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Ligation-independent cloning and self-cleaving intein as a tool for high-throughput protein purification

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ABSTRACT

The rapid production of purified recombinant proteins has become increasingly important for countless applications. Many purification methods involve expression of target proteins in fusion to purification tags, which often must be removed from the target proteins after purification. Recently, engineered inteins have been used to create convenient self-cleaving tags for tag removal. Although intein methods can greatly simplify protein purification, commercially available expression vectors still rely on conventional restriction/ligation cloning methods for target gene insertion. We have streamlined this process by introducing Ligation-Independent Cloning (LIC) capability to our intein expression plasmids, which provides a simple method for constructing self-cleaving tag-target gene fusions. In this work, we demonstrate efficient gene insertion via this system, as well as target protein expression and purification consistent with previously reported results. Through this newly developed system, arbitrary protein genes can be rapidly incorporated into self-cleaving tag expression vectors, and their products purified using convenient platform methods.

Introduction

The need for rapid and reliable methods to produce purified recombinant proteins has become more urgent as the study of gene products for various applications increases. During the last two decades, several methods have been developed for streamlined molecular cloning and protein purification. In particular, Invitrogen's Gateway® [1] and TOPO® [2] cloning technologies have provided a means for parallel cloning of a single target gene into several vectors. Although these methods are highly efficient, they require the purchase of commercial kits, which can increase the cost of finding the optimal vector for expressing a specific target protein. A more inexpensive cloning technique is ligation-independent cloning (LIC)[1]. This method allows the simple and precise cloning of an amplified gene into an expression vector by taking advantage of the 3′–5′ exonuclease activity of T4 DNA Polymerase (Fig. 1). In comparison to conventional molecular cloning, LIC relies on extended segments of complementary DNA to facilitate DNA strand annealing and highly efficient ligation *in vivo*, thereby eliminating the need for conventional restriction endonuclease digestion and *in vitro* ligation [3].

Several university researchers and commercial companies have incorporated LIC into simplified protein purification systems. The majority of these systems employ affinity purification methods, which require an affinity tag to be expressed in fusion to the target protein. The expressed fusion protein is then passed over a compatible affinity resin, which selectively binds the affinity tag and allows all other cell components to pass through. Once the fusion protein is purified and recovered, the affinity tag is often cleaved to produce a pure native product protein. Many of the existing LIC/protein purification systems encode polyhistidine (His_6) purification tags, and include convenient protease target sequences for tag removal [4–6]. Although these systems allow simple cloning and purification of proteins, the protease enzymes can be expensive and must also be separated from the product protein in an additional downstream purification step.

One alternative to the use of proteases for tag removal is self-cleaving inteins. Inteins are naturally occurring proteins that excise themselves from a native host protein through self-cleavage and ligation of their flanking peptide bonds. Over the last 20 years, inteins have been reengineered to exhibit isolated N-, C- or double terminal cleavage for the purification of proteins [7–11]. In practice, inteins are expressed as part of a fusion protein, where the intein separates the affinity tag from the target protein. This fusion is then purified using the included tag, after which the tag

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[1] *Abbreviations used:* LIC, ligation-independent cloning; MBP, Maltose Binding Protein; CBD, Chitin Binding Domain; ELP, Elastin-Like polypeptide; AMPD, 2-amino-2-methyl-1,3-propanediol.

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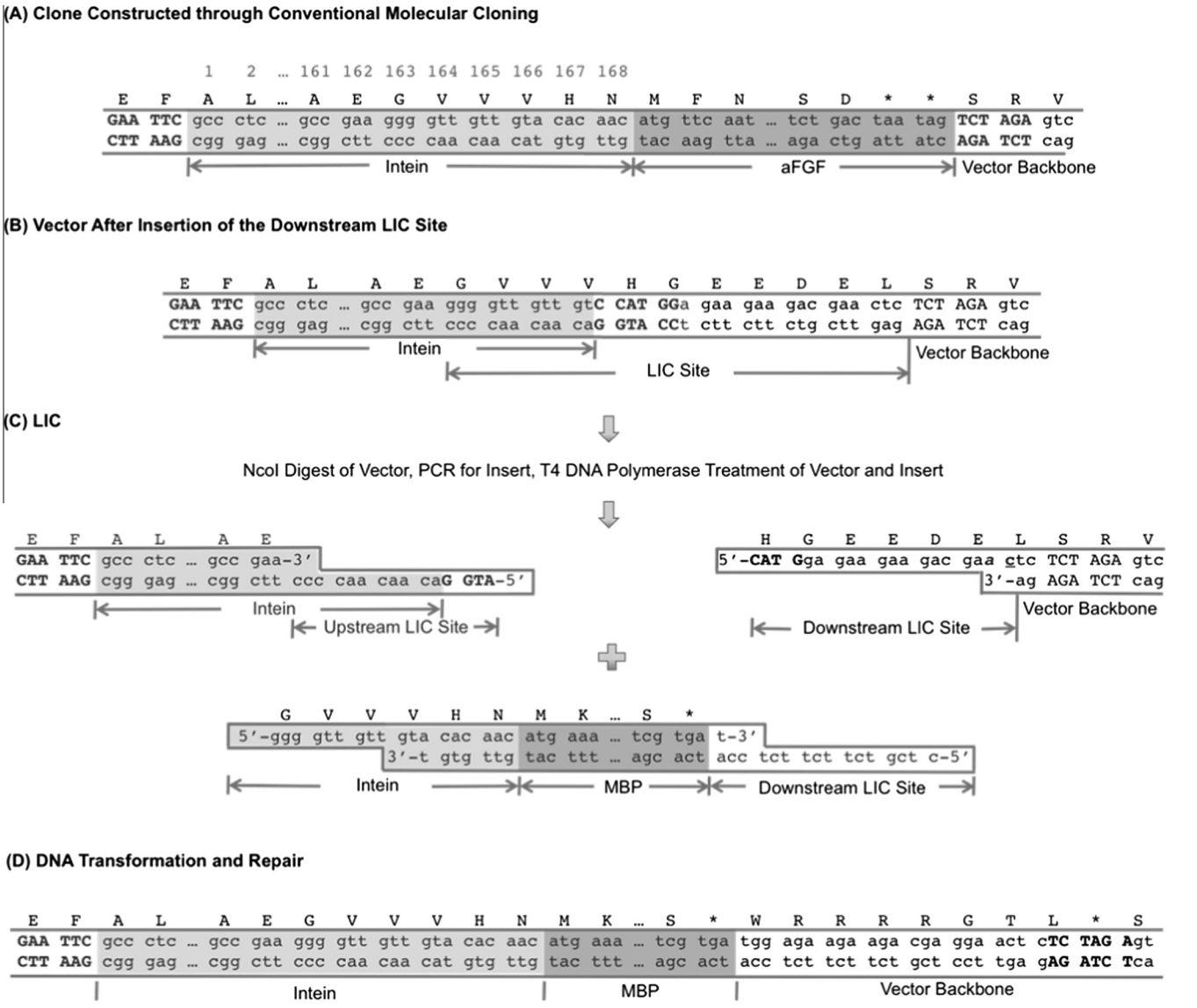

Fig. 1. Ligation-independent cloning procedure in the LIC-intein system. Restriction sites are indicated in each sequence using bold letters: EcoRI = GAATTC, XbaI = TCTAGA, NcoI = CCATGG (A) Original ΔI-CM intein vector before modification to include the LIC cloning site. (B) Modified region of the LIC intein vector showing the location of the LIC annealing regions and the introduced NcoI restriction site for vector linearization. Note that this vector does not contain any target protein sequence and that the intein gene has been partially truncated to allow direct fusion of the target protein gene without the introduction of any additional DNA sequence. (C) Intein vector and insert DNA shown after vector linearization and treatment with T4 DNA polymerase as described in the text. Overlapping complementary sequences are designed to facilitate stable annealing *in vitro* for subsequent ligation *in vivo* after transformation. The single mismatch is indicated by an italicized lowercase letter. The single gap is indicated by an underlined lowercase letter. There are two vector overhangs: (1) GGTA on the 5′ upstream end and (2) CAT on the 5′ downstream end. (D) Final intein-target protein junction after LIC and transformation. In this case, MBP is shown as an example target protein.

is induced to self-cleave. This method thus provides all the power and generality of affinity tag methods, but produces a native target without the need for proteolytic tag removal.

We have previously reported the engineered ΔI-CM self-cleaving intein, derived from the *Mycobacterium tuberculosis* RecA intein [12,13]. Its three primary advantages are its small size (18 kDa), its pH and temperature sensitivity for cleavage, and the ability to produce a native target protein without additional amino acids at the N-terminus [14]. In previous work, the ΔI-CM intein was reengineered for use with Invitrogen's Gateway® and TOPO® cloning technologies [15,16]. These systems were successfully used to express and purify proteins using conventional affinity methods via Maltose Binding Protein (MBP) and Chitin Binding Domain (CBD) tags, as well as non-chromatographic purification methods via the Elastin-Like polypeptide (ELP) tag.

In this work, two new vectors were constructed that facilitate LIC with intein-mediated protein purification. The utility of the modified intein system was verified by the expression and purification of two different target proteins using both conventional affinity and non-chromatographic purification methods. In addition, this newly developed system yielded 70% successful gene-insertion, comparable to other LIC systems, with negligible affect on intein cleavage efficiency. By combining LIC with the ΔI-CM self-cleaving intein, we have developed an inexpensive, streamlined method for simplified cloning and rapid expression and purification of proteins.

Materials and methods

Plasmid construction (pET:CBD-I-LIC, pET:ELP-I-LIC)

A pET vector encoding the Chitin Binding Domain (CBD), the ΔI-CM intein and the acidic Fibroblast Growth Factor (aFGF) genes