Last modified: October 2024; see revision notes at end
Last reviewed: October 2024
Version 1.2.2

Protocol: pDNA Library Amplification

Brief Description

This protocol is for the amplification of pDNA libraries for the creation of additional material. This protocol should produce around 500 µg of DNA per 20,000 constructs in a library. If more DNA is desired, this protocol can be repeated multiple times using the same source material. Please note that each new "generation" of library will likely have a wider distribution and overall poorer quality than its parent due to stochastic processes. Therefore, it is recommended that no library be amplified past a second generation (i.e. two rounds of subsequent amplification) and that different generations never be mixed. There are also some libraries that we do not recommend amplifying (see Note at the bottom of the document for more information). Always verify the quality of the library via Illumina sequencing before use.

Materials, Reagents & Instrumentation

- Library plasmid DNA (pDNA) (200 ng per 20,000 constructs, rounded up)
- 100 µL electrocompetent cells per 20,000 constructs (STBL4™, Thermo Fisher Scientific, Cat# 11635-018)
- Sterile Eppendorf tubes
- Electroporation cuvettes (0.1 cm gap, Bio-Rad, Cat#165-2089)
    - 3 cuvettes per 20,000 library constructs, rounded up
- Electroporator (MicroPulser™, Bio-Rad Cat# 1652100)
- 10 mL SOC (1X SOC, New England BioLabs, Cat# B9020S)
- Round-bottom 14 mL Falcon polypropylene test tubes (Thermo-Fisher, Cat# 14-959-11B)
- Bioassay plates (500 cm^2, LB agar + antibiotic)
    - 3 bioassays required per 20,000 constructs, rounded up

- 1 Petri dish, LB agar with Carb (50 µg/mL)
- Sterile tubes, various sizes
- Biospreader (Bacti Cell Spreader, VWR International, Cat# 60828-684)
- LB broth
- Hi-Speed Plasmid Maxi Kit (Qiagen HiSpeed Maxi, Cat# 12663)
    - 1 maxi column required per 20,000 constructs, rounded up
- For libraries in pRDA_478 or pRDA_256:
    - Qiaquick Gel Extraction kit (Qiagen cat# 28704)

A) Day 1 (PM)

1. Determine how much pDNA and competent cells are needed to put through transformation. This is done by the following:
    a. Take the number of constructs in the library to be amplified and divide by 20,000. Round up the result to the nearest whole integer (e.g. for a 35K construct library this would be two. For a 41K construct library this would be three).
    b. This is how many aliquots of 200 ng of pDNA and 100 µL of STBL4 cells are needed. (e.g. the amplification of a 35K library would need 2x 200 ng pDNA and 2x 100 µL of Stbl4 cells).

➢ *Note: For high efficiency transformation, for every 100 µL cells, keep the DNA volume below 10 µL. If more than 10 µL of the DNA solution is needed to reach 200 ng, the solution will need to be concentrated.*

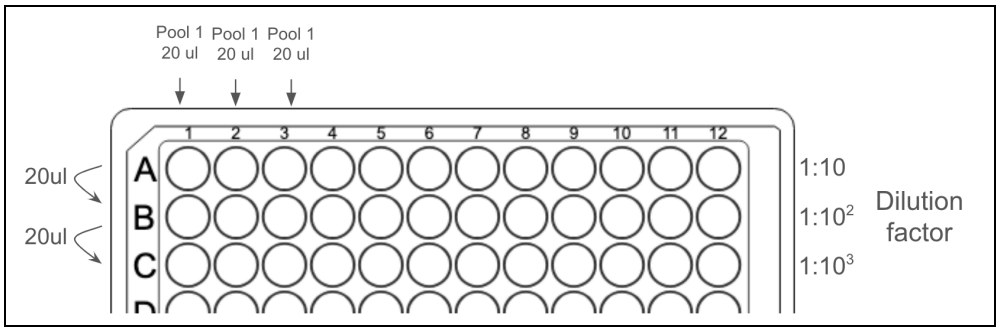
2. Thaw STBL4 cells on ice.
3. In separate sterile Eppendorf tubes, mix each aliquot of 200 ng pDNA with 100 µL of STBL4 cells. Seal the tubes and mix by gently flicking.
4. Add 35 µL of cell mixture to a cuvette, electroporate using the Ec1 setting (1.8 kV), immediately add 500 µL SOC medium, and then transfer to a round-bottom 14 mL tube containing 2.5 mL SOC medium.

➢ *Note: Every aliquot of pDNA/STBL4 cells will require three rounds of electroporation and recovery into the tube of SOC.*

5. Repeat step 4 until all of the pDNA/cell aliquots are electroporated and placed into separate tubes.
6. Shake the tubes of SOC with the cells for 1 hour at 30 °C.
7. In the meantime, pre-warm the bioassay plates + one additional bioassay for colony counts.

➢ *Note: Every tube of SOC + cells will get plated onto one full bioassay plate.*

8. After 1 hour, to get an accurate colony count, make a 1:10 dilution from each tube (we recommend 20 µL culture into 180 µL SOC).
9. Make 2 serial dilutions for each transformation tube.



10. Plate each dilution onto a labeled colony count bioassay by pipetting 10 µL onto the agar and tilting the plate to spread out the droplet over some more surface area. Do this for every transformation tube.
11. Plate the remainder of each 3 mL culture on each of the bioassays. Distribute evenly by shaking vigorously with glass beads.
12. Incubate at 30 °C for 16 – 18 h. Make sure that the bioassays are oriented with the lid down to prevent condensation on the cells.

➢ *Note: For **sgRNA** libraries, growth at 37 °C for 14 – 16 h instead of 30 °C is acceptable when using STBL4 cells. Many other competent cells, though, will show noticeably higher recombination rates at 37 °C.*

➢ *Note: For **ORF** and **shRNA** libraries, we strongly recommend keeping the temperature at 30 °C.*

B) Day 2 (AM)

1. After 16 - 18 h of growth, count the colonies on the colony count bioassay at the dilution factor you can most easily count. For example, for a library of <20,000 guides:

   Library's total colony yield = (Electroporation 1 count + electroporation 2 count + electroporation 3 count)*10^(dilution factor)*3000 µL

   This number should be 1000x greater than the number of constructs in the library to ensure good library representation.

➢ *Note: It often takes more than 16 h to visualize all colonies. If there are fewer than 100x colonies per construct the library may be missing guides/suffer from poor representation. If the colony counts are too low, it is recommended to begin again from the beginning.*

2. If continuing, prepare an ice bucket with a 50 mL conical tube. If your library has more than 3 bioassays to pool together, you will need a bigger vessel (such as a 250 mL conical). You will then mix and divide between 50 mL conicals, one for every three bioassays scraped.
3. To harvest colonies, add 15 mL LB media onto each bioassay plate.
4. Scrape colonies with a biospreader and collect the media into your sterile conical tube, pooling together all colonies of the same pool.

5. Add 10 mL to one of the three bioassays, scrape the remainders. Transfer that onto the next bioassay. Scrape the remainders, and transfer to the last bioassay. This will allow you to harvest as much bacteria as possible with a small amount of volume.
6. Spin down the conicals, pour off media, and confirm that a compact pellet has formed at the bottom (if weighed, the pellet should be more than 1 g). The contents of each conical will undergo one maxiprep.
7. Purify each cell pellet/conical via maxiprep according to the manufacturer's instructions, with the following modifications:
    a. Add P1, P2, P3 directly to the conical and centrifuge for 10 minutes at 3000 g to pellet lysed debris before adding lysate to plunger.
    b. Chill the isopropanol in the freezer before eluting the column into it. Elute from the column directly into the tube of isopropanol.
    c. Elute from the filter cartridge using 1 mL of TE buffer.
    d. When eluting from the filter cartridge allow the TE buffer to drip through the cartridge using atmospheric pressure until the dripping stops. Then push the plunger through.
8. Sequence the library via Illumina to confirm maintenance of representation. See Protocol: PCR of sgRNAs/shRNAs/ORFs from gDNA for Illumina Sequencing for details.

➢ *Note*: *We highly discourage amplification of (1) high-complexity libraries such as the Clonesifter and Cell Counter libraries, since they will experience transformation bottlenecks, and (2) libraries cloned into slow-growing vectors since they are prone to contamination and recombination.*

Revisions from previous versions:

- Added note regarding libraries that should not be amplified.
- Changed the ratio of required pDNA for amplification to the number of constructs in library to improve quality.
- Updated the transformation plating protocol to keep individual electroporations separate until the harvesting step to help identify any isolated "fails."
- Updated the colony count plating method.
- Added notes on low colony counts.
- Modified the maxiprep protocol to improve yield.