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Multichange Isothermal Mutagenesis: a new strategy for multiple site-directed mutations in plasmid DNA

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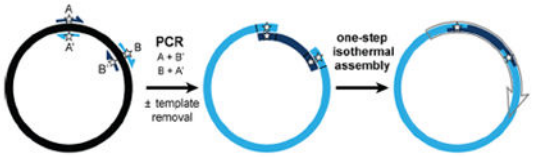
Abstract



Multichange ISOthermal (MISO) mutagenesis is a new technique allowing simultaneous introduction of multiple site-directed mutations into plasmid DNA by leveraging two existing ideas: QuikChange-style primers and one-step isothermal (ISO) assembly. Inversely partnering pairs of QuikChange primers results in robust, exponential amplification of linear fragments of DNA encoding mutagenic yet homologous ends. These products are amenable to ISO assembly, which efficiently assembles them into a circular, mutagenized plasmid. Because the technique relies on ISO assembly, MISO mutagenesis is additionally amenable to other relevant DNA modifications such as insertions and deletions. Here we provide a detailed description of the MISO mutagenesis concept and highlight its versatility by applying it to three experiments currently intractable with standard site-directed mutagenesis approaches. MISO mutagenesis has the potential to become widely used for site-directed mutagenesis.

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Author Contributions

L.A.M., Y.C., and M.T. are co-first authors of this work and wrote the manuscript. J.C., L.A.M., and M.T. created the figures. L.A.M., J.C., A.M.N., Y.C., L.D., and M.T. contributed experimental data. The work was performed in the lab of J.D.B.

The authors declare no competing financial interest.

Supporting Information

Supplementary figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

Keywords

site-directed mutagenesis; one-step isothermal assembly; QuikChange; point mutation; insertion; deletion

Site-directed mutagenesis (SDM) is one of the most frequently used techniques in molecular biology. The QuikChange reaction, developed by Stratagene (La Jolla, CA), is the standard approach for introducing point mutations into plasmids. Its widespread use to make specific coding changes in proteins has driven fundamental discoveries in the fields of genetics, biology, and biochemistry. QuikChange uses reverse complementary mutational primers to replicate a parent plasmid, introducing mutation(s) at the site of primer binding. The DNA replication step is a linear cyclic amplification reaction in which a *pfu* polymerase copies the entire plasmid, stopping upon reaching the primer's 5′ end; the newly synthesized DNA is nicked and cannot serve as a template in later cycles but rather anneals to form nicked double-stranded, mutagenized molecules. Enzymatic digestion of methylated template DNA produced in *E. coli* using DpnI reduces the background of wild type parental molecules in the reaction.

Since its invention, only modest improvements to the QuikChange reaction have been proposed,[1-3] and much inefficiency still exists in this system. First, while performed on a thermal cycler, DNA replication is linear not exponential. Second, strand displacement by the polymerase results in exponential amplification of the plasmid and encodes a competing byproduct that cannot transform *E. coli.* Third, it is difficult to introduce mutations at more than one location in any single reaction. Fourth, the size of the template is limiting because the method relies on replication of the entire plasmid. Fifth, because the entire plasmid must be copied, resequencing all nonselectable coding regions is obligatory. Sixth, background depletion by DpnI can be difficult because a large amount of DNA (50 ng) is recommended for the QuikChange reaction and hemimethylated heteroduplex DNA is resistant to DpnI digestion.[1,4] Seventh, because QuikChange primers are perfectly complementary, primer dimerization is highly favorable; long nick-bridging primers may minimize this and improve amplification.[1] Finally, deletions and insertions larger than a single codon are generally beyond the scope of the classic QuikChange reaction; however, modified QuikChange protocols attempt to address this shortcoming.

Herein we describe a simple and robust protocol for site directed mutagenesis that overcomes all of the above challenges of the standard QuikChange reaction. We call our approach Multichange ISOthermal (MISO) mutagenesis since it is capable of introducing multiple DNA modifications in a single reaction and incorporates a DNA assembly strategy named onestep isothermal (ISO) assembly.[5] MISO mutagenesis is a completely different strategy from QuikChange; however, it still leverages the elegant design of QuikChange primers, which are reverse complementary sequences, usually 40 bp in length, that encode desired base changes centrally. In the simplest application of MISO mutagenesis, two pairs of QuikChange primers are inversely partnered to exponentially amplify two linear, double-stranded PCR products (Figure 1a). The resulting PCR products encode the desired mutations at each end and moreover share ~40 bp of terminal homology. The one-step

isothermal (ISO) reaction works by using a master mix of three enzymes to seamlessly assemble DNA pieces whose ends contain 30–40 base pairs of overlapping sequence (Figure 1b). Briefly, a 5′ exonuclease chews back double-stranded DNA molecules to expose complementary single-stranded DNA overhangs. Homologous segments then specifically anneal. Next, a polymerase fills in the gapped molecules, and a ligase covalently seals nicks. Thus, PCR products with homologous ends, such as those generated through inverse partnering of QuikChange primers, may be enzymatically joined *in vitro* using the one-step ISO assembly protocol to generate the desired mutagenized plasmid.

To demonstrate a robust capability for multi-site directed mutagenesis, we tested MISO mutagenesis with a set of 6 QuikChange primers encoding 8 base changes. These primers were originally designed to incorporate eight lysine-to-arginine point mutations into a 6.5-kb plasmid using a combination of iterative QuikChange reactions and overlap extension PCR.[6,7] We inversely partnered the six primer pairs (Figure 2a) to exponentially amplify six double-stranded DNA fragments, ranging in size from 140 bp to 5.3 kb. The fragments were gel purified, subjected to one-step ISO assembly, and transformed into competent *E. coli* cells. As a control, one 140-bp fragment was omitted from the reaction, and the resulting assembly produced no colonies. Colony PCR reactions on 96 individual transformants using two diagnostic primer pairs (Supplementary Figure 1a and b) revealed that 92/96 clones had assembled correctly (Supplementary Figure 1c). Sequencing of 24 correctly assembled constructs revealed that 100% contained all 8 desired mutations. Thus, in a single round of experimentation we successfully generated a lysine-free version of a protein of interest for assessment of post-translational modification status.[8] These data support MISO mutagenesis as a tremendously improved strategy for multi-site directed mutagenesis.

Another limitation of QuikChange is the size of the template plasmid. As the reaction mandates replication of the entire construct, the upper limit for QuikChange is ~7–10 kb. Further, many large plasmids carry a significant fraction of coding region, and any that cannot be functionally validated following QuikChange must be entirely resequenced to verify accuracy. Here we demonstrate additional versatility of MISO mutagenesis to overcome these two issues by coupling MISO mutagenesis with a traditional restriction digestion. To introduce a single point mutation into a 15.3-kb plasmid of which ~9 kb encodes protein sequence (Figure 2b),[9] we identified unique restriction enzymes sites flanking the desired base substitution by 200 bp and 800 bp and designed primers to anneal beyond these boundaries. In individual PCR reactions, we inversely partnered these primers with two QuikChange-style primers encoding the mutation. Separately, the backbone was digested with the appropriate restriction enzymes, and the three fragments were gel purified, thereby quickly generating three overlapping pieces of DNA amenable to one-step ISO assembly. We confirmed introduction of the mutation in five out of five unique transformants by sequencing. Further, one of these constructs was sequence verified at both overlapping junctions. Here, not only did MISO mutagenesis allow efficient installation of the mutation of interest, it also greatly reduced the amount of sequence validation required.

DNA modifications of biological relevance are not limited to base substitutions. Rather, the introduction of insertions and deletions into plasmids is often desirable, for instance, to generate fusion proteins, co-expression systems, or to delete protein domains. To this end,

we next used MISO mutagenesis to couple the introduction of a point mutation with simultaneous deletion of a DNA segment and insertion of a 1-kb sequence into a series of yeast shuttle vectors (Figure 2c).[10] Briefly, we needed to recode a single BsaI site within the *bla* gene, remove two BsmBI sites from a noncoding region of the vector backbone, and construct a new cloning site with BsaI sites flanking a red fluorescent protein (RFP) to generate new host plasmids amenable to Golden Gate assembly.[11] We designed QuikChange primers for BsaI recoding plus three additional pairs of primers with overlapping overhangs (Figure 2c), which were used to exponentially amplify four fragments with homologous ends. Gel purification of all amplification fragments prior to one-step ISO assembly yielded ~99% red colonies, and 20 were miniprepped and the assembly tested by restriction digests with BsaI and BsmBI (Supplementary Figure 2). We discovered that 19/20 clones yielded the expected digestion pattern; the single incorrect clone derived from an assembly error in which one RFP-flanking BsaI site was not intact (Supplementary Figure 2). This highlights that one-step assembly reactions are prone to mis-assemblies and junctions must be sequence verified. Thus, after functional verification by restriction digestion, we further sequenced the BsaI-RFP junctions of 8 correctly assembled clones to verify elements that could not be interrogated by digest; no undesired mutations were found. Taken together, the application of MISO mutagenesis presents a simple strategy for making many types of biologically relevant DNA modifications in a single round of experimentation.

MISO mutagenesis overcomes many specific technical problems associated with traditional QuikChange mutagenesis. Since primers are inversely partnered in separate reactions, the problem of primer dimerization is circumvented. Further, MISO mutagenesis affords exponential rather than linear amplification, thus allowing easy verification of product generation by gel electrophoresis. Background is reduced almost completely through template removal by gel purification and/or DpnI digestion. Like QuikChange, however, MISO mutagenesis is limited by DNA sequences that are challenging to amplify by PCR, or may be toxic, unstable, or otherwise not tolerated in bacteria. The error rate associated with oligonucleotide synthesis is another problem common to both approaches. Limitations specific to MISO mutagenesis derive largely from the ISO-assembly step. First, as demonstrated here, mis-assembly errors can occur during one-step ISO assembly, and it is likely that mis-assemblies will be exacerbated by repetitive or GC-rich sequences in homologous regions. It is possible that implementation of alternative enzymatic assembly strategies may overcome some types of assembly errors.[12,13] Second, the introduction of mutations that are relatively close together (e.g., 50–80 bp) may be difficult to achieve using MISO mutagenesis, as this would require exceedingly long complementary mutagenic primers to encode both mutations or alternatively generating a very short PCR product. Thus, MISO mutagenesis is likely best applied when desired mutations are close enough to be encoded together on one primer or distant enough to generate a reasonably sized PCR product using two mutagenic primers. Finally, the number of pieces that can be put together by one-step ISO assembly defines the upper limit of DNA modifications introduced during a single round of MISO mutagenesis. Of course, most of these limitations can be overcome by performing MISO twice.

Overall, we believe that MISO mutagenesis is a versatile and efficient strategy for making the most common types of DNA sequence modifications in plasmids. This approach is accessible and cost-effective for all laboratories as it seamlessly incorporates QuikChange-style primers, which are both familiar and ubiquitous in molecular biology, and requires no expensive primer purification. Implementation of the one-step ISO assembly protocol is also straightforward as step-by-step instructions to prepare the three enzyme reaction mixture have been comprehensively detailed.[12] Alternatively, kits with all the required reagents preassembled are available from NEB. Thus, MISO mutagenesis represents an excellent solution for laboratories that infrequently perform SDM in plasmid DNA to those where it is routine. Notably, single mutations can also be installed using MISO mutagenesis by inversely partnering QuikChange-style primers with a second pair of non-mutagenic primers or by employing the approach outlined in (Figure 2b). Indeed, we have used this approach to successfully salvage failed QuikChange reactions (data not shown).

METHODS

Primer Design

Primers used in this study are listed in Supplementary Table 1. For mutagenic primers, 40 nucleotide exact reverse complementary sequences were designed with the base substitution placed centrally, as per the QuikChange manual (Stratagene). In the case of insertions/deletions, the desired construct was assembled *in silico* using the free plasmid editor ApE (http://biologylabs.utah.edu/jorgensen/wayned/ape/), keeping track of the junction locations. Primers were then designed to consist of two parts: an annealing sequence (20–30 nt, $T_m \approx$ 55 °C) and an 'overhanging' sequence to generate the homologous region. In our hands, the minimal homologous region for ISO assembly is ~30 bp, although 40 bp is recommended,[5,12] and we have succeeded in assembling fragments that overlap by as much as 200 bp. As an example, the primers used to generate Figure 2c are diagramed in Supplementary Figure 3. In all cases, overlapping regions were confirmed to be unique to each assembly reaction.

PCR Amplification of DNA Fragments

Phusion Polymerase (NEB, F530L) was used to generate all PCR products described here, although any high fidelity polymerase is appropriate for use. PCR reactions were prepared as follows: 5–10 ng template DNA, 200 μM concentration of each dNTP (Takara, 4030), 0.2 μM concentration of each primer (Supp. Table 1), 1x Phusion HF buffer, 0.02 U/μL Phusion DNA polymerase in a final volume of 50 μL. Applied Biosystem Veriti 96-Well Thermal Cyclers were used for amplifications with an extension time of 30 s/kb. PCR products were either gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, D4002) as per the manufacturer's instructions or purified using the DNA Clean & Concentrator kit (Zymo Research, D4003) with or without DpnI treatment (NEB, R0176) for 1 h at 37 °C.

One-Step ISO Assembly

One-step ISO assembly reagents (5X ISO Buffer and Reaction Master Mix) are described in detail elsewhere.[12] PCR products were combined in equimolar amounts in 5 μL and mixed with 15 μL of Reaction Master Mix by gentle tapping. One-step isothermal assembly was

performed at 50 °C in a preheated PCR block for 30 min, and 2 μL of each assembly reaction was transformed into 50 μL of competent DH5α *E. coli* cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

MISO	multichange isothermal
ISO assembly	one-step isothermal assembly
PCR	polymerase chain reaction

DNA	DNA
nt	nucleotide
bp	base pair

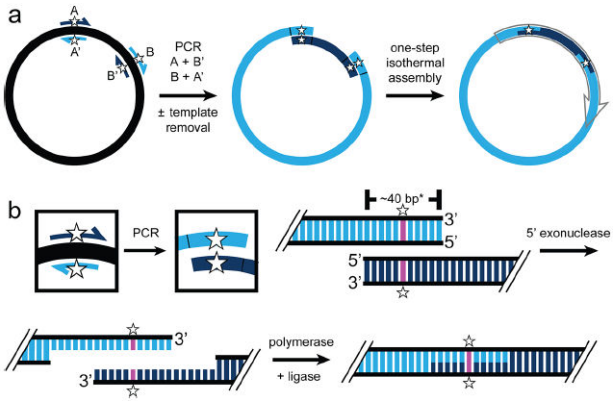

Figure 1.

Overview of Multichange ISOthermal (MISO) mutagenesis. (a) QuikChange-style primer pairs (A, A′; B, B′) encode reverse complementary 40-nucleotide primers with a base substitution (star). Inverse partnering of primer pairs [A+B′] and [B+A′] in separate PCR reactions yields exponential amplification of two linear pieces of DNA with homologous ends. After template removal (DpnI digestion or gel purification), the mutagenized plasmid is assembled using one-step isothermal assembly.[5] (b) One-step isothermal assembly relies on the concerted action of three enzymes. A 5′ exonuclease chews back double-stranded DNA, exposing complementary single strands that anneal. Then a polymerase fills in the gaps, and a ligase seals the nick.

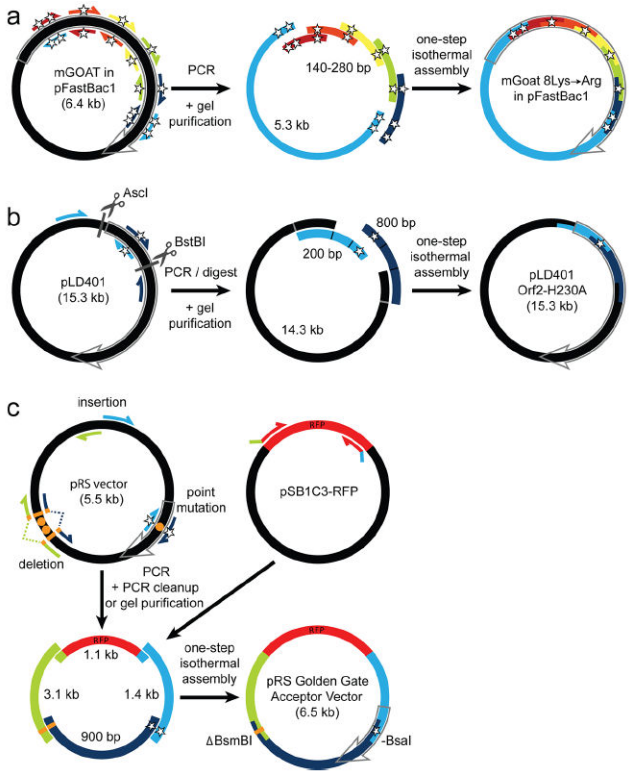

Figure 2.

Three applications of MISO mutagenesis. (a) Simultaneous introduction of eight point mutations into the mGOAT coding sequence. Six different pairs of QuikChange-style primers were partnered (shown by colors) to generate six PCR products ranging in size from 140 bp to 5.3 kb and encoding eight lysine-to-arginine substitutions. One-step isothermal assembly reaction efficiently generated the desired lysine-free construct (see Supplementary Figure 1). (b) Introduction of a single point mutation into an existing 15.3-kb construct without vector segment amplification. QuikChange-style primers were partnered with non-mutagenic primers complementary to plasmid ends to generate two PCR products (200 bp, 800 bp). Separately, pLD401 was digested with AscI and BstBI, and the large vector fragment was gel purified. The three DNA fragments, with homologous ends, were subjected to one-step isothermal assembly to construct the mutagenized plasmid. Only the region of the plasmid produced by PCR required confirmatory resequencing. (c) Simultaneous introduction of a base substitution, deletion, and insertion into yeast shuttle vectors. One standard set of QuikChange primers (starred) plus three other primer pairs were partnered (shown by color) to generate four overlapping PCR products. One-step ISO assembly allowed deletion of two BsmBI sites from a noncoding region, recoding of one BsaI site in the *bla* gene, and insertion of a BsaI-flanked RFP gene to generate a new cloning site. [Open gray arrows = coding sequence; stars = point mutations; scissors = unique restriction enzyme sites; orange circles = undesirable restriction enzyme recognition sites; dashed lines = deleted region.]