DOI 10.1002/biot.200800198 Biotechnol. J. 2009, 4, 544–553

Technical Report

Sticky PCR: A PCR-based protocol for targeted protein engineering

Montarop Yamabhai

School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand

This article describes a simple but powerful PCR-based protocol for the generation of cohesive ends on linear DNA fragments, permitting the precise engineering of DNA constructs for a variety of applications. These include the introduction of deletion mutations, domain swapping, creating hybrid DNA fusions, or targeted protein engineering. This novel method can also facilitate the cloning of large or complex DNA fragments into a relevant cloning vector independent of the use of internal restriction endonuclease sites. The protocol involves the amplification of the required fragments by polymerase chain reaction through the use of two sets of overlapping desalted oligonucleotide primers. The subsequent mixing, denaturation and re-annealing of these products present correct cohesive terminal ends for ligation. There is no requirement for special vectors, enzymes or bases, suggesting that this protocol provides a unique way of engineering constructs in a rapid and cost-effective way for specific applications, such as precise deletion or swapping of various domains of the epidermal growth factor receptor to determine their role in membrane localization.

Received 11 September 2008
Revised 12 February 2009
Accepted 17 February 2009

Keywords: Cloning · Mutagenesis · PCR · Protein engineering

1 Introduction

Protein engineering is essential for the tailoring of proteins, with conformational changes introduced through the use of three approaches: random design, rational design, and semi-rational design [1]. All of these techniques involve the manipulation of the protein-encoding DNA sequence through the use of standard molecular biology techniques. Random design is useful for the engineering of proteins where there is limited knowledge of the structure-function relationship [2], whereas rational and semi-rational methodologies require an understanding of the structure and/or function of the protein of interest [1, 3].

The key protocol for the rational design of enzymes and other proteins is site-directed mutagenesis [4], where specific base pair(s) of the protein-encoding gene are identified and modified through the use of overlapping polymerase chain reaction (PCR) or commercially available strategies [5]. This is particularly useful for the targeting of short DNA sequences, but is problematic when longer sequence alterations are required for protein engineering purposes. An alternative protocol is the use of restriction endonuclease-based digestion and cloning. This relies heavily on the availability of cohesive restriction endonuclease sites for facilitating ligation to a relevant cloning/expression vector, meaning that a carefully designed strategy is of paramount importance. A third alternative is the use of synthetic oligonucleotides which may be custom synthesized, although this is also problematic when a large DNA-encoding sequence is selected for modification [5].

Correspondence: Dr. Montarop Yamabhai, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima, 30000, Thailand
E-mail: montarop@sut.ac.th, montarop@gmail.com
Fax: +66-44-224150

Abbreviations: CR2, cysteine-rich 2; **EGFR**, epidermal growth factor receptor; **GFP**, green fluorescent protein; **LDLR**, low-density lipoprotein receptor; **scFv**, single-chain variable fragment

Here, I discuss a novel alternative for permitting the precise engineering of proteins for application in a variety of biotechnological processes. These modifications are permitted through the use of 'sticky PCR', which also enables the analysis of the functional properties of individual domains/motifs within a protein of interest by facilitating the deletion or swapping of individual amino acids and enabling the encoded protein to be phenotypically analyzed. This provides an alternative to the more traditional method of restriction mapping. In this article, the application of this method for permitting the precise deletion of various domains of the epidermal growth factor receptor (EGFR), or swapping its transmembrane domain with that of low-density lipoproteins (LDLR) to investigate their role in membrane targeting, were demonstrated. Furthermore, I also discuss how this protocol may be used for the subcloning of large or complex DNA fragments (inclusive of antibody-encoding sequences) that contain internal restriction sites into any commonly used vector (*i.e.* pEFGP or pFLAG-based).

2 Materials and methods

Oligonucleotide primers were purchased from Sigma-Aldrich (St. Louis, MO). Standard-quality primers (desalted) were found to be suitable for use in this experimental work, although, where necessary, PAGE-purified primers were selected. Most of the primers were 5′ phosphorylated (5′ PO_4) to increase the ligation efficiency. A list of all primers used in this study is shown in Table 1. All restriction endonucleases were purchased from New England Biolabs (MA, USA). PCR-based amplifications were performed with *Pfu* DNA polymerase (Promega, WI, USA). The cloning vector pEGFP-N1 was purchased from Clontech (CA, USA) and pAPHisFlag [6] was a modified version of pFLAG-CTS (Sigma-Aldrich). The *Escherichia coli* strains DH5αF′ (Life Technologies) and Top10 (Invitrogen, CA, USA) were used for cloning and propagation.

2.1 PCR amplification

All PCR reactions were performed in a thermocycler (MJ Research, Pierce, Perkin Elmer) and in accordance with the manufacturer's recommendations. The concentration of the template selected was between 50 and 100 ng, with primer concentrations ranging from 0.1 to 1.0 μM. PCR reactions (50 μL) were also performed in the presence of dNTPs (10 mM), 1.25 U of *Pfu* polymerase and a suitable reaction buffer (supplied with the polymerase). All amplifications were performed as follows: initial DNA denaturation at 95°C for 0.5–1 min followed by 30 cycles of denaturation at 95°C for 0.5–1 min, annealing at 50–65°C for 30 s and extension at 72°C for 2 min for every 1 kb. The final extension step was for 10 min at 72°C. All PCR products were separated on a 1% w/v agarose gel stained with 1 μg/mL ethidium bromide and visualized under UV irradiation. PCR products were purified by commercially obtained kits (Qiagen, Germany) for further re-annealing.

2.2 Denaturation and re-annealing of the PCR products

Approximately equal amounts of PCR products were mixed together in a PCR tube and heated at 95°C for 5 min, and then the denatured products were briefly mixed in a vortex mixer. The re-annealing was done in a thermal cycler machine by reducing the temperature slowly from 95 to 25°C. There is no specific protocol for re-annealing; the total time for re-annealing is usually 2–3 h. The annealing could also be done by moving the PCR tubes from a number of different heated water baths, such as 80, 65, 50, and 37°C.

2.3 Ligation into expression vector

Ligation reactions were performed according to the recommendations outlined in references [5, 7], with the relevant restriction endonuclease used to digest the vector and a molar ratio of linearized vector to re-annealed insert of approximately 1:15. The amount of vector used for each ligation ranged between 20 and 200 ng. Ligations were typically performed for 1–2 h at 25°C or for 18 h at 16°C in the presence of T4 DNA ligase in a final volume of 10–15 μL. T4 DNA ligase was heat-inactivated (65°C for 15 min) before transformation into either electrocompetent (1–2 μL of ligation reaction) or chemically competent (5–10 μL of ligation reaction) *E. coli* cells [7]. Transformed cells were propagated for 1 h before spreading onto Luria-Bertani (LB) agar (tryptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L; bacto agar 15 g/L) containing 100 μg/mL ampicillin (for pFLAG-based vector) or 50 μg/mL kanamycin (for pEGFP vector).

2.4 Three-component ligation

The three-component ligation mixture consisted of: (1) linearized vector digested with two corresponding restriction enzymes; (2) the N-terminal re-annealed insert cut with the first enzyme, and

Table 1. List of primers used in this study.

Name	Sequence (5'→3')	Construct
fw-N	CTGTGCCTCGAG*CGCCACC*ATGCGACCCTCCGGGACG	EGFR mutants (Fig. 2)
N-long.a	GCAAAGGAAGACGAGGAGCACGATGGGGAGGACAATGGACAGAGCCCTCACGGACGGG ATCTTAGGCCCATTCGTTGG	EGFR domain swapped (Fig. 2A)
N-short.a	GATGGGGAGGACAATGGACAGAGCCCTCACGGACGGGATCTTAGGCCCATTCGTTGG	EGFR domain swapped (Fig. 2A)
C-long.a	GTGCTCCTCGTCTTCCTTTGCCTGGGGGTCTTCCTTCTATGGCGAAGGCGCCACATCGTTCGG	EGFR domain swapped (Fig. 2A)
C-short.a	CTGGGGGTCTTCCTTCTATGGCGAAGGCGCCACATCGTTCGG	EGFR domain swapped (Fig. 2A)
N-long.b	CCCCACCATCCCAGTGGCGATGGATGAAATTATCACATCTCCATCACTTATCTC	EGFR domain deletion (Fig. 2B)
N-short.b	TGAAATTATCACATCTCCATCACTTATCTC	EGFR domain deletion (Fig. 2B)
C-long.b	TCCATCGCCACTGGGATGGTGGGG	EGFR domain deletion (Fig. 2B)
C-short.b	GCCCTCCTCTTGCTGCTGGTG	EGFR domain deletion (Fig. 2B)
rv-C	CTGTGCGGTACCCATGCTCCAATAAATTCACTGC	EGFR mutants (Fig. 2)
fw-long.a	AATTC*CGCCACC*ATGGCTCAGTTTGGAACTCCG	*X. laevis* Ints cloning (Fig. 3A)
fw-short.a	C*CGCCACC*ATGGCTCAGTTTGGAACTCCG	*X. laevis* Ints cloning (Fig. 3A)
rv-long.a	GATCCTATATATTTATATATTTTACATTCATATCAGGACAAAATCGAGGTGC	*X. laevis* Ints cloning (Fig. 3A)
rv-short.a	CTATATATTTATATATTTTACATTCATATCAGGACAAAATCGAGGTGC	*X. laevis* Ints cloning (Fig. 3A)
fw-long.b	TCGAGGCGGCCCAGCCGGCCATGGCC	Human scFv cloning (Fig. 3B)
fw-short.b	GGCGGCCCAGCCGGCCATGGCC	Human scFv cloning (Fig. 3B)
rv-long.b	GTACCGTGATGGTGATGATGATGTGCGGCCGC	Human scFv cloning (Fig. 3B)
rv-short.b	CGTGATGGTGATGATGATGTGCGGCCGC	Human scFv cloning (Fig. 3B)

Sequences that are part of restriction sites or of the LDLR transmembrane domain are underlined. The Kosak sequence is italicized. *X. laevis* Ints is an Intersectin mRNA (*ints-A*) of the African clawed frog *Xenopus laevis*.

(3) the C-terminal re-annealed insert cut with the second restriction endonuclease. Equal amounts of the first and second re-annealed inserts were used, with the approximate molar ratio of linearized vector and both re-annealed inserts being 1:8:8. Subsequent ligation steps were as discussed above.

3 Results

3.1 Precision mutagenesis

A key contributory factor for successfully introducing mutations using the 'sticky PCR' technique was the availability of carefully designed oligonucleotide primers. As demonstrated in this article, a number of EGFR mutations were precisely generated as shown in Fig. 1. These types of mutations are difficult to generate using commercially obtained mutagenesis kits or other conventional methods as the site of the mutagenized domain is relatively large. A schematic representation of the precise mutagenesis strategy used to replace the transmembrane domain of the EGFR by a domain from an LDLR [8] is shown in Fig. 2A. Two sets of sticky primers were designed for the generation of two sticky PCR products. The first set was for the N terminus of the gene (I), and the second primer

set (II) was for the C terminus. For the N-terminal DNA fragments, the forward primer (fw-N) was designed according to the conventional PCR cloning method, where extra bases containing *Xho* I recognition sites for cloning into pEGFP-N1 were incorporated. The reverse (anti-sense) primers consisted of two primers that were complementary to the middle of the gene for the generation of sticky ends. Primer N-long.a consisted of the sequence that was complementary to the 27 bases of the extracellular component of the EGFR and an additional 51 bases encoding the sequence of the LDLR transmembrane domain. Primer N-short.a was similar to primer N-long.a, but was 21 bp shorter. The sticky PCR product for the N terminus of the construct was generated from the amplification and subsequent re-annealing of the product amplified from primers fw-N & N-long.a and fw-N & N-short.a. For the C-terminal DNA fragments, the reverse (anti-sense) primer (rv-C) was designed according to the conventional PCR cloning method where extra bases containing *Kpn* I recognition sites for cloning into pEGFP-N1 were incorporated. The forward primers consisted of two primers that were complementary to the middle of the gene for the construction of sticky ends. Primer C-long.a consisted of the sequence that was complementary to the 21 bases of the cytosolic part of the EGFR and 42 additional bases encoding the sequence of the transmembrane domain of the LDLR for replacement. Primer C-short.a was similar to primer B, but was 21 bp shorter. Sticky PCR products of the C terminus were generated from PCR amplification and re-annealing using primers C-long.a & rv-C and

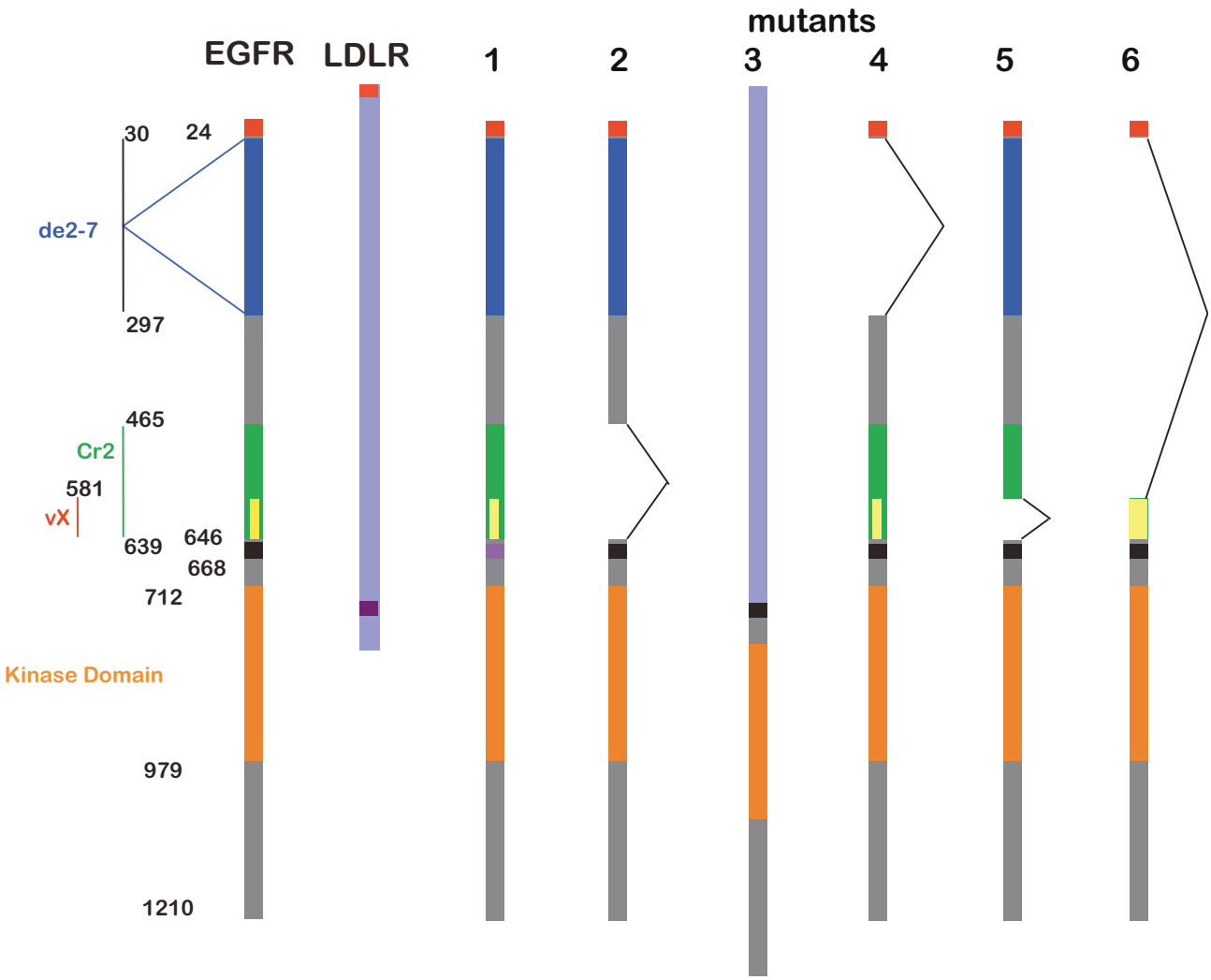

Figure 1. Various targeted EGFR mutations generated by the sticky PCR-based protocol. Various domains of the EGFR are indicated by the color code. Black indicates the transmembrane domain, green indicates the CR2 domain, blue indicates the de2–7 domain, yellow indicates the vX domain, orange indicates the kinase domain. The LDLR (purple) and its transmembrane domain (dark purple) are also shown. The figure is drawn to scale. The primer design for the construction of the first two mutants is explained in detail in this article.

primers C-short.a & rv-C. After PCR amplification and re-annealing, the sticky PCR products of the N terminus and the C terminus were digested with *Xho* I or *Kpn* I restriction enzymes, respectively. The PCR products were then purified, combined and ligated with linearized vector using T4 DNA ligase. Thus, the ligation reaction consisted of three components: (1) linearized vector, (2) sticky products of the N-terminal part, and (3) sticky products of the C-terminal part. This strategy can also be applied for creating fusions between different protein domains (*i.e.* mutant no. 3 in Fig. 1).

For the generation of precise deletion mutations, a schematic representation of the precise mutagenesis strategy used for the construction of an EGFR mutant without the cysteine-rich 2 (CR2) domain is demonstrated in Fig. 2B. Two sets of sticky primers were designed for the generation of

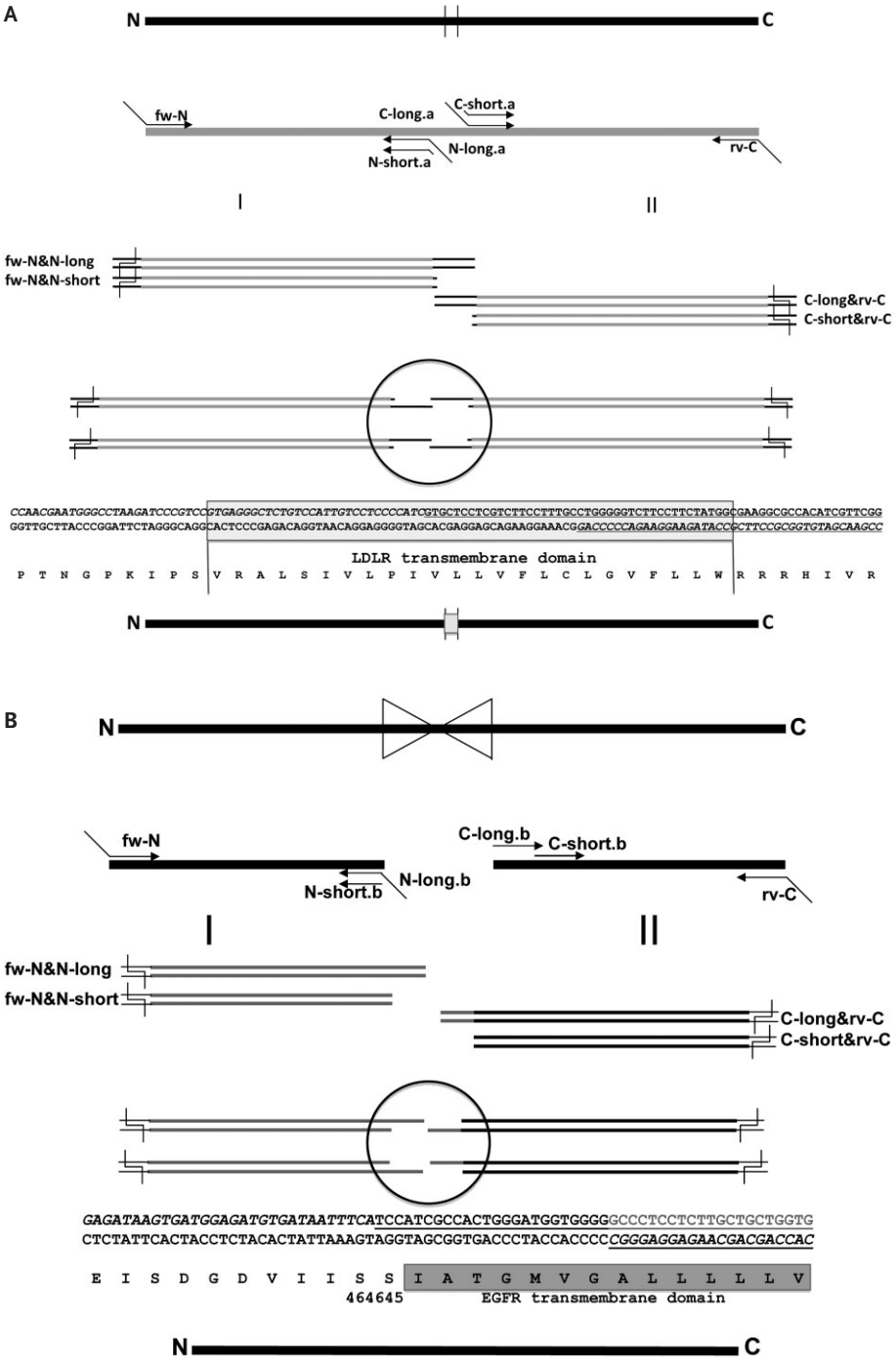

Figure 2. Primer design for precise mutagenesis. Two types of precision mutagenesis using the sticky PCR strategy are demonstrated: domain swapping (Fig. 2A) and domain deletion (Fig. 2B). Primer design and PCR products before and after re-annealing are illustrated. Annotated sequences of the ligated sticky PCR products in the middle of the gene are shown. Each font type represents different primers used; underlined fonts represent primers for C-terminal parts, and italicized fonts represent shorter primers.

two sticky PCR products. The first set (I) was for the N terminus of the gene, and the second primer set (II) was for the C terminus. For the N-terminal DNA fragments, the forward primer (fw-N) was designed as described above. The reverse (anti-sense) primers consisted of two primers that were complementary to the middle of the gene for the generation of sticky ends. Primer N-long.b consisted of the sequence that was complementary to the 27 bases encoding amino acids 455–464 and an additional 24 bases encoding the amino acid sequence 645–652. Primer N-short.b was similar to N-long, but had no additional 24 bases. The sticky PCR product for the N-terminus of the construct was generated from the amplification and subsequent re-annealing of the product amplified from primers fw-N & N-long.b and primers fw-N & N-short.b. For the C-terminal DNA fragment, the reverse (anti-sense) primer (Rv-C) was designed as described above. The forward primers consisted of two primers that were complementary to the middle of the gene for the construction of sticky ends. Primer C-long.b consisted of the sequence that was complementary to the 24 bases encoding the amino acid sequence 645–652, whereas primer C-short.b encoded the amino acid sequence 653–659. Sticky PCR products of the C terminus were generated from PCR amplification and re-annealing using primers C-long.b & Rv-C and primers C-short.b & Rv-C. The re-annealed products were then subjected to the three-component ligation as described above. Automated DNA sequencing was done to verify all of these constructs. This strategy has been successfully used to generate various EFGR mutations to study the mechanism of membrane localization [8].

3.2 Directional cloning into a regular vector

In addition to precision engineering of a protein as described above, the sticky PCR strategy is also a simple and powerful method for cloning large or complex DNA inserts. Depending on the primers selected, it is possible to generate all four combinations of cohesive overhangs in the insert, namely, 5′ and 5′, 3′ and 3′, 5′ and 3′, 3′ and 5′. Two pairs of oligonucleotide primers were required for each restriction site, one for the longer protruding end and the second one for the shorter end. It is important to determine the nature of restriction digestion to verify whether a 5′ or 3′ overhang is generated, as this would determine the pair of primers selected for the amplification step. Figure 3 illustrates two examples of primer design for cloning into *Eco* RI/*Bam* HI (Fig. 3A) and *Xho* I/*Kpn* I (Fig. 3B). After restriction digestion, *Bam* HI, *Eco* RI, and *Xho* I generate 5′ overhangs, whereas *Kpn* I presents a 3′ overhang. Thus, the final products that would be compatible with the linearized vector would be different. After the correct set of primers was used to amplify two PCR products, the PCR products were cleaned, and equal molar amounts of the PCR products would then be mixed, re-annealed, and used for the subsequent ligation step.

In this study, two types of cloning were demonstrated. The first experiment demonstrated the cloning of a relatively long insert (3813 bp) of *Xenopus laevis* Intersectin cDNA [9] containing two internal *Bam* HI recognition sites at positions 3516 and 3597, which would not allow cloning by conventional methods. Primers were designed for cloning the *Xenopus* Intersectin cDNA (GenBank accession number NM_001087486) into the *Eco* RI and *Bam* HI cloning sites of pEGFP-N1 to generate the green fluorescent protein (GFP)-fusion construct (Fig. 3A). In the second experiment, the complex DNA fragments of a single-chain variable fragment (scFv) against Aflatoxin B1 from phage display libraries (Yamo1 [10] and Tomlinson I+J, MRC HGMP Resource Centre, Cambridge, UK) were subcloned (*Xho* I and *Kpn* I) into the pAP-HisFlag expression vector for generating scFv-alkaline phosphatase fusions and used as one-step detection probes [6, 11] (Fig. 3B).

3.3 Cloning efficiency

An illustration of the sticky PCR-based protocol for facilitating targeted protein engineering and molecular cloning is illustrated in Fig. 4. The cloning efficiencies of different constructs are summarized in Table 2. The efficiency of the three-component ligation is less than that of normal ligation, but sufficient to obtain the right construct from a single experiment. The results shown in Table 2 also compared certain factors that could affect the cloning efficiency, including the 5′ PO_4 group on the insert, the purity, and the transformation method. We found that 5′ PO_4 and PAGE-purified primers could increase the transformation efficiency; however, there is no significant difference in the number of corrected constructs when using either chemically competent or electrocompetent cells for transformation. These results are in accordance with other cloning procedures [5].

4 Discussion

'Sticky' PCR provides an easy and powerful method for protein engineering, especially for the generation of mutants that are more problematic to

Biotechnol. J. 2009, 4, 544–553

generate through the use of more conventional protocols which require prior restriction mapping knowledge. The implementation of this protocol successfully requires the careful design of primers for the amplification of blunt-ended products by proof-reading DNA polymerase, such as *Pfu* polymerase. The use of this method still requires restriction enzymes for cloning. However, since half of the genes will be digested with corresponding enzymes separately, it is possible to have internal restriction sites that appear in the multiple cloning sites of the selected vector. Since the efficiency of this type of cloning is not as high as for a normal ligation reaction, a greater number of colonies are needed for screening. Moreover, it is postulated

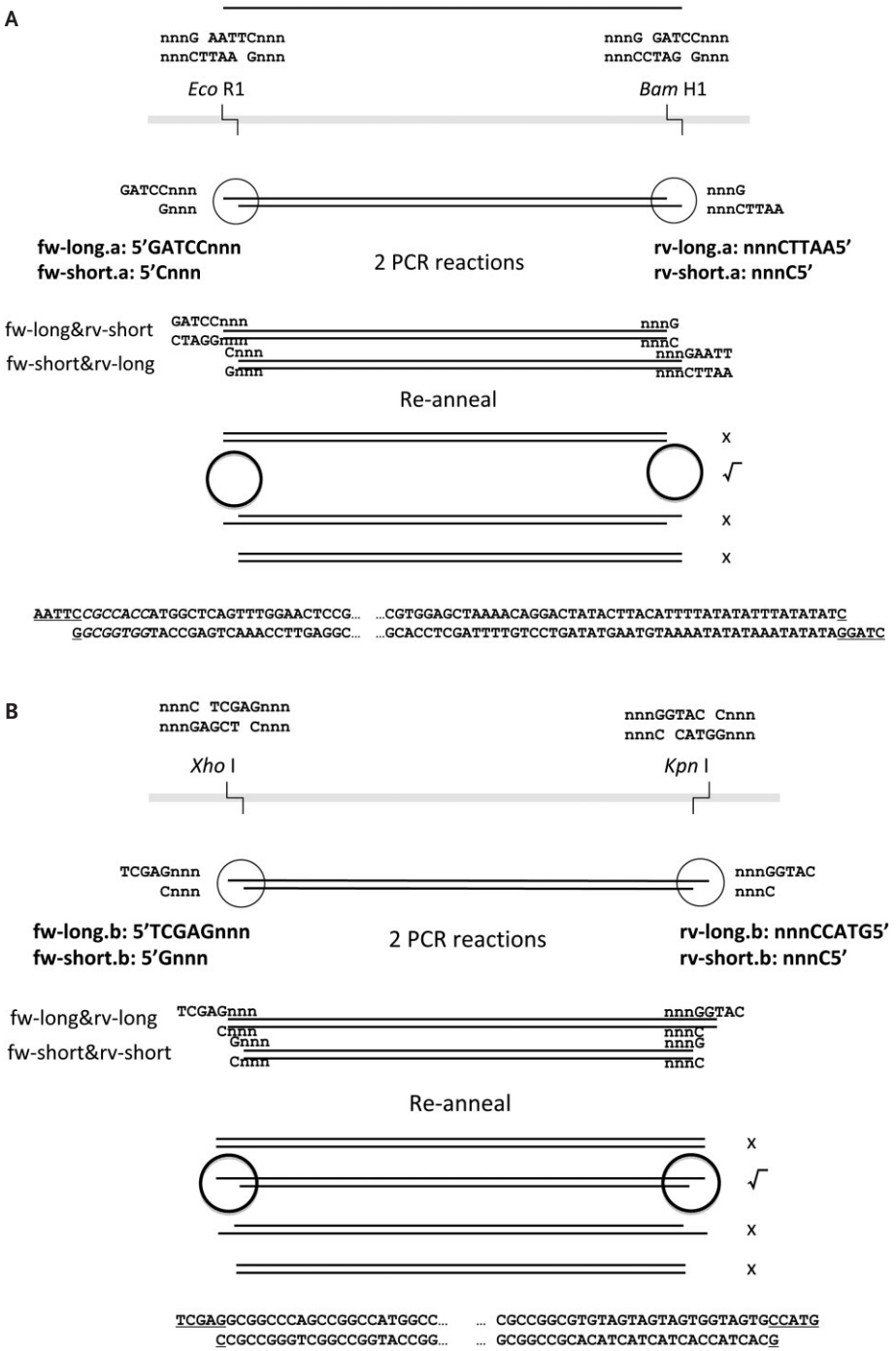

Figure 3. Primer design for molecular cloning. Primer design for permitting PCR cloning into *Eco* RI/*Bam* HI (A) and *Xho* I/*Kpn* I (B) cloning sites of the linearized vector is demonstrated. The 'nnn' refers to the sequence of the DNA to be cloned, and normally between 18 and 21 base pairs are required for successful PCR amplification. The DNA sequences of the correct cohesive ends of the re-annealed products for the cloning of the *Xenopus* Intersectin (A) and scFv genes (B) are illustrated. See text for a detailed explanation.

that the ligation of four components may be permitted by this method.

The quality of the primers also influences the success rate of the engineering experiment, as we found that PAGE-purified primers yielded higher cloning efficiency. This grade of primer is normally recommended by the manufacturer of a particular polymerase. However, we found that the use of a regular desalted primer with the appropriate 5′ phosphorylation was sufficient for most engineering work.

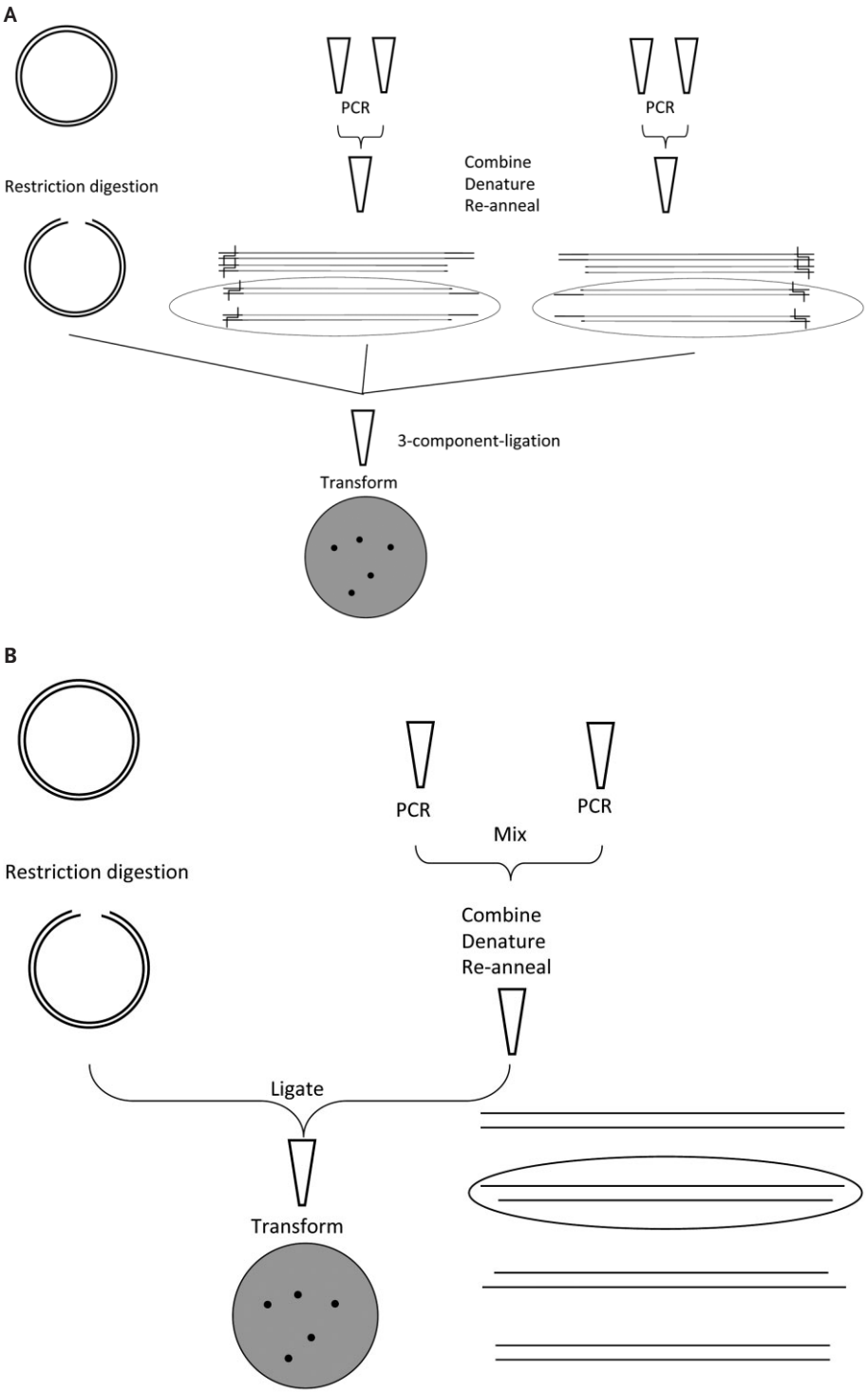

Figure 4. Schematic representation of protein engineering by the sticky PCR method. (A) Strategy for the efficient generation of a difficult mutation *via* the sticky PCR method followed by a three-component ligation. Using this strategy, two sets of primers are used to amplify two parts of sticky PCR products. Two out of four possible re-annealed products contain correct cohesive ends for the ligation reaction. After restriction digestion with appropriate restriction enzymes, the two parts of the sticky PCR products are combined with the linearized vector and ligated as normal. (B) Sticky PCR cloning of large or complex DNA fragments without restriction digestion of DNA inserts. One out of four possible re-annealed products contains the correct cohesive ends for a ligation reaction. This method can be done without using extra materials, except for a second set of PCR primers, and proof reading (*Pfu*) DNA polymerase to generate blunt-ended PCR products. See text for more detailed explanations.

Table 2. Cloning efficiency using the sticky PCR method.

Type of construct	Primer/ligation	Transformation method	Number of transformants[a)]	Number of correct constructs[b)]
Long insert with internal restriction site(s)[c)]	Desalted, 5' PO_4, two-component	Chemical	25 (4/4)	2/2
Complex insert[d)]	Desalted, two-component	Chemical	8 (3/4)	2/2
Complex insert[d)]	Desalted, two-component	Electroporation	79 (3/4)	2/2
Domain swapping[e)]	Desalted, 5' PO_4, three-component	Chemical	12 (6/10)	2/6
Domain deletion[f)]	PAGE-purified, 5' PO_4, three-component	Chemical	18 (8/10)	4/6

a) The number of correct clones checked by restriction digestion/the number of clones picked for assay.
b) The number of correct constructs confirmed by automated DNA sequencing/the number of clones submitted for analysis.
c) cDNA of Intersectin from *X. laevis*.
d) Human scFv from phage display library.
e) Mutant human EGFR construct that had the transmembrane replaced with the one from the LDLR.
f) Mutant human EGFR construct that had the CR2 domain deleted.

The application of 'sticky' PCR for molecular cloning has previously been postulated by two publications [12, 13] and provides an alternative cloning strategy to those described in references [14–25] and commercially available kits. The advantage of this method is that it may be performed in any molecular biology laboratory without the need for a specific vector, special DNA modifications or kits, which can often be expensive. The joining of compatible sticky ends is a very efficient way to recombine DNA and, hence, greatly enhance cloning efficiency [24]. Moreover, this method can also be used in ligation-independent cloning (LIC) if the length of the 'sticky' ends is more than 8–12 nucleotides [14].

This research was supported by the Royal Thai government and Suranaree University of Technology. The author would like to thank Prof. Dr. Bernie Weisblum and Prof. Richard G. W. Anderson for inspiration, and Miss Podjamas Pansri and Miss Kuntalee Rangnoi for technical assistance. Reviewer No. 2 is acknowledged for invaluable comments and suggestions during the preparation of this manuscript.

The authors declared no conflict of interest.

5 References

[1] Chica, R. A., Doucet, N., Pelletier, J. N., Semi-rational approaches to engineering enzyme activity: Combining the benefits of directed evolution and rational design. *Curr. Opin. Biotechnol.* 2005, *16*, 378–384.

[2] Jackel, C., Kast, P., Hilvert, D., Protein design by directed evolution. *Annu. Rev. Biophys.* 2008, *37*, 153–173.

[3] Bloom, J. D., Meyer, M. M., Meinhold, P., Otey, C. R. *et al.*, Evolving strategies for enzyme engineering. *Curr. Opin. Struct. Biol.* 2005, *15*, 447–452.

[4] Carter, P., Site-directed mutagenesis. *Biochem. J.* 1986, *237*, 1–7.

[5] Sambrook, J., Russell, D. W. (Eds.), *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor, New York 2001.

[6] Yamabhai, M., BAP-fusion: A versatile molecular probe for biotechnology research, in Richter, F. W. (Ed.), *Biotechnology: Research, Technology & Applications*, Nova Science Publishers, New York 2008, pp. 327–345.

[7] Davis, L., Kuehl, M., Battey, J., *Basic Methods in Molecular Biology*, Prentice Hall International (UK) Limited, London 1994.

[8] Yamabhai, M., Anderson, R. G., Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts. *J. Biol. Chem.* 2002, *277*, 24843–24846.

[9] Yamabhai, M., Hoffman, N. G., Hardison, N. L., McPherson, P. S. *et al.*, Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J. Biol. Chem.* 1998, *273*, 31401–31407.

[10] Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., Yamabhai, M., A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol.* 2009, *9*, 6.

[11] Yamabhai, M., Kay, B. K., Mapping protein-protein interactions with alkaline phosphatase fusion proteins. *Methods Enzymol.* 2001, *332*, 88–102.

[12] Wang, T.-F., Wang, A. H.-J., Preparation of sticky-end PCR products and ligation into expression vectors for high-throughput screening of soluble recombinant proteins. *CSH Protocols* 2006, doi:10.1101/pdb.prot4189.

[13] Zeng, G., Sticky-end PCR: New method for subcloning. *Biotechniques* 1998, *25*, 206–208.

[14] Aslanidis, C., de Jong, P. J., Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res.* 1990, *18*, 6069–6074.

[15] Shuldiner, A. R., Scott, L. A., Roth, J., PCR-induced (ligase-free) subcloning: A rapid reliable method to subclone polymerase chain reaction (PCR) products. *Nucleic Acids Res.* 1990, *18*, 1920.
[16] Haun, R. S., Serventi, I. M., Moss, J., Rapid, reliable ligation-independent cloning of PCR products using modified plasmid vectors. *Biotechniques* 1992, *13*, 515–518.
[17] Hsiao, K., Exonuclease III induced ligase-free directional subcloning of PCR products. *Nucleic Acids Res.* 1993, *21*, 5528–5529.
[18] Yang, Y. S., Watson, W. J., Tucker, P. W., Capra, J. D., Construction of recombinant DNA by exonuclease recession. *Nucleic Acids Res.* 1993, *21*, 1889–1893.
[19] Kaluz, S., Flint, A. P., Ligation-independent cloning of PCR products with primers containing nonbase residues. *Nucleic Acids Res.* 1994, *22*, 4845.
[20] Rashtchian, A., Novel methods for cloning and engineering genes using the polymerase chain reaction. *Curr. Opin. Biotechnol.* 1995, *6*, 30–36.
[21] Zhou, M., Hatahet, Z., An improved ligase-free method for directional subcloning of PCR amplified DNA. *Nucleic Acids Res.* 1995, *23*, 1089–1090.
[22] Li, C., Evans, R. M., Ligation independent cloning irrespective of restriction site compatibility. *Nucleic Acids Res.* 1997, *25*, 4165–4166.
[23] Berrow, N. S., Alderton, D., Sainsbury, S., Nettleship, J. *et al.*, A versatile ligation-independent cloning method suitable for high-throughput expression screening applications. *Nucleic Acids Res.* 2007, *35*, e45.
[24] Liu, Z., Hetero-stagger cloning: Efficient and rapid cloning of PCR products. *Nucleic Acids Res.* 1996, *24*, 2458–2459.
[25] Tillett, D., Neilan, B. A., Enzyme-free cloning: A rapid method to clone PCR products independent of vector restriction enzyme sites. *Nucleic Acids Res.* 1999, *27*, e26.

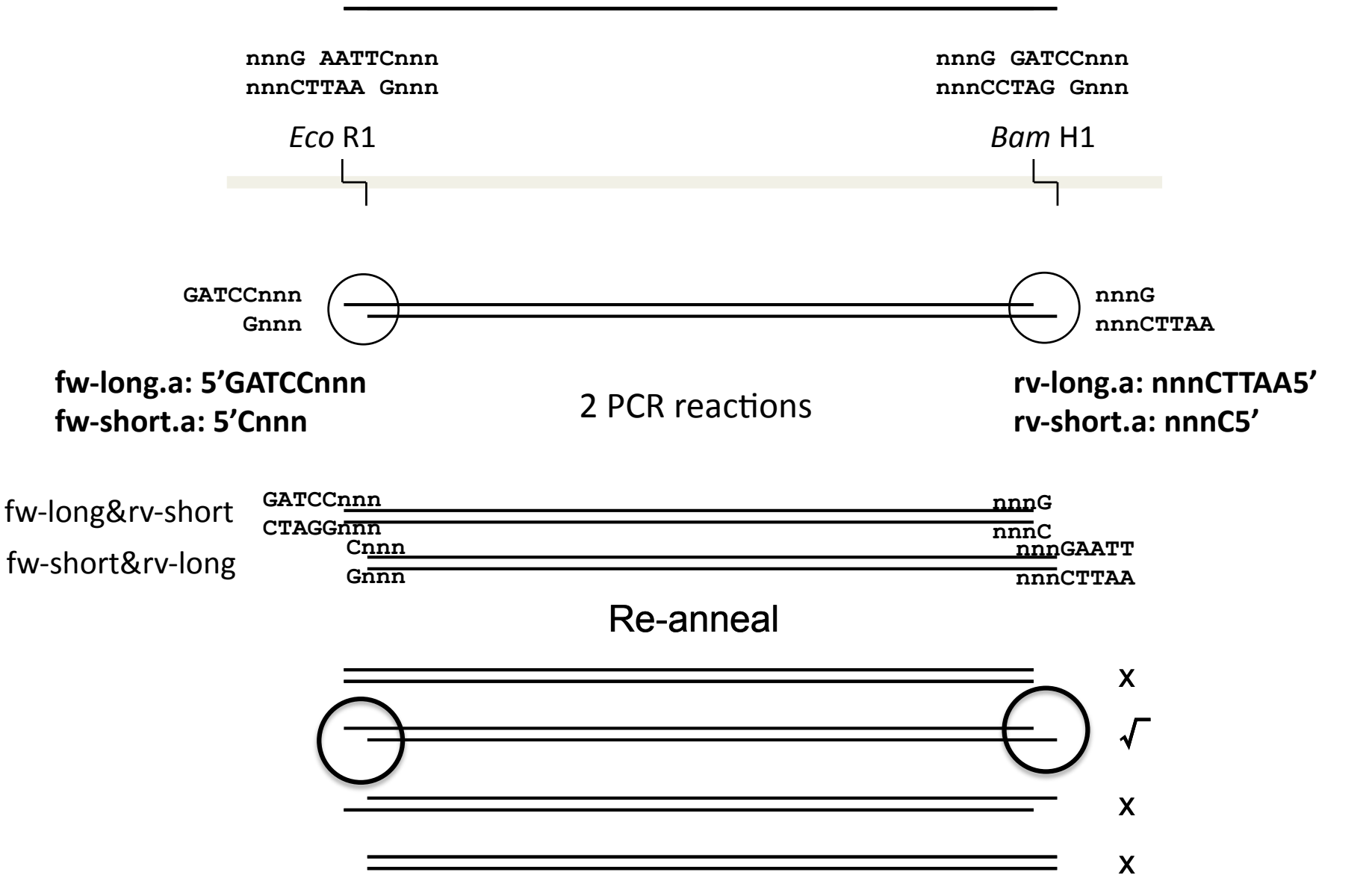
nnnG AATTCnnn
nnnCTTAA Gnnn
nnnG GATCCnnn
nnnCCTAG Gnnn
Eco R1
Bam H1
GATCCnnn
Gnnn
nnnG
nnnCTTAA
fw-long.a: 5'GATCCnnn
fw-short.a: 5'Cnnn
2 PCR reactions
rv-long.a: nnnCTTAA5'
rv-short.a: nnnC5'
fw-long&rv-short
fw-short&rv-long
GATCCnnn
CTAGGnnn
Cnnn
Gnnn
nnnG
nnnC
nnnGAATT
nnnCTTAA
Re-anneal
X
√
X
X
AATTCCGCCACCATGGCTCAGTTTGGAACTCCG...
GGCGGTGGTACCGAGTCAAACCTTGAGGC...
...CGTGGAGCTAAAACAGGACTATACTTACATTTTATATATTTATATATC
...GCACCTCGATTTTGTCCTGATATGAATGTAAAATATATAAATATATAGGATC

Figure 3A