Supplemental Protocol

ONE-STEP OVERLAP PCR (OSO-PCR) TO MAKE READY-TO-ELECTROPORATE SINGLE GUIDE RNA (sgRNA) EXPRESSION CASSETTES – *updated 09/21/2016*

Companion manuscript:

Evaluation and rational design of guide RNAs for efficient CRISPR/Cas9-mediated mutagenesis in *Ciona*

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Primers for OSO-PCR ready to be ordered can be obtained from the CRISPOR sgRNA prediction and design website (http://crispor.tefor.net), which also checks for known single-nucleotide polymorphisms (SNPs) and potential off-targets in the genome. You can also check for polymorphisms using the Kyoto University Ghost Database genome browser (http://ghost.zool.kyoto-u.ac.jp/cgi-bin/gb2/gbrowse/kh/). You should avoid sgRNAs targeting known SNPs or naturally occurring indels, since Cas9 cutting depends on perfect target sequence match. To design OSO-PCR primers *de novo,* follow the instructions:

1- Select your target, as identified by online tools such as CRISPOR (see above).

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                              target        PAM
...TCAACCCAACTGAGGGTTGGACAACAGGTGGAGCAACAGT...
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2- A target (the protospacer) is given as N(20). If the target sequence contains too many T's (three or more T's clustered together tend to terminate transcription), or if it spans many known naturally-occurring polymorphisms, or has a high number of potential off-targets, discard it.

3- For transcription initiation from U6 promoter, replace the first base of the target with a "G", to give a G+(N)19 sequence.

GCTGAGGGTTGGACAACAGG

4- Append "GTTTAAGAGCTATGCTGGAAACAG" to the 3' end of the sequence. This entire sequence is now the forward primer used to PCR the sgRNA scaffold part of the cassette ("OSO forward" primer)

GCTGAGGGTTGGACAACAGGGTTTAAGAGCTATGCTGGAAACAG

5- Copy reverse complement of G+N(19), append "ATCTATACCATCGGATGCCTTC" to the 3' end of this. This is now the reverse primer to PCR the U6 promoter part of the cassette ("OSO reverse" primer)

CCTGTTGTCCAACCCTCAGCATCTATACCATCGGATGCCTTC

6- Set up a PCR reaction using the following components in the exact amounts described. The amounts/concentrations/proportions are critical for the one-step overlap reaction to occur seamlessly. Also, it is very important to eliminate all sources of contamination, otherwise you may re-amplify sgRNAs already in heavy use in the lab. Template plasmids are available from Addgene (https://www.addgene.org/Lionel_Christiaen/):

For 50 ul reaction:

1.5 ul 10mM dNTPs
1 ul 50mM MgSO4
10 ul 10X Pfx Buffer
1 ul U6>XX plasmid at 15 ng/ul
1 ul X>sgRNA(F+E) plasmid at 15 ng/ul
1.5 ul 20 uM U6 forward primer (5'- TGGCGGGTGTATTAAACCAC -3')
1.5 ul 20 uM sgRNA reverse primer (5'- GGATTTCCTTACGCGAAATACG -3')
1 ul **2 uM** OSO forward primer (designed in step 4, or obtained from CRISPOR)
1 ul **2 uM** OSO reverse primer (designed in step 5, or obtained from CRISPOR)
30 ul H2O
0.5 ul Pfx platinum

PCR program:

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94° - 3'
94° - 30"   |
50° - 30"   | X 30
68° - 3'    |
68° - 5'
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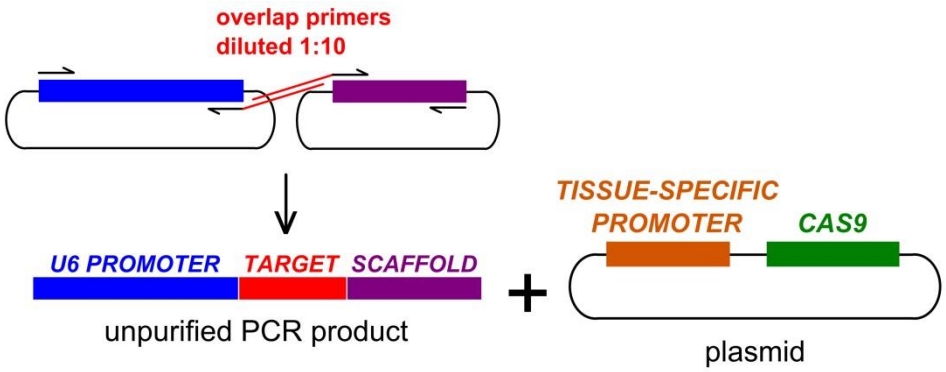

The 1:10 dilution of your custom overlap target-specific primers will force the "fusion" of the entire cassette later in the reaction, when these primers are depleted from the solution through incorporation into the PCR products.

7- Run 2 ul of the PCR reaction on a gel. There should be a strong band at ~1.2 kbp. If the band is only 1 kbp, the fusion did not occur. The success rate in our hands is ~94%. If possible, run alongside positive control (PCR on verified sgRNA plasmid template using same primers).

OSO-PCR products can be electroporated as is, un-purified. 25 ul appears to be sufficient to recapitulate effects of sgRNAs delivered by traditional plasmid electroporation, but this volume can be adjusted accordingly. If you need to clone the cassette into a plasmid, you can use the product as template for additional PCRs using the outer primers with added overhangs for restriction enzyme or Clontech In-Fusion cloning.