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# Overlap extension PCR

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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

Linear assembly of PCR fragments.
Can be used to quickly and efficiently fuse promoters, terminators, fusion proteins etc. without time-consuming sub-cloning steps.

## Materials

STEP MATERIALS

- Q5 High-Fidelity DNA Polymerase - 100 units **New England Biolabs** **Catalog** #M0491S
- Gel and PCR Clean-up kit **Macherey-Nagal** **Catalog** #740609.250

## Protocol materials

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## Before start

Before starting, thoroughly design and plan your experiment *in silico,* ideally using cloning software such as SnapGene.
Make sure all primers anneal only once in the actual template (e.g. when using a whole genome) and separate parts are able to anneal to each other after PCR.
I recommend going through the whole cloning process *in silico* prior to ordering primers to avoid mistakes.

## Primer design

1 Before starting, thoroughly design and plan your experiment *in silico,* ideally using cloning software such as SnapGene.
Make sure all primers anneal only once in the actual template (e.g. when using a whole genome) and separate parts are able to anneal to each other after PCR.
I recommend going through the whole cloning process *in silico* prior to ordering primers to avoid mistakes.

Design primers for all fragments. Primers for overlap extension PCR are designed in a similar manner as for Gibson assembly, except that the outermost parts do not contain overhangs to each other.

- Annealing parts of primer: Solid arrow in scheme; Should bind to template only once with a $T_m$ of 55-65 ˚C
- Overhang parts of primer: Dotted arrow in scheme; total overlap between two fragments should have a $T_m$ of at least 55 ˚C to avoid unspecific binding

Note

It can be beneficial to already add overhangs to the desired vector backbone to the primers flanking the fused part (i.e., P1 and P6 in the scheme) to facilitate downstream cloning.

This method will yield a linear fragment.

Protocol

NAME

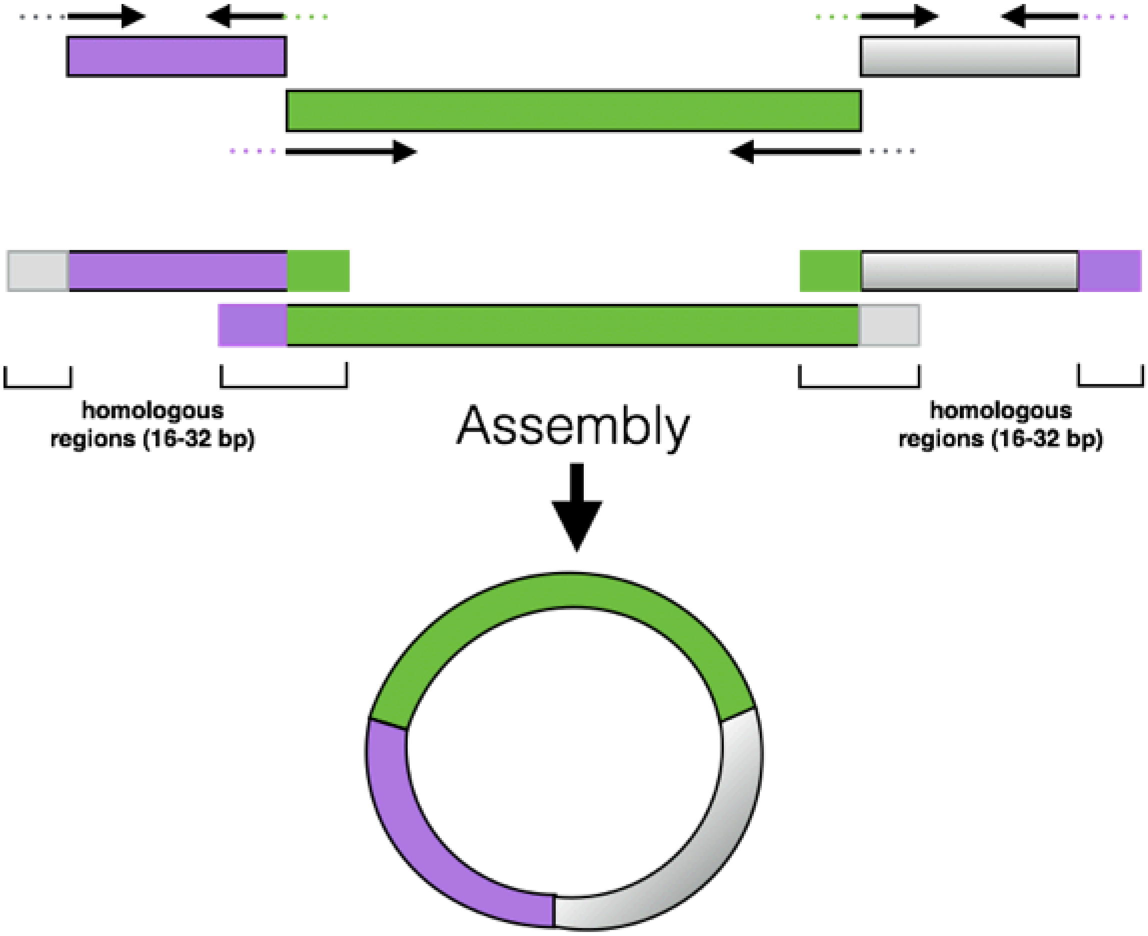
**Primer design for assembly-based cloning**

CREATED BY

Anna Behle

PREVIEW

1.1



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

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

1.3
- 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.

1.4
- 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.

1.5
- 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)

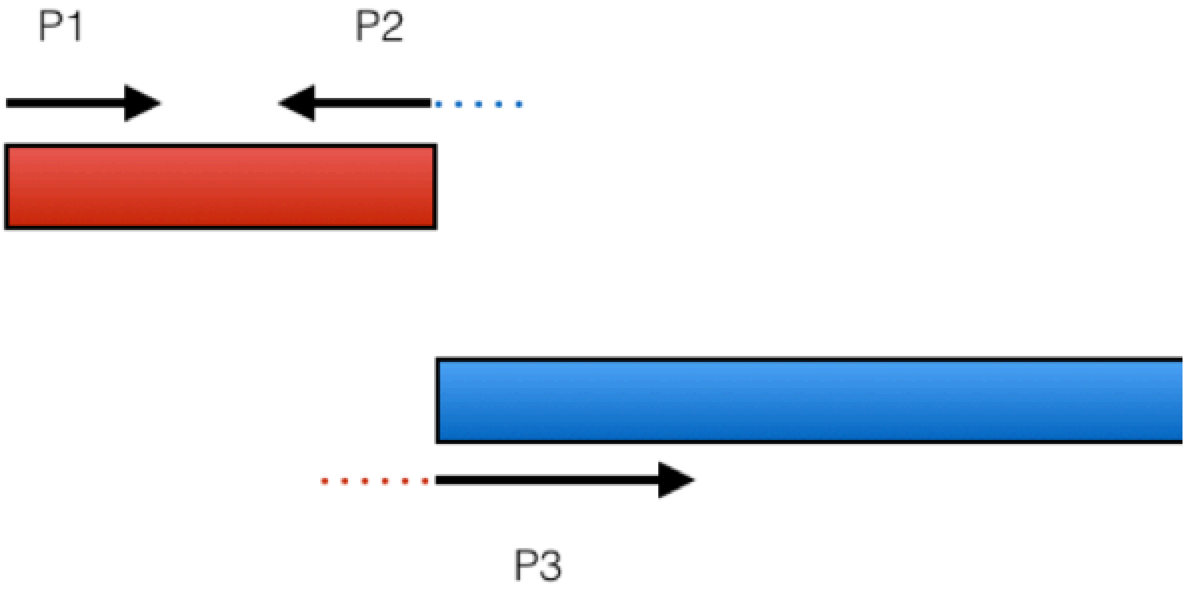
- Hairpin structures: Check the Tm of the structure. It should be lower than the annealing temperature used in the PCR reaction.
- Primer dimers: Recommended ΔG is -9 kcal/mol or more positive values. However, for primers containing palindromic sequences, such as restriction sites, a more negative ΔG is not uncommon. These primers can still work.

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

1.7 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.

## PCR of parts

2



Prepare separate PCR reactions of each part that needs to be assembled.

Note

Since the primers contain long overhangs, it might be necessary to try different annealing temperatures. I generally recommend starting with the annealing temperature corresponding only to the part of the primer which anneals to the template and disregarding the overhang for now.

Q5 High-Fidelity DNA Polymerase - 100 units **New England Biolabs** **Catalog #**M0491S

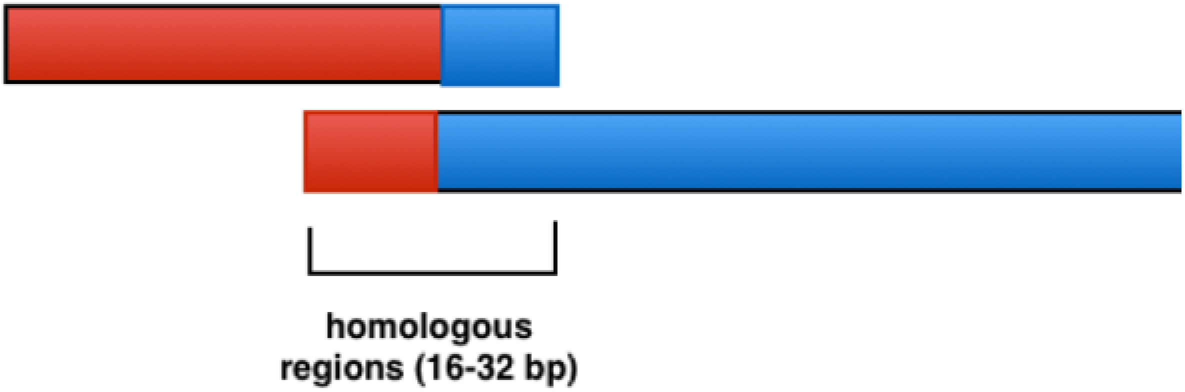
| | Component | Amount [µL] |
|---|---|---|
| | 5x Q5 buffer | 5 |
| | 5x High GC buffer | 5 |
| | dNTP mix, 10 mM each | 0.5 |
| | Primer fwd, 10 µM | 0.5 |
| | Primer rev, 10 µM | 0.5 |
| | template DNA | 0.5 |
| | Q5 Polymerase | 0.25 |

Gel-purify fragment of correct size.

Gel and PCR Clean-up kit **Macherey-Nagal** **Catalog #**740609.250

## Overlap PCR

3



- Prepare PCR mixture, **without primers**. Instead of a template, add your PCR parts. Use a large volume, i.e. 1/2 to 3/4 of the total PCR reaction. Make sure to use a molar ratio of ~1:1.

Note

A large volume can be beneficial because often, the concentration of gel-exctracted PCR fragments is low. I recommend using 50 ng of the larger PCR fragment. This step can be varied, but overloading the template DNA can lead to more unspecific product.

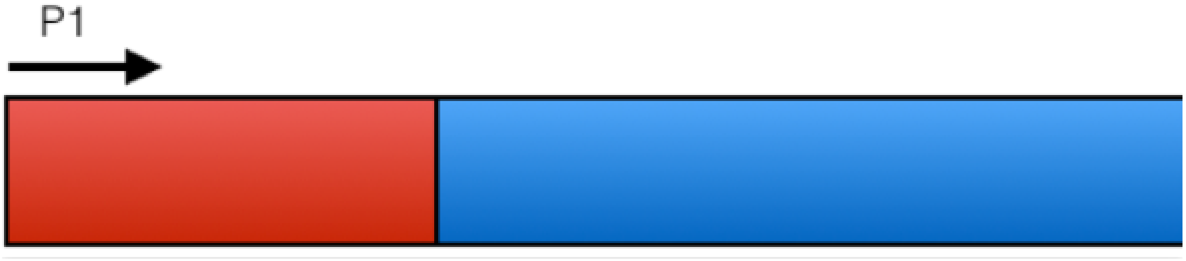
Example using Q5-Polymerase:

| | |
|---|---|
| 5x buffer | 5 µL |
| 5x high GC buffer | 5 µL |
| dNTPs | 0.5 µL |
| template | 4 µL fragment 1 + 5 µL fragment 2 + 5 µL fragment 3 |
| Q5 Polymerase | 0.5 µL |
| $H_2O$ | to 24 µL |

- Run your PCR at 15 cycles, using the annealing temperature of the homologous regions.
- Remove PCR from cycler. Immediately proceed with Step 4.

## Extension PCR

4



- Add the two primers flanking the outer parts as you would in a normal PCR.
- Rerun your PCR at 30 cycles, this time using an annealing temperature matching your flanking primers.

- Important: Gel-extract your overlap extension product, as this method can result in non-specific side products!
- PCR reaction will likely yield multiple bands (e.g. the fragments you started out with), as well as a smear around the desired band.

This method, when successful, yields a strong band of the correct size that can be used downstream for standard cloning methods such as Gibson Assembly.