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# Generation of Combinatorial CRISPR Libraries

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Works for me

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## 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 &amp; 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.

## 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. H2O to 100ul

Incubate @ 55°C overnight

### **Dephosphorylation**

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

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

37C for 45'

Then 5' 80C

Then 4C

Gel purification: take the 8.3kb band.

## 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>

	Reagent 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° 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 *BbsI*

Plasmid 5ug  
Buffer 2.1 10X 5ul  
Enzyme *BbsI* 10000 U/ml 3ul  
H2O up to 50ul total

For 37°C 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 2 hours

### **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

Incubate @ 37°C for 1h

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

### **Digestion of Gibson product**

Digest with *BbsI*  
DNA 50ul  
Buffer 2.1 6ul  
*BbsI* 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 <i>BbsI</i> 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