Gene disruption or upregulation by Crispr

Things need to be ordered

1) gRNA-Cas9 lenti vector: Addgene #52961 (puro)
   https://www.addgene.org/52961/
   or #98291 (hyg) https://www.addgene.org/98291/
2) 2nd generation lentiviral) packaging plasmid: Addgene #12260
   https://www.addgene.org/12260/
3) envelope plasmid pVSVG (pMD2.G): Addgene #8454
   https://www.addgene.org/8454/
   or Box14#742

(Optional GFP lenti viral vector Addgene #19319)

$65x3+$40 (Shipping $60)

2) BsmBI (Esp3I): Fermentas #ER0451 \14000

3) XL1-Blue (recombinase free strain) competent cells: Stratagene \
or Sbtl3 competent cells: Invitrogen #C3737-03 \54000

4) T4 kinase: Takara (2021S) or NEB (M0201) \9000

5) T4 ligase: Promega (M1801) or NEB (M0202) \7000

6) gRNA cording oligos: Operon, PCReady (50μM) \710 each

7) hU6f primer : Operon, PCReady (50μM) \710
   GGACTATCATATGCTTACCGT 53oC

8) Restriction enzymes, BamHI, NdeI

9) For Crispr A
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5526071/
   Vector 1: LentiMPHv2 Addgene #89308 (hyg)
   https://www.addgene.org/89308/
   Vector 2: LentiSAMv2 Addgene #75112 (BSD)
   https://www.addgene.org/75112/ ←sgRNA can be inserted b/w BsmBI site, but note that this vector doesn't contain a stuffer sequence

Optional

10) pCS2-Cas9n, pCS2-Cas9wt vectors: ask Kinoshita-san@Kyoto Univ.

11) gRNA vector: Addgene #43860

Crispr mediated gene disruption or up-regulation (Organelle Lab)

A la Feng Zhang *et al*, **Nature Methods** (2014), PMID: 25075903
"Improved vectors and genome-wide libraries for CRISPR screening"
Feng Zhang *et al*, **Nature Methods** (2014), PMID: 28333914
"Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening"
Feng Zhang *et al*, **Nature Protocol** (2013), PMID: 24157548
"Genome engineering using the CRISPR-Cas9 system"

<u>sgRNA+Cas9 Vector:</u> Addgene #52961 (puro) or #98291 (hyg), or #75112 (BSD) (U6 promoter, for lenti virus)

```
BsmBI—BsmBI  ~1.8kb

TGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGG
ACGAAACACCGGAGACGGTTGTAAATGA………….1.8kb……….CTTTTTTGTACGTCTCTGTTTTAGAGCT
AGAAATA
```

- *Bsm*BI 1st site
  ```
  5' AA|CACC GGAGACG 3'
  3' TT GTGG|CCTCTGC 5'
  ```
- *Bsm*BI 2nd site
  ```
  5' CGTCTCT|GTTT TA 3'
  3' GCAGAGA CAAA|AT 5'
  ```

<u>Target design tool ~~(http://crispr.mit.edu) ← discon.~~</u>
use this site: https://crispr.cos.uni-heidelberg.de
Choose target gene sequence w/ <500bp length
Choose U6 @In vitro transcription
Chose human or what ever @species
Pick a couple of target sequences not to be bothered by off-target effects

Optional: check DSB and repair prediction @
http://www.rgenome.net/mich-calculator/

<u>Given the target sequence</u>

Add "G" at 5'-end for better transcription by U6 promoter
(Unnecessary if the target 5'-end starts with "G")
Remove PAM (3'-end 3 nucleotides, "NGG")
Add "G" at 3'-end to compensate for G removal by *Bsm*BI digestion
Then, add BsmBI cut ends

```
Target      5'     AATGATCGAGACCGCATAGTGGG 3'
```
- ```
  Oligo 1     5'caccGAATGATCGAGACCGCATAGTg         3'
  Oligo 2rc          CTTACTAGCTCTGGCGTATCTcCAAA
  ```
  ```

- Oligo 2 5’aaaccACTATGCGGTCTCGATCATTC 3’

Other reagents should be ordered or made

- Order oligo 1 and oligo 2 (Eurofins, PCReady, @50μM)
- Order *Bsm*BI (Esp3I, Fermentas #ER0451 or 0452, this comes w/ 10xTango buffer)

Note that BsmBI from other company may not be compatible for this use due to optimum temp.

- Use T4 ligase (Promega, M1801 or NEB, M0202) instead of T7 ligase
- Make 10xDTT stock sol’n. (10mM, 1M stock should be somewhere)
- Make 10xATP stock sol’n. (10mM, make new, ATP 5.5mg/ml DW)
- *Bsm*BI needs DTT (Tango buffer doesn’t contain DTT)
- T4 ligase and *Bam*HI seem to be OK w/ Tango buffer

Annealing

Reaction mixture:

- Oligo 1 (50μM) 2μl
- Oligo 2 (50μM) 2μl
- 10mM ATP 1μl
- 10xT4 kinase buffer 1μl
- T4 kinase (Takara) 1μl ←or NEB, M0201
- DW ~3μl /10μl

Reaction:

1 37oC, 30min
2 95oC, 5min
3 -5oC/min till 25oC

Dilute this mixture @1:200 just before use

*Bsm*BI digestion and ligation

Reaction mixture:

- sgRNA+Cas9 vector (#52961 or 98291, or 75112) 50ng (xμl)
- ±diluted, annealed oligo 1μl
  (minus oligo should be background control)
- 10mM DTT 1μl
- 10mM ATP 1μl
- 10xTango buffer 1μl
- *Bsm*BI 0.5μl
- T4 ligase 0.5μl
- DW ~5μl /10μl

Reaction

1 37oC 5min

2 21oC 5min
3 back to 1, cycle x6

<u>Plasmid prep</u>

XL1-Blue (better option: Sbtl3) competent cells for transformation
Transformation, miniprep (#52961 vector is Amp r)
Double digest miniprep'ed sgRNA-oligo w/ *Nde*I and *Bam*HI
Run 1% agarose-TAE gel longer for better separation
OK clone should be 6+4.6kb+2.3kb
The original vector should be 6+6.5+2.3kb
Note that #75112 (LentiSAMv2) doesn't contain the stuffer 2.3kb.
Send clones for sequencing to Macrogen
w/ U6 forward primer (GGACTATCATATGCTTACCGT)

Preparation of lentivirus particles

The vector #52961 contains Puro r and wildtype 5'LTR
Use 2nd generation packaging system (see below).
A293T cells should be co-transfected with below
pVSVG (box14#742) or pMD2.G (box19#960), env
psPAX2 (addgene #12260, box24, 1202), packaging
lenti-sgRNA vector (addgene #52961, box24, 1201 or
control lentivector just expressing GFP
(addgene #19319, box24, 1203)

Day 0
Plate 293T cell line intoT25 or T75 flask

Day 1
Transfection ←in case using T75, use 3-times amounts of ingredients
A293T cell line (~85% confluent, T25)
↓ replace media, 4ml DMEM+10%FBS
↓ Transfection
mix below, incubate for 15min, r.t.
200 μl Serum free DMEM
↓ +x μl pVSVG 1.5μg (box14#742)
↓ +x μl packaging plasmid 3μg (box24#1202
↓ +xμl lentiplasmid 4μg
↓ +20μl 1mg/ml PEI Max
↓ +transfection mixture above, 37oC, O/N

Day 2
Discard media, and change to 5ml DMEM-10%FBS @37oC

Day 3
Collect media (Sup1), and add 5ml DMEM-10%FBS @37oC
Check GFP expression, if lower than 30%, redo transfection
Plate cells for stable line in 6well ←in case of Crispr A, plate cells stably expressing LentiMPHv2 (https://www.addgene.org/89308)

Day 4
Collect media (Sup2), combine Sup1+Sup2
↓
Centrifuge pooled Sup 1+2 @1000rpm, 1min
↓
Filter through 0.45μm pore

↓
*

Add 4ml + 2.4µl Polybrene (10mg/ml stock, filtered) to cells in 6well
↓
Spin 6well plate w/ tape tightly @1700rpm, 60min, r.t. →37oC

Day 5
Split cells and culture cells with drug (puro 2 or hyg 800, or BSD8) for selection

Day 6
Change media (puro 0.8 or hyg 400, or BSD8)

Day 7
Change media (no drug)

Day 8 or 9
Split cells or change media (no drug), Plate cells for immunofluorescence to check target gene expression
Further sup collection from 293-T

Keep culture cells until they stably grow, make frozen stocks

In some gene KO, cells don't grow very well. At Day 8 time point, leave them for 1 week~10 days

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Lenti plasmid — Expression should be checked by regular transient transfection before making viruses
Use 2nd generation packaging system (see below, b/c of wildtype 5'LTR)

Control lenti plasmid ex) addgene #19319 (GFP expressing vector)

pVSVG plasmid — from Clontech or Addgene #8454 (2nd 3rd generation)
packaging plasmid — from Addgene #12260 (2nd generation)

Media for A293T: Should be warmed @37oC
DMEM+10%FBS

Polybrene — 10mg/ml filtered stock@-20oC (Millipore)
Use @0.6mg/1ml
←might be toxic for some cell lines,

use Protamine Sulfate

*viral sup can be concentrated using Lenti-X concentrator (Clontech # 631231)

Optimization of drug conc. for selection
Don't just add drugs to cells in culture
Add drugs when cells splitting
Expression level should be dependent on drug conc.

Drug conc. for HeLa
Puro 2→0.8μg/L
Hyg 8→400
BSD 8→7

Virus sup storage
For short term @4oC
For longer term @-20oC

Crispr mediated gene activation

A la Feng Zhang *et al*, **Nature Protocol** (2017), PMID: 28333914
"Genome-scale CRISPR-Cas9 Knockout and Transcriptional Activation Screening"
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5526071/

vector 1: Lenti-MPH v2 (#1587, https://www.addgene.org/89308) <Hyg r
vector 2: Lenti-SAM v2 (#1586, https://www.addgene.org/75112/) <BSD r

Clone targeting oligos into vector 2 (Lenti-SAM v2 @BsmBI sites)
Targeting sites can be b/w 0~-200bp (see the paper above)
Note: Lenti-SAM v2 doesn't have spacer.

Make lenti viruses, see elsewhere, using vector 1 and vector 2
Co-infect to target cells
Select expressing cells w/ Hyg and BSD
Alternatively, lenti-MPH v2 expressing cell line can be established first.
All in one vector is available # 167934 https://www.addgene.org/167934/