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Product Information

Human Whole Genome SAM CRISPRa Pooled Lentiviral Library Kit Puro
Product Number HSAMPURO
Mouse Whole Genome SAM CRISPRa Pooled Lentiviral Library Kit Puro
Product Number MSAMPURO
Human Whole Genome SAM CRISPRa Pooled Lentiviral Library Kit Zeo
Product Number HSAMZEO
Mouse Whole Genome SAM CRISPRa Pooled Lentiviral Library Kit Zeo
Product Number MSAMZEO
dCas9-VP64-Blasticidin SAM CRISPRa Helper Construct 1 Lentiviral Transduction Particles
Product Number SAMVP64BSTV
MS2-P65-HSF1-Hygromycin SAM CRISPRa Helper Construct 2 Lentiviral Transduction Particles
Product Number SAMMS2HYGV
Custom CRISPRa SAM gRNA Lentiviral Transduction Particles
Product Number CRISPRSAMV
SAM CRISPRa Helper Construct Kit Lentiviral Transduction Particles
Product Number SAMHELPERV

Storage Temperature –80 °C

dCas9-VP64-Blasticidin SAM CRISPRa Helper Construct 1 Plasmid DNA
Product Number SAMVP64BST
MS2-P65-HSF1-Hygromycin SAM CRISPRa Helper Construct 2 Plasmid DNA
Product Number SAMMS2HYG
SAM CRISPRa Helper Construct Kit Plasmid DNA
Product Number SAMHELPERP
Custom CRISPRa SAM gRNA Plasmid DNA
Product Number CRISPRSAMD

Storage Temperature –20 °C

CRISPR SAM Technical Bulletin

Product Description

CRISPR technology has revolutionized genome editing by radically simplifying retargeting methods for DNA binding proteins. Since CRISPR systems can be programmed using minimal genetic information (19–20 bp), they are compatible with high-throughput cloning methods and have been rapidly adapted to drug screening workflows (Shalem et al., 2014; Wang et al., 2014; Koike-Yusa et al., 2014).

Sigma-Aldrich has strong core scientific and production capabilities in both genome editing (Chen et al., 2017; Geurts et al., 2009; Chen et al., 2011; Ji et al., 2014; Sampson et al., 2015) and genetic screening using lentiviral shRNA and whole genome CRISPR libraries (Metzakopian et al., 2017; Ji et al., 2007; Duan et al., 2010; Guin et al., 2014; Whitworth et al., 2012). These core capabilities have been combined to create new reagents, services, and expertise to support CRISPR-based genetic screening.

This bulletin describes the reagents and protocols for the SAM CRISPR activator, pooled, whole genome, human and mouse lentiviral libraries. Designed by scientists at the Broad Institute, and optimized and manufactured at Sigma-Aldrich, the SAM CRISPR library allows researchers to explore gene function through strong genome-wide activation of targets. Strong antibiotic selection markers enrich for cells transduced by CRISPR SAM. After enrichment for phenotype of interest (often growth or death), next generation sequencing and deconvolution of data reveals which gene targets have been enriched or depleted in association with the desired phenotype.

Synergistic Activation Mediator (SAM) is a strong transcriptional activation protein complex. The Sigma-Aldrich CRISPR SAM system uses nuclease-dead SpCas9 (dCas9) and guide RNA as a cargo delivery system to bring SAM effector domains to target gene promoters, enabling site-specific transcriptional activation of the gene of interest (Konermann et al., 2015; Joung et al., 2017). The three plasmid components of the SAM library are:

dCas9-VP64-Blast
gRNA-MS2 aptamer-Puro^R OR gRNA-MS2 aptamer-Zeo
MS2-p65-HSF1-Hygromycin

The transcriptional activator VP64 fused to nuclease-dead SpCas9 (dCas9) forms a ribonucleoprotein complex (RNP) with the CRISPR guide RNA (gRNA). The stem- and tetra-loop sequences in the gRNA scaffold have been modified into minimal hairpin RNA aptamers, which selectively bind dimerized MS2 bacteriophage coat proteins. MS2 coat protein is fused to the p65 subunit of NF-kappaB (NFκB) and the activation domain of human heat-shock factor 1 (HSF1). The guide RNA contains two aptamers, each capable of binding two MS2 coactivator proteins, effectively recruiting four coactivators for every CRISPR targeting activator complex. The dCas9-VP64, MS2-p65-HSF1 helper constructs and gRNA are delivered as three separate plasmids/lentiviral particles (see Figure 1). A detailed protocol for screening and deconvolution can be found in Joung et al., 2017. Additional screening guidelines can be found in Metzakopian et al., 2017.

In contrast to arrayed gRNA libraries containing each clone in a separate plate well, pooled gRNA libraries contain thousands of individual gRNAs in a single tube. The Sigma-Aldrich SAM CRISPR library is provided in 3 pools. Non-targeting (i.e., negative) control gRNAs are included within each pool for use as a baseline in the statistical characterization of changes in gRNA frequency as measured by deep sequencing. Negative control gRNAs share minimal homology with the target genome and should not undergo significant changes in representation throughout control cell treatments. Together, these controls create many options for upfront experimental design and downstream data analysis via MAGeCK, RSA, RIGER, KS-statistics, edgeR, or other methods for qualifying “hits” relevant to drug-gene interactions.

Figure 1.
Vector Maps – Complete SAM CRISPR Lentiviral Plasmid System.

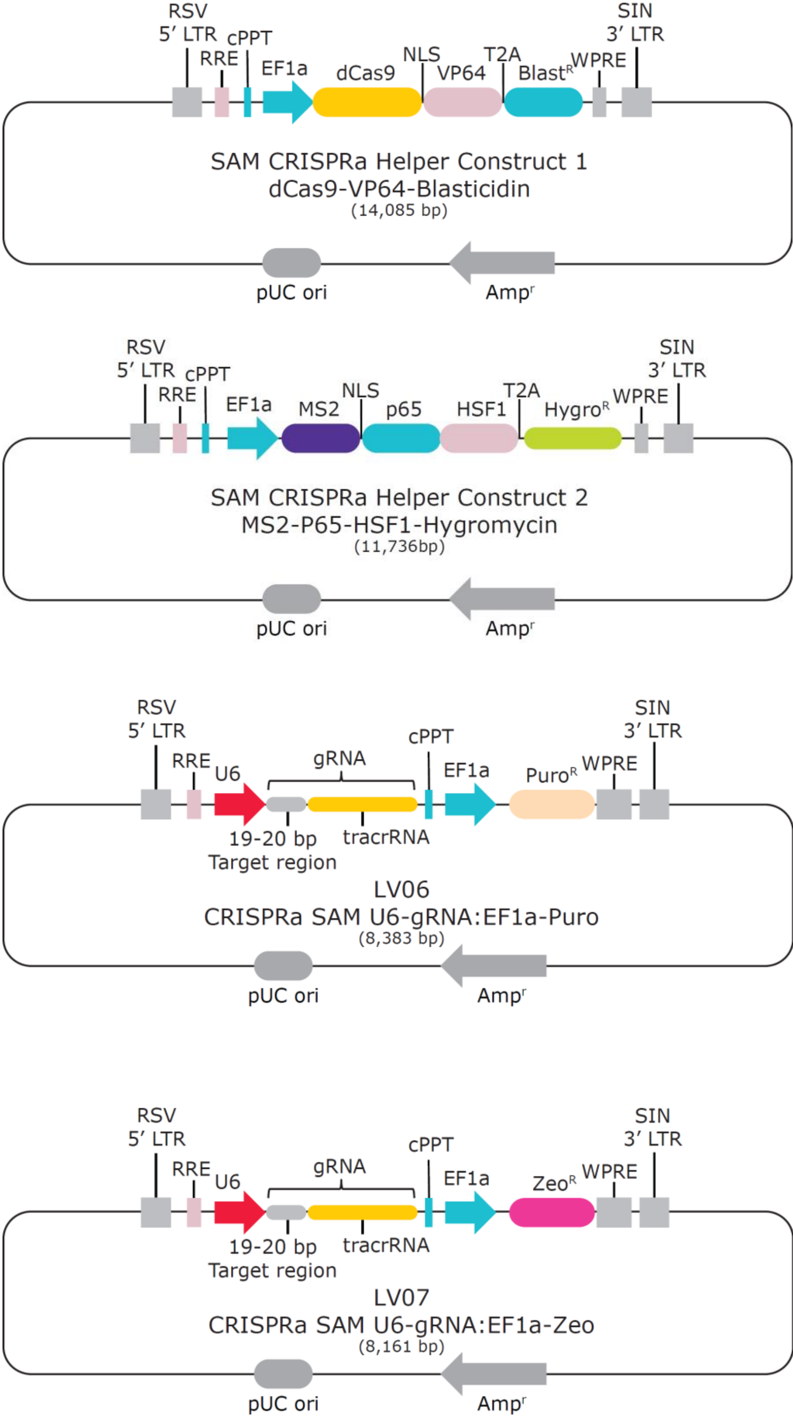

Table 1.
Summary of SAM LentiCRISPR Products

Product Number	Vector Name	Species	Target Class	Total number of gRNAs	dCas9 ?	Selection
HSAMPURO		Human	Whole Genome	70,771	Yes	Puro
MSAMPURO		Human	Whole Genome	70,771	Yes	Zeo
HSAMZEO		Mouse	Whole Genome	69,716	Yes	Puro
MSAMZEO		Mouse	Whole Genome	69,716	Yes	Zeo
CRISPRSAMV	LV06 (Puro) or LV07 (Zeo)	Any	Custom gRNA	1	No	Puro or Zeo
CRISPRSAMD	LV06 (Puro) or LV07 (Zeo)	Any	Custom gRNA	1	No	Puro or Zeo
SAMVP64BST			dCas9-VP64	N/A	Yes	Blast
SAMMS2HYG			MS2-p65-HSF1	N/A	No	Hygro

Components
Product Numbers HSAMPURO, MSAMPURO, HSAMZEO, MSAMZEO
The **Whole Genome Lentiviral CRISPR SAM Pooled Library** was originally designed and assembled by Konermann et al. ["Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex." Nature, 517 (2015)]; it was further modified and optimized by Sigma-Aldrich. One kit contains the following components:

- 3 gRNA sub-pools targeting known human or mouse protein-coding genes* and negative controls**, each provided as 200 μl of lentiviral particles divided into 8 × 25 μl aliquots
- 200 μl EF1a:dCas9-VP64-2A-Blast lentiviral particles, divided into 8 × 25 μl aliquots
- 200 μl EF1a:MS2-p65-HSF1-2A-Hygro lentiviral particles, divided into 8 × 25 μl aliquots

* Each sub-pool contains ~23,500 gRNAs, with 3 gRNAs per RefSeqID. The whole genome library contains ~70,000 gRNAs, in total, targeting 19,000 unique gene symbols (including alternative isoforms encompassing 23,500 RefSeqIDs).

** Each sub-pool contains ~100 negative (non-targeting) controls. Due to the nature of CRISPR activator screening experiments, positive controls must be determined empirically, taking into account the specific phenotype to be explored in the screen.

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices. Though the lentiviral transduction particles produced are replication incompetent, it is recommended that they be treated as **Risk Group Level 2 (RGL-2)** organisms in laboratory handling. Follow all published RGL-2 guidelines for laboratory handling and waste decontamination.

Biosafety Features
Replication incompetence is an important safety feature of Sigma-Aldrich CRISPR Lentiviral Particles. Users should consult and observe their own institutional guidelines when working with viral systems.

Storage and Stability
Upon receipt, immediately store the product at –80 °C. Avoid freeze/thaw cycles, as this will severely reduce transduction efficiency.

Protocol for Lentiviral Transduction and Screening

CRISPR SAM library screening begins with generation of stable dCas9+MS2 SAM helper cells. Next, provided lentiviral gRNA library pools are transduced, followed by a phenotypic enrichment or selection screen. To interpret screen results genomic DNA (gDNA) is then harvested from selected cells, and guides are PCR amplified for Next Generation Sequencing (NGS) analysis. Statistical analysis of screen data will reveal enrichment/depletion of gRNA. Informatics files provided with library may be used to identify corresponding gene targets and to generate candidate gene list. Candidate gRNAs can be transduced individually to further validate screen data.

It is recommended to use cells at low passage number for transduction experiments. Prior to transduction and screening, a kill curve should be established for all required antibiotics in each target cell line and stock used. Lentiviral infection rate, or CFU-based titer, should also be determined for the specific experimental conditions. This preparatory work should take ~2 weeks to complete. The experimental work, or the library screen, should take roughly 2–4 weeks, depending on cell growth rates. It is generally recommended to use BSL2 safety guidelines when working with lentivirus. All contaminated media and supplies should be decontaminated with 10% bleach solution. An overview of the complete experimental protocol is provided in the following sections. For a detailed lentiviral transduction protocol, see:
www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/lentiviraltransdprotocol.pdf

Preparatory Work

1. Perform a 7-day kill curve in each target cell stock for each antibiotic used (see Appendix, entry A).
    - For HSAMPURO or MSAMPURO, the sensitivities to puromycin (puro), blasticidin (blast), and hygromycin (hygro) must be determined.
    - For HSAMZEO or MSAMZEO, the sensitivities to zeo, blast, and hygro must be determined.
2. Determine the lentiviral infection rate (transforming units per ml, TU/ml) for each target cell type (see Appendix, entry B).

Generation of Stable dCas9+MS2 SAM Helper Cells

Day 1 - Seeding Cells

1. Seed 100,000 low passage-number cells in fresh growth medium.
2. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 2 - Transduction

1. Remove growth medium from each well of the plate(s) prepared on day 1.
2. Add 8 μg/ml polybrene-containing medium to each well. Gently swirl the plate to mix.
3. Transduce the cells with both helper constructs: using the equation below, determine and add the appropriate volume of lentiviral particles for each construct. The desired MOI is <0.7. Leave at least one well for selection of non-transduced cells, one well for cells with virus but without selection, and one well for cells without virus or selection agent.

$$\text{Volume of Viral Particles } (\mu l) = 1000\ \mu l \times \frac{\text{Number of Cells} \times \text{Desired MOI}}{\text{Viral Titer } (TU/ml)}$$

4. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 3 - Medium Change

1. Remove the virus-containing medium from each well of the plate(s). Sterilize discarded virus medium and contaminated supplies using a 10% bleach solution for 24 hours.
2. Add fresh growth medium (without polybrene) to each well.
3. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 4 - Dual-Antibiotic Selection

1. Remove the medium from each well.
2. Feed cells with fresh growth medium containing the appropriate concentrations of blasticidin and hygromycin, as determined in the kill curves prior to transduction.

Days 5–10 - Continued Antibiotic Selection

1. Continue to select for transduced cells using concentrations and timelines established in the kill curve prior to transduction.
2. Replace the antibiotic-containing medium as necessary during the selection process (usually every 2 to 3 days). The non-transduced control cells should die completely after 7-day selection with antibiotic.
3. Following selection and subsequent expansion, split selected cells; bank a frozen stock for future culture (CryoStor® cell cryopreservation medium, Product Number C2874), and grow the rest for transduction with the gRNA library. It is not necessary for selected cells to remain under blasticidin and hygromycin selection during the gRNA library screen.

Transduction of the gRNA Library

Day 1 - Seeding Cells

1. Determine the number of cells to seed in each flask using the equation below. The target MOI should be <0.1–0.3 to ensure that the majority of cells integrate a single gRNA cassette. The desired coverage is 300–500× cells per transduced guide. *For example, assuming 24,000 guides per subpool, a coverage of 500×*, and a desired MOI of 0.2, one would require 60,000,000 cells for transduction of 1 subpool.*

   ** Sample calculation based on 500× coverage as described in Konermann et al., 2015 and Joung et al., 2017.*

$$\text{Number of cells} = \frac{\text{Number of Guides} \times \text{Desired Coverage}}{\text{Desired MOI}}$$

2. Seed the appropriate number of cells per flask in fresh growth medium. We recommend seeding enough cells for 2–4 biological replicates per sample from independent library transductions.
3. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 2 - Transduction

1. Remove medium from each flask prepared.
2. Add 8 μg/ml polybrene-containing medium to each flask. Gently rock the flask to mix.
3. Using the equation below, determine and add the appropriate volume of lentiviral particles. *For example, transduction of 60,000,000 cells at an MOI of 0.2 would require 80 μl of virus with a CFU functional titer of 1.5E+08 TU/ml.* Be sure to leave some wells as negative controls for selection, and remember to transduce 2–4 biological replicates for each sample.

$$\text{Volume of Viral Particles } (\mu l) = 1000\ \mu l \times \frac{\text{Number of Cells} \times \text{Desired MOI}}{\text{Viral Titer } (CFU/ml)}$$

4. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 3 - Medium Change

1. Remove the virus-containing medium from each flask.
2. Add fresh medium (without polybrene).
3. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 4 - Antibiotic Selection

1. Remove the medium.
2. Feed cells with fresh growth medium containing the appropriate concentration of puro or zeo, as determined in the kill curve prior to transduction.

Days 5-10 - Continued Antibiotic Selection

1. Continue to select for transduced cells using concentrations and timelines established in the kill curve prior to transduction (4 days for puro or 7 days for zeo).
2. Replace the puromycin-containing medium as necessary during the selection process (usually every 2 to 3 days). The non-transduced control cells should die completely after 4-day selection with antibiotic.
3. Following selection and subsequent expansion, we recommend splitting the cells to bank a frozen stock of selected, transduced cells for future culture (CryoStor cell cryopreservation medium, Product Number C2874). We recommend doing this in case you need to restart the experiment.

Day 11 - Enrichment Screen

1. Expand cells for at least 3 samples at 300–500× coverage.
2. Harvest one group of cells representing time-point zero for guide representation (frozen cell pellet is acceptable). The remaining two samples are cultured for your control and experimental conditions.
   a. Harvest cells using AccuMax™ (Product Number A7089) instead of Trypsin to reduce the carry-over of cell-free DNA.
3. Culture enough cells during each passage to maintain average guide coverage of 300–500×. Perform the enrichment screen using the selection agent or phenotypic assay of choice.

Harvesting Genomic DNA for Screening Analysis

Harvest cells using AccuMax (Product Number A7089) instead of Trypsin to reduce the carry-over of cell-free DNA. Capture genomic DNA for each sample using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Product Number G1N70).

PCR Amplification of gRNA cassettes for NGS

Nested amplification is the preferred method for this type of library generation. Direct amplification from integrated vector sequence in genomic DNA can lead to many nonspecific bands, often close in size to the intended target. Additionally, amplification of cell-free DNA carried over during gDNA harvesting can result in significant NGS read noise. Therefore, an initial outer PCR should be performed using primers detailed in Table 2. While library generation can be accomplished with a single round of amplification, this may require extensive PCR optimization and could lead to wasted reads in the sequencing reaction. Additionally, the length and cost of the final PCR primers that include any NGS platform-specific sequences, phase-shifting diversity sequences, and unique demultiplex barcodes, along with the number of PCR reactions required to consume the harvested unique genomes, would increase the cost of direct amplification step.

On both rounds of PCR, cycle number should be kept to a minimum. This is an important principle, since PCR-introduced errors and biases should be strictly avoided. To compensate for the resulting low yield of PCR amplicons, a large number of PCR reactions should be performed, and the final products combined, purified, and concentrated. Perform enough PCR reactions such that all of the harvested gDNA is amplified. Each 50 μl reaction can hold up to 2.5 μg of gDNA.

Example Methods adapted from Joung et al., 2017 Nature Protocols

1. For all reactions of a single sample, make a single master mix (see Table 3, below) including outer PCR primers (see Table 2, below), template DNA, and JumpStart™ Taq ReadyMix (Product Number P2893).
2. Aliquot reactions into each PCR well and amplify using the cycle conditions detailed in Table 4, below.

Table 2.
Outer PCR Primers (SAM vector specific)

Primer (all per sample)	**Sequence 5′-3′**
NGS-outer-fwd	TAGGAGGCTTGGTAGGTTTAAGAAT
NGS-outer-rev	CTACTATTCTTTCCCCTGCACTGTA

Table 3.
Outer PCR Reaction Mix

Reagent	**1× (multiplied by N reactions)**
2× Jumpstart Taq ReadyMix	25 μl
5 μM NGS-outer-fwd Primer	2.5 μl
5 μM NGS-outer-rev Primer	2.5 μl
gDNA Template	2.5 μg
Water	to volume
	50 μl Reaction Total

Table 4.
Outer PCR Reaction Conditions

95 °C	2 min	
95 °C	30 sec	**25**×
55 °C	30 sec	
72 °C	20 sec	
72 °C	2 min	

3. Purify the 633 bp outer PCR amplicons using GenElute PCR Clean-Up Kit (Product Number NA1020). This will be the template for the inner nested PCR.
4. Perform PCR amplification of the guide cassette from purified PCR amplicons, adapted from the guidelines outlined in Joung et al., 2017 Nature Protocols (Feng Zhang lab).
5. Combine each of the 10 Forward Diversity Primers (see Table 5) for every sample. (e.g., 95 μl of water plus 0.5 μl of each 100 μM fwd primer = 5 μM fwd primer mix).
6. For all reactions of a single sample, make a single master mix (see Table 7), including template DNA, forward primer mix, and corresponding reverse primers (see Table 6).
7. Aliquot reactions into each PCR well and amplify using the conditions detailed in Table 8.

Table 5.
Example Inner PCR Forward Primers (With Diversity Sequences. SAM vector sequence underlined)

Primer (all per sample)	**Sequence 5′-3′**
NGS-Lib-Fwd-1	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTTAAGTAGAGGCTTTATATATCT TGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-2	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTATCATGCTTAGCTTTATATATC TTGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-3	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTGATGCACATCTGCTTTATATAT CTTGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-4	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTCGATTGCTCGACGCTTTATATA TCTTGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-5	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTTCGATAGCAATTCGCTTTATAT ATCTTGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-6	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTATCGATAGTTGCTTGCTTTATA TATCTTGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-7	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTGATCGATCCAGTTAGGCTTTAT ATATCTTGTGGAAAGGACGAAACACC
NGS-Lib-Fwd-8	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTCGATCGATTTGAGCCTGCTTTA TATATCTTGTGGAAAGGACGAAACAC C
NGS-Lib-Fwd-9	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTACGATCGATACACGATCGCTTT ATATATCTTGTGGAAAGGACGAAACA CC
NGS-Lib-Fwd-10	AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTTACGATCGATGGTCCAGAGCTT TATATATCTTGTGGAAAGGACGAAAC ACC

Table 6.
Example Inner PCR Reverse Primers (With Sample Adapters. SAM vector sequence underlined)

Primer (one per sample)	Sequence 5′-3′ (demultiplex barcodes in BOLD)
NGS-Lib-SAM-Rev-1	CAAGCAGAAGACGGCATACGAGAT **TCGCCTTG** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-2	CAAGCAGAAGACGGCATACGAGAT **ATAGCGTC** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-3	CAAGCAGAAGACGGCATACGAGAT **GAAGAAGT** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-4	CAAGCAGAAGACGGCATACGAGAT **ATTCTAGG** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-5	CAAGCAGAAGACGGCATACGAGAT **CGTTACCA** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-6	CAAGCAGAAGACGGCATACGAGAT **GTCTGATG** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-7	CAAGCAGAAGACGGCATACGAGAT **TTACGCAC** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT
NGS-Lib-SAM-Rev-8	CAAGCAGAAGACGGCATACGAGAT **TTGAATAG** GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT

Table 7.
Inner PCR reaction mix

Reagent	1×
2× Jumpstart Taq ReadyMix	25 μl
5 μM Fwd Primer Mix	2.5 μl
5 μM Rev Primer	2.5 μl
Purified outer PCR product	100 ng
Water	to volume
	50 μl Reaction Total

Table 8.
Inner PCR reaction conditions

95 °C	2 min	
95 °C	30 sec	
55 °C	30 sec	**20×**
72 °C	20 sec	
72 °C	2 min	

8. Purify the 270–280 bp inner PCR amplicons using GenElute PCR Clean-Up Kit (Product Number NA1020). Quantify the PCR product and run 2 μg of product on a 2% (wt/vol) agarose gel. Perform gel extraction of the proper band using GenElute Gel Extraction Kit (Product Number NA1111). Elute in nuclease-free water (Product Number W4502). Quantify the DNA.
9. Follow the NGS sequencing preparation and data deconvolution guidelines outlined by Joung et al., 2017 Nature Protocols, or by the manufacturer's protocol specific to your NGS platform.

Validation of Candidate Genes for Screening Phenotype

Transduce gRNA constructs for each candidate gene or gRNA to be validated from the 'top hits' determined by statistical analysis of the screen data. A parsimonious candidate list will give priority to genes that show a similar trend for 2–3 guides per gene. Activation of expression is reported by Konermann et al., 2015 to maximize around 4 days post transduction.

Day 1 - Seeding Cells

1. Seed 100,000 stable CRISPR SAM helper cells in fresh medium.
2. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 2 - Transduction

1. Remove the medium from each well of the plate(s) prepared on Day 1.
2. Add 8 μg/ml polybrene-containing medium to each well. Gently swirl the plate to mix.
3. Transduce the cells with a single guide construct: using the equation below, determine and add the appropriate volume of lentiviral particles for each construct. The desired MOI is <0.5. Leave at least one well for selection of non-transduced cells, one well for cells with virus but without selection, and one well for cells without virus or selection agent.

$$Volume\ of\ Viral\ Particles\ (\mu l) = 1000\ \mu l \times \frac{Number\ of\ Cells \times Desired\ MOI}{Viral\ Titer\ (CFU/ml)}$$

4. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 3 - Medium Change

1. Remove the virus-containing medium from each well of the plate(s). Sterilize discarded virus medium and contaminated supplies using a 10% bleach solution for 24 hours.
2. Add fresh growth medium (without polybrene) to each well.
3. Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 4 - Antibiotic Selection

1. Remove the medium from each well.
2. Feed cells with fresh growth medium containing the appropriate concentrations of either puro or zeo, as determined in the kill curves prior to transduction.

Days 5 - 10 - Continued Antibiotic Selection

1. Continue to select for transduced cells using concentrations and timelines determined in the kill curve (4 days for puro or 7 days for zeo).
2. Replace the antibiotic-containing medium as necessary during the selection process (usually every 2 to 3 days). The non-transduced control cells should die completely after 4- or 7-day selection with antibiotic.
3. Following selection and subsequent expansion, split selected cells; bank a frozen stock for future culture (optional, CryoStor cell cryopreservation medium, Product Number C2874), and grow the rest for screen validation. It is not necessary for selected cells to remain under antibiotic selection during validation. Continue culturing cells until desired number are present for gene expression or phenotypic analysis.

Days 10+ - Screen Validation

1. Subject cells to enrichment screen to validate survival/death/expression/phenotype.

Analysis of Gene Expression

1. Harvest cells using AccuMax (Product Number A7089) instead of Trypsin to reduce the carry-over of cell-free DNA.
2. Purify total RNA from selected cells using GenElute Mammalian Total RNA Miniprep Kit (Product Number RTN70). Elute in water.
3. Perform qRT-PCR analysis to determine relative gene expression changes compared to controls, using Quantitative RT-PCR ReadyMix™ (Product Number QR0200).

Troubleshooting Guide

Problem	Cause	Solution
Low transduction efficiency	Cell density was not optimized: too low or too high.	To determine optimal density and transduction efficiency of tested cells, first use one aliquot of lentivirus included in the kit (start with one of the helper constructs). When adding cells to the wells make sure they are mixed well. Keep mixing them periodically to ensure uniform number of cells in each well.
	Cell culture conditions were not optimized.	Use low passage number cells. Confirm that cells are 90% viable upon transduction.
	Cells are sensitive to polybrene.	Test cells for sensitivity by including control cells incubated with medium containing polybrene (8 μg/ml). If cells are sensitive to polybrene, omit this reagent. Cells can still be transduced but with lower efficiency.
	Polybrene was not included during transduction.	Transduce in the presence of polybrene when possible since it enhances the transduction efficiency of most cell types.
	Selection concentration is not optimal for cell selection.	For each new cell type used, it is recommended to perform a selection agent kill curve to determine the lowest concentration of antibiotic needed to efficiently select transduced cells.
Too many guides mapped in NGS results (High NGS noise)	Carryover of cell-free DNA	To reduce the amount of cell-free genomic DNA carried over from dead cells, passage and harvest cells using AccuMax or another Trypsin-alternative possessing DNase activity.
		Perform a nested PCR on harvested gDNA to prevent amplification of guides in cell-free DNA protected by a nucleosome.

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APPENDIX

A. Puromycin Titration (Kill Curve) - *Preparatory*

The sensitivity of a cell to a given antibiotic varies not only for each cell type, but for each cell stock and passage number. To determine the appropriate antibiotic concentrations for a library screen, a titration experiment or kill curve, must be performed for each target cell stock and each antibiotic used. For HSAMPURO or MSAMPURO, the sensitivities to puro, blast, and hygro must be determined. For HSAMZEO or MSAMZEO, the sensitivities to zeo, blast, and hygro must be determined. The protocol below details the materials and methods for a typical puro titration.

Note: The example protocols below use puromycin. If using HSAMZEO or MSAMZEO, please substitute zeo in place of puro and refer to zeo manufacturer's instructions.

Materials Required

- Target cells in culture
- Puromycin, 10 mg/ml solution (Product Number P9620)
- Blasticidin S (Product Number 15205)
- Hygromycin B, 45-60 mg/ml solution (Product Number H0654)
- Complete growth medium specific for target cells
- Hank's Balanced Salt Solution (Product Number H6648)
- 1× Trypsin-EDTA solution (Product Number T3924)

Methods

1. Plate 2×10^4 cells per well in one 24 well plate with 0.5 ml fresh medium; incubate overnight.
2. On the next day, test a range of concentrations from 0.5-10 μg/ml, in 0.5 μg/ml increments and add the corresponding volume of puro to each well of this plate following the planned layout. Leave one well as a negative control (no puro).
3. Examine the viability of all wells every 2 days.
4. Culture for 3–7 days, depending on the growth rate of the cell type and expected length of time that cells will be under selection during an experiment. Replace the medium containing puromycin every 2–3 days. The minimum concentration of puromycin that causes complete cell death after 4 days of treatment should be used for that cell type and experiment.

Note: Excess antibiotic can cause undesired phenotypic responses in many cell types. Always use the lowest dose that results in 100% cell death at the end of the time-course.

B. Determination of Infection Rates (TU/ml) - *Preparatory*

Lentiviral infection rates are affected by many factors, including target cell characteristics; it is important to determine the infection rate for every target cell type and stock used. The infection rate is equal to the number of Colony Formation Units (CFU) that results from transducing 1 ml of a control lentivirus sample (CFU = transforming units per ml), TU/ml); it is highly recommended that the control lentivirus be in the same vector as that used in the actual experiment. This infection rate, or lentiviral titer, can be used to calculate the number of transducing lentiviral particles per cell, or the Multiplicity of Infection (MOI). Ultimately, the titer and MOI will be used to calculate the volume of lentivirus to use in transduction for each experimental.

Note: Lentiviruses are quite labile. Multiple freeze-thaw cycles and prolonged exposure to ambient temperatures will decrease the lentiviral titer.

Materials Required

- Target cells in culture
- 2 mg/ml Hexadimethrine bromide (Polybrene, Product Number H9268)
- 10 mg/ml Puromycin (Product Number P9620)
- Crystal Violet Solution (Product Number HT90132)
- Dulbecco's Phosphate Buffered Saline (Product Number D8662)

Methods

Day 1 - Seeding Cells

1. Seed 10^5 cells per well in fresh medium on three 6 well plates. It is recommended to use cells at low passage number for transduction experiments.
2. Place the plates in an incubator set to 37 °C with 5% CO_2 for 24 hours.

Day 2 Transduction

1. Thaw one vial of SAM library lentiviral particles (25 μl) on ice. Mix by gently tapping the tube several times. Keep them stored on ice when not in use.
2. Prepare 25 ml of 8 μg/ml polybrene-containing complete growth medium.
3. Prepare 2.5 ml 10-fold serial dilutions of virus over a range of 10^{-2} to 10^{-9} in 15 ml conical vials; use steps 3a–h, below, as a guide. Mix gently by inverting the tubes 3 to 4 times.
   a. Mix 25 μl of lentivirus with 2,475 μl polybrene-containing medium to achieve a 10^{-2} dilution.
   b. Mix 250 μl of 10^{-2} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-3} dilution.
   c. Mix 250 μl of 10^{-3} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-4} dilution.
   d. Mix 250 μl of 10^{-4} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-5} dilution.
   e. Mix 250 μl of 10^{-5} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-6} dilution.
   f. Mix 250 μl of 10^{-6} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-7} dilution.
   g. Mix 250 μl of 10^{-7} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-8} dilution.
   h. Mix 250 μl of 10^{-8} dilution with 2,250 μl of polybrene-containing medium to achieve a 10^{-9} dilution.
4. Remove the medium from each well of the three 6 well plates prepared on Day 1.
5. Add 1.0 ml of polybrene-containing medium to two wells on one 6 well plate - these will be used as negative controls.
6. Add 1.0 ml of each of the lentivirus dilutions to the remaining wells of the three plates. Duplicate the transduction for each lentiviral dilution.
7. Place plates in an incubator set to 37 °C with 5% CO_2 for 24 hours

Day 3 - Medium Change

1. Remove the medium containing the lentiviral particles from each well.
2. Add 2.0 ml of fresh medium (without polybrene) to each well.
3. Place the plates in an incubator set to 37 °C with 5% CO_2 for 24 hours

Day 4 - Antibiotic Selection

1. Remove the medium from each well.
2. Feed cells with fresh medium containing the appropriate concentration of puromycin determined in the puromycin kill curve.

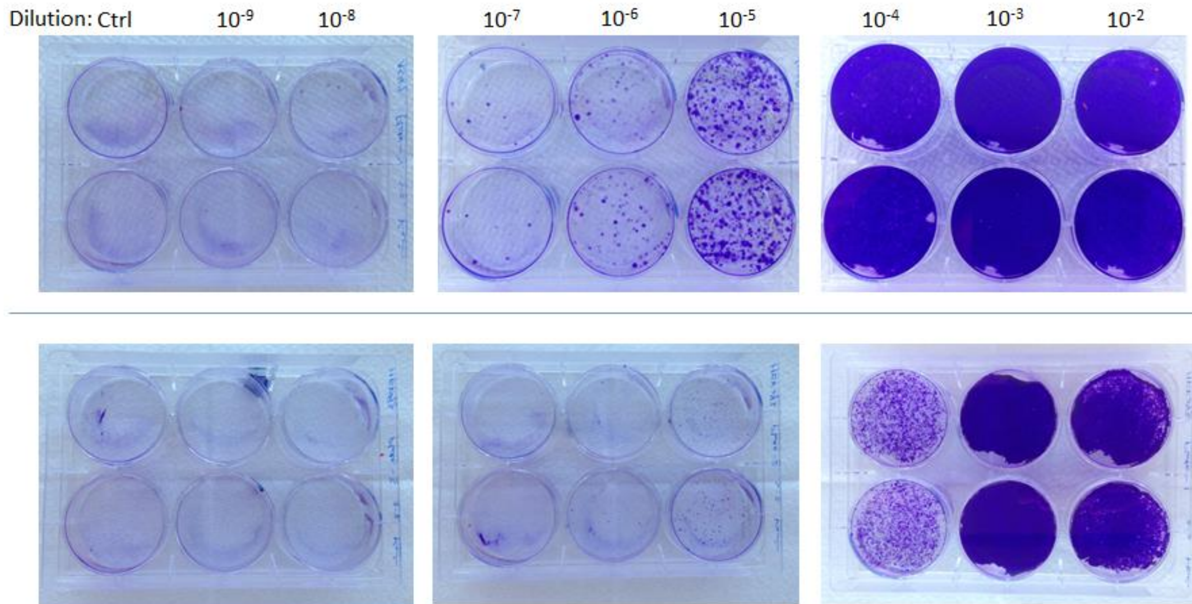
Days 5–8 - Continued Antibiotic Selection

1. Observe each well for cell death daily.
2. Replace the puromycin-containing medium as necessary during the selection process (usually every 2 to 3 days). The non-transduced control cells should die completely after 4-day selection with antibiotic.

Day 9 - Staining and Counting Colonies

1. Remove the medium and gently wash each well with 3.0 ml of Dulbecco's phosphate-buffered saline (PBS).
2. Add 1.0 ml of crystal violet solution per well and incubate 10 minutes at room temperature.
3. Remove the crystal violet solution and wash each well twice with 3.0 ml of PBS per wash.
4. Invert the plates on paper towels and let them dry for about 1 hour.
5. Count the blue-stained colonies (see Figure 2) using a microscope at a magnification of 40×. Only count wells that contain 10–300 colonies; any counts with more or less are not significant.
6. Calculate the infection rate by multiplying the number of colonies per well by the dilution factor. This value is the lentiviral titer, given in units of TU/ml.

Figure 2.
Example data for colony staining and counting to determine lentiviral TU/ml based on puromycin resistance.



CFU count data

Plate Replicate	Dilution Factor	# colonies	Titer	Average	Stdev
1	1.00E+02	DNC	DNC	1.50E+08	5.59E+07
	1.00E+03	DNC	DNC		
	1.00E+04	DNC	DNC		
	1.00E+05	DNC	DNC		
	1.00E+06	189	1.89E+08		
	1.00E+07	11	1.10E+08		
	1.00E+08	DNC	DNC		
	1.00E+09	DNC	DNC		
2	1.00E+02	DNC	DNC	1.84E+08	4.95E+06
	1.00E+03	DNC	DNC		
	1.00E+04	DNC	DNC		
	1.00E+05	DNC	DNC		
	1.00E+06	187	1.87E+08		
	1.00E+07	18	1.80E+08		
	1.00E+08	DNC	DNC		
	1.00E+09	DNC	DNC		
		plates 1 and 2 combined		1.67E+08	3.786E+07

Note: If there are too many or too few colonies to count in a given well, record DNC in the # colonies field.

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