Primer design for assembly-based cloning

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Abstract

This protocol summarizes the process of primer design for assembly-based cloning methods, such as Gibson or AQUA-cloning.

Well-designed primers are necessary for efficient cloning of any kind.

This protocol can also be used to design primers for overlap extension PCR of linear fragments. Alternatively, overhangs can also include restriction sites for restriction/ligation based cloning.

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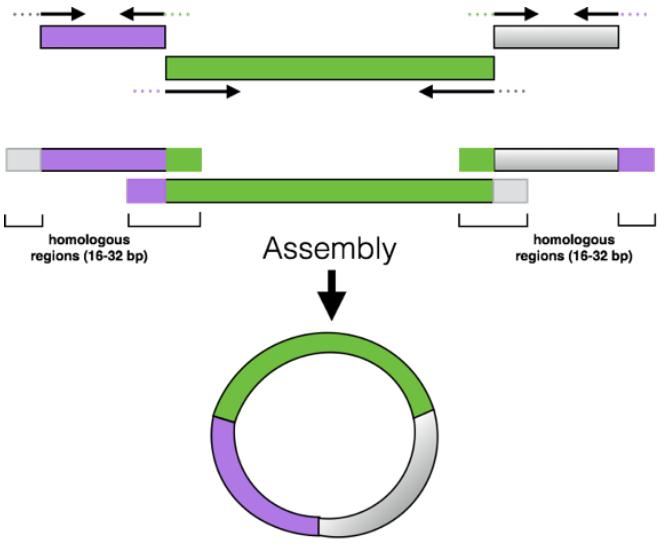
Protocol

Experimental design/overview

Step 1.



Black arrows: Annealing part of primer.

Dotted lines: overhang part of primer (color coded).

Step 2.

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Create in silico vector maps for template and target vectors using SnapGene or a similar tool.

Annealing part of primer:

Step 3.

- 18-21 bp in length, 40-60 % GC-content, T_m = 55-65 °C. T_m- values of two primer pairs (annealing part) should not differ by more than 3 °C.
- Single nucleotide repeats of four or more should be avoided when possible.
- A 3'-GC-clamp (1 or more G's or C's at the 3'-end of the primer) can be beneficial for annealing.

Overhang part of primer:

Step 4.

- 16-32 bp in length - the longer, the better! 40-60 % GC-content (total primer)
- For Tm >72 °C, an annealing temperature of 72 °C can be used.

Quality control

Step 5.

- Check primers for Tm, hairpin structures and primer dimers using online tool of choice (but stick with the same one after that, e.g. http://eu.idtdna.com/calc/analyzer)
- Hairpin structures: Check the Tm of the structure. It should be lower than the annealing temperature used in the PCR reaction.
- Primer dimers: Recommended ΔG is -9 kcal/mol or more positive values. However, for primers containing palindromic sequences, such as restriction sites, a more negative ΔG is not uncommon. These primers can still work.

Quality control

Step 6.

Before ordering, make sure your primers **bind in the template** you plan on using.

Quality control

Step 7.

After PCR, fragments should be separated using gel-electrophoresis and extracted using a kit of choice. This minimizes contamination with template backbones that could yield false-positive clones.

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