gRNA design and CRISPR vector construction

Vectors and protocol by: Thomas Jacobs

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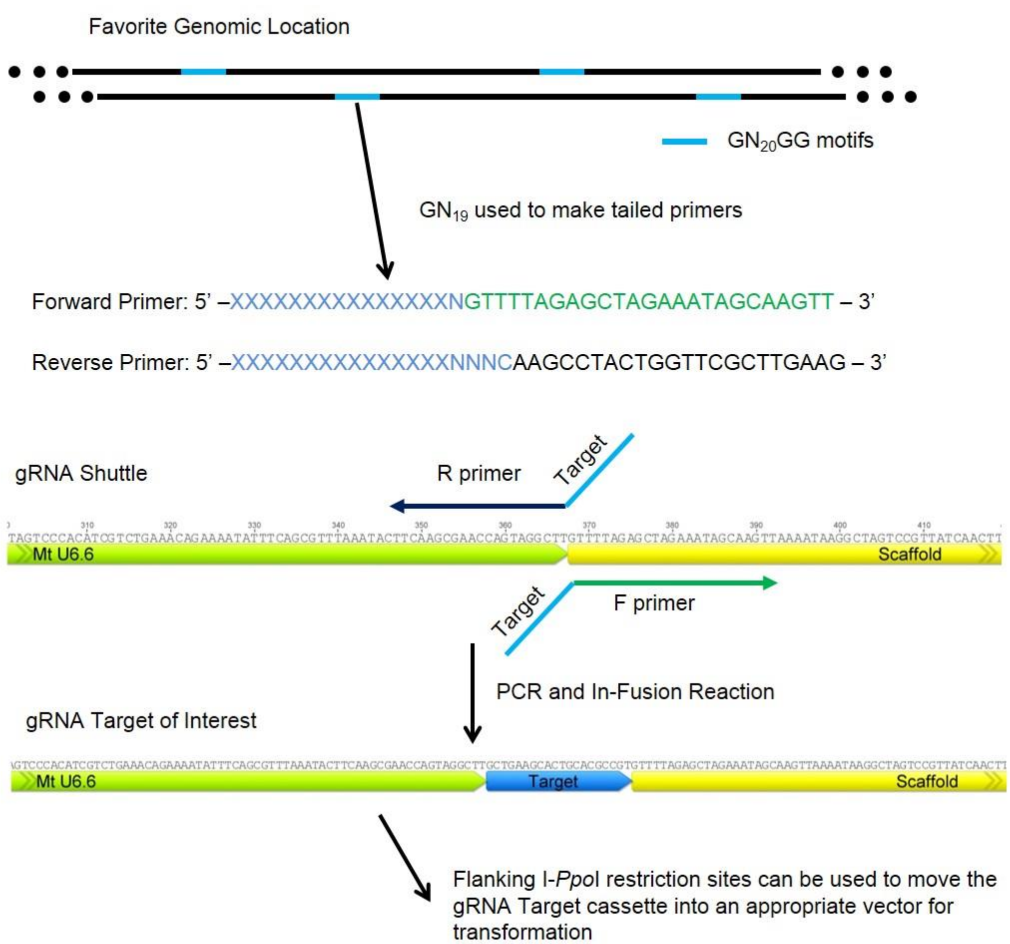
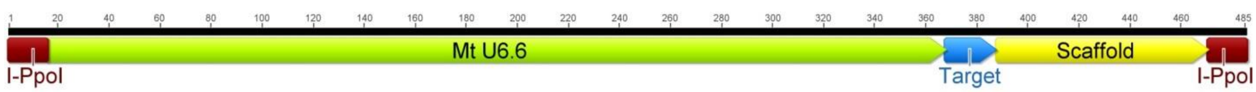


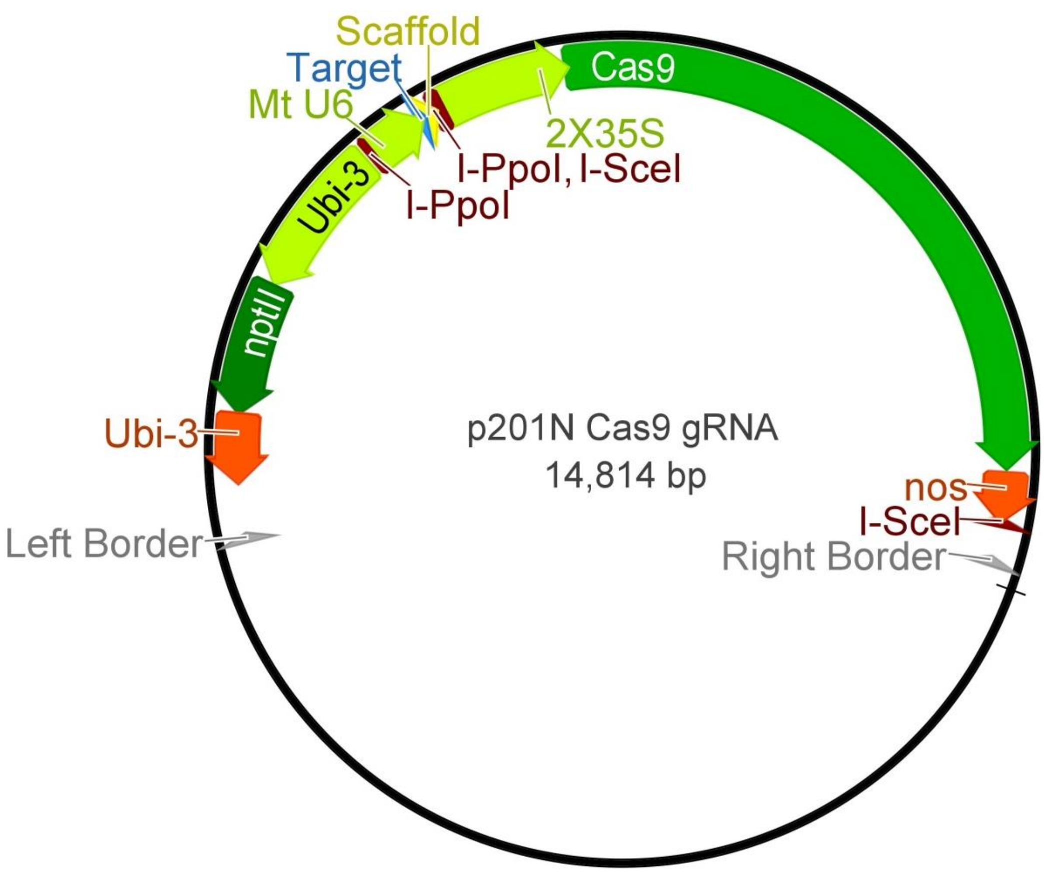
Forward Primer: 5' –xxxxxxxxxxxxxxxNGTTTTAGAGCTAGAAATAGCAAGTT– 3'

Reverse Primer: 5' –xxxxxxxxxxxxxxxNNNCAAGCCTACTGGTTCGCTTGAAG– 3'

Search your gene-of-interest for GN20GG motifs. If you know the protein, go for regions with important motifs, else pick the 5' end as reading-frame deletions should mess up the whole gene. The GN19 portion of the target motif is then incorporated into the shuttle primers as diagramed above. There is some flexibility in the length of the gRNA, recent pubs show shorter gRNAs (2 bases shorter) are as effective and may be more specific (1). The forward and reverse primers share 15bp of the gRNA sequence. PCR with these primers and the pUC gRNA shuttle template, results in a linear plasmid with 15bp of homology on the ends. InFusion cloning (http://www.clontech.com/US/Products/Cloning_and_Competent_Cells/Cloning_Kits/Cloning_Kits-HD-Liquid) can then be used to recombine those 15bp and make a circular plasmid. I use the appendix A protocol since you can use the PCR product without cleanup*. This can then be used for bacterial transformation, select with ampicillin. Check the clones for the correct gRNA by Sanger sequencing of the plasmid with M13R. Then cut the gRNA shuttle vector with the meganuclease I-PpoI (promega) and move that into the cas9 vector also cut with I-PpoI. Note: TSAP does not work in the promega I-PpoI buffer, so we routinely use promega buffer D. The enzyme cuts just fine and we can remove the phosphate in one step. Select for the binary with kanamycin. For a diagnostic digest of the final vector I like to use HF-EcoRV and HF-StyI. This last step is important. I think the size of the plasmid makes it more prone to re-arrangements during the cloning process. I have seen quite a few. For p201G Cas9 (GFP plant selection) the digested fragments should be 4941, 1743, 1544, 1381, 1178, 995, 831, 695, 460, 318, 174, 147. Also check if the gRNA target introduces a restriction site.

Vectors to use





1.**Fu, Y.F., J.D. Sander, D. Reyon, V.M. Cascio, and J.K. Joung.** 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature Biotechnology *32*:279-284.

* It should be noted that this primer scheme should also work with overlap-extension PCR (with appropriate flanking primers, below). I have not tried this. It would have the potential advantage of skipping the shuttle-vector transformation and cloning step. The final PCR product, digested with I-PpoI, could go right into the binary vector.

IPpoI gRNA F (5'-3')

ATATACTCTCTTAAGGTAGCATGCCTATCTTATATGATCAATGAGG

IPpoI gRNA R (5'-3')

TGCTACTCTCTTAAGGTAGCAAAAAAAGCACCGACTCGGTG