Article

Engineering a minimal 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 pICOz, a 1185 bp fully functional high-copy 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 on a GeneAmp 9700 (PE Biosystems) thermocycler. 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 (internal sequencing facility) and restriction enzyme digestion (Promega). Genbankfiles for pUCmu and pICOz in supplemental data.

2.3. Determination of relative yield of plasmid variants

The plasmids pUC18, pUC18deltaLacZ, pUCmu and pICOz were grown in quadruplicates and plasmids were prepped from 3 ml culture per plasmid and replicate using HQ Mini Plasmid Purification Kit (Invitrogen). DNA concentrations were determined by NanoDrop ND-8000 (ThermoFisher).

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3. Results

3.1. Generation of a minimal cloning plasmid with an extended multiple cloning site

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, NruI, SfiI, NdeI, NcoI, MscI; Figure 2) that were added to the multiple cloning site (MCS). This resulted in pUC18deltaLacZ, which is 496 bp smaller than the pUC18 parental plasmid.

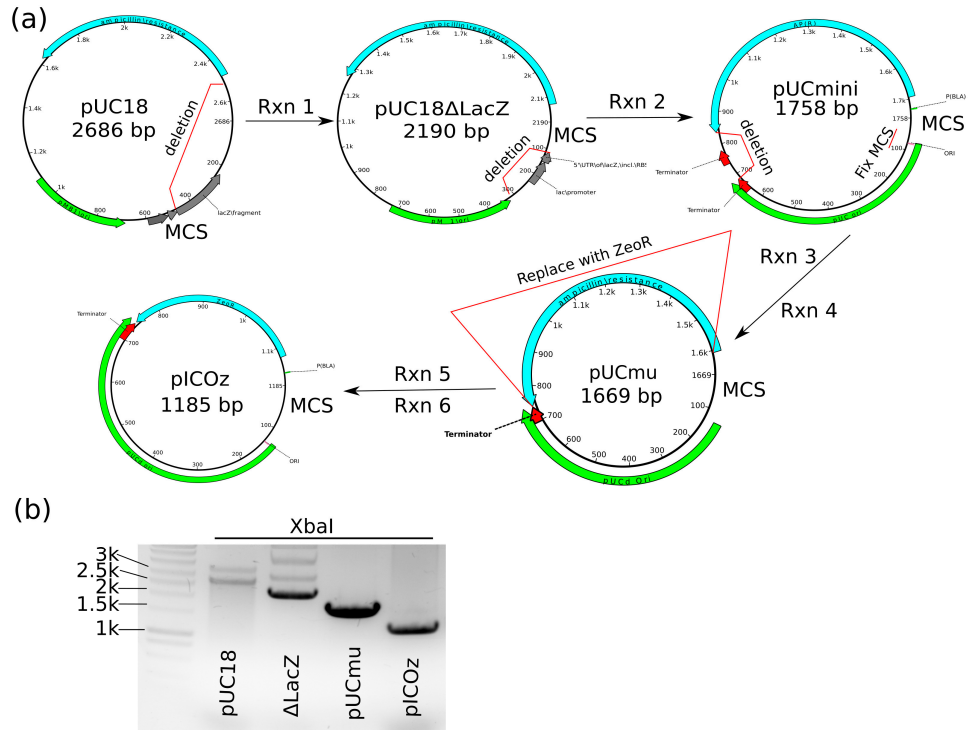

Figure 1. Overview of the cloning scheme for generation of a minimal vector backbone. (**a**) Graphical overview of the modifications made in each step in the miniaturization process. Rxn = reaction. Red lines indicate area that was modified in the following reaction.; Green plasmid elements indicate the annotated Ori in the plasmid; Grey plasmid elements are "useless" annotated elements; Blue plasmid elements indicate antibiotic selection marker; Red plasmid elements indicate annotated prokaryotic transcription terminators. (**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 ; Figure 2). 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.

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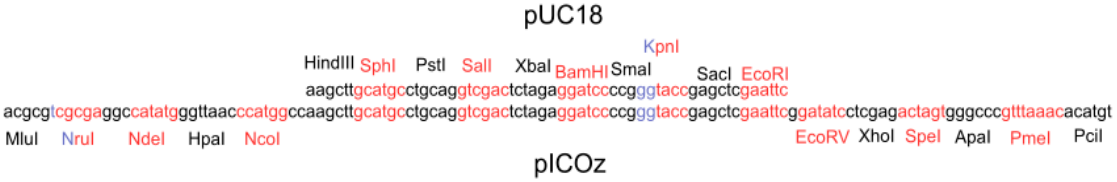

Figure 2. Comparisons of the original pUC18 MCS compared to the extended MCS in pUCmu and pICOz. Restriction sites are indicated in alternating red and black text for clarity. Overlapping nucleotides for two restriction sites are indicated in purple.

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%.

3.2. Determination of absolute and relative yield of the minimal cloning plasmids

In order to verify that the deletions had not eliminated anything that would impact plasmid copy number and total yield, we did several parallel heat shock *E. coli* transfections and mini plasmid preparations of four constructs (pUC18, pUC18deltaLacZ, pUCmu and pICOz).

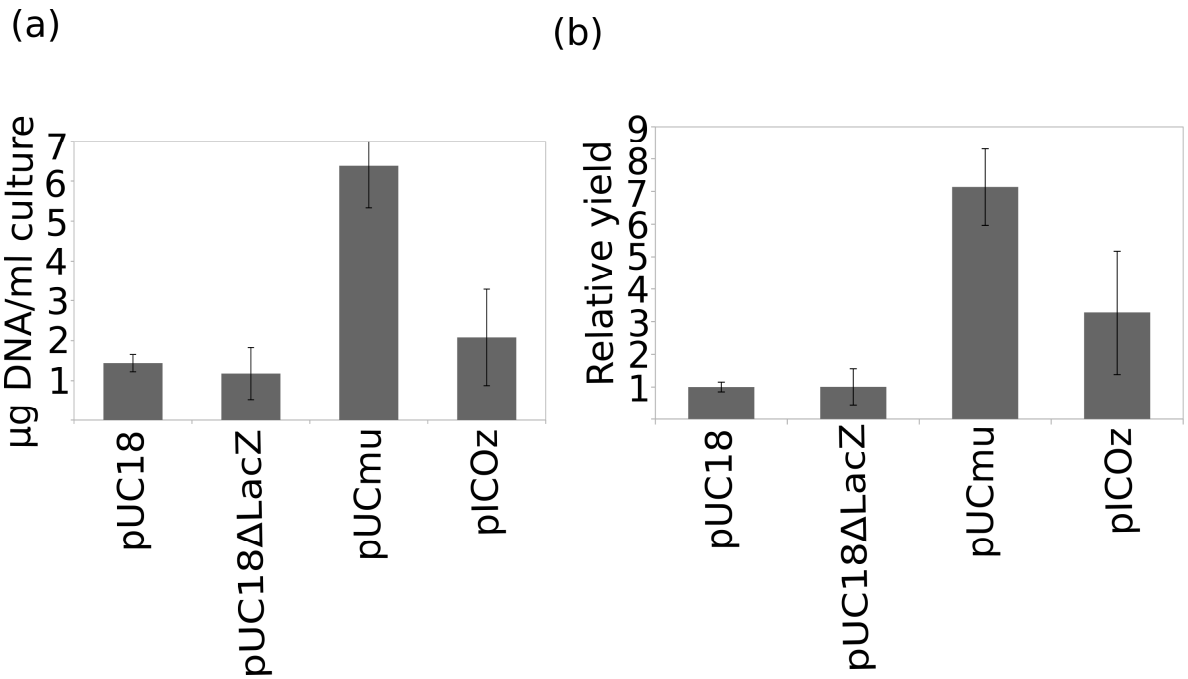

Figure 3. (a) Absolute yield (µg/ml over night culture) for the four constructs. (b) Plasmid size-adjusted relative yield (average pUC18 yield set to 1) and divided by relative size (pUC18 = 1, pUC18deltaLacZ = 0.81, pUCmu = 0.62 and pICOz = 0.44). The plasmid size-adjusted yield should

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be correlated with number of plasmid molecules extracted/ml of over night culture. Error bars represent 95% confidence intervals (Student's t distribution [17]).

Surprisingly, pUCmu shows a very high yield both in absolute amount of DNA produced from an over night culture (Figure 3a) and even more so when factoring in that this plasmid is much smaller than the pUC18 parental plasmid (Figure 3b). This yield gain in pUCmu is lost in pICOz, possibly because of less efficient growth of the bacteria under Zeocin selection in low-salt LB medium. The pICOz plasmid does however still out-perform pUC18 both in absolute yield and plasmid size-adjusted relative yield (Figure 3b).

4. Discussion

4.1. Generation of a minimal cloning plasmid – how small can we go?

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 [18]. 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 [19,20]. 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 [21], 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+coding domain sequence (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*.

4.2. Practical use-cases of a small cloning plasmid

There are several potential use-cases for a tiny plasmid backbone. One very useful use-case is for example to use pICOz as an intermediate cloning plasmid for PCR-mediated mutagenesis and then clone the generated mutant into the expression vector. This has three major advantages: 1) The PCR only needs to amplify a small product (insert + 1 kb) rather than the entire expression vector (typically insert + 5-6 kb for mammalian expression vectors), 2) there is no risk that the PCR amplification has caused any unintended modifications to the expression vector and 3) since most expression vectors are ampicillin resistant, cloning from the zeocin-resistant intermediate pICOz

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vector reduces the risk of contamination from the parental pICOz vector and ligation into the expression vector can be done even without purification of the digest.

The pICOz and pUCmu vector backbones can also be interesting as expression vector backbones in some cases. However, adding a complete mammalian expression casette and SV40 origin of replication defeats the purpose of the tiny backbone since the few kb saved on the backbone will make little difference on the final size of the expression vector. There are however use cases with much smaller expression casettes, like for example CRISPR/Cas9 guideRNA expression from the U6 promoter and terminator (for example: pU6mu LMBP 09491 and pU6z LMBP 11144). Expression of these guide RNAs are also preferably transient, so we do not need the SV40 origin of replication. We have experienced that some cells are more easily transfected with small expression plasmids (for example, electroporation of Jurkat T cells), which means that these small expression plasmids can have a practical use case.

There are however many more potential use cases of these small vector back bones in many fields of molecular biology and we encourage creative use of this tool to develop novel downstream applications.

4.3. Future distributed development and the importance of sharing

We 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 [22], and genetic material shows many commonalities with software in that it can be copied and reproduced very easily [23]. 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 [24,25]. 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 [26–28], 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 [29–31].

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+} [32], Cu^{2+} [33], Ag^{+} [34] or high-salt [35] resistance) could be highly interesting to explore for *E. coli* cloning vectors.

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