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Life Science

Convenient and Quick Isolation and Analysis of Genome-Edited Cells Using PERFLOW Sort and MultiNA

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1. Introduction

With the growing popularity of genome editing techniques, a major concern for researchers is: how efficiently intended edits can be achieved on cells or organisms, and how the type of editing made can be identified by a high-throughput process. These processes of concern, however, are described in papers as if they are very simple procedures. For example, in a paper which describes established genome-edited cells and their characterization analysis, the establishment of a mutated cell line is often summarized in a sentence like the one indicated below (the description about the transfection amount and incubation time is omitted).

"Genome-editing tools were delivered by the ** method, and single-cell cloning was performed to obtain ** and ** clones of heterozygously and homozygously mutated cells, respectively."

Unless it is a technological treatise, the data presented for this process would be the genotyping results of the several cell lines which were used as target mutant cells in the final analysis. A laboratory leader who has read such a paper may without careful consideration instruct a researcher to create cells by inducing mutations into the ** gene in the same way as in this paper. However, the cell line and gene delivery method to use may not be the same, and even if they are the same, depending on the target gene, the targeting efficiency changes and there is no guarantee that the same mutant cell line can be established. Furthermore, the process of single-cell cloning or mutant cell screening involves complicated procedures which do not appear in papers.

This Application Note introduces a pipeline for establishing genome-edited cells efficiently together with an actual example to help researchers resolve such working-level concerns.

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The example introduced here is based on the paper[1] published in the "Genes to Cells" journal. If you need more detailed information, please refer to the journal as well. In addition, regarding genome editing on animal cells, two examples—rice-fish (medaka)[2] and clawed frog (*Xenopus tropicalis*)[3]—were presented in previous Application Note articles. This article explains a simple and rapid method for the isolation and analysis of genome-edited cells focusing on the genome editing of cultured cells, using two analytical instruments, PERFLOW Sort and MultiNA.

2. Overview of Experiment Workflow

In this experiment, CRISPR-Cas9 RNPs (Cas9 protein and crRNA/tracrRNA complex) were introduced into HCT116 cells, derived from human colon adenocarcinoma, and the mutant cells were isolated and analyzed. The general workflow of the experiment is shown in Fig. 1. The detailed procedure of preparing genome editing tools and delivering them into cells is omitted in this article, but Cas9 proteins and crRNA/tracrRNA have become commercially available recently and there is no need to prepare Cas9 RNPs by yourself. In addition, reagents for efficiently delivering Cas9 RNPs into cells are also on the market. By using such reagents, RNPs can be introduced simply at a low cost without the need to use an expensive electroporator. Note that, however, depending on the cell line or target gene locus, a high mutagenesis efficiency may not be obtained, requiring the preparation of genome editing tools and delivery method according to the purpose.

The general flow after delivery of genome editing tools into cells is as follows: First, mutations in the cell population are evaluated before cloning to determine the efficient mutagenesis, and single-cell cloning is performed and then each clone is genotyped. From the next section, the details of each step are explained based on an example experiment.

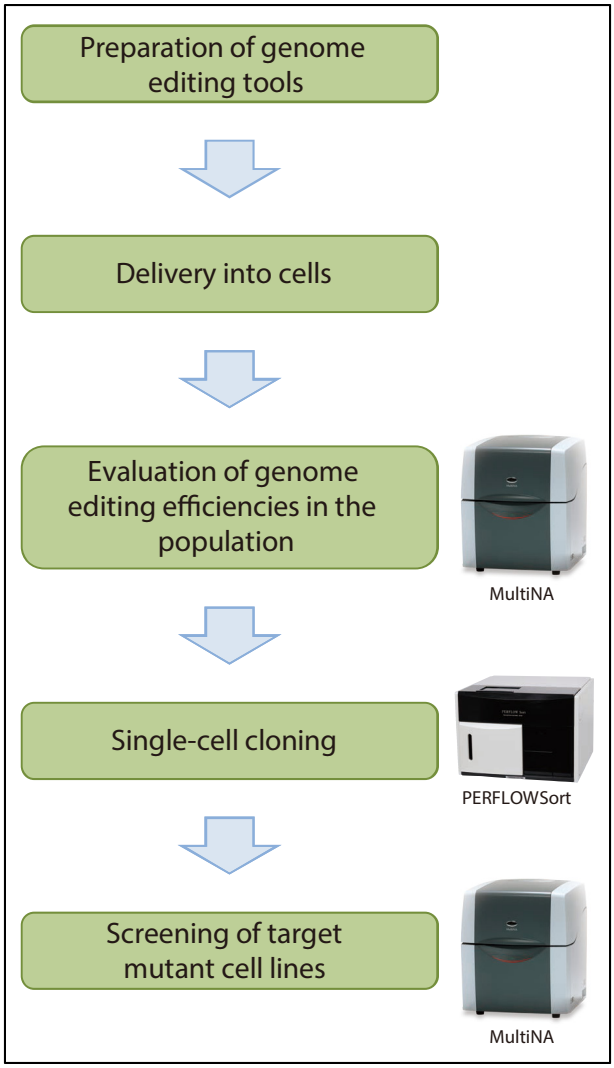
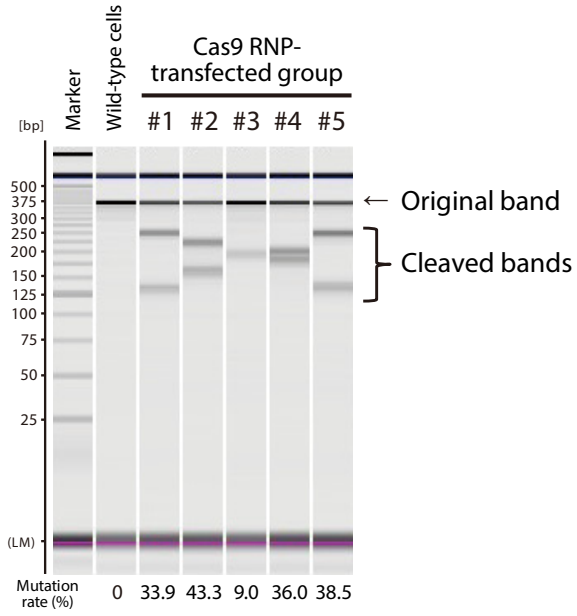


Fig. 1 The General Workflow of the Experiment

3. Population Analysis of Genome-Edited Cells

Cloning is indispensable to establish homogeneous gene knockout cells and knockin cells. Considering the time required to obtain a sufficient number of cells for analysis from single cells and the effort of screening knockout and knockin cell lines, the efficiency of gene knockout and knockin in the population must be confirmed before proceeding to cloning. Cloning without this confirmation may adversely affect the later processes, such as a huge amount of cells needing to be genotyped to obtain the "right" cloned cells or no target clones are obtained, resulting in starting over again from transfection. In particular, in the case of an experiment system which cannot ignore any influence of off-target mutations, it is vital to investigate the frequency of off-target mutations at this stage.

In this example, donor DNA is not used but insertion and deletion mutations are induced by utilizing endogenous repair errors so that the frequency of mutations can be quantified with a method called the Cel-I assay. With the Cel-I assay, the genome area containing the relevant CRISPR-Cas9 target sequence is PCR-amplified from the population where cells having wild-type alleles not mutated and alleles mutated in various patterns are mixed. When the PCR products are heat-degenerated and re-annealed, annealed products of wild-type alleles and mutant alleles, or alleles with different mutations are produced according to the content of mutant alleles. Since these products have mismatches, when digested with a mismatch-specific nuclease, i.e. Cel-I nuclease, a cleavage occurs at the mismatch site, generating short fragments. By quantifying the ratio of fragmented products, the mutation rate can be estimated.[4] If agarose gel electrophoresis or polyacrylamide gel electrophoresis is used at this time, quantitation is indirect with gel photos using the image analysis software, which reduces the reliability of the results. On the other hand, with micro-chip electrophoresis using the MultiNA, the amount of DNA contained in each band can be quantified directly ; therefore, a higher quantitation accuracy can be obtained.



Gel Image

Sample	Size (bp)	Density
Cas9 RNP (–)	(Lower Marker)	2334.18
	332	36.52
	351	84.64
	372	1039.25
	(Upper Marker)	659.91
Cas9 RNP #1	(Lower Marker)	2296.34
	127	92.72
	132	153.9
	244	427.61
	350	33.12
	371	523.14
	(Upper Marker)	701.95

Peak Table
(Excerpts of results for wild-type samples and Cas9 RNP #1)

The results of MultiNA analysis of Cel-I assay products are shown. The density values in red in the peak table indicate the density of the original band, and those in blue indicate that of the cleaved bands. Mutation rates were calculated from these values.

Fig. 2 Population Analysis of Genome-Edited Cells

In the example shown in Fig. 2, the cleaving activity at the HCT116 cells is evaluated for five types of crRNA designed to the human *HPRT1* gene locus. In the case of electrophoresis with the MultiNA, not only a simulated gel image is visualized but also a peak table containing the quantified density values of amplified products in each size can be obtained, which can directly be used to calculate the efficiency of mutagenesis. Furthermore, these values can be used not only for the Cel-I assay but also for genotyping [5], [6] with the heteroduplex mobility assay (HMA) and with the RGEN-RFLP method by analyzing restriction fragment length polymorphism (RFLP) using Cas9 RNPs.

4. Single-Cell Cloning Using PERFLOW Sort

After the efficiency of mutagenesis in the cell population is calculated by population analysis, single-cell cloning is performed to obtain homogeneous mutant cell lines. There may be factors that depend on the cell type, whether they are adherent cells or suspension cultured cells, differentiated cells or stem cells. In general, the limiting dilution or colony separation technique is applied [7] for manual single-cell cloning. With these techniques, the number of single-cell clones that can be isolated is probabilistic and there is no guarantee that the obtained clones are derived from single cells. In some cases, cloning may need to be performed again. In addition, if the cell lines to establish are numerous, another problem is that an enormous load will be put on the operator in terms of time and effort. Cloning using a cell sorter is also widely used; however, when handling delicate cells such as genome-edited cells, the damage by sorting may affect the later proliferation, even though single cell isolation itself is efficient.

To reduce the damage caused by sorting, a damage-less cell sorter, PERFLOW Sort, was used for single-cell cloning in this experiment. Although PERFLOW Sort is damage-less, it enables analyses with transmitted light, side-scattered light and multicolor. In this experiment, gating with transmitted light and side-scattered light was performed before cloning (Fig. 3). Using this flow cytometer, highly reliable single-cell sorting is possible in a short time with less effort, enabling simultaneous establishment of genome-edited cell lines under various conditions.

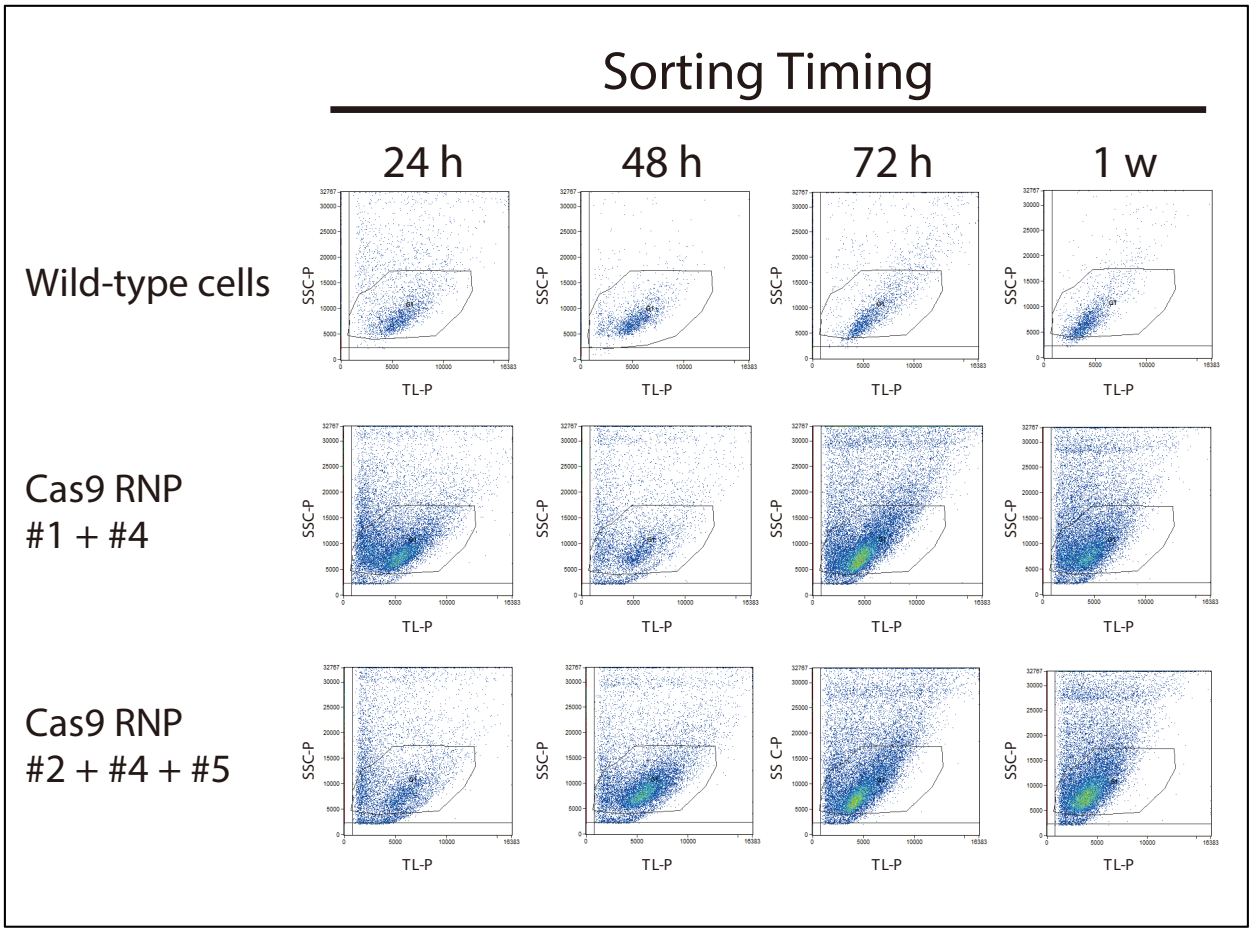


Fig. 3 PERFLOW Sort Sorting Plot

5. High-Throughput Analysis of Cloned Genome-Edited Cells

Even if cloning is done by a high-throughput process as described above, it would be difficult to handle clones in large quantity at one time if a great effort must be expended for genotyping. Therefore, a technique that enables handling of multiple samples with less effort is also desired for analyses following the cloning. The Cel-I assay described above enables the quantitative measurement of mutagenesis efficiency; however, the cost per sample is high, making it unsuitable for genotyping multiple samples. Furthermore, since the alleles after cloning are basically fixed, there is no mixture of various types of alleles as used for population analysis. If all alleles of the target gene have a homogeneous mutation, no mismatch is created even by using the Cel-I assay, which means that no cleaved band can be detected.

Among the other genotyping methods, the RFLP and RGEN-RFLP techniques using restriction enzymes are effective for detection even in homogeneous mutations. Although the former is low-cost, it cannot be used if the recognition sequence of the restriction enzyme does not exist on the site of mutagenesis. The latter can be used basically for every target sequence, but Cas9 RNPs are relatively costly. In particular, when delivering CRISPR-Cas9 in plasmid, Cas9 RNPs need to be prepared separately for genotyping. As a simple and general-purpose method with a low cost, the HMA technique is available. With the HMA technique, genotyping can be completed simply by running electrophoresis of PCR products, which is the quickest and easiest among other techniques. As with the Cel-I assay, if the population consists of homogeneous alleles only, a false negative may be given. However, it can be said that, despite some "omissions", the HMA technique is most suitable when screening numerous samples.

In this example, as described up to this point, an experiment targeting the *HPRT1* gene locus in the HCT116 cell was performed. In the analysis of single-cell cloning, the HMA technique was used after cleaving multiple target sequences at the same time to generate mutant alleles in various patterns. By running electrophoresis using the MultiNA, a high separation performance was obtained at a high throughput (Fig. 4). For the purpose of this experiment, clones in which multiple bands were observed are indicated by asterisks in Fig. 4. However, in practice, not only these clones but also those exhibiting bands different from the band size of wild-type alleles are considered to be mutated cell clones. Among the clones having apparently the same pattern of bands as that of wild-type alleles, there is a possibility that mutant clones slightly different in length may be harbored. From this band pattern, we can instantly recognize that mutant cells are obtained at an extremely high efficiency.

To confirm the results of genotyping, direct sequence analysis of each clone was also performed. As an example, Fig. 5 shows the results of sequence analysis of clones enclosed with black, blue and green lines in Fig. 4. Supporting the results of HMA analysis using the MultiNA, the wild-type clone exhibits a clean peak of wild-type allele, the clone with one short-size band exhibits a clean peak of mutant allele, and the clone with multiple bands exhibits overlapping peaks of multiple alleles. As demonstrated, in the analysis of single-cell cloning, two-step verification—picking up candidate clones by screening with the MultiNA and performing sequence analysis of screened clones—would be an ideal genotyping strategy in terms of efficiency and credibility.

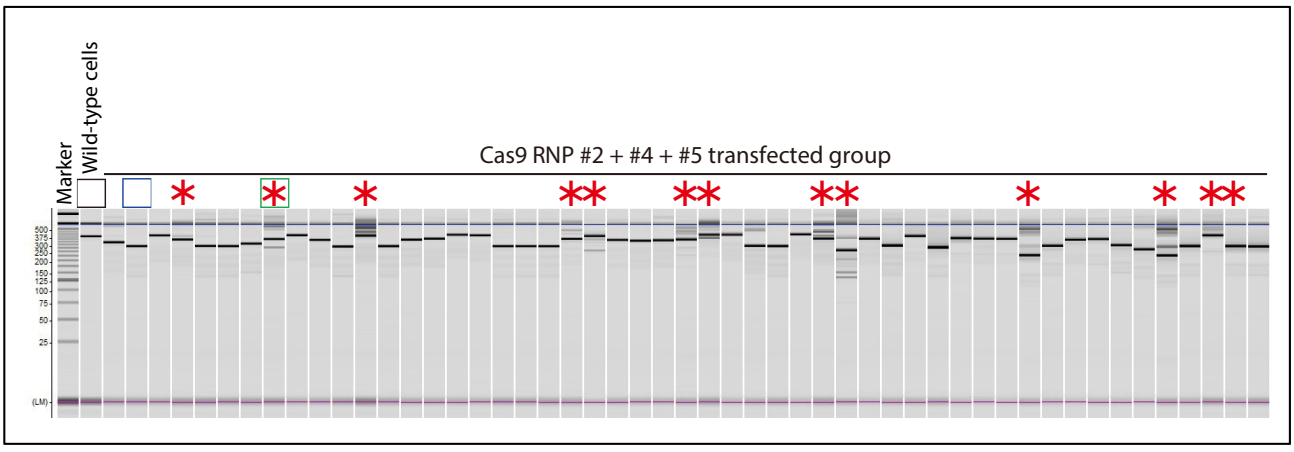


Fig. 4 High-Throughput HMA Analysis of Cloned Genome-Edited Cells

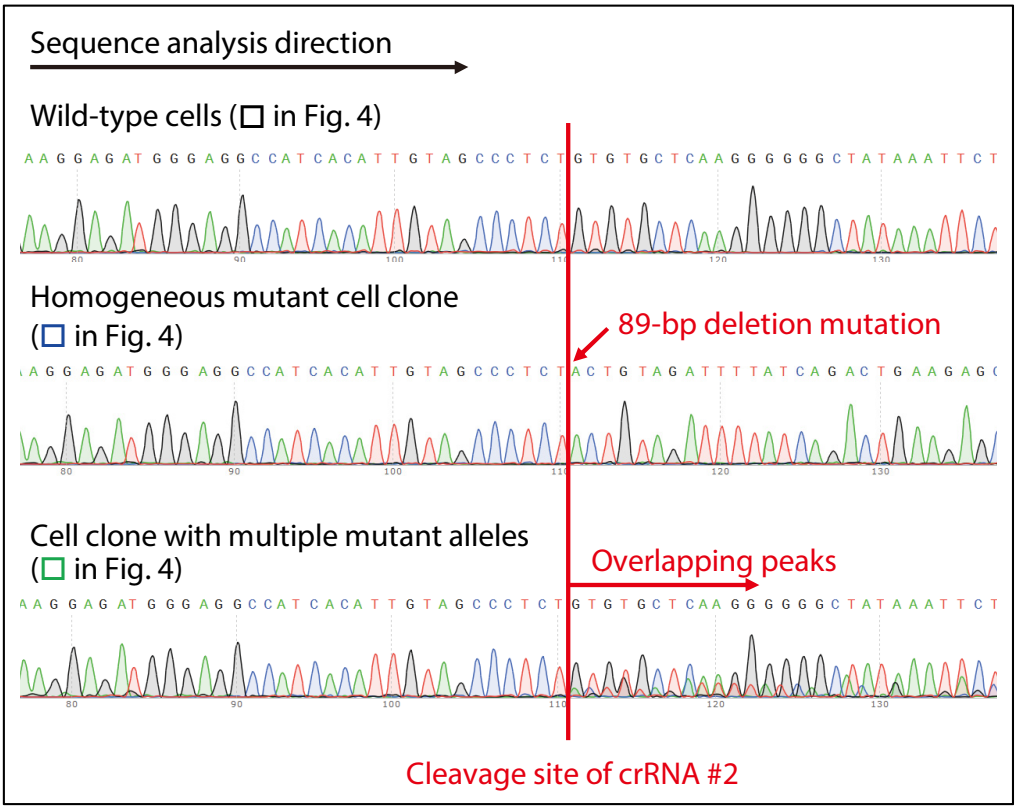


Fig. 5 Direct Sequence Analysis

6. Conclusion

This article introduced a workflow to establish cell lines to which insertion and deletion mutations were induced by transfecting Cas9 RNPs. In genome editing, not only gene knockout by this procedure but also insertion of a foreign sequence such as a reporter gene, delivery by base substitution, wide-area deletion in the chromosomal region, and other genome modifications can be made. Efficient establishment of cell lines and genotyping techniques are needed in various research fields on a daily basis. We hope this Application Note will help many researchers who utilize genome editing.

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PERFLOW Sort Damage-less Cell Sorter

For cell genome editing and hybridoma production, cells need to be isolated. As cells are fragile, limiting dilution by manual operation is generally used.

PERFLOW Sort reduces physical damage to the sample to the maximum and enables culturing and proliferation after sorting, making cloning efficient.

Damage-less sorting

A non-dripping (mechanical flow) system is employed without using high hydraulic pressure, ultrasonic waves or intense magnetic fields.

Large-size cells and cell aggregation sorting enabled

Large-size cells such as megakaryocytes that cannot be put through an ordinary flow cytometer can also be sorted.

New optical system - The world's first transmitted light detection

An optical fiber flow cell is employed. Precise size measurement is possible compared with using front-scattered light.

PERFLOW Sort Damage-less Cell Sorter

MCE-202 MultiNA Microchip Electrophoresis System

MultiNA is the new automatic analytical platform to solve the points to be desired in agarose gel electrophoresis. The presence or absence and size of DNA/RNA can be detected quickly and simply.

This is suitable for the genotyping of various samples from cultured cells to mice and detection of microorganisms and viruses.

MCE-202 MultiNA
Microchip Electrophoresis System

High analytical performance

Microchip electrophoresis with the MultiNA achieves separation performance, sensitivity, repeatability and quantitativity superior to agarose cell electrophoresis.

Full automatic analysis of up to 108 samples

Just by setting samples and reagents, a maximum of 108 samples can be analyzed automatically. Pretreatment and electrophoresis are processed in parallel, and one analysis takes only 80 seconds.*

Pursuit of operating ease

Analytical operation of the MultiNA is very easy. Simply create an analysis schedule, set samples and reagents, and click the START button.

Analysis cost reduction

Highly functional microchips can be used repeatedly to reduce the running cost.

* When four microchips are used for DNA standard analysis (DNA-1000 kit / premixed). The initial rinsing time and post rinsing time are not included. This does not apply to the first analysis.

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