Bioengineering

Volume 2, Issue 3, 126-143.
DOI: 10.3934/bioeng.2015.3.126
Received date 8 June 2015
Accepted date 17 July 2015
Published date 23 July 2015

http://www.aimspress.com/

Research article

OneClick: A Program for Designing Focused Mutagenesis Experiments

Mark Warburton [1], Hossam Omar Ali [2,3], Wai Choon Liong [2], Arona Martin Othusitse [2], Amir Zaki Abdullah Zubir [2,4], Steve Maddock [1], and Tuck Seng Wong [2,*]

[1] Department of Computer Science, The University of Sheffield, Regent Court, 211 Portobello, Sheffield S1 4DP, England.
[2] ChELSI Institute and Advanced Biomanufacturing Centre, Department of Chemical and Biological Engineering, The University of Sheffield, Mappin Street, Sheffield S1 3JD, England.
[3] A*STAR Institute of Materials Research and Engineering (IMRE), 3 Research Link, Singapore 117602.
[4] School of Clinical Dentistry, The University of Sheffield, 19 Claremont Crescent, Sheffield S10 2TA, England.

* **Correspondence:** Email: t.wong@sheffield.ac.uk; Tel: +44-114-2227591; Fax: +44-114-2227501.

Abstract: OneClick is a user-friendly web-based program, developed specifically for quick-and-easy design of focused mutagenesis experiments (*e.g.*, site-directed mutagenesis and saturation mutagenesis). Written in Perl and developed into a web application using CGI programming, OneClick offers a step-by-step experimental design, from mutagenic primer design to analysis of a mutant library. Upon input of a DNA sequence encoding the protein of interest, OneClick designs the mutagenic primers according to user input, *e.g.*, amino acid position to mutate, type of amino acid substitutions (*e.g.*, substitution to a group of amino acids with similar chemical property) and type of mutagenic primers. OneClick has incorporated an extensive range of commercially available plasmids and DNA polymerases suitable for focused mutagenesis. Therefore, OneClick also provides information on PCR mixture preparation, thermal cycling condition, expected size of PCR product and agar plate to use during bacterial transformation. Importantly, OneClick also carries out a statistical analysis of the resultant mutant library, information of which is important for selection/screening. OneClick is a unique and invaluable tool in the field of protein engineering, allowing for systematic construction of a mutant library or a protein variant and simplifying molecular biology work. The program will be constantly updated to reflect the rapid development in

the fields of molecular biology and protein engineering.

Keywords: protein engineering; enzyme engineering; directed evolution; biocatalysis; focused mutagenesis; site-directed mutagenesis (SDM); saturation mutagenesis; QuikChange

1. Introduction

Since the first use of site-directed mutagenesis to modify the active sites of tyrosyl-tRNA synthetase (TyrRS) and β-lactamase in the 1980s [1,2,3], protein engineering has continued to contribute to our understanding of protein structure, folding and enzymatic mechanism [4]. Many engineered proteins are now applied in industry and disease treatment (*e.g.*, lipases, proteases, carbohydrases and antibodies).

Molecular methods to engineer proteins can broadly be classified into 4 categories: 1) random mutagenesis, 2) focused mutagenesis (*e.g.*, site-directed mutagenesis, saturation mutagenesis), 3) DNA recombination and 4) combined approach [5,6]. A recent survey of 100 directed evolution articles, published between 2011–2013, revealed that random mutagenesis is the most popular method of choice, used in 47% of the papers reported [5]. This is followed by focused mutagenesis, which was applied in 23% of the papers surveyed [5]. Among these focused mutagenesis experiments, the QuikChange method of linear whole-plasmid amplification was most frequently used (46%), followed by overlapping PCR or 4-primer method (22%) and QuikChange derivatives (16%).

Many molecular biology kits are now commercially available for focused mutagenesis. Notable examples include QuikChange II and QuikChange Lightning from Agilent Technologies, Q5® from New England Biolabs, GeneArt® from Thermo Fisher Scientific, *etc*. The popularity of these kits has also prompted the manufacturers to develop computational tool to facilitate the design of mutagenic primers (Table 1). Most of these tools are specific to the kits they accompany (*e.g.*, type of mutagenic primer and DNA polymerase).

Table 1. Programs available for designing mutagenic primers.

Program	Company	Weblink
NEBaseChanger v1.2.2	New England Biolabs	http://nebasechanger.neb.com/#
QuikChange Primer Design	Agilent	https://www.genomics.agilent.com/primerDesignProgram.jsp
GeneArt@ Design Tool	Life Technologies (Thermo Fisher Scientific)	http://www.lifetechnologies.com/order/oligoDesigner/mutagenesisplus
PrimerX		http://www.bioinformatics.org/primerx/index.htm

AMUSER–1.0	http://www.cbs.dtu.dk/services/AMUSER/

Over the years, many clever mutagenic primer designs have been proposed to increase PCR efficiency of focused mutagenesis [5]. Further, more sophisticated DNA polymerases (*e.g.*, higher fidelity, processivity and extension rate) have been introduced, which are well suited for whole-plasmid amplification and for difficult-to-amplify plasmids. However, the field of protein engineering is lacking a design platform that encompasses all these options that are readily available for focused mutagenesis.

In this article, we describe the development and demonstrate the application of our OneClick program, which is an algorithm to design the entire process of a focused mutagenesis experiment (*i.e.*, from mutagenic primer to bacterial transformation) and to analyze the resultant mutant library.

2. Materials and Method

2.1. Implementation

A PERL script was written to read the input DNA sequence, analyze it, translate it into its corresponding protein sequence, analyze the protein sequence, design mutagenic primers and mutagenesis experiment, and provide statistical analyses. This was subsequently developed into a Perl CGI web application, using HTML5, CSS3 and JavaScript with the jQuery library to receive client-side-validated input from webforms. Perl CGI was used to parse input data, perform server-side validation, and keep track of intermediate (session) data throughout each step of the application. The Template Toolkit assisted with the creation of web pages and the display of output data.

2.2. IUPAC notations

In OneClick, IUPAC notations are used in reporting a primer sequence (Table 2).

Table 2. IUPAC notations used in OneClick.

Symbol	Description	Nucleobase			
A	Adenine	A			
C	Cytosine		C		
G	Guanine			G	
T	Thymine				T
W	Weak interaction	A			T
S	Strong interaction		C	G	
M	Amino	A	C		
K	Keto			G	T
R	Purine	A		G	
Y	Pyrimidine		C		T
B	Not A (B comes after A)		C	G	T

D	Not C (D comes after C)	A		G	T
H	Not G (H comes after G)	A	C		T
V	Not T or U (V comes after U)	A	C	G	
N	Any nucleotide	A	C	G	T

2.3. Types of amino acid substitution and amino acid classification

OneClick allows 5 types of amino acid substitution (Table 3). In type (ii) substitution, amino acids are grouped into 4 categories, depending on the chemical nature of their side-chains: (iia) aliphatic, (iib) aromatic, (iic) neutral and (iid) charged, identical to the categorization used in the Mutagenesis Assistant Program (MAP; [7,8]). In type (v) substitution, a smart library comprises 12 amino acids [9]. This set of amino acids is a balanced mix of polar and nonpolar, aliphatic and aromatic, and negatively and positively charged amino acids, while excluding structurally/chemically similar amino acids.

Table 3. Types of amino acid substitution in OneClick.

Amino acid substitution	Codon	Amino acid
(i) Substitution to a specific amino acid	[1]	User's choice
(ii) Substitution to a group of amino acids with similar chemical property		
(iia) Aliphatic	VBC	G, A, V, L, I
(iib) Aromatic	TDK	F, Y, W
(iic) Neutral	HNK	C, M, P, S, T, N, Q
(iid) Charged	VRW	D, E, H, K, R
(iii) Substitution to a group of amino acids defined by user	[2]	User's choice
(iv) Substitution to all 20 canonical amino acids	NNK	G, A, V, L, I, F, Y, W, C, M, P, S, T, N, Q, D, E, H, K, R
(v) Substitution to a smart library	NDT	G, V, L, I, F, Y, C, S, N, D, H, R

[1]Most frequent codon is assigned based on expression host chosen.

[2]Codon is assigned to maximize the occurrence of desired amino acids. Rare codon is avoided.

2.4. Mutagenic primer type and primer design

OneClick is capable of designing 4 types of mutagenic primer, which have comprehensively been summarized in a recent review article (Figure 1; [5]): (i) overlapping primers, (ii) partially overlapping primers, (iii) non-overlapping primers and (iv) primers for overlapping PCR (4-primer method). The design rules for each mutagenic primer type are summarized in Table 4.

For mutagenic primer types (i) and (ii), a user is allowed to choose between 1-stage PCR and 2-stage PCR (Figure 2; [10,11]). 2-stage PCR was developed to circumvent the formation of primer-dimer, thereby enhancing PCR efficiency [5]. In a 2-stage PCR, two single-primer PCRs are run in parallel in the first stage. In the second stage, both reactions are mixed, redistributed and

thermocycled.

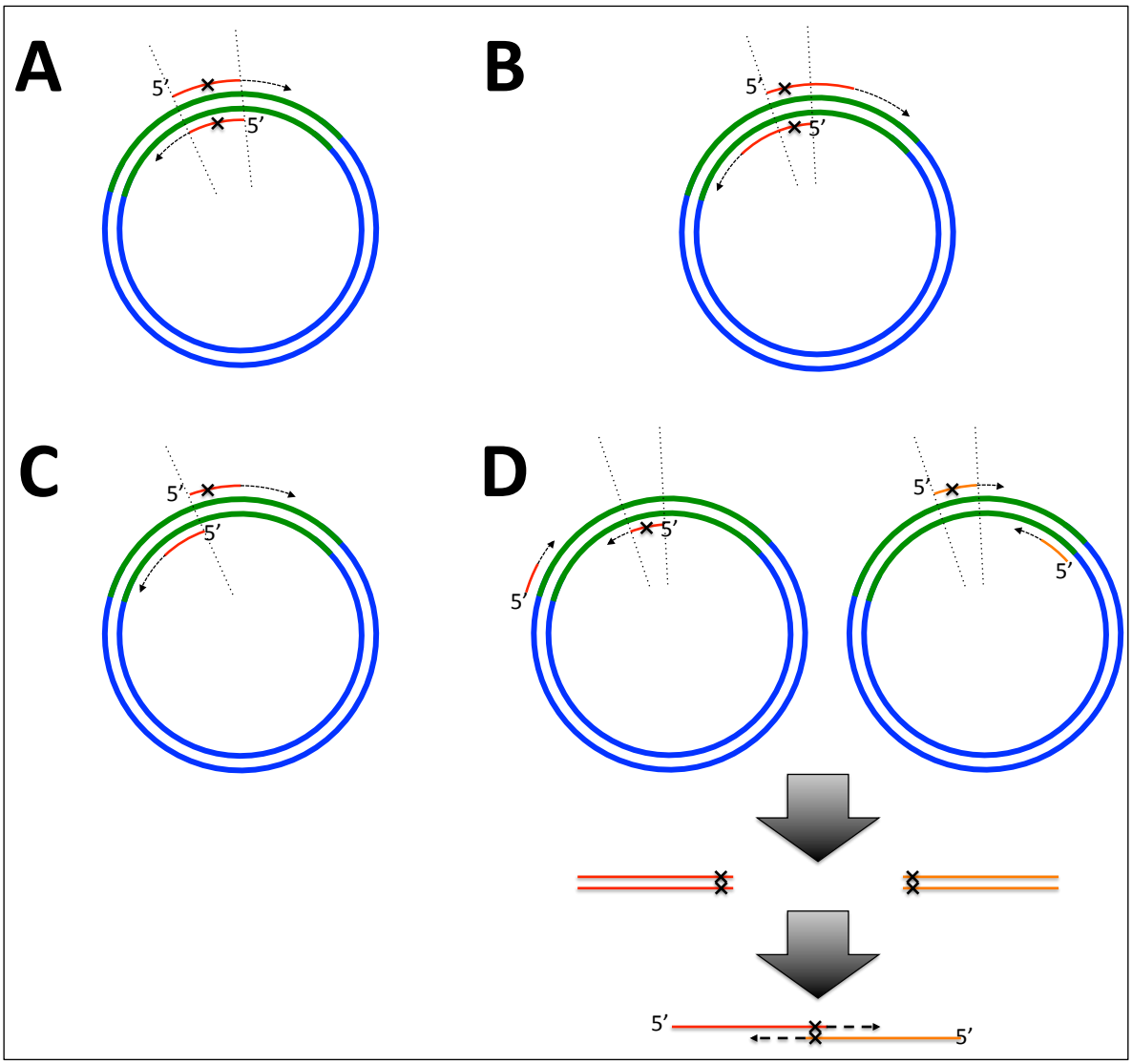

Figure 1. Mutagenic primer types in OneClick: (*A*) overlapping primers, (*B*) partially-overlapping primers, (*C*) non-overlapping primers and (*D*) primers for overlapping PCR (4-primer method).

2.5. Calculation of molecular weight and melting temperature of primer

Molecular weight of primer is calculated using the formula: $MW = A_n \times 313.21 + T_n \times 304.2 + C_n \times 289.18 + G_n \times 329.21 - 61.96$ [12]. This equation assumes that there is no 5'-phosphate group. Melting temperature of a primer is calculated using the formulae: for primer length <14 bases, $T_m = (wA + xT) \times 2 + (yG + zC) \times 4$ [13], for primer length >13 bases, $T_m = 64.9 + 41 \times (yG + zC - 16.4)/(wA + xT + yG + zC)$ [14], where w, x, y, z are the numbers of A, T, G, C bases in the sequence, respectively. Both equations assume that the annealing occurs

under the standard conditions of 50 nM primer, 50 mM Na^+ and pH 7.0 [12]. For primer with mismatch (es), melting temperature is calculated using the formula: $T_m = 81.5 + 0.41\times(\%GC) - 675/N - (\%mismatch)$, where N, %GC and %mismatch are the primer length, percentage of GC content and percentage of mismatches, respectively.

Table 4. Mutagenic primer design rules used in OneClick.

Mutagenic primer type	Sequence (5'-3')[1]	Length	Number of overlapping nucleotides
(i) Overlapping primers			
Primer 1	$(M)_{15}X_1X_2X_3(N)_{15}$	33	33 (overlaps with primer 2)
Primer 2	RC: $(M)_{15}X_1X_2X_3(N)_{15}$	33	33 (overlaps with primer 1)
(ii) Partially overlapping primers			
Primer 1	$(M)_9X_1X_2X_3(N)_{34}$	46	21 (overlaps with primer 2)
Primer 2	RC: $(M)_{34}X_1X_2X_3(N)_9$	46	21 (overlaps with primer 1)
(iii) Non-overlapping primers			
Primer 1	$(M)_{10}X_1X_2X_3(N)_{25}$	38	0
Primer 2	RC: $(P)_{25}$	25	0
(iv) Primers for overlapping PCR (4-primer method)			
Primer 1	$(M)_{12}X_1X_2X_3(N)_{15}$	30	21 (overlaps with primer 2)
Primer 2	RC: $(M)_{21}X_1X_2X_3(N)_6$	30	21 (overlaps with primer 1)
Primer 3	aaaaxxxxxx$(A)_{18}$	28	0
Primer 4	RC: $(B)_{18}$TAAyyyyyybbbb	31	0

[1]$X_1X_2X_2$: Codon targeted for mutagenesis; M: DNA sequence upstream of the codon targeted for mutagenesis; N: DNA sequence downstream of the codon targeted for mutagenesis, P: DNA sequence upstream of sequence M; A: 5'-end of sense strand; B: 3'-end of sense strand; RC: Reverse complement; xxxxxx and yyyyyyy: restriction sites defined by user; aaaa and bbbb: additional bases added to ensure efficient restrictive digestion.

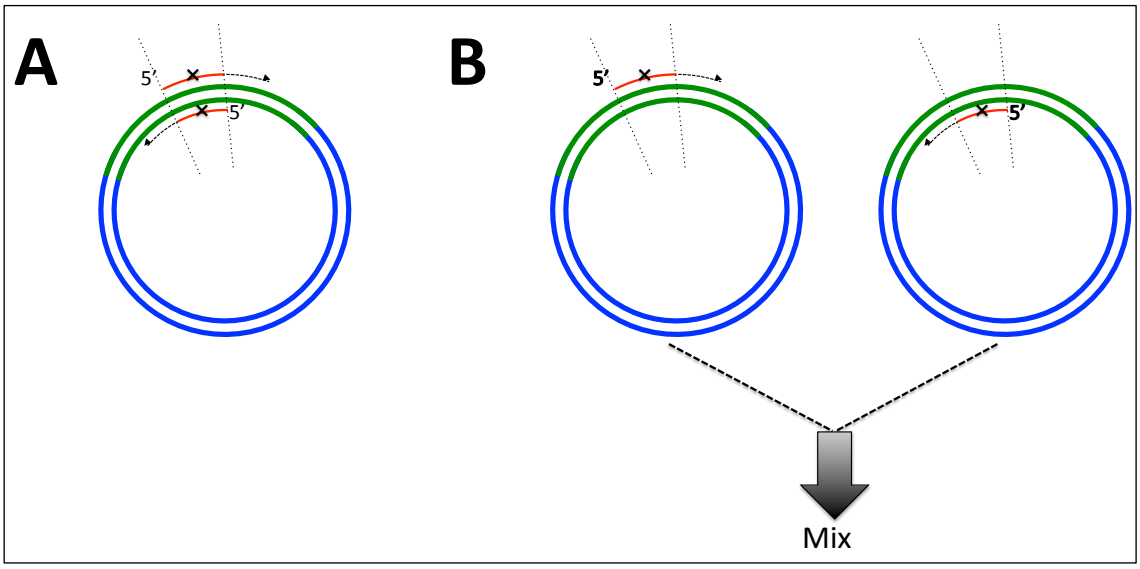

Figure 2. (*A*) 1-stage PCR and (*B*) 2-stage PCR.

2.6. Library of plasmids

OneClick has incorporated a library of 235 plasmids, including pET, pGEX, pQE, pASK-IBA, pBAD, pRSET, pMAL, pGEM, pUC and pBluescript. These plasmids are available from Novagen/Merck (99 plasmids), GE Healthcare (13 plasmids), Qiagen (51 plasmids), IBA (32 plasmids), Invitrogen (12 plasmids), New England Biolabs (6 plasmids), Promega (12 plasmids) and others (10 plasmids). Plasmid size and selection marker of each plasmid are used to determine PCR elongation time and recommend the type of agar plate to use during bacterial transformation, respectively.

2.7. Library of DNA polymerases

OneClick has a library of 14 high-fidelity DNA polymerases, which are suitable for focused mutagenesis (*i.e.*, whole-plasmid amplification). These DNA polymerases are available from Agilent Technologies (Pfu, PfuTurbo, PfuTurboCxHotstart, PfuUltra and PfuUltra II Fusion HS), New England Biolabs (Q5 and Phusion), Invitrogen (AccuPrimePfx), GE Life Sciences (FideliTaq), Toyobo Life Sciences (KOD Plus), GM Biosciences (TaqMaster), Roboklon (Pfu Plus!), Roche (PwoSuperYield) and TaKaRa (PrimeSTAR HS). PCR mixture preparation and thermal cycling condition recommended by the manufacturers are information that has already been built into the OneClick program.

2.8. Availability

OneClick is made publicly available at http://tucksengwong.staff.shef.ac.uk/OneClick/. The program is compatible with commonly used web browsers, *e.g.*, Firefox, Safari, Chrome and Internet Explorer.

2.9. DNA sequences

DNA sequences encoding cytochrome P450 BM3 and human lymphotactin (hLtn or XCL1) were obtained from the National Centre for Biotechnology Information (NCBI), with accession numbers of J04832.1 and NM_002995.1, respectively.

2.10. Materials

All chemicals used were of analytical grade. Plasmid pCMV6-hLtn harbouring *hLtn* gene (catalogue number: SC309015) was purchased from OriGene Technologies (Rockville, USA). PfuTurbo DNA polymerase was purchased from Agilent Technologies (Santa Clara, USA). Restriction enzyme DpnI, deoxyribonucleotides (dNTPs) and DNA ladder were purchased from New England Biolabs (Ipswich, USA). Primers used in this study, Fwd (5'-CAAGCCACA**GAC**GTGAGAGACGTGGTCAGGAGCATGGACAGGAAAT-3') and Rev (5'-GTCTCTCAC**GTC**TGTGGCTTGTGGATCAGCACAGACTTTTAGGCCA-3'), were synthesized by Eurofins Genomics (Ebersberg, Germany). In both primers, overlapping regions were underlined and mutagenic codon was highlighted in bold.

2.11. Site-directed mutagenesis of hLtn

Two PCR mixtures (labelled as TubeF and TubeR; each 50 μL) were prepared. TubeF contained 1× Cloned Pfu reaction buffer, 200 μM dNTPs, 50 ng plasmid pCMV6-hLtn, 20 pmol Fwd primer and 2.5 U PfuTurbo DNA polymerase. TubeR contained 1× Cloned Pfu reaction buffer, 200 μM dNTPs, 50 ng plasmid pCMV6-hLtn, 20 pmol Rev primer and 2.5 U PfuTurbo DNA polymerase. Both mixtures were thermocycled using the following conditions: (i) 2 min initial denaturation at 95 °C and (ii) 10 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 55 °C, 5 min 18 sec elongation at 72 °C. After the first PCR stage, the two mixtures were mixed, redistributed and thermocycled using the conditions: (i) 20 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 55 °C, 5 min 18 sec elongation at 72 °C and (ii) 10 min final extension at 72 °C. Forty units of DpnI were subsequently added to each PCR mixture and incubated at 37 °C for 2 hours to remove parental pCMV6-hLtn template. PCR product was analysed on 1% (w/v) DNA agarose gel and transformed into *E. coli* DH5α using the standard calcium chloride method. Cells were plated on TYE agar plate supplemented with 50 μg/mL kanamycin. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Hilden, Germany) and sequenced by Eurofins Genomics.

3. Results

3.1. The OneClick program

OneClick is a user-friendly program, developed specifically to facilitate the design of focused mutagenesis experiments. The name *OneClick* was chosen mainly to signify the simplicity of the program. To design a focused mutagenesis experiment, the user only needs to follow three basic steps, as depicted in Figure 3. In Step 1, the user is required to upload a DNA sequence encoding for the protein of interest in the FASTA format or copy-and-paste the DNA sequence into the text field

provided. In Step 2, the user will be guided through the entire mutagenic primer design process. Finally in Step 3, the user will perform mutagenesis experiment and analyze experimental data as suggested by OneClick. On the entry page of OneClick (Figure 4), detailed instructions on how to use OneClick are given. Users are also encouraged to provide feedback to program developers. As with most bioinformatics tools available, help functions are provided to guide the user in selecting the most appropriate design option. Therefore, OneClick caters to both newcomers in protein engineering (learn to design) and experienced researchers (quick-and-easy design).

In the following section, we demonstrate the application of OneClick by designing two mutagenic primer pairs, one for cytochrome P450 BM3 and one for human lymphotactin.

3.2. Primer design for cytochrome P450 BM3

Cytochrome P450 monooxygenases (P450s) are enzymes with enormous potential in the production of oxychemicals, owing to their unparalleled regio- and stereo-selectivities [15]. Cytochrome P450 BM3 (CYP102A1; denoted as BM3 herein), isolated from *Bacillus megaterium*, has long been serving as a key model system [16]. Phenylalanine 87 (F87) of BM3, a residue closed to the heme in the substrate-binding pocket, plays critical roles in governing BM3's substrate selectivity, regio- and stereo-selectivity [17–21], H_2O_2-dependent oxidation [22], and organic

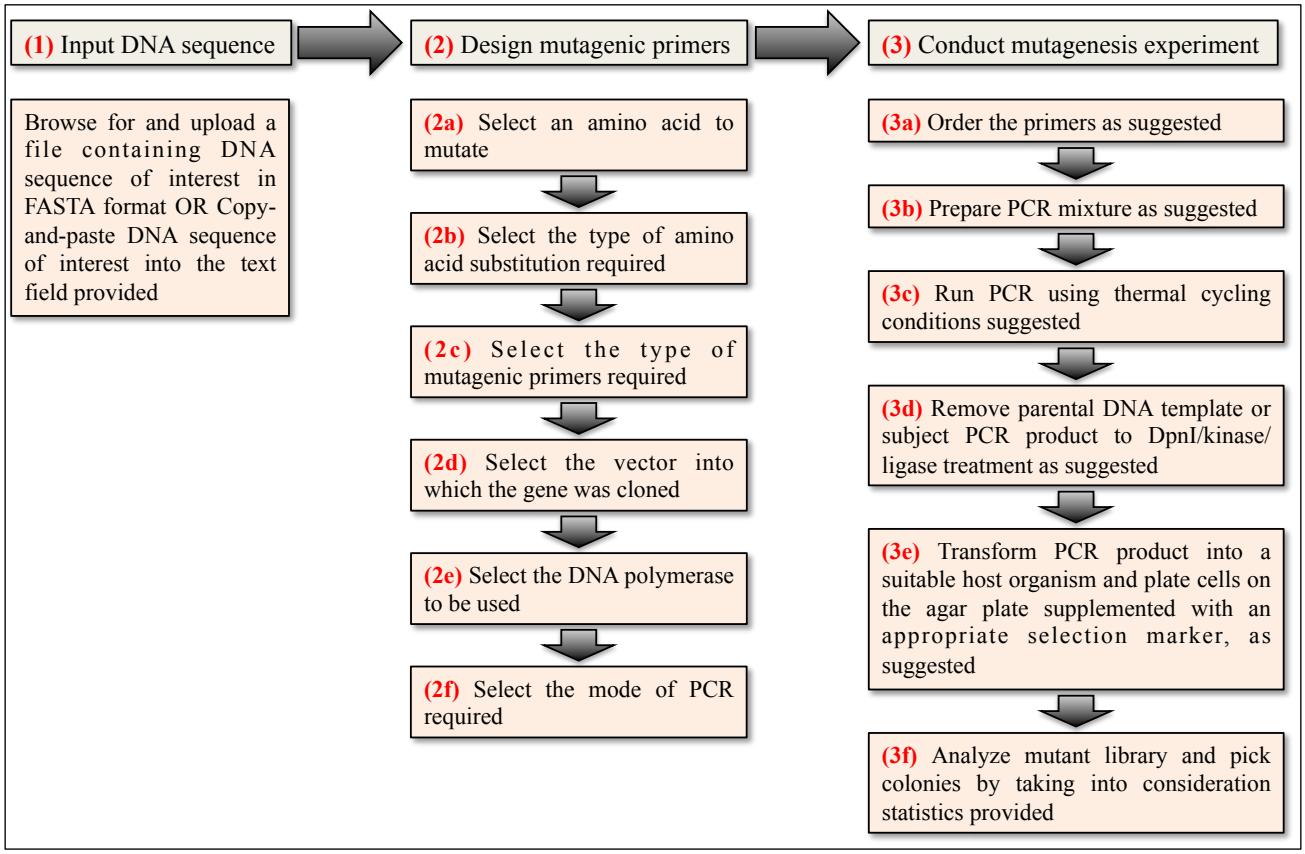

Figure 3. Workflow of OneClick program.

cosolvent tolerance [23,24,25]. Figures 4–6 illustrate the process of designing mutagenic primers and

mutagenesis experiment to mutate residue 87 of BM3 from phenylalanine (an aromatic amino acid) to aliphatic amino acids (G, A, V, L, I). In Step 1, *DNA sequence encoding BM3* was copied-and-pasted into the text field provided (Figure 4). Subsequently in Step 2 (Figure 5), *87* was inputted as the amino acid position to be mutated and *substitution to a group of amino acids with similar chemical property* was selected as the type of amino acid substitution. Next, *aliphatic amino acids (G, A, V, L, I)* was chosen as the chemical group to aim for. In this particular example, *partially overlapping primers*, *pET-24a(+)*, *PfuTurbo DNA Polymerase (Agilent Technologies)* and *2-stage PCR* were selected as the type of mutagenic primers, the plasmid in which the gene is cloned, the DNA polymerase to be used and the PCR run mode, respectively. Upon inputting all information required (information in italic) and by clicking the OneClick button, a comprehensive report was generated in Step 3 (Figure 6), providing a guide on how to conduct the mutagenesis experiment. The report contained all necessary information including the sequences and properties of mutagenic primers, preparation of PCR mixture and thermal cycling condition (in this case, protocol recommended by Agilent Technologies), treatment of PCR product to remove parental DNA template (DpnI digestion), the size of the PCR product expected (8458 bp), the type of agar plate to use during bacterial transformation (supplemented with kanamycin), and statistical analysis of the mutant library. The degenerate codon VBC in the mutagenic primer represents a set of 9 codons (ACC, AGC, ATC, CCC, CGC, CTC, GCC, GGC, GTC). Within this set, 5 of them encode desired amino acids (56%), which are GGC (G), GCC (A), GTC (V), CTC (L) and ATC (I). The remaining 4 codons (44%), on the other hand, encode undesired amino acids [ACC (T), AGC (S), CCC (P), CGC (R)]. Clear advantages of using degenerate mutagenic primers are cost saving and simplified experiment.

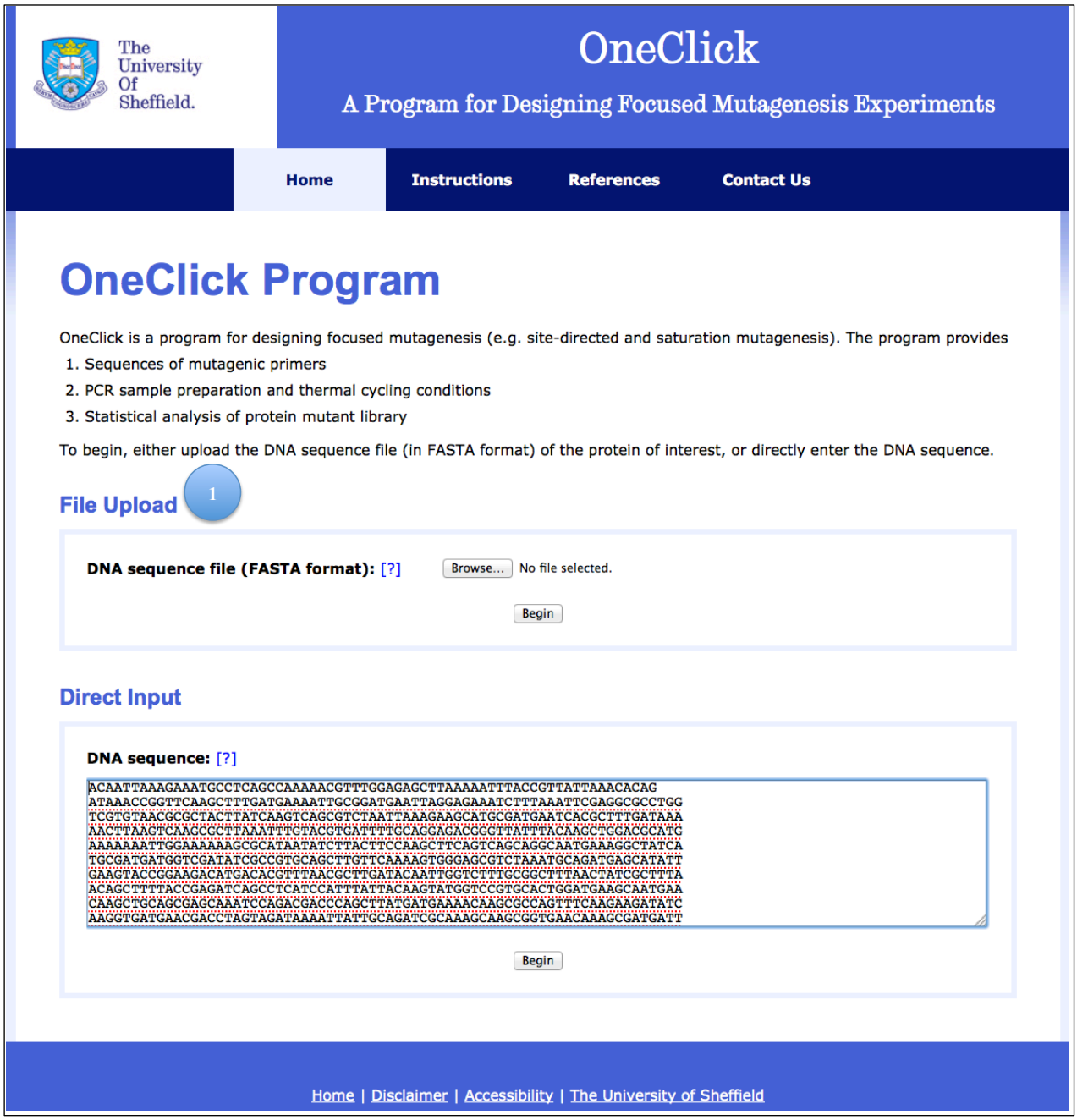

Figure 4. The entry page of OneClick. A user could either browse for the DNA sequence file in the FASTA format or copy-and-paste the DNA sequence into the text field provided. The symbol [?] indicates help functions.

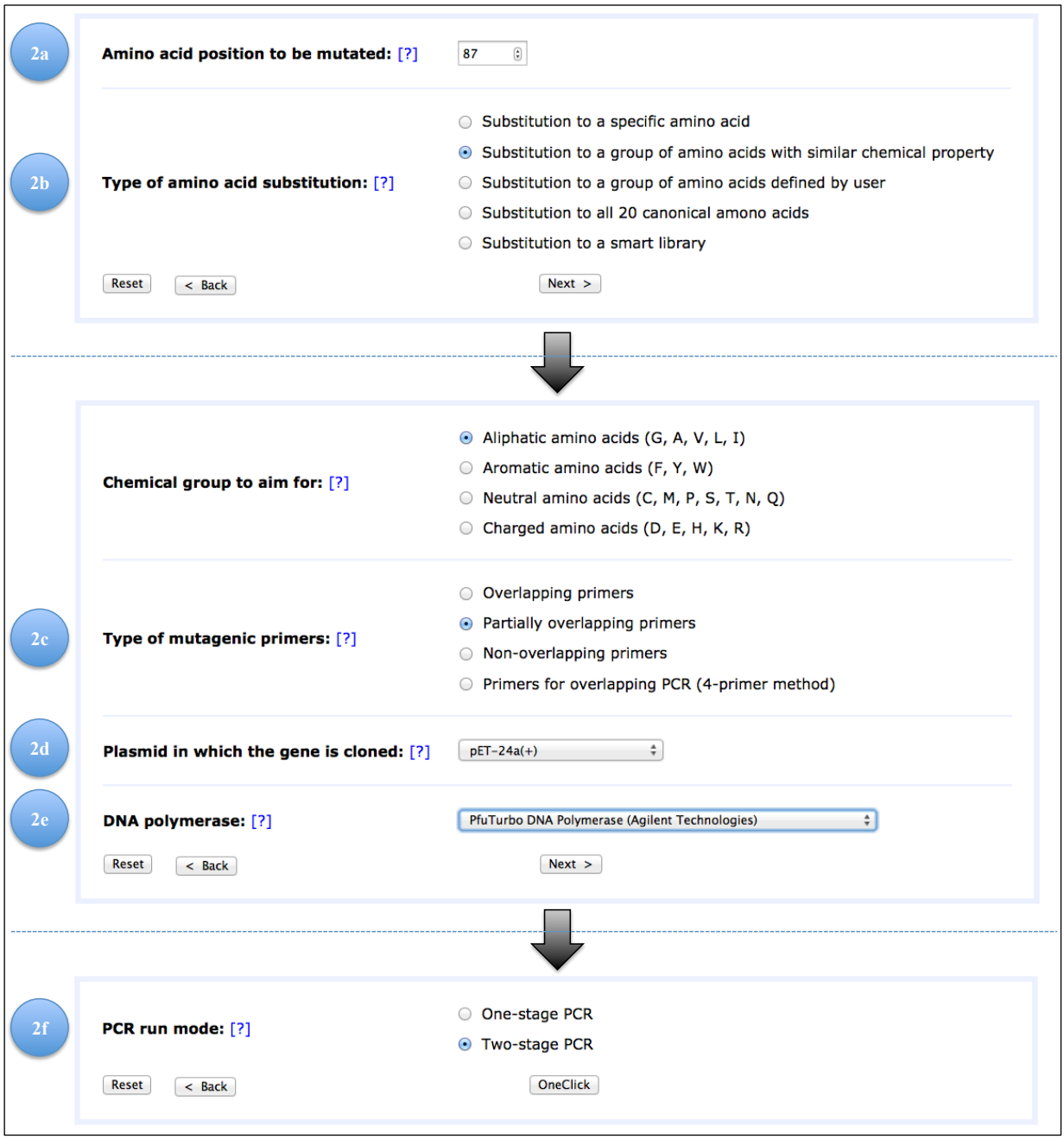

Figure 5. A user is guided through the design of mutagenic primers. The symbol [?] indicates help functions.

3.3. Primer design for site-directed mutagenesis of hLtn

To demonstrate the reliability of OneClick, we tested the program on the plasmid pCMV6-hLtn harboring *hLtn* gene, which we have not worked on previously. *hLtn* encodes human lymphotactin or XCL1, the defining member of the C-class chemokine [26]. Being a metamorphic protein, hLtn folds into two unrelated native state structures. Ltn10 is monomeric, structurally closely resembling other chemokines. The dimeric Ltn40 revealed a drastic structural change. To engineer a conformationally-restricted lymphotactin variant, we introduced the amino acid substitution W55D

to lock the protein in Ltn40 conformation. Using mutagenic primers and experiment designed by OneClick (see section 2.11), we successfully created this protein variant. Figure 7 provides clear indication that whole-plasmid amplification worked effectively with the mutagenic primers and experimental conditions suggested by OneClick, judging on the thick product band. DNA sequencing confirmed the identity of the protein variant. In brief, OneClick provided a right-first-time design in this case.

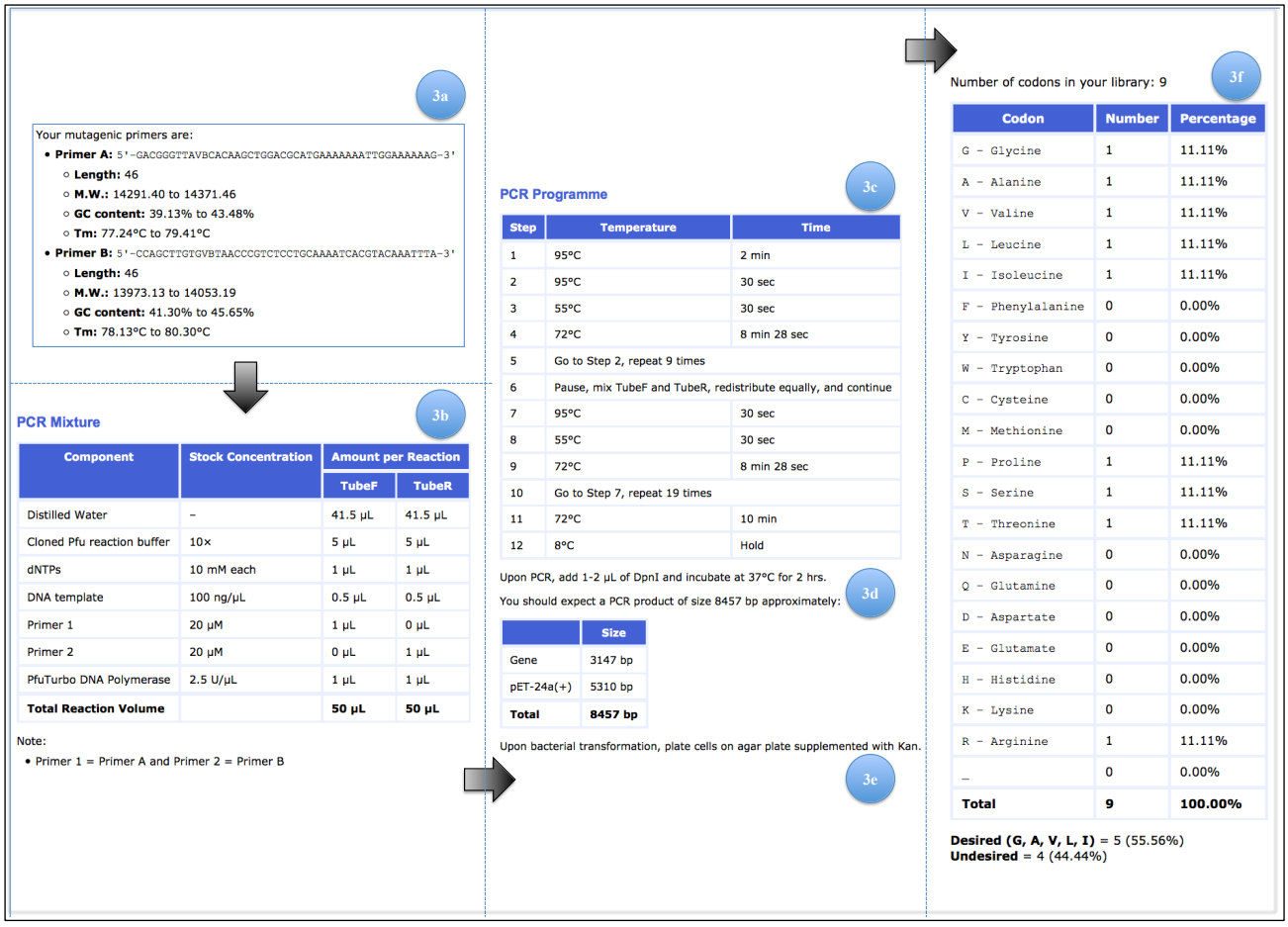

Figure 6. Output generated by OneClick: (*top left*) sequence and properties of mutagenic primer pair, (*bottom left*) preparation of PCR mixtures, (*top center*) cycling condition and PCR product treatment, (*bottom center*) expected size of PCR product and agar plate to use during bacterial transformation, (*right*) mutant library analysis.

4. Discussion

4.1. Unique features of OneClick

Table 5 compares the features and functionalities of 6 mutagenic primer design programs; all are publicly available. Most programs adopt a DNA sequence-based design. A user is prompted to identify the nucleotide position to mutate based upon the inputted DNA sequence and select a

mutation code from the IUPAC notation list (A/C/G/T/W/S/M/K/R/Y/B/D/H/V/N) given in Table 2. OneClick, on the other hand, uses a protein sequence-based design. The input DNA sequence is first translated into its corresponding protein sequence and the user is asked to identify the amino acid position to mutate. OneClick is the only program that offers a selection of amino acid substitution types.

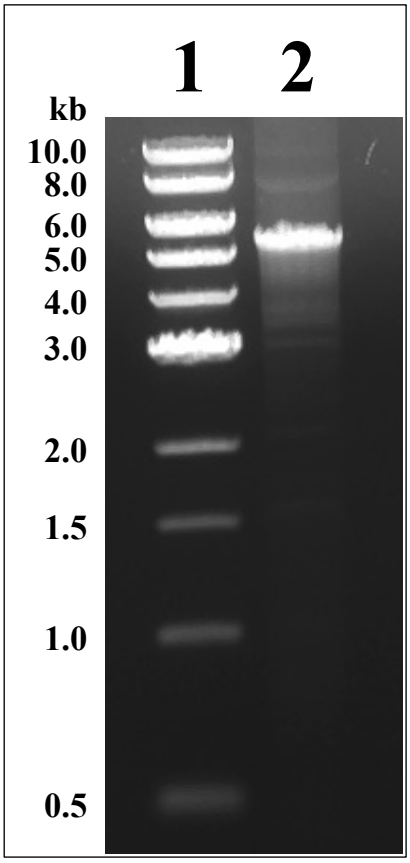

Figure 7. PCR product analysis on 1% (w/v) DNA agarose gel: (*1*) 1 kb DNA ladder (New England Biolabs) and (*2*) PCR product.

Worthy of note, OneClick has incorporated a large plasmid library (235 plasmids in OneClick compared to 134 plasmids in NEBaseChanger v1.2.2). While conducting a focused mutagenesis experiment, researchers always need to check the plasmid size (to determine the elongation time) and the selection marker (to decide what agar plate to use to plate the transformants), and calculate the expected PCR product size. OneClick provides all this information. Further, OneClick is the only program that contains a library of DNA polymerases, which enables it to suggest PCR mixture preparation and thermal cycling condition. Unlike NEBaseChanger v1.2.2 that provides a generic protocol specific to Q5 DNA polymerase (*e.g.*, elongation time of 20–30 seconds/kb), OneClick generates detailed and unambiguous experimental protocol.

Last but not least, OneClick analyses the mutant library created. This information is important for several reasons: 1) to verify the sequence of mutagenic primer designed, 2) to estimate the number of mutant clones to pick or screen, and 3) to quantity genetic diversity within the mutant library. In creating a mutant library involving multiple sites (*e.g.*, CASTing [27]) for instance, this

information is even more crucial.

Worthy of note, one limitation of OneClick is that it is not possible to introduce mutations in the terminal 12 amino acids, as this would then require the surrounding plasmid information. This is also the case for most primer design programs available.

Table 5. Comparison of programs for designing mutagenic primers.

Information provided	O[1]	N[1]	Q[1]	G[1]	P[1]	A[1]
Protein sequence-based design	✓	×	✓	×	✓	×
DNA sequence-based design	×	✓	✓	✓	✓	✓
Instructions/help function	✓	✓	✓	✓	✓	✓
Selection of amino acid substitution types	✓	×	×	×	×	×
Selection of mutagenic primer types	✓	×	×	×	✓	×
Plasmid library	✓	✓	×	×	×	×
DNA polymerase library	✓	×	×	×	×	×
Primer sequence	✓	✓	✓	✓	✓	✓
Primer properties	✓	✓	✓	✓	✓	✓
Preparation of PCR mixture	✓	✓	×	×	×	×
PCR thermal cycling condition	✓	✓	×	×	×	×
PCR product treatment	✓	✓	×	×	×	×
Expected size of PCR product	✓	×	×	×	×	×
Agar plate to use during bacterial transformation	✓	×	×	×	×	×
Mutant library analysis	✓	×	×	×	×	×

[1]O: OneClick, N: NEBaseChanger v1.2.2, Q: QuikChange Primer Design, G: GeneArt@ Design Tool, P: PrimerX, A: AMUSER – 1.0

4.2. Optimization of a focused mutagenesis experiment

Despite focused mutagenesis being a well-established technique, researchers often encounter situations where PCR does not work, product band is barely visible in DNA agarose gel or mutation is not incorporated. In Table 6, we provide suggestions for optimizing a focused mutagenesis experiment based on the experience we have accumulated over the years.

4.3. Future development of OneClick

In OneClick version 1.0, as described in this article, we have endeavored to incorporate functionalities that are needed or frequently used in protein engineering. For the next version, we aim to enhance the program by 1) including multi site-directed mutagenesis, 2) allowing insertion/deletion (which is less frequently used compared to substitution), 3) allowing a user to input his/her own plasmid, 4) expanding the selections/libraries of mutagenic primer types, plasmids and DNA polymerases, and 5) providing the option to send the sequences of mutagenic primers directly to synthetic oligonucleotide suppliers. We will also consider and incorporate suggestions from OneClick users, if deemed appropriate.

Table 6. Suggestions for optimizing a focused mutagenesis experiment.

Observation	Possible cause(s)	Suggestion(s)
PCR does not work	Wrong mutagenic primer design	Use OneClick or other mutagenic primer design programs.
	Difficult to amplify plasmid	Change the plasmid backbone or use overlapping PCR/4-primer method.
	Wrong PCR mixture preparation	Use OneClick or refer to manufacturer's guide.
	Wrong thermal cycling condition	Use OneClick or refer to manufacturer's guide. Check thermocycler settings.
Product band is barely visible	Formation of primer-dimer	Use 2-stage PCR
Mutation is not incorporated	Wrong mutagenic primer design	Use OneClick or other mutagenic primer design programs.
	Concentration of DNA template too high	Reduce the amount of DNA template.
	Incomplete DpnI digestion	Increase the amount of DpnI or prolong the DpnI digestion.

5. Conclusion

OneClick is a powerful program for designing focused mutagenesis experiments. It is developed specifically to serve the protein engineering community. The program will evolve as the field of protein engineering advances.

Acknowledgments

We thank ChELSI and EPSRC (EP/E036252/1) for financial support. HOA and AZAZ are supported by the A*STAR-Sheffield Research Attachment Program (Singapore and UK) and the Majlis Amanah Rakyat (MARA; Malaysia), respectively. WCL and AMO are sponsored by High Commissions of Malaysia and Botswana, respectively.

Conflict of Interest

All authors declare that there are no conflicts of interest.

References

1. Dalbadie-McFarland G, Cohen LW, Riggs AD, et al. (1982) Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function. *Proc Natl Acad Sci U S A* 79: 6409–6413.
2. Sigal IS, Harwood BG, Arentzen R (1982) Thiol-beta-lactamase: replacement of the active-site serine of RTEM beta-lactamase by a cysteine residue. *Proc Natl Acad Sci U S A* 79: 7157–7160.
3. Winter G, Fersht AR, Wilkinson AJ, et al. (1982) Redesigning enzyme structure by site-directed mutagenesis: tyrosyl tRNA synthetase and ATP binding. *Nature* 299: 756–758.
4. Brannigan JA, Wilkinson AJ (2002) Protein engineering 20 years on. *Nat Rev Mol Cell Biol* 3: 964–970.
5. Tee KL, Wong TS (2013) Polishing the craft of genetic diversity creation in directed evolution. *Biotechnol Adv* 31: 1707–1721.
6. Wong TS, Zhurina D, Schwaneberg U (2006) The diversity challenge in directed protein evolution. *Comb Chem High Throughput Screen* 9: 271–288.
7. Wong TS, Roccatano D, Zacharias M, et al. (2006) A statistical analysis of random mutagenesis methods used for directed protein evolution. *J Mol Biol* 355: 858–871.
8. Verma R, Wong TS, Schwaneberg U, et al. (2014) The Mutagenesis Assistant Program. *Methods Mol Biol* 1179: 279–290.
9. Reetz MT, Kahakeaw D, Lohmer R (2008) Addressing the numbers problem in directed evolution. *Chembiochem* 9: 1797–1804.
10. Edelheit O, Hanukoglu A, Hanukoglu I (2009) Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnol* 9: 61.
11. Wang W, Malcolm BA (1999) 2-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. *Biotechniques* 26: 680–682.
12. Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res* 35: W43–46.
13. Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 5: 109–118.
14. Wallace RB, Shaffer J, Murphy RF, et al. (1979) Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA: the effect of single base pair mismatch. *Nucleic Acids Res* 6: 3543–3557.
15. Bernhardt R, Urlacher VB (2014) Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. *Appl Microbiol Biotechnol* 98: 6185–6203.
16. Munro AW, Leys DG, McLean KJ, et al. (2002) P450 BM3: the very model of a modern flavocytochrome. *Trends Biochem Sci* 27: 250–257.
17. Chen CK, Shokhireva T, Berry RE, et al. (2008) The effect of mutation of F87 on the properties of CYP102A1-CYP4C7 chimeras: altered regiospecificity and substrate selectivity. *J Biol Inorg Chem* 13: 813–824.
18. Vottero E, Rea V, Lastdrager J, et al. (2011) Role of residue 87 in substrate selectivity and regioselectivity of drug-metabolizing cytochrome P450 CYP102A1 M11. *J Biol Inorg Chem* 16: 899–912.

19. Graham-Lorence S, Truan G, Peterson JA, et al. (1997) An active site substitution, F87V, converts cytochrome P450 BM-3 into a regio- and stereoselective (14S,15R)-arachidonic acid epoxygenase. *J Biol Chem* 272: 1127–1135.
20. Li QS, Ogawa J, Schmid RD, et al. (2001) Residue size at position 87 of cytochrome P450 BM-3 determines its stereoselectivity in propylbenzene and 3-chlorostyrene oxidation. *FEBS Lett* 508: 249–252.
21. Oliver CF, Modi S, Sutcliffe MJ, et al. (1997) A single mutation in cytochrome P450 BM3 changes substrate orientation in a catalytic intermediate and the regiospecificity of hydroxylation. *Biochemistry* 36: 1567–1572.
22. Li QS, Ogawa J, Shimizu S (2001) Critical role of the residue size at position 87 in H2O2-dependent substrate hydroxylation activity and H2O2 inactivation of cytochrome P450BM-3. *Biochem Biophys Res Commun* 280: 1258–1261.
23. Kuper J, Tee KL, Wilmanns M, et al. (2012) The role of active-site Phe87 in modulating the organic co-solvent tolerance of cytochrome P450 BM3 monooxygenase. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 68: 1013–1017.
24. Kuper J, Wong TS, Roccatano D, et al. (2007) Understanding a mechanism of organic cosolvent inactivation in heme monooxygenase P450 BM-3. *J Am Chem Soc* 129: 5786–5787.
25. Wong TS, Arnold FH, Schwaneberg U (2004) Laboratory evolution of cytochrome p450 BM-3 monooxygenase for organic cosolvents. *Biotechnol Bioeng* 85: 351–358.
26. Volkman BF, Liu TY, Peterson FC (2009) Chapter 3. Lymphotactin structural dynamics. *Methods Enzymol* 461: 51–70.
27. Reetz MT, Wang LW, Bocola M (2006) Directed evolution of enantioselective enzymes: iterative cycles of CASTing for probing protein-sequence space. *Angew Chem Int Ed Engl* 45: 1236–1241.

AIMS Press