BROAD INSTITUTE | GENETIC PERTURBATION PLATFORM

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

Brief Description:

This protocol allows you to clone oligos to generate shRNAs or sgRNAs on a small scale.

-For sgRNAs, pXPR vectors with a single BsmBI or BbsI cloning site are most common, two types of pXPR vectors can be used:

1. pXPR_003 (or “lenti guide”) will only contain an sgRNA and is to be used in a cell line that already expresses Cas9.
2. pXPR_023 (also referred to as “lenti CRISPR” or “all-in-one”) already contains Cas9 sequence prior to cloning an sgRNA

-For shRNA, pLKO vectors with AgeI / EcoRI cloning sites are most common.

Always confirm the restriction site scheme for any vector, to ensure proper expression of the intended insert.

Materials

- TB media (terrific broth; American Bioanalytical,)
- Carbenicillin (Sigma)
- T4 DNA ligase, 400units/ul (NEB)
- 10X ligase buffer (NEB)
- Restriction enzymes (NEB)
- Qiaquick gel extraction kits (Qiagen)
- DH5αMax Efficiency Cells (Invitrogen, or Gene Choice)
- SOC Media (Invitrogen)
- LB agar plates (with appropriate antibiotic)

- Big dyes (ABI)

For sgRNAs continue to Part A.
For shRNAs, continue to Part B.

Part A. sgRNAs

Additional materials

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

Vector preparation

Circular stuffed pXPR vector is digested by BsmBI:

- In a 1.5ml eppendorf tube, add:
  20ug of circular pXPR vector
  20ul NEB buffer 3.1
  Appropriate amount of H2O
  10ul BsmBI

  200ul total reaction

  Incubate at 55°C for 4-6 hours.

- Fragments are purified by a 1% agarose gel (120v for 3 hours). Add 40ul 6x loading dye into the 200ul BsmBI digestion and load 15ul sample in each well. Restriction control (i.e. intact plasmid) is loaded in a single well.

- The open-vector band is excised and the vector is extracted using Qiaquick Gel Extraction kit (Qiagen). We recommend that the eluted DNA from Qiagen column be cleaned by isopropanol precipitation to remove the high salts. The extracted DNA is suspended into a final volume of 2,000ul of TE buffer (about 6 ng per ul). The opened vector is then stored frozen.

2. Oligonucleotides features

Oligos should be designed to have following configuration. The forward and reverse strands pair with each other and result in 2 overhangs (5'CACC as one, 5'AAAC 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'

3. Anneal oligos

- Re-suspend oligos in ddH20 (100uM)
- In an eppendorf tube, add:
  1.5 µL of forward oligo (100uM)
  1.5 µL of reverse oligo (100uM)
  5 µL of 10x NEB buffer 3.1
  42 µL ddH20

  50ul total

- Incubate for 4 min at 95°C.
- Incubate for 10 min in 1L beaker filled with 70C water and allow the water in the beaker to cool to RT (*Note: This will take a couple of hours, but it is important that you do this slowly! One may set up a thermal cycler to achieve the same*.)

Part B. shRNAs

Additional material

- Stuffed-pLKO vector, pLKO_TRC001 or pLKO-TRC005 (TRC)
- Hairpin oligonucleotides (IDT)
- 10X Buffer 2 (NEB)

Vector preparation

Circular stuffed pLKO vector is digested by AgeI and EcoRI:

- In a 1.5ml eppendorf tube, add:
  20ug of circular stuffed-pLKO vector
  20ul NEB buffer I
  Appropriate amount of H2O
  6ul AgeI
  6ul EcoRI

  200ul total reaction

- Set up two controls as well; controls will be loaded in the purification gel:

A. AgeI control:

1ug circular vector
2ul NEB buffer 1
Appropriate amount of H2O
1ul AgeI

20ul

B. EcoRI control:

1ug circular vector
2ul NEB buffer 1
Appropriate amount of H2O
1ul EcoRI

20ul

Incubate at 37°C for 4-6 hours.

- Fragments are purified by a 1% agarose gel. Add 40ul 6x loading dye into the 200ul AgeI/EcoRI digestion and load 15ul sample in each well. Restriction controls, intact plasmid, and linear size markers are loaded in a single well each.

- The 7kb open-vector band is excised and the vector is extracted using Qiaquick Gel Extraction kit (Qiagen). The extracted DNA is suspended into a final volume of 2000ul of TE buffer (about 6 ng per ul). The opened vector is then stored frozen.

  Note: When stuffed-pLKO is used to prep the linear vector, its 1.9kb stuffer facilitates the high-grade purification by separating any incompletely digested vectors from double-digestion products. As a result, the cloning background (ie. empty vector) is no longer a concern.

2. Hairpin oligonucleotides features

Oligos should be designed to have following configuration. 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'

3. Anneal oligos

- Re-suspend oligos in ddH20 (100uM)
- In an eppendorf tube, add:
  1.5 µL of forward oligo (100uM)
  1.5 µL of reverse oligo (100uM)
  5 µL of 10x NEB buffer 2
  42 µL ddH20

  50ul total

- Incubate for 4 min at 95°C.
- Incubate for 10 min in 1L beaker filled with 70°C water and allow the water in the beaker to cool to RT (*Note: This will take a couple of hours, but it is important that you do this slowly! One may set up a thermal cycler to achieve the same*.)

Steps 4-6 are common to both sgRNAs and shRNAs protocols

4. Ligation

Be sure to include a control reaction without annealed oligis. Each ligation contains the following to be added in the order listed:

Note: Users may make a serial dilution e.g. 1ul straight, 1:10, 1:100, 1:1000, to capture an appropriate stoichiometry (between oligo and vector) in the ligation reaction.

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

20ul total

Ligation proceeds at 16°C for 3-4 hours.

5. Transformation/plating/picking colonies

- 2ul ligation reaction mix is added to 25ul competent cells (DH5α). The mix is placed on ice for 30min.
- Heat shock at 42°C for 90 seconds, followed by incubation on ice for 2min.
- The transformed cells are recovered with 400ul SOC at 37°C for 1 hour.
- ~150ul transformants are plated onto agar dishes containing 100ug/ml carbenicillin.
- Incubate agar dishes upside down at 37°C overnight.
- Pick 2-4 colonies each into 1-2 ml TB + 100ug/ml carbenicillin.
- Cells grow at 37°C (300rpm) for no more than 17 hours.
- Glycerol stock can be made by mixing 150ul glycerol with 850ul culture. Stored at -80°C.

6. Sequencing protocol

A. Primer sequence:

5’-GAT ACA AGG CTG TTA GAG AGA TAA TT-3’

B. DNA prep: DNA is prepped by TempliPhi from glycerol stock, or by alkaline lysis prep from overnight cultures. Adjust the concentration to 40ng/ul.

C. Sequencing Reaction: Each sequencing reaction contains ~50ng DNA template. We use 4:1 ratio of bigDye3.1:dGTP bigDye to read through the hairpin structures.

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. Sequencing reactions are cleaned up by ethanol precipitation. The dried fragments are eluted into 10ul of 0.1 mM EDTA, detected 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).