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Generating a gRNA construct for a CRISPR KO experiment

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ABSTRACT

This protocol is designed to clone a gRNA of choice into the PX458 plasmid to nucleofect at a later stage and select single cell clones on the basis of GFP fluorescence. The GFP fluorescence and Cas9 expression are transient and the cells are left without a selection marker, making them more appropriate for mouse in vivo experiments where foreign antigens (fluorescent or antibiotic-resistance proteins) might be an issue.

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Cas9, cloning, BbsI

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MATERIALS TEXT

pSpCas9(BB)-2A-GFP (PX458) **addgene Catalog #48138**

1 Design suitable gRNA to target exon of interest (Use Benchling or Deskgen tools to generate guide(s))
Benchling tool can generate oligo sequences that include BbsI sticky ends for cloning into PX458. Order the oligos from Sigma/IDT. Order dry individual oligos and anneal in the lab or order already annealed.
*If performing annealing in house: Mix equal volumes (20 uL + 20 uL) of 100 uM stock solutions of oligos in 1X Annealing buffer (recipe below) and follow annealing protocol.
Use the following thermal profile on the thermocycler:
a. Heat to 95 °C and maintain the temperature for 2 min.
b. Cool to 25 °C over 45 min (decrease by 15C every 10 min)
c. Cool to 4 °C for temporary storage.
Centrifuge the PCR tube briefly to draw all moisture away from the lid.
Annealing Buffer Composition (1X)
10 mM Tris, pH 7.5 - 8.0
50 mM NaCl
1 mM EDTA

2 Linearise Cas9-GFP plasmid with BbsI. CutSmart buffer, 37 C. Column purify after. (see details: https://international.neb.com/products/r3539-bbsi-hf#Product%20Information)

3 Clone the gRNA sequence into Cas9-GFP vector (PX458) by using BbsI restriction digest and ligation of annealed oligos according to the ligation protocol below:
Vector: Insert molar ratios between 1:1 and 1:10 are optimal for single insertions (up to 1:20 for short adaptors).
Use https://nebiocalculator.neb.com/#!/ligation to calculate the required molar ratio (use 1:7 to begin with)
1. Set up the following reaction in a microcentrifuge tube on ice.
(Quick Ligase should be added last)
COMPONENT
Quick Ligase Reaction Buffer (2X)* 10 uL
Vector DNA (8 kb), x uL (50 ng)
Annealed oligos (25 uM, find MW to calculate dilution to have 1 ng), 1 uL

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Nuclease-free Water, up to 20 uL
Quick Ligase, 1 uL
*The Quick Ligase Reaction Buffer should be thawed and resuspended at room temperature.
2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. Incubate at room temperature (25°C) for 5 minutes.

4 Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells. Alternatively, store at -20°C.
Do not heat inactivate – heat inactivation dramatically reduces transformation efficiency.

5 Transformation protocol:

Stellar Competent Cells (Use 14 mL round-bottom tubes)
50 uL of cell suspension + 2.5 uL of DNA mix
Incubate on ice for 30 min, heat shock for 45 s at 42 degrees, keep on ice 1-2 min
Add 450 uL SOC medium (warmed up to 37 degrees previously)
Incubate for 1 h at 37 degrees (30 min is also fine if in a rush)
Plate 50 uL on an Amp agar plate
Leave in the incubator at 37C

Alternatively, use any other competent cells and transformation protocol of choice.

Make sure to have a ligation control plate (digested vector with no ligase) to confirm that ligation worked, and to assess the background of any undigested plasmid. In my experience, the ligation works extremely well.
Pick a few clones the following day and grow for mini-preps. Isolate DNA by mini-prep and confirm by Sanger sequencing (You can use universal U6 Fwd primer from Genewiz).

N.B. For the next stage, the expression of GFP does not mean editing took place. Each clone should be tested by an appropriate method (WB, FACS, sequencing) to make sure the location of interest was targeted and the protein of interest was lost.

The same procedure can be applied to clone a gRNA into the Cas9 plasmid with puromycin selection.

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