## Cloning gRNAs into EF1a-Triplex-28-M13-28-pA

The EF1a-Triplex-28-M13-28-pA harbors a PaqCI-flanked cloning site in the 3' UTR of the transcript (currently containing M13 sequence as a placeholder) that can be used to insert any desired Cas9 gRNA sequence. To do so, proceed with the following protocol:

1) Restrict 2 µg of the plasmid with PaqCI to remove M13 sequence and create sticky ends.
2) Run the restricted product on a 0.8% agarose gel and clean up the restricted product with a gel extraction kit (we use the Thermo Scientific GeneJET kit K0691).
3) Anneal two oligos to ligate into the cut vector. The two oligos to be annealed are reconstituted at 100 µM in water. 12.5 µl of each is then combined with 25 µl of a buffer containing 20 mM Tris-HCl (pH7.5) and 100 mM NaCl (25 µM each oligo, 10 mM Tris-HCl, 50 mM NaCl final concentrations). To anneal, the oligo mixture is heated to 95°C for 5 min in a thermal cycler, then cooled at the rate of 5°C per min until the temperature reaches 65°C, and then the program is ended and the oligos are allowed to cool to room temperature. For example, if you wanted to generate a construct targeting the P1 promoter in our manuscript, you would hybridize 5'-ctaagaaaTAGTCGCGTGTAGCGAAGCAgtt-3' and 5'-ctaaaacTGCTTCGCTACACGCGACTAtttc-3', which includes the 20 nucleotide guide RNA sequence that is just adjacent to the PAM site in the promoter and PaqCI-compatible sticky ends (lowercase letters), to generate this double-stranded insert:

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5'- ctaagaaaTAGTCGCGTGTAGCGAAGCAgtt -3'
      ||||||||||||||||||||||||||||
   3'-ctttATCAGCGCACATCGCTTCGTcaaaatc-5'
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4) Ligate the insert into the backbone. We use the NEB Quick Ligation Kit (M2200L). We limit the total amount of DNA to 100 ng and maintain the molar ratio of insert:plasmid at 3:1 for inserts > 200 bp and 10:1 for inserts < 200 bp. The ligation mixture is incubated on a thermal cycler with 20 cycles of 15°C for 20 sec and 35°C for 20 sec, followed by a final incubation at 22°C for 5 min.
5) Transform bacteria (we use NEB5-alpha, C2987I), isolate individual colonies on LB agar plate containing Ampicillin (100 µg/ml), miniprep the plasmid, and confirm the sequence of the plasmid with targeted Sanger sequencing or whole-plasmid sequencing (we use Primordium).