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Protocol: Cloning of individually synthesized oligos for sgRNA (CRISPR) or shRNA constructs

Brief Description:

This protocol allows you to clone individually synthesized oligos (either in 96-w plates, or in tubes) to generate sgRNAs or shRNAs on a small scale. The protocol assumes cloning sites common in FGC/GPP vectors, but could of course be adapted for other vectors. Always confirm the restriction site scheme for any vector, to ensure proper cloning and expression of the intended insert. Vector maps can be found on the GPP website.

Materials and reagents required:

- TB media (terrific broth; American Bioanalytical)
- Carbenicillin (Sigma)
- T4 DNA ligase, 400 units/µl (NEB)
- 10X ligase buffer (NEB)
- Restriction enzymes (NEB)
- Qiaquick gel extraction kits (Qiagen)
- DH5 (Max Efficiency Cells (Invitrogen, or Gene Choice)
- SOC Media (Invitrogen)
- LB agar plates (with appropriate antibiotic)

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Part A. sgRNA cloning

Additional materials

- pXPR vector (e.g. pXPR_003, pXPR_023 etc.)
- Oligonucleotides (IDT)
- 10X Buffer 3.1 (NEB)

Step 1: Vector preparation

Circular pXPR vector, typically containing a "stuffer" at the cloning site to yield easily distinguishable sizes for the desired double-cut versus single-cut products, is digested by BsmBI.

A. In a 1.5 mL eppendorf tube, add:

   20 µg of circular pXPR vector
   20 µL NEB buffer 3.1
   10 µL BsmBI
   Appropriate amount of H2O
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   200 µL total reaction

B. Incubate at 55°C for 4-6 hours.
C. Add 40 µL 6x loading dye into the 200 µL BsmBI digestion. Load 15 µL of digested sample across multiple wells and 15 µL of restriction control (i.e. intact plasmid) in a single well. Run fragments on a 1% agarose gel at 120v for 3 hours to purify.
D. Excise the open-vector band and extract using the QIAquick Gel Extraction kit (Qiagen). We recommend that the eluted DNA from Qiagen column be cleaned by isopropanol precipitation to remove the high salts.
E. Suspend the extracted DNA in a final volume of 2,000 µL of TE buffer (about 6 ng per µL) and store at -20°C.

NOTE: *When colonies will be sequenced by NGS, the stuffer cannot be too long or cloning background colonies will not PCR efficiently and will go undetected. Our typical long stuffer of 1.9kb has this problem.*

To detect cloning background properly by PCR and sequencing, you will you need to use vectors with a shorter stuffer. HOWEVER, as noted above, vectors with short stuffers have the disadvantage that is difficult to separate double-digested from single-digested vector, potentially leading to a high cloning background.

One alternative to deal with these problems is: (1) digest with 3-cuts, 2 for ligation sticky ends, 1 in the middle of the stuffer (two hours will do). (2) run gel just enough to remove the small and chopped up stuffer (1 hour, 1% gel). This way, the 3rd cut in the stuffer will (a) reduce the cloning background, and (b) allow reduction of the restriction digest-time and gel run-time, as we just need to get rid of the released short stuffer pieces.

STEP 2. Oligonucleotide design

Oligos should be designed such that the forward and reverse strands pair with each other and result in 2 overhangs (5'CACC as one, 5'AAAC as the other) that can be ligated to the processed pXPR vector.

Forward oligo: 5' CACCG----20 bp target --- 3'
Reverse oligo: 5' AAAC-----20 bp ---------C 3'

For example:

Forward oligo: 5' CACCGAAGAAGGGCCGTACCCGAAA 3'
Reverse oligo: 5' AAACTTTCGGGTACGGCCCTTCTTC 3'

NOTE: *Don't forget the 'G/C' that is needed to precede the guide sequence – it marks the transcription start site.*

STEP 3. Anneal oligos

A. Re-suspend oligos in ddH20 (40 µM).
B. In strip tubes or 96-well plate that fits a PCR cycler, add, per tube (or well):

   1.5 µL of forward oligo (40 µM)
   1.5 µL of reverse oligo (40 µM)
   5 µL of 10x NEB buffer 3.1
   42 µL ddH20
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   50 µL total

C. Using a PCR cycling machine, incubate 95°C for 5 min, then 70 °C for 5 min, followed by lowering the temperature by 5 °C in every 5 min interval, until room temperature and hold.

Part B. shRNA cloning

Additional materials

- Stuffed-pLKO vector, pLKO_TRC001 or pLKO_TRC005
- Hairpin oligonucleotides (IDT)
- 10X Buffer 2 (NEB)

STEP 1. Vector preparation

Circular stuffed pLKO vector is digested by AgeI and EcoRI.

A. In a 1.5 mL eppendorf tube, add:

   20 µg of circular stuffed-pLKO vector
   20 µl NEB buffer I
   Appropriate amount of H2O
   6 µl AgeI
   6 µl EcoRI
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   200 µl total reaction

B. Set up two controls, which will be loaded in the purification gel:

   a. AgeI control:

      1 µg circular vector
      2 µl NEB buffer 1
      Appropriate amount of H2O
      1 µl AgeI
      ----
      20 µl

   b. EcoRI control:

      1µg circular vector
      2 µl NEB buffer 1
      Appropriate amount of H2O
      1 µl EcoRI
      ----
      20 µl

C. Incubate at 37°C for 4-6 hours.
D. Add 40 µL 6x loading dye into the 200 µL AgeI/EcoRI digestion. Load 15 µL of digested sample across multiple wells, and add 15 µL of the restriction controls,

intact plasmid, and a linear size marker each in a single well. Run fragments on a 1% agarose gel at 120v for 3 hours to purify.

E. Excise the open-vector band (7kb) and extract using the QIAquick Gel Extraction kit (Qiagen). Suspend the extracted DNA in a final volume of 2,000 µL of TE buffer (about 6 ng per µL) and store at -20°C.

NOTE: *When colonies are sequenced by Sanger reads: The 1.9kb stuffer of pLKO.1 and pLKO.5 facilitates the high-grade purification by separating any incompletely digested vectors from double-digestion products. As a result, the cloning background (i.e. empty vector) is no longer a concern.*

When the candidate colonies need NGS verification, you need to use vectors with 'short' stuffer, as the cloning background of vectors with long stuffer will not PCR well and will therefore go undetected. HOWEVER vectors with short stuffer can give trouble in separating double digested vectors from singly-digested one, which will lead to cloning background.

Here is a solution: (1) digest with 3-cuts, 2 for ligation sticky ends, 1 in the middle of the stuffer (two hours will do). (2) run gel just enough to remove the small and chopped up stuffer (1 hour, 1% gel). This way, the 3rd cut in the stuffer will (a) reduce the cloning background by which ever its cut rate, (b) allow us to shorten the restriction digest-time and gel run-time, as we just need to get rid of the released short stuffer pieces.

STEP 2. Hairpin oligonucleotide design

Oligos should be designed such that the forward and reverse strands pair with each other and result in 2 overhangs that can be ligated to the processed pLKO vector. To avoid insert chimerism, we do not recommend adding phosphates to oligo strands.

Forward oligo: 5' CCGG---21 bp Sense---CTCGAG---21 bp Antisense---TTTTTG3'
Reverse oligo: 5' AATTCAAAAA---21 bp Sense---CTCGAG---21 bp Antisense---3'

STEP 3. Anneal oligos

A. Re-suspend oligos in ddH20 (40 µM).
B. In strip tubes or 96-well plate that fits a PCR cycler, add, per tube (or well):

   1.5 µL of forward oligo (40uM)
   1.5 µL of reverse oligo (40uM)
   5 µL of 10x NEB buffer 2
   42 µL ddH20
   ----
   50 µl total

C. Using PCR cycling machine, incubate 95 °C for 5 min, then 70 °C for 5 min, followed by lowering the temperature by 5 °C every 5 min interval, until room temperature and hold.

Part C. Ligation/Transformation/Sequencing -- common to both sgRNA and shRNA

STEP 4. Ligation

A. For the ligation reaction, add the following together, in the order listed:

NOTE: *Be sure to include a control reaction without annealed oligos. Users may make a serial dilution e.g. 1 µl straight, 1:10, 1:100, 1:1000, to capture an appropriate stoichiometry (between oligo and vector) in the ligation reaction.*

1 µL of annealed oligo pair (or water in a control reaction)
3 µL of prepped open vector (~20ng)
2 µL 10x ligase buffer (NEB)
13 µL of H2O
1 µL T4 ligase (NEB)
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20 µL total

B. Proceed with the ligation at 16°C for 3-4 hours.

STEP 5. Transformation/plating/picking colonies

A. Add 2 µL of the ligation reaction mix to 25 µL competent cells (DH5). Place the mix on ice for 30 min.
B. Heat shock at 42°C for 45 seconds, followed by incubation on ice for 2 min.
C. Recover the transformed cells with 400 µL SOC at 37°C for 1 hour.
D. Plate ~150 µL transformants onto agar dishes containing 100 µg/ml carbenicillin.
E. Incubate agar dishes upside down at 37°C overnight.
F. Pick 2-4 colonies each into 1-2 mL TB + 100 µg /ml carbenicillin.
G. Cells Grow cells at 37°C (300rpm) for no more than 17 hours.
H. Glycerol stock(s) can be made by mixing 150 µL glycerol with 850 µL culture. Store at -80°C.

STEP 6. Sequencing

The colonies are verified by either Sanger or next-gen sequencing.

6.1) Sanger sequencing:

A. Primer sequence: 5'-GAT ACA AGG CTG TTA GAG AGA TAA TT-3'
B. DNA prep: Prep the DNA by TempliPhi from glycerol stock, or by alkaline lysis prep from overnight cultures. Adjust the concentration to 40 ng/ µL.
C. Sequencing Reaction: Each sequencing reaction contains ~50 ng DNA template. We recommend a 4:1 ratio of bigDye3.1:dGTP bigDye to read through the hairpin structures, and regular bigDye3.1 for guides.

D. Cycled as following:
   96°C/45'' -- [96°C/15'' - 50°C/15'' - 60°C/3' 30''] x 34 cycles -- hold at 4°C
E. Clean up the sequencing reactions by ethanol precipitation. Elute the dried fragments into 10 µL of 0.1 mM EDTA and detect with ABI 3730xl, with POP7, 36cm array (Injection voltage 1.2, Injection time 15 sec, Run time 2450 sec, Oven temperature 60, Run voltage 8.5).

6.2) Next-gen sequencing:

We start out with a pre-made, well-barcode PCR primer plate called the P7_index2 plate, where each well has a unique 8-mer index sequence incorporated in the Illumina P7 primer. In addition, we have a set of P5 primers, each of which has a 6-mer barcode (to serve as the plate barcode.) We currently have 48 plates of P5 primers, with each plate having a unique plate barcode in every well.

Pre-made PCR plates have 5 µL of P7_index2 primer (2 uM stock) and 5 µL P5 primer (2 uM stock). The PCR plates are numbered by the same numbering of the plate barcodes (1 through 48).

Each PCR well will have:

Primers	10 µL (already in the PCR plate)
Glycerol:	1 µL (pipette from top, avoid clumps)
Ex-taq buffer:	2 µL (in master mix)
dNTP (20 uM)	1.6 µL (in master mix)
H2O:	5.25 µL (in master mix)
Ex-Taq:	0.15 µL (in master mix)
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	20 µL (total)

PCR cycling:

95C: 3 min
94C: 30 sec
63C: 30 sec
72C: 20 sec
back to step 2: 24x
72C: 5 min
4C: infinite
(lid: 105°C)

A. Take 5 µL of PCR product from each well into a trough containing PB buffer (as part of Qiagen PCR cleanup kit). Calculate in such a way that after pooling all PCR products, the volume of PB buffer is 5x of PCR reactions that are pooled.

NOTE: *It is VERY important that any PCR reactions after pooling, albeit rare, will yield data that look like template contamination. Pooling PCR samples into PB buffer quenches PCR reactions.*

B. Mix well. Take 4 mL of the pool to put through PCR clean up columns (2 columns). Elute each column with 50 µL EB each and combine the two for 100 µL.
C. **(Optional)** Get 54 µL of the DNA sample and add 6 µL 10x PCR buffer and mix. Then put the sample through AMPure XP cleanup (e.g. 1.8x volume of beads, RT for 5 min, separate beads and supernatant by magnate, remove the supernatant, wash the beads with 200 µL 70% ethanol twice) and elute the final product in 50 µL water.
D. Quantify the DNA.
E. Submit the sample for NGS, e.x. MiSeq (consulting with your plasmid maps to know where the primers land, where the read starts, and the region you need the reads to cover):
    i. Read 1: 50 bp for single guide, 300 bp for multiple guide
    ii. Index 1 read = 8 bp
    iii. Phix = 30-40%

Part D: Oligo configurations for AsCas12a guides

Cas9 guide libraries:

Most common cases of our Cas9 CRISPR vectors use Esp3I to open the vectors and produce 5'-CACC and 5' –AAAC sticky ends. Therefore, the oligos need to be configured in such a way that upon annealing the 2 strands, it will produce 2 sticky ends matching those of the opened vectors. In below example, we need to order the mixture of 2 oligos shown.

Sticky ended insert that is ligated into the vector:

5'-CACCGAAGAAGGGCCGTACCCGAAA -3'
3'- CTTCTTCCCGGCATGGGCTTTCAAA-5'

NOTE: *Don't forget the 'G/C' that is needed to precede the guide sequence – it marks the transcription start site.*

AsCas12a guide libraries:

The AsCas12a (a.k.a Cpf1) system allows multiple guides to be synthesized in a single oligo and to be cloned by Golden Gate reaction in a similar manner as single guide libraries. In this system, each guide is preceded with a 20-nt 'direct repeat (DR). Here are the versions of direct repeat sequences:

DR0: TAATTTCTACTCTTGTAGAT
DR1: TAATTTCTACTGTCGTAGAT
DR2: TAATTTCTACTATCGTAGAT
DR3: AAATTTCTACTCTAGTAGAT

After cloning, the construct will have this configuration:

U6 promoter-DR0-guide1-DR1-guide2-DR2-guide3-DR3-guide4-TTTTTTGAAT

The DR0 preceding the first guide is provided by the vector. The oligos to be synthesized will have following configurations:

For single-guide:

Underlined: sgRNA target
Green: direct repeat (DR)

Sticky ended insert that is ligated into the vector:

5' AGATCCTATCATCGTACCTCCTGGTTGTTTTTT 3'
3' GGATAGTAGCATGGAGGACCAACAAAAAACTTA 5'

For dual-guides:

Underlined: sgRNA target
Green: direct repeat (DR)

Sticky ended insert that is ligated into the vector:

5' AGATACGGCAGGTTGGAGCAGAAAGAATAATTTCTACTGTCGTAGATCCTATCATCGTACCTCCTGGTTGTTTTTT 3'
3' TGCCGTCCAACCTCGTCTTTCTTATTAAAGATGACAGCATCTAGGATAGTAGCATGGAGGACCAACAAAAAACTTA 5'

Revision notes:

- Added next-gen sequencing protocol
- Added oligo configurations for AsCas12a constructs of single and multiple guides