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

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

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## 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*-EF1α-BSD (205150), and Lenti-UbC-*LARGE1*-EF1α-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

- Lenti-X GoStix App **Takara Bio Inc.** Step 27
- .1 cm Cuvettes **Bio-Rad Laboratories Catalog #**1652089 Step 21.2
- Lipofectamine 3000 **Invitrogen - Thermo Fisher Catalog #**L3000001 In 2 steps
- Ham's F-10 Nutrient Mix **Thermo Fisher Catalog #**11550043 Step 47.1
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9647 In 3 steps
- psPAX2 **addgene Catalog #**12260 Step 6.1
- Q5 Reaction Buffer **New England Biolabs Catalog #**B9027SVIAL In Materials, Materials
- Q5 High-Fidelity DNA Polymerase **New England Biolabs Catalog #**M0491SVIAL In Materials, Materials
- autoMACS Rinsing Solution **Miltenyi Biotec Catalog #**130-091-222 Step 32
- PureLink™ Genomic DNA Mini Kit **Thermo Fisher Catalog #**K182002 Step 41
- 0.1% Gelatin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G9391 Step 46
- psPAX2 **addgene Catalog #**12260 Materials
- TransIT-LT1 Transfection Reagent **Mirus Bio Catalog #**MIR 2300 Step 6.1
- PureLink Genomic DNA Mini Kit **Invitrogen Catalog #**K182002 Materials
- Antibiotic-Antimycotic (100X) **Thermo Fisher Scientific Catalog #**15240062 In 2 steps
- Opti-MEM **Gibco - Thermo Fisher Catalog #**31985062 Step 6.1
- .45µm PES filter **Thermo Scientific Catalog #**165-0045 Step 7.1
- IIH6C4 Antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05-593 In 2 steps
- Ammonium chloride ( ≥ 99.5 %) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9434 Step 61
- Nunc™ Thermanox™ Coverslips, 15mm diameter **Thermo Fisher Catalog #**174969 Step 45
- Fetal Bovine Serum **R&D Systems Catalog #**S11150 Step 47.1
- SE Cell Line Nucleofector Solution **Lonza Catalog #**V4XC-1032 In 3 steps
- Lenti-X GoStix Plus **Takara Bio Inc. Catalog #**631280 Step 9
- Puromycin Dihydrochloride **Gibco - Thermo Fisher Catalog #**A1113803 Step 13
- 4D-Nucleofector **Lonza** Step 2
- Automated Cell Counter **Bio-Rad Laboratories Catalog #**TC20 Materials
- pMD2.G **addgene Catalog #**12259 Step 6.1
- BamHI-HF **New England Biolabs Catalog #**R3136S Materials
- UltraPure Distilled Water **Thermo Fisher Scientific Catalog #**10977015 Step 35
- Microscope Slides **Fisher Scientific Catalog #**22-037-246 Step 52

Human BD Fc Block™ **Becton Dickinson (BD) Catalog #**564220 Step 33.2

Lenti-X Concentrator **Takara Bio Inc. Catalog #**631232 Step 7.2

Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**158127 Step 48.2

Antifade Mounting Medium with DAPI **Vector Laboratories Catalog #**H1500 Step 52

40 µm Cell Strainer **Falcon Catalog #**352340 Step 39.4

DMEM **Gibco - Thermo Fisher Catalog #**11995065 In 2 steps

Skeletal Muscle Differentiation Medium **PromoCell Catalog #**C-23061 Step 47.3

DpnI **New England Biolabs Catalog #**R0176S Step 18.1

Versene Solution **Thermo Fisher Catalog #**15040066 Step 29

Viobility 405/452 Fixable Dye **Miltenyi Biotec Catalog #**130-130-420 Step 30

DPBS (10X), no calcium, no magnesium **Thermo Fisher Catalog #**14200166 Step 35

Rabbit anti-Mouse IgM FITC Secondary Antibody **Invitrogen Catalog #**31557 In 2 steps

Q5 High GC Enhancer **New England Biolabs Catalog #**B9028AVIAL In Materials, Materials

1x DPBS **Gibco - Thermo Fisher Catalog #**14190144 In 14 steps

10 mM dNTPs **New England Biolabs Catalog #**N0447 In Materials, Materials

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

Gene Pulser II **Bio-Rad Laboratories** Step 21.3

MspJI **New England Biolabs Catalog #**R0661S Step 18.1

Blasticidin S HCl **Gibco - Thermo Fisher Catalog #**A1113903 Step 13

MACS BSA Stock Solution **Miltenyi Biotec Catalog #** 130-091-376 Step 32

Basic Fibroblast Growth Factor **Merck Millipore (EMD Millipore) Catalog #**GF003AF-MG Step 47.1

NucleoSpin Gel and PCR Clean-Up Kit **Takara Bio Inc. Catalog #**740609 In 8 steps

pMD2.G **addgene Catalog #**12259 Materials

SpCas9 2NLS Nuclease **Synthego** Materials

Synthetic Single Guide RNA Kit **Synthego** Materials

Dexamethasone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D2915 Step 47.1

Endura Electrocompetent Cells **Lucigen Catalog #**60242-1 Step 21

Purelink Midiprep Kit **Invitrogen - Thermo Fisher Catalog #**K210014 Step 23

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

10m

1.2 Spin down 150k cells at 100 x g, 00:10:00 , resuspend in 5 µL supplemented
SE Cell Line Nucleofector Solution **Lonza Catalog #**V4XC-1032 . Add the RNP complexes to the cells.

10m

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: CATCACCCTCAACCTTCTGGTC
FKRP-GT-R: CATCAGGTACTAGGGCCACAAACTC
LARGE1-GT-F: GGCAATCGGGACTTTGGACA
LARGE1-GT-R: GCCTCGCCATGTAGTAAGGG

## 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 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 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 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 [M] 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 [M] 5 µg/mL
Blasticidin S HCl **Gibco - Thermo Fisher Catalog #**A1113903 .
If construct contains PuroR, add final concentration [M] 1 µg/mL
Puromycin Dihydrochloride **Gibco - Thermo Fisher 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 moleuclar 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 µL |
| | Oligo library | |
| | Plasmid template | |
| | Q5 Enh buffer | 10 µL |
| | Q5 polymerase* | 2 µL |
| | Q5 Rxn buffer | 10 µL |
| | Water | To 50 µL* |

| | 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 |
| | 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 µL |
| Q5 Enh buffer | 10 µL |
| 10 mM dNTPs | 1 µL |
| Purified Step3 product | |
| Plasmid template | |
| Q5 polymerase* | 2 µL |
| Water | To 50 µL |

| | Temperature | Time |
|---|---|---|
| | 98 °C | hot start |
| | 98 °C | 5 min |
| | 72 °C | 5 s |
| | 66 °C | 20 s |
| | 72 °C | Elongation time* |
| | 12 °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 µL |
| | MspJI | 0.5 µL |
| | CutSmart | 5 µL |
| | Enzyme activator | 1 µL |
| | Purified Step4 product | 500 ng |
| | Water | To 50 µL |

| | Temperature | Time |
|---|---|---|
| | 37 °C (Lid: 60 °C) | 1 hr |
| | 12 °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 µL |
| Q5 Enh buffer | 10 µL |
| 10 mM dNTPs | 1.5 µL |
| Purified Step5 product | 100 ng |
| 10 µM F2 | 2.5 µL |
| 10 µM R2 | 2.5 µL |
| Q5 polymerase | 1 µL |
| Water | To 50 µL |

| 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 uL 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.

| | |
|---|---|
| XbaI | 1.5 µL |
| BamHI-HF | 1.5 µL |
| Plasmid template | 3 µg |
| CutSmart | 5 µL |
| Water | To 50 µL |

| | |
|---|---|
| 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 µF; 200 Ohms; 1800 volts. Avoid bubbles.

21.4 Add sample to 900 µL 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 µL 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 [M] 100 µg/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 µL |
| Q5 Enh buffer | 10 µL |
| 10 mM dNTPs | 1 µL |
| Plasmid | ~300 ng |
| 10 µM F primer | 2.5 µL |
| 10 µM R primer | 2.5 µL |
| Q5 polymerase | 0.5 µL |
| Water | To 50 µL |

| Temperature | Time |
|---|---|
| 98 ºC | hot start |
| 98 °C | 3 min |
| 98 °C | 6 s |

| | Temperature | Time |
|---|---|---|
| | 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 µL |
| | Q5 Enh buffer | 10 µL |
| | 10 mM dNTPs | 1 µL |
| | Plasmid | ~50 ng |
| | 10 µM F primer | 2.5 µL |
| | 10 µM R primer | 2.5 µL |
| | Q5 polymerase | 0.5 µL |
| | Water | To 50 µL |

| | 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 ºC | hot start |
| 98 ºC | 3 min |
| 98 ºC | 6 s |
| 72 ºC | 15 s |
| 72 ªC | 8 s |
| Repeat 3-5 for 19 more cycles | |
| 72 °C | 5 min |
| 12 ºC | 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 µL of lentivirus per block.

## Staining for FFC and FACS

28 Wash cells twice with 1x DPBS **Gibco - Thermo Fisher 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 Catalog #**14190144 supplemented with 30 µL
Viobility 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 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 Catalog #**14190144 . 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). 15m

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 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 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 |
| | 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 |
| | 10µM PCR2-R | | 2.5 µL |
| | gDNA | | 0.2-0.5 µg |
| | Nuclease-Free Water | | To 50 µL |

| | 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 |
| | 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 [M] 11 ng/µL. Perform the following reaction:

| | Reagent | Catalog | Time |
|---|---|---|---|
| | Q5 Reaction Buffer | NEB, B9027SVIAL | 10 µL |

| | Reagent | Catalog | Time |
|---|---|---|---|
| | 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 |
| | 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 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 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 [M] 2 % (w/v) Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9647 in 1x DPBS **Gibco - Thermo Fisher Catalog #**14190144 at 4 °C for 20:00:00 . 20h

51 Wash cells in 1x DPBS **Gibco - Thermo Fisher 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 Catalog #**14190144 at Room temperature for 02:00:00 . Keep cells in the dark. 2h

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 Catalog #**14190144 . Place facedown on slides over drops of DAPI and keep at Room temperature in the dark for 00:30:00 . 30m

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

12h

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 . 1d

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 Catalog #**14190144 , and add fresh medium to the well. 1h

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 . 1d

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 Catalog #**14190144 , and add fresh medium to the well. 1h

57.5 The next day, collect the newly generated viral particles (referred to as ppVSV-LASV-GPC-Generation1). Perform titration to determine viral titer.

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 2e5 TCID50/mL (MOI ~0.5). Add [M] 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