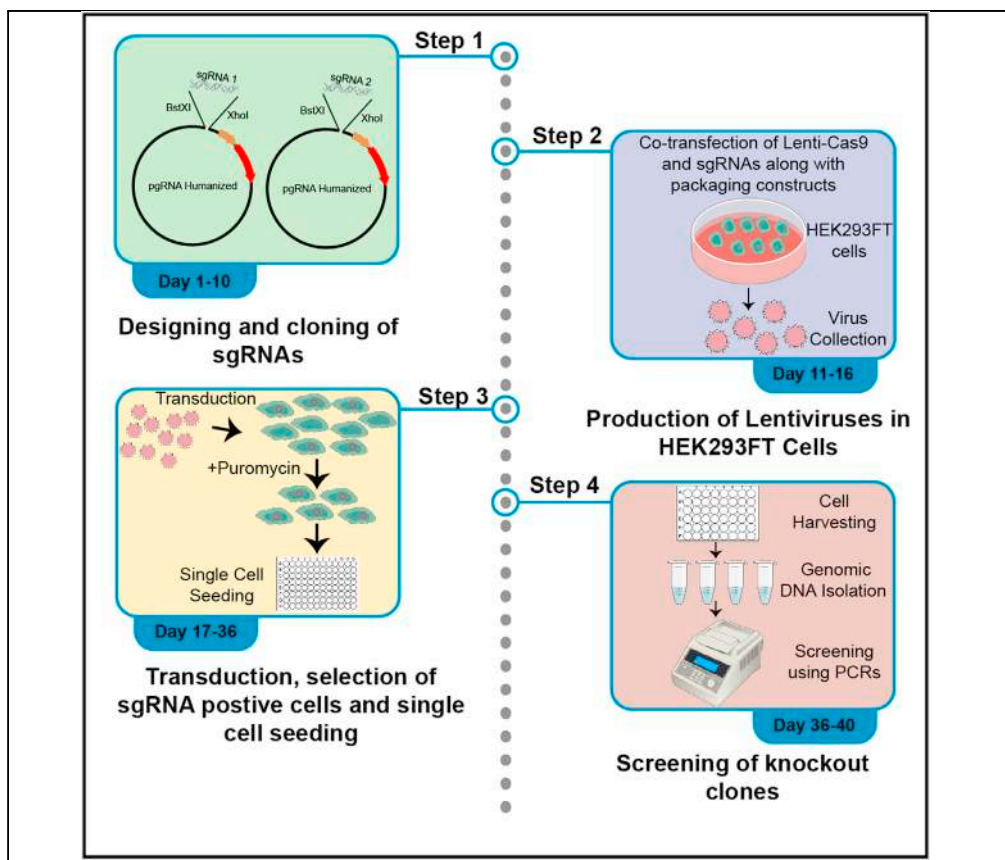


## Protocol

# Optimized protocol to create deletion in adherent cell lines using CRISPR/Cas9 system



The CRISPR/Cas9 system is a powerful tool for genome editing and is adaptable for a wide range of applications. Here, we have put together a step-by-step protocol for generating knockout cell lines (coding or non-coding region) using CRISPR/Cas9 tool. The protocol below has been tested on adherent cell lines such as HeLa and MCF7. However, it may easily be adapted to other adherent cell lines with minor variations.

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### Highlights

CRISPR-Cas9 protocol optimized for deletions in adherent and immortalized cell lines

The protocol covers complete flow from sgRNA cloning to the validation of deletion

This simple protocol can be used in low-resource settings

Farooq & Notani, STAR  
Protocols 2, 100857  
December 17, 2021 © 2021  
The Authors.  
<https://doi.org/10.1016/j.xpro.2021.100857>



## Protocol

## Optimized protocol to create deletion in adherent cell lines using CRISPR/Cas9 system

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## SUMMARY

The CRISPR/Cas9 system is a powerful tool for genome editing and is adaptable for a wide range of applications. Here, we have put together a step-by-step protocol for generating knockout cell lines (coding or non-coding region) using CRISPR/Cas9 tool. The protocol below has been tested on adherent cell lines such as HeLa and MCF7. However, it may easily be adapted to other adherent cell lines with minor variations.

For complete details on the use and execution of this protocol, please refer to Farooq et al. (2021).

## BEFORE YOU BEGIN

⌚ Timing: ~ 3 days

The CRISPR/Cas9 system employs single guide RNAs (sgRNAs) to direct Cas9, an endonuclease protein, to a specific region of DNA (Adli, 2018). When Cas9 is recruited, it creates a double-stranded break in the DNA at the specified region (Jinek et al., 2013). A pair of sgRNAs flanking a region on either side would cause the deletion of that region. This system has been engineered to accommodate a wide range of applications. Currently, this technology is used for genome editing, transcriptional activation and repression, epigenetic modifications, and live DNA/RNA imaging, among other things (Gilbert et al., 2013; Maeder et al., 2013; Amabile et al., 2016; Qin et al., 2017). This protocol explains how to create specific knockout cell lines step by step. It has been tested on adherent cancerous cell lines such as HeLa (Farooq et al., 2021). However, with minor modifications, it can be easily applied to other adherent cell lines.

The plasmids used in the process will be described first, followed by the design and cloning of sgRNAs. Then we go over how to make viral particles, how to use these viruses to transduce cells, and how to isolate and expand single cells. Finally, we discuss how to use PCRs to screen and validate single cell colony deletion. (Please refer to graphical abstract for these steps).

## Plasmids

In this protocol following plasmids are used: lentiCas9-Blast for Cas9 (Addgene #52962); pgRNA-humanized (Addgene #44248) for sgRNA cloning; pCMV-VSVG (Addgene #8454) and psPAX2 (Addgene #12260) for packaging of viruses.

Prepare the plasmids (lentiCas9-Blast; pgRNA-humanized; pCMV-VSVG and psPAX2);



To improve transfection efficiency and obtain high-titer viral particles, high-quality plasmid preparation is needed. To amplify the plasmids, we use the DH5 $\alpha$  E. coli strain. We prepare plasmids with the QIAGEN Plasmid Midi Kit. The supplier's protocol that comes with the kit should be followed when preparing the plasmids.

**Alternatives:** Although we used the DH5 $\alpha$  strain, we recommend either Stbl3 (Thermo C737303) or NEB Stable E. coli strain (NEB C3040I) for lentiviral plasmid amplification. Lentiviral constructs have a proclivity for recombination, and these strains have been modified to have a lower recombination rate. Furthermore, when compared to DH5 $\alpha$ , these strains produce higher yields for lentiviral constructs.

### Coat the cell culture dishes with poly-D-lysine

Add 1 mL of 0.1 mg/mL poly-D-lysine to 35 mm culture dish. Swirl the dish to completely cover it. Incubate for 2–3 min at room temperature (25°C). Allow the dish to dry completely after removing the poly-D-lysine solution. Wash the dish twice with 1 mL of DPBS and allow it to dry completely.

### Genomic DNA isolation buffer

⌚ Timing: ~ 2 h

The recipe for the genomic DNA isolation buffer is as follows:

Genomic DNA Isolation Buffer Recipe		
Reagent	Final concentration	Amount
5M NaCl	100 mM	1 mL
1M Tris-Cl (pH 8.0)	10 mM	0.5 mL
0.5M EDTA (pH 8.0)	25 mM	2.5 mL
10% SDS	0.5%	2.5 mL
Proteinase K	0.1 mg/mL	X $\mu$ l
ddH <sub>2</sub> O	n/a	43.5 mL
Total	n/a	50 mL

The genomic DNA isolation buffer should be stored at room temperature (25°C) without proteinase K. It can be stored for two weeks.

⚠ CRITICAL: Proteinase K is a labile enzyme. It must be added to the buffer freshly.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and viral strains		
DH5 $\alpha$ Competent Cells	Thermo	18265017
Chemicals, peptides and recombinant proteins		
DMEM	Gibco	10569-010
FBS	Gibco	26140079
Penicillin-Streptomycin	Gibco	15070-063
DPBS	Gibco	D8537
Puromycin	Gibco	A11138-03
Poly-D-Lysine	Sigma	P6407
0.25% Trypsin-EDTA (1 $\times$ )	Gibco	25200-072

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BstXI	NEB	R0113L
XhoI	NEB	R0146S
Buffer 3.1	NEB	B7203S
1 Kb Plus DNA Ladder	NEB	N3200
Ampicillin sodium salt	Sigma	A0166
Hexadimethrine bromide (Polybrene)	Sigma	H9268
LB broth	HiMedia	M575
LB broth with agar	Sigma	L3272
Lipofectamine 2000	Invitrogen	11668-019
T4 DNA Ligase	NEB	M02-02M
Opti-MEM	Gibco	31985-070
Kapa Taq DNA Polymerase	Kapa Biosystems	KK1014
Agarose	Sigma	A9539
KOD Hot Start DNA Polymerase	Merck	71086-4
Proteinase K, recombinant, PCR Grade	Roche	17106500
Phenol:chloroform:isoamyl (PCI) alcohol	Ambion	AM9732
T4 DNA Ligase	NEB	M0202L
T4 DNA Ligase Buffer	NEB	B0202S
<b>Critical commercial assays</b>		
QIAGEN Plasmid Midi Kit	QIAGEN	12143
QIAprep Spin Miniprep Kit	QIAGEN	27106
QIAquick Gel Extraction Kit	QIAGEN	28706
QIAquick PCR Purification Kit	QIAGEN	28106
QIAamp DNA Mini Kit	QIAGEN	51304
<b>Experimental models: cell lines</b>		
HeLa	ATCC	CCL-2
HEK293FT	Thermo	R70007
<b>Oligonucleotides</b>		
Universal reverse oligo	Sigma	CTAGTACTCGAGAAAAAAG CACCGACTCGGTGCCAC
sgRNA sequencing oligo	Sigma	CCCTGCCCGGTTAATTTGC
PCRin oligos	Sigma	N/A
PCRout oligos	Sigma	N/A
63-Nucleotide-long DNA guide sequence	Sigma	N/A
<b>Recombinant DNA</b>		
pgRNA-humanized	Addgene	#44248
lentiCas9-Blast	Addgene	#52962
pCMV-VSVG	Addgene	#8454
psPAX2	Addgene	#12260
<b>Others</b>		
96-Well cell culture plate	Thermo	167314
48-Well cell culture plate	Thermo	150687
10 cm Cell culture dish	Thermo	150466
6 well Cell culture plate	Thermo	140675
35 mm Dishes	Thermo	150318
15 mL Centrifuge tube	Tarsons	546021
50 mL Centrifuge tube	Tarsons	546041
1.5 mL Centrifuge tube	Tarsons	500010
Petri dish 90 mm	Tarsons	460090
0.45 Micron filters	Millipore	SLHV033RS
Reagent reservoirs	Thermo	8094

## STEP-BY-STEP METHOD DETAILS

### Designing and cloning of sgRNAs in pgRNA-humanized vector

⌚ Timing: [~10 Days]

To delete a specific genomic DNA region, a pair of sgRNAs targeting either side of the deletion region is required. First, we will go over how to use the CRISPOR tool to design guide sequences complementary to the flanks of the genomic region to be knocked out. Once the guide sequences are designed, we will walk you through the process of cloning them in the pgRNA-humanized vector.

1. **Designing guide sequences for region of interest.** The steps outlined below can be used to obtain the nucleotide sequence of the target region:
  - a. Search the region of interest in the designated assembly of UCSC genome browser (<https://genome.ucsc.edu/>).
  - b. Select your target region.
  - c. Obtain the flanking region sequence of targeted DNA by selecting the flanks.
  - d. Click on 'View' option.
  - e. In the dropdown menu, click on 'DNA' and then click on 'Get DNA'.

⚠ **CRITICAL:** We recommend designing at least two separate pairs of guide sequences for each target.

**Note:** The size of the flanks would depend on the objective of experiment. For deletion of a gene or a non-coding region, larger flanks (50–100 bp) can be selected. For smaller deletions like deletion of transcription factor binding motifs, guide sequences should be designed very close to region to be deleted, thus limiting the flexibility of selecting the flanks.

⚠ **CRITICAL:** It is not necessary to target the complete gene in order to knock it out. Targeting one or a few exons along with promoter is sufficient to perturb the gene.

- f. Paste this sequence into the CRISPOR tool (<http://crispor.tefor.net>) (Concordet and Haeussler, 2018) and choose the correct genome (organism) and PAM that is recognized by the Cas9 protein in use.

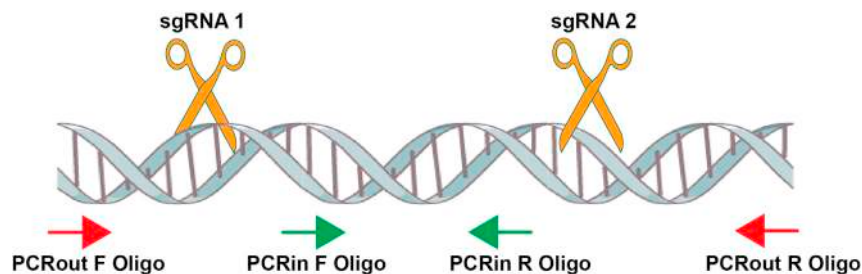
**Note:** The Cas9 protein used in the study recognizes NGG as the PAM site.

- g. Look for guide sequences with high specificity and few predicted off-targets.
  - h. Add the following nucleotide sequences to your guide sequence:  
GGAGAACCACCTTGTGGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAA-TAGCAAGTT (where (N)<sub>20</sub> is the guide sequence).
  - i. Get the 63-nucleotide long DNA guide sequence synthesized.

**Note:** 63 nucleotide long guide sequences can be synthesized in desalted form. We routinely purchase these oligos from local vendors but they can be purchased from IDT (Integrated DNA Technology).

**Note:** We have successfully created lines with deletions ranging from 1 Kb to 3 Kb with this protocol. However, it can be used to knockout smaller or larger regions as well.

2. **Oligos designing for validation of knockout clones.** To screen the colonies for deletion, oligos for PCR validation are to be designed. Design two oligo sets, one within the target region (PCRin) and the other outside the target region (PCRout), as shown in the [Figure 1](#). Get the designed surveyor oligos synthesized.



**Figure 1. Schematic illustrating the design of oligos for PCR validation of knockout clones**

**Note:** Oligos can be designed manually or with the help of a primer designing tool. The knockout amplicon of less than 500 bp is recommended.

3. Cloning of guide sequences in pgRNA-humanized vector
  - a. For sgRNA PCR amplification, the designed 63 nucleotides long guide sequence is used as a forward oligo, universal reverse oligo (See [key resources table](#)) is used as a reverse oligo and the pgRNA-humanized vector as a template (Figure 2). To amplify the full-length sgRNA, set up a 100  $\mu$ L PCR reaction with any proofreading polymerase. For KOD DNA Polymerase following reaction is used:

Component	Volume	Final concentration
10 $\times$ Buffer	10 $\mu$ L	1 $\times$
25 mM MgSO <sub>4</sub>	6 $\mu$ L	1.5 mM
dNTPs (2 mM each)	10 $\mu$ L	0.2 mM
10 $\mu$ M Forward Oligo (guide sequence with overhangs)	3 $\mu$ L	0.3 $\mu$ M
10 $\mu$ M Universal Reverse Oligo	3 $\mu$ L	0.3 $\mu$ M
Template (pgRNA-humanized vector)	X $\mu$ l	30 ng
KOD Polymerase	2 $\mu$ L	0.02 U/ $\mu$ L
Water	X $\mu$ l	
Total Volume	100 $\mu$ L	

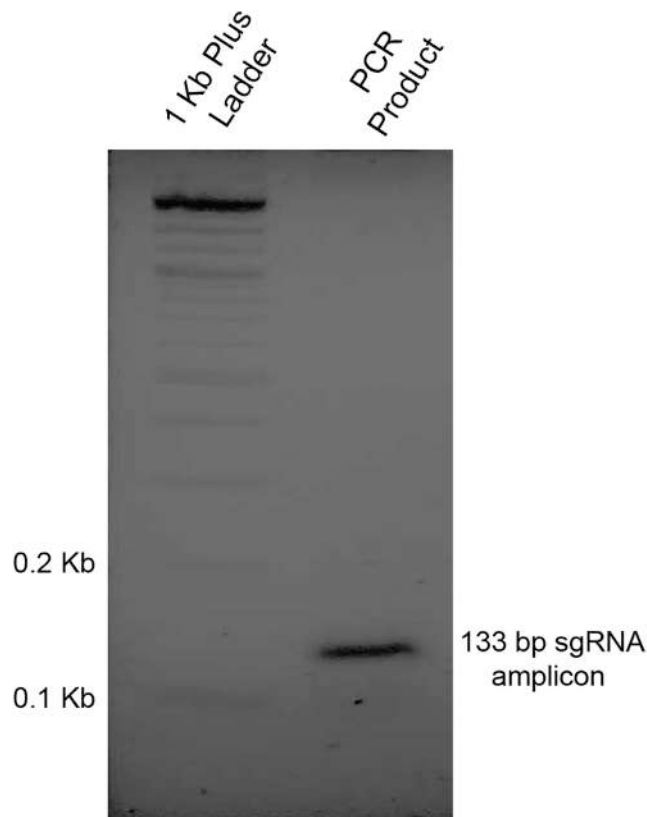
**Note:** Universal reverse oligo can be used for amplification of any sgRNA that is to be cloned in pgRNA-humanized vector.

- b. Run 5  $\mu$ L of the PCR product on 1.8% agarose gel. An amplicon of 133 bp size will be visible (Figure 3).
- c. Clean up the remaining PCR product with the QIAquick PCR Purification Kit after verifying the success of PCR.
- d. Elute the DNA in 25  $\mu$ L of nuclease-free water.
- e. Set up the digestion of purified PCR product with BstXI and XhoI restriction enzymes as follows:

Component	Volume	Final concentration
Purified PCR Product	20 $\mu$ L	X ng
BstXI	1 $\mu$ L	10 units
XhoI	1 $\mu$ L	20 units
Buffer 3.1	2.5 $\mu$ L	1 $\times$
Water	X $\mu$ l	N/A
Total volume	25 $\mu$ L	

- f. Similarly, set up the digestion of pgRNA-humanized vector with BstXI and XhoI restriction enzymes to linearize it:





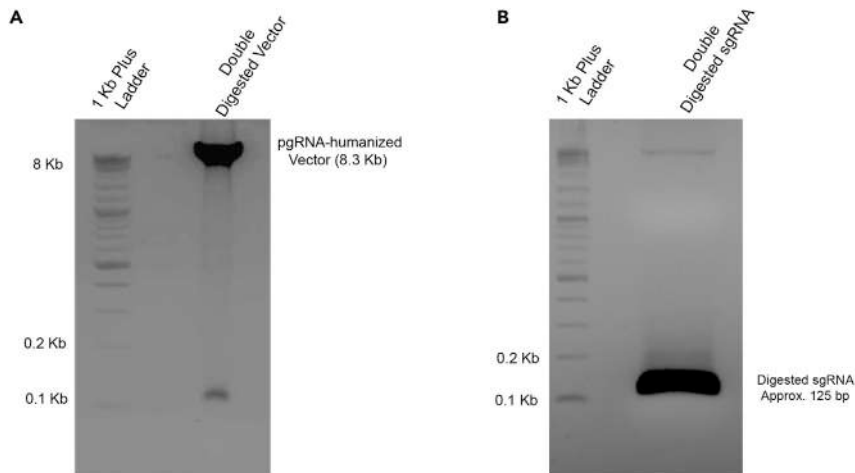
**Figure 3. Agarose gel image showing the amplified full length sgRNA. The amplicon is visible at 133 bp**

Component	Volume	Final concentration
Digested pgRNA humanized vector	X $\mu$ l	50 ng
Digested sgRNA	X $\mu$ l	2.25 ng (3:1 Ratio)
T4 DNA Ligase Buffer	2 $\mu$ L	1 $\times$
T4 DNA Ligase (NEB)	0.5 $\mu$ L	200 U
Water	X $\mu$ l	N/A
Total Volume	20 $\mu$ L	

**Note:** Remember to set up a control ligation reaction that doesn't include the digested sgRNA.

- l. Incubate the ligation samples overnight (14–16 h) at 16°C.
- m. Transform the ligated plasmid into DH5 $\alpha$  strain of E. coli and plate it on agar plate with 100  $\mu$ g/mL ampicillin.
- n. Next day, inoculate at least 10 colonies from the agar plate into tubes containing 3 mL LB broth supplemented with 100  $\mu$ g/mL of ampicillin. Allow the bacteria to grow at 37°C for overnight (14–16 h) with constant shaking.
- o. Using the QIAGEN Plasmid Mini kit, isolate the plasmid from the inoculated colonies. Follow the protocol that comes with the kit.
- p. For screening the positive clones, set up a digestion reaction (as in Step 3e) with BstXI and XhoI (scale down the reaction to 15  $\mu$ L).

**Note:** In order to clearly see the release, minimum 1  $\mu$ g of plasmid DNA should be taken for restriction digestion.



**Figure 4. Agarose gel images showing A) linearized pgRNA-humanized vector and B) sgRNA double digested with BstXI and XhoI**

- q. Run the digested samples on 1.8% agarose gel (Figure 5).
- r. Perform Sanger sequencing on clones that show approx. 125 bp fragment release. Confirm that the clone contains your desired sgRNA with no mutations.

⚠ **CRITICAL:** Test whether your sgRNAs work before proceeding for the rest of the protocol. Transfect the cell line of interest with lentiCas9 Blast and pgRNA-humanized containing your sgRNAs. Select the cells with puromycin (3  $\mu\text{g}/\text{ml}$ ) for 48 h, harvest the cells and isolate genomic DNA and set up PCRs with PCRout oligos. Presence of both wild type and knockout (faint) bands confirms that the sgRNAs are functional. In that case one can proceed with the rest of the protocol.

### Production of lentiviruses in HEK293FT cells

⌚ **Timing:** ~6 days

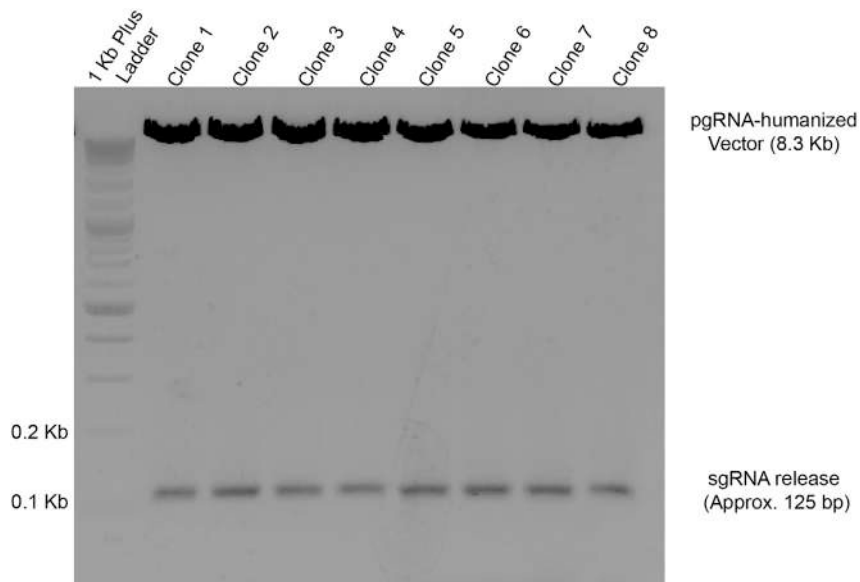
This section describes how lentiviruses carrying sgRNA constructs and lentiCas9-Blast vector are made. Once the lentiviruses are ready, the subsequent steps will guide you to transduce the cells.

4. **Cell culture.** HEK293FT and HeLa cell lines are maintained/cultured in DMEM supplemented with 10% FBS (v/v) and 5% PenStrep (v/v) at 37°C with 5% CO<sub>2</sub>. Passage both cell lines every 3 days.

**Note:** Early passage (less than 30) HEK293FT cell line should be used for viral production.

⚠ **CRITICAL:** HEK293FT cells should be cultured on poly-D lysine-treated plates before transfection because these cells detach from the untreated plate surface following transfection.

5. Cell preparation for transfection (Late Evening)
  - a. Aspirate medium and add 2 mL of 0.25% Trypsin-EDTA to 10 cm dish of HEK293FT cells. Incubate the plate for 1–2 min at 37°C.
  - b. Remove trypsin from the plate carefully to avoid disturbing the cells. Gently tap the plate to dislodge the cells.
  - c. Collect and resuspend cells in 3 mL of fresh culture media.



**Figure 5. Post screening gel image showing the approx. 125 bp release from 8 different positive clones**

d. Seed  $0.8 \times 10^6$  cells on poly-D lysine-coated 35 mm dishes  $\sim 14$  h before transfection. Place the plate back into incubator.

**△ CRITICAL:** At the time of transfection, the cells should be 60–65% confluent. Both low and high confluency will reduce the transfection efficiency, and as a result, also the viral titer.

#### 6. Plasmid transfection (Early Morning)

Transfection mixture of DNA and Lipofectamine 2000 is to be prepared in Opti-MEM medium as follows:

- Add 100  $\mu$ L Opti-MEM and 3  $\mu$ g (0.6  $\mu$ g each of psPAX2, pCMV-VSVG, pgRNA-humanized sgRNA1, pgRNA-humanized sgRNA2 and lentiCas9-Blast) DNA to tube A.
- Add 150  $\mu$ L Opti-MEM and 6  $\mu$ L lipofectamine 2000 to tube B.
- Mix the contents of both tubes thoroughly by pipetting.
- Add the contents of tube B to tube A and mix thoroughly.
- Short spin tube A to collect the liquid from the tube walls.
- Incubate the transfection mixture at room temperature (25°C) for 25 min.
- Meanwhile, remove the spent medium from the plate to be transfected and add 0.75 mL of fresh Opti-MEM to it.
- Add the transfection mixture (tube A) to the cells after 25 min. Swirl the plate to evenly distribute the mixture. Incubate the cells for 6 h at 37°C.
- Remove the transfection mixture from the cells after 6 h of transfection. Add 2 mL fresh media to the plate and incubate it for 48 h in the incubator.

**Note:** The above-mentioned transfection protocol is for 35 mm dishes. If larger dishes are used, the recipe should be scaled up accordingly.

#### 7. Viral Particle collection and transduction

- After 48 h, take the plates out of the incubator and transfer the medium containing viral particles to a sterile 15 mL falcon tube (Falcon A).
- Store the Falcon A at 4°C and incubate the cells for next 24 h with 1 mL of fresh media at 37°C.

- c. Seed  $0.7 \times 10^6$  HeLa cells (or any cell line of interest) in 35 mm dish approx. 14 h prior to transduction.
- d. Remove the media from HEK293FT cell plate after 24 h and transfer it to Falcon A.
- e. Filter the entire viral particle-containing media by passing it through 0.45-micron syringe-driven filters. Collect the filtered media in fresh sterile 15 mL tubes.
- f. Check the volume of filtered media you have and add 8  $\mu\text{g}/\text{mL}$  polybrene.
- g. Add 2 mL of this filtered media to HeLa cells in 35 mm dishes (or any other cell line of interest).
- h. Remove the medium after 12–14 h of transduction. Incubate the cells for 24 h with 2 mL fresh media.

**Note:** If you can't work with/make viruses, cells can be transfected with lentiCas9 Blast and pgRNA-humanized vector with lipofectamine. Because the efficiency of transfection is less than transduction, in that situation approximately 45%–60% of cells will die after puromycin selection.

### Selection of sgRNA-positive cells and seeding of single cells

⌚ Timing: ~20 days

The selection of cells transduced with sgRNAs, followed by single cell seeding in the 96 well plates is discussed in this section.

8. Selection of cells transfected with sgRNAs
  - a. To select the sgRNA positive cells, add 3  $\mu\text{g}/\text{mL}$  puromycin to the transduced cells for 48 h.
  - b. Remove the media and wash the cells twice with DPBS to remove any dead cells.
  - c. Add 0.3 mL trypsin and incubate at 37°C for 1–2 min.
  - d. Carefully remove the trypsin and tap the plate to dislodge the cells.
  - e. Collect the cells and resuspend them in 2 mL of fresh media without puromycin.

**Note:** Puromycin concentration varies from cell line to cell line. Use the concentration which is desired for cell line of your interest.

9. Single cells seeding in 96 well plates
  - a. Using a cell counter, count the number of cells per mL of media. Count the cells at least three times and if the difference between the three readings is small, the average should be used to minimize the variation.
  - b. Add the media and cells to the sterile reagent reservoirs.
  - c. Dilute the cells such that 100  $\mu\text{L}$  of DMEM contains only one cell.
  - d. Using the 100  $\mu\text{L}$  multi-channel pipette, add 100  $\mu\text{L}$  of DMEM-containing cells to the 96 well plate.

**⚠ CRITICAL:** Seed at least five 96 well plates to get clones with homozygous deletion for aneuploid cell lines.

**Note:** Instead of seeding single cells by dilution method, cell sorters can be used to seed cells in 96 well plates. Sorting can be done using the mCherry marker, which is coded from the pgRNA humanized vector along with puromycin.

- e. The next day, observe each well under light microscope at 20 $\times$  resolution to label the wells containing single cell.
- f. Disregard the wells that have no cell or more than one cell.
- g. Allow the single cells to form colonies.
- h. Change the media every 3–4 days, taking care not to lose the cells.

**Note:** It takes approx. 15–20 days for HeLa cells to form colonies that are big enough to be processed further.

△ **CRITICAL:** If the single cells aren't visible the following day, wait 2–3 days for the cells to form minute colonies that can be easily seen under a microscope. Mark the wells that contain single colonies.

**Note:** At least 25–30 wells in a 96-well plate would contain single cells. From five 96-well plates, 100–125 single clones are expected for final screening.

### Screening of knockout clones

⌚ **Timing:** ~ 5 days

This final section will cover the genomic DNA isolation from the cells, followed by PCRs to screen for the colonies carrying homozygous knockout of the region of interest.

#### 10. Genomic DNA isolation

- Add 50  $\mu$ L of trypsin to the colonies and transfer them to 48-well plates with 0.5 mL media. Allow the cells to grow for 2–3 days.
- Trypsinize the cells once they are 80–90% confluent by adding 100  $\mu$ L trypsin to each well. Resuspend the cells in 1 mL media.
- Transfer half of the cells to 1.5 mL tubes for screening, and the other half should be seeded in the same wells.
- Spin the tubes at 600 rcf for 5 min. Discard the supernatant and remove the excess media from the tubes by inverting them on a tissue paper.
- Resuspend the pellet in 300  $\mu$ L of genomic DNA isolation buffer. Pipette thoroughly until the sample's viscosity is reduced. Incubate the samples overnight (14–16 h) at 50°C.
- Add an equal volume of Phenol:Chloroform:Isoamyl (PCI) alcohol to the sample and vortex for 1 min.
- Spin the tubes at 13500 rcf for 12 min at 4°C.
- Collect the aqueous phase with care without disturbing the interphase, and transfer it to a new 1.5 mL tube.

**Note:** Genomic DNA should also be isolated from wild type cells. The nature of clones must be determined by comparison to this control.

△ **CRITICAL:** If the aqueous phase appears viscous, add more digestion buffer to the sample and repeat the PCI extraction.

- To the sample, add 1/10th volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of absolute ethanol. Vortex the tubes and place them in a –20°C freezer for an hour.
- Pellet the precipitated DNA by centrifuging the tubes for 12 min at 4°C at 13500 rcf.
- To remove residual salt, wash the pellet twice with 70% ethanol. Decant the ethanol and allow the pellet to air dry for 15–20 min.
- Dissolve the DNA pellet in 100  $\mu$ L TE buffer.

**Note:** If necessary, RNA contaminants can be removed by treating the sample with RNase A.

⏸ **Pause point:** Extracted DNA can be stored at –20°C for further processing.

#### 11. Validation of knockout clones

**Note:** In our study, we used Kapa Taq DNA polymerase for validation PCRs. Other Taq-based polymerases can also be used, but the supplier's protocol must be followed in that case.

- a. Prepare the PCR mix for the validation of clones (PCRout) as follows:

Components	15 $\mu$ L reaction	Final concentration
10 $\times$ Buffer A or B	1.5 $\mu$ L	1 $\times$
10 mM dNTP	0.3 $\mu$ L	200 $\mu$ M
10 $\mu$ M Forward Oligo	0.6 $\mu$ L	0.4 $\mu$ M
10 $\mu$ M Reverse Oligo	0.6 $\mu$ L	0.4 $\mu$ M
5 U/ $\mu$ L Kapa Taq DNA Polymerase	0.06 $\mu$ L	1 U
Water	Up to 15 $\mu$ L	N/A

- b. Add 2  $\mu$ L of genomic DNA obtained from Step 10I to the PCR mix.

$\Delta$  **CRITICAL:** The concentration of DNA in manually isolated samples cannot be accurately measured. If the desired amplicon is not observed, more volume of template DNA can be taken.

- c. Adjust the PCR to the following parameters:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	30 S	40 cycles
Annealing	T <sub>m</sub> - 5°C	30 S	
Extension	72°C	1 min/kb	
Final extension	72°C	1 min/kb	1

- d. Run all of the samples on an agarose gel (Figures 6A and 6B).

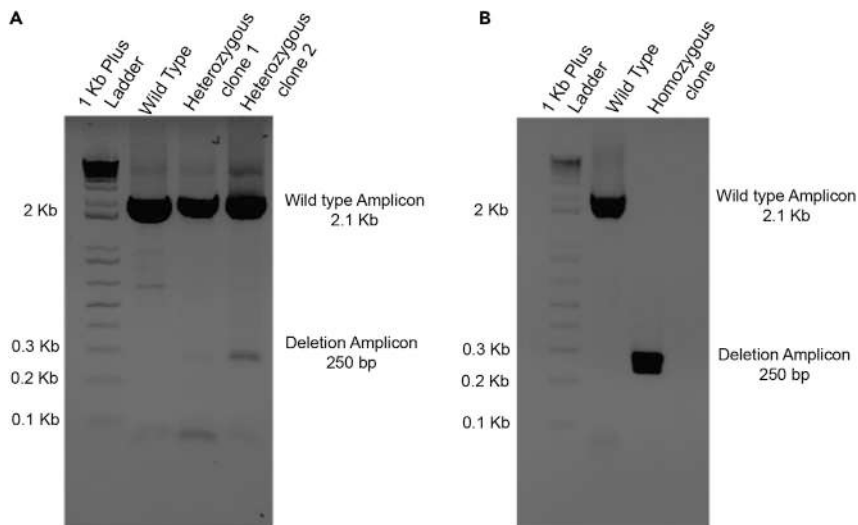
$\Delta$  **CRITICAL:** Determine the percentage of gel based on the expected size of amplicon.

Amplicon size	Gel percentage
>3 Kb	0.8%
1–3 Kb	1%
500 bp – 1 Kb	1.2%
100 bp – 500 bp	1.5%
<100 bp	2%

**Note:** Only the wild type amplicon will be visible in the control sample. Clones with no deletion will have an amplicon similar to the control. Heterozygous clones will produce two distinct amplicons: 1. wild type and 2. knockout amplicon. The homozygous deletion clones show a single amplicon of expected size after deletion.

- e. Complement the above PCR results with another round of PCRs with PCRin oligo set to confirm the outcome of the clones.

**CRITICAL:** The control sample will only show one amplicon of the expected molecular weight. Clones with no deletion and heterozygous deletions will also produce an amplicon of the same size as the control sample. Clones with homozygous deletion will not show any amplification.



**Figure 6. Post PCR validation (PCRout) gel images**

(A) Gel image showing amplicons from wild type cells and heterozygous deletion clones.

(B) Gel image showing amplicon from wild type cells and homozygous deletion clone (Figure 6B is used from Farooq et al., 2021).

**Optional:** If the knockout region codes for a protein, the clones can be further validated using western blotting. qRT PCR can be used to test the expression of regions that are transcribed into RNA.

**△ CRITICAL:** The PCR product from the validated knockout clones should be sent for Sanger sequencing to rule out the possibility of nucleotide insertion or more than expected deletion at the junction of deleted region (Figure 7). Sequence multiple clones for further molecular characterization of deleted line.

## EXPECTED OUTCOMES

We were able to generate knockout lines of both non-coding elements and coding genes using this method. This protocol has been tested primarily with HeLa cell lines, but it also works well with MCF7 cell lines. This protocol can also be applied to other adherent cell lines. The efficiency of obtaining knockout clones, however, would differ from cell line to cell line. The efficiency of this protocol would also be affected by the cell ploidy level.

## LIMITATIONS

This protocol requires co-transfecting at least five different plasmids, reducing the likelihood of obtaining a knockout clone. This limitation can be overcome by using a different vector system that can hold multiple sgRNAs. This would vastly improve protocol efficiency.

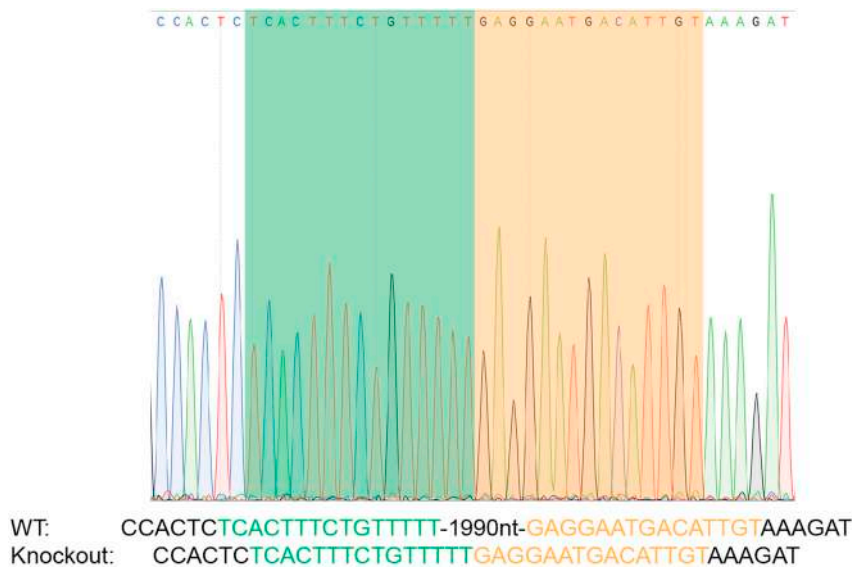
## TROUBLESHOOTING

### Problem 1

No deletion amplicon observed (step 11)

### Potential solution

There are numerous reasons why a knockout amplicon is not obtained. One possible explanation is that one of the sgRNAs in a pair is ineffective. Rule out this possibility by using the second set of sgRNAs. If the second set of sgRNAs also fails to produce a deletion amplicon, double-check



**Figure 7. A representative Sanger sequencing chromatogram showing the absence of the deleted region.**  
 Used from [Farooq et al., 2021](#).

your sgRNA design. Cas9 from various bacterial strains requires a different PAM site. Check to see if you selected the correct bacterial strain when designing the sgRNAs.

### Problem 2

No homozygous knockout line obtained after screening (step 11)

#### Potential solution

There are a variety of explanations why a homozygous clone cannot be obtained. One possible explanation is that the clones obtained are not from a single cell. When seeding the cells in a 96-well plate, try breaking up the clumps into single cells. Even, when labeling the wells that contain only single cells, be very careful. Another possibility is that your region of interest is critical for cell viability, and the cells with the homozygous deletion do not survive during the process.

### Problem 3

All cells are dead after antibiotic selection (step 8)

#### Potential solution

Puromycin concentration varies between cell lines. Check the optimal puromycin concentration for your cell line ahead of time. This issue could also be caused by a low virus titer. Before transduction, determine the viral titer. If you have a low viral titer, try concentrating viruses from multiple dishes. Low titer can occur for a variety of reasons, including: 1. Plasmid transfection efficiency is low. This could be due to poor plasmid quality. It can also occur as a result of too high or too low cell confluency. 2. The HEK293FT cells in poor health at the time of transfection would also affect the transfection efficiency. 3. The cell line of interest does not tolerate constitutive expression of Cas9 protein. This can be avoided by transiently delivering lentiCas9 blast to the cells. Furthermore, using vectors in which Cas9 expression is inducible rather than constitutive would also help. In such cases, vectors like pCW-Cas9 (#50661) should be used.

### Problem 4

During screening, the PCR does not work for all samples (step 11).

### Potential solution

This could be because the samples carry high salt and residual phenol that inhibit the PCR reaction. Purify these samples using Phenol-chloroform-Isoamyl alcohol once more. The pellet should be washed twice with 70% ethanol. This would aid in the removal of salt.

### Problem 5

Sanger sequencing reveals the presence of a few random nucleotides at the junction of deleted region (step 11).

### Potential solution

These added nucleotides are likely the result of DNA repair process. Send more homozygous knockout clones obtained during screening for Sanger sequencing. Choose the clone that does not have any random nucleotide mutations or has the fewest of all.

### Problem 6

PCRout does not show any wildtype amplicon in some samples, whereas PCRin shows an amplicon of the desired size (step 11).

### Potential solution

This implies that the clones are heterozygous in nature. The region of interest is deleted from some copies of the chromosomes (A problem with aneuploid cells) but not from others. PCRin assay should not show any amplicon for a clean homozygous clone. Clones with only knockout amplicon in PCRout (wild type amplicon should be absent) and no amplicon in PCRin assays should be chosen.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dimple Notani ([dnotani@ncbs.res.in](mailto:dnotani@ncbs.res.in))

### Materials availability

No unique reagent was generated in this study.

### Data and code availability

No unique datasets or data were generated in this study.

## ACKNOWLEDGMENTS

We acknowledge support of the Department of Atomic Energy, Government of India, under project no.12-R&D-TFR-5.04–0800, Department of Biotechnology (BT/PR38396/GET/119/305/2020), and intramural funds from NCBS-TIFR. D.N. is an EMBO Global Investigator. We also acknowledge the funding support from Wellcome-IA: IA/1/14/2/501539 (to D.N.). U.F. is supported by CSIR (Govt. of India) senior research fellowship. We are grateful to Henrietta Lacks and to her family members for their contributions to biomedical research

## AUTHOR CONTRIBUTIONS

The protocol is written by U.F. with feedback from D.N. Authors thank DN Lab members for input.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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