

Jul 08, 2024

Saturation Mutagenesis-Reinforced Functional Assays (SMuRF) for alpha-dystroglycan glycosylation enzymes (using FKRP and LARGE1 as examples)

DOI

<https://dx.doi.org/10.17504/protocols.io.8epv5x1yhg1b/v1>

Kaiyue Ma¹, Shushu Huang¹, Jenny Xu¹, Angela Lek¹, Monkol Lek¹

¹Yale University



Jenny Xu

Yale University

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.8epv5x1yhg1b/v1>

Protocol Citation: Kaiyue Ma, Shushu Huang, Jenny Xu, Angela Lek, Monkol Lek 2024. Saturation Mutagenesis-Reinforced Functional Assays (SMuRF) for alpha-dystroglycan glycosylation enzymes (using FKRP and LARGE1 as examples). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.8epv5x1yhg1b/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



Protocol status: Working

We use this protocol and it's working

Created: July 07, 2023

Last Modified: July 08, 2024

Protocol Integer ID: 84629

Keywords: coding single nucleotide variant, insights for disease gene, genetic variant, single nucleotide variant, causing genetic variant, disease gene, performing deep mutational, interpretation of unresolved variant, genes fkrp, deep mutational, saturation mutagenesi, streamlining functional assay, clinical variant interpretation, dystroglycan glycosylation enzyme, critical protein structural regions susceptible to missense disruption, gene, smurf to neuromuscular disease, genome, unresolved variant, related gene, functional assay, charting variant effects hamper, genetics, critical protein, dystroglycanopathy, reinforced functional assay

Abstract

Interpretation of disease-causing genetic variants remains a challenge in the field of human genetics and rare disease. Current costs and complexity of performing deep mutational scanning for charting variant effects hampers crowd-sourcing approaches toward genome-wide resolution of variants in all disease-related genes. Our framework, Saturation Mutagenesis-Reinforced Functional assays (SMuRF), addresses these issues by modularizing DMS components, offering simple and cost-effective saturation mutagenesis, as well as streamlining functional assays to enhance interpretation of unresolved variants. Applying SMuRF to neuromuscular disease genes *FKRP* and *LARGE1*, we have generated functional scores for over 99.8% of all possible coding single nucleotide variants (SNVs), providing an additional line of evidence for clinical variant interpretation in dystroglycanopathies. Data generated from SMuRF enables severity prediction, resolve critical protein structural regions susceptible to missense disruptions, and provide training datasets for development of computational predictors. In summary, our approach provides a framework for enabling variant-to-function insights for disease genes in a manner that is accessible for crowd-sourcing implementation across standard research laboratories.

Materials

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kaiyue Ma (kaiyue.ma@yale.edu).

Materials Availability

- Plasmids generated in this study have been deposited to Addgene: Lenti-*DAG1* (205149), Lenti-UbC-*FKRP*-EF1 α -BSD (205150), and Lenti-UbC-*LARGE1*-EF1 α -BSD (205151)

Data and Code Availability

- NGS raw data have been deposited at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) and are publically available as of the date of publication. Accession numbers are listed in the Key Resources Table.
- All original code has been deposited on Github (<https://github.com/leklab>) and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell Lines

- Wildtype HAP1 (C631) and *DAG1*-KO HAP1 (HZGHC000120c016) cells (male lacking Y chromosome) were ordered from Horizon Discovery. All HAP1 cells were cultured at 37°C in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, 12440053) with 10% Fetal Bovine Serum (FBS, R&D Systems, S11150) and 1x Antibiotic-Antimycotic (Anti-anti, Gibco, 15240062). The medium was replaced every 2 days, unless otherwise stated. HAP1 cells tend to grow into multi-layers; hence, to keep the cells in optimal status, TrypLE Express Enzyme (Gibco, 12605010) was used to passage the cells to maintain the cells in healthy confluency (30-90%). HAP1 cells used in SMuRF were immortalized using lentivirus packaged with pLV-hTERT-IRES-hygro (Addgene, 85140), a gift from Tobias Meyer.
- HEK293T cells (female) were cultured at 37°C in DMEM (Gibco, 11995065) with 10% FBS and 1x Anti-anti. The medium was replaced every 2 days, unless otherwise stated.
- MB135 cells (female) were cultured at 37°C in Ham's F-10 Nutrient Mix (Gibco, 11550043) with 20% FBS, 1x Anti-anti, 51 ng/ml dexamethasone (Sigma- Aldrich, D2915) and 10 ng/mL basic fibroblast growth factor (EMD/Millipore, GF003AF-MG). The medium was replaced every 2 days, unless otherwise stated. MB135 cells were differentiated in Skeletal Muscle Differentiation Medium (PromoCell, C-23061) with 1x Anti- anti. The differentiation medium was replaced every 4 days, unless otherwise stated.

CRISPR RNP nucleofection

 Synthetic Single Guide RNA Kit **Synthego**

 SpCas9 2NLS Nuclease **Synthego**



Lentiviral packaging

HEK293T cells

 psPAX2 **addgene Catalog #12260**

 pMD2.G **addgene Catalog #12259**

Lentiviral plasmid

Polybrene

PALS-C cloning for saturation mutagenesis

 Q5 Reaction Buffer **New England Biolabs Catalog #B9027SVIAL**

 Q5 High GC Enhancer **New England Biolabs Catalog #B9028AVIAL**

 10 mM dNTPs **New England Biolabs Catalog #N0447**

 Q5 High-Fidelity DNA Polymerase **New England Biolabs Catalog #M0491SVIAL**

 BamHI-HF **New England Biolabs Catalog #R3136S**

 NEBuilder HiFi DNA Assembly Master Mix **New England Biolabs Catalog #E2621**

QC of plasmid pools/saturation mutagenesis

HEK293T cells

HAP1 cells

 Q5 Reaction Buffer **New England Biolabs Catalog #B9027SVIAL**

 Q5 High GC Enhancer **New England Biolabs Catalog #B9028AVIAL**

 10 mM dNTPs **New England Biolabs Catalog #N0447**

 Q5 High-Fidelity DNA Polymerase **New England Biolabs Catalog #M0491SVIAL**

 Automated Cell Counter **Bio-Rad Laboratories Catalog #TC20**

Staining for FFC and FACS

15 mL tubes

NGS library construction

 PureLink Genomic DNA Mini Kit **Invitrogen Catalog #K182002**

Immunofluorescence

MB135 cells

24-well plates

Packaging and infection of rVSV/ppVSV



rVSV-LASV-GPC viral particles, ppVSVDG-VSV-G viral particles, and LASV-GPC plasmid (Dr. Melinda Brindley)
HEK293T cells



Protocol materials

- SE Cell Line Nucleofector Solution **Lonza Catalog #V4XC-1032**
- 4D-Nucleofector **Lonza**
- .45µm PES filter **Thermo Scientific Catalog #165-0045**
- Lenti-X GoStix Plus **Takara Bio Inc. Catalog #631280**
- Blasticidin S HCl **Gibco - Thermo Fisher Scientific Catalog #A1113903**
- Puromycin Dihydrochloride **Gibco - Thermo Fisher Scientific Catalog #A1113803**
- Lenti-X GoStix App **Takara Bio Inc.**
- 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144**
- Versene Solution **Thermo Fisher Catalog #15040066**
- Viability 405/452 Fixable Dye **Miltenyi Biotec Catalog #130-130-420**
- MACS BSA Stock Solution **Miltenyi Biotec Catalog # 130-091-376**
- autoMACS Rinsing Solution **Miltenyi Biotec Catalog #130-091-222**
- DPBS (10X), no calcium, no magnesium **Thermo Fisher Catalog #14200166**
- UltraPure Distilled Water **Thermo Fisher Scientific Catalog #10977015**
- IIH6C4 Antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #05-593**
- PureLink[®]; Genomic DNA Mini Kit **Thermo Fisher Catalog #K182002**
- Nunc[®]; Thermanox[®]; Coverslips, 15mm diameter **Thermo Fisher Catalog #174969**
- 0.1% Gelatin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G9391**
- Fetal Bovine Serum **R&D Systems Catalog #S11150**
- Antibiotic-Antimycotic (100X) **Thermo Fisher Scientific Catalog #15240062**
- Dexamethasone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D2915**
- Basic Fibroblast Growth Factor **Merck Millipore (EMD Millipore) Catalog #GF003AF-MG**
- Ham's F-10 Nutrient Mix **Thermo Fisher Catalog #11550043**
- Skeletal Muscle Differentiation Medium **PromoCell Catalog #C-23061**
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9647**
- Rabbit anti-Mouse IgM FITC Secondary Antibody **Invitrogen Catalog #31557**
- Antifade Mounting Medium with DAPI **Vector Laboratories Catalog #H1500**
- Microscope Slides **Fisher Scientific Catalog #22-037-246**
- Ammonium chloride (≥ 99.5 %) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9434**
- Endura Electrocompetent Cells **Lucigen Catalog #60242-1**



- ✕ Purelink Midiprep Kit **Invitrogen - Thermo Fisher Catalog #K210014**
- ✕ .1 cm Cuvettes **Bio-Rad Laboratories Catalog #1652089**
- ✕ Gene Pulser II **Bio-Rad Laboratories**
- ✕ NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609**
- ✕ MspJI **New England Biolabs Catalog #R0661S**
- ✕ DpnI **New England Biolabs Catalog #R0176S**
- ✕ Human BD Fc Block™ **Becton Dickinson (BD) Catalog #564220**
- ✕ 40 µm Cell Strainer **Falcon Catalog #352340**
- ✕ TransIT-LT1 Transfection Reagent **Mirus Bio Catalog #MIR 2300**
- ✕ psPAX2 **addgene Catalog #12260**
- ✕ pMD2.G **addgene Catalog #12259**
- ✕ Opti-MEM **Gibco - Thermo Fisher Scientific Catalog #31985062**
- ✕ DMEM **Gibco - Thermo Fisher Scientific Catalog #11995065**
- ✕ Lenti-X Concentrator **Takara Bio Inc. Catalog #631232**
- ✕ Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #158127**
- ✕ Lipofectamine 3000 **Invitrogen - Thermo Fisher Catalog #L3000001**
- ✕ 10 mM dNTPs **New England Biolabs Catalog #N0447**
- ✕ Q5 Reaction Buffer **New England Biolabs Catalog #B9027SVIAL**
- ✕ PureLink Genomic DNA Mini Kit **Invitrogen Catalog #K182002**
- ✕ Q5 High-Fidelity DNA Polymerase **New England Biolabs Catalog #M0491SVIAL**
- ✕ Automated Cell Counter **Bio-Rad Laboratories Catalog #TC20**
- ✕ BamHI-HF **New England Biolabs Catalog #R3136S**
- ✕ Q5 High GC Enhancer **New England Biolabs Catalog #B9028AVIAL**
- ✕ psPAX2 **addgene Catalog #12260**
- ✕ pMD2.G **addgene Catalog #12259**
- ✕ SpCas9 2NLS Nuclease **Synthego**
- ✕ Synthetic Single Guide RNA Kit **Synthego**
- ✕ NEBuilder HiFi DNA Assembly Master Mix **New England Biolabs Catalog #E2621**



Create FKRP-KO and LARGE1-KO cell lines via CRISPR RNP nucleofection

20m

1 Prepare RNP complexes in

SE Cell Line Nucleofector Solution **Lonza Catalog #V4XC-1032**

1.1 Combine 18 μ L supplemented

10m

SE Cell Line Nucleofector Solution **Lonza Catalog #V4XC-1032** , 6 μ L of **[M]** 30 micromolar (μ M) sgRNA, and 1 μ L of **[M]** 20 micromolar (μ M) Cas9 protein at Room temperature . Incubate at Room temperature for 00:10:00 .

1.2 Spin down 150k cells at 100 x g, 00:10:00 , resuspend in 5 μ L supplemented

10m

SE Cell Line Nucleofector Solution **Lonza Catalog #V4XC-1032** . Add the RNP complexes to the cells.

2 Transfer the mixed samples to the wells of the 16-well Nucleocuvette Strips

Perform nucleofection with 4D-Nucleofector **Lonza**

Use EN-138 for HAP1; CA-137 for MB135

3 Allow the nucleofected cells to recover in the growth medium

Plate the nucleofected cells sparsely and allow them to form monoclonal clusters

Pick monoclonal cells under microscope

4 Determine indel event of each clonal line via targeted Sanger sequencing.

Primers used:

FKRP-GT-F: CATCACCTCAACCTTCTGGTC

FKRP-GT-R: CATCAGGTACTAGGGCCACAACTC

LARGE1-GT-F: GGCAATCGGGACTTTGGACA

LARGE1-GT-R: GCCTCGCCATGTAGTAAGG

Lentivirus packaging

5 Grow HEK293T cells to 90% confluency in a 10-cm dish with 10 mL HEK cell growth media.



6 Create the plasmid mixture and perform packaging

6.1 Mix  1.5 mL  Opti-MEM **Gibco - Thermo Fisher Scientific Catalog #31985062** ,  10 µg  psPAX2 **addgene Catalog #12260** ,  2 µg  pMD2.G **addgene Catalog #12259** ,  9 µg lentiviral plasmid, and  50 µL  TransIT-LT1 Transfection Reagent **Mirus Bio Catalog #MIR 2300** .

6.2 Incubate at  Room temperature for  00:15:00 .

15m

6.3 Add mixture dropwise to HEK293T cells.

6.4 Add  3.5 mL  DMEM **Gibco - Thermo Fisher Scientific Catalog #11995065** to the 10 cm dish.

6.5 Incubate at  37 °C for  72:00:00 in a cell incubator.

3d

7 Collect and concentrate packaged virus.

7.1 Remove supernatant and filter through a  .45µm PES filter **Thermo Scientific Catalog #165-0045** .

7.2 Add  5 mL  Lenti-X Concentrator **Takara Bio Inc. Catalog #631232** to filtered supernatant.

7.3 Incubate on a rocker at  4 °C  Overnight .

3d

8 Collect concentrated virus.

8.1 Transfer mixture to 50 mL tube.



8.2 Centrifuge at  1800 x g, 4°C, 01:00:00 .

1h

8.3 Discard supernatant.

8.4 Resuspend pellet in  200 µL

 DMEM **Gibco - Thermo Fisher Scientific Catalog #11995065** .

9 Titrate lentivirus with  Lenti-X GoStix Plus **Takara Bio Inc. Catalog #631280** per manufacturer's instructions. For long-term storage, store lentivirus in cryovials at  -80 °C .

Transduction

10 Plate cells to transduce in wells.
(Perform pre-experiments following the steps below to decide optimal MOI/drug concentration)

11 One day later, refresh medium and add final concentration  8 µg/mL polybrene.

12 Add lentivirus for a spinfection at  800 x g, 30°C, 01:00:00 .

1h

13 After one day, refresh medium and start drug selection if applicable. Perform drug selection for 7 days-14 days, using a well of un-transduced cells as a negative control.

If construct contains BSD, add final concentration  5 µg/mL

 Blasticidin S HCl **Gibco - Thermo Fisher Scientific Catalog #A1113903** .

If construct contains PuroR, add final concentration  1 µg/mL

 Puromycin Dihydrochloride **Gibco - Thermo Fisher Scientific Catalog #A1113803** .

PALS-C cloning for saturation mutagenesis



- 14 Synthesize 64-bp ssDNA oligos for each variant of all possible CDS SNVs using Twist Bioscience
- 15 Calculate plasmid template input weight:
Coverage = 10^6
Oligo library input weight = (gene cds length * coverage * block number) * (relative molecular mass of one oligo)/(Avogadro constant)
Plasmid template input weight = [(oligo library input weight) * (plasmid length) * 2]/(oligo length)
- 16 Anneal primers carrying degenerate nucleotides to the plasmid template and extend towards the 5' end. Conditions as below:

2m 10s

Reagent	Volume
10 mM dNTPs	1 μ L
Oligo library	
Plasmid template	
Q5 Enh buffer	10 μ L
Q5 polymerase*	2 μ L
Q5 Rxn buffer	10 μ L
Water	To 50 μ L*

Temperature	Time
98 °C	hot start
98 °C	4 min
Annealing temperature*	20 s
72 °C	Elongation time*
12 °C	hold

Notes:

Q5 polymerase is likely to be limiting factor--optimize volume if necessary.

Determine annealing temperature/elongation time based on the product that is most difficult to amplify. If different blocks require vastly different conditions, multiple

reactions can be performed.  70 °C and  00:00:40 were used for FKRP, and  68 °C and  00:01:30 were used for LARGE1.

16.1 Perform PCR purification using

 NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609** per manufacturer's instructions.

17 Isolate products using block-specific primers.

17.1 PALS-C step 2: use universal F1 primer and block-specific adaptor primer R1s to amplify variant strands using the conditions below.

Reagent	Volume
Q5 Rxn buffer	10 µL
Q5 Enh buffer	10 µL
10 mM dNTPs	1 µL
Purified Step1 product	*
10 µM Universal F1	2.5 µL
10 µM Block specific R1	2.5 µL
Q5 polymerase	0.5 µL
Water	To 50 µL

Temperature	Time
98 °C	hot start
98 °C	3 min
98 °C	8 s



Temperature	Time
Annealing temperature	20 s
72 °C	Elongation time
Repeat 3-5 for 34 more cycles	
72 °C	5 min
12 °C	hold

*The input should be decided based on the position of the block. The more distant a block is from the 5' side, the more input is required. Evenly distributed input for all 6 blocks of FKRP generated enough yield for subsequent steps, while the 3' side blocks of LARGE1 required extra input. Step1-2 should be repeated if product yield is insufficient for subsequent steps.

17.2 Perform PCR purification on 19.1 products using



NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609** .

17.3 PALS-C Step 3: Use type2S enzyme to remove the block-specific adaptor via restriction enzyme digest.

Reagent	Volume
Type2S enzyme*	2 µL
Purified Step2 product	1.2 µg
Reaction buffer	5 µL
Water	To 50 µL

Temperature	Time
Reaction temperature (Lid: 60 °C)	50 min
12 °C	hold



*BsmBI was used for FKRP and BsaI was used for LARGE1. The type2S enzymes were picked to avoid the presence of their recognition sites within the CDS.

17.4 Perform gel purification using

 NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609** .

18 PALS-C Step 4: Add WT template and extend variant strands towards 3' end.

Reagent	Volume
Q5 Rxn buffer	10 μ L
Q5 Enh buffer	10 μ L
10 mM dNTPs	1 μ L
Purified Step3 product	
Plasmid template	
Q5 polymerase*	2 μ L
Water	To 50 μ L

Temperature	Time
98 $^{\circ}$ C	hot start
98 $^{\circ}$ C	5 min
72 $^{\circ}$ C	5 s
66 $^{\circ}$ C	20 s
72 $^{\circ}$ C	Elongation time*
12 $^{\circ}$ C	hold

*Q5 polymerase is likely to be the limiting factor of Step4, volume of which requires optimization.

*Optional: the purpose is to enhance the annealing of all strands.

*The elongation time should be sufficient for the shortest strand to be elongated to the R2 primer site



18.1 PALS-C Step 5: Use type2M enzyme

 MspJI **New England Biolabs Catalog #R0661S** and

 DpnI **New England Biolabs Catalog #R0176S** to remove templates.

	Reagent	Volume
	DpnI	0.5 μ L
	MspJI	0.5 μ L
	CutSmart	5 μ L
	Enzyme activator	1 μ L
	Purified Step4 product	500 ng
	Water	To 50 μ L

	Temperature	Time
	37 $^{\circ}$ C (Lid: 60 $^{\circ}$ C)	1 hr
	12 $^{\circ}$ C	Hold

18.2 Perform column purification per manufacturer's instruction.

18.3 PALS-C Step 6: Use Primer F2 and primer R2 to amplify full-length strand.

	Reagent	Volume
	Q5 Rxn buffer	10 μ L
	Q5 Enh buffer	10 μ L
	10 mM dNTPs	1.5 μ L
	Purified Step5 product	100 ng
	10 μ M F2	2.5 μ L
	10 μ M R2	2.5 μ L



Reagent	Volume
Q5 polymerase	1 μ L
Water	To 50 μ L

Temperature	Time
98 °C	hot start
98 °C	5 min
98 °C	6 s
Annealing temperature	20 s
72 °C	Elongation time
Repeat 3-5 for 34 more cycles	
72 °C	5 min
12 °C	Hold

19 Perform electrophoresis and cut correct bands from gels.
IMPORTANT: Use 20 μ L or less of water to dissolve after gel purification, or use vacuum concentrator to evaporate less water

20 Insert purified Step6 product into plasmid backbone.

20.1 PALS-C Step 7: Prepare backbone.

	Xbal	1.5 μ L
	BamHI-HF	1.5 μ L
	Plasmid template	3 μ g
	CutSmart	5 μ L
	Water	To 50 μ L



	37 °C (Lid: 60 °C)	40 min
	12 °C	Forever

20.2 Perform Gibson assembly.

	NEBuilderMaster Mix	20 µL
	Backbone	210 ng
	Purified Step6 product	140 ng
	Water	To 30 µL

	50 °C (Lid: 60 °C)	60 min
	12 °C	Forever

21 Deliver Gibson assembly products to

 Endura Electrocompetent Cells **Lucigen Catalog #60242-1** via electrotransformation.

21.1 PALS-C Step 8: Assemble the following reaction for each block.

	Reagent	Volume
	Electrocompetent cells	40 µL
	Assembly reaction	4 µL
	Water	160 µL

21.2 Split sample into 2 pre-chilled

 .1 cm Cuvettes **Bio-Rad Laboratories Catalog #1652089**

- 21.3 Use  Gene Pulser II **Bio-Rad Laboratories** @ 25 μ F; 200 Ohms; 1800 volts. Avoid bubbles.
- 21.4 Add sample to  900 μ L recovery media per cuvette. 1h
- 21.5 Combine transformed bacteria from both cuvettes in one tube. Shake at  250 rpm, 37°C, 01:00:00 .
- 21.6 Add 1/500 volume of the bacteria to  200 μ L LB broth and plate it on an ampicillin LB agar plate for quick estimation of complexity
- 22 Seed all remaining bacteria in  150 mL LB broth with  100 μ g/mL ampicillin. Grow bacteria overnight. (Standard 37 °C 16hrs condition can be used but 30°C 20hrs is preferred)
- 23 Extract plasmid using  Purelink Midiprep Kit **Invitrogen - Thermo Fisher Catalog #K210014**
- 24 Calculate colony forming units.

QC of plasmid pools and saturation mutagenesis

- 25 Perform QC on plasmid pools using GENEWIZ Amplicon-EZ service.
- 25.1 For the plasmid pool of each FKRP block, perform the following reaction.

Reagent	Volume
Q5 Rxn buffer	10 μ L
Q5 Enh buffer	10 μ L
10 mM dNTPs	1 μ L
Plasmid	~300 ng



Reagent	Volume
10 μ M F primer	2.5 μ L
10 μ M R primer	2.5 μ L
Q5 polymerase	0.5 μ L
Water	To 50 μ L

Temperature	Time
98 °C	hot start
98 °C	3 min
98 °C	6 s
70 °C	15 s
72 °C	5 s
Repeat 3-5 for 32 more cycles	
72 °C	5 min
12 °C	hold

For the plasmid pool of each LARGE1 block, perform the following reaction:

Reagent	Volume
Q5 Rxn buffer	10 μ L
Q5 Enh buffer	10 μ L
10 mM dNTPs	1 μ L
Plasmid	~50 ng
10 μ M F primer	2.5 μ L
10 μ M R primer	2.5 μ L
Q5 polymerase	0.5 μ L
Water	To 50 μ L



Temperature	Time
98 °C	hot start
98 °C	3 min
98 °C	6 s
Annealing temperature	15 s
72 °C	7 s
Repeat 3-5 for 33 more cycles	
72 °C	5 min
12 °C	Hold

Annealing temperature	Blocks
61 °C	1, 4, 6, 7
64 °C	5, 9, 10
66 °C	2, 3, 8

Perform electrophoresis and gel purification (using



NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609**

). Send

products for sequencing.

Mix purified products and perform the following reaction:

Reagent	Volume
Q5 Rxn buffer	10 µL
Q5 Enh buffer	10 µL
10 mM dNTPs	1 µL
Mixed purified products	100 ng
10 µM NGS-PCR3-F	2.5 µL
10 µM NGS-PCR3-R	2.5 µL
Q5 polymerase	1 µL
Water	To 50 µL

Temperature	B
98 °C	hot start
98 °C	3 min
98 °C	6 s
72 °C	15 s
72 °C	8 s
Repeat 3-5 for 19 more cycles	
72 °C	5 min
12 °C	Forever

Perform PCR purification (

 NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609**)and send sample for sequencing.

Lentiviral packaging

26 Perform lentiviral packaging on one 10-cm dish of HEK293T cells. Use small-scale pre-experiments to determine viral dosage for optimal separation

27 Using GoStix Value as quantified by the  Lenti-X GoStix App **Takara Bio Inc.** , scale the dosage for each block.

For each block, plate 600k HAP1 cells or 200k MB135 cells in a well of a 6-well plate. After transduction and drug selection, for FACS, expand this number to 30M+.

Package lentiviral pools of all blocks at the same time using reagents and helper plasmids from the same batch to avoid batch effects. Use 1e3 - 1e4 GV x μ L of lentivirus per block.

Staining for FFC and FACS

28 Wash cells twice with

 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** .



- 29 Digest cells with Versene Solution **Thermo Fisher Catalog #15040066** and count with an Automated Cell Counter (Bio-Rad, TC20).
- 30 Spin down 30M cells at 700 x g, 4°C, 00:15:00 . Resuspend in 3 mL **15m**
 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** supplemented with 30 µL Viability 405/452 Fixable Dye **Miltenyi Biotec Catalog #130-130-420** .
- 31 Perform all following steps in the dark. Rock sample for 00:30:00 . **30m**
- 32 Add 7 mL PEB buffer (1 volume of MACS BSA Stock Solution **Miltenyi Biotec Catalog # 130-091-376** , 19 volumes of autoMACS Rinsing Solution **Miltenyi Biotec Catalog #130-091-222**) to the tube.
- 33 Spin cells down at 700 x g, 4°C, 00:15:00 . **15m**
- 33.1 Remove and discard supernatant.
- 33.2 Resuspend in 3 mL 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** supplemented in 30 µL Human BD Fc Block™ **Becton Dickinson (BD) Catalog #564220** .
- 34 Rock sample gently at room temperature for 00:30:00 . **30m**
- 35 Add 7 mL 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** . **15m**
Spin cells down at 700 x g, 4°C, 00:15:00 . Resuspend in 3 mL MAGIC buffer (5% FBS; 0.1% NaAz w/v; 10% 10× DPBS (10X), no calcium, no magnesium **Thermo Fisher Catalog #14200166** ; UltraPure Distilled Water **Thermo Fisher Scientific Catalog #10977015**) supplemented with 1:200



 IIH6C4 Antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #05-593**

(discontinued, or the same antibody made in Dr. Kevin Campbell's lab).

36 Rock sample gently at  4 °C for  20:00:00 .

20h

37 Add  7 mL MAGIC buffer.

.

37.1 Spin down at  700 x g, 4°C, 00:10:00 .

10m

37.2 Discard supernatant.

37.3 Resuspend in  3 mL MAGIC buffer supplemented with 1:50

 Rabbit anti-Mouse IgM FITC Secondary Antibody **Invitrogen Catalog #31557** .

38 Rock sample gently at  4 °C for  20:00:00 in the dark.

20h

39 Add  7 mL  1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** to the sample.

39.1 Spin down at  700 x g, 4°C, 00:10:00 .

10m

39.2 Remove supernatant.

39.3 Resuspend in  4 mL

 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144**

39.4 Filter resuspended cells using  40 µm Cell Strainer **Falcon Catalog #352340** .

NGS library construction

40 Spin down the cells harvested from FACS at  800 x g, 4°C, 00:10:00 .

10m

41 Harvest gDNA from each sample using

 PureLink®; Genomic DNA Mini Kit **Thermo Fisher Catalog #K182002**

42 Step 1: Use primers specific to the lentiviral backbone to amplify the lentiviral CDS sequences of each sample

42.1 Perform the following reaction:

	Primer name	Sequence
	PCR1-F	GATCGTCACTTGGTACCGGTTCTAGA
	PCR1-R (FKRP)	TGGCACTTTTCGGGGGATCCTC
	PCR1-R (LARGE1)	TGGCACTTTTCGGGGGATCCCT

	Reagent	Catalog	Volume/weight
	Q5 Reaction Buffer	NEB, B9027SVIAL	10 µL
	Q5 High GC Enhancer	NEB, B9028AVIAL	10 µL
	10 mM dNTPs	NEB, N0447	1 µL
	Q5 High-Fidelity DNA Polymerase	NEB, M0491SVIAL	1 µL
	10µM PCR1-F		2.5 µL
	10µM PCR1-R		2.5 µL
	gDNA		0.3-1 µg
	Nuclease-Free Water		To 50 µL

	Step	Temperature	Time
	Step1	98 °C	Hot start



Step	Temperature	Time
Step2	98 °C	3 mins
Step3	98 °C	8 s
Step4	68 °C	20 s
Step5	72 °C	45 s
Step 3-5, 35 cycles		
Step6	72 °C	5 mins
Step7	12 °C	hold

- 42.2 Perform electrophoresis in 1% agarose gel. Expected band sizes are 1534 bp for FKRP and 2317 bp for LARGE1. Perform gel purification using  NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609** and elute in  25 µL nuclease-free water.

43 Step 2: Isolate blocks.

- 43.1 Perform the following reaction. See Supp. Method 8 for primers.

Reagent	Catalog	Volume/Weight
Q5 Reaction Buffer	NEB, B9027SVIAL	10 µL
Q5 High GC Enhancer	NEB, B9028AVIAL	10 µL
10 mM dNTPs	NEB, N0447	1 µL
Q5 High-Fidelity DNA Polymerase	NEB, M0491SVIAL	1 µL
10µM PCR2-F		2.5 µL



	Reagent	Catalog	Volume/Weight
	10 μ M PCR2-R		2.5 μ L
	gDNA		0.2-0.5 μ g
	Nuclease-Free Water		To 50 μ L

	Step	Temperature	Time
	Step1	98 °C	Hot start
	Step2	98 °C	3 mins
	Step3	98 °C	6 s
	Step4	Annealing temperature	15 s
	Step5	72 °C	7 s
	Step 3-5, 25 cycles		
	Step6	72 °C	5 mins
	Step7	12 °C	hold

Annealing temperatures:

	Temperature	Primers
	61 °C	LARGE1-blk1, LARGE1-blk4, LARGE1- blk6, LARGE1-blk7
	64 °C	FKRP-blk1, LARGE1-blk5, LARGE1- blk9, LARGE1-blk10
	66 °C	LARGE1-blk2, LARGE1-blk3, LARGE1- blk8

	Temperature	Primers
	68 °C	FKRP-blk6
	72 °C	FKRP-blk2, FKRP-blk3, FKRP-blk4, FKRP-blk5

43.2 Perform PCR purification with

 NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609** . Elute

with  40 µL nuclease-free water.

44 Perform adaptor addition.

44.1 Mix purified PCR2 products (200 ng each) and dilute to 11 ng/µL . Perform the following reaction:

	Reagent	Catalog	Time
	Q5 Reaction Buffer	NEB, B9027SVIAL	10 µL
	Q5 High GC Enhancer	NEB, B9028AVIAL	10 µL
	10 mM dNTPs	NEB, N0447	1 µL
	Q5 High-Fidelity DNA Polymerase	NEB, M0491SVIAL	1 µL
	10µM PCR3-F		2.5 µL
	10µM PCR3-R		2.5 µL
	Mixed sample		23 µL

	Step	Temperature	Time
	Step1	98 °C	Hot start
	Step2	98 °C	3 mins



	Step	Temperature	Time
	Step3	98 °C	6 s
	Step4	72 °C	15 s
	Step5	72 °C	8 s
	Step 3-5, 25 cycles		
	Step6	72 °C	5 mins
	Step7	12 °C	Infinite

Set 3 *  50 µL reactions.

Perform  NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #740609**
and elute with  50 µL nuclease free water.

- 44.2 Send PCR3 product for next generation sequencing and use GENEWIZ Amplicon-EZ service to check quality and coverage. Sequence using Hiseq X service.

Immunofluorescence

- 45 Place

 Nunc[®]; Thermanox[®]; Coverslips, 15mm diameter **Thermo Fisher Catalog #174969**

in a 24-well plate.

- 46 Coat coverslips in

 0.1% Gelatin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G9391** and immediately remove. Air-dry.

- 47 Plate MB135 cells

- 47.1 Resuspend 250k MB135 cells in  .5 mL growth medium (

 Ham's F-10 Nutrient Mix **Thermo Fisher Catalog #11550043** with 20%



Fetal Bovine Serum **R&D Systems Catalog #S11150** , 1x

Antibiotic-Antimycotic (100X) **Thermo Fisher Scientific Catalog #15240062** ,

[M] 51 ng/mL

Dexamethasone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D2915** , and

[M] 10 ng/mL

Basic Fibroblast Growth Factor **Merck Millipore (EMD Millipore) Catalog #GF003AF-MG**

)

47.2 Seed cells into each well.

47.3 One day later, change out the medium for

Skeletal Muscle Differentiation Medium **PromoCell Catalog #C-23061** with 1x

Antibiotic-Antimycotic (100X) **Thermo Fisher Scientific Catalog #15240062** .

47.4 Differentiate cells for 3-7 days until myotubes are formed.

48 Fix cells.

48.1 Wash cells with 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** .

48.2 Fix with 4%

Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #158127** for

00:10:00 at Room temperature .

10m

49 Block cells with [M] 2 % (w/v)

Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9647** in

1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** at

Room temperature for 01:00:00

1h

50 Incubate with 1:200

IIH6C4 Antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #05-593** in

20h



[M] 2 % (w/v)

⊗ Bovine Serum Albumin Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9647 in

⊗ 1x DPBS Gibco - Thermo Fisher Scientific Catalog #14190144 at 🌡 4 °C for

🕒 20:00:00 .

- 51 Wash cells in ⊗ 1x DPBS Gibco - Thermo Fisher Scientific Catalog #14190144 and incubate in 1:100

⊗ Rabbit anti-Mouse IgM FITC Secondary Antibody Invitrogen Catalog #31557 in

[M] 2 % (w/v)

⊗ Bovine Serum Albumin Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9647 in

⊗ 1x DPBS Gibco - Thermo Fisher Scientific Catalog #14190144 at

🌡 Room temperature for 🕒 02:00:00 . Keep cells in the dark.

- 52 Drop ⊗ Antifade Mounting Medium with DAPI Vector Laboratories Catalog #H1500 onto ⊗ Microscope Slides Fisher Scientific Catalog #22-037-246

- 53 Wash coverslips with ⊗ 1x DPBS Gibco - Thermo Fisher Scientific Catalog #14190144 . Place facedown on slides over drops of DAPI and keep at 🌡 Room temperature in the dark for

🕒 00:30:00 .

- 54 Image on a Revolve ECHO microscope
(DAPI - EX:380/30 EM:450/50 DM:425)
(FITC - EX:470/40 EM:525/50 DM:495)

Packaging and infection of rVSV/ppVSV

- 55 Transfect HEK293T cells with LASV-GPC plasmid

- 56 Transduce cells with ppVSVΔG-VSV-G viral particles. Resulting particles will be referred to as ppVSV-LASV-GPC-Generation1.



- 56.1 Seed HEK293T cells in a well of a 6-well plate. Incubate @ 37 °C Overnight .
- 56.2 Once cells reach 70-90% confluency, transfect cells using 4 µg LASV-GPC plasmid and Lipofectamine 3000 **Invitrogen - Thermo Fisher Catalog #L3000001** per manufacturer's instructions. Incubate @ 37 °C 24:00:00 .
- 56.3 Add ppVSVΔG-VSV-G (MOI=0.5). Calculate viral dose given estimated cell number of ~2 M.
- 56.4 01:00:00 later, remove medium, wash with 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** , and add fresh medium to the well.
- 56.5 The next day, collect the newly generated viral particles (referred to as ppVSV-LASV-GPC-Generation1). Perform titration to determine viral titer.
- 57 Infect LASV-GPC transfected HEK293T cells with ppVSV-LASV-GPC-Generation1 to produce ppVSV-LASV-GPC-Generation2. This reduces residual VSV-G in pseudotyped particles. ppVSV-LASV-GPC-Generation2 used moving forward.
- 57.1 Seed 6M HEK293T cells in a 10-cm dish. Incubate @ 37 °C Overnight .
- 57.2 Transfect cells using 30 µg LASV-GPC plasmid and Lipofectamine 3000 **Invitrogen - Thermo Fisher Catalog #L3000001** per manufacturer's instructions. Incubate @ 37 °C 24:00:00 .
- 57.3 Add ppVSV-LASV-GPC-Generation1 (MOI=0.1) to the well. Determine viral dose using estimated cell number of ~12M.
- 57.4 01:00:00 later, remove medium, wash with 1x DPBS **Gibco - Thermo Fisher Scientific Catalog #14190144** , and add fresh medium to the well.
- 57.5 The next day, collect the newly generated viral particles (referred to as ppVSV-LASV-GPC-Generation1). Perform titration to determine viral titer.

1d

1h

1d

1h



58 Determine 50% tissue culture infectious dose using Spearman-Karber method.

59 Determine MOI of ppVSV (performed at MOI 1-3)

60 Perform transduction and blasticidin drug selection as in FACS assay

61 Divide cells into 2 ~1M groups. Infect one group with rVSV at a concentration of 2×10^5 TCID₅₀/mL (MOI ~0.5). Add 5 millimolar (mM) final concentration



Ammonium chloride (≥ 99.5 %) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9434**

during infection and recovery.

62 60:00:00 later, refresh medium. Allow cells to recover for 12:00:00 (cell count ~1M).

3d

63 Harvest cells