

# Next generation DNA assembly tools

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## ABSTRACT

Being the cradle of the synthetic biology era, a variety of life sciences disciplines find themselves in the need of applications that include the integrated analysis of complex pathways, the construction of new biological parts and the re-design of existing, natural biological systems. All these areas require the precise and concerted assembly of multiple DNA fragments of various sizes, including chromosomes. Current cloning approaches lack one or more of these requirements. Here we present a set of technologies that allow the seamless, simultaneous, and highly efficient assembly of genetic material, designed for a wide size dynamic range (10s to 100,000s base pairs). The assembly can be performed either *in vitro* or within the living cells and the DNA fragments may or may not share homology at their ends. These approaches are particularly relevant for the study of phage and bacteria, as these organisms are frequently used as chassis or vehicles for the generation of biological circuits.

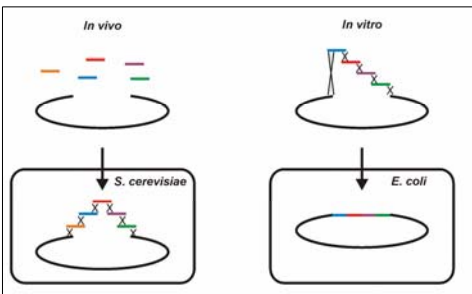
## SIGNIFICANCE AND PURPOSE

The recombinant DNA technology was initiated more than 40 years ago, soon after the discovery of DNA ligase and restriction endonucleases. Since then and with the subsequent benefit of PCR, it has been predominantly applied to the construction and manipulation of relatively uniform DNA molecules.

In anticipation of an imminent paradigm shift, largely due to the emergence of the synthetic biology field, our goal is to offer a comprehensive solution to generate any DNA molecule up to high level genetic systems starting from digital sequences stored in a computer.

## APPROACHES

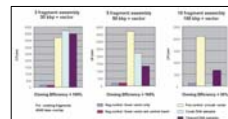
Figure 1. *In vitro* and *in vivo* recombining



Our strategy relies on homologous DNA recombination promoted by purified enzymes *in vitro* and completed in *E. coli*, or performed entirely *in vivo* within a yeast cell.

## RESULTS IN VIVO

Figure 2. Fragment assembly in yeast



Initial studies were performed using 10-kbp fragments, previously cloned into pACYC184. Fragments sharing 80 bp of overlapping sequence were excised from the vector by NotI digestion. Excised molecules were used crude or purified over a silica spin-column, then combined with linearized pDEST22, transformed into *S. cerevisiae* MAV203 cells and plated onto CSM agar plates lacking tryptophan.

Figure 3. BAC/YAC Vector



To maximize plasmid capacity and make it compatible with transfer and screening processes, a new BAC-YAC episome was designed to incorporate the F' ori elements, oriT and the lambda COS site.

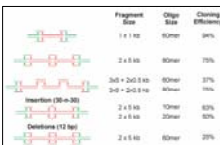
Table 1. More Fragments, shorter overlaps

#	#kbp (Pre-exist)	#kbp (PCR)	Overlap (bp)	Insert (ng)	Eff. (%)
10	10 x 10	0	80	100	50
20	8 x 10	12 x 0.5-2.5	80	100	58
20	8 x 10	12 x 0.5-2.5	80	200	83
1	0	1 x 0.7	30	100	100
1	0	1 x 1.0	80	200	100
1	0	1 x 1.0	30	200	100
10	0	10 x 0.5	30	200	92

\*Linearized plasmid pYES1L not counted as a fragment.

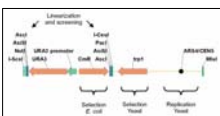
Further assemblies were performed using pre-existing fragments (as above), PCR-amplified fragments, or both. Recipient plasmid was pYES1L. Other variables were overlap span and amount of DNA.

Figure 4. Oligo Stitching



Our approach can also be applied to "stitch" fragments without end-homology. The homology is provided in trans by overlapping oligonucleotides. This feature, allows editing the fragment junctions, thereby generating required imperfections such as insertions or deletions.

Figure 5. Conversion Cassette

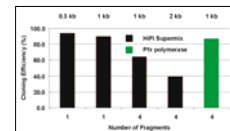


A blunt 4 kbp DNA fragment coding for the yeast replication elements and selection markers can be cloned into *E. coli* vectors to make them yeast-compatible.

## RESULTS IN VITRO

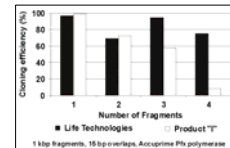
We sought to develop an *in vitro* cloning method that is seamless, multi-fragment capable, vector-independent. Homologous recombination turned to be the most viable approach (Fig 1, right panel).

Figure 6. Cloning up to 4 fragments into a vector



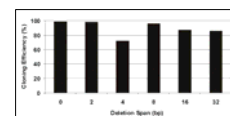
Fragments were amplified using a standard PCR polymerase (PCR SuperMix HiFi) or a proofreading thermostable DNA polymerase (Pfx) and recombined into *Fst-KpnI* linearized pUC19

Figure 7. Comparative Audit



The kit performance was compared to an existing product. Both kits perform similarly for 1 or 2-fragment assembly, but our formulation works more efficiently with 3 or 4 fragments (1 kbp each).

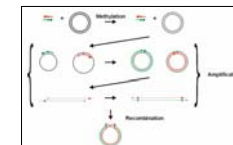
Figure 8. End Editing



The recombination can take place up to 32 bp away from the end of the linearized product. This attribute is useful to generate cloning variants from a single linearized vector.

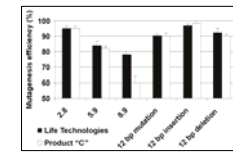
## RESULTS MUTAGENESIS

Figure 9. Overview



Synthetic biology relies on modulation of gene expression and protein engineering, requiring even single nucleotide modification. In this context, we developed a high efficiency site-directed mutagenesis kit.

Figure 10. Comparative Audit



The kit performance was compared to an existing product by reverting a mutation in a *lacZ alpha* gene. Kits perform similarly in most tested scenarios. Our kit exhibited better performance with large plasmids.

## OLIGO DESIGNER

Figure 11. Design Software



The DNA assembly experimental design is facilitated by an on-line webtool that generates DNA oligonucleotides for PCR and/or bridging, advises on the potential existence of internal homology, and delivers final construction in GenBank format.

## SUMMARY

The gene assembly and mutagenesis tools in combination with DNA synthetic capabilities provide a comprehensive solution to generate, manipulate, and assemble any DNA molecule starting from digitized sequence information.

- Seamless *in vitro* assembly system to piece together up to 5 fragments and up to 13 kbp.
  - Terminal homology of 15 bp overlaps
  - 30-min reaction, single tube, isothermal reaction
  - Vector-independent
- Seamless *in vivo* assembly to piece together up to 11 fragments and up to 110 kbp.
  - Terminal homology of 30 bp overlaps
  - Shuttle vector: yeast assembly and *E. coli* propagation
  - Stitching oligo option allows reuse of fragments and end editing
  - Yeast - *E. coli* transfer (10 min), no liquid culture required
- On-line design tools to facilitate the processes above
- High efficient site directed mutagenesis approach to mutate up to 12 nt closely spaced.

## ACKNOWLEDGEMENTS

We thank the invaluable contribution of Jaime M Humara and Don Selway

## PRODUCTS

For information, check [www.invitrogen.com/DNAAssembly](http://www.invitrogen.com/DNAAssembly)

Web tool: [www.invitrogen.com/DesignDNAAssembly](http://www.invitrogen.com/DesignDNAAssembly)

## Catalog products

- GENEART® Seamless Cloning and Assembly Kit (A13288)
- GENEART® Linear pUC19L Vector for Seamless Cloning (A13289)
- GENEART® High-Order Genetic Assembly System (A13285)
- GENEART® High-Order Genetic Assembly Systems (with Yeast Growth Media) (A13286)
- GENEART® High-Order Linear pYES1L Vector (A13287)
- CSM Media for Mav203 Yeast Cells (A13292)
- GENEART® High-Order Vector Conversion Cassette (A13291)
- GENEART® Site-Directed Mutagenesis System (A13282)