

## BBF RFC 88 Yeast Golden Gate: Standardized Assembly of *S. cerevisiae* Transcriptional Units

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### **BBF RFC 88: Yeast Golden Gate: Standardized Assembly of *S. cerevisiae* Transcriptional Units**

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#### **1. Purpose**

RFC88 describes a new standard for the assembly of basic *Saccharomyces cerevisiae* transcriptional units (TUs) consisting of a promoter/5'untranslated region (UTR), open reading frame (ORF), and 3'UTR/terminator. Note that we use the term "promoter" here to refer to both the promoter and the 5' UTR, which we currently define as a single part. Future iterations of this standard will incorporate subdivision of currently defined parts e.g. into promoter and 5' UTR. The standard makes use of the type IIS restriction enzyme BsaI to generate standardized and user-defined 'signature overhangs', thus enabling directional and seamless TU assembly. RFC88 is supported by the Yeast Standardized Collection of Parts for Expression (SCoPE), a repository of subcloned and sequence verified parts compatible with this assembly standard. The Yeast SCoPE is currently populated by a large number of *S. cerevisiae* promoters and terminators that facilitate expression and characterization of non-native ORFs.

#### **2. Relation to other BBF RFC's**

RFC28, RFC53, and RFC61 all describe the use of Type IIS restriction enzymes for the purpose of directional assembly of DNA fragments.

#### **3. Copyright notice**

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#### **4. Description**

##### **a. BsaI, a type IIS restriction enzyme.**

RFC88 utilizes the Type IIS restriction enzyme BsaI, which has a six base pair non-palindromic recognition sequence and cuts at positions +1/+5. Because the enzyme cleavage site is distal to its recognition sequence, the overhang may be user-specified during the design stage. Importantly, if oriented correctly the six base pair BsaI recognition sequence can effectively be eliminated during digestion and ligation, thus allowing two parts with complementary overhangs to assemble without an intervening restriction enzyme site 'scar'. Note that in the instance that a part must contain an internal BsaI site, it is possible to substitute the enzyme BsmBI, which generates overhangs with exactly the same characteristics.



Figure 1. BsaI, a Type IIS restriction enzyme, recognizes a six base pair sequence (red text, 5' GGTCTC 3') and cuts distally at positions +1 and +5. If oriented as shown here, BsaI digestion generates user defined, 5' sticky end overhangs (blue text, N's) flanking the part sequence (Part).

### b. Signature overhangs for directional and seamless TU assembly

To enable directional assembly of a TU, we have assigned non-palindromic, 'signature overhangs' to each of the three types of parts. The 5' and 3' ends of all promoters encode the signature overhangs 5'-CAGT-3' and 5'-AATG-3', respectively. Similarly, ORF signature overhangs are designated 5'-AATG-3' and 5'-TGAG-3' and terminators 5'-TGAG-3' and 5'-TTTT-3'. All acceptor vectors encode the overhangs 5'-CAGT-3' and 5'-TTTT-3'. Note that all overhang sequences are given as top strand sequences for clarity. Signature overhang sequences are flanked by BsaI sites oriented so as to eliminate themselves during the digestion reaction and expose the required complementary sticky ends between promoter/ORF and ORF/terminator, and between acceptor vector/promoter and terminator/acceptor vector. Further, for virtually seamless assembly of the TU, start and stop codons are encoded within the signature overhangs that assemble on either side of the ORF.

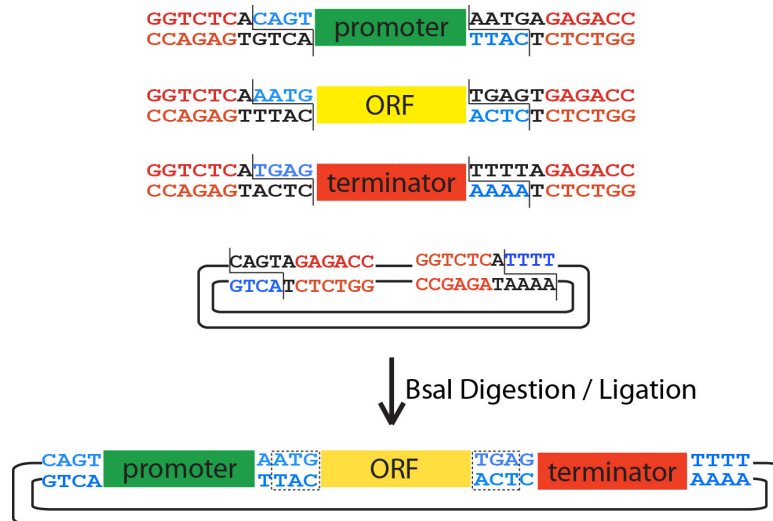


Figure 2. Near-seamless and directional assembly of promoter/5'UTR, ORF, and 3'UTR/terminator into an acceptor vector using signature overhangs. BsaI sites, shown in red letters, flank the signature overhangs, shown in blue letters. Following BsaI digestion and ligation, a functional TU is assembled. The start and stop codons for the ORF are encoded by the ligated sticky ends (dashed boxes).

### c. 'One-pot' assembly of a TU in an Acceptor Vector

The assembly of a TU may be carried out by simultaneous digestion and ligation, referred to here as a one-pot reaction. Donor constructs, encoding the promoter, ORF, and terminator parts, are introduced into a single reaction vessel along with BsaI and T4 DNA ligase. Further included is an acceptor vector specifically designed to encode signature

overhangs, exposed by BsaI digestion, that are compatible with the 5' and 3' ends of the promoter and terminator, respectively. The combination of digestion and ligation leads to essentially reversible digestion of BsaI sites except when components of the TU/acceptor vector ligate; in this instance the BsaI site is eliminated. Over time the reaction generates more and more of the desired product. We eliminate background of intact donor molecules entirely by (i) a 5 minute at 50°C BsaI digestion to linearize residual acceptor vector molecules and (ii) encoding a different drug resistance marker in the acceptor vector. Finally, the parent acceptor vector encodes RFP between the BsaI sites. The RFP gene in the acceptor vector yields red colonies such that assembly of the TU in place of RFP is readily identified visually. Other easily screenable or visual markers may be substituted for RFP and similarly, other combinations of drug markers could be used instead of Kan and Amp.

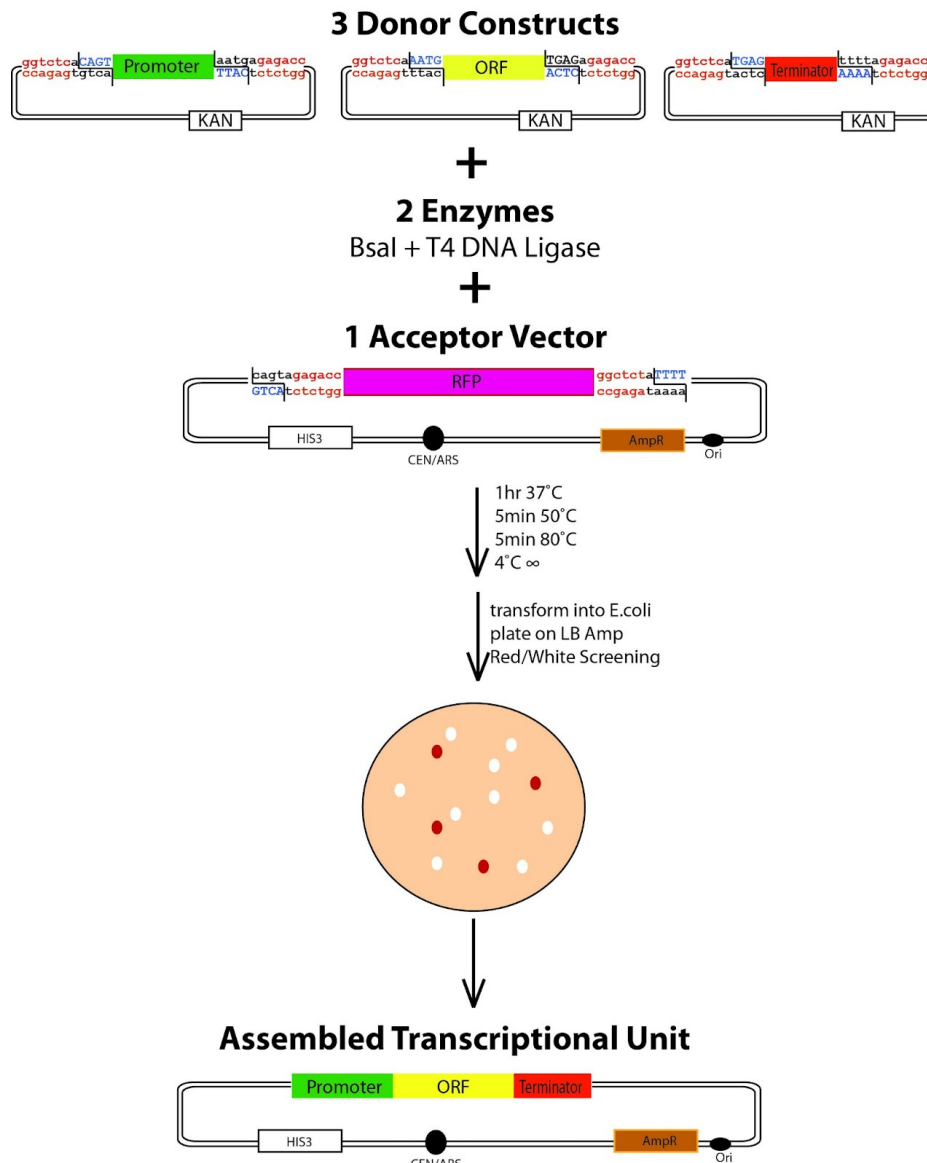


Figure 4. An Overview of the Yeast Golden Gate Assembly Protocol

## 5. Advantages of the New Standard

Biobrick standard assembly requires a step-by-step process using restriction sites. Biobricks also use four restriction enzyme sites whereas Yeast Golden Gate uses only one restriction enzyme site. By enforcing the use of two distinct drug resistances in donor and acceptor vectors, our method avoids the use of gel electrophoresis and extracting bands from the gel. Because it is a one-pot reaction with both the restriction enzyme and ligase active at the same time, and avoids electrophoresis it is inherently safer, less prone to human error, and amenable to automation. The colonies may be distinguished by red-white selection. Successful ligation will result in white colonies whereas re-ligated acceptor vectors will retain the RFP and present as red colonies. In practice, due to the 5 minute 50°C step that follows the 37°C incubation, a condition in which BsaI is active and the ligase is not, the background of red colonies is driven below the limit of detection in most instances.

## 6. Protocol

- 1) Measure the concentration of each part to be included in the reaction.
- 2) Introduce the following into a single PCR tube:
  - 100 ng ampicillin resistant acceptor vector
  - equimolar amounts of promoter, ORF, and terminator parts\* sub-cloned into kanamycin resistant vector backbones
  - 0.75µl 10X NEB T4 DNA ligase buffer
  - 0.75µl 100X BSA
  - 0.5µl BsaI (NEB)
  - 0.5µl T4 ligase, 2 million cohesive end units/mL (NEB)
  - add water to a final volume of 7.5µl
- 3) Program the thermocycler and run as follows:
  - 37°C for 60 minutes
  - 50°C for 5 minutes\*
  - 80°C for 5 minutes
  - 4°C ∞
- 4) Transform 2.5µl of each assembly reaction into 50µl of competent *E. coli*.
  - Combine DNA and cells and incubate on ice for 20 minutes.
  - Heat shock each for 45 seconds at 42°C, then immediately place on ice for 2 minutes.
  - Add 125µl LB and incubate at 30°C for 1 hour.
  - Spread the transformation mix on an LB plate containing the appropriate antibiotic and incubate overnight at 30°C.
  - Select white colonies for further characterization.

\*Internal Bsal sites will need to be removed by design or mutagenesis before a part can be employed, e.g. by introducing a silent mutation to each Bsal site in a CDS. BsmBI can be also used instead of Bsal, however, a BsmBI vector would need to be used in this variation.

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