TYPE IIS MEDIATED PARALLEL DNA ASSEMBLY
THE ONE STEP DIGESTION-LIGATION PROTOCOL

1. REFERENCES

- Patron et al (2015) Standards for Plant Synthetic Biology: A Common Syntax for Exchange of DNA Parts
- Patron (2016) DNA assembly for plant biology
- Engler et al (2008) A one pot, one step, precision cloning method with high throughput capability. DOI: 10.1371/journal.pone.0003647
- Engler et al (2011) Generation of families of construct variants using golden gate shuffling. DOI: 10.1007/978-1-61779-065-2_11
- Werner et al (2012) Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system DOI: 10.4161/bbug.3.1.18223
- Sarion-Perdigones et al (2011) GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. DOI: 10.1371/journal.pone.0021622

2. EQUIPMENT NEEDED

- Purified plasmid DNA containing the parts to be assembled
- Purified DNA of the acceptor plasmid in which parts will be assembled
- Sterile distilled water
- 10 x T4 ligase buffer (New England Biolabs, 500 mM Tris-HCl; 100 mM $MgCl_2$; 10 mM ATP; 100 mM DTT; pH 7.5 @ 25°C)
- Bovine Serum Albumin (1 mg mL^{-1}). Note that New England Biolabs supply this at 10 mg mL^{-1}
- T4 DNA ligase (New England Biolabs; 400U/µL)
- *BsaI* (Eco31I) (ThermoFisher 10 U μL^{-1})
- A thermal cycler
- An electroporator and electrocompetent cells (>10^9 colony forming units μg^{-1}) or, alternatively, a water bath set to 42°C and chemically competent cells.
- LB-agar plates with appropriate antibiotics, X-gal and IPTG
- LB with appropriate antibiotics

3. STEPS IN PROCEDURE

Assembly of standard parts in the common syntax into transcriptional units.

This protocol describes the assembly of standard parts in the common syntax to form complete transcriptional units. Parts are assembled into an acceptor plasmid such as a MoClo Level 1 acceptor or a GB Level alpha (α) acceptor. Level 1 acceptors from the MoClo Plant Toolkit and GB Level alpha (α) acceptors are binary vectors and can be used for *Agrobacterium* mediated delivery to plant tissues. If all DNA parts and the acceptor plasmid are compatible with the following three rules, parts can be assembled together in the one-pot, one-step reaction described.

1. The acceptor plasmid must have a different antibiotic resistant to the plasmids in which the DNA parts are housed. The common syntax states that standard DNA parts should be housed in plasmids that confer resistance to chloramphenicol or spectinomycin and flanked by a pair of convergent *BsaI* recognition sequences. DNA parts are assembled together into transcriptional units in acceptor plasmids that confer resistance to kanamycin or ampicillin/carbenicillin and contain a pair of divergent *BsaI* recognition sequences flanking a cloning selection gene such as *LacZα.* It is also possible to assemble linear DNA fragments; such fragments must also be flanked by a convergent pair of *BsaI* recognition sequences. The use of linear fragments rather than plasmid DNA generally impacts the efficiency of the assembly reaction.
2. Other than the pairs of *BsaI* recognition sites used in the cloning reaction, all plasmid backbones and the parts themselves, must be free from additional recognition sequences for *BsaI*. For multigene assembly using the MoClo or GB plasmid toolkits, parts should additionally be free from recognition sequences for *BpiI* and *BsmBI*.
3. When released from their plasmids by digestion with *BsaI*, parts must have unique sets of compatible overhangs as described in the common syntax (Figure 3). This will ensure that parts are assembled in the correct order and build a functional transcriptional unit.

Protocol:

1. Mix 20 fmol (1 nM final concentration) of the acceptor plasmid with 40 fmol of each of the plasmids containing the DNA parts to be assembled. An example assembly reaction is given in Table 1. The volume of the combined plasmid DNA must be 15 µL or less for a 20 µL reaction. If the volume is greater 15 µL then it is preferable to concentrate the DNA stocks rather than to increase the total reaction volume. The number of moles of DNA can be calculated using the following equation:

   moles dsDNA (mol) = mass of dsDNA (g)/((length of dsDNA (bp) x 617.96 g/mol) + 36.04 g/mol)

   e.g. for 20 fmol of 5 kb DNA = 61.79 ng are required

2. Add sterile distilled water to the DNA mixture to bring the total volume to 15 µL
3. Add the following reagents to the DNA mixture in order: 2 µL 10x T4 DNA Ligase Buffer, 1 µL T4 DNA Ligase (200U / µL), 1 µL Bovine Serum Albumin (1 mg ml^{-1}) 1 µL *BsaI* (10U / µL).
4. Mix the reaction gently by pipetting and incubate in a thermal cycler. The exact thermal cycling conditions can be varied to account for the optimal temperature specified by the enzyme manufacturer and, either fewer cycles with longer incubation

steps, or a greater number of cycles with shorter incubation steps may be more successful on some thermal cycling machines. A guide program is 26 cycles of 37°C for three minutes followed by 16°C for four minutes. Following these 26 cycles, complete the reaction with a five-minute incubation at 37°C followed by a final five-minute incubation at 80°C.

5. Mix 2 µL of this assembly reaction with 20 µL electrocompetent cells (or 4 µL with 20 µL chemically competent cells) and transform according to the manufacturer's instructions.
6. Both MoClo Level 1 and GB Level α plasmid acceptors contain a *lacZ*α cloning gene for blue/white selection. Positive clones can therefore be selected by plating the transformed cells on Luria broth (LB) agar supplemented with IPTG (0.5 mM) and X-gal (0.02 mg ml^{-1}) and the appropriate antibiotic to select for the acceptor plasmid. Select two to six white colonies after 16-20 hours incubation at 37°C. Grow a small liquid culture in LB supplemented with appropriate antibiotics from each colony to obtain sufficient plasmid DNA for confirmation of the correct assembly by restriction analysis and/or sequencing. The assembly reaction will be most efficient if all reagents and DNA molecules are present in the correct proportions. When assembling transcriptional units from standard parts housed in plasmids, >90% of colonies should typically be white and contain the correct assembly. Efficiency will be lower if linear fragments are included and will also decrease as the number and size of the DNA parts increases.

Table 1 An example Type IIS assembly reaction in which five standard parts, initially housed in separate plasmids, are assembled into a plasmid acceptor in a one-pot, one-step reaction mediated by *BsaI* and T4 DNA ligase. The acceptor plasmid and inserts are present at a 2:1 molar ratio. An active version of this is available as an excel file in this folder!

Plasmid and overhangs created by digestion with *BsaI*	**Plasmid Type**	**Plasmid Size (bp)**	**Part Size (bp)**	**[DNA Stock] (ng/µL)**	**DNA stock to add to reaction (fmol)**	**DNA stock to add to reaction (µL)**
Level 1 Acceptor GGAG-CGCT	acceptor	5500	n/a	100	20	0.7
Standard Part GGAG_CCAT	insert	4287	2500	124	40	0.9
Standard Part CCAT_CCAT	insert	3500	1287	110	40	0.8
Standard Part CCAT_AATG	insert	4200	500	120	40	0.9
Standard Part AATG-TTCG	insert	3328	1200	89	40	1.0
Standard Part TTCG-CGCT	insert	2960	328	101	40	0.8
					Total DNA Volume	5.1
					dH_2O	9.9
					T4 DNA Ligase Buffer 10x	2.0
					T4 DNA Ligase (200U/µL)	1.0
					Bovine Serum Albumin	1.0
					BsaI (5U/µL)	1.0
					Total Reaction Volume	20.0

Assembly of Transcriptional Units into Multi-gene constructs using MoClo
The MoClo plant toolkit (Addgene kit # 1000000044) can be used to assemble up to six transcriptional units into a Level 2 or M acceptor. Level 2 and M acceptors are binary vectors and can be used for *Agrobacterium* mediated delivery. Level M acceptors can be assembled together into Level P acceptors and are therefore used when the final construct will contain more than six transcriptional units. Level 1 acceptors contain by a separate set of fusion sites flanking the cloning site for transcriptional units that will produce 4 base pair overhangs after digestion with *BpiI.* These define the order of the transcriptional units in Level 2 or M assemblies (Figure 4A). It is essential to have planned in advance which Level 1 acceptor each set of standard parts is assembled into to allow transcriptional units to be joined in the desired order. All level 2 and M acceptors have the same 3' fusion site (GGGA) (Figure 4A). This allows two, three, four, five or six Level 1 transcriptional units to be assembled together with the aid of an End-linker to bridge between the final Level 1 assembly and the GGGA fusion site (Figure 4A). The MoClo toolkit contains End-linkers that correspond to each Level 1 position.

Once the correct Level 1 assemblies, Level 2 or M acceptors and End-linkers have been selected, the reaction is identical as for the assembly of standard parts into Level 1 acceptors other than that 1 µL of *BpiI* (10U/µL) is included in place of 1 µL *BsaI* (10U/µL).

Making new standard parts
The cost of DNA synthesis has been steadily decreasing over the past few years and in the majority of cases it is both cost and time-efficient to obtain new standard parts of the desired sequence from a commercial vendor of synthetic DNA (e.g. Gen9, Twist Bioscience, GenScript, GeneArt IDT). The requested sequences must be free from *BsaI* recognition sites other than the convergent pair flanking the part that produce the correct overhangs for the type of part. For multigene assembly using MoClo or GB, parts must additionally be free from recognition sequences for *BpiI* and *BsmBI*. Many commercial vendors will provide a choice of plasmid backbones with different antibiotic resistances and it is generally possible to select one that confers the correct antibiotic resistance and that is free from recognition sequences for *Bsa*I.

Alternatively, a desired DNA sequence can be amplified by polymerase chain reaction (PCR) from genomic or complementary DNA and converted to a standard part by cloning into a Universal Acceptor Plasmid such as pUAP1 (Addgene plasmid #63674) or pUDP2 (Addgene plasmid #68161). It is also possible to simultaneously mutate internal instances of *BsaI*/*BpiI*/*BsmBI.*

1. Amplify the DNA of interest using oligonucleotide primers with 5' tails that (a) introduce *BpiI* (or *BsmBI* if using pUDP2) recognition sequences to allow a one-step digestion-ligation reaction with pUAP1 and (b) the correct fusion sites from the plant common syntax to define the type of part. For non-coding parts, primer design needs no further rules. However, to keep new protein-coding parts in frame the following additional rules must be adhered to:

    When the 5' fusion site is AATG: The last three bases code for the AUG start codon (methionine). The first base of the part should therefore be the first position of the first codon following the native ATG.

    When the 5' fusion site is AGCC, AGGT, or TTCG: The last three bases of these overhangs code for alanine, glycine and serine, respectively. To keep the part in frame, the first base of the part should be the first position of the desired codon.

When the 5' fusion site is CCAT: The last two bases contribute code for AU to the AUG start codon (methionine). The part must begin with a G and be followed by the first position of the desired codon.

When the 3' fusion site is GCTT: A stop-codon should be encoded adjacent to this fusion site.

When the 3' fusion site of a coding part is TTCG: A stop-codon should not be included. The last three bases (TCG) code for serine. The T in the first position of this fusion site will therefore be in the third position of the last codon of the part. There are two options:
(a) Include two additional base pairs to complete this codon. Typically, GG, TC, or AG would be used to code for one of the smaller amino acids: glycine (GGU) or serine (UCU or AGU).
(b) Remove the last base pair of the last codon before the stop codon and allow it to be replaced with the first T from the TTCG fusion site. The specific amino acid coded by this change should be assessed for any impact on structure or function.

2. If the sequence of interest contains a recognition sequence for *BsaI/BpiI/BsmBI* this can be mutated by amplifying the sequence of interest in two or more parts producing multiple amplicons that can be assembled together using bespoke fusion sites in which mutations to disrupt the enzyme recognition site are introduced (Figure 5).
3. The linear PCR amplicons are mixed with the pUAP/ pUDP2 plasmid at a 2:1 molar ratio. The laboratory protocol for the assembly of compatible linear fragments into pUAP/ pUDP2 are exactly the same as those described above for the assembly of standard parts, with the important exception that either 1 µL of *BpiI* (10U/µL) or 1 µL of *BsmBI* (10U/µL) are included in place of *BsaI.*
4. pUDP2 contains a *lacZ*α cloning gene for blue/white selection but pUAP1 contains an RFP expression cassette. Colonies housing pUAP1 plasmids without an insert appear bright pink on LB agar plates while colonies with plasmids in which an insert has replaced the RFP gene appear white. Plasmids from white colonies should be confirmed by sequencing to ensure that mutations were not introduced by PCR amplification. Subsequently, these parts can be used in assembly reactions as described above.

Critical Parameters

1. The molar ratio of parts in the reaction is not to be underestimated, particularly when inserts differ substantially in size, when the number of parts being assembled is greater than six, and when total size of the assembly is greater than ~12 kb.
2. The quality of the enzymes is critical for this protocol and care should be taken with both storage and use to ensure that they are not inactivated or degraded by contamination or heat. T4 DNA ligase buffer containing ATP should be thawed on the bench and not at 37°C (to prevent breakdown of ATP). Once thawed, it should be placed on ice. If you use a manufacturer that does not include ATP in T4 ligase buffer, you must supplement it with ribo ATP; deoxyribo ATP (dATP) will not work.
3. In large assemblies, the presence of impurities and inhibitors in the DNA can be critical. Efficiency is generally improved when DNA is prepared using a high-purity plasmid purification method or kit. Many so-called midi-prep and maxi-prep kits contain columns that yield DNA that is relatively free from impurities.

Troubleshooting
In all cases it is useful to perform a transformation control with an intact plasmid to assess the efficiency of transformation and the competency of the cells. It is also useful to perform a control assembly reaction from parts that are known to assemble together reliably in order to assess the functionality of the enzymes and reagents.

1. If no or very few white colonies are seen on the agar plate after incubation, but a large number of coloured (e.g. blue or pink) colonies are present indicating that the acceptor plasmids does not contain an insert, it is likely that the restriction enzyme (*BsaI*, *BpiI* or *BsmBI*) is not working efficiently.
2. If no or very few colonies of any colour are seen on the agar plates after incubation either (a) the competent cells are inefficient – assess competency using a control plasmid (b) the wrong antibiotic was added to the LB-agar plates (c) the T4 ligase was not added or is not working efficiently or the ligase buffer is missing ATP (d) the parts to be assembled have incompatible overhangs - double check the design and re-sequence the parts and acceptor if necessary (e) one or more of the restriction enzyme recognition sites is mutated or missing - double check the design and re-sequence the parts and acceptor if necessary.

Anticipated Results
When assembling transcriptional units from standard parts, if all reagents are working and the molar ratios and DNA concentrations are properly calculated, >90% of colonies should contain the correct assembly. It is unusual to see a large number of white colonies on the plate that contain plasmids in which parts are incorrectly assembled: incorrect assemblies usually result in a drastic reduction of white colonies. Both the total number of colonies and the percentage of correct assemblies will decline as the constructs increase in size.

Time Considerations
A suggested timeline is to set up an assembly reaction in the morning of the first day and run the thermal cycling reaction starting in the early part of the day. The reaction can then be transformed and plated in the afternoon allowing an overnight incubation. The following day colonies can be selected and, if desired, insert size can be screened by conducting PCR on the colonies. Overnight cultures can be set up on the second afternoon and on the third day the plasmid DNA can be purified from the overnight cultures to provide DNA for restriction analysis, sequencing and use in further assembly reactions.