Clone Manager 11

Golden Gate Cloning Method

This method provides for the directional assembly of two, or many, fragments in a single reaction. Fragments can be joined in a scarless, or seamless, fashion. Recombinant clones can be used to provide a source of functional cassette modules which can be combined into a final recombinant (example: the MoClo modular cloning system).

The Golden Gate cloning method employs a mix of a type II-S restriction enzyme and ligase in a single reaction tube. Type II-S restriction enzymes have an asymmetric recognition sequence and cut outside of the recognition sequence. By positioning a pair of recognition sites outside the region of interest, and pointing inwards, the insert can be excised without including any extraneous sequence. Thermal cycling between the temperatures optimal for restriction enzyme digestion and ligation allows for high cloning efficiency by preventing the re-ligation of the original clones or vector.

Since type II-S enzymes cut outside of the recognition sequence, the exact nature of the overhang is determined by the adjacent sequence. The typically used type II-S restriction enzyme cuts to produce a four-base overhang. Because the enzyme cuts outside of the recognition sequence, the overhang produced can be controlled by the adjacent sequence or the sequence of the PCR primers used for amplification. This provides 256 sequence combinations that can be used to uniquely define the order of assembly of insert fragments using simple ligation.

Clone Manager Wizard

The Golden Gate wizard can be started from the main Clone menu. The wizard is based on the standard cloning ligation window and provides access to all of the standard ligation operations in addition to the specific design options to support the Golden Gate cloning method.

The wizard assumes that any molecule selected either has a suitable pair of type II-S restriction enzyme sites to excise the fragment to be cloned or else will use PCR amplification to introduce the pair of sites.

When the Golden Gate wizard is started, the first dialog page will allow you to enter the default settings for the type II-S restriction enzyme being used and defaults for design of PCR primers used to amplify fragments. Each of these settings can be customized when designing individual PCR primers.

Settings:

The most important setting is the type II-S restriction enzyme that you want to use. It is generally advised that the same enzyme be used for all Golden Gate components since the reaction is typically performed in a single tube. However, you can use more than one enzyme if necessary and Clone Manager will check that there are no unexpected recognition sites for the additional enzyme in any of the fragments being cloned. You will be alerted to the presence of internal sites but this may not be a problem as these cuts will simply re-ligate during the thermal cycling of the reaction. Available enzymes

can be selected from the drop-down list and include all enzymes with at least a 6 base recognition sequence that produce at least a 4 base overhang and cut within 4 bases of the end of the recognition sequence.

The other settings relate to how PCR primers will be designed. A PCR primer consists of two parts: the homologous region and the 5' extension. The homologous region is determined by the region of each molecule being cloned and will be defined when each fragment is added to the wizard. The 5' extension adds the bases for the recognition sequence of the desired type II-S enzyme together with additional bases that provide the base overhang that will be used to ligate to the adjacent fragment.

The first data entry field defines the 5' end of the primer. This sequence is before the restriction enzyme recognition sequence and will not be included in the amplified product. Its purpose is to provide a few bases flanking the recognition sequence to enable the enzyme to bind and cut efficiently (a link for more information is provided in the references).

The second data entry field is the restriction enzyme that you intend to use. This defines the next bases that will be added to the primer's 5' extension.

The third field provides the ability to enter additional bases that will precede the region to be amplified and is the place where a great deal of customization can be introduced. Customization will typically be deferred until you are designing an individual fragment to be cloned. It is recommended that you enter one base here and check the box labelled 'Match overhang of adjacent fragment'. The one base is required, for the most commonly used restriction enzymes, to fill the gap between the end of the restriction enzyme recognition sequence and its cut position. This base will not appear in the final recombinant. Checking the 'Match' box enables the wizard to automatically design appropriate overhangs to enable the correct assembly of fragments in the order you specify.

Add Molecule:

When you start the Golden Gate wizard, the currently active molecule will automatically be selected as the first molecule you want to use for cloning. You can add more molecules by using the toolbar button to add a molecule or the drop-down button to replace an existing molecule. Other toolbar buttons allow you to re-organize or modify the fragments and the order in which they will be joined.

When a new molecule is selected, Clone Manager will inspect the molecule and look for a suitably positioned pair of type II-S restriction enzyme sites. If found, your molecule will automatically be cut to produce the fragment for cloning. You will be asked to confirm the selection if the sites found are not for your preferred enzyme or there are more than one pair of sites available. Should you want to perform a different operation, you can click the toolbar Undo button.

If a pair of type II-S sites are not found, then Clone Manager will assume that you will be using PCR amplification. The PCR wizard will automatically appear and allow you to design the fragment that you want to clone. Should you not want to use PCR amplification, you can click the cancel button to close the PCR wizard.

The fragment to clone will now appear in the parts list across the bottom of the window. The display will show the overhang ends for the fragment and its relationship to adjacent fragments. Fragments will be assembled into the final recombinant in the order they appear in this window.

Toolbar buttons are available to review, or change, the last PCR step or undo the last construction step.

PCR Wizard:

This wizard will automatically be started when you add a new molecule that does not contain a suitable pair of type II-S restriction enzyme sites. It can also be explicitly started from the toolbar to review, or customize, the design of the PCR primers. This is especially important if you want to introduce extra bases between the fragments you are joining.

The first page of the wizard is used to define the region of the molecule that you want to clone. The region is defined using coordinates from the upper strand but you can select which strand you want to clone. Selecting to clone the complement strand will effectively invert the selected region to give you the reverse complement sequence. The features button allows access to your molecule's features table to allow quick selection of region and strand.

If you have the Professional edition of Clone Manager you will also have access to the primer criteria used to evaluate and design primers. This wizard assumes that you are using PCR amplification to subclone from an existing cloned recombinant and uses rather loose design criteria. If you will be cloning from a genomic sample then you can make the criteria more restrictive. However, you will have more control if you use the standard main menu Primer, PCR Design wizard to design more selective primers and directly create the amplified product that you want to clone.

The next two pages of the PCR wizard allow you to design the primers for the left and right ends of the fragment region you are cloning. Each page contains the same primer design fields but the second page for designing the right-hand primer will show the amplification results in the reverse compliment direction to reflect the content of the final amplified product after cutting with the restriction enzyme.

A PCR primer consists of two logical parts: homologous bases and the 5' extension. The homologous bases are defined by the region of the molecule that you selected on the first page of the wizard and whether you are designing the forward primer at the left-hand or the reverse primer at the right-hand end. The 5' extension adds the bases for the recognition sequence of the desired type II-S enzyme together with additional bases that provide the base overhang that will be used to ligate to the adjacent fragment.

As described above for the default settings, the 5' start and restriction enzyme used will be automatically preset. The field for additional bases will be disabled if you are using the default automatic match functionality as the wizard will automatically enter the appropriate base sequence to provide an overhang that allows ligation to the adjacent fragment. You can customize this field by unchecking the match box. This will enable you to enter extra bases into the primer sequence and these bases will appear in the final recombinant between the two adjacent fragments. You can also edit this field if you need to enter a specific overhang sequence. This may be needed if you are assembling a large number of fragments and the automatically generated overhangs are not unique.

The bases entered into the additional bases field can be divided into three parts: the first part is required by the type II-S enzyme being used so that the enzyme will not cut within the region to be cloned. For example, BsaI has a 6 base recognition sequence but cuts after 7 bases. A seventh base needs to be provided as part of the additional bases field. This base will not appear in the final cloning fragment but may affect the efficiency of the designed primer.

The second part of the additional bases field is the most important and defines the actual bases that will constitute the overhang. These bases must match the overhang created by the adjacent fragment. This sequence can be automatically determined by the wizard if the checkbox 'Match' is checked.

The match function assumes that you want to join fragments in a scarless, or seamless, fashion without any extra bases being inserted. To achieve this, the wizard will design a reverse primer for the right end of the previous fragment that does not include any additional bases for the overhang. This will create an overhang that is the last 4 bases of the region to be cloned. The forward primer of the adjacent fragment will be designed with a 4 base overhang that matches the previous overhang. In this fashion, ligation of the two fragments will restore the complete sequence of both fragments with no additional bases between them. If the adjacent fragment was not designed by the PCR match function, then the wizard will automatically enter the bases required to match the overhang of the adjacent fragment.

The third part of the additional bases field is optional and allows you to enter any additional bases that you want to include in the final assembled recombinant. These base(s) will appear between the fragments being joined.

As you enter your design into the available data entry fields, you will be shown the results of the design. You will see the actual sequence of the primer (gaps are used to separate the fields so that you can clearly see where each base is coming from) including the first 15 bases of the region homologous to the region to be amplified and cloned.

The next display block shows the double strand sequence of the fragment after PCR amplification and cutting with the type II-S restriction enzyme. The two columns show the amplified region and the bases that are added by the 5' extension of the primer. Note that the order of these columns is reversed for the right-hand primer since that is amplifying the target from the reverse direction. If this is the first fragment that you are entering, and you are using the auto match wizard function, the nature of the overhang will not yet be known. This will be updated when you complete the PCR wizard.

Also shown is the cohesive end that will created. This uses the convention that the upper strand bases are shown and the caret symbol indicates the position of the cut. For example, a cohesive end of ^AATC will indicate a 4-base overhang of the upper strand 5' end. Similarly, AATC^ will indicate a 4-base overhang of the upper strand 3' end.

Assembly design display:

As you enter, and design, each fragment that you want to include in your Golden Gate project, the display will update to show you an overview. Across the bottom of the window will be a list of iconic fragments shown in the order that they will be assembled. The icons will show a brief description of the fragment together with the overhangs that will be generated. Between each fragment will be an icon that confirms that the overhangs between adjacent fragments are compatible for ligation. If the failure icon is shown then ligation is not possible and you will need to resolve the incompatibility before continuing.

Above the ordered list of fragments is a more detailed iconic map of the selected fragment including its size and sequence of the ends.

Ligation of product:

When you have entered all of the fragments for the cloning, you can complete the wizard and generate the final recombinant by clicking the ligate button which is the final button on the toolbar. The default action assumes that you want to circularize the final product if possible. More options are available from the drop-down menu on this button. Also included as an option is to display construction details which

will report how each fragment is prepared. This information is also saved in the notes field of the final recombinant.

If you have the Professional edition of Clone Manager, you will also have the ability to design and save suitable primers. Primer design will use the criteria for 'General Primer Pair' which uses rather loose design criteria assuming that you are using PCR amplification to subclone from an existing cloned recombinant. Saving the primer sequences will enable you to use them for analysis or other purposes. You can also export the primer sequences which may be convenient for ordering the primers.

References:

Engler, C., Kandzia, R., & Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. *PloS one*, *3*(11), e3647.

Engler C, Marillonnet S. Golden Gate cloning. Methods Mol Biol. 2014;1116:119-31. doi: 10.1007/978-1-62703-764-8_9. PMID: 24395361.

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Cleavage close to the end of DNA Fragments: https://www.neb.com/tools-and-resources/usage-guidelines/cleavage-close-to-the-end-of-dna-fragments