CRISPR/Cas9 Ribonucleoprotein Nucleofection for Genome Editing in Primary Human Keratinocytes: Knockouts, Deletions, and Homology-Directed Repair Mutagenesis

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Keratinocytes are the most abundant cell type in the human epidermis, the outermost layer of the skin. For years, primary human keratinocytes (HKs) have been used as a crucial tool for studying the pathogenesis of a wide range of skin-related diseases. To mimic the physiological and pathological behavior of human skin, organotypic 3D skin models can be generated by *in vitro* differentiation of HKs. However, manipulation of HKs is notoriously difficult. Liposome-mediated gene delivery often results in low transfection rates, and conventional electroporation results in high mortality, is difficult to optimize, and requires high cell numbers without necessarily achieving maximum efficiency. Additionally, HKs have a short lifespan *in vitro*, with a limited number of cell divisions before senescence, even when cultured on a feeder layer. Therefore, the possibility to use an efficient CRISPR/Cas9 system in HKs is not without challenge in terms of transfection technology and clonal selection. In this article, we provide detailed protocols to perform efficient gene knock-out (KO) or genomic deletion in a small number of HKs without clonal selection of edited cells. By nucleofecting ribonucleoprotein complexes, we efficiently generate KO cells as well as deletion of specific genomic regions. Moreover, we describe an optimized protocol for generating site-specific mutations in immortalized keratinocytes (N/TERT2G) by exploiting the homology-directed repair system combined with rapid single-clone screening. These methods can also be applied to other immortalized cells and tumoral cells of epithelial origin. Together, these protocols provide a comprehensive and powerful tool that can be used to better understand the molecular mechanisms underlying different skin diseases. © 2024 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Knock-out generation by indel mutation in primary human keratinocytes using nucleofection of ribonucleoprotein (RNP) complex
Basic Protocol 2: Deletion of specific genomic region using RNPs via nucleofection
Basic Protocol 3: Use of homology-directed repair system to introduce site-specific mutations

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INTRODUCTION

Keratinocytes are the predominant cell type in the human epidermis, accounting for >90% of all epidermal cells (Mestrallet et al., 2021). Primary human keratinocytes (HKs), which retain their ability to differentiate *in vitro*, are a critical tool for studying epidermal physiology and, when combined with the most advanced genome editing tools, can be used to understand gene function and uncover the pathogenesis of a wide range of skin-related disorders.

To date, CRISPR/Cas9 is the most straightforward genome editing tool that can be applied to a wide range of cell types. Discovered in bacteria and archaea as a defense mechanism against phage infection and plasmid transfer (Barrangou et al., 2007), the ability of the CRISPR (clustered, regularly interspaced, short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system to specifically target DNA was first successfully employed as a genome editing tool in mammalian cells nearly a decade ago (Cong et al., 2013; Ran et al., 2013). Since then, the CRISPR/Cas9 technology has been progressively improved, providing a highly accurate and programmable platform for enabling local gene deletion or tailored sequence alterations in a variety of cell types and organisms.

The CRISPR/Cas systems are commonly categorized into two classes based on differences in structure and organization of the Cas genes (Jinek et al., 2012). Whereas class I CRISPR/Cas systems consist of multiprotein effector complexes, class II systems include only a single effector protein. Among these systems, the most commonly used subtype is the type II CRISPR/Cas9 system, which in most cases is based on a single Cas protein derived from *Streptococcus pyogenes* (SpCas9) directed against a particular DNA sequence (Jiang et al., 2013).

Mechanistically, CRISPR/Cas9-mediated genome editing requires a Cas9–guide RNA (gRNA) complex containing Cas9 endonuclease, CRISPR RNA (crRNA) needed for target DNA sequence identification, and a *trans*-activating CRISPR RNA (tracrRNA), which serves as a binding scaffold for the Cas nuclease. A synthetic fusion of crRNA and tracrRNA into a single molecule generates the single gRNA (sgRNA), which is composed of a 20-bp sequence complementary to the target DNA (mimicking the function of the crRNA component) and an RNA scaffold sequence that binds the Cas9 protein (mimicking the function of the tracrRNA component). By changing the nucleotide sequence of the crRNA in the sgRNA, the CRISPR/Cas9 system makes it possible to target and modify almost any desired genomic locus.

Once introduced into the target cell, the sgRNA can bind to complementary DNA near protospacer-adjacent motifs (PAMs), which are short guanine-enriched sequences adjacent to the 3' end of the DNA target site that enable Cas9 site-specific double-strand DNA breaks (DSBs) (Chylinski et al., 2013). SpCas9 requires "NGG" as a

PAM sequence, which is commonly found in the genomes of most organisms, enabling the use of CRISPR/Cas9 technology in a wide range of species (Jiang & Doudna, 2017).

The target cell usually repairs damage through one of two endogenous mechanisms: non-homologous end joining (NHEJ) (Hefferin & Tomkinson, 2005) and homology-directed repair (HDR) (Liang et al., 1998). NHEJ operates by recognizing DSBs, processing the DNA ends, and ligating them, infrequently resulting in insertions or deletions (indels) that can disrupt gene function (Stinson & Loparo, 2021; Takata et al., 1998). Within an open reading frame, this error-prone mechanism can eventually lead to a frameshift, creating a premature stop codon, thus resulting in gene KO. Alternatively, the HDR pathway is capable of repairing DSBs using a sister chromatid as a repair template, and for this reason, it is considered an error-free system. Unlike HDR, NHEJ does not require a homologous template, which simplifies the editing process. However, NHEJ cannot be used for precise modifications such as gene correction. For this purpose, it is possible to use the HDR system to introduce specific genetic changes by providing an exogenous donor DNA template with homology to sequences flanking the DSB. This approach allows specific modifications, such as gene correction, insertion of specific sequences, and targeted gene integration (Fichter et al., 2023; Xue & Greene, 2021).

The possibility to exploit these two distinct DNA repair pathways makes CRISPR/Cas9 a highly flexible genome editing technology. The choice between the HDR and NHEJ pathways must be carefully made to achieve the desired outcome in genome editing procedures, underscoring the importance of strategic design and implementation in genetic engineering experiments.

Although CRISPR/Cas9 has been successfully applied in a plethora of cell lines, its employment in primary cells, including HKs, can be challenging. One of the main reasons is related to the fact that primary cells maintained *in vitro*, including HKs, have a short lifespan with a limited number of cell divisions before undergoing senescence, even when cultured on feeder cells, thus reducing the possibility of long-term clonal selection after gene editing. Moreover, HKs are notoriously refractory to genetic manipulation with conventional methods. For example, lipid-based transfection consistently shows low efficiency of DNA uptake and expression.

Viral gene delivery vectors are usually more efficient than non-viral vectors, but there are some limitations. Lentiviral transduction has been used widely as a delivery system for CRISPR/Cas9 components (Fenini et al., 2018). Despite good transduction efficiency and low immunogenicity (Dong & Kantor, 2021), the random integration of Cas9- and gRNA-expressing plasmid into the host genome can disrupt essential genes or regulatory elements, leading to unplanned genomic changes. Moreover, the constitutive expression of both Cas9 protein and gRNA increases the risk of off-target effects (Lino et al., 2018; Papapetrou & Schambach, 2016). Additionally, the antibiotic resistance–based selection methods associated with the screening of recipient cells results in low efficiency considering the short lifespan of HKs (Fenini et al., 2018).

A non-viral transfection method, different from lipid-based transfection, should be used for HKs to increase delivery efficiency, reduce immunological responses, and minimize side toxic effects. In this regard, nucleofection has been reported as the most efficient non-viral transfection approach for primary cells (Aluigi et al., 2006). Unlike other transfection methods that preferentially transfer DNA into the cytoplasm and only allow it to enter the nucleus during cell division, the nucleofector allows DNA to be delivered directly into the nucleus, making nucleofection independent of cell division (Distler et al., 2005).

Performing genome editing experiments requires careful consideration of not only the delivery method selected but also the proper design and optimization of editing tools. In fact, regardless the delivery system used, the strategy of using plasmid-expressing Cas9/sgRNA has some limitations compared to direct delivery of the Cas9 protein and sgRNA as a ribonucleoprotein (RNP) complex. Indeed, the RNP complex method results in fewer off-target effects and faster editing. With this system, the nuclease is active for 24 to 48 hr, whereas plasmids remain active for several days, increasing the probability of cutting in regions other than the targeted one. Moreover, the use of an RNP complex is associated with reduced experimental time, as the pre-assembled complex is introduced into the cell nucleus and starts cutting immediately, without having to wait for the plasmid to be transcribed and the Cas9 protein to be translated (Kim et al., 2014).

Here, we provide a detailed genome editing approach for HKs by using the Cas9 RNP complex to generate KO cells (Basic Protocol 1), deleting specific genomic regions (Basic Protocol 2), and introducing site-specific mutations exploiting the HDR system (Basic Protocol 3).

The approaches described below show multiple benefits. First, editing a small number of primary HKs overcomes the common issue of limited cell number and constant skin donor availability. In addition, with Cas9/gRNA RNP complex nucleofection, we avoid the generation of viral vectors (the production of which can be harmful to the user) while maintaining a significantly higher efficiency than transfection with lipid- or virus-based methods. Moreover, cells continue to grow at similar rate after nucleofection due to the minimal cell toxicity of this technique. These approaches have been successfully used with primary HKs and cell lines of epithelial origin; however, these methods can be easily adapted to other primary or immortalized cell types.

Overall, we describe the processes of selection of optimal sgRNAs using online tools, how to properly reconstitute the reagents needed for editing, how to maintain and prepare the primary HKs for nucleofection, how to generate Cas9/sgRNA RNP complexes for both KO and genomic deletions, and subsequent assessment of genome editing. Finally, we provide information on how to rapidly perform and screen precise gene-editing correction in HKs.

STRATEGIC PLANNING

The design of gRNA and selection of the right Cas9 protein need to be tailored specifically to each gene of interest and experimental condition. In the protocols described here, we rely on user-friendly and optimized online tools provided by Integrated DNA Technologies (IDT) for both gRNA design and Cas9 selection.

Several types of Cas9 protein have been described. However, to maximize laboratory utility, the selection of a single enzyme that can be applied to different genome editing strategies is an essential step. In fact, one of the most common Cas9 proteins used is the Alt-R™ S.p. Cas9 Nuclease, which is a recombinant *S. pyogenes* Cas9 nuclease, purified from *Escherichia coli*, containing a nuclear localization sequence (NLS), C-terminal 6-His tag, and DSB induction capacity. Many companies provide Cas9 proteins for genome editing experiments (e.g., Addgene, Thermo Fisher Scientific, Sigma-Aldrich, NEB, OriGene). We used a Cas9 variant with improved specificity and reduced off-target effects that has been engineered (Alt-R™ S.p. HiFi Cas9 Nuclease V3 from IDT) as a fair compromise for various applications, from indel mutations to HDR-mediated genome editing. Accordingly, there are many sgRNA design tools available, such as CHOPCHOP (Labun et al., 2016), CRISPR Design Tool – MIT (Hsu et al., 2013), CRISPRscan (Moreno-Mateos et al., 2015), CRISPOR (Concordet & Haeussler, 2018), and the Broad Institute's GPP Web Portal (https:// portals.broadinstitute.org/gpp/public/

analysis-tools/sgrna-design (Doench, 2016). We used the Custom Alt-R™ CRISPR-Cas9 guide RNA IDT online tool to generate CRISPR/Cas9 gRNAs. The tool can be used with any species, taking into consideration possible off-target effects in human, mouse, rat, zebrafish, or *Caenorhabditis elegans* genes. By directly inserting the gene symbol or the accession number of the gene of interest, it is possible to search for pre-designed IDT-recommended gRNAs. We routinely design guides by using the FASTA sequence of the gene of interest and selecting the proposed gRNA accordingly to the best ON-target and OFF-target scores. Higher ON-target and OFF-target scores suggest an efficient and specific gRNA. It is possible to design either crRNA or sgRNA according to the researcher's needs. In fact, the decision between sgRNA and crRNA depends largely on the number of genome editing experiments to be performed. For a single genome editing experiment, sgRNA offers a cost-effective and ready-to-use solution. On the other hand, for multiple gRNA applications, it may be advisable to use separate crRNAs and tracrRNA stocks to reduce costs. For all the experimental procedures performed by our group, we used sgRNA. In particular, to generate precise genomic deletion, two independent sgRNAs flanking the region of interest have been designed. For indel mutations and HDR-mediated genome editing, one sgRNA may be sufficient; however, it is advisable to test two different sgRNAs for efficiency and to avoid any sgRNA-specific off-target effects.

HDR Design

To generate site-specific mutations exploiting the HDR system, an additional donor oligonucleotide needs to be designed, containing 5' and 3' homology arms to the region flanking the DSB and the desired modification between those two homology arms. Specific inserts in the 1- to 2-kb range can be introduced efficiently using homology arms of 200 to 300 bp in length. For insertions of shorter length, shorter homology arms of 30 to 60 nt can be used. In this case as well, there are many donor oligo design tools available, such as CRISPOR (Concordet & Haeussler, 2018) and DNARepairDesigner (Paix et al., 2017). We used the online Alt-R™ CRISPR HDR Design Tool from IDT. This system can be used to create single-nucleotide changes, longer nucleotide substitutions, and specific deletions or insertions. The tool also provides a preview translation option, useful to verify if the sequence of interest will be in frame at the protein level. The addition of silent mutations in the PAM sequence is highly recommended to prevent Cas9-mediated re-cutting after donor incorporation. It is also recommended to test different gRNA and HDR donor oligos. Select the gRNA with the higher ON-target and OFF-target scores. The IDT online Alt-R™ CRISPR HDR Design Tool provides two different HDR donor oligos, one that replaces the positive strand of genomic DNA (positive HDR donor oligo) and another that replaces the negative strand of genomic DNA (negative HDR donor oligo). In our experience, we observed no differences regarding cell viability and mutagenesis efficiency in HKs using positive and negative HDR donor oligos.

HDR-mediated genome editing requires a single-clone selection step to obtain a homogeneous population that will be further screened by DNA sequencing. However, the identification of clones having the modification of interest can be a time-consuming step and will require several cell doublings. Using an immortalized HK cell line (N/TERT2G), we set up a pre-screening approach to preliminary sub-select potential mutant clones by designing an HDR donor oligo carrying a restriction site sequence that results in a silent mutation. PCR for the genomic region of interest, followed by enzymatic digestion and agarose gel electrophoresis, allows simple and clear pre-selection of putative genome-edited clones. This method reduces the number of clones undergoing DNA sequencing.

The efficiency of CRISPR/Cas9-mediated genome editing can also be affected by the delivery system used. Nucleofection is a highly efficient technology used to easily achieve delivery of RNP. However, to verify electroporation efficiency and viability of the cells of

interest, a preliminary experiment using a plasmid expressing a green fluorescent protein to test different nucleofector programs should be performed.

Nucleofection is an advanced technique designed to facilitate the introduction of molecules into the cell nucleus, especially for those cells resistant to traditional transfection methods. Among the leading nucleofection systems available today are the Amaxa Nucleofector by Lonza, the Neon Transfection System by Thermo Fisher Scientific, and the GenePulser Xcell by Bio-Rad.

For our experiments, we used an Amaxa 4D-Nucleofector by Lonza, and we tested several manufacturer-specified programs (DS119, DG138, DS138, CM137, and ED138) for evaluating efficiency in HKs. We found that the best efficiency/survival ratio for primary HKs was achieved using the DS138 program. However, we noticed that for other epithelial cell lines, in particular the squamous cell carcinoma cell line SCC13 (Rheinwald & Beckett, 1981), the program ED138 could be used to improve nucleofection efficiency without excessively affecting cell viability. Gene editing in primary HKs is usually completed within 72 hr following RNP nucleofection. However, depending on the stability of the protein of interest, a longer time may be required before the loss of the gene product can be detected.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 1

KNOCK-OUT GENERATION BY INDEL MUTATION IN PRIMARY HUMAN KERATINOCYTES USING NUCLEOFECTION OF RIBONUCLEOPROTEIN (RNP) COMPLEX

The aim of this protocol is to provide a guideline for knocking out a gene of interest by introducing indel mutations in difficult-to-transfect cells such as primary HKs and to provide instructions on how to thaw, passage, and maintain HKs before inducing genetic modifications (Fig. 1A). Culture of HKs on feeder cells is a key step for providing a supportive environment to promote cell growth and maintain an undifferentiated state. However, this protocol has also been tested on HKs cultured in EpiLife Medium (Gibco, MEPI500CA) with no differences in genome editing efficiency. To achieve high CRISPR/Cas9 genome editing efficiency (>90%) in a small number of HKs without clonal selection of the targeted cells, this protocol employs transient nucleofection of an RNP complex consisting of Cas9 protein and sgRNA containing chemical modifications to stabilize the RNA and increase its resistance to nuclease activity. This is delivered into the cells using a Lonza 4D-Nucleofector. The described protocol can be also applied for KO generation in N/TERT2G (Dickson et al., 2000) or in cancer cell lines of epithelial origin (such as SCC13, SCC12, or A431).

Materials

3T3-J2 mouse embryonic fibroblast cells (Rheinwald & Green, 1975)
3T3-J2 cell medium (see recipe), 37°C
0.25% (w/v) trypsin-EDTA (Gibco, 25200072), 37°C
Thawed or freshly isolated primary HKs
Keratinocyte growth medium (see recipe), 37°C
1× phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} (D-PBS - Ca^{++}/Mg^{++}; Euroclone, ECB4004L), 37°C
Fetal bovine serum (FBS; Euroclone, ECS 500L)

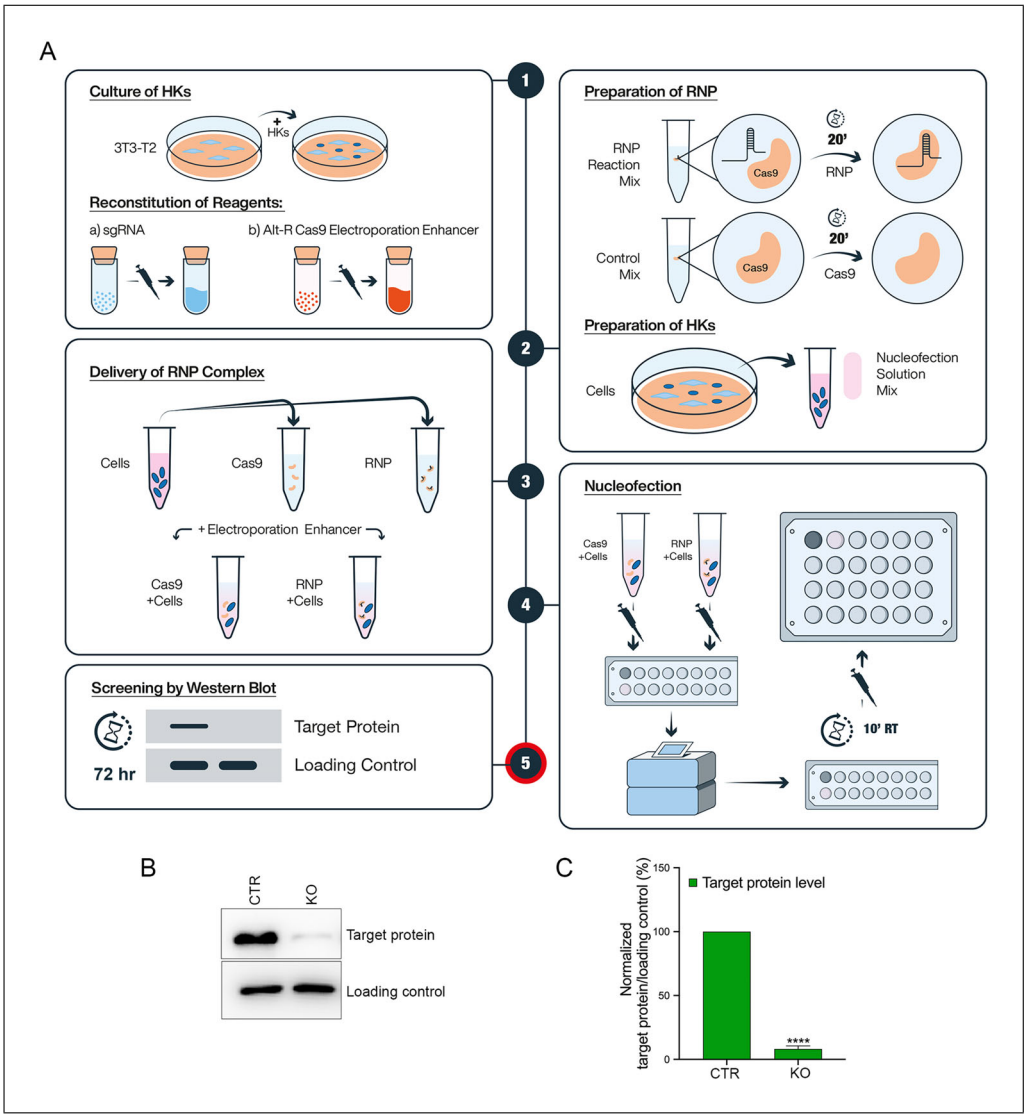

Figure 1 Graphical representation of the experimental procedure and results of Basic Protocol 1. (**A**) Sequential steps of the protocol. The red circles highlights the unique part of the specific basic protocol. Images were created using Adobe Illustrator. (**B**) Representative western blot of the target protein in extracts derived from human keratinocytes transfected with Cas9 protein (CTR) or Cas9/sgRNA RNP complex (KO). (**C**) Densitometric analysis of the western blots in (B). Normalized expression of the target protein compared to loading control and relative to CTR (n = 3). Data are the mean ± SD. Two-tailed paired Student's *t*-test; ns, non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

Synthetic modified sgRNA oligonucleotides, lyophilized (Alt-R™ CRISPR-Cas sgRNA, IDT, *www.idtdna.com*)
WFI water, for cell culture (Gibco, A12873-01), 4°C
Alt-R™ Cas9 Electroporation Enhancer, 10 nmol, lyophilized (IDT, 1075916)
Alt-R™ S.p. HiFi Cas9 Nuclease V3, 500 µg (IDT, 1081061)
Amaxa P3 Primary Cell 4D-Nucleofector X Kit S (Lonza, V4XP-3032), including P3 Primary Cell Nucleofector Solution, Supplement 1, and 16-well nucleocuvette strip

100-mm plastic cell culture plates (Corning, 430167)
Irradiator (RS-2000 Biological Irradiator, Rad Source Technologies)
Hemocytometer
Phase-contrast microscope

15-ml tubes (Corning, CC430790)
Standard tabletop centrifuge
15-ml tubes (Corning, CC)
1.5-ml tubes, sterile (Corning, CC3621)
24-well cell culture plates (Corning, 3526)
Amaxa 4D-Nucleofector Core Unit (Lonza, AAF-1003B)
Amaxa 4D-Nucleofector X Unit (Lonza, AAF-1003X)

Additional reagents and equipment for analysis (western blot, DNA extraction, PCR amplification, and agarose gel electrophoresis and imaging)

Preparation of feeder cells

1. Seed 3T3-J2 mouse embryonic fibroblast cells at a density of 10,000 cells/cm^2 in a 100-mm plastic cell culture plate using 3T3-J2 cell medium. Upon confluency, passage cells (approximately every 3 days) with pre-warmed (at 37°C) 0.25% trypsin-EDTA at a 1:3 ratio.

2. To induce non-fatal growth arrest, irradiate a sub-confluent (∼80%) 100-mm plate of 3T3-J2 cells with 50 Gy in 3T3-J2 cell medium.

   Using the RS-2000 Biological Irradiator, it is possible to irradiate three 100-mm plates of 3T3-J2 cells simultaneously. Because the dose rate for level 4 of the irradiator, where the cell plates fit, is 2.9 Gy/min, the Gy exposure time is ∼18 min.

   An alternative method for feeder preparation is to incubate 80% confluent 3T3-J2 cells with freshly prepared 3 µg/ml mitomycin C for 2.5 hr.

3. After inactivation, collect the cells by trypsinization and count the cells using a hemocytometer and a phase-contrast microscope. Then, centrifuge them in a 15-ml tube for 5 min at 250 × *g* at room temperature.

4. Discard the supernatant and resuspend the 3T3-J2 cell pellet in 3T3-J2 cell medium.

   Alternatively, resuspend the 3T3-J2 cell pellet in cold freezing medium [90% FBS, 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, D2650-100 ml)] at a concentration of 1 × 10^6 cells/ml for freezing. If feeder cells are required regularly, a practical and efficient method is to prepare a bulk of mitotically inactivated feeder cells and store them cryopreserved. Batches of irradiated 3T3-J2 cells can be stored for ≤2 weeks at –80°C. For longer storage, liquid nitrogen is required.'

5. Plate mitotically blocked 3T3-J2 cells at 20,000 cells/cm^2 in 100-mm plates using 3T3-J2 cell medium and incubate them overnight at 37°C and 5% CO_2.

   When time is of the essence, it is possible to reduce the seeding time of mitotically blocked 3T3-J2 cells to 6 hr.

Culture of primary human keratinocytes

6. The day after plating the proliferation-incompetent feeder cells, seed thawed or freshly isolated primary HKs into a 100-mm plate containing the feeder cells, using a density of 3200 cells/cm^2 in keratinocyte growth medium.

   Cells will reach confluency and be ready for splitting ∼5 days post-plating.

   If frozen cells are used, thaw them rapidly in a 37°C water bath until approximately half of the medium has thawed. Take care not to completely submerge the tube and be sure to sterilize the tube with 70% (v/v) ethanol before opening.

7. To split HKs, remove the keratinocyte growth medium from the cell plate.

8. Wash the cells once using 5 ml pre-warmed (at 37°C) 1× D-PBS -Ca^{++}/Mg^{++}.

9. Add 1 ml pre-warmed (at 37°C) 0.25% trypsin-EDTA and incubate for 1 min at 37°C to remove the feeder cells.

   The volume of trypsin-EDTA solution must be adjusted according to the cell plate size used.

10. Remove trypsin-EDTA solution containing feeder cells.

11. Wash once with 1 ml trypsin-EDTA solution to ensure that all feeder cells are removed. Check cell detachment under a phase-contrast microscope to ensure that only feeder cells were removed.

12. Remove the trypsin-EDTA solution and then detach the primary HKs by adding 1 ml fresh pre-warmed (at 37°C) 0.25% trypsin-EDTA and incubating for 4 min at 37°C. Gently tap the plate on a surface to fully dissociate the cells and check under the phase-contrast microscope to confirm that all cells are detached.

13. Inactivate the trypsin-EDTA solution by adding the same volume (v/v) of 100% FBS and then collect the cells in a 15-ml tube.

14. Count the cells using a hemocytometer.

    It is highly recommended to verify cell morphology and viability using trypan blue (1:1 cell suspension/trypan blue). Only count small viable (unstained) circular keratinocytes.

15. Centrifuge cells for 5 min at 250 × *g* at room temperature.

16. During centrifugation, remove 3T3-J2 medium from a 100-mm plate containing proliferation-incompetent 3T3-J2 cells (see step 5), wash once with 5 ml pre-warmed (at 37°C) 1× D-PBS -Ca^{++}/Mg^{++}, and add 8 ml keratinocyte growth medium.

17. Plate the primary HKs from step 15 at 3200 cells/cm^2 on the mitotically blocked 3T3-J2 cells.

Reconstitution of reagents

18. Reconstitute the synthetic sgRNA:

    a. Spin down tubes containing lyophilized synthetic modified sgRNA oligonucleotides (Alt-R™ CRISPR-Cas sgRNA) to ensure that the RNA is collected at the bottom.
    b. Dissolve the sgRNA by adding 20 µl cold WFI water to 2 nmol sgRNA to a final concentration of 100 µM. Gently pipet up and down to ensure full reconstitution.

       WFI water can be substituted with 1× TE buffer.

    c. Store dissolved sgRNA for ≤1 year at −20°C prior to use.

19. Reconstitute Alt-R™ Cas9 Electroporation Enhancer:

    a. Spin down tubes containing lyophilized Alt-R™ Cas9 Electroporation Enhancer.
    b. Dissolve the dried Alt-R™ Cas9 Electroporation Enhancer by adding 100 µl cold WFI water to 10 nmol dried Alt-R™ Cas9 Electroporation Enhancer to a final concentration of 100 µM. Gently pipet up and down to ensure full reconstitution.

       WFI water can be substituted with 1× TE buffer.

    c. Store dissolved Alt-R™ Cas9 Electroporation Enhancer for ≤1 year at −20°C prior to use.

Preparation of ribonucleoprotein complex

20. To allow sgRNA and Cas9 complex assembly, prepare RNP reaction mix and Control mix in 1.5-ml tubes under sterile conditions:

RNP reaction mix:

Reagent	Volume
Alt-R™ CRISPR-Cas sgRNA (stock 100 µM; see step 18)	1.2 µl (final concentration 120 pmol)
Alt-R™ S.p. HiFi Cas9 Nuclease V3 (stock 62 µM)	1.7 µl (final concentration 104 pmol)
D-PBS -Ca^{++}/Mg^{++} (1×)	2.1 µl
Final volume	*5 µl*

Control mix:

Reagent	Volume
Alt-R™ S.p. HiFi Cas9 Nuclease V3 (stock 62 µM)	1.7 µl (final concentration 104 pmol)
D-PBS -Ca^{++}/Mg^{++} (1×)	3.3 µl
Final volume	*5 µl*

The volumes were optimized to nucleofect a small number of cells (see below) using the 16-well nucleocuvette strip.

For the Control mix, we suggest using only Cas9 protein to take into account possible undesirable effects mediated by the nuclease activity.

21. Incubate the RNP reaction mix and the Control mix at room temperature for 20 min.
22. During the RNP assembly, proceed with cell preparation (see steps 23 to 27).

Preparation of primary human keratinocytes for the genome editing experiment

23. The day before the experiment, plate mitotically blocked 3T3-J2 cells (see step 4) at 20,000 cells/cm^2 in a 24-well cell culture plate using 3T3-J2 cell medium and incubate them overnight at 37°C and 5% CO_2.
24. Remove the keratinocyte growth medium from the cell plate and proceed with steps 8 to 14.
25. Collect 2×10^5 cells for each nucleofection reaction in a new 15-ml tube.

For example, if you need 2×10^5 cells for KO generation by indel mutation and another 2×10^5 cells as a control reaction, start with 4×10^5 cells in a 15-ml tube.

26. Centrifuge 4×10^5 cells for 5 min at 250 × *g* at room temperature.
27. During centrifugation, remove 3T3-J2 medium from the 24-well plate of irradiated feeders (see step 23), wash once with pre-warmed (at 37°C) 1× D-PBS -Ca^{++}/Mg^{++}, and add 1 ml pre-warmed (at 37°C) keratinocyte growth medium per well.

This protocol could also be applied to a lower number of cells. We successfully tested editing of 1×10^5 cells without changing the proportion of the reagents used.

28. During centrifugation, proceed to preparation of the Nucleofection Solution mix (see steps 29 and 30).

Preparation of Nucleofection Solution mix

29. Determine the volume of the Nucleofection Solution mix needed according to the number of nucleofection reactions to be carried out (at least one RNP reaction and one Control reaction).
30. In a sterile 1.5-ml tube, prepare the Nucleofection Solution mix (using components of the Amaxa P3 Primary Cell 4D-Nucleofector X Kit S) as follows:

Reagent (for RNP and Control reactions = 4×10^5 cells)	**Volume**
P3 Primary Cell Nucleofector Solution	32.8 µl
Supplement 1	7.2 µl
Final volume	*40 µl*

The Amaxa P3 Primary Cell 4D-Nucleofector X Kit S provides all the reagents needed to prepare the Nucleofection Solution mix. The kit also provides pmaxGFP™ Vector, useful to test cell viability and nucleofection efficiency.

31. After centrifugation (see step 26), carefully remove all the medium and resuspend the cells in the Nucleofection Solution mix (4×10^5 cells are resuspended in 40 µl Nucleofection Solution mix).

    Long-term exposure to Nucleofection Solution mix might be toxic for the cells. Therefore, avoid leaving cells in Nucleofection Solution mix for >30 min.

Delivery of ribonucleoprotein complex through nucleofection

32. Prepare two independent Nucleofection Reaction mixes for cells undergoing genome editing and for control cells:

Reagent	**Volume**
2×10^5 cells (in Nucleofection Solution mix; see step 31)	20 µl
RNP reaction mix (sgRNA+Cas9) or Control mix (only Cas9) (see step 21)	5 µl
Alt-R™ Cas9 Electroporation Enhancer (stock 100 µM; see step 19)	1 µl
Final volume	*26 µl*

    The addition of Alt-R™ Cas9 Electroporation Enhancer to the Nucleofection Solution mix increases delivery efficiency.

33. Carefully transfer each Nucleofection Reaction mix into a well of the 16-well nucleocuvette strip provided with the Amaxa P3 Primary Cell 4D-Nucleofector X Kit S, avoiding bubbles.

    If there are air bubbles, gently tap the nucleocuvette strip and/or use a needle to pop the bubbles.

34. Switch on the Amaxa 4D-Nucleofector Core and X Units, select the 16-well nucleocuvette strip module, and choose the position of the loaded well.

35. Open the tray, transfer the 16-well nucleocuvette strip into the Amaxa 4D-Nucleofector machine, and select the electroporation program and the pulse code (for primary HKs, use Primary Cell P3 program and DS138 pulse code).

    Make sure that the larger gap in the strip lid is at the top of the strip to ensure perfect complementarity between the holder and the strip.

36. Press start and wait until the electroporation is completed.

    At the end of the run, the screen should display a "+" over the wells that were successfully electroporated.

37. Carefully remove the 16-well nucleocuvette strip from the Amaxa 4D-Nucleofector machine and incubate 10 min at room temperature.

38. Carefully add 75 µl keratinocyte growth medium to each well of the nucleocuvette strip containing the 26 µl of Nucleofection Reaction mix. Slowly pipet up and down 2 to 3 times and then transfer the entire contents to one well of the 24-well plate containing mitotically blocked 3T3-J2 cells (see step 27). Repeat this step twice total to ensure collection of all edited cells.

39. Incubate the plate overnight at 37°C, 5% CO_2.

40. The following day, replace the keratinocyte growth medium with fresh medium to remove dead cells, debris, and the leftover Nucleofection Reaction mix, and especially the Alt-R™ Cas9 Electroporation Enhancer, which can be toxic to the cells.

    Cell survival is usually highest if cells are kept more dense after nucleofection and not sparsely plated. We have nucleofected and plated 1 × 10^5 cells in one well of a 24-well

plate or plated 2 × 10^5 cells in a 12-well plate (Corning, 3513) without loss of cell viability.

Genome editing screening after knock-out generation through indel mutations

41. Harvest and analyze cells ≥72 hr post-nucleofection.

    Western blot analysis can be performed to verify genome editing efficiency (Fig. 1B and 1C).

    The time at which loss of protein expression will be observed depends on the half-life of the protein. For proteins with long half-lives, it may be necessary to wait longer to see the best result.

    If necessary, a single-clone isolation followed by genomic DNA extraction, PCR amplification of the region of interest, and sequencing could be performed to verify the generation and efficiency of the KO.

BASIC PROTOCOL 2

DELETION OF SPECIFIC GENOMIC REGION USING RNPs VIA NUCLEOFECTION

Here, we describe a simple method that can be used for highly efficient and fast deletion of specific genomic regions (Fig. 2A). This method allows up to 90% of targeted genomic deletion in primary HKs in only 3 days without clonal selection. Although the main steps are in common with Basic Protocol 1, this protocol outlines some key steps in the pipeline that are critical for efficient deletion of a genomic segment using two independent RNP complexes and nucleofection-mediated delivery.

Materials

See Basic Protocol 1.

Assembly of two ribonucleoprotein complexes to delete a specific genomic sequence

1. To delete a specific genomic region, design two sgRNAs flanking the region of interest to delete.

   We used the IDT website (www.idtdna.com) to generate sgRNA sequences.

2. For optimal genome editing, to assemble two independent RNPs, one containing the sgRNA located upstream of the region to delete + Cas9 protein (RNP1) and the other one containing the sgRNA located downstream of the region to delete + Cas9 protein (RNP2), prepare independent reaction mixes of RNP complexes (RNP1 and RNP2) to maximize the frequency of the modification as well as a Control mix lacking the sgRNA:

   RNP1 reaction mix:

Reagent	Volume
Alt-R™ CRISPR-Cas sgRNA1 "start" (stock 100 µM; see step 1)	1.2 µl (final concentration 120 pmol)
Alt-R™ S.p. HiFi Cas9 Nuclease V3 (stock 62 µM)	1.7 µl (final concentration 104 pmol)
D-PBS -Ca^{++}/Mg^{++} (1×)	2.1 µl
Final volume	*5 µl*

   RNP2 reaction mix:

Reagent	Volume
Alt-R™ CRISPR-Cas sgRNA2 "end" (stock 100 µM; see step 1)	1.2 µl (final concentration 120 pmol)
Alt-R™ S.p. HiFi Cas9 Nuclease V3 (stock 62 µM)	1.7 µl (final concentration 104 pmol)
D-PBS -Ca^{++}/Mg^{++} (1×)	2.1 µl
Final volume	*5 µl*

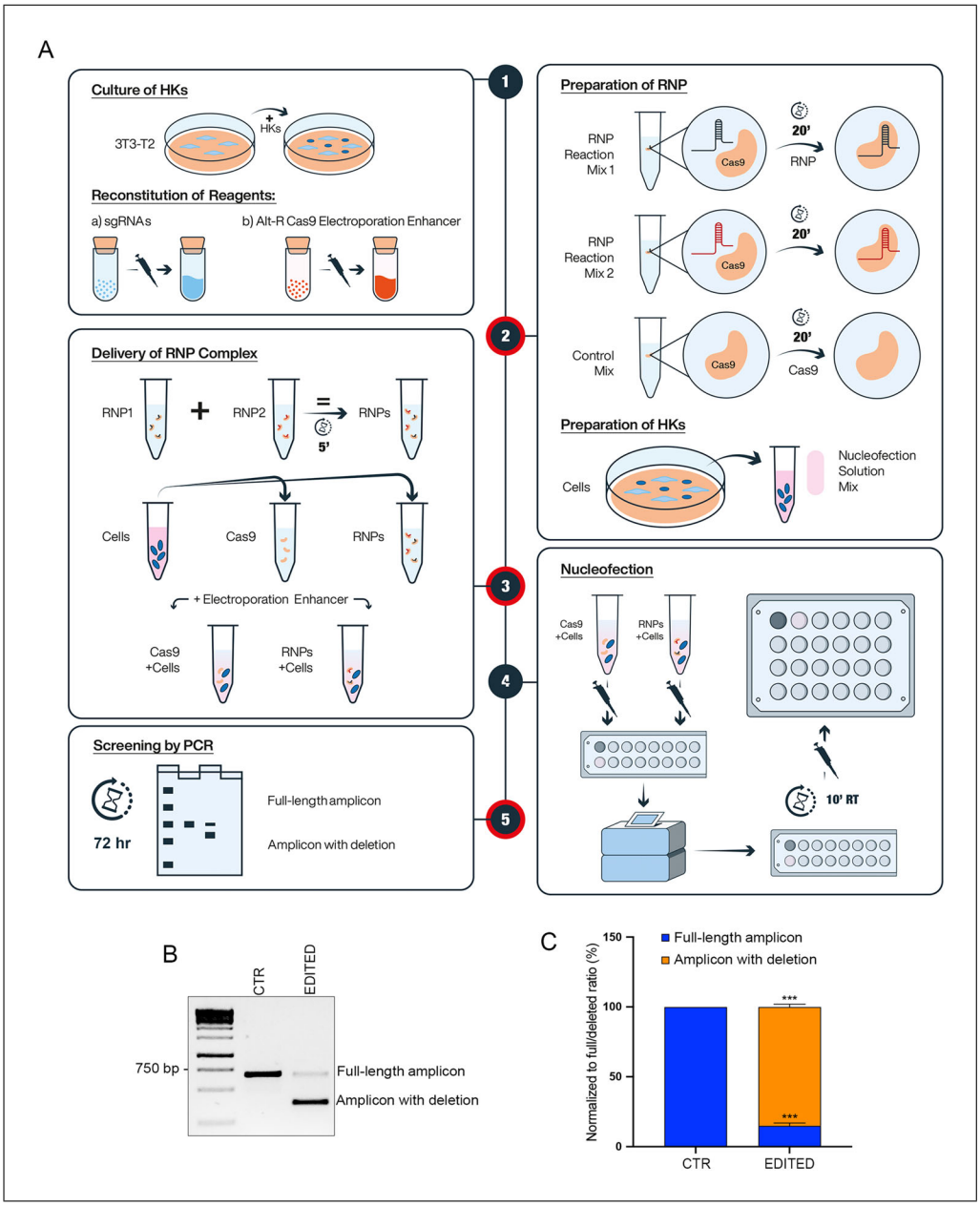

Figure 2 Graphical representation of the experimental procedure and results of Basic Protocol 2. (**A**) Sequential steps of the protocol. The red circles highlight the unique parts of the specific basic protocol. Images were created using Adobe Illustrator. (**B**) Representative DNA agarose gel showing efficient deletion of a DNA segment in primary HKs transfected with two Cas9/sgRNA RNP complexes (EDITED) compared to Cas9 protein only (CTR). (**C**) Normalized levels of deleted amplicon compared to full-length amplicon (n = 3). Data are the mean ± SD. Two-tailed paired Student's *t*-test; ns, non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

Control mix:

Reagent	Volume
Alt-R™ S.p. HiFi Cas9 Nuclease V3 (stock 62 µM)	1.7 µl (final concentration 104 pmol)
D-PBS -Ca^{++}/Mg^{++} (1×)	3.3 µl
Final volume	*5 µl*

We have tested several RNP complex mix combinations: sgRNA1+sgRNA2+Cas9, sgRNA1+sgRNA2+2XCas9, sgRNA1+Cas9, and sgRNA2+Cas9. We found that using two independent RNP complexes formed by sgRNA1+Cas9 and sgRNA2+Cas9 provides the highest editing efficiency. In fact, in the first case, despite the reduction in cost by

using a smaller amount of Cas9 protein, we found less efficient deletion of the region of interest, probably due to competition between sgRNAs for the Cas9 protein. In the second case, despite doubling the amount of Cas9 protein that could theoretically bind the sgRNAs with the same efficiency, we still found an inefficient deletion of the region of interest.

3. Incubate the RNP1 reaction mix, the RNP2 reaction mix, and the Control mix separately at room temperature for 20 min.

4. During the RNP assembly, prepare the cells to be nucleofected as described in Basic Protocol 1, steps 23 to 27.

Preparation of Nucleofection Solution mix for specific genomic deletion

5. Prepare the Nucleofection Solution mix according to the number of nucleofection reactions to be carried out (at least one RNP reaction and one Control reaction).

6. In a 1.5-ml tube under sterile conditions, prepare the Nucleofection Solution mix (using components of the Amaxa P3 Primary Cell 4D-Nucleofector X Kit S) as follows:

Reagent (for RNP and Control reactions = 4 × 10^5 cells)	**Volume**
P3 Primary Cell Nucleofector Solution	32.8 µl
Supplement 1	7.2 µl
Final volume	*40 µl*

After independent formation of RNP1 and RNP2, these complexes are mixed together. Therefore, for each experiment, you will still have one Control mix and one RNP mix composed of the two pre-mixed RNPs (RNP1 and RNP2).

7. After centrifugation (see Basic Protocol 1, step 26), carefully remove all the medium and resuspend the cells in the Nucleofection Solution mix (4 × 10^5 cells are resuspended in 40 µl Nucleofection Solution mix).

Long-term exposure to Nucleofection Solution mix might be toxic for the cells. Therefore, avoid leaving cells in Nucleofection Solution mix for >30 min.

Delivery of ribonucleoproteins through nucleofection for specific genomic deletion

8. After separate assembly of the two RNPs, mix them together and incubate for 5 min at room temperature.

9. Prepare the Nucleofection Reaction mix and Control Reaction mix as follows:

Nucleofection Reaction mix:

Reagent	**Volume**
2 × 10^5 cells (in Nucleofection Solution mix; see step 7)	20 µl
RNP1+2 reaction mix (sgRNA1+Cas9 and sgRNA2+Cas9; see step 8)	10 µl
Alt-R™ Cas9 Electroporation Enhancer (stock 100 µM; see Basic Protocol 1, step 19)	1 µl
Final volume	*31 µl*

Control Reaction mix:

Reagent	**Volume**
2 × 10^5 cells (in Nucleofection Solution mix; see step 7)	20 µl
Control mix (only Cas9; see step 2)	5 µl
D-PBS -Ca^{++}/Mg^{++} (1×)	5 µl
Alt-R™ Cas9 Electroporation Enhancer (stock 100 µM; see Basic Protocol 1, step 19)	1 µl
Final volume	*31 µl*

Do not exceed a final volume of Nucleofection Solution mix of 35 µl; in fact, a higher volume in the strip could severely reduce nucleofection efficiency.

10. Carefully transfer each Nucleofection Reaction mix and Control Reaction mix into separate wells of the 16-well nucleocuvette strip provided with the Amaxa P3 Primary Cell 4D-Nucleofector X Kit S, avoiding bubbles.
11. Switch on the Amaxa 4D-Nucleofector Core and X Units, select the 16-well strip module, and choose the position of the loaded well.
12. Open the tray, transfer the 16-well nucleocuvette strip into the Amaxa 4D-Nucleofector machine, and select the electroporation program and the pulse code (for primary HKs, use Primary Cell P3 program and DS138 pulse code).
13. Press start and wait until the electroporation is completed.
14. After nucleofection, incubate the strip for 10 min at room temperature to maximize the entry of the RNPs into the nucleus and to reduce cellular stress.
15. Add 70 µl keratinocyte growth medium to each well of the nucleocuvette strip containing the 31 µl of Nucleofection Reaction mix, slowly pipet up and down 2 to 3 times, and transfer the entire contents (100 µl) to one well of the 24-well plate containing mitotically blocked 3T3-J2 cells (see Basic Protocol 1, step 23). Repeat this step twice total to ensure collection of all edited cells.
16. Incubate the plate overnight at 37°C, 5% CO_2.
17. The following day, exchange the keratinocyte growth medium with fresh medium to remove the Nucleofection Reaction mix, and particularly the Alt-R™ Cas9 Electroporation Enhancer, which can be toxic for the cells.

Genome editing screening after specific genomic deletion

18. Harvest and analyze cells 72 hr post-nucleofection.

    For specific genomic deletion, it is very useful to perform PCR screening on genomic DNA using primers that discriminate the wild-type sequence (with the generation of a longer amplicon) from the deleted sequence (having a shorter amplicon) (Fig. 2B and 2C).

    Protein extraction and western blot analysis can also be performed to verify genome editing efficiency.

USE OF HOMOLOGY-DIRECTED REPAIR SYSTEM TO INTRODUCE SITE-SPECIFIC MUTATIONS

BASIC PROTOCOL 3

This protocol outlines all the steps and the critical points to site-specifically mutate a genomic region of interest (Fig. 3A). Whereas for KO generation (Basic Protocol 2), the CRISPR/Cas9 system exploits the NHEJ repair mechanism, mutagenesis with a precise modification at a specific location in the genome requires the use of a donor oligo DNA to favor the HDR mechanism. The HDR machinery uses the donor template to accurately repair the DSBs mediated by the Cas9 protein, incorporating the desired genetic modifications into the genome. HDR can be used to introduce a wide range of genetic modifications, such as point mutations and gene insertion or correction. Although the mechanism of HDR is statistically less efficient than NHEJ, this protocol outlines an optimized method to achieve nucleotide substitution with impressive efficiency as compared to previous technologies. However, a single-clone selection step is essential to identify the clones carrying the mutation of interest. We optimized a protocol to reduce cell mortality associated with the editing/single-cloning process and rapidly evaluate the efficiency of genome editing. On the basis of the genome sequence, we introduced a restriction site by adding a silent mutation into the donor template. With this strategy, it is possible to pre-screen single clones by DNA extraction, PCR, enzymatic digestion, and agarose gel electrophoresis before proceeding with DNA sequencing.

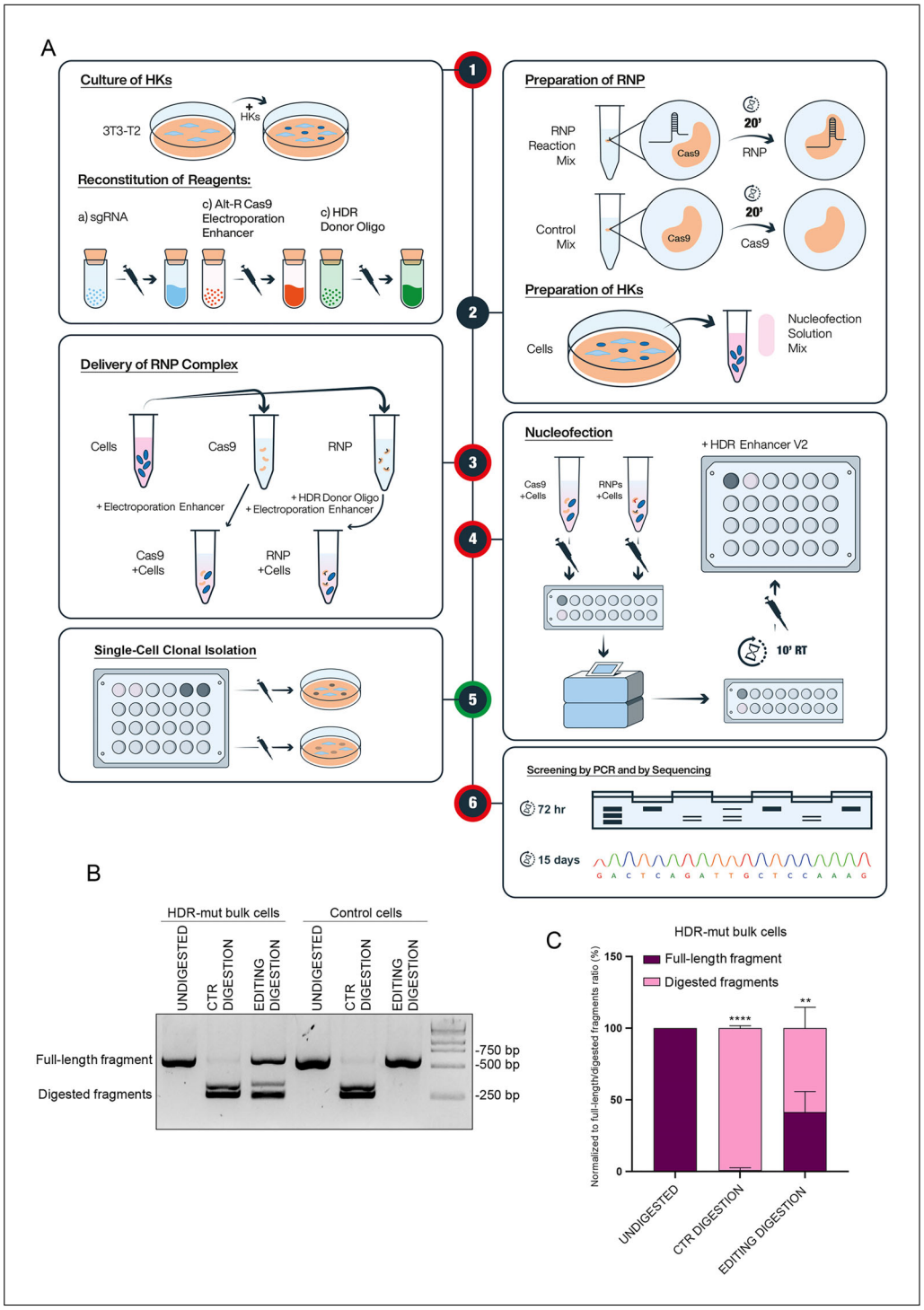

Figure 3 Graphical representation of the experimental procedure and results of Basic Protocol 3. (**A**) Sequential steps of the protocol. The red circles highlight the unique parts of the specific basic protocol, and the green circle indicates the extra step of single-clone isolation necessary for assessment of the desired modifications. The images were created using Adobe Illustrator. (**B**) Representative DNA agarose gel showing undigested and digested DNA fragments in primary HKs transfected with Cas9/sgRNA RNP complex and Donor template (HDR-mut bulk cells) compared to Cas9 protein only (control cells). For both conditions, a control enzymatic digestion (CTR DIGESTION) and an enzymatic digestion that discriminates the integration of the donor template (EDITING DIGESTION) is shown. (**C**) Normalized levels of digested amplicon compared to full-length amplicon (n = 4). Data are the mean ± SD. Two-tailed paired Student's *t*-test; ns, non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

Additional Materials *(also see Basic Protocol 1)*

Alt-R™ HDR Donor Oligo, 2 nmol, lyophilized (IDT, *www.idtdna.com*)
Alt-R™ HDR Enhancer V2, 3 mM, 30 µl (IDT, 10007910)
Alt-R™ CRISPR-Cas sgRNA (IDT, *www.idtdna.com*)
Silicone grease (Molykote high-vacuum silicone grease, Sigma-Aldrich, Z273554)
QIAamp DNA Micro Kit (Qiagen, 56304)

Cloning cylinders, 6 × 8 (Pyrex, 3166-6)
48-well cell culture plate
Tweezers, sterile
Inverted microscope
Additional reagents and equipment for enzymatic digestion and Sanger sequencing

Reconstitution of reagent

1. Reconstitute lyophilized Alt-R™ HDR Donor Oligo:
   a. Spin down the tube containing Alt-R™ HDR Donor Oligo.
   b. Resuspend 2 nmol Alt-R™ HDR Donor Oligo using 20 µl cold WFI water to a final concentration of 100 µM. Gently pipet up and down to ensure full reconstitution.
   c. Store dissolved Alt-R™ Cas9 HDR Donor Oligo for ≤1 year at −20°C prior to use.

Preparation of a plate containing Alt-R™ HDR Enhancer V2

2. To increase site-specific mutagenesis efficiency, prepare keratinocyte growth medium containing 1 µM Alt-R™ HDR Enhancer V2 by adding 0.5 µl Alt-R™ HDR Enhancer V2 in 1.5 ml keratinocyte growth medium.

3. Prepare RNP according to Basic Protocol 1, steps 20 and 21, and prepare feeders and primary HKs as in Basic Protocol 1, steps 23 to 27. During centrifugation of primary HKs, remove 3T3-J2 medium from the 24-well plate containing irradiated feeders.

   For HDR-mediated genome editing, we used 1 × 10^5 cell per reaction for a total of 2 × 10^5 cells (control + HDR).

4. Wash once with pre-warmed 1× D-PBS -Ca^{++}/Mg^{++}.

5. Add 0.5 ml of the previously prepared, pre-warmed (at 37°C) keratinocyte growth medium with 1 µM Alt-R™ HDR Enhancer V2 (see step 2) to each well.

Preparation of Nucleofection Solution mix

6. During centrifugation of primary HKs (see step 3), also prepare Nucleofection Solution mix (using components of the Amaxa P3 Primary Cell 4D-Nucleofector X Kit S) in a 1.5-ml tube under sterile conditions as follows:

Reagent (for RNP+Donor and Control reactions = 2 × 10^5 cells)	**Volume**
P3 Primary Cell Nucleofector Solution	32.8 µl
Supplement 1	7.2 µl
Final volume	*40 µl*

7. After centrifugation, carefully remove all the medium and resuspend the cells in the Nucleofection Solution mix (2 × 10^5 cells are resuspended in 40 µl Nucleofection Solution mix).

   Long-term exposure to Nucleofection Solution mix might be toxic for the cells. Therefore, avoid leaving cells in Nucleofection Solution mix for >30 min.

Delivery of ribonucleoprotein complex through nucleofection

8. Prepare two independent Nucleofection Reaction mixes, for control cells and for cells undergoing genome editing. To induce site-specific mutagenesis, add resuspended Alt-R™ HDR Donor Oligo to the mixes as follows:

Nucleofection Reaction mix:

Reagent	Volume
1×10^5 cells (in Nucleofection Solution mix; see step 7)	20 µl
RNP reaction mix (sgRNA+Cas9; see step 3)	5 µl
Alt-R™ Cas9 Electroporation Enhancer (stock 100 µM)	1 µl
Alt-R™ HDR Donor Oligo (100 µM; see step 1)	1.2 µl
Final volume	*27.2 µl*

Control Reaction mix:

Reagent	Volume
1×10^5 cells (in Nucleofection Solution mix; see step 7)	20 µl
Control mix (only Cas9; see step 3)	5 µl
Alt-R™ Cas9 Electroporation Enhancer (stock 100 µM)	1 µl
D-PBS -Ca^{++}/Mg^{++} (1×)	1.2 µl
Final volume	*27.2 µl*

The IDT tool suggests testing both positive and negative strands of Alt-R™ HDR Donor Oligo separately to verify possible differences in editing efficiency. We observed no significant differences in efficiency or survival between positive and negative strands of Alt-R™ HDR Donor Oligos.

9. Carefully transfer each Nucleofection Reaction mix and Control Reaction mix into separate wells of the 16-well nucleocuvette strip provided with the Amaxa P3 Primary Cell 4D-Nucleofector X Kit S, avoiding bubbles.

   If there are air bubbles, gently tap the nucleocuvette strip and/or use a needle to pop the bubbles.

10. Switch on the Amaxa 4D-Nucleofector Core and X Units, select the 16-well strip module, and choose the position of the loaded well.

11. Open the tray, transfer the 16-well nucleocuvette strip into the Amaxa 4D-Nucleofector machine, and select the electroporation program and the pulse code (for primary HKs, use Primary Cell P3 program and DS138 pulse code).

    Make sure that the larger gap in the strip lid is at the top of the strip to ensure a perfect fit between the holder and the strip.

12. Press start and wait until the electroporation is completed.

    At the end of the run, the screen should display a "+" over the wells that were successfully electroporated.

13. Carefully remove the nucleocuvette strip from the 4D-Nucleofector machine and incubate for 10 min at room temperature.

14. Carefully add 75 µl pre-warmed keratinocyte growth medium with 1 µM Alt-R™ HDR Enhancer V2 (see step 2) to each well of the nucleocuvette containing the 27.2 µl of Nucleofection Reaction mix or Control Reaction mix.

15. Slowly pipet up and down 2 to 3 times.

16. Divide the contents of each Nucleofection Reaction mix and Control Reaction mix into two wells of a 24-well plate containing mitotically blocked 3T3-J2 cells (see step 3) by plating 50 µl/well.

Dividing each Nucleofection Reaction and Control Reaction mix into two wells allows simultaneous analysis of the bulk population (from one edited well) and the single-clone screen (from the other well). For preliminary screening of the bulk population, process the cells as described in steps 47 to 54 below. The only major difference will be to detach the bulk-modified cell population from the plate and then proceed by processing the cell pellet.

17. Incubate the plate overnight at 37°C, 5% CO_2.

18. The day after, exchange the medium with fresh keratinocyte growth medium.

After performing the HDR-mediated genome editing experiment, the next step is to derive a clonal population of the edited cells by single-cell cloning (see steps 19 to 46) in order to identify the clones carrying the mutation of interest. However, dilution of cells immediately after nucleofection increases cell mortality. To avoid this issue, we incubate the cells for 72 hr before proceeding with clonal selection.

Single-cell clonal isolation

19. Autoclave the cloning cylinders and the silicone grease.

20. Plate mitotically blocked 3T3-J2 cells from step 3 at 30,000 cells/cm^2 in a 100-mm plate using 3T3-J2 cell medium and leave them overnight in a cell culture incubator set to 37°C and 5% CO_2. Prepare a total of six 100-mm plates.

A higher number of feeder cells is required to sustain single-cell expansion. This is because, over the extended period required for keratinocyte clones to grow, which takes ∼15 days, some mitotically blocked 3T3-J2 cells undergo cell death. If needed, additional 3T3-J2 could be added every 4 to 5 days to guarantee the growth support provided by the feeders.

21. At 72 hr post-nucleofection, before splitting edited primary HKs to obtain a homogenous population of cells, remove keratinocyte growth medium from the cell plate.

22. Wash the cells once using 0.5 ml pre-warmed (at 37°C) 1× D-PBS -Ca^{++}/Mg^{++}.

23. Add 0.3 ml pre-warmed (at 37°C) 0.25% trypsin-EDTA and incubate for 1 min at 37°C to remove only feeder cells.

24. Remove trypsin-EDTA solution containing feeder cells.

25. Wash once with 0.3 ml trypsin-EDTA solution to ensure that all feeder cells were removed. Check cell detachment under a phase-contrast microscope to ensure that only feeder cells were removed.

26. Remove the trypsin-EDTA solution and then detach the primary HKs by adding 0.3 ml fresh pre-warmed (at 37°C) 0.25% trypsin-EDTA and incubating for 4 min at 37°C. Gently tap the plate on a surface to fully dissociate the cells and check under the phase-contrast microscope to confirm that all cells are detached.

27. Inactivate the trypsin-EDTA solution by adding the same volume (v/v) of 100% FBS and then collect the cells in a sterile 1.5-ml tube.

28. Count cells using a hemocytometer.

29. Dilute cells to 50 cells per 100-mm plate.

The dilution rate might vary accordingly to the cell type and culture conditions. For optimal experimental results, we suggest testing several dilutions. In our case, we tested 50, 100, 500, and 1000 cells per 100-mm plate. We found that 50 cells per 100-mm plate guarantees single clone formation while avoiding cross-contamination between different clones.

30. Plate edited and diluted primary HKs on mitotically blocked 3T3-J2 cells (see step 20).
31. Change the keratinocyte growth medium every 3 days.
32. Verify that individual clones are visible after 7 to 10 days.
33. At 15 days post-nucleofection, prepare to proceed with isolation of the single clones (see steps 34 to 46).
34. Plate mitotically blocked 3T3-J2 cells (see step 3) at 20,000 cells/cm^2 in a 48-well cell culture plate using 3T3-J2 cell medium and leave them overnight in a cell culture incubator set to 37°C and 5% CO_2.
35. Using an inverted microscope, identify well-isolated colonies.

    The clones will appear as opaque white spots to the naked eye. Draw a circle with a marker on the outer bottom of the plate to outline the contours of each clone and facilitate the positioning of the cloning cylinder.
36. Remove the medium.
37. Gently wash with 5 ml pre-warmed (at 37°C) 1× D-PBS -Ca^{++}/Mg^{++}.
38. Apply a thin layer of sterile silicone grease (see step 19) to the bottom edge of each sterile cylinder (see step 19).
39. Using sterile tweezers, gently place a cloning cylinder around the colony of interest.

    Make sure that the cylinder is tightly attached to the cell plate.
40. Carefully add 30 µl pre-warmed (at 37°C) 0.25% trypsin-EDTA and incubate for 7 min at 37°C.

    A longer trypsin-EDTA incubation is required to detach single clones from the plate.
41. During the incubation, remove 3T3-J2 cell medium from the 48-well plate (see step 34) and add 0.5 ml keratinocyte growth medium.
42. Inactivate the trypsin-EDTA solution by adding the same volume (v/v) of 100% FBS and transfer each clone into a well of the 48-well plate containing mitotically blocked 3T3-J2 cells.

    No centrifugation step is applied at this stage to avoid cell loss. The excess of trypsin-EDTA/FBS mixture is removed the day after by replacing the medium with fresh medium (see step 44).
43. Incubate the plate overnight at 37°C, 5% CO_2.
44. The day after, exchange the medium with fresh keratinocyte growth medium.

    The number of clones to isolate depends on the efficiency of genome editing. With this protocol, we have achieved 50% to 60% efficiency of the desired modification. However, we still suggest isolating at least 50 to 100 clones to have the possibility to sub-select clones with similar phenotypic characteristics.
45. Split the clones when they reach 80% confluence.
46. Plate each clone at a density of 3200 cells/cm^2 in a new plate of mitotically blocked 3T3-J2 cells (see step 3). Collect the remaining cells for clone screening.

Screening of single clones

47. Centrifuge the cells of each clone in a 15-ml tube for 5 min at 250 × *g*.
48. Gently remove keratinocyte growth medium from pellets.

49. Wash with 0.5 ml pre-warmed 1× D-PBS -Ca^{++}/Mg^{++}.

50. Centrifuge 5 min at 250 × *g*.

51. Proceed with cell lysis and DNA extraction using a QIAamp DNA Micro Kit.

    For optimal DNA extraction from a small number of cells, we suggest use of this specific kit.

52. After DNA extraction, perform PCR amplification of the genomic region containing the site-specific modification using appropriately designed primers.

    In the case of preliminary screening based on restriction enzymes, a PCR mix in triplicate for each DNA sample will be necessary, as explained in step 53.

53. Proceed by performing enzymatic digestions of the PCR amplification product obtained for each clone.

    We suggest using three different PCR amplicon mixes for each clone: an amplicon mix that will not be digested as a control, an amplicon mix digested using a restriction enzyme that cuts in the same way both the HDR-mutated and wild-type DNA, and an amplicon mix to be digested with the restriction enzyme that cuts only if the donor DNA has been integrated into the genome (Fig. 3B and 3C).

    The enzymatic digestion screening is an efficient method to reduce time and cost, but DNA sequencing of positively digested clones is an essential step to verify insertion of the desired modifications.

54. Extract a small amount of DNA from control and positively digested clones resulting from the enzymatic digestion and sequence by Sanger sequencing.

    The single-cell clonal isolation and the screening have to be performed not only on the HDR-modified primary HKs but also on the control nucleofected cells.

REAGENTS AND SOLUTIONS

3T3-J2 cell medium

88 ml high-glucose Dulbecco's modified Eagle's medium (DMEM; Euroclone, ECM0101L)
10 ml FBS (Euroclone, ECS 500L)
1 ml penicillin-streptomycin (Euroclone, ECB3001D)
1 ml L-glutamine (Euroclone, ECB3000D)
Store ≤4 weeks at 4°C

Keratinocyte growth medium

64.4 ml high-glucose DMEM (Gibco, 61965-026)
22 ml Ham's F12 (Gibco, 31765-027)
10 ml FBS, characterized (Cytivia, Cha1115L)
1 ml L-glutamine (Euroclone, ECB3000D)
1 ml penicillin-streptomycin (Euroclone, ECB3001D)
250 µl adenine (Sigma-Aldrich, A2786)
125 µl insulin human recombinant zinc (4 mg/ml; Gibco, 12585-014)
10 µl hydrocortisone (4 mg/ml; Millipore, 386698-25 mg)
50 µl apo-transferrin, human (Sigma-Aldrich, T2252-100 mg)
2 µl triiodothyronine (T3; 100 mM; Sigma-Aldrich, T6397)
10 µl cholera toxin (10 mM; Sigma-Aldrich, C8052-5mg)
10 µl epidermal growth factor (0.1 mg/ml; Corning, 354052)
100 µl ROCK inhibitor Y27632 (Enzo Life Sciences, EN270333M005)
Filter with a 0.2-µm filter (Filter Stericup-GV 0.22 µm, PVDF, 500 ml, Sigma-Aldrich, SCGVU05RE)
Store ≤15 days at 4°C

COMMENTARY

Background Information

We developed these protocols given the need for a rapid, efficient, and reproducible editing strategy for difficult-to-transfect cells such as HKs. In fact, prior to the advent of advanced genome editing technologies such as CRISPR/Cas9, genetic modification of HKs faced significant technical barriers (low transduction/transfection efficiency, difficulty in long-term clonal selection, high off-target effects). The main advantages of the methods described here are the high efficiency, the reduced number of cells used for each reaction, and the small volume of reagents needed, which improve the cost-efficiency ratio. These techniques may also be applied to other cell lines of epithelial origin.

Critical Parameters

The protocols described here can be adopted in different laboratories to manipulate HKs with high efficiency. In this regard, establishing high quality for both 3T3-J2 feeder cells and the HK culture is a critical step. HK colonies should never exceed 80% confluence to avoid terminal differentiation. No visible colonies undergoing spontaneous differentiation should be present in the cell plate. The addition of the ROCK inhibitor Y27632 (Enzo Life Sciences, EN270333M005) is essential to extend the lifespan of HKs. Early passages (p1 to p10) should be used for both 3T3-J2 cells and HKs.

Cells should be routinely tested for mycoplasma. Proper sterile cell culture techniques are necessary to prevent contamination during co-culture. Before starting the experiment, cells should be split once after thawing. We have optimized the use of these protocols for small volumes of cells in 16-well nucleocuvette strips. We were able to achieve comparable genome editing efficiency using both 1×10^5 and 2×10^5 cells without scaling up Cas9 and sgRNA volumes. In general, we observe editing efficiencies >80% for both KO and genomic deletion experiments, although the efficiency might vary depending on the protein of interest.

All the genome editing reagents (Cas9, sgRNA, enhancers, and donor oligos) must be slowly thawed on ice to avoid degradation. Cas9/sgRNA RNPs should be prepared immediately before transfection; they cannot be pre-annealed and stored at –80°C.

Troubleshooting

Please see Table 1 for a list of common problems for these protocols, their causes, and potential solutions.

Understanding Results

Each protocol includes a figure summarizing the experimental procedure (Figs. 1A, 2A, and 3A) and the results that can be obtained using the protocol. Figure 1B shows an example of western blot analysis used to verify KO efficiency in HKs. As expected, the control cells still have a high expression level of the protein of interest, as Cas9 could not proceed with the cut without the gRNA. On the other hand,

Table 1 Troubleshooting Guide for CRISPR/Cas9 RNP Nucleofection in Primary Human Keratinocytes

Problem	Possible cause	Solution
Low cell number not associated with cell death	Defective cell count	Use an automatic cell counter
Yellow medium at 48 or 72 hr after nucleofection	Cell density is too high	Change the medium every day
High cell death	Long-term exposure to nucleofection solution	Work more rapidly and change the medium after ≤15 hr post-nucleofection
	Defective cell count	Use an automatic cell counter
	Stressed cells	Refresh the cell medium
	Program not ideal for this cell type	Test different nucleofection programs
Low editing efficiency	Air bubbles in the nucleocuvette	Gently tap the nucleocuvette strip and/or use a needle to pop the bubbles
	Volume higher than 35 µl per nucleocuvette	Remove all the medium before resuspending the cell pellet in the nucleofection solution

CRISPR/Cas9 bulk targeted cells show very low levels of the protein of interest. The presence of a faint band corresponding to the desired molecular weight is due to the fact that no selection method was used to isolate the edited cells. Editing efficiency can be measured using Bio-Rad Image Lab or similar image analysis software (Fig. 1C). Figure 2B shows an example of PCR analysis used to verify a specific genomic deletion. Control cells that were uniquely nucleofected with Cas9 protein produce a clean band corresponding to the PCR amplicon containing the sequence of interest. Accordingly, the cells nucleofected with RNPs (RNP1+RNP2) show efficient deletion of a DNA segment (shorter amplicon). The amplicon without deletion appears to have the same length as the control band. The higher band intensity of the shorter amplicon with the deletion compared to the control band indicates a highly efficient genomic deletion. Again, the presence of a band corresponding to the control band is probably due to the fact that no selection method was used to isolate the edited cells. The ratio of amplicon intensity of edited/control band provides information on editing efficiency in bulk. Quantification intensity can be measured using Bio-Rad Image Lab or similar image analysis software (Fig. 2C). Figure 3B shows a representative PCR analysis followed by enzymatic digestion and an agarose gel run of the digested fragment, which is useful to assess HDR-mediated mutagenesis in bulk. HDR-mutated bulk cells and control cells (nucleofected only with Cas9 protein) are analyzed 72 hr post-nucleofection by DNA extraction, PCR amplification of the genomic region of interest, and enzymatic digestions. Two independent digestion reactions are performed for both HDR-mutated bulk and control cells: a "control digestion" performed with an enzyme that cuts in the edited and control cells in the same way and an "editing digestion" in which only the edited DNA is cut. The agarose gel shows full-length fragment and digested fragments. The presence of full-length fragment in the editing digestion of HDR-mutated bulk cells is due to a percentage of cells (~40%) that have not integrated the site-specific modification. For this reason, single-clone isolation is required for site-specific mutagenesis experiments. The quantification intensity of full-length and digested fragments can be measured using Bio-Rad Image Lab or similar image analysis software (Fig. 3C).

Time Considerations

Exposure of sub-confluent 3T3-J2 cells to 50 Gy takes ~1 hr (this time might vary depending on the equipment used). The cryopreservation process, including trypsinization, counting, and freezing, typically takes 1 to 2 hr. Mitotically blocked feeder cells should be plated 24 hr before adding HKs to allow them to adhere and form a supportive layer. After plating, HKs typically require 24 to 48 hr to adhere properly to the culture substrate before starting cell division. Reconstitution of the reagents for the genome editing experiment takes 10 min. For all the protocols, CRISPR/Cas9 RNP preparation and nucleofection take ~1 hr. If we exclude the time required for the analysis, each protocol can be performed in 3 days:

- Day 0: preparation of feeder cells
- Day 1: preparation of reagents and CRISPR/Cas9 RNP nucleofection
- Day 2: incubation of cells to allow CRISPR/Cas9 editing to occur
- Day 3: collection of cells and preparation of samples for further analysis.

For the analysis of KO generation by indel mutations and deletion of a specific genomic region (Basic Protocols 1 and 2), 1 to 2 days will be needed. For site-specific mutations using the HDR system (Basic Protocol 3), a longer time is required (10 to 15 days).

Author Contributions

Martina Bamundo: Data curation; methodology; formal analysis; writing—original draft. **Sara Palumbo:** Formal analysis; writing—original draft. **Ludovica D'Auria:** Data curation; visualization. **Caterina Missero:** Funding acquisition; writing—review and editing. **Daniela Di Girolamo:** Conceptualization; data curation; formal analysis; methodology; supervision; validation; writing—original draft; writing—review and editing.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data, tools, and materials (or their source) that support the protocols are available from the corresponding author upon reasonable request.

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