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# Generation of Combinatorial CRISPR Libraries

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**Protocol status:** Working
**We use this protocol and it's working**

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## Disclaimer



## Troubleshooting

## Summary

1 This protocol is a step by step protocol based on the method originally described by: Vidigal, J.A. & Ventura, A. Rapid and efficient one-step generation of paired gRNA CRISPR-Cas9 libraries. *Nature Communications* **6**, 8083 (2015).

Throughout:

All PCR clean-ups done with Monarch PCR & DNA cleanup kit
**https://www.neb.com/products/t1030-monarch-pcr-dna-cleanup-kit-5-ug**

All gels run with Sybr green, not ethidium bromide, and no gels exposed to UV light to avoid DNA damage.

## Vector Preparation

2 **Backbone digestion and preparation (lenti-guide puro)**

Digest 5-10ug of your vector of choice:
1. lenti-guide puro 10ug (Addgene #52963)
2. 10X buffer 3.1 10ul
3. BsmBI 10000 U/ml 8ul
4. H20 to 100ul

Incubate @ 55°C overnight

**Dephosphorylation**

https://www.neb.com/protocols/1/01/01/vector-dephosphorylation-protocol

- 49ul of digestion
- Antartic phosphatase 5ul
- Antartic phosphatase buffer 10X 6ul

37C for 45'
Then 5' 80C
Then 4C

Gel purification: take the 8.3kb band.

## PCR and oligos

3 **PCR amplification of oligonucleotides**

Supplied by TWIST bioscience

Diluted library to final concentration of 0.0045ng/ul

Do 14xPCR – each PCR 50ul

https://www.neb.com/protocols/1/01/01/pcr-protocol-m0530

| | | Regent amount for 14x reactions |
|---|---|---|
| | Oligo | 14 |
| | HF phusion buffer | 140 |
| | dNTP | 14 |
| | F primer | 17.5 |
| | R primer | 17.5 |
| | Phusion HF polymerase | 7 |
| | H2O | 490 |
| | | 700 |

PCR program

a.98C 30′

i.98 10′′

ii.68 $C^{o}$ 35′

iii.72 C 30′′16X

b.72C 10′

c.4C 4ever

PCR cleanup and elute in 30ul

**Digestion of pdonor_SU6**

Digest 5ug of pDonor_sU6 (plasmid #741) with *Bbs*I

Plasmid 5ug
Buffer 2.1 10X 5ul
Enzyme BbsI 10000 U/ml3ul
H20 up to50ul total

For 37C for three hours
Gel purification: take the 415bp band
**Gibson Ligation**
https://www.neb.com/products/t1030-monarch-pcr-dna-cleanup-kit-5-ug
Donor fragment 405ng
Oligo amplicon 432ng
2xGibson MM 30 ul (NEBuilder® HiFi DNA Assembly Master Mix, E2621S)
H2O to 60 ul

Incubate @ 50°C for 2hours
**Nuclease Digestion**

http://www.epibio.com/enzymes/nucleases-glycosylases-dna-binding-proteins/dna-exonucleases/plasmid-safe-atp-dependent-dnase?details

Add to each Gibson reaction
- 10x Plasmid Safe Buffer 9 ul
- ATP (25mM) 9 ul
- Plasmid Safe nuclease 3 ul
- H2O 9 ul

àincubate @ 37°C for 1h

Clean up using PCR purification kit and elute in 50ul water

**Digestion of Gibson product**

Digest with *Bbs*I
DNA 50ul
Buffer 2.1 6ul
*Bbs*I 4ul

Incubate @ 37°C for 2.5h

Run digestion on 2.5% gel and cut ~480 bp band
Gel extract and elute the completed insert in 30ul

**Ligation**

https://www.neb.com/products/m2200-quick-ligation-kit

Neb Quick ligation kit; M2200S; scale up as appropriate

| | | Amount | |
|---|---|---|---|
| | Digested&purified lenti-guide puro | 25ng | |
| | sgRNA insert BbsI purified | X | |
| | ligase buffer | 5ul | |
| | Ligase | 0.5ul | |
| | H2O to 10.5ul | | |

Incubated at 25C for 15minutes (thermocycler)
Prior to library creation controls done with vector only (lentiguide puro) to assess background.
For library used final volume of 300ul for ligation
PCR purification and elution in 30ulwater(for library)

## Transformation

4 **Transformation**

https://www.neb.com/products/c3020-neb-10-beta-electrocompetent-e-coli
25ul bacteria/2.5ul ligation product/transformation; electroporated as per website protocol. 10 electroporations. 1hour in pre-warmed SOC shaking.
Plated at dilutions (1:1000; 1:10000) onto warmed ampicillin plates. The remainder inoculated into ampicillin containing LB broth 3 litres at 100ug/ml and shaken at 225rpm/37C overnight.
Following morning pooled spun down in Avanti centrifuge (6000g for 10 minutes)
Bacterial pellet processed using maxi/mega prep (Qiagen) ~1.5g bacteria/mega prep