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Golden Gate Cloning - Loop and MoClo - part and primerdesign guidelines

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We use this protocol and it's working

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Abstract

This protocol is largely based on the original Loop and uLoop protocol **dx.doi.org/10.17504/protocols.io.yxnfxme**by the corresponding authors. This protocol provides general guidelines for primer design for introducing parts into the Loop system of assembly.

Golden Gate ligation/cloning/assembly, first developed by Engler et al., is a technique used to join DNA fragments in a specific order.
It employs Type IIS restriction enzymes. These enzymes recognize a specific sequence and cut outside of the recognition sequence leaving overhangs. This has 2 implications:

1. Cut sites have directionality (i.e., the recognition sequence can be to the left or right of the cut site depending on the strand the sequence is in)
2. Cut sites' overhangs are not the same and will depend on the surrounding sequence to the recognition sequence.

To simplify and optimize the Golden Gate assembly, multiple standardized systems have been developed to allow for easy interchange of parts and maximize efficiency of assembly.

For example, BsaI enzyme cuts leaving a 4 base pair overhang. An overhang of 4 bases AAAT is less efficient than one of 4 bases ACGT.

For standardized assembly, typical elements of DNA assemblies (e.g., promoter, coding sequence, terminator, etc.) are called "parts" and have defined 5' and 3' overhangs, so that no matter which promoter you choose, it can be ligated with a coding sequence because all promoters have the same overhangs. All of these elements are put in one pot, one step reaction alongside an acceptor vector, whose overhangs must match the first overhang of the parts (in order) (for example the 5' overhang of a promoter) and the last overhang of the parts (the 3' of the terminator).

In acceptor vectors, the enzyme recognition sequences are diverging (cut sites facing away from each other) and are found inside the "insert" (typically a lacZ or other selection/screening gene) (in reality, the insert is defined by the presence of the cut sites), because we are interested in the backbone and not the actual insert. In donor vectors (which hold the parts/elements), the recognition sites are found convergently, because the insert is what we are interested in keeping in the final assembly.

For any assembly, you will need any number of parts so long as all overhangs match up and can join together to form a plasmid. After digestion with the enzyme, we have a linear backbone, linear parts. If our reaction is correct, they will find each other in the solution, be joined if the overhangs match, and then be permanently ligated by ligase enzyme, as cut sites are removed, in the backbone of donor parts and insert of acceptor parts. For every final assembly, there is no longer any cut sites of that enzyme present in the vector, ensuring high efficiency and "no backsies".

For more information read the original papers:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2574415/ Original Golden Gate
https://pubs.acs.org/doi/10.1021/sb500366v Yeast MoClo kit
https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.15625 Loop
https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.13532 Plant MoClo
https://currentprotocols.onlinelibrary.wiley.com/doi/full/10.1002/cpmb.115 Full description of Golden Gate assembly

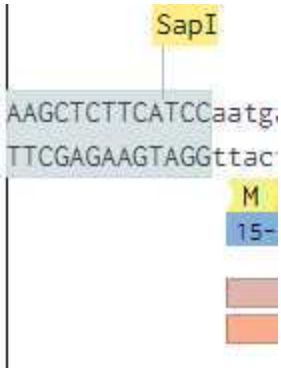
Designing L-1 parts

1 If you are not familiar with Golden Gate, please read description for Golden Gate summary.

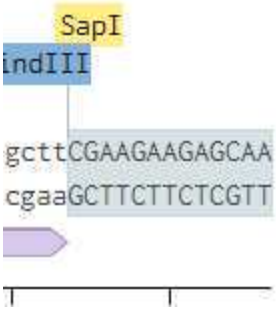
Purchase your primers to create a L-1 part through PCR or synthesize the part. See below for guidelines on designing primers and synthesis parts to ensure system compatibility.

1.1 A L-1 part is a linear fragment which has "adapters", or sequences which flank the part of interest. For the example, we will use the coding sequence for the antibiotic resistance gene, specR.

The adaptor consists of 4 sections



5' adaptor for Loop incorporation

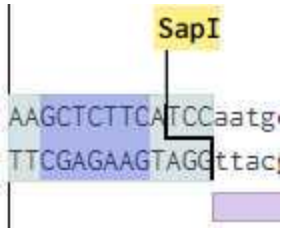


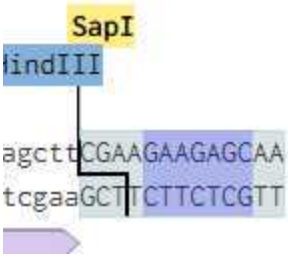
3' adaptor for Loop incorporation (ensure that when you are purchasing primers, you submit the **reverse complement of the final desired sequence,** as primers are purchased 5'-3')

Section 1: 2-4 bp overhang clamp. Serves the purpose of facilitating enzyme binding to the part and is typically AA/AT (AA above). This sequence can be modified with little problem to accommodate for primers' Tm if needed (see later - PCR)

Section 2: recognition sequence(s): These hold the recognition sequences for enzymes and range from 5-7 base pairs. Note how 5' cutsites are to the right of the sequence and 3' to the right, this ensures that when digested, the part is removed in the form of an insert with the 5' overhang being on the forward strand, while the 3' overhand is on the reverse strand. Conventionally, the identity of the overhangs is always read on the 5' strand.

In the loop system, the enzyme which goes from L-1 > L0 is SapI





Section 3: defined cut site for integration to L0 acceptor vector. In the above images, 5' = TCC and 3' = CGA. L-1 parts will be digested alongside an acceptor vector which has defined overhangs matching these, and thus will only take 1 part at a time. This enables a L-1 part to be transported and propagated in the form of a plasmid.

Section 4:
For Loop, you need to include 4 bases immediately to downstream of the defined cut site (when designing primers, the forward primer will have the defined overhang (for a coding sequence, as in the example, it is AATG), but the reverse primer **is submitted as a reverse complement, so ensure that the 4 bases are also reverse complement.**

By convention, the 3' overhangs are read from the forward strand. For example, the defined overhangs for a coding sequence part in the plant MoClo system are: AATG-GCTT
Read more on Moclo here: https://moclo.readthedocs.io/en/latest/index.html
Although once digested, the 3' overhang does not contain the sequence GCTT and instead has the complement, CGAA.

Ensure that when you are designing parts you do not make the mistake of forgetting to reverse complement the defined overhangs, as you will end up with a part that (for example) has overhangs as: AATG-CGAA and this will not ligate.

It is critical that the 4 bases overhang you define conform to whatever system you plan to use and match the identity of the part that you are trying to make. If this is not correct, higher order assemblies cannot occur, either by mismatch, or by incorrect order of assembly.

2 If you are synthesizing a part, you can just submit the part for synthesis with the appropriate adaptors, and double check your sequence.

Make sure you:

1. Codon optimize your sequence for whatever organism you are working with
2. Remove transit peptides/NLS or whatever
3. There are no illegal BsaI/SapI/BpiI/BsmBI recognition sites inside the sequence. Even though you technically only need to ensure that there are no sites of the enzymes that you will use to form the assembly, if there is ever a need to port the part to other assembly systems, its better to cover all your bases.

3 If creating a part by PCR, add to the 3' end of the primer a string of 18-28 bases which will be complementing to the template (plasmid, gDNA which contains the part). Stick to typical primer formatting guidelines (50% GC content, shorter is better). When calculating the Tm, only use the part of the primer which will anneal to the template (do not include the adaptor). Use NEB tm calculator (https://tmcalculator.neb.com/#!/main.

Double check the homo and heterodimer deltaGs on IDT oligo analyzer tool (https://www.idtdna.com/calc/analyzer). For this, include the entire primer. Sometimes, the adaptor sequence forces a highly negative deltaG (try to stay above -9).

Purchase the primers and ensure the **3' adaptor is submitted in 5'-3' direction as reverse complement.**

4 Amplify your part through PCR using high fidelity polymerases.

5 After PCR, check your sample through gel electrophoresis and gel purify the sample.
We use the Qiagen kit with success (QIAEX II Gel Extraction Kit (150) Cat. No. / ID: 20021, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaex-ii-system)

Ligation

6 Follow the protocol by Pollak et al for ligation

https://www.protocols.io/view/loop-and-uloop-assembly-14egn6rml5dy/v5

Parts generated through this method ligate into pL0R, the universal level 0 acceptor vector in the Loop system.