



HAL
open science

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

Marjolaine Noirclerc-Savoie, Benoit Gallet, Florent Bernaudat, Thierry Vernet

► To cite this version:

Marjolaine Noirclerc-Savoie, Benoit Gallet, Florent Bernaudat, Thierry Vernet. Large scale purification of linear plasmid DNA for efficient high throughput cloning. *Biotechnology Journal*, 2010, 5 (9), pp.978. 10.1002/biot.201000132 . hal-00575229

HAL Id: hal-00575229

<https://hal.science/hal-00575229v1>

Submitted on 10 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



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

Journal:	<i>Biotechnology Journal</i>
Manuscript ID:	biot.201000132.R1
Wiley - Manuscript type:	Technical Report
Date Submitted by the Author:	09-Jul-2010
Complete List of Authors:	Noirclerc-Savoye, Marjolaine; Institut de Biologie structurale Gallet, Benoit; Institut de Biologie Structurale Bernaudat, Florent; Institut de Biologie Structurale Vernet, Thierry; Institut de Biologie Structurale
Primary Keywords:	linear vector
Secondary Keywords:	high throughput cloning
Keywords:	membrane protein, Mystic, Mono Q™



1
2
3
4
5
6 1 Technical Report
7

8 2
9

10 3 **Large scale purification of linear plasmid DNA for**
11
12
13
14 4 **efficient high throughput cloning**
15
16

17 5
18

19
20 6 Marjolaine Noirclerc-Savoye^{a,b,c}, Benoit Gallet^{a,b,c}, Florent Bernaudat^{a,b,c} and
21
22 7 Thierry Vernet^{a,b,c}
23
24

25
26 8 ^a CEA, Institut de Biologie Structurale Jean-Pierre Ebel, 41 rue Jules Horowitz
27 9 38027 Grenoble, France
28

29 10 ^b CNRS, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France
30

31
32 11 ^c Université Joseph Fourier, Institut de Biologie Structurale Jean-Pierre Ebel,
33 12 Grenoble, France
34

35 13
36

37 14
38

39
40 15 Keywords: linear vector, high throughput cloning, membrane protein, Mystic,
41

42 16 Mono QTM
43
44

45 17
46

47 18 Correspondence : Dr. Marjolaine Noirclerc-Savoye, Laboratoire d'Ingénierie des
48

49 19 Macromolécules, Institut de Biologie Structurale, 41 rue Jules Horowitz, 38027
50

51
52 20 Grenoble, France.
53

54 21 Fax. (33) 4 38 78 54 94.
55

56 22 E-mail : marjolaine.noirclerc@ibs.fr
57
58
59
60

1

2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2 In this report we describe a rapid, simple, and efficient method for large
3 scale purification of linear plasmid DNA to answer demand from high throughput
4 gene cloning. The process is based on the separation of the linear vector from
5 small DNA fragments by anion exchange chromatography. Gene cloning
6 experiments by restriction/ligation or the In-Fusion™ technique confirmed the
7 high quality of the linearized vector as 100% of the genes were successfully
8 cloned.

9

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

1
2
3
4
5
6 1 previously described [18-20], showing a correlation between the size of linear
7
8 2 DNA fragments and the salt concentration needed for elution. For a given size,
9
10 3 substantial retardation of the d(A+T) rich fragments has been reported [19]. Anion
11
12 4 exchange chromatography has also been used for plasmid adsorption, purification
13
14 5 and quantification [9, 15, 21]. We report here the use of anion exchange
15
16 6 chromatography for the large-scale purification of linear cloning vector and
17
18 7 separation from small restriction fragments. This application was first suggested
19
20 8 by Westman et al. [18], but to our knowledge, has never been described in the
21
22 9 literature.
23
24
25
26

27 10 Our protocol has been developed using two expression vectors specifically
28
29 11 constructed for an automated HTP approach to test the influence of purification
30
31 12 tags on the protein behavior: That is the presence or absence of His-tag for
32
33 13 soluble proteins, and of the His-Mistic-tag for membrane proteins. Membrane
34
35 14 proteins constitute up to 30% of all proteins [22, 23] and are involved in
36
37 15 numerous cellular processes. There are two bottlenecks in membrane protein
38
39 16 structural analysis: high-yield protein production and crystallization. Recently, a
40
41 17 novel membrane-associated protein discovered in *Bacillus subtilis*, Mistic, has
42
43 18 been shown to enhance the expression of recombinant membrane proteins in *E.*
44
45 19 *coli*, when fused to their N-terminus [24-26].
46
47
48
49

50 20 This article describes the purification of linearized expression vectors in
51
52 21 sufficient quantity and quality to efficiently perform hundreds of gene cloning in
53
54 22 parallel. This method offers a rapid and simple way to standard molecular
55
56 23 biology laboratories to prepare linear plasmid DNA on a large scale, and is a good
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 alternative to multiple small scale preparations or expensive preparation from
2 commercial suppliers.
3

For Peer Review

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 *NdeI-BamHI* fragment (Figure 1). For pLIM14, a synthetic gene *mistic-ccdB* (Geneart) was first inserted as an *NdeI-BamHI* fragment into pET41c. The exchange of the *XbaI-NdeI* 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.

1
2
3
4
5
6 1 To improve yield, the culture was separated in 4 fractions (250 ml each)
7
8 2 treated in parallel. Cells were harvested by centrifugation (6200 g, 15 min, 4°C)
9
10 3 and each pellet was resuspended in 40 ml of GTE buffer (50 mM glucose, 25 mM
11
12 4 Tris/HCl pH 8, 10 mM EDTA) and 4 ml of lysozyme (25 mg/ml). After 10 min at
13
14
15 5 room temperature, 20 ml of a freshly prepared solution of 0.3M NaOH and 2%
16
17 6 SDS were added. The mixture was gently mixed by inverting the tubes 5 times,
18
19 7 and DNA denaturation was allowed to proceed 10 min on ice. Thirty ml of 3 M
20
21 8 potassium acetate pH 5.5 were then added and mixed thoroughly by inverting the
22
23 9 tubes 5 times, to allow the renaturation of plasmid DNA and the precipitation of
24
25 10 chromosomal DNA, SDS-protein complexes and other cellular debris. This
26
27 11 mixture was incubated 10 min on ice before centrifugation (19,000 g, 10 min).
28
29 12 Supernatants containing plasmid DNA were pooled and passed through a funnel
30
31 13 filter before the addition of RNase A to a final concentration of 50 mg/L and
32
33 14 incubation at 37°C for 20 min. Plasmid DNA was precipitated with the addition
34
35 15 of 0.6 volume of isopropanol and recovered after 20 min at room temperature by
36
37 16 centrifugation (15,000 g, 10 min). DNA pellet was washed once with 2 ml of
38
39 17 70% ethanol, dried in a vertical laminar air-flow hood, and dissolved in 5 ml of 5
40
41 18 mM Tris/HCl pH 8.5.
42
43
44
45
46
47
48
49

50 **2.3 Large scale plasmid digestion**

51
52
53 21 One hundred micrograms of plasmid DNA were digested in a 5 ml final
54
55 22 volume in the presence of 500 µL of buffer 4 (NEB), 50 µL of *Bam*HI HF (1000
56
57 23 U, NEB) and 100 µL of *Nde*I (2000 U, NEB) or 100µL of *Nco*I (1000 U, NEB), at
58
59
60

1 37°C for 5 hours. Prior the addition of NaCl to a final concentration of 600 mM,
2 five µL of digestion product were analyzed by agarose gel electrophoresis.

3

4 **2.4 Linear vector isolation by Mono Q™ anion exchange chromatography**

5 Chromatography was performed with an Äktabasic UPC apparatus (GE
6 Helthcare) and monitored at 254 nm. The DNA sample was loaded at 1ml/min
7 onto a Mono Q™ 5/50 GL, (GE Healthcare) column equilibrated with buffer A
8 (10 mM Tris/HCl pH 8, 1 mM EDTA, 700 mM NaCl). After a wash at 0.5
9 ml/min with 10 ml of buffer A, a 64 ml linear gradient of 0 to 10% buffer B (10
10 mM Tris/HCl pH 8, 1 mM EDTA, 2 M NaCl) was applied at 0.15 ml/min. Peak
11 fractions of 0.5 ml were precipitated individually with the addition of 0.6 volume
12 of isopropanol. After 20 min room temperature, DNA pellets were recovered by
13 centrifugation (21,000 g, 10 min, 4°C), washed with 70% ethanol and redissolved
14 in 50 µL of 5 mM Tris/HCl pH 8.5. Two µL of these fractions were analyzed on
15 1% agarose gel. Fractions containing pure linear vector were pooled and assayed
16 in gene cloning experiments. Homogeneity and the sequence extremities of the
17 purified linear vector were checked by sequencing using the T7prom and T7term
18 primers.

19

20 **2.5 Target genes amplification**

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

1
2
3
4
5
6 1 primer extensions were respectively 5'-AAGGAGATATACCATATG-3' and 5'-
7
8 2 TACAGAATTCGGATCCTTA-3', for pLIM14_NdeI_BamHI, 5'-
9
10 3 ACCATCACCATCATATG-3' and 5'-TACAGAATTCGGATCCTTA-3' and for
11
12 4 pLIM14_NcoI_BamHI, 5'-
13
14 5 CGGGCGGTAGCCATGGGGAAAATCTGTATTTTCAGGGC-3' (containing
15
16 6 protease TEV site) and 5'-TACAGAATTCGGATCCTTA-3'. These extensions
17
18 7 allow both restriction/ligation (using the underlined restriction sites) and InFusion
19
20 8 methods (relying on the extended sequence complementary to the vector).
21
22 9 Primers were ordered at 20 μ M in a 96-wells microplate (MWG). Preparation of
23
24 10 the 0.5 μ M primers mix and of the PCR reaction was fully automated on a
25
26 11 Microlab Star robot (Hamilton). Reactions were performed with 100 ng of
27
28 12 chromosomal DNA template, 10 μ L of primer mix 0.5 μ M, 4 μ L of dNTPs 2.5
29
30 13 mM, 10 μ L of HF buffer 5X (Finnzyme), 0.5 μ L Phusion enzyme (2U/ μ L,
31
32 14 Finnzyme) in a 50 μ L volume, using a Trobot PCR machine (Biometra) with the
33
34 15 following parameters : 98°C, 1 min; 30 cycles of [98°C, 30 sec; 50°C, 30 sec;
35
36 16 72°C, 1 min]; 72°C, 8 min.

37
38
39 17 Five μ L of each PCR product were analyzed on 1% agarose gel. Satisfying
40
41 18 PCR products (single or few multiple bands) were purified on a Nucleofast plate
42
43 19 (Macherey-Nagel) and eluted with 40 μ L of water, using a Microlab Star
44
45 20 automaton (Hamilton).

21 22 **2.6 Cloning with the In-Fusion™ Advantage System**

1
2
3
4
5
6 1 Five μL of the PCR products were treated with Cloning Enhancer reagent
7
8 (In-Fusion™, Clontech) according to the supplier's recommendations. The In-
9
10 2 Fusion™ reactions were performed by mixing 100 ng of linear vector with 2 μL
11
12 3 of each treated insert (3 μL for the 3501 bp-long insert) in 10 μL final volume.
13
14 4
15 5 After the addition of 40 μL of TE, 2.5 μL of each reaction were used to transform
16
17 6 50 μL of NEB5 α competent *E. coli* cells (Biolabs). The transformed cells were
18
19 7 pelleted by centrifugation (5200 g, 5 min) and spread onto LB (kanamycin 50
20
21 8 mg/L, glucose 10 mM) agar plates and incubated overnight at 37°C.
22
23
24
25
26

27 2.7 Cloning by restriction/ligation

28
29 11 Forty μL of PCR products were mixed with 5 μL buffer 4 10X (NEB), 0.5
30
31 12 μL *Nde*I (10 U) or *Nco*I (5 U) and *Bam*HI (5U) enzyme (NEB). After 2 h at
32
33 13 37°C, fragments were purified on a Nucleofast plate (Macherey-Nagel) and eluted
34
35 14 with 40 μL of water. Two μL of each insert were mixed with 100 ng of linear
36
37 15 vector in the presence of 1 μL of 10X T4 DNA ligation buffer, 1 μL of T4 DNA
38
39 16 ligase (3 U/ μL , Promega), in a final volume of 10 μL . After 3 hours at room
40
41 17 temperature, ligation reactions were stopped at 65°C (10 min). Five μL were used
42
43 18 to transform 50 μL of DH5 α competent *E. coli* cells. The transformed cells were
44
45 19 then pelleted before spreading on LB (kanamycin 50 mg/L, glucose 10 mM) agar
46
47 20 plate and incubated overnight at 37°C.
48
49
50
51
52

53 21

54 22 2.8 Plasmid screening by restriction analysis

1
2
3
4
5
6 1 Isolated transformants were used to inoculate 2 ml of 2xTY (50 mg/L
7
8 2 kanamycin) medium in a 24-wells plates sealed with a porous film. Cultures were
9
10 3 grown for 20 h at 37°C with agitation (200 rpm). Cells were pelleted and
11
12 4 plasmids were prepared on a Qiagen BioRobot 8000 using the Nucleospin robot
13
14
15 5 96 kit (Macherey-Nagel) and eluted with 125 µL of EB buffer. Eight µL were
16
17
18 6 used for restriction analysis with 2.5 U of each enzyme in a final volume of 10
19
20 7 µL; profiles were analyzed on 1% agarose gel. Two positive clones for each gene
21
22 8 were sent for full length sequencing with the universal T7 prom and T7 term
23
24 9 primers (Beckman Cogenics). Sequencing with internal primers was necessary
25
26
27 10 for genes longer than 2 Kb.
28

29
30 11
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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. subtilis* 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 *NdeI* and *BamHI* 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 *NcoI* and *BamHI* releases 5455 bp

1 and 331 bp fragments. The larger fragments are the linearized vectors, the small
2 fragments contain the *ccdB* gene. These digestions allow the in frame insertion of
3 any gene amplified with specific primers (see Materials and Methods and Figure
4 1).

6 **3.2 Large scale plasmid purification**

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

14 **3.3 Large-scale linear plasmid purification by Mono Q™ anion exchange 15 chromatography**

16 This procedure succeeded with the three linearized pLIM vectors. The
17 purification of pLIM13 digested with *NdeI* and *BamHI* is presented in details
18 below as an example.

19 One hundred micrograms of pLIM13 were digested and the NaCl
20 concentration was brought to 600 mM prior to the chromatography to avoid
21 adsorption of low charge density impurities on the Mono Q™ column [15]. The
22 advantage of a Mono Q™ resin is the fast and strong binding of the DNA (Figure
23 2) [21].

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

9 **3.4 Quality control of linear plasmid**

10 One hundred nanograms of each vector (pET41c, pLIM13, and pLIM14)
11 were transformed into NEB5 α and DH5 α . In both strains, approximately one
12 thousand transformants were obtained with pET41c and none with pLIM13 as
13 expected due to the presence of the *ccdB* gene in the latter plasmid. To our
14 surprise, thousand transformants were also obtained with the pLIM14. As *ccdB* is
15 still present, we hypothesize that the normally cytosolic CcdB protein is not
16 functional when fused to Mystic and targeted to the membrane. However, this
17 finding allowed us to evaluate the homogeneity of the purified linear vector, as we
18 could compare the number of transformants obtained with the native and the
19 linearized plasmid. Linearized pLIM14 yielded no transformant showing the
20 absence of undigested parental vector.

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

1
2
3
4
5
6 1 (25 μ g), 43% (38 μ g) or 73% (69 μ g) for pLIM13_ *NdeI_BamHI*,
7
8 2 pLIM14_ *NdeI_BamHI* and pLIM14_ *NcoI_BamHI*, respectively. Individual
9
10 3 fraction precipitations and pooling are probably responsible for the loss of
11
12 4 material.
13
14
15
16
17

18 **3.5 Efficient cloning with linear plasmid**

19
20 7 The suitability for high throughput cloning of the purified
21
22 8 pLIM13_ *NdeI_BamHI* linearized vector was tested by the insertion 24
23
24 9 independent genes in parallel. The gene size varied between 387 bp and 3501 bp.
25
26
27 10 Eleven genes were cloned by restriction/ligation and 13 using the In-Fusion™
28
29 11 technique. Genes containing restriction sites within their sequence were treated
30
31 12 by the In-Fusion™ method.
32
33

34 13 The automated PCR amplification of the 24 genes was performed with the
35
36 14 Phusion enzyme on a Hamilton robot. PCR products were analyzed on agarose
37
38 15 gel (Figure 2) and showed the successful amplification of all the genes using the
39
40 16 same PCR parameters. Purification on a Nucleofast plate eliminated primer
41
42 17 dimers and allowed buffer exchange.
43
44

45
46 18 Genes to be treated by ligation were first digested with the two appropriate
47
48 19 restriction enzymes, and purified again on Nucleofast to eliminate the short
49
50 20 fragments released by the digestion. Genes destined to In-Fusion™ cloning were
51
52 21 directly incubated with the Cloning Enhancer according the manufacturer's
53
54 22 recommendations.
55
56
57
58
59
60

1
2
3
4
5
6 1 With both cloning methods, 100 ng of linear vector were mixed to 2-3 μ L of
7
8 2 insert. We have deliberately chosen to standardize the volume of insert,
9
10 3 independently of their size, in order to develop an efficient high throughput
11
12 4 process for a wide range of gene sizes, as the amplification delivers PCR
13
14 5 fragments in amounts that are not limiting. After ligation or In-Fusion™
15
16 6 recombination, transformation suspensions were plated on agar containing
17
18 7 kanamycine, and glucose to repress the expression leakage.
19
20
21

22 8 Between 20 and 100 transformants were obtained, without relationship
23
24 9 between the size of the insert and the number of transformants. For the negative
25
26 10 control (linear pLIM13 plasmid without insert), around 10 and 70 transformants
27
28 11 were obtained. These transformants resulted from aberrant recombination events.
29
30 12 To evaluate the cloning success rate, 2 to 8 transformants were analyzed for each
31
32 13 of the 24 genes by plasmids miniprep followed by restriction mapping.
33
34
35

36 14 The 24 genes were successfully cloned in parallel into the expression vector
37
38 15 indicating the good quality of the linear vector preparation (Table 1). Cloning
39
40 16 efficiencies were not correlated to the size of the gene, although the most difficult
41
42 17 gene to clone was the longest (3501 bp). Except in the latter case, 50% to 100%
43
44 18 of positive clones were obtained for all other genes. The negative clones resulted
45
46 19 apparently from aberrant recombination events, such as the clones obtained from
47
48 20 the negative controls. The mean cloning efficiency by restriction/ligation was
49
50 21 63%, whereas that obtained by In-Fusion™ was 80% including the lower yield
51
52 22 obtained with the longest gene. The systematic full length gene sequencing
53
54
55
56
57
58
59
60

1
2
3
4
5
6 1 performed on two positive clones of each gene, showed the absence of mutation
7
8 2 with a single exception likely originating from a PCR error.
9

10
11 3

12 13 4 **4 Concluding remarks**

14
15 5 Large amount of linear expression vector is obtained by separating the
16
17 6 vector from its insert by anion exchange chromatography. The cloning results
18
19 7 indicate that the quality of the linear vector purified by Mono Q™ is compatible
20
21 8 with different high throughput cloning techniques. However, the In-Fusion™
22
23 9 technique is, in our view, better adapted to high throughput as this system
24
25 10 catalyzes the joining of DNA pieces in a manner that is independent of the
26
27 11 sequence of the cloned gene.
28
29
30
31

32
33 12

34
35 13 We thank André Zapun for critical reading of the manuscript. This work
36
37 14 was partly supported by the European grant LSMH-CT-EUR-INTAFAR 2004-
38
39 15 512138 and the ANR-FORM-080124-01-01. This work was performed on the
40
41 16 RoBioMol Platform at the IBS (Grenoble), which belongs to the Partnership for
42
43 17 Structural Biology (PSB).
44
45

46
47 18

48
49 19 The authors have declared no conflict of interest.
50
51
52
53
54
55
56
57
58
59
60

5. References

- [1] Busso, D., Delagoutte-Busso, B., Moras, D., Construction of a set Gateway-based destination vectors for high-throughput cloning and expression screening in *Escherichia coli*. *Analytical biochemistry* 2005, 343, 313-321.
- [2] Geertsma, E. R., Poolman, B., High-throughput cloning and expression in recalcitrant bacteria. *Nature methods* 2007, 4, 705-707.
- [3] Qin, H., Hu, J., Hua, Y., Challa, S. V., *et al.*, Construction of a series of vectors for high throughput cloning and expression screening of membrane proteins from *Mycobacterium tuberculosis*. *BMC biotechnology* 2008, 8, 51.
- [4] 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 research* 2007, 35, 1-12.
- [5] Klock, H. E., White, A., Koesema, E., Lesley, S. A., Methods and results for semi-automated cloning using integrated robotics. *Journal of structural and functional genomics* 2005, 6, 89-94.
- [6] Berrow, N. S., Alderton, D., Owens, R. J., The precise engineering of expression vectors using high-throughput In-Fusion PCR cloning. *Methods in molecular biology (Clifton, N.J)* 2009, 498, 75-90.
- [7] Weeks, S. D., Drinker, M., Loll, P. J., Ligation independent cloning vectors for expression of SUMO fusions. *Protein expression and purification* 2007, 53, 40-50.
- [8] Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning. A laboratory manual*, 1989.

- 1
2
3
4
5
6 [9] Ferreira, G. N., Monteiro, G. A., Prazeres, D. M., Cabral, J. M., Downstream
7
8 processing of plasmid DNA for gene therapy and DNA vaccine applications.
9
10 *Trends in biotechnology* 2000, *18*, 380-388.
11
12 [10] Heilig, J. S., Elbing, K. L., Brent, R., Large-scale preparation of plasmid
13
14 DNA. *Current protocols in molecular biology / edited by Frederick M. Ausubel ...*
15
16 *[et al* 2001, *Chapter 1*, Unit1 7.
17
18 [11] Stadler, J., Lemmens, R., Nyhammar, T., Plasmid DNA purification. *The*
19
20 *journal of gene medicine* 2004, *6 Suppl 1*, S54-66.
21
22 [12] Levy, M. S., O'Kennedy, R. D., Ayazi-Shamlou, P., Dunnill, P., Biochemical
23
24 engineering approaches to the challenges of producing pure plasmid DNA. *Trends*
25
26 *in biotechnology* 2000, *18*, 296-305.
27
28 [13] Chakrabarti, A., Sitaric, S., Ohi, S., A procedure for large-scale plasmid
29
30 isolation without using ultracentrifugation. *Biotechnology and applied*
31
32 *biochemistry* 1992, *16*, 211-215.
33
34 [14] Hines, R. N., O'Connor, K. C., Vella, G., Warren, W., Large-scale
35
36 purification of plasmid DNA by anion-exchange high-performance liquid
37
38 chromatography. *BioTechniques* 1992, *12*, 430-434.
39
40 [15] Diogo, M. M., Queiroz, J. A., Prazeres, D. M., Chromatography of plasmid
41
42 DNA. *J Chromatogr A* 2005, *1069*, 3-22.
43
44 [16] Prazeres, D. M., Schluep, T., Cooney, C., Preparative purification of
45
46 supercoiled plasmid DNA using anion-exchange chromatography. *J Chromatogr*
47
48 *A* 1998, *806*, 31-45.
49
50
51
52
53
54
55
56
57
58
59
60

- 1 [17] Smrekar, F., Podgornik, A., Ciringier, M., Kontrec, S., *et al.*, Preparation of
2 pharmaceutical-grade plasmid DNA using methacrylate monolithic columns.
3 *Vaccine*, 28, 2039-2045.
- 4 [18] Westman, E., Eriksson, S., Laas, T., Pernemalm, P. A., Skold, S. E.,
5 Separation of DNA restriction fragments by ion-exchange chromatography on
6 FPLC columns Mono P and Mono Q. *Analytical biochemistry* 1987, 166, 158-
7 171.
- 8 [19] Muller, W., Fractionation of DNA restriction fragments with ion-exchangers
9 for high-performance liquid chromatography. *European journal of biochemistry /*
10 *FEBS* 1986, 155, 203-212.
- 11 [20] Wells, R. D., Hardies, S. C., Horn, G. T., Klein, B., *et al.*, RPC-5 column
12 chromatography for the isolation of DNA fragments. *Methods in enzymology*
13 1980, 65, 327-347.
- 14 [21] Tiainen, P., Galaev, I., Larsson, P. O., Plasmid adsorption to anion-exchange
15 matrices: comments on plasmid recovery. *Biotechnology journal* 2007, 2, 726-
16 735.
- 17 [22] Wallin, E., von Heijne, G., Genome-wide analysis of integral membrane
18 proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci* 1998,
19 7, 1029-1038.
- 20 [23] Boyd, D., Schierle, C., Beckwith, J., How many membrane proteins are
21 there? *Protein Sci* 1998, 7, 201-205.
- 22 [24] Kefala, G., Kwiatkowski, W., Esquivies, L., Maslennikov, I., Choe, S.,
23 Application of Mystic to improving the expression and membrane integration of

- 1
2
3
4
5
6 1 histidine kinase receptors from Escherichia coli. *Journal of structural and*
7
8 2 *functional genomics* 2007, 8, 167-172.
9
10 3 [25] Roosild, T. P., Greenwald, J., Vega, M., Castronovo, S., *et al.*, NMR
11
12 4 structure of Mystic, a membrane-integrating protein for membrane protein
13
14 5 expression. *Science (New York, N.Y)* 2005, 307, 1317-1321.
15
16 6 [26] Roosild, T. P., Vega, M., Castronovo, S., Choe, S., Characterization of the
17
18 7 family of Mystic homologues. *BMC structural biology* 2006, 6, 10.
19
20 8 [27] Bahassi, E. M., Salmon, M. A., Van Melderren, L., Bernard, P., Couturier, M.,
21
22 9 F plasmid CcdB killer protein: ccdB gene mutants coding for non-cytotoxic
23
24 10 proteins which retain their regulatory functions. *Molecular microbiology* 1995,
25
26 11 15, 1031-1037.
27
28 12 [28] Afif, H., Allali, N., Couturier, M., Van Melderren, L., The ratio between
29
30 13 CcdA and CcdB modulates the transcriptional repression of the ccd poison-
31
32 14 antidote system. *Molecular microbiology* 2001, 41, 73-82.
33
34 15 [29] Bernard, P., Couturier, M., Cell killing by the F plasmid CcdB protein
35
36 16 involves poisoning of DNA-topoisomerase II complexes. *Journal of molecular*
37
38 17 *biology* 1992, 226, 735-745.
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 **Figure legends**

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

9
10 **Figure 2. Linear plasmid purification by Mono Q™ anion exchange**
11 **chromatography and PCR amplification of the 24 genes used in cloning**
12 **experiments.** (A) Chromatogram showing the purification of pLIM13 digested
13 with *NdeI* and *BamHI* monitored at 254 nm. Plasmids were loaded on a Mono
14 Q™ 5/50 GL column. DNA was eluted with a 64 ml linear gradient of 700 mM
15 (0% B) to 900 mM (10 % B) of NaCl at 0.15 ml/min. Arrows indicate the
16 fractions analyzed. (B) 1% Agarose gel analysis of the fractions corresponding to
17 the elution peaks shown in (A): M, DNA ladder; 1, loaded sample; 2, flow
18 through (pooled fractions 2-4); 3 to 6, fractions corresponding to the *ccdB* insert;
19 7 to 15, fractions corresponding to the linear vector. (C) 1% Agarose gel analysis
20 of the pooled elution fractions from the chromatographic separations of
21 pLIM14_ *NdeI*_ *BamHI*: 1, Mistic-*ccdB* insert; 2, linear vector; and
22 pLIM14_ *NcoI*_ *BamHI*: 3, *ccdB* insert; 4, linear vector. (D) 1% Agarose gel
23 analysis of the PCR amplification of 24 independent genes. M, DNA ladder; 1 to

- 1
- 2
- 3
- 4
- 5
- 6 1 24, PCR amplification obtained in the conditions described in Materials and
- 7
- 8 2 Methods section and successfully cloned into pLIM13_ *NdeI*_ *Bam*HI:
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19
- 20
- 21
- 22
- 23
- 24
- 25
- 26
- 27
- 28
- 29
- 30
- 31
- 32
- 33
- 34
- 35
- 36
- 37
- 38
- 39
- 40
- 41
- 42
- 43
- 44
- 45
- 46
- 47
- 48
- 49
- 50
- 51
- 52
- 53
- 54
- 55
- 56
- 57
- 58
- 59
- 60

For Peer Review

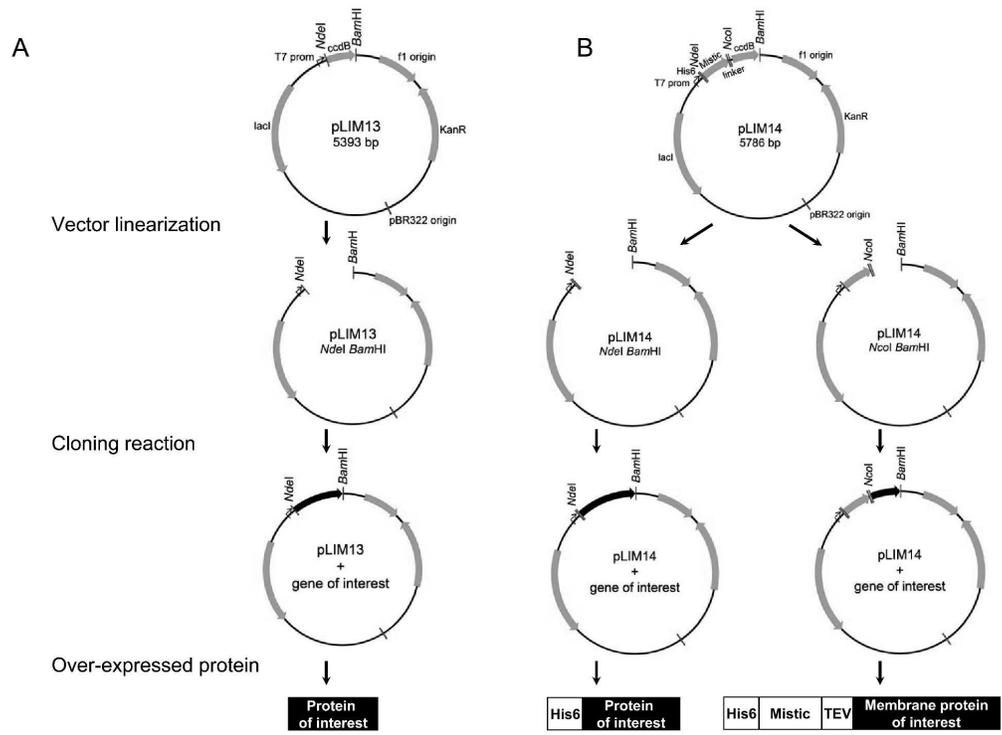
1 **Table 1. Gene cloning using pLIM13_***NdeI-Bam***HI linear vector by**
 2 **restriction/ligation or InFusion techniques**

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)

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

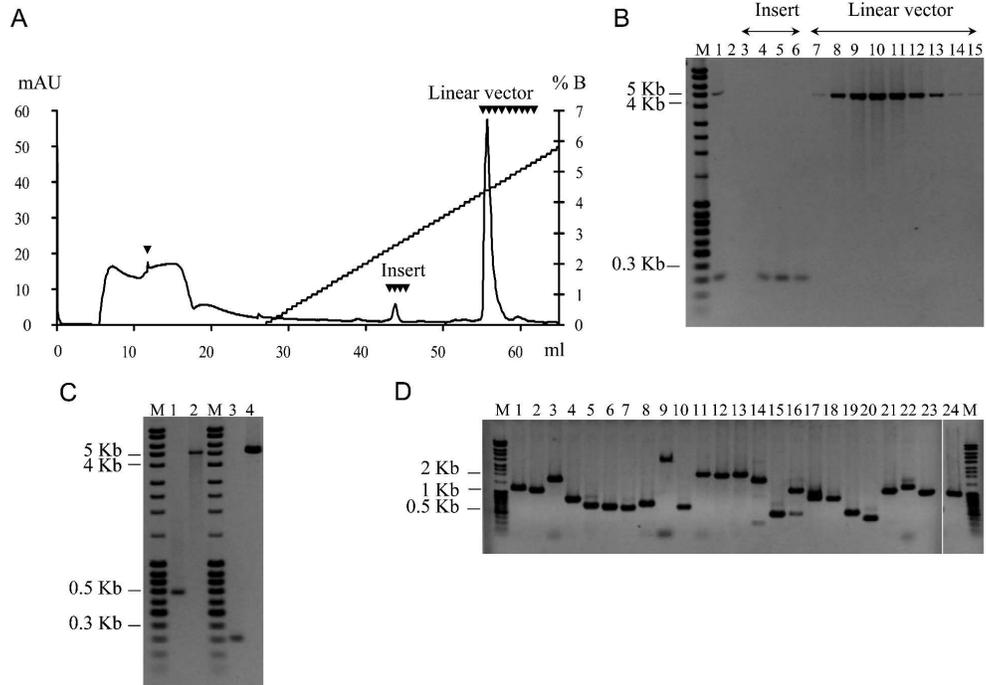


245x180mm (600 x 600 DPI)

Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



250x179mm (600 x 600 DPI)