

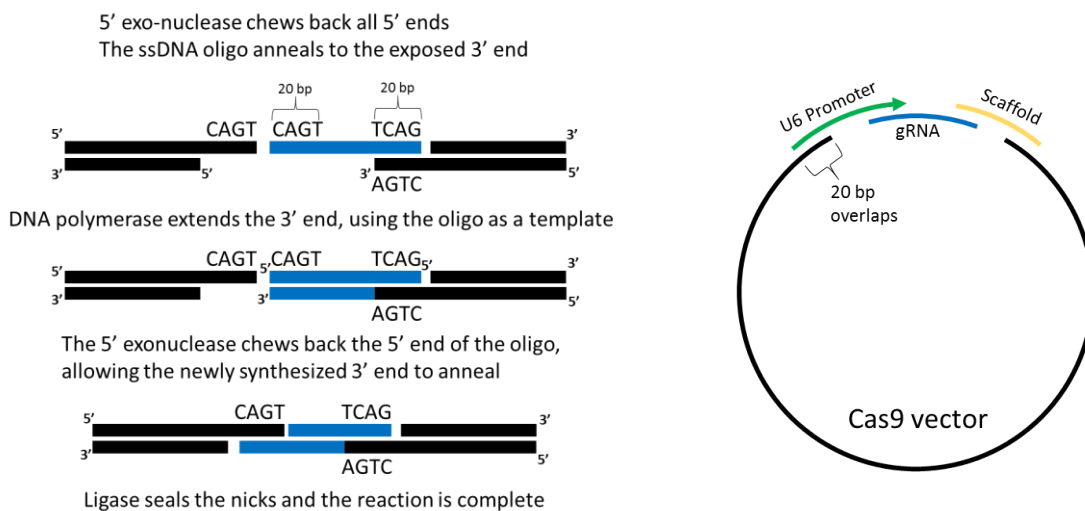
NEBuilder® Assembly of CRISPR vectors using ssDNA oligos

By: Thomas Jacobs, Boyce Thompson Institute for Plant Research, April 2015

Email questions to: tbj26@cornell.edu

This protocol is a follow-up to the one reported in [Jacobs et al., 2015](#). It is superior in several ways: new gRNAs are created and inserted into final CRISPR vectors in a single cloning step, hands-on time is minimal, and multiple gRNAs can be created in a single reaction. Restriction enzymes are only used for vector preparation.

Rationale: The NEBuilder assembly mix contains three enzymes; 5' exo-nuclease, DNA polymerase, and ligase. DNA fragments with 15-40 bp overlaps on their ends are mixed together with assembly mix and are recombined to form a single molecule. The 5' exo-nuclease chews back 5' ends, allowing complementary ends to anneal. The DNA polymerase fills in the gaps, and the ligase seals the nicks, producing a single molecule. This approach allows ssDNA oligos to be used as well.



The primers listed are for the [p201 Cas9 vectors](#) (Addgene 59175-59178). Four DNAs are mixed together, each with 20-bp overlaps; U6 promoter, gRNA ssDNA 60-mer oligo, scaffold, and p201 vector. The U6 and scaffold DNAs are made by PCR and the p201 vector is digested. Pools of oligos can be used in a single reaction, thus reducing the cost per gRNA. I have observed even distributions of inserts from a single reaction.

Materials:

- NEBuilder (E5520) or NEB Gibson assembly (E2611). I have frequently observed SNPs immediately adjacent to the overlaps with Gibson; NEBuilder is highly recommended.
- Competent cells. I have had success with chemical and electro competent DH5α and 10β.
- Restriction enzymes *SpeI* and/or *SwaI*.

Primers, underline is 20-bp overlap

SwaI_MtU6F: GATATTAATCTCTTCGATGAAATTTATGCCTATCTTATATGATCAATGAGG

MtU6R: AAGCCTACTGGTTCGCTTGAAG

ScaffoldF: GTTTTAGAGCTAGAAATAGCAAGTT

SpeI_Scaffold R: GTCATGAATTGTAATACGACTCAAAAAAAGCACCGACTCGGTG

For 201G and 201B, use in place of Swa1_MtU6F

35SSpeI_MtU6F: CGTGCTCCACCATGTTGGGAATGCCTATCTTATATGATCAATGAGG

ssDNA gRNA Oligo design and prep

Oligos to the sense or antisense strand work. I have noticed more colonies with antisense.

For a sense oligo: TCAAGCGAACCAGTAGGCTT—GN₁₉—GTTTTAGAGCTAGAAATAGC

For antisense oligo: GCTATTTCTAGCTCTAAAAC—N₁₉C—AAGCCTACTGGTTCGCTTGA

Add 1 µL of each 100 µM oligo to 500 µL 1X NEB buffer 2 or 2.1. I've pooled 11 oligos with success.

Vector prep

For p201N and H Cas9, use *SwaI* digestion followed by cleanup and *SpeI* digestion. NEB recommends vector digest with two enzymes to limit vector re-circularization (it happens). p201G and B Cas9 do not carry a *SwaI* site in the MCS, and will need a single *SpeI* digest (and/or *I-PpoI*, will require different overlaps). Digest at least 2 µg and check on a gel. Heat inactivation or cleanup is not required after *SpeI* digest. Dephosphorylation is not required.

U6 and scaffold amplicon prep

PCR amplify MtU6 (377 bp) and scaffold (106 bp) using their respective primers with a high-fidelity polymerase. pUC gRNA shuttle can be used as a template since it is amp resistant, so any plasmid carryover will be selected against. Run large reactions to get a lot of product for future use. Ensure single bands on a gel and clean up (cut bands if necessary).

NEBuilder reaction

Set thermal cycler to hold at 50°C.

p201 vector (14,349 bp)	~100 ng	0.011 pmol *
MtU6 amplicon (377 bp)	50 ng	0.2 pmol
Scaffold amplicon (106 bp)	12 ng	0.2 pmol
ssDNA oligo (60-mer)	1 µL	0.2 pmol
2x Mix	5-10 µL	

H₂O to final volume of 10-20 µL (20 is recommended, I've gone as low as 10)

* NEBuilder protocol calls for equimolar amounts of DNA, at 0.2 pmol each for 4 or more fragments. This amount of vector works fine in my hands. I also tried using less of the PCR amplicons, but efficiency was reduced.

Mix and place in cycler for 1 hour. Transform 2 µL into 50 µL chemical competent cells or 1 µL into electrocompetent cells. Plate on LB Kan₅₀.

Next day, screen colonies with Ubi3p218R (ACATGCACCTAATTTCACTAGATGT) and ISceIR (GTGATCGATTACCCTGTTATCCCTAG) primers (for 201N and H). Insert is 725 bp, no insert is 310 bp. Plasmid prep and diagnostic digest with HF-EcoRV and HF-Styl. Fragments are 4192, 2001, 1743, 1544, 1381, 995, 831, 702, 460, 423, 318, 174.