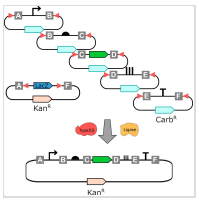

Apr 01, 2022

Golden Gate Assembly

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dx.doi.org/10.17504/protocols.io.x54v9yr3mg3e/v1

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The Golden Gate technique allows the assembly of genetic sequences from libraries of standardized basic components, which are cleaved from their donor vectors and concatenated in the acceptor vector in a defined order. This reaction is subsequently transformed into competent bacterial cells that are grown overnight to reveal positive colonies carrying the correctly assembled vector.

The cleavage of the components is done by a TypeIIS restriction enzyme, which recognizes asymmetric DNA sequences and cleaves outside of this site, leaving overhangs in a directional way (Figure 1A). This property allows the creation of ordered assembly positions by defining synthetic syntaxes of these overhangs (Figure 1B).

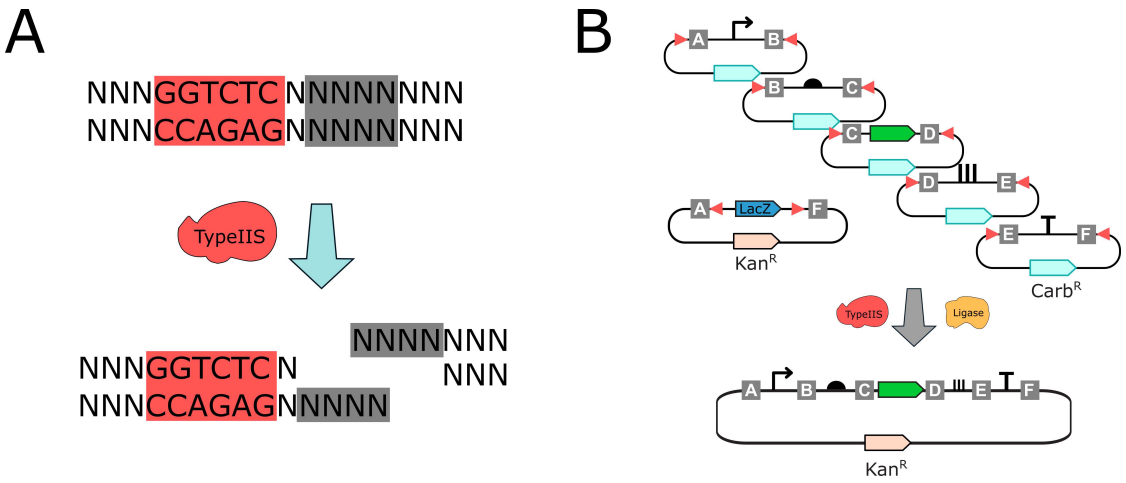

Figure 1: Golden Gate Assembly. **A**: Cleavage of TypeIIS restriction enzyme with recognition site indicated in red and overhangs indicated in gray. **B**: Ordered assembly of DNA pieces into an acceptor vector.

Within the different methodologies and libraries based on Golden Gate, some methods, such as uLoop Assembly (Pollak et al, 2020), allow the cyclic assembly of increasingly large and complex vectors in which the products of a reaction are the substrates of the next level reactions (**Figure 2**). By this means it is possible to build libraries of easily reusable components to perform assemblies in a combinatorial way of odd and even assembly levels.

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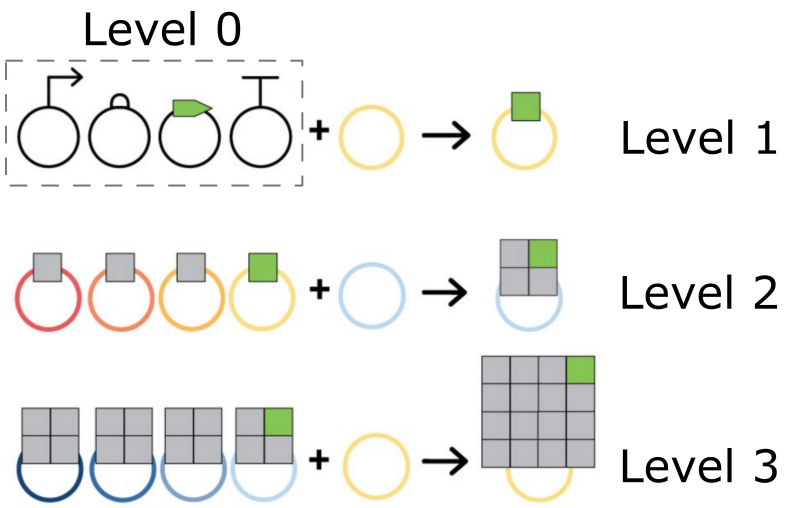

Figure 2: Loop Assembly of complex vectors by cycling assembly of combinatorial components. Odd acceptor vectors are indicated in hot colors and Even vectors in cold colors.

The standardized DNA components have to be flanked by the enzyme recognition sites and the proper syntax according to the desired position of assembly. These DNA pieces can be obtained from previously created libraries or custom made.

The reaction operates cycling between 37°C and 16°C. At the first temperature, the restriction enzyme cuts the DNA pieces. These pieces are concatenated by their overhangs sequence homology and sealed by the catalysis of a ligase enzyme at the second temperature. Once a piece is correctly assembled it cannot be cutted again, and cycle by cycle, the pieces are concatenated in the final assembly product.

DOI

dx.doi.org/10.17504/protocols.io.x54v9yr3mg3e/v1

Isaac Núñez, Tamara Matute, Fernan Federici 2022. Golden Gate Assembly. **protocols.io** https://dx.doi.org/10.17504/protocols.io.x54v9yr3mg3e/v1

DNA Assembly, Golden Gate, DNA fabrication, Assembly, Restriction Enzymes, TypeIIS, Synthetic Biology

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Apr 01, 2022

Apr 01, 2022

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Reagents

Molecular grade H_2O
T4 DNA Ligase [400 U/µL] (NEB)
BsaI-HF®v2 [20 U/µL] (NEB)
SapI [10 U/µL] (NEB)
T4 DNA Ligase Buffer 10X (NEB) – It comes with T4 DNA Ligase
CutSmart Buffer 10X (NEB) – It comes with SapI
Purified BSA 20 mg/mL (NEB)
X-Gal 20 mg/mL in DMSO

T4 ligase buffer has to be aliquoted upon arrival to avoid degradation by thaw and dethaw. **10 µL** aliquots are recommended.

Materials

0.2 µL tubes
Pipette tips

Equipment & Tools

Thermocycler
P2 Pipette
P10 Pipette
Ice Bucket

Preparation of the DNA components 14h 20m

1 Prepare plasmids stock solutions of the desired components to be used. 14h

This step is done by overnight growing of the strains in LB supplemented with proper antibiotics and performing the purification by any standard miniprep protocol. We use
Wizard® Plus SV Minipreps DNA Purification System
Promega Catalog #A1460

2 Measure the concentration of purified plasmids. 5m

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Typically concentrations range from [M]**50-800 ng/µL** depending on the plasmid.

3 Perform dilutions of the plasmids to working concentrations. 15m
It is:

- [M]**15 fmol/µL** for donor vectors
- [M]**7.5 fmol/µL** for receiver vectors

Molar concentration can be computed from mass concentration accord the next equation:

$$X\left[\frac{fmol}{\mu L}\right] = \frac{Concentration\left[\frac{ng}{\mu L}\right] * 10^6\left[\frac{fg}{ng}\right]}{650\left[\frac{fg/fmol}{bp}\right] * length[bp]}$$

Then, dilutions should be made in molecular grade H_2O using the standard equation:

$$v_x = \frac{cf\left[\frac{fmol}{\mu L}\right] * vf[\mu L]}{\left[X\frac{fmol}{\mu L}\right]}$$

c_f: 7.5 or 15 fmol/µL
v_f: Final dilution volume. Tipically 50µL to create a stock of the component
v_x: Volumen of the component at X concentration to be taken

Golden Gate Reaction 3h 21m

4 Prepare the **DNA mix**. In a pcr tube, mix **1 µL** of each component to be assembled (donor vectors) and **1 µL** of the acceptor vector. 5m

The total volume of the DNA mix should be **5 µL** considering four components and one acceptor, however the number of components may be different. If this is the case, the volume difference must be corrected by adjusting the volume of water in the 2X Reaction Master Mix (next step).

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5 Prepare the **2X Reaction Master Mix** (BsaI or SapI) **On ice** according to the next table: 5m

To avoid too small volume pipetting and variations, we recommend to do a master mix adding [1.1 * each component] and then divide into each tube.

BsaI mix is for *odd level* assemblies and SapI mix for *even level* assemblies.

Step 5 includes a Step case.
2X M.Mix BsaI
2X M.Mix SapI

step case

2X M.Mix BsaI

Component	Volume (µL)
H_20 (HPLC grade)	3,00
T4 DNA Ligase Buffer 10X (NEB)	1,00
BSA [1mg/mL]	0,50
T4 DNA Ligase [400 U/µL] (NEB)	0,25
BsaI-HF®v2 [20 U/µL] (NEB)	0,25

*volumes per reaction

6 Mix the **5 µL** of the DNA mix (step 4) with **5 µL** of the 2X Reaction Master Mix (step 5). 1m

Spin the tube to ensure the whole reaction is at the bottom of the tube and free of bubbles.

7 Bring the tubes to a thermocycler and run it according the next program: 3h 10m

Cycling parameters	
3 minutes at 37°C	Repeat 25X
4 minutes at 16°C	
5 minutes at 50°C	
10 minutes at 80°C	

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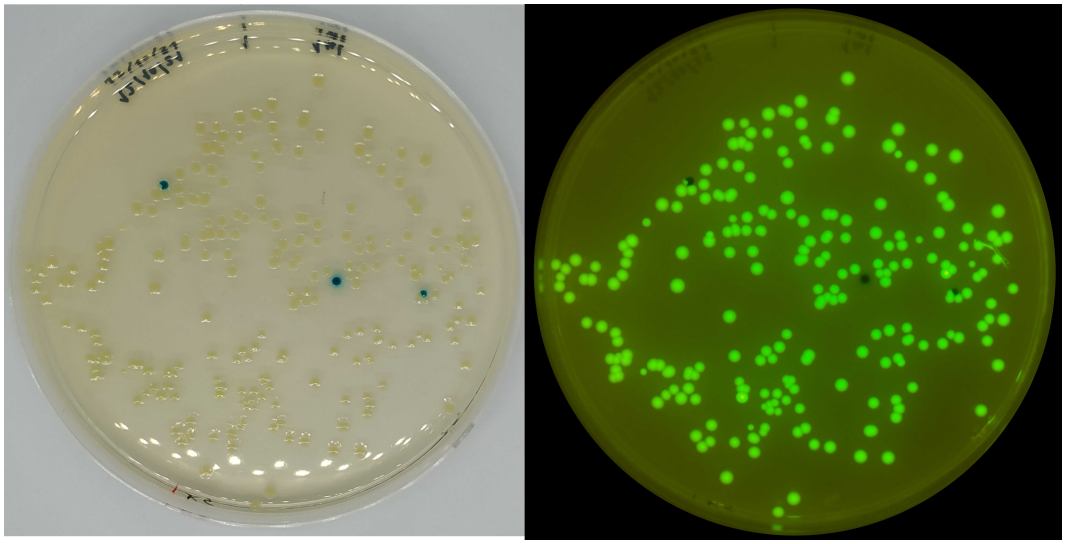
You can short-store the reactions at **4 °C** or **-20 °C** for long storage.

Transformation and colony selection 14h 33m

8 Directly transform **5 µL** of the reaction into **50 µL** chemo-competent cells. 2h 30m

9 Plate the transformant cells into plates supplemented with the proper antibiotic plus X-Gal **40 µg/mL** and growth overnight at **37 °C** 12h

10 Identify the colonies carrying the proper assembled vectors based on blue - white screening of the grown colonies (blue colonies carry intact acceptor vector and should not be selected), plus any other particular screening criteria related to your assembly (i.e. fluorescence expression, colony PCR, sequencing, etc). 3m



Golden gate transformation results. LacZ(+) colonies become "blue" by reacting with X-Gal and white colonies should carry properly assembled vectors.

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