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Homologous Recombinatorial Cloning Without the Creation of Single-Stranded Ends

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ABSTRACT

We describe a new type of molecular cloning that complements the available strategies for homologous recombinatorial cloning. Purified, linear double-stranded DNA molecules with homologous ends are simply mixed in water and they transform readily into *E. coli*. Insert and linear vector need as few as ten base pairs of homologous sequence at their ends and essentially no incubation or enzyme treatments are needed for creating recombinants from linear fragments. Our method outcompetes most existing cloning methods in simplicity and affordability and is well-suited for high-throughput applications.

1 INTRODUCTION

2 Since the advent of recombinant DNA technology, molecular cloning has become an
3 indispensable part of biological research. As the traditional restriction enzyme and ligase-based
4 cloning suffers from restricted flexibility and poor efficiency, several alternatives have been
5 developed [listed in 1]. Many of the current methods for high-throughput cloning are based on site-
6 specific or homologous recombination and the terms are used as described [2]. Commercial high-
7 throughput cloning methods such as Gateway® cloning (Life Technologies) and In-Fusion® PCR
8 cloning (Clontech) are costly and in addition Gateway® cloning requires the presence of predefined
9 sequences for recombination which remain as scars in the assembled DNA sequences. Another
10 strategy is to use type IIS restriction enzymes as in the Golden Gate assembly [3] but these enzymes
11 are not completely sequence-independent. In addition, many existing vectors are not compatible
12 with the Golden Gate assembly since they comprise type IIS enzyme restriction sites.

13 The exploitation of homologous recombination or complementary annealing has spurred the
14 development of a variety of cloning methods including enzyme-free cloning [4], ligation-
15 independent cloning (LIC) [5], polymerase incomplete primer extension (PIPE) method [6],
16 sequence- and ligation-independent cloning (SLIC) [2], Gibson assembly [7] and several variations
17 of the mentioned methods [8–10]. The connecting concept behind these methods is the creation of
18 terminal single-stranded DNA regions, which can anneal to complementary sites of another single-
19 stranded DNA molecule. Depending on the approach, the DNA is made single-stranded either by
20 variations of PCR as in enzyme-free cloning and PIPE or enzyme treatment as in SLIC and Gibson
21 assembly. Homologous recombination has the advantage that there are no specific sequence
22 requirements for the insert or vector. In addition, these ligation-independent cloning techniques
23 have low costs because the methods use only commonly available enzymes [1]. Depending on the
24 nature of the DNA material, the different approaches vary in their efficiencies to generate correct

1 recombinant plasmids [11]. However, the different ligation-independent cloning techniques are
2 highly interconvertible so different methods can be applied for the same starting material.

3 Here we show that there is no requirement for creating single-stranded DNA for homologous
4 recombinatorial cloning. We show that purified DNA fragments created by simple restriction
5 enzyme treatment can form recombinants with PCR amplified fragments. Complementary ends of
6 the putatively double-stranded DNA seem to interact *in vitro* and potentially form circular hybrids
7 which are readily transformed into chemically competent bacteria. We named our method
8 exonuclease and ligation-independent cloning (ELIC) since its design resembles ligation-
9 independent cloning with the exception of omitting exonuclease or other treatments for
10 recombination. Our method can be readily implemented in the common laboratory practices ranging
11 from every-day cloning to high-throughput applications.

1 MATERIALS AND METHODS

2 Materials

3 *Escherichia coli* strains XL1-Blue (F'[:Tn10 *proAB*⁺ *lacI*^q Δ(*lacZ*)M15] *recA1 endA1 gyrA96*
4 (Nal^R) *thi-1 hsdR17*(r_k⁻m_k⁺) *supE44 relA1 lac*] (Stratagene) and TOP10 (F⁻ *mcrA* Δ(*mrr-hsdRMS-*
5 *mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1*
6 *nupG* λ⁻) (Invitrogen) were used in this study. Chemically competent cells were prepared as
7 described [12]. Plasmids pUC19 [13], pKQV4 [14], and yeast shuttle vectors pRS413 and pRS416
8 [15] have been described elsewhere. Plasmids comprising cDNAs encoding human P5 (huP5) and
9 mouse GRP94 (mGRP94) proteins were generously provided by Lloyd Ruddock and Linda M.
10 Hendershot, respectively. All restriction endonucleases and Phusion® polymerase were purchased
11 from commercial suppliers (Thermo Fisher Scientific) and used according to the recommended
12 protocols. Primers were synthesized by Eurofins MWG Operon (Germany) and full sequences are
13 listed in Supplementary Table 1. Sequencing was performed at Haartman Institute Sequencing Unit
14 (University of Helsinki, Finland).

15 Preparation of linear vector and insert DNA fragments

16 All PCR reactions were done with Phusion® polymerase according to standard protocols
17 including a final extension step at +72 °C for 10 min for filling-in possible single stranded ends. All
18 PCR fragments were separated on agarose gels and extracted using NucleoSpin Extract kit
19 (Macherey-Nagel) before they were used in ELIC-reactions.

20 The *LacZ* gene was amplified with PCR using pUC19 as template and the primer pairs described
21 in Supplementary Table 1. Primers were designed to encompass sequences of varying length
22 homologous to the recipient vector at their 5' ends. The lengths of homologous sequences ranged
23 between 10 and 40 bps. The four nucleotides of the 5' staggered end of XmaI treated vector were
24 included in the number of homologous base pairs and the sequence of the restriction site was added

1 to each primer. When using SmaI in the linearization of the recipient plasmid the number of
2 homologous base pairs is reduced by two.

3 The amplification of mGRP94 (AA 1-802), huP5 (AA 20-440) and yeast PDI AA 1-22 was
4 conducted in two rounds: first with primers complementary to the target gene with an adapter
5 sequence at their 5' ends and then with a primer pair complementary to the adaptor sequence with
6 an 5' end homologous to the recipient plasmid sequence. Exception to this was the 5' primer for
7 huP5 and 3' primer for yeast PDI, which were the same in both reaction rounds. The length of
8 homologous area was around 30 bp in ends complementary to the yeast shuttle vector pRS416-
9 Tef/pRS413-Gal and 19 bp between huP5 (AA 20-440) and yeast PDI AA 1-22. All primers with
10 their specific features are listed in Supplementary Table 1.

11 The vector pKQV4 was digested with SmaI (FastDigest, Thermo Fischer Scientific) or Cfr9I
12 (XmaI) (Thermo Fischer Scientific) for 16 hours at +37 °C and was subsequently gel-purified from
13 0.7 % agarose with NucleoSpin Plasmid kit (Macherey-Nagel). Yeast shuttle vectors were digested
14 with SpeI and XhoI (FastDigest, Thermo Fischer Scientific), also 16 hours at +37 °C before gel-
15 purification. DNA concentrations were measured on Take3-platform with Eon Microplate
16 Spectrophotometer (BioTek). Purity of DNA was controlled using 260/280 ratio.

17 **Blue-white screening with two fragments assembly ELIC**

18 40 ng of vector was mixed with insert at the indicated molar ratios in a final volume of 10 µl
19 ddH₂O and incubated at +22 °C for one hour (unless otherwise noted). 50 µl of chemically
20 competent XL1-Blue cells was added and the mixture was incubated on ice for 30 min. After heat
21 shock for 1 min at +42 °C and three minutes of cooling on ice, 250 µl LB was added and cells were
22 allowed to recover at +37 °C, 230 rpm shaking for 30-60 min. The transformation mixture was
23 plated onto LB Agar plates supplemented with ampicillin, isopropyl β-D-1-thiogalactopyranoside,
24 and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Amp IPTG/X-GAL, *E. coli* FastMedia,
25 Fermentas). Plates were incubated at +37 °C for 24 hours after which the colonies were scored.

1 **Three fragments assembly ELIC**

2 100 ng of linear yeast shuttle vector pRS413-Gal, 120 ng of P5 gene fragment and 60 ng of PCR
3 fragment encoding yeast PDI AA 1-22 (molar ratios 1:5:34) were mixed and water was added to a
4 final volume of 10 µl. The mixture was incubated at +22 °C for 60 min and transformed into
5 chemically competent TOP10 cells as described above. Six colonies were randomly chosen and
6 grown over night at +37 °C, 230 rpm in LB supplemented with ampicillin. Plasmids were purified
7 with NucleoSpin Plasmid-kit (Macherey-Nagel) and digested with *Eco31I* (FastDigest, Fermentas)
8 for two hours at +37 °C followed by restriction analysis on 0.7% agarose.

9 **Testing of *in vitro* recombination**

10 60 ng of linear yeast shuttle vector pRS416-Tef and 120 ng of GRP94 insert (molar ratio 1:4.5)
11 with homologous ends were mixed and incubated at +22 °C for 75 min. As controls, the same
12 amounts of individual fragments were also incubated separately for the same time in 10 µl sterile
13 ddH₂O. 1 x FastDigest Green buffer (Fermentas) was added to the reaction mixture and the mixture
14 was separated on a 0.7% agarose gel run at 40 V.

15

1 **RESULTS**

2 **As low as eight base pairs homologous area is enough to create recombinants**

3 In order to establish and optimize the exonuclease and ligation-independent cloning (ELIC)
4 method, an experimental design based on blue-white screening was established, which allowed fast
5 and reliable evaluation of the efficiency of ELIC. The experimental design is shown in Figure 1A.
6 The *LacZ* gene was amplified from pUC19 using PCR, which included a final extension step of 10
7 min for filling-in all potential single-stranded DNA ends using a primer pair depicted in Figure 1B.
8 A defined number of base pairs homologous to the target sequence in plasmid pKVQ4 were added
9 to the 5' end of each oligonucleotide (Figure 1B). All DNA fragments and digested vector were gel-
10 purified before they were mixed and transformed. Expression of correctly oriented fragments
11 inserted into the IPTG inducible expression vector leads to the formation of blue colonies on media
12 containing IPTG and X-GAL. White colonies represent the vector background. Several factors that
13 might influence the efficiency were tested including the length of homologous regions, type of the
14 restriction site (blunt versus sticky ends), molar ratios of vector and insert, and incubation time
15 before transformation. In addition, the applicability of the method in multiple fragment assembly
16 was studied with two inserts and a linearized yeast shuttle vector.

17 Most of the existing recombinatorial cloning methods rely on the presence of single-stranded
18 DNA ends. For linearization of the recipient plasmid, we therefore selected a restriction site for
19 which two isoschizomers exist: *XmaI*, which creates four-nucleotide sticky ends and *SmaI*, which
20 creates blunt ends (Figure 1B). The four nucleotides of the sticky end were counted to the number
21 of complementary base pairs; therefore the number of bps homologous to the target sequence is
22 reduced by two when cutting with *SmaI*. A number of *LacZ* fragments with terminal homologous
23 regions to the target plasmid varying in length between 10 and 40 bps were created using PCR.
24 After following the procedure shown in Figure 1A, the colonies were identified and counted. No
25 blue colonies were encountered in the absence of insert DNA in any of the control plates. The

1 amount of white colonies was of similar magnitude in all plates and control plates with insert only
2 did not contain any colonies. In general, the amount of blue colonies was higher if the vector was
3 treated with XmaI generating sticky ends (Figure 1C) compared to SmaI treated vector generating
4 blunt ends (Figure 1D). Recombinant plasmids were formed for each length of homologous area
5 tested; although the efficiency of ELIC correlated with the length of homologous region (Figure 1C
6 and 1D). After blunt end restriction only eight base pairs of homologous area were sufficient to
7 create recombinants. The optimal length of homologous area was found to be between 23 and 30
8 bps as seen in Figure 1C and 1D. One of the prerequisites for the successful applicability of ELIC is
9 also whether the sequence at the site of recombination is maintained. Therefore, the primers were
10 designed to retain the restriction site at the junction of the sequences, thus SmaI could be used for
11 restriction analysis (Figure 1B). A single blue colony originating from the recombination of each of
12 the inserts with different length of homologous region with the target plasmid was chosen and
13 subjected to restriction analysis. Restriction analysis revealed that the area of recombination was
14 fully maintained (Figure 1E).

15 Based on the number of white colonies formed, which is representing the vector background, the
16 use of SmaI for restriction proved to be better and more consistent results were obtained; thus SmaI
17 was chosen as enzyme for optimizing the reaction conditions. Therefore, in further experiments the
18 vector was treated with SmaI and the homologous area was kept at 23 bps. First, different
19 vector:insert molar ratios were tested. Increasing the molar ratio of vector versus insert up to 1:4
20 resulted in increasing numbers of blue colonies (Figure 2A). Higher surplus of insert did not further
21 increase the efficiency. Next we tested whether the incubation time has any effect on the efficiency
22 of ELIC. So far, vector and insert were incubated at room temperature for one hour after mixing.
23 Interestingly, shortening the incubation time from 60 minutes to no incubation at all prior to
24 transformation did not have any considerable effect on the formation of recombinants (Figure 2B).

In order to verify the integrity of the DNA sequences around the sites of integration, four different recombinant plasmids were sequenced and a 100% identity between target and query sequence was achieved (data not shown). In addition to *E. coli* XL1-Blue strain, we have also used *E. coli* TOP10 cells for ELIC and both strains are compatible with this cloning procedure.

Our research group has successfully used ELIC for the creation of over 50 constructs with insert fragments ranging from 0.1 up to 4.5 kb. We have collected a summary of a subset of our successful cloning reactions representing varying insert sizes and contents (Table1). In summary, in the event of single insert recombination the overall efficiency of recombinant formation was calculated to be 80 % meaning that from all the plasmids screened 80% show correct banding pattern in restriction analysis. However, as seen in Table 1, the efficiency of ELIC varies between reactions as for some inserts the efficiency is close to 100%.

Multiple inserts can be assembled albeit with reduced efficiency

The next step was to test whether multiple fragment assembly would be feasible using ELIC. Yeast shuttle vector pRS413-Gal was digested with *Spe*I and *Xho*I and two inserts were prepared by PCR. The fragments contained 19-30 bps homologous overlaps at their ends and should thus assemble directionally as illustrated in Figure 3A. Insert fragments were of very different size, being 0.1 and 1.3 kb long while the linear vector was 5.6 kb. Fragments were mixed, incubated for one hour and transformed into *E. coli* TOP10 cells. The addition of inserts to the vector enhanced the formation of recombinants four fold, since a total of 16 colonies appeared compared to the vector only –plate with four colonies. Six of these recombinants were analysed with restriction analysis using *Eco*31I, which cuts once in two of the fragments and twice in the third (Figure 3A). Three out of six plasmids yielded the predicted restriction pattern (Figure 3B). One of the plasmids (labelled with asterisk in Figure3B) was sequenced and the obtained sequence was 100 % identical to the predicted construct. Moreover, this experiment revealed that fragments of even small sizes

1 can be cloned efficiently. The correct assembly of three fragments has been successful in 50% of all
2 the plasmids screened as judged from restriction analysis.

3 One limiting factor for assembling multiple fragments is that the interaction between two DNA
4 sequences is not completely specific, e.g. in the Golden Gate assembly misalignment can occur
5 between the short overhang sequences [16]. Therefore, we created an insert with only one end
6 complementary to the vector and tested for the formation of recombinants. This reaction occurred
7 with a very low efficiency compared to the same insert having complementary regions at both ends.
8 Four colonies were obtained using a single homologous region compared to over 60 colonies
9 obtained with both ends complementary to the target sequence while without insert no colonies
10 appeared. Restriction analysis revealed that plasmids in two out of the four colonies indeed
11 contained the insert (data not shown).

12 **Vector and insert seem to interact already *in vitro***

13 Although we avoid making any conclusions about the mechanism of ELIC, it has been shown that
14 double stranded homologous areas at the ends of two DNA molecules can form four-way DNA
15 structures through strand exchange [17, 18]. If recombination takes place at both insert fragment
16 ends, bacteria might be able to take up the circular form, which resembles a replication
17 intermediate, and complete it to a recombinant plasmid. To test if hybridization occurs, vector and
18 insert with 30 of flanking base pairs were mixed and incubated for 75 minutes. Control reactions of
19 insert only and vector only were set-up. After the incubation, the reaction mixtures were analysed
20 using agarose gel electrophoresis under mild conditions (see Materials and Methods). Interestingly,
21 besides of the two original DNA fragments, an additional band was detectable when insert and
22 vector were pre-incubated together, which was not present in either vector or insert only
23 preparations (Figure 4). This band matches the size of a linear combination of vector and insert.
24 This might indicate that the two fragments have annealed via one of their homologous ends, while
25 the double annealed circular form is probably present in too low quantities to be seen.

DISCUSSION

Ligation-independent cloning techniques can be used to introduce DNA fragments into any desired plasmids cheaply, reliably and with high efficiencies. Several different LIC protocols have been described so far from which many rely on creating recessed ends using an exonuclease activity [2, 5, 9]. Here we describe a new, simple cloning procedure similar to other LIC methods but without the need to create single-stranded ends. We term our method exonuclease and ligation-independent cloning, ELIC. We show that the annealing of homologous ends of molecules can happen between two supposedly double-stranded fragment ends and that the frequency of this event is high enough for effective recombinatorial cloning. To our knowledge and according to Stevenson *et al.* (2013) this has not been shown to occur while using restriction enzyme treated vector and purified fragments, although a similar procedure has been represented for PCR-amplified fragments in complex reaction mixtures [19]. There are many advantages of preparing the vector by simple restriction compared to PCR-amplification which include the lower error rate of copying *in vivo* and avoidance of unwanted PCR products resulting from unspecific primer annealing. In essence, our method is a streamlined version of one-step SLIC [9]. A fair comparison of the efficiencies of different protocols in terms of number of correct clones in relation to transformed DNA from the published literature is difficult, since the efficiency is highly dependent on transformation method and quality of competent cells. For most purposes ELIC is convenient and easy, complementing the available cloning strategies as a simple entry point to ligation-independent cloning.

Our data shows that as little as 8 to 10 bps of homologous regions are sufficient for formation of recombinants, which is very similar to minimal sequence length requirements of effective single-strand annealing [9, 20]. We verified the junctions of various constructs using DNA sequencing. Sequence analysis of the junctions and adjacent sequences revealed that recombination was site-specific and did not result in the introduction of mutations into the DNA sequence. There has been no noticeable difference in cloning efficiency between small and large inserts unlike reported for

1 some other LIC methods [11] and assembly of two fragments has a mean success rate of 80%.
2 However, the rate of success varies between the inserts and with some inserts the efficiency is close
3 to 100% which is similar as reported for other methods [9, 11]. As in one-step SLIC [9], also three
4 fragments can be assembled simultaneously, although the efficiency is in this case reduced to 50%.
5 ELIC has been successfully applied using a range of different target vectors, including yeast shuttle
6 bacterial expression vectors and binary plant expression vectors.

7 In the light of high-throughput cloning, ELIC has many desirable features needed for large-scale
8 projects [21]: it is easy to use, reliable, flexible and the reagent costs are at a minimum since only
9 one round of PCR and one restriction enzyme is needed unlike other LIC methods mentioned,
10 which utilize multiple rounds of PCR or additional enzymes [2, 4, 5, 9]. Linear DNA fragments
11 generated by PCR or DNA synthesis possessing short complementary sequences to their targets are
12 sufficient as such to assemble easily into circular DNA constructs irrespective of any sequence
13 requirements for their complementary ends. The only requirement in ELIC is the careful design of
14 primers for amplification of the insert, thus our method is easily implemented to every-day cloning
15 procedures.

16

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4

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1 **FIGURE LEGENDS**

2 **Figure 1. Experimental design and the amount of recombinants formed with various**

3 **lengths of homologous area.** A schematic view of the experimental design is shown in (A). Vector
4 pKQV4 is linearized with one of the two isoschizomers, XmaI or SmaI creating a sticky or blunt
5 end, respectively. A *LacZ* gene with homologous ends to the vector is prepared by PCR. The sites
6 of homologous recombination are indicated with grey-shaded rectangles. The linearized vector and
7 *LacZ* gene PCR fragments are subsequently gel-purified, mixed in water, incubated and transformed
8 into chemically competent cells. Colonies containing the desired recombinant plasmid can be
9 detected by the formation of blue colour. (B) The nucleotide sequences of the region of
10 recombination. The depicted areas represent the 5' and 3' ends of the PCR amplified fragment and
11 the adjacent plasmid region after the recombination event. The nucleotides of the vector are
12 underlined. The primers contain 17 bps complementary to the target gene at their 3' end, six
13 nucleotides of the restriction site on top of which 5-35 bps complementary to pKQV4 were added at
14 the primer 5' end. After recombination, the restriction site (XmaI/SmaI) is recovered in both ends of
15 the insert. Effects of the length of complementary region on the number of blue colonies generated
16 after treatment of vector with XmaI (C) or SmaI (D) was assessed for lengths ranging from 8 to 40
17 bps. The assay was repeated three times and the mean and standard deviation are depicted as
18 columns and error bars, respectively. The molar ratio of vector and insert was kept constant at 1:6.
19 The four nucleotides of the sticky end are included in the number of complementary base pairs.
20 Optimal length of homologous region seems to be between 23 and 30 bps. Slightly higher number
21 of colonies were obtained after treatment of destination vector with XmaI generating a staggered
22 end. (E) Restriction analysis of recombinant plasmids isolated from six randomly picked blue
23 colonies using SmaI. A single colony from each different length of homologous region was
24 analysed and the plasmids showed the expected banding pattern.

1 **Figure 2. The effects of different amounts of insert and the incubation time on formation of**
2 **blue colonies.** (A) The molar ratio of inserts in respect to 40 ng of SmaI digested vector pKQV4
3 was varied between 0 and 6. As expected no blue colonies were formed in the absence of insert. The
4 number of blue colonies was highest at vector to insert ratio of 1:4. (B) The effect of incubation on
5 formation of blue colonies was tested. Vector and insert were mixed and incubated for 0 to 60
6 minutes prior to transformation. The duration of incubation did not have a considerable effect on the
7 amount of blue colonies formed although there was some variation between the samples. Columns
8 represent the mean from three repetitions and the error bars show the standard deviation.

9
10 **Figure 3. Multiple fragments assembly using ELIC.** (A) A schematic illustration of the three
11 fragments assembly: yeast vector pRS413-Gal and DNA fragments encoding signal peptide (“SP”,
12 yeast PDI amino acids 1-22) and amino acids 20-440 of human P5 protein form the recombinant
13 plasmid pEK by recombining at the overlapping sequences at their ends. The three regions of
14 homologous recombination are marked with black and the sizes of the DNA pieces are labelled
15 below the corresponding fragments. All fragments contained at least one Eco31I-site as shown and
16 the expected fragments after restriction analysis are indicated. The expected banding pattern of
17 correctly assembled clone after digestion with Eco31I is given below. An additional 3.8 kB
18 fragment representing part of vector backbone is generated (not marked). (B) Six randomly picked
19 colonies out of 16 colonies were screened with restriction analysis using Eco31I. The plasmids in
20 lanes 2 to 4 have four correctly sized bands indicative for the desired recombinant. The plasmid run
21 on lane 2 (marked with asterisk) was sequenced and the sequence proved to be as expected.

22
23 **Figure 4. Analysis of *in vitro* recombination product by agarose gel electrophoresis.** Vector and
24 insert were mixed in water, incubated for 75 min and analysed on a 0.7% agarose gel at 40 V.
25 Control samples with vector only (“v”) and insert only (“i”) were incubated at identical conditions.

- 1 In the mixture lane (“v+i”) a faint band of approximately 8 kb appears (arrow) which is absent in
- 2 both control lanes. This fragment corresponds to the size of a linear vector-insert combination.

1 TABLES

2 **Table 1. A selection ELIC cloning reactions representing varying insert sizes and content.**

insert content	insert length (kb)	vector length (kb)	GC content insert (%)	Efficiency (%)	transformants/ μg of vector
ORF ¹⁾ from <i>Mus musculus</i>	2.4	5.5	43.4	100	125
ORF from <i>M. musculus</i>	2.4	5.5	43.4	100	625
ORF from <i>Homo sapiens</i>	1.5	6.6	56.5	100	225
ORF from <i>H. sapiens</i>	1.5	6.6	45.4	100	50
Two genes (Promoters and terminators from <i>S. cerevisiae</i> , ORFs from <i>H. sapiens</i>)	4.5	5.5	50.6	83	1000
Promoter region from <i>S. cerevisiae</i>	0.5	5.2	39.3	100	615
Promoter region from <i>S. cerevisiae</i>	0.4	5.2	33.7	100	750
ORF from <i>H. sapiens</i>	3.0	5.5	55.5	40	200
ORF from <i>H. sapiens</i>	1.5	5.6	45.4	100	1000
ORF from <i>H. sapiens</i>	0.5	5.5	59.8	100	100
Promoter region from <i>Rhodospiridium</i> <i>toruloides</i>	0.8	7.8	58,3	100	2000

Promoter from <i>R. toruloides</i>	1.5	7.8	65,1	67	1500
Two inserts: promoter from <i>R. toruloides</i> , ORF and terminator from <i>S. cerevisiae</i> vector	0.8 + 0.9	6.8	59	50	970
Two inserts: promoter from <i>R. toruloides</i> , ORF and terminator from <i>Pichia pastoris</i>	1.5 + 0.7	6.8	62,5	100	190

1 ¹⁾ ORF: open reading frame.