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Golden Gate Protocol

N.J. Hillson[1,2]

[1]JBEI, [2]LBNL

1 Works for me dx.doi.org/10.17504/protocols.io.6t4heqw

Mike Fero

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

C. Engler, R. Gruetzner, R. Kandzia and S. Marillonnet, "Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes," PLOS ONE, p. https://doi.org/10.1371/journal.pone.0005553, 14 May 2009.
C. Engler, R. Kandzia and S. Marillonnet, "A one pot, one step, precision cloning method with high throughput capability," PLOS ONE, vol. 3, no. 11, p. 3647, 2008.

1 Measure the DNA concentration (ng/ml) of each assembly piece.

2 Add 100 ng of the linearized vector backbone and equimolar amounts of the other assembly pieces to a 15 ml total volume assembly reaction mixture as follows:

linearized vector backbone (100 ng)
+ each additional assembly piece (to equimolar with backbone)
+ 1.5 ml 10X NEB T4 Buffer
+ 0.15 ml 100X BSA*
+ 1 ml BsaI
+ 1 ml NEB T4 Ligase, 2 million cohesive end units / mL
+ _____dH_2O to
15 ml

NOTE: It is essential to use a High Concentration Ligase
* BsaI is only 10% active at 37 C without the addition of BSA.

3 Perform the assembly reaction in a thermocycler as follows:
either (following Engler 2009):
3 min @ 37 C }
4 min @ 16 C } 25 cycles
5 min @ 50 C }
5 min @ 80 C } 1 cycle
or, alternatively (modified from Engler 2008)
1 hour @ 37 C 1 cycle
5 min @ 50 C }
5 min @ 80 C } 1 cycle

NOTE: If any of the assembly pieces contain an internal BsaI site(s), it would be first preferable to silence the internal BsaI site(s) through point mutation(s); a second option, if the internal BsaI site overhang(s) are not cohesive with the other assembly overhangs, is to adjust the thermocycling parameters to terminate after a ligation step (e.g. skip the final cycle at 50 and then 80 C).

4 Transform 5 ml of the assembly reaction into 100 ml of competent E. coli and/or run a diagnostic agarose gel to check for successful assembly.

Oligonucleotide phosphorylation and annealing

5 Oligonucleotides (oligos) come in tubes (dehydrated) or as liquid stocks (may only be available in 96-well plate format).

6 De-hydrated oligos will have a specified quantity (usually in nanomoles) on the tube. Reconstitute the dehydrated oligos with sterile (or RNAase, DNAase free) water to a 100 µM stock. As a rule of thumb, add (ten times the number of nanomoles of DNA in the tube) µL water to make a 100 µM stock.

7 Oligo phosphorylation:

8 Set up separate phosphorylation reactions for each forward (FW) and reverse (RV) oligos

100 µM oligo stock	2 µL
10X T4 DNA ligase buffer	2 µL
Sterile water	15 µL
T4 Polynucleotide Kinase	1 µL
Total	20 µL

9 Incubate the reaction mixture at 37 C for 1 hour and heat inactivate the T4 PNK at 65 C for 20 minutes. Store the phosphorylated oligos at -20 C till further use.

10 NEB PNK enzyme is supplied with its PNK buffer and it does not contain the ATP required for the phosphorylation reaction. To simplify the reaction step, use T4 ligase buffer (instead of PNK buffer). T4 ligase buffer provides the appropriate amount (1mM ATP) of phosphate for the phosphorylation reaction.

11 Use fresh T4 ligase buffer (<1 year old) and avoid repeated freeze/thaw cycles of the buffer. The DTT present in T4 ligase buffer oxides naturally and its oxidation is accelerated by repeated freeze/thaw cycles or excessive heating.

12 Annealing the phosphorylated FW and RV oligos:

FW oligo	5 µL
RV oligo	5 µL
Sterile water	90 µL
Total	100 µL

13 Incubate the phosphorylated oligos at 95 C for 3 minutes. If handling >10 annealing reactions, using a thermocycler is convenient. In that case, choose a program step without a heated lid (to ease removal).

14 Cool the reaction slowly at room temperature for ~ 30 min to 1 hr, check the concentration with a nanodrop and store the annealed products at -20 C.