BBF RFC : Recombination Based Part Assembly

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Overview

Here we propose a new recombination-based assembly standard, optimized to allow for efficient cloning of mammalian vectors.

Motivation

Standard biobrick assembly is a useful tool in the process of cloning an expression vector; however, it is not an optimal procedure. Issues stemming from the use of restriction digestions prevent this process from working well with mammalian constructs; it is also a fairly time-intensive process. Our goal was to find a new assembly standard that would allow for efficient cloning of the longer sequences encountered in mammalian constructs, and attempt to improve on the efficiency of the previous system.

Recombination cloning is a quick and efficient process, already widely used in scientific community as a protocol for vector assembly. Invitrogen has standardized and simplified this process; their system, Gateway® Cloning, involves the use of two different bacteriophage recombination enzymes to allow for the assembly of an expression vector from two 'part'-containing vectors. This process is extremely robust (up to 99% recombination efficiency), and circumvents many of the more laborious steps involved in traditional restriction cloning, such as separate ligation and digestion procedures.

MammoBlock Flanking Sequence Standards

One of the advantages of the MammoBlock standard is the variety of construction methods available to assemble the component parts. In standard BioBrick assembly, 'components' are created with flanking restriction sites, then digested and ligated into the appropriate backbone. This approach can still be used to create MammoBlock parts; however, other methods including recombination cloning can be easily adapted to create the appropriate entry vectors.

The restrictions on appropriate MammoBlock parts only involve the sequence of the final entry vector; we require that the entry vector contain the construct of interest, flanked by two specific Gateway© recombination sites. There are two entry vector categories, one for promoter and one for gene inserts. A specific pair of recombination sites is associated with each entry vector standard; see the specifications below.

L4R1 Promoter Entry Vector

The promoter entry vectors are characterized by attL4 and attR1 recombination sites flanking the insert; this design places the promoter directly in front of the gene after a multisite Gateway© reaction. We require that a MammoBlock L4R1 promoter entry vector have the following sequence structure around the insert. Note that here we define the 'part' as the entire region between the flanking attL4 and attR1 sites.

```
5' _attL4 site_--------Insert--------_attR1_site_3'
```
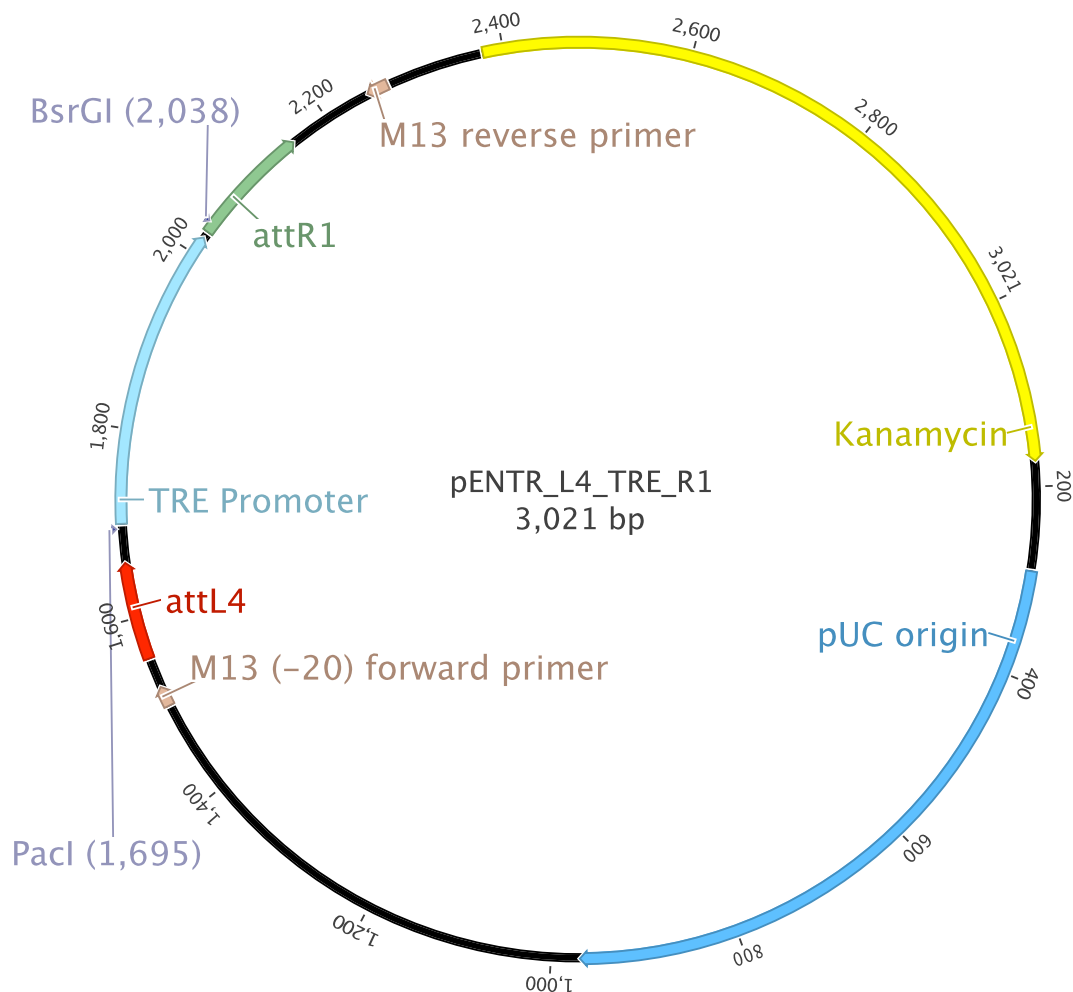
att_L4 Recombination Site Sequence:

5'CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATAAG
CAATGCTTTTTTATAATGCCAACTTTGTATAGAAAAGTTG 3'

att_R1 Recombination Site Sequence:

5'CCAAGTTTGTACAAAAAAGTTGAACGAGAAACGTAAAATGATATAAATATCAATATA
TTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAAACACAACATATGCA
GTCACTATG 3'

Below is the L4R1 MammoBlock promoter entry vector for the inducible TRE promoter. We submit this vector, with biobrick designation pSB1K15 as the first MammoBlock backbone for promoter entry vectors.

Insert 'Part' Sequencing Primers for pSB1K15:

M13 (-20) forward primer: `5'CATTTTGCTGCCGGTC 3'`
M13 reverse primer: `5'CAGGAAACAGCTATGAC 3'`

pSB1K15 Bacterial Antibiotic Resistance: Kanamycin

L4R1 Entry Vector Assembly

One noteworthy feature of the the L4R1 backbone plasmid above is the existence of convenient directional restriction sites between the recombination sites and the promoter sequence; these are not a required feature of MammoBlock backbones, but they can simplify entry vector construction. The following is an optional method for restriction cloning promoters into the pSB1K15 backbone.

Insert 'parts' for this cloning procedure should be fabricated in the following format:

```
           PacI                                    BsrgI
5'AGTCTTAATTAA—Insert—CGACCCAAGTTTGTACAAGTC 3'
3'TCAGAATTAATT—Insert—GCTGGGTTCAAACATGTTCAG 5'
                       attR1 fragment
```

BsrgI cuts inside the attR1 recombination site; the fragment in the digested insert replaces the cut portion of attR1.

Digestion of pSB1K15 with PacI and BsrgI yields 'sticky' ends of the form

```
5'AGTTTGGTTAAT          GTACAAAAAAGT3'
3'TCAAACCAAT                 TTTTTTCA5'
```

between the attL4 and attR1 recombination sites, respectively. Cutting the insert with PacI and BsrgI and ligating into digested vector yield the final product:

```
5'AGTTTGGTTAATTAA—Insert—CGACCCAAGTTTGTACAAAAAAGT3'
3'TCAAACCAATTAATT—Insert—GCTGGGTTCAAACATGTTTTTTCA5'
```

Note that this cloning step is directional; the ligation will yield a promoter in the correct orientation.

L1L2 Gene Entry Vector

MammoBlock gene entry vectors are defined by the presence of attL1 and attL2 recombination sites flanking the gene insert. We require that MammoBlock L1L2 Gene Vectors contain the following sequence structure around the insert (or 'part'), which allow for insertion of the gene directly in behind the promoter during the Gateway© reaction :

```
5' _attL1 site_--------Insert--------_attL2 site_3'
```
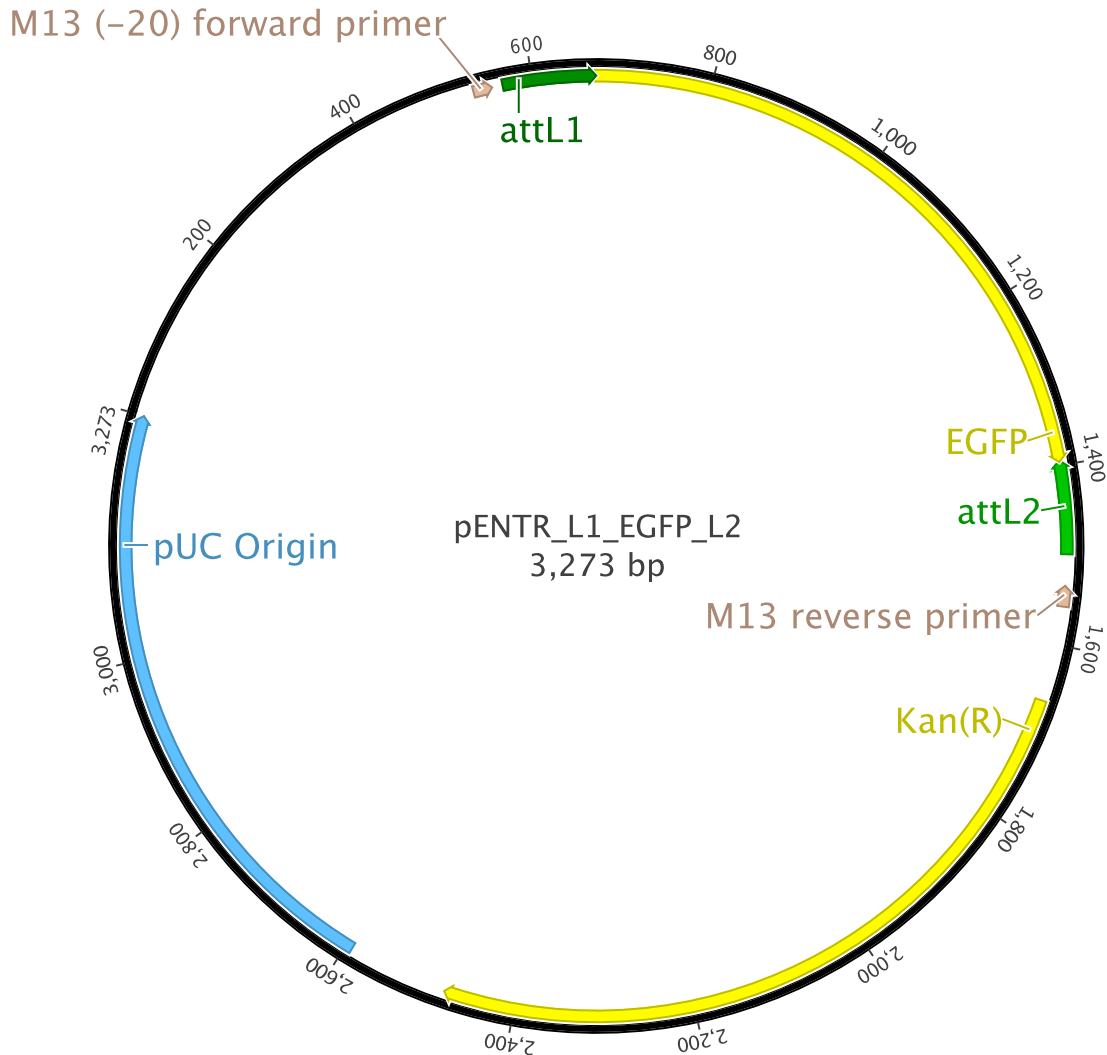
att_L1 Recombination Site Sequence:

```
5'CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAG
CAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT3'
```

att_L2 Recombination Site Sequence:

5'ACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTT
GCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG3'

An example is shown below of an L1L2 MammoBlock part containing the gene
fluorescent protein EGFP. We submit this vector, with biobrick designation pSB1K16 as
the first MammoBlock backbone for promoter entry vectors.

Insert 'Part' Sequencing Primers for pSB1K16:

M13 (-20) forward primer: 5'CATTTTGCTGCCGGTC 3'
M13 reverse primer: 5'CAGGAAACAGCTATGAC 3'

pSB1K16 Bacterial Antibiotic Resistance: Kanamycin

MammoBlock-Compatible Destination Vectors

Expression vectors are assembled from MammoBlocks by combining an L4R1 Promoter part with an L1L2 Gene part and an appropriate destination vector in a multisite Gateway© reaction. The only strict sequence requirement for the destination vector is the presence of attR4 and attR2 recombination sites. Other plasmid features useful for bacterial and mammalian cloning are listed below

Basic Cloning:
1) The presence of a E. Coli killing gene (e.g. ccdB) , flanked by attR4 and attR2 recombination sites. This allows for the selection of correct plasmids during the transformation step following the recombination reaction.
2) A bacterial origin of replication.

Mammlian Cloning:

3) Resistance genes against various mammalian-toxic antibiotics (e.g. hygromcin, puromycin.)
4) A lentiviral origin of replication and lentiviral packaging sequences, for use in the creation of virus for infection into stable cell lines.

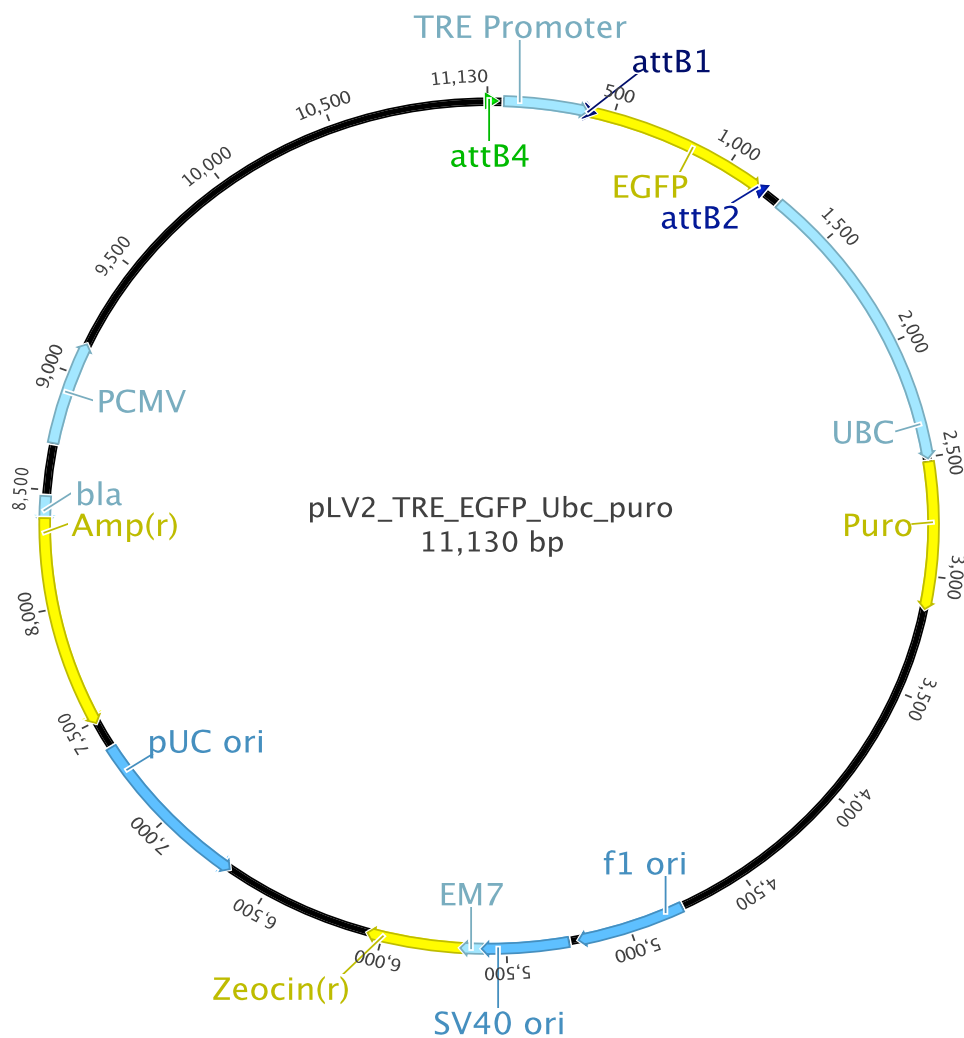# MammoBlock Expression Vector Assembly

A MammoBlock L4R1 Promoter Part, L1L2 Gene Part, and appropriate destination vector are combined in a multi-site Gateway© reaction to yield the final expression vector.

Recombination Cloning Background:
Two recombination reactions form the basis for the assembly cloning. The enzymes involved function in the lysogenic cycle of the temperate lambda bacteriophage. During the lysogenic cycle, lambda bacteriophage integrates its genome into the host E. coli genome. The enzymes that control this integration are integrase (Int), excisionase (Xis) and integration host factor (IHF). Integrase can mediate either excision or integration of the genome, catalyzed by IHF. The recombination sites involved are termed attB, attP, attL and attR; during integration, Int catalyzes the recombination of attB and attP sites to form an attL and attR sites flanking the integrated genome. The opposite reaction takes place for excision; the Int catalyzes the recombination of the attL and attR sites, yielding a liberated phage genome with an attP site, and the original host genome with an attB site The reaction equilibrium normally favors the attB and attP recombination. The excisionase Xis catalyzes excision by binding to the attR site and shifting the equilibrium to favor the reverse reaction.

Assembly Reaction:
The actual assembly reaction involves combing equimolar amounts of the part vectors with a mix of Integrase, IHF, and Excisionase enzymes to catalyze the recombination reaction. After 12-16 hours, the reaction is stopped with the addition of Proteinase K, and the product is then transformed into bacterial cells, for miniprepping to yield the final construct. The product of an assembly recombination reaction between the L4R1 TRET promoter vector and the L1L2 EGFP gene vector is shown below.

pLV2_TRE_EGFP_Ubc_puro
11,130 bp

Note: this plasmid is optimized for mammalian cloning; it contains appropriate selection markers and lentiviral sequences. The Ubc constitutive promoter controls expression of the puromycin resistance gene. The attB sites between the TRE promoter and the EGFP gene insert are the result of recombination between the attL and attR sites.

Conclusion

We present MammoBlock recombination cloning as the mammalian standard for part-based assembly of expression vectors. The variety of methods for creating entry vectors, the robust nature of the reaction, and the quick time for completion combine to make this assembly a quick and efficient counterpart to the BioBricking standard.

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Relation to other BBF RFCs

BBF RFC# does not update or replace any earlier BBF RFC. It does not affect RFC 45, which addresses mammalian standardization in the context of traditional biobrick protocols.

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