Topic Introduction

# Construction of Multifragment Plasmids by Homologous Recombination in Yeast

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Over the past decade, the focus of cloning has shifted from constructing plasmids that express a single gene of interest to creating multigenic constructs that contain entire pathways or even whole genomes. Traditional cloning methods that rely on restriction digestion and ligation are limited by the number and size of fragments that can efficiently be combined. Here, we focus on the use of homologous-recombination-based DNA manipulation in the yeast *Saccharomyces cerevisiae* for the construction of plasmids from multiple DNA fragments. Owing to its simplicity and high efficiency, cloning by homologous recombination in yeast is very accessible and can be applied to high-throughput construction procedures. Its applications extend beyond yeast-centered purposes and include the cloning of large mammalian DNA sequences and entire bacterial genomes.

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## INTRODUCTION

DNA cloning has been a fundamental aspect of molecular biology for decades. However, with the widespread availability of whole-genome sequences and the increased interest in synthetic and systems biology, the focus of cloning has evolved from inserting a single gene of interest into a vector to creating large, multigenic constructs that express entire pathways. With this new focus comes the need for cloning strategies that allow for efficient multifragment assembly.

Traditionally, cloning was based on the introduction of restriction sites onto the ends of a DNA sequence of interest via the tails of a primer used in the polymerase chain reaction (PCR). After digestion of the PCR product and the vector with the proper restriction enzyme(s), ligation and transformation into *Escherichia coli*, the resulting plasmid could be obtained (Cohen et al. 1973). As the used restriction sites cannot be present in the sequence of interest and as the cloning efficiency drops as the number of fragments increases, it quickly becomes problematic to clone large or multiple DNA fragments using this method.

In homologous-recombination-based cloning techniques, the primers add a sequence to the ends of the amplified product that is homologous to the incorporation site in the vector. The homologous sequences can recombine into a single DNA molecule either in vitro using specific enzymes (Aslanidis and de Jong 1990; Gibson et al. 2009) or in vivo using homologous-recombination-competent microorganisms such as the yeast *Saccharomyces cerevisiae* (Ma et al. 1987; Raymond et al. 1999; Gibson 2009). Homologous-recombination-based cloning strategies do not rely on the availability of restriction sites, and so any set of DNA fragments can be joined together independent of their sequence. This

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eliminates both the main disadvantage and various time-consuming steps of ligase-based cloning and increases the overall efficiency of the process.

## IN VITRO AND *ESCHERICHIA COLI*–BASED RECOMBINATION METHODS

In vitro recombination relies on the creation of single-stranded DNA overhangs by an enzyme with exonuclease activity such as T4 DNA polymerase or T5 exonuclease (Aslanidis and de Jong 1990; Gibson et al. 2009) or by commercially available kits such as In-Fusion by Clontech or Gateway Cloning by Life Technologies (Benoit et al. 2006; Liang et al. 2013). Complementary single-stranded sequences can then anneal in vitro, followed by transformation into *E. coli* to seal the remaining nicks in the DNA (Fig. 1A). Alternatively, the commonly used bacterium *E. coli* can be used as a host for in vivo recombination reactions. As homologous recombination is not very proficient in wild-type *E. coli* strains, several mutant strains such as *recBC sbcA* strains and strains overexpressing Redαβ or RecET prophage proteins have been used to increase the recombination efficiency (Zhang et al. 1998; Wang 2000; Fu et al. 2012). Although these in vitro and *E. coli*

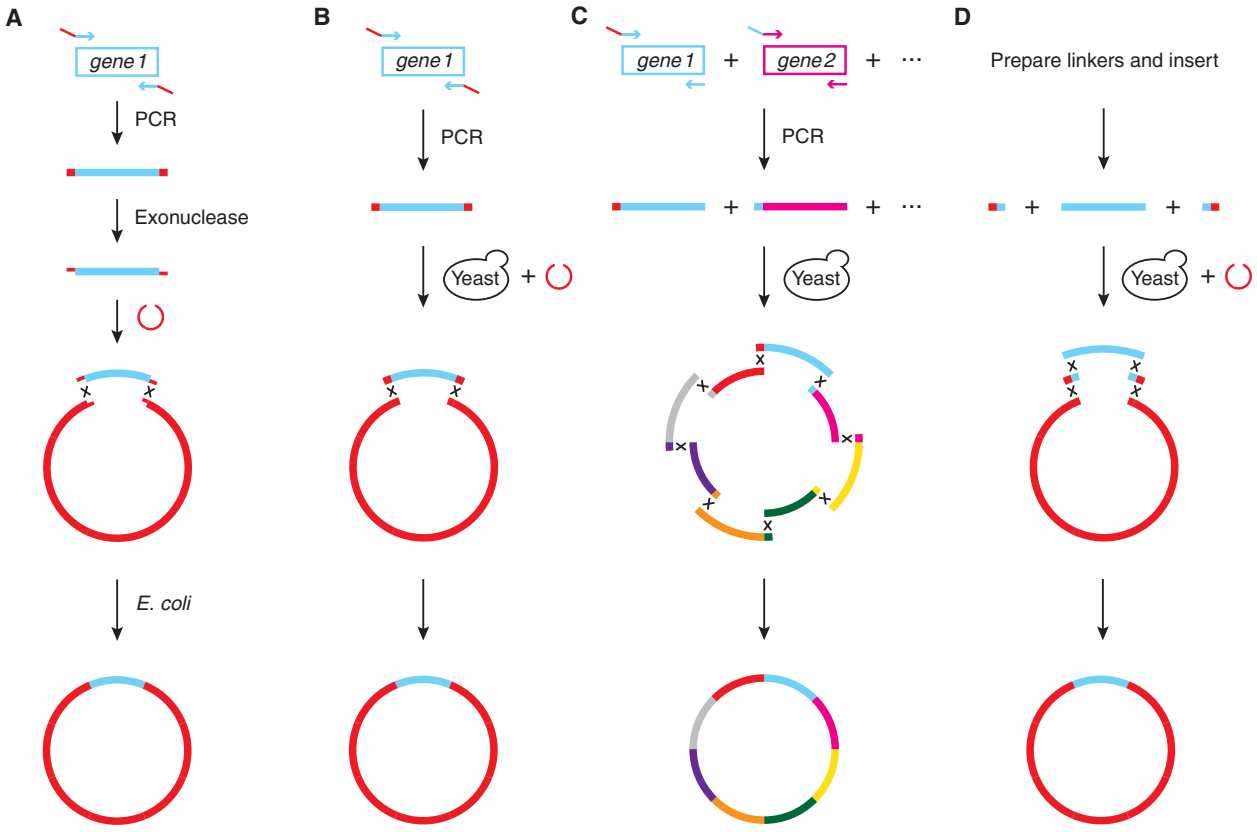


**FIGURE 1.** Cloning by homologous recombination. (*A*) In vitro homologous recombination of a linearized yeast plasmid and an insert through the creation of complementary single-stranded DNA overhangs by means of an exonuclease. (*B*) In vivo recombination of a linearized yeast plasmid and an insert in budding yeast using homologous double-stranded DNA sequences introduced by PCR. (*C*) In vivo recombination of multiple fragments in budding yeast using homologous double-stranded DNA sequences introduced by PCR. Instead of using a linearized yeast plasmid as one fragment, the selection marker(s) and replication origin(s) can be introduced on different DNA fragments. (*D*) In vivo recombination in budding yeast using homologous sequences present on separate DNA-linker fragments. In this case, the DNA linkers have homology regions to both the acceptor vector and the insert and are useful for cloning inserts from different sources when PCR amplification is difficult or undesirable.

methods are very straightforward for regular two-fragment cloning, the overall efficiency of these recombination strategies in multifragment reactions remains low, and there are limitations to the size of the fragments that can be cloned.

## RECOMBINATIONAL CLONING USING *S. CEREVISIAE*

The homologous recombination pathway in *S. cerevisiae* efficiently repairs double-strand DNA breaks and can be exploited to join multiple DNA fragments together (Fig. 1B–D). These DNA fragments can be oligonucleotides, PCR products, synthetic DNA, or parts of plasmids or chromosomes. Although homologous recombination in yeast is extremely practical when the downstream applications are also in yeast, its application extends beyond yeast-specific research questions. For example, yeast can be used to assemble constructs that, owing to their size, the number of DNA fragments or the nature of the sequence, are difficult to clone by other methods (Kouprina et al. 2003; Gibson et al. 2008a,b; Vieira et al. 2010; Noskov et al. 2011).

One of the first applications of homologous recombination in yeast for the construction of plasmids from DNA fragments was described by the Botstein laboratory (Ma et al. 1987). In these early experiments, large homology regions of several-hundred base pairs were used. However, it quickly became clear that as little as 30- to 50-bp homology regions are sufficient to induce recombination, which opened the door for PCR-mediated strategies in which homology regions are introduced in the primers (Fig. 1B) (Baudin et al. 1993; Manivasakam et al. 1995; Hua et al. 1997; Oldenburg et al. 1997).

As recombinational repair is far more prominent in yeast than ligation or nonhomologous end-joining of a linearized plasmid, the background of nonrecombinant vector is generally very low (Ma et al. 1987; Hudson et al. 1997; Raymond et al. 1999; Tsvetanova et al. 2011). Up to 25 large (17–35 kb) DNA fragments have been assembled efficiently into one vector after cotransformation in yeast without direct selection for any of the fragments except the vector backbone (Gibson et al. 2008b). If a low recombination rate is anticipated, for example owing to the low concentration or large size of an insert, loss of a (counter)selectable marker in the vector can be used to screen for recombinants among the resulting transformants (Ma et al. 1987; Gunyuzlu et al. 2001; Noskov et al. 2002; Raymond et al. 2002; Kitazono 2009). Alternatively, the yeast selection marker and replication origin can be introduced on different DNA fragments, thereby separating the elements necessary for survival and eliminating background transformants caused by self-closure of a vector backbone (Fig. 1C) (Kuijpers et al. 2013).

## PCR-FREE RECOMBINATION IN YEAST

Several recombination-based methods have been described for cloning DNA fragments from one vector into another (Ma et al. 1987; Erickson and Johnston 1993; Prado and Aguilera 1994; Gunyuzlu et al. 2001; Iizasa and Nagano 2006). These methods rely on homology regions between the two used plasmids that can either recombine directly to yield the plasmid of interest (Ma et al. 1987; Erickson and Johnston 1993; Iizasa and Nagano 2006) or recombination can be directed by the use of double-stranded DNA linker molecules (Gunyuzlu et al. 2001). These DNA-linkers have homology regions to both the acceptor vector and the insert and can also be used to clone inserts from different sources when PCR amplification is difficult or undesirable (Fig. 1D) (Raymond et al. 1999; DeMarini et al. 2001; Gunyuzlu et al. 2001; Tsvetanova et al. 2011). Furthermore, single-stranded DNA can serve as a DNA-linker (DeMarini et al. 2001; Raymond et al. 2002) or even as the insert itself, as was shown by the assembly of 38 overlapping single-stranded oligonucleotides into one plasmid after cotransformation with linearized vector in yeast (Gibson 2009).





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## APPLICATIONS OF RECOMBINATIONAL CLONING IN YEAST

Large genomic fragments from either yeast or more-complex genomes can be captured in a circular vector or on yeast artificial chromosomes (YACs) by cotransforming yeast cells with linearized vector and the genomic DNA of the organism of interest (Orr-Weaver and Szostak 1983; Fairhead et al. 1996; Noskov et al. 2002). Clones that carry up to 2 Mb of human DNA sequences have been reported (Marschall et al. 1999). An example of the use of homologous recombination in yeast for multifragment heterologous cloning is the construction of a single vector that expresses the complete zeaxanthin biosynthetic pathway by assembling cassettes of eight genes from other microorganisms combined with *S. cerevisiae* promoters and terminators (Shao et al. 2009). Another highlight of multifragment heterologous cloning by homologous recombination in yeast is the assembly of the full ~1.1-Mb *Mycoplasma mycoides* genome in three stages from 1-kb DNA fragments (Gibson et al. 2010). The *M. mycoides* genome was stable in yeast as a centromeric plasmid and was fully functional after transferring to a bacterial host cell (Gibson et al. 2010).

Yeast homologous recombination can easily be adapted to introduce point mutations or partial deletions, add a carboxy- or amino-terminal tag, or create chimeric genes using either yeast genes or DNA sequences from other organisms (Pompon and Nicolas 1989; Muhlrad et al. 1992; Marykwas and Passmore 1995; Storck et al. 1996; Oldenburg et al. 1997; Raymond et al. 1999). For example, Hudson and others cloned fusions of nearly all yeast open-reading frames (ORFs) to the activation domain of the transcriptional regulator Gal4p, which can be used in large-scale yeast two-hybrid analysis (Hudson et al. 1997). Additionally, yeast homologous recombination can be used to create mutants in genomes of other species that do not have the genetic tools available for such a modification directly. This was shown by the creation of a deletion allele of a nonessential restriction endonuclease gene in the complete *M. mycoides* genome by homologous recombination in yeast (Lartigue et al. 2009).

## CONCLUDING REMARKS

Over the past few decades, yeast-based recombinational cloning has evolved from inserting just a single fragment into a yeast–bacterial shuttle vector using homology regions of several-hundred base-pairs, to the impressive creation of large plasmid-free assemblies of many fragments with short homologous sequences. As plasmid construction by homologous recombination in yeast is straightforward and highly efficient, we expect its use and applications to further expand in the future. In the accompanying protocol, we present a method using homologous recombination in the budding yeast to assemble multiple DNA fragments into one construct; see Protocol: **Rapid and Efficient Plasmid Construction by Homologous Recombination in Yeast** (van Leeuwen et al. 2015).

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