Oligo Assembly and Preparation of sgRNA
Selection of targets at end of protocol

Reaction:

Generic oligo [10µM]	1µl
GeneSpecific oligo [10µM]	1µl
Hi-Fi Polymerase Mix 2X	12.5µl
RNAse free H2O	10.5µl
Vt	25µl

Conditions:

1. Denature at 98°C for 2 mins.
2. Anneal at 50°C 10 mins.
3. Extend at 72°C 10 mins.
4. Run 2.5% Agarose Gel, you should see a 120bp band when 3-5ul loaded on gel.

sgRNA Synthesis

Wear gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 30 µl but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

1. Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to the bottoms of tubes. Keep on ice.
2. Assemble the reaction at room temperature in the following order:

2X NTP mix (NEB N0466S)	5 µl
Assembled oligos	1.5 µl
T7 Polymerase mix (M0255A NEB)	1 µl
RNAase free H2O	7.5 µl
Vt	15 µl

3. Mix thoroughly and pulse-spin in a microfuge.
4. Incubate at 37°C for 4 hours or longer for maximum yield. It is safe to incubate the reaction for 16 hours (overnight), however, sgRNA amounts sufficient for many experiments may be synthesized in less than 4 hours. We recommend incubation in a dry air incubator or a thermocycler to prevent evaporation of the sample.

Note that sgRNA synthesis reactions use 10 µl more water than standard HiScribe T7 Quick High Yield RNA Synthesis Kit reactions. The volume of NTP Buffer Mix and T7 RNA Polymerase Mix, however, remains the same.

DNase treatment to remove DNA template. Typical RNA synthesis reactions using the HiScribe T7 Quick High Yield RNA Synthesis Kit can generate large amounts of RNA, at

concentrations up to 10 mg/ml. As a result, the reaction mixture can be quite viscous. It is easier to perform DNase treatment after diluting the reaction.

5. To remove template DNA, add 2 µl of DNase I (RNase-free) to each 15 µl reaction, followed by 33 µl nuclease-free water, mix by pipetting up and down and incubate for 15 minutes at 37°C.
6. Mix 2ul of RNA sample with 3ul of RNA Dye and incubate at 65°C for 3-5min.
7. Proceed with analysis of transcription products on a 2.5% gel.

Precipitation/purification of guide-RNA (sgRNA)

Use Zymo RNA Clean and Concentrator-5 kit as manufacture's instruction

Check sgRNA quality on gel; 1-2ul on 2.5% agarose gel; add RNA gel loading dye and heat to 65 for 3-5 mins. Prior to loading.

Nanodrop the RNA concentration, aliquot and store at -80°C.

Injections:

sgRNA (200-400 ng/µl)	1 µl
Cas9 protein	1 µl
3 µM stop codon cassette oligo	1 µl
phenol red injection dye	0.3 µl

Picking CRISPR targets:

1. Finding targets: use program CHOPCHOP
    a. Under options make sure "5' requirements for sgRNA" is GG
    b. Target 2nd or 3rd exon
    c. Ideally find a target near a unique restriction site
    d. Save all sequences, target and primer sets
2. Order oligo with T7-target-anneal for tracrRNA
    a. Naming convention: T7-Dr-gene-rank
    b. Sequence ATTTAGGTGACACTATA-$\mathbf{N_{20}}$-GTTTTAGAGCTAGAAATAGCAAG
        i. **MAKE sure target starts with GG**
    c. You will combine this with the generic oligo in the assembly step
        i. Generic oligo sequence: AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
3. If there are no targets with good restriction sites, use the 3x Stop donor construct
    a. From Schier lab at Harvard
    b. The stop codon cassette oligonucleotide contains two 20 base homology arms that flank the predicted Cas9-mediated breakpoint. These homology arms surround the stop codon cassette.
    c. Stop codon cassette v2, which has the longer sequence 5'-GTCATGGCGTTTAAACCTTAATTAAGCTGTTGTAG-3'. This cassette has stop codons in

all frames, and also PmeI and PacI restriction enzyme sites. The stop codon cassette v2 also has the advantage of being a better template for an insert-specific PCR primer.

d. Donor should match the target strand (complementary to the guide RNA), according to Richardson, 2016 Nature Biotech (Richardson, Ray, DeWitt, Curie, & Corn, 2016)
    i. (Paix et al., 2017) disagree and say strand has no effect