Cloning a promoter into Px-PhiYFP-triplex-DR-M13-DR-pA

The Px-PhiYFP-triplex-DR-M13-DR-pA harbors a cloning site downstream of PhiYFP in the 3' UTR (currently containing M13 sequence as a placeholder) flanked by BsmBI sites that can be used to insert any desired Cas12a crRNA sequence. It also has another cloning site upstream of the transcript (currently containing M13 sequence as a placeholder) flanked by BamHI and SacI restriction sites that can be used to insert any desired Cas12a target sequence as an inducible promoter. When replacing elements in this construct, it is important to insert the crRNA first (removing the BsmBI site) and then to build the promoter (which also uses insertion of new elements containing BsmBI sites). To do so, proceed with the following protocol:

1) Restrict 2 µg of the plasmid with BsmBI 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 P2 promoter in our manuscript, you would anneal 5'-agatAGGCTAGCCCAGGACAGTAC-3' and 5'-aattGTACTGTCCTGGGCTAGCCT-3', which includes the 20 nucleotide guide RNA sequence that is just adjacent to the PAM site in the promoter and compatible sticky ends (lowercase letters), to generate this double-stranded insert:

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5'-agatAGGCTAGCCCAGGACAGTAC-3'
        ||||||||||||||||||||
    3'-TCCGATCGGGTCCTGTCATGttaa-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).
6) When you're ready to modify the promoter, restrict 2 µg of the plasmid containing the new crRNA with BamHI and SacI to remove the promoter M13 sequence and create sticky ends (again start with 2 ug).
7) Run on an 0.8% agarose gel and clean up the restricted product with a gel extraction kit.
8) 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. The annealed sequence will contain a 5' sticky end (BamHI site), one Cas12a target site (UPPERCASE) adjacent to a proper PAM site (tttc), a BsmBI site (enabling addition of more target sites to the promoter), and a 3' sticky end (SacI site). For example, if you wanted to generate a construct with the P1 promoter used in our manuscript, you would anneal 5'-gatcctttcAGGCTAGCCATGCTTCGCTAcacgcgactatagatccgagacgacatacatacatgagct-3' and 5'-catgtatgtatgtcgtctcggatctatagtcgcgtgTAGCGAAGCATGGCTAGCCTgaaag-3' to generate this double-stranded insert:

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5’-gatcctttcAGGCTAGCCATGCTTCGCTAcacgcgactatagatccgagacgacatacatacatgagct-3’
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3’-gaaagTCCGATCGGTACGAAGCGATgtgcgctgatatctaggctctgctgtatgtatgtac-5’
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9) Ligate the insert into the backbone with the method described in **Step 4**.
10) Transform bacteria, 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.
11) To add additional sites in the promoter array, perform **Steps 6-10** on the newly generated plasmid until the desired number of target sites is achieved.
12) Once the desired number of target sites is cloned, the BsmBI site can be removed and the 5’ UTR returned to the original sequence by annealing 5’-CGCTAcacgcgactaTAATATTTTCAGCTAGCGGGGGGCTATAAA-3’ and 5’-TTTATAGCCCCCCGCTAGCTGAAAATATTAtagtcgcgtgTAGCG-3’ (these oligos overlap the target site - UPPERCASE) and inserting the annealed product with Gibson assembly after once again linearizing the backbone (with the new promoter) with BsmBI and SacI restriction. The annealed oligos will generate this double-stranded insert:

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5’-CGCTAcacgcgactaTAATATTTTCAGCTAGCGGGGGGCTATAAA-3’
   |||||||||||||||||||||||||||||||||||||||||||||
3’-GCGATgtgcgctgatATTATAAAAGTCGATCGCCCCCCGATATTT-5’
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13) Transform bacteria, 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.