Mobius Assembly Supporting Materials and Protocols

Mobius Assembly has three tiers (Level 0, 1, and 2).

Level 0 cloning

In Level 0 cloning, DNA fragments are PCR amplified and cloned into the Mobius Universal Acceptor Vector (mUAV) to form standard parts (Figure 1).

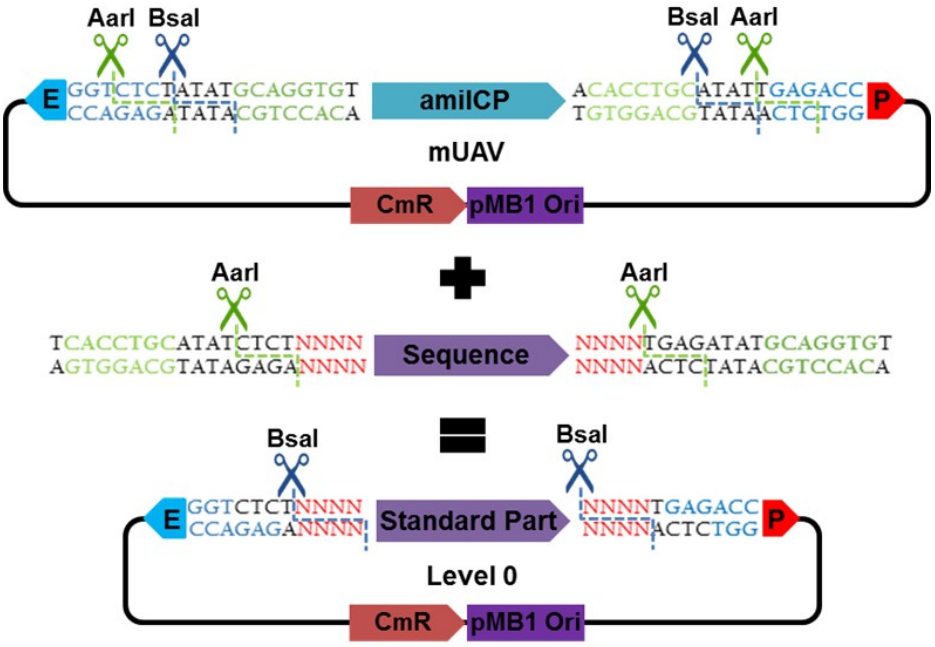

Figure 1. Mobius Assembly standard part generation. (Image from Andreou and Nakayama 2018 PLOS ONE 13(1): e0189892)

Primers for any Level 0 Standard Part should be designed as follows:

FW: TCACCTGCATATCTCTNNNN+(17-30 bp)

RV: (17-30 bp)+NNNNTGAGATATGCAGGTGT

CACCTGC == AarI recognition site

CTCT and TGAG == 5' and 3' Overhang matching to mUAV

NNNN == Standard Phytobrick Overhangs (dependent on the type of parts and final chassis, see Figure 2)

17-30 bp == According to PCR primer design guidelines

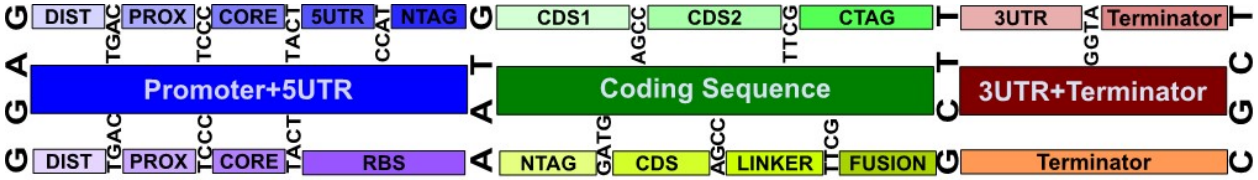

Figure 2. Standard Phytobrick Overhangs (Image from Andreou and Nakayama 2018 PLOS ONE 13(1): e0189892)

Level 1 cloning

In Level 1 cloning, Standard Parts from Level 0 are fused in a Level 1 Acceptor Vector to form a Transcriptional Unit (TU). There are four Level 1 Acceptor Vectors, 1A, 1B, 1Γ and 1Δ. In the following example (Figure 3), a Promoter (Pro), a Coding Sequence (CDS) and a Terminator (Ter) are fused. Successful assembly will result in white colonies because of the replacement of the pink chromoprotein *spisPink*, which is the negative screening marker.

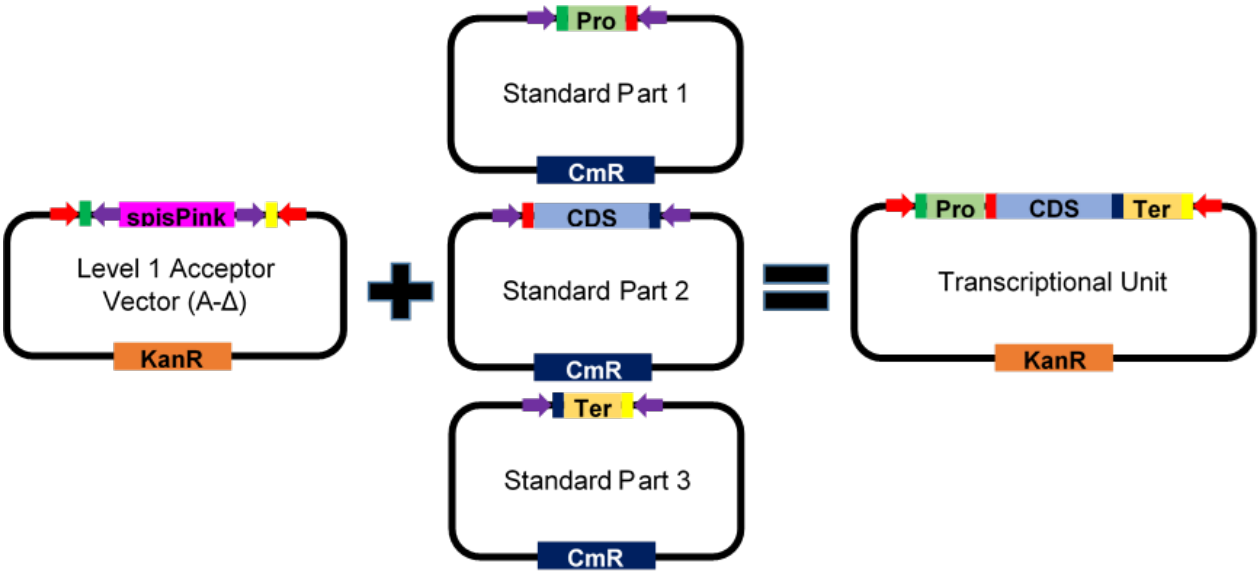

Figure 3. Level 1 assembly of Standard Parts

Level 2 cloning

In Level 2 cloning, (mutli-)TUs from Level 1 are fused in a Level 2 Acceptor Vector with the help of the Auxiliary Plasmids (Figure 4). There are four Level 2 Acceptor Vectors, 2A, 2B, 2Γ and 2Δ. Every Acceptor Vector in Level 2 can take up to four Transcriptional Units (TUs) from Level 1. The Auxiliary Plasmids provide missing overhangs and help the cloning. Successful assembly will result in white colonies because of the replacement of the yellow *sfGFP*, which is the negative screening marker.

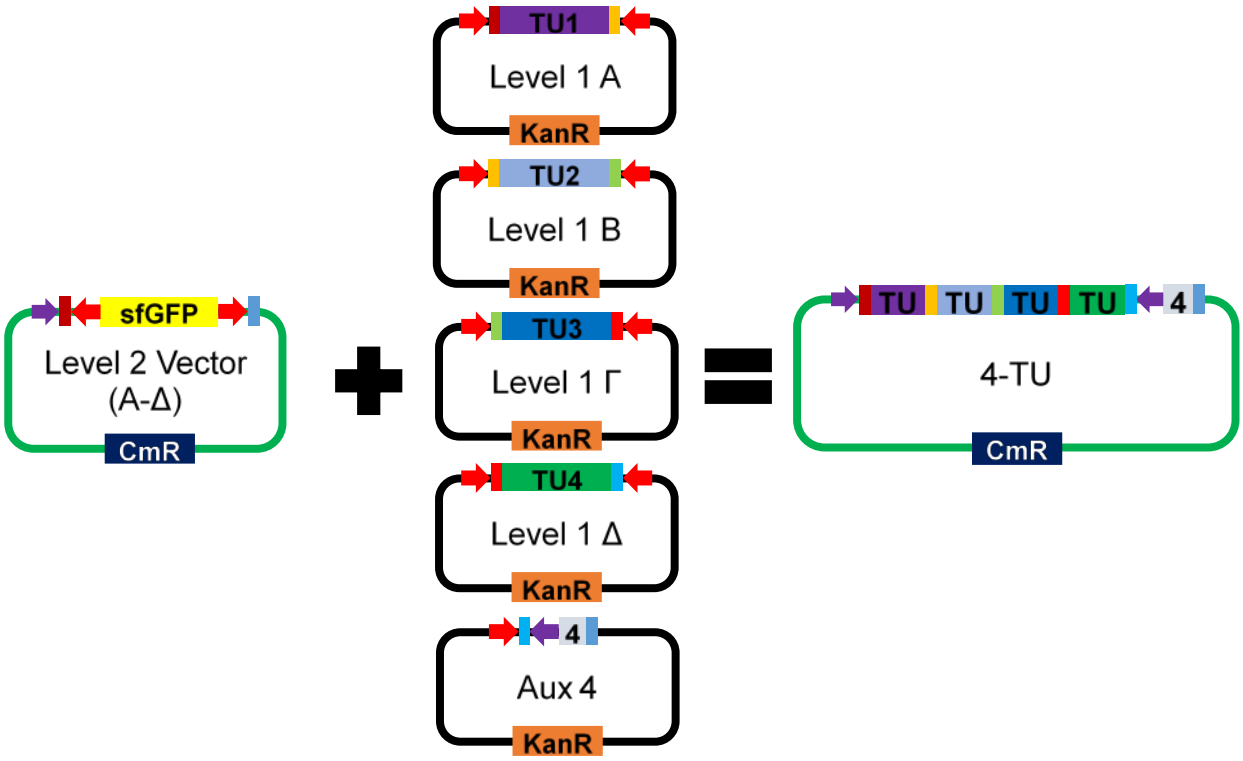

Figure 4. Level 2 assembly of four Level 1 (multi-)TUs

The assembly can continue further by going back to Level 1, where TUs from Level 2 are fused to form a multi-TU. In the following example (Figure 5) four 4-TU constructs (TU1+TU2+TU3+TU4) from Level 2 A, Level 2 B, Level 2 Γ and Level 2 Δ vectors are fused to form a 16-TU construct. Switching back and forth between Level 1 and 2 leads to further expansion of multi-TUs.

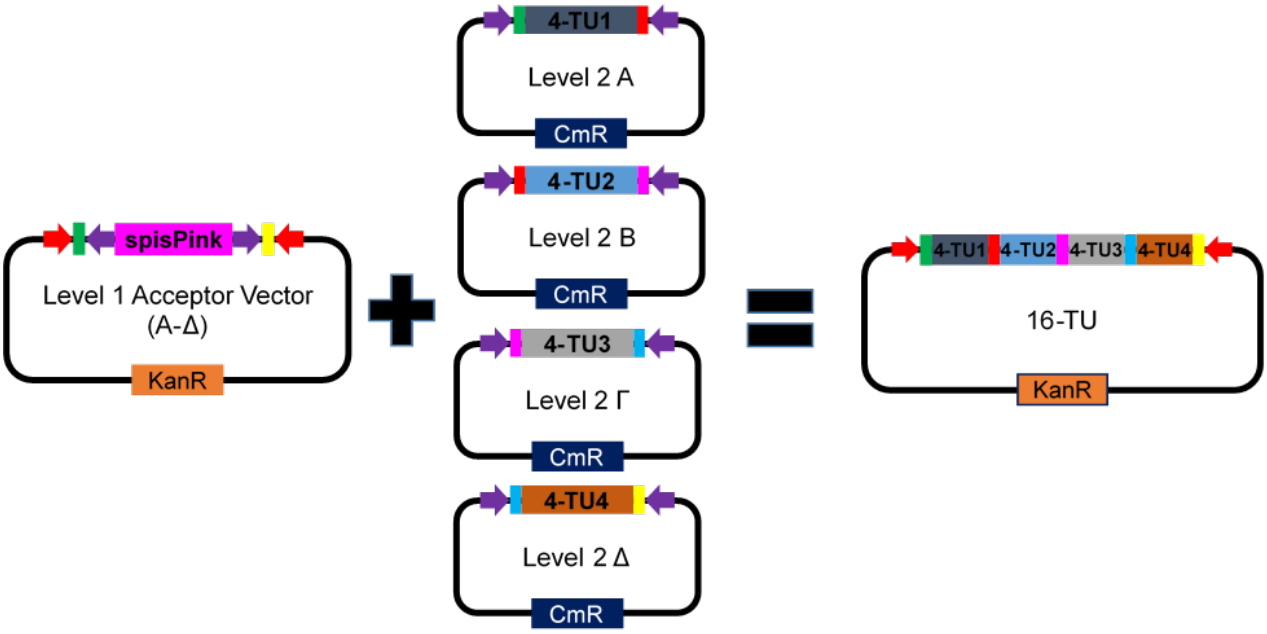

Figure 5. Level 1 assembly of Level 2 multi-TUs

Auxiliary Plasmids

The Auxiliary Plasmids 4A, 4B, 4Γ and 4Δ are used when 4 TUs are assembled in Level 2, and they correspond to the four Level 2 Acceptor Vectors. So, for example if you want to make a 4-TU construct comprising from the TUs ***a***, ***b***, ***c*** *and* ***d***, first you assemble your TUs in Level 1 Acceptor Vectors as follow:

TU *a*	Level 1 Vector A
TU *b*	Level 1 Vector B
TU *c*	Level 1 Vector Γ
TU *d*	Level 1 Vector Δ

Then, TUs ***a***, ***b***, ***c*** *and* ***d*** will be cloned in the Level 2 Vector A to form the 4-TU ***abcd,*** and in the reaction, you will use **Auxiliary Plasmid 4A** (as you assemble 4-TUs in Level 2 Acceptor Vector A)

TU a + TU b + TU c + TU d + Auxiliary 4A	Level 2 Vector A

Should you clone fewer than four TUs in your Level 2 Acceptor Vector, you use the **Auxiliary Plasmids 1, 2 and 3** corresponding to the number of TUs you clone: one, two or three.

Let's say you just want to assemble 3 TUs ***a, b and c***. Again, you will first clone them in Level 1 Acceptor Vectors:

TU *a*	Level 1 Vector A

TU *b*	Level 1 Vector B
TU *c*	Level 1 Vector Γ

As before TUs ***a, b and c*** will be cloned in the Level 2 Vector A to create the 3-TU ***abc*** but this time you will add Auxiliary plasmid 3 in the reaction (as you are assembling 3 TUs).

TU a + TU b + TU c + Auxiliary 3	Level 2 Vector A

A more complex example. Let's say you want to make a 10-TU construct comprising of the TUs ***a, b, c, d, e, f, g, h, i, j***. First you construct each of your TUs in a Level 1 Acceptor Vector:

TU *a*	Level 1 Vector A
TU *b*	Level 1 Vector B
TU *c*	Level 1 Vector Γ
TU *d*	Level 1 Vector Δ
TU *e*	Level 1 Vector A
TU *f*	Level 1 Vector B
TU *g*	Level 1 Vector Γ
TU *h*	Level 1 Vector Δ
TU *i*	Level 1 Vector A
TU *j*	Level 1 Vector B

Next, TUs ***a, b, c, d*** will be cloned in Level 2 Acceptor Vector A (+ Auxiliary 4A, as you assemble 4-TUs in Level 2 Acceptor Vector A), TUs ***e, f, g, h*** in Level 2 Acceptor Vector B (+ Auxiliary 4B, as you assemble 4-TUs in Level 2 Acceptor Vector B) and TUs ***i, j*** in Level 2 Acceptor Vector Γ (+ Auxiliary 2, as you assemble 2 TUs):

TU a + TU b + TU c + TU d + Auxiliary 4A	Level 2 Vector A
TU e + TU f + TU g + TU h + Auxiliary 4B	Level 2 Vector B
TU i + TU j + Auxiliary 2	Level 2 Vector Γ

Lastly, multi-TUs ***abcd***, ***efgh***, and ***ij*** will be assembled in a Level 1 Vector A to form the 10-TU construct

4-TU abcd + 4-TU efgh + 2-TU ij	Level 1 Vector A

Exemption to this rule is when we make constructs of eight or twelve TUs, where in the last Level 2 Acceptor Vector, we **always** add **Auxiliary Plasmid 4Δ** which provides the necessary overhangs for the cloning back to Level 1. I am skipping the cloning in Level 1 and directly showing the assembly in Level 2.

Eight-TU construct:

TU a + TU b + TU c + TU d + Auxiliary 4A	Level 2 Vector A
TU e + TU f + TU g + TU h + Auxiliary 4Δ	Level 2 Vector B

Which back in Level 1 will form the 8-TU ***abcdefgh***:

4-TU abcd + 4-TU efgh	Level 1 Vector A

And twelve-TU construct:

TU a + TU b + TU c + TU d + Auxiliary 4A	Level 2 Vector A
TU e + TU f + TU g + TU h + Auxiliary 4B	Level 2 Vector B
TU i + TU j + TU k + TU l Auxiliary 4Δ	Level 2 Vector Γ

Which back in Level 1 will form the 12-TU ***abcdefghijkl***

4-TU abcd + 4-TU efgh + 4-TU ijkl	Level 1 Vector A

Mobius Assembly protocols

Reaction mix:

Level 0	**Level 1**	**Level 2**
~50 ng mUAV	~50 ng Level 1 Acceptor Vector	~50 ng Level 2 Acceptor Vector
2:1 molar ratio of insert to vector	2:1 molar ratio of insert to vector	2:1 molar ratio of insert to vector
-	-	2:1 molar ratio of Auxiliary Plasmid to vector
1 µl BSA (1mg/ml)	1 µl BSA (1mg/ml)	1 µl BSA (1mg/ml)
1 µl T4 DNA ligase buffer	1 µl T4 DNA ligase buffer	1 µl T4 DNA ligase buffer
0.5 µl AarI	0.5 µl Eco31I (BsaI)	0.5 µl AarI
0.5 µl T4 DNA ligase	0.5 µl T4 DNA ligase	0.5 µl T4 DNA ligase
0.2 µl 50x AarI oligos	-	0.2 µl 50x AarI oligos
to 10 µl H_2O	to 10 µl H_2O	to 10 µl H_2O

Default thermocycling conditions	
5 min 37°C	x5
10 min 16°C	
5 min 37°C	
5 min 80°C	
16°C Hold	

Alternative thermocycling conditions (for large constructs)	
2.5 min 37°C	X40
5 min 16°C	
5 min 37°C	
5 min 80°C	
16°C Hold	

Tips:

➔ To isolate plasmid DNA from any Acceptor Vector, use a single colony from a streaked agar plate and inoculate a bacterial culture. If glycerol stock is used directly as inoculum, the colour of the negative selection marker does not properly develop sometimes.

➔ For isolation of Level 2 Acceptor Vectors (especially with high copy number backbones), we recommend PureYield™ Plasmid Miniprep (Promega) and QIAprep Spin Miniprep Kit (Qiagen). We tested GeneJET Plasmid Miniprep Kit (Thermofisher), but the quantity and purity of the DNA was low, possibly due to the expressed sfGFP that binds on the membrane of the miniprep columns and co-elute with the plasmid DNA.

➔ The freshness of AarI is important for the assembly efficiency. Make aliquots of the enzyme and store accordingly.

➔ Increase the number of digestion/ligation cycles when you deal with assembly of large fragments. We double the cycles when the assembled construct is >10kb and we use the alternative thermocycling conditions when the assembled construct is >20kb.

For more detailed information, see:

Andreou, Andreas I, and Naomi Nakayama. 2018. “Mobius Assembly: A Versatile Golden-Gate Framework towards Universal DNA Assembly.” *PLOS ONE* 13(1): e0189892. https://doi.org/10.1371/journal.pone.0189892.