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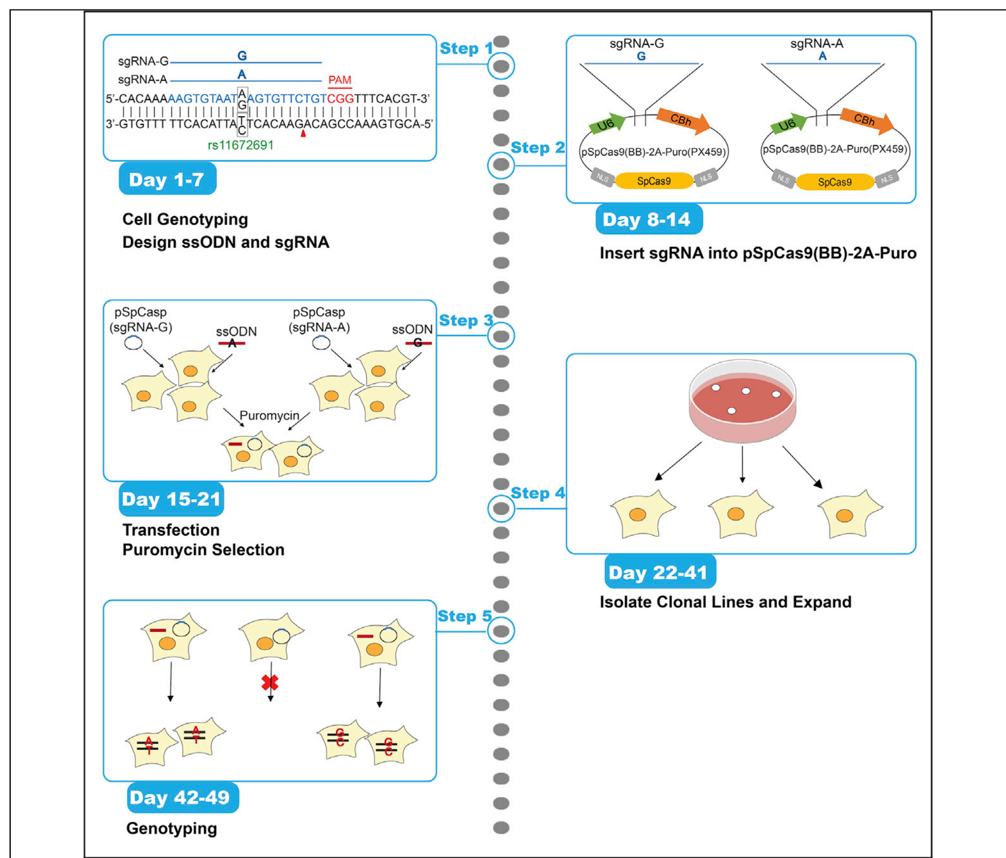
Optimized CRISPR/Cas9-mediated single nucleotide mutation in adherent cancer cell lines



CRISPR/Cas9 is an efficient, accurate, and optimizable genome-editing tool. Here, we present a modified CRISPR/Cas9 genome-editing protocol for single nucleotide mutation in adherent cell lines. The protocol was adapted to focus on ease of use and efficiency. The protocol here describes how to generate a single nucleotide mutation in cultured 22Rv1 cells. We have also used the protocol in other adherent cell types. Thus, the protocol can be applied to assessing the effect of non-coding SNPs in a variety of cell types.

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Highlights

A detailed protocol for CRISPR/Cas9-mediated single nucleotide editing in cultured cells

Protocol to convert an SNP genotype in 22Rv1 cell line

Efficient and easy method yields single cell clones with desired genotypes

Applicable to the study of SNPs in a variety of adherent cell lines

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Optimized CRISPR/Cas9-mediated single nucleotide mutation in adherent cancer cell lines

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SUMMARY

CRISPR/Cas9 is an efficient, accurate, and optimizable genome-editing tool. Here, we present a modified CRISPR/Cas9 genome-editing protocol for single nucleotide mutation in adherent cell lines. The protocol was adapted to focus on ease of use and efficiency. The protocol here describes how to generate a single nucleotide mutation in cultured 22Rv1 cells. We have also used the protocol in other adherent cell types. Thus, the protocol can be applied to assessing the effect of non-coding single nucleotide polymorphisms (SNPs) in a variety of cell types.

For complete details on the use and execution of this protocol, please refer to Gao et al. (2018).

BEFORE YOU BEGIN

This protocol describes below the single nucleotide mutation in cultured 22Rv1 cells (Gao et al., 2018; Zhang et al., 2018). However, we have also used this protocol in cultured breast cancer cell line MCF7 and normal human lung epithelial BEAS-2B cells (Wang et al., 2020). Thus, the main steps of the protocol can be also adapted for other adherent cells. All the oligos used in this experiment were ordered from Eurofins Genomics (Ebersberg, Germany),

Reagent and material check

Timing: ~7 days

1. Read carefully the reference Ran et al., 2013. This will help researchers to design proper sgRNAs and single-stranded DNA oligonucleotides (ssODNs) as repair template for the experiment.
2. Purchase and prepare plasmid (pSpCas9 (BB)-2A-Puro (PX459); AddGene plasmid #62988).

> ⚠ CRITICAL: Midi plasmid prep kit or endotoxin free plasmid extraction kit should be used at plasmid extraction step when these plasmids are prepared for cell transfection.

3. Prepare reagents and cell culture medium that are sufficient for the experiment.

> *Note:* The following experimental procedures use a standard hemocytometer for cell counting.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Stbl3™ chemically competent cells	Thermo Fisher Scientific	N/A
Chemicals, peptides, and recombinant proteins		
T4 DNA ligase	New England Biolabs	Cat#M0202L
BbsI	New England Biolabs	Cat#R3539S
T4 polynucleotide kinase	New England Biolabs	Cat#M0201S
CutSmart buffer, 10×	New England Biolabs	Cat#B7204S
T4 DNA ligase reaction buffer, 10×	New England Biolabs	Cat#B0202A
Alkaline phosphatase buffer 10×	TAKARA	Cat#2250A
Alkaline phosphatase	TAKARA	Cat#2250A
RPMI1640	Merck	Cat#R8758
Fetal bovine serum	Gibco	10099141C
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15140122
Trypsin-EDTA solution	Merck	Cat#T3924-500ML
Opti-MEM™ I Reduced Serum Medium, no phenol red	Thermo Fisher Scientific	Cat#11058021
Lipofectamine 2000	Thermo Fisher Scientific	Cat#11668030
Lipofectamine 3000	Thermo Fisher Scientific	Cat#L3000015
Puromycin	Merck	Cat#P9620
Exonuclease I and FastAP	Thermo Fisher Scientific	Cat#EF0651
2× Phusion Master Mix with HF Buffer	Thermo Fisher Scientific	Cat#F531
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	Cat#10977049
UltraPure™ Agarose	Thermo Fisher Scientific	Cat#16500100
Dulbecco's phosphate buffered saline	Corning	Cat#20-031-CVR
1 Kb Plus DNA Ladder	Thermo Fisher Scientific	Cat#10787018
100 bp DNA Ladder	Thermo Fisher Scientific	Cat#15628019
Ampicillin	Merck	Cat#A5354
QuickExtract DNA extraction solution	Epicentre	Cat#QE09050
LB medium	Thermo Fisher Scientific	Cat#10855021
Critical commercial assays		
GeneJET PCR Purification Kit	Thermo Fisher Scientific	Cat#K0702
GeneJET Gel Extraction Kit	Thermo Fisher Scientific	Cat#K0692
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific	Cat#K0503
PureYield™ Plasmid Miniprep System	Promega	Cat#A1222
QIAGEN Plasmid Midi Kit (25)	QIAGEN	Cat#12143
PureLink™ Genomic DNA Mini Kit	Thermo Fisher Scientific	Cat#K182001
Experimental models: cell lines		
22Rv1	ATCC	Cat#CRL-2505
Oligonucleotides		
Sequencing primer: primer: U6-Fwd primer: GAGGGCCTATTTCCCATGATTCC	Ran et al., 2013	N/A
sgRNA: sgRNA-A-top: CACCGAAGTGTA ATAAGTGTTCTGT	Gao et al., 2018	N/A
sgRNA: sgRNA-A-bottom: AAACACAGA ACACTTATTACACTTC	Gao et al., 2018	N/A
sgRNA: sgRNA-G-top: CACCGAAGTGTA ATGAGTGTTCTGT	Gao et al., 2018	N/A
sgRNA: sgRNA-G-bottom: AAACACAGA ACACTCATTACACTTC	Gao et al., 2018	N/A
Genotyping primer: rs11672691-Forward: CCAGCGATTAAGGGTCTCGT	Gao et al., 2018	N/A
Genotyping primer: rs11672691-Reverse: TCCCATAAAATGGCCACGCTC	Gao et al., 2018	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ssODN: rs11672691-A-F: CATGTTC ATTATAAGTGAAGTAAATGAGCT CACAAAAAGTGTAATAAGTGTTC TGTCGATTTCACGTAAAGATATA TAATGGCATCGTCCC	Gao et al., 2018	N/A
ssODN: rs11672691-A-R: GGGACG ATGCCATTATATATCTTTACGTGA AATCGACAGAACACTTATTACAC TTTTTGTGAGCTCATTTACTTCAC TTATAATGAACATG	Gao et al., 2018	N/A
ssODN: rs11672691-A-F: CATGTTC ATTATAAGTGAAGTAAATGAGCTC ACAAAAAGTGTAATGAGTGTTCTG TCGATTTCACGTAAAGATATATAA TGGCATCGTCCC	Gao et al., 2018	N/A
ssODN: rs11672691-A-R: GGGACG ATGCCATTATATATCTTTACGTGAA ATCGACAGAACACTCATTACACTT TTTGTGAGCTCATTTACTTCACTT ATAATGAACATG	Gao et al., 2018	N/A
Recombinant DNA		
pGL3-Basic	Promega	Cat#E1751
pSpCas9 (BB)-2A-Puro (PX459)	Addgene	Cat#48139
Others		
MicroAmp™ Clear Adhesive Film	Thermo Fisher Scientific	Cat#4306311
Multiplate PCR Plates 96-well, clear	Bio-Rad	Cat#MLL9601
96-well cell culture plate	Corning	Cat#3599
24-well cell culture plate	Corning	Cat#3524
15 mL Centrifuge tube	Corning	Cat#430791
1.5 mL Centrifuge tube	SARSTEDT	Cat#72.690.001
10 ul Bulk tip	Sartorius	Cat#790014
200 ul Bulk tip	Sartorius	Cat#790240
1000 ul Bulk tip	Sartorius	Cat#791004
10 cm Pipette	Corning	Cat#4488
10 cm Cell culture plate	Greiner	Cat#664160

Note: The reagents recommended in the list can be substituted with the same reagent from other suppliers except 2× Phusion Master Mix with HF Buffer and pSpCas9 (BB)-2A-Puro (PX459).

STEP-BY-STEP METHOD DETAILS

Genotyping 22Rv1 cells

Timing: ~4–6 days

The genotype of the SNP rs11672691 in 22Rv1 cells should be examined first. The results are essential for researchers to design proper sgRNA and ssODNs.

1. Design genotyping primers
   a. The SNP site centered 600 bp genomic DNA sequence was used to design genotyping primers.
   b. Enter the DNA sequence into Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome)(Ye et al., 2012) to design proper primers (amplicon length 200–400 bp, Tm value 60°C ± 3).
   c. Three paired primers were synthesized from Eurofins Genomics (Ebersberg, Germany)

d. A PCR reaction mixture as step 3 was setting up for testing primer specificities In brief, PCR reaction was carried out as step 4, and the PCR product was run on a 1.5% agarose gel. The primers that can yield for a single size of PCR fragment will be selected, and the PCR products are confirmed Sanger sequencing.

⚠ CRITICAL: If the PCR cycling condition does not work well for the primers, the gradient PCR should be taken into consideration to find the optimal annealing temperature.

2. Extract genomic DNA from 22Rv1 cells using genomic DNA extraction kit, and dilute the DNA concentration to 5 ng/μL.
3. Set up 50 μL PCR reaction mix as the following PCR reaction mixture.

Reagent	Final concentration	Amount
Genotyping Primer (5 μM)	0.5 μM	5 μL
Genomic DNA (5 ng/μL)	1 ng/μL	10 μL
2 × Phusion High-Fidelity PCR Master Mix with HF Buffer	1 ×	25 μL
ddH_2O		10 μL
Total		50 μL

4. Carry out PCR under the following PCR cycling conditions, run the target DNA band in 1.5% agarose gel, purify the PCR fragment from the gel and send for genotyping.

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	5 min	1
Denaturation	98°C	15 s	30–34 cycles
Annealing	60°C	20 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

⚠ CRITICAL: To keep the high fidelity of PCR product, PCR cycle number should be less than 35 in the conditions.

Inserting sgRNA into the vector pSpCas9 (BB)-2A-Puro

⏲ Timing: ~7 days

Prior to insert sgRNA into plasmid pSpCas9 (BB)-2A-Puro, make sure to design and order proper sgRNA from a qualified company (e.g., Eurofins Genomics, (Ebersberg, Germany))

5. Preparation of the sgRNA oligos for vector insertion
   a. Design sgRNAs that have cut site near the SNP using the online sgRNA design tool: https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design.
   b. 100 μM top and bottom sgRNA were ordered from Eurofins Genomics (Ebersberg, Germany) and mixed gently in the 0.2 mL tubes according to Table 1.
   c. Put the tubes into the thermocycler for oligo annealing: 37°C for 30 min; 95°C for 5 min; ramp down to 25°C at 5°C per min.
   d. Dilute the annealed oligos 200× using ddH_2O.

6. Digest plasmid pSpCas9 (BB)-2A-Puro.

Table 1. The volume of sgRNA and chemicals for annealing mixture

Reagent	Final concentration	Amount
sgRNA top (100 μM)	10 μM	1 μL
sgRNA bottom (100 μM)	10 μM	1 μL
T4 ligation buffer, 10×	1×	1 μL
T4 PNK		1 μL
ddH_2O		6 μL
Total		10 μL

a. Prepare the mixture in the 1.5 mL tubes according to Table 2, and incubate the tubes at 37°C 12–16 h;
b. 42 μL of the mixture was transferred to a new tube, 5 μL alkaline phosphatase buffer 10× and 3 μL alkaline phosphatase were added, incubate the tube at 37°C for 15 min; 50°C for 15 min.
c. Run the digested product on 1% agarose gel, purify the plasmid by GeneJET Gel Extraction Kit, and dilute the digested plasmid to 25 ng/μL for next step.

7. Ligate the sgRNA and vector according to Table 3, incubate at 16°C 12–16 h.

⚠ CRITICAL: 1 μL of ddH_2O instead of sgRNA in the ligation system served as a negative control. If there are many colonies on the negative control plate after step 8, the digest efficiency of the plasmids in step 6 should be lower. Thus, all the reagents in step 6 are recommended to be quality-checked. If the numbers of colonies on the experimental plate are rare as the negative control plate, all the reagents from step 7 should be assured in good quality. Alternatively, 2 μL of sgRNA can be added to enhance ligation efficiency.

8. Transformation. Transfer 10 μL of ligation product into 100 μL of competent Stbl3 strain, incubate the mixture on ice for 1 h, heat-shock at 42°C for 90 s and return it immediately on ice for 3 min. The add 500 μL of LB medium (without antibiotic) and incubate at 37°C for 30 min, centrifuge the tube at 1500 × *g* 5 min, and remove 500 μL supernatant. Next, resuspend the bacteria in the rest of supernatant and spread it onto an LB plate containing 100 μg/mL ampicillin. Put the plate into the incubator 14–16 h at 37°C.
9. Pick single clone and place it in the tube containing 3 mL LB medium with 100 μg/mL ampicillin, then put the tube in the shaker at 37°C 12–16 h.

⚠ CRITICAL: More clones from one plate can be picked up at the same time to ensure get positive clone.

10. Plasmids were purified and verified by using U6-Fwd primer for sequencing.

Cell culture and transfection

⏲ Timing: ∼7 days

Table 2. The amount of vectors, enzymes, and chemicals for plasmid digestion

Reagent	Final concentration	Amount
Vector	0.04 μg/μL	2 μg
CutSmart buffer, 10×	1 ×	5 μL
ddH_2O		41 μL
BbsI		2 μL
Total		50 μL

Table 3. The amount of sgRNA, vector and chemicals for ligation mixture

Reagent	Final concentration	Amount
ddH_2O		6 μL
Diluted sgRNA		1 μL
Vector 25 ng/μL	2.5 ng/μL	1 μL
T4 ligation buffer, 10×	1 ×	1 μL
T4 ligase		1 μL
Total		10 μL

22Rv1 cells were cultured according to the instructions from ATCC. One 10 cm plate of 70%–80% confluency cells was prepared for this experiment. Cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) at 37°C with 5% CO_2. At the same time, the SNP centered repair templates should be designed and ordered (ssODNs) from Eurofins Genomics (Ebersberg, Germany).

11. Prepare cells for transfection
    a. Remove the old medium and rinse the cells once by DPBS. Add 3 mL of Trypsin to 10 cm plate, put the plate in the cell culture incubator for 5 min at 37°C. 5 mL warm (37°C) RPMI-1640 medium was added in the plate to inactivate trypsin. Pipette the cells gently and transfer cell suspension into 15 mL tubes and spin at 600 × *g*, 5 min. The supernatant was removed, 4 mL fresh medium was added, and the cells were dissociated by gently and repeatedly pipetting. 0.5 mL of cells was added into 5 mL warm medium, homogenize cells in medium by pipetting, and then 0.5 mL of the mixture was transferred to each well in a 24-well plate. Notably, one more well should be added as control cells, and put in the cell culture incubator at 37°C with 5% CO_2.
    b. 12 h later, the medium was replaced with 0.5 mL fresh warm medium.

⚠ CRITICAL: Low density cells should be used to increase the transfection efficiency. Cells with 40%–50% confluency is ideal for transfection.

12. Plasmid transfection
    a. The Lipofectamine 2000 and DNA were diluted, respectively, with OptiMEM according to Table 4. After 5 min incubation, combine the mixture #2 with mixture #1, incubate at 20°C –25°C- for 20 min.
    b. Add the DNA-Lipofectamine 2000 complexes to each well, mix gently by rocking.
    c. 12 h later, the medium was changed with 0.5 mL fresh warm medium. At 24 h post transfection, puromycin selection can be applied at a final concentration of 0.7 μg/ mL. 48 h post transfection, cells should be passaged using medium with 0.7 μg/ mL puromycin.

Optional: The plasmid with the SNP centered DNA fragment can also be used as repair template. The length of the DNA fragment is better less than 1 kb. During the transfection step, 350 ng of the repair vectors instead of ssODN is enough for one well of 24-well plate.

Table 4. Volume of plasmids, ssODN and chemicals for transfection mixture

Mixture	Reagent	Experimental group	Control group
#1	OptiMEM	100 μL	100 μL
	Lipofectamine 2000	4 μL	4 μL
#2	OptiMEM	100 μL	100 μL
	pSpCas9 (BB)-2A-Puro	350 ng	
	ssODN 10 μM	2 μL	2 μL

⚠ CRITICAL: 24-well plate is proper for single clone selection. 22Rv1 cells should be passaged every 4 days. The amount of vector pSpCas9 (BB)-2A-Puro should not be more than 0.5 μg, too much amount of plasmid will cause cell death.

Isolating clonal lines

⏲ Timing: ~20–30 days

After three days, when the control cells all died in the medium with 0.7 μg/mL puromycin, the experiments are ready for isolating single cell clones.

13. Seeding single cells in 10 cm plate.
    a. Remove the old medium, rinse the cells twice by DPBS, then twice by 0.5 mL trypsin immediately at 20°C–25°C. Aspirate 450 μL trypsin out of the well. Put the plate in the cell incubator at 37°C.
    b. Check the cell status under microscope after 6–10 min, until all cells detached from dish, 1 mL warm 1640 medium supplemented with 10% (vol/vol) FBS was added into each well for inactivating trypsin.
    c. Dissociate cells by pipetting and transfer all the cell suspension into 1.5 mL tube.
14. Dilute the cells 20 × using DPBS, count the cells with hemocytometer.
15. Take 100 or 200 cells from one tube and seed in 10 cm plate. Rest of the cells were seeded into 6-well plate. Check the density of cells in 10 cm plate in 1 h and observe cells under microscope magnifying 10×. Then randomly select five fields and count the cell numbers, with about 4–8 cells in total representing the best density. Dilute the cells again if the density of the cells is high or add more cells into 10 cm plate when the density of the cells is too low.

⚠ CRITICAL: To increase the efficiency, fluorescence-activated cell sorting (FACS) is not recommend for seed single cell into 96-well plate, because cells are quite fragile after sorting and easily died during extended period of cultivation.

16. Put the plates in the cell incubator at 37°C with 5% CO_2 for 18–25 days until the clones grow bigger enough for next step. The old medium was changed, and cells were checked every 5 days.

Optional: Cells maintained in 6-well plate can be cryopreserved in liquid nitrogen in case need them for future use.

⚠ CRITICAL: Lower amount of trypsin is useful to well separate adherent cells. In order to well dissociate adherent cells, do not shake or vibrate the plate before the cells were detached in step 13.

Pick single cell clones for genotyping

⏲ Timing: ~1–3 h

When the clones are visible to the naked eyes, they are ready to be picked up from the 10 cm plate (Figure 1).

17. Add 20 μL trypsin into each well of 96-well plate.
18. Take out the 10 cm plate from cell incubator, discard the old medium completely, add 4 mL DPBS into the plate. Be sure that the DPBS will not spill out while rotating the plate.
19. Single clone isolation.

a. Use the 10 μL pipette tips to pick clones with moderate size. When pick clones from 10 cm plate, we recommend aspirate 5 μL trypsin first in the pipette tip, and then pipette it out on the clone and hold the push-button.
b. Scrap the clone from the plate by the pipette tips, then slowly release the push-button on the pipette to carefully aspirate the cell clone in the tip.
c. Transfer these single clones quickly into the wells of a 96-well plate.

⚠ CRITICAL: Change the position after selecting one single clone or remember to rotate the plate frequently to prevent the plate dry out.

Optional: Cloning cylinders is an alternative choice for picking single clones.

20. 24–36 single clones from each plate with different genotyped (GG or AA repair template) were picked up and incubated at 37°C for 5 min. Then 30 μL medium supplemented with 10% (vol/vol) FBS was added into each well of 96-well plate to inactivate trypsin. Pipetting the cells to separate the adherent cells.
21. After that, transfer 10 μL cell suspension from each well into a 96-well PCR plate for step 23.
22. 100 μL fresh medium was added into each well. The 96-well plate was put back to the cell incubator. 24 h later, change the old medium of the 96-well plate with fresh medium to remove the containing trypsin.

⚠ CRITICAL: To avoid the cells exposure in trypsin too long time, we recommend continue step 20 after picked 24 single clones into one 96-well plate. If there are more clones, use a new plate to repeat steps 19 and 20.

Genotyping cell clones

Timing: ~4–5 days

After collecting all the samples from step 21, it is ready for DNA extraction and genotyping.

23. Add 20 μL QuickExtract solution to each well of PCR plate (from step 21) with 10 μL cell suspension. Mix by pipetting gently, then put the plate in PCR machine and run the following PCR program.

Steps	Temperature	Time	Cycles
1	65°C	6 min	1
2	98°C	2 min	1
Hold	4°C	forever	

Pause point: Extracted DNA can be stored at –20°C for 12 months.

24. Prepare PCR mix with 4 μL DNA from step 23 as follows .

Reagent	Final Concentration	Amount
Genotyping Primer (5 μM)	0.5 μM	5 μL
Genomic DNA from step 23		4 μL
2 × Phusion High-Fidelity PCR Master Mix with HF Buffer	1 ×	25 μL
ddH_2O		16 μL
Total		50 μL

⚠ CRITICAL: The amount of DNA cannot be accurately measured. A test PCR of 10 μL system is necessary to ensure that you can get target band before using 50 ul PCR reaction system.

Optional: The amount of DNA can also be measured by the Qubit fluorometer if the results of PCR testing are not good. Moreover, this may help researchers to find reasons why there is no band from PCR.

25. Run the PCR cycling conditions of step 4.
26. Run the PCR product in 1.5% agarose gel, cut the gel and purify DNA using the GeneJET Gel Extraction Kit.

Optional: If your genotyping primer is quite specific, the primer dimers can be removed from the PCR product and send directly for sequencing.

⚠ CRITICAL: Genotype the clones as soon as possible, before the cell clones become confluence in 96-well plate. The clones in 96-well plate should be checked every day. If genotyping results were not obtained timely, the confluent cells need to be transferred to a 24-well plate for further culture until the genotyping results came out.

Passage cell clones

Timing: ~14–30 days

The culture expansion of the cells with successfully converted SNP genotypes should be done after the genotyping step.

27. When cells reach 50%–70% confluence, remove the old medium, and rinse the cells twice by DPBS. Add 50 μL of Trypsin, incubate at 37°C for 5 min. 100 μL medium was added to inactivate trypsin, dissociate cells by pipetting and transfer all the cell suspension into the wells prefilled with 500 μL fresh medium in 24-well plate.

⚠ CRITICAL: The cells in the 96-well plate usually grow very slowly, and remember to change the medium every three days until the cells are confluent for passaging. Earlier transfer of the cells to bigger plate may cause cell death when the density of the cells is too low.

EXPECTED OUTCOMES

The expected outcomes are showing in Figure 2. Using this method, the mutated efficiency at rs11672691 in 22Rv1 cells is approximately 0.1%–0.5%. We have also applied this method to convert the genotype of rs2853669 in 22Rv1 cells and MCF7 cells, respectively, resulting in the efficiency about 1%–2% in 22Rv1 cells and 3%–5% in MCF7 cells. Thus, the efficiency of this method varies at different SNP sites and cell lines. This is probably due to the differences in local genomic sequence feature, chromatin structure, and cellular properties.

LIMITATIONS

We failed many times to change the genotype of SNPs located at the exons. This may indicate that the mutated efficiency is lower when the SNPs located at the exonic genomic region. If the SNP located in the non-conserved region such as *TERT* promoter, the conversion efficiency is quite higher than that of location at the highly conserved region.

TROUBLESHOOTING

Problem 1

Genotyping 22Rv1 cells:

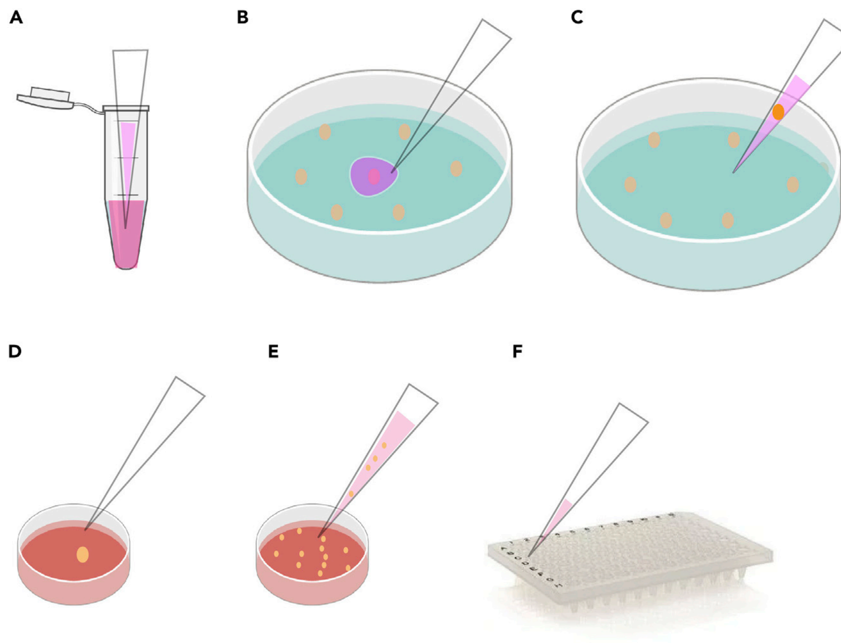

Figure 1. Workflow of picking single clone for genotyping
(A) 5 μL trypsin was aspirated in the pipette tip.
(B and C) Scrap the clone from the plate by the pipette tips (B), then slowly release the push-button on the pipette to carefully aspirate the clone in the tip (C).
(D) Transfer the single clone into one well of a 96-well plate with 20 μL trypsin and incubate 5 min at 37°C.
(E and F) Inactivate trypsin and separate the adherent cells by pipetting (E), transfer 10 μL cell suspension from each well into a 96-well PCR plate (F).

Primers with low specificity for genotyping (step 1)

Potential solution

The genotype of the SNP should be determined in advance in the tested cells with CRISPR/Cas9-mediated SNP genotype conversion. The results of SNP genotype are needed for designing proper sgRNA and ssODNs. If the sequencing results did not show a single peak, the primer for genotyping should be re-designed and tested. Make sure to design the specific primers as this is critical for the final genotyping results.

Problem 2

Inserting sgRNA into the vector pSpCas9 (BB)-2A-Puro:

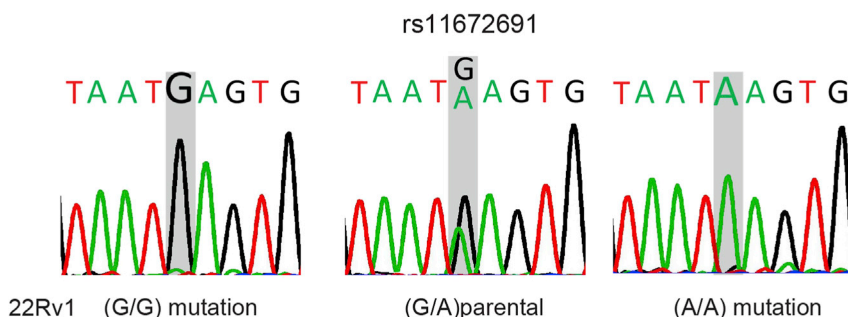

Figure 2. The genotype at rs11672691 in 22Rv1 cells was successfully converted from G/A to G/G and A/A, respectively

Table 5. The concentration gradient tests for puromycin in different cell lines

	Puromycin concentration (μg/mL)					
Wells with pSpCas9 (BB)-2A-Puro	1	0.9	0.8	0.7	0.6	0.5
Control wells	1	0.9	0.8	0.7	0.6	0.5

Low ligation efficiency (steps 6 and 7).

Potential solution

Digest plasmid pSpCas9 (BB)-2A-Puro with BbsI, incubate the tubes at 37°C for 12–16 h. If the time for digestion is shorter, plasmid pSpCas9 (BB)-2A-Puro will not be completely digested. If so, it is not easy to successfully ligate the sgRNA and vector.

Problem 3

22Rv1 cell culture and transfection:

Many dead cells were observed after transfection (step 12).

Potential solution

Forget to change media 12–18 h after the addition of the transfection mixture will result in cell death due to the toxicity of Lipofectamine 2000. So, ensure that the medium was replaced with fresh warm medium at 12 h post transfection.

Problem 4

22Rv1 cell culture and transfection:

Low transfection efficiency (step 12).

Potential solution

Lipofectamine 3000 instead of Lipofectamine 2000 can be used to increase the transfection efficiency. Reverse transfection should also be tried to enhance transfection efficiency.

Problem 5

22Rv1 cell culture and transfection:

All cells are dead after puromycin selection step or control cells still alive after three days puromycin selection. (step 12)

Potential solution

For different cell lines, the working concentration of puromycin should be tested in advance. The titration experiment can be set up according to the following formula in Table 5 and performed to select a suitable dose of puromycin that could kill all the control cells in three days without harming the cells with pSpCas9 (BB)-2A-Puro.

Problem 6

Pick single clone for genotyping:

The picked single clonal cells died after transferred into 96-well plate. (step 19)

Potential solution

When the clone is visible to the naked eye, single clone is ready to be picked out from the 10 cm plate. Make sure to pick the moderate size clones by using the pipette with 10 μL tip. It is not easy to proliferate for too small size clones after transferring clones from 10 cm plate into 96-well

plate. Then the cells are likely to go apoptosis. In addition, the clones with too big size are unlikely to be single clones, maybe the mixture of two or more single clones.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gong-Hong Wei (gonghong_wei@fudan.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No new datasets or code were generated in the preparation of this manuscript.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.G. and G.-H.W.; investigation, P.G., X.D., Y.W., and G.-H.W.; writing – original draft, P.G. and G.-H.W.; writing – review & editing, P.G., X.D., Y.W., and G.-H.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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