Chapter 15

Joint Universal Modular Plasmids: A Flexible Platform for Golden Gate Assembly in Any Microbial Host

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Abstract

Modular cloning standards based on Golden Gate DNA assembly allow for construction of complex DNA constructs over several rounds of assembly. Despite being reliable and automation-friendly, each standard uses a specific set of vectors, requiring researchers to generate new tool kits for novel hosts and cloning applications. JUMP vectors (Valenzuela-Ortega and French, bioRxiv 799585, 2019) combine the robustness of modular cloning standards with the Standard European Vector Architecture and a flexible design that allows researchers to easily modify the vector backbone via secondary cloning sites. This flexibility allows for JUMP vectors to be used in a wide variety of applications and hosts.

Key words Synthetic biology, Molecular cloning, DNA assembly, Single-pot assembly, Golden Gate cloning, PhytoBricks, JUMP

1 Introduction

Synthetic biology aims to solve problems by approaching biology as an engineering discipline. Simple DNA elements (or parts) are characterized and combined to generate biological systems with new features [1]. However, the degree to which functionality and behavior of new sequences built from basic parts can be predicted depends on their molecular context, a phenomenon widely known as context dependency [2]. Considering this, generation of new biological systems requires an iterative design–build–test cycle and robust methodologies for building complex DNA constructs [3]. DNA assembly standards based on Golden Gate methods are ideal for process automation and basic part reusability and sharing. In Golden Gate cloning [4], DNA parts are flanked by sites recognized by type IIS restriction enzymes, which cut outside the recognition site, leaving a user-defined fusion site. Parts are ligated in an ordered manner and restriction sites are removed during assembly.

Various hierarchical assembly standards based on Golden Gate have been published [5–10], collectively known as modular cloning

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(or MoClo) standards or tool kits. In MoClo standards, the product of one assembly level can be used as a part in the next level assembly, which is possible due to the use of alternate restriction enzymes and selection markers in the destination vectors of different assembly levels (Fig. 1a). Basic (or level 0) parts must be "domesticated," a process that removes internal restriction sites and adds flanking restriction sites with the appropriate overhang, normally also introducing the part into a plasmid for amplification and distribution. After a part has been domesticated, MoClo standards are PCR-independent, allow for the sharing and reuse of parts in different assemblies, and allow for generation of complex constructs after multiple assembly rounds without PCR.

Different MoClo standards differ in restriction enzymes, fusion sites, and vectors, but they share the same design paradigm (Fig. 1a). Each tool kit offers a set of vectors that are arranged in a rigid format with a backbone containing an origin of replication, selection markers, and a modular cloning site where multiple parts from lower level vectors are combined and can then be transferred to higher level backbones. The first limitation of this paradigm is the nature of the vector: each host and application has its own specific vector, which has led to the proliferation of MoClo tool kits. The second limitation comes from the fact that more assembly steps are needed for complex constructs, where there are common assembled modules for multiple genes or sequences (Fig. 1b). Some tool kits include common elements or auxiliary factors in the vector backbone, simplifying the use of the tool kit for a specific application. For example, plant tool kits include left and right border sequences flanking the cloning site to allow for *Agrobacterium*-mediated plant transformation [5, 6, 8, 9]. Another interesting additional feature is the "secondary module" in the EcoFlex tool kit [7]. Multiple transcriptional units (TUs) can be introduced in the secondary module, using an alternative type IIS nuclease, followed by the assembly of two or three TUs in the main site. Moore et al. showed that secondary sites increase assembly efficiency by decreasing the number and size of parts in an assembly, which is highly desirable when generating libraries. While the potential of secondary sites was demonstrated, in EcoFlex this feature is restricted to special level 2 vectors that can only receive two or three TU in the main site. Furthermore, the TUs must be preassembled into a level 2 vector before subcloning them into the secondary site, limiting the flexibility and usefulness of this feature.

The Joint Universal Modular Plasmids (JUMP) offer a flexible platform to overcome the limitations of current modular cloning systems. JUMP vectors have been designed to be easily modified: orthogonal assemblies allow for introduction of any sequence or assembly into the vector backbone (Fig. 1c) without affecting the main modular cloning site. Additionally, JUMP vectors are based on the Standard European Vector Architecture (SEVA) [11] and

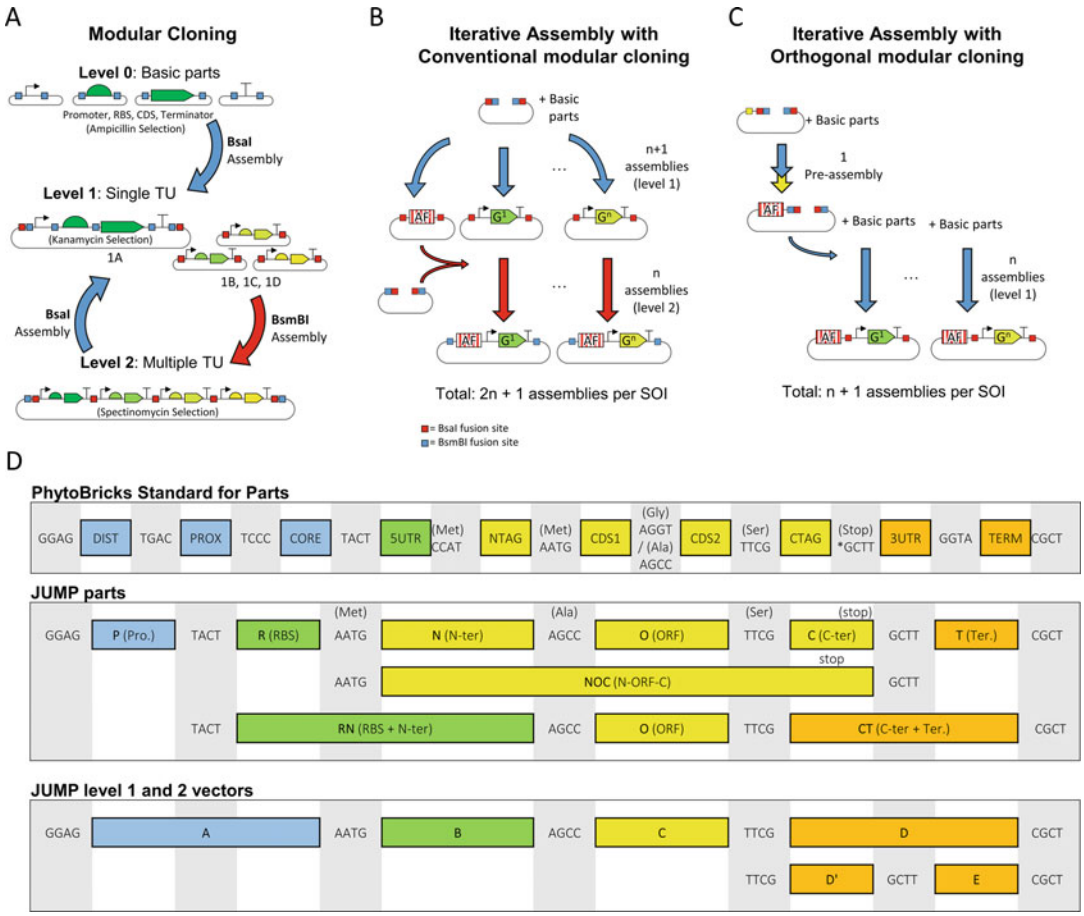
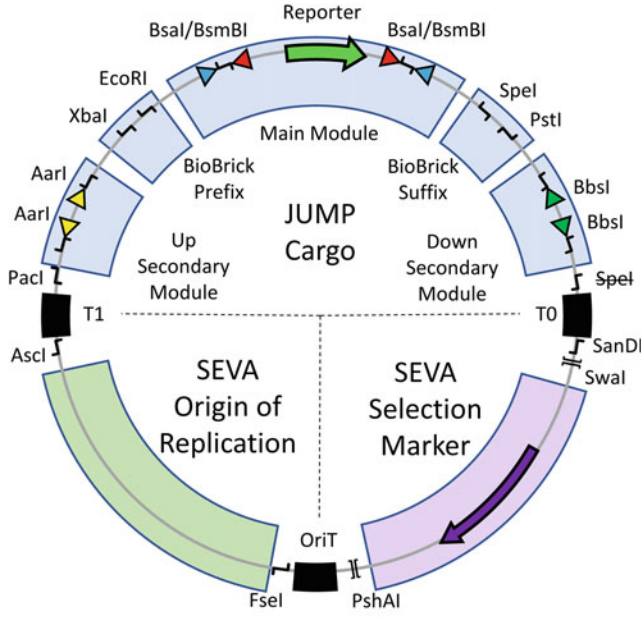


Fig. 1 Modular cloning and PhytoBrick standardization of parts. (**a**) Basic parts are contained in level 0 vectors, which are assembled to form a single transcription unit (TU) in a level 1 vector. To simplify cloning and screening, the level 1 destination vector contains a different selection marker than level 0 vectors and a reporter gene replaced by the assembled TU. Multiple level 1 assembly products can be combined in a level 2 vector, due to the use of an alternative type IIS restriction enzyme and selection marker. In JUMP vectors and some MoClo standards level 1 plasmids can be used as level 3 assembly destination vectors. (**b**) Conventional modular cloning approach with iterative assemblies. The sequence of interest (SOI) is assembled in parallel with the auxiliary factors (AF) needed to test the SOI, then combined in a second assembly tier. (**c**) Orthogonal modular cloning applied in iterative assemblies. The AF is preassembled in a secondary site of the destination vector thus reducing number of assemblies needed per SOI. (**d**) PhytoBricks standard of fusion sites [11]; format of JUMP parts provided in tool kit; fusion sites of level 1 and level 2 JUMP vectors

are compatible with PhytoBricks basic parts (*see* Fig. 1d) as well as BioBricks. The Standard European Vector Architecture (SEVA) [11] is a large collection of origins of replication (OriV) and antibiotic selection markers (AbR) with a standardized format that allows for simple exchange of vector elements (Fig. 2a). JUMP vectors contain a special SEVA "cargo" that includes the main Golden Gate sites for modular cloning (Main Module), BioBricks Prefix and

A



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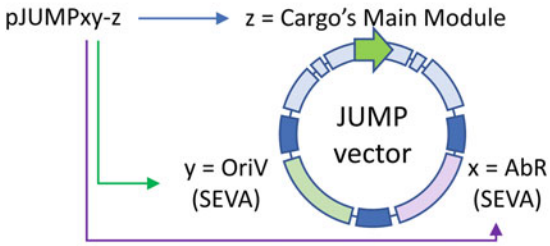

C

AbR	Antibiotic Selection	Notes
1	Ampicillin /Carbenicillin	Default level 0
2	Kanamycin	Default level 1
3	Chloramphenicol	Broad host range marker
4	Spectinomycin /Streptomycin	Default level 2

D

OriV	Origin of replication	Notes
1	R6K	Narrow-host range, (*pir*-dependent)
2x	RK2	Low copy number, broad host range. contains BsmBI, AarI, BtgZI sites
3	pBBR1	Medium-low copy, Broad host range
4	pRO1600 /ColE1	Shuttle E.coli/Pseudomonas *sp*
5	RSF1010	Broad host range
6	p15A	Medium-low copy number
7	pSC101	Low copy number
7ts	pSC101-ts	Thermosensitive replication, low copy
8	pUC	High copy number
9	pBBR322-ROP	Medium-high copy number (Default level 1 and 2)

Fig. 2 Design of joint universal modular plasmids**.** (**a**) JUMP as a modification of the SEVA design. In SEVA (Standard European Vector Architecture) plasmids, three common short DNA sequences flank three variable regions: origin of replication, marker, and "cargo," which might be any expression cassette. The invariable regions are two transcription terminators (T1 and T0, which flank the cargo) and origin of conjugation (oriT). Invariable regions also contain rare cutting sites, forbidden in the sequence of variable regions. This design means that all variable regions are exchangeable between vectors. JUMP is designed as special cargo of SEVA vectors to allow for compatibility with future OriVs and AbRs of the collection. The cargo contains Upstream (Up) Module (outward AarI); BioBricks prefix (XbaI, EcoRI); Main Module (BsaI and BsmBI outward and inward

Suffix, and the orthogonal Secondary Sites (Upstream and Downstream Module, before and after the Main Module, respectively). JUMP secondary sites are compatible with modular cloning done in the Main Module by having the same acceptor sites as the Main Module in all vectors. These secondary sites are also completely orthogonal due to the use of alternative type IIS restriction sites.

A collection of JUMP plasmids has been constructed [12] and deposited in Addgene for distribution. The collection contains bacterial PhytoBricks basic parts of common use in synthetic biology, vectors to domesticate additional basic parts, and cloning vectors to perform assembly at any level with ten origins of replication and four selection markers. The nomenclature of JUMP vectors is based on that of SEVA (Fig. 2b) for antibiotic selection (Fig. 2c) and origin of replication (Fig. 2d). The full list of parts can be found and ordered here: https://www.addgene.org/browse/article/28203402/. Sequences of the plasmids can be found in the supplementary material of Valenzuela-Ortega and French [12].

2 Materials

Molecular biology enzymes and DNA samples should be stored at −20 °C and kept on ice during use. PCR, digestion, and assembly reactions should be prepared on ice and then incubated as specified. We recommend using bioinformatics software to plan assembly and screening. Examples of software to assist plasmid design include Vector NTI, SnapGene, and Genome Compiler.

2.1 General Materials

1. Waterbath or heat block for heat-shock transformation.
2. Microcentrifuge.
3. Shaking incubator for liquid cultures.
4. Static incubator for agar cultures.
5. Thermocycler for PCR and Golden Gate incubations.
6. 1.5 mL microcentrifuge tubes.
7. 0.2 mL PCR tubes.
8. Ultrapure water.

Fig. 2 (continued) for level 1, respectively, and vice versa for level 2); BioBrick suffix (SpeI, PstI); and Downstream (Down) Module (outward BbsI). SEVA's canonical SpeI site was removed to allow for BioBricks compatibility. (**b**) JUMP nomenclature is conservative with SEVA's style for origins of replication (OriV) and selection marker (AbR) vector regions, while cargo nomenclature is replaced by JUMP's Main Module index. (**c**) Antibiotic selection markers used in JUMP vectors. (**d**) Origins of replication in JUMP vectors

2.2 JUMP Plasmids

1. All necessary parts for the assembly, in level 0 donor vectors. (New parts may be prepared as described below, Subheading 3.2).
2. Destination vector (level 1 for a single transcription unit, or level 2 for multiple transcription units) with suitable replication origin and antibiotic resistance marker for the ultimate host strain to be used.
3. If the final destination vector is level 2 (i.e., will contain multiple level 1 transcription units): core level 1 assembly plasmids. (These are not required if the final construct, to be introduced into the final host, is level 1, that is, contains a single transcription unit.)

2.3 Media and Strains

1. Chemically competent *Escherichia coli* cloning strains such as JM109 (NEB #E4107) or DH5α (NEB #C2987I) (*see* **Note 1**).
2. Helper strain for tri-parental conjugation, if required for introduction of the final construct into the final host (*see* **Note 2**).
3. Autoclaved LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and LB agar (as above plus 15 g/L agar).
4. Antibiotics for selection of plasmids. Level 0: ampicillin or carbenicillin (final concentration 100 μg/mL); level 1, kanamycin (50 μg/mL); level 2, spectinomycin (50 μg/mL) alternative selection vectors, chloramphenicol (18 μg/mL) (*see* **Note 3**).

2.4 Molecular Biology Reagents for Assembly Procedure

1. BsaI-HFv2 (NEB) (*see* **Note 4**).
2. BsmBI (NEB) (*see* **Note 4**).
3. T4 DNA ligase (NEB).
4. 10× T4 DNA ligase buffer (NEB). It is recommended to store this buffer in small aliquots at −20 °C.
5. Shrimp alkaline phosphatase (NEB) (only for assemblies in secondary sites).
6. AarI (ThermoFisher #ER1581) (only for assembly in Upstream Module) (*see* **Note 4**).
7. BbsI-HF (NEB) (only for assembly in Downstream Module) (*see* **Note 4**).
8. T4 polynucleotide kinase (NEB) (for phosphorylation of "dummy" linkers if needed).

2.5 PCR for Amplification of Parts and Clone Screening

1. Oligonucleotide primers from Sigma-Aldrich or a similar supplier.
2. High Fidelity Q5 polymerase (NEB) for amplifying new parts (*see* **Note 5**).
3. GoTaq® G2 Green 2× Master Mix (Promega) for colony screening.

4. Nuclease-free water.
5. TE buffer (QIAGEN).

2.6 Agarose Gel Electrophoresis

1. 50× TAE buffer (Formedium).
2. SYBR Safe (ThermoFisher) or SafeView Nucleic Acid Stain (NBS Biologicals).
3. Safe Imager™ Blue-Light Transilluminator (Invitrogen) with amber filter unit, or similar.

2.7 DNA Purification

1. QIAprep Spin Miniprep Kit (QIAGEN) for plasmid DNA miniprep.
2. QIAquick PCR Purification Kit (QIAGEN) (if purification of PCR reactions is required).
3. QIAquick Gel Extraction Kit (QIAGEN) (if extraction of DNA fragments from agarose gels is required).

3 Methods

3.1 Planning the Assembly

1. Select final destination vector based on the final intended host organism. JUMP vectors, as distributed by Addgene, offer ten different origins of replication for different purposes (*see* Fig. 2d). When only one transcription unit (TU) is needed, it can be built as a level 1 assembly using the destination vector with the OriV of interest. However, to build assemblies with more transcription units, intermediate assemblies should be built using the "core set" of level 1 and level 2 vectors with OriV #9.
2. Decide whether to assemble in main or secondary sites. While all transcription units and genetic elements can be combined in Main Modules (Subheading 3.7), if some of the elements are reused in multiple assemblies they can be introduced in a secondary site to reduce the total number of assemblies (*see* Fig. 1b, c). Figure 3 shows a flowchart to plan assemblies. Briefly, after deciding on the final destination vector type (OriV), elements to be assembled are divided into common elements (shared among multiple assemblies) and unique genetic devices or sequences of interest (SOI). Common elements can be prepared using modular cloning (if assembly is needed) and then introduced in a secondary site via conventional restriction-ligation-transformation cloning or two-step assembly (*see* Subheading 3.7).
3. Plan individual assembly steps. When planning a level 2 assembly with multiple TU, the level 1 assemblies used to prepare each individual TU should be done in different level 1 destination vectors (*see* Fig. 1d), which will allow for assembly in a level

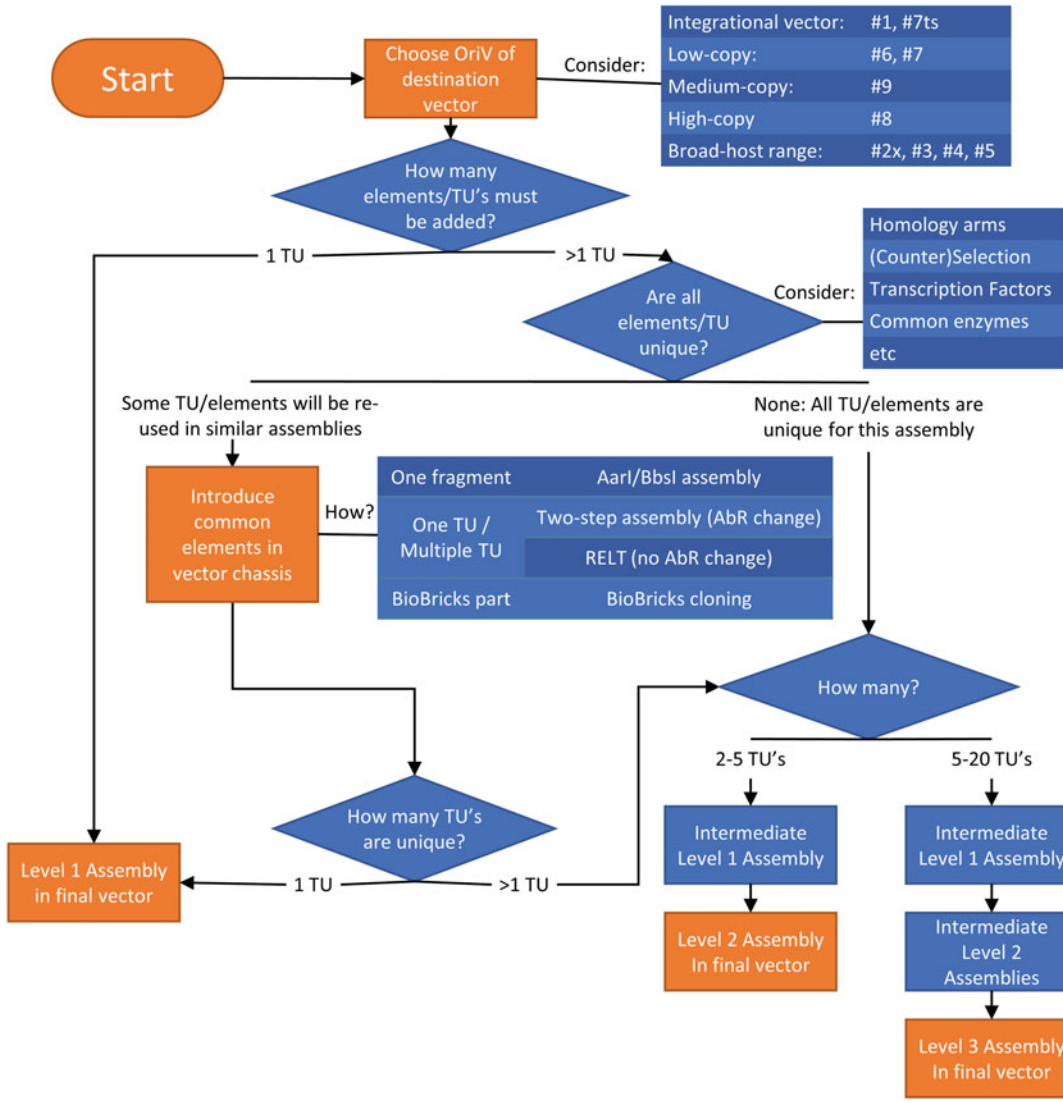

Fig. 3 JUMP assembly-planning guide. After choosing the OriV of the final vector (*see* Fig. 2d for more details), the elements and transcription units (TU) needed in the assembly can considered as "unique" for the assembly or common. The destination level is chosen depending on the number of "unique" TU, and any number of "common" elements can be introduced in secondary sites. Common elements can be introduced in JUMP backbones in multiple ways: if assemblies come from modular cloning they can be assembled in the destination site using the two-step assembly (Subheading 3.8) if the selection markers in the donor and destination vectors are different, or with conventional cloning (restriction endonuclease–ligase–transformation or RELT). If the common elements are available as BioBricks, they can be introduced within JUMP vector's BioBricks Prefix or Suffix. If sequences are designed ad hoc to be introduced in a secondary site, and the inserts are flanked by AarI or BbsI sites, they can be assembled directly via Golden Gate using AarI/BbsI

2 destination vector in the required order. Multiple level 2 assemblies can be combined in a level 1 vector (level 1 vectors are equivalent to a level 3 destination vector, and level 2 vectors are equivalent to level 0).

4. Select the correct parts. Ensure that the parts assembled contain compatible fusion sites. JUMP is designed to use PhytoBricks standardized parts, but researchers should be sure that the selected parts complete a correct assembly (*see* Fig. 1d to find possible alternative part combinations). "Dummy" linkers can be used to replace parts (e.g., when the number of parts assembled is not sufficient to have a complete assembly). Dummy linkers can be prepared with annealed oligonucleotides as explained in Subheading 3.6. Design software may be useful (*see* **Note 6**).
5. Design any new parts required (i.e., parts which are not already in the JUMP kit). Parts must be flanked by sites recognized by the restriction enzyme used during the assembly (BsmBI for insertion into level 0 vectors, and BsaI for assembly in level 1 vectors) and must not have internal sites for these enzymes. Only BsaI and BsmBI sites must be removed from parts to allow assemblies with JUMP vectors (including those into secondary sites). Optionally, AarI, BbsI, and BtgZI sites can be removed to allow direct assembly using those enzymes and allow compatibility with other plasmid tool kits. Generation of new parts from existing sequences is known as domestication. Guidelines for correct part domestication are described in Subheading 3.2.

3.2 Domestication of New Parts (If Required)

DNA molecules used as assembly parts can either have a linear form (e.g., PCR products) or be circular plasmids (Fig. 4a). We recommend domesticating parts into plasmid vectors, such as the Universal Part acceptor pJUMP19-Uac. This allows users to ensure the sequence is correct, store and amplify the part, and share it with other labs. To store the part in a level 0 plasmid, it must be made in a linear form to introduce it into the part acceptor plasmid (level 0 assembly); therefore, it is convenient to generate it in such way that it can be used for both storage in a level 0 vector and direct assembly in a level 1 vector.

1. Design the part sequence. To allow level 0 and level 1 assemblies, parts should be flanked by overlapping BsaI (for level 1 assembly) and BsmBI sites (for level 0 assembly) as shown in Fig. 4a. Digestion with these enzymes should yield the correct 4-base overhang for the particular part type (Fig. 1d).
2. Decide whether to make the part by synthesis, PCR, or annealing oligonucleotides. Small parts (up to ~100 bases) can be generated with oligonucleotides containing the whole part sequence and flanking restriction sites, which should be annealed to each other before assembly (*see* Subheading 3.5). Alternatively, oligonucleotide-based parts can be generated without flanking restriction sites leaving 4 base pair overhangs after annealing, which should correspond to the fusion site of

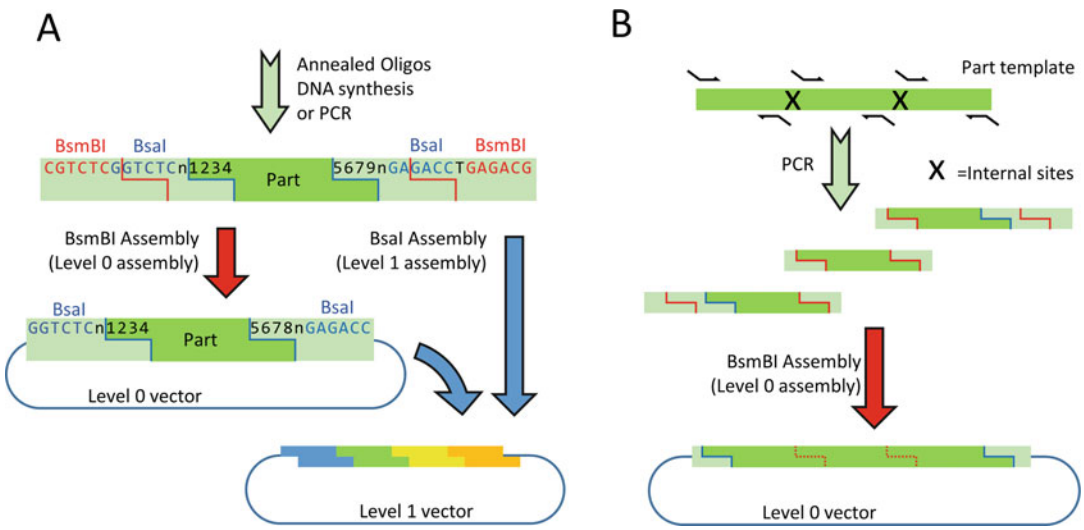

Fig. 4 Domestication of basic parts. (**a**) Domestication of parts using a Universal Acceptor plasmid (such as pJUMP18-Uac). Linear fragments with overlapping BsaI and BsmBI sites can be used for both level 1 assembly and for domestication Golden Gate reaction to introduce the basic part into a level 0 vector. (**b**) PCR-based domestication allows for removal of internal forbidden sites during cloning. Parts are amplified as multiple subpart fragments with primers that introduce silent single-point mutations removing internal sites with flanking BsmBI sites in subpart fragments. BsmBI is used to assemble the subparts in the Universal Acceptor plasmid

the part. Parts made this way must be phosphorylated during the preparation step using T4 polynucleotide kinase. To generate longer parts, users can order the whole sequence as a synthetic DNA fragment (from suppliers such as Integrated DNA Technologies Ltd. or Twist Bioscience) or amplify it via PCR with primers that introduce the flanking restriction sites and fusion sites corresponding to the part type (*see* Fig. 2a). PCR procedure is described in Subheading 3.4.

3. If parts are to be made by PCR, and the natural sequence contains BsaI or BsmBI sites: the domestication PCR can be used to remove internal restriction sites from parts, as described for GoldenBraid 2.0 [5] (Fig. 4b). Briefly, the part template is amplified between the ends and internal sites, generating subpart fragments that end in BsmBI sites. The BsmBI sites generate fusion sites that allow all subparts to be introduced into a part acceptor vector using BsmBI assembly. Single-base mutations are introduced in the fusion sites between subpart fragments, removing internal restriction sites without changing amino acid sequence when the part codes for a protein sequence. Note that this is unnecessary if parts are synthesized or made from oligonucleotides, since the "forbidden" internal restriction sites may be removed in the sequence design stage before ordering.

4. Decide on a suitable level 0 vector for part storage. For general purposes, Universal Part acceptor pJUMP19-Uac should be used. Optionally, researchers who wish to characterize the function of promoters and terminators can domesticate these parts in special level 0 vectors that permit part characterization (*see* **Note 7**).
5. Test the purity of DNA following synthesis and reconstitution or preparation. DNA must be free of contaminants that might inhibit the endonuclease or ligase present in the assembly reaction, and free of unwanted DNA molecules that might interfere with the assembly. Purification kits (for plasmid miniprep, PCR reaction cleaning or gel-extraction) give a suitable DNA purity to perform assemblies, but it is advisable to check the absorbance spectrum of the purified parts to estimate concentration and ensure that the ratio of absorbance at 260 nm/230 nm and 260 nm/280 nm correspond to pure DNA (2.0–2.2 and 1.8, respectively).

3.3 Preparation of Parts and Vectors by Plasmid DNA Miniprep

1. Grow the plasmid-bearing cells. *E. coli* cultures must be incubated at 37 °C, or 30 °C when a thermosensitive vector is used. Shake liquid cultures appropriately to ensure sufficient aeration and homogeneity of culture.
2. Perform plasmid DNA miniprep. We used the QIAprep Spin Miniprep Kit (QIAGEN), following the manufacturer's protocol, adjusting culture and elution volumes depending on the copy number of the plasmid. For vectors with low-copy number (oriV #2, #3, #5, #6, #7, #9), 10 mL of overnight culture is used for the miniprep and DNA is eluted twice in the same 50 μL of nuclease free water. For medium-copy number vectors (oriV #9), use 10 mL culture and elute DNA in 50 μL of nuclease free water twice (total 100 μL). For high-copy number vectors (oriV #4, #8) use 5 mL culture and elute DNA in 50 μL of nuclease free water twice (total 100 μL).
3. Check DNA concentration and purity. If the DNA concentration is too low for analysis of an assembly clone, DNA can be concentrated (*see* **Note 8**).

3.4 Preparation of Parts by PCR Amplification

1. Perform the PCR reaction. For part amplification, a high-fidelity polymerase should be used to avoid introducing unwanted mutations. We routinely use Q5 High-Fidelity Polymerase according to the manufacturer's protocol.
2. Check the product by agarose gel electrophoresis.
3. Purify DNA. If the only band amplified corresponds to the expected size, the PCR can be cleaned using a PCR-reaction cleaning kit according to the manufacturer's protocol. If multiple bands appear in the PCR amplification (including primer

dimers), the band of the correct size should be purified from an agarose electrophoresis gel using a gel-purification kit. We used the QIAquick Gel Extraction Kit (QIAGEN) repeating the wash step with PE buffer, eluting DNA in 50 μL of nuclease free water and concentrating it in 10 μL (*see* **Note 5**). Note that gel-purification should only be applied when multiple bands appear in a gel. This type of purification will result in lower DNA concentrations and higher level of chemical impurities than with the PCR-purification kit.

3.5 Preparation of Parts by Annealing Oligonucleotides

1. Prepare 100 μM stock solution of each oligonucleotide in TE buffer or nuclease-free water. This should be stored at −20 °C.
2. Combine the two strands. For oligonucleotide parts with restriction sites on both ends, combine 5 μL of each oligonucleotide (100 μM stock solution) and 40 μL of nuclease free water. For oligonucleotides without restriction sites, which are designed to anneal so as to leave the correct four-base overhangs, combine 5 μL of each oligonucleotide (100 μM), 34 μL of nuclease-free water, 5 μL of T4 DNA ligase buffer, and 1 uL T4 DNA polynucleotide kinase (PNK) and incubate for 30 min at 37 °C.
3. Anneal the two strands by heating at 95 °C for 5 min and cool down slowly to room temperature. This can be done using a heat-block and switching it off to allow gradual cooling, or in a thermocycler adjusted to cool at a controlled speed (such as 0.2 °C per second).
4. Prepare working solutions. We recommend diluting 20-fold in TE buffer (to a concentration of 0.5 μM) and storing at −20 °C until used.

3.6 Modular Cloning Assembly in Main Module

Assemblies in the Main Module of JUMP vector work as conventional Golden Gate assemblies, regardless of the level. For an assembly in a JUMP vector of a certain level, the destination vector is combined with insert parts from one level lower: level 1 assembly products are inserts for level 2 vectors; level 2 assembly products (and basic parts) are inserts for level 1 vectors (which also work as acceptors for level 3 assemblies).

1. Prepare assembly reactions. Parts are assembled in equimolar concentrations as shown in Table 1. BsaI is used for level 1 and level 3 assemblies and BsmBI for level 0 and level 2 assemblies. Note that, when calculating the molar concentration of a molecule, the molecular weight is that of the whole molecule, including the backbone carrying the inserts.
2. Carry out the assembly reaction as shown in Table 2. For difficult assemblies (higher number of parts, parts of larger size) or when a higher colony number is desirable (combinatorial assemblies to generate libraries), the incubation protocol can be extended (Table 3).

Table 1
Golden Gate assembly reaction setup

Reagent	Initial Conc.	Vol. (μL)	Final Conc.
Backbone DNA	x	x	20 fmol/20 μL
Insert DNA	x	x	20 fmol/20 μL
T4 ligase buffer	10×	2	1×
T4 DNA ligase (enzyme)	400 U/μL	0.25	100 U/20 μL
BsmBI/BsaI-HF	10/20 U/μL	1 (or 0.5)	10–20 U/20 μL
H_2O (nuclease free)	–	x	–
Total		20	

Table 2
Golden gate assembly incubation conditions

BsaI-HF	BsmBI	Incubation	
37 °C	42 °C	15 min	
37 °C 16 °C	42 °C 16 °C	3 min 3 min	×30 cycles
37 °C	55 °C	15 min	
80 °C	80 °C	5 min	
10 °C	10 °C	Hold	

Table 3
Golden Gate assembly incubation conditions for difficult assemblies

BsaI-HF (overnight)	BsmBI (overnight)	Incubation	
37 °C	42 °C	15 min	
37 °C 16 °C	42 °C 16 °C	5 min 5 min	×60 cycles
37 °C	55 °C	60 min	
80 °C	80 °C	5 min	
10 °C	10 °C	Hold	

3. Transform *E. coli* with 1–2.5 μL of assembly reaction, and plate 10% of the transformation on LB agar containing the antibiotic corresponding to the destination vector.

3.7 Two-Step Assembly in Secondary Modules

All JUMP vectors enable introduction of flanking sequences on either side of the Main Module. Conventional cloning allows introduction of any sequence using AarI (in the Upstream Module) or BbsI (in the Downstream Module). Moreover, because the receiver fusion sites of both secondary sites are the same as the ones used by the Main Module across all JUMP vectors (GGAG and CGCT), the sequences introduced in secondary sites can originate from other JUMP vector Main Modules. To maximize the utility of this design principle, we have developed a two-step assembly method to efficiently assemble TU from basic parts in either secondary site.

1. Assemble inserts, without destination vector, using the methodology described in Subheading 3.6, but increasing concentration to 40 fmol of each part per 20 μL reaction. We recommend using the long overnight protocol to increase efficiency.
2. While the assembly reaction is running, predigest the destination vector (Table 4). Incubate at 37 °C for as long as the half-assembly is running followed by 20 min at 65 °C to inactivate the restriction enzyme. A predigested backbone can be used multiple times with different insert half-assemblies.
3. Both first-step reactions can be stored frozen, but to store them for long periods, reactions should be purified with the PCR-purification kit.
4. Ligate insert-assembly and predigested destination vector as shown in Table 5 and incubate for 1 h at 16 °C.
5. Transform *E. coli* (*see* **Note 1**) with 1–2.5 μL of the assembly reaction, and plate 10% of the transformation on LB agar containing the antibiotic corresponding to the destination vector.

Table 4
Backbone digestion setup conditions

Upstream Predigestion	Downstream Predigestion
200 fmol of destination vectors	
1 μL AarI enzyme	1 μL BbsI-HF enzyme
0.4 μL AarI's oligo[a] 50×	–
2 μL AarI Buffer 10×	2 μL Cutsmart Buffer 10×
0.5 μL Shrimp alkaline phosphatase Nuclease-free water to a final volume of 20 μL	

[a]Correct AarI digestion of DNA substrate requires the addition of an oligonucleotide provided with the enzyme (ThermoFisher)

Table 5
Ligation setup for two-step assembly

20 fmol of assembly product (10 μL of insert half-assembly reaction)
20 fmol of predigested backbone (2 μL of reaction)
2 μL of T4 ligase buffer 10×
1 μL of T4 DNA ligase enzyme
Nuclease-free water to a final volume of 20 μL

3.8 Selection of Correct Assemblies

1. Choose colonies which have grown on the correct antibiotic and in which the marker gene has been inactivated, suggesting possible replacement by the new insert. The default reporter in the Main Module of all JUMP vectors is a constitutive sfGFP cassette, but we have built and added alternative vectors with a *lacZ'α* gene (included in the Addgene distribution) to allow for white–blue screening when assembling GFP-coding genes. Note that secondary modules do not contain a marker gene.
2. Check candidate colonies by colony PCR. We recommend a thorough screening process because, based on our experience, plasmid cotransformation is a persistent problem in all modular cloning systems (*see* **Note 9**). Test between 4 and 8 colonies of correct phenotype (i.e., without sfGFP reporter). For assemblies in secondary modules (where no reporter gene is present), there may be no way of distinguishing undigested destination vector clones, and it may be necessary to analyze more colonies.
3. Resuspend each colony in 40 μL sterile dH_2O. When all colonies to be analyzed have been resuspended, vortex them for a few seconds.
4. Prepare primers. Use either cargo-flanking primers or one primer specific for the insert and one specific for the vector. SEVA primers PS1 (AGGGCGGCGGATTTGTCC) and PS2 (GCGGCAACCGAGCGTTC) bind in all SEVA and JUMP vectors amplifying the cargo region. Flanking primers should only be used when the sequence of the insert is of a different length to that in the parental destination vector.
5. Prepare reaction mixtures. Prepare master-mix with all reagents except template (Table 6), place 8 μL of Mastermix in a PCR tube and then add 2 μL of template (cell suspension).
6. Perform PCR as in Table 7.
7. Analyze the colony PCR product by agarose gel electrophoresis.
8. Grow cultures of 2–4 colonies that gave a positive result on the colony PCR. The colony resuspension can be used to inoculate the overnight culture to perform a plasmid DNA miniprep.

Table 6
Reaction setup for colony PCR

GoTaq Green Master Mix (Promega)			
Reagent	**Initial Conc.**	**Vol. per reaction (μL)**	**Final Conc.**
Gotaq Green 2× MasterMix	2×	5	1×
Primer F	10 μM	0.2	0.2 μM
Primer R	10 μM	0.2	0.2 μM
H_2O (nuclease free)	–	2.6	–
Template	–	2	–

Table 7
Reaction incubation for colony PCR

95 °C	5 min	×1
95 °C 60 °C[a] 68 °C	20 s 30 1 min/kb	×30 cycles
68 °C	5 min	×1
10 °C	Hold	

[a]Adjust annealing temperature for each primer pair. 60 °C is suitable for primers PS1/PS2

9. Test for cotransformation. We recommend confirming that no part-donor plasmid has been cotransformed by inoculating LB agar plates with 2–5 μL (*see* **Note 6**) of the colony resuspension. Cotransformants will show growth with the antibiotic corresponding to the donor vector.
10. The next day, take a sample of the overnight cultures for possible propagation of the clones, and store it at 4 °C.
11. Perform the plasmid DNA miniprep (*see* Subheading 3.3).
12. Restriction test. Verify the plasmids by digesting at least 200 ng of the plasmid DNA with restriction enzymes that will generate a different pattern for the correct assembly from that expected for the parental destination vector. Analyze by gel electrophoresis.
13. Sequence. Sanger sequencing offers definitive confirmation of assemblies. SEVA primers PS1 and PS2 flank cargo and will be sufficient to sequence the ends of assemblies. We only recommend sequencing of the whole assembled sequence for newly domesticated parts, as the PCR-free Golden Gate assembly procedure is not expected to introduce new mutations.

4 Notes

1. Homemade chemically competent *E. coli* cells were prepared based on the protocol of Chung et al. [13]. Briefly, LB medium prewarmed at 37 °C is inoculated with a fresh overnight culture of the strain of interest (inoculum–culture = 1:100), and incubated at 37 °C with shaking at 200 rpm. When the OD reaches 0.5, the culture is transferred to ice for 30–60 min, and then centrifuged in a prechilled centrifuge (at 4 °C) for 10 min at 3900 × *g*. Supernatant is gently decanted and the pellet is resuspended in prechilled sterile TSS (TSS–culture = 1:10). TSS ("Transformation and Storage Solution) is LB broth containing 10% (w/v) polyethylene glycol 4000, 5% (v/v) dimethyl sulfoxide, and 50 mM $MgCl_2$.
2. Our triparental conjugation was based on the methodology published by de Lorenzo and Timmis [14]. We used a helper *E. coli* strain carrying plasmid RK600 [14], which carries *tra* genes for conjugation. Briefly, 0.1 mL of overnight culture of donor, recipient and receiving strain were mixed with 5 mL of fresh LB, incubated for 5 h at 37 °C without agitation (30 °C when using mesophilic bacteria or thermosensitive vectors), then 100 μL were streaked for colony isolation on a selective medium.
3. Chloramphenicol selection is done with 18 μg/mL chloramphenicol, which is about half of the concentration commonly used with *E.coli*. We used the broad-host chloramphenicol resistance gene from pSEVA3b61 [15] and found that higher chloramphenicol concentrations inhibited growth of cells carrying this marker.
4. Isoschizomers of these restriction enzymes are available and may also work, but protocol conditions here have been optimized for the enzymes listed.
5. Other polymerases can be used, but we recommend using High Fidelity polymerases to reduce introduction of mutations in parts. Alternatively, DNA synthesis can be obtained from Integrated DNA Technologies Ltd. among other suppliers.
6. Some bioinformatics software packages (such as SnapGene) allow for in silico construction of assemblies, which helps in confirming that chosen inserts match and provides a simple way of obtaining the sequence map of the newly built plasmid.
7. Promoters and terminators can be domesticated into the universal acceptor or into the specialized acceptors pJUMP19-Pac and pJUMP19-Tac. These vectors have inserts in secondary sites that allow direct visualization of the activity of these two types of parts. Vector pJUMP19-Pac has eGFP without

promoter in the Downstream Module, which will be expressed by any promoter introduced in the Main Module. Vector pJUMP19-Tac has the same eGFP in the Downstream Module plus a constitutive promoter in the Upstream site. The domestication of a terminator in the Main Module of pJUMP19-Tac will be indicated by transcription termination before the eGFP and will be shown by a decrease in fluorescence.

8. We routinely increased the concentration of DNA samples using a vacuum concentrator (Eppendorf™ Concentrator Plus). Alternatively, other common methodologies such as ethanol precipitation can be used to concentrate nucleic acids [16].
9. We have found that plasmid cotransformation occurs very frequently upon transformation with Golden Gate assembly mixes: unselected donor plasmids can be introduced and replicate without the presence of their specific selecting antibiotic. We have found that this also occurs using other MoClo tool kits and, in some assemblies, cotransformant colonies can be more than 10% of the total.

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