Application Note

Homology-based cloning methods in Geneious Prime
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Introduction

PCR combined with restriction enzyme-based cloning is without doubt the most widely-used cloning technique in molecular biology. This is mainly due to the relatively easy implementation and troubleshooting as well as reduced cost. However, this technique involves many steps, especially for preparing all the parts to be assembled, being quite laborious and often ineffective. Other PCR-based methods have been developed to overcome some of these limitations, for example homology-based cloning methods, for which restriction enzymes are not needed. These methods rely on recombination of homologous regions of the parts to be assembled.

In addition to full support for performing in silico restriction cloning, Geneious Prime also provides a tool for simulating a number of homology-based cloning methods. These methods are described in this Technical note, where we show how it is possible to design experiments using exonuclease-based recombination (3’ or 5’ exonuclease) or using homologous recombination.

All homology-based cloning methods in Geneious Prime are performed using the Gibson Assembly tool, available from the Cloning menu in the Toolbar. This tool supports:

- Peer-reviewed and proprietary protocols based on 3’ exonuclease activity (e.g. SLIC and In-Fusion® Cloning)
- Other protocols that do not rely on exonucleases, but on polymerase overlap extension (e.g. CPEC), ex vivo homologous recombination (e.g. SLiCE) or in vivo homologous recombination.

When required the Gibson Assembly tool will design and output primers suitable for generating overlapping parts required for homology-based assembly.

All methods described in this note start with the same linearised destination vector and inserts and result in the same final circular construct.

Data

For each of the use-cases detailed below we use example data and settings for simultaneous cloning of two PCR fragments into a plasmid vector as reported by Jacobus and Gross1. The vector (backbone) used in all examples is pUC19 (GenBank accession number M77789), while the two insertion fragments were derived from the yeast Schizosaccharomyces japonicus var. versatilis ura4 gene (GenBank accession number AATM00000000.2). In brief, the strategy recombines two fragments of the S. japonicus ura4 gene, to create a ura4 deletion mutant, and places it into pUC19.

![Figure 1. Homology-based cloning methods interface, found under Cloning -> Gibson assembly. Options used for Gibson assembly are shown, including modified primer options.](image)
Sequences were downloaded within Geneious Prime by searching the Nucleotide NCBI database via the Sources panel, and specifying each sequence accession number. The two ura4 intragenic regions (Target 1 and 2) were extracted from the S. japonicus scaffold KE651167 after first reverse-complementing the CDS region to orient it in the forward direction. The target regions were annotated on the ura4 CDS and extracted as two separate documents. Target 1 comprises the beginning of the CDS (first 293 bp), while Target 2 the CDS end (last 300 bp).

Before performing in silico homology-based cloning, the vector pUC19 was linearised by PCR amplification using the primers dest-f (5’-GCGCAGCCTGAATGGCGAATGG) and dest-r (5’-GGAGAGGCGGTTTGCGTATTGGG), as described by Jacobus and Gross 1. In Geneious, primers were annotated onto pUC19 using Test with saved primers and then in silico PCR was carried out using Extract PCR product. Both operations are available from the Primers menu in the Geneious Toolbar.

Note that homology-based cloning methods require a linearised backbone, which can be obtained via PCR, as described above, or by simulated digestion with restriction enzymes using Cloning -> Find Restriction Sites followed by Cloning -> Digest into Fragments.

**Gibson Assembly**

Gibson assembly is a one-pot reaction that uses T5 exonuclease to digest bases from 5’ ends to expose single-stranded overhangs that will anneal with complementary overlapping fragments2.

Gibson assembly was performed using three input files (pUC19 PCR product, Target 1 and Target 2), selecting pUC19 PCR product as Backbone, 5’ Exonuclease and 30 bp as minimum overhang under Primer Options, as shown in Fig.1.

The Geneious Prime Gibson Assembly tool designs primers with complementary overhangs and generates the final construct (Fig.2). A report with all the information about the construct parts and the list of primers is generated. It is also possible to Save intermediate products, which include PCR products and digested sequences, as individual documents.

The same options used for Gibson assembly can be applied to other 5’ exonucleases approaches, such as the proprietary GeneArt® Seamless Cloning by Life Technologies. Only the Primer Options should be changed according to the recommended overhang length. For example, for GeneArt® Seamless Cloning the recommended overhang length is 15 bp, whilst for Gibson assembly it is usually 25 to 40 bp.

**In-Fusion® Cloning**

A widely-used proprietary method is In-Fusion® Cloning by Clontech, which employs a 3’ exonuclease activity to generate single strand complementary overhangs at 5’ ends. Also the peer-reviewed SLIC (Sequence and Ligase Independent Cloning)3 method is based on the 3’ exonuclease activity of the T4 polymerase, recommending a 25 bp overhang, while In-Fusion® recommends a shorter 15 bp overhang.

To simulate In-fusion® cloning the same input molecules used for Gibson cloning were used. The Exonuclease was set to 3’ Exonuclease, and as we were simulating an In-Fusion® Cloning reaction, the Min overhang length was set to 15 bp in the Primer Options.

The resulting output from this operation is identical to that shown in Fig. 2 with primers having a 15 bp extension.

**In vivo homologous recombination**

In Geneious Prime it is also possible to design in silico cloning experiments that do not rely on exonucleases but on homologous recombination mechanisms or polymerase-based overlap extension. In this case, the Gibson Assembly option Exonuclease is set to No Exonuclease.

For simulating in vivo homologous recombination in E. coli as described by Jacobus and Gross1, we set a 30 bp as minimum overhang length, as this was identified by the authors as the most effective overlap length. The operation returns the same final circular construct as in Fig. 2 and generates primers with 30 bp extensions.

The option of avoiding the use of an exonuclease is also compatible with strategies such as CPEC (Circular Polymerase Extension Cloning)4, which amplifies the whole strand with a polymerase, and SLiCE (Seamless Ligation Cloning Extract)5, which uses a ex vivo cell extract to recombine DNA molecules using short-end overlaps.
Assembly of already homologous parts

The Geneious Prime Gibson Assembly tool is also able to identify and assemble correctly different parts that already have overlapping homologous ends (no primer design is required) and follow the specified criteria (e.g. minimum overlapping homology).

The Gibson tool was run with the same settings as in the \textit{in vivo} homologous recombination (No Exonuclease, primers with 30 bp overlap). The pUC19 PCR product was used as backbone. The \textbf{Target 1 - PCR product} and \textbf{Target 2 - PCR product}, generated from the \textit{in vivo} homologous recombination described above, were used as inserts (to obtain intermediate PCR products in the output of the previous analysis, make sure to select \textit{Save intermediate products} from the Gibson options).

This analysis returned the same final construct as the other analyses and the Assembly Report highlights that no primers need to be designed as overhangs are already compatible (Fig. 3).

Summary

Geneious Prime provides a wide selection of cloning tools, which include the most common applications, such as Restriction Cloning, Gateway Cloning, Golden Gate, TOPO Cloning and Gibson Assembly. The latter is a flexible tool for performing not only Gibson Assembly, but also many other homology-based cloning methods as showcased in this technical note.

References


