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Version 1.2.2

Protocol: Amplification of pDNA Libraries

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. Always verify the quality of the library via Illumina sequencing before use.

Materials and reagents required:

- Library plasmid DNA (pDNA) (200 ng per 20,000 constructs, rounded up)
- 100 μL electrocompetent cells (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

Protocol:

Day 1 (PM):

1. Determine how much pDNA and competent cells are needed to put through transformation. This is done by the following:
    - ⇒ 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 (i.e. for a 35k construct library this would be two. For a 41k construct library this would be three).
    - ⇒ This is how many aliquots of 200 ng of pDNA and 100 µL of STBL4 cells are needed. (i.e. 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. Pre-warm the bioassays and petri dish.
    - ⇒ *Note: every tube of SOC and cells will need one full bioassay plate.*
8. After 1 hour, combine all of the cultured cells together in a sterile tube of the appropriate size and gently mix. If the final volume is more than 18 mL (>40k constructs), place the tube of culture on ice.
9. To get an accurate colony count, plate a diluted sample of the culture onto a petri dish: mix 3 µL of the combined culture into 897 µL of LB, then repeat, for a final dilution of 1:90,000. Plate 100 µL of the final dilution onto the petri dish using glass beads or a biospreader.
10. Plate 3 mL of the combined culture on each of the bioassays. Distribute evenly with a biospreader or glass beads.
11. Incubate at 30˚C for 16 – 18h. 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 – 16h instead of 30˚C is acceptable when using STBL4 cells. Many other competent cells, though, will show noticeably higher recombination rates at 37˚C.*

Day 2 (AM):

1. After 16 - 18h of growth, count the colonies on petri dish. Total colony yield = Count x 300 x 300 x 10 ÷ 0.1. This number should be at least 1000x greater than the number of constructs in the library.
    - ⇒ *Note: it often takes more than 16h to visualize all colonies. If there are less 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 50 mL conical tubes.
3. Use a biospreader to scrape the bioassays into the prechilled 50 mL conicals with cold LB (generally two rounds of 15 mL LB should be enough). Each 50 mL conical can hold three bioassay's worth. If the library requires more than three bioassays, scrape all the bioassays together into a large flask, mix, then divide between 50 mL conicals, one for every three bioassays scrapped.
4. 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.
5. Purify each cell pellet/conical via maxiprep according to the manufacturer's instructions, with the following modifications:
    - ⇒ Add P1, P2, P3 directly to the conical and centrifuge to pellet lysed debris before adding to plunger.
    - ⇒ Chill the isopropanol in the freezer before eluting the column into it. Elute from the column directly into the tube of isopropanol.
    - ⇒ Elute from the filter cartridge using 1 mL of TE buffer.
    - ⇒ 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.
6. Sequence the library via Illumina to confirm maintenance of representation. See **Protocol: sgRNA/shRNA/ORF PCR for Illumina Sequencing** for details.

Revisions from previous protocol versions:

- Changed the ratio of required pDNA for amplification to the number of constructs in library to improve quality
- Modified the maxiprep protocol to improve yield
- Added notes on low colony counts