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Pooled Library Amplification

Addgene The Nonprofit Plasmid Repository[1]

[1]Addgene

1 Works for me dx.doi.org/10.17504/protocols.io.ba79ihr6

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ABSTRACT

This protocol describes pooled library amplification. To see the full abstract and additional resources, visit https://www.addgene.org/protocols/pooled-library-amplification/.

EXTERNAL LINK

https://www.addgene.org/protocols/pooled-library-amplification/

GUIDELINES

Required Quality Control (QC):

Pooled libraries can be challenging and expensive to ensure adequate quality, but this upfront cost will save headaches and expense later.

At a minimum, we recommend the use of a diagnostic digest and high-throughput next-generation sequencing (NGS). Select a restriction enzyme for digest that will cut the shared plasmid backbone a single time and visualize that digestion on an agarose gel (see protocol here). Lentiviral plasmids can recombine between their LTRs resulting in a smaller plasmid containing only the elements required for bacterial propagation (origin of replication and antibiotic selection). This recombination, at a low rate, is not typically a problem as these sequences are not efficiently packaged into lentiviral particles. See below for options if the recombinant band makes up a significant proportion of the DNA pool.

Workflow Timeline:

Day 1: Transform, recover, set up overnight growth (Estimated time **02:00:00** - **03:00:00**)

Transformation should be performed at the end of the day to ensure that growth times are limited.

Day 2: Harvest cells and purify DNA (Estimated time **03:00:00** - **04:00:00**)

Cells should be harvested first thing in the morning to ensure that outgrowth and competition are limited.

Tips and Troubleshooting

What do I do if my transformation efficiency is not high enough?
Repeat the experiment with more cells, more DNA, gentler practices, and colder reagents (not including the recovery media!). Do not proceed with Maxipreps or NGS until adequate transformation efficiency is obtained. Ensure that electrocompetent cells are being used. Chemically competent cells will not provide adequate transformation efficiency! Ensure that no arcing is taking place during electroporation. Arcing would manifest as a loud pop often accompanied by some light in the cuvette during the electroporation step.

I got a heavy recombined band by digest

Recombination is thought to be in part related to selective pressure. These recombined plasmids are much smaller and are a smaller nutritional burden on the bacteria. Bacteria that contain a recombinant plasmid likely grow faster in the closed system of a crowded plate or culture. Try restricting growth to the bare minimum by growing at 30˚C, limiting growth time further, or potentially spreading less bacteria on a given plate (ex by increasing the total number of bioassay plates used). Scaling up the experiment using a Gigaprep can be used to obtain sufficient intact pooled library even in the face of a recombined fraction but care must be taken to perform adequate NGS based QC to ensure no change in representation compared to the pre-amplified stock. A last option is to perform a gel extraction of either the original sample or the amplified sample, followed by reamplification of the DNA, but please note that NGS should be performed to ensure representation is maintained.

Maxipreps - Less is more

Do not overload the Maxipreps as yield can dramatically plateau and sometimes fall off entirely if the column or reagents are significantly overloaded. If you find that your pellet outstrips the capacity listed in the Maxiprep protocol, scale the reagent volume and column number as needed. The use of Mega or Gigapreps is acceptable when scaling up or reducing the number of tandem purifications. Consult the manufacturers' handbooks for appropriate volumes and numbers of tandem purifications.

MATERIALS TEXT

Equipment

- Table top centrifuge
- BioRad Electroporator (MicroPulser TM , Bio-Rad 1652100)

Reagents

- **200 µl** electrocompetent cells (Default: 4 tubes of Endura Duos, Lucigen, 60242-1)

- Alternatives include Stbl4 cells or other ultra-high efficiency electrocompetent cells that are suitable for unstable or recombination-prone DNA.
- The use of electrocompetent cells is essential to ensure high efficiency uptake of plasmid library DNA.
- This quantity of cells is sufficient for libraries up to 200,000 individual plasmids. For larger libraries scale the number of cells and DNA accordingly.

Pro-tip

Use of extremely high efficiency (1x10^10 cfu/µg) commercially prepared electrocompetent cells is strongly recommended.

- **800 ng** pooled library DNA (**100 ng** plasmid DNA per **25 µl** electrocompetent cells)

Less library DNA can be used but this dramatically increases the chances of individual plasmids being lost from the pool and/or increasing the skewness of the pool.

- 8 electroporation cuvettes (BioRad, Micropulser, 0.1 cm)
- **20 ml** SOC recovery media (Lucigen, 80026-1)
- 8X LB Agar + Antibiotic 245 mm bioassay plates (Molecular Devices, X6023)

Pro-tip

Pour these plates at least one day in advance to allow adequate time to fully gel and to dry slightly. We routinely use

350 ml of LB Agar per bioassay plate.

- **14 ml** Vented Falcon Tubes (BD Biosciences, 352059)
- 3X LB Agar + Antibiotic 65 mm Petri dish (VWR, 11019-552)
- 4 Maxipreps (Qiagen HiSpeed Max, Catalog #12663)
- Tips (**1000 µl** , **200 µl** , **10 µl**)
- Bacti Cell Spreaders (VWR, 60828-680)
- **5 ml** and **10 ml** Serological pipettes
- Ice slurry (Ice bucket with ice and water to create slurry)
- **100 ml** LB house-made

At Addgene we use premixed LB (VWR 101414-072) for convenience but any brand or house-made LB that supports normal growth is expected to work

- **50 ml** Falcon Conical tubes (Fisher, 14-432-22)

Reagent Preparation (optional)

1. Prepare, sterilize, and pour all LB Agar + Antibiotic plates.
2. Prewarm **12 ml** recovery media at **37 °C** (for at least **00:15:00**).
3. Prewarm 3X LB Agar + Antibiotic plates at **37 °C** .
4. Prewarm 8X LB Agar + Antibiotic Bioassay plates.
5. Prechill Micropulser cuvettes on ice.
6. Thaw 4 tubes of electrocompetent cells on ice for **00:15:00** - **00:20:00** or until completely thawed.
7. Chill a box of **200 µl** micropipette tips in a **-20 °C** freezer.
8. Aliquot **3 ml** SOC into each of four **14 ml** Vented Falcon Tubes and have **1 ml** SOC per electroporation readily available for post-electroporation recovery of cells.
9. Ensure access to autoclaved, sterile reagents for all steps.

SAFETY WARNINGS

See SDS of listed reagents for safety warnings and hazards.

Day 1

1 Add **200 ng** DNA to each **50 µl** aliquot of thawed Endura Duos on ice. Flick gently to mix

2 Electroporate cells (one at a time for a total of eight electroporations):

Electroporator Conditions: Bio-Rad Micropulser Ec1 0.1 cm cuvette, 1.8 kV, 1 pulse.

3 Aliquot **25 µl** DNA-Endura into pre-chilled cuvette.

4 Pulse.

Electroporation involves the use of high voltages, please use caution when activating pulse and follow all specifications described in the equipment manual.

5 Immediately add **1 ml** SOC to cuvette.

6 Remove all liquid from cuvette and add to **14 ml** vented Falcon Tube containing **3 ml** SOC.

7 Repeat for each of the **25 µl** aliquots of cell/DNA mixture used (8 in the described protocol) using a new vented falcon tube for every two transformations. Each of the four **14 ml** vented falcon tubes should contain a total of **5 ml** (**3 ml** SOC + **2 ml** transformed Endura from two separate transformations).

Pro-Tip
Do not pipette repeatedly or mix when removing SOC containing transformed DNA-Endura from cuvette.

8 Shake four **14 ml** Vented Falcon Tubes at **30 °C** - **37 °C** , **225 rpm** for **01:00:00** .

9 After the 1 hour shaking period, pool and gently mix the four tubes.

10 Perform sequential 1:100 dilutions of the cells (add **10 µl** of the pool to **990 µl** LB then perform a second and then third 1:100 dilution). Plate **100 µl** of each dilution onto a prewarmed Petri dish.

11 Incubate plates at **30 °C** overnight.

12 Plate **2.5 ml** of the transformed cells on each of the eight bioassay plates (two plates per tube). Distribute evenly with a sterile spreader until all liquid has been absorbed by the agar. This usually takes **00:01:00** - **00:02:00** .

Critical
Be careful not to rip or shred the agar. Do so by gentle spreading. Some spreaders have a sharp edge that can scrape plates more abrasively at a certain angle.

13 Incubate plates upside down at **30 °C** overnight.

Critical
Ensure at this stage that no unabsorbed media drips onto the lid. Let plates remain agar side up until dried before overnight incubation if needed.

14 Place **100 ml** sterile LB at **4 °C** .

Day 2

15 Before beginning, prechill at least four conical tubes on ice and ensure access to sterile scraper and cold LB.

Pro-Tip
Prepare one to two more conical tubes on ice in case you need to spread out the harvested cells further than four Maxipreps worth.

16 Count colonies on the most dilute Petri dish.

- Total colony yield = count x 100 x 100 x 100 ÷ 0.1.
- This number should be at least 1000x greater than the number of perturbations in the library.
- *Example: At least 10 colonies on the most diluted plate for 1000x coverage of a library of 100,000 plasmids.*
- Colonies may appear small and require extra incubation time in order to be enumerated accurately.
- Frequently the number of colonies can be too great to count. Ideally, dilutions would have been sufficient to enumerate single colonies but as long as one can ensure more than the required colonies are present. If colonies are not present on the most dilute plate, count the second plate (ie. the 1:10,000 dilution).

17 After **12:00:00** - **18:00:00** of growth, use the spreader and cold LB to scrape bioassay plates and remove bacteria.

18 Use one scraper for all plates.

19 Use two **10 ml** pipettes (one for dispensing cold LB and one for removing LB containing bacteria from the plate).

Pro-Tip
Scraped bacteria in LB can clog the pipette either mix gently up and down avoiding introducing bubbles or pour off plates into conical tubes as needed.

20 Add **10 ml** cold LB to each plate for each scrape and use spreader to scrape plates.

Pro-Tip
Pushing motion is better than pulling motion and take care not to split or gouge agar during the scraping process.

21 Add each scrape into a **50 ml** conical tube on ice pooling the scrapings of two plates into each tube. Keep each tube on ice while scraping.

22 Repeat addition of **10 ml** cold LB and scrape for each plate until agar is clear (all bacteria have been removed). This should produce **25 ml** cold LB-bacteria per plate.

23 Centrifuge tubes (**4000 x g, 4°C 00:15:00**) to pellet bacteria.

24 Decant LB and weigh pellet. - The total weight of each pellet should be ~ **1 g** - **2 g** .

Pro-Tip
Make sure to weigh the empty tube beforehand! If you've already gone too far, weigh an identical empty tube. It will be close enough for the purpose of a Maxiprep.

25 Purify plasmid DNA using the Qiagen HiSpeed Maxi Kit (one conical is its own Maxiprep).

Critical
Do not freeze pellets for later purification. Immediately purify them! Commercial Maxipreps rely on incremental, ordered cell lysis. *E. coli* cells are subject to lysis by freeze-thaw if not suspended in cryoprotectant like Glycerol or DMSO solutions.

26 Quantify the individual Maxipreps by Nanodrop, Picogreen, or Qubit.

Each different DNA measurement type can report slightly different concentrations of DNA. In our hands fluorescent dye methods tend to report lower values than Nanodrop, although most protocols have been designed with Nanodrop as the quantification method.

27 If all Maxipreps appear to contain sufficient DNA for use, pool samples, and continue with analysis.

“Sufficient” DNA will vary upon intended use. Typically yields range from several hundred micrograms to a milligram of total plasmid DNA.