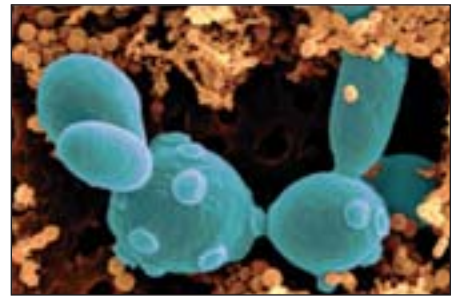
Bench philosophy (24): Yeast recombinational cloning

Smart cloning



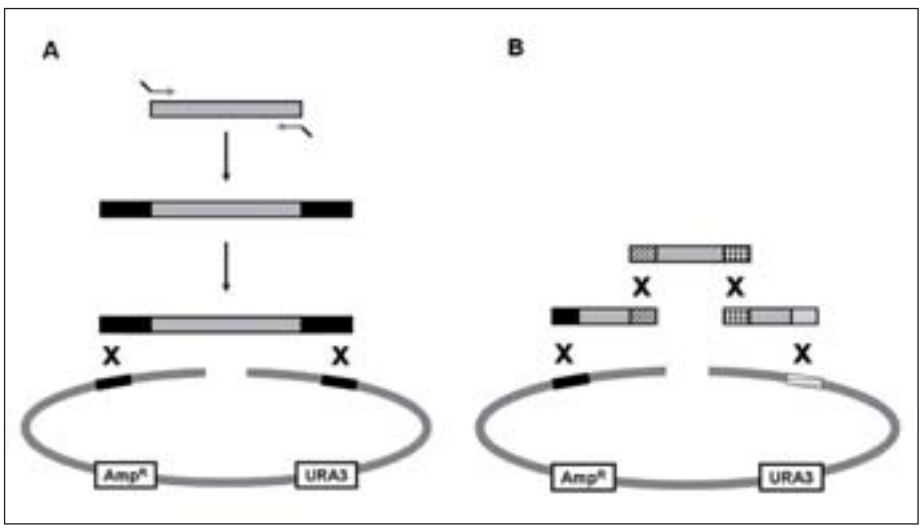
Yeasts have developed a highly efficient homologous recombination system. Researchers may take advantage of it, to easily clone their gene of interest.

The logical next step after genome sequencing in the fascinating journey of exploring the underlying basis of living organisms, is trying to globally understand gene function and also, how gene expression is regulated. It is then easy to appreciate that this functional genomics step would benefit deeply from methodologies that allow for high-throughput cloning (of ORFs or regulatory sequences, such as promoters) and vector tailoring.

Molecular cloning is routine in any basic research molecular biology laboratory. Traditionally, this involves PCR-amplifying the region of interest using primers harbouring customised restriction sites, followed by digestion of the amplicon and the target vector, ligation and transformation into *E. coli*. Sometimes, you can use the PCR product directly in a digestion reaction with the proper enzymes (after column-purifying the PCR product) but cloning into pGEMT vector system is also frequently employed. This involves a TA tailing step (as high fidelity enzymes, used for cloning, typically produce blunt ends), bacterial transformation and screening. Once you digest your PCR product (directly or from pGEMT), you'll have to digest the target vector with the same enzymes, set up a ligation reaction (the exact conditions of which may need some tweaking) and then use an aliquot of the reaction to transform bacteria. Note that you have to make sure that the enzymes you select do not cut within your region of interest. Ideally, you'll use two different enzymes to linearise the target vector to avoid re-circularisation. Further, this methodology is dependent on the availability of restriction sites at the particular location on the target vector where you want to clone your fragment. You can then evaluate cloning success by doing colony PCR of the antibiotic-resistant colonies resulting from the transformation and, ultimately, sequencing.

Other approaches for cloning PCR products include blunt-end cloning, TA cloning, ligation-independent cloning and others. All these require post-PCR enzymatic treatments (e.g. restriction enzyme digestions, ligations, processing by T4 DNA polymerase, etc.), are time consuming and need a lot of manual input. These then, are not an ideal choice for high-throughput analyses or when the design of complex constructs (involving bringing together four or more DNA fragments) is required.

Recombinational cloning methods overcome many of the practical limitations of traditional cloning and have been used for several high-throughput studies and vector tailoring. In this article, I will try to briefly describe a highly efficient, cheap and simple method, useful for the cloning of PCR products and vector construction, based on *in vivo* recombination using either baker's yeast (*S. cerevisiae*) or bacteria (*E. coli*). I will mainly describe the methodology using yeast, as it is the one we have been using the longest in our laboratory, but the same principles apply to *E. coli*.



A) A segment of interest is PCR-amplified with primers designed to contain 20-40 nt of homology to the region of the vector where recombination is to occur. The vector is linearised with restriction enzymes, which generate a gap that will be repaired after integration of the segment by homologous recombination in yeast (see text for details). B) YRC can be used to assemble complex constructs as multiple overlapping inserts can be co-transformed into yeast.

Simple strategy

The cloning strategy we use routinely in our lab is called Yeast Recombinational Cloning (YRC, also referred to as "gap-repair cloning"), a strategy which has been around for some time (Ma *et al.*, *Gene* 58, 201-216, 1987) and has been refined over the years (Oldenburg *et al.*, *Nucleic Acids Res.* 25, 451-452, 1997; Gibson, *Nucleic Acids Res*. 37, 6984-6990, 2009). The concept behind this strategy is simple: homologous recombination in yeast can be used to generate a DNA fragment from overlapping independent parts. So, if the segment you'd like to clone into a particular vector bears homology to defined vector sequences, it can be directly integrated into the linearised vector, after co-transformation into yeast, by *in vivo* recombination through the endogenous double-strand break repair pathway.

The highly efficient recombination system in yeast was initially exploited for genetic studies in this organism and it has since been used for the creation of ORF libraries and construction of yeast plasmids, among other applications, extending beyond yeast biology to other organisms.

Initially, it was shown that a DNA restriction fragment containing appropriate sequence homology to a linearised target vector and spanning the gap, could serve as a substrate for such "gap-repair" cloning (it's called gap repair, as you'll repair

the restriction enzyme-derived gap in the linearised vector once the fragment of interest is incorporated through homologous recombination). An important advance in the use of such recombination-based methods for gene cloning and plasmid construction in yeast, involved the use of PCR (rather than restriction assays) to generate the DNA fragment to be used. As it was shown that the length of sequence homology needed to promote efficient recombination between the segment of interest and the vector was small (~20-40 nt), it was quickly realised that these sequences could be included as part of the PCR primers used to amplify the segment of interest. Thus, any sequence could be cloned into any vector, as long as they share an appropriate region of sequence homology, which can be added to the amplicon through proper primer design. Such "recombination-mediated PCR-directed" plasmid construction strategy arose then as a simple and flexible cloning alternative.

How exactly do you generate a particular construct through YRC? The linearised target vector containing a yeast-compatible selectable marker (e.g. URA3) is co-transformed into yeast with the PCR fragment of interest. As mentioned, this fragment contains typically 20-40 nt of homology at each end, to the region of the vector at which recombination is to occur (we generally use 30 nt, which works perfectly). These nucleotides are added to the fragment as part of the primers. PCR products do not need to be purified before transformation; an aliquot taken directly from the PCR reaction tube can be used. The same applies to the linearised vector. By homologous recombination, some of the linearised vectors are recircularised (i.e. the gap is repaired due to the integration of the segment of interest into the vector) and the plasmid can now be propagated in yeast. Recombinants are then selected as Ura^+ transformants (in this example, see Figure A). Yeast colony PCR on the Ura^+ transformants can be used to check for correct plasmid assembly. Note that the linearised vector will not lead to Ura^+ transformants, as such vectors cannot be propagated. Plasmids are then obtained from yeast and propagated in *E. coli*.

This protocol may take longer (in days) than the traditional approach (as you'll have to wait for the transformed yeast to grow in selective media) but requires much less handling time; it has significantly less steps, uses less restriction enzymes (as you'll only use them to linearise the target vector), uses no ligase (as no *in vitro* ligation step is needed) and does not require any sort of purification of the reagents (PCR product and linearised vector). The only methods involved are digestion, PCR and yeast transformation, the latter of which is very simple, efficient and requires reagents typically found in a molecular biology lab. Last but not least, it is technically simple and economical.

Ideal cloning system

An ideal cloning system has to assure high fidelity, ease of use, reliability and flexibility, along with maintaining the integrity of the cloned segment, i.e. it must not add unwanted sequences to the fragment of interest. All of these criteria are fulfilled by YRC. In our hands, cloning efficiency and fidelity are around 90 % and errors mainly arise from PCR amplification, an aspect shared with other high-throughput cloning methods.

Notably, there are no particular sequence requirements for the homologous recombination tags, so in principle, any sequence can be cloned into any vector. Furthermore, recombination can be targeted several base pairs away from the restriction enzyme-generated gap, which expands the number of sites and choices of restriction enzymes to be used for vector linearisation. This is an important advantage over traditional cloning methods. Also, there is no cloning size bias and linkers can be used for large fragments that cannot be amplified by PCR or for bridging non-homologous fragments. The method outlined here is particularly helpful for vector tailoring and the generation of complex constructs, as multiple inserts can be used, provided they overlap each other (Figure B). This includes, but it is not limited to, the generation of chimeric ORFs, epitope-tagging, the study of promoter regulatory elements, etc. Further, this provides an easy alternative to overlap extension PCR for site-directed mutagenesis. This multiple insert feature has important applications for synthetic biology (Gibson, *Nucleic Acids Res*. 37, 6984-6990, 2009). As stated, yeast recombinational cloning is amenable to high-throughput approaches and upscaling and has been successfully used for functional genomics in fungi other than yeast (Colot *et al.*, *PNAS* 103, 10352-10357, 2006; Larrondo *et al.*, *Eukaryot. Cell* 8, 800-804,2009)

Even though the method described above has several advantages, many general-purpose vectors lack the sequences required for use in yeast (i.e. selectable marker and replication sequences) and then cannot be used in YRC. Hence, a similar method in *E. coli* would be very valuable. Moreover, the constructs would be immediately obtained in bacteria. Indeed, *E. coli in vivo* recombinational cloning is possible and uses common laboratory strains (such as DH5alpha, although efficiency is higher using other strains, like KC8). This approach, as YRC, is also technically simple and cheap and high efficiency can be obtained using relatively short recombination tags (20 nt). Notably, bacterial recombinational cloning has been used for high-throughput cloning of ORFs with great success (Parrish *et al.*, *J Proteome Res*. 3, 582-586, 2004).

In summary, *in vivo* recombinational cloning is a convenient and useful cloning approach with several advantages over traditional and commercial cloning systems. Its ease of use, reduced cost, flexibility, high fidelity and efficiency make this method a feasible cloning alternative for the study of gene function and regulation of gene expression.

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