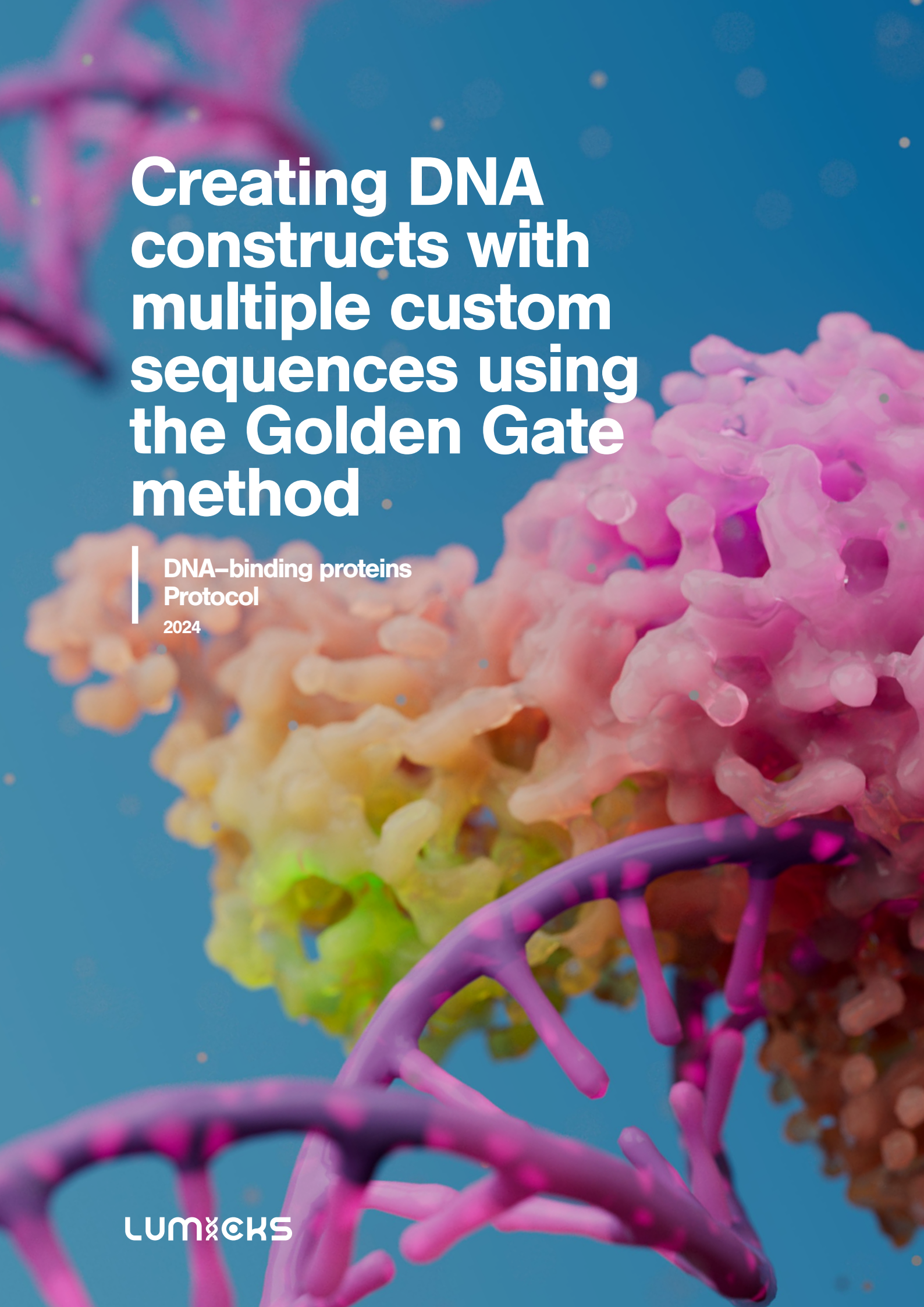

Creating DNA constructs with multiple custom sequences using the Golden Gate method

DNA–binding proteins
Protocol

2024

LUMICKS

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Introduction

Golden Gate technology is a powerful tool that enables the ligation of multiple DNA fragments in any order of choice into a vector backbone (NEBridge® Golden Gate Assembly | NEB). This method takes advantage of Type IIS restriction endonucleases' ability to cleave DNA outside of the recognition sequence.

Since the overhang sequence is not determined by the restriction enzyme, it is possible to design the DNA fragments in a way that digestion generates specific overhangs for each DNA fragment. These overhangs can then be used to facilitate the simultaneous and orderly ligation of multiple DNA fragments. The DNA ends can be designed in a way that the resulting construct does not contain any restriction enzyme recognition sites or scar sequences but only a sequence of interest.

LUMICKS developed a protocol that combines Golden Gate technology with the LUMICKS DNA tethering kit, to create custom constructs to use for C-Trap experiments. In our protocol, the two biotinylated DNA handles from the DNA tethering kit form the two outmost fragments of the final DNA construct assembly. This configuration allows subsequent tethering in the C-Trap. Between the two DNA handles, up to 12 DNA fragments containing sequences of interest (SOI) can be inserted, as illustrated in **Figure 1**.

This protocol was optimized using 12 DNA fragments, each 2.1 kb in length, which were assembled to form a 25.2 kb DNA sequence. This sequence, ligated to the 6.3 kb handles, results in a total tether length of approximately 37.8 kb.

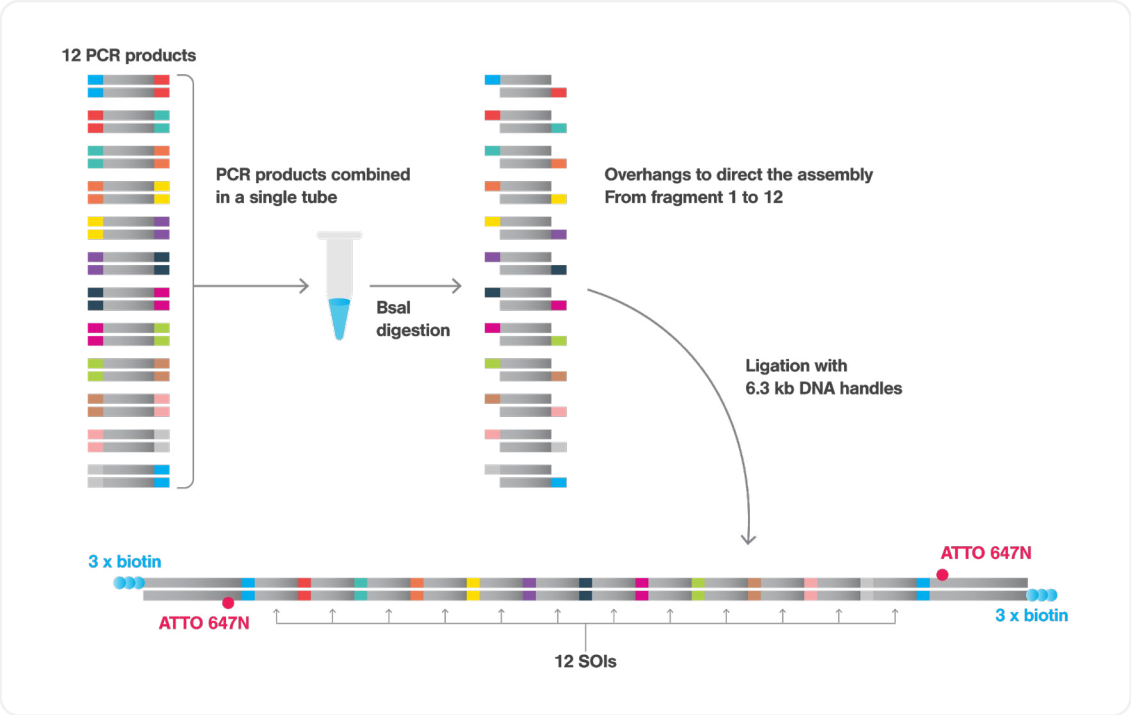

Figure 1 Schematic illustration of the workflow using Golden Gate technology to design DNA constructs with multiple custom sequences for C-Trap experiments. 12 different fragments, each containing a sequence of interest, are first amplified in individual PCRs. A one-pot BsaI digestion then exposes complementary overhangs. In the subsequent ligation step, all complementary fragments are ligated and biotinylated DNA handles (from LUMICKS DNA tethering kit) are ligated to the ends forming the complete DNA construct assembly.

Required materials

To successfully execute this protocol, you need the following material:

- LUMICKS DNA tethering kit (SKU 000026-1)
- Promega DNA purification kit (Catalogue A9281) – or similar DNA purification kit
- NEB BsaIv2-HF (NEB R3733) – or similar Type IIS restriction enzyme kit
- Amicon Ultra-0.5 mL Centrifugal Filters 30 kDa cutoff (Millipore UFC503008)

Step-by-step protocol

Step 1: Primer design

BsaI restriction sites are introduced into the SOI by PCR. Table 1 specifies a set of primers to assemble 12 DNA fragments. Alternatively, use Golden Gate Assembly tools such as the NEBridge® for primer design.

The primers of each PCR are designed such that the DNA ends will form fragment-specific overhangs upon digestion with BsaI. This later allows the orderly assembly of the 12 fragments by ligation. The upstream overhang of fragment 1 and the downstream overhang of fragment 12 are designed to be complementary to the DNA handles of the LUMICKS DNA tethering kit.

Table 1 Forward and reverse primers for SOI amplification by PCR. The 6 nt sequence in red is the BsaI digestion site, while the 4 nt sequence in green forms complementary overhangs after BsaI digestion. The additional 6 nt upstream the BsaI digestion site allows efficient digestion of the ends of the PCR products. The specified primers need to be complemented with the annealing sequence of the SOI, indicated by dashes, to be amplified during PCR.

	FW primer	RV primer
Fragment 1	5' ggctacggtctcgcaac-----> 3'	5' ggctacggtctcctagg-----> 3'
Fragment 2	5' ggctacggtctcctcta-----> 3'	5' ggctacggtctcgagta-----> 3'
Fragment 3	5' ggctacggtctcatact-----> 3'	5' ggctacggtctcggata-----> 3'
Fragment 4	5' ggctacggtctcgtatc-----> 3'	5' ggctacggtctctagat-----> 3'
Fragment 5	5' ggctacggtctctatct-----> 3'	5' ggctacggtctctctca-----> 3'
Fragment 6	5' ggctacggtctcttgag-----> 3'	5' ggctacggtctctccag-----> 3'
Fragment 7	5' ggctacggtctctctgg-----> 3'	5' ggctacggtctctcgct-----> 3'
Fragment 8	5' ggctacggtctctagcg-----> 3'	5' ggctacggtctcattga-----> 3'
Fragment 9	5' ggctacggtctcatcaa-----> 3'	5' ggctacggtctcctcac-----> 3'
Fragment 10	5' ggctacggtctccgtga-----> 3'	5' ggctacggtctcaactc-----> 3'
Fragment 11	5' ggctacggtctcagagt-----> 3'	5' ggctacggtctcaatag-----> 3'
Fragment 12	5' ggctacggtctcactat-----> 3'	5' ggctacggtctctacca-----> 3'

Step 2: PCR and DNA purification on gel

Use a PCR kit of choice to amplify the DNA fragments in individual PCR reactions, one for each fragment. Run PCR products on an agarose gel and purify using the Promega Wizard® SV Gel and PCR Clean-Up System (Catalogue A9281). We advise to perform an extra air-dry step to completely remove ethanol as also described in detail in Step 4.

Step 3: BsaI digestion

Perform BsaI digestion to form the complementary overhangs for subsequent ligation. We recommend using BsaI-HFv2 (NEB).

Note that this protocol has been optimized using 12 DNA fragments of 2.1 kb each, i.e. ~3.5 nM per fragment in the digestion mix. Adjust the amount of DNA based on the length of your DNA fragments.

1. Determine the concentration of each PCR product, using a NanoDrop™ or similar spectrophotometer.
2. Pool the 12 PCR products in an equimolar ratio. Use for example 1 µg for each DNA fragment to create a DNA fragments mix.
3. Digest the DNA fragments mix using BsaI-HFv2 (NEB).

Below is an example of a Bsalv2-HF reaction assembly using the NEB kit. Incubate the mix at 37°C for 3 hours.

Components	Digestion mix
DNA fragments mix	12 µg (1 µg per fragment of 2.1 kb)
10x buffer	20 µl
Bsalv2-HF	12 µl
Milli-Q water	to 200 µl

Step 4: DNA purification

Purify the digested DNA fragments after BsaI digestion. We recommend using the Promega Wizard® SV Gel and PCR Clean-Up System (Catalogue A9281). We advise adding an additional air-dry step to completely remove ethanol and an additional elution step to improve the DNA yield of the purification.

1. Add 200 µl of DNA binding solution to the product of the BsaI digestion reaction (200 µl).
2. Transfer the mix to a minicolumn.
3. Incubate for 1 minute.
4. Centrifuge at 16,000 x g for 1 minute.
5. Wash with 700 µl wash buffer and centrifuge at 16,000 x g for 1 minute. Discard the flow-through.
6. Wash with 500 µl wash buffer and centrifuge at 16,000 x g for 5 minutes. Discard the flow-through.
7. Transfer the column into a new, clean collection tube and centrifuge at 16,000 x g for 1 minute.
8. Perform an extra air-dry step by leaving the column at room temperature for 10-30 minutes.
9. Transfer the column into a new, clean collection tube.
10. For the first elution, add 30 µl of Tris-HCl 10 mM pH 8 to the minicolumn. Incubate for 1 minute, then centrifuge at 16,000 x g for 1 minute.
11. Keep the same collection tube and add 15 µl of Tris-HCl 10 mM pH 8 to the minicolumn.
12. The final elution volume should be ~35 µl.

Digesting a total amount of 12 µg of 12 fragments of 2.1 kb each, we consistently obtain a final concentration of 300-500 ng/µl of DNA.

Step 5: Ligation of Golden Gate assembly and DNA handles

In the last step, the 12 DNA fragments containing the SOIs and the two DNA handles (6.3 kb) are ligated. This is achieved by mixing all DNA components in an equimolar ratio (as described in the protocol of the DNA tethering kit) at 0.05 pmol per fragment.

1. Prepare the ligation mix by first mixing 2 µl of 10x DNA ligase buffer with 6 µl of Milli-Q water.
2. Add the 12 DNA fragments and the two DNA handles in an equimolar ratio (0.05 pmol of each fragment and 0.05 pmol of each DNA handle). Mix gently by pipetting up and down.
   When for example using 12 fragments of 2.1 kb, you require a total of 830 ng of DNA fragments to obtain a total of 0.6 pmol.
3. Add 1 µl of DNA ligase and again mix gently by pipetting up and down.
4. Incubate the reaction at 22°C for 16 hours. Note that this incubation time is significantly longer than described in the DNA tethering kit protocol. Longer incubation is necessary for the successful ligation of all 12 DNA fragments and two DNA handles.
5. After incubation, add EDTA (pH 8) to a final concentration of 30 mM to the reaction.
6. Heat-inactivate the DNA ligase at 65°C for 10 minutes.
7. Perform buffer exchange using Amicon Ultra-0.5 mL" Centrifugal Filters (30 kDa cutoff) as described in the protocol of the DNA tethering kit.

Components	Ligation mix
10 x DNA ligase buffer	2 µl
Milli-Q water	6 µl
DNA fragments mix	2 µl (0.6 pmol, 830 ng for 12x 2.1 kb)
DNA handle 1	5 µl
DNA handle 2	5 µl
DNA ligase	1 µl

Step 6: DNA tethering in the C-Trap

To tether the fragment assembly in the C-Trap please follow the instructions as provided in the protocol of the DNA tethering kit (pages 10, 11).

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