Oct 29, 2023 Version 1

sgRNA library re-amplification in liquid culture V.1

DOI
dx.doi.org/10.17504/protocols.io.n92ldmr7xl5b/v1

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Atlas of Variant Effects ...

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Protocol Citation: Erik Haussner, Michael Böttcher 2023. sgRNA library re-amplification in liquid culture. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.n92ldmr7xl5b/v1**

Protocol status: Working
We use this protocol and it's working

Created: September 15, 2023

Last Modified: October 29, 2023

Protocol Integer ID: 87835

Keywords: sgRNA, reamplification, Plasmid pool, Library, Electroporation, amplification, sgrna libraries in liquid culture, sgrna library, sgrna, liquid culture, order from addgene, distribution of library element

Abstract

In this protocol, we describe a stepwise procedure for the re-amplification of sgRNA libraries in liquid culture. In our hands, this protocol works reliably to amplify pre-cloned sgRNA libraries (e.g. order from Addgene) in a way that preserves the distribution of library elements.

Materials

- 100 µL ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog** #11635018
- Up to 400 ng of Sample
- Electroporator and Electroporation cuvettes **Biozym Catalog** #748010

Protocol materials

- Electroporation cuvettes **Biozym Catalog #**748010
- ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018
- NucleoBond Xtra Midi kit for transfection-grade plasmid DNA **Macherey-Nagal Catalog #**REF 740410.50
- GelRed™ Nucleic Acid Gel Stain, 10,000X in Water **Gold Biotechnology Catalog #**G-725
- 1% Agarose gel **Catalog #**/
- TriTrack DNA Loading Dye (6X) **Thermo Fisher Catalog #**R1161
- 1 kb Plus DNA-Ladder **Thermo Fisher Scientific Catalog #**10787018
- 1 kb Plus DNA-Ladder **Thermo Fisher Scientific Catalog #**10787018
- ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018
- Electroporation cuvettes **Biozym Catalog #**748010
- SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #**B9020S
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- Liquid LB medium
- ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018
- ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018
- 1.5 mL Eppendorf tubes
- Liquid LB medium
- Liquid LB medium
- LB agar plates with the proper antibiotic (e.g. Kanamycin)
- Liquid LB medium
- LB agar plates with the proper antibiotic (e.g. Kanamycin)
- LB agar plates with the proper antibiotic (e.g. Kanamycin)
- 1x TBE buffer
- 1% Agarose gel **Catalog #**/
- Agarose Low Melt **Carl Roth Catalog #**6351.4
- Electroporation cuvettes **Biozym Catalog #**748010
- SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #**B9020S

Troubleshooting

Before start

Keep your original stock safe and aliquoted. For large plasmids with complementary sequences such as LTR sites, keep in mind that repeated reamplification from an already reamplified stock may lead to an accumulation of recombined plasmids and a poorer distribution of library elements.

Library transformation

25m

1 Prepare Sample

ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018

Electroporation cuvettes **Biozym Catalog #**748010 and

SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #**B9020S for electroporation.

5m

1.1 Thaw

ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018

00:05:00 on ice.

5m

1.2 Pre-cool Electroporation cuvettes **Biozym Catalog #**748010 by placing it on ice.

1.3 Pre-warm

SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #**B9020S at

37 °C .

2 Add 100 ng Sample into 25 µL

ElectroMAX™ Stbl4™ Competent Cells **Thermo Fisher Catalog #**11635018

, carefully mix by pipetting up and down.

10m

3 Add 25 µL of the plasmid/cell mix into a cuvette, electroporate at 1.2 kV, 25 uF and 200 ohm or alternative setting (see note below). Directly after electroporation, add 1 mL of pre-warmed

SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #**B9020S .

10m

Note

The electroporator setting may vary from model to model and should be checked along with the test plasmids provided in the kit of the STBL4 cells.

Safety information

Make sure that any water or ice residue is removed from the cuvettes before inserting them into the electroporator to avoid arching.

Library recovery

1h 15m

4 After electroporation, add the 1 mL resuspended cells in a 14 ml culture tube and incubate the cells in a thermoshaker 600 rpm, 37°C, 01:00:00 .

1h 15m

Note

In general, an incubation temperature of 37°C is optimal for cell recovery. Since E. coli tend to recombine plasmids with complementary sequences (e.g. LTRs), recovery temperature can be reduced to 30°C. This may however, result in a lower total number of recovered cells.

Determination of transformation efficiency

16h 30m

5 Use a small fraction of your cells to determine the electroporation efficiency of the reamplification.

Note

In this step, much depends on the size of the particular plasmid and the number of elements in the library. Therefore, the dilution factor must be chosen based on properties of the library and the scale of the electroporation. Smaller plasmids yield significantly more colonies than large ones, and an upscaled plasmid input at the electroporation step may result in higher dilutions being required to achieve a countable number on the respective agar plates after plating.

5.1 **For 1:10,000 dilution:** 5m

Prepare 1.5 mL Eppendorf tubes . Take 10 µL of recovery culture and dilute in 990 µL of Liquid LB medium (1:100 dilution). Take 100 µL of 1:100 dilution and dilute in 900 µL of Liquid LB medium (1:1,000) and plate 100 µL on LB agar plates with the proper antibiotic (e.g. Kanamycin) (1:10,000 dilution).

5.2 **For 1:1,000,000 dilution:** 5m

Take 10 µL of the 1:1,000 dilution and dilute in 990 µL of Liquid LB medium and plate 100 uL on a pre-warmed LB agar plates with the proper antibiotic (e.g. Kanamycin) (1:1,000,000 dilution).

Note

When preparing the dilution series, always mix stock solutions well by flicking the tube before diluting, to resuspend sedimented cells. Distribute the plated cells evenly over the plate by e.g. using glas beads.

6 Place the plates in an incubator at 37 °C Overnight . 16h

Library extraction and quality control 16h

7 Use rest of recovery to inoculate up to 500 mL of Liquid LB medium with an added selection marker specific antibiotic like ampicillin in an Erlenmeyer flask for Overnight culture. 600 rpm, 30°C 16h

Determination of transformation efficiency 15m

8 On the next day, check for overall coverage via colony counting on LB agar plates with the proper antibiotic (e.g. Kanamycin) . The overall colony count should be 1000x the element number of your library. 15m

Note

Below we provide a simplified example for how to determine transformation coverage.

Example calculation of coverage: On the 1:10,000 dilution plate we count 100 colonies. This gives us 100*10,000 = 1,000,000 total colonies. This total colony number is divided by the number of elements (e.g. sgRNAs) in the respective library. For a library the size of 1,000 sgRNAs, the coverage would 1,000,000/1,000=1,000x. For larger libraries, e.g. the size of the genome-wide Brunello library (80,000 sgRNAs), we would count the 1:1,000,000 dilution plate. In this case, 80 counted colonies would mean 80,000,000 total colonies which divided by the library size (80,000 sgRNAs) would again return a transformation coverage of 1,000x.

Library preparation and QC 1h

9 Follow the protocol instructions of the NucleoBond Xtra Midi kit for transfection-grade plasmid DNA **Macherey-Nagal Catalog #**REF 740410.50 for transfection-grade plasmid DNA for Midi Prep. Follow the protocol instructions of the for transfection-grade plasmid DNA for Midi Prep. 25m

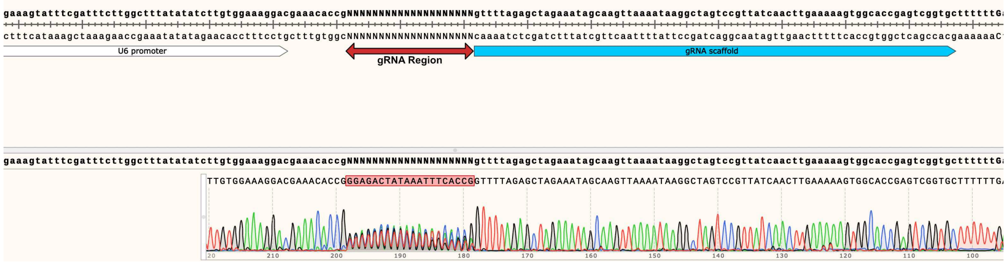
10 Determine your final Sample concentration via NanoDrop or Qubit measurement.

Equipment	
new equipment	NAME
Qubit 2.0 Fluorometer instrument	BRAND
Q33226	SKU
with Qubit RNA HS Assays	SPECIFICATIONS

Equipment	
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	NAME
UV-Vis Spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU

11 Send a sample of your reamplified Sample for Sanger sequencing.

Below we show an example chromatogram of an expected sequencing result. We recommend using sequencing primers 50-100 nt upstream of the sgRNA region. You should see clean traces up- and downstream of the SPACER region, and a noisy 20 nt signal in the SPACER region, due to the sgRNA diversity in your library.



Example of a Sanger sequencing result.

Note

! To validate the distribution of elements in your library, we strongly recommend performing next generation sequencing of your plasmid pool before proceeding with downstream experiments. To do so, follow the NGS protocol provided with your library, using the plasmid pool as template, instead of the genomic DNA (as you would in CRISPR screens).

QC: Plasmid recombination check

3h 15m

12 Since sgRNA library plasmids can recombine during E.coli re-amplification, it is recommended to check for recombination via linearization of 200 ng of your reamplified Sample via a restriction digest within the backbone of your library vector.

1h

13 Prepare a 1% Agarose gel **Catalog #**/ by melting 1 g of Agarose Low Melt **Carl Roth Catalog #**6351.4 in 100 mL 1x TBE buffer

20m

14 Let the required amount for casting cool down till it is approxametly 50 °C and add 1 µL of GelRed™ Nucleic Acid Gel Stain, 10,000X in Water **Gold Biotechnology Catalog #**G-725 per ml of melted 1% Agarose gel **Catalog #**/

30m

15 Pour the warm, still liquid gel into an electroporation chamber and wait until it has cooled down.

15m

16 Mix your liearized reamplified Sample with TriTrack DNA Loading Dye (6X) **Thermo Fisher Catalog** #R1161 and prepare 1 kb Plus DNA-Ladder **Thermo Fisher Scientific Catalog** #10787018 for gel loading. 5m

17 Add your linearized reamplified Sample alongside with the prepared 1 kb Plus DNA-Ladder **Thermo Fisher Scientific Catalog** #10787018 onto the gel and run it for 01:00:00 at 120 V. 1h

18 Check the plasmid size on your gel using UV excitation. 5m

Expected result

In the best case, only one band will be present, corresponding to the size of the respective vector. In case of recombination, one or more additional bands will be present. For optimal downstream results, the band of the intact vector should be dominant.