



5'-NNNNNNNNNNNNNNNNNNNNNNNNNNNGG-3' into two 61-mer oligonucleotides as indicated below. The region marked in green corresponds to the above-mentioned 20-mer. The red region corresponds to reverse complements the green 20-mer.

4. Insert\_F: 5'-

TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGNNNNNNNNNNN  
NNNNNNNNNNN-3'

5. Insert\_R: 5'-

GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACNNNNNNNNNNNN  
NNNNNNNNNC-3'

6. Anneal the two oligos and extend these to make a 101 bp double-strand DNA fragment using Phusion polymerase.
7. Linearize the gRNA cloning vector (Addgene #41824) with *Afl* II and incorporate the 101 bp DNA fragment into the linearized vector using Gibson assembly.
8. Confirm the sequence of gRNA with M13 reverse primer.
9. For construction of retroviral vectors expressing gRNA, gBlock can be cleaved from a gRNA vector constructed using the above-mentioned gRNA cloning vector with appropriate restriction enzymes (eg. [*Xho* I + *Hind* III], *Eco*R I).
10. Insert the cleaved gBlock into pSIR-based self-inactivating retroviral vectors.

Vectors & Sites of insertion

pSIR-neo (Addgene #51128):	eg. [ <i>Xho</i> I + <i>Hind</i> III]
pSIR-GFP (Addgene #51134):	eg. [ <i>Xho</i> I + <i>Hind</i> III], <i>Eco</i> R I
pSIR-DsRed-Express2 (Addgene #51135):	eg. [ <i>Xho</i> I + <i>Hind</i> III], <i>Eco</i> R I
pSIR-hCD2 (Addgene #51143):	eg. <i>Eco</i> R I

**Note**

Binding of the CRISPR complex may affect chromatin structure such as nucleosome positioning and abrogate normal genome activities such as gene expression. Although the effects of binding need to be tested empirically for each locus, the author's group has guidelines to avoid potential aberrant effects caused by binding of the CRISPR complex.

(a) For analysis of promoter regions near transcription start sites (TSSs), the gRNA binding site should be several hundred base 5' to the transcription start site so that the

binding would not inhibit transcription or disrupt nucleosome positioning. In contrast, for identification of binding molecules of genomic regions with distinct boundaries such as enhancer or silencer, the binding site of gRNA can directly be juxtaposed to the regions because of less probability of inhibition of their function. (b) The binding site should not be conserved among species because conserved regions are often binding sites of conserved binding molecules. In this regard, however, since multiple gRNA can be generated easily, a trial-and-error approach can work.

## **References**

- 1 Mali, P., Yang, L., Esvelt, K. M., et al. (2013) RNA-guided human genome engineering via Cas9, *Science* 339, 823-826.