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Technical Report

Large scale purification of linear plasmid DNA for efficient high throughput cloning

Marjolaine Noirclerc-Savoye[a,b,c], Benoit Gallet[a,b,c], Florent Bernaudat[a,b,c] and Thierry Vernet[a,b,c]

[a] CEA, Institut de Biologie Structurale Jean-Pierre Ebel, 41 rue Jules Horowitz 38027 Grenoble, France

[b] CNRS, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France

[c] Université Joseph Fourier, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France

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Correspondence : Dr. Marjolaine Noirclerc-Savoye, Laboratoire d'Ingénierie des Macromolécules, Institut de Biologie Structurale, 41 rue Jules Horowitz, 38027 Grenoble, France.

Fax. (33) 4 38 78 54 94.

E-mail : marjolaine.noirclerc@ibs.fr

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In this report we describe a rapid, simple, and efficient method for large scale purification of linear plasmid DNA to answer demand from high throughput gene cloning. The process is based on the separation of the linear vector from small DNA fragments by anion exchange chromatography. Gene cloning experiments by restriction/ligation or the In-Fusion™ technique confirmed the high quality of the linearized vector as 100% of the genes were successfully cloned.

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1 Introduction

Investigation of large numbers of proteins requires high throughput (HTP) gene cloning approaches to prepare constructs that can be used for comprehensive structural and functional analysis of proteins. Most methods do not require a ligation step and these include Gateway cloning [1], Ligation Independent Cloning (LIC) [2, 3] or In-Fusion™ [4], even though classical ligation-dependent cloning has also been adapted to HTP [5].

Restriction/ligation and In-Fusion™ techniques are popular as they do not require additional base pairs that convert into additional amino acids at the protein extremities. The former method may be limited by the availability of unique restriction sites. The latter one is more flexible as it allows in vitro assembly through homologous recombination of fragments, providing that they include extremities matching the sequences of the cloning site [4]. In that way, PCR products can be cloned into any vector at any site of linearization. The success rate of these techniques is strongly dependent on the quality of the linear vector. For HTP approaches, the production of large batches of high quality linear vector is of paramount importance. Small-scale purification is often based on gel electrophoresis and spin column [6, 7], two steps that are incompatible with the production of large amount of DNA.

Plasmid DNA purification [8] has been the subject of many studies, allowing large scale and high grade plasmid preparation [9-13], including the use of anion exchange chromatography [14-17]. The separation of multiple small restriction fragments by anion exchange chromatography on FPLC columns was

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previously described [18-20], showing a correlation between the size of linear DNA fragments and the salt concentration needed for elution. For a given size, substantial retardation of the d(A+T) rich fragments has been reported [19]. Anion exchange chromatography has also been used for plasmid adsorption, purification and quantification [9, 15, 21]. We report here the use of anion exchange chromatography for the large-scale purification of linear cloning vector and separation from small restriction fragments. This application was first suggested by Westman et al. [18], but to our knowledge, has never been described in the literature.

Our protocol has been developed using two expression vectors specifically constructed for an automated HTP approach to test the influence of purification tags on the protein behavior: That is the presence or absence of His-tag for soluble proteins, and of the His-Mistic-tag for membrane proteins. Membrane proteins constitute up to 30% of all proteins [22, 23] and are involved in numerous cellular processes. There are two bottlenecks in membrane protein structural analysis: high-yield protein production and crystallization. Recently, a novel membrane-associated protein discovered in *Bacillus subtilis*, Mistic, has been shown to enhance the expression of recombinant membrane proteins in *E. coli*, when fused to their N-terminus [24-26].

This article describes the purification of linearized expression vectors in sufficient quantity and quality to efficiently perform hundreds of gene cloning in parallel. This method offers a rapid and simple way to standard molecular biology laboratories to prepare linear plasmid DNA on a large scale, and is a good

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alternative to multiple small scale preparations or expensive preparation from commercial suppliers.

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2 Materials and Methods

2.1 pLIM13 and pLIM14 plasmids for high throughput cloning

The pLIM13 and pLIM14 vectors developed by the RoBioMol platform are derived from the commercial pET41c (Novagen) (Complete plasmid sequences are available upon request). For pLIM13, the toxic *ccdB* expression cassette [27] was PCR amplified from pDEST17 (Invitrogen) and inserted into pET41c as an *Nde*I-*Bam*HI fragment (Figure 1). For pLIM14, a synthetic gene *mistic-ccdB* (Geneart) was first inserted as an *Nde*I-*Bam*HI fragment into pET41c. The exchange of the *Xba*I-*Nde*I fragment of pET41c vector for one encoding a His6-tag resulted in pLIM14 (Figure 1).

These pLIM plasmids are designed so that the linearization with two restriction enzymes releases the toxic *ccdB* expression cassette (Figure 1). pLIM13 was designed to express proteins without tag, whereas pLIM14 was designed to express His6- or His6-Mistic-tagged proteins, depending on the restriction sites used to clone the corresponding genes (Figure 1).

2.2 Large scale plasmid purification

The pLIM13 and pLIM14 plasmids were prepared as previously described [10, 16] with some modifications. A 5 ml 2xTY + kanamycin (50 mg/L) overnight culture of *E. coli* DB3.1 strain (Invitrogen) containing pLIM13 or pLIM14 was used to inoculate 1 L of 2xTY + kanamycin (50 mg/L) into a 3 L flask. The culture was grown to saturation at 25°C for 36 hours, with 200 rpm shaking.

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To improve yield, the culture was separated in 4 fractions (250 ml each) treated in parallel. Cells were harvested by centrifugation (6200 *g*, 15 min, 4°C) and each pellet was resuspended in 40 ml of GTE buffer (50 mM glucose, 25 mM Tris/HCl pH 8, 10 mM EDTA) and 4 ml of lysozyme (25 mg/ml). After 10 min at room temperature, 20 ml of a freshly prepared solution of 0.3M NaOH and 2% SDS were added. The mixture was gently mixed by inverting the tubes 5 times, and DNA denaturation was allowed to proceed 10 min on ice. Thirty ml of 3 M potassium acetate pH 5.5 were then added and mixed thoroughly by inverting the tubes 5 times, to allow the renaturation of plasmid DNA and the precipitation of chromosomal DNA, SDS-protein complexes and other cellular debris. This mixture was incubated 10 min on ice before centrifugation (19,000 *g*, 10 min). Supernatants containing plasmid DNA were pooled and passed through a funnel filter before the addition of RNase A to a final concentration of 50 mg/L and incubation at 37°C for 20 min. Plasmid DNA was precipitated with the addition of 0.6 volume of isopropanol and recovered after 20 min at room temperature by centrifugation (15,000 *g*, 10 min). DNA pellet was washed once with 2 ml of 70% ethanol, dried in a vertical laminar air-flow hood, and dissolved in 5 ml of 5 mM Tris/HCl pH 8.5.

2.3 Large scale plasmid digestion

One hundred micrograms of plasmid DNA were digested in a 5 ml final volume in the presence of 500 µL of buffer 4 (NEB), 50 µL of *Bam*HI HF (1000 U, NEB) and 100 µL of *Nde*I (2000 U, NEB) or 100µL of *Nco*I (1000 U, NEB), at

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37°C for 5 hours. Prior the addition of NaCl to a final concentration of 600 mM, five µL of digestion product were analyzed by agarose gel electrophoresis.

2.4 Linear vector isolation by Mono Q™ anion exchange chromatography

Chromatography was performed with an Äktabasic UPC apparatus (GE Helthcare) and monitored at 254 nm. The DNA sample was loaded at 1ml/min onto a Mono Q™ 5/50 GL, (GE Healthcare) column equilibrated with buffer A (10 mM Tris/HCl pH 8, 1 mM EDTA, 700 mM NaCl). After a wash at 0.5 ml/min with 10 ml of buffer A, a 64 ml linear gradient of 0 to 10% buffer B (10 mM Tris/HCl pH 8, 1 mM EDTA, 2 M NaCl) was applied at 0.15 ml/min. Peak fractions of 0.5 ml were precipitated individually with the addition of 0.6 volume of isopropanol. After 20 min room temperature, DNA pellets were recovered by centrifugation (21,000 *g*, 10 min, 4°c), washed with 70% ethanol and redissolved in 50 µL of 5 mM Tris/HCl pH 8.5. Two µL of these fractions were analyzed on 1% agarose gel. Fractions containing pure linear vector were pooled and assayed in gene cloning experiments. Homogeneity and the sequence extremities of the purified linear vector were checked by sequencing using the T7prom and T7term primers.

2.5 Target genes amplification

Target genes were amplified by PCR using specific primers, designed with the Vector NTI software (Invitrogen), containing 5' extensions related to the vectors used for cloning. For pLIM13_NdeI_BamHI, the forward and the reverse

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primer extensions were respectively 5'-AAGGAGATATACATATG-3' and 5'-TACAGAATTCGGATCCTTA-3', for pLIM14_NdeI_BamHI, 5'-ACCATCACCATCATATG-3'and 5'-TACAGAATTCGGATCCTTA-3' and for pLIM14_NcoI_BamHI, 5'-CGGGCGGTAGCCATGGGGAAAATCTGTATTTTCAGGGC-3'(containing protease TEV site) and 5'-TACAGAATTCGGATCCTTA-3'. These extensions allow both restriction/ligation (using the underlined restriction sites) and InFusion methods (relying on the extended sequence complementary to the vector). Primers were ordered at 20 µM in a 96-wells microplate (MWG). Preparation of the 0.5 µM primers mix and of the PCR reaction was fully automated on a Microlab Star robot (Hamilton). Reactions were performed with 100 ng of chromosomal DNA template, 10 µL of primer mix 0.5 µM, 4 µL of dNTPs 2.5 mM, 10 µL of HF buffer 5X (Finnzyme), 0.5 µL Phusion enzyme (2U/µL, Finnzyme) in a 50 µL volume, using a Trobot PCR machine (Biometra) with the following parameters : 98°C, 1 min; 30 cycles of [98°C, 30 sec; 50°C, 30 sec; 72°C, 1 min]; 72°C, 8 min.

Five µL of each PCR product were analyzed on 1% agarose gel. Satisfying PCR products (single or few multiple bands) were purified on a Nucleofast plate (Macherey-Nagel) and eluted with 40 µL of water, using a Microlab Star automaton (Hamilton).

2.6 Cloning with the In-Fusion™ Advantage System

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Five µLof the PCR products were treated with Cloning Enhancer reagent (In-Fusion™, Clontech) according to the supplier's recommendations. The In-Fusion™ reactions were performed by mixing 100 ng of linear vector with 2 µL of each treated insert (3 µL for the 3501 bp-long insert) in 10 µL final volume. After the addition of 40 µL of TE, 2.5 µL of each reaction were used to transform 50 µL of NEB5α competent *E. coli* cells (Biolabs). The transformed cells were pelleted by centrifugation (5200 *g*, 5 min) and spread onto LB (kanamycin 50 mg/L, glucose 10 mM) agar plates and incubated overnight at 37°C.

2.7 Cloning by restriction/ligation

Forty µL of PCR products were mixed with 5 µL buffer 4 10X (NEB), 0.5 µL *Nde*I (10 U) or *Nco*I (5 U) and *Bam*HI (5U) enzyme (NEB). After 2 h at 37°C, fragments were purified on a Nucleofast plate (Macherey-Nagel) and eluted with 40 µL of water. Two µL of each insert were mixed with 100 ng of linear vector in the presence of 1 µL of 10X T4 DNA ligation buffer, 1µL of T4 DNA ligase (3 U/µL, Promega), in a final volume of 10 µL. After 3 hours at room temperature, ligation reactions were stopped at 65°C (10 min). Five µL were used to transform 50 µL of DH5α competent *E. coli* cells. The transformed cells were then pelleted before spreading on LB (kanamycin 50 mg/L, glucose 10 mM) agar plate and incubated overnight at 37°C.

2.8 Plasmid screening by restriction analysis

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Isolated transformants were used to inoculate 2 ml of 2xTY (50 mg/L kanamycin) medium in a 24-wells plates sealed with a porous film. Cultures were grown for 20 h at 37°C with agitation (200 rpm). Cells were pelleted and plasmids were prepared on a Qiagen BioRobot 8000 using the Nucleospin robot 96 kit (Macherey-Nagel) and eluted with 125 µL of EB buffer. Eight µL were used for restriction analysis with 2.5 U of each enzyme in a final volume of 10 µL; profiles were analyzed on 1% agarose gel. Two positive clones for each gene were sent for full length sequencing with the universal T7 prom and T7 term primers (Beckman Cogenics). Sequencing with internal primers was necessary for genes longer than 2 Kb.

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3 Results and discussion

HTP cloning requires large quantities of high quality linearized vector. The commonly used method of purification is preparative gel electrophoresis followed by spin column extraction [6]. Both steps are poorly suited to large amounts of DNA. To overcome this limitation, we have scaled up the separation of restriction fragments by anion exchange chromatography [18].

3.1 Construction of expression vectors for soluble or membrane proteins expression

Two expression plasmids were designed to express soluble or membrane proteins, with the same backbone vector. The pLIM13 vector allows expression of proteins without tag, whereas pLIM14 is for His6- or His6-Mistic-fusion for soluble or membrane proteins respectively. The Mistic protein from *B. subtillis* was shown to increase the yield by targeting fused proteins more efficiently to the membrane in *E. coli* [24], (F. Bernaudat, personal observations). The toxic *ccdB* gene was inserted between the restrictions sites used for linearization (Figure 1). The CcdB protein is a poison of the DNA-topoisomerase II complexes killing bacterial strains not expressing the antidote CcdA [28], or not containing the gyrA462 mutation [28, 29]. The interest of *ccdB* is to select against the parental vector in *E. coli* sensitive strains.

Digestion of pLIM13 with *Nde*I and *Bam*HI produces two fragments of 5082 bp and 311 bp. Digestion of pLIM14 with the same enzymes gives 5103 bp and 683 bp fragments, whereas digestion with *Nco*I and *Bam*HI releases 5455 bp

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and 331 bp fragments. The larger fragments are the linearized vectors, the small fragments contain the *ccdB* gene. These digestions allow the in frame insertion of any gene amplified with specific primers (see Materials and Methods and Figure 1).

3.2 Large scale plasmid purification

The protocol for purification of large amount of pLIM13 and pLIM14 was derived from known procedures [10, 16], and yielded 1.8 mg of each low copy vector from 1 L of the CcdB resistant DB3.1 *E. coli* strain grown in 2xTY. The purified plasmid was mostly supercoiled, with the presence of a minor nicked DNA form and no detectable RNA or protein contamination. The yield was sufficient for the preparation of 18 batches of linear vector as described below.

3.3 Large-scale linear plasmid purification by Mono Q™ anion exchange chromatography

This procedure succeeded with the three linearized pLIM vectors. The purification of pLIM13 digested with *Nde*I and *Bam*HI is presented in details below as an example.

One hundred micrograms of pLIM13 were digested and the NaCl concentration was brought to 600 mM prior to the chromatography to avoid adsorption of low charge density impurities on the Mono Q™ column [15]. The advantage of a Mono Q™ resin is the fast and strong binding of the DNA (Figure 2) [21].

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A salt gradient was employed to displace the different nucleic acids that should elute in order of increasing charge density [15]. The charge density of DNA is function of its length and conformation. Long fragments, being more folded, have higher charge densities and are eluted later. The separation was visualized by the detection of two peaks and confirmed by the agarose gel analysis (Figure 2). Fractions containing the linearized expression vector were pooled. A third early peak corresponded to the elution of restriction enzymes

3.4 Quality control of linear plasmid

One hundred nanograms of each vector (pET41c, pLIM13, and pLIM14) were transformed into NEB5α and DH5α. In both strains, approximately one thousand transformants were obtained with pET41c and none with pLIM13 as expected due to the presence of the *ccdB* gene in the latter plasmid. To our surprise, thousand transformants were also obtained with the pLIM14. As *ccdB* is still present, we hypothesize that the normally cytosolic CcdB protein is not functional when fused to Mistic and targeted to the membrane. However, this finding allowed us to evaluate the homogeneity of the purified linear vector, as we could compare the number of transformants obtained with the native and the linearized plasmid. Linearized pLIM14 yielded no transformant showing the absence of undigested parental vector.

The quality and quantity of the linear vectors isolated by Mono Q™ chromatography are suitable for high throughput cloning, even if yields vary with the vectors and the digestions. Yields after digestions and purification were 26%

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(25µg), 43% (38µg) or 73% (69µg) for pLIM13_*Nde*I_*Bam*HI, pLIM14_*Nde*I_*Bam*HI and pLIM14_*Nco*I_*Bam*HI, respectively. Individual fraction precipitations and pooling are probably responsible for the loss of material.

3.5 Efficient cloning with linear plasmid

The suitability for high throughput cloning of the purified pLIM13_*Nde*I_*Bam*HI linearized vector was tested by the insertion 24 independent genes in parallel. The gene size varied between 387 bp and 3501 bp. Eleven genes were cloned by restriction/ligation and 13 using the In-Fusion™ technique. Genes containing restriction sites within their sequence were treated by the In-Fusion™ method.

The automated PCR amplification of the 24 genes was performed with the Phusion enzyme on a Hamilton robot. PCR products were analyzed on agarose gel (Figure 2) and showed the successful amplification of all the genes using the same PCR parameters. Purification on a Nucleofast plate eliminated primer dimers and allowed buffer exchange.

Genes to be treated by ligation were first digested with the two appropriate restriction enzymes, and purified again on Nucleofast to eliminate the short fragments released by the digestion. Genes destined to In-Fusion™ cloning were directly incubated with the Cloning Enhancer according the manufacturer's recommendations.

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With both cloning methods, 100 ng of linear vector were mixed to 2-3 µL of insert. We have deliberately chosen to standardize the volume of insert, independently of their size, in order to develop an efficient high throughput process for a wide range of gene sizes, as the amplification delivers PCR fragments in amounts that are not limiting. After ligation or In-Fusion™ recombination, transformation suspensions were platted on agar containing kanamycine, and glucose to repress the expression leakage.

Between 20 and 100 transformants were obtained, without relationship between the size of the insert and the number of transformants. For the negative control (linear pLIM13 plasmid without insert), around 10 and 70 transformants were obtained. These transformants resulted from aberrant recombination events. To evaluate the cloning success rate, 2 to 8 transformants were analyzed for each of the 24 genes by plasmids miniprep followed by restriction mapping.

The 24 genes were successfully cloned in parallel into the expression vector indicating the good quality of the linear vector preparation (Table 1). Cloning efficiencies were not correlated to the size of the gene, although the most difficult gene to clone was the longest (3501 bp). Except in the latter case, 50% to 100% of positive clones were obtained for all other genes. The negative clones resulted apparently from aberrant recombination events, such as the clones obtained from the negative controls. The mean cloning efficiency by restriction/ligation was 63%, whereas that obtained by In-Fusion™ was 80% including the lower yield obtained with the longest gene. The systematic full length gene sequencing

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performed on two positive clones of each gene, showed the absence of mutation with a single exception likely originating from a PCR error.

4 Concluding remarks

Large amount of linear expression vector is obtained by separating the vector from its insert by anion exchange chromatography. The cloning results indicate that the quality of the linear vector purified by Mono Q™ is compatible with different high throughput cloning techniques. However, the In-Fusion™ technique is, in our view, better adapted to high throughput as this system catalyzes the joining of DNA pieces in a manner that is independent of the sequence of the cloned gene.

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The authors have declared no conflict of interest.

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Figure legends

Figure 1. pLIM vector maps. Linearization of the vectors was performed using the restriction sites mentioned. PCR-amplified genes were inserted either by restriction ligation or by the In-Fusion™ method. (A) pLIM13 for the overexpression of untagged proteins; (B) pLIM14 for the overexpression of His-tagged (cloned between the *Nde*I and *Bam*HI sites) or His-Mistic-tagged proteins (cloned between *Nco*I and *Bam*HI). The T7 promoter induces expression in *E. coli* harbouring the λ (DE3) prophage.

Figure 2. Linear plasmid purification by Mono Q™ anion exchange chromatography and PCR amplification of the 24 genes used in cloning experiments. (A) Chromatogram showing the purification of pLIM13 digested with *Nde*I and *Bam*HI monitored at 254 nm. Plasmids were loaded on a Mono Q™ 5/50 GL column. DNA was eluted with a 64 ml linear gradient of 700 mM (0% B) to 900 mM (10 % B) of NaCl at 0.15 ml/min. Arrows indicate the fractions analyzed. (B) 1% Agarose gel analysis of the fractions corresponding to the elution peaks shown in (A): M, DNA ladder; 1, loaded sample; 2, flow through (pooled fractions 2-4); 3 to 6, fractions corresponding to the *ccdB* insert; 7 to 15, fractions corresponding to the linear vector. (C) 1% Agarose gel analysis of the pooled elution fractions from the chromatographic separations of pLIM14_*Nde*I_*Bam*HI: 1, Mistic-*ccdB* insert; 2, linear vector; and pLIM14_*Nco*I_*Bam*HI: 3, *ccdB* insert; 4, linear vector. (D) 1% Agarose gel analysis of the PCR amplification of 24 independent genes. M, DNA ladder; 1 to

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24, PCR amplification obtained in the conditions described in Materials and Methods section and successfully cloned into pLIM13_*Nde*I_*Bam*HI:

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Table 1. Gene cloning using pLIM13_*Nde*I-*Bam*HI linear vector by restriction/ligation or InFusion techniques

Gene number	Plasmid	Technique used	Insert size (bp)	% of positive clones (Nb clones tested)
1	pLIM13	Ligation	1233	50% (4)
2	pLIM13	Ligation	1131	50% (4)
3	pLIM13	Ligation	1683	75% (4)
4	pLIM13	Ligation	816	50% (4)
5	pLIM13	Ligation	657	50% (4)
6	pLIM13	InFusion	627	87% (8)
7	pLIM13	InFusion	600	100% (8)
8	pLIM13	Ligation	672	75% (4)
9	pLIM13	InFusion	3501	37% (8)
10	pLIM13	InFusion	603	100% (8)
11	pLIM13	InFusion	1950	50% (8)
12	pLIM13	InFusion	1887	75% (8)
13	pLIM13	Ligation	1962	100% (4)
14	pLIM13	InFusion	1557	75% (8)
15	pLIM13	Ligation	456	100% (4)
16	pLIM13	InFusion	1119	55% (9)
17	pLIM13	InFusion	843	100% (3)
18	pLIM13	InFusion	837	87% (8)

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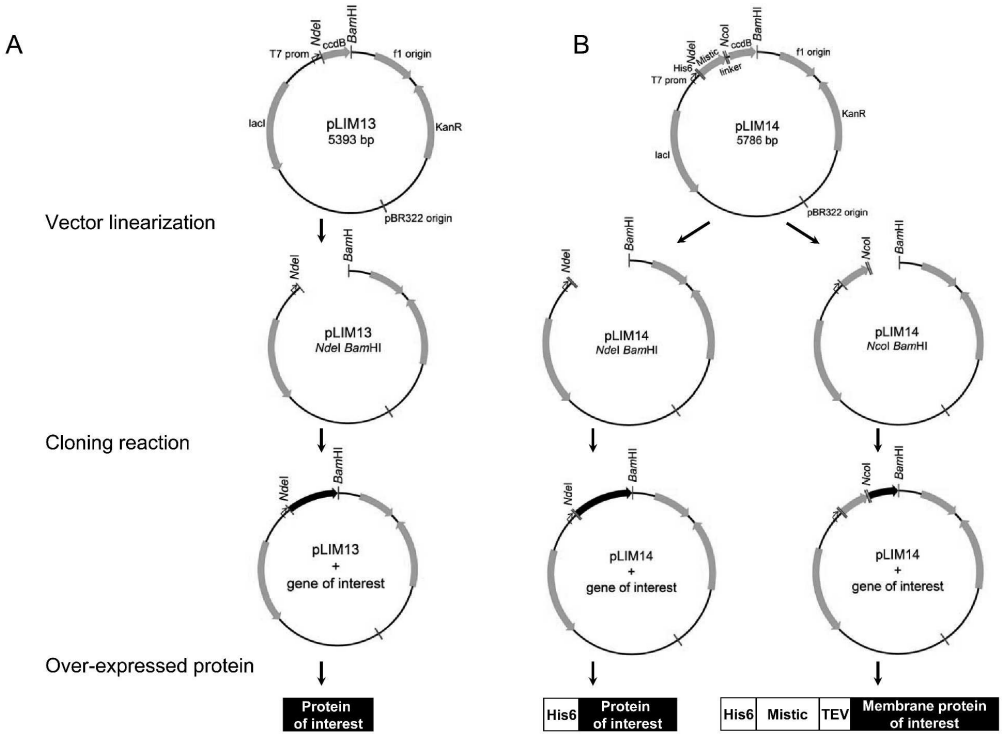
19	pLIM13	Ligation	489	50% (4)
20	pLIM13	Ligation	387	50% (2)
21	pLIM13	InFusion	1116	100% (3)
22	pLIM13	InFusion	1302	100% (8)
23	pLIM13	Ligation	1038	50% (2)
24	pLIM13	InFusion	1059	100% (8)

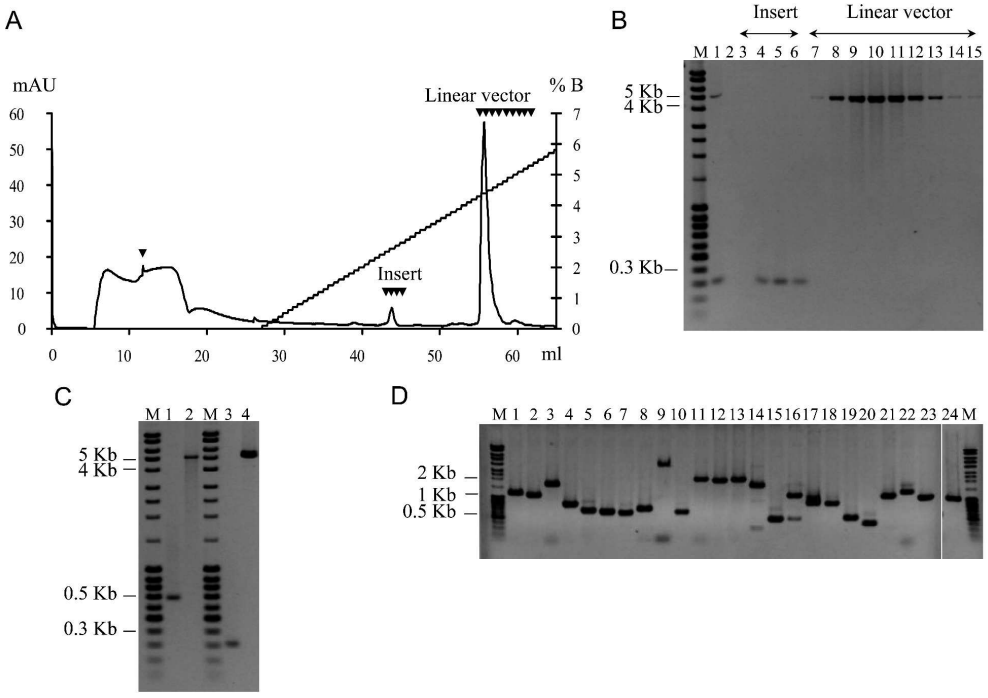
1

For Peer Review



245x180mm (600 x 600 DPI)

Review



250x179mm (600 x 600 DPI)

Review