GuEST-List Cloning Protocol

Materials

- CROPseq-style vector, Twist oligo insert pool, and amplification primers (See “Cloning Schematic” slides, attached CROPseq backbone sequences, and oligo reference sheet)
- 0.1 M DTT Solution (Invitrogen 707265ML)
- FastDigest Esp3I (Thermo Fisher FD0454)
- FastDigest BamHI (Thermo Fisher FD0054)
- FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific EF0654)
- Zymo DNA Clean & Concentrator-25 Kit (Zymo Research D4033)
- 1% Agarose Gel + Electrophoresis System (we use homemade gels with BioRad #1704486)
- Razor blade (ex, Electron Microscopy Sciences 71960)
- Zymoclean Gel DNA Recovery Kit (Zymo Research D4007)
- SPRIselect DNA Size Selection Reagent (Beckman Coulter B23317)
- 2x KAPA HotStart ReadyMix (Kapa Biosystems 07958935001)
- 2% E-Gel EX Agarose Gel (Thermo Scientific G402022)
- 100% Molecular Biology Grade Ethanol (eg, Sigma-Aldrich E7023-500ML)
- Gibson Assembly Master Mix (New England Biolabs E2611S)
- 100% Molecular Biology Grade Isopropanol (eg, Sigma Aldrich I9516-25ML)
- GlycoBlue Coprecipitant (Thermo Fisher AM9516)
- 5M NaCl (Thermo Fisher AM9759)
- TE Buffer (Thermo Scientific J75793.AE)

Procedure Part I: Prepare stock of cut backbone

1. Set up a restriction digest master mix with CROPseq backbone plasmid as indicated in below table. Divide reaction master mix across 4 PCR tubes in a strip with 60 uL reaction in each tube.

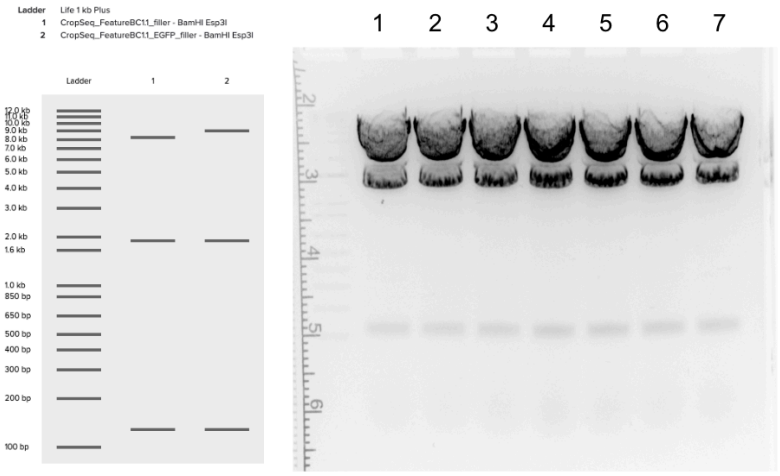
Reagent	1x Reaction	4.1x Reaction
400 ng/uL CROPseq plasmid	25 uL	102.5 uL
20 mM DTT (dilute fresh)	3 uL	12.3 uL
Esp3I	2 uL	8.2 uL
BamHI	2 uL	8.2 uL
10x FastDigest Buffer	6 uL	24.6 uL
Water	22 uL	90.2 uL

2. Digest at 37 °C for 2 hours.
3. After 2 hrs, spike in 30 uL of FastAP spike-in mix to each tube as indicated in below table. This prevents self-ligation in the event you have low efficiency cutting with BamHI for some reason.

Reagent	1x Reaction	4.1x Reaction
FastAP	3 uL	12.3 uL
10x FastDigest Buffer	3 uL	12.3 uL
Water	24 uL	98.4 uL

4. Digest at 37 °C for an additional 45 minutes.
5. Once FastAP incubation is complete, purify using the Zymo-25 kit. Use 2 columns per restriction digest reaction, elute 25 uL per column. Pool together.

6. Load and run a gel to physically separate the backbone from the other digested fragments. I will typically load 20 uL of the sample from step 5 into each lane of a 1% agarose gel prepared with the BioRad MiniGel system, but this may depend on the volume of the wells your lab uses.



7. Using a clean razor blade, cut the largest band and place into eppendorf tubes. Can proceed directly with gel purification from here or store the slices at 4 °C overnight.
8. Purify the backbone from the gel. I use the Zymo gel kit with the following modifications:
    a. Run one column per lane (may require multiple spins to load all the material)
    b. Perform the gel digestion for 1.5 hours with 600 rpm agitation.
    c. Follow all modifications and footnotes indicated in the Zymo protocol for large DNA. In particular, warm the elution buffer to 60 C.
    d. Elute into a volume of 25 uL.
9. Pool together eluted fractions. Perform a 2x SPRI cleanup, and elute into 70 uL.
10. Nanodrop concentration. A minimum yield of 3 ug is recommended to proceed.

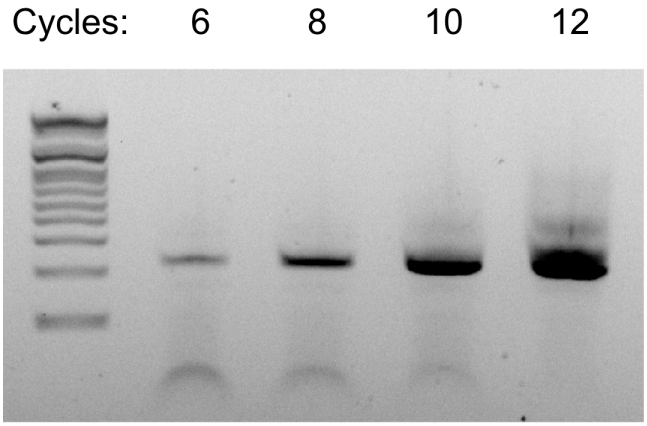
Procedure Part II: Amplification of insert pool

1. Follow the manufacturer's instructions for resuspension of Twist pool to 10 ng/uL.
2. Run a series of test PCRs with 6, 8, 10, and 12 cycles according to the following specifications.

Reagent	1x Reaction
2x KAPA Buffer	12.5 uL
10 uM FW Primer	0.75 uL
10 uM RV Primer	0.75 uL
Oligo Pool (10 ng/uL)	1 uL
NFW	10 uL
Total volume: 25 uL	

Thermocycler Program		
1 cycle	95 °C	3 min
6-12 cycles	98 °C	20 s
	55 °C	15 s
	72 °C	15 s
1 cycle	72 °C	1 min
Hold	4 °C	∞
Lid temp: 105 °C		

3. Visualize the results of the PCRs on a 2% e-gel to determine the optimal number of cycles. Choose the number of cycles yielding the largest amount of product without the formation of larger, secondary products (from this example, we chose to proceed with 8 cycles).



4. Clean up the PCR reactions with a 1.4x SPRI cleanup. Elute each reaction into 20 uL. Qubit to measure concentration.
5. Calculate how many PCR reactions with your optimal cycle number will be required for a minimum yield of 405 ng at ≥ 3.7 ng/uL. Repeat the PCR specified in step 2 with an appropriate number of cycles and reactions.
6. Cleanup the productive reactions with a 1.4x SPRI, elute each into 20 uL, and pool.

Procedure Part III: Gibson Reaction

1. Prepare 10 mL of 70% EtOH. Put at -20 °C to chill.
2. Set up a total of 4 x 60 uL reactions with a 5:1 molar ratio of insert to backbone according to the following tables (note volumes will depend on the precise concentrations of the products from Part I and Part II, also masses will differ if you are using different insert/backbone structures).

Reagent	1x Reaction with CROPseq Vector	1x Reaction with CROPseq + GFP Vector
Digested Vector	750 ng	750 ng
Insert Library	101.25 ng	92.55 ng
2x Gibson Mix	30 uL	30 uL
NFW	up to 60 uL	up to 60 uL

3. Allow to incubate for 1 hour at 50 °C.
4. Thaw GlycoBlue and spin down.
5. Prepare the following extraction mix for each reaction. Do not prepare a master mix. Add to each Gibson reaction in a 1.5 mL eppendorf.

Reagent	1x Reaction
Reagent-grade isopropanol	60 uL
GlycoBlue	1 uL
5M NaCl	1.2 uL

6. Incubate for 15 min at RT.
7. Spin down pellet at full speed for 15 min at RT.
8. Wash DNA with 1 mL of “frozen” 70% EtOH from step 1, pipetting up and down to disturb pellet.
9. Spin at full speed for 5 minutes at RT.
10. Remove ethanol and repeat wash.
11. Air dry pellet for 15-30 minutes.
12. Resuspend in 6 uL of TE. Incubate at 4 °C overnight or 55 °C for 10 minutes.
    a. The clean plasmid pool is now ready to be transformed into bacteria for amplification, according to the protocol “GuEST-List Library Amplification Protocol”.