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Design and construct retroviral sgRNA plasmids for gene knockout

1. Vector backbone

pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP (Addgene, #102796)

2. Design sgRNA oligos

I. Access CRISPick website (Broad institute)

https://portals.broadinstitute.org/gppx/crispick/public

II. Select Reference Genome: human or mouse?

- CRISPRa/i…NCBI reference data set
- CRISPRko…Ensembl data set

III. Select Mechanism

- CRISPRko

IV. Select the type of Enzyme

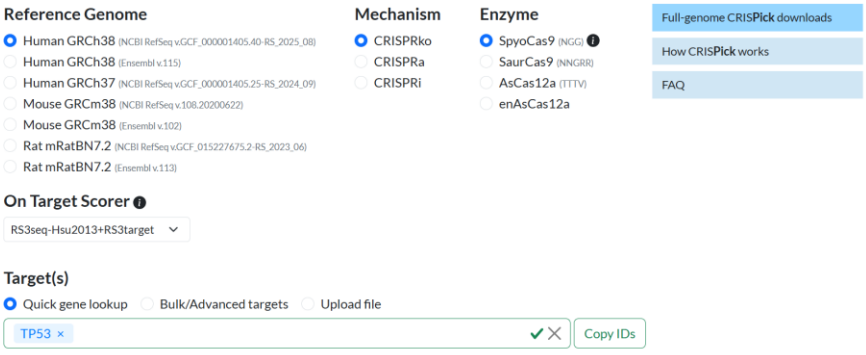
- SpyoCas9(NGG)

V. On Target Scorer

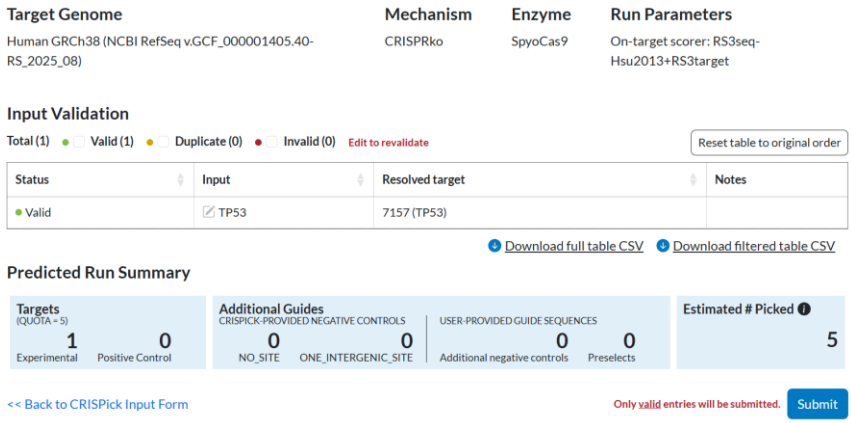
Use Hsu(2013) if the tracrRNA in your vector starts with GTTTT.

(pLKO5.sgRNA.EFS.tRFP657, pLKO5.sgRNA.EFS.GFP, lentiGuide-Puro start with GTTTT, and pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP)

VI. Input Transcript IDs: Put RefSeq transcript ID



Click validate and then click submit



Click picking results

VII. Target Sequences

原則として上位 1, 2 の “target sequences (sgRNA sequences)”を使う (Pick order)

VIII. Design oligos

Oligo 1: CACCG(Target Sequence)GT

Oligo 2: TAAAAC(Reverse Complement of Target Sequence)C

e.g.
Target Sequence: AATCACGAAATGGCCAACGG
Reverse Complement: CCGTTGGCCATTTCGTGATT
(http://www.bioinformatics.org/sms/rev_comp.html)

Oligo1: CACCGAATCACGAAATGGCCAACGGGT
Oligo 2: TAAAACCCGTTGGCCATTTCGTGATTC

3. Cloning Oligos into pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP

(1) Digesting pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP vector

Vector (2 μg)	2 μl
10x rCutSmart™ Buffer	2 μl
BbsI-HF (NEB)	1 μl
DDW	15 μl
Total	20 μl

Incubate at 37°C for 2 hours
Inactivate at 65°C for 20 min and run 1% agarose gel

(2) Annealing oligos

Forward oligo (100 μM)	1 μl
Reverse oligo (100 μM)	1 μl
10x NEBuffer™ r2.1 (NEB) or 10x T buffer (Takara)	5 μl
DDW	43μl
Total	50 μl

Vortex, spin down
95°C 4 min
70°C 10 min
Take out the heat block and cool it down to room temperature (at least 1 hour)
*Do not directly put it on ice!

(3) Run 1% agarose gel (100V, 25 min) with step 3-1 digested vectors

Expected fragment size:

pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP: **7457 bp (cut vector)** **,** 22 bp

-> Cut 7-8 kb digested vector and perform gel extraction

-> Elute DNA with 25 μl DDW

(4) Ligation

*10x dilution for annealed oligos: 5 μl mixture (from step 3-2) + 45 μl DDW

10x diluted oligos	3 μl
Purified vector (step 3-3)	1 μl
2x mighty ligation mix (Takara, 6023)	4 μl
Total	8 μl

Incubate at 16°C for 1-3 hours

(5) Transformation (DH5α)

-> Take 2 μl of ligation product (step 3-4) into 25 μl of DH5α

-> on ice 30 min -> heat shock 42°C for 45 sec -> on ice 3 min -> add 80 μl SOC

-> all for plating with Amp plate

(6) Mini-culture

Pick up 6 colonies each group for mini-culture (2 ml LB+2 μl Amp)

(7) Mini-prep

Dissolve DNA with 30 μl TE

(8) Enzyme digestion check

Mini-prep DNA	1 μl
10x rCutSmart™ Buffer	2 μl
BbsI-HF (NEB)	0.4 μl
BsiWI-HF (NEB)	0.4 μl
DDW	16.2 μl
Total	20 μl

Correct expected fragment size: 7500 bp (single cut)

Run 1% agarose gel (100V, 20 min)

(9) Select a correct plasmid for sequencing

U6 primer: gagggcctatttcccatgatt