

Supplementary File 1

Protocol for gene targeting with Cas9

I) Digesting the plasmid with BpII:

66ul TE
20ul plasmid (~ few ug) from a standard miniprep that has a yield of 50-100ng/ul
10ul Tango buffer
2ul 50x SAM
2ul BpII enzyme
100ul total
Incubate overnight at 37C.
After the digest, gel purify the cut plasmid.

II) duplexing the oligos:

a) Order the oligos the following way:

Example target sequence: 5'CCCAGGTATTGTTAGCGGTTTGAACGCTGCAGG 3'
Select the 20 nt sequence flanked by an NGG sequence (Cas9 PAM). This 20 nt sequence will be the gRNA sequence that will be inserted in the BpII site in bRA plasmid. Add **GTTTT** to the 3' end of the forward oligo. Take the reverse complement of the 20nt sequence and add **GATCA** to the 3' end of the reverse oligo (see the colored sequences below). When these complementary sequences are duplexed, these additional sequences will serve as overhangs that are complementary to the BpII sites.

Oligo1 (Forward) for the example target sequence:

5'GTTAGCGGTTTGAACGCTGC**GTTTT**3'

Oligo2 (Reverse) for the example target sequence :

5'GCAGCGTTCAAACCGCTAAC**GATCA**3'

b) Resuspend each of the oligos in 1X TE to bring the concentrations to 100uM

c) Duplexing the oligos:

5ul Oligo1 (100uM)

5ul Oligo2 (100uM)

2ul of ligase buffer

8ul TE

20ul total

Heat up the oligos to 100°C, using a water or sand bath or PCR machine for 5 minutes and let it cool down to room temperature.

Duplexed oligos in the above concentration (25uM) are more than 1000-fold concentrated than the BpII-digested plasmid (5ng/ul). So you have to serially dilute the duplexed oligos more than 1000-fold before setting up the ligation reaction.

III) Ligation:

3ul gel purified vector (~5ng/ul)

5ul ~1000-fold diluted duplexed insert

1ul 10x ligase buffer

1ul ligase

10ul total (incubate for 1 hour at room temperature)

IV) Transformation into bacteria (standard protocol)

Thaw 100 ul competent cells on ice.

Add it directly onto 10ul ligation reaction.

Incubate on ice for 15mins, heat shock at 42C for 90 seconds, then incubate on ice for 5 minutes.

Recover the cells in 500 ul LB at 37 C for 45 minutes and plate onto LB-amp plates.

Note: It is a good idea to set up a control reaction with no gRNA insert to see the frequency of self-ligation. The colonies that pop up on the control plate can also be used as negative control for the colony PCR indicated below.

V) Check the insertion of gRNA into bRA plasmids by colony PCR using the following primers

a) Forward gRNA oligo (5'NNN...NNN**GTTTT 3'**)

b) AGCTGAATGAAGCCATACCAAACGA (reverse complement to the β -lactamase gene). The percent of PCR positive colonies is ~50-70%.

VI) Sequence verify

Pick two colonies confirmed by PCR, and extract the plasmids. Send them for sequencing for further confirmation. Universal M13 F primer can be used for sequencing. Check for the disruption of BpII cut site and make sure that gRNA sequence is inserted.

Integration of mutations to the genome by using Cas9. In our experience, variations of this method can be used to introduce small (~100 bp) or large (~800 bp and probably larger) deletions, insertions, or point mutations. For de novo mutations, 80mers or gblocks (larger dsDNA oligomers) can be used. But any DNA sequence that has homology to the site that you want to manipulate can serve as a template for Cas9-directed gene manipulation. For example, this template sequence can be amplified from a plasmid and a PCR product can be transformed in the cell together with the Cas9 plasmid.