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Journal of DNA Research

DIRECTIVE PUBLICATIONS

Case Report

Gem-Gate: An Affordable, Adaptable Method For Biobrick Construction.

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Abstract

For many synthetic biologists, the ability to quickly and modularly assemble DNA components is essential. Golden Gate assembly can accomplish this, but it frequently necessitates the purchase and delivery of fresh primers for every component and assembly arrangement. For Golden Gate assembly, we present a minimal set of primers that can be used to amplify any DNA from the Registry of Standard Biological Parts. Although these primers pair improperly, they add type IIS restriction enzyme sites in a manner that reduces assembly scars and bind to regions that are common to the backbone plasmid for these components. This method speeds up and lowers the cost of redesigning assembly procedures, which can allow more scientists and students have access to synthetic biology

Keywords : *Golden Gate; BioBrick; registry; PCR; iGEM; universal; primers.*

INTRODUCTION

As part of the International Genetically Engineered Machine competition (iGEM), thousands of undergraduate students have been designing new genetic circuits annually for the past ten years [1]. These designs are put together using BioBricks, which are a set of standardized DNA components. The iGEM registry of standard biological parts (parts.igem.org, latest accessed 14 February 2023) has pertinent information for each BioBrick, which is designed and characterized to enable assembly of larger constructions and anticipate their behavior. There are several methods for assembling two or more pieces together, such as Gibson assembly, Golden Gate assembly, or standard 3A assembly. An advantage of Golden Gate assembly is that it can allow for flexible and modular assembly strategies,These designs are put together using BioBricks, which are a set of standardized DNA components. The iGEM registry of standard biological parts (parts.igem.org, latest accessed 14 February 2023) has pertinent information for each BioBrick, which is designed and characterized to enable assembly of larger constructions and anticipate their behavior.

Type IIS restriction enzymes, like BsaI or BsmBI, are utilized to create these overhangs since they cut next to their recognition sequence. Golden Gate cloning comes in a wide variety of forms, such as MoClo, Mobius Assembly, Golden-Braid, Gold-Bricks, Jump Vectors, and Loop assemble [2–8]. These tactics, which are occasionally tailored for usage in particular organisms, increase versatility because of shared sequences found in a collection of plasmids [9].

The majority of Golden Gate assembly initiatives need the design and synthesis of primers unique to each BioBrick or assembly step. As an alternative, specific vectors that complement the previously outlined tactics and offer a repertoire of BsaI recognition sites need to be acquired. Even if the cost of DNA synthesis has significantly decreased recently, buying new primers is still a substantial investment in some situations, such as labs in underdeveloped areas or synthetic biology courses in high schools and community colleges. A distribution kit of BioBricks, frequently containing thousands of the most helpful pieces in the registry, is sent to these universities as part of iGEM, and students are tasked with putting them together in practical combinations.

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Received: 14-Feb-2025, **; Editor Assigned:** 15-Feb-2025 **; Reviewed:** 06-Mar-2025, **; Published:** 14-Mar-2025.
Citation: Chloe Boer. GEM-Gate: An Affordable, Adaptable Method for BioBrick Construction. Journal of DNA Research. 2025 March; 1(1).

Only a small number of these BioBricks can be easily utilized in MoClo or other current Golden Gate assembly strategies, despite the fact that many of them are compatible with Golden Gate assembly (as demonstrated by iGEM's type IIS assembly standard, RFC1000). To employ these BioBricks, primers must be designed and ordered, which increases the possibility of mistakes and causes a slight lag in the assembly project's progress.

In the case of one particular BioBrick, BBa_B0034 (a ribosome binding site, or RBS), this problem has already been addressed. A novel PCR-based method that used a primer with a short 3′ region matching the pSB1C3 backbone was created in order to speed up the addition of this common RBS to a coding region [10]. In order to guarantee hybridization during PCR, this primer additionally included a 5′ anchor and the ribosome binding site. This method motivated us to develop a method for adding a Golden Gate cloning sequence to any BioBrick. Our primers incorporate a BsaI recognition site and a distinct overhang in place of an RBS just upstream of the BioBrick. We outline the usefulness of these primers (henceforth referred to asA novel PCR-based method that used a primer with a short 3′ region matching the pSB1C3 backbone was created in order to speed up the addition of this common RBS to a coding region [10]. In order to guarantee hybridization during PCR, this primer additionally included a 5′ anchor and the ribosome binding site.

SUPPLIES AND PROCEDURES

PCR assays

PCR reactions were typically conducted in the manner described below: 7.5 µL of distilled H2O (dH2O), 0.5 µL of template (usually at 0.1–1 ng/µL), and 1 µL of each primer at 10 µM were combined with 10 µL of Q5 polymerase mix (New England Biolabs, Ipswitch, MA, USA). Cycles of 10″ at 94°C, 20″ at 57–60°C, and 30–60″ at 72°C were used to amplify the products. Following purification, the products were either sequenced or added to assembly processes (see to the supplemental procedures). As necessary, reactions were scaled.

Gel electrophoresis PCR products were run for 30 to 45 minutes at 12 V/cm on a 0.8% agarose gel in LAB buffer (10 mM lithium acetate and 10 mM boric acid) with ethidium bromide. Gel pictures2.1. PCR assays PCR reactions were typically conducted in the manner described.

Golden Gate Installation

The Golden Gate cloning kit (New England Biolabs) was used to build the products, which were typically as follows: DNA fragments representing the processed vector and BioBricks were combined in the proper proportions. After adding dH2O, T4 DNA Ligase buffer, and NEB Golden Gate Enzyme mix to this mixture, it was incubated (or scaled) in accordance with the supplier's instructions before being transformed and sequenced.

Sequencing, Miniprep, and Transformation

Using either CCBM80 buffer (10 mM KOAc, 80 mM CaCl2, 20 mM MnCl2, 10 mM MgCl2, and 10% glycerol; pH 6.4) or TSS solution (10% PEG-8000, 5% DMSO, and 30 mM MgCl2 in LB), 2 µL of the assembled product was converted into chemically competitive Escherichia coli DH5-alpha. Commercially accessible substitutes wereThe Golden Gate cloning kit (New England Biolabs) was used to build the products, which were typically as follows: DNA fragments representing the processed vector and BioBricks were combined in the proper proportions. After adding dH2O, T4 DNA Ligase buffer, and NEB Golden Gate Enzyme mix to this mixture, it was incubated (or scaled) in accordance with the supplier's instructions before being transformed and sequenced.

2.4. Using either CCBM80 buffer (10 mM KOAc, 80 mM CaCl2, 20 mM MnCl2, 10 mM MgCl2, and 10% glycerol; pH 6.4) or TSS solution (10% PEG-8000, 5% DMSO, and 30 mM MgCl2 in LB), 2 µL of the assembled product was converted into chemically competitive Escherichia coli DH5-alpha. Some tests also employed commercially available alternatives (NEB 5-Alpha, New England Biolabs). Following a 45-inch heat shock and recovery, cells were incubated on LB plates with 30 µg/mL chloramphenicol for an entire night at 37 °C. Following colony subculturing, plasmid DNA was column purified using a QIAgen QuickLyse kit in Hilden, Germany, and examined using the proper primers for Sanger sequencing.

FINDINGS

The GEM-Gate primers in our first iteration (V1) closely resemble the previously published design for adding ribosome binding sites (Figure 1). Our primers add an 11 bp region that contains a BsaI recognition site and one of six distinct overhang sequences in place of a ribosome binding site. Although these overhangs can be changed as needed, our first choices were predicated on the pSB1C3 prefix and suffix, the 3A assembly locations, and the currently used Golden Gate assembly-based techniques. The Ligase fidelity viewer (Table S4) was used to further validate them [11–13].

Since the primer design depended on the existence of a start codon, one disadvantage of our original strategy was that it restricted the selection of BioBricks to coding regions. As a result, we created V2, a different version of the primers that has an even shorter 3′ area of homology to the template and does not hybridize to the start codon. Regretfully, this primer variant was less reliable and occasionally failed to amplify the target (Figure 2). Given that allele-specific PCR diagnostic techniques are based on mismatches near the 3′ end of a

primer, which can significantly reduce PCR performance, this is not wholly unexpected [14,15].

The possible disadvantage of both of these systems is the possibility of improperly amplifying composite BioBricks. For instance, amplification of the BioBrick BBa_K081014 should start at the beginning of the BioBrick since it comprises an RBS and the coding sequence for red fluorescent protein (RFP). Nevertheless, the 3′ end of the primer may also bind to the scar sequence that separates the RFP and RBS, effectively eliminating the RBS from the finished product. We also tested primers with an extended 3′ region of homology (V3) to lessen this undesired binding. These primers consistently provided effective amplification and were strong. However, assembling these primers would result in significantly bigger scar sequences that don't meet BioBrick's requirements.

All of the earlier iterations were predicated on the previously published RBS addition primer design, which looped out the sequence to be added. We also experimented with an alternative strategy, where the Golden Gate sequences that were introduced stood in for template mismatches (V6). These primers, in our experience, were nearly as reliable as the V3 set without adding an unwanted scar sequence (Figure 2). Unless otherwise specified, this primer sequence iteration was used for all of our subsequent amplification and assembly.

These primers were used to amplify a variety of BioBricks; the majority produced the intended result, while occasionally certain templates and primer combinations were unable to amplify the target. When internal primers were used to sequence the resulting products, the Golden Gate sequences were typically present as anticipated (Figure S1). We aimed to overcome the challenge of amplifying resistant BioBricks (see Text S1 for a comprehensive list) to increase the applicability of our approach. The control VF2 and VR primers were able to successfully amplify these troublesome templates, but the forward and reverse GEM-Gate primers were unable to do so, most likely because of the peculiar target binding mechanism these primers use.

However, if VF2 or VR was paired with just one GEM-Gate primer (i.e., AATT_F6 paired with VR), we were ultimately successful in amplifying every BioBricks test. The result would serve as the template for a follow-up PCR, where the required reverse GEM-Gate primer would be used in place of VR and a forward primer that binds to the 5′ end of the V6 primers would be used in place of VR (Figure S2). The required Golden Gate sequence is added to one end of this two-stage method, and then that sequence is added to the other end. This method also adds flexibility since, depending on the assembly technique being employed, a variety of reverse primers can be selected to be utilized in the second PCR reaction.

We successfully amplified and assembled two BioBricks—the lac operon promoter, BBa_R0010, and the previously stated BBa_K081014 (RFP)—to illustrate the usefulness of this strategy. Sequencing verified the junction between these BioBricks and produced the anticipated "scar" sequence, which is made up of the overhang found in the chosen primers. Furthermore, the resultant colonies had a distinctive red color, indicating that this scar sequence had no effect on or eliminated the anticipated function (Figure S3). We used BioBricks or an incorrect primer selection as a control. During assembly, primers that result in incompatible overhang sequences may amplify their target as anticipated but fail to form colonies. Similarly, the uncommon BioBricks with internal BsaI sites (such K081012, which encodes GFP) were not selected correctly and could not assemble.

Additionally, we looked for sequences that would match the hybridizing scar sequence and the BsaI overhang in the iGEM DNA distribution kit BioBricks. Off-target annealing was anticipated to be negligible in the majority of situations, and no such sites were found (Tables S2 and S3). To find out if undesirable products will build up, PCR was performed on a number of representative BioBricks, including the handful that were anticipated to be problematic. Preliminary findings showed that amplified BioBricks typically produced a single product as anticipated, occasionally with a small, low molecular weight product (likely primer dimers; see Figure S6).

Sometimes, rather than the anticipated BsaI recognition site and overhangs, we saw template sequences at the end of the PCR products (Figure S7). One explanation was that the primer-template mismatch, which has been shown to occur within 6–8 nucleotides of a primer's 3′ end, was fixed in favor of the template by the proof-reading polymerase used in our experiment [16]. As a result, we created a different version of the primers (V7) that had an additional three nucleotides recessed for overhangs and possible mismatches. Although these novel primers leave a slightly bigger scar, they are strong and effective in assembling many fragments (Figures S8–S11). Similar to the last primer set, attempts to assemble incompatible overhangs did not result in a sizable colony count.

TALK

This method allowed us to effectively amplify many BioBricks and put together a promoter and various reporter gene coding sequences. As is common with most Golden Gate-based methods, the introduction of extra genes and primer overhangs should enable larger, more sophisticated assemblies, even though the pilot tests described here only built a maximum of four DNA pieces (three BioBricks and a plasmid backbone). An alternative to the conventional Golden Gate or Gibson assembly, GEM-Gate should enable researchers to move from a design to colonies in as little as 24 hours. Conventional

assembly will sometimes take one to three extra days if a priming purchase from a commercial provider is needed (Figure S5). In time-sensitive research environments (like an iGEM team), this delayThis method allowed us to effectively amplify many BioBricks and put together a promoter and various reporter gene coding sequences. As is common with most Golden Gate-based methods, the introduction of extra genes and primer overhangs should enable larger, more sophisticated assemblies, even though the pilot tests described here only built a maximum of four DNA pieces (three BioBricks and a plasmid backbone).

Additionally, the GEM-gate method gives scientists more freedom. Each overhang can produce a distinct fusion by using the same primer set and shuffling that BioBrick got (Figure 3). In fact, the identical gene can be assembled in either a forward or reverse orientation thanks to the careful selection of these primers (Figure S6). One significant warning regarding the choice of overhang sequences in the primers is that some of them, such "AATT," are troublesome palindromes that attempt to exploit 3A assembly sites and result in ineffective assembly. As previously stated, we were able to successfully merge three fragments using the GEM-gate method.

Therefore, we present a limited collection of primers that can enable a laboratory to create an infinite number of BioBrick combinations. We showed how amplification and the unique binding of these primers may be accomplished, and how this can serve as a precursor to Golden Gate assembly. This "GEM-Gate" method eliminates the need for costly and time-consuming big primer orders. Access to this technology has been further expanded by recent work by others that has made it easier to obtain the restriction enzymes and polymerases needed for Golden Gate assembly [17]. Even if there are other methods and plasmids that make use of Golden Gate assembly, these still call for acquiring particular primers or plasmids. We anticipate that the GEM-gate method will facilitate the acceleration of synthetic biologists' work inWe showed how amplification and the unique binding of these primers may be accomplished, and how this can serve as a precursor to Golden Gate assembly. This "GEM-Gate" method eliminates the need for costly and time-consuming big primer orders.

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