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Efficient Cloning and Electro-transformation of Large Eukaryotic DNA Fragments

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Introduction

Genes of higher eukaryotes can be hundreds of kilobases in size. The need to rapidly clone and manipulate DNA molecules in that range has become acute in conjunction with the Human Genome Project. Cosmid vectors are constrained to 40–45 kb by the packaging limit of phage lambda. Recently, three systems have been developed that extend this range: yeast artificial chromosome (YAC)-based vectors (>500 kb limit),[1] phage P1-based vectors (100 kb limit),[2] and *E. coli* F factor-based vectors (limit as yet untested).[3–5] YAC clones have proven very successful, but are difficult to manipulate due to the extreme fragility of large linear DNA molecules. Bacterial episomes, being supercoiled, don't suffer that constraint. Unfortunately, F factor vectors have been limited by the low efficiency of transformation of large DNA molecules into *E. coli*. Recently, through the use of electroporation, efficiencies of 10^{10} transformants/µg DNA of small plasmids have been achieved. A systematic analysis of large DNA molecules has been reported,[5] and is summarized here.

Materials and Methods

E. coli strain JS4[6] was grown at 37 °C in LB broth; in mid-log phase (OD_{600}=0.6) flasks were placed in ice water and chilled for 15 minutes. Cells were harvested, washed in ice-cold 1 mM HEPES, pH 7.0 (first wash 1x, second wash 0.5x of original culture volume), and resuspended at 10^{11} cells/ml in 10% glycerol. 40 µl aliquots were flash-frozen in dry ice/ethanol and stored at -70 °C. Cells were thawed on ice, DNA (2 µl in TE) was added, and transferred to ice-cold 0.2 cm gap Bio-Rad electroporation cuvettes. A single pulse was delivered (Gene Pulser® II apparatus used with the Pulse Controller II) at 25 µF, 2.50 kV and 200 ohms (time constant=4.5–4.7 msec). Cells were immediately resuspended in 1 ml of SOC (room temperature) and incubated at 37 °C for 45 minutes. Dilutions were plated on LB plates containing appropriate antibiotics. Plasmid DNA concentrations were determined by absorbance readings at OD_{260} and verified by gel electrophoresis.

Results

We have investigated the relationship between plasmid size and electroporation efficiency in *E. coli* and found that even very large plasmids can be efficiently transfected. Six plasmids were used: pUC19 (2.6 kb),[7] cosmid tsA/Y/1d4 (44 kb),[8] and F factor clones pMBO52 (52 kb), pMBO74 (74 kb), pMBO99 (99 kb) and pMBO136 (136 kb).[3] The average efficiencies (number of colonies per µg DNA) were: pUC19, 2 x 10^9; tsA/Y/1d4, 6.1 x 10^7; pMBO52, 6.6 x 10^6; pMBO74, 3.2 x 10^6; pMBO99, 2.7 x 10^6; pMBO136, 1.7 x 10^6 (Figure 1). The data points can be fitted to a straight line when both axes are plotted on a logarithmic scale. The relation can be written as $E=1.2 \times 10^6 M^{-0.4248}$, where E is efficiency (colonies/fmole), and M is plasmid size (kb).

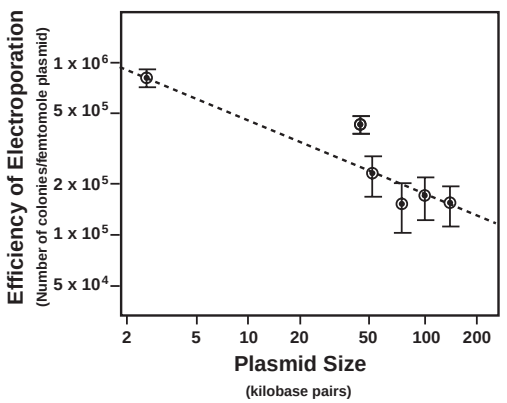

Fig. 1. Molar efficiency of electroporation as a function of plasmid size. Antibiotic selection was: pUC19 and tsA/Y/1d4, ampicillin resistance; pMBO52-pMBO136, chloramphenicol resistance. The efficiency of plating on chloramphenicol was 20–30% of that on ampicillin. Error bars are the standard deviations of six independent electroporation experiments.

Supercoiled and relaxed episomes were electroporated with equal efficiency. Rearrangements incurred during electroporation were analyzed at the level of restriction digestion; none were detected. To exploit these observations we have constructed a novel mammalian-*E. coli* shuttle vector, designated pFRS1 (F Replicon Shuttle, Figure 2), which contains three functional units: 1) the *E. coli* F factor origin of replication, partitioning, and copy number control region, 2) a marker selectable both in *E. coli* and mammalian cells (the neo cassette from plasmid pRSVneo), and 3) the polylinker from the pBluescript SK™ cloning vector. The vector stably confers resistance to kanamycin in *E. coli*, and resistance to G418 in mammalian cells. The plasmid is approximately 12 kb in size, and, like its F factor parent, is maintained at low copy number.

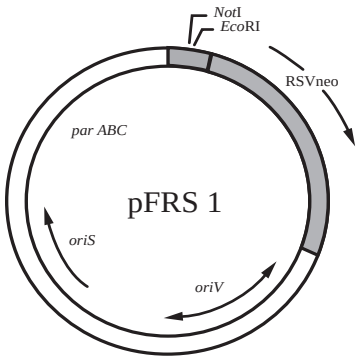

Fig. 2. The pFRS1 vector. The plasmid was assembled from three fragments: 1) a 393 bp fragment from pBluescript KS, *Pvu*II (coordinate 525 nt), to *Ase*I (coordinate 918 nt); 2) a 2,925 bp fragment from pRSV*neo*DRI (pRSV*neo* which had the *Eco*RI site in the LTR, coordinate 89 nt, removed by filling in), *Nde*I (coordinate 582 nt), to *Bam*HI (coordinate 3,397 nt); 3) an 8,717 bp fragment from the *E. coli* F sex factor plasmid, *Hin*dIII (coordinate 41.1 F map units, or 847 nt in the sequence of the *Eco*RI f5 fragment, GenBank accession M12987) filled in and *Bcl*I linkered, to *Eco*RI (coordinate 49.9 F map units, or 9,564 nt, as above) blunted with mung bean nuclease.

Discussion

The new vector system should accept inserts well in excess of 100 kb. The efficiencies of electroporation are well above the minimum required to construct representative libraries of complex eukaryotic genomes. At this time, the only vector system available for the cloning of very large DNA fragments uses YAC vectors. *E. coli* has several advantages over yeast as an organism for the passive propagation of foreign DNA. *E. coli* grows faster, DNA can be rapidly and efficiently extracted, and yields, even of single copy plasmids, are significantly higher. In addition, circular supercoiled DNA molecules are physically quite stable and easy to handle. The most crucial issue, however, is the genetic stability of foreign DNA. Use of *E. coli* offers the distinct advantage of the very considerable body of knowledge concerning its recombination pathways. In several instances the instability of foreign DNA in *E. coli* has been overcome by the judicious choice of appropriate, multiply marked, recombination-deficient strains.[9–11]

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