

Reusable rapid assembly of genetic parts for *Neurospora crassa*

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1. Purpose

This BBF RFC provides a method in which standardized parts can be easily created for *Neurospora Crassa*.

2. Relation to other BBF RFCs

BBF RFC 82 works in conjunction with BBF RFC 10 in the definition of a part and the final part plasmid assembly.

3. Copyright Notice

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4. Genetic Construct Definitions

The fungus *Neurospora Crassa* has enormous potential for the bioconversion of waste biomass and cheap feedstocks into useful bio-based products. *N. Crassa* lives naturally off of dead plant material and is known to contain 23 cellulase genes, several lignin metabolizing enzymes and amylases in addition to a molecular system for plant cell wall adhesion.

Although under-exploited as a genetic model organism, *N. Crassa* has been sequenced (Nature, 422, 859–868. 2003) and the tools exist for cell transformation and efficient gene elimination, replacement and addition through homologous recombination. Here we propose a modular approach for the rapid assembly of gene replacement cassettes as a means to accelerate the pace of metabolically engineering the organism.

Any genetic construct to be inserted into *Neurospora Crassa* requires the following parts, assembled in the following order.

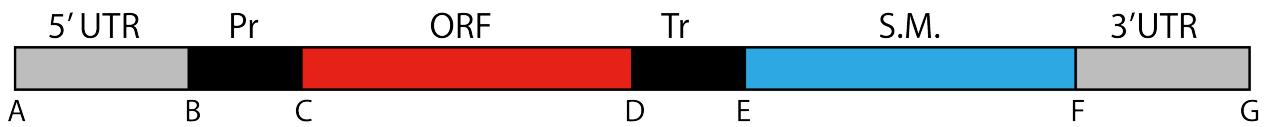
1. A 5' homologous recombination region
2. The gene of interest. Can be broken down into:
 - a. Promoter
 - b. Open Reading Frame
 - c. Terminator
3. A reporter gene. Most commonly a fungicide or antibiotic resistance.
4. A 3' homologous recombination region

Homologous recombination regions are defined as ~1000 bp sections of DNA which correspond to sections of DNA in the *N. crassa* genome flanking the point of insertion. The gene of interest is defined as whatever gene is to be inserted into *N. crassa*. A reporter gene is defined as any gene that allows you to select for transformants. The most commonly used reporter genes are fungicide or antibiotic resistances (ex. Hygromycin B).

5. Genetic Construct Sequence

To assemble these genetic constructs necessary for transformation, we propose a standardized method of assembly where all parts under the same definition carry the same overhangs.

Shown below are the modular requirements for a flexible integration cassette including hypothetical 5' and 3' untranslated regions (UTR) for site-specific insertion, a selectable marker (SM) and an open reading frame for a hypothetical gene (ORF) flanked by a promoter (Pr) and transcriptional terminator (Tr).



This system of modular ends allows for a simple plug-and-play method of assembly. All parts can be interchanged with any other parts that fall under the same definition, allowing for greater efficiency and variability in assembling genetic constructs.

6. Ends

The ends of each of the six elements are demarcated by seven unique 5' overhangs (four bases in length labeled A-G) which are generated by the asymmetric cutter Bsal from outside of the sequence of interest. Pieces are produced from either PCR products or released from a host plasmid. The model we have chosen for proof of principle is the replacement of FAD1 with the TesA' fatty acid thiolesterase from *E. coli* as a means of increasing F.A. synthesis for biodiesel production.



Registry parts include Bba_K612001 to Bba_K612008. Note that these form a sufficient and useful set for other ORF insertions but can be expanded accordingly to meet the sophistication of the effort.

Ends are listed as complimentary pairs. A can only pair with A', B can only pair with B', and so on.

A	B
5' - (N) nNNNN	TGGGNNNN (N) n-3 '
3' - (N) nNNNNACCC	NNNN (N) n-5 '
A'	B'
C	D
5' - (N) nNNNN	ACAANNNN (N) n-3 '
3' - (N) nNNNNNTGTT	NNNN (N) n-5 '
C'	D'
E	F
5' - (N) nNNNN	ATCCNNNN (N) n-3 '
3' - (N) nNNNNNTAGG	NNNN (N) n-5 '
E'	F'
G	
5' - (N) nNNNN	GCCTNNNN (N) n-3 '
3' - (N) nNNNNCGGA	NNNN (N) n-5 '
G'	

7. Characterization

A key requirement of this approach is that upon ligation, ends must find partners precisely without promiscuity. To test this requirement and select ends that meet this criterion we subdivided the coding sequence of the alpha subunit of beta galactosidase into nine segments ending four base 5'overhangs that were selected to eliminate duplication. Also included were two segments encoding a promoter (Bba J23001) and one segment encoding the transcriptional terminator Bba 1002 as shown below:

-----Promoter (BBa J23100)-----Met->
 GCCTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCAAGTTACGTAGGAGGACAGCTATGGCATGATTACG
 AACTGCCGATCGAGTCAGGATCCATGTCACGATCGTTCAAGTGCATCCTCTGCGATACCCGTACTAATGCG

CTGGCCGTCGTTTACAACGTCGTGACTGGAAAACCTGGCGTTACCCAACCTTAATCGCCTTGCAGCACATCCTC
 GACCGGCAGAAAATGTTGCAGCACTGACCCCTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTAGGAG

CTTCGCCAGCTGGCGTAATAGCGAAGAGGGCACGCACCGATGCCCTCCAAACAGTTGCGCAGCCTGAATGGCGA
 GAAAGCGGTCGACCGCATTATCGCTCTCCGTGCGTAGCGGGAAAGGGTTGTCACCGTGGACTTACCGCT

ATGGCGACTGATGCGGTATTTCTCCTTACGCATCTGCGGTATTCACACCGCATATGGTGCACCTCTCAGTACA
 TACCGCTGACTACGCCATAAAAGAGGAATGCGTAGACACGCCATAAAGTGTGGCGTATACCACGTGAGAGTCATGT

-----Terminator (Bba 1002)-----
 ATCTGCTCTGATGCCTGACAAAAAACCCGCTCGGGGGTTTTTCGCG

TAGACGAGACTACGGACTGTTTTGGGGCGAAGCCGCCAAAAAGCGACCC

Segments were produced from desalted phosphorylated oligonucleotides (IDT) that had been annealed over a two-hour temperature gradient from 70 to 25 degrees. Plasmids that contained the BetaGal cassette were then assembled using the BioBytes sequential assembly method on magnetic beads as described by the Alberta iGEM 2010 team. Briefly, all segments were mixed and ligated simultaneously at 50-fold molar excess to a bead-bound origin of replication derived from pSB1C3 at the restrictive temperature of 37 degrees celsius. Following several washes, the KanR gene was ligated and capped for recircularization. The construct was then released from the beads and to avoid bias in colony selection, transformants were plated onto media lacking XGal. Ten colonies were then selected for sequencing. Notably, the DNA from all ten colonies contained the complete alpha-BetaGal cassette attesting to the precision of the BioByte assembly method. Only one -1 frameshift was detected in the first promoter segment at position 15, representing an accuracy of synthesis of 1 mistake in 3000 base pairs. The fact the no mutations were detected at the boundaries of segment ligation illustrates that any of the overhangs defined above were suitable for the *N. Crassa* gene insertion design described above.

Ends A/A' and G/G' have already been well characterized in Team Alberta's 2010 iGEM team's assembly method, BioBytes 2.0.

8. Strains

In wildtype *N. crassa*, both homologous recombination and non-homologous end rejoining machinery are present. Homologous recombination allows any genetic insert flanked by homologous recombination regions to be inserted very accurately into the *Neurospora crassa* genome (Ninomiya et. al), with near 100% transformation rates. However, non-homologous end-rejoining machinery inserts genetic constructs randomly into the *N. crassa* genome.

Mus strains of *Neurospora crassa* are to be used for all genetic manipulations. Mus strains have their non-homologous end-rejoining machinery knocked out, and are able to be accurately and stably transformed.

9. Construction

Because all parts in the assembly carry unique overhangs on both of their ends, assembly can be carried out in whatever way you see fit. Ligations can be performed with all parts in the construct added at once. They can also be assembled sequentially using the BioBytes bead assembly construction method, as outlined in Team Alberta's 2010 iGEM project, Genomikon.

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12. References

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Yuuko Ninomiya, Keiichiro Suzuki, Chizu Ishii, and Hirokazu Inoue. Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining PNAS 2004 101 (33) 12248-12253