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Note

A universal cloning method based on yeast homologous recombination that is simple, efficient, and versatile

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ABSTRACT

Cloning by homologous recombination (HR) in *Saccharomyces cerevisiae* is an extremely efficient and cost-effective alternative to other methods of recombinant DNA technologies. Unfortunately, it is incompatible with all the various specialized plasmids currently used in microbiology and biomedical research laboratories, and is therefore, not widely adopted. In an effort to dramatically improve the versatility of yeast gap-repair cloning and make it compatible with any DNA plasmid, we demonstrate that by simply including a yeast-cloning cassette (YCC) that contains the 2-micron origin of replication (2 μm *ori*) and the *ura3* gene for selection, multiple DNA fragments can be assembled into any DNA vector. We show this has almost unlimited potential by building a variety of plasmid for different uses including: recombinant protein production, epitope tagging, site-directed mutagenesis, and expression of fluorescent fusion proteins. We demonstrate the use in a variety of plasmids for use in microbial systems and even demonstrate it can be used in a vertebrate model. This method is remarkably simple and extremely efficient, plus it provides a significant cost saving over commercially available kits.

Complex molecular genetic techniques demand efficient and cost-effective DNA cloning methods that can be universally incorporated into any laboratory workflow. This has led to numerous methods for ligase-dependent and independent cloning. Some notable examples include TOPO-TA (Life Technologies) cloning that uses Topoisomerase I to directly insert a PCR-generated DNA fragment into a pre-processed vector. Another popular and versatile commercial method is the Gateway system (Life Technologies) that allows an existing cloned piece of DNA to be transferred from vector to vector. More recently, a number of in vitro cloning methods devised by academic labs have been described. For example, sequence and ligation-independent cloning (SLIC) uses RecA-mediated recombination and was one of the first in vitro recombination methods (Li and Elledge, 2007). The Gibson assembly method combines attributes of a 5′ exonuclease, a thermostable DNA polymerase, and T4 DNA ligase and appears to be a great advancement in constructing large DNA sequences for synthetic biology applications, but is also being widely adopt for smaller cloning experiments (Gibson et al., 2009). SLiCE (Seamless Ligation Cloning Extract) relies on a bacterial extract and is perhaps the best (and least expensive) alternative to routine recombination-mediated cloning, but long oligonucleotides are needed to achieve high efficiencies unless the engineered bacterial PYY strain is used (Zhang et al., 2012). In addition, residual plasmid template in the PCR reaction needs to be removed by *Dpn*I digestion and all fragments have to undergo a gel extraction and purification. CPEC (Circular Polymerase Extension Cloning) is essentially the splicing by overlap extension (SOE) method that is modified to create a plasmid (Horton et al., 1989; Quan and Tian, 2009). Unfortunately, many of these methods have the major drawback of forcing laboratories to purchase expensive kits, maintain a steady supply of reagents, or are complicated to the extent that they require time-consuming optimization and troubleshooting.

A popular and highly efficient cloning alternative utilizes the ease and simplicity of homologous recombination in *Saccharomyces cerevisiae*. Yeast recombination cloning allows assembly of multiple DNA fragments in a single step (Oldenburg et al., 1997) and many variations on the initial method of yeast recombination/plasmid shuffle/gap repair have been described. It is extremely efficient, requiring only 29 nucleotides of overlapping sequences that can be added to the synthesized oligonucleotides. Despite this, yeast cloning is still limited in its applications because it requires a yeast compatible shuttle vector and many experiments demand specific plasmids with specialized genetic elements for defined applications. Therefore, we set out to make the yeast cloning applicable to any DNA cloning experiment by including a yeast-cloning cassette (YCC). This any-gene-any-plasmid (AGAP) cloning represents a significant improvement that facilitates universal adoption regardless of the plasmid backbone and allows anyone the ability to clone any gene (or combination of DNA fragments) into any conceivable vector in a single step without the need for ligase or

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molecular cloning kits. We demonstrate the usefulness and versatility of AGAP cloning by generating plasmids for recombinant protein production in *Escherichia coli*, epitope tagging and site-directed mutagenesis in the opportunistic pathogen *Staphylococcus aureus*, and we further demonstrate it can be used to create a construct to express a fluorescent fusion protein in the vertebrate model zebrafish (*Danio rerio*).

A detailed step-by-step protocol of the yeast transformation and other methods needed for AGAP cloning, including the strains and oligonucleotides, can be found in the supplemental material. All the strains used in this report are listed in Supplemental Table 1. *S. cerevisiae* strain FY2 was used for construct assembly and grown at 30 °C in YPD medium and plated on YMD medium for transformations. *S. aureus* and *E. coli* strains were routinely grown at 37 °C on solid (1.5% agar) or liquid TSB or Luria–Bertani media, respectively. Anaerobic growth was conducted on solid media in an anaerobic jar. Oxygen was replaced by a 95% N_2 5% H_2 headspace using a COY anaerobic chamber. When included in the growth medium, antibiotics were present in the following concentrations; ampicillin 150 μg/mL; chloramphenicol, 30 μg/mL; tetracycline, 3 μg/mL; or spectinomycin, 100 μg/mL.

The plasmids generated in this report were constructed in the following manner and all of the oligonucleotides are listed in Supplemental Table 2. To create the pET24a-Nono, the *Nono* CDS was first obtained by generating a cDNA library from total Trizol extracted RNA isolated from Zebrafish (*Danio rerio*) using Superscript III (Invitrogen) with an anchored oligo dT. The *Nono* CDS was then PCR amplified using oligonucleotides pet24a-NONO-F and pet24aNONO-R. The pET24a vector (EMD Millipore) was digested with *Xho*I, *Eco*RI and *Sph*I and then heat-inactivated. The YCC was PCR-amplified from pRS426 using oligonucleotides pET-URA-F and pET-URA-R. The resulting 4 fragments were then transformed into yeast using the detail protocol listed above and plated on YMD plates. Amplification of the YCC with flanking meganuclease sites was also performed as a proof a concept. The resulting vector was ultimately transformed into BL21(DE3)RiL and induced with 0.4 M IPTG for 4 h. The cells were lysed using a French pressure cell and the protein was purified by Ni-NTA chromatography (Qiagen).

A construct containing a N-terminus flag tagged version of the *srrA* gene was created as follows. The pLL39 vector was linearized with *Bam*HI and *Sal*I. The start codon for the *srrA* ORF was removed and replaced by a 3X flag sequence. *S. aureus* strain LAC chromosomal DNA was used as template for PCR amplification of the *srrAB* promoter and *srrAB* genes. Following yeast transformation and plasmid isolation from *E. coli* the pLL39_FLAG_srrAB construct was confirmed by sequencing and positive clones were retained. The pLL39 vector is a single-copy integration vector system for *S. aureus* that contains an origin of replication for *E. coli* but, it can only be maintained in *S. aureus* by chromosomal integration. The pLL39_FLAG_srrAB construct was transformed into RN4220 containing pLL2787 and integrated onto the chromosome of *S. aureus* at the Φ11 *attB* site. Correct vector integration was verified using PCR with the Scv8 and Scv9 primers and chromosomal DNA for template. The integrated pLL39 and pLL39_FLAG_srrAB constructs were transduced from RN4220 into an *srrAB* double deletion mutant created in the *S. aureus* LAC background using transducing phage α80 creating strains JMB2579 and JMB2592, respectively. The *srrAB* deletion was verified using PCR with the srrABup5EcoRI and srrABdwn3SalI oligonucleotides and genomic DNA as template.

The *FLAG_srrA*$_{D53A}$ site-directed mutant was made as follows. A forward and a reverse oligonucleotide were designed that contained an internal mismatch to direct the D53 ➔ A53 mutation (Fig. 2). These primers were used to PCR amplify the *srr* promoter, *FLAG_srrA*, and *srrB* DNA sequences using chromosomal DNA from strain JMB2592 as template. The alleles were integrated onto the chromosome of RN4220 and transduced into an *srrAB*Δ mutant as described above. The directed mutation was confirmed by DNA sequencing.

Detection of FLAG-tagged SrrA was performed by Western blot analysis from overnight cultures of strains JMB2579, JMB2592, and JMB3946 that were diluted 1:100 into 5.0 mL of liquid TSB. Culture aliquots were harvested at specified time points and 500 μL aliquots of the cultures were removed to assess FLAG-SrrA protein abundance. Cells were isolated by centrifugation and suspended in 200 mM Tris–HCl, pH 8.0. Cells were lysed by the addition of 20 μg of lysostaphin and DNaseI and incubation at 37 °C for approximately 30 min. Lysates were clarified by centrifugation and protein concentrations of the cell free lysates were determined using a micro BCA assay. Proteins were separated using SDS-PAGE chromatography (40 μg protein/lane). Proteins were transferred to a PVDF membrane and incubated with primary anti-FLAG antibody (1:4000 dilution) and subsequently secondary HRP-conjugated anti-mouse antibody (1:12,000 dilution). The blots were developed and visualized using chemiluminescent detection.

For genetic complementation analysis, strains were grown overnight in TSB (~18–20 h) to stationary phase. 2.0 μL of culture was spotted on a TSB agar plate and streaked for colony isolation. Control plates contained nitrate as an electron acceptor. Plates were incubated anaerobically, at 37C for ~18 h. Growth was analyzed for each strain with respect to the small colony variant phenotype for an *srrAB* deletion mutant.

The pCMV-Nono-eGFP plasmid was constructed in a similar manner. The *Nono* CDS was PCR-amplified from the same cDNA library used above with oligonucleotides CMVeGFP-NONO-F and CMVeGFP-NONO-R. The YCC was amplified from pRS426 using CMVeGFP-URA-F and CMVeGFP-URA-R. The pEGFP-C1 plasmid (Clonetech) was digested with *Ase*I and *Eco*RI and then heat inactivated. The 4 fragments were transformed into yeast FY2 using YMD plates as selection. The vector was recovered in *E. coli*, linearized, then injected into zebrafish embryos and examined by fluorescence microscopy.

All zebrafish husbandry and experimental procedures complied with policies set forth by the NIH Office of Laboratory Animal Welfare and approved by Rutgers University Institutional Animal Care and Use Committees (IACUC).

Conceptually, the AGAP cloning method is remarkably straightforward and is based on yeast gap-repair/plasmid shuffle (Ma et al., 1987). Multiple DNA fragments containing at least 29 nucleotides of overlapping homologous sequence are mixed with a linearized vector backbone along with the YCC. The YCC contains the yeast *2* μm origin of replication (*2* μm *ori*) and the *ura3* gene amplified from pRS426. DNA fragments are obtained by PCR amplification using oligonucleotides containing at least 29 nucleotides of sequence identity between each fragment. The amplified YCC, DNA fragments, and linearized plasmid are assembled by transformation into a yeast strain lacking a functional *ura3* gene, which provides the selective pressure to facilitate appropriate construct assembly. Plasmids are then recovered from yeast DNA by transforming into *E. coli*. The YCC and DNA fragments can be assembled in tandem or at separate plasmid locations. Homing meganuclease sites or unique restriction endonuclease sites flanking the YCC can also be included for subsequent removal if desired, but in routine applications, this is unnecessary.

A general outline of the procedure is shown in Fig. 1. Prior to beginning AGAP cloning, one must find a minimum of two unique restriction endonuclease sites within the vector and then double digest the plasmid with both enzymes (This example uses *Xho*I and *Eco*RI). One enzyme is also sufficient, but may cause elevated background. The restriction endonuclease recognition sequences can both be located in the multiple cloning site (MCS) and the YCC can be inserted in tandem with the fragment(s). Alternatively, the YCC can be inserted at a separate location (as shown in the figure). If the YCC is inserted in a location separate from the MCS, then care must be taken to ensure no important elements within the vector (regulatory regions, replication origins, selectable markers, etc.) are disrupted by the insertion. The 29 nucleotides of sequence identity (adjacent to the enzyme cut site) needed for homologous recombination is added to the 5′ end of the synthesized oligonucleotide. Once the vector is digested and the fragments amplified, the mixture of linearized DNA fragments is transformed into yeast using the protocol contained in the Supplemental material.