iGEM Type IIS Standard Guidebook.

iGEM Uppsala 2020

A short compilation of general information, design experiences and laboratory work to help you jump into Type IIS assembly method

Since 2009, the Uppsala University team has been one of the recurring participants of the iGEM competition. Throughout the years, however, the MoClo or related Type IIS-based assembly techniques have not yet been used by the Uppsala teams. Therefore, in the beginning of the 2020 season there was no prior knowledge of Type IIS cloning or internal material resources such as custom cloning vectors.

This position gave us the opportunity to test the iGEM resources, physical as well as theoretical, for the new Type IIS standard RFC1000. To share our experience and to help other iGEM teams walking the same path in the future, we compiled all the useful information in this guidebook. We write about our good and bad experiences as well as the small contributions we made to develop and support the Type IIS iGEM standard. Enjoy!

"To all iGEM teams considering using this standard, without prior experience and relying on the iGEM Distribution Kit but having doubts: You can do it!"

Björn, Dori, Hui, Núria and Tereza from UofUppsala 2020

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1. Introduction

As Tom Knight said in his paper introducing the BioBrick standard assembly (Knight, 2003): *"The lack of standardization in assembly techniques forces each DNA assembly reaction to be both an experimental tool for addressing the current research topic, and an experiment in and of itself"*. Standards are essential for development of technologies. By accepting one standard, people can use and combine components from different manufacturers and easily share parts and information between different groups.

BioBrick standard assembly RFC10 has revolutionized the practice of molecular biologists around the world and shaped the whole generation of synthetic biologists - iGEMers. It enabled the shift of focus from assembly itself to the challenge of engineering complex biological systems. Implementation of the BioBrick standard led to the creation of an enormous database of standardized biological parts.

However, the BioBrick standard has limitations in terms of efficiency and generation of cloning scars between the assembled parts. In these aspects, this standard has been overcome by methods of modular cloning based on the use of Type IIS restriction enzymes, which enable reliable assembly of multiple parts in a single reaction and flexibility to design seamless joints.

Despite the cloning techniques using Type IIS enzymes being older than the BioBrick standard (Rebatchouk *et al.*, 1996), they gained worldwide popularity later on with the introduction of the Golden Gate (Engler *et al.*, 2009, 2008) seamless one-pot cloning and the MoClo (Weber *et al.*, 2011), a sophisticated strategy for the construction of large assemblies from basic functional modules. After the introduction of these techniques, a plethora of derived toolkits appeared. Some are specialized for assembling eukaryotic transcription units in plants (Sarrion-Perdigones *et al.*, 2013) or yeasts (Lee *et al.*, 2015) others for prokaryotes such as *E. coli* (Moore *et al.*, 2016) or cyanobacteria (Vasudevan *et al.*, 2019). Based on this, many slightly different standards were suggested, such as MoClo (Weber *et al.*, 2011), GoldenBraid2.0 (Sarrion-Perdigones *et al.*, 2013), or the common standard for plants (Patron *et al.*, 2015) and the related iGEM PhytoBricks.

"Approximately half of the benefits provided by having a standard is lost when there are two different standards."

In 2018 the Loop assembly was developed (Pollak *et al.*, 2019). This method is simpler compared to MoClo (Weber *et al.*, 2011), as it significantly reduces the number of vectors required for the assembly. Due to recursive design, just two sets of four vectors can be used for assembly of higher-level constructs.

Since 2019, iGEM fully supports the Type IIS Loop assembly and thus the basic set of assembly vectors have been included in the Distribution Kit 2019.

2. How does it work? General Basics

2.1. Enzyme classes

The most commonly used restriction enzymes cleave the DNA inside their palindromic recognition sequence. These are classified as Type IIP enzymes, where the P stands for "palindromic", and they include the BioBrick assembly enzymes EcoRI, XbaI, PstI and SpeI.

In contrast, Type IIS restriction enzymes cleave the DNA at the specific distance and direction from the recognition sequence (Figure 1) and therefore are said to have a "shifted specificity". Type IIS assembly techniques are built upon the use of these enzymes.

Figure 1: Schematic representation of the cleavage by the Type IIS enzyme SapI. The SapI restriction enzyme binds to the recognition sequence (blue) and cleaves the DNA while generating the 5' overhang.

2.2. Scars

BioBrick assembly uses pairs of Type IIP enzymes which recognize different hexamer sequences but produce compatible overhangs. After the ligation of the overhangs, a non-palindromic hexamer sequence is formed, which cannot be cleaved by either enzyme. This site is called a scar and it is formed at the junction between any two parts. Type IIS enzymes cleave any sequence which is at a certain distance and direction from the recognition sequence. Therefore, it is possible to design overhangs so that the two sequences are joined without any scar. Type IIS assembly techniques allow the seamless joining of parts which cannot be done with Type IIP enzymes.

2.3. Efficiency

Moreover, the ability of a single Type IIS enzyme to generate many different overhangs allows joining of multiple fragments in a single reaction, which makes it very time efficient. Recently, a set of overhangs allowing joining of up to 24 fragments was developed (Potapov *et al.*, 2018a).

2.4. A Compromise on Seamlessness for Standardization

Standardization simplifies sharing and manufacturing of biological parts and enables automatization. Instead of designing without scars, iGEM Type IIS Loop assembly standard defines the fusion sites, scars, between the parts so that the parts of the same kind (for example promoters) with different sequences generate the same overhangs when cut by the Type IIS enzyme.

3. iGEM Type IIS Loop Assembly Explained

3.1. One Step of the Assembly

3.1.1. Four pieces

Type IIS iGEM standard allows for fast and efficient construction of large assemblies in a few consecutive cloning steps. In one cloning step (Figure 2), four components are cut out from their donor backbones and assembled into the acceptor backbone. The acceptor backbone carries a different antibiotic selection marker than the donor backbones and the products are selected for using the acceptor backbone's antibiotic.

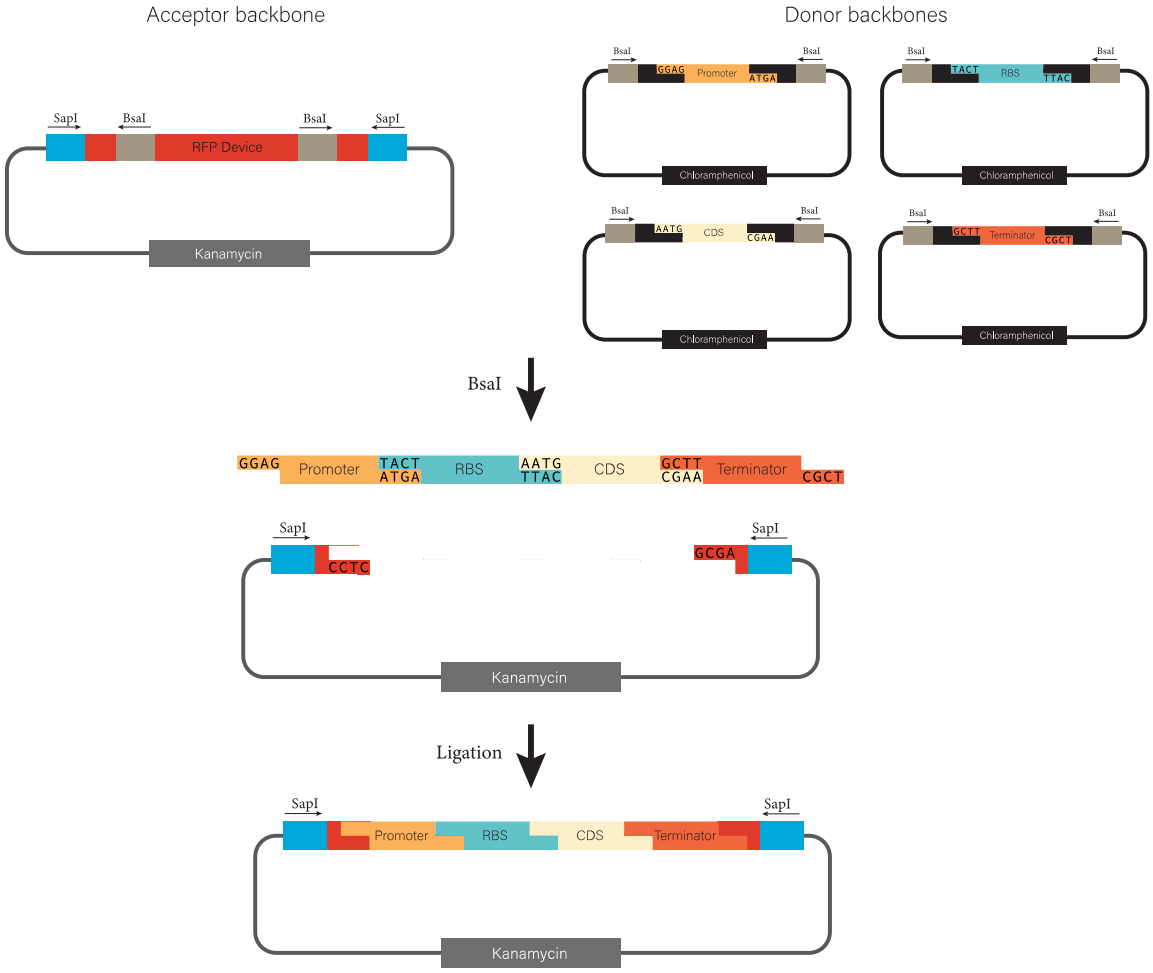

Figure 2: One cloning step in the Type IIS iGEM Loop assembly. Using one restriction enzyme (BsaI), four parts (here Promoter, RBS, CDS and Terminator) are cut out of their original backbones and assembled into the acceptor backbone while replacing the red fluorescent protein (RFP) selection device. The product of this cloning step is a transcriptional unit (TU) in the acceptor backbone, which is flanked by the recognition sites of the second Type IIS enzyme, SapI. Note that the direction of the SapI recognition site is the same as the direction of the BsaI site in the donor backbones. Therefore, in the following cloning step, the TU will be cleaved out and further assembled together with other three TUs in an analogous procedure using SapI digestion. The structure of the acceptor backbone for this cloning step can be imagined as the acceptor backbone shown in this figure (top left) with swapped BsaI and SapI recognition sites. Notes: CDS – coding sequence, RBS – ribosome binding site

3.1.2. Red and White Screening

The acceptor backbone originally contains the red fluorescent protein (RFP) selection device and the four pieces are assembled in its place. The RFP selection device is basically a transcription unit of constitutively expressed red fluorescent protein. This protein makes the cells turn visibly red and therefore allows for the selection of positive colonies by eye.

After transformation, antibiotic selection pressure is applied so that only the cells harboring acceptor backbones are able to survive. The positive colonies will have the acceptor backbone with assembled parts and will give rise to white colonies. If a portion of the original acceptor backbone ends up not cleaved during the cloning reaction, the RFP selection device allows us to distinguish them from the positive colonies, as they will turn red after 16-18 h incubation (Figure 3).

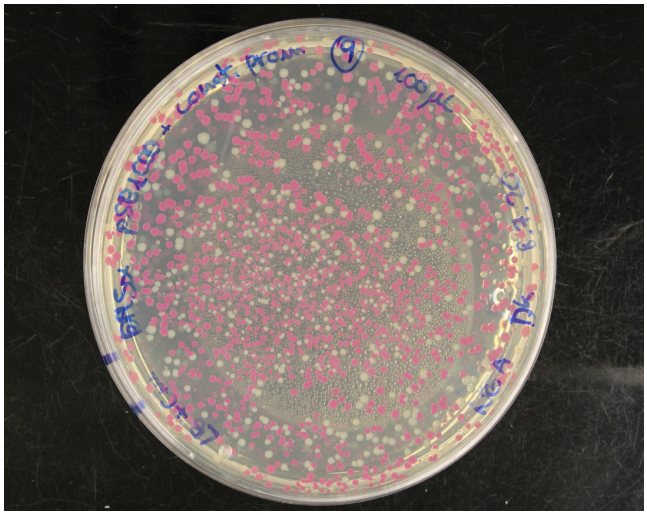

Figure 3: Red and white screening. While positive colonies with assembled parts are white, the remnants of the un-cleaved acceptor backbone give rise to the red colonies.

"Since the expression, maturation and accumulation of red fluorescent protein requires time, sometimes you cannot easily distinguish red and white colonies after overnight incubation and prolonged incubation might be needed to get a clear result."

3.1.3. Orientation of the Recognition Site

Different orientation of the recognition sites for BsaI in donors compared to the acceptor backbone is very important. In both cases, the element in between the two sites is cleaved out. However, while the BsaI recognition site remains in the donor backbones after the cleavage, in the acceptor backbone, it is cleaved out together with the RFP cassette. Therefore, when the parts ligate back to the donor backbones during the cloning reaction, they are cleaved again, but as soon as they ligate to the acceptor backbone, they become uncleavable by BsaI. This shifts the balance of the reaction towards the products and makes it very efficient.

3.2. Creation of the Loop

In the beginning, it was mentioned that the Type IIS iGEM Loop assembly enables the construction of large assemblies in just a few steps. One step of the loop was described in general terms in the previous section (Figure 2). If it were the first step, a promoter, a ribosome binding site, a coding sequence and a terminator would be assembled into a transcriptional unit (TU).

By repeating an analogous cloning procedure but using another restriction enzyme and a different acceptor backbone, four TUs can be assembled into a multi-transcriptional unit (MTU). And after that, in yet another analogous cloning step, four MTUs can be assembled, forming a construct composed of 16 transcriptional units. Details of these steps are outlined in the following section.

3.2.1. Steps Two and Three

The second cloning step of the Loop assembly is very similar to the first one. However, there are three major differences.

First, the TUs which will be assembled are each in a different donor plasmid. These plasmids are always four and each is a product of a separate cloning reaction. In the first step, the parts are all in the same plasmid, the universal acceptor (details in the section Backbones for Loop Assembly below).

Second, a different Type IIS enzyme is used. After the first step, TUs are flanked by SapI recognition sequences in the same orientation as BsaI in the original donor backbones (Figure 2). This enables using the plasmids with TUs as donors in the next cloning step, when the SapI restriction enzyme is used.

Third, a different acceptor backbone is used. It contains the RFP selection device, but in contrast to the acceptor used in the first step (Figure 2), the positions of BsaI and SapI recognition sites are swapped. Therefore, when the four TUs assemble into this backbone, the product is a multi-transcriptional unit (MTU) flanked by BsaI sites.

In the third cloning step, BsaI is used again and four MTUs are assembled into the same acceptor backbone which was used in the first step. This third step will create a construct consisting of 16 TUs flanked by SapI recognition sites. From this step onwards, the Loop is created. Odd-numbered cloning steps will use BsaI to create constructs flanked by SapI recognition sites, and even-numbered steps will use SapI and obtain constructs flanked by BsaI recognition sites. In the next section, all backbones used for the iGEM Type IIS Loop assembly will be introduced in detail.

3.4. Backbones for Loop Assembly

As outlined in the previous chapters, Loop assembly consists of two alternating cloning steps. In one step BsaI is used, in the other SapI is used. Therefore, there are two sets of four vectors, termed pOdd (pSB1K01, pSB1K02, pSB1K03 and pSB1K04 together pSB1K0#) and pEven (pSB3C1#: pSB3C11, pSB3C12, pSB3C13 and pSB3C14) and the cloning steps can be referred to as Odd and Even cloning steps. Additionally, a special backbone, pSB1C00, is used for maintaining the library of basic parts.

"For every backbone in the iGEM Type IIS Loop assembly, both restriction enzymes, BsaI and SapI, are used at some point. The difference is whether they are used to remove the RFP device and "get the parts in" or to release the part and serve as a donor."

3.4.1. Maintaining the parts: Level 0 - pSB1C00

The Level 0 backbone, pSB1C00, serves for maintaining basic parts which are also called Level 0 parts. There are four kinds of basic parts: Promoters, 5' UTR / RBS (5' untranslated regions or ribosome binding sites), Coding sequences (CDS) and Terminators. SapI digestion is used to “get the part in" to the backbone and BsaI is used to release the part (Figure 4).

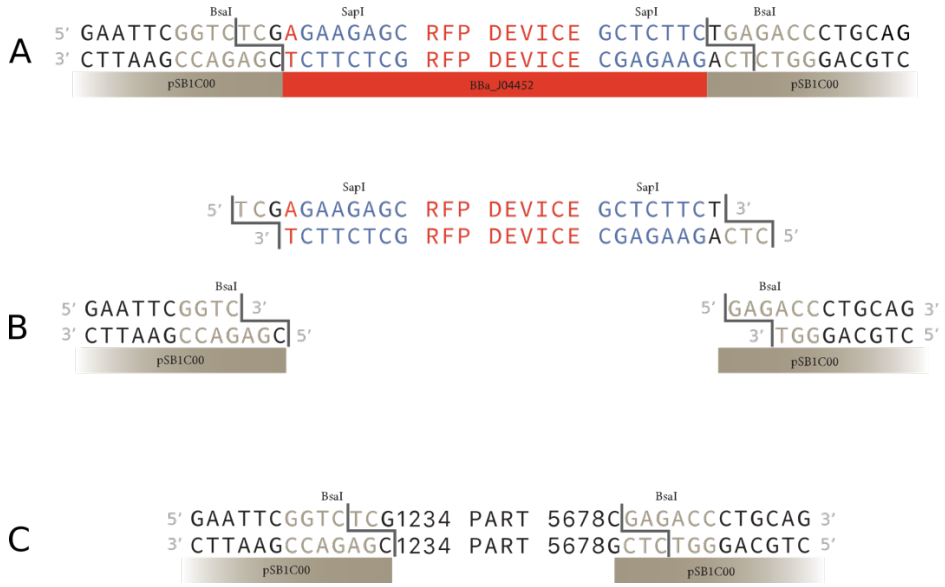

Figure 4: Universal acceptor for Level 0 parts, pSB1C00. (A) Upon digestion by SapI, the RFP selection device (BBa_J04452) is cleaved out. **(B)** During this process, the flanking BsaI recognition site is disrupted. **(C)** When one of the basic parts is inserted, the BsaI site is restored. Moreover, the fusion site sequences (1234 and 5678), which are inside of the part, are now next to the BsaI site, where they will be cut by BsaI. This structure enables the existence of a single Level 0 acceptor for different kinds of basic parts. When the parts are released by BsaI in the next cloning step, they have different fusion sites (1234 and 5678). In contrast to this arrangement, the backbones for higher levels contain the fusion sites inside of the backbone (see Figure 5). Therefore, there are four different backbones for each level which differ in fusion sites.

While there are four kinds of basic parts, there is only one Level 0 backbone and therefore it is often called a universal acceptor. In contrast to that, there are four backbones for higher level assemblies. This is possible due to the unique arrangement of the BsaI and SapI sites in pSB1C00, which are closer to each other in pSB1C00 (Figure 4, top) compared to other backbones. The fusion sites (Table 1) will be the overhangs of parts when they are cut out of pSB1C00. These fusion sites are not part of pSB1C00 but are inserted with the part and their identity determines what kind of part it is, and which position it will take up in the transcriptional unit.

Assemblies to pSB1C00 will usually consist of a linear fragment being inserted to the backbone. Thus, the fusion sites in this fragment can be easily added by PCR or synthesized. This is the reason why there can be only one Level 0 acceptor. For higher level assemblies, these fusion sites have to be in the backbones, so sets of four backbones are needed.

Table 1: Fusion sites of the parts cleaved out from the Level 0 backbone pSB1C00. Upon digestion by BsaI, parts with different fusion sites are cut out of pSB1C00. Fusion sites are compatible and determine the order in which the parts are assembled into the Level 1 acceptor backbone.

Fusion site 5′		Fusion site 3′
GGAG	Promoter	TACT
TACT	RBS	AATG
AATG	CDS	GCTT
GCTT	Terminator	CGCT

3.4.2. Transcriptional units: Level 1 - pSB1K0#

Level 1 backbones serve for the assembly of TUs. There are four variants of this backbone (pSB1K01, pSB1K02, pSB1K03 and pSB1K04) which differ in six nucleotides, the fusion sites (Figure 5). BsaI is used to “get the parts in" to the Level 1 backbone and SapI is used to release the TU for the assembly of Level 2 constructs. TUs with different overhangs (Table 2) are released from the four backbones, from TU1 to TU4. Four basic parts can be assembled into any of the Level 1 backbones. However, the choice of the backbone determines the order of TUs in the MTU assembled in the next cloning step (Table 2).

Figure 5: General structure of the pOdd Level 1 backbones, pSB1K0#. Upon digestion by BsaI, the RFP selection device (BBa_J04454) is cleaved out and the basic parts are ligated in its place while forming a transcriptional unit (TU). The TU is flanked by three-nucleotide fusion sites (here NNN, see Table 2) and the SapI recognition sites. In the next cloning step, four TUs are cut out of the pSB1K01, pSB1K02, pSB1K03 and pSB1K04 and assembled according to their compatible fusion sites into one of the pEven backbones pSB3C1# (see section 3.4.3.).

Table 2: Fusion sites of the pOdd Level 1 backbones, pSB1K0#. Upon digestion by SapI, four transcriptional units (TUs) differing in fusion sites are released from the Level 1 backbones pSB1K01, pSB1K02, pSB1K03 and pSB1K04. According to their compatible fusion sites, the TUs are assembled TU1-TU2-TU3-TU4 while forming a multi-transcriptional unit.

Backbone	Fusion site 5′		Fusion site 3′
pSB1K01 / pOdd1	ATG	TU1	GCA
pSB1K02 / pOdd2	GCA	TU2	TAC
pSB1K03 / pOdd3	TAC	TU3	CAG
pSB1K04 / pOdd4	CAG	TU4	GGT

"If you are wondering about how the system works in case you need to assemble less than four TUs, you can find more about that in the section: How about less than four parts? - Dummies "

3.4.3. Multi-transcriptional units: Level 2 - pSB3C1#

Level 2 backbones serve for the assembly of MTUs. There are four variants of this backbone (pSB3C01, pSB3C02, pSB3C03 and pSB3C04), which differ in eight nucleotides, the fusion sites (Figure 6). SapI is used to “get the parts in" to the Level 2 backbone and BsaI is used to release the MTU for the assembly of Level 3 constructs. MTUs with different overhangs analogous to the overhangs of Level 0 part (Table 1) are released from the four backbones, from MTU1 to MTU4.

"Since pOdd vectors pSB1K0# are high copy number plasmids and therefore might be unstable for large assemblies, we constructed new medium and low copy number vectors for Level 3 assemblies. You can read more about them in the next chapter."

Level 2 backbones pSB3C1# are an improved version of the set of pSB3C0# vectors that can be found in the 2019 iGEM Distribution Kit. pSB3C0# backbones contain an illegal BsaI site. Since BsaI is only used to release the parts before the next assembly, the presence of the illegal BsaI site only breaks the backbone which is no longer needed. We have shown that the pSB3C0# set can be used without major issues.

However, an additional BsaI site competes for the restriction enzyme with other sites, which could compromise the cloning efficiency. Therefore, we recommend using the improved set of pSB3C1#, in which the site was removed by PCR mutagenesis as described in our Wet lab page.

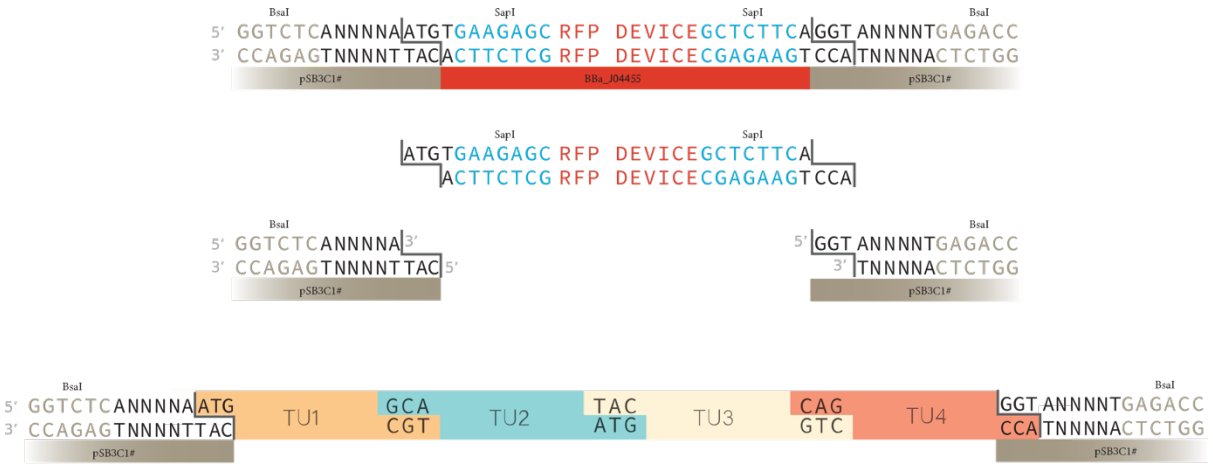

Figure 6: Cloning into the pEven Level 2 backbones, pSB3C1#. Upon digestion by SapI, the RFP selection device (BBa_J04455) is cleaved out and the four transcriptional units assemble in its place, forming a multi-transcriptional unit (MTU). The MTU is flanked by four-nucleotide fusion sites (here NNNN), which are identical to the ones used for basic parts (see Table 1). Upon digestion by BsaI, four MTUs are cut out of the pSB3C01, pSB3C02, pSB3C03 and pSB3C04. These four MTUs can be assembled into one of the pOdd vectors (see Figure 5) in the next cloning step. To increase stability, new medium and low copy number pOdd backbones might be useful, especially for the large assemblies. These backbones are described in the following chapter.

3.4.4. Higher level assemblies: New Level 3 - pSB3K0#, pSB4K0#, pSB4A0#

Level 3 backbones serve for the construction of assemblies composed of between 5 and 16 TUs. In theory, Level 1 pOdd backbones could be used for Level 3 assembly. However, while assembling from pSB3C1# to pSB1K0# is not a problem *in silico*, it is highly likely that it would not work *in vivo.* The reason for this is that the copy number of the currently available pOdd vectors is high (100-300 copies per cell).

High copy number is advantageous, since it allows for higher yield from plasmid extraction, which makes it easier to obtain enough DNA for sequencing and subsequent cloning. However, the metabolic burden caused by the expression of multiple transcriptional units in a high copy number plasmid can be too overwhelming for the cell, which might make the plasmid unstable and prone to mutation or loss (Jones *et al.,* 2000).

In summary, one of the problems of iGEM Type IIS standard in the 2020 season is the lack of suitable pOdd backbones for Level 3 assemblies. Therefore, an important avenue of improvement that we took is the introduction of new pOdd backbones.

Three sets of Level 3 plasmids have been designed in order to suit different situations. Each set comprises four plasmids which have the RFP device for pOdd plasmids (BBa_J04454) flanked by the corresponding Type IIS fusion sites and restriction sites.

The first set, pSB3K0#, is derived from the pSB3K3 plasmid. These pOdd backbones have the same copy number as the existing Level 2 backbones (10-12 copies).

The other two sets are tailored for the situations where the copy number of 10-12 copies is still too high. Very low copy number plasmid sets (~5 copies) were designed. One set, pSB4K0#, is derived from the pSB4K5 plasmid. These plasmids keep the alternating Cm-Kan resistances on Type IIS backbones. However, there might be a situation in which a very low copy number plasmid has to be co-transformed with a high copy number plasmid. For this reason, pSB4A0# were derived from the pSB4A5 plasmid. This last set can be co-transformed with any of the already existing Level 1 and Level 2 backbones, allowing even triple transformation as an option for special cases.

"If you are in need of low copy number backbones for iGEM Type IIS assembly, you can look into the details about how we constructed the backbones using gene synthesis and Gibson assembly in our Wet lab page. "

4. Designing an assembly

4.1. How do I design compatible parts?

The first step of the iGEM Type IIS Loop assembly is to introduce basic parts into the Level 0 backbone. As previously described, the Level 0 backbone is dedicated for the cloning and amplification of four basic part types, such as promoter, UTR, coding sequence (CDS) and terminator. To be able to clone these parts into the backbone, the addition of a prefix and a suffix are required. These contain a Sap1 recognition site and a so-called fusion site, which is specific to the type of the basic part. The latter is important as this will determine the order of the parts within the transcriptional unit when cloned into Level 1 backbone. Thus, the fusion sites are designed so that the basic parts will be assembled in the Level 1 backbone in the following order: promoter, RBS, CDS, terminator. To build up a standardized system, each fusion site is defined by the iGEM Type IIS assembly standard RFC1000 (see Table 1). The sequences can be either synthetized with the prefix and suffix, or they can be added as overhangs by PCR.

"To easily design your own basic parts, you can use templates for each part type, which can be found in the Type IIS part collection."

4.2.**What if there are more than four?**

In other occasions, more than four parts are needed to be cloned into the backbone. In this case, more than four parts can be introduced to the system in two different ways, either by just synthesizing them together or by creating new fusion sites and using the Loop assembly method.

4.2.1. Ordering them together

When two parts are ordered together, they can act like one part during the assembly. They are designed to have one fusion site in the beginning of the sequence and one fusion site at the end of the sequence, but instead of having only one part in between, there are two. This way, more than four parts can be introduced into the backbone.

However, a big disadvantage is that the modularity of the system gets compromised. These two parts cannot be used separately, they can only move together. This system has an advantage when the fused parts are not used separately.

4.2.2. New fusion sites and seamless cloning

To keep the modularity of the system, new fusion sites can be created. This system has advantages since each part is flexible to move around and can be used in different constructs. What should be taken into account when creating new fusion sites is whether the ligation of more fragments will be efficient or not. It is known that ligases can sometimes ligate the DNA when there is a mismatch, and this depends on the ligase and the specific mismatch (Potapov *et al.*, 2018b).

In two studies by Potapov *et al.* (Potapov *et al.*, 2018a, 2018b), the rate of mismatched ligation of different overhangs by T4 DNA ligase was tested. In the case of three base overhangs (Potapov *et al.*, 2018b), there is a weak trend towards lower fidelity with the increase of GC content in the overhang. It is rare, however, that it ligates when the mismatch is in the middle base. Therefore, it is important to make high AT fusion sites that differ in the middle base when possible and avoid TNA overhangs since they ligate very inefficiently. In the case of four base overhangs (Potapov *et al.*, 2018a), it is also good to avoid high GC content and TNNA overhangs. For an expanded iGEM Type IIS standard, we recommend using the unused MoClo fusion sites (Weber *et al.*, 2011) or those from the expanded MoClo, whose fidelity was tested by New England Biolabs (refer to Online Resources [1]). One could also make use of the characteristics of Type IIS enzymes to design seamless cloning. This is a good option for fusion proteins when one of its domains should be easily interchangeable.

An example of this design is the pelB + B-F10 + ehaA construct (BBa_K3425070). This is a fusion protein that brings a nanobody, B-F10, to be displayed in the outer membrane of *Escherichia coli*. It is important that the nanobody can be easily exchanged for different nanobodies, in order to change the specificity of the system and enable detection of different target molecules.

In this case, the three parts combined constitute a coding sequence. Thus, the fusion sites for CDS (see Table 1) are at the 5’ end of pelB and the 3’ end of ehaA. B-F10, the nanobody, overlaps with both pelB and ehaA by four nucleotides, which define the custom fusion site.

Each of these parts is cloned to pSB1C00 and then assembled as one CDS when assembling the transcription unit to Level 1. For more information on the design of this construct you can check our Parts page or BBa_K3425042, BBa_K3425043, BBa_K3425044 and BBa_K3425070 in the iGEM registry.

4.3. How about less than four parts? - Dummies

In case of the iGEM standardized Type IIS assembly, exactly four parts can be cloned into the backbone. For the occasions when the experiment requires to have less than four transcriptional units or four multi-transcriptional units in the construct, we have designed a set of dummies. Eight dummies (see Table 3) were designed to be cloned into each backbone so that they fill up for any position when they are assembled into a higher-level construct.

Table 3: Table of dummies (DY) and respective backbones. Dummies are parts which serve as placeholders for assemblies of less than four parts at any level.

Part number	Part name	Backbone
BBa_K3425023	pSB1K01-DY	pSB1K01
BBa_K3425024	pSB1K02-DY	pSB1K02
BBa_K3425025	pSB1K03-DY	pSB1K03
BBa_K3425026	pSB1K04-DY	pSB1K04
BBa_K3425027	pSB3C11-DY	pSB3C11
BBa_K3425028	pSB3C12-DY	pSB3C12
BBa_K3425029	pSB3C13-DY	pSB3C13
BBa_K3425030	pSB3C14-DY	pSB3C14

Previously, we designed two universal dummies based on an existing Modular Assembly method (Binder *et al.*, 2014). Thus, TU-DY BBa_K3425021 was cloned into each of the pSB1K0# plasmids in order to mimic TUs 1 to 4, and MTU-DY BBa_K3425022 was cloned into each of the pSB3C1# plasmids to become placeholders for MTUs 1 to 4. The result of these cloning reactions were eight backbones with the same sequence between all the iGEM Type IIS standard fusion sites.

In order to check whether these would work as dummy parts, all four pSB1K0#-DY mimicking four TUs were cloned together into pSB3C11. Similarly, all four dummies mimicking MTUs pSB3C1#-DY were cloned into pSB1K02. Sequencing (Figures 7 and 8) revealed that only one out of six clones contains the expected sequence (the last clone of Figure 7). The remaining five clones contain deletions or insertions of various sizes, all of them in the area of the “multi-dummy” sequence.

Figure 7. Detail of the sequencing result of the cloning of four dummies (TU-DY^4) into pSB3C11. At the upper part, dummy parts, fusion sites and BsaI recognition sequences are annotated. At the bottom, three sequencing reads corresponding to three different clones are shown.

Figure 8. Detail of the sequencing result of the cloning of four dummies (MTU-DY^4) into pSB1K02. At the upper part, dummy parts, fusion sites and BsaI recognition sequences are annotated. At the bottom, three sequencing reads corresponding to three different clones are shown.

This is probably due to the short and repetitive sequence generated by the dummies, which might have caused polymerase slippage. This phenomenon can occur when there are reiterated sequences, even if they are imperfect (Levinson and Gutman, 1987). Since the fusion sites at the ends, next to the BsaI and SapI recognition sequences, are present in all the clones it is likely that the cloning was successful and the polymerase slippage happened afterwards, during the growth of the transformed cells.

Dummy parts are only supposed to be fillers that allow the cloning reaction to ligate properly when you have less than four parts to assemble. In this sense, these dummy sequences work as intended. However, it is not ideal to have unpredictable mutations occurring systematically. Therefore, longer and unique dummies were designed *in silico* by retrieving random 8bp DNA sequences from the Random DNA Generator (refer to Online Resources [2]) and adding the corresponding fusion sites (Table 1).

5. Experimental

In Type IIS iGEM Loop assembly, four fragments are assembled into the backbone in a one-pot reaction. This reaction combines two steps: restriction digestion and ligation and therefore both the ligase and the restriction endonuclease are present in the reaction mixture.

Thermal cycles are set so that the optimal temperature for digestion (37 °C) alternates with the optimal temperature for ligation (16 °C) 26 times (Table 4). After that, the mix is shortly incubated at 50 °C and then the enzymes are heat inactivated at 80 °C.

Table 4: Cycling conditions for Type IIS iGEM Loop assembly. These cycling conditions were used by the authors of Loop assembly (Pollak *et al.*, 2019).

Temperature	Time	
37 °C (Restriction digestion)	3 min	26 cycles
16 °C (Ligation)	4 min	
50 °C	5 min	
80 °C (Enzyme heat inactivation)	10 min	
4 °C (Storage)	∞	

During the first cycle, part of the cleaved substrate re-ligates back into the original backbones. However, since multiple cycles are included in the protocol and the re-ligated substrate contains the restriction sites, it is cleaved again during next cycles. The product, on the other hand, lacks the restriction sites and is not cleaved. This shifts the balance of the reaction towards the products and makes the reaction highly efficient.

"Once, by accident we set the thermocycler so that digestion was done for 3 minutes and only the ligation step was repeated 26 times. It worked anyway, just the number of positive colonies we found after the transformation was very low."

5.1.Mixing the reaction

The reaction mixture contains T4 DNA ligase, one of the restriction enzymes (either BsaI or SapI, depending on the stage of the assembly), bovine serum albumin (BSA) as an additive stabilizing the enzymes, water and DNA (Table 5). The molar ratio of the parts to be inserted (donor parts / donor plasmids) to the acceptor plasmid is 2:1. Therefore 15 fmol of each part and 7.5 fmol of the acceptor vector are used in the assembly.

Table 5: Preparation of the reaction mixture for Type IIS iGEM Loop assembly. The same reaction setup was used by the authors of Loop assembly (Pollak *et al.*, 2019). Tip: Especially when preparing the enzyme master-mix, volume can be split into two parts: 5 µL containing buffers and enzymes and the remaining 5 µL with all DNA components. Notes: BSA – bovine serum albumin.

Component	Amount (For 1 reaction)
T4 DNA ligase buffer 10x	1 µL
BSA (1 mg/mL)	0.5 µL
SapI (5 U/µL) / BsaI (5 U/µL)	0.5 µL
T4 DNA ligase (400 U/µL)	0.25 µL
Parts (15 fmol/µL)	1 µL
Acceptor vector (7.5 fmol/µL)	1 µL
H_2O	Till 10 µL
Total Amount	10 µL

5.2.Calculations

The concentration of DNA in the lab is routinely measured in ng/µL. However, the required concentrations for the cloning reaction are given in fmol/µL. To convert the units from ng/µL to fmol/µL, the length of the DNA needs to be considered.

By dividing the length of the part to be inserted by 100, the concentration in ng/µL corresponding to the required 15 fmol/µL is obtained:

15 [fmol/µL] = length of the donor plasmid with the part / 100 [ng/µL]

By dividing the length of the acceptor vector by 200, the concentration in ng/µL corresponding to the required 7.5 fmol/µL is obtained:

7.5 [fmol/µL] = length of the acceptor plasmid / 200 [ng/µL]

5.2.1. An example calculation

For example, to assemble a transcription unit consisting of the Juniper GFP reporter, the constitutive promoter BBa_J23108 (with added Type IIS fusion sites) with an RBS and terminator into the Level 1 backbone pSB1K01, the following stock solutions would be prepared (Table 6). All parts are in the Level 0 backbone pSB1C00.

Table 6: Example calculation of the stock solutions of all DNA components for assembly of a transcription unit. Tip: If you are in doubt how exactly the different parts look inside of the backbones, have a look at some of the complete .dna files on the Parts page.

Part	Length of the part [nt]	Length in pSB1C00 [nt]	Required [ng/µL]
Promoter – BBa_J23108	35	2096	20.96
RBS - BBa_K3425033	20	2081	20.81
Juniper GFP - BBa_K3425048	705	2766	27.66
Terminator – BBa_B0015	129	2190	21.90
pSB1K01 with mRFP1 device	3286	-	16.43

5.3.Transformation, Red and White Screening

After the cloning reaction is completed, it can be stored at 4 ºC or directly used for transformation (5 µL) following a standard protocol and plating on selective LB plates with the antibiotic the acceptor plasmid is resistant to (either chloramphenicol or kanamycin). After 16-18 hours, colonies should appear. The positive colonies carrying the parts assembled in the acceptor backbone are white, while the negative colonies of the uncut acceptor vector are red, due to the presence of the RFP selection device. The cloning reaction thus can be analyzed without the use of any advanced equipment.

"Have fun and don't get lost in the LOOP"

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9.1. Online resources

[1]
New England Biolabs, Expanded “assembly standards” for MoClo | NEB. [online]
https://international.neb.com/tools-and-resources/usage-guidelines/expanded-assembly-standards-for-moclo-goldenbraid2-and-other-modular-golden-gate-assembly-methods
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[2]
Random DNA Generator [online]
https://www.faculty.ucr.edu/~mmaduro/random.htm
(Accessed October 16, 2020)