Golden Gate Cloning @ VIB

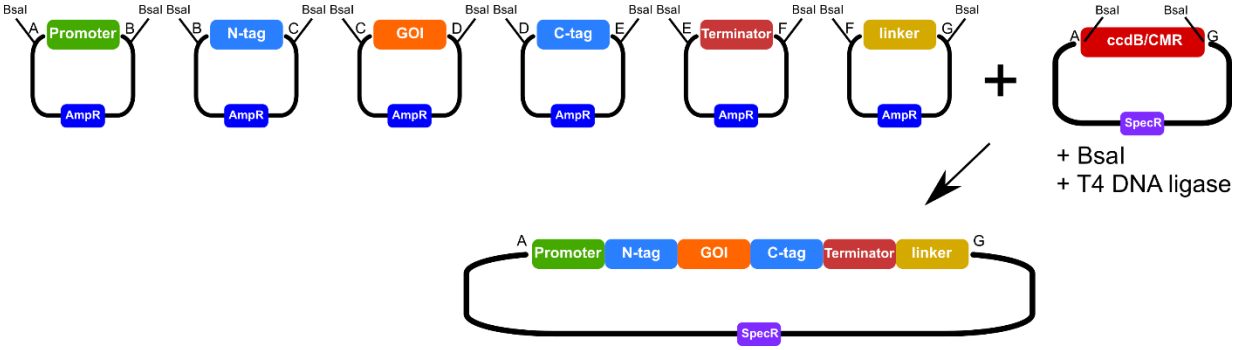
Version 1.0. Updated July 16, 2019

Contents

Article I. Introduction

The general cloning strategy of Golden Gate cloning with the GreenGate method is presented below. Six DNA elements can be assembled into a destination vector using a Golden Gate reaction with the restriction enzyme BsaI and T4 DNA ligase. This results in a final plasmid with the DNA elements in a pre-defined order. The assembly is fast (2 hours), efficient (hundreds of colonies), and inexpensive (€ 1/rxn).

We have been developing and curating a collection of Golden Gate (GG) entry and destination plasmids for PSB. There are also some slight variations on the method that can be useful in certain experimental setups. This manual will go step-by-step on the basics of cloning with Golden Gate, the materials needed, how new modules can be generated and quality control.



Article II. Empty entry vectors

There are six base plasmids (Table 1) in a standard pUC19 plasmid. They have the general structure of

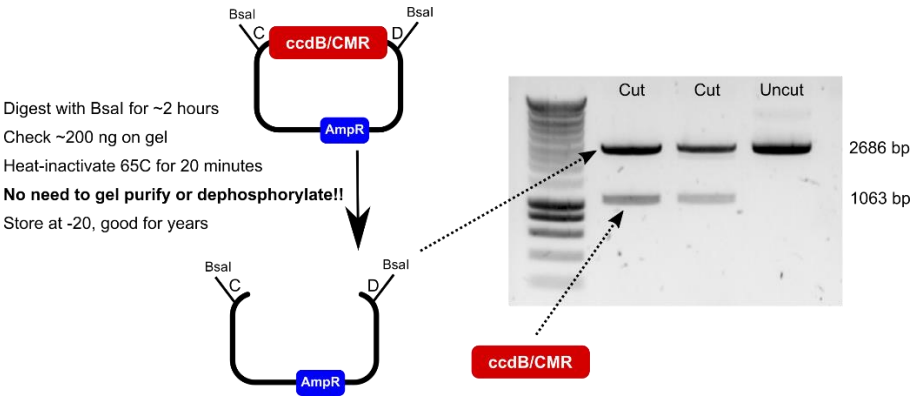
BsaI – Left overhang – *ccd*B/CMR – Right overhang – BsaI

Plasmid	AKA	Collection #	L overhang	R overhang	Function
pGGA000	AB	7-43	ACCT (A)	AACA (B)	Promoters
pGGB000	BC	7-44	AACA (B)	GGCT (C)	N-tags
pGGC000	CD	7-45	GGCT (C)	TCAG (D)	CDS
pGGD000	DE	7-46	TCAG (D)	CTGC (E)	C-tags
pGGE000	EF	7-47	CTGC (E)	ACTA (F)	Terminator
pGGF000	FG	7-48	ACTA (G)	GTAT (H)	Linker/selection

Due to the *ccd*B suicide gene, these plasmids come in DB3.1 cells. Ampicillin (carbenicillin) or chloramphenicol can be used for bacterial selection. Note, we have tried re-transforming these into ccdB-survival cells, but get no useful colonies from this. Should you need to re-transform, use DB3.1.

We recommend making stock BsaI digests for each of the modules. Digest a few micrograms with BsaI for a few hours, verify digestion on gel with an aliquot of the reaction (~200 ng, see figure below), and then heat inactivate the rest of the reaction at 65°C for 20 minutes. These stocks are stable at -20°C for years.

Note: There is no need to column purify the vector or to dephosphorylate. If the *ccd*B insert is re-ligated, the plasmids will kill the *E. coli* host. The overhangs are also not compatible with one another so self-ligation is not a concern.



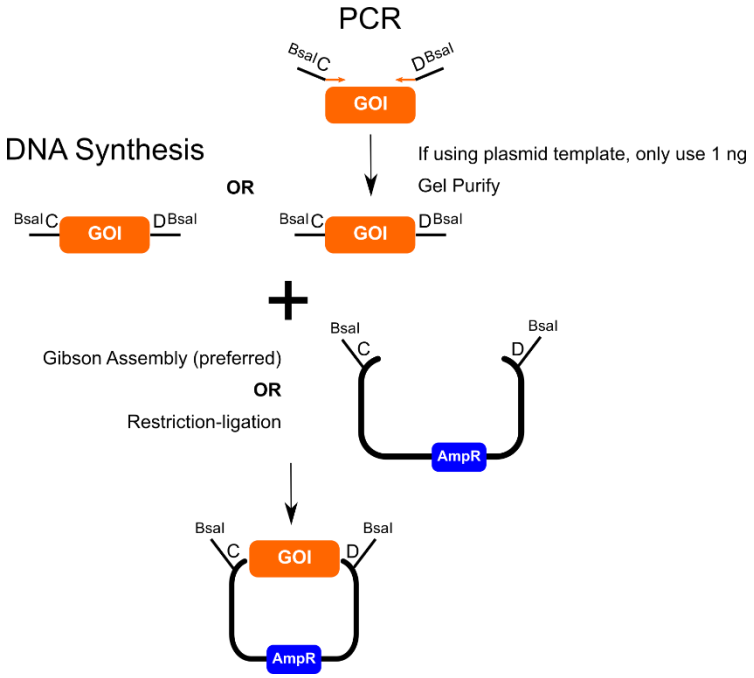
Article III. Entry clone design rules

We strongly discourage deviations from these design rules as it reduces compatibility between groups.

- The six empty entry vectors are designed to assemble a single expression cassette (table 1). Promoters go into AB, N- and C-terminal tags into BC and DE, respectively, your gene of interest into CD, terminators in EF and linkers or selection cassettes in FG.
- N-tags in the BC module must contain a start codon.
- The CDS in the CD module should not contain a stop codon. Start codons are fine.
- To maintain the reading frame in the CD and DE elements, 2 bp are added just before the sequence. For example,
  GGTCTCA*GGCT*CCATG
  The BsaI restriction site is in bold, the C overlap GGCT in italics, the framing sequences underlined, and the start codon highlighted in green.
  This results in a GS between the linker and CDS and should not pose a problem. The S can be swapped with another amino acid if need be.
- The DE module must contain a stop codon.
- Restriction sites to avoid: BsaI must not be present in any element. We also suggest the removal AarI sites (it's not very common) as this can allow for some extra features we have built into the system (more below). To remove these restriction sites, have the sequence synthesized with silent mutations or use site-directed mutagenesis.

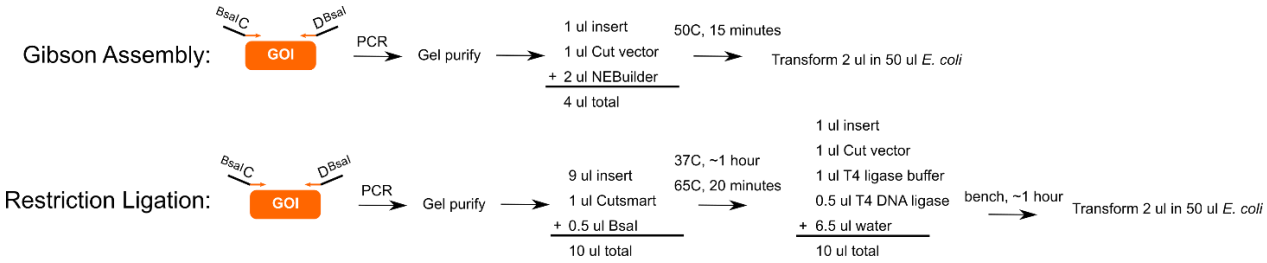
We strongly discourage deviations from these design rules as it reduces compatibility between groups.

Article IV. New entry clones



DNA synthesis or PCR can be used to generate new Golden Gate entry clones. In most cases, if a sequence exists, either as a plasmid, cDNA, genomic fragment, *etc.*, it takes less time and is less expensive to PCR amplify the fragment and clone. If you want to generate a novel sequence, or there are technical limitations restricting the use of PCR, DNA synthesis can be used.

Once the fragment has been generated, there are two options to clone it into an empty vector; Gibson assembly or restriction-ligation. We highly encourage the use of Gibson assembly as it takes less time (~2 hours less) and the cost is the same.



Section 4.01 Gibson assembly

The simplest/fastest/cheapest way to clone new modules is via Gibson assembly. Synthesize or PCR amplify your sequence of interest with at least 20 bp of homology with the entry vector. Below are the 20-bp overlaps to use when synthesizing fragments.

	FOR SYNTHESIS
AB	5′- AGAAGTGAAGCTT**GGTCTC**A*ACCT* – insert – *AACAT***GAGACC**GAATTCTCGCCCT
BC	5′- AGAAGTGAAGCTT**GGTCTC**A*AACA* – insert – *GGCTT***GAGACC**GAATTCTCGCCCT
CD	5′- AGAAGTGAAGCTT**GGTCTC**A*GGCTCC* – insert – *TCAGT***GAGACC**GAATTCTCGCCCT
DE	5′- AGAAGTGAAGCTT**GGTCTC**A*TCAGCT* – insert – *CTGCT***GAGACC**GAATTCTCGCCCT
EF	5′- AGAAGTGAAGCTT**GGTCTC**A*CTGC* – insert – *ACTAT***GAGACC**GAATTCTCGCCCT
FG	5′- AGAAGTGAAGCTT**GGTCTC**A*ACTA* – insert – *GTATT***GAGACC**GAATTCTCGCCCT

	Primers For PCR
AB-F	5′- AGAAGTGAAGCTT**GGTCTC**A*ACCT* – insert forward
AB-R	5′- AGGGCGAGAATTC**GGTCTC**A*TGTT* – insert reverse
BC-F	5′- AGAAGTGAAGCTT**GGTCTC**A*AACA* – insert forward
BC-R	5′- AGGGCGAGAATTC**GGTCTC**A*AGCC* – insert reverse
CD-F	5′- AGAAGTGAAGCTT**GGTCTC**A*GGCT*CC – insert forward
CD-R	5′- AGGGCGAGAATTC**GGTCTC**A*CTGA* – insert reverse
DE-F	5′- AGAAGTGAAGCTT**GGTCTC**A*TCAG*CT – insert forward
DE-R	5′- AGGGCGAGAATTC**GGTCTC**A*GCAG* TTA* – insert reverse
EF-F	5′- AGAAGTGAAGCTT**GGTCTC**A*CTGC* – insert forward
EF-R	5′- AGGGCGAGAATTC**GGTCTC**A*TAGT* – insert reverse
FG-F	5′- AGAAGTGAAGCTT**GGTCTC**A*ACTA* – insert forward
FG-R	5′- AGGGCGAGAATTC**GGTCTC**A*ATAC* – insert reverse

* The DE-R primer should contain a stop codon. Other stop codons can be used.

If you PCR-amplify your fragment, we highly recommend gel purifying the insert. It is the cleanest way to clone and saves a lot of time doing colony PCRs to check for full-length inserts. That being said, un-purified PCR products can be used in a Gibson reaction, at no more than 25% of the mixture. Only do this if there are no primer dimers or additional bands on the gel. Note: We prefer the use of the Zymo gel purification kit over Thermo's. See the reagents list below for details.

Our typical Gibson assembly reaction is as follows:

1 µL insert (we never quantify) + 1 µL digested, heat-inactivated base vector (50 ng) + 2 µL NEBuilder. Combine everything in a PCR tube.

Incubate at 50°C for 15 minutes. Transform 2 µL into 50 µL of *E. coli* DH5α cells.

Section 4.02 Restriction ligation

For those of you who do not wish to embrace the future, restriction ligation can also be used to generate new entry modules.

The same gene synthesis fragments can be used as they contain BsaI sites. The primers can be further shortened. It is advised to add 5 nt to the end of the oligos so the restriction enzyme has "somewhere to sit" on your DNA. I use TATAT, any other T/A combination can be used. G/C is avoided so as not to increase the melting temperature/ form hairpins.

	FOR PCR
AB-F	5′- TATAT**GGTCTC**A*ACCT* – insert forward
AB-R	5′- TATAT**GGTCTC**A*TGTT* – insert reverse
BC-F	5′- TATAT**GGTCTC**A*AACA* – insert forward
BC-R	5′- TATAT**GGTCTC**A*AGCC* – insert reverse
CD-F	5′- TATAT**GGTCTC**A*GGCT*CC – insert forward

CD-R	5′- TATAT**GGTCTC**A*CTGA* - insert reverse
DE-F	5′- TATAT**GGTCTC**A*TCAG*CT - insert forward
DE-R	5′- TATAT**GGTCTC**A*GCAG* TTA* - insert reverse
EF-F	5′- TATAT**GGTCTC**A*CTGC* - insert forward
EF-R	5′- TATAT**GGTCTC**A*TAGT* - insert reverse
FG-F	5′- TATAT**GGTCTC**A*ACTA* - insert forward
FG-R	5′- TATAT**GGTCTC**A*ATAC* - insert reverse

Perform PCR with any high-fidelity enzyme. Gel purify the PCR product.

Digest with BsaI at 37°C for 1-2 hours.

Component	**Amount µL**
PCR product	8.5
Cutsmart	1
BsaI-HF®v2	0.5
Total	**10**

Heat-inactivate the enzyme at 65°C for 20 minutes.

Assemble the ligation reaction.

Component		**Amount µL**
Digested, heat-inactivated PCR product		X
Entry module	50 ng	1
10 XT4 ligation buffer (w/ 10 mM ATP)		1
T4 Ligase	200 U*	0.5
H2O		To 10 ul
Total		**10**

*The number of units will vary for different enzyme sources. 0.5 – 1 µL is sufficient.

Incubate on the bench for 1-2 hours. Less time is possible if the reaction is efficient. If you get no colonies, the reaction can be performed overnight at 16°C.

Transform 2-4 µL into 50 µL of *E. coli* DH5α cells.

Section 4.03 Quality control

After transformation of your new entry clones into *E. coli*, grow up individual colonies in 2-4 mL of LB with 100 mg L^{-1} Carbenicillin. Generally, there should be no need to perform a colony PCR. However, if you are new to these methods or you have empty or truncated inserts or primer dimers, then a colony PCR is a good idea. Use primers outside the cloning sites such as the following 1651 and 1652. Alternatively, M13F/R primers can be used.

1651 AGGGTTTTCCCAGTCACGACGTT
1652 CAGGCTTTACACTTTATGCTTCCGGC

After plasmid prep, quantify by nanodrop and dilute the plasmid to 100 ng/ul. This dilution will make all downstream work much simpler for yourself. Sanger sequence with oligos 1651 and/or 1652. M13F/R primers can be used, but we often find that the very ends of the inserts are missed. Oligos 1651 and 1652 were specifically designed to overcome this limitation and they work very reliably with Eurofins Mix2Seq. Simply send 2 µL of 10 µM primer with 15 µL of the diluted plasmid. If your fragment is > ~1200 bp, internal primers will be needed for sequencing.

Once you have confirmed your entry clone by Sanger, the quality check is done. Stock your plasmid and be sure to make a glycerol stock. Also give a copy to the Plant Genome Editing group so we can add it to the collection.

Article V. Modifying existing entry modules

There usually comes a time when you will need to make a slight change to an existing clone; one or a few SNPs, some additional amino acids, changing the overhangs, *etc*.

Section 5.01 Site-directed mutagenesis with Gibson

There are various methods to perform site-directed mutagenesis of a clone. Here is our preferred method using Gibson assembly. In the figure below, three case examples are given. One for a single site mutation, one when sites far apart need to be changed, and one for insertions into the clone. PCR is the basis for all changes. Design primers with 20-bp overlaps to allow for the assembly of the fragments with Gibson. After gel purification, mix 1 µL of each component. Add an equal amount of NEBuilder to the mix, incubate at 50°C for 15 minutes*, and transform 2 µL into 50 µL of *E. coli* cells.

*Incubate for up to 1 hour when assembling 3 or more fragments.

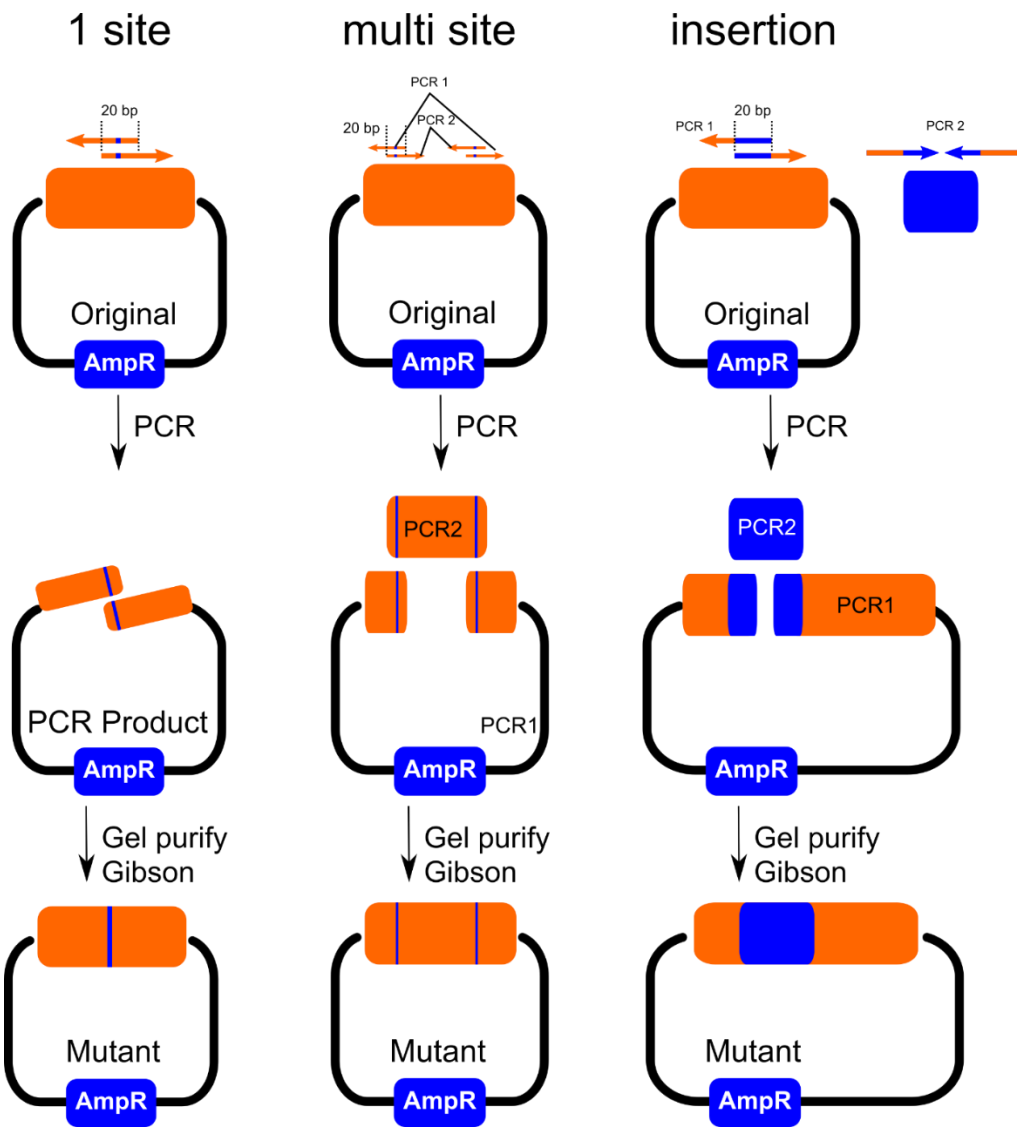
1 site

Component	**Amount µL**
PCR product	1
H_2O	1
NEBuilder	2
Total	**4**

Multisite

Component	**Amount µL**
PCR product 1	1
PCR product 2	1
NEBuilder	2
Total	**4**

IMPORTANT: As the original plasmid and final plasmid contain the same selection (ampicillin), care must be taken to prevent the carryover of the original plasmid. Be sure to use only 1 ng, or less, of plasmid as template for the PCR reaction and gel purify the product. You can also run some template plasmid in a lane next to your PCR products to make sure you get good separation before gel purifying. If these recommendations are followed, there should be no need for colony PCR.



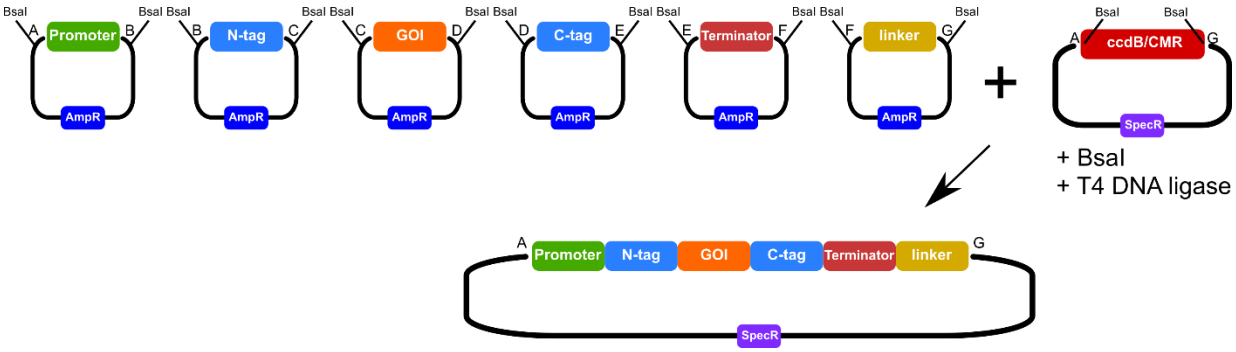
Section 5.02 Swapping entry modules

Article VI. New destination vectors

Coming soon

Article VII. Golden Gate Assembly

Your clones have been sequenced verified and stocked. You are now ready to do the Golden Gate assembly. We find it useful to spell out the six modules plus destination vector as given below. This will ensure that a module is not forgotten and your assembly will work.



Section 7.01 Golden Gate Assembly conditions

Mix the following components in a PCR tube

Component		**Amount**	**Volume (µL)**
A-B entry vector	Promotor	100 ng	1
B-C entry vector	N-tag or Linker	100 ng	1
C-D entry vector	Nuclease	100 ng	1
D-E entry vector	C-tag or Linker	100 ng	1
E-F entry vector	Terminator	100 ng	1
F-G entry vector	**Variable***	100 ng	1
Destination vector		100 ng	1
10X CutSmart Buffer		1X	1.5
10 mM ATP		1 mM	1.5
T4 DNA ligase		200 U	0.5
BsaI-HF®v2		10 U	0.5
H_2O			4
Total			15

* The F-G entry vector used depends on your final goal (end-point expression construct only, performing additional assemblies, selectable marker, etc.). To make an end-point plasmid, the F-linkerII-G module is perfect. If you will need to perform additional assemblies into this plasmid, go for pGG-F-AarI-SacB-AarI-G.

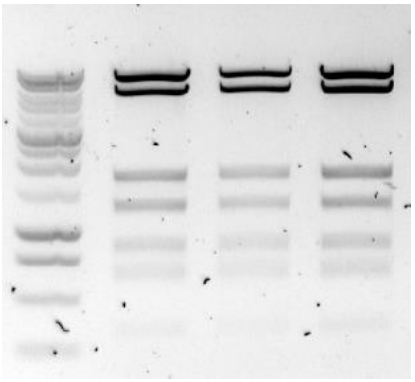
Golden Gate reaction conditions

Reaction conditions are flexible. We regularly use 2 minute steps for the 37°C and 16°C steps with 20 cycles. This reduces the reaction time to ~1 hour and 45 minutes. For very simple reactions, try a single step at 37°C for 1 hour followed by 55°C for 5 minutes. For difficult reactions, increase the reaction time and number of cycles. Don't just take our word for it, check out the NEB protocol.

37°C	2 min	20 x
16°C	2 min	
50°C	5 min	
80°C	5 min	
16°C	∞	

Transform 5 µL of the reaction mixture into 50 µL *ccdB*-sensitive DH5α *E. coli* cells via heat shock. The transformed cells are plated on LB medium containing 100 µg mL^{-1} spectinomycin.

Validate the vector by restriction digest. Ideally the restriction digest will result in a ladder of fragments that are easily sized. For example:



Article VIII. Expanding the kit

Section 8.01 AarI linkers

Coming soon

Section 8.02 Golden Gateway

Coming soon

Section 8.03 Golden Gibson

Coming soon

Article IX. Troubleshooting

Coming soon

Article X. Reagents

Section 10.01 Enzymes and related consumables

Product	Concentration	Provider	Product Number
CutSmart Buffer	10X	NEB	With enzyme
T4 DNA ligase*	400,000 U/mL	NEB	M0202L
BsaI-HF®v2**	20,000 U/mL	NEB	In stock room
BbsI-HF	20,000 U/mL	NEB	In stock room
ATP	10 mM	Thermo	
NheI	10 U/mL	Promega	In stock room
Buffer B	10X	Promega	With enzyme
Acetylated BSA	10 µg/µL	Promega	With enzyme
AarI	2 U/mL	Thermo	ER1581
Buffer AarI	10X	Thermo	With enzyme
Oligonucleotides	50X (0.025 mM)	Thermo	With enzyme
Q5® High-Fidelity DNA Polymerase	2,000 U/mL	NEB	
Q5® Reaction Buffer	5X	NEB	With enzyme
10 mM dNTP's	10 mM	NEB	
One Shot™ ccdB Survival™ 2 T1R Competent Cells		Thermo	See Mansour
ZymocleanTM Gel DNA Recovery Kit		Zymo	D4002

*We have tested T4 DNA ligases from multiple vendors (NEB, Thermo, and Invitrogen). At this time, we have not observed a clear difference in efficiency.

**We have also used the standard BsaI and BsaI-HF versions. We do not observe any differences. Since the cost is the same, we opt for HFv2.

Section 10.02 Antibiotics

Ampicillin (or Carbenicillin)	100 mg L^{-1}
Kanamycin	50 mg L^{-1}
Spectinomycin	100 mg L^{-1}
Gentamicin	25 mg L^{-1}
Chloramphenicol	30 mg L^{-1}

Article XI. Protocols

Section 11.01 Tom's E. coli transformation protocol

This is based on recommendations from NEB (scroll to product information>advantages and features). This should give the highest yield of transformants, but shortcuts can be taken at the underlined steps.

Add cloning reaction mix to 50 µL of chemical competent *E. coli*. Keep on ice 20-30 minutes (can be less). Heat shock at exactly 42°C for 30 seconds. Immediately chill on ice for 5 minutes. Add 300 µL pre-warmed (37°C) SOC (not necessary, but helps). Shake at 37°C for 20-60 minutes (20 minutes is minimum; for AmpR, can be skipped entirely). Plate 75 µL on pre-warmed (37°C) (not necessary, but helps) LB w/ antibiotic plates.

Section 11.02 High-fidelity PCR

Mix the following components in a PCR tube:

Component	**Amount**	**Volume (µL)**
template	1 ng*	1
FW primer (10 µM)	0.5 µM	1
REV primer (10 µM)	0.5 µM	1
dNTP's	200 µM	0.2
Q5® Reaction Buffer	1X	2
Q5® High-Fidelity DNA Polymerase	1U	0.1
MQ		4.7
		10

Reaction conditions:

98°C	30 sec	
98°C	10 sec	34 x
T_a**	30 sec	
72°C	1 min	
72°C	2 min	
16°C	∞	

* Use at most 1 ng of plasmid template. As little as a few fg is also possible. When using genomic DNA, 1 µL of a prep is sufficient.

** My default T_a is 60°C. This works for 95% of amplicons. You may need to go higher.

Section 11.03AarI restriction digest

AarI digestion is performed according to the manufacturer's recommendations.

Component	Amount	Volume
Plasmid DNA (100 ng/µL)	1 µg	10.0 µL
H_2O		7.1 µL
Buffer AarI	1x	2.0 µL
Oligonucleotides	0.5 µM	0.4 µL
AarI	1 U	0.5 µL
		20.0 µL

Incubating at 37°C for ~2 hours or up to overnight. Heat inactivate the AarI enzyme for 20 min at 65°C, or directly run on a 0.8% agarose gel. Include the undigested vector as a control.

Article XII. Common Sequences

Coming soon