Center for Skeletal Research

MGH Endocrine Unit

Bone Cells Core

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pSpCas9(BB)-2A-GFP (PX458) Addgene Cloning Protocol for CRISPR/Cas9

(IMPORTANT** read note at the bottom of page 2)
Same vector is also available with PURO resistance cassette (PX459)

1) Anneal oligos:
Dilute oligos to 100 µM

Annealing master mix, 8 µL to each tube:
1 µL 10x T4 ligase buffer
1 µL T4 PNK
6 µL ddH_2O
1 µL forward guide (100 µM)
1 µL reverse guide (100 µM)
10 µL total volume

Run product on PCR machine (37°C for 30 min., 95°C for 5 min, ramp down to 25°C at 5°C/min)
Dilute annealed oligo solution 1:200 – add 1 µL of annealed oligos to 199 µL ddH_2O

2) Ligation

Ligation master mix, 18 µL to each tube:

0.5 µL of 200 ng/µL PX458 vector stock (100 ng)
2 µL 10x Tango buffer
1 µL 10 mM DTT
1 µL 10 mM ATP
1 µL FastDigest BbsI
0.5 µL T7 DNA Ligase
12 µL ddH_2O
2 µL diluted oligo complex (1:200)
20 µL total volume

Run on PCR (37°C for 5 min, 21°C for 5 min, repeated 6 times)

3) Digest residual linearized DNA (optional, but highly recommended)

Digestion master mix, 4 µL to each tube:
1.5 µL 10x PlasmidSafe buffer
1.5 µL 10 mM ATP
1 µL PlasmidSafe exonuclease
11 µL ligation reaction from above
15 µL total volume

37°C for 30 min, followed by 70°C for 30 min on PCR machine

4) Transform bacteria

Use 3 µL of digest reactions + 25 µL (1/2 of tube) of NEB 5-α Competent *E. Coli* (High Efficiency), then follow NEB transformation protocol.

NEB Transformation Protocol
1. Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice for 10 minutes.
2. Add **3 µl** of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex.**
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette **475 µl** of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm amp selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting
10. Spread **100 µl** of each sample onto an amp selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

After incubation, select colonies with p1000 pipette tip and place in 1.5 mL of LB Broth + 1x ampicillin in 15 mL tubes (can leave caps closed tightly).

Incubate 15 mL tubes overnight at 37°C, shaking at 250 RPM.

After overnight incubation, pick 10-20 colonies (or more) and perform minipreps using Qiagen kit (27106), following Qiagen protocol.

Submit minipreps for sequencing (10 µL miniprep DNA + 10 µL sequencing primer) to validate targets.
The plasmid is now ready for transfection for CRISPR/Cas9

** These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.
pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138)

link to Addgene
https://www.addgene.org/48138/