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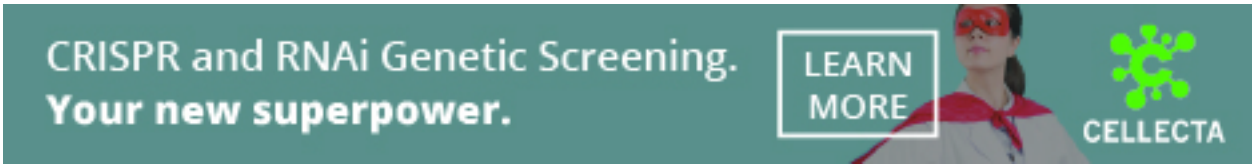

Rapid PCR Site-directed Mutagenesis

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In vitro site-directed mutagenesis is an invaluable technique for studying protein structure–function relationships, gene expression, and vector modification. Several methods for performing site-directed mutagenesis have appeared in the literature, but these methods generally require single-stranded DNA (ssDNA) as the template.[1–4] PCR-mediated methods have been developed that allow for the use of double-stranded DNA (dsDNA). PCR-mediated methods use denaturation by heat to separate complementary strands of DNA and thus allow for the use of double-stranded molecules.[5–7] Often, these procedures require the use of multiple pairs of primers to perform the method. To circumvent the disadvantages of preexisting PCR-based methods of site-directed mutagenesis (SDM), we have developed a PCR–SDM method that allows site-specific mutation in virtually any double-stranded plasmid, thus eliminating the need for subcloning into M13-based bacteriophage vectors and for ssDNA rescue.[8] The PCR–SDM system uses a single mutagenesis primer set and generates mutants with >50% efficiency in ~3 hr. The protocol is simple to perform and uses either miniprep plasmid DNA or cesium chloride-purified DNA.

The PCR–SDM system (see Fig. 1) uses increased template concentration and reduced cycling number to decrease potential second-site mutations during the PCR. The polymerase adjunct, *Taq* Extender PCR additive, is added to the PCR to increase reliability.[9] The *Dpn*I endonuclease (target sequence, 5′-G^{m6}ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest parental DNA and select for mutation-containing amplified DNA. DNA isolated from almost all *Escherichia coli* strains is Dam methylated and therefore susceptible to *Dpn*I digestion. DNA isolated from *dam*-deficient *E. coli* or other host organisms can be methylated in vitro using *dam* methylase. Cloned *Pfu* DNA polymerase is used prior to end-to-end ligation of the linear template to remove any bases extended onto the 3′ ends of the product by *Taq* DNA polymerase.[10,11] The recircularized vector DNA incorporating the desired mutations is then ligated and transformed into *E. coli.* A single buffer (SDM buffer) has been developed that can be used for all of the steps involved in the procedure.

REAGENTS REQUIRED

Taq DNA polymerase (various suppliers)
Cloned *Pfu* polymerase (Stratagene; cat. no. 600153)
Taq Extender PCR additive (Stratagene; cat. no. 600148)
*Dpn*I restriction endonuclease
T4 DNA ligase
ATP (10 mM)
dNTP mixture (25 mM total; 6.25 mM each nucleotide triphosphate)
Competent *E. coli* cells
Mutagenesis primers
Template DNA
SDM buffer [20 mM Tris HCl (pH 7.5), 8 mM $MgCl_2$, 40 μg/ml BSA]
Falcon 2059 tubes

PRIMER CONSIDERATIONS

Mutagenesis primers introduce chosen mutations, and mutagenesis primer oligonucleotides for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenesis primers:

1. Both the mutagenesis and the second primers must anneal to different strands of the plasmid.

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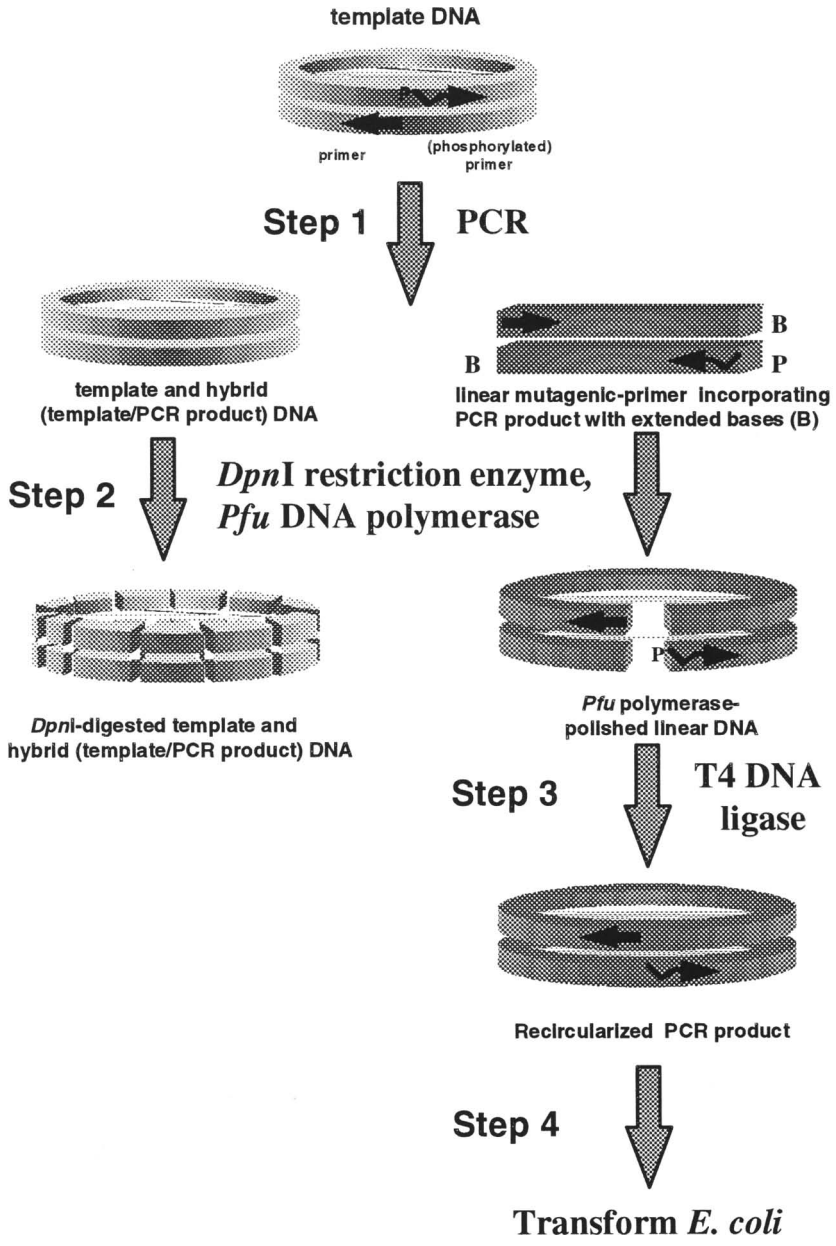

FIGURE 1 Schematic of the method for SDM using PCR. Template DNA is treated by the PCR–SDM protocol for a limited number of PCR cycles. The resulting mixture of template, newly synthesized, and hybrid parental/newly synthesized DNA is treated with *Dpn*I (target site 5′G^{m6}ATC) and *Pfu* DNA polymerase. (P) 5′ phosphate; (B) 3′-terminal extended base(s). The end-polished PCR product is then intramolecularly ligated together and transformed into *E. coli*. Specific Methods: Template DNA (~0.5 pmole) is added to a 25-μl PCR cocktail containing 1× SDM buffer, 15 pmoles of each primer, 250 μM each dNTP, 2.5 units *Taq* DNA polymerase; and 2.5 units of *Taq* Extender (Stratagene). The PCR cycling parameters were 1 cycle of 4 min at 94°C, 2 min at 50°C, and 2 min at 72°C, followed by 8 cycles of 1 min at 94°C, 2 min at 56°C, and 1 min at 72°C (*step 1*). The parental template DNA and the linear, mutagenesis primer incorporating newly synthesized DNA are treated with *Dpn*I (10 units) and *Pfu* DNA polymerase (2.5 units, Stratagene). This results in the *Dpn*I digestion of the in vivo-methylated parental template and hybrid DNA[14] and the removal, by *Pfu* DNA polymerase, of the *Taq* DNA polymerase-extended base(s) on the linear PCR product. The reaction is incubated at 37°C for 30 min and then transferred to 72°C for an additional 30 min (*step 2*). SDM buffer (115 μl, containing 0.5 mM ATP) is added to the *Dpn*I-digested, *Pfu* DNA polymerase-polished PCR products. The solution is mixed, and 10 μl is removed to a sterile microcentrifuge tube and T4 DNA ligase (4 units) is added. The ligation is incubated for >60 min at 37°C (*step 3*). The ligase-treated DNA is then transformed into competent *E. coli* (*step 4*).

2. The distance between the primers is not crucial, but the primers should not overlap.
3. Primers should be >20 nucleotides in length (shorter primers may be used; see 4, below).
4. The mismatched portions should be at the 5′ end of one or both of the primers with ⩾15 bases of correct sequence on the 3′ side.
5. The primers should be capable of synthesizing a PCR product (see PCR Considerations, below).
6. One or both of the primers must be 5′ phosphorylated.[12]

PCR CONSIDERATIONS

It may be desirable to optimize the PCR reaction before beginning the PCR–SDM procedure. Researchers should establish the PCR conditions needed to synthesize full-length products. *Taq* Extender (Stratagene) is a PCR adjunct that increases the efficiency and reliability of *Taq* DNA polymerase-generated PCR products. Pilot PCR reactions should be performed with reduced (50 ng) template concentrations and increased cycle numbers (30–40 cycles). Other reagent concentrations (including the primer set) should be kept constant as described below in the protocol section. These concentrations should be used to optimize denaturing, annealing, and extension times. The parameters that generate full-length, linear template molecules as the major amplification product should be used in the PCR–SDM method. After these conditions have been established, the template concentration should be increased, the cycle number reduced, and the PCR–SDM protocol followed.

INTRODUCTION OF TRANSLATIONALLY SILENT MUTATIONS

For easy analysis and later manipulations, it is often desirable to incorporate translationally silent restriction sites into the mutagenesis primer during site-specific mutagenesis.[13,14] Tables 1 and 2 can be used for reverse translation of protein-coding regions to determine where, in a particular sequence, a translationally silent restriction site can be inserted.

THE PCR–SDM PROTOCOL

For an overview of the PCR–SDM protocol, see Figure 1.

Prepare the PCR–SDM reaction mixture by adding the following reagents to a reaction tube:

0.5 pmole of template DNA [0.5 pmole template = 0.33 μg/kb × size of template (kb)]
2.5 μl of 10× SDM buffer
1 μl of dNTP mix
15 pmole of each primer [15 pmoles of primer = 5 (ng/base) × size of primer (base)]
ddH_2O to a final volume of 24 μl

Note: (1) It is critical that one or both primers contain a 5′ phosphate. The primer(s) can be phosphorylated either with T4 polynucleotide kinase or synthesized with a 5′-terminal phosphate. (2) Template DNA must contain methylated G^{m6}ATC sequences; if not, in vitro methylation with Dam methylase must be done prior to initiation of PCR–SDM.

Step 1: PCR

1. Add 2.5 units each of *Taq* DNA polymerase and 2.5 units of *Taq* Extender PCR additive.

TABLE 1 Introducing Restriction Endonuclease Sites by Silent Mutations

R.E.[a]	Target sequence	Reading frame 1 (amino acids) 1	2	Reading frame 2 (amino acids) 1	2	3	Reading frame 3 (amino acids) 1	2	3
*Alw*441	GTGCAC	V	H	CRSG	A	LPHQR	LSXWPQRMTKVAEG	C	T
*Apa*I	GGGCCC	G	P	WRG	A	LPHQR	LSXWPQRMTKVAEG	G	P
*Bam*HI	GGATCC	G	S	WRG	I	LPHQR	LSXWPQRMTKVAEG	D	P
*Bcl*I	TGATCA	X	S	LMV	I	IMTNKSR	FSYCLPHRITNVADG	D	HQ
*Bgl*II	AGATCT	R	S	XQKE	I	FLSYXCW	LSXPQRITKVAEG	D	L
*Bsp*MII	TCCGGA	S	G	FLIV	R	IMTNKSR	FSYCLPHRITNVADG	p	DE
*Cla*I	ATCGAT	I	D	YHND	R	FLSYXCW	LSXPQRITKVAEG	S	IM
*Eco*RI	GAATTC	E	F	XRG	I	LPHQR	LSXWPQRMTKVAEG	N	S
*Eco*RV	GATATC	D	I	XRG	Y	LPHQR	LSXWPQRMTKVAEG	I	S
*Hin*dIII	AAGCTT	K	L	XQKE	A	FLSYXCW	LSXPQRITKVAEG	S	FL
*Hpa*I	GTTAAC	V	N	CRSG	X	LPHQR	LSXWPQRMTKVAEG	L	T
*Kpn*I	GGTACC	G	T	WRG	Y	LPHQR	LSXWPQRMTKVAEG	V	p
*Mlu*I	ACGCGT	T	R	YHND	A	FLSYXCW	LSXPQRITKVAEG	R	V
*Msc*I	TGGCCA	W	P	LMV	A	IMTNKSR	FSYCLPHRITNVADG	G	HQ
*Nae*I	GCCGGC	A	G	CRSG	R	LPHQR	LSXWPQRMTKVAEG	P	A
*Nar*I	GGCGCC	G	A	WRG	R	LPHQR	LSXWPQRMTKVAEG	A	P
*Nco*I	CCATGG	P	W	SPTA	M	VADEG	FSYCLPHRITNVADG	H	G
*Nde*I	CATATG	H	M	SPTA	Y	VADEG	FSYCLPHRITNVADG	I	CXW
*Nhe*I	GCTAGC	A	S	CRSG	X	LPHQR	LSXWPQRMTKVAEG	L	A
*Nru*I	TCGCGA	S	R	FLIV	A	IMTNKSR	FSYCLPHRITNVADG	R	DE
*Pst*I	CTGCAG	L	Q	SPTA	A	VADEG	FSYCLPHRITNVADG	C	SR
*Pvu*I	CGATCG	R	S	SPTA	I	VADEG	FSYCLPHRITNVADG	D	R
*Pvu*II	CAGCTG	Q	L	SPTA	A	VADEG	FSYCLPHRITNVADG	S	CXW
*Sal*I	GTCGAC	V	D	CRSG	R	LPHQR	LSXWPQRMTKVAEG	S	T
*Sma*I	CCCGGG	P	G	SPTA	R	VADEG	FSYCLPHRITNVADG	P	G
*Spe*I	ACTAGT	T	S	YHND	X	FLSYXCW	LSXPQRITKVAEG	L	V
*Sph*I	GCATGC	A	C	CRSG	M	LPHQR	LSXWPQRMTKVAEG	H	A
*Sst*I	GAGCTC	E	L	XRG	A	LPHQR	LSXWPQRMTKVAEG	S	S
*Sst*II	CCGCGG	P	R	SPTA	A	VADEG	FSYCLPHRITNVADG	R	G
*Stu*I	AGGCCT	R	P	XQKE	A	FLSYXCW	LSXPQRITKVAEG	G	L
*Xba*I	TCTAGA	S	R	FLIV	X	IMTNKSR	FSYCLPHRITNVADG	L	DE
*Xho*I	CTCGAG	L	E	SPTA	R	VADEG	FSYCLPHRITNVADG	S	SR
*Xma*III	CGGCCG	R	P	SPTA	A	VADEG	FSYCLPHRITNVADG	G	R

Adapted from refs. 13 (for Macintosh-based computer) and 14 (for IBM PC-based computers). Supplementary material containing these computer programs written for an Apple MacIntosh computer is available from the Quantum Chemistry Program Exchange (QCPE), Department of Chemistry, Indiana University, Bloomington, Indiana 47405 (program no. QMAC006).
[a](R.E.) Restriction endonuclease.

Note: These enzymes can be mixed together and stored as a 1:1 (vol/vol) mixture at −20°C for at least 3 months.

2. Overlay with 20 μl of mineral oil, and thermal cycle the DNA using at least 7, but no more than 12 cycles. (For an initial general reaction, the parameters in Table 3 are suggested; see also PCR Considerations, above).

Step 2: Digesting and Polishing the PCR–SDM Product

1. Following PCR, place the reaction on ice for 2 min to cool the reaction to ≤37°C.

2. Add the following components directly to the 25-μl amplification reaction below the mineral oil overlay.

Note: It is important to insert the pipet tip below the mineral oil overlay when adding additional components to the reaction tube in the digestion, polishing, and ligation steps.

1 μl of the *Dpn* I restriction enzyme (10 units)
1 μl of cloned *Pfu* DNA polymerase (2.5 units)

TABLE 2 The Genetic Code

Amino acid	Abbreviation (letters) 1	3	Codon[a]
Alanine	A	Ala	(GCN)
Arginine	R	Arg	(CGN) or (AGR)
Asparagine	N	Asn	(AAY)
Aspartic acid	D	Asp	(GAY)
Cysteine	C	Cys	(TGY)
Glutamine	Q	Gln	(CAR)
Glutamic acid	E	Glu	(GAR)
Glycine	G	Gly	(GGN)
Histidine	H	His	(CAY)
Isoleucine	I	Ile	(ATH)
Leucine	L	Leu	(CTN) or (TTR)
Lysine	K	Lys	(AAR)
Methionine	M	Met	(ATG)
Phenylalanine	F	Phe	(TTY)
Proline	P	Pro	(CCN)
Serine	S	Ser	(TCN) or (AGY)
Threonine	T	Thr	(ACN)
Tryptophan	W	Trp	(TGG)
Tyrosine	Y	Tyr	(TAY)
Valine	V	Val	(GTN)

[a](N) Any base; (R) purine; (Y) pyrimidine; (H) A, C, or T.

3. Gently mix and spin the reaction in a microcentrifuge tube for 1 min. Immediately incubate the reaction at 37°C for 30 min.
4. Incubate the reaction at 72°C for an additional 30 min.

Step 3: Ligating the PCR–SDM Product

1. Add the following components to the *Dpn*I, cloned *Pfu* DNA polymerase-treated product:

100 µl of ddH_2O
10 µl of 10× SDM buffer
5 µl of 10 mM ATP

2. Gently mix and spin the reaction in a microcentrifuge tube for 1 min. **Optional:** An 8-µl aliquot may be stored and analyzed by standard agarose gel electrophoresis. To verify the integrity of the PCR–SDM product, a single band should be apparent.
3. Remove 10 µl of the above reaction to a sterile microcentrifuge tube and add 1 µl of T4 DNA ligase (4 units). **Note:** There seem to be considerable differences in efficiency of various lots of T4 DNA ligase to ligate blunt-ended DNA molecules. Different lots from the same manufacturer may yield anywhere from 30% to 70% mutagenesis efficiency in this assay. Once a useful lot

TABLE 3 PCR–SDM Cycling Parameters

Segment	Cycles	Temperature (°C)	Time (min)
1	1	94	4
		50	2
		72	2
2	8	94	1
		56	2
		72	1
3	1	72	5

has been identified, it is recommended it be held in reserve for use in PCR–SDM.

4. Incubate the reaction for 1 hr at 37°C.

Step 4: Transforming into Competent Cells

Rapid Transformation Protocol (used for XL1-Blue-competent cells from Stratagene)

1. Gently thaw the competent cells on ice, and aliquot 80 μl of the cells to a prechilled Falcon 2059 polypropylene tube.
2. Add 2 μl of the ligase-treated DNA to the cells, swirl gently, and incubate for 30 min on ice.
3. Heat-pulse for 45 sec at 42°C and place on ice for 2 min.

Note: This heat pulse has been optimized for the Falcon 2059 tubes.

4. Immediately plate the entire volume of transformed cells.
5. Incubate the plates overnight.

CONCLUSIONS

The advantages to the PCR–SDM method include increased template concentration to allow reduced cycling, the use of *Taq* Extender, which provides increased reliability in generating longer PCR products, the use of *DpnI* restriction endonuclease to reduce parental molecules, the use of *Pfu* DNA polymerase to remove undesired base extensions, and the efficient method for blunt-ended ligation. The complete protocol is extremely fast and does not require cleanup or precipitation procedures between steps. PCR–SDM can be used for both large deletions and insertions.

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