A Simple, Robust, and Cost-effective Method for Genotyping Small-scale Mutations

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Abstract

Background and objectives: Genotyping is an important tool for studying gene functions in animals or detecting genetic variants in humans. Various methods using low to high concentrations of agarose or polyacrylamide gel electrophoresis have been developed for genotyping. These methods rely on the detection of large-size differences (20–2,000 bp) of targeted PCR products between a wild-type gene and a mutant gene. Endonuclease digestion was introduced to identify heterozygous mutations, but it was not possible to differentiate the wild-type from the homozygous mutants with the same or similar size. This study thus developed a novel, simple, and reliable test for genotyping animals or cells following genetic modifications.

Methods: We developed an improved and simple method that used 2% agarose gel electrophoresis following T7E1 or Surveyor endonuclease digestion to firstly separate the heterozygous mutations from the wild-type or homozygous mutations. By adding a wild-type PCR product to a potentially homozygous product, which would form heteroduplexes, we could then separate the wild-type from a homozygous mutation with a nearly identical size or only a single base pair substitution without Sanger sequencing.

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Results: We verified this method in genotyping zebrafish mutants with a 2-8-bp deletion or insertion and mouse mutants with a 1- or 8-bp substitution. The wild-type, heterozygous, and homozygous mutations ranged 1–8 bp were clearly differentiated on agarose gel. Sanger sequencing also confirmed our genotyping results.

Conclusions: This novel and improved genotyping method may have a broad application in many clinical and research laboratories for rapid and economical genotyping of patients and animals with a small area deletion or single base pair substitution, particularly in the era of gene editing or in those with naturally occurring mutations.

Introduction

Genotyping is an important and essential process for conducting the research of genes and gene variants associated with human diseases. Polymerase chain reaction (PCR) coupled with agarose[1-3] or SDS-polyacrylamide gel electrophoresis (PAGE)[4] has been extensively used for genotyping single genes with a large deletion or insertion of nucleotides ranging from 20 to 2,000 bp.[1-3] However, due to the limited resolution of the gel electrophoresis-based methods, it might be difficult to determine the genotype by simply relying on the size difference of the targeted PCR products between the wild-type and mutant genes if the area of change in the nucleotide is small.[5] As such, a high-resolution melting curve analysis would allow for discrimination between the wild-type and heterozygous genotypes based on the fluorescence changes during the DNA melting phase after the PCR.[6-7] This method requires a strict primer design as 100-bp amplicons are optimal for the detection of a nuclease-induced small insertion or deletion.[7,8] However, this method may not appear to be sensitive enough to differentiate between the wild-type and small-scale homozygous mutations given that the melting curves would be very close to each other.[8] For a single or two base pair substitution, deletion, and insertion, it is nearly impossible to know the genotype without sequencing. Therefore, Sanger sequencing is the gold standard method for identifying such small area mutations. Nevertheless, it would still require purification of high-quality DNA, which would be time-consuming and costly for a large number of samples. Thus, there is an unmet need for a simple, rapid, reliable, and low-cost method for genotyping animals in a routine research laboratory.

T7 endonuclease I (T7E1)[9-11] and Surveyor[12,13] endonuclease recognize and cleave heteroduplexes with more than two base pairs and single nucleotide mismatch, respectively. The introduction of the endonuclease digestion has allowed for separation of a heterozygous mutant from a wild-type or homozygous mutant genotype as a result of the heteroduplex formation in amplified PCR products after denaturation and reannealing. However, the endonuclease digestion alone does not distinguish a wild-type genotype from a homozygous mutation because the amplified PCR product of either genotype does not form the heteroduplex that is required for endonuclease cleavage.

Here, we describe a new, simple, robust, and low-cost method using routine agarose gel electrophoresis of the PCR products following T7E1 or Surveyor nuclease digestion for genotyping animals with a small-scale insertion, deletion, or substitution in the targeted sequence, which is in many cases difficult to differentiate from the original wild-type sequence. Such a method may be widely used in many research laboratories for genotyping animals.

Materials and methods

Genomic DNA extraction and PCR

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (US). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kansas Medical Center, Kansas City, KS, USA. Tissue samples were obtained from the animals to be genotyped and wild-type controls. Tail fin cuttings and tail clippings were obtained from zebrafish and mice, respectively. Genomic DNA was extracted using a cell lysis buffer containing protease plus (Biotool, Jupiter, FL, USA). PCR was performed using Invitrogen Platinum™ II Hot-Start Green PCR Master Mix (2x) for 20-μL reactions with gene specific primers (Table 1). The PCR program was as follows: A temperature of 95°C for 4 m for initial denaturation, then 35 cycles at 95°C for 30 s for denaturation, 57°C for 30 s for annealing, 72°C for 30 s for extension, and the final extension was conducted at 72°C for 10 m.

Heteroduplex formation and digestion

The PCR product (10 μL) of each sample was then denatured and reannealed with or without addition of the wild type PCR product using a PTC-200 Peltier Thermal Cycler following the heating cycle: 95°C for 2 m, −2°C/s to 85°C, and −0.1°C/s to 25°C. The resulting products were digested using T7E1 (New England BioLabs, Ipswich, MA, USA) for mutations 2bp or using a Surveyor (Integrated DNA Technologies, Coralville, IA, USA) for a single point mutation.

Agarose gel electrophoresis

After digestion, the samples were resolved via 2% agarose gel electrophoresis. Eight μL of each sample was loaded into the gel and run in 1x Tris-acetate-EDTA (TAE) running buffer.

Sanger sequencing

For verification of this genotyping method, Sanger sequencing of the targeting PCR products was also performed in McLab (South San Francisco, CA, USA).

Results

The new assay design

Figure 1 outlines a general protocol for the new genotyping method. The first step was to amplify the targeted sequences using a routine PCR from genomic DNA isolated from animals. The PCR products were denatured and annealed in the absence or presence of a

wild-type PCR product and digested with T7E1 or Surveyor endonuclease. The digested materials were then separated using 2% agarose gel stained with GelRed nucleic acid staining dye (Fisher Scientific, Hampton, NH< USA). As predicted, in the absence of an added wild-type PCR product, it was easy to identify the heterozygous animal from the wild-type and homozygous mutant animals because the heteroduplexes that formed in the PCR products of the heterozygous samples were efficiently cleaved by T7E1 or Surveyor endonuclease; in the presence of an added wild-type sequence, the homozygous mutant animals could be easily separated from the wild-type animals as the added wild-type sequence resulted in the formation of heteroduplexes with the homozygous mutant sequence, but not with its own wild-type sequence that would be sensitive to proteolysis by endonuclease.

Identification of an 8-bp deletion mutation in adamts13 using agarose gel electrophoresis following T7E1 digestion

To begin assessing the utility of this new genotyping strategy, we began with genotyping zebrafish containing an 8-bp deletion mutation in zebrafish *adamts13* (a disintegrin and metalloprotease with thrombospondin type 1 repeat, 13) (Fig. 2a).[8] Following the PCR amplification, T7E1 digestion with or without addition of a wild-type PCR product resulted in two cutting bands (~230 bp and ~310 bp) in the heterozygous mutant products, but not in the wild-type and homozygous mutant products (Fig. 2b). The addition of a wild-type PCR product to the potential homozygous nutant sequence resulted in efficient cleavage of the artificial heteroduplexes formed between the added wild-type sequence and the homozygous mutant sequence (but not in the wild-type samples) (Fig. 2c). Sanger sequencing confirmed the accuracy of the wild-type (Fig. 2d) and mutant (Fig. 2e) sequences of zebrafish *adamts13*. These results demonstrated that the new method could quickly and accurately identify all three genotypes in animals with an eight-nucleotide deletion.

Identification of a 2-bp deletion mutation in ankrd26 using agarose gel electrophoresis following the T7E1 digestion

To further confirm the robustness of this new assay, we took advantage of another existing zebrafish line in the lab with a 2-bp deletion in a gene encoding ankyrin repeat domain-containing protein-26 (*ankrd26*) (Zheng *et al.*, 2021a, unpublished) (Fig. 3a). Following the heterozygous-to-heterozygous breeding, we anticipated to generate wild-type, heterozygous, and homozygous mutant animals. As shown, the heterozygous animals were easily differentiated from the wild-type or homozygous mutant animals as there were two resulting cutting bands in the PCR products of the heterozygous animals following the T7E1 endonuclease digestion (Fig. 3b). However, there was no difference with regard to the size of the PCR products between the wild-type and homozygous mutant sequence and no resulting cutting band observed in those PCR samples following the T7E1 digestion. Again, we added a wild-type PCR product to either a prospective wild-type or homozygous mutant PCR product to create an artificial heterozygous PCR product. These artificial heteroduplexes became sensitive to digestion by the T7E1 endonuclease (Fig. 3c). This provided an easy and reliable method to separate the wild-type from the homozygous mutant animals. All the genotyping (delGC) results based on this new method were also confirmed by Sanger

sequencing with 100% accuracy (Figs. 3d, e). These results suggest that the new method allows quick and accurate genotyping animals with two nucleotide deletion.

Identification of a 7-bp insertion mutation in vwf using agarose gel electrophoresis following the T7E1 digestion

We created *a* zebrafish line with a 7-bp insertion in *vwf* (Von Willebrand facor) (Fig. 4a), resulting in *vwf*^{-/-} phenotype.[8] As shown, two cut bands (~210 bp and ~350 bp) were detected following the T7E1 digestion in the PCR products from the heterozygous animals with a 7-bp insertion in *vwf* (Fig. 4b). Again, addition of a wild-type PCR product to a potential homozygous mutant PCR product resulted in an expected cut pattern of the artificial heteroduplexes (Fig. 4c). Sanger sequencing confirmed 100% accuracy of the genotyping results with the new method (Fig. 4d, e). These results suggest that the new method is capable of discriminating between three genotypes in animals with a seven-nucleotide insertion.

Identification of the substitution mutations of dot1l using agarose gel following the T7E1 nuclease digestion

We further explored the possibility that this genotyping method could discriminate substitution mutations of the same length. A scattered 8-bp substitution mutation in a disruptor of telomeric silencing 1-like *(dot1l)* was created in a mouse (Fig. 5a) (Rumi *et al.*, unpublished data). Following the T7E1 digestion of the targeted gene PCR products, we could clearly distinguish the heterozygous mutant from the wild-type and homozygous mutant samples based on two expected cutting bands (~205 bp and ~405 bp) in the heterozygous samples on the agarose gel (Fig. 5b). The same digestion pattern was found in the potential homozygous mutant samples following the addition of a wild-type PCR product (Fig. 5c). Again, Sanger sequencing confirmed the 8-bp scattered substitution in 100% of the samples genotyped for the targeted *Dot1l* mutation using our new method (Fig. 5d, e). These results demonstrated that the new method could easily identify all three genotypes in animals with the eight scattered nucleotide substitution.

Identification of a single base pair substitution mutation of cfh using agarose gel following the Surveyor nuclease digestion

Surveyor nuclease cleaves any site with a mismatched base pair with high efficiency.[12] To assess if the new method could genotype animals with a single nucleotide substitution, we used Surveyor nuclease as a digestion enzyme for the PCR product of the murine complement factor H (*cfh*) (Fig. 6a).[14,15] As shown, two faint, but distinct cut bands of ~200 bp and ~350 bp were detected after the Surveyor nuclease digestion in three PCR samples (Fig. 6b), thus indicating the heterozygous genotype of these animals. Similarly, addition of a wild type PCR product to a potentially homozygous *cfh* mutant resulted in the cleavage of the heteroduplexes by the Surveyor protease (Fig. 6c). Again, all samples with a specific mutation identified by our new method were verified by Sanger sequencing with 100% concordance (Fig. 6d, e). These results indicated that our new genotyping method was able to discriminate between all three genotypes in animals with a single nucleotide substitution without the need for Sanger sequencing.

Discussion

A variety of genotyping methods have been described in the past, including agarose gel electrophoresis, SDS-PAGE, melting curve analysis, enzyme digestion, and Sanger sequencing, etc.[9,15-17] These methods have intrinsic limitations in either lacking sensitivity or costing too much. Zhu *et al.* described a method using 15% polyacrylamide gel for detecting the digestion fragment by the T7E1 endonuclease.[18] Even with this high-resolution gel, this method could not differentiate the wild type from a homozygous mutant with a small deletion. Bhattacharya and Meir also described a method using 4–6% agarose (or high concentration) gel electrophoresis for genotyping mice with a deletion of greater than two base pairs following the T7E1 endonuclease digestion.[4] Their high concentration agarose gel electrophoresis took too long to run and generated excessive heat that resulted in the diffusion of the PCR products into the gel and inability to accurately genotype the animals with nucleotide substitutions.

Here, we describe a novel, robust, and cost-effective method for genotyping animals or cells using routine 2% agarose gel electrophoresis and T7E1 or Surveyor endonuclease digestion. We could detect the difference between the heterozygous from the wild-type and homozygous mice and zebrafish with a small deletion, insertion, or substitution. Upon spiking the wild-type PCR product, we could reliably differentiate the sequence between the wild-type and the homozygous mutant. The addition of the wild-type sequence to the homozygous mutant sequence to create artificial heteroduplex is sensitive to be cleaved by the T7E1 endonuclease.

However, the detection of a sequence with only one substitution remains to be a challenge. While Surveyor nuclease has shown to be able to cleave the heteroduplexes formed between a wild-type sequence and those with only one nucleotide substitution, its cleavage efficiency would still remain limited.[13] In our preliminary result, the cleavage efficiency of a single point mutation of *cfh* by Surveyor nuclease was much less than that with an indel mutation by T7E1 with only the faint cleavage band visible. Therefore, further optimization of Surveyor nuclease or experimental conditions would be needed to develop more robust genotyping methods for detecting the difference of a gene with a single point mutation.

Conclusions

We developed a rapid, cost-effective, and highly accurate genotyping method using the normal agarose gel electrophoresis following the T7E1 or Surveyor nuclease digestion, with and without the added wild-type sequence. This new method would significantly accelerate the routine molecular biology laboratory to reliably genotype animals and cells with various gene mutations at a record speed at a minimal cost.

Funding

Data sharing statement

The technical appendix, gene sequences, and dataset used in support of the findings of this study are included within the article.

Abbreviations:

ADAMTS13	A disintegrin and metalloprotease with thrombospondin type 1 repeat 13
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
T7E1	T7 endonuclease I
TAE	Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer

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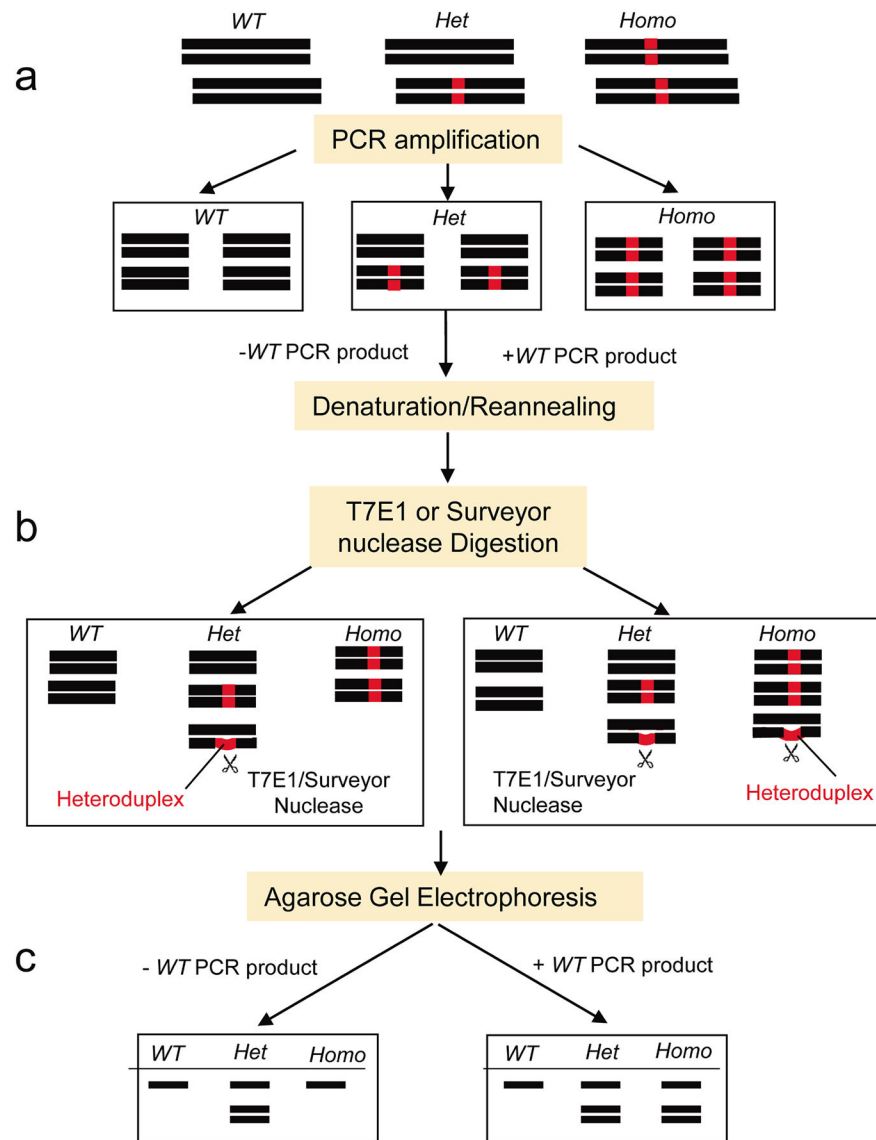
Fig. 1. An outlined protocol for the new genotyping method.
Genomic DNA was released from the samples following tissue lysis. The cell lysate was then directly used for the PCR to amplify the target gene region. The PCR product was split into two aliquots. In one aliquot, a wild-type (*WT*) control PCR product was added; in another aliquot, no *WT* control PCR product was added. The PCR products were then denatured at 95°C and reannealed. Heteroduplexes of the DNA fragments were expected to form in the heterozygous (*Het*) mutant products in this step. However, when mixed with a *WT* control PCR product, the artificial heteroduplexes of DNA were also expected to form in the homozygous (*Homo*) mutant samples, but not in the wild-type samples. T7 endonuclease I (T7E1) recognized and cleaved the heteroduplexes with a mismatch 2 base pairs but the Surveyor nuclease could cleave a single base mismatch and indels up to at least 12 nucleotides. Thus, we could clearly differentiate the *WT*, *Het*, and *Homo* animals with various sizes of mutations based on the cleaved products revealed on 2% agarose gel electrophoresis.
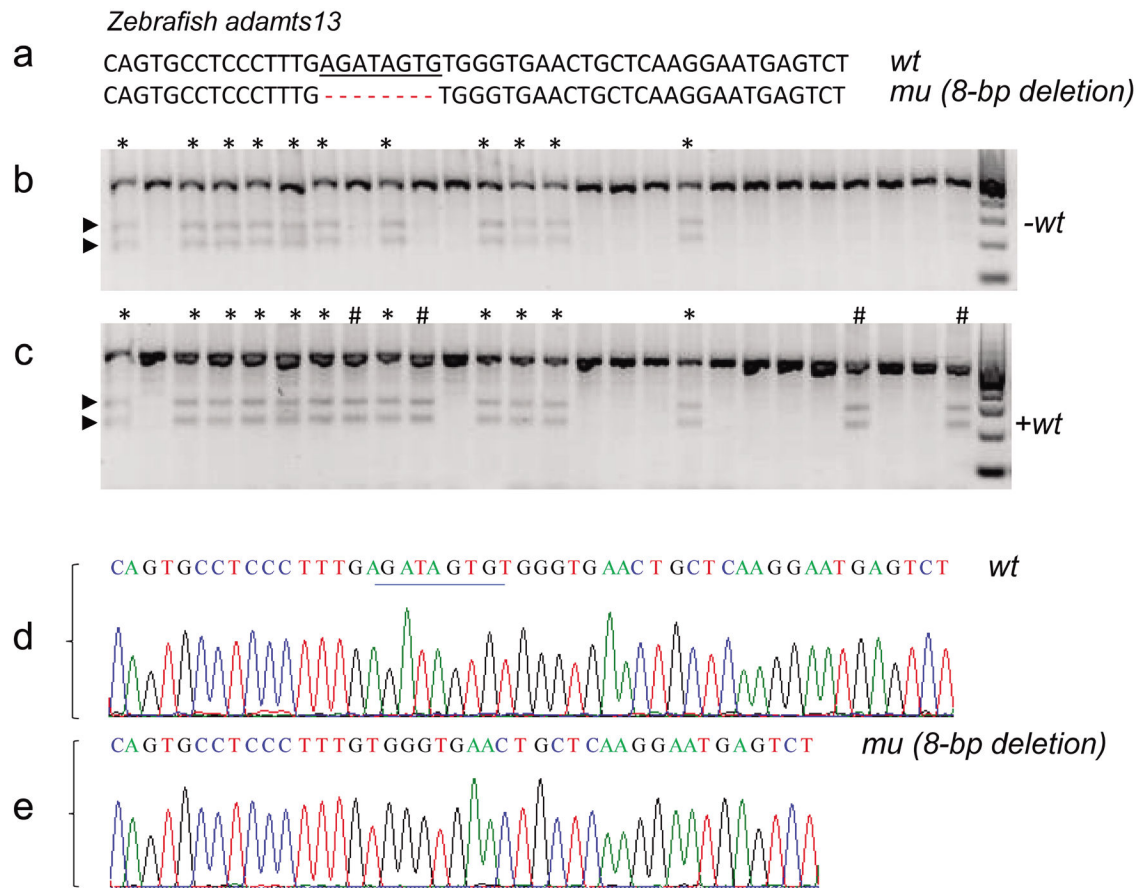
Fig. 2. Genotyping *wild-type*, heterozygous, and homozygous 8 bp-deletion mutation in zebrafish *adamts13*.

(a) The expected wild type (*wt*) and mutant (*mu*) (8-bp deletion) sequences of zebrafish adamts13. (b–c) are the T7E1 digestion patterns of the PCR products in the absence (−*wt*) and presence (+*wt*) of the *wt* PCR product, respectively. Here, * and # indicate the presence of the cleavage bands (arrowheads) in the absence and presence of a *wt* sequence, respectively. (d–e) show the Sanger sequence tracings of *wt* and *mu* (−8 bp), respectively.
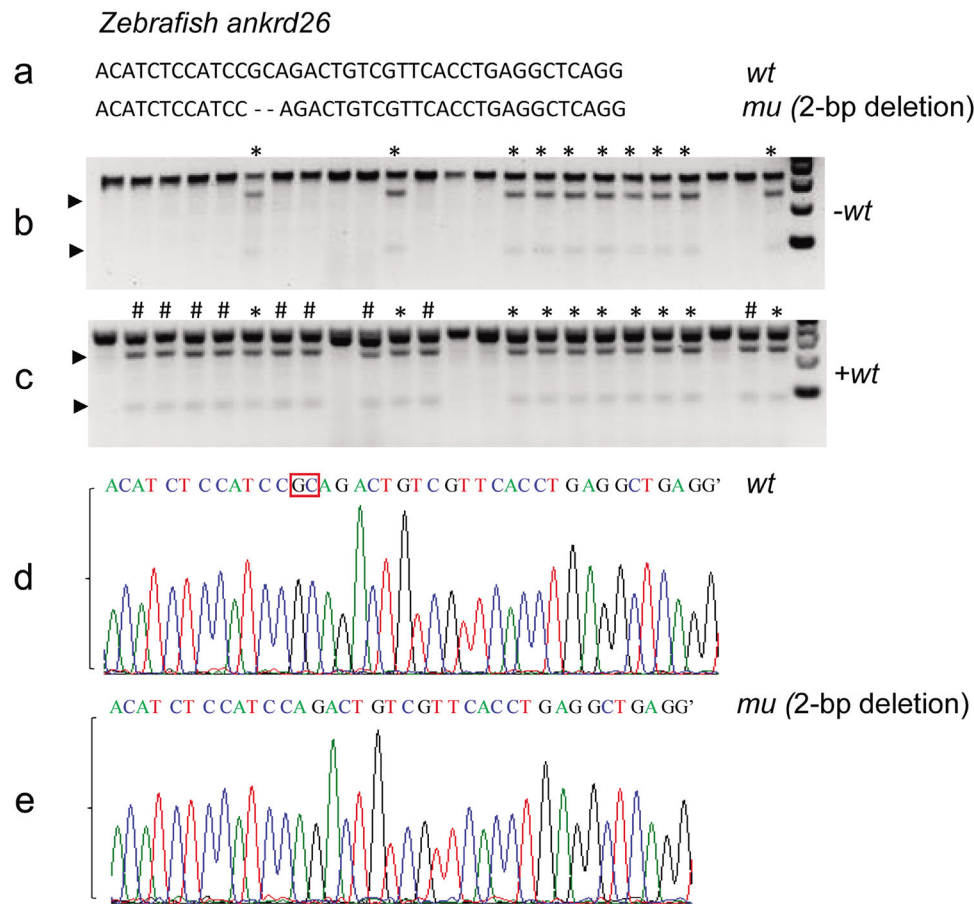
Fig. 3. Genotyping wild-type, heterozygous, and homozygous zebrafish with a 2 bp-deletion in *ankrd26*.

(a) The expected DNA sequences of the wild-type (*wt*) and mutant (*mu*) (i.e., 2 bp-deletion) ankrd26. (b–c) The T7E1 digestion patterns of the PCR products in the absence and presence of a *wt* PCR product, respectively. Here, * and # indicate the presence of the cleavage bands (arrowheads) in the absence (−) and the presence (+) of a *wt* PCR sequence, respectively. (d–e) Sanger sequencing results confirmed the genotyping results (−2 bp) revealed by the agarose gel electrophoresis following T7E1 digestion.
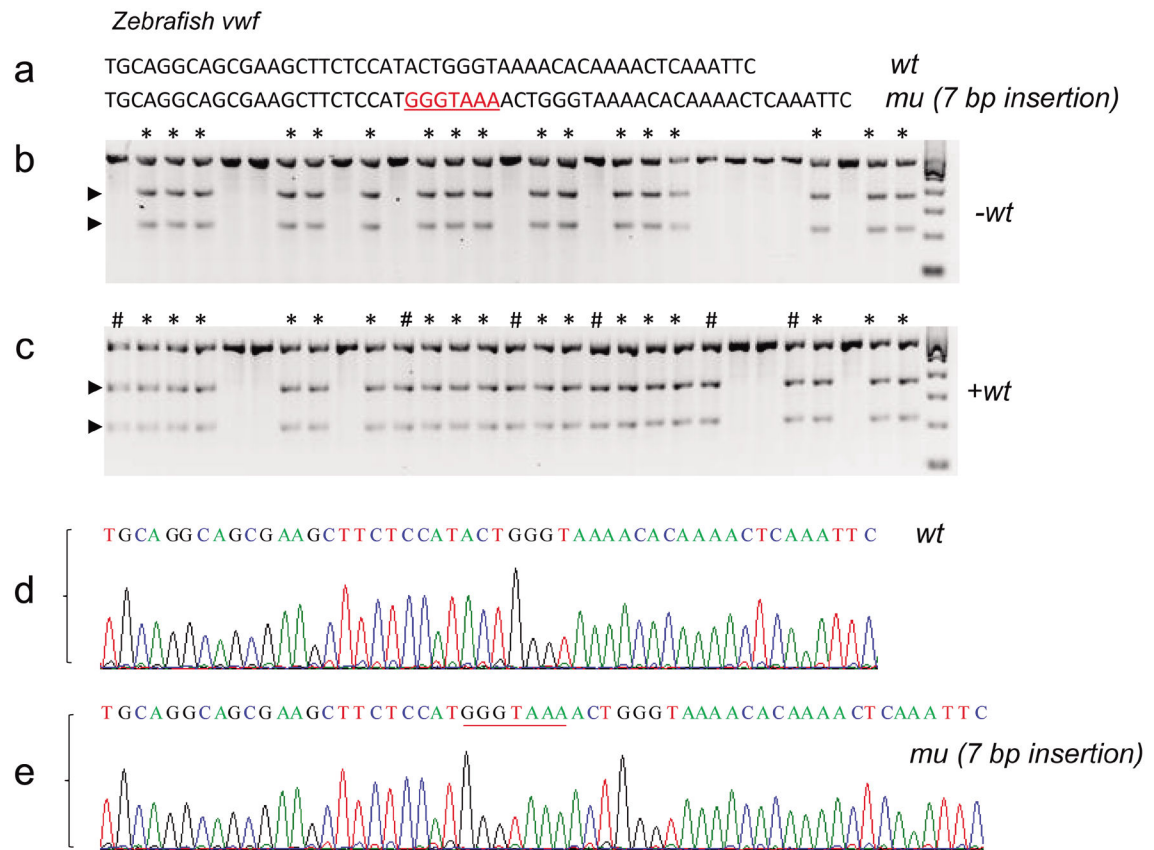
a

Zebrafish vwf

TGCAGGCAGCGAAGCTTCTCCATACTGGGTAAAACACAAAACTCAAATTC   *wt*

TGCAGGCAGCGAAGCTTCTCCAT<u>GGGTAAA</u>ACTGGGTAAAACACAAAACTCAAATTC   *mu (7 bp insertion)*

b

-wt

c

+wt

d
TGCAGGCAGCGAAGCTTCTCCATACTGGGTAAAACACAAAACTCAAATTC   *wt*

e
TGCAGGCAGCGAAGCTTCTCCAT<u>GGGTAAA</u>ACTGGGTAAAACACAAAACTCAAATTC   *mu (7 bp insertion)*

Fig. 4. Genotyping wild-type, heterozygous and homozygous zebrafish with 7 bp-insertion in *vwf*.
(a) The expected DNA sequences of the wild-type (wt) and mutant (mu) (*i.e.*, 7 bp-insertion) in zebrafish von Willebrand factor (*vwf*). (b–c) are the T7E1 digestion patterns of the PCR products in the absence (−) and the presence (+) of a wt PCR product, respectively. Here, * and # indicate the presence of the cleavage bands (arrowheads) in the absence and presence of a wt PCR sequence, respectively. (d–e) Sanger sequencing results confirmed the genotyping results (+7 bp-insertion) revealed by the agarose gel electrophoresis following the T7E1 digestion.

Fig. 5. Genotyping wild-type, heterozygous, and homozygous mice with 8 bp-substitution in _dot1l_.

(a) The expected DNA sequences of the wild-type (_wt_) and mutant (_mu_) (i.e., 8 bp-substitution) in _dot1l_. (b–c) The T7E1 digestion patterns of the PCR products in the absence (−) and the presence (+) of a _wt_ PCR product, respectively. Here, * and # indicate the presence of the cleavage bands (arrowheads) in the absence and presence of a _wt_ PCR sequence, respectively. (d–e) Sanger sequencing of the PCR products confirmed the genotyping results (i.e., 8 bp- substitution) above.
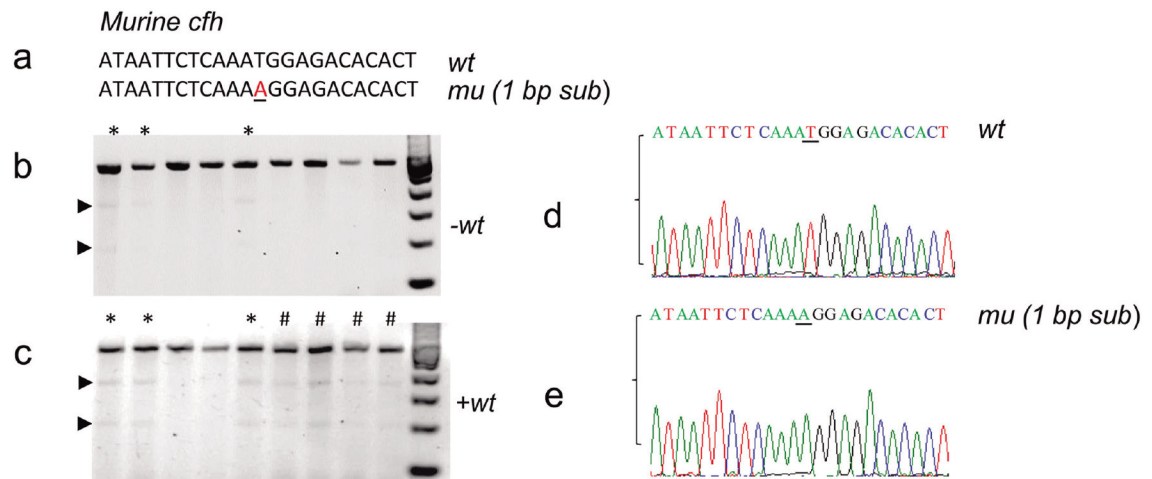
Fig. 6. Genotyping wild-type, heterozygous, and homozygous mice with a single nucleotide substitution in _cfh_.
(a) The expected DNA sequences of the wild-type (_wt_) and mutant (_mu_) murine _cfh_. (b–c) The Surveyor nuclease digestion patterns of various PCR products in the absence (−) and the presence (+) of a _wt_ sequence, respectively. Here, * and # indicate the presence of the cleavage bands (arrowheads) in the absence (i.e., heterozygous) and the presence (i.e., homozygous) of a _wt_ PCR sequence, respectively. (d–e) Sanger sequencing confirmed the _wt_ and _mu_ (one nucleotide substitution) sequences revealed above.

Table 1.

Primers used for the PCR amplification

Gene name	Gene symbol	Primer sequences
Ankyrin repeat domain protein, 26	*ankrd26*	Forward: TTATTCTTAGAGAATGGGCG Reverse: CCTTATCCAGCTGGTTTAAA
A disintegrin-like metalloproteinase with thrombospondin motif type 1 member, 13	*adamts13*	Forward: AGATTCACTGAAACCTCAAG Reverse: CAGCTGCTCGCAACACATATC
von Willebrand factor	*vwf*	Forward: GCTTTGGTCTACACTAGATG Reverse: TAAATACAGCAAGATGTGCATG
Disruptor of telomeric silencing 1-like	*dot1l*	Forward: CTGTGGGGCAGTTTGTACCT Reverse: CTCCACAAGGGACAGCATGT
Complement factor H	*cfh*	Forward: ATTGACCAGCTACAGACAGTATCA Reverse: CATGCATGTGCCTTTCTAAAACA