Published in final edited form as:

Methods Mol Biol. 2013 ; 978: 217–228. doi:10.1007/978-1-62703-293-3_16.

Rational protein sequence diversification by multi-codon scanning mutagenesis

Jia Liu and T. Ashton Cropp*

Department of Chemistry, Virginia Commonwealth University, Richmond, 23284

Summary

A new method for protein sequence diversification is based on generating random codon mutations to an encoding DNA. This allows for the scanning of user-defined amino acid changes to any protein of interest, and is an alternative to traditional directed evolution strategies. This chapter describes the procedures required to apply this technology to any protein of interest. The resulting libraries can then be screened for new or improved protein function.

Keywords

directed evolution; protein mutagenesis; green fluorescent protein; library

1. Introduction

Nearly all random protein diversification approaches rely on generating single nucleotide changes in an encoding DNA sequence. While this is quite easy to do in the lab using methods such as error-prone PCR(1) and/or DNA shuffling(2), such changes are limited by the redundancy of the genetic code. Indeed many nucleotide changes do not result in protein changes and simply dilute out functional diversity. Likewise, there are many amino acid substitutions that require three consecutive nucleotide changes and are thus much less likely to occur during random mutation. We recently reported(3) an alternative approach that randomly changes DNA sequence “codon at-a-time” rather than “nucleotide at-a-time”, which ensures a high percentage of functional diversity. Importantly the codon can be chosen as a single amino acid or group to offer precise diversity. Moreover the method is easily adaptable to multiple codons, for example “two codons at-a-time” libraries. The methodology is complementary to other methods and, depending on the experiment, might even be combined with, for example, DNA shuffling to further expand protein sequence space.

Multi-codon scanning mutagenesis is based on the development of an asymmetric mini-Mu transposon. Transposon mutagenesis is used to essentially deliver randomly placed primer binding sites to facilitate polymerase chain reactions. The overall method, which is outlined in Figure 1, consists of cloning a gene of interest into a special targeting plasmid, pIT-GOI. This template is then subjected to a round of transposon mutagenesis to create a library of

*To whom correspondence should be addressed. Tel:(804)828-3597; tacropp@vcu.edu.

new plasmids. These then serve as a template for two inverse-PCR reactions which add, and then remove, DNA sequence scars. The final result is a library of new expression vectors, each containing a new gene with one, two, or three new in-frame codon mutations.

Depending on the experiment design, generating such libraries can be accomplished in approximately three weeks. Below outlines the experimental procedures required using the model protein superfolder green fluorescent protein (sfGFP) as an example.

2. Materials

1. Intein targeting plasmid (pIT) (3) (*see* Note 1 and ²).
2. pUC18-transposon (3) (*see* Note 3).
3. Gene of interest (*see* Note 4 and ⁵).
4. Engineered pTrcHisA vector (Invitrogen Corporation, Carlsbad, CA) (3) or other appropriate expression vector (*see* Note 6)
5. .HyperMu MuA transposase and 10× transposition reaction buffer (Epicentre Biotechnologies, Madison, WI). Store at −20 °C.
6. 10 mM Tris-HCl buffer, pH 8.5 at 25 °C.
7. TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.5 at 25 °C.
8. 500 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 at 25°C.
9. 50× TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid and 100 mL 500 mM EDTA (pH 8.0 at 25°C) and distilled water up to 1 L.
10. Primers
 - Forward and reverse primers for cloning the gene of interest into pIT vector (*see* Note 7).
 - Forward and reverse primers for cloning the gene of interest with random transposon insertions into a desired expression vector.

¹pIT-sfGFP (3) plasmid and other necessary DNA stocks can be sent upon request.

²pIT vector contains a DNA sequence upstream fused to the gene of interest. This DNA sequence encodes the “head” reporter tatSS signal peptide and the N-terminal domain of *Saccharomyces cerevisiae* vacuolar membrane ATPase (VMA) intein. The tatSS signal peptide will direct a fused peptide or protein into the periplasm of Gram negative bacteria, which is necessary for the function of many proteins such as β-lactamase. The VMA intein N-terminal domain (VMAI-N) is part of a split intein splicing system, the function of which requires the insertions of asymmetric transposon carrying the VMA intein C-terminal domain (VMAI-C). Importantly, the leading peptide (tatSS+VMAI-N) usually disrupts the native function of a fused protein. Hence, activity assay can not be directly performed in the context of pIT vector.

³Prior to transposition reaction, the asymmetric transposon is released from the pUC18-transposon vector by *Bgl* II digestion. This creates a 5'-GATC overhang at each end of transposon that can increase the efficiency of transposition (4).

⁴*Bpm* I, *Bsg* I, *Nhe* I and *EcoR* I restriction sites should be removed from the gene of interest to accommodate subsequent processes.

⁵It is advisable to optimize the codons in the gene of interest to accommodate protein expression in host cells.

⁶This methodology has been successfully applied on pTrcHisA vector (3). If another expression vector is used, some subsequent experimental conditions, such as PCR reactions, may need to be re-optimized. *Bsg* I and *Bpm* I sites should be removed from this vector to accommodate subsequent processes.

⁷The forward primer should contain an *Nhe* I site for the cloning. The nucleotides in the primers should be adjusted such that the gene of interest will be in frame fused with the leading peptide. The reverse primer should contain an *EcoR* I site after stop codon.

- Primers for EI-PCR reactions (*see* Table 1 for sequence)

Dissolve primer stocks in TE buffer at a final concentration of 100 μ M. Store the stock primers at -20°C and dilute to 10 μ M prior to use.

11. dNTPs stock solution, 10 mM each of dATP, dTTP, dGTP and dCTP. Store as 20 μ L aliquots at -20°C .

12. Phusion DNA polymerase and 5 $\times$ HF Phusion buffer (New England Biolabs, Ipswich, MA). Store at -20°C .

13. FastDigest *Bpm* I (Fermentas, Glen Burnie, MD), *Bsg* I (NEB), *Nhe* I, *EcoR* I and other desired restriction enzymes. Store at -20°C .

14. Large (Klenow) fragment of DNA polymerase I (NEB). Store at -20°C .

15. T4 DNA ligase (Fermentas). Store at -20°C .

16. 1% (w/v) agarose gels: add 1 g agarose to 100 mL 1 $\times$ TAE. Microwave to thoroughly melt prior to use (*see* Note 8).

17. QIAquick PCR purification kit (Qiagen, Valencia, CA).

18. QIAEX II gel extraction kit (Qiagen, Valencia, CA).

19. Chemically or electro-competent *Escherichia coli* DH10B cells. Store at -80°C .

20. 1000 $\times$ antibiotic stock solutions: ampicillin (100 mg/mL) and kanamycin (50 mg/mL). Store as 1 mL aliquots at -20°C .

21. 1000 $\times$ inducing reagent for protein expression, such as 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG). Store as 1 mL aliquots at -20°C .

22. Luria Bertani (LB) medium (sterilized): 10 g tryptone, 5 g yeast extract, 10 g sodium chloride and distilled water up to 1 L.

23. SOC medium (sterilized): 20 g tryptone, 5 g yeast extract, 10 mM sodium chloride, 25 mM potassium chloride and distilled water up to 1 L. Autoclave and then add sterile magnesium chloride to 10 mM and glucose to 20 mM.

24. 2 $\times$ YT medium (sterilized): 16 g tryptone, 10 g yeast extract, 5 g sodium chloride and distilled water up to 1 L, pH 7.2.

25. LB agar plates with appropriate antibiotics.

⁸Cautions should be taken if agarose DNA gels are to be stained by ethidium bromide.

3. Methods

3.1. Generation of pIT-GOI plasmid containing gene of interest

1. Digest 2 µg of the pIT-sfGFP (3) vector using *Nhe* I/*Eco*R I. Gel-purify the 3.2 Kbp fragment corresponding to pIT backbone using QIAEX II gel extraction kit (*see* Note 9).
2. Amplify the gene of interest using PCR to introduce *Nhe* I and *Eco*R I sites at the 5' and 3' ends, respectively. Ensure that the gene of interest is in-frame with the targeting sequence of pIT- vector backbone, as indicated in Figure 1.
3. Purify the PCR product using with the QIAquick PCR purification kit.
4. Digest the PCR product using *Nhe* I/*Eco*R I and gel-purify the digestion product using QIAEX II gel extraction kit.
5. Ligate the purified digestion products of pIT vector and gene of interest using T4 DNA ligase (*see* Note 10). Name the recombinant plasmid as pIT-GOI.

3.2. Transposition reaction

1. Prepare asymmetric transposon DNA by digesting pUC18-transposon plasmid with *Bgl* II as follows: 2 µg DNA, 10 U of *Bgl* II (Fermentas) and 1×buffer O (Fermentas) in a 50 µL solution (*see* Note 11). Incubate the reaction at 37 °C for 5 h.
2. Purify the 1.3 Kbp fragment using QIAEX II gel extraction kit (Qiagen) and determine its concentration by comparison with a DNA standard. Store the purified transposon DNA at −20 °C prior to use.
3. Perform a 20 µL transposition reaction as follows: 450 ng of pIT-GOI plasmid DNA, 1.3 molar excess of asymmetric transposon DNA (*see* Note 12), 1 unit of HyperMu MuA transposase (Epicentre), 50 mM Tris-acetate, pH 7.5, 150 mM potassium acetate, 10 mM magnesium acetate and 4 mM spermidine. Incubate the reaction at 30 °C for 4 hours. Stop the reaction by addition of 2 µL 0.1% SDS, followed by heat-inactivation at 70 °C for 10 min. Cool the reaction on ice.
4. Transform each 1 µL of transposition product into 50 µL of electro-competent *E. coli* DH10B cells (*see* Note 13 and ¹⁴). Recover the cells in 500 µL SOC at 37 °C for 1 h. Plate

⁹When gel slices are incubated in solubilization buffer (Qiagen), temperatures above 50 °C are not recommended. Higher temperatures denature double-stranded DNA and reduce the yield of gel-purification.

¹⁰It is important to ensure that the gene of interest is placed in frame with the N-terminal fusion peptide carried in pIT vector.

¹¹It is not recommended to use excess DNA (e.g., > 2 µg) in *Bgl* II digestion. In our experience, excess DNA may result in incorrect or incomplete digestion that will reduce the efficiency of transposon integration.

¹²Transposon DNA should be maintained at a molar excess (*versus* pIT-GOI plasmid). Higher transposon concentrations may lead to the formation of unstable pIT-GOI-transposon complexes containing multiple transposon insertions. These unstable complexes will compromise transposition reactions.

¹³The time constant for each electroporation experiment should be monitored. A time constant above 4.0 ms is preferred for efficient transformation. Low time constant (below 3.0 ms) is indicative of too little resistance in medium and is frequently caused by too much residual salts. If this is the case the ligation product can be purified by spin column or ethanol precipitation. Alternatively, less ligation product can be used (for example, adding 0.5 µL reaction to per 50 µL competent cells).

¹⁴When using competent cells with a competency of 5×10⁷ CFU/µg DNA (CFU, colony forming units), 20 µL transposition product typically results in >10,000 colonies. If desired, multiple 20 µL transposition reactions can be performed at once, pooled, ethanol precipitated, and then transformed.

transformed cells on LB agar supplemented with 50 µg/mL kanamycin and 40 µg/mL ampicillin (*see* Note 15). Grow the plates at room temperature for 48 h (*see* Note 16 and ¹⁷).

5. For a gene of L bps in length, collect at least $3 \times (L+1,500)$ colonies from the transposition reaction (*see* Note 18). Resuspend the collected cells in LB medium supplemented with 50 µg/mL kanamycin and 40 µg/mL ampicillin. Save at least 5 tubes of 1 mL stock cells with an OD₆₀₀ of ~1.0 in 10% glycerol at -80 °C (*see* Note 19). Extract the plasmid DNA from the remaining cells to obtain the pIT-GOI-transposon library.

3.3. Transfer of gene of interest with random transposon insertions into pTrcHisA vector

1. Digest 2 µg of the transposon library DNA using *Nhe* I/*Eco*R I.
2. Use QIAEX II gel extraction kit to gel purify the DNA fragment corresponding to the gene of interest with transposon insertions (*see* Note 20).
3. Ligate the purified DNA fragment into pTrcHisA pre-cut with *Nhe* I/*Eco*R I using T4 DNA ligase.
4. Transform the ligation products into electro-competent *E. coli* DH10B cells. Recover the cells in 500 µL SOC at 37 °C for 1 h and then plate the cells on LB agar supplemented with 100 µg/mL ampicillin.
5. Collect $3 \times L$ colonies (*see* Note 21). Resuspend the collected cells in LB medium supplemented with 100 µg/mL ampicillin. Save at least 5 tubes of 1 mL stock cells with an OD₆₀₀ of ~1.0 in 10% glycerol at -80 °C. Extract the plasmid DNA from the remaining cells to obtain the pTrcHisA-GOI-transposon library.

¹⁵The asymmetric transposon carries a reading-frame selection marker that consists of VMA intein C-terminal domain (VMAI-C) and TEM-1 β-lactamase (BLA). This fusion peptide has a stop codon after BLA encoding sequence but does not bear a start codon, such that its expression is strictly dependent on the insertion of transposon into an open reading frame. When the asymmetric transposon inserts in frame with the gene of interest (as well as with the leading peptide), VMAI-N from the pIT vector will be in the same reading-frame with VMAI-C from the asymmetric transposon. In this case, VMA intein will self-splice and assemble the tatSS signal peptide and BLA, conferring cells ampicillin resistance.

¹⁶Incubation at a reduced temperature is critical for a non-biased intein-mediated splicing (5) High temperatures may disrupt the folding of VMA intein when a destabilizing fusion peptide is introduced between VMAI-N and VMAI-C by transposon insertion. Compromised intein stability at high temperatures could be the main cause of a biased pattern of mutations.

¹⁷The randomness of transposon insertions can be verified by restriction digestion or sequencing. The asymmetric transposon used in this study contains two closely placed *Mly* I sites at the 3' end (~50 bp apart). Individual colonies from the transposon library can be digested using *Mly* I together with another unique restriction site on the pIT-GOI plasmid. Depending on the position of transposon insertion, two DNA fragments with variable sizes should be expected (3).

¹⁸To calculate the required colonies for a full coverage of library, the number of allowed transposon insertion sites and the site preference of transposase (6) must be considered. The pIT vector backbone roughly contains 1,500 possible sites for transposon insertions (2,900 of total length minus 600 bps of replication origin and 800 bps of kanamycin resistance gene). There are thus $(L+1,500)$ possible insertion sites in pIT-GOI plasmid. Three-fold redundant colonies, $3 \times (L+1,500)$, are required to cover 95% of all the possible insertion events, assuming a non-biased insertion pattern (7). To compensate for the site preference of insertions, we assume that additional 3-fold redundancy is sufficient (8). Therefore, $3 \times 3 \times (L+1,500) = 9 \times (L+1,500)$ colonies are sufficient to give a 95% coverage of all the transposon insertions. Among these, only one third is expected to survive ampicillin selection due to the reading-frame selection system. Therefore, $3 \times (L+1,500)$ colonies should be collected at this point.

¹⁹For future experiments, the whole tube (1 mL) of stock cells should be used for inoculation to maintain the library diversity.

²⁰The transposon can insert both inside and outside of the gene of interest. In the former case, *Nhe* I/*Eco*R I digestion will result in two DNA fragments corresponding to pIT vector backbone and gene+transposon. If transposon inserts outside of the gene of interest, pIT+transposon and gene of interest bands are expected.

²¹Because transposon insertions outside of the gene of interest have been purged from the library, only $3 \times L$ colonies are required at this point to maintain a full coverage of library.

3.3. Introduction of new codons and “anchor” sequence

One, two and three codon mutations can be generated using the same pTrcHisA-GOI-transposon library DNA as the template but different primer pairs in the PCR amplification. The optimum template concentrations and annealing temperatures have been individually determined for each PCR reaction (3).

1. Set up several 100 μ L PCR reactions containing **X** pg/ μ L pTrcHisA-GOI-transposon library DNA, 0.5 μ M each of forward and reverse primers (see below), 0.2 mM dNTPs, 0.005 U/ μ L of Phusion DNA polymerase (NEB), 1 $\times$ Phusion HF buffer (NEB, providing 1.5 mM MgCl₂) and additional 2 mM MgSO₄. **X** indicates the optimum template concentrations and are 100, 125 and 200 pg/ μ L for one, two and three codon mutations, respectively (*see* Note 22). The primer pairs are FF1/ FR1, FF2/ FR2 and FF2/ FR3 for one, two and three codon mutations, respectively (*see* Table 1 for sequence).
2. Cycle the PCR reactions using the following conditions: initial denaturation at 98 °C for 2 min, 18 cycles of 98 °C for 10 s, **Y** °C for 30 s and 72 °C for 2 min (*see* Note 23), and a final extension at 72 °C for 10 min. **Y** represents the optimum annealing temperatures and are 57 °C, 59 °C and 65 °C for one, two and three codon mutations, respectively (*see* Note 22).
3. Purify the PCR product using QIAquick PCR purification kit (Qiagen).
4. Digest the purified PCR product of one codon library with *Bpm* I in a 50 μ L reaction containing 500 ng DNA, 1 U FastDigest *Bpm* I (Fermentas) and 1 $\times$ FastDigest buffer (Fermentas) (*see* Note 24). Incubate the reaction at 30 °C for 4.5 h. Heat-inactivate the reaction at 65 °C for 20 min. Digest the purified PCR product of two and three codon libraries with *Bsg* I in a 50 μ L reaction containing 500 ng DNA, 6 U *Bsg* I (NEB), 80 μ M *S*-adenosylmethionine (SAM) and 1 $\times$ buffer 4 (NEB) (*see* Note 25). Incubate the reaction at 37 °C for 4.5 h. Heat-inactivate the reaction at 65 °C for 20 min.
5. For each 50 μ L of the above heat-inactivated digestion product, add 1.7 μ L of 1 mM dNTPs and 0.5 U of Klenow fragment (NEB). Incubate the end-blunting reaction at 25 °C for 15 min (*see* Note 26). Stop the reaction (50 μ L) by adding 1.1 μ L of 0.5 M EDTA, pH 8.0, followed by heat-inactivation at 75 °C for 20 min (*see* Note 27).
6. Gel-purify the Klenow-treated digestion product using QIAEX II gel extraction kit (Qiagen).

²²It is recommended to monitor the quality of the PCR product. Due to the random transposon insertions, the “head” of a PCR product may overlap with the “tail” of another product, resulting in homologous recombination (9). This side product, shown as a smear on agarose DNA gels, should be avoided. In our experience, the side product becomes predominant once the concentration of the desired PCR product exceeds 2 ng/ μ L.

²³Phusion DNA polymerase requires 15–30 s for the extension of 1 Kbp DNA. The extension time need be adjusted according to the length of the gene of interest.

²⁴Although Fermentas provides standard versions of *Bpm* I (*Gsu* I), in our hands the FastDigest enzyme gives better cleavage efficiency.

²⁵Fresh SAM is critical for complete *Bsg* I digestion. Liquid SAM stocks can be stored at –80 °C for no longer three months.

²⁶Longer incubation time or higher temperatures are not desired, as overdigestion (such as removal of extra nucleotides) may occur in these cases.

²⁷It is important to quench the reaction first with EDTA prior to heat-inactivating the Klenow enzyme. Heat-inactivation without quenching frequently results in removal of extra nucleotides.

7. Re-circularize the purified DNA into plasmid in a 10 μ L ligation reaction containing 2.5 ng/ μ L DNA, 0.5 Weiss unit/ μ L T4 DNA ligase (Fermentas), 40 mM Tris-HCl, pH 7.8 at 25 $^{\circ}$ C, 10 mM MgCl₂, 10 mM DTT and 0.5 mM ATP (*see* Note 28). Incubate the reaction 16 $^{\circ}$ C overnight. Heat-inactivate the reaction at 70 $^{\circ}$ C for 7 min (*see* Note 29). Cool the reaction on ice.

8. Transform 1 μ L of the ligation product (*see* Note 30) into 50 μ L electro-competent *E. coli* DH10B cells. Plate the transformed cells on LB agar supplemented with 100 μ g/mL ampicillin.

9. Collect desired number of colonies to maintain library diversity (*see* Note 31). Save cell stocks as above and extract the plasmid DNA from the remaining cells to obtain the pTrcHisAGOI-anchor libraries for each of one, two and three codon scanning libraries.

3.4. Removal of primer sequence and generation of seamless, in-frame codon mutations

The second EI-PCR/digestion process is conceptually the same to the first one as described above. The goal of this process is to remove the reverse primer sequence (“anchor” sequence) left in the gene of interest from the first PCR, generating seamless codon mutations.

1. Set up several 100 μ L PCR reactions containing **X2** pg/ μ L pTrcHisA-GOI-anchor library DNA, 0.5 μ M each of forward and reverse primers (*see* below), 0.2 mM dNTPs, 0.005 U/ μ L of Phusion DNA polymerase (NEB), 1 $\times$ Phusion HF buffer (NEB, providing 1.5 mM MgCl₂) and additional 2 mM MgSO₄. **X2** represents the optimum template concentrations and are 200, 125 and 200 pg/ μ L for one, two and three codon scanning libraries, respectively. The primer pairs for one, two and three codon scanning libraries are SF0/ SR, SF0/ SR and SF1/ SR, respectively (Table 1)

2. Cycle the PCR reactions using the following conditions: initial denaturation at 98 $^{\circ}$ C for 2 min, 18 cycles of 98 $^{\circ}$ C for 10 s, **Y2** $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 2 min (*see* Note 22), and a final extension at 72 $^{\circ}$ C for 10 min. **Y2** represents the optimum annealing temperatures and are 67 $^{\circ}$ C, 63 $^{\circ}$ C and 66 $^{\circ}$ C for one, two and three codon scanning libraries, respectively.

3. Purify the PCR product using QIAquick PCR purification kit (Qiagen).

4. Digest the purified PCR product of all the three libraries with *Bsg* I as described above.

5. Treat the digestion product with Klenow fragment as described above.

²⁸It is recommended to use 0.5 mM ATP in blunt-end ligations for higher end-joining efficiency.

²⁹Heat-inactivation of T4 DNA ligase has been reported to increase the efficiency of following transformation (10). This is particularly important if electro-transformation is to be used (11). Alternatively, the ligation product can be purified using commercial spin columns.

³⁰Multiple 10 μ L ligation reactions can be performed, pooled, ethanol-precipitated and then transformed into competent cells.

³¹At this stage, the introduced codons and protein size should be taken into account for calculating the library diversity. For example, if NNK codon is being scanned through a protein consisting of 200 amino acids, 3 (redundancy factor) $\times$ 32 (NNK codon combination) $\times$ 200 = 19,200 colonies should be collected.

6. Gel-purify the Klenow-treated digestion product using QIAEX II gel extraction kit (Qiagen).
7. Ligate and transform the gel-purified product as described above.
8. Collect sufficient colonies to build the final library. Save cell stocks as above and extract library DNA for future experiments.

Acknowledgments

The authors thank the National Institutes of Health (GM084396) for financial support.

4. Notes

References

1. Liu J, Cropp TA. A method for multi-codon scanning mutagenesis of proteins based on asymmetric transposons. *Protein Eng Des Sel.* 2012; 25:67–72. [PubMed: 22184456]
2. Haapa S, et al. An efficient and accurate integration of mini-Mu transposons *in vitro*: a general methodology for functional genetic analysis and molecular biology applications. *Nucleic Acids Res.* 1999; 27:2777–84. [PubMed: 10373596]
3. Gerth ML, et al. A second-generation system for unbiased reading frame selection. *Protein Eng Des Sel.* 2004; 17:595–602. [PubMed: 15331775]
4. Kim YC, Morrison SL. N-terminal domain-deleted Mu transposase exhibits increased transposition activity with low target site preference in modified buffers. *J Mol Microbiol Biotechnol.* 2009; 17:30–40. [PubMed: 19033677]
5. Patrick WM, et al. User-friendly algorithms for estimating completeness and diversity in randomized protein-encoding libraries. *Protein Eng.* 2003; 16:451–7. [PubMed: 12874379]
6. Poussu E, et al. Probing the alpha-complementing domain of *E. coli* beta-galactosidase with use of an insertional pentapeptide mutagenesis strategy based on Mu *in vitro* DNA transposition. *Proteins.* 2004; 54:681–92. [PubMed: 14997564]
7. Williams R, et al. Amplification of complex gene libraries by emulsion PCR. *Nat Methods.* 2006; 3:545–50. [PubMed: 16791213]
8. Michelsen BK. Transformation of *Escherichia coli* increases 260-fold upon inactivation of T4 DNA ligase. *Anal Biochem.* 1995; 225:172–4. [PubMed: 7778774]
9. Ymer S. Heat inactivation of DNA ligase prior to electroporation increases transformation efficiency. *Nucleic Acids Res.* 1991; 19:6960. [PubMed: 1762931]

Figure 1.

(a) Depiction of the overall procedure described in this protocol. (b) pIT-sfGFP (available upon request) is used to generate pIT-GOI by cloning gene of interest into the *NheI* and *EcoRI* sites. The reading frame of the target protein must be maintained as shown. (c) The transposon (available upon request) is made ready for the reaction by *Bgl*II, *Bam*HI digestion.

Table 1

List of primers for EI-PCR reactions

Name	Sequence (<i>Bsg</i> I and <i>Bpm</i> I sites underlined)	Use
FF1	TACTTTTCTGGAGCCGTCGGA	First PCR, Fwd, remove one codon.
FF2	TTTTTCGTGTGCAGTCGGA	First PCR, Fwd, remove two codons.
FR1 ^a	MNNAATCAACGACTTTGCGCCGCTAAG	First PCR, Rev, introduce one codon.
FR2 ^a	MNNMNNNAATCAACGACTTTGCGCCGCTAAG	First PCR, Rev, introduce two codons.
FR3 ^a	MNNMNNMNNNAATCAACGACTTTGCGCCGCTAAG	First PCR, Rev, introduce three codons.
SF0	TAACGTGCAGTTACAAGTCGTTGATT	Second PCR, Fwd, cleave to new codons.
SF1	CATCGTGCAGATGCGCCGCTAAG	Second PCR, Fwd, remove one codon.
SR	CTTCGTGCAGTAAATGCGCCGCTAAG	Second PCR, Rev, cleave to target gene.

^a Note: These reverse primers will deliver one to three NNK (N: A, T, G, C; K: G, T) degenerate codons into the gene of interest. MNN (M: A, C) codons, the reverse complementary sequence of NNK, are designed in these primers because the new codons will be delivered in a reverse complementary manner.