Chapter 18

Using CRISPR/Cas9 Technology for Manipulating Cell Death Regulators

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Abstract

Clustered, regularly interspaced, short palindromic repeats (CRISPR)/Cas9 technology has been demonstrated to be a useful tool for generating targeted mutations in cell lines and mice. However, the use of CRISPR/Cas9 in a constitutively expressed manner can often result in low targeting efficiencies and lethality due to mutations in essential genes. Here, we describe the use of an inducible lentiviral vector platform, enabling rapid transduction and enrichment of CRISPR/Cas9 positive cells and high levels of targeted mutations upon induction.

Key words CRISPR/Cas9, Inducible, Lentiviral, Guide RNA

1 Introduction

CRISPR together with the Cas9 endonuclease originating from *Streptococcus pyogenes* have been extensively used for genome editing in a wide variety of eukaryotic and prokaryotic organisms [1–4]. The adaptation of the CRISPR/Cas9 system for genome editing features the use of a 20 nt single guide RNA (sgRNA) sequence to direct Cas9 endonuclease activity to any stretch of DNA sequence that has a protospacer adjacent motif (PAM) consisting of the nucleotides NGG, whereby N can be any of the DNA bases [5]. Cas9 endonuclease activity results in double stranded breaks, which stimulates the cellular DNA repair machinery to repair these breaks by the highly error prone non-homologous end-joining (NHEJ) pathway. The NHEJ repair process results in insertion/deletion (InDel) mutations, which can disrupt the open reading frame of a target gene and lead to its functional inactivation.

Here, we demonstrate the use of an inducible CRISPR/Cas9 lentiviral platform that can efficiently deliver Cas9 and sgRNA encoding vectors in a wide range of mouse and human cells [6]. In this system, sgRNAs are under the regulatory control of a

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tetracycline inducible H1 promoter. The exposure of cells to doxycycline (dox) relieves the Tet repressor from the H1 promoter, resulting in robust sgRNA expression in a temporally regulated manner. We also present a next-generation sequencing method to validate and characterize CRISPR/Cas9 mediated InDels in a high throughput and accurate approach. These techniques can be easily adapted to study apoptotic gene function in vitro and in vivo.

2 Materials

2.1 Vectors and sgRNA Cloning

1. pFgh1tUTG vector for dox-inducible expression of sgRNAs.
2. pFUCas9mCherry vector for constitutive expression of the Cas9 endonuclease.
3. Viral packaging vectors pMDL, pRSV-rev, and pVSV-G.
4. Oligonucleotide annealing buffer—Combine 2 μl of 1 M Tris–HCl (pH 7.5), 2 μl of 1 M $MgCl_2$, 0.5 μl of 3 M NaCl, and 9.5 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). This makes 14 μl of oligonucleotide annealing buffer, sufficient for one oligonucleotide annealing reaction.
5. T4 DNA ligation buffer.
6. Phenol/chloroform/isoamyl alcohol solution pH 7.8–8.2.
7. Chloroform/isoamyl alcohol solution.
8. 3 M NH_4OAc, pH 5.5.
9. 100 % ethanol.
10. 80 % ethanol.
11. Electrocompetent STBL4 bacterial cells.
12. 50–100 mg/ml Ampicillin stock solution.
13. Qiaprep Spin Miniprep Kit or equivalent.
14. DNAse-free water.
15. PNKinase.
16. 10× restriction enzyme buffer.
17. BsmB I restriction enzyme.
18. Bam H I restriction enzyme.
19. TE buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.
20. T4 DNA Ligase.
21. 20 mg/ml Glycogen.
22. Phospohorylated oligonucleotides.
23. Luria agar plates with ampicillin, 50–100 μg/ml final concentration.

2.2 Virus Production and Transduction

1. 293FT cells.
2. 10 % *D*ulbecco's *M*odified *E*agle *M*edia (DMEM), containing DMEM supplemented with 10 % fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-alanyl-L-glutamine.
3. HBS buffer: 0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2PO_4. Adjust pH to 6.95–7.05 with NaOH.
4. Polybrene.
5. 0.5 M $CaCl_2$.
6. 10 cm tissue culture plates.
7. 0.45 μm syringe filters.
8. 6-well tissue culture plates.
9. 10 mg/ml doxycycline stock concentration (10,000×).
10. LSR IIW or FACSCalibur flow cytometer or equivalent.

2.3 Validation of Knock Out Cell Lines

1. Standard Western blotting reagents, including polyacrylamide gels, electrophoresis tanks, transfer membranes, and ECL detection reagents.
2. Next-generation sequencing reagents, including MiSeq reagent kit v2, AMPure XP DNA clean-up, and size selection beads or equivalent.
3. MiSeq Forward indexing oligonucleotide:
   5′CAAGCAGAAGACGGCATACGAGATCCGGTCTC GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNN NNNGTGACCTATGAACTCAGGAGTC)3′.
4. MiSeq Reverse indexing oligonucleotide:
   5′AATGATACGGCGACCACCGAGATCTACACTCTT TCCCTACACGACGCTCTTCCGATCTNNNNN-NNNCTGAGACTTGCACATCGCAGC3′
   The sequence NNNNNNNN is where unique 8 bp indexes are inserted into forward and reverse indexing oligonucleotides.
5. GoTaq Green mix polymerase.
6. DNAse-free water.
7. Forward primer: 5′**GTGACCTATGAACT-CAGGAGTC***CGAGGCTGCTTTTCTTCG* 3′
   (bold: overhang; italics: gene specific).
8. Reverse primer: 5′**CTGAGACTTGCACATCGC-AGC***AACTCGTCCTCCTCCTCCTC* 3′
   (bold: overhang; italics: gene specific).
9. Magnetic rack.

2.4 Functional Assays

1. 5.0 mg/ml ionomcycin stock (1000×).
2. 50 μg/ml Etoposide stock (1000×).
3. Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 and Propidium Iodide (PI) or equivalent.

3 Methods

3.1 Designing sgRNAs

1. Use the Optimized CRISPR Design website, crispr.mit.edu for sgRNA design. Select the genome of the organism to be targeted (e.g., mm9 for the mouse genome). Enter a DNA sequence, up to 250 nucleotides long, of the gene to be targeted by Cas9. A list of candidate sgRNAs will be generated, ordered by specificity and efficiency. The higher the sgRNA score, the greater the specificity with fewer off-targets. Select sgRNAs with a score greater than 75 and select at least two sgRNAs per target gene (*see* **Note 1**).
2. Order selected sgRNA sequences with a 5′ "TCCC" 4 bp overhang for the complementary sequence and a 5′ "AAAC" 4 bp overhang for the reverse complementary sequence. For instance, an sgRNA with sequence "GGCAACTATGGCTTCCACCT" should be ordered as (Forward: 5′**TCCC**GGCAACTATGGCTTCCACCT 3′) along with (Reverse: 5′ **AAAC**AGGTGGAAGCCATAGTTGCC 3′) (*see* **Note 2**).

3.2 Cloning of sgRNAs into the dox-Inducible pFgh1tUTG Lentiviral Vector

1. Resuspend oligonucleotides in DNAse-free water to a concentration of 100 μM. Anneal oligonucleotides, by adding 3 μl of forward sgRNA oligonucleotide (containing 4 bp overhang) and 3 μl of reverse sgRNA oligonucleotide (containing 4 bp overhang) to 14 μl of oligo annealing buffer in a PCR tube. Heat PCR tube to 96 °C for 5 min and place on ice immediately.
2. Phosphorylate annealed oligonucleotides by adding 5 μl of annealed oligonucleotides to 12 μl DNAse-free water, 2 μl of T4 DNA ligation buffer and 1 μl of PNKinase. Incubate mixture for 20 min at 37 °C followed by heat inactivation for 10 min at 70 °C. Add 1 μl of phosphorylated oligonucleotides to 99 μl of DNAse-free water to produce a 1:100 diluted oligonucleotide solution (*see* **Note 3**).
3. Generate the overhangs for inserting the annealed oligos into the pFgh1tUTG vector, by cutting the vector with BsmB I. Add 10 μl of 10× restriction enzyme buffer to 5 μg of pFgh1tUTG vector, 4 μl (10 U/μl) of BsmB I restriction enzyme and adjust reaction volume to 100 μl with DNAse-free water.
4. Incubate the mixture from **step 3** above at 55 °C for 4 h followed by 80 °C for 20 min to heat inactivate the restriction enzyme. Add 1 μl (50 ng) of digested pFgh1tUTG to 19 μl of DNAse-free water to produce a 1:20 diluted pFgh1tUTG solution.

5. Ligate sgRNA oligonucleotides into the cut pFgh1tUTG vector, by adding 3 μl of diluted pFgh1tUTG solution from **step 4** above to 3 μl of diluted phosphorylated oligonucleotide solution from **step 2**, 2 μl of 10× ligation buffer, 0.8 μl DNA ligase, and 11.2 μl DNAse-free water. Leave overnight at room temperature.
6. Next day add 180 μl of TE buffer to the 20 μl ligation reaction, followed by 200 μl of phenol/chloroform/isoamyl alcohol solution and vortex well. Centrifuge at 13,000 RPM (17,900 × *g*) in a bench top centrifuge for 5 min.
7. Transfer 200 μl of the top aqueous phase into a new microfuge tube, add 200 μl of chloroform/isoamyl alcohol solution, and vortex well. Centrifuge at 13,000 RPM (17,900 × *g*) in a bench top centrifuge for 5 min.
8. Transfer 200 μl of the top aqueous phase from **step 7** above into a new microfuge tube, add 20 μl of 3 M NH_4OAc, 1 μl (20 μg) glycogen, and vortex well.
9. Add 500 μl 100 % ethanol, vortex well, and incubate solution at −20 °C overnight.
10. Next day centrifuge at 13,000 RPM (17,900 × *g*) in a bench top centrifuge for 15 min at 4 °C. Discard supernatant by carefully aspirating.
11. Wash the DNA pellet by adding 500 μl of 80 % ethanol and vortexing briefly. Centrifuge at 13,000 RPM (17,900 × *g*) in a bench top centrifuge for 15 min at 4 °C and discard supernatant and allow pellet to air dry for 15 min at room temperature.
12. Resuspend purified DNA pellet in 5 μl of DNAse-free water and use all of the resuspended DNA to transform electrocompetent STBL4 bacterial cells.
13. Plate out transformed bacterial cells on ampicillin (100 μg/ml) supplemented L-Broth agar and incubate plates at 37 °C overnight in an incubator.
14. After incubation, select six to eight colonies per sgRNA and complete a miniprep protocol for each colony according to manufacturer's instructions.
15. Digest minipreps from **step 10** with Bsmb I and BamH1. If the sgRNA sequence has been successfully ligated, this double digestion will linearize the plasmid. Release of a 1.2 kb fragment indicates unsuccessful cloning (*see* Fig. 1).
16. Perform Sanger sequencing for each miniprep using sequencing primer CAGACATACAAACTAAAGAAT for the sequencing reaction and run samples on a DNA analyzer (Applied Biosystems, ABI 3730XL). Verify that the sgRNA sequence of interest is correct.

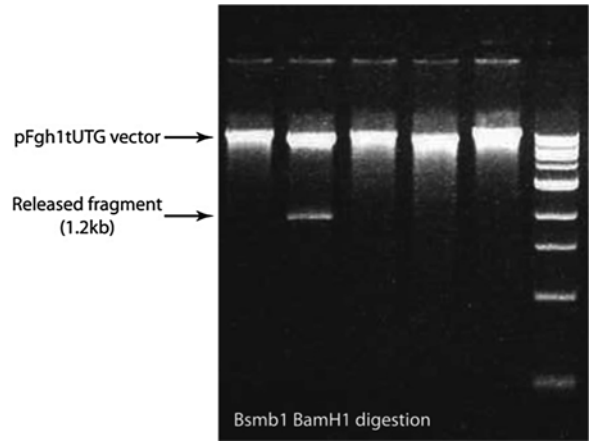

Fig. 1 Diagnostic digest for successful sgRNA cloning into the pFgh1tUTG vector. The successful ligation of the sgRNA into the pFgh1tUTG vector removes the Bsmb1 cloning site, resulting in a single linearized band when double digested with Bsmb1 and BamH1. If the ligation was unsuccessful, Bsmb1 and BamH1 double digestion of the empty pFgh1tUTG vector will release a 1.2 kb fragment (*lane 2*)

3.3 Virus Production and Transduction of Cells

1. Culture 3×10^6 293FT cells in 10 ml of 10 % DMEM media on a 10 cm dish at 37 °C in an incubator overnight.
2. The following day, aspirate media and replace with fresh 10 % DMEM.
3. Make lentiviral packaging cocktail, by adding 5 μg of pMDL vector, 2.5 μg of pRSV-REV vector, 3 μg of pVSV-G vector, 10 μg of pFgh1tUTG vector and/or pFUCas9mCherry vector, 250 μl of 0.5 M $CaCl_2$, 500 μl of HBS, and 250 μl of DNAse-free water. Vortex the solution for 10 s and incubate for 10 min at room temperature (*see* **Note 4**).
4. Transfect 293FT cells with lentiviral packaging constructs, by adding lentiviral packaging cocktail from **step 3** above, drop wise over 293FT cells while swirling the dish constantly. Incubate cells overnight at 37 °C in an incubator (viral particles will form in the media overnight).
5. Seed cells to be transduced in a 6-well plate such that they reach 40–50 % confluence the following day (*see* **Note 5**).
6. On the following day, collect the virus-containing supernatant from **step 4** above and filter sterilize using a 0.45 μm syringe filter to remove any contaminating 293FT cells.
7. Add fresh 10 % DMEM media to the 293FT cells and incubate overnight at 37 °C for collecting a second batch of viral complexes.
8. Supplement 3 ml of filtered viral supernatant from first batch (**step 6**) with 8 μg/ml polybrene (*see* **Note 6**). Add this onto target cells cultured in 6-well plates.

9. Spin the plate at 2200 rpm (~500×*g*) for 2 h at 32 °C. Place cells at 37 °C overnight in an incubator.
10. The following day, remove virus-containing media from the 6-well plate and reinfect cells with a second batch of viral particles as described in **step 8**. 293FT cells can then be discarded.
11. Replace virus-containing media from 6-well plates with fresh media. Passage cells when confluent.

3.4 Flow Cytometric Analysis, dox Induction and Sorting of Cells

1. The pFgh1tUTG sgRNA vector and pFUCas9mCherry vector feature a GFP and a mCherry selection marker, respectively, allowing efficient identification of transduced cells by flow cytometry. Following viral transduction and expansion, assess the cell lines by flow cytometry using GFP and mCherry detection parameters.
2. If GFP and mCherry double positive cells represent less than 50 % of the cell population, sort GFP and mCherry double positive cells using a cell sorter and reassess GFP and mCherry double positivity after cells have expanded.
3. Repeat cell sorting if necessary until GFP and mCherry double positive cells represent more than 85 % of the cell population (*see* Fig. 2).
4. Induce expression of sgRNAs in cell lines, by adding dox in cell culture media to a final concentration of 1 μg/ml. Culture cells in dox supplemented media for 3 days before replacing culture media with fresh dox-free media. By this stage, sgRNAs would have complexed with constitutively expressed Cas9 protein and generated InDels in target gene sequences.
5. The cell lines can now be expanded and analyzed as a bulk population or further sorted (gate on GFP mCherry double positive cells) in 96-well plates to generate clonal cell populations (*see* **Note 7**).

3.5 Validation of Knock Out Cell Lines

1. If an antibody is available to detect the gene product of interest, perform Western immunoblotting analysis on material derived from expanded single-cell clonal cell lines or from bulk cell populations (*see* **Note 8**).
2. To validate the precise genome editing outcome induced by CRISPR/Cas9, perform next-generation sequencing of cell lines (*see* **Note 9** and Fig. 3). Start by generating gene-specific oligonucleotides (with 60 °C annealing temperatures) that flank the sgRNA used, producing an amplicon approximately 300 bp long. Extend gene-specific oligonucleotides with the overhangs GTGACCTATGAACTCAGGAGTC for the forward oligonucleotide and CTGAGACTTGCACATCGCAGC for the reverse oligonucleotide. Position each overhang at the

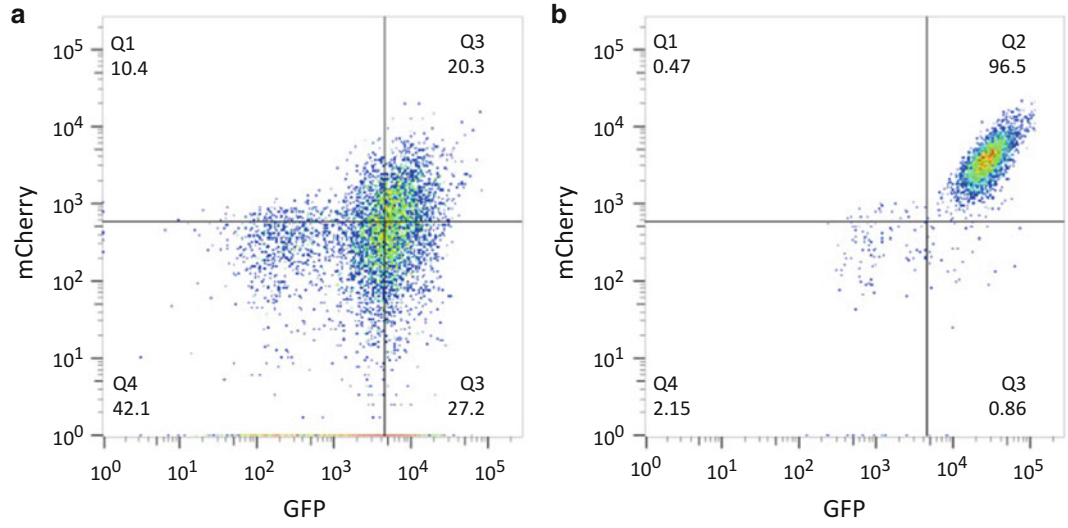

Fig. 2 Flow cytometric analysis and enrichment of pFgh1tUTG and pFUCas9mCherry double transduced cells. (**a**) Flow cytometric analysis of HeLa cells 1 week after transduction with pFgh1tUTG and pFUCas9mCherry vectors. Double positive cells transduced with both vectors were enriched by flow cytometric cell sorting, gating on the GFP and mCherry high population (Q2). (**b**) Flow cytometric analysis of HeLa cells 1 week after sorting and enrichment

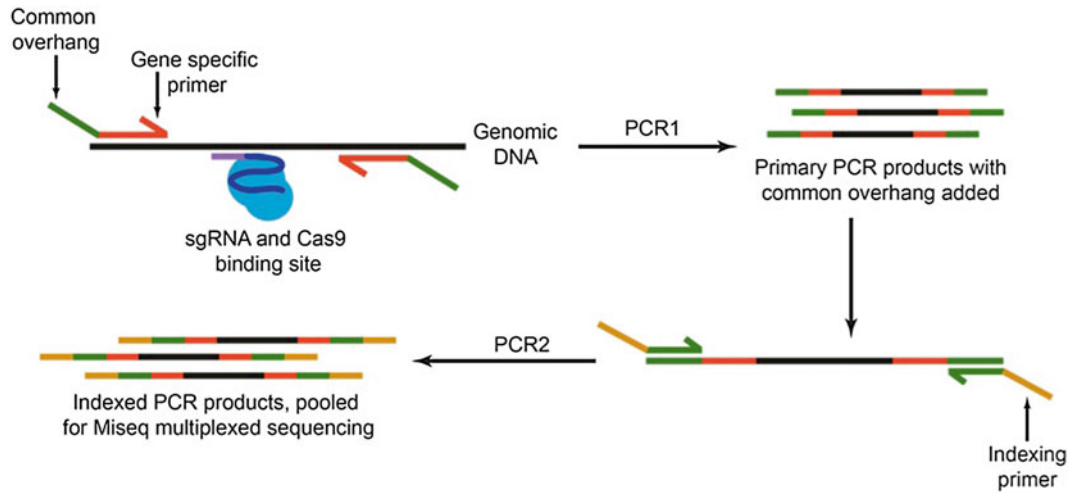

Fig. 3 Overview of the next-generation sequencing protocol

5′ end of each oligonucleotide. For instance, the following overhangs (green) are positioned at the 5′ position of the gene-specific oligonucleotides (red):

Forward primer: 5′ **GTGACCTATGAACTCAGGAGTC**-CGAGGCTGCTTTTCTTCG 3′

Reverse primer: 5′ **CTGAGACTTGCACATCGCAGC**AAC-TCGTCCTCCTCCTCCTC 3′.

3. Perform the first PCR reaction by combining 1 μl of DNA (100 ng/μl) with 10 μl of GoTaq Green mix polymerase (2× concentration, Promega), 0.5 μl of 10 μM forward oligonucleotide, 0.5 μl of 10 μM reverse oligonucleotide, and 8 μl of DNAse-free water. Run the PCR reaction for 18 cycles (95 °C 2 min, 60 °C 30 s, 72 °C 30 s) (*see* **Note 10**).
4. To purify amplicons from the first PCR reaction, add 20 μl of Agencourt AmPure XP beads (Beckman Coulter) to each PCR reaction. Incubate for 10 min at RT to allow beads to bind DNA amplicons. Place PCR plates on a magnetic rack and leave until magnetic beads have been fully attracted to the magnet. Aspirate and discard supernatant from each well. Add 150 μl of 80 % ethanol to each well and gently pipette to wash magnetic beads. Remove ethanol and repeat ethanol wash one more time. Remove ethanol and allow magnetic beads to air dry at room temperature (*see* **Note 11**).
5. To elute DNA, remove PCR plate from magnetic rack, add 30 μl of DNAse-free water to each well and pipette to mix. Once magnetic beads appear homogeneous, place PCR plate back onto a magnetic rack and allow beads to accumulate toward the magnet. Transfer 10 μl of clear eluted DNA solution to a fresh PCR plate and add 10 μl of GoTaq Green mix (2× concentration, Promega), 0.5 μl of 10 μM forward indexing oligonucleotide, and 0.5 μl of 10 μM reverse indexing oligonucleotide. Run PCR reactions for 24 cycles (95 °C 2 min, 60 °C 30 s, 72 °C 30 s).
6. Combine all PCR reactions in a clean trough and mix well by pipetting. Pipette a 100 μl aliquot in a 1.5 ml tube and add 100 μl of magnetic beads. Perform purification of amplicons as described in **step 4**. Resuspend air-dried magnetic beads in 100 μl of DNAse-free water. Quantify DNA concentration and molarity using a Qubit Fluorometer (Life technologies) and 2200 Tapestation (Agilent technologies), respectively.
7. Dilute dual indexed library pool to a 2 nM concentration and proceed with library preparation using the Miseq Reagent Kit V2 (Illumina) according to manufacturer's instructions. Perform sequencing with a 281 cycle forward read followed by a 44 cycle second index read.
8. Conduct functional assays—for an example of a functional assay (*see* **Note 12** and Fig. 4).

4 Notes

1. Beware of sgRNAs that have a high degree of complementarity with predicted off-targets in gene exons as they can cause unintended frameshift mutations. The 12 nucleotides of the sgRNA

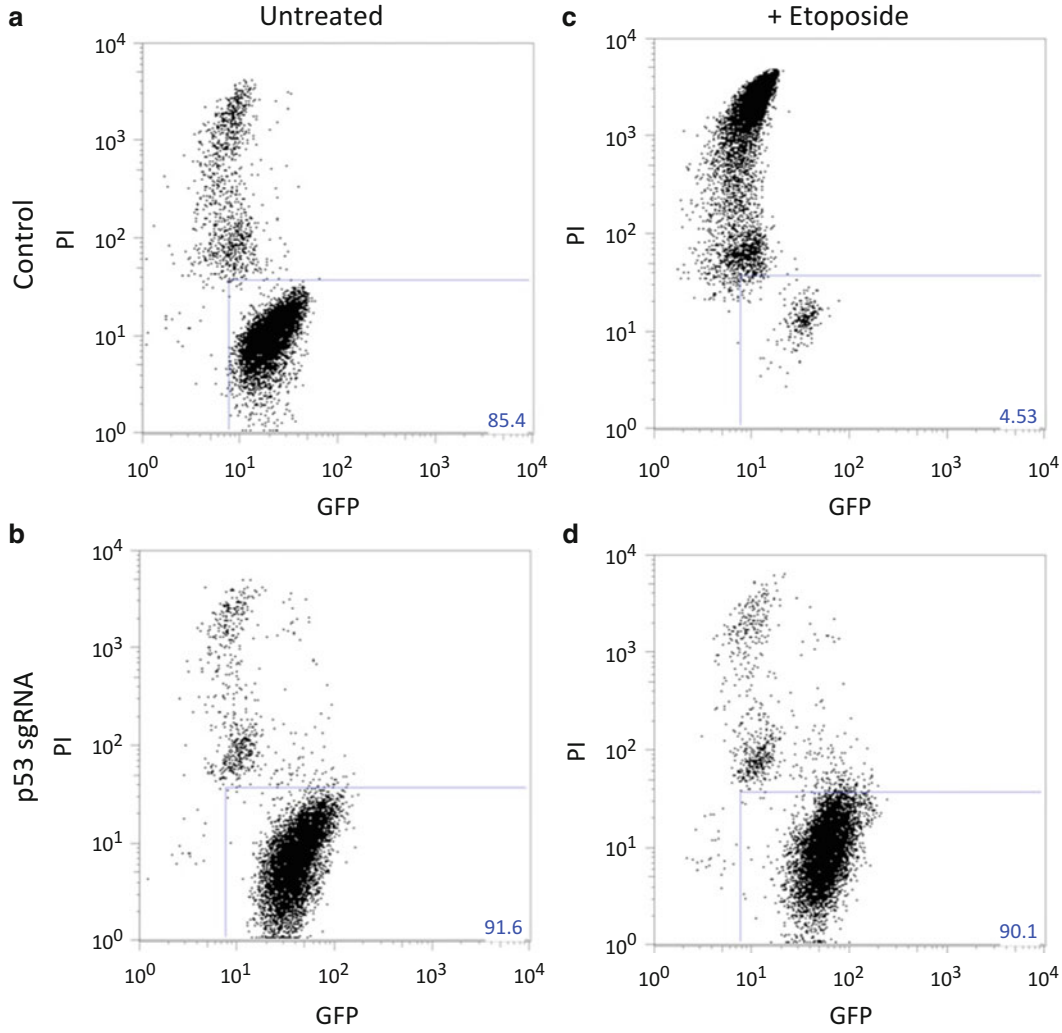

Fig. 4 PI cell viability assay of control and p53 knockout Eμ-myc murine lymphoma cell lines following 0.05 μg/ml etoposide treatment for 24 h. (**a** and **b**) Untreated control and p53 knockout Eμ-myc murine lymphoma cell lines displaying high levels of PI negative viable cells. (**c**) Control cell lines display a significant increase in PI positive dead cells in response to etoposide treatment compared to (**d**) p53 knockout Eμ-myc murine lymphoma cell lines which display resistance to etoposide treatment

adjacent to the PAM site form the seed region of the sgRNA. In particular, mismatches in this seed region reduce the chance of Cas9 activity at potential off-targets.

Ensure that sgRNAs target essential exons used by all protein coding splice variants of the target gene. Targeting a proximal exon increases the chance of frameshift mutations caused by InDels. Gene sequences coding for functional domains such as catalytic domains are also particularly good targets and increase the chance of nonfunctional gene products being

produced due to CRISPR/Cas9 induced InDels. Multiple sgRNAs can also be used in tandem to delete entire exons. Ordering at least two sgRNAs per gene target allows validation of phenotypes observed and reduces the likelihood that cellular phenotypes are due to off-targets. An alternative sgRNA design site is E-CRISP (http://www.e-crisp.org/E-CRISP/). CCTop (http://crispr.cos.uni-heidelberg.de/index.html) also offers an alternative sgRNA off-target prediction tool.

2. When ordering sgRNA sequences, ensure that the PAM site is *not* included.
3. If experiencing difficulty with cloning, try diluting oligonucleotide solution 1:1000 and 1:10,000. Use these dilutions in parallel to the 1:100 diluted solution during the sgRNA ligation step.
4. Transducing cells with the pFUCas9mCherry vector alone first will yield a stock of Cas9 positive cells that can then be transduced with any sgRNA of interest. However, if time is limited, the pFUCas9mCherry vector and the pFh1tUTG sgRNA vector can be simultaneously transduced.
5. Depending on cell type, target cells can also be cultured in 24-well or 48-well plates.
6. Polybrene increases viral transduction efficiencies but may be highly cytotoxic to certain cell types. Protamine sulfate can be used as an alternative to polybrene.
7. If mutation of the target gene causes a lethal cellular phenotype, analyze the bulk cell population immediately after dox induction. A clonal population can also be derived prior to dox treatment.
8. For negative controls, include samples that were (a) not transduced with viral vectors and (b) transduced with viral vectors but not treated with dox. Depending on the cell lines used, gene of interest and efficiency of transduction, it may be necessary to screen 5–20 single-cell clonal lines to isolate sufficient knockout cell lines. At least two knockout single-cell clonal lines per sgRNA should be used to perform functional assays.
9. Gene-specific primers featuring a common overhang sequence flank the predicted Cas9 cutting site in the first PCR reaction. Primary PCR products from this reaction are added to a secondary PCR reaction along with primers featuring indexing barcodes. The final indexed PCR products are then pooled for multiplexed sequencing on the Illumina Miseq next-generation sequencing platform.

10. A proof-reading polymerase can be used in this PCR reaction if required. Performing the PCR reactions in a 96-well plate format allows the use of multichannel pipettes and speeds up the completion of the protocol.
11. The magnetic beads will assume a flaky appearance when completely dry.
12. If proapoptotic genes have been targeted and mutated, this can be functionally validated using several cell death assays. For instance, the loss of Bim can be validated by the gain of resistance to 5 μg/ml ionomycin treatment. Similarly, the loss of p53 can be validated by the gain of resistance to 0.05 μg/ml etoposide treatment. Quantify cell viability by flow cytometry using Annexin V-PI staining.

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