Gibson Assembly of CRISPR vectors

Tsai lab, UGA, August 2019

Adapted from Jacobs and Martin (2016) JoVE (110), e53843, doi:10.3791/53843 (2016), with modifications.

Materials:

p201N-Cas9 binary vector (Addgene #59175): this is the vector backbone.

pUC-gRNA shuttle vector (Addgene #47024): this one serves as template for U6 promoter and scaffold.

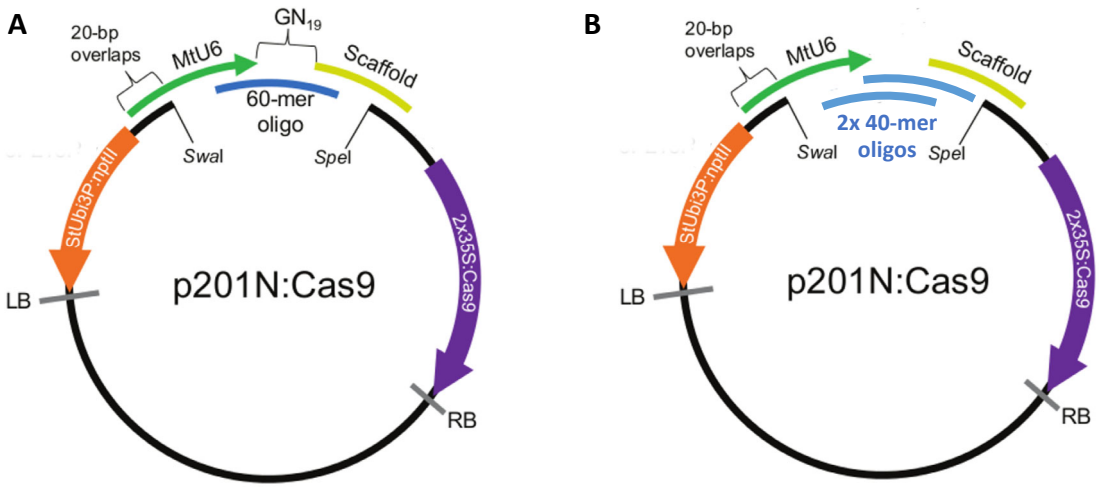
Gibson Assembly Master Mix (NEB E2621S or E2621L). We have also used SGI-DNA kit with success.

Competent cells

Restriction enzymes: SwaI or SpeI

Long-range, high-fidelity Taq polymerase: Q5 (NEB M0492S) or PrimeSTAR GXL (Takara R051A)

Principle: The Gibson assembly method (Gibson et al. 2009 Nat Methods 6: 343-345) makes use of three enzymes, **5' exonuclease**, **DNA polymerase**, and **ligase**, to assemble overlapping DNA fragments in a single isothermal reaction. Any DNA fragments, regardless of sequences, with 15-40 bp overlaps on their ends can be joined together into a plasmid. The linear fragments are first chewed back by exonuclease to expose single-stranded overhangs with complementary sequences. Annealing of the overhangs provides priming sites for DNA polymerase to extend and fill the gaps, followed by ligase repair of the nicks to form a single molecule. A combination of dsDNA fragments and ssDNA oligos can be used.



Panel A shows the original design from Jacobs and Martin (2016) for 4-fragment assembly with p201N vector, MtU6, scaffold (SF, sometimes called hairpin or hp) and a 60-mer oligo containing 20-mer each of U6-gRNA-SF.

Panel B is our preferred design, using two 40-mer oligos (gRNA.U6.F and gRNA.SF.R). These oligos double as gene-specific primers (gRNA) for subsequent cloning verification (along with vector primer).

Tom Jacobs has used pools of oligos (scheme **A**) in a single reaction and observed even distribution of inserts. This can reduce the cost of Gibson mix per gRNA construct. We have not tried this for scheme **B**.

Abbreviated vector sequence at the cloning sites

>p201N-Cas9 (color coded for NPTII-**StUbiP**-SwaI-SpeI-I-PpoI-2X35SP-Cas9)

(NPTII)…aatcaaaatcgttaattatctagatcaagatatatgccctttcctaatgtatttgatacatgcacctaatttcactagatgt
atcttttctatttttaaattatgaatagttaatttttccatatgtgtatttgatacatacttcatgactttaaaaaattaattatatac
cagatatatgtatttaaaatttgttatgtatttaaagtatgtatatgattattc**gatattaatctcttcgatgaaatttaaat**cgataact
atgtgctttggatcgatctgcccactagtgagtcGTATTACAATTCATGACTCTCTTAAGGTAGCCAAAATCGATATCTAGGGATAACAGG
GTAATCGATCACTAGaattcggcgcgccgggcccaacatggtggagcacgacactctcgtctactccaagaatatcaaagatacagtctca
gaagaccaaagggctattgagacttttcaacaaagggtaatatcgggaaacctcctcggattccattgcccagctatctgtcacttcatca
aaaggacagtagaaaaggaaggtggcacctacaaatgccatcattgcgataaaggaaaggctatcgttcaagatgcctctgccgacagtgg
tcccaaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgaacat
ggtggagcacgacactctcgtctactccaagaat…(Cas9)

>pUC-gRNA (color coded for HindIII-MtU6-//-Scaffold/hairpin-EcoRI, // is the gRNA insertion site between U6 and SF)

AGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTATTACCCTGTTATCCCTAGATATCGATTTTGGCTACC
TTAAGGTAGCATGCCTATCTTATATGATCAATGAGGCATTTAATTGGGTGCATATGATGGTGAAAAAAGGTGCAGCTCCTGGCTTGGGAAT
GATGACTCATGTGGAATTTGGTCTTAAATTTATCACATCCTTTTGGGATGTGATGATTGTATCACTTGTTCATTTTGCAAAGACAAGGTGC
ACTGCTACAAACTTTGGTTTAATCTGAAATAAAACAAAACTCACTGAGAGGAAGATGCATCCCAGTAGGTGAAAGTCGAGAAGGATTTGCA
TGTTACTATTACACTTGCTTTTTAGTCCCACATCGTCTGAAACATAAAATATTTCAGCGTTTAAATACTTCAAGCGAACCAGTAGGCTTGT
TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTGCTACCTTAA
GAGAGTCATGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCC
CCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAA

>p201N-4CL1gRNA (from successful Gibson assembly)

(NPTII)…aatcaaaatcgttaattatctagatcaagatatatgccctttcctaatgtatttgatacatgcacctaatttcactagatgt
atcttttctatttttaaattatgaatagttaatttttccatatgtgtatttgatacatacttcatgactttaaaaaattaattatatac
cagatatatgtatttaaaatttgttatgtatttaaagtatgtatatgattattc**gatattaatctcttcgatgaaatttATGCCTATCTTA
TATGATCAATGAGG**CATTTAATTGGGTGCATATGATGGTGAAAAAAGGTGCAGCTCCTGGCTTGGGAATGATGACTCATGTGGAATTTGGT
CTTAAATTTATCACATCCTTTTGGGATGTGATGATTGTATCACTTGTTCATTTTGCAAAGACAAGGTGCACTGCTACAAACTTTGGTTTAA
TCTGAAATAAAACAAAACTCACTGAGAGGAAGATGCATCCCAGTAGGTGAAAGTCGAGAAGGATTTGCATGTTACTATTACACTTGCTTTT
TAGTCCCACATCGTCTGAAACATAAAATATTTCAGCGTTTAAATACTTCAAGCGAACCAGTAGGCTT**GAGGATGTTGAAGTCTGGA**GTTTT
AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTgagtcGTATTACA
ATTCATGACTCTCTTAAGGTAGCCAAAATCGATATCTAGGGATAACAGGGTAATCGATCACTAGaattcggcgcgccgggcccaacatggt
ggagcacgacactctcgtctactccaagaatatcaaagatacagtctcagaagaccaaagggctattgagacttttcaacaaagggtaata
tcgggaaacctcctcggattccattgcccagctatctgtcacttcatcaaaaggacagtagaaaaggaaggtggcacctacaaatgccatc
attgcgataaaggaaaggctatcgttcaagatgcctctgccgacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaa
agaagacgttccaaccacgtcttcaaagcaagtggattgatgtgaacatggtggagcacgacactctcgtctactccaagaat…(Cas9)

Primers for cloning (always 5′ → 3′ unless noted):

Vector	p201N-SwR	**atttcatcgaagagattaatatc**gaataatc (SwaI site is lost)
	p201N-SpF	gagtcGTATTACAATTCATGACTC (immediately after SpeI)
MtU6	SwaI.MtU6F	**gatattaatctcttcgatgaaatttATGCCTATCTTATATGATCAATGAGG**
	MtU6R	AAGCCTACTGGTTCGCTTGAAG
SF	SpeI.hpR	GTCATGAATTGTAATACgactcAAAAAAAGCACCGACTCGGTG
	hpF	GTTTTAGAGCTAGAAATAGCAAGTT
gRNA	gRNA.U6.F	TCAAGCGAACCAGTAGGCTT**GAGGATGTTGAAGTCTGGA**
example	gRNA.SF (reverse complement below)	GAGGATGTTGAAGTCTGGAGTTTTAGAGCTAGAAATAGCAAGTT
	gRNA.SF.R	AACTTGCTATTTCTAGCTCTAAAAC**TCCAGACTTCAACATCCTC**

Primers for colony PCR and sequencing confirmation:

StUbiP-R	acatgcacctaatttcactagatg	+	gRNA.SF.R (gene-specific)	= 618 bp
2X35Shyb	ctccaccatgttcacatcaatc	+	gRNA.U6.F (gene-specific)	= 567 bp

Vector preparation: Use SpeI and/or SwaI pre-cut p201N vector as template for PCR. This will prevent original vector 'bleed-through' during *E. coli* transformation and resulting in false positive colonies.

Restriction digestion (you may use only one restriction enzyme, but vector could self-ligate during Gibson assembly)

p201N (500 ng)	__ µl
NEBuffer 2.1 (10X)	5 µl (50 mM NaCl final)
HF SpeI	1 µl (add 0.3 µl every 30 min) **incubate at 37°C**
ddH_2O	to 50 µl
NaCl (5M)	2 µl (add 50 mM NaCl to 100 mM final)
HF SwaI	1 µl (add 0.3 µl each, every 30 min then overnight) **incubate at 25°C**

Check 1 µl on a gel for completion of digestion. **No need to heat-inactivate enzymes or perform any clean-up.** Store as is (10 ng/µl) at -20°C. Dilute an aliquot to 1 ng/µl for use as template (diluted DNA does not keep well with multiple freeze-thaw cycles). **Test a 10 µl reaction first before you scale up.**

PCR with Q5 2X Master Mix

Pre-cut vector (1 ng/µl)	1 µl
2X Q5 master mix (add this last)	25 µl
p201N-SwR (5 µM)	5 µl
p201N-SpF (5 µM)	5 µl
ddH_2O	to 50 µl

PCR conditions: 98°C/30 sec, 30 cycles of
[98°C/10 sec, TmC*/15 sec, 72°C/10 min**]
hold at 25°C (DNA is stable, no need to hold at cool temp)
*Use the NEB Tm calculator to determine the annealing temp.
**Q5 extension time is 40-50 sec/kb for long amplicons.

or

PCR with PrimeSTAR GXL Premix

Pre-cut vector (1 ng/µl)	1 µl
2X GXL premix (add this last)	25 µl
p201N-SwR (5 µM)	2 µl
p201N-SpF (5 µM)	2 µl
ddH_2O	to 50 µl

98°C/30 sec,
[98°C/10 sec, 68°C/10 min]* x30 cycles
hold at 25°C (DNA is stable, no need to hold at cool temp)
*Can also be: [98°C/10 sec, 60°C/15 sec, 68°C/10 min] x30
See Takara PrimeSTAR GXL manual for detail.

Check 1 µl on a gel for QC. **Clean** amplification is needed for successful cloning (no smearing). We had used Q5 with success in the past, but recent results have been inconsistent especially for large vectors. GXL works well.

Vector cleanup: Perform ethanol precipitation and resuspend in ddH_2O to 50 ng/µl.
NEVER use any column or gel purification kit for binary vector purification. The yield is usually too low.

U6 and scaffold fragment preparation: Amplify MtU6 and scaffold by PCR using their respective primers with a high-fidelity enzyme. The pUC-gRNA shuttle vector (uncut) can be used as a template since it is Ampicillin resistant and any carryover will be selected against (by Kanamycin).

PCR with Q5

Vector (1 ng/µl)	1 µl
2X Q5 master mix (add this last)	10 µl
For. primer (5 µM)	2 µl
Rev. primer (5 µM)	2 µl
ddH_2O	to 20 µl

PCR conditions:
98°C/30 sec,
[98°C/10 sec, TmC*/10 sec, 72°C/10 sec**] x30 cycles
hold at 25°C (DNA is stable, no need to hold at cool temp)
*Use the NEB Tm calculator to determine the annealing temp.
**For short amplicons from simple templates, extension is ~10 sec/kb.

QC on a gel to ensure single bands. Column-purify the fragments (or by EtOH precipitation).

Note, PCR products can be used directly **without further purification** especially for the vector (use gel QC to estimate yield against molecular weight ladders), as long as it is relatively pure (no non-specific products) and is **less than 20%** of the final Gibson assembly volume. We have done this successfully when vector prep is difficult.

Gibson assembly: Perform 4-fragment assembly (vector, U6, SF, and oligo duplex) in a 10 ul reaction (**half rx**).

Assembly with the NEBBuilder HiFi master mix kit (vector: other fragments = 1:20)

p201N vector (14,832 bp), fixed at **50 ng***	1 µL (50 ng)	= 0.005 pmol
MtU6 (377 bp)	1 µL (25 ng)	= 0.1 pmol
Scaffold (106 bp)	1 µL (6 ng)	= 0.1 pmol
U6-gRNA oligo, 0.1 µM	1 µL	= 0.1 pmol
gRNA-SF oligo, 0.1 µM	1 µL	= 0.1 pmol
2X Mix	5 µL	
H2O	to 10 µL	

*Because obtaining high quality binary vector is often the rate-limiting step, we fix binary vector at 50 ng for all of our assembly reactions. Use NEBBioCalculator to calculate the corresponding DNA moles.

Set thermal cycler to hold at 50°C for 1 hr.

***E. coli* transformation:** Use 3 µl of Gibson reaction for transformation.

1. Quickly transfer a competent cell tube from -80 onto ice. If you only have a single reaction, add the reaction mixture to the top of still-frozen competent cells. After thawing, mix gently by tapping the tube 2-3 times and let it sit on ice for 30 min (do not mix). For up to 3 reactions going at once, allow the competent cells to thaw on ice briefly, and gently pipet 1/3 into a new tube that already contains the Gibson reaction. Mix gently by pipetting up and down 2-3 times. Incubate on ice for 30 min. **DO NOT disturb the tube(s) from this point on, be gentle!** Be sure to record the *E. coli* strain in your bacterial clone record.
2. Heat shock at 42°C for 30 sec (up to 2 min OK). Because the incubation time is very short, it is often easier to warm up water in a beaker using the microwave and adjust temp to 42°C (with cold water if overheated). Hold the floating rack in place during the incubation (not swirling around).
3. Transfer the tube to ice for 2 min. Add 500 µL of room temp SOC or LB media to each tube. Incubate at 37°C with shaking for 1 hr.
4. Warm selection (Kan) plates to room temp in a laminar flow. Spread 100 µl cells onto the plate and incubate overnight at 37°C. You may spread different volumes (e.g., 50 µl and 200 µl) to better capture varying levels of assembly/transformation efficiency.

Colony PCR and Sanger sequencing

Colony Lysis buffer	**stock**	10ml
1% Triton X-100	100%	100 µl
20 mM Tris-HCl, pH8.5	500 mM	400 µl
2 mM EDTA	100 mM	200 µl
ddH_2O		9.3 ml

1. Aliquot 50 µl of lysis buffer into each tube.
2. Number the colonies. Pick a part of the colony with a pipet tip and mix with lysis buffer by pipetting up and down several times. Re-incubate the plate at 37°C for a few hrs.
3. Incubate at 95°C (or using boiling water on a hot plate) for 10 min. Centrifuge for 2 min at max. speed at room temp.
4. Use 1 µl lysate for colony PCR (10 µl reaction). Stored the rest at -20°C (optional, transfer supernatant to new tubes).
5. Prepare **a master mix** for PCR using GoTaq or other standard Taq (with 10% extra to account for pipetting error).
6. Check 2-5 µl on a gel.

		x 22 (example for 20 colonies)	
Lysate	1 µl	-	PCR conditions:
2X GoTaq master mix (add this last)	5 µl	110	95°C/2 min,
For. primer (5 µM)	0.3 µl	6.6	[95°C/30 sec, Tm*/30 sec, 72°C/30 sec**] x30 cycles
Rev. primer (5 µM)	0.3 µl	6.6	hold at 25°C (DNA is stable, no need to hold at cool temp)
ddH_2O	3.4 µl	74.8	*Use primer Tm-2°C.
			**Extension time is 1 min/kb