Two step PCR method to exchange the resistance cassette of a vector

Nils Cremer and Anne Diehl

Department of NMR-supported structural Biology, Leibniz-Forschungsinstitut für Molekulare
Pharmakologie (FMP), Robert-Rössle-Str. 10, 13125 Berlin, Germany

Correspondence: diehl@fmp-berlin.de

Keywords: ligation independent cloning, exchange antibiotic resistance, protein expression

Abstract: 50 words

Manuscript: 859 words

21 **Abstract**

22 For co-transformation of two plasmids, both have to possess different antibiotic selection markers. If
 23 that is not the case, normally the gene of interest (GOI) is subcloned into another vector. Here we
 24 introduce a fast and easy method to exchange the antibiotic resistance cassette (ARC) in only two
 25 PCR steps.

26 **Method Summary**

27 To shuttle the antibiotic resistance cassette (ARC) from one vector to another, one can amplify the
 28 ARC of interest and use the resulting PCR-product as a primer pair for the next amplification step.
 29 Simply remove parental DNA template by *DpnI* digestion, transform PCR product directly in *E. coli*
 30 *cells*, select transformants on an appropriate agar plate and isolate target vector by plasmid
 31 preparation.

32

33

34

35

36

37

38

39

40

41

42 Progress in structural biology depends on properly folded und functional recombinant proteins. After
 43 completion of the big structural genomics projects the low hanging fruits for *E. coli* expression have
 44 been almost entirely achieved. The challenge now is to produce difficult-to-express proteins in an
 45 improved *E. coli* environment, as this host is the easiest and cheapest one to handle with respect to
 46 NMR-labelling. One possibility is to optimize *E. coli* protein homeostasis by initiation of stress by
 47 sigma factor 32 (1). The appropriate plasmid pBAD.Sigma32.I54N (Figure 1A) carrying ampicillin
 48 resistance can be obtained via Addgene from Jeff Kelly. Sigma 32 causes the up-regulation of
 49 proteostasis network components like chaperones, folding enzymes or proteases. We have used that
 50 system surprisingly well for stable expression of an intrinsic unfolded protein with improved yield.
 51 Encouraged by that, we now want to use it for soluble expression of proteins with a high tendency to
 52 form inclusion bodies. Unfortunately most of our constructs have ampicillin as the selection marker,
 53 making co-transformation with pBAD.Sigma32.I54N impossible due to resistance being the same.
 54 Instead of re-cloning all our constructs we decided to change the antibiotic resistance cassette (ARC)
 55 of pBAD.Sigma32.I54N. A common pET26b vector (69862, Merck, Germany) was chosen as a
 56 template for the kanamycin ARC (Figure 1B). Initially both ARCs with their flanking regions were
 57 analyzed using alignments with respect to homolog regions in both directions. Indeed a reverse
 58 complimentary sequence downstream the ampicillin ARC of pBAD.Sigma32.I54N is identical to a
 59 sequence upstream the kanamycin ARC of pET26b and vice versa. Therefore two XL primers were
 60 synthesized (BioTez, Berlin, Germany) according to Table 1. With these primers the ampicillin ARC
 61 downstream of the sigma 32 and with the same translational orientation will be replaced by the
 62 kanamycin ARC in the opposite direction (Figure 1C). The reverse orientation is recommended for
 63 the kanamycin ARC (2).

64 The forward primer with 56 nucleotides matches to 100 % in both vectors and the reverse primer
 65 with 55 nucleotides fits to 71 % (39 nucleotides underlined part Table 1) to pET26b and 100 % to
 66 pBAD.Sigma32.I54N. If those identical stretches do not exist, the XL-primer can be designed by taking
 67 in 5'-3' direction 25-30 nucleotides from the ARC flanking site of the target vector (in our case

68 pBAD.Sigma32.I54N) combined with 25-30 nucleotides of the donor vector (in our case pET26b) to
 69 amplify first the ARC of interest and then introduce the resulting PCR product into the target vector
 70 via a second PCR. The possibility of changing the orientation of the ARC is then given via a
 71 combination of up- and downstream parts of donor and target vector, respectively, in the forward
 72 primer and vice versa for the reverse primer.

73 For both PCR reactions the KOD Hot Start DNA Polymerase Kit (71086, Merck, Germany) was used.
 74 The first PCR with pET26b as template and donor for the kanamycin ARC was performed as follows in
 75 a 50 µl set up: 1x KOD buffer; 2 mM MgSO₄; 0.2 mM each of the 4 NTP's; 0.3 µM of both primer;
 76 0.5 ng/µl pET26b, 0.02 U/µl KOD Polymerase. The following PCR program was used:

77	95 °C	5:00 min	
78	94 °C	0:30 min	← 5x
79	45 °C	1:00 min	
80	72 °C	2:00 min	
81	94 °C	0:30 min	← 13x
82	45/50 or 55 °C	1:00 min	
83	72 °C	2:00 min	
84	68 °C	8:00	
85	4 °C	hold	

86 The expected PCR product of about 1000 bp was separated on a 0.8 % agarose gel (Figure 2a) and
 87 purified (LSKGEL050, Montage Gel Extraction Kit, Millipore, USA) for use as a primer pair in the final
 88 PCR step.

89 The second PCR to exchange the ampicillin ARC by the amplified kanamycin ARC in
 90 pBAD.Sigma32.I54N was set up as the first PCR, but with the double amount of KOD polymerase and
 91 6 µl of 1 kB PCR product with 40 ng/µl (final 7.2 nM) as primers. The cycling program was extended.

92	95 °C	5:00 min	
93	94 °C	0:30 min	← 5x
94	60 °C	1:00 min	
95	68 °C	6:00 min	

96	94 °C	0:30 min	← 13x
97	55 °C	1:00 min	
98	68 °C	6:00 min	
99	68 °C	24:00 min	
100	4 °C	hold	

101 After digestion of the template plasmid with *DpnI* at 37°C for 1 hour and heat inactivation for 5 min
 102 at 80 °C 2 µl of the treated PCR product was used to transform NovaBlue GigaSingles competent cells
 103 (71227, Merck, Germany) according to NovaBlue transformation protocol (3). Within 12 to 18 hours
 104 transformants grew on kanamycin but not on ampicillin selection plates.

105 In principle this result was proof enough that the kanamycin resistance cassette has been inserted
 106 correctly into the target vector. Additionally, using Sanger sequencing (Source Bioscience, UK) we
 107 demonstrated that pBAD.Sigma32.I54N-kana still carries the gene for Sigma factor.

108 Furthermore we analyzed plasmid preparations of six clones by restriction digest (Figure 2B).

109 The robustness of the method is very good, as the first PCR was successful in a broad window of
 110 annealing temperatures, many transformants were produced, six analyzed clones possessed all the
 111 expected digestion patterns and code for sigma32. An unwanted CT doubling in the middle of the
 112 reverse primer had not influenced the result negatively, as untranslated flanking regions were used
 113 as primer pairing positions and some mismatches are tolerated.

114

115 Author contributions

116 N.C. performed the experiments. A.D. design the work, including the primer. N.C and AD wrote the
 117 manuscript.

118

119 Acknowledgement

120 pBAD.Sigma32.I54N was a gift from Jeffery Kelly (Addgene plasmid # 59982). We thank Catherine L.
121 Worth (FMP) for reading the manuscript.

122

123 **Competing interests**

124 The authors declare no competing interests.

125

126

127

128

129 **References**

- 130 **1. Zhang X, Liu Y, Genereux JC, Nolan C, Singh M and Kelly JW. (2014) The Heat-Shock**
131 **Response Transcriptional Program Enables High-Yield and High-Quality Recombinant Protein**
132 **Production in Escherichia coli. ACS Chem Biol. 2014 Jul 22. 10.1021/cb500447**
- 133 **2. Novagen: pET System Manual TB055 8th Edition 02/99**
- 134 **3. NovaBlue GigaSingles TB360**

135

Table 1. Primer for 1. PCR and their corresponding sites in vectors used.

primer	sequence	pET26b	pBAD.Sigma32.I54N
forward	5'CTTTGATCTTTTCTACGGGGTCTGACGCTC AGTGAACGAAACTCACGTTAAGG3'	3872-3926	4000-3946
reverse	5'GCGTTTCTACAACTCTTTGTTTATTTTC TAAATACATTCAAATATGTATCCGC3'	4854-4816	2868-2923

Figure 1. Plasmid maps (Vector NTI, Fisher Scientific , UK). A) pBAD.Sigma32.I54N with ampicillin ARC, B) pET26b donor of the kanamycin ARC und C) the resulting pBAD.Sigma32.I54N-kana. Used restriction sites are indicated.

Figure 2. Analysis by agarose gels A) Products of 3 parallel set ups for the first PCR with different annealing temperatures (Ta) analyzed in a 1.5 % agarose gel. Lane 1: 18 cycles with Ta 45 °C; Lane 2: 5 cycles with Ta at 45 °C, followed by 13 cycles with Ta of 50 °C; Lane 3: 5 cycles with Ta at 45 °C, followed by 13 cycles with Ta of 55 °C; M1: 1kB Marker (SM0331, Thermo Fisher Scientific) B) Restriction digest of pBAD.Sigma32.I54N and pBAD.Sigma32.I54N-kana analyzed by 0.8 % agarose gel. Marker and all “fast digest enzymes” from Thermo Fischer Scientific Lane 1: pBAD.Sigma32.I54N (4812 bp) digested with *Bam*HI (FD0054) and *Sma*I (FD0663) is only linearized as expected as it possess no cleavage site for *Sma*I; Lane 2a-f: 6 clones of pBAD.Sigma32.I54N-kana digested with *Bam*HI and *Sma*I showed the expected fragments of 2878 and 1934 bp; Lane 3a und b: 2 clones of pBAD.Sigma32.I54N-kana digested with *Bam*HI and *Ava*I (FD0384, cleaves inside the kanamycin cassette and within the regulator gene *araC*) formed 3 fragments (1932, 1795, 1085 bp) as predicted; M1: 1kB Marker (SM0331) M2 Fast ruler Middle range with 5000, 2000, 850 400 and 100 bp (SM1113); M3 Lambda DNA/ *Eco*130I (SM0161).

A

B

C