

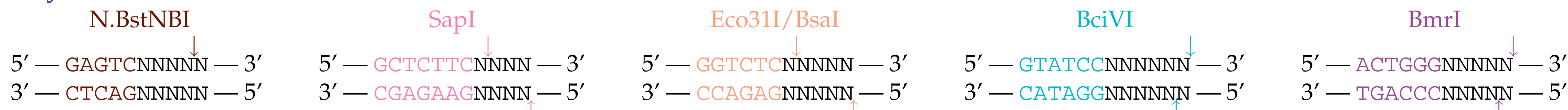
Motivation

Construction of complex biological systems can require assembling many modules together. BioBricks++ is a modular assembly scheme using commercially available offset and nicking restriction enzymes. BioBricks++ allows for more operations than the BioBricks assembly scheme including:

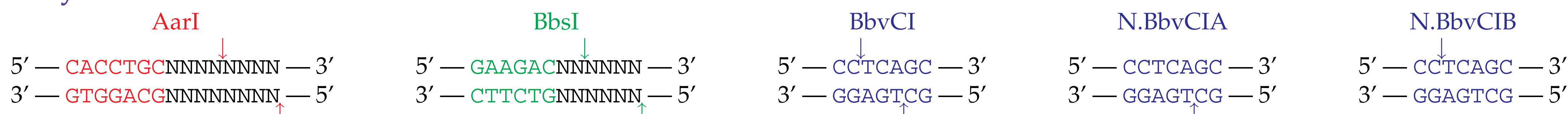
- Any two modules can be assembled together in either order.
- The assembly of two modules can be made seamless, with no extra intervening sequence inserted between the modules.
- The direction of modules can be easily and seamlessly reversed.
- A small number of bases can be removed from either end of a module, allowing for operations such as protein fusions or addition of tags.

Restriction Enzymes

Enzymes for Plasmids



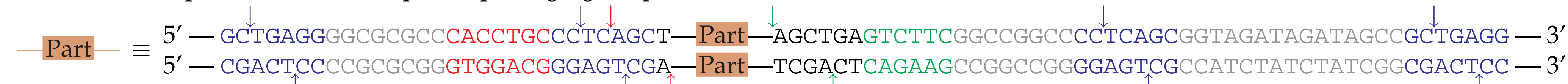
Enzymes for Parts



Standard Part Specification

A valid BioBricks++ part:

- Does not contain the sequences for the restriction enzymes AarI, BbsI, or BbvCI.
- Has a standard prefix and suffix sequence packaging the part:



Derivative Part Forms

Part Cut with BbsI and N.BbvCIA (Prefix-Sticky Form):



Part Cut with BbsI (blunted) and N.BbvCIA (Prefix-Blunt Form):



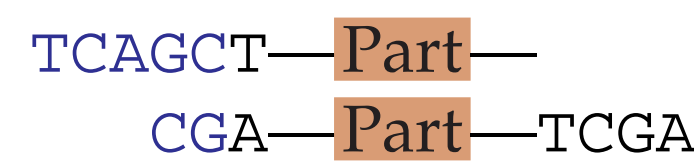
Part Cut with AarI and N.BbvCIB (Suffix-Sticky Form):



Part Cut with AarI (blunted) and N.BbvCIB (Suffix-Blunt Form):



Part Cut with BbsI and BbvCI (Transfer Form):



Entry Operations

Desired operation: [Part] \Rightarrow —Part—

Initial part can be obtained from direct synthesis or PCR. Appropriate ends can be added to allow cloning into pReverse after digestion with BbsI and BbvCI. Some other methods of entering a part into the appropriate form follow:

TA Entry Cloning

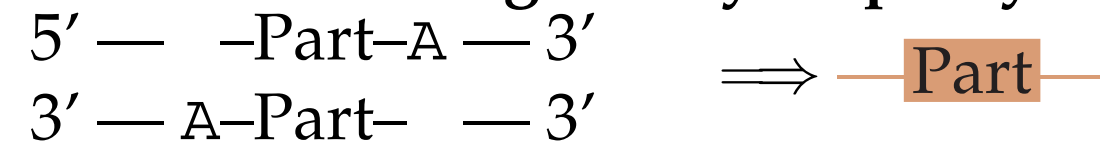
pEntryTA Plasmid Cloning Site:



pEntryTA Plasmid Cut with BciVI and BmrI:



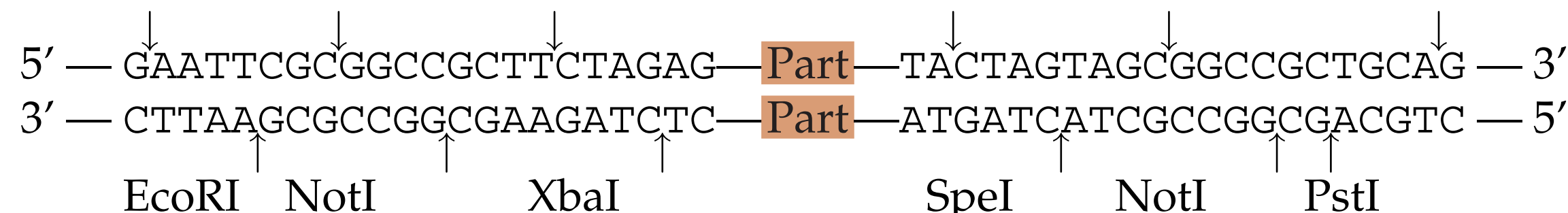
Addition of Single A by Taq Polymerase and Ligation with Cut pEntryTA:



Directional cloning is possible by dephosphorylating one end of insert and one end of the plasmid.

BioBricks Conversion

BioBricks Part:



BioBricks Part Cut with NotI and SpeI:



pBioBricksUpgrade Plasmid Cloning Site:



pBioBricksUpgrade Plasmid Cut with Eco31I:



Ligation of Cut BioBricks Part with Cut pBioBricksUpgrade:

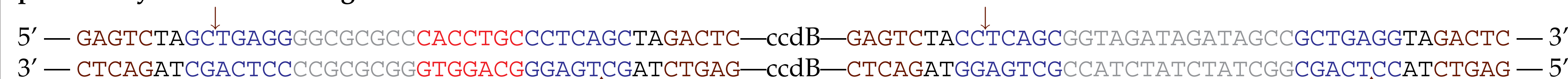


Part Operations

Assembly

Desired operation: —Part1— + —Part2— \Rightarrow —Part1-Part2—

pAssembly Plasmid Cloning Site:



pAssembly Plasmid Cut with N.BstNBI:



Ligation of Part1(Prefix-Blunt Form), Part2(Suffix-Blunt Form), and Cut pAssembly:



Two parts have been seamlessly assembled.

Part Reversal

Desired operation: —Part— \Rightarrow —Part—

pReverse Plasmid Cloning Site:



pReverse Plasmid Cut with Eco31I and SapI:



Ligation of Part(Transfer Form) and Cut pReverse:



The part has been seamlessly reversed.

End Base Removal

With the appropriate plasmid, up to 6 bases can be removed from the end and up to 10 bases can be removed from the front of a part.

Desired operation: —Part— \Rightarrow —Part—

pRemove(-6) Plasmid Cloning Site:



pRemove(-6) Plasmid Cut with BciVI (blunted) and then N.BstNBI:



Ligation of Part(Prefix-Blunt Form) and Cut pRemove(-6):



Ligation Cut with BbsI (blunted) and then N.BbvCIA:



Six bases have been removed from the end of the part and it is in the Prefix-Blunt Form ready to be used in an assembly.

Advantages

- Long overhangs (25bp) are used on both ends during assembly, allowing for ligation independent cloning. Also, chromosomal or other random DNA contamination will not be an issue with the long overhangs.
- Only three relatively long recognition sequences are reserved for parts (1 6-bp and 2 7-bp). In random DNA, a restricted sequence appears with a greater than 50% probability in about 945bp.
- No oligos specific to any parts need to be synthesized to perform any of the operations. This removes the necessity to *think* about any of the operations. All operations are done identically each time. The amount of time, materials, and cost required is independent of the size of the module, i.e. $O(1)$, assuming that cloning takes a constant amount of time. In contrast, direct DNA synthesis or PCR assembly is at least $O(n)$.

Disadvantages

- Different plasmids are needed for different operations. Also, the number of N.BstNBI sites (asymmetric 5-bp sequence) that need to be eliminated from plasmids may be large. However, plasmid construction only needs to be done once.
- The long overhangs used can make it more difficult to separate fragments via standard gel electrophoresis.
- Only existing components can be manipulated. It is not possible to build modules unrelated to the modules existing in the standard form.

Implementation

- All potentially overlapping dam, dcm, EcoBI, and EcoKI methylation sites were checked for and ensured to never occur with any enzymes sensitive to methylation, avoiding potential problems with restriction digests.
- The base plasmid used has a kanamycin resistance gene. It is low copy when grown in a *lacI^q* strain and can be induced to very high copy by IPTG.
- Many site-directed single base mutations were introduced throughout the original base plasmid to remove restriction sequences.
- Other plasmids, such as pAssembly, pReverse, and pBioBricksUpgrade were constructed from the base plasmid.
- Many of the basic operations have been tested and work as expected.