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## Generation of Combinatorial CRISPR Libraries

 [Nature Communications](#)

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**Protocol status:** Working

**We use this protocol and it's working**

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## Disclaimer

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## 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. 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.



## 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>

	Reg ent amo unt for 14x reac tions
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 *Bbsl*

Plasmid 5ug

Buffer 2.1 10X 5ul

Enzyme *Bbsl* 10000 U/ml 3ul

H<sub>2</sub>O 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)

H<sub>2</sub>O 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
- H<sub>2</sub>O 9 ul

→ incubate @ 37°C for 1h

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

### **Digestion of Gibson product**

Digest with *Bbsl*

DNA 50ul

Buffer 2.1 6ul

*Bbsl* 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 (lenti-guide puro) to assess background.

For library used final volume of 300ul for ligation

PCR purification and elution in 30ul water (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