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Application Note

No.36A

Genome Editing and Creating Mutant Strains in Medaka
– Application of MultiNA™ –

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LifeScience

1. Introduction

In recent years, various genome editing technologies have been developed, such as the TAL effector nuclease (Transcription Activator-Like Effector Nuclease) and CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein) systems, which allow DSB (Double-Strand Breaks) to be introduced at any desired sequence in the genome. The two are fundamentally different. TAL effector nuclease is an artificial nuclease of chimeric protein which has a nuclease domain. CRISPR/Cas systems utilize an RNA-guided RGEN (RNA-guided endonuclease).[1,2,3)]

Following those developments, a broad variety of tools based on such technology have been developed, and advancements are being made day by day.

One of the most significant contributions of this technology is that has now become possible to perform selective gene targeting (gene disruption) using a wider variety of organisms. Until now this was only possible with limited model organisms such as mice, in which ES cells are present. So far, there have been reports of gene disruption with nematodes, drosophila, silkworms, crickets, sea squirts, frogs, newts, and so on.[4)] The mechanism by which gene targeting using DSB takes place is shown in Fig. 1. After DSB occurs, cells attempt to perform repairs. There are two main paths that can be followed when performing repairs. With one, known as homology-directed repair, normal sister chromosome is used as a template for performing the repair. In this case, chromosomes are restored to their original state, eliminating any damage. As a result, mutations are not introduced. On the other hand, with non-homologous end joining, chromosomes are joined as a stopgap measure to address the chromosome breakage. At this time, a mutation in the form of insertion or deletion (in/del) of the base is introduced, and genetic disruption occurs.

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Although gene targeting itself has been made possible, in reality, the establishment of mutant strains requires an assessment of the activity of the TAL effector nuclease- or CRISPR/Cas-treated individuals, the selection of individuals in which the desired mutation has been introduced, the selection of individuals that have the same mutation, and the selection of individuals having the same mutation in both alleles, all of which entail a great deal of work and expense.
For such assessment and selection, we employed the heteroduplex mobility assay (HMA) method, described later in this document. HMA is conventionally performed using polyacrylamide gel electrophoresis. However, this method requires much time and effort to prepare the gel and process multiple individuals.
The MultiNA™ microchip electrophoresis system is an analytical instrument that is capable of conducting electrophoresis automatically. Analysis requires only the setting up of the necessary reagents and samples.
Here, we introduce efficient methods for producing mutant strains by using the MultiNA™ for assessing and selecting mutant individuals.[5, 6, 7]

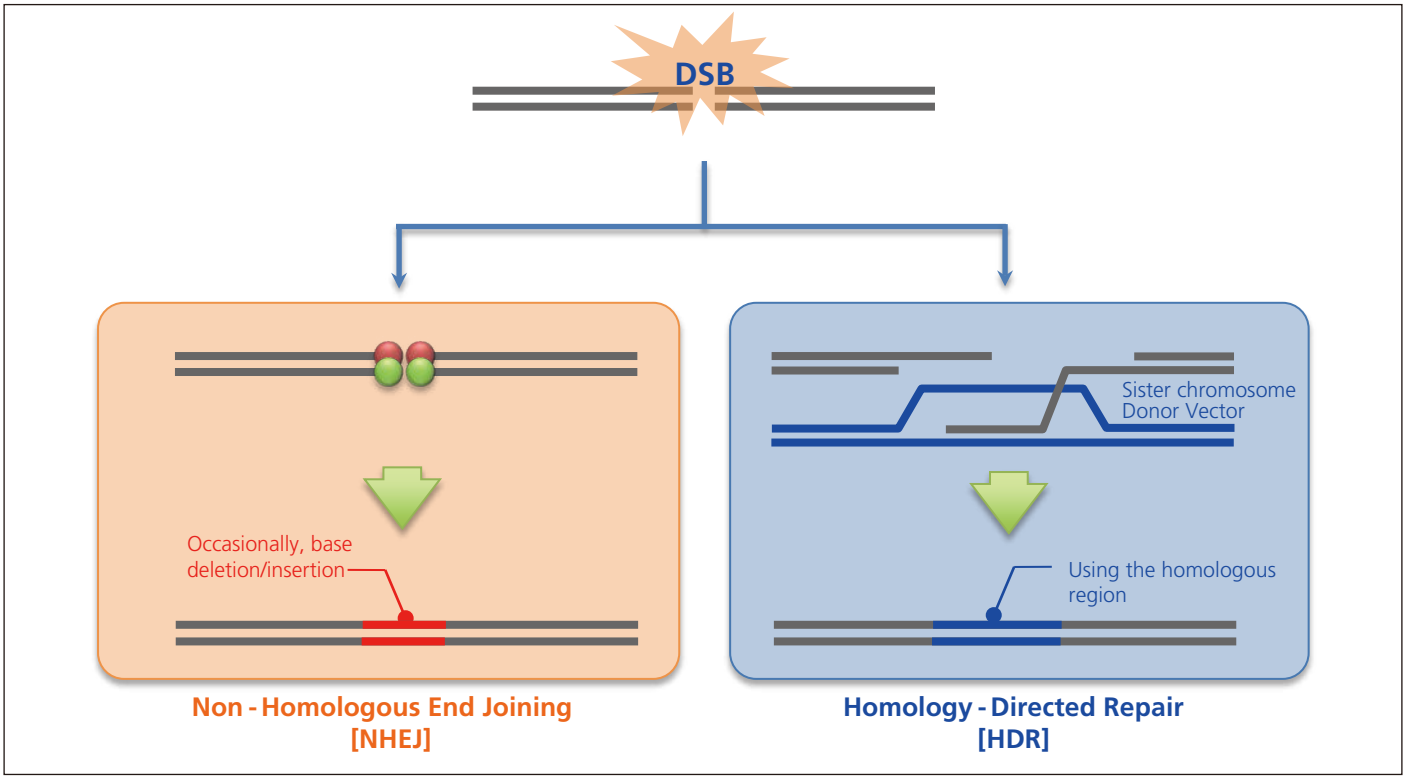


Fig. 1 Mechanism of Gene Targeting Using DSBs (Double-Strand Breaks) of Chromosomes and Repair Method

2. Workflow of Creation of Mutation and Mutant Strains in Medaka (Oryzias latipes: Small Teleost Fish)

The basic workflow is shown in Fig. 2. The process in which the MultiNA™ is used is described from step 2 below. For information regarding step 1, including the design of the TAL effector nuclease or CRISPR/Cas system or the synthesis of the RNA, refer to the websites below or other literatures.[4]

- TAL effector nuclease assemble protocol
http://www.shigen.nig.ac.jp/medaka/strain/laboManual.jsp

- Design of sgRNA
http://viewer.shigen.info/cgi-bin/crispr/crispr.cgi

- Search for off-target sites for Medaka
http://viewer.shigen.info/medakavw/crisprtool/

- Information for Medaka (Oryzias latipes)
NBRP medaka HP: http://www.shigen.nig.ac.jp/medaka/

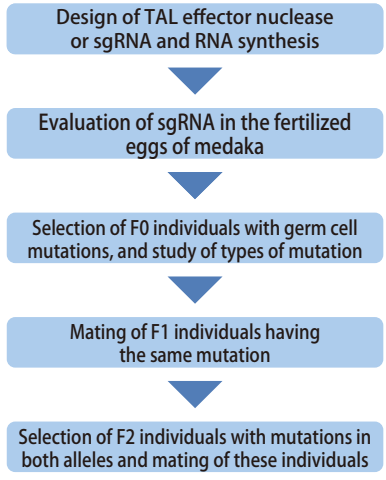


Fig. 2 Workflow for Establishing a Gene-Targeting Medaka Strain

3. Using HMA to Check the Individuals in Which TAL effector nuclease or CRISPR/Cas Were Applied for Mutation

When electrophoresis is performed on double-stranded DNA of identicallength, completely complementary homoduplex DNA exhibits mobility that is dependent on molecular weight. However, with heteroduplex DNA, in which mismatch occurs in some areas, the steric structure of the area with a mismatch differs from that of homoduplex DNA, which impedes the speed of migration.
HMA makes use of this phenomenon to detect whether or not there is a mutation.

After the mutation has been induced in an individual, PCR has been conducted for the area in the vicinity of the deletion/insertion. The PCR product is denatured, then reannealed to form a heteroduplex product. Then, by checking the migration pattern of the sample using the MultiNA™, the presence of short deletions can be verified by means of the structural change, which would be difficult to determine solely by comparing differences in chain length.

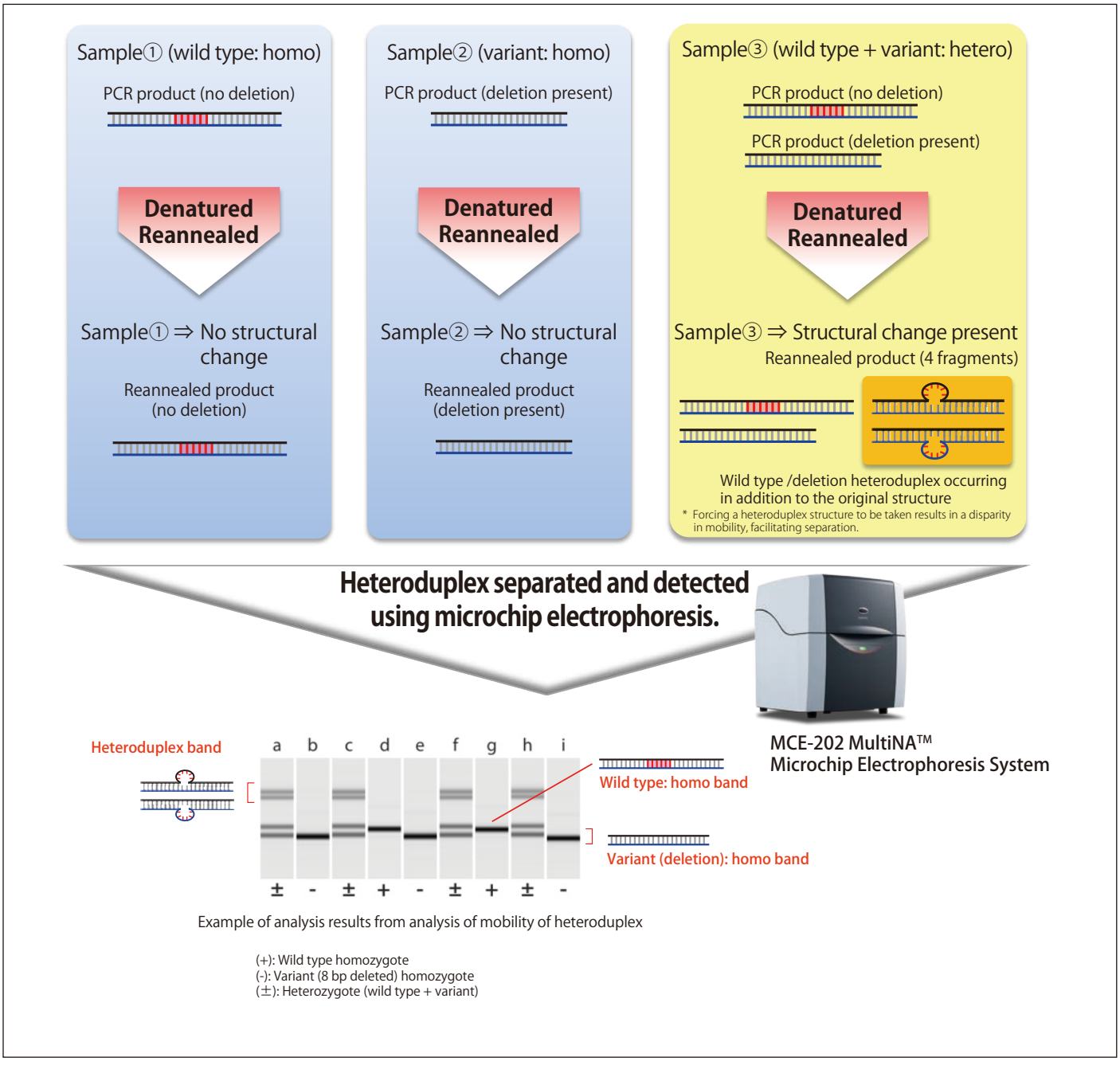
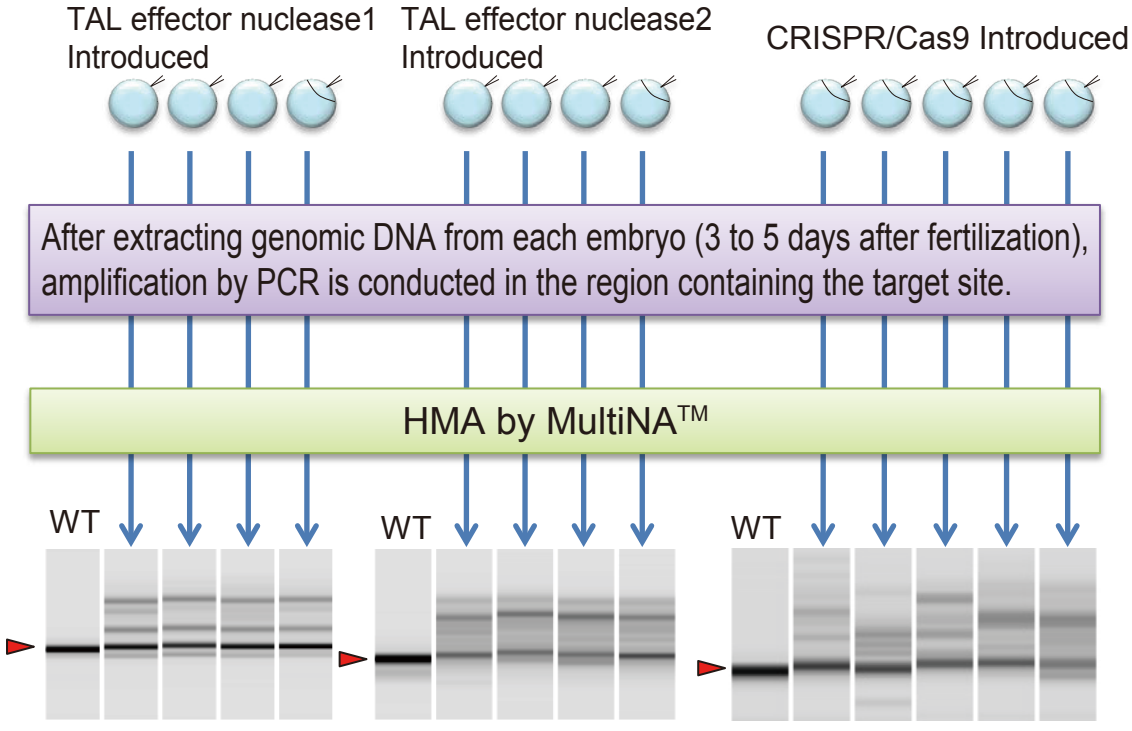


Fig. 3 HMA Principles and Analysis Procedures

4. Evaluation of Mutagenesis Activity in Fertilized Eggs of Medaka Where TAL effector nuclease or RGEN Were Introduced

TAL effector nuclease or RGEN is introduced by microinjection into fertilized eggs at the one-cell stage. After breaking the egg membrane of each embryo 3 to 5 days after fertilization by adding an alkaline solution (25 mM NaOH, 0.2 mM EDTA), the eggs are heated at 95 °C for 10 minutes. An equal volume of 40 mM Tris, pH 8.0 is added to them to neutralize the pH, and using them as templates. PCR amplification is conducted for fragments of 100 to 250 bases containing the target site. (With the MultiNA™, results with better resolution are obtained using PCR products with fewer than 250 bases.)

The PCR products are analyzed by the MultiNA™ using the DNA-500 kit. In the generation where TAL effector nuclease or RGEN were introduced, a wide range of PCR bands are seen due to the multiple types of mutations (in/del) introduced. Fig. 4 shows the results obtained using two different types of TAL effector nucleases that were produced for the same gene. When multiple bands are observed, that individual contains a mutation. Multiple bands were confirmed in all of the embryos into which TAL effector nuclease1 and TAL effector nuclease2 were introduced. However, regarding bands seen in the wild type (WT) (indicated by arrows in Fig. 4), those bands in the embryos in which TAL effector nuclease2 was introduced are thinner, and more numerous than those in which TAL effector nuclease1 was introduced. From this, it is clear that there is higher mutagenesis activity in the TAL effector nuclease2 individuals. In fact, after sub-cloning the PCR products from these embryos, it was revealed in confirmation of the base sequences, that mutations were introduced in 30 % of the embryos in which TAL effector nuclease1 was introduced, as opposed to 53 % of those in which TAL effector nuclease2 was introduced. Also, similarly as with TAL effector nuclease, this mutation evaluation can be conducted using the MultiNA™ when RGEN is used.



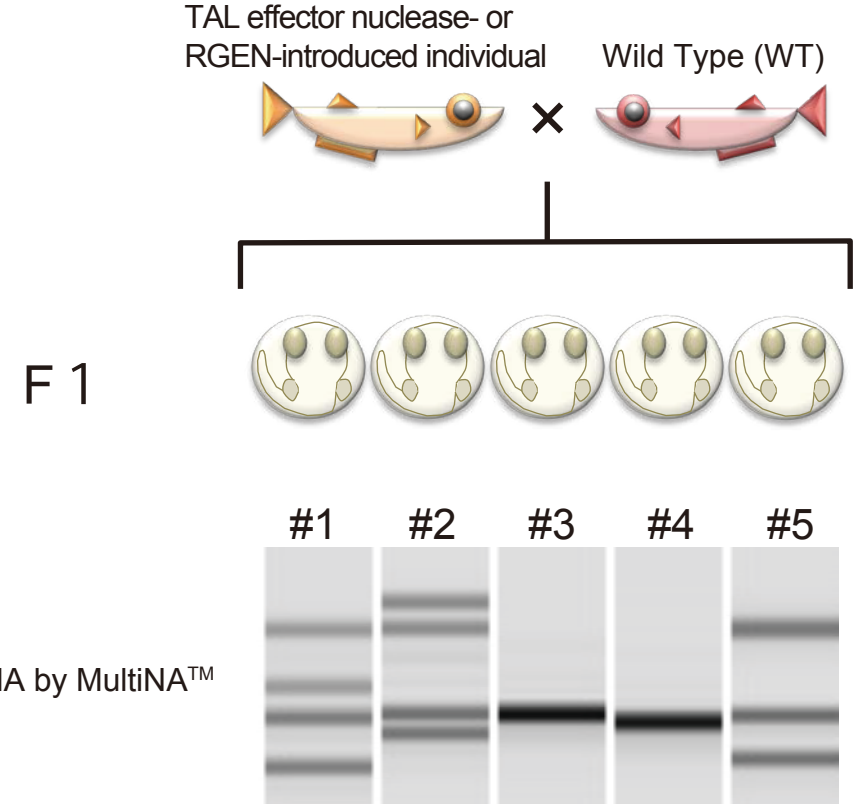
WT: Embryos in which neither TAL effector nuclease nor CRISPR/Cas9 was introduced

Genomic DNA was prepared from each of the medaka embryos in which different TAL effector nuclease and RGEN were introduced, and following PCR, HMA was conducted by the MultiNA™.

Fig. 4 Study of Mutagenesis Activity of TAL effector nuclease or RGEN

5. Selection of F0 Individuals with Germ Cell Mutations, and Study of Types of Mutation

Fertilized eggs in which mutagenesis activity due to the introduction of TAL effector nuclease or RGEN was confirmed were cultured, and the offspring were raised to adult fish. As a mosaic pattern is introduced in the F0 generation (but not introduced into all cells), we investigated whether or not mutations were introduced into germ cells of F0 individuals, and further, how such mutations may have been introduced.
As shown in Fig. 5, after the introduction of TAL effector nuclease or RGEN, a mature individual was crossed with a wild-type (WT) individual to obtain fertilized eggs (F1 generation). Using these embryos, the MultiNA™ was then applied to conduct HMA on the PCR products. If mutations are introduced into the germ cells of F0, multiple bands will be observed in the HMA since mutation is present in the hetero (one of the alleles) in the F1 generation that was crossed with the wild type. In the mutational analysis of F0, as multiple types of mutations are introduced into the same individual, many bands are observed. However in F1 individuals, the number of bands was limited because the mutation was limited to one type in a single individual, and further, a specific pattern is observed depending on each mutation. In Fig. 5, 4 types of band patterns are observed. As only one band is seen in each of #3 and #4, it can be determined that these are individuals in which the mutation was not introduced. On the other hand, multiple bands are observed in each of #1, #2 and #5, and further, their patterns vary depending on the embryo. Then, in the analysis of these nucleotide sequences, it was found that there were deletions of 15 bases, 7 bases and 14 bases, respectively. Thus, by conducting pattern analysis by HMA using the MultiNA™, it is possible to distinguish between the types of mutation in each individual.

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WT              CATACCCTCCAACAGGGAGTAAAACGCCTCAAGATCCCACCCAG
#1(del-15)      CATACCCTCCAAC---------------TCAAGATCCCACCCAG
#2(del-7)       CATACCCTCCAACAGGGAG-------CCTCAAGATCCCACCCAG
#5(del-14)      CATACCCTCCAACAGGGAG-------------ATCCCACCCAG
```

Fig. 5 Selection of F0 Individuals with Germ Cell Mutations, and Study of Types of Mutation

6. Selection and Mating of F1 Individuals Having the Same Mutation

As described above, it is possible to confirm the introduction of a mutation in F0 germ cells, so next, we bred F1 individuals, cut off each of their tail fins, placed each individual in a separate cup to permit their continued growth. We then extracted the genomic DNA from each tail fin, and conducted HMA using the MultiNA™ as described in the previous section (for embryos). Multiple band patterns are observed in the F1 individuals, as shown in Fig. 6. Excluding the wild types, these can be classified into 4 patterns, A, B, C and D. Among these, it is clear that the C pattern is the same band pattern of #2 of Fig. 5. That is, these are individuals with a deletion of 7 bases.

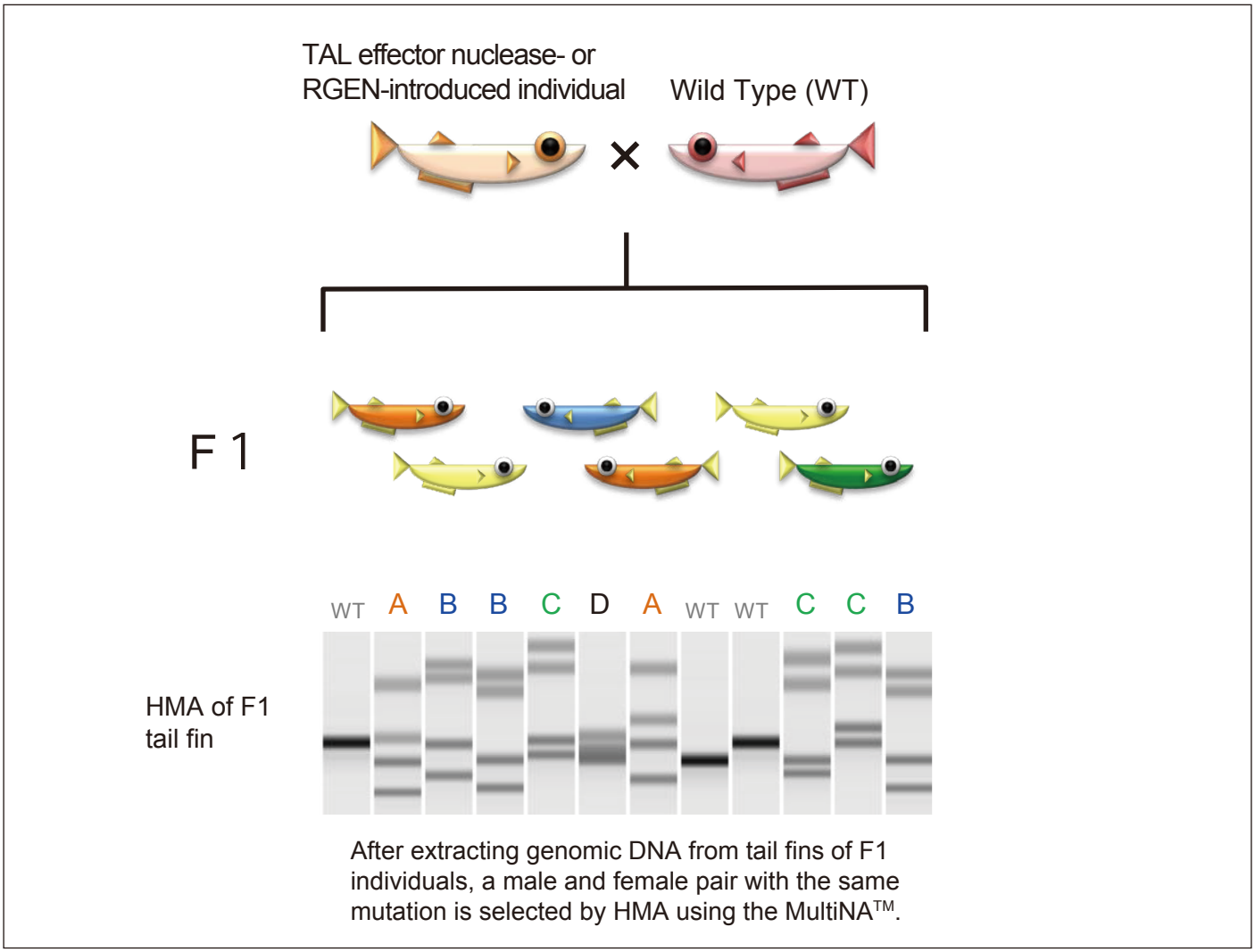

Fig. 6 Selection of F1 Individuals with the Same Mutation

7. Selection of F2 Individuals with Mutations in both Alleles and Establishment of Mutant Strains

To establish mutant strains, mating is conducted between male and female of the C pattern in Fig. 6. By crossing these F1 individuals, the F2 generation is expected to emerge with a ratio of 1:2:1 of wild type, heterozygous mutants, and homo mutants (KO individuals), respectively. First, HMA is conducted as described above (1st HMA).

Here, heterozygous mutants showed multiple bands so that they were easily distinguished from the wild type and homozygous mutants. On the other hand, the wild type and homozygous mutant each display only one band. When there are mutations involving relatively large insertion and deletion of bases, these bands can be distinguished according to size using the MultiNA™. In this case, since the variant is a 7-base deletion, the mobility is faster than that of wild-type band.

Therefore, lanes 3 and 6 can be assumed to be homozygous mutants, and lanes 2, 7 and 8, wild-type. However, in cases where the degree of variation is small, or there is an insertion or deletion of only one base, it can be difficult to judge which is the homo mutant. In such a case, or when a more reliable distinction between a wild-type and homozygous mutant is required, second HMA may be conducted as follows.

To a PCR product showing a single band pattern, add a PCR product separately prepared from a wild type. Then heat at 95 °C for 5 minutes, gently return to room temperature, and re-anneal. If HMA (2nd HMA) is conducted using the MultiNA™, whereas the wild type (lane 2', 7', 8') shows a single band, the homo mutant (lane 3', 6') shows multiple bands for the newly formed heteroduplex. Thus, conducting this 2nd HMA permits a clear distinction between the wild type and the homozygous mutant.

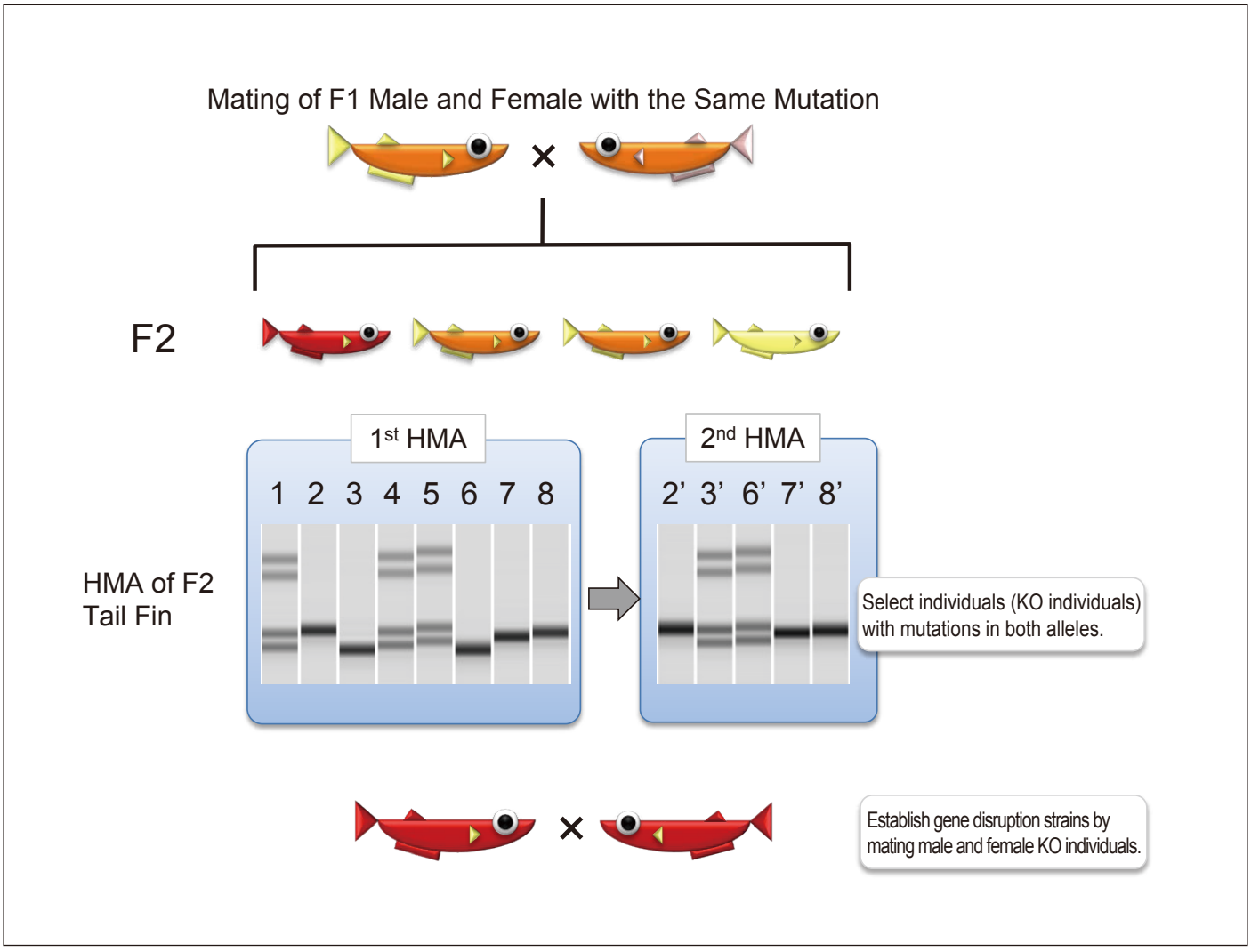

Fig. 7 Establishment of KO Strains by Selection and Mating of F2 Individuals with Mutations in both Alleles

8. Conclusion

Gene targeting using TAL effector nuclease or RGEN can be applied to many types of organisms, including both animal and vegetable. However, finding variants of interest from among TAL effector nuclease- or RGEN-treated targets requires time and effort. By using the MultiNA™ as introduced here, mutants can be obtained quickly and efficiently with greatly reduced effort.
When using the MultiNA™, excellent results can be obtained using PCR products that are designed to be smaller than 250 bases.
When the size of PCR products becomes large, heteroduplex resolution decreases, making it difficult to distinguish between mobility changes originating from different mutations.

References

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7) Ansai S and Kinoshita M. (2014) Targeted mutagenesis using CRISPR/Cas system in medaka. Biology Open, 3, 362-371.

MCE-202 MultiNA™ Microchip Electrophoresis System

Agarose gel electrophoresis is a method which involves a long series of operations, including the formulation of reagents, gel preparation, electrophoresis, and acquisition of the imaged result, not to mention cleanup following the analysis. Further, sensitivity, separation, repeatability, as well as objectivity are all lacking to some degree.

The MCE-202 MultiNA™ Microchip Electrophoresis System resolves all of the shortcomings of agarose gel electrophoresis.

MCE-202 MultiNA™ Microchip Electrophoresis System

Excellent Analytical Performance

Microchip electrophoresis by the MultiNA™ achieves enhanced separation, sensitivity, reproducibility and quantitative accuracy compared with agarose gel electrophoresis.

Automated Analysis of 1 to 108 Samples

Up to 108 samples can be analyzed automatically, and though parallel processing with an analysis time of just 80 seconds per sample including pretreatment. *1

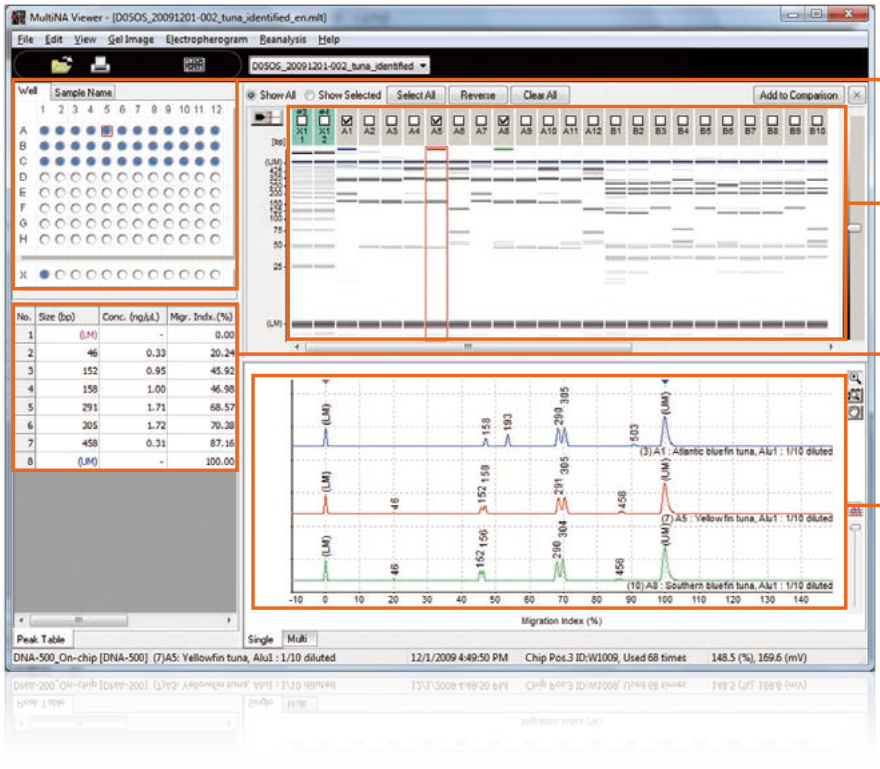
Begin Analysis in Just Three Steps

Extremely simple operation. Once the analysis schedule has been created, simply load the samples and reagents and click the Start button

Reducing analysis cost

The low running cost is achieved using the high-performance microchip which is used for multiple samples

*1 Analysis time using four microchips in parallel with the DNA-1000 kit, excluding initial washing to start the run.



Displaying Analysis Results in the MultiNA Viewer™

Sample Well Display

Displays the analysis progress status in different colors.

Gel Image

Can be saved as image data (jpg, bmp, tif).

Peak Table

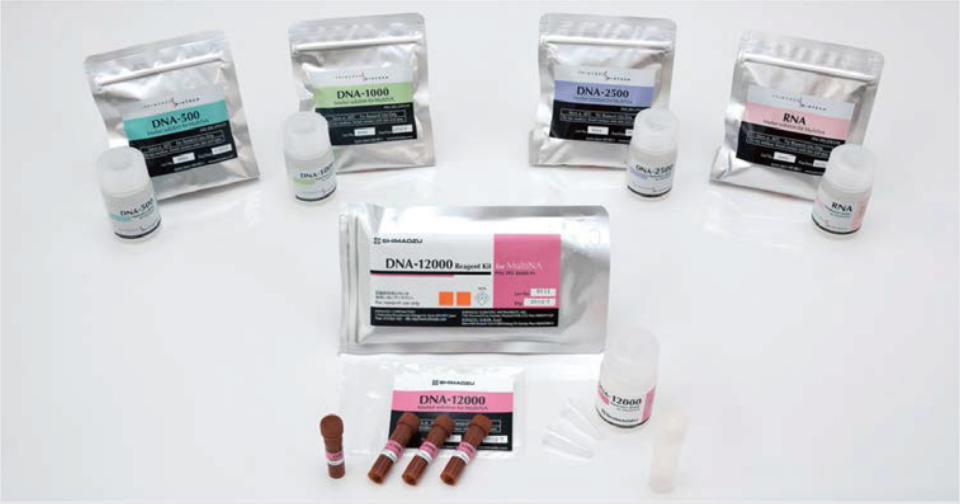
Predicted size values and concentrations can be saved to a csv file.

Electropherogram

Can be saved as image data (jpg, bmp, tif).

■MultiNA™ Reagent Kits

Reagent kits are designed to work optimally for different size ranges and sample types.

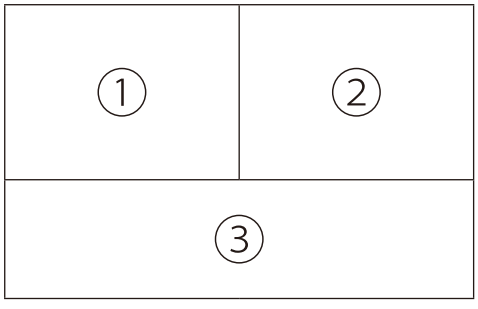


■Microchip

The microchip is common to all reagent kits.

■RA Chip Cleaning Kit

Fluorescent dye and reagent components can be adsorbed onto the wall of the microchip flow channel, thus reducing the separation performance and lowering the number of reuses. Cleaning of the microchip using the CHIP CLEANING KIT-RA eliminates the adsorbed components and improves (or restores) the separation performance of the microchip.



Cover picture

1) 5-day embryo of wild-type black medaka
2) This shows the F0 embryos of transgenic medaka in which the causative gene is destroyed using TAL effector nuclease. The black-colored pigment deletion condition varies depending on the individual as a result of mosaic pattern introduction in the F0 generation.
3) The embryos in (1) and (2) have grown up to become these individuals. The black one is the wild type (TAL effector nuclease not applied). As for the white one, since the causative gene (slc45a2) was almost completely destroyed due to TAL effector nuclease (melanophores not formed), it became the color of the transgenic medaka.

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