

Cloning of pooled sgRNAs into lentiviral vector

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1) Vector Preparation: pLG1 library vector

a) Digest 5 ug of vector with Thermo Scientific FastDigest BstX1 and Blp1 for 1h at 37°C. Set up reaction as follows:

100 uL Reaction

- | | |
|-------------------------------|----------------|
| • 10x FastDigest Green Buffer | 10 uL |
| • Vector | (5ug) |
| • Blp1 | 2.5 uL |
| • BstX1 | 2.5 uL |
| • Water | Fill to 100 uL |

If you plan to use the vector often I recommend setting up multiple reactions (4 to 8).

b) While digest is incubating, make 0.8% agarose gel and use wide gel combs. Stain gel and excise linearized vector.

c) Gel purify excised vector using an appropriate gel purification kit, e.g. Machery-Nagel's NucleoSpin Gel & PCR Cleanup kit (Catalog # 740609)

2) Insert Preparation: PCR

a) Set 6 X 100 uL reactions and an additional "NO template" negative control. While 6, 100 uL reactions is excessive you will have plenty of back-up insert.

	<u>100 uL reaction</u>	<u>6 x 100uL reactions</u>
• Water	71 uL	426 uL
• 5X Phusion HF Buffer	20 uL	120 uL
• DMSO (100%)	3 uL	18 uL
• dNTPs (10 mM)	2 uL	12 uL
• P(F) 100 uM	0.5 uL	3 uL
• P(R) 100 uM	0.5 uL	3 uL
• Template (0.1 pM/uL)	2 uL	12 uL
• HF Phusion	1 uL	6 uL

PCR conditions

- 98°C, 30s
- 15 cycles
 - 98°C, 15s
 - 56°C, 15s
 - 72°C, 15s

- 72°C, 10min
- 7°C hold

b) Combine reactions in a 5 mL tube (or similar). To verify correct amplification, run 5 uL of combined reactions on a 10% acrylamide gel (or similar), at 120V for ~20-30 minutes.

c) While gel is running purify the remaining ~595 uL of each PCR using Qiagen's MinElute Reaction Cleanup Kit (or similar) (Catalog #28204), using 1 column per pooled sample:

- Add 4X sample volume of Qiagen Binding Buffer PB to combined PCR.
- Add 1/100th volume 3M NaOAc.
 - Combined PCR= ~600 uL, Add 2.4 mL PB and 30 uL NaOAc
- Once PCR is bound to column, Wash 2X w/ 750 uL of PE
- Perform one dry spin at max speed for 2 minutes
- Elute in 20 uL of EB (warm media to 70C and wait 5 minutes before eluting)

***NOTE: The MinElute Kit does NOT come with Buffer PB, must be purchased separately (Catalog #19066)**

Your recovery should be between ~0.75 ug to 1.5ug (3, 100 uL rxns), ~1.5ug to 3ug (6, 100 uL rxns). If your recovery is low you will need to optimize your PCRs before proceeding with the digest.

d) At this point the gel should be finished. **The expected insert size is 84bp.** Once you have verified your Insert PCR is correct (you see a nice bright band at 84bp), you can proceed and digest your insert.

3) Insert Digest:

a) Digest 1 ug of purified insert. Incubate digest at 37°C for 2-6 hours. Following digest insert will be ~33bp (84=26+33+25).

30 uL Digest

- | | |
|-------------------------------|---------------|
| • Insert | (1 ug) |
| • 10x FastDigest Green buffer | 3 uL |
| • Bstx1 | 2.5 uL |
| • Blp1 | 2.5 uL |
| • Water | Fill to 30 uL |

b) Gel purification

- To purify, run the digest on a 10% acrylamide gel. Load 15 uL of digest into each of 2 adjacent wells, leaving an empty spacer well between different samples. Run the gel at

~120V for ~30-40 minutes, or until the yellow dye has traveled at least 75% of the total lane length

- While gel is running prepare tubes and tips for rapid extraction:
- When the gel has completed running, stain with EtBr or SYBRsafe and excise insert using UV or blue light transilluminator. Change blades between samples to avoid cross contamination. Place excised gel piece inside 0.5mL nonstick tube prepared above, and proceed with Rapid Extraction.

c) Rapid Extraction

- Pierce a 0.5mL nonstick tube (Ambion, Catalog #AM12350) with a 18.5 gauge needle and place inside a 1.5 mL nonstick tube
- Excise gel piece and place it inside of 0.5ml tube with a hole.
- Spin tubes at 20,000 x g for 3 min. This will crush the gel as it is forced through the hole. Check for gel pieces in the 0.5 mL tube. Residual pieces should be transferred to large tube. If you cannot tap the residual pieces use a pipette tip.
- Add 200 uL of water to gel pieces, and incubate for 30min~1 hour at 70°C
- Vortex gel slurry for 30s and use cut p1000 tips to transfer gel mixture to Costar Spin-X columns.
- Spin tubes for 3 min at 20,000 x g to recover the elution mixture free of gel debris.
- Transfer eluate to a new 1.5 mL nonstick tube
- Isopropanol Precipitation
 - **200 uL of eluate (insert is here)**
 - **2uL of glycoblu (ThermoFisher #AM9515) (this will help visualize your pellet)**
 - **25 uL 3M NaOAc or 3M NaCl**
 - Mix Well (invert 10-15X)
- Add 0.6 mL 100% EtOH, mix well
- Incubate at -20°C, for 2hr to overnight.
- Pellet: Spin 30 min at 20,000 x g at 4°C , remove supernatant
- Wash pellet 2X with ice cold 80% EtOH
- Air Dry
- Resuspend pellet in 25 uL water or Qiagen EB

d) If you nanodrop your sample you will observe a contamination peak at 230. This is from the glycoblu. I generally observe a nanodrop concentration of ~20-25 ng/uL with glycoblu, and without glycoblu ~10-15 ng/uL. From Qubit, a fluorometer, (DNA high sensitivity assay) I observe ~0.5-2 ng/uL. As the Qubit is more sensitive I use these concentrations. Proceed to ligation.

4) Ligation

As a first pass, you may want to set up 3 conditions (vector:insert), 1:2, 1:1, 2:1. Adding too much insert can cause sgRNA concatemers, which are observed at 1:3. The molar ratios of 1:1 worked well for us (500 ng vector:1.9 ng insert).

a) 20 uL ligation

- Vector (500 ng)
- Insert (0.9 ng)
- 10x Ligase buffer 2 uL
- T4 ligase 1 uL
- Water Fill to 20uL

**Include a negative control with no insert to check for background from vector.
Incubate at 16C for 16 hours**

b) Ligation Reaction Clean-Up: EtOH Precipitation

Removing the excess salt in the ligation reaction is crucial for electroporation.

EtOH Precipitation

- Bring 20 uL ligation reaction to 200 uL with water.
- Add 2uL of glycoblue (this will help you visualize your pellet)
- Add 1/10 reaction volume of 3M NaOAc, 20 uL. Mix Well.
- Add 3X reaction volume of 100% EtOH, 600 uL. Invert 10X to mix.
- Place at -20°C, 2h to overnight.
- Pellet: Spin 30 min at 20,000 x g at 4°C
- Remove supernatant and wash pellet 2X with ice cold 80% EtOH
- Air Dry
- Resuspend pellet in 20 uL of water
From a 20 uL ligation, we observe ~15 ng/uL (from Qubit)

5) Test Transformation

A series of test transformations will be performed to verify correct inserts and successful ligations with low background. Two serial transformations will be performed, as a single transformation of the ligation generates many mixed peaks during Sanger sequencing of colony PCR product.

a) *Primary transformation with high efficiency chemically competent cells (ex, Stellar cells (Clontech, # 636736, 108),*

- Add 0.5uL ligation to 20 uL Stellar cells, and gently tap to mix.
- Incubate on ice for 30 minutes
- Heat-shock 45s @ 42°C
- Incubate on ice for 2 min
- Add 300 uL pre-warmed SOC and incubate for 1 hour at 37°C.
- Plate 200 uL on pre-warmed plates (save the remaining recovery at RT or 4C)
- Incubate O/N at 37°C

b) *Plasmid prep:*

If your negative control shows <5% background colonies, it is safe to proceed. Scrape plates with 1.5 mL of LB and purify plasmid (mini-prep).

c) *Secondary transformation with subcloning efficiency chemically competent DH5a, 106)*

- Add 1ul of prepared plasmid to 15-20uL of DH5a and gently tap to mix.
- Incubate on ice for 2 minutes
- Heat-shock 30s @ 42°C
- Incubate on ice for 2 min
- Add 980 uL SOC
- Plate 2 dilutions of recovery, 1/50x (~20uL) and 1/200x (~5uL), on separate plates for each sample (save the remaining recovery at RT or 4C). The goal is to achieve very sparse colonies for colony PCR
- Incubate O/N at 37°C

d) *Pick the best plates for each sample. Sequence 10-20 colonies from each plate. The Sanger sequencing primer sequences are below; you can sequence directly from a colony PCR with the primer pair (expected size 804bp) or miniprep the plasmids first. Either primer will allow you to sequence through the sgRNA protospacer and adjacent restriction sites. To verify your test transformation:*

- Align the Sanger sequence output to the library vector map in your favorite cloning software (e.g. ApE) to ensure the sgRNAs were cloned properly
- The 19bp corresponding to the unique protospacer sequence should not align it the vector. Search for this sequence in the list of sgRNAs in your oligo pool. If 60-80% of the sequences match to the correct library (you will likely see some oligo synthesis errors and sequencing errors), it is safe to proceed with the large-scale transformation.
 - No sequences should perfectly align to the library vector at the protospacer region. This would indicate that the parental vector was incompletely digested and the stuffer sequence is present in the library.
 - It is also extremely unlikely that any sgRNA appears multiple times in a random set of 10 colonies. Repeated sequences are an indication that one species from the oligo pool is very overrepresented.

sgRNA-LIB_colonyPCR_F gcgccaattctgcagacaaa 57.8 °C
 sgRNA-LIB_colonyPCR_R CCTTCTCTAGGCACCGGTTTC 58.2 °C

6) Large-Scale Transformation

a) *Before starting, plan out the large-scale transformation We will be using large plates (Fisher, # NC9372402) with LB/Carbenicillin (75% of normal concentration).*

- *The ideal colony density is 100,000 colonies per plate; aiming for a 30x coverage of your library, this yields ~3333 unique oligos per plate. Consequently, you will want ~15 plates for every 50,000 unique oligos,*
- *We want to use ~100ul of MegaX cells per 15 plates. Scale accordingly and add some additional cells.*
- *Each electroporation cuvette holds a maximum of 90 uL.*
- *The total recovery media is 600uL x total number of plates per desired group.*

An example spreadsheet is shown:

Total Elements	# Plates	# Plates	MegaX (uL)	Total MegaX	#Cuvettes	Total Recovery Media (mL)
26060	7.818	8	53.33333	60	1	4.8
32670	9.801	10	66.66667	70	1	6
33810	10.143	10	66.66667	70	1	6
24570	7.371	8	53.33333	60	1	4.8
25390	7.617	8	53.33333	60	1	4.8
26290	7.887	8	53.33333	60	1	4.8
40290	12.087	12	80	90	2	7.2

b) *Pre-warm LB/carbenicillin plates 3-4 hours ahead of time. Thaw Mega-X cells on ice and pre-chill cuvettes on ice.*

- In a 1.5 mL eppendorf tube, mix appropriate volume of Mega-X and ligation product (~100 ng of ligation per 20 uL of MegaX). Tap gently 3X to mix
- Incubate on ice for 30 min
- Transfer the mix to prechilled cuvette using long gel-loading tips. Be sure there are no bubbles in your MegaX/dna mix
- Electroporate at 2.0 kV, 200 ohms, 25 uF in 0.1 cm cuvette (Gene Pulser Xcell, Bio Rad) You should observe a TC between 4.4ms and 4.6ms. Values outside this range are NOT successful electroporations. You may need to use less ligation per cuvette reaction, and/or double check for bubbles.
- Following electroporation, rinse each cuvette twice with 600 uL of recovery media or S.O.C and transfer to prepared cell culture tube. Recover at 37C at ~250RPM for 1.5-2hrs.
- Plate 600 uL per plate.

- Incubate at 37°C for 18h and make sure that colonies are grown well on all plates.

7) Scrape Plates

Add 15-25 mL LB for the initial scrape. Transfer to a clean collection tube. Add an additional 5-15 mL LB and transfer to collection tube. If you are scraping multiple plates, place your collection tube on ice. Once you are finished scraping, spin at 4,000 rpm for 20 min. The pellet should be tight. Pour off media and freeze pellet or proceed with plasmid preparation.

8) Plasmid Preparation

Qiagen or Sigma midi, maxi, mega or giga plasmid prep kits. Make sure columns are dry prior to the final elution. Wet columns will give a low recovery.

9) Prepare Samples for Illumina Sequencing

Illumina sequencing of the resulting plasmids is highly recommended to ensure the resulting plasmid libraries from this protocol are correct, have a large fraction of sgRNAs that match perfectly to the expected library, and the relative fractions of each sgRNA are tightly distributed. You only need ~100 sequencing reads per sgRNA in the library to be able to validate the library (ex. 20M reads for the entire CRISPRi genome-scale library), so you can include your samples as a <5% spike-in to other sequencing runs.

To sequence your libraries, follow Step 3 of the sequencing sample prep protocol (<https://weissmanlab.ucsf.edu/CRISPR/IlluminaSequencingSamplePrep.pdf>) with the following modifications:

- For the PCR, perform one 100uL PCR per library. If you have cloned multiple sublibraries, PCR each individually with a different sample index to allow you to detect any cross-contamination or sample mix-ups (although be careful to not introduce this during the PCR!).
- Substitute the genomic DNA input into the PCR with 100ng library plasmid.
- Only perform 15 cycles of PCR.