Takara Bio USA, Inc.

Guide-it™ CRISPR Genome-Wide sgRNA Library System User Manual

Cat. Nos. 632646 & 632647
(090122)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

A. Summary

The **Guide-it CRISPR Genome-Wide sgRNA Library System** (Cat. No. 632646) is a complete system for producing high yields of lentiviruses encoding both Cas9 expressing and sgRNA library expressing components necessary for performing pooled, genome-wide phenotypic screens in human cell lines.

CRISPR/Cas9 gene editing technology uses two key components that form a complex: Cas9 endonuclease and a single guide RNA (sgRNA) that directs Cas9 to a specific target site in genomic DNA (Jinek et al. 2012). The Cas9 cleaves the genomic DNA at the target gene, which is then repaired by non-homologous end joining resulting in a knockout phenotype. CRISPR is very well suited for genome-wide knockout screens due to the ease of generating guide RNAs and the specificity of Cas9-sgRNA complexes to knock out expression of target genes.

Loss-of-function (LoF) genetic screens can be applied to identify novel protein functions by knocking out genes across a population of cells, applying selective pressure, and then identifying genes that are either enriched or depleted in the selected cell population relative to a control population. The CRISPR/Cas9 system has helped researchers utilize the potential of this novel in vitro screening method for the identification of key proteins in biological processes (Doench et al. 2016; Doench, 2018). Briefly, target cells are first transduced with Cas9 and selected for stable, high Cas9 expression. The cells with high expression of Cas9 are then transduced with the sgRNA library and subsequently screened for loss-of-function phenotypes. The genomic DNA from cells exposed to selective pressure is then extracted from the selected cells and analyzed using next-generation sequencing (NGS) for enrichment or depletion of sgRNAs, as compared to a reference, unselected cell population. See Figure 1 for an example of the steps involved in a pooled library screen.

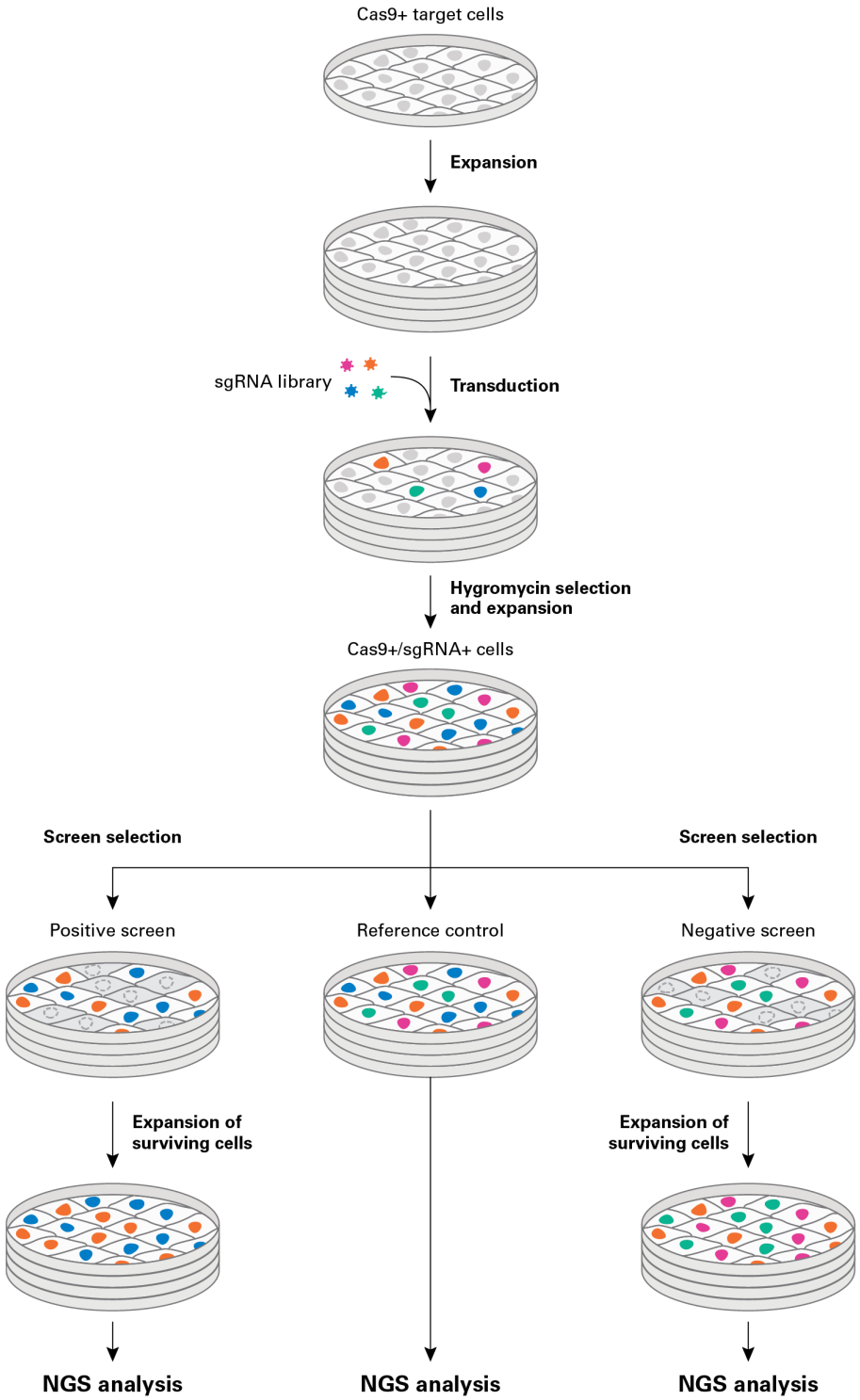


Figure 1. Depiction of pooled CRISPR/Cas9 screens. A selected cell line expressing Cas9 is transduced with an sgRNA library and then selected for positively transduced cells and expanded. The cells can then be subjected to either a positive or a negative screen. In the case of a positive screen, cells harboring edits that result in resistance to or increased survival under the specific screen selection pressure will be identified due to their increased abundance in the screened population relative to a reference control population. In the case of a negative selection screen, on the other hand, most cells will survive the selection agent, and only those cells in which the targeted genetic manipulation results in increased sensitivity to or reduced survival under the screen selection pressure will be lost during the screen and identified due to their diminished frequency or absence in the screened population relative to the reference control population.

B. Guide-it CRISPR Genome-Wide sgRNA Library System Features

Guide sequence selection

The potency of the Guide-it CRISPR Genome-Wide sgRNA Library System comes from the selection of highly active guides. This system contains sgRNAs from the Brunello library which were chosen using algorithms that maximize on-target specificity and activity while minimizing off-target activity (Doench et al. 2016). Each gene in the human genome is represented by four highly active sgRNAs, which allows for smaller scale screening while maintaining representation and potency. A complete list of all sgRNAs in our library and their representation data can be found on our website at takarabio.com/genomewidelibrary.

Guide representation

To ensure that every sgRNA is represented within the library, the sgRNA content of the library plasmids is verified in every lot by NGS to contain >90% of the sgRNAs within a 10-fold distribution range (see guide representation data under the [Image data] tab on the product page). We have additionally measured representation in a transduced cell population and guaranteed an extremely high correlation between the starting plasmid material and the transduced cell population (Spearman and Pearson's correlation > 0.95).

sgRNA scaffold design

We have also modified the sgRNA scaffold in the pLVXS-sgRNA-mCherry-hyg Vector to improve the Cas9-sgRNA interaction, ensuring high editing efficiency (Figure 2). Due to the nature of the Cas9-sgRNA complex, it is important to maximize the efficiency of binding of the sgRNA to the Cas9 endonuclease by using this modified scaffold (Chen et al. 2013).

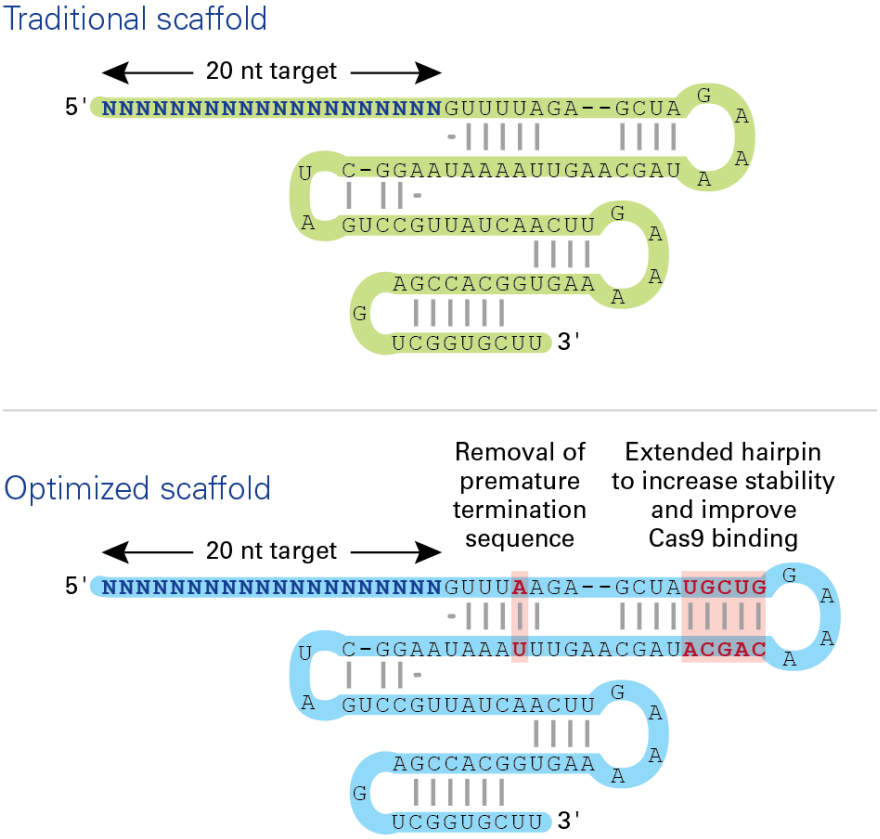


Figure 2. Optimized sgRNA scaffold design. pLVXS-sgRNA-mCherry-hyg Vector includes an improved sgRNA scaffold design for increased editing efficiency.

Lentiviral production

Representation of the sgRNA library during lentiviral production is maintained by utilizing our high-titer packaging formulation, Lenti-X™ Packaging Single Shots (VSV-G). Lenti-X Packaging Single Shots (VSV-G) are pre-aliquoted, lyophilized, single tubes of Xfect™ Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids and provide the highest titers and widest tropism for viral transduction. In the Guide-it CRISPR Genome-Wide sgRNA Library System, both the sgRNA library and the Cas9 plasmid are formulated with the Lenti-X Packaging vectors and Xfect transfection reagent such that only water is required to produce a highly efficient transfection mix for transfecting Lenti-X 293T Packaging Cells to produce high-titer sgRNA libraries (Figure 3).

pLVXS-sgRNA-mCherry-hyg Vector

The lentiviral vector into which the sgRNA library is cloned has several features for optimum performance and safety:

- The library sgRNAs are highly expressed from an optimized human U6 promoter to ensure efficient Cas9 loading and editing.
- A dual marker system permits the identification and titration of transduced cells using fluorescence (mCherry) or by hygromycin drug selection.
- The vector is self-inactivating (SIN), ensuring a high level of safety during production and use.

Lenti-X 293T cells

Getting the most from your lentiviral packaging system requires a host HEK 293T cell line that is easily transfected, and supports a high expression of viral proteins. Our Lenti-X 293T Cell Line has been clonally selected to meet these requirements, allowing you to produce the highest possible unconcentrated lentiviral titers (up to 10^8 IFU/ml) when combined with our premium high-titer Lenti-X Packaging Mix.

Guide-it CRISPR Genome-Wide sgRNA Library System Workflow

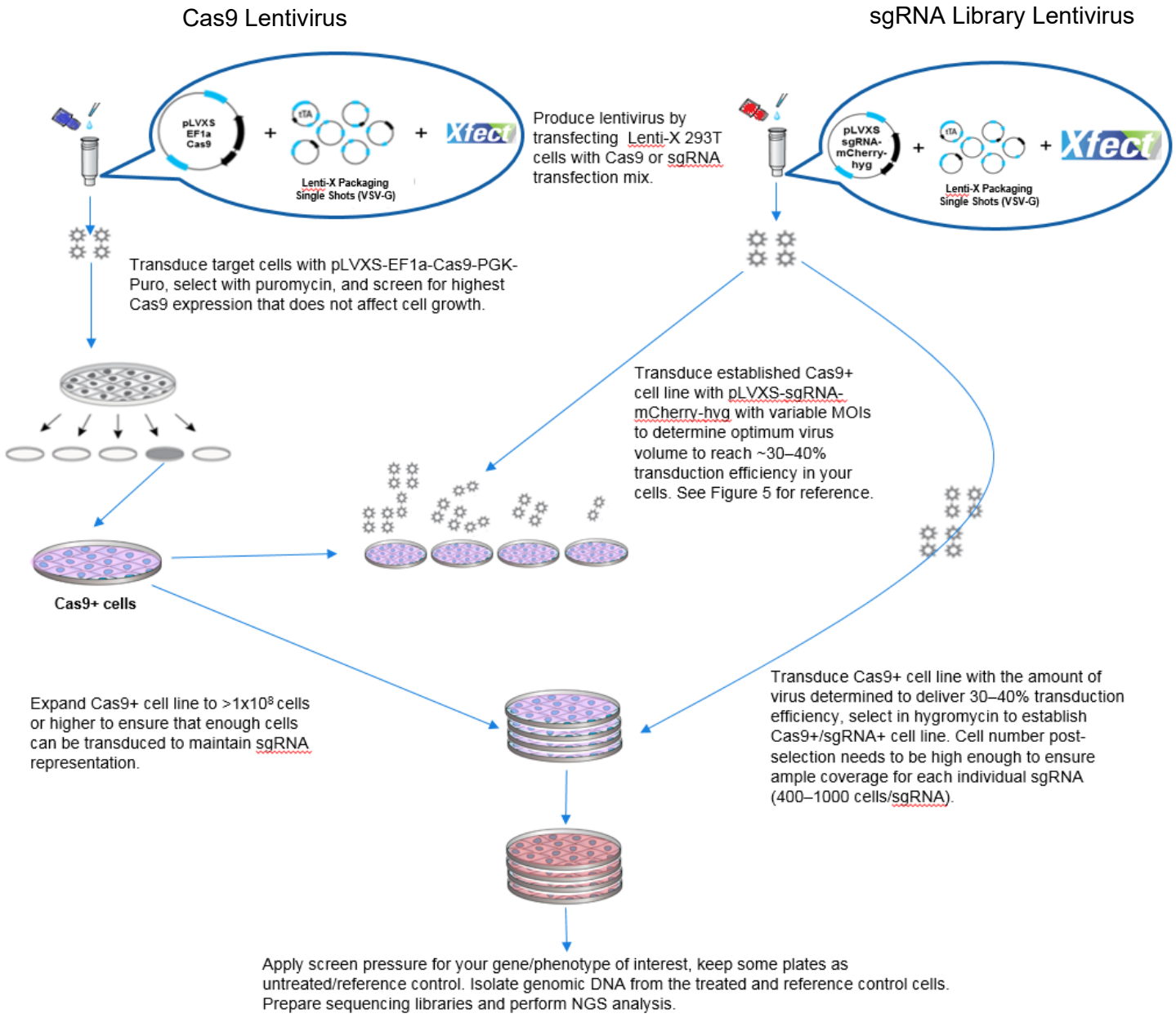


Figure 3. Protocol overview for the Guide-it CRISPR Genome-Wide sgRNA Library System.

II. List of Components

- Store the Cas9 and sgRNA library transfection mixes at –20°C in the supplied foil pouch containing the desiccant sachet.
- Make sure to return any unused lyophilized transfection mixes to the supplied foil pouch containing the desiccant sachet, and store at –20°C.

Table 1. Guide-it CRISPR Genome-Wide sgRNA Library System components.

Guide-it CRISPR Genome-Wide sgRNA Library System	Cap color	632646 (5 screens)
Guide-it Genome-Wide sgRNA Library Transfection Mix* (Cat. No. 632650)		
Guide-it Lentiviral sgRNA Library Transfection Mix (Genome-Wide)†	Red	2 x 5 vials
Guide-it Cas9 Lentiviral Transfection Mix* (Cat. No. 632648)		
Guide-it Cas9 Lentiviral Transfection Mix†	Blue	5 vials
pLVXS-sgRNA-mCherry-hyg Vector* (Cat. No. 632649)		
pLVXS-sgRNA-mCherry-hyg Vector (0.5 µg/µl) †		10 µl
Lenti-X 293T Cell Line (Cat. No. 632180)		
Lenti-X 293T Cell Line (2.0 x 10^6 cells/tube)‡		1 ml
Lenti-X GoStix Plus (Sample-Not for Sale) *§ (Cat. No. 631279)		
Lenti-X GoStix Plus		3 tests
Chase Buffer		3 ml
p24 Control		5 tests

*Not sold separately.
†Store at –20°C.
‡Store cells in liquid nitrogen (–196°C) or in a –150°C freezer.
§Store at room temperature.

III. Additional Materials Required

The following reagents are required for this protocol but not supplied.

A. Tetracycline-Free Fetal Bovine Serum

Tetracycline-free FBS should be used in the transfection medium and in the medium used to collect the virus. The following functionally tested tetracycline-free sera are available from Takara Bio:

Cat. No.	Serum Name
631106	Tet System Approved FBS (500 ml)
631107	Tet System Approved FBS (50 ml)
631367	Tet System Approved FBS (3 x 500 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)
631368	Tet System Approved FBS, US-Sourced (3 x 500 ml)

B. Antibiotics for Selecting Stable Cell Lines

pLVXS-EF1a-Cas9-PGK-Puro and pLVXS-sgRNA-mCherry-hyg Vectors have puromycin and hygromycin resistance markers, respectively, for selection of stable clones or populations. Use the following recommended antibiotic concentrations:

Table 2. Recommended antibiotic concentrations for selecting and maintaining stable cell lines.

		Recommended concentration (µg/ml)	
Cat. No.	**Antibiotic**	**Selecting colonies***	**Maintenance**
631309	Hygromycin B (1 g)	50–400	100
631305	Puromycin (25 mg)	0.25–10	0.25
631306	Puromycin (100 mg)	0.25–10	0.25

*When selecting for single colonies, the appropriate dose must be determined empirically for your specific cell line. Test a dosage range using dishes of untransfected cells and choose the dose that kills all the cells in 3–5 days (puro) or 7–10 days (hygro). If all the cells die in less than 24 hours, you should use a lower dose. See Appendix C for more information.

C. Mammalian Cell Culture Supplies

Medium for Lenti-X 293T Cells:

- 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma-Aldrich, Cat. No. D5796); 10% Fetal Bovine Serum (FBS)
- Culture medium, supplies, and additives specific to your target cells
- Trypsin/EDTA (Sigma-Aldrich, Cat. No. T4049)
- Cell freezing medium, with or without DMSO (Sigma-Aldrich, Cat. Nos. C6164 or C6039), for freezing stable clones or populations
- 10 and 15 cm cell culture dishes

D. Transduction Enhancers

Polybrene is a cationic agent that enhances lentivirus to cell contact through charge neutralization. Transduction efficiency is typically enhanced several-fold with the inclusion of polybrene. Higher transduction efficiencies allow for the use of less virus or for transduction of more cells.

- Polybrene (hexadimethrine bromide; Sigma-Aldrich, Cat. No. H9268)

RetroNectin® reagent is a multivalent molecule that simultaneously binds virus particles and cell surface proteins, maximizing cell-virus contact. RetroNectin reagent is recommended for increasing the transduction efficiency of suspension cells and stem cells; visit takarabio.com for details.

Cat. No.	Transduction Enhancer	Size
T110A	RetroNectin Precoated Dish	10 dishes
T100B	RetroNectin Recombinant Human Fibronectin Fragment	2.5 mg
T100A	RetroNectin Recombinant Human Fibronectin Fragment	0.5 mg

E. NGS Analysis

Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit (Cat. No. 632647; 10 rxns)

- Store Guide-it CRISPR Genome-Wide Library PCR Kit components at –20°C
- Store NucleoBond CB 500 and NucleoSpin Gel and PCR Clean-Up at room temperature

Guide-it CRISPR Genome-Wide Library PCR Kit (Cat. No. 632651; 20 rxns)

- 100 µl LVXS P5 primer pool with index 1 (10 µM)
- 100 µl LVXS P7 primer with index 2 (10 µM)
- 100 µl LVXS P7 primer with index 3 (10 µM)
- 100 µl LVXS P7 primer with index 4 (10 µM)
- 100 µl LVXS P7 primer with index 5 (10 µM)
- 30 µl TaKaRa *Ex Taq*® (5 U/µl)
- 500 µl 10X *Ex Taq*™ Buffer (Mg^{2+} plus)
- 400 µl dNTP Mixture (2.5 mM each)

NucleoBond CB 500 (Cat. No. 740509; 10 preps)

- 2 x 125 ml Buffer G1
- 1 x 125 ml Buffer G2
- 2 x 125 ml Buffer N2
- 2 x 125 ml Buffer N3
- 1 x 125 ml Buffer N5
- 1 x 8 ml Proteinase Buffer PB
- 1 x 40 mg Proteinase K (lyophilized)
- 2 x 15 g Saccharose
- 10 NucleoBond AXG 500 columns
- 5 Plastic Washers

NucleoSpin Gel and PCR Clean-Up (Cat. No. 740609.10; 10 preps) (Not sold separately)

- 10 ml Binding Buffer NT1
- 6 ml Wash Buffer NT3
- 13 ml Elution Buffer NE
- 10 NucleoSpin Gel and PCR Clean-up Columns (yellow rings)
- 10 Collection Tubes (2 ml)

F. Secondary Screen

For cloning sgRNAs used in the secondary screening protocols, use the DNA Ligation Kit, Mighty Mix—a single solution, ready-to-use 2X ligation mix that enables high efficiency ligations.

Cat. No.	Product	Size
6023	DNA Ligation Kit, Mighty Mix	100 rxns

G. Competent Cells

Use our Stellar™ Competent Cells for cloning your sgRNA into the pLVXS-sgRNA-mCherry-hyg Vector for performing secondary screens.

Cat. No.	Concentrator	Size
636763	Stellar Competent Cells	10 x 100 µl
636766	Stellar Competent Cells	50 x 100 µl
636767	Stellar Competent Cells (96-well plate)	96 x 20 µl
636764	Stellar Competent Cells (dam-/dcm-)	10 transformations

H. Molecular Biology Supplies

- TE buffer or molecular biology-grade, nuclease-free water
- PCR reaction tubes
- Micropipette tips (with hydrophobic filters)
- 1.5 ml microfuge tubes
- 1.5 ml DNA LoBind Tube (Eppendorf, Cat. No. 022431021)
- 50-ml conical tubes (Corning, Cat. No. 352070 or equivalent)
- 0.2-µM sterile syringe filters (Thermo-Fisher Scientific, Cat. No. 723-2520 or equivalent)
- BsmBI-v2 (10 units/µl; NEB, Cat. No. R0739S)
- DNase I (5 U/µl; Takara Bio, Cat. No. 2270A)
- 1 kb DNA Ladder (Dye Plus) (100 rxns; Takara Bio, Cat No. 3426A)
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- Qubit Assay Tubes (Thermo Fisher Scientific, Cat. No. Q32856)
- Agilent High Sensitivity DNA Kit (Agilent Technologies Cat. No. 5067-4626)
- MiSeq® Reagent Kit v3 (150 cycles) (Illumina, Cat. No. MS-102-3001)
- NextSeq® 500/550 mid output V2 kit (150 cycles) (Illumina, Cat. No. FC 404-2001)
- PhiX Control v3 (Illumina, Cat. No. FC-110-3001)

IV. Additional Materials Recommended

The following additional materials are recommended for this protocol but not required.

A. Lentivirus Production

Lenti-X Packaging Single Shots are a fourth-generation lentiviral packaging system that provides an extremely simple and consistent one-step method for producing high-titer lentivirus. No additional transfection reagent is needed because Lenti-X Packaging Single Shots consist of pre-aliquoted, lyophilized, single tubes of Xfect Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids. Lenti-X Packaging Single Shots can be used in combination with the pLVXS-sgRNA-mCherry-hyg Vector supplied with this kit for performing transductions in secondary screens.

Cat. No.	Lentiviral Packaging
631275	Lenti-X Packaging Single Shots (VSV-G) (16 rxns)
631276	Lenti-X Packaging Single Shots (VSV-G) (96 rxns)

B. Lentiviral Titer Determination

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. Various technologies are available from Takara Bio USA, Inc.; visit takarabio.com for details.

Cat. No.	Lentiviral Titration Technology
632200	Lenti-X p24 Rapid Titer Kit (96 rxns)
631235	Lenti-X qRT-PCR Titration Kit (200 rxns)
631280	Lenti-X GoStix Plus (20 tests)

C. Lentivirus Concentration

Use Lenti-X Concentrator to simply increase your available titer up to 100-fold or reduce sample volume, without ultracentrifugation—visit takarabio.com for details.

Cat. No.	Concentrator
631231	Lenti-X Concentrator (100 ml)
631232	Lenti-X Concentrator (500 ml)

D. Assays for Determining Editing Efficiency

These items are required for determining the efficiency of gene editing and the nature of the edits once candidate gene targets are identified. These can be used to quickly verify editing in secondary screens.

Cat. No.	Product	Size
631443	Guide-it Mutation Detection Kit	100 rxns
631448	Guide-it Mutation Detection Kit	25 rxns
632611	Guide-it Genotype Confirmation Kit	100 rxns
631444	Guide-it Indel Identification Kit	10 rxns

E. Cas9 Antibody

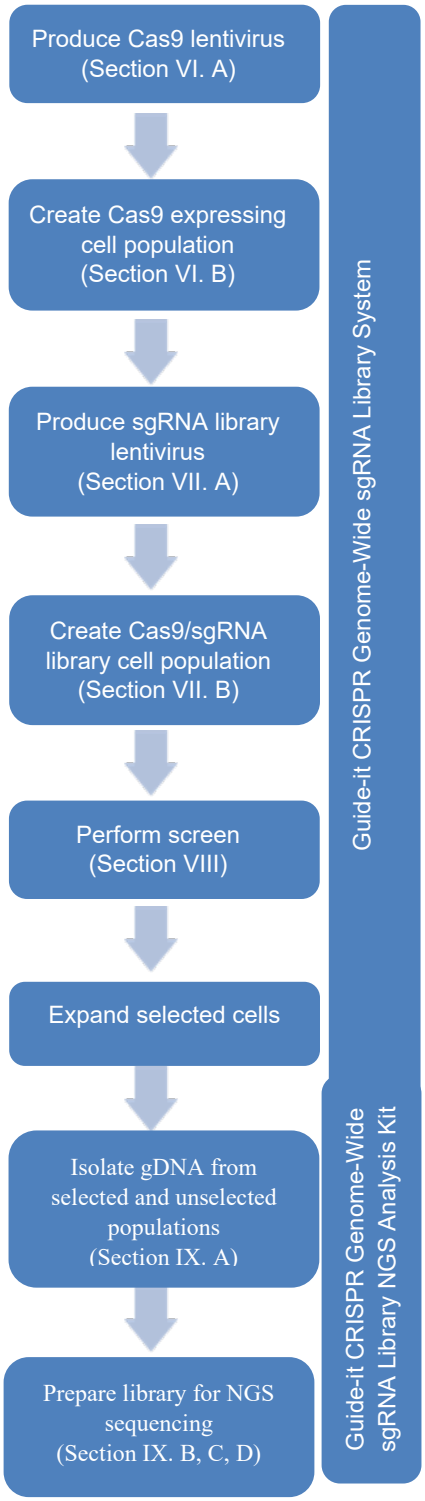
Guide-it Cas9 antibodies are raised against recombinant Cas9 protein from *Streptococcus pyogenes*. These antibodies recognize the modified version of Cas9 included with the Guide-it CRISPR Genome-Wide sgRNA Library System and are suitable for Cas9 detection by Western blot. We recommend this antibody if you want to confirm Cas9 expression in your transduced cells by Western blot.

Cat. No.	Product	Size
632628	Guide-it Cas9 Monoclonal Antibody	100 µl
632627	Guide-it Cas9 Monoclonal Antibody	3 x 100 µl
632607	Guide-it Cas9 Polyclonal Antibody	100 µl
632606	Guide-it Cas9 Polyclonal Antibody	3 x 100 µl

V. Protocol

Please read each protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

A. Protocol Overview



Produce Cas9 lentivirus by adding water to the Guide-it Cas9 Lentiviral Transfection Mix (Blue cap) and adding the contents to Lenti-X 293T cells.

Transduce target cells with different amounts of Cas9 lentivirus and select the populations in puromycin. Re-plate cells in equal amount and monitor the cells' growth rate over one to two weeks. Choose a Cas9 population transduced with the highest MOI that does not exhibit any change in cell growth due to sensitivity to Cas9 expression. Make a master cell bank from this line.

Produce sgRNA lentivirus by adding water to the Guide-it Genome-Wide sgRNA Library Transfection Mix and adding the contents to Lenti-X 293T cells.

Establish the amount of sgRNA lentivirus required to transduce the Cas9 expressing cell line to 30–40% positive cells by selection in hygromycin (or fluorescence). Scale up cell number to ensure proper representation (400–1,000 cells/sgRNA).

Expand and plate cells according to the desired screening method. Be sure to maintain an untreated reference control population.

Expand the cells from the screening method, ensure representation is maintained (400–1,000 cells/sgRNA), and harvest cells.

Isolate gDNA from all populations associated with the screen using the NucleoBond CB 500 reagents included with the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit.

Using the included sequencing primers, amplify the sgRNA sequences from the gDNA using the Guide-it CRISPR Genome-Wide Library PCR Kit and then purify the amplicons using the included NucleoSpin Gel and PCR Clean-Up kit.
Perform NGS analysis.

Figure 4. User manual protocol workflow.

B. General Cell Culture

This user manual provides only general guidelines for mammalian cell culture techniques. For users requiring more information on mammalian cell culture, transfection, and creating stable cell lines, we recommend the following general reference:

Freshney, R.I. (2016). *Culture of Animal Cells: A Manual of Basic Technique, 7th Edition* (Wiley-Blackwell, Hoboken, NJ).

Antibiotic kill curves for puromycin and hygromycin resistance

To successfully select cells transduced with Cas9 or the sgRNA library, you need to know the concentration of antibiotic that kills untransduced cells of a given type within a given amount of time. A protocol for establishing proper concentrations of antibiotic can be found in Appendix C at the end of this User Manual.

C. Protocol: Starting Lenti-X 293T Cell Line Cultures from Frozen Stock

Frozen cells should be cultured immediately upon receipt, or as soon as possible thereafter. If culturing is significantly delayed after receipt, decreased cell viability may result. For HEK 293-based cell lines, we recommend using collagen-coated plates or flasks for efficient culturing of frozen stocks. Vessels coated with compounds other than collagen may also provide suitable growth substrates (e.g. poly-L-lysine). Once recovered, the cells may be cultured directly on tissue culture plastic. However, if adherence is poor, we recommend using only collagen-coated vessels.
To prevent osmotic shock and maximize cell survival, perform the following:

1. Warm ~25 ml of complete culture medium in a 37°C water bath. See Section III. C for medium composition.
   NOTE: Be sure to use Tet System Approved Fetal Bovine Serum (Cat. Nos. 631101, 631105, 631106 or 631107) when using these cells with the Guide-it Cas9 Lentiviral Transfection Mix (Cat No. 632648) and the Guide-it Genome-Wide sgRNA Library Transfection Mix
2. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. **All the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions.** Unscrew the top of the vial slowly and using a pipet transfer the contents of the vial to a 15-ml conical centrifuge tube containing 1 ml of pre-warmed medium. Mix gently.
3. Slowly add an additional 4 ml of fresh, pre-warmed medium to the tube and mix gently.
4. Add an additional 5 ml of pre-warmed medium to the tube, mix gently. Centrifuge at 100*g* for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium.
   NOTE: This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.
5. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% CO_2 as appropriate) for 24 hr.
6. The next day, examine the cells under a microscope. If the cells are well-attached and confluent, they can be passaged for use. If most of them are not well-attached, continue culturing for another 24 hr. Complete attachment of newly thawed cultures of HEK 293-based cell lines may require up to 48 hr.
7. Once the culture has been started and the cells are growing normally, you should prepare frozen aliquots to provide a renewable source of cells. Consult the Lenti-X 293T Cell Line Protocol-At-A-Glance for a cell freezing protocol.

D. Safety Guidelines for Working with Lentiviruses

IMPORTANT: The protocols in this user manual require the production, handling, and storage of infectious lentivirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of lentiviruses.

The National Institutes of Health and the Centers for Disease Control and Prevention have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 (BSL-2) facility for work involving these viruses and others like it. The VSV-G pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

IMPORTANT: For the reasons mentioned above, due caution must be exercised in the production and handling of any recombinant lentivirus.

For more information on Biosafety Level 2 agents and practices, download the following reference, available on the web at http://www.cdc.gov/biosafety/publications/bmbl5/:

> Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (December 2009) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH.

Biosafety Level 2: The following information is a brief description of Biosafety Level 2. ***It is neither detailed nor complete.*** Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with lentiviruses.

Summary of Biosafety Level 2:

- **Practices:**
  - Standard microbiological practices
  - Limited access to work area
  - Biohazard warning signs posted
  - Minimize production of aerosols
  - Decontaminate potentially infectious wastes before disposal
  - Use precautions with sharps (e.g., syringes, blades)
  - Biosafety manual defining any needed waste decontamination or medical surveillance policies
- **Safety equipment:**
  - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is un-recirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- **Facilities:**
  - Autoclave available for waste decontamination
  - Chemical disinfectants available for spills

VI. Creation of Cas9-Expressing Cell Line

A. Protocol: Cas9 Virus Production

This protocol describes transfection and Cas9 lentivirus production with the Guide-it Cas9 Lentiviral Transfection Mix—a pre-aliquoted, lyophilized, single tube of Xfect Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids (VSV-G pseudotype) and a self-inactivating Cas9-expressing lentivirus vector, pLVXS-EF1a-Cas9-PGK-Puro (Appendix B). Refer to the pLVXS-EF1a-Cas9-PGK-Puro Vector Information for details (takarabio.com/manuals).

The Guide-it Cas9 Lentiviral Transfection Mix provides a simple method for transfecting Lenti-X 293T cells with lentiviral vector DNA. The amount of reagent, packaging, and Cas9 vector in each tube is optimized for lentivirus production in a 10-cm dish. Transfections can be carried out entirely in the presence of serum.

NOTE: The use of tetracycline-free FBS is critical for achieving high titers with this technology.

1. Approximately 24 hr before transfection, seed 4–5 x 10^6 Lenti-X 293T cells/10-cm plate in 8 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C, 5% CO_2 overnight. Continue to incubate the cells until you are ready to add the transfection mixture in Step 4. The cells should be 80–90% confluent at the time of transfection.
2. Add 600 µl of sterile water to a tube of Guide-it Cas9 Lentiviral Transfection Mix (blue cap), replace the cap, and vortex well at a high speed for 20 sec. The pellet should dissolve completely.

   NOTE: In some cases, some insoluble material may be visible after vortexing. This material does not have a negative effect on transfection efficiency or virus yields.
3. Incubate the rehydrated transfection mix for 10 min at room temperature to allow nanoparticle complexes to form. After the 10-min incubation, centrifuge the tube for 2 sec to bring the liquid to the bottom of the tube.

   NOTE: Sample tubes can be inserted into 1.5 ml microfuge tubes for a brief centrifugation.
4. Add the entire 600 µl of nanoparticle complex solution dropwise to the 8 ml of cell culture prepared in Step 1. Rock the plate gently back and forth to mix.

   NOTE: It is normal for the medium to change color slightly upon addition of the nanoparticle complex solution.
5. Incubate the cells in a 37°C incubator supplied with 5% CO_2.

   NOTE: A 4-hr incubation with Xfect-DNA nanoparticles is sufficient for optimal transfection. Incubation may be continued overnight for convenience but does not generally increase transfection efficiency or titer.
6. After 4 hr to overnight, add an additional 6 ml of fresh, complete growth medium and incubate at 37°C, 5% CO_2 for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection; however, multiple harvests can be collected at both 48 and 72 hr and pooled without loss of titer.
7. Harvest the lentiviral supernatants and pool similar stocks, if desired (a 48-hr sample may be stored at 4°C until a 72-hr sample is harvested).

   CAUTION: ***Supernatants contain infectious lentivirus.***

8. Centrifuge briefly (500*g* for 10 min) or filter through a 0.2-µm filter to remove cellular debris.

   NOTE: The filter used should be made of cellulose acetate or polysulfone (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the lentivirus membrane and destroys the virus.

9. Verify virus production using Lenti-X GoStix Plus (for details, see the Lenti-X GoStix Plus Protocol-At-A-Glance). Alternatively, titrate the virus stock, then use the virus to transduce target cells, or store aliquots at –80°C.

   NOTES:
   - Titers can drop as much as 2–4 fold with each freeze-thaw cycle.
   - Titers of Cas9 expressing lentivirus are often lower due to the large size of the vector. In such cases, we recommend the use of Lenti-X Concentrator (Cat. No. 631231)

10. For protocols and tips describing how to transduce your target cells or create frozen stocks, see the Lenti-X Lentiviral Expression Systems User Manual.

B. Protocol: Transduction and Selection of Cas9-Expressing Cells

This protocol describes the process of creating a cell line that stably expresses Cas9 endonuclease. In many cell lines or types, overexpression of Cas9 can affect cell growth and proliferation. For robust and consistent editing results, it is important to establish the highest level of Cas9 expression that is not detrimental to cell growth. Lentiviral copy number is directly related to expression levels; therefore, it is helpful to transduce with a wide range of MOIs. The steps outlined below describe a process where serial dilutions of Cas9 lentivirus are used to transduce your cell line, followed by puromycin selection (see kill curve protocol, Appendix C). Once stable lines are established from the dilutions, they are re-plated and monitored for ~2 weeks so that differences in cell number can be assessed. The goal is to identify the cells with the highest amount of Cas9 expression that still grow well.

The following protocol is a general method for transducing adherent cell lines, such as HT-1080 or A375, using Polybrene. Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically, but generally falls within a range of 2–12 µg/ml. However, excessive exposure to Polybrene (>24 hr) can be toxic to cells. This protocol can be used as a starting point for determining the optimal transduction conditions for your target cells. Refer to the Lenti-X Lentiviral Expression System User Manual, Appendix B for additional references and alternative infection methods. For cells that are difficult to transduce (e.g. suspension cells) or that might be sensitive to Polybrene, **RetroNectin Reagent** (Cat. Nos. T100A & T100B) can be used to greatly improve transduction speed and efficiency.

1. Plate target cells in a 6-well plate in complete growth medium 12–18 hr before transduction. The cells should be 50–60% confluent at the time of transduction.
2. Thaw aliquots of pLVXS-EF1a-Cas9-PGK-Puro lentiviral stock prepared in the previous protocol or use filtered virus stocks freshly prepared from packaging cells (Section VI.A).
3. Add polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).
4. Transduce target cells with a wide range of MOIs using the Cas9 lentivirus stock. Start with MOIs as high as 50–60 and then perform serial dilutions (see Figure 5, below). If possible, centrifuge the cultures to improve transduction efficiency.

NOTE: CULTURE CENTRIFUGATION INCREASES INFECTION EFFICIENCY. Centrifuging the plate at 1,200*g* for 60–90 min at 32°C can significantly increase infection efficiency. A room-temperature centrifuge is acceptable if a 32°C unit is not available.

5. Transduce the cells for 8–24 hr. If you are concerned that exposure to either the polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing medium and replace it with fresh growth medium, as appropriate.

   CAUTION: ***Discarded medium contains infectious lentivirus.***
7. Continue to incubate the cells for 24 hr to allow for cell recovery and expression of Cas9 and puromycin *N*-acetyl-transferase.
8. Passage cells for all dilutions and begin selection with puromycin until all non-transduced cells are dead. (See Appendix C for instructions on how to determine a suitable amount of puromycin to apply to cells).
9. When cells have accumulated at sufficient levels for counting, count and plate all transduced populations at equivalent density (~40–50% confluency) and continue culturing, passaging, and counting the cells as needed, so that growth curves for each transduced population can be established.

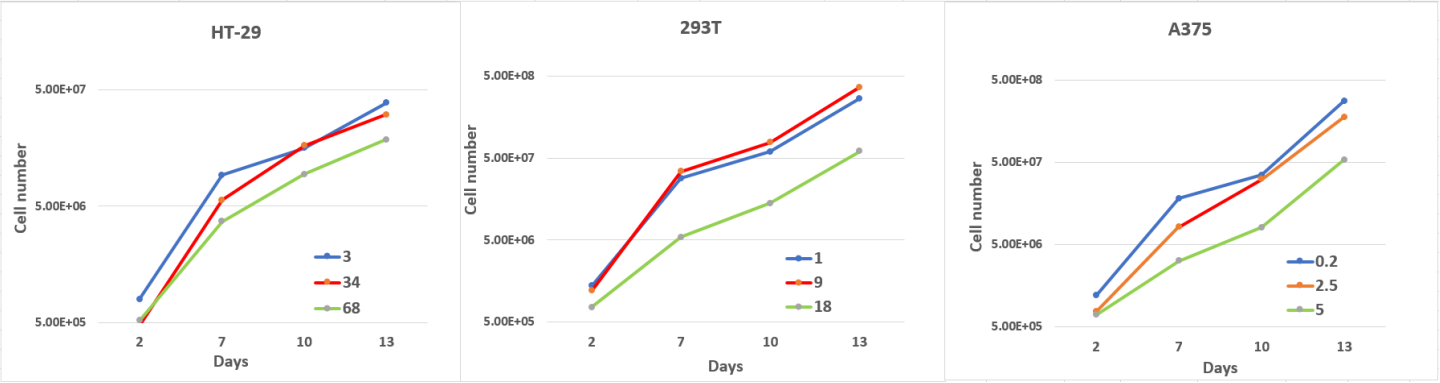


Figure 5. Growth curves for HT-29, 293T, and A375 Cas9+ cell lines produced with varying Cas9 lentiviral MOIs.

10. When sufficient curves are established, choose the cells transduced with the highest amount of virus that does not affect cell growth or viability. Optimal MOIs vary by cell type and will depend on the cells' tolerance for Cas9 expression and ability to be transduced easily. In Figure 5 above, for example, the samples of HT-29, 293T, and A375 cells produced with MOI of 34, 9, and 2.5, respectively, should be used. Certain cell lines may not be affected by constitutive Cas9 expression, whereas others may be extremely sensitive.
11. Once a population is chosen, expand the cells and freeze down a master cell bank for later use.
12. At this point, depending upon the size of your screen, it is a good time to begin scaling up the Cas9+ cell culture to achieve the cell numbers required for transduction with the sgRNA library.

NOTE: We recommend that once a Cas9 expressing line is generated, its editing efficiency is checked using a previously tested sgRNA.

VII. Transduction of Cas9-Expressing Cells with the sgRNA Library Virus

A. Protocol: sgRNA Library Virus Production

This protocol describes transfection and sgRNA lentivirus production with the Guide-it Genome-Wide sgRNA Library Transfection Mix, a pre-aliquoted, lyophilized, single tube of Xfect Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids (VSV-G pseudotype) and self-inactivating sgRNA-expressing lentivirus vector pLVXS-sgRNA-mCherry-hyg (Appendix B). Refer to the pLVXS-sgRNA-mCherry-hyg Vector Information for details (takarabio.com/manuals).

The Guide-it Genome-Wide sgRNA Library Transfection Mix provides a simple method for transfecting Lenti-X 293T cells with lentiviral vector DNA. The amount of transfection reagent, packaging plasmids, and sgRNA library vector in each tube is optimized for lentivirus production in a 10-cm dish. Transfections can be carried out entirely in the presence of serum.

NOTE: The use of tetracycline-free FBS is critical for achieving high titers with this technology.

This protocol is for lentiviral sgRNA library production in at least two 10-cm plates. The quantity of plates required is determined by virus titer, the number of target cells, and the amount of virus required to transduce the target cells and maintain adequate library representation (see Table 3).

1. Approximately 24 hr before transfection, seed (in duplicate) 4–5 x 10^6 Lenti-X 293T cells/10-cm plate, in 8 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C, 5% CO_2 overnight. Continue to incubate the cells until you are ready to add the transfection mixture in Step 4. The cells should be 80–90% confluent at the time of transfection.
2. Add 600 µl of sterile water to each of the 2 tubes of Guide-it Genome-Wide sgRNA Library Transfection Mix (red cap), replace the caps, and vortex well at a high speed for 20 sec. The pellet should dissolve completely.

   NOTE: In some cases, some insoluble material may be visible after vortexing. This material does not have a negative effect on transfection efficiency or virus yields.
3. Incubate the rehydrated transfection mix for 10 min at room temperature to allow nanoparticle complexes to form. After the 10-min incubation, centrifuge the tube for 2 sec to bring the liquid to the bottom of the tube.

   NOTE: Sample tubes can be inserted into 1.5 ml microfuge tubes for a brief centrifugation.
4. Add an entire 600-µl volume of nanoparticle complex solution dropwise to each of the 8-ml cell cultures prepared in Step 1. Rock the plate gently back and forth to mix.

   NOTE: It is normal for the medium to change color slightly upon addition of the nanoparticle complex solution.
5. Incubate the cells in a 37°C incubator supplied with 5% CO_2.

   NOTE: A 4-hr incubation with Xfect-DNA nanoparticles is sufficient for optimal transfection. Incubation may be continued overnight for convenience but does not generally increase transfection efficiency or titer.
6. After 4 hr to overnight, add an additional 6 ml of fresh complete growth medium and incubate at 37°C, 5% CO_2 for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of

transfection, however multiple harvests can be collected at both 48 and 72 hr and pooled without loss of titer. This is especially helpful when performing large scale transductions.

7. Harvest the lentiviral supernatants and pool similar stocks, if desired (a 48-hr sample may be stored at 4°C until a 72-hr sample is harvested).

   CAUTION: ***Supernatants contain infectious lentivirus.***

8. Centrifuge briefly (500*g* for 10 min) or filter through a 0.2-µm filter to remove cellular debris.

   NOTE: The filter used should be made of cellulose acetate, or polysulfone (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the lentivirus membrane and destroys the virus.

9. Verify virus production using Lenti-X GoStix Plus (for details, see the Lenti-X GoStix Plus Protocol-At-A-Glance). Alternatively, titrate the virus stock, then use the virus to transduce target cells, or store aliquots at –80°C.

   NOTE: Titers can drop as much as 2–4 fold with each freeze-thaw cycle.

10. For protocols and tips describing how to transduce your target cells or create frozen stocks, see the Lenti-X Lentiviral Expression Systems User Manual.

B. Protocol: Transduction of Cells with the sgRNA Library Virus

A robust screen is best achieved by ensuring that proper sgRNA representation is maintained throughout lentivirus production, cell transduction, and subsequent cell culture. A hit is an identified sgRNA that is either enriched (positive screen) or depleted (negative screen) after application of selection pressure during the screen. Identification of multiple guides targeting the same gene increases the confidence in these hits.

1. Titration of Virus on Target Cells

A key goal in producing a Cas9+/sgRNA+ cell population for screening is to maintain sgRNA representation while having each cell express only one sgRNA. Therefore, it is important to establish the amount of virus required to achieve approximately 30–40% transduction efficiency in your Cas9+ cell line. Transduction efficiencies of 30–40% (equivalent to MOI of ~0.4 to 0.6) are expected to yield the greatest quantities of cells expressing a single sgRNA while maintaining a reasonable culture size prior to selection in hygromycin (Miles et al. 2016). This titration is performed in small scale and cells are analyzed for mCherry expression by FACS or selected for hygromycin resistance to determine the percentage of cells transduced.

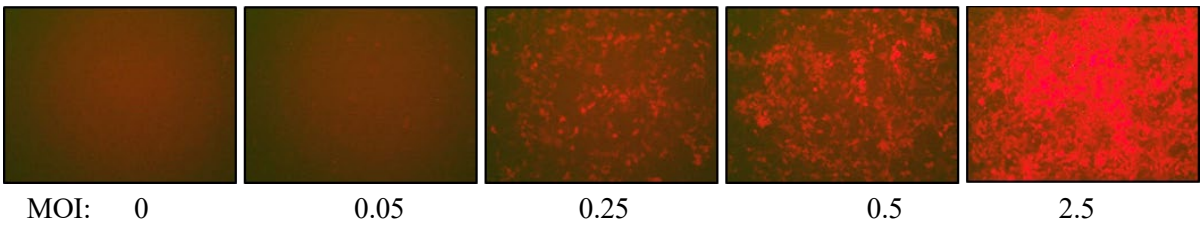


Figure 6. Relationship between sgRNA library transduction efficiency and MOI in Cas9+ A375 cells assayed for mCherry expression.

1. Plate Cas9+ target cells in a 12-well plate in complete growth medium 12–18 hr before transduction. The cells should be 50–60% confluent at the time of transduction. Do not include puromycin in your culture during this procedure.
2. Thaw an aliquot of pLVXS-sgRNA-mCherry-hyg lentiviral stock prepared in the previous protocol or use filtered virus stocks freshly prepared from packaging cells (Section VII.A).
3. Add polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).
4. Transduce target cells with different amounts of the sgRNA library lentivirus stock. We recommend using a half-log dilution range to cover from 0 to >50% transduction efficiency.

   NOTE: Use transduction conditions that will exactly mimic those to be used during the large-scale transduction (>1 x 10^8 cells).
5. Analyze transduced cells for fluorescence or re-plate in a larger culture vessel and select in hygromycin to determine your selection titer.
6. Once the amount of virus corresponding to a 30–40% transduction efficiency is established, scale up to meet the requirements of your large-scale transduction. See Table 3 below for cell numbers required for maintaining guide representation and NGS analysis.

2. Target Cell Number Determination

Achieving complete sgRNA representation in the transduced cell population requires a minimum of 200 times more cells than the complexity of the library to ensure that the guides with the lowest abundance are represented; however, we recommend a range between 400–1,000 times the number of guides. Therefore, for the Guide-it CRISPR Genome-Wide sgRNA Library containing >76,000 sgRNAs, >3 x 10^7 cells would have to be transduced.

Also, because the target MOI is in the 0.4–0.6 range, it is recommended to plate 2–3 times more cells to ensure that all cells are carrying only one sgRNA construct per cell. This would equate to ~1 x 10^8 Cas9+ cells plated for the transduction process with the goal of having >3 x 10^7 cells transduced after selection in hygromycin (see Table 3).

Table 3. Example of calculations for determining cell number and virus amounts.

Parameter	Guide-it sgRNA Library
Number of guides	76,612
Genes targeted	19,114
Cell number required for ~400X representation	~3.1 x 10^7
Cell number to transduce	~1.00 x 10^8
Transduction efficiency (MOI)	0.40
Fold coverage/sgRNA (400–1000 cells/sgRNA)	400

Moreover, it is important to culture cells properly, and in sufficient numbers so that guide representation is maintained. This is especially critical when performing screens that result in depletion of sgRNAs (negative screens) or those in which subtle increases or decreases in guide frequency need to be detected (i.e., screens other than for viability or proliferation). Therefore, it is recommended that when splitting cells during or after screening, the number of remaining cells should exceed the sgRNA count by at least 1,000X. With the Guide-it CRISPR Genome-Wide

sgRNA Library System, this would mean that a minimum of 7.6 x 10^7 cells should be retained after each split.

3. Transduction of Target Cells with sgRNA Library Lentivirus

Once the appropriate cell number and amount of virus for transduction of the sgRNA library have been determined, scale the Cas9+ target cell population to the appropriate size and transduce the cells with the sgRNA library virus. The transduction conditions must mimic exactly what was used for the titration experiment outlined above (Section VI.B) (i.e., if the titration was performed using spinoculation, the large-scale transduction must also use spinoculation).

In addition, it is also important to mimic the conditions used to establish your hygromycin kill curve for your cell line in terms of the hygromycin amount, cell density, and the time under selection (Appendix C).

VIII. Screen Recommendations

A. Positive Screens

Positive screens identify genes that are sensitive to the selection mechanism, such that when these genes are knocked out, the cells survive the selection. In this type of a screen, most cells are lost and only cells which contain sgRNAs targeting genes which make the cell sensitive to the selection agent will survive. The expected result is that the remaining cells will be enriched for these sgRNAs. In these types of screens, it is important to culture the cells long enough to allow for the loss of the edited targets and manifestation of the resulting phenotype prior to sequencing. In our experience, 10 days to 2 weeks is generally sufficient. Because these types of screens can be rather robust, a typical recommended NGS read depth is ~1 x 10^7 reads (See Section IX.E).

B. Negative Screens

Negative screens identify genes that are essential for survival under the selective pressure provided by the screening conditions. Cells expressing sgRNAs that trigger null or loss-of-function edits in these genes will be lost from the population upon application of screening conditions, such that cells expressing other sgRNAs are overrepresented. These types of screens are often more challenging, as most cells survive the screen, and parameters need to be tightly controlled to ensure that statistically significant changes can be detected. To detect subtle changes in sgRNA representation in these negative screens, NGS analysis may require read depths of up to ~1 x 10^8 reads (See Section IX.E).

IX. Sample Preparation for NGS (Using the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit)

The Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit is designed to be used for NGS library preparation in combination with the Guide-it CRISPR Genome-Wide sgRNA Library System. After conducting an sgRNA library screen, cell populations are harvested, genomic DNA (gDNA) is isolated, and sgRNA sequences are amplified from the integrated proviruses contained in the gDNA. Amplicons are then analyzed using NGS to determine which guide RNAs have increased or decreased in frequency due to the selective pressure applied in the screen. This kit enables you to perform 20 PCR reactions (~10 sequencing library preparations) and contains all the components needed to produce a sequencing-ready library, including a gDNA purification kit, primers, the PCR enzyme and buffers, and a PCR clean-up kit.

- Guide-it CRISPR Genome-Wide Library PCR Kit (Cat. No. 632351) (Not sold separately)
- NucleoBond CB 500 (Cat. No. 740509)
- NucleoSpin Gel and PCR Clean-Up (Cat. No. 740609.10) (Not sold separately)

A. Protocol: gDNA Isolation and Purification

High-yield, high-quality genomic DNA extraction is important to effectively maintain representation and amplify sgRNA sequences from cells isolated during sgRNA pooled screens. The NucleoBond CB 500 purification kit provided in the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit contains NucleoBond AXG Columns and appropriate buffers to purify high molecular weight genomic DNA from cell cultures. It is important that the DNA purification is scaled to not overload the columns which can reduce sample diversity. The population of cells should contain ~400–1,000 cells per sgRNA and population size be maintained throughout the screen (100–200 million cells) including purification of gDNA. This protocol is for purifying gDNA from up to 1 x 10^8 cells per column. Be sure to include a reference control with your screen sample.

1. Prepare working solutions one day in advance before performing the protocol:
   a. Add and dissolve Saccharose in Buffer G1. Store at 4°C.
   b. Add Buffer PB to dissolve lyophilized Proteinase K and store at –20°C.
2. Before starting the purification:
   a. Chill water (TC graded) and Buffer G1 on ice.
   b. Fast cool centrifuge to 4°C.
   c. Prepare an air incubator for 50°C incubation.
3. Harvest up to 1 x 10^8 cells from your screen as well as the reference control plates (Figure 1) in separate tubes and wash cells twice with PBS.
4. Resuspend each cell pellet in 40 ml of water.
5. Add 10 ml of G1 Buffer to each of the cell suspensions.
6. Mix the suspensions by inverting the tubes 6–8 times and incubate the mixture for 10 min on ice.
7. Centrifuge the mixtures at 4°C **(important)** for 15 min at 1,500*g*.
8. Discard the supernatant. A small white pellet will be visible.
9. Add 2 ml of Buffer G1 (ice-cold) and 6 ml of water (ice-cold) into each tube. Resuspend the pellets by vortexing for ~5–10 sec.

10. Centrifuge the mixtures at 4°C **(important)** for 15 min at 1,500*g*. Discard the supernatant.
11. Add 10 ml of Buffer G2 into each tube and completely resuspend the pellets by vortexing for 15–30 sec.
12. Add 200 µl of Proteinase K (20 mg/ml) into each tube and incubate the mixtures for 60 min at 50°C.
13. Prepare NucleoBond AXG 500 Columns on a rack. Equilibrate columns with 5 ml of Buffer N2.
14. When the 50°C incubation is complete, add 10 ml of Buffer N2 (RT) to each of the sample tubes. Vortex the mixtures for 15 sec at maximum speed.
15. Load the samples onto the column. Allow them to enter the resin by gravity flow.
16. Wash the columns with 8 ml of Buffer N3 three times.
17. Elute gDNA from each column with 8 ml of Buffer N5 into a 50-ml tube which is capable of high-speed centrifugation.
18. Add 5.6 ml or 0.7 vol of isopropanol (RT) to each tube. Mix well and incubate for 1 hr at RT.
19. Centrifuge the tubes at 9,000*g* for 35 min at 4°C.
20. Discard the supernatant, add 10 ml of 70% EtOH to each tube, and vortex briefly.
21. Centrifuge the tubes at 9,000*g* for 15 min at RT.
22. Discard supernatant completely and air dry the DNA pellets.
23. Add 2 ml of nuclease-free water to each tube and shake tubes on an orbital shaker at RT overnight. Make sure that the DNA pellets are totally soaked in water.
24. On the next day, measure O.D. to determine DNA concentration and yield. Typical yields are 300–500 µg.

B. Protocol: PCR amplification

To accurately identify sgRNAs present within the gDNA from cells isolated from pooled screens, high-quality NGS library production is essential. The TaKaRa *Ex Taq* DNA Polymerase provided in the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit combines the proven performance of Takara *Taq* polymerase with the proofreading activity of an efficient 3' to 5' exonuclease for high-sensitivity, high-efficiency PCR reactions.

The sequencing primers included in the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit contain all the features necessary for amplification and analysis of the sgRNA sequences from the gDNA, including Illumina P5 and P7 flow cell attachment sequences and primer sites for pLVXS vector amplification and Illumina sequencing. Additional features include barcodes to help with deconvolution during analysis, and primer staggering to maintain complexity when selected populations become dominated by a smaller number of sgRNAs. These features are outlined in Figure 7 below.

Name (Tube label)	Stock	Sequence (5' --> 3')
P5 0 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAAACACC*G
P5 1 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACACC*G
P5 2 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTTGTGGAAAGGACGAAACACC*G
P5 3 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTTGTGGAAAGGACGAAACACC*G
P5 4 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACTTGTGGAAAGGACGAAACACC*G
P5 6 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCACCTTGTGGAAAGGACGAAACACC*G
P5 7 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCAACTTGTGGAAAGGACGAAACACC*G
P5 8 nt index 1	10 µM	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGACCCTTGTGGAAAGGACGAAACACC*G
P7 index 2	10 µM	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTACAGCATCTGCGTTTGTCTATGGTGA*T
P7 index 3	10 µM	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTACAGCATCTGCGTTTGTCTATGGTGA*T
P7 index 4	10 µM	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTACAGCATCTGCGTTTGTCTATGGTGA*T
P7 index 5	10 µM	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTACAGCATCTGCGTTTGTCTATGGTGA*T

P5 flow cell attachment sequence, P7 flow cell attachment sequence, Barcode region, Illumina sequencing primers, Stagger region, Vector primer binding sequence, * Phosphorothioate Bond

Figure 7. Primer features in the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit. Contact technical support for this table in an excel format.

We recommend that the cycle number used for library amplification be optimized for yield without over-amplification to avoid PCR-related biases. Use the protocol below as a guide.

PCR Set-Up for Processing of gDNA (Screened Populations and Reference Control)

NOTES:

- It is recommended that gDNA obtained from screened and reference control populations are processed in parallel, as described in the following protocol.
- Refer to Figure 8 (below) for an overview of the PCR optimization process before proceeding.

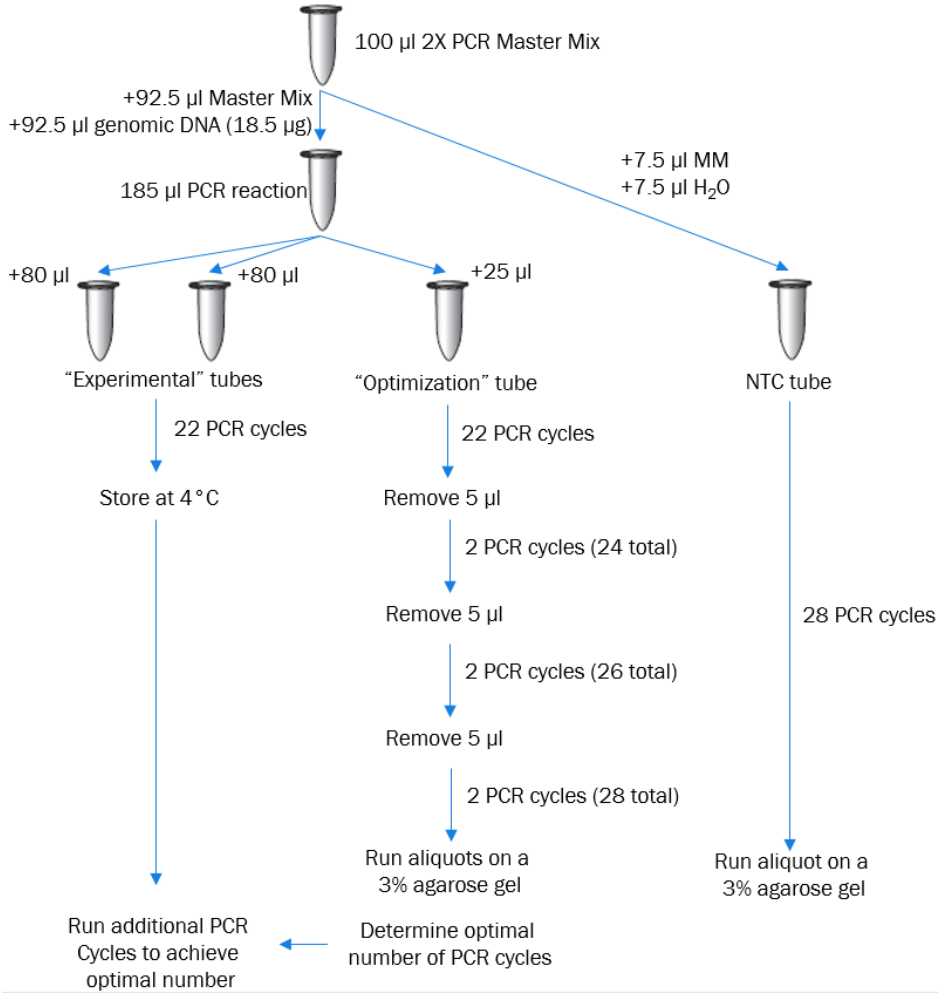


Figure 8. Optimization of PCR parameters for optimal yield during library amplification.

1. Dilute genomic DNA in nuclease-free water to a final concentration of 0.2 µg/µl.
2. Prepare PCR mix inside a PCR hood, after cleaning the surface with DNase Away and 70% Ethanol.
3. Following the recipe in the table below, prepare enough 2X master mix for amplification of gDNA obtained from your screened population(s) and reference control population in separate 1.5 ml tubes *without* adding gDNA template. Mix well by vortexing and spin the tubes briefly in a microcentrifuge.

20 µl	*Ex Taq* Buffer (Mg^{2+} plus)
16 µl	dNTP Mixture (2.5 mM each)
10 µl	P5 primer mix (10 µM)
3 µl	*Ex Taq* polymerase
10 µl	P7 primer (10 µM)
41 µl	PCR-grade water
100 µl	**Total volume**

4. To prepare a non-template control (NTC) reaction, combine 7.5 µl of the master mix with 7.5 µl of water in a separate 0.5 ml PCR tube.
5. For each gDNA sample to be analyzed (screened population[s] and reference control) prepare two "Experimental" reactions and one "Optimization" reaction as follows (see Figure 8, above, for context):
   a. Add 92.5 µl (18.5 µg) of gDNA template to a corresponding 92.5-µl volume of 2X master mix. Vortex to mix and spin briefly.
   b. Aliquot two 80-µl volumes of PCR mixture prepared in the previous step into two 0.5 ml PCR tubes (these are the "Experimental" reactions).
   c. Aliquot the remaining 25-µl volume of PCR mixture into a separate 0.5 ml PCR tube (this is the "Optimization" reaction).
6. Bring the tubes containing Experimental, Optimization, and NTC reactions to a hot-lid thermal cycler. Do not place the tubes in the wells until the block reaches 95°C.
7. Add the tubes to the thermal cycler and run the following program:

95°C	1 min	
22 cycles:		
	95°C	30 sec
	53°C	30 sec
	72°C	30 sec

8. Upon completion of 22 cycles, remove all Experimental and Optimization tubes from the thermal cycler, but leave the NTC tube on the cycler.

   NOTE: The NTC reaction will be kept on the thermal cycler for a total of 28 cycles.

9. Store the Experimental tubes at 4°C.
10. From each Optimization tube (25 µl reaction volume), transfer 5 µl of product to a clean microcentrifuge tube (for agarose gel analysis).
11. Return the Optimization tubes to the thermal cycler and run 2 additional cycles with the remaining 20 µl of PCR mixture (to a total of 24 cycles).

12. Upon completion of the 2 additional cycles, remove the Optimization tubes from the thermal cycler and transfer 5 µl of product from each Optimization tube to a clean microcentrifuge tube (for agarose gel analysis).
13. Return the Optimization tubes to the thermal cycler and run 2 additional cycles with the remaining 15 µl of PCR mixture (to a total of 26 cycles).
14. Upon completion of the 2 additional cycles, remove the Optimization tubes from the thermal cycler and transfer 5 µl of product from each Optimization tube to a clean microcentrifuge tube (for agarose gel analysis).
15. Return the Optimization tubes to the thermal cycler and run 2 additional cycles with the remaining 10 µl of PCR mixture (to a final total of 28 cycles).
16. Analyze 2 µl of PCR product from each stopping point for the Optimization reaction (22, 24, 26, and 28 cycles, respectively) and the 28-cycle NTC reaction on a 3% agarose gel in 1X TAE along with 500 ng of 1 kb DNA Ladder. See Figure 9, below, for an example.
17. Confirm the expected band size for the PCR product: ~310 bp for gDNA samples, there should be no bands for the NTC.

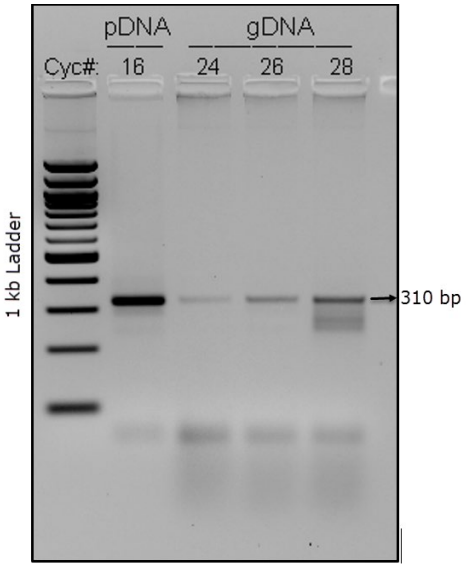


Figure 9. Example of agarose gel from sgRNA target amplification during PCR cycle optimization.

18. Determine the optimal cycle numbers for further processing of the Experimental reactions (for example, in Figure 9, the 24-cycle Optimization reaction provided sufficient yield for purification and sequencing without background).

    NOTE: It is possible that the optimum number of PCR cycles will differ for gDNA samples obtained from the screened and reference control populations.

19. Once the optimal PCR cycle numbers for the various samples are identified, remove the tubes containing the 22-cycle Experimental reactions from 4°C storage, and, if necessary, transfer them back to the thermal cycler and run additional cycles until optimal numbers of cycles are performed for each sample type.

    NOTE: Don't forget to halt the PCR reaction and remove samples, as needed, if the optimum number of cycles differs for gDNA samples obtained from the screened and reference control populations.

20. Once any additional cycles are completed, pool the Experimental PCR products for each sample type, respectively, and analyze 2 µl of each pooled PCR product on a 2% agarose gel in 1X TAE buffer, along with 500 ng of a 1 kb DNA Ladder.
21. Confirm that PCR products for each Experimental sample are consistent with the results for the Optimization samples, and suitable for further processing and analysis.

C. Protocol: PCR Product Purification and Quantitation

Load the entire PCR product (~158 µl) for each sample from Section B, Step 17 above, on a 2% agarose gel. Excise DNA fragments of the desired size (~310 bp) from the gel and proceed to column purification.

DNA cleanup and quantitation

1. Purify the desired DNA fragments using the NucleoSpin Gel and PCR Clean-Up kit according to its user manual.
2. Elute DNA in 20 µl of NE Buffer.
3. Add 2 µl of purified DNA to 98 µl of nuclease-free water to make a 1:50 diluted sample.
4. Measure DNA concentrations using the Qubit dsDNA HS Assay Kit.
5. Dilute a small portion of the samples to 0.5 ng/µl. Analyze 1 µl (0.5 ng) on an Agilent High Sensitivity DNA Chip.
6. Expected results: a dominant DNA peak with an approximate size of 310 bp (see Figure 10).

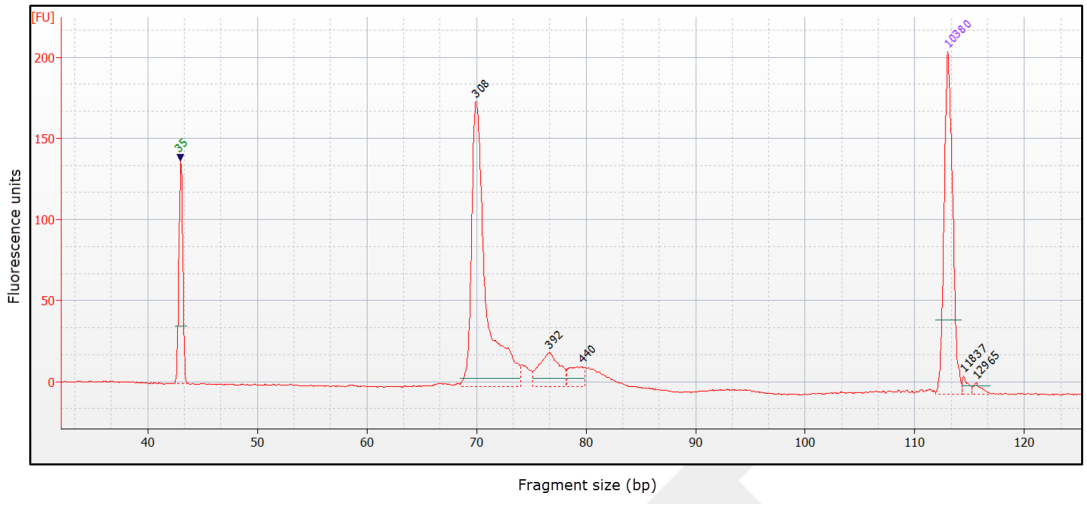


Figure 10. Example of bioanalyzer plot for sgRNA target amplified from gDNA from transduced cells.

D. Protocol: NGS Library Preparation

We have performed NGS of pooled amplified sgRNAs from the Guide-it CRISPR Genome-Wide sgRNA Library System on both the Illumina® NextSeq500 and MiSeq sequencers. The protocol outlined below is for the MiSeq instrument using the MiSeq Reagent Kit v3 (150 cycles) and provides up to 25 million reads, which provides ample coverage for a single library sample.

Preparing the library sample:

1. Dilute a small portion of each library sample to 0.8 ng/µl (4 nM) in nuclease-free water to a total volume of at least 5 µl in a 1.5 ml DNA LoBind tube. Set on ice.

2. In a fresh 1.5 ml tube, add 2 µl of 2 M NaOH to 18 µl of nuclease-free water to adjust the final concentration to 0.2 M.
3. Combine 5 µl of 4-nM library sample with 5 µl of 0.2 M NaOH in a 1.5 ml DNA LoBind tube. Vortex briefly to mix.
4. Incubate for 5 min at RT to denature the sample.
5. Add 990 µl of HT1 to the denatured sample to make a total volume of 1,000 µl. This results in 20 pM denatured DNA in 1 mM NaOH.
6. Further dilute the denatured sample in HT1 to a final concentration of 13 pM with ~5% PhiX Control DNA spike-in to aid in increasing complexity of the sample:

618 µl	Denatured library DNA (20 pM)
32 µl	Denatured PhiX Control (20 pM)
350 µl	Pre-chilled HT1
1,000 µl	**Total volume**

7. Keep sample on ice until ready to load.
8. Load 600 µl of the sample into cartridge.

E. Analysis

Once sequencing is completed, reads must be trimmed using software (such as cutadapt) so that the sgRNA sequence (20 bp) can be mapped to the reference library or controls and the fold-change determined. Figure 11 depicts the sgRNA library amplicon and its reference sequences that can be used for this purpose.

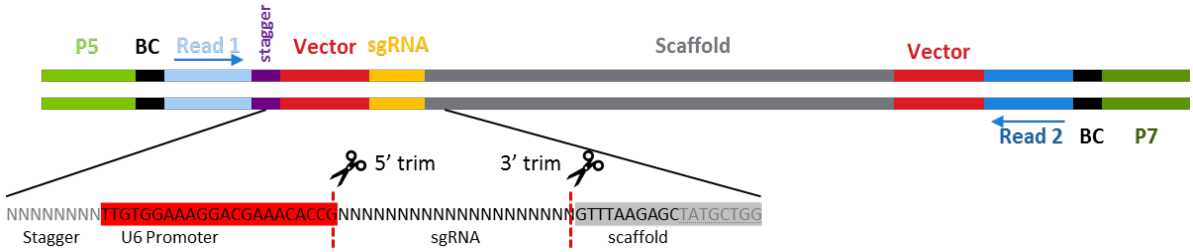


Figure 11. Structural features of final libraries generated with the Guide-it CRISPR Genome-Wide sgRNA Library NGS Analysis Kit. The adapters added contain sequences allowing clustering on any Illumina flow cell (P5 shown in light green, P7 shown in dark green), Illumina TruSeq® indexes in black, and the regions recognized by sequencing primers Read Primer 1 (Read 1, light blue) and Read Primer 2 (Read 2, dark blue). In addition, the 5′ primer pool contains additional bases to aid in providing complexity. Sequences that can be used to aid in trimming are identified.

Based on data from our screens as well as others, >1 x 10^7 reads should be sufficient to adequately sequence a complex library of ~1 x 10^5 sgRNAs at the plasmid or baseline infected cell population level. For a positive selection screen under strong selective pressure, useful results can be obtained with a few million reads. For negative selection screens, however, most cells survive and therefore changes in representation may be subtle. This may require much deeper sequencing of >1 x 10^8 reads.

For a standard screen in which sgRNA activity results in cytotoxicity, it is good practice to use the transduced, unselected line (reference control) as a baseline control. The baseline cell population will take into account the original plasmid representation used to make the virus as well as the representation achieved post transduction. We have found that the frequency distributions of sgRNAs encoded in the

plasmid library are virtually identical to the distribution observed in efficiently transduced cell line when gDNA is harvested soon after transduction.

X. Secondary Screens

Once analysis of the sequencing data from a screen has been completed, it is necessary to validate any hits identified using an assay similar to the original screen. The Guide-it CRISPR Genome-Wide sgRNA Library System includes the pLVXS-sgRNA-mCherry-hyg Vector into which sgRNAs that provided hits can be cloned and used to create isogenic lines. These cells can then be used in downstream secondary screens to separate true-positive hits from false-positive hits.

A. Cloning of Candidate sgRNAs into the pLVXS-sgRNA-mCherry-hyg Vector

1. Preparing the pLVXS-sgRNA-mCherry-hyg Vector for Cloning

The lentiviral backbone used in this library, pLVXS-sgRNA-mCherry-hyg, contains two BsmBI restriction sites. These sites are used to linearize the vector in order to clone a candidate sgRNA.

1. Set up the vector digest as follows:

4 µl (2 µg)	pLVXS-sgRNA-mCherry-hyg Vector DNA
2.5 µl	10X Buffer
1 µl	BsmBI-v2
17.5 µl	Water
25 µl	**Total volume**

2. Incubate at 55°C for 8 hr. Then heat inactivate at 80°C for 20 min.
3. Analyze 2 µl of the digest on a 1% agarose/ TAE gel along with a 1 kb DNA Ladder to confirm complete digestion. Expected band sizes are 9,064 bp and 30 bp.
4. Run the remaining digest (~23 µl) on 1% agarose/ TAE gel. Excise the desired DNA fragment (9,064 bp) from the gel and proceed to column purification using the NucleoSpin Gel and PCR Clean-Up kit.
5. Measure O.D. to determine DNA concentration.
6. Adjust final DNA concentration to 7.5 ng/µl.

2. Designing and Ordering Oligos

To clone your sgRNA into the linearized pLVXS-sgRNA-mCherry-hyg Vector, you must order or synthesize a pair of oligos corresponding to the target-specific sequence of the sgRNA of interest, including short 5′ overhang sequences complementary to the vector backbone. Only the 20-nucleotide sequence corresponding to the target needs to be cloned into the linearized pLVXS-sgRNA-mCherry-hyg vector; the vector already contains the sgRNA scaffold sequence.

1. The sense oligo (Oligo 1) corresponds to the 20-nucleotide sgRNA sequence chosen from the sgRNA library Master Sheet, plus the 5′ overhang sequence, cacc. Do **not** include the PAM sequence. The sgRNA library Master Sheet can be obtained on our website at takarabio.com/genomewidelibrary.

   Oligo 1: 5′-cacc XXX XXX XXX XXX XXX XXX XX-3′

2. The antisense oligo (Oligo 2) is the complementary sequence of Oligo 1, plus the 5′ overhang sequence, aaac.

   Oligo 2: 5′-aaac YY YYY YYY YYY YYY YYY YYY-3′

NOTE: If the first base at the 5′ end of the sgRNA sequence (Oligo 1) is not a guanine, it is necessary to add an extra guanine at this end, as shown below:

Oligo 1: 5′-cacc **G**XXX XXX XXX XXX XXX XXX XX-3′

Oligo 2: 5′-aaac YY YYY YYY YYY YYY YYY YYY**C**-3′

3. Protocol: Annealing Oligos

1. Resuspend each target oligo completely in TE buffer or molecular biology-grade, nuclease-free water such that the concentration is 100 µM. Prepare 100 ml Oligo Annealing Buffer (10 mM Tris, 20 mM NaCl, adjust pH to 8.0 with HCl). If you wish, you can store the excess Oligo Annealing Buffer at
   –20°C for future use.

2. Mix the oligos in a 200-µl PCR tube as follows:

1 µl	Oligo 1 (100 µM)
1 µl	Oligo 2 (100 µM)
8 µl	Oligo Annealing Buffer
10 µl	**Total volume**

NOTE: The concentration of each of the oligos is now 10 µM.

3. Denature the oligos at 95°C using a thermal cycler, and then reanneal by slowly reducing the temperature according to the following cycling conditions:

95°C	2 min
15 min slope from 95°C to 25°C	
25°C	Forever

4. Mix 1 µl of the annealed oligo solution with an additional 99 µl of Oligo Annealing Buffer to make a 100 nM (fmol/µl) solution.

5. Store the annealed oligos at –20°C until use.

4. Protocol: Cloning the sgRNA Targeting Sequence Into pLVXS-sgRNA-mCherry-hyg Vector

1. Thaw the necessary reagents at room temperature and set up the reaction as follows:

2 µl	Linearized pLVXS-sgRNA-mCherry-hyg Vector(7.5 ng/µl)*
3 µl	Target-specific annealed oligos (100 fmol/µl)†
5 µl	DNA Ligation Mighty Mix
10 µl	**Total Volume**

*From restriction digest above
†From Step 5 above

2. Incubate the reaction mix at 16°C for 30 min.

3. Meanwhile, thaw one vial of Stellar Competent Cells on ice.

4. Add the entire 10-µl ligation mixture to the competent cells and mix gently by tapping.
5. Allow the mixture to stand on ice for 30 min.
6. Heat-shock the cells at 42°C for 45 sec and immediately place on ice. Incubate for 2 min.
7. Add 1 ml of SOC medium and incubate at 37°C for 1 hr with vigorous shaking.
8. Plate an appropriate amount of the culture on pre-warmed (37°C) LB plates containing ampicillin (final concentration 100 µg/ml).
9. Incubate the plates at 37°C overnight.
10. Pick single colonies and inoculate into an appropriate amount of LB medium containing ampicillin (final concentration 100 µg/ml).
11. Incubate with shaking overnight at 37°C.
12. Purify your plasmid DNA from the competent cells. We highly recommend NucleoSpin Plasmid (Takara Bio, Cat. No. 740588.50) for rapid, high-yield, and high-purity purification. For transfection-grade plasmid preparation, use NucleoBond Xtra Midi (Takara Bio, Cat. No. 740410.10) or NucleoBond Xtra Maxi (Takara Bio, Cat. No. 740414.10).
13. Perform sequencing analysis according to your preferred sequencing protocol.

B. sgRNA Virus Production

For production of lentiviruses encoding candidate sgRNAs, we recommend using Lenti-X Packaging Single Shots (VSV-G) (Cat. No. 631275), which provide an extremely simple and consistent one-step method for producing high-titer lentivirus. Follow the Lenti-X Packaging Single Shots Protocol-At-A-Glance.

C. Transduction for Secondary Screening

Once individual lentiviruses are produced for each of the candidate sgRNAs identified in the primary screen, these lentiviruses can then be used to transduce the Cas9 expressing cell line used in the primary screen in individual wells. These Cas9+/sgRNA+ cells are then selected on hygromycin to ensure all cells are positive for the sgRNA and to allow the target sequence to be cut and reduction/elimination of protein expression. The cells are then screened under the same conditions as the primary screen. Individual editing results can be analyzed/confirmed using the Mutation Detection Kit (Cat. No. 631448) for identifying editing efficiency or identification of actual indels using the Guide-it Indel Identification Kit (Takara Bio, Cat. No. 631444).

XI. References

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Appendix A. Troubleshooting Guide

Table 4. Troubleshooting Guide for the Guide-it CRISPR Genome-Wide sgRNA Library System.

Problem	Possible Explanation	Solution
A. Lenti-X 293T packaging cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure described in Section V. C, and/or consult the Lenti-X 293T Cell Line Protocol-At-A-Glance.
	Incorrect culture medium	Use DMEM with additives listed in Section III. C. Use 10% Tet System Approved FBS (Tc-free).
	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Slow growth	Incorrect culture medium	Use DMEM with additives listed in Section III. C. Use 10% Tet System Approved FBS (Tc-free).
Cells do not attach to plate.	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Cells appear morphologically different.	Passage of cell culture is too high (old cells)	Thaw/purchase new aliquot of Lenti-X 293T cells.
B. Virus production		
Poor transfection efficiency (as determined by GOI or marker expression in the Lenti-X 293T cell line)	Cells plated too densely	Plate 4–5 x 10^6 cells/100 mm plate, or fewer if the cells divide rapidly. Use at 50–80% confluency.
	Transfection is toxic to cells	Use the optimized conditions specified in the Lenti-X Packaging Single Shots Protocol-At-A-Glance.
	Cells analyzed too soon after transfection	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<10^5 cfu/ml)	Serum in medium contains tetracycline contaminants	Use **Tet System Approved FBS** (Cat. Nos. 631101, 631105, 631106 & 631107) in the 293T culture medium.
	Poor transfection efficiency	See section above for poor transfection efficiency. Concentrate the virus using centrifugation or use the **Lenti-X Concentrator** (Cat. Nos. 631231 & 631232) to increase your available titer up to 100-fold without ultracentrifugation.
	Virus was harvested too early	Harvest virus 48–72 hr after the start of transfection.

Problem	Possible Explanation	Solution
	Polybrene is missing or at a suboptimal concentration	Add polybrene (4 µg/ml) during transduction or optimize the concentration (2–12 µg/ml).
	Virus was exposed to multiple freeze-thaw cycles	Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaw cycles.
	Suboptimal selection procedure during titration	Perform an antibiotic kill curve on the cell line prior to using it for titration.
C. Transduction of target cells		
Poor transduction efficiency	Low titer	See Section VI. B or use the Lenti-X Concentrator (Section IV. C) to increase your available titer up to 100-fold without ultracentrifugation.
	Transduction protocol not optimized	See the Lenti-X Lentiviral Expression Systems User Manual for references to help with optimizing transduction protocols.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection.
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin reagent (Cat. No. T100B) and the RetroNectin-Bound Virus transduction protocol.
		Excessive exposure to polybrene: optimize amount (titrate) or shorten time of exposure to viral supernatant.
	Viral supernatant contains transduction inhibitors	Use RetroNectin reagent or RetroNectin reagent-coated plates in the RetroNectin-Bound Virus transduction protocol, which allows virions to bind the RetroNectin substratum and be washed free of inhibitors prior to target cell infection.
Infection is toxic to target cells	MOI too high (i.e., too much virus used)	Titrate the virus; dilute virus stock.
	Polybrene toxicity	Reduce or optimize polybrene concentration; reduce infection time.
	Packaging cell supernatant or medium is toxic to cells	Dilute virus stock using target cell culture medium; harvest virus from packaging cells using target cell medium.
Cells die/grow slowly after transduction with Cas9 lentivirus	Cells are sensitive to Cas9 expression	Titrate Cas9 lentivirus on target cells to ensure one copy per cell or choose an alternate cell line that is less sensitive to Cas9 expression.

D. Genomic DNA isolation/PCR		
Low yield during gDNA purification	Overloaded column	Do not exceed the maximum cell number for the AXG500 column (~1x10^8 cells); use multiple columns for more cells.
	Lost gDNA pellet	Handle with care
Additional bands present after amplification	Cycle number not optimized	Optimize cycle number
Low/no yield in PCR	Cycle number not optimized	Optimize cycle number
E. Library		
Low editing efficiency	Low Cas9 expression	Titrate Cas9 lentivirus to ensure highest possible expression that is not detrimental to cell health/viability; test with sgRNA with known editing efficiency.
	Transduction protocol not optimized	Use a small portion of the lentivirus library to optimize transduction efficiency in target cells.
Low representation in selected population	Transduction protocol not optimized	Use a small portion of the lentivirus library to optimize transduction efficiency in target cells.
	Over passaging of transduced population	Over culturing of the Cas9+/sgRNA+ population can lead to a bias in the starting population. Perform screen as soon as cells have reached the appropriate number for selection.

Appendix B. Vector Information

For complete descriptions of the vectors provided with each system, refer to the respective Certificate of Analysis available at takarabio.com.

Appendix C. Establishment of Puromycin and Hygromycin Kill Curves

Prior to using G418, hygromycin, or puromycin to establish stable and double-stable cell lines, it is important to titrate your selection agent stocks to determine the optimal concentration for selection with the particular host cell line being tested. This is also important because of lot-to-lot variation in the potency of these drugs. Therefore, you should titrate each new lot of antibiotic to determine the optimal concentration. We recommend that you perform two experiments for each drug: (1) a titration to determine the optimal drug concentration, and (2) an experiment to determine the optimal plating density. This step is recommended even if you are using premade cell lines.

Titrate at fixed cell density:

1. Plate 2 x 10^5 cells each in six 10-cm tissue culture dishes containing 10 ml of the appropriate complete medium plus varying amounts (0, 50, 100, 200, 400, 800 µg/ml) of hygromycin or G418. For puromycin, add the drug at 0, 1, 2.5, 5, 7.5, and 10 µg/ml.

2. Incubate the cells for 10–14 days, replacing the selective medium every four days (or more often if necessary).
3. Examine the dishes for viable cells every two days.

For selecting stable transformants, use the lowest concentration that begins to give massive cell death in ~5 days and kills all the cells within two weeks. Also see Section III. B for recommended starting dosages.

Determine optimal plating density:

Once you have determined the optimal drug concentration determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluency before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, large cells that double rapidly have a lower optimal plating density than small cells that double slowly.

1. Plate cells at several different densities in six 10-cm tissue culture dishes containing 10 ml of the appropriate selective medium. Suggested densities (cells/10-cm dish): 5×10^6, 1×10^6, 5×10^5, 2×10^5, 1×10^5, and 5×10^4.
2. Incubate the cells for 5–14 days, replacing the selective medium every four days.
3. Examine the dishes for viable cells every two days.
4. For selecting stable transformants, use a plating density that allows the cells to reach ~80% confluency before massive cell death begins (at about day 5). This is the cell density at which cells should be plated for selection of stable transformants.

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