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Tutorial

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Software-Supported USER Cloning Strategies for Site-Directed Mutagenesis and DNA Assembly

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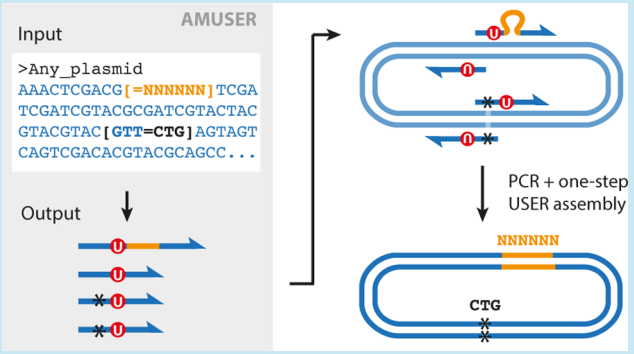
Supporting Information

ABSTRACT: USER cloning is a fast and versatile method for engineering of plasmid DNA. We have developed a user friendly Web server tool that automates the design of optimal PCR primers for several distinct USER cloning-based applications. Our Web server, named AMUSER (Automated DNA Modifications with USER cloning), facilitates DNA assembly and introduction of virtually any type of site-directed mutagenesis by designing optimal PCR primers for the desired genetic changes. To demonstrate the utility, we designed primers for a simultaneous two-position site-directed mutagenesis of green fluorescent protein (GFP) to yellow fluorescent protein (YFP), which in a single step reaction resulted in a 94% cloning efficiency. AMUSER also supports degenerate nucleotide primers, single insert combinatorial assembly, and flexible parameters for PCR amplification. AMUSER is freely available online at http://www.cbs.dtu.dk/services/AMUSER/.



KEYWORDS: *DNA assembly, USER cloning, primer design, site-directed mutagenesis, point mutation, Web server*

A fundamental practice of synthetic and molecular biology is the construction and tuning of genetic systems. Although *de novo* synthesis of genes and genetic constructs is becoming an increasingly feasible option, it is often not the preferred path when it comes to manipulating and combining DNA parts directly available at hand in the lab. The USER (or "uracil excision") cloning method[1] is a powerful method for nucleic acid engineering with several applications in plasmid and protein engineering. Using a fast and simple protocol, USER cloning has proved successful for directional scar-less assembly of multiple DNA fragments[2] as well as for site-directed mutagenesis,[3] including mismatch mutations, insertions, and deletions.[4]

The principle of USER cloning is based on PCR-amplification with primers containing a deoxyuracil (hereafter referred to as "uracil") residue in the place of a thymine residue. During PCR, the uracil residue is incorporated at each of the termini of the amplified PCR products. Next, by an enzymatic uracil excision reaction, unique 3′ overhangs of ∼8 nucleotide are generated at each PCR product allowing seamless and directional assembly of multiple parts. Following PCR, this single step uracil excision reaction and assembly can be completed in less than an hour. The entire reaction mixture containing the assembled and circularized construct(s) can be directly transformed hereafter without the need of ligation (see Supporting Information for a USER cloning protocol). The independence of restriction enzymes, ligase, or specialized vectors makes the USER method both highly flexible, fast, and inexpensive.

In addition to being a pronounced method for DNA assembly, another advantage of the USER cloning method is its applicability for site-directed mutagenesis (Figure 1 b–f).[4] Nucleotide manipulations are introduced during PCR, and hence, all sequence modifications will be incorporated in the PCR fragments. This means that after the mutated fragments

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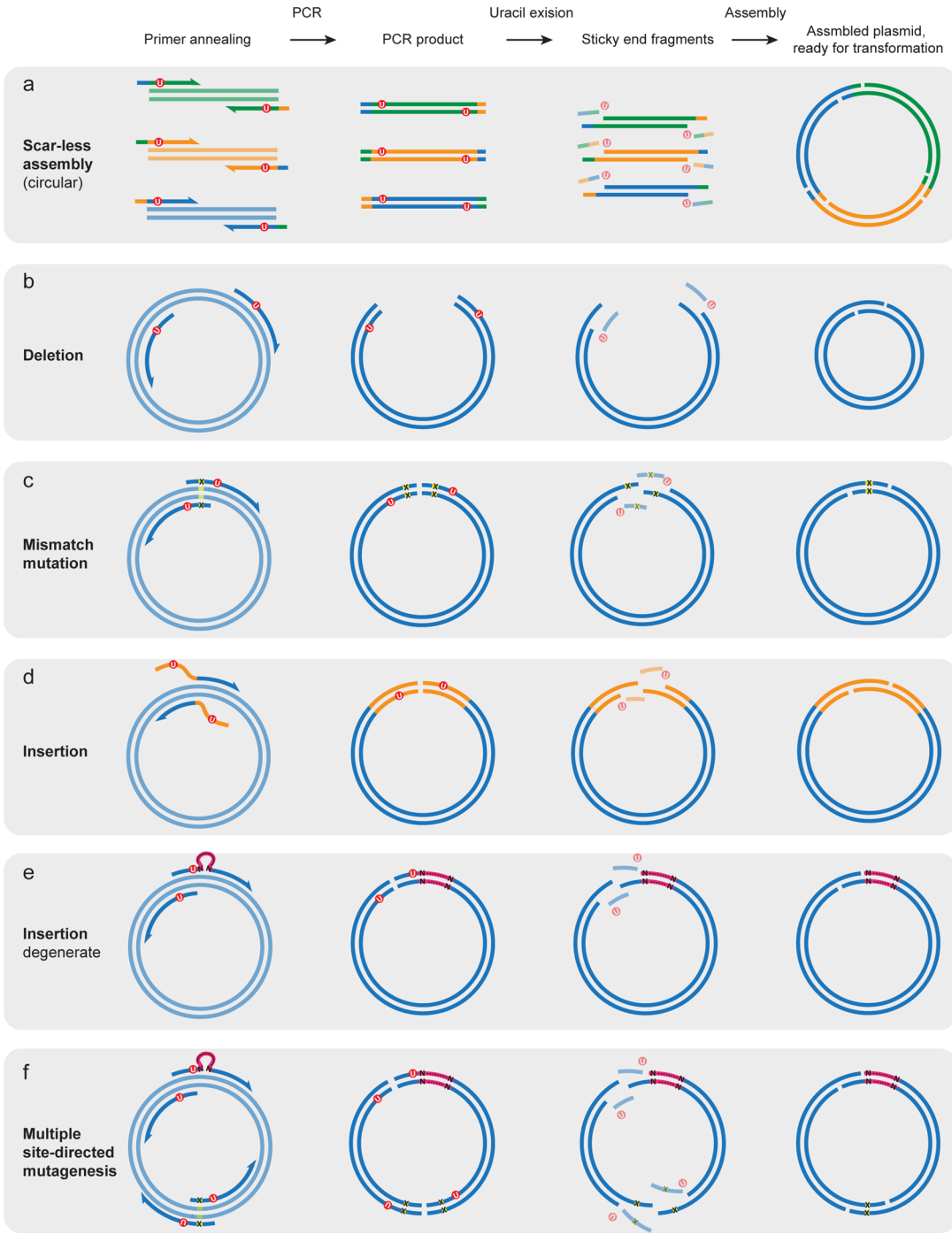

Figure 1. Plasmid engineering possibilities supported by the AMUSER Web server. With USER cloning, the same experimental protocol (outlined at the top) can be used for a number of DNA engineering purposes solely by altering the design of the PCR primers. Following PCR amplification with primers containing a uracil residue (U), fragments are treated with USER enzyme generating 7−13 nucleotide sticky-end overhangs that allows for seamless and directional assembly. Following PCR and DNA fragment purification, the single-tube uracil excision and assembly reaction can be accomplished in 30 min. The mix can be transformed directly into chemically competent cell without the need for ligation. (a) Directional and scar-less assembly of multiple fragments. (b) Sequence deletions are achieved by omitting the region to be deleted during PCR. (c) Introduction of mismatch mutations is accomplished by adding sequence modifications in the primer tails. (d) Sequence insertions of up to 100 bp can be achieved with long primer tails. (e) Insertions and mismatch mutations with random sequences are accomplished by adding degenerate nucleotides in the primer-tails and ensuring that the degenerate nucleotides are positioned outside the assembly overhang regions. (f) Multiple modifications can be performed in a single reaction.

have been generated by PCR, the remaining step is DNA assembly, which follows the same experimental protocol as any other USER cloning assemblies. Following this principle, we here demonstrate how site-directed mutagenesis of a plasmid can be performed at multiple sites simultaneously in a single experimental setup. The number of sites in a plasmid that can be altered in a single step equals the number of fragments that must be assembled following PCR (Figure 1 f). Hence, the maximum sites that can be mutated in a single step is determined by the success of assembly, and USER cloning has

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previously proved successful for assembly of up to 7 parts.[5] Modifications can be performed with degenerate nucleotides, too (Figure 1 e), and in contrast to the QuickChange protocol (Stratagene), introduction of degenerate nucleotides does not complicate the protocol nor increase the risk of failure. By virtue of this flexibility simply facilitated by primer-design, USER cloning is a highly versatile method for performing virtually any type of site-directed mutagenesis.

Whereas the possibilities of DNA engineering are many, assessing the optimal cloning strategy and designing primers remains a complex and highly critical step of the cloning process. Manual primer design is tedious and prone to errors due to the numerous factors that must be taken into account, such as defining the overhang regions, making compatible ends that can be joined in an orderly fashion, ensuring uniform melting temperature (Tm), and avoiding hairpins and primer dimer formation. This is a significant limitation that can lead researchers to stick to well-known, but inferior and time-consuming procedures. Manually designed primers for complex cloning tasks might contain primer design errors that discourage further use of the technique. Furthermore, high-throughput experiments are extremely time-consuming to plan when all molecular details of the primers must be designed manually. To address these challenges, design tools have been developed for a number of cloning techniques that assists the experimental protocol generation, for example GeneDesign,[6] Gibthon (http://gibthon.org), and J5.[7] However, none of these tools provide support for the USER cloning method. We have previously reported PHUSER, a Web server tool to support primer-design for simple DNA assembly by USER cloning (USER fusion).[8]

Here, we report the AMUSER Web server, a user friendly software tool aimed at molecular biologists for automated design of primers for advanced USER cloning based DNA engineering. In addition to directional scar-less multipart assembly as we previously addressed, AMUSER enables advanced options for site-directed mutagenesis at multiple sites, introduction of degenerate nucleotides and construction of single insert combinatorial libraries by strategic design of specific USER cloning primers. In-depth knowledge of the USER cloning methods' possibilities and limitations is not required: starting with nothing but the sequence(s) of the parts to be engineered the user simply provides the order of assembly, whether any nucleotide modifications must be performed, and whether the desired output construct should be circular, linear, or compatible with a USER cassette. The service can be accessed through our Web based user interface at http://www.cbs.dtu.dk/services/AMUSER/.

AMUSER WEB SERVER

Input Format. As input, the Web server accepts one or multiple sequences in FASTA format. Sequences can be added either by copying/pasting into the input window or by uploading a text file. The input sequence must match the DNA sequence that will serve as template for PCR. To facilitate site-directed mutagenesis, a number of special input characters are supported as described in the sections below. Before the sequences are processed, a basic check of the input integrity (FASTA headers, IUPAC compliant nucleotide characters, and valid AMUSER operators, e.g. the "+" sign) is performed.

Output Construct Options. When two or more sequences are submitted as input, PCR primers are designed to result in a scar-less assembly of the sequences in the order they are listed in the input window. As it is essential that all overhangs in the sequences intended for the same PCR are unique, an AMUSER algorithm exhaustively tests all possible primer combinations and selects only primer pairs with unique overhangs. The decision for removal of all but one of the identical primer overhangs is based on how well each possible pair comply with the thermodynamic features of good PCR primers (length, Tm, GC content, presence of GC clamp at 3′-end, the risk of primer dimer formation, the risk of intraprimer complementarity, and the presence of polyN stretches).[9] The Web server facilitates primer design for seamless fusion of the sequence(s) into a standard or custom USER cassette, assembly into a linear fragment, or assembly of the input sequences to a circular construct. Circular assembly (or cassette-free cloning[10]) has become the standard practice for many since all parts including the backbone can now be generated with high fidelity PCR.

Site-Directed Mutagenesis. As a powerful and useful feature in DNA engineering, the Web server offers the possibility of designing primers for site-directed mutagenesis. Any desired manipulation of a sequence (i.e., deletion, mismatch mutation, or insertion (Figure 1 b–f)) can be introduced in the input query by typing the desired change in squared brackets:

[template sequence = desired sequence]

For example, if the investigator wishes to replace an ATT with GGG this can submitted by typing [ATT=GGG] at the specific position in the sequence. If the ATT sequence is to be deleted, the "desired sequence" part is left empty: [ATT=]. Finally, if the GGG is to be inserted only, the "template sequence" part is left empty: [=GGG] (more input query examples are given in Chart 1). Computationally, this is performed by removing the targeted region, virtually breaking up the affected sequence(s) at the mutation site(s), and designing primers with the desired mutation in the primer tails. Following PCR, the resulting fragments will include the specified changes introduced into the primers. Several sites can be targeted in one or multiple sequences by the introduction of more squared brackets in a single input. If two or more mutations are introduced within 40 bp of each other, all mutations are included in a single primer set to avoid the need for amplification of very short DNA sequences. To minimize the length of primers when performing insertions, the Web server distributes the insertion sequence to the tails of both the forward and reverse primer (Figure 1 d).

Degenerate Nucleotides. For insertions and mismatch mutations, the Web server supports the introduction of degenerate nucleotides as defined by the IUPAC nomenclature, thus allowing the user to perform site-directed mutagenesis with random or semi random nucleotides. In order to avoid degenerate nucleotide in the assembly overhang, AMUSER disregards all A–T segments that include degenerate nucleotides (Figure 1 e,f). Degenerate nucleotide modifications at multiple sites in a single submission is supported.

Single Insert Combinatorial Assembly. For the planning of multiple experiments, the Web server enables primer design for single-insert combinatorial assembly. This feature is useful for, for example, a situation where a researcher needs to seamlessly subclone multiple genes in the same vector backbone. Figure 2 illustrates such an example where three parts are to be cloned individually into the same site in a vector. In order to reduce cost and time of the experiment, the Web server strategically designs primers such that a minimum set of

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Chart 1. AMUSER Web Server Input Examples

Scar-less assembly of 3 parts[a]

```
>part1
ACTGCAGCGACGATATATATGGCATCAGCTGCAA
>part2
ACTACGCATCGAGGATAGGCTAGAGGGATTTAAA
>part3
CCGATGGGCATGGTTAATTTAATGCGTACGGGAT
```

Deletion of "ATATATAT" in part1

```
>part1
ACTGCAGCGACG[ATATATAT=]GGCATCAGCTGCAA
```

Insertion of "GGG" in part1

```
>part1
ACTGCAGCGACGATATATAT[=GGG]GGCATCAGCTGCAA
```

Replacement of "ATATATAT" with "GGG"

```
>part1
ACTGCAGCGACG[ATATATAT=GGG]GGCATCAGCTGCAA
```

Combinatorial insertion of part1, part2 and part3 into a vector

```
>vector
TTTGACGGATGGCTAAGCTGGTTTCAGCATAATAT
+
>part1
ACTGCAGCGACGATATATATGGCATCAGCTGCAA
>part2
ACTACGCATCGAGGATAGGCTAGAGGGATTTAAA
>part3
CCGATGGGCATGGTTAATTTAATGCGTACGGGAT
+
```

[a]Note, for representation reasons, the shown example sequences are significantly shorter than the sequences typically used for cloning experiments (e.g., a vector is typically 2−4 kb).

primer pairs and a minimum number of PCR reactions are needed. To accomplish this, the Web server designs the primers such that the 3′ overhangs are A−T regions within the vector sequence (Figure 2 b). This ensures that the overhangs of the vector backbone (the fixed sequence) are always complementary with the 3′ overhangs of any of the insert sequences (varying sequences) without leaving any scars at the junction sites. The combinatorial assembly option is activated by entering the '+' symbol before and after the set of FASTA sequences that are to be cloned in combination (see example in Chart 1). Currently, the Web server allows only a single + list entry.

PCR Settings. The primer pair for each fragment to be amplified is designed based on a number of quality scoring parameters, one of which is a resulting primer Tm between 55 and 72 °C. By default, the Web server designs primer pairs with Tm values that are optimized individually for each primer pair. Alternative options are also available and can be specified by choosing "advanced PCR settings". Salt and primer concentration are factors influencing Tm and may vary among individual experiments. The Web server allows the user to adjust these parameters to obtain the most realistic Tm calculation (calculated using the nearest neighbor method).[11] Furthermore, for each fragment to be amplified, the user can specify a desired Tm and AMUSER will aim at designing primers as close as possible to this temperature. This allows for multiple PCR amplifications to be performed at the same Tm and hence in a single PCR program, thus reducing the time of the amplification procedure.

Web Interface. The Web server query page is simple and intuitive and guides the user through the necessary steps: sequence input, assembly information, and PCR settings. Detailed options for USER cassette assembly and PCR settings are hidden and will appear as a drop down menu only when relevant. Following submission of the input, the user will be presented with the results page displaying a list of designed primers, a graphical overview of the design process, and primer-evaluation parameters. The primer list can be directly copied into an oligonucleotide ordering form at the user's preferred vendor. The primer description contains fragment name and Tm so the following PCR reactions are easily set up.

Experimental Demonstration. In order to demonstrate both the software as well as the USER cloning method for multiple site-directed mutagenesis and DNA assembly, we performed site-directed mutagenesis of GFP to YFP and swapped the vector backbone—all in a single step reaction. Here, we show how this can be achieved in less than a day and at the cost of only four primer pairs (insofar as the DNA fragments and vectors are at hand)—significantly faster and cheaper than synthesis of the entire construct.

Three amino acid substitutions (V68L, Q69K, and T203Y) are required in the mut3 variant of GFP[12] to produce a change in fluorescence emission from green (510 nm) to yellow (527 nm).[13] We aimed to express the YFP from the pZE21[14] backbone containing a number of vector elements different from the original backbone holding the GFP (pJBA28[15]). With the parts available to us, three amino acid substitutions and a backbone transfer were required to build the construct.

We used AMUSER to design primers for the construction. First, the FASTA files containing the sequences that would serve as template for PCR, namely *gfpmut3* and the pZE21 backbone, were pasted into the Web server input window. V68 and Q69 are adjacent amino acids encoded by GTTCAA, and hence, the V68L and Q69K mutation can be achieved by changing GTTCAA to CTGAAG. The T203Y can be accomplished by changing CTA to TAC at the corresponding position. These nucleotide mismatch mutations were typed directly in the FASTA sequence in squared brackets as shown in Figure 3b (see Supporting Information for full input query). Circular assembly was selected under the "Output construct" menu.

Using AMUSER, we generated four pairs of PCR optimized uracil containing primers for amplification of the *gfpmut3* gene as three fragments and the pZE21 backbone in one fragment and with the primer-tails containing the sequence modifications (see Supporting Information for primer sequences). In the graphical output, primers are mapped to their complementary regions of their template sequence making it easy to visually inspect the junctions at molecular detail and validate the design (Figure 4).

Following synthesis and delivery of the uracil primers, the pZE21 vector backbone and the three *gfpmut3* parts were successfully amplified by PCR (Figure 3d). Since no unspecific bands were visible when evaluating the PCR by gel electrophoresis, we directly purified and mixed the DNA, removed template DNA by a DpnI reaction (digests methylated DNA only) and created 3′ assembly overhangs by uracil excision using the USER enzyme mixture. Next we allowed the four-fragment mixture to assemble for 15 min at room temperature and used a small volume to directly transform chemically

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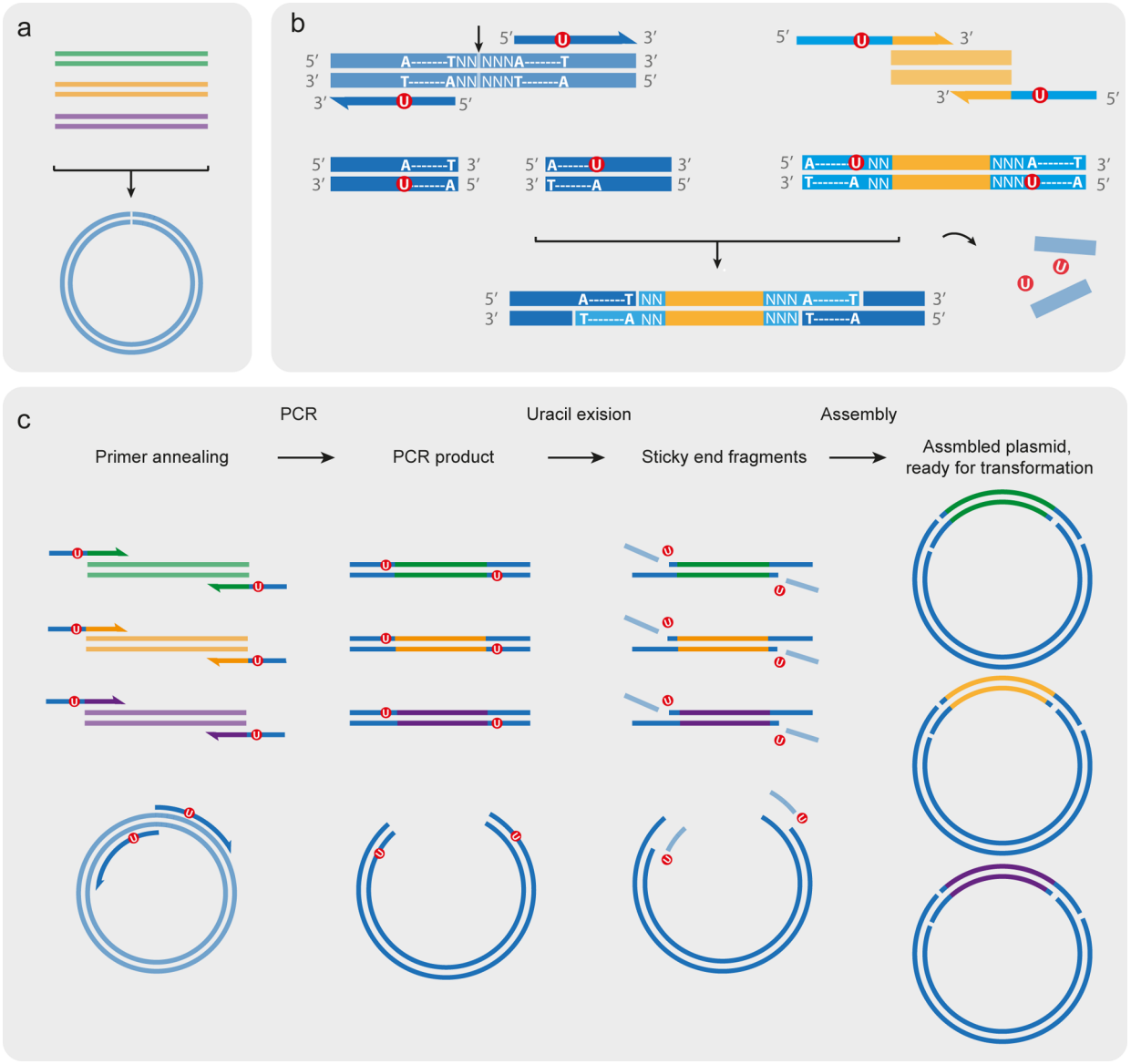

Figure 2. Single insert combinatorial assembly design supported by the AMUSER Web server. (a) The feature can be applied if multiple parts are to be cloned into the same site in a vector. (b) By designing the primers such that the assembly overhang is an A−T region within the vector sequence, seamless combinatorial assembly can be achieved for any sequence to be inserted. With this strategy, the vector backbone need only to be amplified once and the resulting fragment combines seamlessly to any insert with matching overhangs. (c) Schematic representation of the experimental steps for generating the final constructs.

competent *E. coli* cells (see Methods for details). The entire procedure from receiving the primers to incubation of transformed cells took only 4 h.

Transformants were evaluated by inspecting the agar plate containing the colonies. Conveniently, colonies expressing GFP, YFP, or no fluorescent protein could be distinguished visually without the use of UV light (Figure 3f). Out of 846 colonies, we counted 798 (94%) yellow colonies, 47 (6%) white colonies, and 1 green colony. Sequencing of the plasmid of one colony of each phenotype (green fluorescence, no fluorescence, and yellow fluorescence), revealed that the green fluorescent colony contained a nonmutated GFP inserted on the pZE21 backbone, while the sequenced white colony (no fluorescence) contained the expected correct mutations but included an additional frameshift mutation at the very beginning of the *yfp* gene. The sequenced yellow fluorescent colony contained the expected mutations only. The experimental example demonstrates the ability of the AMUSER Web server to highly accelerate the design of functional primers by automation and demonstrates how the USER cloning method can be applied for advanced plasmid engineering in a single, fast, and efficient experimental step.

DISCUSSION

The USER cloning method represents a powerful cloning method. By a single experimental protocol, practically any plasmid engineering goal can be achieved. With the recent development of polymerases that are not inhibited by uracil, such as PfuX7,[16] PfuTurbo Cx Hotstart DNA Polymerase (commercially available from Stratagene), and Phusion U Hot Start DNA Polymerase (commercially available from Thermo Scientific), USER cloning has furthermore become compatible with high fidelity PCR.[1] Traditionally, specialized vectors containing a USER cassette was recommended (NEB

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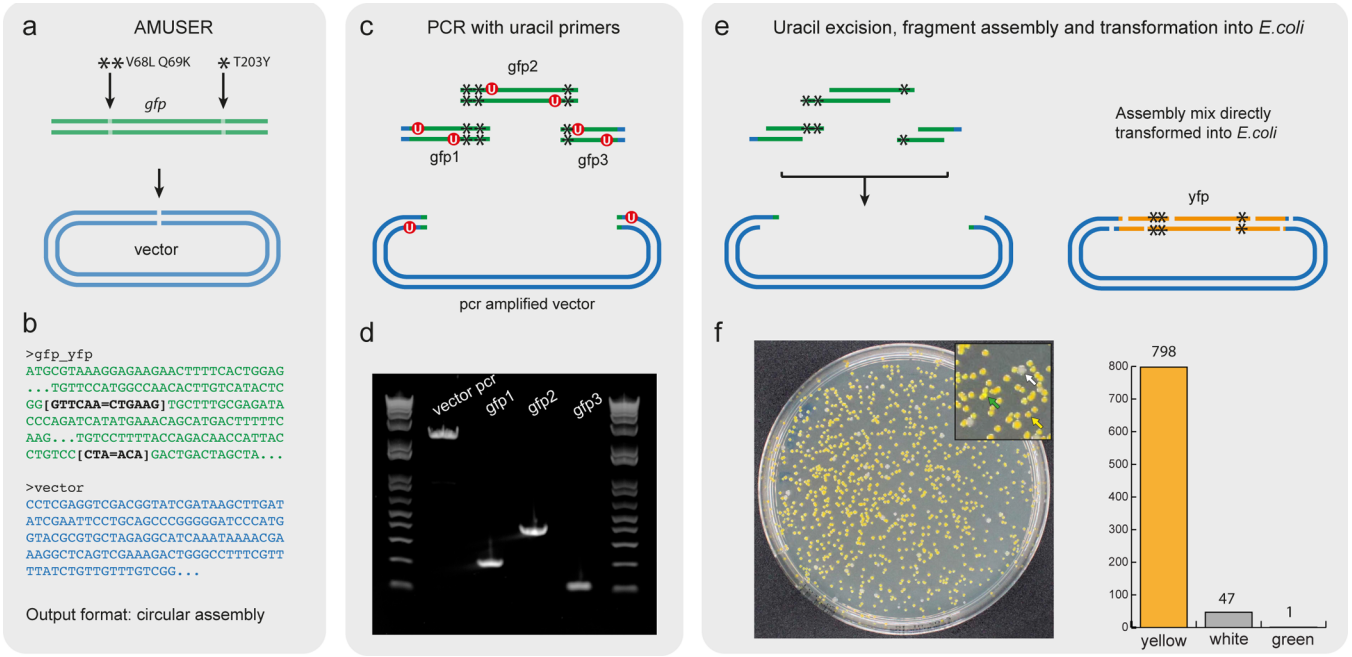

Figure 3. Experimental demonstration of the AMUSER Web server by site-directed mutagenesis of green fluorescent protein (GFP) to yellow fluorescent protein (YFP). (a) Three amino acid substitutions at two loci are required for mutating the GFP to YFP. In addition to the two mutations, the *gfp* gene was subcloned to a new vector backbone. (b) Two FASTA sequences were submitted to the Web server, and the desired nucleotide modifications (GTTCAA to CTGAAG) and (CTA to ACA) were entered in the *gfp* sequence in squared brackets at the exact positions (see Supporting Information for full query sequence). "Circular assembly" was chosen as the output construct. (c) Schematic representation of all fragments including vector backbone after PCR amplification with the designed uracil primers. (d) PCR evaluation by gel-electrophoresis showing successful amplification of all parts. (e) The PCR fragments were purified and mixed, and USER enzyme was added for generation of 3′ overhangs. Assembly of all four fragments produced the desired construct encoding a yellow fluorescent protein. (f) Transformed *E. coli* colonies after 24 h incubation at 37 °C. Colored arrows indicate fluorescence color of colony (white, green, or yellow). Successful clones produce yellow colonies.

overview of your final construct after cloning (circular):

GFPmut3 - part 1 of 3 (3' end) | insert1 | GFPmut3 - part 2 of 3 | insert2 | GFPmut3 - part 3 of 3 | pZE21 | GFPmut3 - part 1 of 3 (5' end)

graphic overview of DNA fragments and primers:

fusion region and related primers for joining of **GFPmut3 - part 1 of 3** and **GFPmut3 - part 2 of 3:**

```
                                                  5'-AAGTGCU
                                                     TTGCGAGATACCCAGATC-3'
5'-[...]GAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTCTGAAGTGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAG[...]-3'
                                  3'-GTGATGAAAGCCAATACCA
                                                  GACUTCACGA-5'
```

fusion region and related primers for joining of **GFPmut3 - part 2 of 3** and **GFPmut3 - part 3 of 3:**

```
                                                5'-ACCAATCU
                                                   GCCCTTTCGAAAGATCCC-3'
5'-[...]CTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCTACCAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCT[...]-3'
                                  3'-TCTGTTGGTAATGGACAGG
                                                  AUGGTTAGA-5'
```

fusion region and related primers for joining of **GFPmut3 - part 3 of 3** and **pZE21:**

```
                                              5'-ATAACCU
                                                 CGAGGTCGACGGTATCGA-3'
5'-[...]TTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAACCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCG[...]-3'
                              3'-ACCGTACCTACTTGATATGTT
                                              UATTGGA-5'
```

fusion region and related primers for joining of **pZE21** and **GFPmut3 - part 1 of 3:**

```
                                              5'-ACCGATGCGU
                                                 AAAGGAGAAGAACTTTTCA-3'
5'-[...]ACATCAGCAGGACGCACTGACCGAATTCATTAAAGAGGAGAAAGGTACCGATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGA[...]-3'
                          3'-CTTAAGTAATTTCTCCTCTTTCCA
                                              UGGCTACGCA-5'
```

Figure 4. Assembly details from the AMUSER Web server report. The primers are mapped to the final construct sequence. Mismatch mutations are marked as "insert1" and "insert2", respectively.

manualE5500), but with high fidelity PCR, vector backbone amplification can now be performed at low risk of introducing mutations, thus enabling full independence of specialized vectors or unique restriction sites. Still, it should be noted

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that for inserting genes on large vector backbones (e.g., those intended for eukaryotic organisms) USER cassettes can be highly useful.[17,18] The USER cloning method can be easily adapted to any molecular biology laboratory. Only two nonstandard enzymes are needed: the USER enzyme mixture and a USER compatible polymerase, as mentioned above. In return, there is no need for restriction enzymes, ligase, or expensive kits for site-directed mutagenesis, and engineering can be performed in any vector at any site. The uracil primers can be purchased from most oligo providers, however, typically at a 2−3 fold higher cost than standard primers.

Other fast and elaborate restriction enzyme free techniques for DNA assembly includes CPEC,[19] SLIC,[20] Gibson isothermal assembly,[21] and others (reviewed by Ellis et al. 2011[22]). One advantage of USER cloning compared to other sequence independent overlap cloning techniques is that relatively short overhangs of only ∼8 nucleotide are required. In contrast, the now widely used Gibson isothermal assembly recommends >20 bp overhangs, which results in longer PCR primers that are potentially more costly and prone to misannealing, as well as secondary structure and primer dimer formation.

Similar to other PCR based methods, USER cloning is limited by DNA sequences that are challenging to amplify by PCR such as DNA with a high degree of secondary structures or DNA with multiple repeating sequences. Another limitation to the method is the dependency of an A−T segment at the assembly junction site. In some cases, there may be no A−T segments of preferred length (7−15 bp) near the assembly site, which complicates the procedure, and in rare cases precludes use of the method. When performing site-directed mutagenesis at multiple sites, mutations that are closely positioned (e.g., 90 bp apart) can likewise be challenging, as it either requires PCR of unusually short segments or require very long tails of the mutagenic primers (48 bp tail at minimum per primer for a 90 bp insertion). Moreover, the number of sites that can be targeted in a single experiment are limited by the number of fragments that can be assembled in a single round.

Here, we have presented a software-supported method for DNA engineering and assembly, by strategic design of primers for USER cloning. The tool allows the user to submit DNA engineering queries in a simple Web interface and have the molecular details of the experiment automatically prepared. The AMUSER Web server rapidly evaluates multiple cloning strategies for the submitted query and uses a scoring matrix to select the optimal strategy. This relieves the investigator from the tedious process of assessing the details of all possible strategies, which greatly reduces the risk of errors in the actual experiment. It is our belief that the simple experimental design facilitated by the Web server can help alleviate the challenges to advanced applications of USER cloning, making the method far more accessible to new users. Furthermore, by supporting both DNA assembly and site-directed mutagenesis, the AMUSER Web server enables the versatility of USER cloning and facilitates acceleration of DNA engineering in general.

METHODS

Software. The main script is written in Perl with a Python wrapper for parsing data and input options to the main script and the html interface. The Web server has been tested in Chrome 36.0, Firefox 29.0, and Safari 7.0 on Mac OSX and Linux. A command line version of the software for advanced users is available for download from the Web site.

Primer Design for Experimental Demo. The DNA sequences of *gfpmut3* and the pZE21[14] backbone were submitted to the Web server (http://www.cbs.dtu.dk/services/AMUSER/) in FASTA format. Desired nucleotide manipulations were typed in squared brackets at the exact positions in the sequence (see Supporting Information for full input query). In "Step 2: Output Construct", the "circular" option was chosen, and in "Step 3, PCR Experimental Conditions" the "Default" options were used. Primers were purchased from Integrated DNA Technologies (IDT).

PCR Amplification of DNA Fragments. The PfuX7 DNA polymerase[16] was used to generate PCR fragments with high fidelity, although PfuTurbo Cx Hotstart DNA Polymerase (Agilent Technologies, 600410) and Phusion U Hot Start DNA Polymerase (Thermo Scientific, F-555S) are also usable. The above-mentioned polymerases are genetically engineered variants carrying a point mutation that prevents PCR inhibition by uracil residues. Each PCR reaction was performed in a total volume of 50 μL with 10 ng template DNA, 200 μM of each dNTP, 0.3 μM of each primer, 1× Phusion HF buffer, and 0.02 U/μL DNA polymerase. Reaction mixtures were heated to 98 °C for 30 s followed by 30 cycles at 98 °C for 10 s, 59 °C for 15 s, and 72 °C for 50 or 30 s/kb. PCR results were evaluated by gel electrophoresis. PCR products were purified using Qiagen MiniElute PCR Purification Kit (Qiagen, 28004) and was eluted in water.

One-Step USER Assembly. The four purified PCR products (including the vector backbone) were combined in equimolar amounts (∼0.3 pmol each) and 0.5 μL DpnI enzyme and 1 μL buffer (NEB, R0176) was added to a final volume of 9 μL. Following 30 min incubation at 37 °C, 1 μL of USER enzyme (NEB, M5505), which is active in both PCR and DpnI buffer, was added and the mixture was incubated for another 15 min at 37 °C, followed by 15 min at room temperature to allow DNA assembly. The assembly reaction solution (5 μL) was transformed into 50 μL chemically competent *E. coli* DH10b cells. Sanger sequencing was performed at Beckman Coulter (U.K.). See Supporting Information for a general USER cloning protocol.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org. The Web server requires no login and is available online free of charge at http://www.cbs.dtu.dk/services/AMUSER/.

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Author Contributions

Primer design strategies were developed by H.J.G. and M.T.B. Software workflows and algorithms were developed by L.R.O. with assistance from J.B.J. User Interface design and Web server integration performed by F.O.B. and R.W. Experimental work was designed by H.J.G. and M.O.A.S., and was performed by H.J.G. The manuscript was prepared by H.J.G. with contributions from all authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

USER, uracil specific excision reagent; PCR, polymerase chain reaction; DNA, Deoxyribonucleic acid; bp, base pair; HF, high fidelity; U, deoxyuridine; T, deoxythymidine; A, deoxyadenine; Tm, melting temperature; ng, nanogram; *E. coli*, *Escherichia coli*; μL, microlitre

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