AQUA cloning

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Abstract

Protocol for seamless, scarless, homology-based cloning of vectors using an enzyme-free technique.

The method was published by Zurbriggen et al., 2015, doi: 10.1371/journal.pone.0137652

It relies on the endogenous homologous recombination machinery of *E. coli* lab strains.

Citation: Anna Behle, Nicolas Schmelling AQUA cloning. **protocols.io**
dx.doi.org/10.17504/protocols.io.gs9bwh6
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Before start

Make sure to recover high concentrations of fragments.

Protocol

Materials and equipment

Step 1.

Material	Volume/Mass	Company	Serial no.
Q5 Polymerase	Dependent on reaction	NEB	M0491L
Nucleo-Spin Gel & PCR clean up	250 reactions	Macherey-Nagel	740609.250
NEB5a competent cells	25 µL	NEB	C2987I
Sterile reaction tubes	1.5 mL		

Primer design:

Step 2.

PROTOCOL

. Primer design for assembly-based cloning

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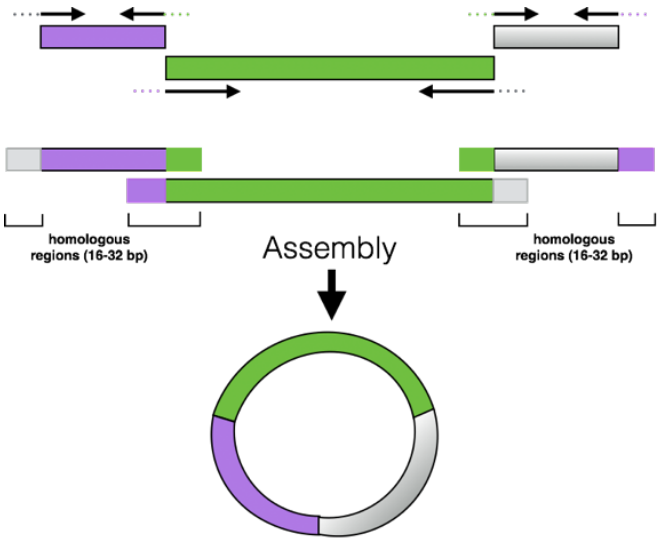
Experimental design/overview

Step 2.1.



Black arrows: Annealing part of primer.

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

Step 2.2.

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

Annealing part of primer:

Step 2.3.

- 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 2.4.

- 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 2.5.

- 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 2.6.

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

Quality control

Step 2.7.

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.

Preparation of PCR fragments

Step 3.

Create PCR products of all the fragments that are to be assembled, using the appropriate primers. Homologous overhangs should range between 16 and 32 bp.

Optional: Preparation of plasmid backbone

Step 4.

Optional when using pSHDY or other large backbone: Digest backbone using appropriate restriction enzymes. (*Note: AQUA assembly using digested backbone is less efficient.)*

Recovery of fragments

Step 5.

Gel-extract PCR and backbone fragments using the Nucleo-Spin kit. Make sure to recover high amounts. (*Note: PCR clean-up alone is insufficient due to backbone templates yielding false positives.*)

Assembly reaction

Step 6.

In a 10 to 15 µL reaction, mix fragments with molar ratios of 3:1 (insert:vector) or more for 2-fragment assembly or equimolar ratios for 3 or more fragments.

ANNOTATIONS

Dennis Dienst 10 Oct 2017

How about the absolute amount of DNA? According to Beyer et al. - '12 ng of linearized vector per 1 kb'. Further experiences?

In my hands, assembly of divergent fragments (1500bp: 2700bp: 4700 bp) works well with gradual/linear ratios (e.g. 1:2:4), based on that 12ng-of-backbone-suggestion

Step 7.

Incubate reaction at RT for 1 hour.

DURATION
01:00:00

Step 8.

Transform appropriate reaction volume into chemically competent DH5α cells. *(Note: Best results are achieved using commercially available competent .)*

REAGENTS
NEB 5-alpha Competent E.coli (High Efficiency) - 20x0.05 ml C2987H by New England Biolabs

Step 9.

Centrifuge transformation mix at 2500 rpm for 2 minutes. Remove supernatant; resuspend pellet in 100 µL LB.

Plate entire amount of cells on LB-plate containing appropriate antibiotics. Incubate over night at 37 °C.

DURATION
00:02:00

Control

Step 10.

Test clones using restriction analysis or colony PCR. (*Note: For colony PCR, use primers that amplify a region spanning two assembled fragments to avoid false positives.*)