	1-4, 13-21
X	WO 93 13216 A (HARVARD COLLEGE) 8 July 1993 (1993-07-08) the whole document	1-4, 13-21
X	H. OHMORI: "A new method for strand discrimination in sequence-directed mutagenesis" NUCLEIC ACIDS RESEARCH, vol. 22, no. 5, 1994, pages 884-885, XP002142036 IRL PRESS LIMITED,OXFORD,ENGLAND cited in the application the whole document	1-4, 13-21
A	WO 91 06643 A (CANADA MAJESTY IN RIGHT OF) 16 May 1991 (1991-05-16) the whole document	
A	WO 97 20950 A (STRATAGENE INC ;CHILDRENS MEDICAL CENTER (US)) 12 June 1997 (1997-06-12) cited in the application the whole document	

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/01075

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 319 759 A (ABBOTT LAB) 14 June 1989 (1989-06-14) the whole document	
A	THOMAS D C ET AL: "HETERODUPLEX REPAIR IN EXTRACTS OF HUMAN HELA CELLS" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 266, no. 6, 25 February 1991 (1991-02-25), pages 3744-3751, XP000652436 ISSN: 0021-9258 the whole document	
A	OWEN J L ET AL: "A HIGHLY EFFICIENT METHOD OF SITE-DIRECTED MUTAGENESIS USING PCR AND UDG CLONING" FOCUS,DE,HERRSCHING, vol. 16, no. 2, 1994, pages 39-44, XP000775814 the whole document	
P,X	US 5 955 363 A (LEWIS MARTIN K ET AL) 21 September 1999 (1999-09-21) column 9, line 4 - line 25	1-4, 6-22,24

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/GB 00/01075

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9802537 A	22-01-1998	US 5780270 A	14-07-1998
		AU 3666397 A	09-02-1998
		EP 0938552 A	01-09-1999
WO 9313216 A	08-07-1993	CA 2126438 A	08-07-1993
		EP 0620858 A	26-10-1994
		JP 8500721 T	30-01-1996
		US 5354670 A	11-10-1994
WO 9106643 A	16-05-1991	US 5071743 A	10-12-1991
		AT 125297 T	15-08-1995
		CA 2071892 A	28-04-1991
		DE 69021073 D	24-08-1995
		EP 0497798 A	12-08-1992
		JP 5502788 T	20-05-1993
WO 9720950 A	12-06-1997	US 5789166 A	04-08-1998
		CA 2239879 A	12-06-1997
		EP 0871778 A	21-10-1998
		US 5932419 A	03-08-1999
EP 0319759 A	14-06-1989	DE 3877337 A	18-02-1993
		DE 3877337 T	19-08-1993
		JP 2002348 A	08-01-1990
US 5955363 A	21-09-1999	NONE	