Chapter 7

Production and Validation of Lentiviral Vectors for CRISPR/Cas9 Delivery

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Abstract

Genetic information transferred by HIV-1-based lentiviral vectors as single-stranded RNA is converted to double-stranded DNA by reverse transcription and subsequently inserted into the genome of recipient cells. Integration into the genome allows stable, long-term expression of genes-of-interest driven by promoter sequences contained within the vector. This technology can be used as a standard method for production of cells stably expressing Cas9 protein and single guide RNA (sgRNA), the key components of the CRISPR genome editing system. Here, we provide a protocol for production and validation of VSV-G-pseudotyped lentiviral vectors for delivery of the CRISPR system and generation of knockout cell lines.

Key words CRISPR/Cas9, Lentiviral, sgRNA, Genome-wide screening, Forward genetics

1 Introduction

Human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vectors, originally developed by Naldini and coworkers [1, 2], remain powerful carriers of genetic information. Several recent reports describe the effective use of lentiviral vectors for therapeutic gene transfer, including gene delivery to hematopoietic stem cells for treatment of immunodeficiences and leukodystrophies [3–6], but for many researchers, lentiviral vector systems are key tools in everyday studies of molecular biology and genetics. Also for genome editing and delivery of the components of the CRISPR/Cas9 system, lentiviral vectors have become cherished vehicles.

Already in the 1990s, shortly after the invention of lentivirus-based vectors, initial biosafety concerns induced the development of safer lentiviral vector systems, resulting in the third-generation lentiviral vector system [7], which is essentially the system currently used in many laboratories. This system features a chimeric long terminal repeat (LTR) containing a constitutive promoter upstream of the vector transcript. In a later version of this vector, the U3 region in the downstream LTR was partially deleted, facilitating

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formation of proviral DNA devoid of HIV-1-derived promoter elements [8, 9]. These vectors, referred to as "self-inactivating" (SIN) lentiviral vectors, allowed integration of vector-derived DNA with expression of the transgene driven strictly by an internal promoter of choice. Early on, cell lines for production of lentiviral vectors were developed [10], but state-of-the-art lentiviral vector production today is based on transfection of four plasmids (three packaging constructs and a transfer vector) into easy-to-transfect HEK293T cells. Here, we provide a protocol for production and validation of lentiviral vectors transferring *Streptococcus pyogenes* SpCas9 and single guide RNA (sgRNA) expression cassettes.

2 Materials

2.1 Production of Lentiviral Vectors

1. Lentiviral packing and transfer plasmids (Addgene IDs: 12253, 12251, 12259)
2. 2.5 M $CaCl_2$.
3. 2× Hepes Buffered Saline (2×HBS), pH 7.2.
4. dH_2O.
5. HEK293T cells.
6. 15 mL falcon tubes.
7. p15 dishes.
8. 0.45 μM filter.
9. Centrifuge tubes.
10. General reagents and appliances for cell culturing.
11. Ultracentrifuge (We run the samples in a SW28 rotor using the Beckman coulter centrifuge tubes, reference number: 358126).
12. PBS.
13. Parafilm.

2.2 Titration of Lentiviral Particles

1. General reagents and appliances for cell culturing.
2. qPCR master mix.
3. qPCR primer and probe sets for amplification of the Woodchuck Hepatitis Virus post-regulatory element (WPRE) and segments of the albumin gene.
4. pAlbumin available from Addgene (www.addgene.org; Addgene ID: 22037).
5. 96-well optical plate.
6. Optical film.
7. Lightcycler Real-Time PCR system.

8. Antibiotic (depending on the resistance gene in the transfer plasmid).
9. 0.6% methylene blue solution.
10. Regular H_2O.
11. Optional: polybrene.

2.3 Generation of Knockout Cell Lines Using CRISPR/Cas9

1. pLentiCas9-blast [11], pLentiGuide-Puro [11], and pLenti-CRISPR v2 [11] available from Addgene (www.addgene.org; Addgene IDs: 52962, 52963, 52961).
2. Blasticidin (final concentration suitable for your cell line).
3. Puromycin (final concentration suitable for your cell line).
4. General reagents and appliances for cell culturing.

2.4 Assessment of CRISPR Gene Disruption Efficiency by TIDE Analysis

1. Reagents for PCR.
2. Primer set to amplify a fragment encompassing the sgRNA target region.
3. 1% agarose gel.
4. General equipment to run an agarose gel.
5. Gel extraction kit.
6. Barcodes to send samples for sequencing, or in-house Sanger-sequencing equipment.

3 Methods

3.1 Production of Lentiviral Vectors

Initially you will need HEK293T cells and an estimate of the yield of lentiviral vectors required for a certain application. You will also have to consider whether or not you will need to further purify and concentrate viral particles harvested in growth medium from transfected HEK293T cells (we refer to this harvest as a "crude" preparation) by ultracentrifugation.

The production of lentiviral vectors takes 4–6 days depending on the number of harvests from transfected producer cells and whether you will need to up-concentrate the vector preparation by ultracentrifugation.

This protocol below is based on production of lentiviral vectors in p15 dishes. You may want to scale it down and use p10 dishes (*see* **Note 1**).

1. Day 1, seed 1×10^7 HEK293T cells per p15 dish in 25 mL medium.
2. Day 2, change medium on the HEK293T cells (25 mL DMEM) 1 h prior to transfection.
3. Prepare the transfection mix according to Table 1 (*see* **Note 2**).

Table 1
Amount of each plasmid used for preparing the transfection mix

Plasmid	per p15 dish (μg)
pRSV-REV	7.26
pMD.2G	9.07
pMDIg/p-RRE	31.46
Transfer plasmid	31.46

Co-transfect the HEK293T cells with the lentiviral packaging and transfer plasmids. We use the third generation lentiviral packing plasmids available at Addgene (www.Addgene.org). Names of the plasmids that we use and plasmid amounts are provided in Table 1.

4. Add dH_2O to the pDNA mix to a final volume of 1089 μL and mix.
5. Add 121 μL 2.5 M $CaCl_2$ and mix by pipetting up and down.
6. Add 1210 μL 2×HBS buffer to the pDNA-$CaCl_2$ solution.
7. Vortex the solution thoroughly (10–15 s) immediately after adding the 2×HBS.
8. Incubate the transfection mix at room temperature for 15 min (*see* **Note 3**). Add the 2.42 mL transfection mix dropwise to the cells and gently swirl the dish to distribute the transfection mix.
9. Day 3, remove the medium from the p15 dish and add 19 mL fresh medium (*see* **Note 4**).
10. Check the transfection efficiency (optional): on day 3 you can evaluate the transfection efficiency by fluorescence microscopy. If you estimate the transfection efficiency to be below 75%, consider starting over. Examples of GFP expression 24 and 48 h after successful transfection with pCCL/PGK-eGFP as the transfer plasmid are provided in Fig. 1.
11. Day 4, pool the supernatant from the p15 dishes if you have more than one dish transfected with the same pDNA mix.
12. If you are planning on doing a second harvest on day 5, add 19 mL fresh medium and incubate for another 24 h (*see* **Note 5**).
13. Filter the pooled supernatant through a 0.45 μm filter (*see* **Note 6**).

3.2 Concentration by Ultracentrifugation

1. Divide the supernatant equally between an appropriate number of centrifuge tubes (aliquote approximately 24 mL per tube leaving space for the sucrose solution).

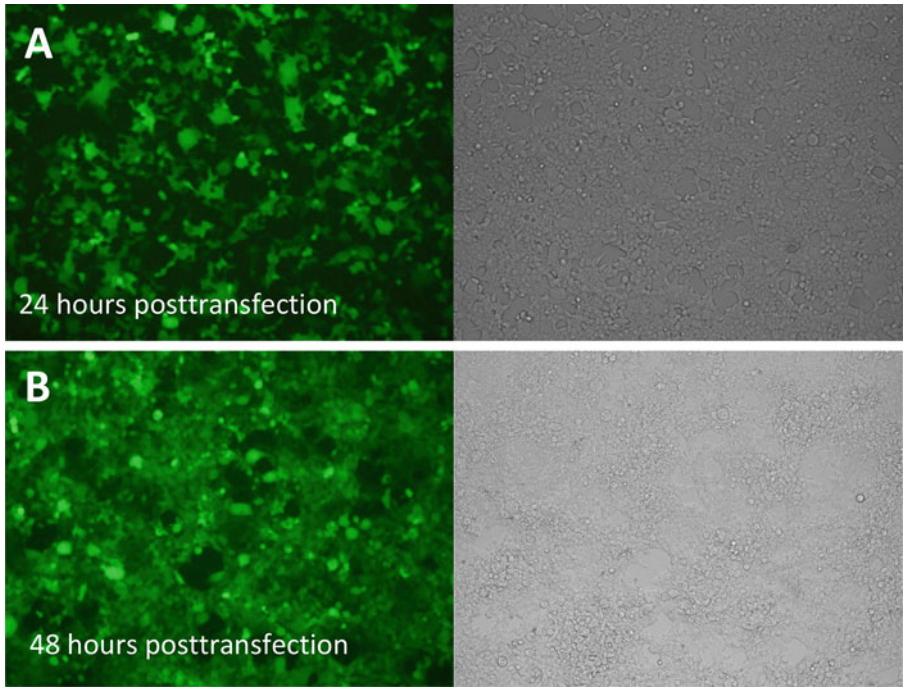

Fig. 1 Expected level of GFP expression 24 and 48 h after DNA transfection. HEK293T cells were seeded in a p15 dish and transfected with the plasmids indicated in Table 1 including pCCL/PGK-eGFP as the transfer plasmid. GFP expression, in this case driven by the phosphoglycerate kinase promoter (PGK), was assessed 24 (**a**) and 48 (**b**) hours after transfection by fluorescence microscopy. Right panels show the same area using light microscopy

2. Slowly add 4 mL sucrose solution to the bottom of the centrifuge tubes using a serological pipette (*see* **Note 7**).
3. Weigh the centrifuge tubes and adjust the volumes to ensure balance in the centrifuge.
4. Ultracentrifuge for 2 h at 113,000 × *g* at 4 °C.
5. After centrifugation, discard the supernatant and let the tubes stand upside down for 2 mins.
6. Remove any remaining supernatant from the tube.
7. Add the volume of $PBS^{-/-}$ corresponding to the desired fold up-concentration (*see* **Note 8**).
8. Cover the centrifuge tubes with parafilm and let the virus pellet resuspend overnight at 4 °C.
9. Repeat the protocol for supernatant from day 5 and discard the cells.
10. Pool the virus particles from the first and second harvest, make aliquots in appropriate sizes to avoid repeated thawing and freezing, and store the aliquots at −70 to −80 °C until use.

3.3 Titration of Lentiviral Particles by qPCR

A viral titer is a numerical expression of the quantity of viral units in a given volume and is usually either a physical titer or a transductional (functional) titer. Physical titers can be estimated by p24 ELISA or qPCR on viral RNA. These assays are generally faster to perform than assays used to determine a transductional titer. However, the assays used to determine a physical titer tend to overestimate the titer, as they measure both functional and defective viral particles. Transductional titers are based on serial transductions of the target cells and indicate the amount of infectious virus particles in a given volume. These assays are in general more laborious than the ones used to determine physical titer. However, they are considered to be more accurate and are specific for the cell type in which the assay is performed. In this protocol, we present two ways to determine the transductional titer of a preparation of lentiviral vectors.

Assessing transductional titer by qPCR is a viable option in both suspension and adherent cells. The protocol that we use is adapted from a protocol developed by Didier Trono's lab (https://tronolab.epfl.ch/) and utilizes qPCR to determine the titer of a lentiviral vector batch. This titer assay is based on qPCR measurement of the number of lentiviral DNA copies integrated into the genome of the target cells plus potential episomal DNA intermediates. The number of integration-competent particles in the virus batch can be calculated by normalizing to an endogenous control gene with two copies per cell.

In this set up, the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE), present in most third-generation lentiviral vectors, is used to determine the number of integrated lentiviral DNA copies, and the gene encoding albumin is used as the endogenous control gene.

1. Day 1, seed 1×10^5 target cells per well in a 6-well dish or in another culture format. Prepare one well for each dilution of the vector batch and one extra well for a non-transduced control (*see* **Note 9** if the targets cells are cultured in suspension).
2. Day 2, transduce the target cells with appropriate dilutions of the vector batch (*see* **Note 10**). If the target cells can tolerate polybrene, it is possible to supplement with 8 μg/mL polybrene (final concentration) to increase the transduction efficiency.
3. Count the number of cells in the non-transduced well and re-seed them. The number of cells is used to calculate the titer after the qPCR has been performed; re-seeded cells serve as a control in the qPCR reaction.
4. Day 3, change medium on the transduced cells and on control cells.

Table 2
Sequences of primers and probes used for qPCR

Target sequence	Primer/probe	Primer/probe sequence
WPRE	WPRE_forward	5′-GGCACTGACAATTCCGTGGT-3′
	WPRE_reverse	5′-AGGGACGTAGCAGAAGGACG-3′
	WPRE_probe	5′-FAM-ACGTCCTTTCCATGGCTGCTCGC-BHQ-3′
Albumin	Albumin_forward	5′-GCTGTCATCTCTTGTGGGCTGT-3′
	Albumin_reverse	5′-ACTCATGGGAGCTGCTGGTTC-3′
	Albumin_probe	5′-FAM-CCTGTCATGCCCACACAAATCTCTCC-BHQ-3′

5. Day 6, extract genomic DNA (gDNA) from each individual well using your preferred protocol.
6. Elute gDNA with dH_2O.
7. Store gDNA at −20 °C until use.

3.4 Quantification of WPRE in Proviral DNA

The primer and probe sets for the qPCR are presented in Table 2. The WPRE primer/probe set is used for quantification of the WPRE sequence, which is contained in the vector and thus inserted into the genome of the target cells after lentiviral transduction. The albumin gene primer/probe set is used for quantification of the endogenous albumin gene, which is used to normalize for the amount of gDNA present in the qPCR reaction. The albumin primer/probe set is specific for the human albumin gene, so if your target cells have nonhuman origin, an alternative primer/probe set should be used. The standard curves for WPRE and the albumin gene are made using plasmids containing WPRE and albumin gene sequences (*see* **Note 11**).

1. Adjust the DNA concentrations of the two plasmids containing the WPRE and the albumin gene sequence to 1 μg/μL.
2. The first point of the standard curves should be 1×10^8 molecules in 8 μL corresponding to 1.25×10^7 molecules per μL. Adjust the concentration of the two plasmids for the standard curves accordingly (*see* **Note 12**).
3. Generate the standard curves by making tenfold dilutions until 10 molecules in 8 μL (8 serial dilutions in total).
4. Prepare the gDNA samples. The gDNA sample amount in each qPCR reaction should be between 50 and 100 ng in 8 μL (6.25–12.5 ng/μL).
5. Prepare two master mixes (Table 3), one for quantification of WPRE and one for quantification of the albumin gene according to the following protocol. All samples and standards should be run in technical duplicates as a minimum.

Table 3
Table guide for preparing Maxima qPCR master mix

8.5 μL Maxima qPCR master mix or equivalent
0.17 μL forward primer (10 μM)
0.17 μL reverse primer (10 μM)
0.17 μL probe (10 μM)

6. Place 9 μL of the master mix into the appropriate number of wells in a 96-well optical plate.
7. Add 8 μL DNA sample to each of the appropriate wells.
8. Seal the plate with optical film.
9. Centrifuge the plate at 200 × *g* for 1 min at room temperature to ensure that all liquid is at the bottom of the wells.
10. Place the 96-well plate in the real-time PCR machine and run the appropriate program depending on the fluorochromes (FAM) and quencher (BHQ) used in the TaqMan probes. Run the amplication reaction as follows: Preincubation for 10 mins at 95 °C followed by 45 cycles: 15 s at 95 °C, 1 min at 60 °C.
11. Analyze the amplification using the standard curve method.
12. Calculate for each sample the WPRE copy number per cell using the following formula:

$$\text{Vector copy number (per cell)} \frac{\text{Quantity mean of WPRE sequence}}{\text{Quantity mean of albumin gene}} \times 2$$

(*see* **Note 13**).

13. Calculate the transduction titer using the formula below.

$$\text{Titer}\left(\frac{\text{Integrating particles}}{\text{mL}}\right) = \frac{\text{Number of recipient cells (counted at day 2)} \times \text{Vector copy number per cell}}{\text{Volume of virus in mL}}$$

3.5 Titer Determination of Lentiviral Vectors by Quantification of Colony-Forming Units (CFU)

Another way to determine the number of infectious particles in a lentiviral vector batch is to do a colony-forming units (CFU) assay. Obviously, this method is not an option in suspension cells, but provides a less work-intensive method of assessing the transductional titer on adherent cells. This assay requires that the transfer plasmid contains a selection cassette, which is inserted into the genome of the target cells after lentiviral transduction. If your lentiviral transfer plasmid does not contain a selection cassette, measurement of titer should be performed with qPCR.

1. Day 1, seed 1×10^5 target cells per well in a 6-well dish or in another culture format. Seed one well for each dilution of the vector batch and include an extra well for a non-transduced control for the drug selection.

2. Day 2, transduce the target cells with serial tenfold dilutions of the lentiviral vector batch. If the target cells can tolerate polybrene, it is possible to supplement with 8 μg/mL polybrene (final concentration) to increase the transduction efficiency. Leave one well un-transduced.
3. Day 3, change medium on the target cells.
4. Day 4, start selection for drug-resistant cells at a concentration suitable for the target cells (*see* **Note 14**).
5. When colonies have appeared (typically after 4–6 days depending on the cell type), remove the medium from the wells.
6. Wash the wells with PBS.
7. Add an appropriate volume of 0.6% methylene blue solution to each well.
8. Incubate at room temperature for 15 min.
9. Wash the wells with H_2O and leave to air dry.
10. Count the number of colonies manually and calculate the CFU per mL using the following formula:

$$\frac{\text{CFU}}{\text{mL}} = \frac{\text{Number of formed colonies}}{\text{Volume of virus} - \text{containing supernatant added in mL.}}$$

3.6 Generation of Knockout Cell Lines Using CRISPR/Cas9

The lab of Feng Zhang has developed two lentiviral systems for easy and efficient delivery of the CRISPR/Cas9 system to mammalian cells: a one-vector system plentiCRISPRv2 encoding all elements necessary for genome editing in one vector, and a two-vector system, by which SpCas9 and the sgRNA are delivered on two separate lentiviral vector constructs (plasmids referred to as plentiCas9-Blast and plentiGuide-Puro, respectively). Both systems allow you to clone-in your 20-nucleotide sgRNA sequence of interest in the context of a lentiviral vector plasmid, allowing incorporation of derived vector RNA into lentiviral particles. These vectors are not the only available vectors for lentiviral delivery of the CRISPR/Cas9 system, but the following protocols are based on our experience with these vectors.

The protocols below describe the generation of knockout cell lines based on 2 to 3 weeks of drug selection for cells carrying one or more integrated copies of the vector. However, for some cell types, which can easily be transduced (e.g., HEK293 cells), high indel frequencies can be achieved by transferring vector-containing supernatant to the recipient cells. Hence, depending on the cell type, indel frequencies above 90% can be achieved 5 days post transduction without selection (Fig. 2a). In the shown example, indel rates of 50% were achieved in normal human dermal fibroblasts (NHDF) after 5 days (Fig. 2b). In our hands, we rarely detect indels within the first day after transduction, but observe the largest

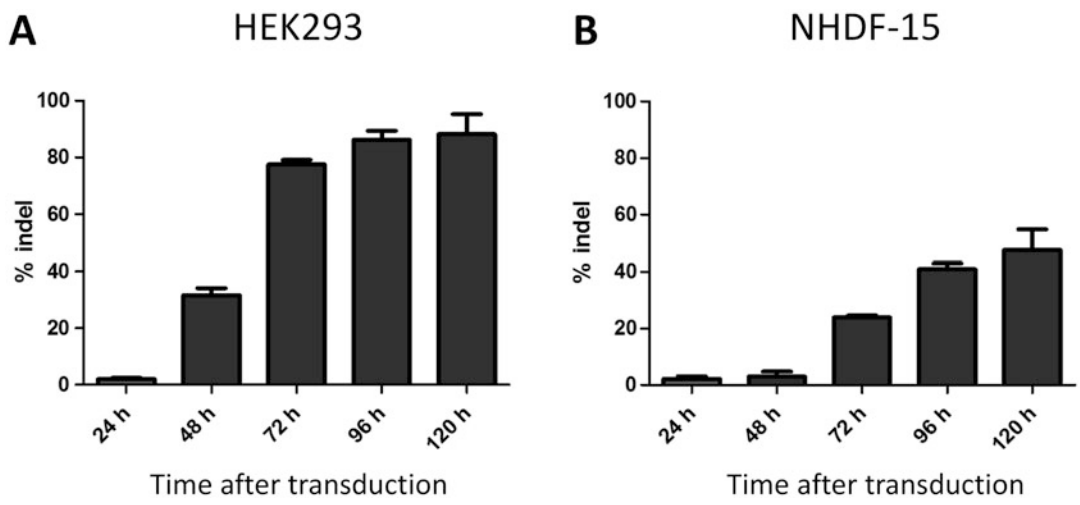

Fig. 2 Gene disruption rates in HEK293 cells and normal human dermal fibroblasts over a period of 5 days. HEK293 (**a**) and NHDF-15 (**b**) cells were transduced with LV/lentiCRISPRv2-sgRNA encoding a sgRNA targeting the *SERPING1* gene. 24 h after transduction the virus-containing medium was removed and the cells were harvested in 24-h intervals, and the indel frequencies were determined by TIDE analysis. Data are depicted as mean + SEM

increase in indel frequency between 48 and 72 h after lentiviral delivery of the CRISPR/Cas9 system (Fig. 2a, b). Thus, under conditions allowing high levels of transduction, the majority of the SpCas9-mediated cleavage occurs within this time frame. Although the preferred methodology may obviously depend on the application, we recommend carrying out drug selection for the time indicated in the protocols. This ensures depletion of non-transduced cells and allows use of lower amounts of vector, leading to reduced risks of integrating more than one lentiviral vector in transduced cells.

3.7 Generation of Knockout Cell Lines Using the One-Vector CRISPR/Cas9 System

For the generation of knockout cell lines using lentiviral delivery of the one-vector CRISPR/Cas9 system, clone the sgRNA sequence of interest into the plentiCRISPRv2 [11] plasmid (resulting in plentiCRISPRv2-sgRNA) and prepare a batch of lentiviral vectors (designated LV/lentiCRISPRv2-sgRNA) according to the guidelines in Subheading 3.1 using plentiCRISPRv2-sgRNA as the transfer plasmid.

1. Day 1, seed 1–2 × 10^5 targets cells per well in a 6-well dish or in another suitable culture format. Seed four wells in total (*see* **Note 15**).
2. Day 2, transduce cells in three of the wells with the LV/lentiCRISPRv2-sgRNA batch. If the target cells can tolerate polybrene, it is possible to supplement with 8 μg/mL polybrene (final concentration) to increase the transduction

efficiency. The fourth well serves as a control for the puromycin selection applied on day 4.

3. Day 3, change medium on the target cells.
4. Day 4, start selection with puromycin in all four wells at a concentration suitable for the target cells.
5. Renew puromycin-containing medium every second or third day, and maintain puromycin selection for 14 days. Passage the cells if needed. At the first passage after transduction, pool the three wells containing transduced cells. Expect the cells in the non-transduced well to die within the first 4–7 days.
6. Day 19, freeze down aliquots of the cell population for later studies and harvest gDNA for validation of CRISPR/Cas9 activity leading to gene disruption.

3.8 Generation of Knockout Cell Lines Using the Two-Vector CRISPR/Cas9 System

The generation of stable SpCas9-expressing cell lines can be accomplished with the plentiCas9-blast vector. Due to the nature of lentiviral infection, it is recommended to transduce at a low multiplicity of infection (MOI) of 0.1–0.3. The low MOI helps control the number of inserted copies of the SpCas9-encoding cassette per cell. This will produce a heterogeneous population of cells, ideally each with a single insertion, reducing the overall heterogeneity of the population in terms of SpCas9 expression levels.

Prior to creation of SpCas9-expressing cells, produce a batch of LV/lentiCas9-blast vectors and assess the titer (*see* Subheadings 3.1–3.5).

Proceed following the general steps described below.

1. Day 1, seed 2–5 $\times$ 10^5 cells per well in a 6-well dish or in another suitable culture format. Seed cells in 4 wells in total.
2. Day 2, transduce 3 wells with LV/lentiCas9-Blast using a viral particle load ensuring low MOI. The needed volume of the vector preparation is determined based on the specific titer in the relevant cell line.
3. Day 3, replace medium with medium containing blasticidin; the fourth well serves as a control for blasticidin selection.
4. From Day 4 to 17, once each well becomes confluent, pool cells from the different wells and proceed by culturing cells in a larger appropriate culture volume. Maintain cells under blasticidin selection and passage as required (*see* **Note 16**).
5. Day 18, freeze down several aliquots of the population and proceed to test the functionality of SpCas9 in the blasticidin-resistant population.
6. (Optional) To establish and characterize individual SpCas9-expressing clones, seed cells individually to start generating expanded cell clones.

3.9 Validation of SpCas9-Expressing Cell Lines and gRNA Transduction

After 2 weeks of blasticidin selection most cell lines should be completely depleted of blasticidin-sensitive cells. Since the blasticidin resistance gene is expressed by the same promoter as SpCas9 and the two genes are fused by a P2A-peptide sequence, any blasticidin-resistant cell should also contain SpCas9 protein. Assessing the functionality and efficiency of SpCas9 in the cells can be done by delivering a sgRNA to the cells (*see* **Note 17**). We recommend doing this by transducing the cells with LV/lentiGuide-Puro, a vector that contains a sgRNA scaffold as well as a selection marker. A high-activity sgRNA (here designated $sgRNA^X$) should be cloned into plentiGuide-Puro for this step.

1. Day 1, seed 2–5 $\times$ 10^5 cells per well in a 6-well dish or in another suitable culture format. Seed 4 wells in total.
2. Day 2, transduce 3 wells with LV/LentiGuide-Puro-$sgRNA^X$.
3. Day 3, apply puromycin to all wells; the fourth well will serve as a control for puromycin selection.
4. Day 4–11, maintain puromycin selection and passage cells as usual (*see* **Note 18**).

Day 11. Harvest gDNA for PCR, sequencing and TIDE analysis, as described in Subheading 3.9 (*see* **Note 19**).

3.10 TIDE-Based Validation of CRISPR/Cas9-Mediated Gene Knockout

One way to validate CRISPR/Cas9-mediated editing in the cell lines created in Subheadings 3.6 and 3.7 is by tracking of indels by decomposition (TIDE) [12]. The TIDE software requires two chromatograms of the region targeted by the Cas9/sgRNA complex, (1) one from the cell lines generated in Subheadings 3.6 or 3.7 and (2) one from an untreated control population of the same cell line (or alternatively a population of cells transduced with a control vector). The TIDE software analyzes the two supplied chromatograms by using the height of each peak in the chromatogram to determine the relative abundance of aberrant nucleotides. The output is an indel frequency, which represents the fraction of alleles cleaved by Cas9 and subsequently repaired by NHEJ in a cell population.

1. Extract gDNA from the knockout population created in Subheadings 3.6 or 3.7 and from an untreated population of the same cell line using your preferred protocol.
2. PCR-amplify the sgRNA target region using the gDNA as template (*see* **Note 20**).
3. Run the PCR on a 1% agarose gel and purify the PCR band.
4. Sequence the PCR products; if you follow the recommended primer design, this can be done with your forward primer.

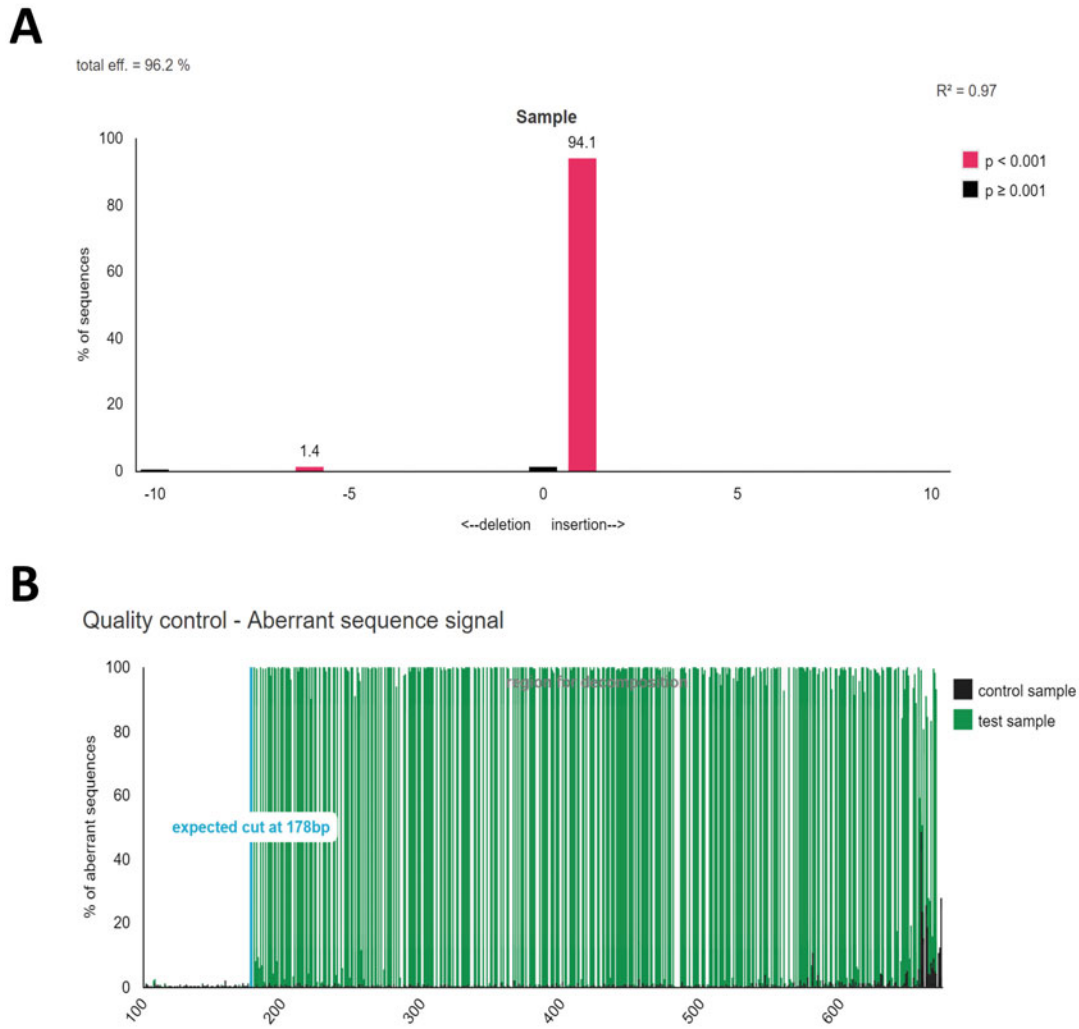

Fig. 3 TIDE analysis performed on primary fibroblasts after lentiviral delivery of the one-vector CRISPR/Cas9 system with a sgRNA targeting the *SERPING1* gene. An example of the output from a TIDE analysis showing an indel frequency of 96.2% with a one base pair insertion as the most common indel (**a**). The TIDE software also returns a quality control of the chromatograms uploaded depicted as % aberrant sequence with an illustration of the expected cut site (**b**)

5. Perform TIDE analysis using the TIDE software (https://tide.nki.nl/) or alternatively ICE analysis (https://ice.synthego.com).

   An example of a TIDE analysis is shown in Fig. 3. This TIDE analysis is performed on primary fibroblasts after lentiviral delivery of the one-vector CRISPR/Cas9 system with a sgRNA targeting the *SERPING1* gene.

4 Notes

1. Production specifications are displayed in Table 4 below.
2. Transfection control: The transfection efficiency is absolutely crucial for production of high-titer vector preparations. If the transfer plasmid does not contain a fluorescent marker gene, it is recommended to include a transfection control. You can either transfect cells in a separate dish with a transfer plasmid expressing a fluorescent marker or include 1 μg of a standard expression plasmid (non-lentiviral) encoding a fluorescent marker in the transfection.
3. When carrying out a large-scale production, it is recommended to split the transfections into several rounds. Do not prepare more than 10 transfection mixtures at once, since incubation of the transfection mixture for prolonged periods of time will reduce the potency of the transfection.
4. If you are not ultracentrifuging the vector-containing supernatant, and the recipient cells grow in another medium than DMEM, change to that medium now. The cells are very confluent at this point, so be gentle when adding the fresh medium.

Table 4
List of production specifications used in this method

Reagent	Amount/volume
Day 1	
HEK293T cells per p10 dish	4×10^6
DMEM	10 mL
Day 2	
DMEM 1 h before transfection	10 mL
pRSV-REV	3 μg
pMD.2G	3.75 μg
pMDIg/p-RRE	13 μg
Transfer plasmid	13 μg
dH_2O in transfection mix	To a final volume of 450 μL
2 M $CaCl_2$	50 μL
2×HBS	500 μL
Day 3	
DMEM	7–10 mL

5. If you are doing a second harvest on day 5, you can store the first harvest at 4 °C, pool it with the second harvest, and then make the aliquots or do the ultracentrifugation at day 5.
6. If you are not up-concentrating the vector preparation by ultracentrifugation, the crude vector-containing supernatant is now ready to use. To avoid repeated thawing and freezing, make aliquots of the crude preparation in appropriate sizes and store the aliquots at −70 to −80 °C until use.
7. When preparing the sucrose cushion aspirate 7 mL of the sucrose solution in a 5 mL serological pipette. Slowly release the sucrose solution into the tube and stop when you reach 3 mL. By doing this, you will have a 4 mL solution in each tube allowing you to keep track of the volume added and avoid disturbing the sucrose cushion by accidentally blowing air into it.
8. We typically add 85 μL to each tube resulting normally in a vector batch with an approximately 300-fold higher concentration of vectors relative to the crude prep.
9. If the recipient cells are suspension cells, you can combine day 1 and day 2 and do seeding and transduction in one step.
10. We normally go for 1000 μL, 200 μL, and 50 μL crude virus (vector-containing supernatant) or the equivalent volume of ultracentrifuged virus in a total volume suitable for your culture.
11. We use the pCCL-PGK-Puro-H1-MCS plasmid [13] for the WPRE standard curve, but any vector containing the WPRE element (like pLentiGuide-Puro or pLentiCRISPRv2) can be used. pAlbumin (acquired from Addgene) is used for the Albumin standard curve.
12. The conversion from grams to number of molecules of pAlbumin is carried out as depicted below. As the size of pAlbumin is 7539 bp plasmid and the estimated molecular weight of a base pair is 660 g/mol, the calculation is as follows:

    Molecular weight of pAlbumin $= 7539\ \text{bp} \times \frac{660 \frac{\text{g}}{\text{mol}}}{\text{bp}} = 5 \times 10^6 \frac{\text{g}}{\text{mol}}$.

    If the concentration of the plasmid stock is 1 μg/μL then

    Molar concentration $= \frac{\frac{1 \times 10^{-6}\text{g}}{\mu L}}{\frac{5 \times 10^6 \text{g}}{\text{mol}}} = 2 \times 10^{-13} \frac{\text{mol}}{\mu L}$

    and the number of molecules per μL $= 2 \times 10^{-13} \frac{\text{mol}}{\mu L} \times 6.02 \times 10^{23} \frac{\text{Molecules}}{\text{mol}} = 1.2 \times 10^{11} \frac{\text{molecules}}{\mu L}$.

    Since $\frac{1.2 \times 10^{11}}{1.25 \times 10^7} = 9.6 \times 10^3$, the first sample of the standard curve for Albumin should be a 9.6×10^3 times dilution of the 1 μg/μL pAlbumin plasmid.

13. As two alleles of the albumin gene are assumed to be present in the genome, the relative quantity mean of WPRE and the albumin gene needs to be multiplied by 2 (indicated by "×2" in the equation above).
14. Keep the cells in selection medium throughout the experiment. When the cells in the non-transduced control well have died off, and the colonies in transduced wells have reached a size visible with the naked eye, the colonies can now be stained and counted.
15. If the target cells are suspension cells, you can combine day 1 and day 2 and do seeding and transduction in one step.
16. Consider expanding your cells throughout the selection period in order to achieve a large cell population for freezing and storage. This will provide aliquots with minimum passage time ready for downstream applications.
17. Alternatively, Cas9 protein levels can be assayed by Western blot using anti-Flag antibodies. Obviously, this only confirms the expression and does not provide an assessment of functionality.
18. By using the two-vector system, the presence of Cas9 protein at a steady state in the cell allows for a more rapid generation of saturated knockout levels after sgRNAs delivery. Thus, one week of puromycin selection will suffice with the two-vector system.
19. With a typical Cas9-expressing population and delivery of a high-activity sgRNA species, indel formation should be near maximum after 1 week of puromycin selection. Expect an indel rate determined by TIDE above 75%.
20. Primer design: When designing primers for the PCR reaction, place the forward primer minimum 200–300 bp upstream of the expected Cas9 cut site to allow enough sequence for alignment with the control sequence. Aim for a PCR amplicon of 700–1000 bp and make sure that the primer set is not able to amplify another region in the genome of the same size.

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