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Article

Engineering a Minimal 1185 Bp Cloning Vector from a Puc18 Plasmid Backbone with an Extended Multiple Cloning Site

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Abstract: Minimal plasmids play an essential role in many intermediate steps in molecular biology. They can for example be used to assemble building blocks in synthetic biology or be used as intermediate cloning plasmids that are ideal for PCR-based mutagenesis methods. A small backbone also opens up for additional unique restriction enzyme cloning sites. Here we describe the generation of a ~1kb fully functional cloning plasmid with an extended multiple cloning site (MCS). To our knowledge, this is the smallest high-copy cloning vector ever described.

Keywords: PCR, recombination, cloning, engineering, biotechnology, synthetic biology, synthetic nucleotide, plasmids, repository, minimalism, *Escherichia coli*, mutagenesis

1. Introduction

Minimal plasmids have many uses and can either be generated synthetically [1] or by iterative deletions in an existing plasmid. The pUC family [2,3] of plasmids have been extensively used as backbone for various cloning and expression vectors [4]. One of the most attractive features of the pUC family of plasmids is that they harbor a mutated pMB1 origin of replication (Ori) which leads to very high-copy replication of the plasmid [5]. Minimalism is an artistic as well as a functional design ideal [6], which provides enhanced robustness and utility in many fields of engineering (often referred to as the KISS principle, an acronym with many different interpretations but with the same essential meaning : keep it small and simple). The ideal is often summarized by a quote from the French poet Antoine de Saint Exupéry [7]: *"It seems that perfection is attained not when there is nothing more to add, but when there is nothing more to remove"*. In the software world, useless code and functions are often referred to as "bloat", and some software projects have simplicity and minimalism as core values in their development philosophy [8] (for example: https://suckless.org/philosophy/). Just like in software, useless and bloated code in plasmids can cause "bugs", like the unintended eukaryotic transcription factor binding sites present in the pUC plasmids [9,10]. We have thus applied the minimalistic philosophy in the design of a high-copy cloning plasmid backbone with as little bloat as possible, which opens up for many improved downstream applications. Minivectors have for example been found to be highly efficient for mammalian cell transfection [11] and *in vivo* for gene therapy [12]. Tiny plasmids are however not only found in a laboratory settings, replicating minimal plasmids down to 746 bp have also been found in nature [13]. There may thus be further room for improvement, which we invite the research community to explore further in an open, distributed manner.

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2. Materials and Methods

2.1. Iterative deletions of pUC18

The plasmid was reduced in size in several different reaction (Rxn) steps by PCR as outlined in Table 1. Primers were ordered from Invitrogen. The *in silico* cloning, graphical vector map generation and sequence analyses was done in UGENE (http://ugene.net/) [14].

Table 1. Overview of primers used in the iterative shrinking of pUC18 into pICOz.

Primer name	Sequence	PCR rxn
PUC18-dLacZ-F	gccgttaacccatggccaagcttgcatgcctgcaggtcg	1 [1]
pUC18-dLacZ-R	ccatatggcctcgcgacgcgttatgtatccgctcatgag	1 [1]
pUCmini-F	gtgggcccgtttaaacacatgtgagcaaaaggccag	2 [1]
PUCmini-R	tagtctcgaggatatccgaattcgagctcggtac	2 [1]
pUCmu-F	taccaatgcttaatcagtgaggca	3 [1]
pUCmu-R	agtagaaaagatcaaaggatcttct	3 [1]
pUcReFix-F	attagctcgagactagtgggcccgtttaaacacatgtgtttttccataggctccg	4 [2]
pUcReFix-R	ctaatctcgaggatatccgaattcgagctcggtacccgggatcctctagagtcgacctg	4 [2]
pICOz-dAMP-F	ttcgtggccgaggagcaggactgacgtagaaaagatcaaaggatctt	5 [3]
pICOz-dAMP-R	aacggcactggtcaacttggccatactcttccttttcaatattat	5 [3]
PICOz-Zeo-F	ataatattgaaaaaggaagagtatggccaagttgaccagtgccgtt	6 [3]
pICOz-Zeo-R	aagatcctttgatcttttctacgtcagtcctgctcctcggccacgaa	6 [3]

[1] Phusion DNA polymerase PCR, phosphorylated primers + T4 ligation. [2] Phusion DNA polymerase PCR, digestion + T4 ligation. [3] Universe DNA polymerase PCR, CloneEZ recombination.

Phusion DNA polymerase (New England Biolabs) PCR reactions were performed with the following general program: 3 min 98 °C denaturation, 35x [10 s 98 °C denaturation, 20 s 57 °C annealing, 20 s/Kbp 72 °C elongation], 10 min 72 °C. The Universe DNA polymerase (Biotool) PCR progam was 5 min 95°C denaturation, 35x [20 s 95 °C denaturation, 20 s 57 °C annealing, 45 s/Kbp 72 °C elongation], 10min 72 °C elongation. Ligation with T4 DNA ligase (Promega) was performed at room temperature over night. CloneEZ (GenScript) reactions were performed for 30 minutes at room temperature. All DNA products were transformed into competent MC1061 *E. coli* by 30 s heat shock at 42 °C.

2.2. Verification, storage and distribution of DNA material

The resulting plasmids were deposited to the ISO 9001:2008 [15] compliant BCCM/GeneCorner culture collection (www.genecorner.ugent.be) under the following accession numbers for re-distribution: pUC18deltaLacZ (LMBP 9213), pUCmini (LMBP 9221), pUCmu (LMBP 9329) and pICOz (LMBP 11103). All plasmids were verified by sanger sequencing and restriction enzyme digestion. Additional plasmids with alternative selection markers not described in this paper are also available: pUCmuk (Kanamycin; LMBP 9630) and pICOb (Blasticidin; LMBP 9607). Also specific-use plasmids, like a minimal cloning/expression vector for CRISPR/Cas9 guide RNA in the pUCmu backbone (pU6mu; LMBP 9491).

3. Results

In order to shrink a high-copy and widely used cloning backbone like pUC18, we sequentially eliminated code segments that we identified as “useless bloat”. The first reaction (Rxn1; Figure 1) eliminated LacZ and some additional sequence upstream of the pBla promoter driving Ampicilin resistance. The eliminated sequence got replaced by some additional restriction enzyme sites (MluI,

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NruI, SfiI, NdeI, NcoI, MscI) that were added to the multiple cloning site (MCS). This resulted in pUC18deltaLacZ, which is 496 bp smaller than the pUC18 parental plasmid.

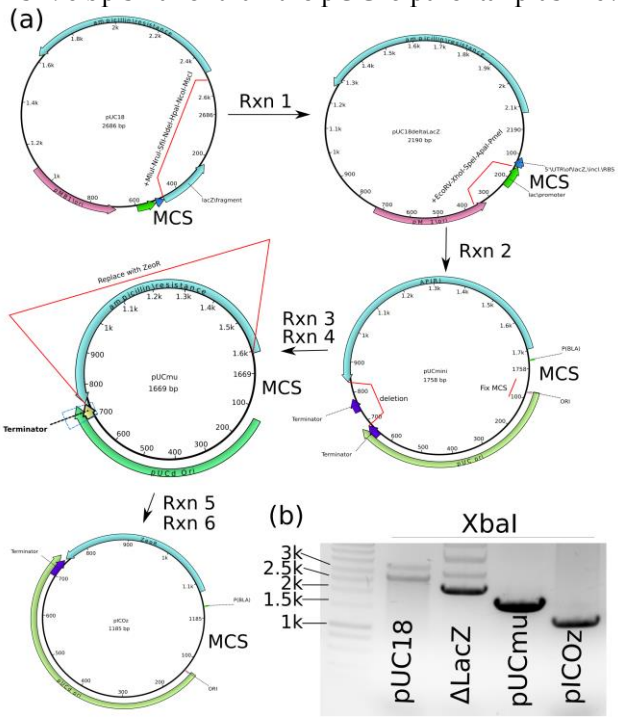

Figure 1. Overview of the cloning scheme for generation of a minimal vector backbone. (**a**) Graphical overview of the modifications made in each step in the miniaturization process. Rxn = reaction. Red lines indicate area that was modified in the following reaction.; (**b**) XbaI digest of 3µg pUC18, ΔlacZ (=pUC18deltaLacZ), pUCmu and pICOz run on a 1% Agarose/TAE gel. Sizes on the SmartLadder (Eurogentec) indicated in Kbp ("k").

In the second reaction (Rxn2, Figure 1), we eliminated additional parts of the LacZ promoter and useless code between the MCS and the origin of replication (Ori) from pUC18ΔLacZ, and this code got replaced by additional restriction enzyme sites in the MCS (EcoRV, XhoI, SpeI, ApaI, PmeI). By random chance, a clone that was picked up showed a deletion in the pUC Ori and part of the MCS without showing any detrimental effects on plasmid yield. This plasmid was kept as pUCmini, which is 432 bp smaller than the pUC18deltaLacZ parental plasmid.

In order to shrink the plasmid further, an additional region with useless code was identified between the ampicillin resistance and the Ori, and a deletion was made to make the resistance marker use a terminator sequence present in the Ori (Rxn 3, Figure 1). Since the pUCmini plasmid also had a deletion in the MCS, we also repaired the MCS to restore the lost restriction enzyme sites (Rxn 4, Figure 1). The resulting pUCmu plasmid ended up being 89 bp smaller than pUCmini but with a complete extended MCS.

After this reaction, no "useless bloat" code could be identified in the sequence and the only way to further shrink the plasmid was by replacing the antibiotic selection with a smaller selectable marker using overlap extension cloning [16] or recombination-based cloning. As a proof-of-concept, we replaced ampicillin resistance with zeocin resistance by amplifying the pUCmu plasmid without the ampicillin resistance sequence (Rxn 5, Figure 1) and recombined this PCR product with a PCR-amplified zeocin resistance gene (Rxn 6) using CloneEZ. In parallel, a PCR-based fusion between the two fragment was equally successful. The resulting pICOz plasmid is 484 bp smaller than the minimal pUCmu parental plasmid.

With these four sequential steps of elimination, we have thus been able to reduce the 2686 bp pUC18 plasmid to the 1185 bp pICOz plasmid which contains more useable cloning sites, a total size reduction of 56%.

4. Discussion

We here describe the generation of a minimal ~1kb fully functional cloning plasmid, which is as far as we currently can get in miniaturization. All useless sequences have been eliminated and there is no additional space between the MCS, Ori and selectable marker (Fig. 1). We now use the smallest widely used resistance marker and the only way to shrink the plasmid further is to do additional deletion/engineering of the Ori or replace the Ori with an alternative, smaller, Ori. By lucky coincidence, we managed to identify a random deletion mutant of the pUC variant of the pMB1 Ori, which shrunk the pUC Ori from 750 bp to 616 bp. A minimal pUC-derived Ori of 674 bp (GenBank: EU496091.1) has been described in the BioBrick system [17]. Sequence comparisons reveal alignment between pICOz and base 3-618 of the BioBrick pUC Ori. The alignment also revealed that the BioBrick pUC contains a few point mutations to eliminate restriction sites in the Ori. Our minimal backbone could in principle very easily be made completely synthetically, where all restriction sites in the backbone in theory could be eliminated and the MCS be further extended. This could be one very interesting future prospect for further development of the pICOz backbone as a standard IGEM (http://igem.org/) "part" for synthetic biology [18,19]. Our random deletion pUC Ori mutant is likely to be the most minimal variant of the pUC Ori that can be generated, since RNAII (27-615 bp on the BioBrick genbank annotation) is absolutely needed. A minimal Ori from pSC101 of only 220 bp has been described [20], but this is a low copy plasmid which usually is less interesting and it is not entirely clear if this minimal element is sufficient to make a self-replicating plasmid. For example the pMB1/pUC Ori often annotaded in the pUC plasmid maps is not the complete sequence required for plasmid replication (Figure 1). In theory, however, it should be possible to shrink pICOz to 789 bp with this minimal Ori if it works. With our 1185 bp, we are however already very close to the absolutely smallest plasmid ever found in nature (746 bp), and we usually need a high-copy plasmid and a selectable marker to have a useful cloning vector, which currently restricts further size optimization. The selectable marker unconditionally adds at least 458 bp (promoter+CDS+terminator), so it might be that we are already at the lowest limits of what can be obtained for a fully functional high-copy cloning vector in *E. coli*.

We do however highly encourage others to try to further improve on our best attempt to generate a minimal core cloning plasmid. Distributed development has been shown to be an extremely powerful force in open source software development [21], and genetic material shows many commonalities with software in that it can be copied and reproduced very easily [22]. An essential component for a functional distributed development of genetic material is the availability of reliable repositories of verified material to be distributed with as few restrictions as possible [23,24]. For plasmids, this means culture collections like Addgene (www.addgene.org) and BCCM/GeneCorner (www.genecorner.ugent.be). One potential uncertainty in plasmid development and other genetic resources is still the intellectual property status of derived material [25–27], which sometimes is covered by a Materials Transfer Agreement (MTA) but not always. A true copyleft for genetic material does not exist, but there are some efforts to bring this very successful cultural and legal philosophy from open source software also to the development of genetic material [28–30].

People developing this backbone further are naturally free to explore and adapt it in any way they see fit. Interesting future perspectives would however be to further explore alternative Ori and selectable markers in this vector backbone to try to shrink it further. Also extending the MCS and making a synthetic "un-cleavable" pICOz backbone is a very interesting idea for future development. Also other optimization aspects than size and cloning properties can however be interesting. For example, non-antibiotic selectable markers (like Zn^{2+} [31], Cu^{2+} [32], Ag^{+} [33] or high-salt [34] resistance) could be highly interesting to explore for *E. coli* cloning vectors.

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Writing-Review & Editing, R.B.; Visualization, J.S.; Supervision, R.B.; Project Administration, R.B.; Funding Acquisition, R.B."

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