Analytical Biochemistry 476 (2015) 1–4

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Positive selection improves the efficiency of DNA assembly

Chang-Ho Baek, Jonathan Chesnut, Federico Katzen *

Thermo Fisher Scientific, Carlsbad, CA 92008, USA

ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form 23 January 2015
Accepted 26 January 2015
Available online 4 February 2015

Keywords:
Positive selection
Cloning
DNA assembly
Synthetic biology

ABSTRACT

With the advent of synthetic biology and cell engineering, the demand for large synthetic DNA fragments has been steadily increasing. Consequently, a number of multi-fragment cloning technologies optimized for the assembly of sizable DNA constructs have been developed. Still, screening for the right clone can be tedious because the high incidence of illegitimate assembly results in a relatively large proportion of missing or shuffled DNA elements. To mitigate this risk, we have developed a strategy that reduces the rate of fragment mis-assembly and is compatible with a variety of cloning methodologies. The approach is based on the positive selection of truncated plasmid markers, which are rendered active by providing their missing sequences during the assembly process. The method has been successfully validated in the context of complex in vivo and in vitro homologous recombination workflows, but it could be readily adapted to other cloning strategies, including those based on restriction endonucleases.

During recent years, there has been a growing interest in the precise and concerted assembly of multiple DNA fragments of diverse sizes, including chromosomes. Although the approaches and workflows rely on a variety of biochemical reagents, types of nucleic acid modifications, sources of DNA, sequence requirements, and host organisms, they all exhibit a common trend: their efficiencies decrease as the number of donor fragments increases (for reviews, see Refs. [1,2]). A mitigation has been proposed where the essential backbone elements, such as the selection marker and the origin of replication, are separated and placed into individual building fragments [3]. The disadvantage of this methodology is that the number of required DNA elements becomes larger, thereby expanding the assembly complexity.

Here we present a technique based on a positive selection approach that improves the efficiency of multi-fragment cloning technologies. The strategy relies on vectors harboring truncated and inactive replication origin and selection markers whose short missing sequences are provided in trans during the cloning procedure. The approach selects those clones that have the correct outermost fragments, reducing the effective combinatorial universe of unwanted constructs and, thereby, increasing the intrinsic cloning efficiency (Fig. 1). In this work we show the proof of concept of the approach in the context of in vivo and in vitro homologous recombination approaches because those are the most common and effective strategies employed to build large and complex DNA constructs without the interference of unwanted sequence scars [4–6].

Materials and methods

Strains and media

Chemically or electro competent *Escherichia coli* and *Saccharomyces cerevisiae* cells DH10B-T1, TOP10, and MaV203 were obtained from Thermo Fisher Scientific. The *E. coli* strain S17-1::λ-*pir* [7] was used to maintain the positive selection vector pASE101. *E. coli* cells were grown in LB medium or LB agar supplemented with the appropriate antibiotics: ampicillin (Amp,[1] 50 μg/ml), kanamycin (Km, 25 μg/ml), and chloramphenicol (Cm, 20 μg/ml). Yeast MaV203 transformants were grown on CSM-Trp agar medium (Thermo Fisher Scientific).

DNA and reagents

Construction details of the positive selection vectors pYES8D and pASE101 (Fig. 1B and C) are provided in the Online supplementary material. Oligonucleotides and DNA fragments used in this study are listed in Supplementary Tables S1 and S2, respectively. Synthetic DNA molecules were supplied by Thermo Fisher Scientific. A subset of these synthetic DNA fragments was cloned

* Corresponding author.
E-mail address: federico.katzen@thermofisher.com (F. Katzen).

[1] *Abbreviations used:* Amp, ampicillin; Km, kanamycin; Cm, chloramphenicol; PCR, polymerase chain reaction.

http://dx.doi.org/10.1016/j.ab.2015.01.021
0003-2697/

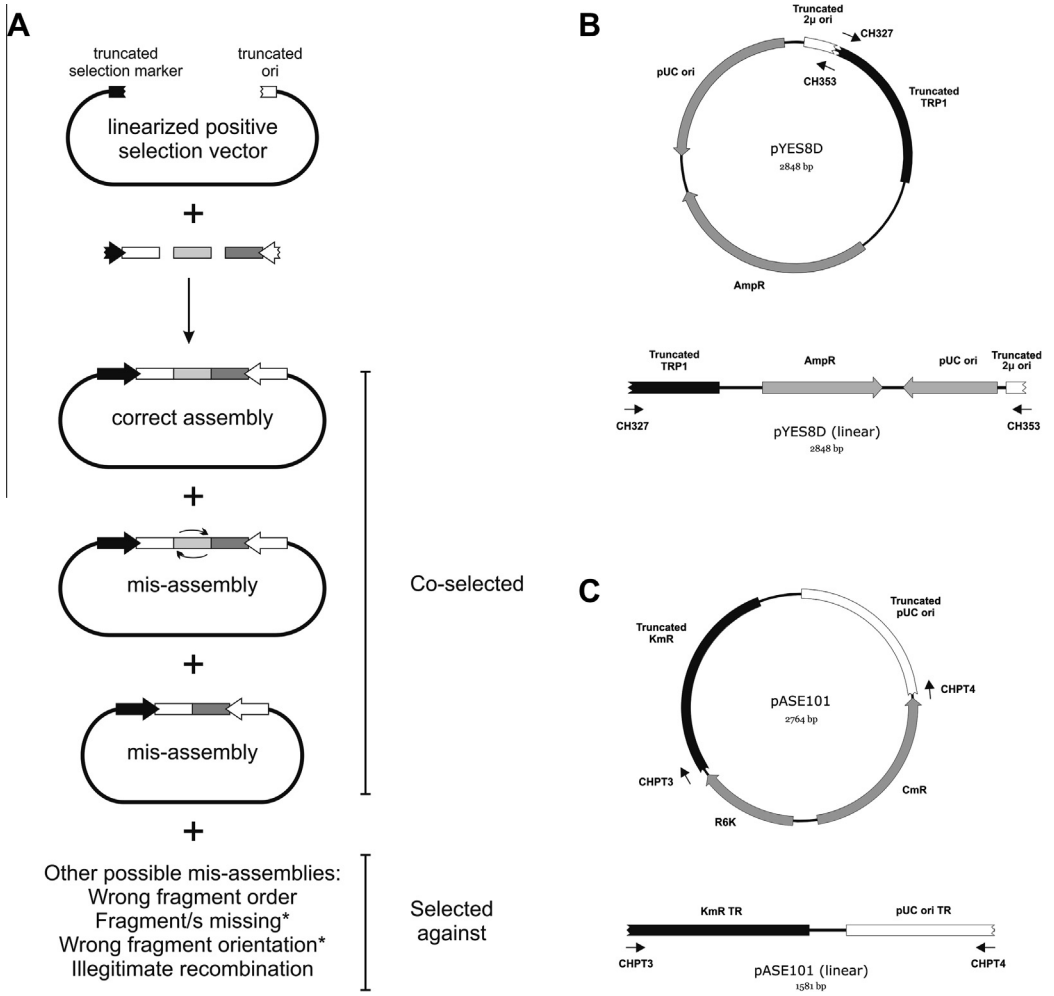

Fig.1. Positive selection assembly approach. (A) The diagram shows the possible outcomes of a three-fragment cloning approach if legitimate and illegitimate assemblies were to occur. Whereas the positive selection strategy selects for the expected clone and two mis-assembled constructs, a variety of other variants could, in theory, be co-selected using standard selection approaches. The asterisks refer to those mis-assemblies not selected for using the positive selection approach. For simplicity, a few vector elements described in the text were not included in the figure. (B, C) Maps of the circular and PCR-amplified pYES8D vector (B) and pASE101 vector (C). Small arrows indicate the position of the amplifying oligonucleotides. For additional information, see text and Supplementary material (Tables S1 and S2).

into pCR–Blunt II-TOPO PCR cloning kit (Thermo Fisher Scientific). When indicated, these pre-cloned DNA fragments were used as templates to produce polymerase chain reaction (PCR)-amplified inserts. DNA assemblies were performed in vivo and in vitro using the homologous recombination cloning kits GeneArt High-Order Genetic Assembly System and GeneArt Seamless PLUS Cloning and Assembly, respectively, following the manufacturer's directions (Thermo Fisher Scientific). Cloning efficiencies were determined by screening either 10 or 12 clones per experiment and were expressed as the ratio of number of correct clones divided by the number of total clones analyzed. Validation was performed by DNA restriction and sequencing.

Results and discussion

Positive selection in S. cerevisiae

It has been shown that when working with large and/or complex DNA fragment assemblies, yeast-based cloning provides a significant advantage over methodologies that use *E. coli* as the propagation organism (for a few examples, see Refs. [8,9]). However, there are certain types of assembly settings where the sought clone is extremely difficult to obtain. Features that make DNA fragments difficult to clone include, but are not limited to, G/C content, potential secondary structures of the intermediate single-strand sequences produced during the assembly procedure, and partial sequence homology shared among the fragments' ends. We used the recombination proficiency of yeast as a model to show the applicability of our approach to established in vivo cloning methodologies. For this purpose, we engineered the positive selection yeast vector pYES8D (Fig. 1B), which encodes two inactive elements required for propagation: (i) a truncated TRP1 gene missing its last 21 bp (5′-GTCAAAAATGCTAAGAAATAG-3′), which in its otherwise active form is required for tryptophan biosynthesis [10], and (ii) an origin of replication from the yeast 2μ episome [11] harboring a 10-bp deletion from one of its ends (5′-AGATAAAC AT-3′). The functionality of these short complementing elements was validated by phenotypic analysis of a series of truncated constructs (data not shown). We chose the smallest truncation that yielded selection to minimize the size of the required complementing sequence. These short sequences can be incorporated into the 5′ end of the corresponding PCR oligonucleotides. The vector in its current form cannot be propagated in yeast unless the missing sequences are provided during the cloning procedure. In addition to the elements above, the β-lactamase gene (AmpR) and the