EZ-editor™ Gene Knockout kit

Product Info

This EZ-editor™ Gene Knockout kit is based on the CRISPR-U™ system, and provides the main ingredients required in the whole process of gene-editing. The EZ-editor™ Gene Knockout kit provides efficient and convenient gene knockout tools for researchers and achieves the fast construction of gene knockout cell lines.

In order to achieve efficient gene knockout, 3 knockout plasmids in this kit are constructed by Ubigene Red Cotton™ CRISPR Gene Editing System after high-throughput data simulation. These knockout plasmids can ensure the comprehensively optimal performance on the cutting site, cutting efficiency, and specificity.

To greatly reduce the experimental cost and shorten the experimental turnarounds. The MicroCell DNA Lysis and PCR Validation in the EZ-editor™ Gene Knockout kit are the developed products for single-cell clones validation in gene-editing experiment, which can validate the single-cell clones at the early stage of single-cell clone growth. The preparation of genomic samples only takes 15-20 minutes. The cells are directly lysed to release the genome and the cell lysate can be used directly in following PCR experiments without purification. Utilizing 96-well plates to simultaneously identify up to hundreds of clones makes high-throughput validation of single-cell clones. Through screening positive single-cell clones at early stage, the experimental turnarounds is shortened by 2-4 weeks, and the experimental cost of culturing non-positive clones are greatly reduced. The preferred PCR validation reagent can be compatible with the factors that may affect the PCR reaction, such as the residual medium and components after cell lysis in the roughly extracted genome samples, and can quickly and accurately identify the

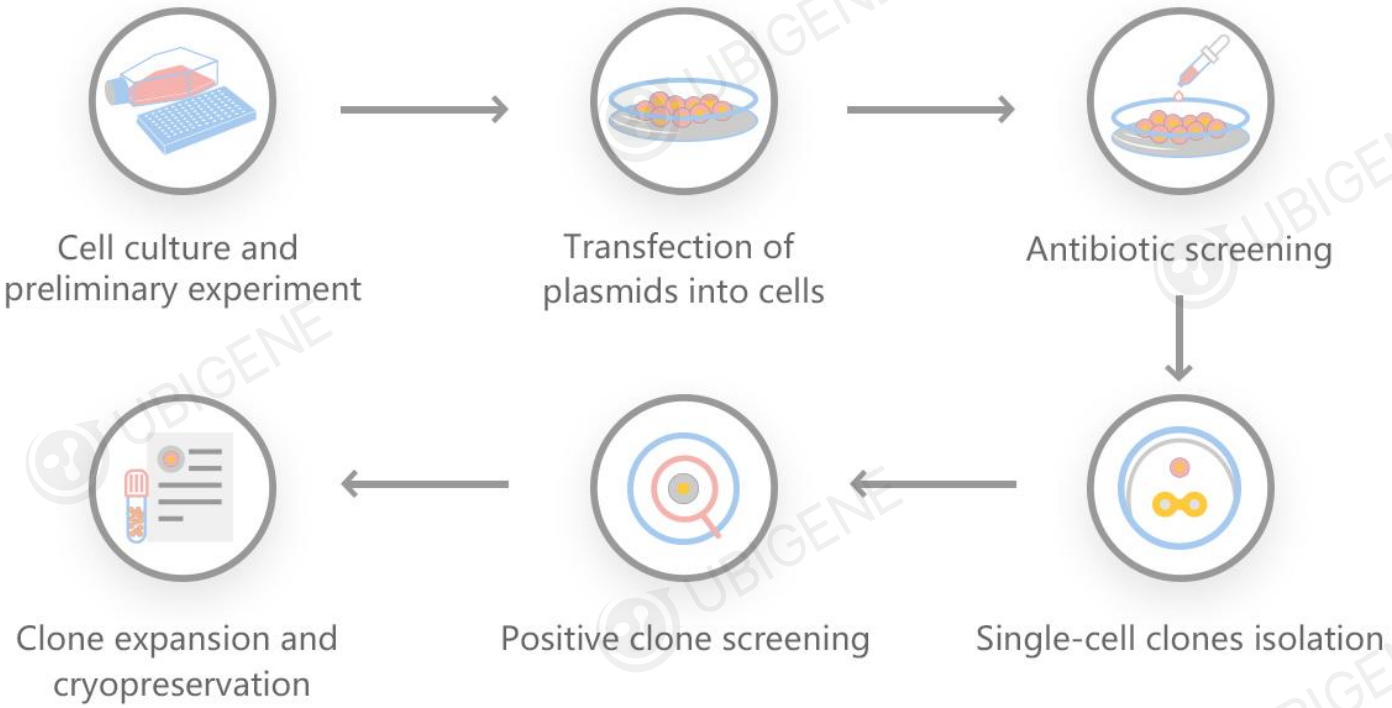
genotype of single-cell clones.

Additionally, Ubigene has released two useful tools (1) the primer design tool for gene editing target site identification (EZ-editor ™ high efficiency primer design platform) <https://en.rc-crispr.com/tools/primer_tool.html> provided in the Red Cotton™ system and (2) EZ-editor™ Genotype Analysis System (GAS) <https://en.rc-crispr.com/tools/gas.html> can assist you more rapidly through the entire single-cell clone validation process.

Kit Components

Components		Quantity	Storage temperature
KO Plasmids	EZ-editor™ gRNA1	50 μg	-20℃
	EZ-editor™ gRNA2	50 μg	-20℃
	EZ-editor™ gRNA3	50 μg	-20℃
Transfection Control	TransF Ctrl	50 μg	-20℃
MicroCell DNA Lysis	Buffer A	10 mL	RT
	Buffer B	1 mL	RT
PCR Validation	CloneAmp Taq Mix (+Dye)	1.25 mL	-20℃
	CloneAmp Ctrl	200 μL	-20℃
	Genotyping Primer F	500 μL	-20℃
	Genotyping Primer R	500 μL	-20℃

Workflow of gene KO experiment



Protocol

Preliminary experiment

1. Exploring the transfection method

The Transfection Control plasmid from the EZ-editor™ Gene Knockout kit carrys EGFP gene (green fluorescence) and can be used to figure out transfection parameters for formal experiment. Table 1 shows the results of transfection test of A375 cell line, it determines that the optimal transfection method is electroporation and the optical electroporation parameter is Program #20.

Transfection method		Fluorescent%	Cell viability
Lipo3000 Lipotamine (Lipo3000)		10%	90%
Electroporation	Program #6	35%	90%
	Program #9	70%	90%

	Program #20	80%	90%

Table 1

Note: The protocol of Lipotamine (Lipo3000) refers to

https://tools.thermofisher.cn/content/sfs/manuals/lipofectamine3000_protocol.pdf.

The Neon transfection system is used in electroporation, programs are the built-in programs.

2. Exploring the concentration for antibiotic screening (Kill curve)

① Digest the cells in the logarithmic growth phase into a single-cell suspension, and inoculate into a 12-well plate. The total volume of the culture medium is 1ml. Incubate the cells in 5% CO2 incubator at 37℃ for 24 hours.

② When the confluence level reaches about 50%, change the medium to a drug screening medium containing different concentrations of Puromycin, the concentration gradient is: 0, 1, 1.5, 2, 3, 4 μg/ml.

③ After 2-3 days, observe the cells under a microscope, and select the lowest concentration of the cells that are all dead as the drug screening concentration for subsequent experiments.

3. Exploring the single-cell clonal formation ability

① Digest the cells in the logarithmic growth phase into a single-cell suspension, and do the cell counting. Dilute the cell suspension according to seeding size of plate. e.g. if seeding size of plate is 1 cell/well, 5 cells/well, 10 cells/well, 20 cells/well, take appropriate amount of cells to dilution concentration of 10 cells/ml, 50 cells/ml, 100 cells/ml, 200 cells/ml.

② Take a certain amount of diluted cell suspension, inoculate into a 96-well plate, giving the total volume of each well of the culture medium is 100 μl. Incubate the cells in 5% CO2 incubator at 37℃ for static culture.

③ After 7-10 days, observe under the microscope whether the cells have a proliferation trend and form cell clusters, and count the number of single-cell clones in each group.

4. Target Sites validation

To ensure the knockout effect of the EZ-editor™ gRNAs, it is recommended to verify the site targeted by the gRNA by sequencing first. The target site can be amplified and sequenced

using the reagents for MicroCell DNA Lysis and PCR Validation from the kit, and then align the sequencing results with the theoretical sequence. If the target site sequence matches the theoretical sequence, feel free to continue the experiment with the gRNA; if the target site has a mutation, you would need to change the gRNA.

Formal Experiment

1. Transfection of KO plasmids (Neon transfection system electroporation)

① Digest the cells in the logarithmic growth phase into a single-cell suspension, take some cells for cell counting and to investigate the cell viability.

② Transfer 1×10^6 cells into a 1.5 mL EP tube, centrifuge at 300×g for 4 minutes, then discard the supernatant.

③ Resuspend the cell pellet with 1 mL PBS, centrifuge at 300×g for 4 minutes, then discard the supernatant.

④ Resuspend the cell pellet with 200μL Buffer R, add 10 μg EZ-editor™ gRNAs and mix well.

⑤ Add 3ml Buffer E2 to the electroporation cuvette, and then put it into the insertion site of the electroporator.

⑥ Use a 100μl electroporation pipette tip to aspirate cells-plasmid mixture, insert it into the electroporation cuvette, set electroporation conditions, and press start.

⑦ After the electroporation is completed, inoculate the cells in a 6-well plate with pre-warmed culture medium and continue to culture.

⑧ Repeat the above ⑥-⑦ steps as duplicates.

2. Antibiotic screening

24-48 hours after transfection, observe the effect of electrotransfection under a microscope, and use the antibiotics-containing culture medium to screen the cells. Antibiotic concentration according to the optimal concentration obtained from the preliminary experiment, the antibiotic screening could take 1-5 days, different cells have differences screening time. Until the cells without fluorescence are all screened dead by antibiotics, stop

the antibiotics addition, change back to the common culture medium (no antibiotics), that is, the gene knockout cell pool.

3. Validation of cutting effect from cell pool

① Rinse down the cells from 1 duplicate with PBS, centrifuged to collect the cells, then discard the supernatant.

② Following the ratio of 10^6Cell/mL MicroCell DNA Lysis, add the MicroCell DNA Lysis BufferA and mix well with cells 10-15 times by pipetting to lyse cells.

③ Transfer 100 μL of the sample from last step into PCR tube, set the program on the PCR machine (95℃, 10 min), then load the sample.

④ Take the lysed sample from PCR machine, add 10 μL MicroCell DNA Lysis Buffer B, mix well by pipetting.

⑤ Take 2 μL of the lysed cell solution of cell pool, prepare the PCR reaction solutions according to Table 2 and 3.

Table 2: Sample preparation method(Experiment group)

Reagent	Volume (Each reaction)
CloneAmp Taq Mix (+Dye), 2x	12.5 μL
Monoclone cell lysate	2 μL
Genotyping Primer F (10 μM)	1 μL
Genotyping Primer R (10 μM)	1 μL
ddH_2O	8.5 μL
Total volume	25 μL

Table 3: Sample preparation method (PCR control group)

Reagent	Volume(Each reaction)

CloneAmp Taq Mix（+Dye），2x	12.5 μL
Monoclone cell lysate	2 μL
CloneAmp Ctrl	2 μL
ddH_2O	8.5 μL
Total volume	25 μL

Note: The band amplified from PCR control group was about 330 bp

⑥ PCR reaction parameter

Table 4. PCR reaction parameter

Step	Temperature	Time	Cycle
Pre-denaturation	95℃	3 min	1 cycle
Cyclic amplification	95℃	15 s	35~40 cycles
	60℃	15 s	
	72℃	1 min/kb	
Extension compensation	72℃	5 min	1 cycle

⑦ PCR products can be either directly perform agarose gel electrophoresis (without loading buffer), or directly used for Sanger sequencing. Sequencing results can be efficiently analyzed and interpreted by using **EZ-editor ™ Genotype Analysis System (GAS),** developed by Ubigene.

4. Single-cell clones formation

① Resuspend the KO cell pool into a single-cell suspension, and do cell counting.

② Dilute cell suspension according to optimal seeding concentration obtained from the

preliminary experiment, inoculate into a 96-well plate. Ubigene' s Cell Monoclonal Culture Medium is recommended to use for this step.

③ Culture the cells in 37℃, 5% CO2 incubator, observe each well after 7-10 days and label the well with single-cell clones.

④ When the clones grow to an appropriate size, split the clones into 2 different 96-well plates. One plate is for subculture, another plate is for validation.

5. Single-cell clones validation

5.1 Sample preparation

① Aspirate the culture medium of the clones. If the sample is suspension cells, centrifuge the cell, then discard the culture medium.

② Add 90 μL MicroCell DNA Lysis Buffer A to each well of the 96-well plate, mix well 10-15 times by pipetting.

③ Transfer the sample from last step to a 96-well PCR plate, covered with a silica membrane. Or transfer to a PCR tube, strips of 8, well sealed by cap

④ Set up the program on PCR machine, 95℃, 10 mins, and load the sample.

⑤ Remove the lysed sample from PCR machine, add 10μl MicroCell DNA Lysis Buffer B, well mixed by pipetting. Lysed sample can be stored at -20℃ or directly used for PCR reaction.

5.2 PCR Validation

① Take CloneAmp Taq Mix (+Dye) and CloneAmp Ctrl from the - 20 ℃ refrigerator, place them in an ice box for thawing, and prepare the PCR reaction solution according to Table 1 and 2.

② PCR reaction parameter sees Table 4.

③ PCR products can be directly performed the agarose gel electrophoresis (without loading buffer), the positive clones can be screened according to the size of bands on the gel.

Sample case: The PCR validation for Cd180 KO Raw264.7 cell line project

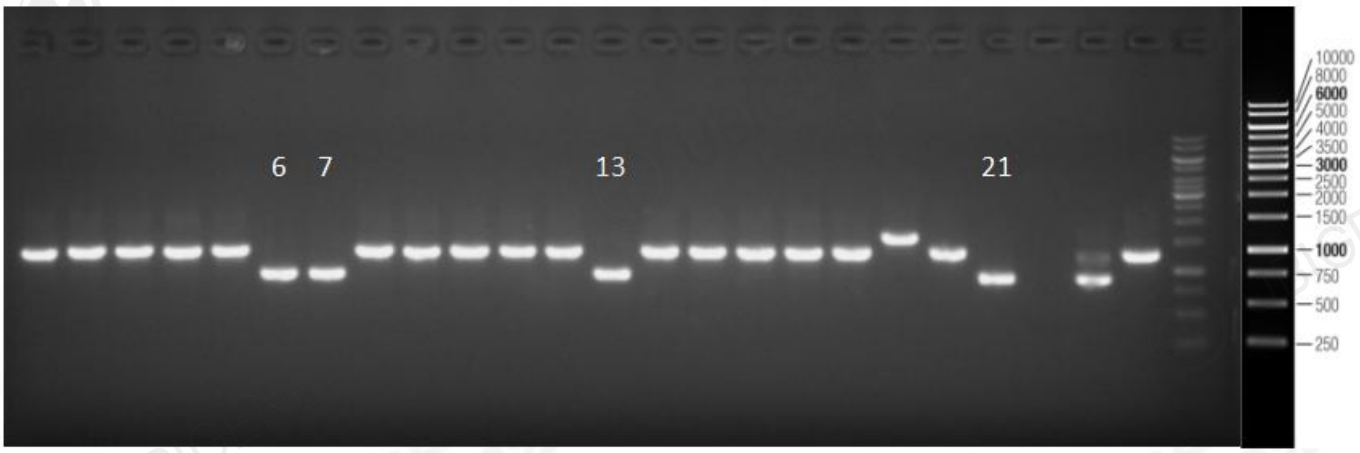


Figure 1. Monoclone validation results of gene Cd180-KO Raw264.7 cell line project. From the validation, a total of 4 positive KO clones were detected (KO theoretical size about 902bp), including 1 single-allelic KO clone (two bands, KO clones size about 902bp, WT band size about 1284bp). Clones 6, 7, 13, and 21 should be selected for genotype validation.

④ Sequencing the positive clones by Sanger sequencing. Sequencing results can be efficiently analyzed and interpreted by using **EZ-editor™ Genotype Analysis System (GAS)**, developed by Ubigene. Or, if using frameshift mutation strategy, the positive clones cannot be distinguished by PCR and agarose gel electrophoresis, then randomly select the clones for sequencing.

6. Clone expansion and cryopreservation

Expand the cell amount of the positive single-cell clones (pass the sequencing validation) by subculturing, and cryopreserve the clones. Or continue to the cell phenotypic analysis experiment.

FAQ

① Transfect the 3 gRNA plasmids together or transfect separately?

EZ-editor™ gRNAs (KO plasmids) are designed for frameshift mutation. Need to be careful of the KO region, if the 3 gRNAs target the same exon, 3 gRNAs can be transfected to the cells together; If the gRNAs target different exons, it is recommended to separately

transfect the gRNAs. In addition, the genotype of the cells transfected by 1 gRNA KO plasmid alone is relatively simple and easy to analyze; While transfecting 3 gRNAs together enables better KO effect, but the genotype of the clones screened would be complicated.

② How to judge whether the cell transfection is successful?

EZ-editor™ gRNA KO plasmids carry green fluorescent gene. If the cells are successfully transfected by the KO plasmids, the green fluorescent marker can be observed by fluorescence microscopy 24 hours after transfection. According to the contrast of the cell image under white light and fluorescence, the fluorescence rate of the cells can be determined, it is considered as good transfection with fluorescence rates greater than 40%. In addition, EZ-editor™ gRNA KO plasmids carry Puromycin resistance, facilitating screening enrichment of transfected cells.

③ Can I use one pair of primers for validation if co-transfecting 3 gRNAs into the cells?

If the 3 gRNAs target the same exon, same pair of primers can be used for validation. If the 3 gRNAs target different exon, it depends on the distance between the target sites of the gRNAs, it is recommended that the PCR fragment does not exceed 2kb.

④ How to adjust if the cell condition is not good, cell confluency is low, transfection efficiency is not high after transfection?

First of all, make sure the cell viability is normal before transfection, it is recommended to use Ubigene Transfection Culture Medium. Then try to adjust the transfection conditions, such as cell amount for transfection, plasmid amount, the voltage of eletroporation, etc.

⑤ How to judge whether the growing clone is single-cell clone?

For adherent cells, especially epithelial cells, they grow in clusters like clones and are nearly circular. If they grow from multiple cells, there will be depressions or irregular boundaries at the junction of the two clone culsters. If there is only one cell cluster in one well of the 96-well plate, the cell cluster in this well is considered as single-cell clone. If there are more than two cell clusters, it is polyclonal.

Suspension cells are easy to be dispersed in the process of moving, so it is difficult to identify single cell clones. Suspension cells will also form clusters when forming clones. Therefore, generally, if there is only one cell cluster in one well of the 96-well plate, the cell cluster in this well is considered as single-cell clone. If there are more than two cell clusters, it is polyclonal. If the cells are scattered, it is unlikely to distinguish whether they are monoclonal or not.

⑥ In the case that: the cell line is difficult to form single-cell clones; the inoculation density is low that the clones do not grow; the inoculation density is high but the cell cluster is less likely to be monoclonal, how can I improve it?

Based on the rich experience on gene-editing, Ubigene is now offering the optimized Cell Monoclonal Culture Medium, which can effectively improve the single-cell clones formation rate after cell transfection.