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Research Article

pBlue and pYellow – TWO NOVEL CLONING VECTORS WITH MARKERS FOR VISUAL SELECTION OF TRANSFORMANTS

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Summary: Two novel plasmid cloning vectors, pBlue and pYellow, were constructed by substituting the *LacZ'* gene fragment of pUC19 with *amilCP* and *amilGFP* chromoprotein coding sequences obtained from the coral *Acropora millepora*. Both sequences were codon-optimized for expression in *Escherihia coli* and, when expressed, produced blue and yellow coloring, respectively. The substitution of the *LacZ'* gene fragment eliminates the need for adding X-Gal to the growth medium while keeping the same mode for positive transformants selection as the one used by the ancestral vector. As a proof of the concept, a 644 bp long fragment from the *dia-1* gene in *D. melanogaster* was cloned in our new vectors pBlue and pYellow using standard restriction and ligation techniques. Positive transformants were selected using the white color of the colonies as an indicator for a successful cloning event. All colonies that contained empty vectors developed the corresponding blue or yellow coloring in the process of cultivation, allowing their visual identification.

Keywords: Cloning vector; positive selection; chromoprotein; restriction free cloning.

Abbreviations: LIC – *L*igation-*I*ndependent *C*loning; RF – *R*estriction *F*ree Cloning.

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INTRODUCTION

The creation of recombinant DNA molecules is one of the milestones of modern molecular biology (Tirabassi, 2014). It relies on a set of techniques collectively known as molecular cloning which allow the insertion of foreign DNA fragments into appropriate host organisms. The foreign fragment needs to be inserted into a vector so it can be maintained by the host organism. Naturally occurring plasmids were among the first used vectors but proved to be unsuitable for the needs of the molecular genetics (Cohen, 1993). A variety of artificial vectors were developed based on the naturally occurring plasmids. They made restriction-ligation cloning a straightforward if rather time consuming

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technique (Balbás, 1986).

One of the major drawbacks of the classical cloning approach is the tendency of linearized vectors to self-circularize without inserting a fragment. This necessitates the addition of a screening step for positive transformants to all cloning procedures. Some of the commonly used cloning vectors, such as the pUC derivatives rely on the use of a polylinker and a selection marker such as the *LacZ'* (Vieira and Messing, 1991). However, those vectors require an inducer (IPTG) and an artificial substrate (X-Gal) to allow color-based selection of positive transformants. In our work, we propose a substitution of the *LacZ'* gene fragment with coding sequences for *amilCP* (blue chromoprotein from coral *Acropora millpora*) and *amilGFP* (green fluorescent protein in coral *Acropora millepora*). Both these genes encode related chromoproteins that lead to development of intensive blue and yellow colors in this organism. The corresponding colors can be easily distinguished with a naked eye. Substituting the *LacZ'* region with chromoprotein sequence of this type will eliminate the need of additional chemical substrate for color formation while preserving the advantages of the original pUC19 vector.

Despite its simplicity, our proposal faces some technical challenges. All commonly used restriction endonucleases in pUC19 are located inside the polylinker region thus eliminating the possibility to clone the chromoprotein coding sequence using classical restriction and ligation dependant cloning procedure. To overcome this issue we focused our attention on the rapidly emerging field of novel cloning methods. In the past few decades many new approaches have emerged that shirk some of the major drawbacks of restriction-ligation cloning (Aslanidis et al., 1990; Erijman et al., 2011; van den Ent and Lowe, 2006). They are cheaper and quicker and allow seamless cloning which eliminates additional amino acids in the protein products of the cloned sequences. The first developed novel approach is ***L***igation-***i***ndependent ***c***loning (LIC) (Eschenfeldt et al., 2009), which gives rise to a variety of homology and PCR based techniques such ***S***equence and ***L***igation ***I***ndependent ***C***loning (SLIC) (Jeong et al., 2012), ***P***olymerase ***I***ncomplete ***P***rimer ***E***xtension (PIPE) (Klock et al., 2009) and Restriction Free cloning (Bryksin et al., 2010).

In this research we selected the restriction free (RF) cloning which allows inserting any fragment in any specific vector location (Chen et al., 2000). It relies on a two stage process with two PCR reactions prior to transformation (Fig. 1). The first PCR creates the so called megaprimer that contains the insert and flanking sequences complementary to the insertion site of the vector. This megaprimer is then used in the second PCR with the chosen vector. To ensure that the recipient cells are transformed only with the construct containing the insert, treatment with *DpnI,* was performed. *DpnI* enzyme discriminates its DNA targets based on their methylation status, allowing elimination of the initial empty plasmid used as PCR matrix. This methodology avoids the use of restriction or modification enzymes as well as expensive cloning kits (Unger et al., 2010).

MATERIALS AND METHODS

Bacterial strains and mediums

E. coli DH5α was used as a recipient in the transformation experiments. It was grown on LB medium without supplements prior to transformation and on LB with ampicillin (100ng/µl) after transformation.

E. coli transformation

E. coli JW1 competent cells were prepared using the $CaCl_2$ procedure described by Sambrook (1989). The produced competent cells were stored at -80°C as 200 µl aliquots. Transformation was carried out according to Sambrook (1989) using 10 ng plasmid DNA.

Primers used in this study

All of the primers and gene constructs used in the current experiment were synthesized by Integrated DNA Technologies (IDT, USA). Their sequences and general characteristics are shown in Table 1. The underlined sequences are complementary to the pUC19 vector, while the bold ones – to the chromoproteins' sequences. All primers for the RF-cloning procedure were prepared using the specialized free online software at http://www.rf-cloning.org.

Codon optimization and gene synthesis

The original protein sequences of *amilCP* and *amilGFP* were obtained from NCBI (AAU06854.1 and AAU06846.1). Coding sequences for efficient chromoprotein expression in *E. coli* were designed from them using the Codon Optimization On-Line Tool (COOL) (Chin et al., 2014). Both resulting DNA fragments, together with all corresponding elements for translational initiation and termination, were synthesized as gBlock fragments by IDT.

Preparation of plasmid DNA

Plasmid DNA from *E. coli* was extracted using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific™) according to the manufacturer's

Table 1. Primers used and their characteristics.

Primer	Sequence	$T°_{an}$	Function
amilCP_pUC19_F	5'-GGTGTCGGGGCTGGCTTAAC**TTATC ATGCCACCACTGGTTTA**-3'	60°C	Amplification of *amilCP*
amilCP_pUC19_R	3'-GAATTCACTGGCCGTCGTTTTACAA **ATGTCGGTGATCGCGAAAC**-5'	60°C	Amplification of *amilCP*
amilGFP_pUC19_F	5'-CGGGTGTCGGGGCTGGCTTAAC**TTA TTATTTCACTTTCAGCGGATTC**-3'	60°C	Amplification of *amilGFP*
amilGFP_pUC19_R	3'-CGAATTCACTGGCCGTCGTTTTACA A**ATGAGCTACAGCAAACAAGGC**-5'	60°C	Amplification of *amilGFP*
BamHIAhdI_Dia1F	5'- TTGGATCCGACAATAAGTCGGACTG CAGAGTTACGG-3'	60°C	Amplification of *dia-1*
EcoRIAhdI_Dia1R	5'- TTGAATTCGACAATAAGTCGCGGTA TCCAACTTCTCGG-3'	60°C	Amplification of *dia-1*

specifications from overnight cultures grown on LB + ampicillin medium.

PCR

Phusion Flash PCR Master Mix (Thermo Fisher Scientific™) was used for amplification of both *amilCP* and *amilGFP* gene constructs under the following conditions: 2x Phusion Flash PCR Master Mix, 0.2 µM amilCP_pUC19_F/amilGFP_pUC19_F forward primer, 0.2 µM amilCP_pUC19_R/amilGFP_pUC19_R reverse primer and 10 ng *amilCP* or *amilGFP* gene constructs in a total volume of 50.0 µl with the following amplification protocol: initial denaturation at 98°C for 30 sec, followed by 24 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec and amplification at 72°C for 30 sec followed by a final amplification step at 72°C for 5 min and hold at 4°C. The PCR products were purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit. The PCR products were used as megaprimers for the RF substitution of *LacZ'* in the pUC19 vector. The DNA concentration of the purified PCR products was determined approximately using 1% agarose electrophoresis with a 100 bp MassRuler™ Express DNA Ladder (Thermo Fisher Scientific™) using a densitometry approach using GeneTools software. The *D. melanogaster* diaphorase 1 gene fragment *(dia-1)* was amplified using the BamHIAhdI_Dia1F and EcoRIAhdI_Dia1R primer pair generating fragment with total size of 644 bp which has *BamH*I and *EcoR*I recognition sites at its ends. The reaction mix included 10x *Taq* buffer (Thermo Fisher Scientific™), 2 mM $MgCl_2$ (Thermo Fisher Scientific™), 0.2 µM primers, 5U/µl *Taq* polymerase and *D. melanogaster* genomic DNA in total volume of 50.0 µl. We used the following amplification protocol: initial denaturation at 94°C for 10 min, followed by 38 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and amplification at 72°C for 1 min followed by a final amplification step at 72°C for 3 min and hold at 4°C. The PCR products were purified using GeneJET Gel Extraction and DNA Cleanup Micro. The DNA concentration of the purified PCR products was determined using 1.2% agarose analytical gel electrophoresis with a Lambda DNA/HindIII molecular weight marker.

Molecular cloning techniques

The substitution of the *LacZ'* gene in pUC19 with the synthesized *amilCP* and *amilGFP* sequences was performed using RF cloning as presented in Fig. 1. Two separate PCRs were performed - one for each substitution. The reaction mix contained 2x Phusion Flash PCR Master Mix, 50 ng megaprimer and 20 ng pUC19 vector in a total volume of 50.0 µl. We used the following amplification protocol: initial denaturation at 95°C for 30 sec followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min and amplification at 72°C for 3 min followed by a final amplification step at 72°C for 7 min and hold at 4°C. The PCR products were purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit. After purification the products were treated with *DpnI* (Thermo Fisher Scientific™) to remove the unmodified pUC19 vector and then transformed into competent *E. coli* cells. Positive transformants were selected based on color development and these results were further confirmed via

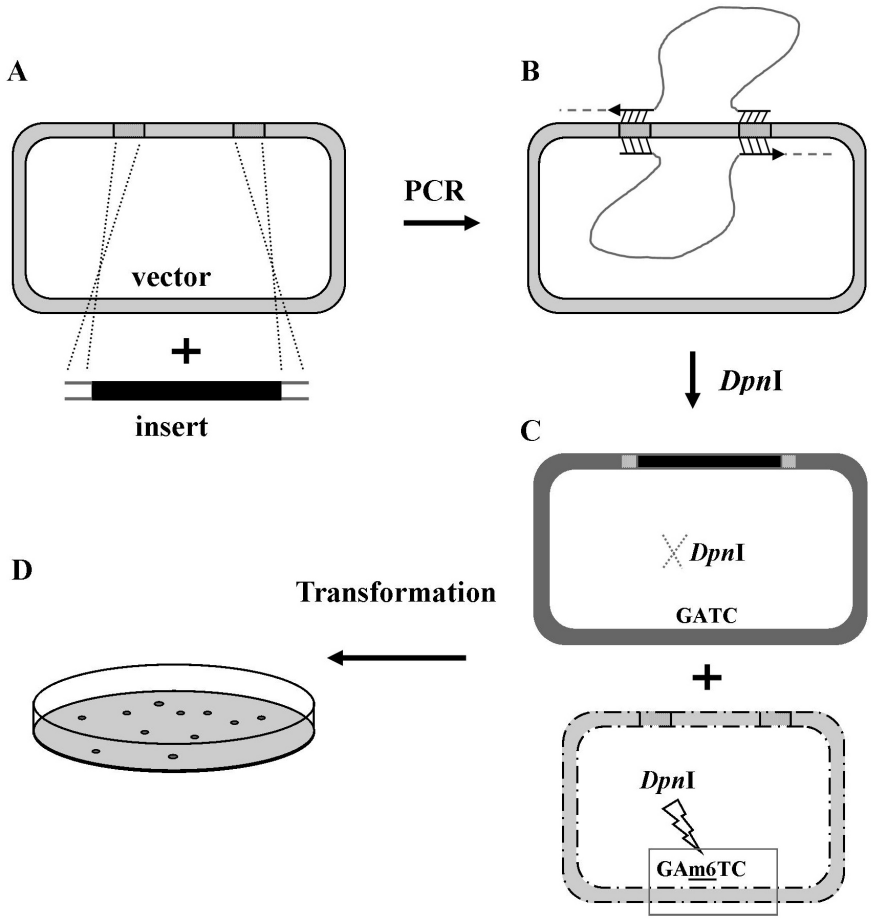

Figure 1. Schematic representation of restriction-free (RF) cloning. **A**. The plasmid used in the RF procedure and the megaprimer which contains the desired insert. The vector molecule is isolated from a dam+ *E. coli* strain. The desired insert is flanked by homologous to the vector insertion site sequences responsible for the proper annealing and precise insertion of the fragment during the second round of PCR. **B.** The megaprimer provides 3'-OH ends for linear amplification reaction. It extends and integrates the desired insert into the vector resulting in a nicked, circular DNA molecule. **C.** The parental vector is digested with *Dpn*I treatment. This enzyme cuts in every available GATC site but only if the sequence is methylated. The newly synthesized molecules from point B are not methylated and thus resistant to *Dpn*I. **D.** The double nicked circular plasmid with the desired insert is transformed into competent cells.

screening using Colony PCR with primers specific for the vectors (Table 1).

Next, a 644 bp long *dia-1* test fragment was cloned in our new vectors, named pBlue and pYellow, respectively. All DNA molecules were double digested with *BamHI* and *EcoRI* to create compatible sticky ends. The restriction was carried out according to the manufacturer's specifications (Thermo Fisher Scientific™) and incubated for 3 h at 37°C. Both vectors were dephosphorylated using alkaline dephosphorylase (Thermo Fisher Scientific™).

Two separate ligation reactions were carried out with different insert: vector ratio – 6:1 for pBlue and 3:1 for pYellow using T4 DNA ligase in 10 µl total volumes and incubated for 22 h at 18°C. The whole volume of the reactions was used to transform the recipient cells.

Screening for successful cloning in the new vectors was carried out with Colony PCR with primers specific for the dia-1 gene.

RESULTS

Codon optimization of chromoprotein sequences from *Acropora millepora* for expression in *E. coli*

Due to the preference of different organisms to specific codons simply cloning a gene from one organism into another usually leads to poor protein expression. The more the evolutionary distance between the host organism and the donor, the more likely it is to come across this problem. The *amilCP* and *amilGFP* genes originate from a coral (*Acropora millepora*). This necessitated a significant optimization of the nucleotide sequence of those genes before they can be cloned into a prokaryotic organism such as *E. coli*. The results of the optimizations we performed are given in the ***Supplementary material*** section. As it can be seen while there were significant differences in the nucleotide sequences of the original and optimized versions, the amino acid sequences remained the same.

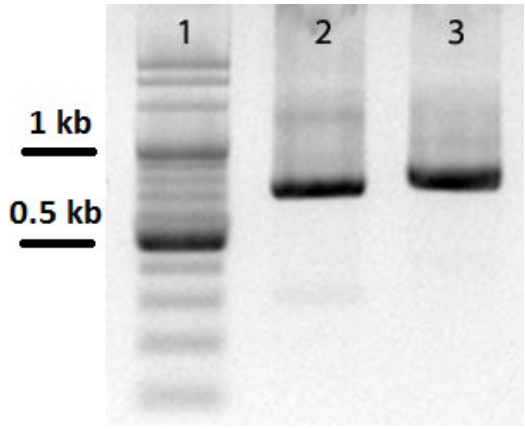

Figure 2. PCR amplification of *amilCP* and *amilGFP* gBlock fragments. Line 1 - 100 bp MassRuler™ Express DNA Ladder (Thermo Fisher Scientific™; 100 – 3000 bp); line 2 – *amilCP* amplification product (714 bp expected size); line 3 – *amilGFP* amplification product (747 bp expected size).

Substitution of *LacZ'* with *amilCP* and *amilGFP* in pUC19 through RF cloning

RF cloning relies on homology between the vector and insert which is introduced in the ends of the insert through the primers during the first round of amplification (Fig. 1). Because of this the primer design is of the utmost importance. Following amplification with the two sets of primers (amilCP_pUC19_F – amilCP_pUC19_R and amilGFP_pUC19_F – amilGFP_pUC19_R, respectively) the products were purified using preparative gel electrophoresis and the resulting megaprimers were visualized on 1% agarose gel (Fig. 2). Distinct bands with the appropriate length corresponding to the lengths of the amplicons can be seen. The concentration was determined to be 50 ng/µl. Both primers used to generate the megaprimer fragments were designed to create a homology with pUC19 downstream of the lac promoter. This sequence homology maintained the substitution of the *LacZ'* gene fragment with a chromoprotein coding sequence. The precise positions of the chromoprotein insertions in pUC19 are 146 and 376. RF

cloning provides improved control of the insertion positions and avoids the creation of "scars" that could create problems during translation. The megaprimers are used in a second round of amplification that creates a mix of unmodified and modified vectors. The methylated by the host cells unmodified vectors used as PCR matrix are removed through *DpnI* digestion prior to the direct transformation with the reaction mixture into chemically competent cells. The resulting colonies show color depending on the type of vector used for their transformation.

Screening and transformation efficiency of pBlue and pYellow

Transformation efficiencies of $3.54x10^5$ cfu/µg for the pBlue and $3.37x10^5$ cfu/µg for the pYellow were calculated. The corresponding efficiency for pUC19 was estimated to be $3.67 x10^5$ cfu/µg.

To prove the successful substitution we used two types of screening. The first type is based on the color of the colonies. Eight random transformants of both plasmids were plated on sector plates with the appropriate medium and left to incubate at 37°C. During the first 24-36 h there was no coloration of the colonies but after storage at 4°C some colonies started to reveal a pale color. All 8 pYellow transformants carried the desired substitution and 4 out of 8 of the pBlue were positive. The second type of screening used colony PCR with one vector primer and one that anneals to the chromoprotein sequence. Seven colonies from both plates were chosen for this round of screening – two blue, three yellow and two with no color. As expected, five of the samples that had color also showed the expected lengths of

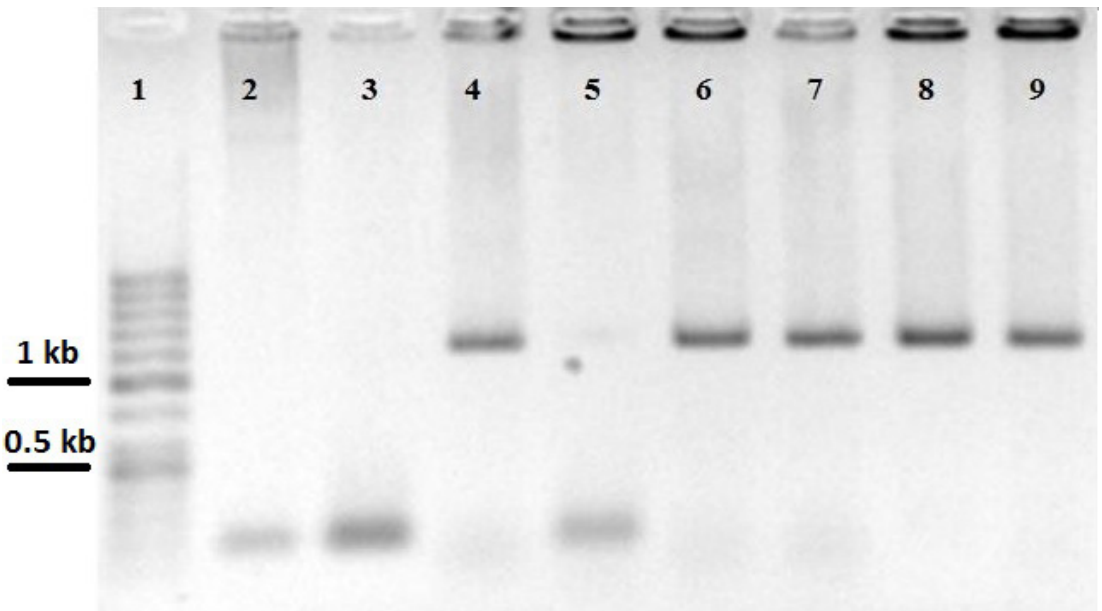

Figure 3. Colony PCR screening for *dia-1* insert in pBlue and pYellow. Line 1 - 100 bp MassRuler™ Express DNA Ladder (Thermo Fisher Scientific™; 100 – 3000 bp); line 2 – empty pBlue vector (control); line 3 – empty pYellow vector (control); lines 4,6 – pBlue-*dia-1* positive clones (1393 bp expected size); line 5 - pBlue-*dia-1* negative clone; 7,8,9 – pYellow-*dia-1* positive clones (1423 bp expected size).

774 and 804 bp for pBlue and pYellow, correspondingly.

Next, we selected and amplified a 644 bp long fragment from the *dia-1* gene in *D. melanogaster*. It was cloned into both new vectors pBlue and pYellow using the classical cloning procedure with restriction and ligation. We selected positive transformants relying on the screening for white colonies resulting from insertional inactivation in a manner similar to pUC19 *LacZ* selection. After transformation, plating and incubation overnight at 37°C, 3 random pBlue colonies and 3 random pYellow colonies were screened using colony PCR for the *dia-1* insert. The results are shown in Fig. 3. As negative controls we used colonies transformed with the new vectors. The results of the colony PCR showed that 5 out of the 6 chosen colonies carried the *dia-1* insert which gave amplicon of 1393/1423 bp (pBlue/pYellow). These results were in agreement with our initial expectations, indicating that positive transformants in both new vectors can be isolated using the white color of the colonies as main selection criteria. All vector features and selection requirements are shown in Fig. 4.

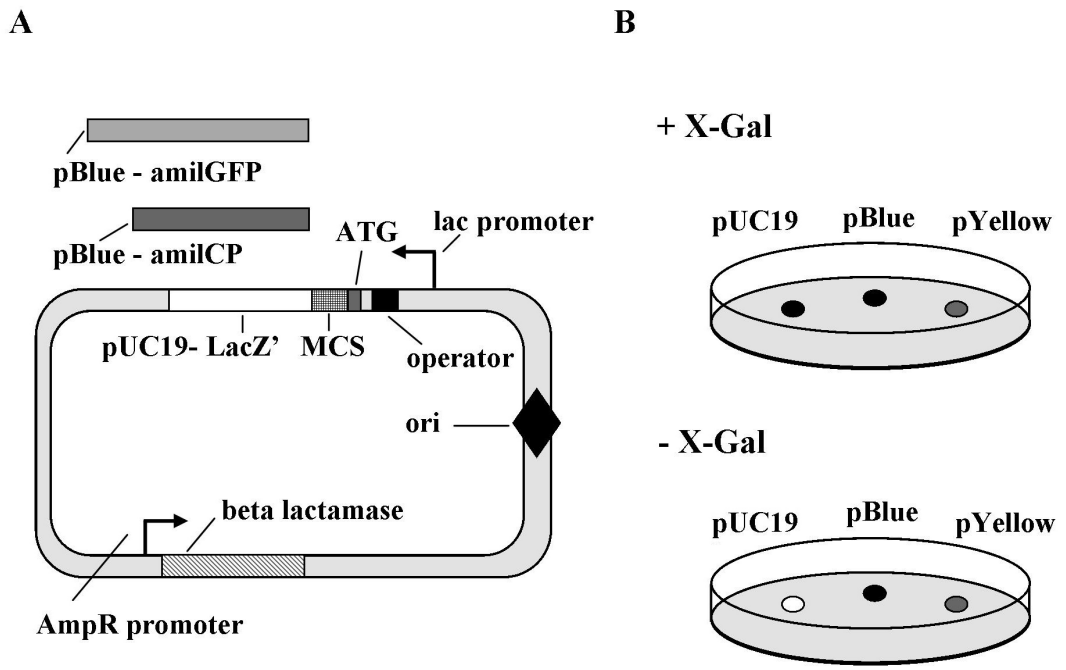

Figure 4. pBlue and pYellow – vector maps and colour based screening procedures. **A.** Vector map of pUC19, pBlue and pYellow. All identical important regions are marked as follows – ori (high copy number pUC origin of replication), lac promoter, ATG (initiation codon for β-gal fragment/chromoprotein), MCS (polylinker region with target sites for restriction endonucleases), AmpR promoter and beta-lactamase (responsible for Ampicilin resistance). The LacZ' found in the original pUC19 vector is given as part of the plasmid and the corresponding AmilCP and AmilGFP sequences in pBlue and pYellow are presented above it. **B.** Difference in colour based selection. All vectors form the corresponding colours on media with X-Gal but only colonies with pBlue and pYellow are colored on media without this compound.

DISCUSSION

Restriction free cloning is an efficient way for fast, cheap and precise way of cloning fragments of a few hundred bases. It can be easily modified to serve different purposes such as substitutions, exchange of fragments or creating DNA constructs without the need for specialized kits and also requires little other than PCR equipment (Bond and Naus, 2012). Here we describe the construction of a couple of working cloning vectors, named pYellow and pBlue. These pUC19 derivatives allow direct color based selection of positive transformants. Their functionality was demonstrated by cloning fragments of the *dia-1* gene into them. The high transformation efficiencies, similar to those shown by the ancestral vector, make pBlue and pYellow useful tools for molecular cloning. The substitution of the *LacZ'* gene fragment with a chromoprotein's coding sequence permits the use of visual color based selection in a wider set of hosts as it does not depend on α-complementation. Moreover, no additional small molecules are required for color formation leading to simpler and cheaper cloning procedures (Langley et al., 1975). The relatively slow color development and the paleness of the two newly developed vectors can be pointed as a partial drawback. Possible explanations for this phenomenon can be the used relatively weak promoter and/or a chromoprotein miss-folding due to the altered N-terminus of the encoded chromoprotein by the added polylinker sequence (Baneyx and Mujacic, 2004). However, the mentioned downsides are compensated by the more elegant screening procedure which does not require the use of specific deletion mutant host strains. Moreover, the selectable growth media requires only addition of ampicillin but not the supplementation of X-gal, making it more cost efficient for preparation.

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SUPPLEMENTARY MATERIAL

>amilCP codon-optimized sequence for expression in *E. coli*; total size 669 bp; 5' ATG and two in frame stop codons at 3' end are included

ATGTCGGTGATCGCGAAACAGATGACCTATAAAGTGTATATGAGCGGCACC-
GTTAACGGTCACTACTTCGAAGTGGAAGGCGATGGCAAAGGTAAACCGTAT-
GAAGGTGAACAAACGGTAAAATTAACGGTGACTAAAGGCGGCCCGCTGCC-
GTTTGCCTGGGATATTCTGTCGCCGCAGTGCCAGTACGGCAGCATCCCTTT-
TACCAAATATCCGGAGGATATCCCGGATTACGTGAAACAAAGTTTTCCG-
GAAGGGTACACCTGGGAGCGTATTATGAACTTTGAAGATGGTGCAGTT-
TGTACCGTCAGCAATGACAGCAGCATTCAGGGCAACTGCTTTATTTAT-
CACGTCAAATTCTCCGGTCTGAACTTCCCGCCGAACGGGCCGGTGATG-
CAGAAAAAAACCCAAGGCTGGGAACCGAATACCGAACGTCTGTTCGCCC-
GCGACGGCATGCTGCTGGGCAATAATTTTATGGCGCTGAAACTGGAAGGCG-
GTGGTCATTATCTGTGCGAATTTAAAACGACCTACAAAGCGAAGAAACCT-
GTTAAAATGCCGGGTTATCATTACGTCGATCGCAAGCTGGATGTCACCAAC-
CATAATAAAGATTATACCAGCGTTGAACAGTGTGAAATTTCCATCGCTCGTA-
AACCAGTGGTGGCATGATAA

>amilGFP codon-optimized sequence for expression in *E. coli*; total size 699 bp; 5' ATG and two in frame stop codons at 3' end are included

ATGAGCTACAGCAAACAAGGCATTGTGCAGGAAATGAAAACCAAATAC-
CATATGGAAGGGTCCGTCAACGGTCATGAATTTACCATTGAAGGC-
GTGGGCACCGGTTATCCTTATGAAGGCAAGCAGATGAGTGAACTGGTGAT-
TATCAAACCTGCTGGTAAACCGTTGCCGTTTTCATTTGATATCCTCTCTTCT-
GTCTTCCAGTACGGCAATCGCTGCTTTACTAAATATCCGGCAGATATGCCG-
GACTATTTTAAACAGGCGTTCCCGGATGGCATGTCGTACGAACGCAGCTTTT-
TATTCGAAGATGGTGCGGTGGCGACCGCCAGCTGGAATATTCGTCTG-
GAAGGTAACTGTTTTATTCATAAAAGCATTTTCCACGGCGTTAACTTCCCC-
GCCGACGGCCCGGTGATGAAAAAAAAGACCATCGACTGGGATAAATC-
GTTTGAAAAAATGACCGTCAGTAAAGAAGTGCTGCGCGGCGATGTCAC-
CATGTTCCTGATGCTGGAGGGTGGCGGCAGCCACCGTTGCCAGTTTCATTC-
CACCTATAAAACGGAAAAGCCGGTAACGCTGCCGCCGAACCACGTTGTT-
GAACATCAGATTGTTCGTACCGATCTGGGGCAAAGCGCGAAAGGCTTCAC-
CGTTAAACTGGAAGCGCACGCCGCAGCGCATGTGAATCCGCTGAAAGT-
GAAATAATAA