VectorBuilder

User Instructions:

Pooled Lentivirus Libraries for In Vitro Screening

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Overview of Pooled Lentivirus Library

Pooled Library-mediated Genetic Screening

Pooled lentivirus libraries are powerful and cost-effective tools for conducting large-scale functional screens of genes or genetic elements. Shown in **Figure 1** is a typical workflow of CRISPR knockout screens employing pooled gRNA lentivirus library. First, Cas9-expressing cells of interest are transduced with the lentivirus library and the positively transduced cells are selected based on marker gene(s) (e.g. drug-selection or fluorescence marker) carried by lentiviral vectors. These positively transduced cells are then divided into two groups: a reference population and an experimental population. The experimental population is subjected to particular selective pressure (e.g. drug treatment or repeated passaging) to screen for cells with the desired phenotype. After screening, cells from both experimental and reference groups are harvested, and the enriched or depleted gRNAs in the experimental group relative to the reference group is identified using Sanger sequencing or next-generation sequencing (NGS). Candidate genes that are theoretically targeted by the enriched or depleted gRNAs can be further investigated through downstream functional studies.

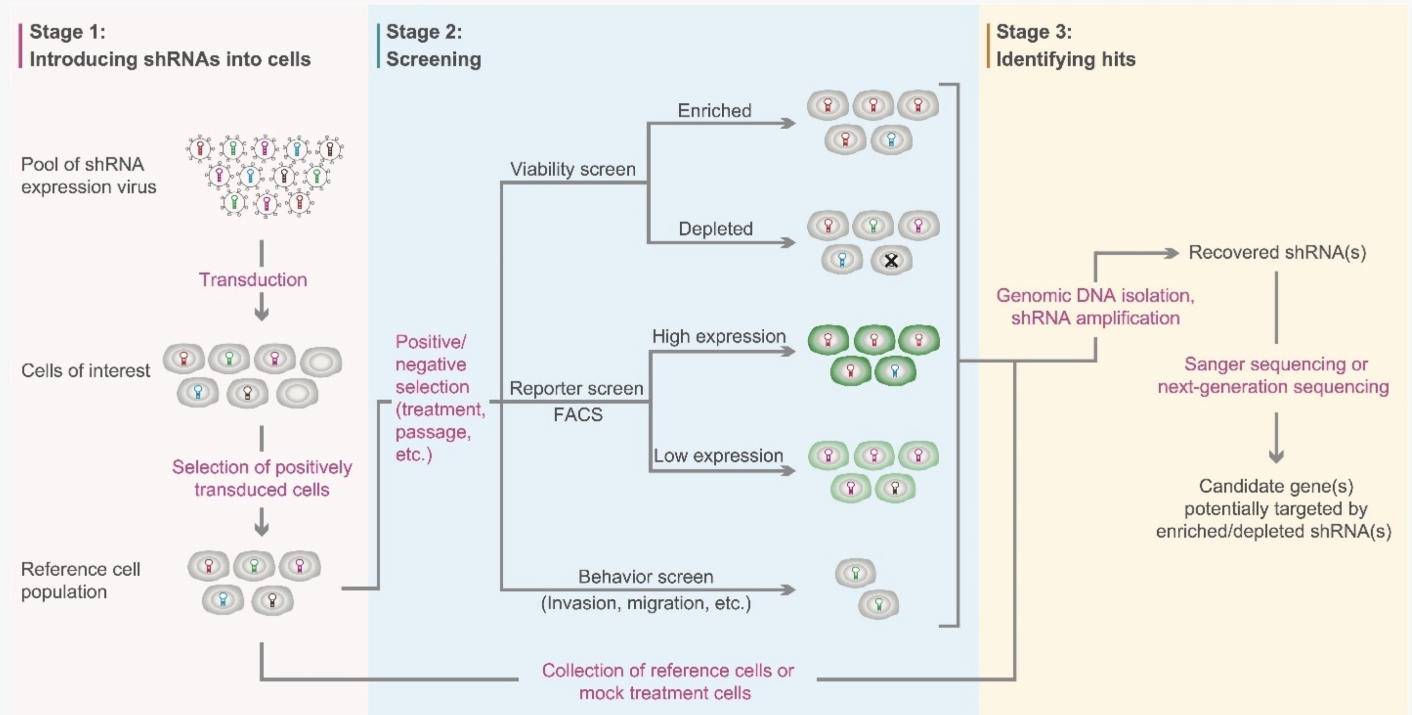

Figure 1. Workflow of CRISPR-based knockout screens employing pooled gRNA lentivirus library. Adapted from Acta Biochim Biophys Sin 44:103-112 (2012).

VectorBuilder offers high-quality pooled lentivirus libraries including dual-gRNA, single-gRNA, and shRNA libraries, available in various scales. These versatile and cost-effective libraries are valuable tools for studying a wide range of biological processes. For more information about our library services and premade library products, please visit our Library Screening page.

Lentivirus Library Backbone

Our libraries are constructed using validated lentivirus backbones designed to achieve the best outcome of screening. **Figure 2** shows two typical designs of lentiviral vectors utilized in our premade dual-gRNA and shRNA libraries.

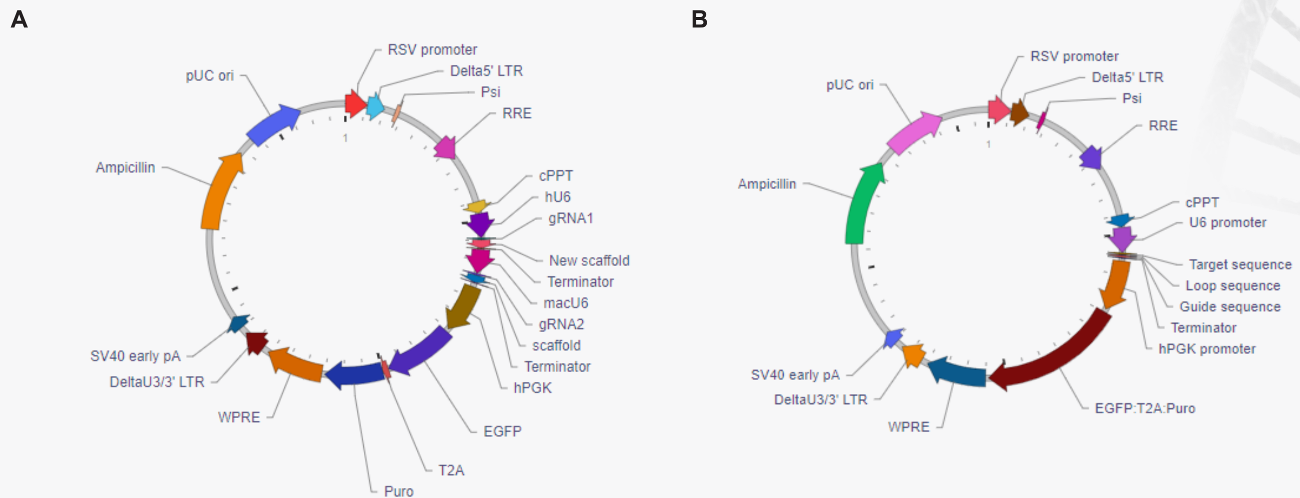

Figure 2. Maps of the dual-gRNA and shRNA library lentiviral vector.

(A) Dual-gRNA library lentiviral vector. The vector includes a pair of gRNA expression cassettes and an EGFP/puromycin dual marker. When introduced into cells, the vectors simultaneously express the paired gRNAs, targeting two separate sites on the same gene. This results in two CRISPR cut sites, leading to large loss-of-function deletions for knockout screens. The two gRNAs in each vector are driven by two different U6 promoters (human U6 and macaque U6) and contain two different gRNA scaffolds (described in Nat Methods. 14:573 (2017)) that are distinct in sequence but equivalent in function. This design reduces unwanted recombination between the two gRNA cassettes and allows PCR amplification and sequencing by NGS of either the upstream or the downstream gRNA, or both, from cells transduced with the library. (B) shRNA library lentiviral vector. The vector contains a shRNA expression cassette and an EGFP/puromycin dual marker. The shRNA is driven by a human U6 promoter, which is a highly efficient system for the stable knockdown of target gene expression across a wide variety of cell types.

Pooled Lentivirus Libraries Offered by VectorBuilder

Deliverable

- **Lentivirus (recommended):** This is the default deliverable for premade libraries and can be purchased when ordering custom library construction. Common scales of the lentivirus libraries are shown in the table below. More lentivirus packaging scales can be found here.

Table 1. Scale and titer for pooled lentivirus libraries.

Scale	Application	Titer	Volume
Medium	Cell culture & In vivo	>10^8 TU/ml	1 ml (10x100 ul)
Plus	Cell culture & In vivo	>10^8 TU/ml	5 ml (50x100 ul)

- **E. coli glycerol stock:** Glycerol stocks are always provided upon ordering custom library construction.
- **Plasmid DNA:** Various scales of plasmid DNA are available for purchase upon ordering custom library construction.

Off-the-shelf Libraries

- **Whole-genome dual-gRNA CRISPR knockout libraries:**

Product Name	No. of Genes	No. of gRNA pairs
Human Whole-Genome Dual-gRNA Library	20,048	91,926
Mouse Whole-Genome Dual-gRNA Library	20,493	90,344

We also offer premade whole-genome single-gRNA CRISPR knockout libraries, whole-genome CRISPRa libraries, whole-genome CRISPRi libraries, and pathway-specific CRISPR knockout libraries. Please visit our CRISPR Libraries page for more information.

- **Whole-genome shRNA libraries:**

Product Name	No. of Genes	No. of shRNAs
Human Whole-Genome Pooled shRNA Library	20,593	105,233
Mouse Whole-Genome Pooled shRNA Library	22,023	105,170
Human Elite Gene Pooled shRNA Library	2,161	12,471
Mouse Elite Gene Pooled shRNA Library	2,233	12,472

For more information, please visit our Pooled shRNA Libraries page.

Storage and Handling

- **Pooled lentivirus:** Our lentivirus is stored in HBSS buffer and is shipped on dry ice. Upon receipt, it should be stored at -80°C for long term (stable for at least 6 months), or -20°C for short-term usage within one week. The shelf life of the lentivirus is approximately one year.

  CAUTION: Repeated freeze-thaw cycles of lentivirus should be avoided, as this can cause a large titer drop.

  Safety precautions: All lentiviruses from VectorBuilder are “self-inactivating”, meaning that they cannot replicate within target cells or infect other cells. This is because the genes responsible for replication have been deleted from the viral genome. However, the virus can, in theory, pose a biohazard risk because it can transduce primary human cells. **We recommend handling the virus according to Biosafety Level 2 (BSL-2) criteria.** All handling, storage, and disposal of biohazard waste must be in accordance with published and institutional criteria.

- **E. coli glycerol stock:** Upon receipt, the E. coli glycerol stocks should be kept at -80°C for long-term storage.

  - Prepare new glycerol stocks: After thawing, add one or multiple vials of the glycerol stocks to a freshly prepared antibiotic-containing LB medium at a volume ratio of 1:40 to 1:50. Shake at 37°C until the optical density (OD) reaches ~1.2. Gently mix the culture with an equal volume of 30% glycerol (15% final glycerol concentration) for long-term storage at -80°C. We recommend preparing multiple glycerol stocks with large volumes (e.g., 10 ml, 40 ml) for storage to avoid repeated amplification.

  - Inoculation and amplification: After thawing, add **one or multiple vials of the glycerol stocks** to a freshly prepared antibiotic-containing LB medium at a volume ratio of 1:40 to 1:50. Shake at 37°C until the OD reaches 1.4-1.6 for plasmid harvest.

    Note:

    a. **To maintain the complexity of the library, always inoculate at least a full vial of the glycerol stock.**

    b. **Harvest the plasmid when E. coli growth is still in the log phase. Overgrowth of E. coli will result in poor uniformity of the library.**

    c. **To avoid skewing the representation of each variant, minimize the number of subculture passages during amplification.**

- **Pooled plasmid DNA:** Our plasmid DNA is stored in 1x TE buffer. Upon receipt, spin the tube at >10,000 x g for 10-30 s. Plasmid DNA can be stored at 4 °C for short term (≤2 weeks). For long-term storage, please store the plasmid DNA at -20°C or -80°C.

  Note: To avoid losing the complexity and uniformity of the library, we do not recommend that you re-transform the plasmid pool.

Protocol for In Vitro Screening Using Pooled Lentivirus Library

This protocol provides comprehensive instructions for optimizing conditions, executing the screening, and preparing samples for subsequent NGS. Before conducting a screening with our pooled lentivirus libraries, we strongly recommend determining the antibiotic kill curve for your cells of interest, calculating the functional titer of the virus for your cells of interest, and calculating the number of cells and lentiviral particles required for transduction. After screening, genomic DNA is isolated, and PCR is performed to prepare samples for NGS analysis.

Note: **This document serves as a guide for preparing and conducting your screens using VectorBuilder's pooled lentivirus libraries. Further refinement and optimization are necessary for different screens and cell types. This guide should be adjusted to your system's optimal transduction conditions, plating density, etc.**

I. Determine the Antibiotic Kill Curve

Different cell types exhibit distinct intrinsic resistance to antibiotics. To achieve a successful screen, it is critical to determine the optimal concentration of the selection agent (antibiotic) for each cell type by conducting a kill curve assay on the cells of interest. In this protocol, we use puromycin as an example.

1. Day before drug treatment (Day 0)
   - Plate target cells in the appropriate medium so that they will be 25-30% confluent at the time of antibiotic treatment. Incubate for 18-20 hours at 37°C in a humidified 5% CO_2 incubator.
2. Day of drug treatment (Day 1)
   - Replace the cell culture media with fresh medium containing gradient concentrations of puromycin. We recommend using concentrations of 0, 0.2, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, and 14 ug/ml for puromycin. We suggest having duplicated wells for each concentration.
3. Day 3 and onward
   - Refresh the puromycin-containing medium for each well.
   - Regularly monitor cell viability under a microscope.
4. Day 5 or longer
   - Determine the percentage of surviving cells compared to the untreated control (0 ug/ml puromycin). Assays including crystal violet staining, cell counting, flow cytometry, or MTT assay can be conducted.
   - Select the lowest puromycin concentration that completely kills your cells as the 'optimal' antibiotic selection concentration for your screen (see point b in the **Note** below for more information).

Note:

a. **Avoid stringent selection by choosing the lowest antibiotic concentration is critical to prevent any bias towards cells containing multiple perturbations.**

b. **After virus transduction, certain cell lines may exhibit varying degrees of sensitivity to the selection agent. It is advisable to select a concentration one level lower than the determined 'optimal' concentration for use in the actual experiment.**

c. **When conducting antibiotic selection, it is essential to account for any pre-existing drug resistance of the cells. For example, a cell line stably expressing Cas9 established prior to the CRISPR screen may have a certain type of drug resistance introduced together with Cas9. Another example is that HEK293T cells have a neomycin resistance due to the introduction of the large T antigen.**

II. Determine the Functional Titer

We recommend assessing the functional titer of the lentivirus library before transduction, as transduction efficiency can vary across different cell lines and conditions. Accurate titration of the library is crucial for correctly calculating the number of cells and viruses needed for screening.

For pooled lentivirus libraries, the functional titer can be determined using various methods based on the library's vector components. If the vector contains a fluorescent marker, fluorescent microscopy or flow cytometry can be employed. For vectors with a drug selection marker, antibiotic selection is applicable. Additionally, the qPCR-based method is suitable for all lentiviruses. All the above mentioned approaches can be applied to VectorBuilder's pooled shRNA or dual-gRNA CRISPR libraries.

1. Day before transduction (Day 0)
    - Plate the target cells in the appropriate culture medium and plates. Incubate for 18-20 hours at 37°C in a humidified 5% CO_2 incubator. For example, when using HEK293T cells, we recommend plating 1 x 10^5 cells per well in a 12-well plate.
2. Day of transduction (Day 1)
    - Prepare a series of lentivirus dilutions with polybrene in sterile microcentrifuge tubes. Prepare a medium containing polybrene without the virus as a control. We recommend having duplicate wells for each dilution, starting with MOIs of 0.2, 0.4, 0.8, 1.5, and 2. The volume of lentivirus can be calculated based on the physical titer provided by VectorBuilder.
    - Remove the growth media from the cell culture and replace it with dilutions of lentivirus and polybrene, including the no virus control.
3. Day 2
    - Remove the virus-containing medium and replace it with fresh complete culture medium. Incubate at 37°C in a humidified 5% CO_2 incubator overnight.

    Note:

    a. **Start transducing cells with the amount of lentivirus recommended above if the cells are readily transduced. For some cell lines, a larger volume may be needed.**

    b. **Lentivirus transduction in some cell types can be enhanced by Polybrene, though it is not necessary for most cell types. Polybrene can be harmful to some cells. If you choose to use Polybrene, you may need to first test your cells for sensitivity to Polybrene at 1-10 ug/ml. The recommended concentration is 5 ug/ml for most cell types.**

4. Day 3

- **Fluorescent microscopy:**
    - Determine the percentage of EGFP-positive cells:

$$\text{Percentage of EGFP-positive cells} = \frac{\text{Number of EGFP-positive cells}}{\text{Total cells}} \times 100$$

    - Determine the corresponding MOI for a specific percentage of EGFP-positive cells using **Table 2** and calculate the functional titer.

$$\text{Functional titer (TU/ml)} = \frac{\text{Total cells at transduction x MOI}}{\text{Virus volume (ml)}}$$

    - Take the average functional titer from multiple dilutions within the linear range for a more accurate titer.
- **Flow cytometry:**
    - Determine the percentage of EGFP-positive cells.
    - Determine the corresponding MOI for a specific percentage of EGFP-positive cells using **Table 2** and calculate the functional titer.

$$\text{Functional titer (TU/ml)} = \frac{\text{Total cells at transduction x MOI}}{\text{Virus volume (ml)}}$$

    - Take the average functional titer with multiple dilutions within the linear range to obtain a more accurate titer.
- **Antibiotic selection (puromycin as an example) :**
    - Refresh the medium with complete culture medium containing the previously determined concentration of puromycin. No virus control wells should be replaced with media without puromycin.
    - Day 4 and onward
        - Refresh the puromycin-containing medium for each well. Regularly monitor cell viability under a microscope.
    - Day 6 or longer (depends on the specific antibiotic used)
        - Determine the survival rate compared to the no virus, untreated control groups using assays including crystal violet staining, cell counting, flow cytometry, or MTT assay.
        - Determine the corresponding MOI at a certain survival rate (% of positive cells) using **Table 2** and calculate the functional titer.

$$\text{Functional Titer (TU/ml)} = \frac{\text{Total cells at transduction x MOI}}{\text{Virus volume (ml)}}$$

- Take the average functional titer from multiple dilutions within the linear range to obtain a more accurate titer.

Note:

a. Determining the functional titer using the expression of a fluorescence marker or antibiotic selection gene is a stringent method, as their expression can be influenced by several factors, such as the promoter strength, the marker sensitivity, and the vector's structure and size. Consequently, these methods may underestimate the functional titer.

b. The number of EGFP-positive or surviving cells does not necessarily correspond to the number of transduction units (TU). In other words, the percentage of EGFP-positive cells or the survival rate does not always equal to the multiplicity of infection (MOI). When the MOI is low, these two numbers are approximately equal. However, at high MOIs, the likelihood of multiple integrations per cell increases. Therefore, for more accurate titer determination, please refer to Table 2 to find the corresponding MOI at a certain percentage of positively transduced cells.

Table 2. The relationship between the percentage of positively transduced cells and MOI.

% of positive cells	5	10	20	30	40	50	60	70	80	90	95
MOI	0.05	0.10	0.23	0.36	0.51	0.69	0.93	1.22	1.64	2.30	3.00

- **qPCR:**
  - Extract genomic DNA using commercially available genomic DNA extraction kits, following the manufacturer's instructions.

    Note: Ensure that the number of cells processed per purification is compatible with the kit's capacity. We recommend performing pilot testing if necessary.
  - Prepare the qPCR reaction mix, including the extracted genomic DNA and primers.

    Note: Two sets of primers are required: one set targets the unique region in the lentiviral genome, and the other pair targets a region in the cellular genome, such as a housekeeping/reference gene.
  - Conduct qPCR under optimal conditions for template and the primers to determine the Ct value for each group.
  - Use reference DNA containing binding sites for both sets of primers to generate a standard curve correlating Ct values with copy numbers.
  - Calculate the copy number of each group based on their respective Ct values based on the standard curve.
  - Determine the copy ratio and functional titer for each sample.

$$\text{Copy ratio} = \frac{\text{Lentiviral genome copy number}}{\text{Cellular genome copy number}}$$

$$\text{Functional Titer (TU/ml)} = \frac{\text{Total cells at transduction x Copy ratio}}{\text{Virus volume (ml)}}$$

- Take the average functional titer from multiple dilutions within the linear range for a more accurate titer.

III. Calculate the Number of Cells and Lentivirus Particles for Transduction

To calculate the number of cells and lentivirus particles required for the screen, the number of biological replicates, fold coverage of the library, and an MOI of transduction need to be determined first.

For pooled genetic screenings, it is recommended to have at least two biological replicates and maintain 100- to 500-fold coverage per replicate of your library. While higher fold coverage is associated with better representation of each variant, it also requires a significantly larger number of cells and virus particles. Achieving a balance between data reliability and resource availability is crucial to ensure the feasibility of the screening. In negative screens with a strong selection pressure, a higher fold coverage is recommended to maintain better gRNA or shRNA representation for essential genes.

An appropriate MOI is selected to minimize the number of cells with multiple integrations into their genome. **Table 3** below demonstrates the percentage of cells with a certain number of lentiviral integrations at different MOIs. An MOI of 0.3 is typically recommended for pooled screens. At an MOI of 0.3, the Poisson distribution predicts that 22% of the cells will have a single lentiviral integration, while only 3% of cells are expected to have more than one lentiviral integration. In cases where screens are constrained by limited material, such as the number of cells available, transduction can be conducted with a slightly higher MOI to reduce the required cell count.

Table 3. Poisson distribution illustrating lentiviral integration in cells at different MOIs.

MOI	Number of lentiviral integration per cell				
	0	1	2	3	4
0.1	90%	9%	0%	0%	0%
0.2	82%	16%	2%	0%	0%
0.3	74%	22%	3%	0%	0%
0.4	68%	27%	5%	1%	0%
0.5	61%	30%	8%	1%	0%
0.6	55%	33%	10%	2%	0%
0.7	50%	35%	12%	3%	0%
0.8	45%	36%	14%	4%	1%
0.9	41%	37%	16%	5%	1%
1.0	37%	37%	18%	5%	2%

Number of cells needed in the screen can be determined using the following formula:

(Number of cells needed at MOI = 1) = Number of biological replicates x Number of variants x Desired coverage

$$(\text{Number of cells needed at MOI} = \chi) = \frac{\text{Number of cells needed at MOI=1}}{\text{Percentage of cells with a single integration at MOI} = \chi}$$

Volume of lentivirus needed (ml):

$$(\text{Number of TU needed at MOI} = \chi) = \frac{\text{Number of cells needed at MOI} = \chi}{\text{MOI}}$$

$$(\text{Volume of lentivirus needed at MOI} = \chi)\ (\text{ml}) = \frac{(\text{Number of TU needed at MOI} = \chi)}{\text{Functional Titer (TU/ml)}}$$

Case study

For VectorBuilder's Human Whole-Genome Dual-gRNA Lentivirus Library containing 91,926 gRNA pairs, how many cells do I need for a 500-fold coverage at MOI = 0.3 in one replicate?

91,926 gRNA pairs x 500-fold coverage = 45,963,000 cells needed for screening at MOI = 1

45,963,000 cells / 22% = 208,672,020 cells needed for screening at MOI = 0.3

Note:

a. **The calculation above is for one replicate. It is recommended to have at least two biological replicates.**

b. **A 15 cm^2 tissue culture plate or a T-75 flask holds approximately $2x10^7$ cells at confluency, so more than 10 plates will be needed for one replicate.**

IV. Conduct Screening

VectorBuilder offers a wide variety of screening services, including CRISPR, shRNA, mutation, and enhancer/promoter libraries screenings. Request design support now to get a free consultation.

Transduce target cells

1. Day before transduction (Day 0)
    - Plate target cells in the appropriate medium so that they will be 30-50% confluent at the time of transduction. Incubate for 18-20 hours at 37°C in a humidified 5% CO_2 incubator.
2. Day of transduction (Day 1)
    - Remove the growth medium from the cell culture and replace it with the appropriate amount of lentivirus with polybrene as calculated previously.
3. Day 2
    - Remove the virus-containing medium and replace it with the fresh complete culture medium. Incubate at 37°C in a humidified 5% CO_2 incubator overnight.
4. Day 3
    - Refresh the medium with complete culture medium containing puromycin of the concentration determined previously.
5. Day 4 and onward
    - Refresh the puromycin-containing medium for each well. Regularly monitor cell viability under a microscope.

The images below show the cells transduced with VectorBuilder’s Human Whole Genome Dual-gRNA Lentivirus Library (MOI=10). The lentiviral backbone contains a dual-marker expression cassette of EGFP and a puromycin resistance gene (Puro), allowing the selection of positively transduced cells by puromycin and visual tracking by green fluorescence.

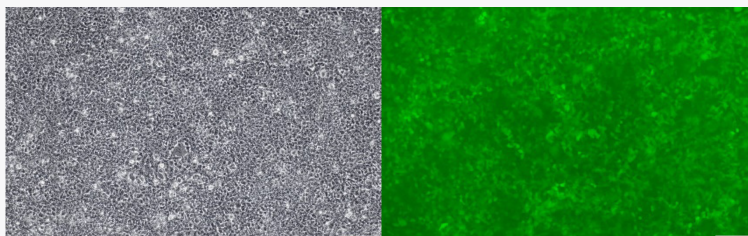

Figure 3. EGFP expression in HEK293T cells transduced with Human Whole Genome Dual-gRNA Lentivirus Library (MOI=10) after 4 days of puromycin selection (1.5 ug/ml). Left: bright field. Right: EGFP. Scale bars, 200 um.

Screening

Transduced cells are first divided into the reference population and the experimental population. The experimental population is subjected to selective pressure (e.g. drug treatment or repeated passage) to identify cells with the desired phenotype. As illustrated in **Figure 1** in the first chapter, there are three major types of screening strategies: 1) viability screens that search for gRNAs that are enriched or depleted in surviving cells when exposed to the selective pressure; 2) reporter screens that look for gRNAs enriched in cells with either high or low reporter expression (e.g. gRNAs targeting transcription factors that modulate reporter gene expression); 3) behavior screens that identify gRNAs affecting genes associated with cell invasion, migration, etc.

Note:

a. **Passaging cells or dividing transduced cells into multiple groups during screening will reduce the fold coverage accordingly. It is essential to maintain the desired fold coverage in each group after removing a portion of the cells.**

b. **Proper coverage should be maintained at the beginning of the screening, which may not be equal to the coverage when seeding the cells. If other procedures such as drug selection or cell differentiation will be performed after library transduction, ensure to consider the fraction of cell death or undifferentiated cells which will cause the actual coverage to decrease accordingly.**

Case study

For VectorBuilder's Human Whole-Genome Dual-gRNA Lentivirus Library, the number of cells needed for 500-fold coverage at MOI = 0.3 in one replicate is about 2.1×10^8, as calculated in the previous case study. This is the minimum number of cells required to maintain a 500-fold coverage of the library. Therefore, following puromycin selection, ensure that the surviving cells reach this number in each replicate. Similarly, if cells are passaged during screening, ensure that the retained cells reach this number.

Post-screening

1. Isolate genomic DNA

   The genomic DNA from all groups is collected following screening using the genomic DNA purification kit of your choice. A typical yield of genomic DNA with widely available commercial kits is approximately 1.5 ug to 15 ug from 1×10^6 cells, depending on the cell type.

Note:

a. **Dissolving genomic DNA in water results in a high degree of impurity. Since the extracted genomic DNA will then be utilized for library preparation, which requires high purity, it is strongly recommended to perform elution in 1×TE buffer.**

b. **After extracting the genomic DNA, we recommend running a small amount (such as 100 ng to 150 ng) on an agarose DNA gel to assess its quality. Please refer to Figure 4 for a comparison between normal genomic DNA (left gel, lanes 1 and 2) and genomic DNA with degradation (right gel, lane 3). Genomic DNA degradation can impede PCR amplification and subsequently lead to sub-optimal NGS data quality.**

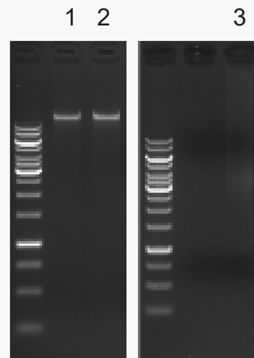

Figure 4. Electrophoresis of 100 ng extracted genomic DNA on a 1% agarose gel for 30 minutes. Lane 1 and 2: Genomic DNA with minimal degradation. Lane 3: Genomic DNA exhibiting severe degradation.

c. **You may also roughly assess the quality of isolated genomic DNA by determining the absorbance ratio of A260/280 and A260/230. High-quality genomic DNA samples should exhibit an A260/280 ratio between 1.8 and 2.0, and an A260/230 ratio exceeding 2.0, suggesting the absence of protein and other organic contaminants.**

d. **We suggest saving a portion of the extracted genomic DNA as backup, given the unpredictability associated with NGS.**

2. Prepare for Illumina sequencing

The next step in pooled library screening involves PCR amplification of the variable sequences such as gRNA, shRNA, or barcode region from the extracted genomic DNA, along with the addition of sequencing adaptors. Different biological replicates or controls can be amplified with distinct sequencing adaptors, enabling their multiplexing on a single sequencing flow cell.

Depending on the library design, different primers should be utilized to amplify the regions of interest for subsequent NGS analysis. Please see below for the primers and PCR conditions we recommend for use with VectorBuilder's dual-gRNA or shRNA lentivirus library:

Dual-gRNA library forward primer:

5'- P5 flowcell attachment sequence Illumina sequencing primer U6 Promoter -3'

5'- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACA -3'

Dual-gRNA library reverse primer:

5'- P7 flowcell attachment sequence Index Illumina sequencing primer Scaffold -3'

5'- CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAACTTGCTATTTCTAGCTCTAA -3'

shRNA library forward primer:

5'- P5 flowcell attachment sequence Illumina sequencing primer U6 Promoter -3'

5'- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACACCG -3'

shRNA library reverse primer:

5'- P7 flowcell attachment sequence Index Illumina sequencing primer backbone+hPGK Promoter -3'

5'- CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGCAACCCCAACTTTTC TATAC -3'

Note:

a. **When choosing the index sequences, we suggest selecting them from those recommended by Illumina.**
b. **Due to the length of the primers, we highly recommend choosing HPLC or PAGE-purified oligos. The quality of the primers is crucial for downstream NGS.**

Each 50 ul PCR reaction can be prepared with 1-2 ug of genomic DNA. Prepare enough PCR reactions to amplify all the isolated genomic DNA to maintain the proper coverage of the library. Each PCR reaction should contain:

Component	Amount
2X PCR master mix	25 ul
Purified genomic DNA	1-2 ug
Forward primer (10 uM)	2.5 ul
Reverse primer (10 uM)	2.5 ul
ddH_2O	
Total	50 ul

Note: **Please ensure choosing a PCR amplification kit suitable for NGS library preparation, such as the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®.**

Once the PCR reactions are prepared, amplify them using the following cycling conditions:

Temperature	Time	Cycles
98℃	3 min	1
98℃	30 s	24-26
60℃	30 s	
72℃	30 s	
72℃	3 min	1
4℃	∞	

Note: The PCR cycle number should be determined before the actual run. The objective is to select the smallest cycle number that yields sufficient product for NGS analysis to minimize bias and uneven variant distribution caused by PCR amplification. This can be achieved by performing a qPCR with the same reaction mix as the actual PCR amplification. The optimal cycle number should be before reaching the plateau phase to avoid skewing variant distribution and generating by-products. Alternatively, a small amount of genomic DNA can be amplified with a gradient of cycle numbers. It is advised to select the lowest (or even lower) cycle number where the correct band just starts to appear in a DNA gel.

The number of PCR reactions needed can be determined based on the mass of genomic DNA (gDNA) required for the scrren:

Mass of gDNA required for the screen = Number of cells used in the screen x Mass of gRNA per genome

For human cells, it is approximately 6.6×10^{-3} ng/genome.

$$\textit{Number of PCR reactions} = \frac{\textit{Mass of gDNA required for the screen}}{\textit{Amount of gRNA per PCR reaction}}$$

The amount of gRNA in each PCR is usually 1-2 ug.

After PCR amplification, run the products on an agarose DNA gel, ensuring that the band size falls within the correct range. Subsequently, we recommend conducting gel purification to isolate the correct fragment, followed by measuring the DNA concentration of the purified sample. We recommend 150 bp paired-end sequencing for the dual-gRNA library and at least 50 bp paired-end sequencing for the shRNA library.

Note: The coverage of NGS should ideally match the coverage used during screening. For instance, if a 500-fold coverage of the library is utilized for screening, it is recommended to also employ a 500-fold coverage for NGS.

FAQ

1. How many screenings can be conducted with VectorBuilder's pooled lentivirus library?

The number of screenings that can be conducted depends on several factors, including the functional titer determined for your cell line of interest, the number of biological replicates, the fold coverage of the library, and the MOI of transduction. For details regarding how to determine these factors, please refer to previous sections in the **Protocol for In Vitro Screening Using Pooled Lentivirus Library**.

2. Why do some variants exhibit low representation after screening?

Insufficient cell number: For pooled genetic screenings, it is recommended to maintain at least a 100- to 500-fold coverage for your library. Higher fold coverage is associated with better representation of each variant, leading to more reliable hit identification.

Fold coverage not maintained during passage: Once a specific fold coverage is determined, it is essential to maintain this coverage if cells require passaging during the screenings. For instance, if only half of the cells are kept in culture while the other half is discarded, the representation of each variant will decrease by half after passaging.

Variants depleted during selection: In viability screenings, certain variants may be depleted under selection pressure. After NGS, the read count of each variant will be analyzed. It is advisable to optimize the selection pressure to avoid excessive cell loss, which may result in false-positive hit identification.

Genomic DNA not collected properly: We recommend extracting genomic DNA from all available cells to avoid skewing variant representation. If only a subset of cells is used for genomic DNA extraction, there is a risk of insufficient representation for certain variants. If subsampling is needed, extracting genomic DNA before subsampling increases the likelihood of achieving a more uniform variant representation compared to directly subsampling from cells. Additionally, the quality of genomic DNA should be assessed after extraction to ensure minimal degradation, as described in the previous section of **Isolating genomic DNA**.

Suboptimal PCR conditions: The PCR cycle number should be determined prior to the actual run. Exceeding the optimal number of PCR cycles, which saturates the reaction, can result in uneven distribution of variants and the formation of by-products.

Non-uniform library: During the amplification of E. coli glycerol stock or virus packaging, maintaining the uniformity of the library is crucial. It is recommended to conduct quality control (QC) of the library before initiating screenings to ensure a uniform representation of variants.

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