MoClo Baculo protocol

History:

Version 1.0, Zhihao Lai 24/01/2025

Content:

1. Recommended reading:

This guide is intended to provide additional information to help users carry out the cloning AFTER they read our related publication:

Zhihao Lai, Sarena F Flanigan, Marion Boudes, Chen Davidovich. "Modular cloning of multigene vectors for the baculovirus system and yeast." *J Mol Biol*. 2025 Jan 13:168943. doi: 10.1016/j.jmb.2025.168943.

2. Plasmids:

- **MoClo Baculo Toolkit (AddGene kit #[TBD]):** This toolkit includes all the plasmids needed to construct multigene expression vectors for the baculovirus system, compatible with the Bac-to-Bac system. Most users who wish to build such vectors would find the MoClo Baculo Toolkit sufficient, so they do not need to obtain the MoClo Yeast Toolkit.
- **MoClo Yeast Toolkit: (AddGene Kit #1000000061):** This toolkit is required in addition to the MoClo Baculo toolkit ONLY if yeast protein expression is desired, or if advanced users who are interested in baculovirus expression wish to have more flexibility in developing the system by using a greater selection of connector parts.
- **biGBac Kit for Rapid Generation of Baculoviral Expression Constructs: (Kit #1000000088):** Up to 4 plasmids from the biGBac kit are required if users wish to build multigene vectors containing more than 6 genes each (see more information below, under “Level 3” plasmids). These plasmids are: pBIG2ab (Addgene ID 80616, allows up to 12 genes), pBIG2abc (Addgene ID 80617, allows up to 18 genes), pBIG2abcd (Addgene ID 80618, allows up to 24 genes), pBIG2abcde (Addgene ID 80619, allows up to 30 genes). While users can obtain individual plasmids based on their needs, we generally find pBIG2ab and pBIG2abc sufficient for most projects.

New plasmids that are included in the MoClo Baculo toolkit beyond what is described in Lai et al. 2025: The MoClo Baculo toolkit includes all the part plasmids that were described in Lai et al. 2025 and can be used exactly as described in that paper. However, we also added into the MoClo Baculo toolkit a few additional plasmids. These new plasmids simplify vector construction and allow MoClo Baculo to serve as a stand-alone toolkit for users who are interested primarily in the baculovirus system without using plasmids from the MoClo Yeast toolkit:

- pM.000: This is an entry vector that is nearly identical to the entry vector pYTK001 from the MoClo Yeast toolkit (Lee et al 2015. ACS Synth. Biol. 2015, 4, 9, 975–986). An exception is that in the pM.000 plasmid, the dropout was changed to an RFP. Hence, users can use the pM.000 vector that is included in the MoClo Baculo toolkit, instead of the pYTK001 vector that was used in Lai et al. 2025 and originated from the MoClo Yeast toolkit .
- pMB.Pos1-6 plasmids: These plasmids allow users to reduce the number of parts required for building Level 1 vectors. Additionally, these parts allow users to build Level 1 and 2 vectors for baculovirus expression without using connector plasmids from the MoClo Yeast toolkit. More about these plasmids in the section “**Design of Level 1 plasmids**”.

- pM.L3456, pM.L456, pM.L56 and pM.L6: These are linker plasmids that allow cloning 2, 3, 4 and 5 transcription units, respectively, into Level 2 vectors aiming for baculovirus expression without the usage of plasmids from the MoClo Yeast toolkit. More about these plasmids in the section "**Design of Level 2 plasmids**".

3. Introduction

Golden Gate cloning: Modular cloning is based on Golden Gate cloning with type IIS restriction enzymes. Details regarding the mechanism of the Golden Gate reaction can be found on the NEB website at:
https://www.neb.com/en-au/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/golden-gate-assembly

Hierarchical Modular Cloning and its application for MoClo Baculo: Modular Cloning (MoClo) is commonly performed hierarchically, where all intermediate products (modules) are plasmids that can be propagated in bacteria, disregarding the intended expression system. The hierarchy of plasmids used for MoClo comprises "Levels", where multiple plasmids of Level *k* can typically be combined in a single Golden Gate assembly to produce a single plasmid of Level *k+1*. The MoClo Baculo toolkit supports the construction of up to four levels of plasmids:

- **Level 0 plasmid:** Each level 0 plasmid contains one or more essential elements for the generation of expression plasmids. Such elements are called "parts" and some examples of them are a tag, a promoter or an open reading frame. Level 0 plasmids cannot be used for protein expression on their own, and are only used to store parts and combine them into more complex plasmids—commonly Level 1 plasmids—through a Golden Gate assembly.
- **Level 1 plasmid**: Each level 1 plasmid contains one Transcription Unit, which is the minimal set of functional elements required to express a single protein. They can be used to generate baculovirus DNA for the expression of a single protein using the Bac-to-Bac system. In that sense, Level 1 plasmids are the equivalent of a pFastBac1 plasmid when the intended application is baculovirus expression. Multiple Level 1 plasmids can be combined using Golden Gate assembly to form a single Level 2 plasmid.
- **Level 2 plasmid**: Each Level 2 plasmid contains up to 6 Transcription Units. They can be used to generate baculovirus DNA using the Bac-to-Bac system for the co-expression of up to 6 proteins.
- **Level 3 plasmid (biGBac)**: Level 3 plasmid is generated through integration with the biGBac system. Specifically, the biGBac-compatible backbones that are provided within the MoClo Baculo toolkit can be used for assembling Level 2 plasmids that are compatible with the biGBac system. Subsequently, 2 to 5 bigBac-compatible Level 2 plasmids can be subjected to a single Gibson Assembly reaction that yields one Level 3 plasmid with up to 30 Transcription Units.

Accordingly, a project workflow would typically include the following steps: New parts (e.g. newly obtained open reading frames) are domesticated into Level 0 plasmids by a Golden Gate assembly reaction using BsmBI. Next, multiple Level 0 plasmids are assembled into one Level 1 plasmid using Golden Gate assembly with BsaI. Then, multiple Level 1 plasmids

are assembled into one Level 2 plasmid with up to 6 Transcription Units, using Golden Gate assembly with BsmBI. If the intended protein complex includes more than 6 subunits, then the construction of Level 2 plasmids is done using the same method, but with bigBac-compatible Level 2 backbone parts that can be subsequently combined using Gibson Assembly into one Level 3 plasmid with up to 30 Transcription Units.

4. Reagents used

We find the reagents below adequate, although they could likely be replaced with equivalent products.

BsaI-HF®v2 (R3733, NEB)
BsmBI-v2 (R0739, NEB)
Hi-T4™ DNA Ligase (M2622, NEB)
NEBridge® Ligase Master Mix (M1100, NEB)
XL1-Blue Competent Cells (200249, Agilent)
NEB® 10-beta Competent *E. coli* (C3019, NEB)
QIAprep Spin Miniprep Kit (27104, Qigen)
10X Lv2 Reaction buffer (Homemade)

- 500 mM Tris-HCl pH 7.5
- 500 mM NaCl
- 100 mM $MgCl_2$
- 1 mg/ml BSA (B9200S, NEB)
- 10 mM ATP (pH 7.0, A26209, Sigma)
- 100 mM DTT

Note: BSA may precipitate due to the presence of reducing agents. This does not affect the performance of the buffer.

5. Optional reagents:

PrimeSTAR® Max DNA Polymerase Ver.2 Master Mix (R047B, TaKaRa); may be used for mutagenesis
Gibson Assembly® Master Mix (E2611, NEB); required for Level 3 plasmid construction and optional for the generation of Level 0 plasmids.
NEBuilder® HiFi DNA Assembly Master Mix (E2621, NEB); may be used for the optional generation of Level 0 plasmids using single-strand DNA

6. Gene synthesis:

We commonly get parts from gene synthesis (Genscript) cloned in a plasmid that has a backbone free of BsaI and BsmBI sites (pUC57-Kan-Key IIS free). However, most plasmids will be sufficient, even if they have some BsaI and BsmBI sites in their backbone. This is because the likelihood that random BsmBI sites would generate cohesive ends that will compete against the desired cohesive ends of the part during a Golden Gate assembly reaction is rather low.

7. Whole plasmid sequencing:

Oxford Nanopore whole plasmid sequencing by Plasmidsaurus

8. Antibiotic selection (1X concentration):

Ampicillin 100 ug/mL
Kanamycin 50 ug/mL
Chloramphenicol 25 ug/mL
Spectinomycin 50 ug/mL
Gentamicin 7 ug/mL

9. General workflow and timeline

Before starting a new project, it is important to read all the sections that are included below, especially the sections titled "**Design of Level 0 plasmids**", "**Design of Level 1 plasmids**", and "**Design of Level 2 plasmids**".

Duration
The whole process from the generation of Level 0 to Level 2 plasmids takes 9 days. If level 0 plasmids are already available in the lab, the entire process up to Level 2 plasmids takes 6 days.

Stop points
The process can be paused at the end of any day for a few days if the plates, cells or reaction mixtures are stored at 4 °C.

Day 1: Golden Gate reaction for Level 0 plasmids using plasmids, gene fragments or PCR products, followed by the transformation of the Golden Gate reaction into *E. coli* (see detailed protocol under "**Generation of Level 0 plasmids**").
Day 2: Pick colonies into liquid media and grow, aiming for a miniprep on the next day (see protocol under "**Generation of Level 0 plasmids**").
Day 3: Miniprep of Level 0 plasmid and carry out restriction digestion to verify correct assembly (see protocol under "**Generation of Level 0 plasmids"**). Subsequently, use the correctly assembled Level 0 plasmids for a Golden Gate reaction to generate Level 1 plasmids (**"Generation of Level 1 plasmids"**).
Day 4: Transformation of Golden Gate reaction into *E. coli* (see protocol under "**Generation of Level 1 plasmids"**).
Day 5: Pick colonies into a liquid culture, aiming for a miniprep on the next day (see protocol under "**Generation of Level 1 plasmids**").
Day 6: Miniprep the Level 1 plasmids and perform restriction digestion to assay for the correct assembly (see protocol under "**Generation of Level 1 plasmids**"). Proceed with the correctly assembled Level 1 plasmids into a Golden Gate assembly reaction to generate a Level 2 plasmid (see protocol under "**Generation of Level 2 plasmids**").
Day 7: Transformation of the Golden Gate reaction into an *E. coli* (see protocol under "**Generation of Level 2 plasmids**").

Day 8: Pick colonies into a liquid culture and grow overnight, aiming for a miniprep the next day (see protocol under "**Generation of Level 2 plasmids**").
Day 9: Miniprep of level 2 plasmid, restriction digestion to determine the correct assembly and then send correct clones for whole plasmid sequencing (see protocol under "**Generation of Level 2 plasmids**").

10. Design of Level 0 plasmids

Generation of level 0 plasmids can be achieved by BsmBI Golden Gate assembly or Gibson Assembly. However, BsmBI Golden Gate is generally the preferred method and should be used when possible. The sequence of the intended part should be devoid of BsaI, BsmBI and PmeI restriction sites. If users would also like to construct yeast expression vectors using the same parts, through compatibility with the MoClo Yeast tool kit, then NotI sites should also be removed from part sequences. In addition to the part sequence, the user has to include in the design also 'integration sequences'. Integration sequences are sequences that are required for the correct assembly of Level 0 and Level 1 plasmids, and they need to be added at the 5' and 3' ends of the DNA sequence that is designed for a given part. These integration sequences must be part of the DNA sequence that is cloned into Level 0 plasmids, disregarding the method used to generate the part. Ahead of the construction of Level 0 plasmids, the intended part can be either in another plasmid (e.g. if obtained in a plasmid from gene synthesis) or as a linear double-strand DNA (e.g. if obtained as a gBlock or PCR product that can assemble DNA oligonucleotides into a double-strand DNA). Either way, the DNA that is used to make Level 0 part plasmids must include the correct integration sequences. The integration sequences are defined based on the part "type", and they are specified in the table below (read Lai et al. 2025 for part type description):

Parts name	Parts position	Upstream integration sequence	Downstream integration sequence	Requirement
Promoter	2	GCATCGTCTCATCGGTCTCAAACG	TCCGGTATGTGAGACCTGAGACGGCAT	Part sequence should be free of BsaI, BsmBI and PmeI sites.
Promoter	2a	GCATCGTCTCATCGGTCTCAAACG	GGAAGATGAGACCTGAGACGGCAT	Part sequence should be free of BsaI, BsmBI and PmeI sites.
N'Tag	2b	GCATCGTCTCATCGGTCTCAAAGATG	TCCGGTATGTGAGACCTGAGACGGCAT	ATG is included (underlined), but no stop codon should be added. Part sequence should be free of BsaI, BsmBI and PmeI sites (and also NotI site if yeast expression is intended), and in frame with the ATG sequence (underlined).
CDS	3	GCATCGTCTCATCGGTCTCATATG	GGATCCTGAGACCTGAGACGGCAT	ATG is included (underlined), but no stop codon should be added. Part sequences should be free of BsaI, BsmBI and PmeI sites (and also NotI site if yeast expression is intended), and in frame with the ATG sequence (underlined)
C'Tag	4a	GCATCGTCTCATCGGTCTCAATCCTCAGGT	TCCGGTGGCTGAGACCTGAGACGGCAT	The part sequence should be free of BsaI, BsmBI and PmeI sites (and also NotI site if yeast expression is intended), and the coding sequence of the C-terminal tag should be in +1 frame (i.e. the first codon of the C-terminal tag starts immediately after the last base of the upstream integration sequence). Stop codon is not required.
Terminator	4b	GCATCGTCTCATCGGTCTCATGGCTAATGA	GGGCTGTGAGACCTGAGACGGCAT	The part sequence should be free of BsaI, BsmBI and PmeI sites.
Terminator	4	GCATCGTCTCATCGGTCTCAATCCTAATGA	GGGCTGTGAGACCTGAGACGGCAT	The part sequence should be free of BsaI, BsmBI, and PmeI sites.

Table 1. Generation of new parts ahead of the construction of Level 0 plasmids.
‘Integration sequences’ are flanking sequences that are added to the 5’ and 3’ ends of the part sequence (“Upstream integration sequence” and “Downstream integration sequence”, respectively), either if it is ordered from gene synthesis or produced in another way. The correct integration sequence must be added to the right part type (see under “part name” and “part position”), as these sequences define the overhangs that are formed after cleavage with BsmBI and BsaI (read more about part types and their assembly in Lai et al 2015). “Requirements” indicate part-specific requirements. Underlined are ATG start codons. BsaI sites and their overhangs are highlighted in dark and light orange, respectively.

All BsmBI, BsaI, and PmeI sites need to be removed from the parts sequences. While the PmeI site needs to be removed only if Level 3 plasmids are intended, it is recommended to remove it as a good practice, so that future projects are not limited to Level 2 plasmids. If yeast expression is intended down the line, then Not I sites need to be removed as well. If using existing plasmids, a single restriction site can be removed via PCR (e.g. using PrimeSTAR® Max DNA Polymerase). When removing restriction sites by mutagenesis, a reduced background can often be obtained if the corresponding restriction enzyme is used as a negative selection before a transformation into *E. coli*.

We generally discourage the use of Gibson Assembly for the construction of Level 0 plasmids. This is because the application of Gibson Assembly for Level 0 vector construction is not as simple as other approaches. However, if users choose to use Gibson Assembly for generating Level 0 plasmids (Gibson Assembly® Master Mix or NEBuilder® HiFi DNA Assembly Master Mix), then the BsmBI site does not need to be included, but BsaI sites and overhangs are essential. Thus, users must include the BsaI overhang and Gibson Assembly homology region, and the open reading frame must be kept the same as described above for other vector building approaches (Table 1).

11. Generation of Level 0 plasmids

1. Set up a plasmid digestion reaction including 30 ng/ul pM.000 plasmid with 1 U/ul BsmBI overnight at 55°C in NEB3.1 buffer. This reaction mixture can be frozen and reused for many Level 0 constructions. The pM.000 plasmid is identical to the pYTK001 plasmid from the MoClo Yeast toolkit, except for the dropout that was changed to an RFP with a weaker bacterial promoter intended to enable a higher yield of plasmid DNA.
2. Prepare Level 0 reaction as in the table below.
3. Incubate the level 0 reaction mixture in a thermocycler using a program as in the table below.

Level 0 Golden Gate reaction mixture	Vol. [ul]
pM.000 digest	1
Part DNA as a gBlock or PCR product (<100 ng), or as a donor plasmid (<50 pmol)	1-2
BsmBI v2	1
NEBridge® Ligase Master Mix	2
MilliQ water	0-1
Total	6ul

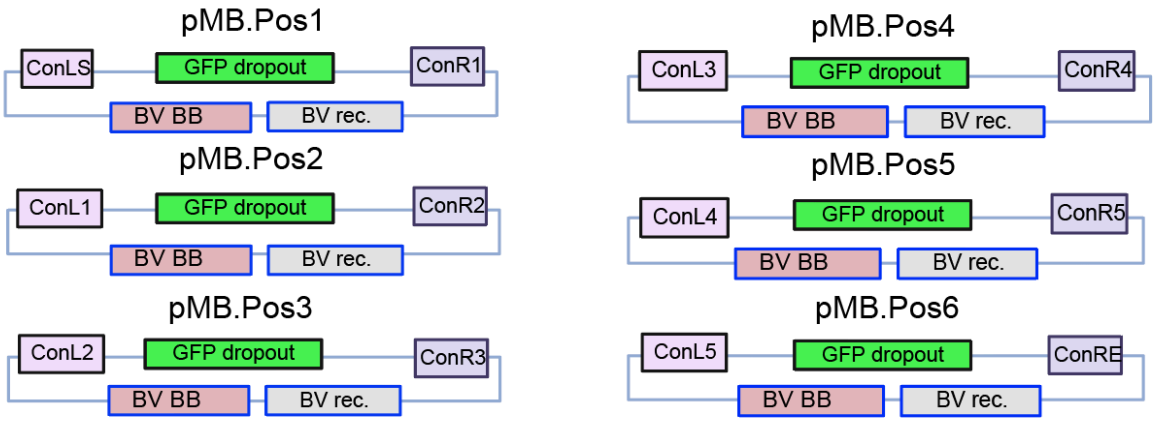
Level 0 Golden Gate reaction thermocycler program		
Temperature [degree C]		Time [min]
42		15
10 cycles	16	5
	42	5
60		10
10		Hold

4. Transform 2 ul to 50 ul XL1-blue, plate on Chloramphenicol plates.
5. Pick a white colony for miniprep.
6. Restriction digestion analysis with BsaI.
7. Proceed to the next step.

12. Design of Level 1 plasmids

As shown by Lai et al 2025, up to 6 transcriptional units (TU) can be inserted into a single Level 2 plasmid, whose assembly will be discussed below. The ligation of these TUs and therefore their order in the downstream Level 2 vector is dependent on the different overhang sequences, as defined by the connector parts used for Level 1 vectors. Since there are up to 6 TUs, we call them “Positions” 1-6, from upstream to downstream. Since the connectors are included in Level 1 plasmids, they cannot be changed upon the transition to Level 2. Therefore, one should take into account the intended architecture of the desired downstream Level 2 plasmid when designing the Level 1 plasmids that will be used to assemble it. To future-proof Level 1 plasmids, it is convenient to use the same connectors for cloning mutually exclusive subunits of a given complex.

A feature of the MoClo Baculo toolkit and this protocol that is advantageous over the method described by Lai et al. 2025 is the simplification of the cloning process of Level 1 plasmids in cases where the downstream application is baculovirus expression. Specifically, we cloned connectors and Tn7 sequences together with the Level 1 vector backbone. The resulting plasmids are called pMB.Pos1-6, and are aimed at the construction of baculovirus Level 1 expression vectors of transcription units that will be assembled into positions 1-6, respectively, in Level 2 plasmids. Hence, all pMB.Pos1-6 plasmids are identical except for the connector sequences, and they are outlined in the figure below:



Therefore, if plasmids pMB.Pos1-6 are used for assembling Level 1 vectors, then the plasmids pMB.001, pMB.003 and the connector parts (ConL/R) do not need to be added into the Level 1 Golden Gate assembly reaction. Instead, the user can add one of the plasmids, pMB.Pos1-6, together with the desired Type 2-4 parts (i.e. promoter, optional N-terminal tag, protein coding sequence, optional C-terminal tag, and terminator). The pMB.Pos1-6 plasmids reduce the number of parts used for the assembly of baculovirus expression Level 1 vectors and, together with the newly added pM.000 entry vector, make MoClo Baculo a

stand-alone toolkit for users who only wish to construct baculovirus expression vectors, without yeast compatibility.

13. Generation of Level 1 plasmids

1. Dilute and store all relevant Level 0 plasmids as 25 fmol/uL in Milli-Q water.
2. Prepare Level 1 reaction as follows:

Level 1 Golden Gate assembly reaction mix	Vol. [ul]
pMB.Pos1-6	1
Promoter	1
CDS	1
Terminator	1
Tag(s)	0-2 (1 ul per tag part, 0 for none).
BsaI	1
3X NEB bridge ligase	4
Milli-Q water	1-3
Total	12

3.

3. Run level 1 reaction in a thermocycler as follows:

Level 1 reaction thermocycler program		
Temperature [degree C]		Time [min]
37		30
30 cycles	16	5
	37	5
37		30
65 (can skip this step if immediately proceeding for a transformation without holding in 10 degrees).		30
10		Hold

4. Transform 3 ul of crude Golden Gate reaction mixture to 50 ul XL1-blue cells and plate on Ampicillin plates.
5. On the next day, pick white colonies and set up a growth for a miniprep.
6. On the next day, carry out a miniprep.
7. Carry out restriction digestion analysis with BsmBI. If the insert is the same size as the backbone, a second enzyme may be needed.
8. If the obtained Level 1 plasmids are intended for the expression of a single protein, then they need to be sequenced. Otherwise, if the produced Level 1 plasmids are intended for the construction of a multi-gene vector, then the plasmids can usually proceed without sequencing for the assembly of a Level 2 plasmid (see next step).

14. Design of Level 2 plasmids

The design of Level 2 plasmids, as described here, is recommended for most users who intend to use the baculovirus system for protein expression, especially if they are new to Modular Cloning. The method we recommend here is based on pre-assembled linker plasmids that are included within the MoClo Baculo Toolkit and were designed to simplify the process of Level 2 vector construction. There are other methods to construct Level 2 plasmids, some of which provide more flexibility to the user, but these other methods require additional parts from the MoClo Yeast toolkit (e.g. connectors and spacer parts) and that are not part of the MoClo Baculo toolkit. Hence, users who would like to experiment with more advanced methods for Level 2 vector construction could source additional parts from the MoClo Yeast toolkit, given its compatibility with the MoClo Baculo toolkit (described by Lai et al. 2025). But as stressed above, the method described below would be suitable for most structural biologists who wish to express and purify protein complexes using the baculovirus system, and the MoClo Baculo toolkit includes all the plasmids required to carry it out.

When constructing Level 2 plasmids using fewer than 6 subunits, linker plasmids must be added to the reaction to compensate for the missing transcription units. For example, if one wishes to express a complex with only two proteins, and these two proteins are aiming to be expressed from two transcription units that will be placed at positions 1 and 2, then a linker plasmid pM.L3456 replaces all the Level 1 plasmids that otherwise would have been placed at positions 3, 4, 5 and 6. These linker plasmids simply include connectors that connect the last transcription unit to the backbone.

We included in the MoClo Baculo toolkit the pre-assembled linker plasmids pM.L3456, pM.L456, pM.L56 and pM.L6. These linker plasmids replace positions 3-6, 4-6, 5-6 and 6, respectively, and therefore allow the cloning of 2, 3, 4 and 5 transcription units into Level 2 plasmids. The inclusion of 6 transcription units in Level 2 plasmids does not require linker plasmids. The expression of a single transcription unit can be done from a Level 1 plasmid, without building a Level 2 plasmid. Therefore, we consider the four linker plasmids that are included in the MoClo Baculo toolkit (pM.L3456, pM.L456, pM.L56 and pM.L6) as sufficient for most users who are interested in baculovirus expression. Specifically, these linker plasmids were designed to allow the assembly of vectors for the co-expression of any number of proteins between 2 to 6 from a Level 2 plasmid, or any number of proteins from 4 to 30 using Level 3 plasmids. However, users who wish to have more flexibility in designing

their own custom linker plasmids can do it using part plasmids from the MoClo Yeast toolkit, which is compatible with the MoClo Baculo toolkit.

We discourage users from skipping positions in cases where one protein is intended to be omitted from the complex. Although technically this can be done (e.g. by replacing a Transcription Unit in position 3 with the pMB.Pos3 plasmid that was described above), in reality, the obtained Level 2 plasmids and their derived bacmids will express GFP in bacterial cells, which may reduce DNA extraction yield.

If the Level 2 plasmids are intended to subsequently be used for Level 3 assembly, through the compatibility with the biGBac system, then the plasmids pMB.BIG1a, pMB.BIG1b, pMB.BIGc, pMB.BIG1d, pMB.BIG1e (in short: pMB.BIG1a-e here) are used to contribute the backbone for Level 2 assembly, instead of the pMB.002 plasmid. pMB.BIG1a-e are part of the MoClo Baculo toolkit and are functionally equivalent to the pBIG1a-e plasmids from the biGBac system (read Lai et al 2025 for more details on constructing Level 3 plasmids using the compatibility between MoClo Baculo to biGBac).

15. Generation of Level 2 plasmids

1. Dilute and store all relevant Level 1 plasmids as 25 fmol/uL in milli-Q water.
2. Prepare Level 2 Golden Gate assembly reaction as follows:

Level 2 Golden Gate assembly reaction mix	Vol. [ul]
Level 1 plasmids	2-6
Linker plasmids	0-1
pMB.002*	1
Hi-T4 ligase	1
BsmBI v2	1
10X Lv2 Rxn buffer	1
MQ	0-3
Total	10ul

*If one wishes to subsequently proceed for Level 3 assembly in the next step, then the pMB.002 plasmid should be replaced here with one of the pMB.BIG1a-e plasmids (compatible with biGbac).

3. Incubate Level 2 Golden Gate assembly reaction in a thermocycler with the following program:

Level 2 reaction		
Temperature, degree		Time, min
50		15
60 cycles	16	5
	50	5
55		30
80 (can skip this step if immediately proceeding for a transformation without holding in 10 degrees)		15
10		hold

4. After the incubation is completed, transform 4 ul crude Golden Gate reaction to 50 ul XL1-blue and plate on spectinomycin plates.
5. On the next day, pick a white colony and culture it, aiming for a plasmid miniprep on the next day.
6. On the next day, perform plasmid miniprep and carry out restriction digestion analysis. We usually use MfeI restriction enzymes, but users can use any other enzyme that can be used to confirm the insertion of transcription units.
7. Subject plasmids that were assembled correctly for a whole plasmid sequencing.
8. If Level 2 plasmids are the intended product, then they can be used for protein expression. If Level 3 plasmids are desired, then proceed to Level 3 plasmid construction using the compatibility between the MoClo Baculo toolkit to the biGBac system.

16. Construction of biGBac Level 3 plasmids

The construction of a Level 3 plasmid from multiple biGBac-compatible Level 2 plasmids is typically required when more than 6 protein subunits are expressed or in cases where open reading frames are extremely long, making multi-gene expression vectors longer than the limitations of Golden Gate assembly (commonly up to 20-30 kbp per plasmid).

Level 3 vector construction is done by a Gibson Assembly reaction, through compatibility with the biGBac system. For more information about the biGBac system, we suggest users who wish to build Level 3 vectors to first read Weissmann et al.:

F. Weissmann, G. Petzold, R. VanderLinden, P.J. Huis in 't Veld, N.G. Brown, F. Lampert, S. Westermann, H. Stark, B.A. Schulman, & J. Peters. biGBac enables rapid gene assembly for

the expression of large multisubunit protein complexes, Proc. Natl. Acad. Sci. U.S.A. 113 (19) E2564-E2569, https://doi.org/10.1073/pnas.1604935113 (2016).

The workflow of Level 3 vector building, below, largely follows Weissmann et al. 2016. However, we find that the purification of digested long DNA (>15 kbp) using QIAquick PCR Purification Kit is very inefficient, as most DNA fragments are not eluted from the DNA binding silica columns. This also exceeds the recommended fragment size by Qiagen (10 kbp). Thus, in Lai et al 2025, we described a method for Level 3 construction without PCR purification of restriction-digested products, as follows:

1. When one minipreps the Level 2 pMB.BIG1a-e plasmids, an extra PE wash is carried out to remove trace salt. Next, plasmids are eluted in Milli-Q water preheated to 60 °C.
2. The pBIG2* plasmids are linearised using PmeI (NEB #R0560) restriction digest reaction at a final plasmid concentration of 10 fmole/µL.
3. Mix 500 fmole of each pMB.BIG1a-e plasmid in a 10 µL reaction of 1x Cutsmart (NEB) containing 1 µL PmeI (NEB #R0560). If the Level 2 plasmids concentration is not high enough, the plasmids mixture is dried using a speedvac and re-dissolved in 9 µL 1x Cutsmart (NEB) with 1 µL PmeI (NEB #R0560).
4. Incubate for digestion: 90 min at 37 °C
5. 1 µL of linearised backbone (pBIG2*) and 1 µL digested pMB.BIG1a-e mix is used to set up a 20 µL Gibson Assembly (NEB #E2611) reaction.
6. Incubate the reaction at 50 °C for 1 hour.
7. 2 uL ligation mix was used to transform 50 uL chemical-competent NEB10-beta cells. Proceed with colony picking and plasmid miniprep while factoring the size of the plasmid (see step 1 above).

* pBIG2 referred here to one of the plasmids pBIG2ab, pBIG2abc, pBIG2abcd or pBIG2abcde from the biGBac kit (Addgene Kit #1000000088). When assembling Level 3 plasmids using two or more pMB.BIG1a-e plasmids, one needs to use the right backbone from the biGBac toolkit, accordingly:

- pBIG2ab for the integration of pMB.BIG1a and pMB.BIG1b.
- pBIG2abc for the integration of pMB.BIG1a, pMB.BIG1b and pMB.BIG1c
- And so forth, as described in Weissmann et al.

17. References

Zhihao Lai, Sarena F Flanigan, Marion Boudes, Chen Davidovich. Modular Cloning of Multigene Vectors for the Baculovirus System and Yeast. *J Mol Biol.* **2025** Apr 1;437(7):168943. doi: 10.1016/j.jmb.2025.168943.

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