This is a simplified protocol for cloning libraries without plating on 24.5cm^2 bioassay plates. Amplification of the library in liquid culture, instead of plating, saves time and labor while maintaining representation that is required for many applications.

Prepare the inserts

Inserts are usually synthesized as DNA oligo pools by commercial suppliers like Twist or IDT. They should include priming sites for amplification. In this protocol, we use Golden Gate cloning. So the oligo sequences as well as the backbone should not have recognition sites for the enzyme used in cloning (in this case BpiI) outside of the designated cloning sites. For more information on how we designed oligo pools for barcode libraries see: https://github.com/amjask/BarcodeLibraries.git

To amplify the oligo pool, ideally 10ng should be used in each 25µl PCR reaction to maintain representation (i.e. 0.4ng/µl final concentration). But in cases where the amount of available pool was limited, we've had successful results with lower amounts (up to 0.05ng/µl final concentration). The numbers below reflect this situation where there is not enough of an oligo pool to follow Twist's standard protocol for amplification. If you have enough to use 0.4ng/µl final concentration in your PCR reaction, modify these numbers accordingly.

To determine the number of PCR amplification cycles, run a qPCR test first. Use 4-5 cycles beyond Cq value. This is usually less than 20 cycles. More PCR cycles can introduce bias, mutations, or rearrangements.

- Resuspend the oligo pool to 10ng/µl in TE buffer pH 8.0

- Make a 1ng/µl working solution

- Set up 20µl qPCR reactions with 1ng of oligo pool as template.

    We use iQ SYBR Green supermix. Reactions include 10µl of iQ SYBR Green supermix, 1.2µl of each primer at 5µM concentration, 1µl of oligo pool working solution, and 7.8µl of PCR grade water. Cycling program was 95°C for 3min + (95°C for 10sec + Tm for 30 sec) x 40 + Melt curve (55 to 95°C). Cq under these conditions is typically ~6 and we usually use 11 cycles for amplification.

- Amplify the oligo pool by PCR. You may need multiple PCR reactions to get enough insert for the next step. Note that restriction sites required for cloning are often added in this PCR step. So design your primers with this in mind. If needed, you can also combine two oligo pools in this step using overlap extension PCR. This way you can have a longer variable region in your library and reduce the cost of oligo synthesis significantly.

Template (1ng/ul)	2.5 µl
Herculase II Fusion DNA Polymerase	0.5 µl

Fwd primer (10 µM)	1.25 µl
Rev primer (10 µM)	1.25 µl
5X buffer	10 µl
dNTP (25mM each)	0.5 µl
dH2O	34 µl
Total	50 µl

PCR program: 95°C for 2min + (95°C for 15sec + Tm for 20 sec + 72°C for 30sec) x 11 + 72°C for 3min.

Run the product on 2% gel, cut and purify by column.

Prepare the backbone

The backbone should be compatible with Golden Gate cloning. For us, BsaI and BpiI enzymes have worked much more efficiently than Esp3I. BpiI sites may be present in your desired vector, for example the lentiviral transfer plasmid. But you may be able to remove them without affecting the function. See Addgene Plasmid #127168 for an example.

To reduce background, you can pre-digest your vector before setting up the cloning reaction. If you do this, purify the digested backbone, but do not do a phosphatase treatment. That will reduce the efficiency. Alternatively, you can include a ccdB negative selection cassette in your backbone, which is removed upon cutting by BsaI or BpiI enzyme.

Golden Gate cloning

Set up the reaction like this:

	1X	33X
NFW	4.25 µl	140.25
10X Buffer G (ThermoFisher)	1	33
10mM ATP	1	33
40% PEG-8000	1.5	49.5

Backbone (20fmol/µl)	0.25	8.25
Insert (60fmol/µl)	1	33
T7 ligase (NEB)	0.5	16.5
BpiI (Thermo)	0.5	16.5
Total	10µl	330µl

Set up 6 x 50µl reactions for each backbone. Spin down and incubate according to this:

(37°C for 5 min + 20°C for 5 min) x 15 + (37°C for 30 min)

Clean up the reactions: we use SPRI beads following the manufacturer's protocol to purify cloning reactions before transformation. 1:1 mix of beads and assembly reaction. At the end, elute with 20 to 30µl nuclease free water for 300µl starting material. DNA concentration is usually between 50 - 100 ng/µl after purification. If your concentration is below 5ng/µl, it's better to troubleshoot and repeat before moving forward with transformation.

Amplification

For transformation, use Endura electrocompetent cells according to their protocol. We usually do a negative control with 5ul of water added to 25ul of cells and a positive control with 1µl of pUC19 DNA (comes with the cells) to 25µl of cells. For the library, add 10µl of the purified reaction to 50µl of the cells. Split them into two parts and electroporate each ~30ul in a separate cuvette. After electroporation, recover the cells in 2ml of recovery media per 25ul of competent cells at 37°C for 1 hour (in bacterial culture tubes).

Set up cultures and dilution plates: After recovery, plate 10^3, 10^4, 10^5, and 10^6 dilution plates. Leave these plates in a 30°C incubator overnight. The number of colonies on these plates helps estimate the number of colony forming units (CFU) in the liquid culture. Ideally you want to have CFUs equal or greater than 100 times the number of variants in your library. For the positive control with pUC19 DNA, you expect about 10^5 CFU.

Culture the rest of the electroporated cells in 100ml of LB media with the appropriate antibiotic at 30°C for 18 hours. Then, spin down the cells in two 50ml tubes and isolate the plasmid DNA using midiprep.

Quality control

Library representation has to be evaluated by next generation sequencing (NGS). This is a time consuming and expensive step, yet necessary. Before that you can do some preliminary tests to make sure your library is worth sending out for NGS. You can take a small sample of the midiprep and perform a restriction digest to make sure the insert size is correct and uniform. You can also miniprep some of the colonies on the serial dilution plates and perform Sanger or full plasmid sequencing to ensure the cloning has generated intended results in most, if not all, cases.

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