

First, cut pSimpleII-U6-tracr-U6-BsmBI-NLS-NmCas9-HA-NLS(s) with BsmBI. No need to SAP treat the vector, because the ends generated by BsmBI are not compatible with each other.

Then look for the following sequence in the genomic region of interest.

NNNGATT is the PAM sequence required by NmCas9. Don't forget to look for NNNNGATT in the complement strand to increase your options. Note, the spacer is 24nt for NmCas9. However, the first nt should always be a 'G' to satisfy the requirement of U6 promoter. That is why only 23nt is highlighted in green in the sequence below.

5'-(NNN NNNNN NNNNN NNNNN NNNNN)₂₃ NNNNGATT -3'

Order oligoes in the following format. Substitute (N)23 in the first oligo with the sequence highlighted in green above. Substitute (N)23 in the second oligo with the **reverse complement** of (N)23 of the sequence highlighted in green above. Then anneal those two oligoes to create an insert that can be cloned into the All-in-one NmCas9 plasmid.

CACCG(N23)GTTGTAGCTCCCTTCTCATTTCG — — — — — Oligo #1
AAAACGAAATGAGAAAGGGAGCTACAAC(N23)C — — — — — Oligo #2

The insert should always be sequenced before using the plasmid. You can use the following primer for sequencing spacer inserts: TAATACGACTCACTATAGG