pgRNA cloning

protocol used in Thomas et al (2020), *Nature Genetics*

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Major steps

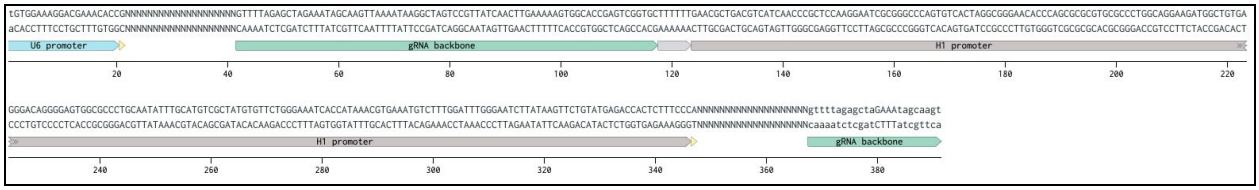
Procedure #1: gene fragment-based strategy

1. Order insert fragment from Genewiz – this is the only company that has been able to synthesize these fragments (the presence of repetitive gRNA backbone sequences has flagged this sequence as not "synthesize-able" when I tried other companies). An example fragment is here. Here's the sequence:

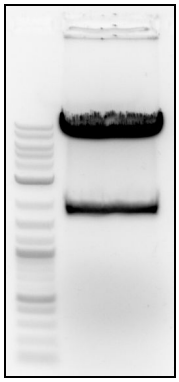
TGTGGAAAGGACGAAACACCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTA
TCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGAACGCTGACGTCATCAACCCGCTCCAAGGAATCGCGGGCCCAGTGTCA
CTAGGCGGGAACACCCAGCGCGCGTGCGCCCTGGCAGGAAGATGGCTGTGAGGGACAGGGGAGTGGCGCCCTGCAATATTTGCATG
TCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAATGTCTTTGGATTTGGGAATCTTATAAGTTCTGTATGAGACCACTCTTTC
CCANNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGT

- Part of U6 promoter
- "G" for efficient transcription initiation
- gRNA sequence
- gRNA backbond
- RNA Polymerase III termination signal
- H1 promoter
- "A" for efficient transcription initiation

Here's a schematic:



2. Digest lentiGuide-Puro backbone (Addgene #52963)



5 µg plasmid
3 µL BsmBI FastDigest (Thermo Esp3I; Cat # ER0451)
3 µL FastAP (Thermo Cat # EF0654)
6 µL 10X FastDigest Buffer
0.6 µL 0.1 M DTT
Water to 60 µL

Digest at 37°C for at least 30 minutes. Run product on a 0.8% agarose gel and excise backbone band (~0.2 kb BsmBI filler will have dropped out).

Purify using the Zymo gel extraction kit (Cat # D4007) following the manufacturer's protocol (add the extra water step that it recommends).

This purified backbone can be stored at -20°C for a long time.

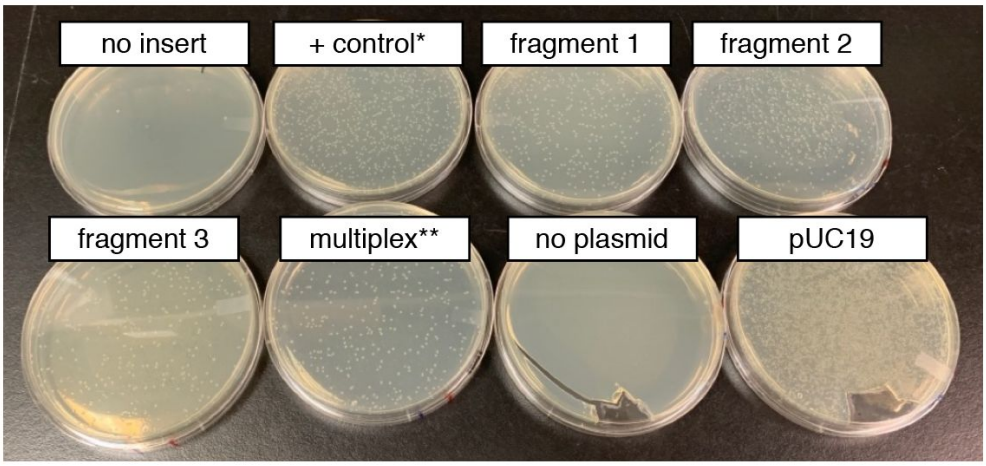
3. Dilute the gene fragment from above to ~5 ng/µL and perform fragment assembly. For this, it is helpful to include a no insert control to make sure there is little/no contaminating backbone.

   Set up reactions as follows:
   5 µL NEBuilder HiFi (NEB Cat # 2621S)
   52.5 ng of plasmid backbone (1:5 insert)
   3.80 ng of insert
   Water to 10 µL

   Incubate at 50˚C for 1 hour.

   This product can be stored at -20˚C for several months.

4. Transform bacteria using manufacturer's protocol. Here's an example of a successful cloning experiment:



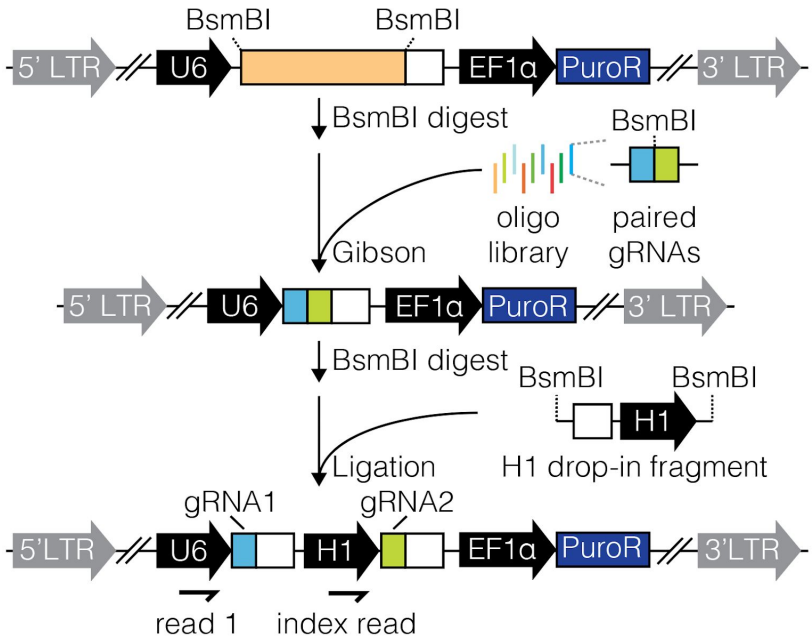
* PCR-amplified pgRNA ultramer without H1 drop-in (i.e., previous protocol)

** fragments 1, 2, 3 mixed together prior to Gibson

Individual colonies can be Sanger sequenced to confirm the identity of the pgRNA construct. For this, I make duplicate "touch plates". I send one for sequencing. After I get the Sanger results back, I grow up a miniprep and extract DNA to make lentivirus.

Procedure #0: original strategy

Overview

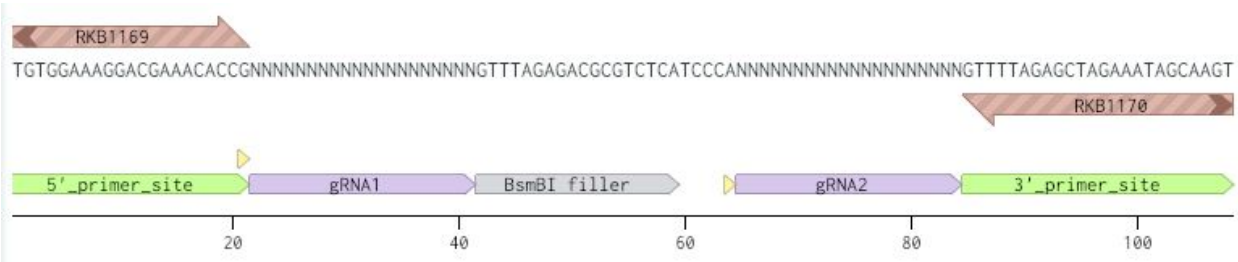


General notes:

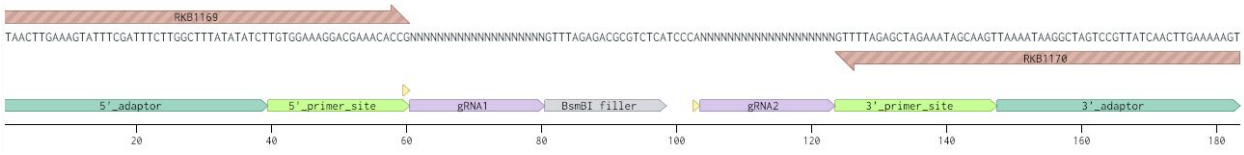
- The overview above is for cloning an oligo library, but the same scheme holds true for a single (or small pool) oligo cloning.
- Backbone is the lentiGuide-Puro plasmid (Addgene #52963).
- pgRNA oligos for the first step are ordered as 108 bp ultramers from IDT.
- Multiple pgRNA oligos can be pooled for multiplexed cloning and then sorted out at the end using Sanger sequencing of bacterial colonies.
- The "H1 drop-in" fragment is ordered as a 376 bp gBlock from IDT.
- NEB Stable cells are used for bacterial propagation.

pgRNA oligo overview

pgRNA oligo ultramer <- LINK



pgRNA oligo ultramer after amplification with RBK1169 & RKB1170 <- LINK



RKB1169 sequence: TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

RKB1170 sequence: ACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

H1 Drop-In sequence: link

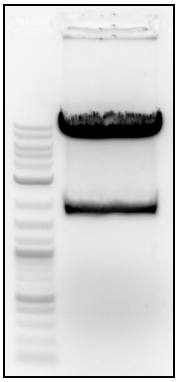
Digest and gel extract pLGP backbone

Set up restriction digest reaction as follows:

- 5 µg plasmid
- 3 µL BsmBI FastDigest (Thermo Esp3I; Cat # ER0451)
- 3 µL FastAP (Thermo Cat # EF0654)
- 6 µL 10X FastDigest Buffer
- 0.6 µL 0.1 M DTT
- Water to 60 µL

Digest at 37°C for at least 30 minutes.

Run product on a 0.8% agarose gel and excise backbone band (~0.2 kb BsmBI filler will have dropped out):



Purify using the Zymo gel extraction kit (Cat # D4007) following the manufacturer's protocol (add the extra water step that it recommends).

This purified backbone can be stored at -20°C for a long time.

PCR amplify and purify oligos

Prepare oligos for PCR:

Single oligo:

1) Resuspend the 4 nmol lyophilized oligo in 400 µL water.
2) Make a 1:10 dilution of this mix (10 µL oligo + 90 µL water).
3) Make a 1:20 dilution (1 µL oligo + 19 µL water) & use 1 µL for PCR.

For multiplexed cloning

1) Resuspend the 4 nmol lyophilized oligo in 400 µL water.
2) Make a 1:10 dilution of this mix (10 µL oligo + 90 µL water).
3) Combine 5 µL of each oligo from each group into a group master mix.
4) Make a 1:20 dilution (1 µL oligo + 19 µL water) & use 1 µL for PCR.

Set up PCR mixture as follows:

25 µL NEBNext High Fidelity 2X Ready Mix (Cat # M0541S)
1.5 µL of 10 µM forward primer (RKB1169)
1.5 µL of 10 µM reverse primer (RKB1170)
1 µL of diluted oligo(s)
Water to 50 µL

Amplification conditions:

95°C	5 min	1 cycle
98°C	20 s	2 cycles
62°C	15 s	-
72°C	30 s	-
98°C	20 s	5 cycles
72°C	45 s	-
72°C	5 min	1 cycle
10°C	hold	

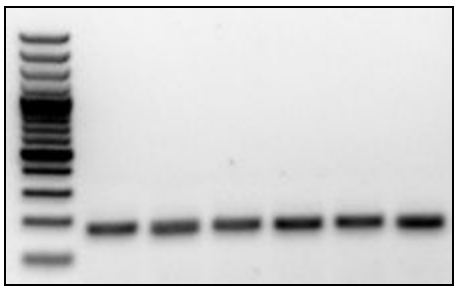
Purify reaction using a 1X SPRI bead cleanup:

1. Vortex SPRI beads and bring to RT for ~30 minutes.
2. Add 1X volumes of SPRI beads for every 1 volume of solution (e.g., 50 µL beads for every 50 µL reaction). Mix thoroughly by pipetting.
3. Incubate for 15 minutes at RT.
4. Place on magnet for ~5 minutes.
5. Take off supernatant leaving ~5 µL behind.
6. Wash with 200 µL of 80% ethanol (make this fresh daily!).
7. Incubate for 30 seconds.
8. Take off all ethanol.
9. Repeat wash once.

10. Dry beads with caps open. This is the trickiest part because you don't want to overdry the beads. The beads should be just slightly wet when you resuspend. This typically takes < 5 minutes of drying.
11. Remove the tube from the magnet and add 22 µL of water.
12. Incubate for 2 minutes at RT.
13. Place on magnet for ~5 minutes.
14. Transfer 20 µL of supernatant to a new tube.

Quantify the purified pLGP backbone and PCR product using a NanoDrop. If the yield is low, multiple PCR reactions can be set up and pooled instead.

The purified product should be a single band:



Having a single band is **very** important! If you see higher molecular weight smears, this can result in many undesired cloning products at the end of the procedure. If these are observed, you can (1) reduce the input oligo amount and/or (2) reduce PCR cycle numbers.

Gibson assembly: combine pgRNA insert & plasmid backbone

Notes:
- This step is usually very efficient.
- For this, be sure to include a no insert control!

Set up reactions as follows:
5 µL NEBuilder HiFi (NEB Cat # 2621S)
52.5 ng of plasmid backbone (1:5 insert)
3.80 ng of insert
Water to 10 µL

Incubate at 50°C for 1 hour.

This product can be stored at -20°C for several months.

Bacterial amplification round #1

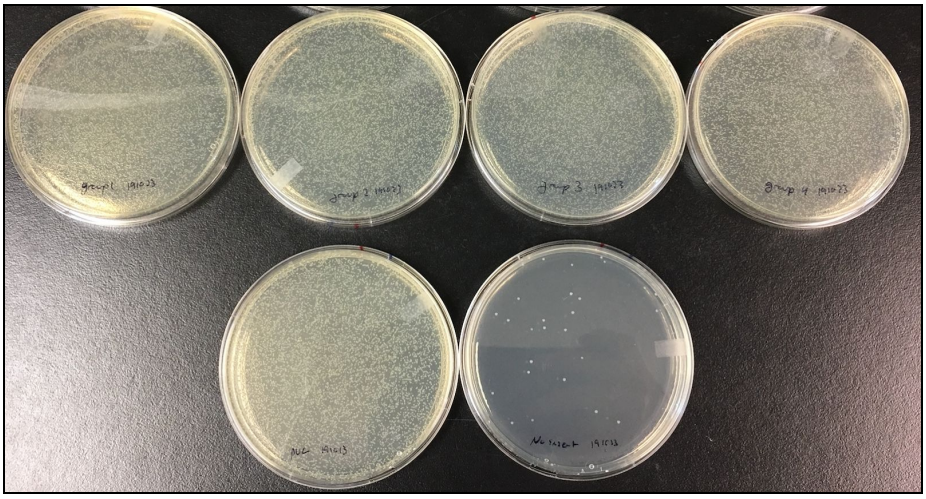
For transformations, two controls are helpful:
1. Gibson no insert control (to ensure backbone did not re-ligate)

2. pUC19 control (to ensure transformation worked)
3. Optional: if available, use a previous pgRNA insert as a positive control

Chemical Transformation:

1. Bring NEB 10-beta/Stable Outgrowth Medium (1 mL per transformation) and LB-Carb selection plates to room temperature. Optional: bring an additional LB-Carb selection plate to room temperature per transformation to help calculate transformation efficiency (see step 8 below).
2. Thaw tube of NEB Stable cells on ice for ~10 minutes. Once thawed, I typically aliquot 20 µL of bacteria into a new tube for each transformation.
3. Add 2 µL of Gibson reaction per transformation.
4. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at exactly 42°C for 30 seconds using a water bath. Do not mix.
6. Place on ice for 5 minutes. Do not mix.
7. Pipette 980 µL of room temperature NEB 10-beta/Stable Outgrowth Medium intro mixture and place at 30°C shaking at 250 rpm for 60 minutes. During this time, place the LB-Carb selection plates at 30°C as well.
8. Mix the cells thoroughly by flicking the tube and inverting. Spread ~100 µL of cells onto selection plate.
9. Incubate plates overnight at 37°C.

Example of a successful transformation:



The next day, bacteria can be collected one of two ways:

(1) Pick individual colonies and grow mini-preps overnight before proceeding to the next step or ...
(2) Add liquid LB media to the plate and scrap the colonies into a single suspension. Perform mini-preps directly from this solution. If you're doing multiplexed cloning, this is the preferred method.

Extract plasmid DNA using the Zymo mini-prep kit (Cat # D4015).

Digest and purify pLGP-2xSpacer

Notes:

- I've found that digesting the backbone overnight can reduce the number of background colonies in the subsequent ligation steps.
- Actually, my most recent cloning results (191029) using one of Austin's positive control backbones suggests that poor BsmBI digestion (NOT the ligation or AP treatment) is the culprit of a failed final step. This underscores the benefit of overnight digestion.

Reaction:

1 µg plasmid
1 µL BsmBI FastDigest (Thermo Esp3I)
1 µL FastAP (Thermo)
4 µL 10X FastDigest Buffer (Thermo)
0.8 µL 0.05 M DTT
Water to 40 µL

Digest at 37°C for at least 1 hour (longer helps to reduce uncut product).

Perform 1X SPRI bead clean-up. Elute in 22 µL water H_2O. Transfer 20 µL to a new tube.

Digest and purify H1 drop-in

Reaction:

25 µL of 10 ng/µL H1 drop-in (IDT GBlock)
2.5 µL BsmBI FastDigest
5 µL 10X FastDigest Buffer
1 µL 0.05 M DTT (dilute stock 0.1M by half to get this)
Water to 50 µL

Digest at 37°C for at least 30 minutes.

Purify product using a 1.8X SPRI bead clean-up. Elute in 15 µL water/rxn.

Ligation: combine H1 drop-in insert & plasmid backbone

Notes:

- This is the most finicky part of the protocol in my hands. Occasionally, I get no colonies on my plates or many background colonies in the no insert control plates. This could be explained by poor BsmBI digestion and/or inefficient ligation. I have often thought the ligase buffer (particularly the ATP?) might go bad after freeze thaws. I think it's best to aliquot the ligase buffer into 1 time use tubes.

- I've found that digesting the backbone overnight can reduce the number of background colonies.
- Here is NEB's protocol.

Reaction:

5 ng insert
100 ng vector
5 µL 2X Quick Ligase buffer
Water to 10 µL
1 µL Quick Ligase (NEB Cat # M2200S)

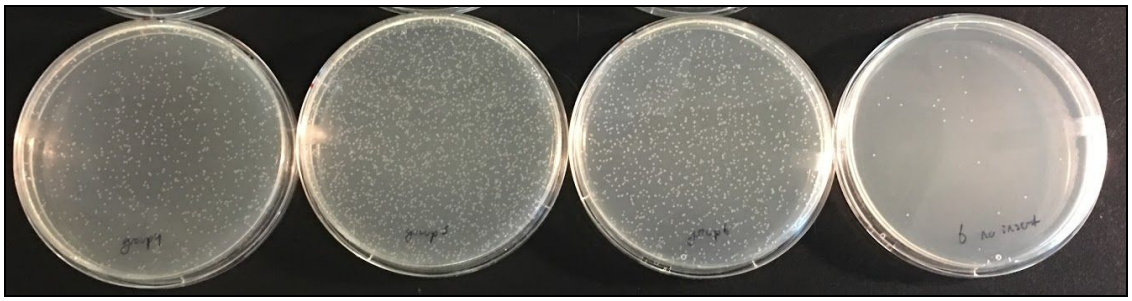
Incubate at room temperature for 15 minutes. The protocol says less is actually better. Don't heat inactivate the enzyme. Either proceed with bacterial transformation or store at -20°C.

Bacterial amplification round #2

Follow transformation and plasmid isolation protocol as above. For multiplexed cloning, individual colonies can be Sanger sequenced to confirm the identity of the pgRNA construct. For this, I make duplicate "touch plates". I send one for sequencing. After I get the Sanger results back, I grow up a miniprep and extract DNA to make virus.

For Sanger sequencing, use RKB1148: GAGGGCCTATTTCCCATGAT

Example of a successful ligation reaction:



Three successful positive ligations on the left. No insert control on right.