Single day construction of multi-gene circuits with 3G assembly

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Supplementary Information

Detailed protocol for 3G assembly:

The 3G assembly protocol can be broken into two sections. The first is a set of one-time preparations that result in a library of UNS adapters, primers to amplify from those adapters, and vectors with UNS1-UNS10 flanking sequences for Gibson assembly. The second is the daily pipeline that is performed to assemble multi-gene circuits.

Initial preparations:

Design of UNS adapter sequences

The first step in the 3G assembly pipeline is the design of UNS adapter sequences. For most Golden Gate systems geared towards use in synthetic biology, there are four standard part types (Promoters, 5' UTRs, CDSs, and Terminators), each with a unique overhang sequence that distinguishes them. In the CIDAR MoClo system, which we use in this paper, promoters are distinguished as 'AB' fragments (they have a 4bp 'A' overhang on the left, and a 4bp 'B' overhang on the right), 5' UTRs as 'BC' fragments, CDSs as 'CD' and terminators as 'DE.'[1] Thus, a standard transcriptional unit assembly, in the absence of a backbone, results in an 'A' overhang on the left and an 'E' overhang on the right. In the CIDAR MoClo system, vectors that are designed to receive transcriptional units can be cut with BsaI to reveal 'A' and 'E' compatible overhangs on the vector which then ligate to the insert transcriptional units.

Our goal is to design adapter sequences that contain either a BsaI cutsite that reveals the binding site for the 'A' overhang on the promoter, or the binding site for the 'E' overhang on the terminator, and a landing pad that can be used for priming in PCR and as regions of homology in Gibson assembly. We use the unique nucleotide sequences (UNSs) designed by Torella *et al.* as our landing pads for our 3G assembly method.[2] Below are example designs of a UNS1_A adapter and a UNS10_E adapter.

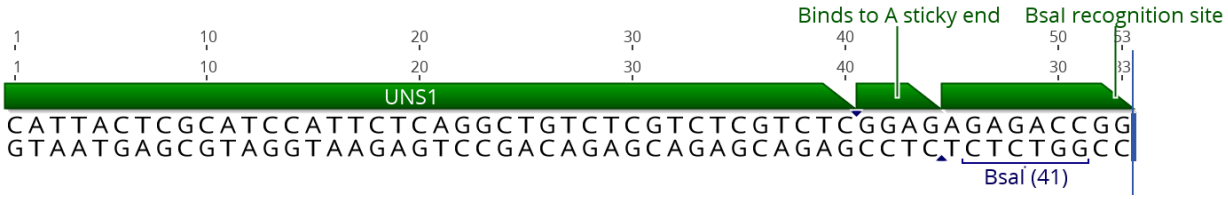

Figure 1: UNS1_A adapter

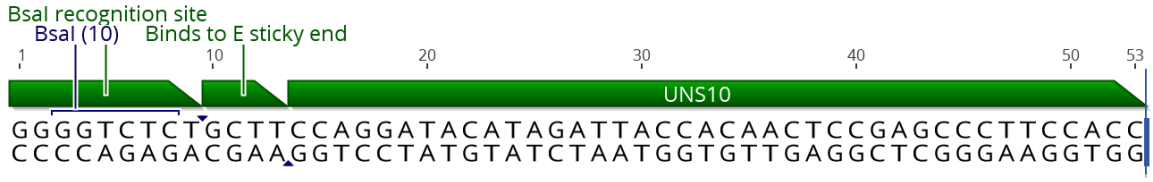

Figure 2: UNS10_E adapter.

These designs are specific to the library developed by Iverson *et al.*[1] For application to other type IIS systems, the sticky end and / or the restriction enzyme used to reveal it will change. However, the entire 40bp UNS sequence will stay the same.

To maximize flexibility, all vectors are flanked by UNS1 and UNS10. Therefore, for every multi-gene assembly the first fragment must have UNS1 on the left and the last fragment must have UNS10 on the right. For example, a one transcriptional unit assembly should look like:

Fragment 1 = UNS1_A – Promoter – UTR – CDS – Terminator – UNS10_E

Multi-transcriptional unit assembly should again have UNS1 on the left of the first fragment and UNS10 on the right of the last fragment, but fragments that are adjacent should share a UNS sequence. For example, in a three transcriptional unit assembly

Fragment 1 = UNS1_A – P1 – UTR1 – CDS1- Terminator1 – UNS2_E
Fragment 2 = UNS2_A – P2 – UTR2 – CDS2 – Terminator2 – UNS3_E
Fragment 3 = UNS3_A – P3 – UTR3 – CDS3 – Terminator3 – UNS10_E

This will result in a final product of:

U1-Transcriptional Unit 1 – U2 – Transcriptional Unit 2 – U3 – Transcriptional Unit 3 – U10

that will then be assembled into a U1-U10 backbone.

To take full advantage of the UNS1 / UNS10 sequences the following adapters must be generated:

1. UNS1_A
2. UNS2_A
3. UNS2_E
4. UNS3_A
5. UNS3_E
6. UNS4_A
7. UNS4_E
8. UNS5_A
9. UNS5_E
10. UNS6_A
11. UNS6_E
12. UNS7_A
13. UNS7_E
14. UNS8_A
15. UNS8_E
16. UNS9_A
17. UNS9_E

18. UNS10_E

This list is reduced by our choice not to use UNS2 in our assemblies. We found that UNS2 and UNS5 would occasionally join together in the final Gibson assembly, so we removed UNS2 from our set of adapters. This leaves:

1. UNS1_A
2. UNS3_A
3. UNS3_E
4. UNS4_A
5. UNS4_E
6. UNS5_A
7. UNS5_E
8. UNS6_A
9. UNS6_E
10. UNS7_A
11. UNS7_E
12. UNS8_A
13. UNS8_E
14. UNS9_A
15. UNS9_E
16. UNS10_E

By designing these adapters, up to eight transcriptional units can be combined in a single day. For even more, custom generation of additional orthogonal UNSs can be performed using publicly available tools.[3]

Each of these adapters is 53bp in total length. We found that the most cost-effective and efficient way to synthesize these sequences was to split each adapter into a top and bottom strand and order them as oligos that we then annealed. All sequences used can be found in Supplementary Table 1.

Annealing UNS adapter oligos:
After receiving single stranded oligos we then anneal them to generate double stranded DNA that can be cut by our type IIS restriction enzyme of choice. We follow IDT's recommended protocol for annealing oligos (https://www.idtdna.com/pages/education/decoded/article/annealing-oligonucleotides).

In brief, we combine the oligos at 5μM in Duplex Buffer (100 mM Potassium Acetate; 30 mM HEPES, pH 7.5). We then heat the mix in a thermal cycler to 94°C for 2 minutes and cool by 1°C every minute. We then dilute the reaction from 5μM to 50nM in 1x TE or nuclease free water. The annealed adapters can now be stored at -20°C until they are needed.

Designing UNS primers:
Based on the UNS adapters we designed above, we also need the following primers:

1. UNS1_Forward
2. UNS3_Forward
3. UNS4_Forward
4. UNS5_Forward
5. UNS6_Forward
6. UNS7_Forward
7. UNS8_Forward
8. UNS9_Forward
9. UNS3_Reverse
10. UNS4_Reverse
11. UNS5_Reverse
12. UNS6_Reverse
13. UNS7_Reverse
14. UNS8_Reverse
15. UNS9_Reverse
16. UNS10_Reverse

All primer sequences were designed for a 64°C annealing temperature using NEB's melting temperature calculator for Q5 (https://tmcalculator.neb.com/#!/main). All primer sequences can be found in Supplementary Table 1.

Preparation of UNS1-UNS10 backbones:
The final preparation is to generate destination vectors that are linear and flanked by the UNS1 and UNS10 sequences on either side. This is easily done by PCRing an existing backbone to linearize it, including overhangs on the primer sequences to add UNS1 on one side of the vector and UNS10 on the other. Before using the UNS1-UNS10 backbones in a Gibson reaction we recommend gel extracting the PCR product to increase efficiency.

Daily pipeline:
Golden Gate assembly:
The first step in the daily pipeline is to assemble the individual transcriptional units of interest, each in their own reaction tube, and add on the adapters we designed above.

As an example, we will design a two transcriptional unit assembly, that will result in the U1 – Transcriptional Unit 1 – U3 – Transcriptional Unit 2 – U10 in our vector backbone.

Reaction 1:
0.5µL UNS1_A (50nM stock)
0.5µL UNS3_E (50nM stock)
0.5µL Promoter 1 (30nM stock)
0.5µL UTR 1 (30nM stock)

0.5µL CDS 1 (30nM stock)
0.5µL Terminator 1 (30nM stock)
0.5µL 10x T4 DNA Ligase Buffer
0.25µL T4 DNA Ligase
0.25µL BsaI
1.0µL nuclease free water

Reaction 2:
0.5µL UNS3_A (50nM stock)
0.5µL UNS10_E (50nM stock)
0.5µL Promoter 2 (30nM stock)
0.5µL UTR 2 (30nM stock)
0.5µL CDS 2 (30nM stock)
0.5µL Terminator 2 (30nM stock)
0.5µL 10x T4 DNA Ligase Buffer
0.25µL T4 DNA Ligase
0.25µL BsaI
1.0µL nuclease free water

Golden Gate conditions:
{37°C for 3 minutes
16°C for 4 minutes}
(9 cycles)
50°C for 5 minutes
4°C hold

Post Golden Gate Amplification:
We now have our individual transcriptional units assembled, and a standard type IIS cloning system would transform our reaction into cells to amplify the product.[1] Instead, in 3G we use the UNS adapters added to each construct to amplify the transcriptional units directly from the Golden Gate reaction. Not only does this have the advantage of saving the time to transform, pick colonies, and miniprep plasmid, PCR keeps any variants included in the Golden Gate reaction (multiple different promoters or UTRs for example), thus allowing the downstream Gibson reaction to generate circuit variants in a single pot.

PCR conditions:
25uL 2x Q5 Hot Start Master Mix
2.5uL 10µM Forward primer
2.5uL 10µM Reverse primer
18.5µL nuclease free water
1.5µL Golden Gate product

Cycling conditions:
98°C for 30 seconds

{98°C for 15 seconds
64°C for 30 seconds
72°C for 30 seconds per kb}
(27 cycles)

72°C for 5 minutes
4°C hold

PCR purification:
PCR products were purified using the QIAquick Gel Extraction kit and eluted in 15μL of elution buffer (Qiagen). We found that gel extraction increases the efficiency of the downstream Gibson reaction.

Gibson assembly:
Gibson assembly was performed using NEBuilder HiFi DNA Assembly Master Mix. All fragments, included vector backbone, were combined at 2nM final concentration in a 5μL reaction and incubated at 50°C for 1 hour.

Transformation:
Transformations were performed using 2μL of the Gibson assembly reaction transformed into 50μL of chemically competent DH5α cells (NEB 5-alpha Competent *E. coli* (High Efficiency)). Cells were outgrown at 37°C in 300μL of SOC, 100 μL of which was plated on LB-Agar and grown overnight at 37°C.

References:
1. Iverson, S. V., Haddock, T. L., Beal, J., and Densmore, D. M. (2015) CIDAR MoClo: improved MoClo assembly standard and new E. coli part library enable rapid combinatorial design for synthetic and traditional biology, *ACS Synthetic Biology 5*, 99-103.
2. Torella, J. P., Boehm, C. R., Lienert, F., Chen, J.-H., Way, J. C., and Silver, P. A. (2013) Rapid construction of insulated genetic circuits via synthetic sequence-guided isothermal assembly, *Nucleic Acids Research 42*, 681-689.
3. Casini, A., MacDonald, J. T., Jonghe, J. D., Christodoulou, G., Freemont, P. S., Baldwin, G. S., and Ellis, T. (2013) One-pot DNA construction for synthetic biology: the Modular Overlap-Directed Assembly with Linkers (MODAL) strategy, *Nucleic Acids Research 42*, e7-e7.

Sequences required for 3G assembly:
To perform 3G assembly using the existing CIDAR MoClo library, UNS adapters corresponding to both A and E overhangs are required. In addition, primer sequences complementary to the UNS adapters are required to amplify the Golden Gate reactions must also be ordered. The table below lists all sequences designed and used for 3G assembly. All primers were ordered without modification and in the smallest scale reaction from Integrated DNA Technologies.

Table 1:

Primer description	Sequence
UNS1_A Top	CATTACTCGCATCCATTCTCAGGCTGTCTCGTCTCGTCTCGGAGAGAGACCGG
UNS1_A Bottom	CCGGTCTCTCTCCGAGACGAGACGAGACAGCCTGAGAATGGATGCGAGTAATG
UNS3_A Top	GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCGGGAGAGAGACCGG
UNS3_A Bottom	CCGGTCTCTCTCCCGACCTTGATGTTTCCAGTGCGATTGAGGACCTTCAGTGC
UNS3_E Top	GGGGTCTCTGCTTGCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG
UNS3_E Bottom	CGACCTTGATGTTTCCAGTGCGATTGAGGACCTTCAGTGCAAGCAGAGACCCC
UNS4_A Top	CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTCGGAGAGAGACCGG
UNS4_A Bottom	CCGGTCTCTCTCCGACTTTGCGTGTTGTCTTACTATTGCTGGCAGGAGGTCAG
UNS4_E Top	GGGGTCTCTGCTTCTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
UNS4_E Bottom	GACTTTGCGTGTTGTCTTACTATTGCTGGCAGGAGGTCAGAAGCAGAGACCCC
UNS5_A Top	GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAGGGAGAGAGACCGG
UNS5_A Bottom	CCGGTCTCTCTCCCTCTAACGGACTTGAGTGAGGTTGTAAAGGGAGTTGGCTC
UNS5_E Top	GGGGTCTCTGCTTGAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
UNS5_E Bottom	CTCTAACGGACTTGAGTGAGGTTGTAAAGGGAGTTGGCTCAAGCAGAGACCCC
UNS6_A Top	CTCGTTCGCTGCCACCTAAGAATACTCTACGGTCACATACGGAGAGAGACCGG
UNS6_A Bottom	CCGGTCTCTCTCCGTATGTGACCGTAGAGTATTCTTAGGTGGCAGCGAACGAG

UNS6_E Top	GGGGTCTCTGCTTCTCGTTCGCTGCCACCTAAGAATACTCTAC GGTCACATAC
UNS6_E Bottom	GTATGTGACCGTAGAGTATTCTTAGGTGGCAGCGAACGAGAAG CAGAGACCCC
UNS7_A Top	CAAGACGCTGGCTCTGACATTTCCGCTACTGAACTACTCGGGA GAGAGACCGG
UNS7_A Bottom	CCGGTCTCTCTCCCGAGTAGTTCAGTAGCGGAAATGTCAGAGC CAGCGTCTTG
UNS7_E Top	GGGGTCTCTGCTTCAAGACGCTGGCTCTGACATTTCCGCTACT GAACTACTCG
UNS7_E Bottom	CGAGTAGTTCAGTAGCGGAAATGTCAGAGCCAGCGTCTTGAAG CAGAGACCCC
UNS8_A Top	CCTCGTCTCAACCAAAGCAATCAACCCATCAACCACCTGGGGA GAGAGACCGG
UNS8_A Bottom	CCGGTCTCTCTCCCCAGGTGGTTGATGGGTTGATTGCTTTGGT TGAGACGAGG
UNS8_E Top	GGGGTCTCTGCTTCCTCGTCTCAACCAAAGCAATCAACCCATC AACCACCTGG
UNS8_E Bottom	CCAGGTGGTTGATGGGTTGATTGCTTTGGTTGAGACGAGGAAG CAGAGACCCC
UNS9_A Top	GTTCCTTATCATCTGGCGAATCGGACCCACAAGAGCACTGGGA GAGAGACCGG
UNS9_A Bottom	CCGGTCTCTCTCCCAGTGCTCTTGTGGGTCCGATTCGCCAGAT GATAAGGAAC
UNS9_E Top	GGGGTCTCTGCTTGTTCCTTATCATCTGGCGAATCGGACCCAC AAGAGCACTG
UNS9_E Bottom	CAGTGCTCTTGTGGGTCCGATTCGCCAGATGATAAGGAACAAG CAGAGACCCC
UNS10_E Top	GGGGTCTCTGCTTCCAGGATACATAGATTACCACAACTCCGAG CCCTTCCACC
UNS10_E Bottom	GGTGGAAGGGCTCGGAGTTGTGGTAATCTATGTATCCTGGAAG CAGAGACCCC
UNS1_Forward	CATTACTCGCATCCATTCTCAGGC
UNS3_Forward	GCACTGAAGGTCCTCAATCG
UNS4_Forward	CTGACCTCCTGCCAGCAATAGT
UNS5_Forward	GAGCCAACTCCCTTTACAACCT
UNS6_Forward	CTCGTTCGCTGCCACCTAAGAA
UNS7_Forward	CAAGACGCTGGCTCTGACATTT
UNS8_Forward	CCTCGTCTCAACCAAAGCAATC
UNS9_Forward	GTTCCTTATCATCTGGCGAATCGGA
UNS3_Reverse	CGACCTTGATGTTTCCAGTGCG
UNS4_Reverse	GACTTTGCGTGTTGTCTTACTAT

UNS5_Reverse	CTCTAACGGACTTGAGTGAGGTTG
UNS6_Reverse	GTATGTGACCGTAGAGTATTCTTAGGTGG
UNS7_Reverse	CGAGTAGTTCAGTAGCGGAAA
UNS8_Reverse	CCAGGTGGTTGATGGGTTGATT
UNS9_Reverse	CAGTGCTCTTGTGGGTCCGAT
UNS10_Reverse	GGTGGAAGGGCTCGGAGTTG

All adapters were annealed following the protocol defined by IDT:
https://www.idtdna.com/pages/education/decoded/article/annealing-oligonucleotides

In figure 2 we tested variants of an inducible cell lysis circuit. The pools of the different promoters and 5' UTRs we used are provided below along with references to the original papers from which they were taken.

Table 2:

Promoters used for LasR	Sequence
J23106[1]	TTTACGGCTAGCTCAGTCCTAGGTATAGTGCTAGC
J23116[1]	TTGACAGCTAGCTCAGTCCTAGGGACTATGCTAGC
J23119[1]	TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC
P11[2]	TTGACAATTAATCATCCGGCTCTTAGTGTTTGTGGA
P13[2]	TTCCCTATTAATCATCCGGCTCGTATAATGTGTGGA
5' UTRs used for LasR	Sequence
BCD2[2]	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTT CGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT
BCD12[2]	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTT CGTACTGAAACATCTTAATCATGCTGCGGAGGGTTTCT
BCD8[2]	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTT CGTACTGAAACATCTTAATCATGCATCGGACCGTTTCT

5' UTRs used for ΦX174E	Sequence
Bujard RBS[3]	GAATTCATTAAAGAGGAGAAAGGTACC
Collins RBS[4]	CAGGACGCACTGACCGAATTCGCATTAAGGAGGTACA
UTR1[5]	AATAATTTTGTTTAACTTTAAGAAGGAGATATA
BCD2[2]	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTT CGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT
BCD12[2]	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTT CGTACTGAAACATCTTAATCATGCTGCGGAGGGTTTCT
BCD8[2]	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTT CGTACTGAAACATCTTAATCATGCATCGGACCGTTTCT

1. Anderson Promoter Library: http://parts.igem.org/Promoters/Catalog/Anderson
2. Mutalik, V. K., Guimaraes, J. C., Cambray, G., Lam, C., Christoffersen, M. J., Mai, Q.-A., Tran, A. B., Paull, M., Keasling, J. D., and Arkin, A. P. (2013) Precise and reliable gene expression via standard transcription and translation initiation elements, *Nature Methods 10*, 354.
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4. Cameron, D. E., and Collins, J. J. (2014) Tunable protein degradation in bacteria, *Nature Biotechnology 32*, 1276.
5. Shin, J., and Noireaux, V. (2010) Efficient cell-free expression with the endogenous E. coli RNA polymerase and sigma factor 70, *Journal of Biological Engineering 4*, 8.