



Article Detection of Indiscriminate Genetic Manipulation in Thoroughbred Racehorses by Targeted Resequencing for Gene-Doping Control

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Abstract: The creation of genetically modified horses is prohibited in horse racing as it falls under the banner of gene doping. In this study, we developed a test to detect gene editing based on amplicon sequencing using next-generation sequencing (NGS). We designed 1012 amplicons to target 52 genes (481 exons) and 147 single-nucleotide variants (SNVs). NGS analyses showed that 97.7% of the targeted exons were sequenced to sufficient coverage (depth > 50) for calling variants. The targets of artificial editing were defined as homozygous alternative (HomoALT) and compound heterozygous alternative (ALT1/ALT2) insertion/deletion (INDEL) mutations in this study. Four models of gene editing (three homoALT with 1-bp insertions, one REF/ALT with 77-bp deletion) were constructed by editing the myostatin gene in horse fibroblasts using CRISPR/Cas9. The edited cells and 101 samples from thoroughbred horses were screened using the developed test, which was capable of identifying the three homoALT cells containing 1-bp insertions. Furthermore, 147 SNVs were investigated for their utility in confirming biological parentage. Of these, 120 SNVs were amenable to consistent and accurate genotyping. Surrogate (nonbiological) dams were excluded by 9.8 SNVs on average, indicating that the 120 SNV could be used to detect foals that have been produced by somatic cloning or embryo transfer, two practices that are prohibited in thoroughbred racing and breeding. These results indicate that gene-editing tests that include variant calling and SNV genotyping are useful to identify genetically modified racehorses.

Keywords: amplicon sequencing; gene doping; gene editing; horse; thoroughbred

1. Introduction

Gene doping is a prohibited practice in both human and horse sports to maintain integrity [1]. The global leader of thoroughbred racing, the International Federation of Horseracing Authorities (IFHA, https://www.ifhaonline.org/, accessed on 1 September 2022), prohibits the administration of genetic materials including transgenes, therapeutic oligonucleotides, and genetically modified cells to horses; further, the IFHA and the International Stud Book Committee (ISBC, https://www.internationalstudbook.com/, accessed on 1 September 2022) prohibit the creation of genetically engineered racehorses. Genetically engineered animals (including embryos) have been recently created in many species, including horses [2,3].



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). The methods for creating genetically modified animals include the introduction of an exogenous gene into the host genome using transposons [4], retroviruses (including lentiviruses) [5], cell-mediated transgenesis [6], and gene editing (homologous recombination [HR] of CRISPR/Cas9) [7,8]. Gene insertion is used in gene therapy to supplement the functions of defective genes [9]. Alternatively, CRISPR/Cas9 can be used to specifically introduce short insertions or deletions (INDELs) to targeted regions via nonhomologous end joining (NHEJ) [10,11]. This facilitates gene knockdown or knockout by introducing a frameshift into the targeted gene.

Pathological models of animals using gene-editing techniques to study diseases and livestock with modified genes related to economic traits have also been developed [12,13]. Gene-editing techniques make it theoretically possible to easily perform illegal gene doping to produce genetically modified racehorses, which pose a threat to the integrity of both the horse racing industry and equestrian sports.

Many methods to detect inserted transgenes using quantitative PCR have been developed for gene-doping control [14–23]. By designing hydrolysis probes targeting the exon/exon junction, transgenes can be detected in a target-specific manner. In recent years, next-generation sequencing (NGS) technology has enabled collection of large amounts of massive parallel sequence (MPS) information. NGS technology has also enabled wholegenome resequencing of species with reference genome sequences [24–26]. Using NGS, transgenes can be detected as deletions of introns [27–29], enabling screening for nontargeted transgenes by comparing the detected intron deletions to annotated gene information (i.e., the intron location in reference sequences). Amplicon sequencing to detect targeted genes using NGS has also been developed for transgene detection [30,31].

Currently, there is no method to detect genome-edited sequences. NGS technology has enabled large-scale whole-genome resequencing (WGR), whole-exome resequencing (WER), and targeted gene or exon resequencing of species with reference genome sequences. WGR and WER are generally used to search for mutations causative of hereditary diseases [32,33], and targeted resequencing is often used to detect mutations in genes associated with cancer [34].

In this study, we developed and validated a method to identify genome-edited sequences using targeted resequencing and examined its applicability by screening geneedited equine cells. We also evaluated the effectiveness of a single-nucleotide variant (SNV) panel to detect foals that have been produced using embryo transfer and somatic cell nuclear transfer, two artificial breeding techniques that are also prohibited in the thoroughbred racing and breeding industry.

2. Materials and Methods

2.1. Animal Ethics and Sample Collection

Blood and hair sample collection was approved by the Animal Care Committee of the Laboratory of Racing Chemistry (approval number 20-4, 13 February 2020). Samples were collected from horses at the Hidaka Training and Research Center (HTRC) of the Japan Racing Association (JRA), Japan Bloodhorse Breeders' Association (JBBA), and donated by individual horse owners by the Japan Association for International Racing and Stud Book (JAIRS).

Hair roots (n = 46) and whole blood (n = 50) were collected from thoroughbred horses (1–15 years old) as a validation set of samples for the developed gene-editing test. Hair roots from 120 thoroughbred horses (<1 years old) were collected as casework examples to test the utility of the parent verification SNV panel.

Blood was collected in BD Vacutainer[®] spray-coated K2EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Hair with roots were pulled from the mane. Blood and hair were stored at -30 °C and 4 °C, respectively, until use.

2.2. DNA Extraction

Genomic DNA was extracted from whole blood (200 μ L) and hair (5 and 10 roots) using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Extracted DNA was quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracts were diluted to 4 ng/ μ L with Milli-Q.

2.3. Construction of Genome Edited Cells

Ear tissues of necropsied horses were provided by the Equine Research Institute, Japan Racing Association (ERI2021-711, 12 October 2021). Fibroblasts were isolated from ear tissue using an explant culture method. The tissue was finely diced (5–10 mm-thickness) using two scalpels and then cultured at 37 °C in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and gentamicin (20 μ g/mL). After cell migration was confirmed, the tissues were removed from the culture medium. Primary cells were passaged once and then harvested for transfection.

The Guide-it CRISPR/Cas9 System (Takara Bio Inc., Kusatsu, Shiga, Japan) was used to edit the *myostatin* (*MSTN*) gene. gRNAs (Guide RNAs) were designed to target locations 66609921-66609940 (5'-TTTCCAGGCGCAGTTTACTG-3') on exon 1, 66607737-66607756 (5'-TACCTTGTACCGTCTTTCAT-3') on exon 2, and 66605475-66605494 (5'-AAATCTCTTCTG GATCGTTT-3') on exon 3 in chromosome 18 of EquCab3.0 (GCA_002863925.1). DNA oligonucleotides of each gRNA sequence were artificially synthesized (Fasmac Co., Ltd., Atsugi, Kanagawa, Japan) and cloned into the pGuide-it plasmid vector, which contained the U6 promoter and sgRNA scaffold to transcribe gRNA, genes encoding Cas9, and green fluorescent protein (GFP).

The constructed pGuide-it vector was electroporated into cultured horse fibroblasts using the Neon[®] Transfection System (Thermo Fisher Scientific). Transgenic cells expressing GFP were selected and a single transgenic cell line representing each edit was isolated in a 24-well culture plate (Corning, Inc., Corning, NY, USA) using the TransferMan[®] Nk-2 Micromanipulation System (Eppendorf AG, Hamburg, Germany).

As some genome edits did not develop into sufficient numbers of cells, whole-genome amplification (WGA) was performed using the REPLI Single Cell Kit (Qiagen) as required.

Finally, genomic DNA was extracted from genome-edited cells or their WGA products using a DNeasy Blood and Tissue Kit (Qiagen). The extracted DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The extracts were diluted to approximately $4 \text{ ng}/\mu L$ using Milli-Q.

2.4. Assay Design for Targeted Genes and SNVs

Fifty-two equine genes (Table 1) selected based on their known functions in several species (primarily horse, human, and mouse) and 147 single-nucleotide variants (SNVs) typically used for DNA typing were targeted in this study [35,36]. Primers for PCR amplification of targeted resequencing were designed by Illumina Concierge using DesignStudio Sequencing Assay Designer (Illumina Inc., San Diego, CA, USA) based on the horse reference genome, EquCab3.0 (GenBank: GCA_002863925.1). Each amplicon ranged from 125 base pairs (bp) to 275 bp in length.

Table 1. Gene name, location, and analyzed exon numbers for the gene-editing test.

Gene Name	Gene Symbol	Chromosome	Exon Number
angiotensin I converting enzyme	ACE	11	25
actinin α 2	ACTN2	1	21
actinin α 3	ACTN3	12	21
activin A receptor type 1B	ACVR1B	6	11
adrenoceptor β 2	ADRB2	14	1
angiotensinogen	AGT	1	4
AKT serine/threonine kinase 1	AKT1	24	13

Gene Name	Gene Symbol	Chromosome	Exon Number
aldehyde dehydrogenase 2 family member	ALDH2	8	13
adenosine monophosphate deaminase 1	AMPD1	5	16
apolipoprotein A2	APOA2	5	3
apolipoprotein A5	APOA5	7	1
bradykinin recentor B2	BDKRB2	24	2
creating kinase M-type	CKM	10	8
clock circadian regulator	CLOCK	3	20
ciliary neurotrophic factor	CNTE	12	20
erythropoietin	FPO	13	5
fibroblast growth factor 1	EGE1	13	3
fibroblast growth factor 2	FGF2	2	3
fibroblast growth factor 7	FGF7	1	3
follistatin	FST	21	6
FTO <i>a</i> -ketoglutarate	151	21	0
dependent dioxygenase	FTO	3	10
growth hormone 1	GHI	11	4
hypoxia inducible factor 1	HIF1A	24	15
subunit α	1011	20	4
insulin-like growth factor 1	IGFI	28	4
insulin-like growth factor 2	IGF2	12	3
binding protein 3	IGFBP3	4	5
interleukin 15 receptor subunit α	IL15RA	29	8
interleukin 1 receptor antagonist	IL1RN	15	4
interleukin 6	IL6	4	5
interleukin 6 receptor	IL6R	5	10
lactase	LCT	18	17
lipoprotein lipase	LPL	2	10
melanocortin 4 receptor	MC4R	8	1
myostatin	MSTN	18	3
methylenetetrahydrofolate reductase	MTHFR	2	12
5-methyltetrahydrofolate-homocysteine	MTR	1	33
5-methyltetrahydrofolate-homocysteine	MTRR	21	16
nouromadin B	NIMP	1	2
nitria avida sunthasa 2	NOS2	1	25
nhosphoopolpyruvato	11035	4	25
carboxykinase 1	PCK1	22	9
pyruvate dehydrogenase kinase 4	PDK4	4	12
peroxisome proliferator activated receptor α	PPARA	28	6
peroxisome proliferator activated receptor delta	PPARD	20	6
peroxisome proliferator activated	PPARG	16	6
$\frac{1}{2} \frac{1}{2} \frac{1}$	PPARCC1 A	3	13
solute carrier family 16 member 1	SI C16A1	5	4
solute carrier family 30 member 8	SI C30A8	9	10
uncoupling protein 2	UCP2	7	8
uncoupling protein 2	UCP3	7	6
vitamin D receptor	VDR	6	8
vascular endothelial	VEGFA	20	7
growth factor A	. 20111	_0	
zinc finger and AT-hook domain containing	ZFAT	9	17

2.5. Library Preparation and Sequencing

Libraries were prepared using AmpliSeq Library PLUS for Illumina (Illumina Inc.) based on the manufacturer's recommendations. AmpliSeq CD Indexes Set A-D for Illumina (384 Indexes, Illumina Inc.) were used to index the samples. Sequencing was performed on the NextSeq 500 sequencing platform (Illumina Inc.) using the NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles, Illumina Inc.).

2.6. Data Analysis, Variant Calling, and SNV Genotyping

Variant detection was performed using the RESEQ pipeline (Amelieff Co., Minato, Tokyo, Japan), which was constructed based on QCleaner (Amelieff Co.), Burrows-Wheeler aligner (BWA, version 0.7.17) [37], Genome Analysis Toolkit (GATK, version 4.0.8.1, Broad Institute, Cambridge, MA, USA) (https://software.broadinstitute.org/gatk/best-practices/, accessed on 5 August 2022), and SnpEff (version v4_0) (http://pcingola.github.io/SnpEff/, accessed on 5 August 2022) [38]. While the original pipeline had a step for excluding duplicated reads using Picard (version 2.13.2) (https://broadinstitute.github.io/picard/, accessed on 5 August 2022), this step was not included in this study.

Briefly, quality control was performed on raw reads using QCleaner, and low-quality bases (<20 phred scores) were removed. Additionally, reads were removed if 80% of their nucleotides had a quality value <20, if they had sequences of over five unknown nucleotides, if they had <32 base length sequences, or if they did not have a mate-pair. Finally, only high-quality sequences were selected.

BWA was used to align the reads using default parameters to the horse reference genome sequence EquCab3.0 assembly from GenBank (GCA_002863925.1). Alignments were converted from the sequence alignment/map (SAM) format to sorted and indexed binary alignment/map (BAM) files using SAMtools (version 1.8) (http://www.htslib.org/, accessed on 5 August 2022).

GATK was used to detect SNVs and INDELs using the default parameters, with the exception of: -minIndelFrac = 2.0. SNVs, and INDELs were annotated using SnpEff. In this analysis, the GATK VariantFiltration program was not used. The obtained variant data and their depth information in the 52 targeted gene regions were extracted from each VCF file and BAM file and then combined as one file using GENOMINEE (Amelieff Co.). Mapped reads and detected variants were visualized using Integrative Genomics Viewer (IGV, version 2.3.97, Broad Institute).

SNVs were genotyped by collecting variant information (REF-allele, ALT-allele, and their depth) using a BED file in which SNV locations were listed. As HomoREF (REF/REF) was not listed in the VCF file, the depth information of HomoREF was collected from the BAM file using GENOMINEE (Amelieff Co.). REF/REF is HomoREF with sufficient depth information (depth > 50).

3. Results and Discussion

3.1. Extraction of Genomic DNA from Hair Roots and Blood Samples

All thoroughbreds are parent-verified as part of the registration process. This would also be a suitable time to perform the gene-editing test, as a condition of registration is an unmodified genome. Currently, hair roots or blood are the routine sample types collected for parentage testing of thoroughbreds. Amplicon sequencing using NGS generally requires purified genomic DNA (e.g., $4 \text{ ng}/\mu L \times 5 \mu L$) as the template DNA, and DNA extracted from blood is known to satisfy library preparation quality and quantity requirements for NGS (Table 2). However, the method for extracting purified genomic DNA required for amplicon sequencing from hair roots has not been properly evaluated. Here, we detail a DNA extraction method from hair roots sufficient for amplicon sequencing.

Sample	Age	Number/ Volume	Mean Concentration (ng/µL)	Standard Deviation
Hair root	0-year-old	5 roots	1.25	0.31
Hair root	0-year-old	10 roots	1.63	0.53
Hair root	2-years-old	5 roots	1.67	0.48
Hair root	2-years-old	10 roots	3.03	0.91
Blood	2-years-old	200 µL	47.2	13.5

Table 2. Mean concentration of genomic DNA extracted from hair roots or blood in 11 horses that were younger than one year or older than two years.

Genomic DNA was extracted from five and ten hair roots of 11 horses that were less than one-year-old or over two-years-old. The mean concentrations of DNA extracted are shown in Table 2. DNA extracts from either five or ten hair roots did not reach 4 ng/ μ L, the required quantity for library preparation for NGS. The genomic DNA was concentrated under reduced pressure to obtain amounts of DNA suitable for NGS analysis. This indicates that a gene-editing test could be performed using the same sample collected for the standard parentage test. In a previous study, an average of 8.5 ng/ μ L (200 μ L) of genomic DNA was extracted from 15 hair roots of horses older than one-year-old [39]. However, to streamline the testing of several samples, it may be better to use a small number of hair roots (i.e., five hair roots) to minimize the time spent cutting samples.

3.2. Construction of Genome Edited Cells

Four different genome-edits targeting the *MSTN* gene were introduced into horse fibroblasts, as shown in Table 3. By designing a gRNA complimentary to sequence in exon 3, a heterozygous edited cell lacking 77 bp at the end of intron 2 and start of exon 3 was constructed (Figure 1A, Edited cell_1). Similarly, gRNAs were designed to target two areas in exon 2, and one in exon 1, resulting in homozygous edited cells with a 1-bp insertion in either exon 2 or 1 (Figure 1B–D, Edited cell_2, 3, and 4). The edited sites were all within the designed gRNA sequences.

Name	Location	Exon/Intron	Genotype	Reference-Allele	Edited-Allele
Edited cell_1	chr18: 66605476-66605554	Exon3-Intron2	Hetero	77-bp insertion	77-bp deletion
Edited cell_2	chr18: 66607750	Exon2	Homo	C	CT
Edited cell_3	chr18: 66607753	Exon2	Homo	Т	TA
Edited cell_4	chr18: 66609936	Exon1	Homo	Т	TA

Table 3. Summary of gene-edited cells constructed using CRISPR/Cas9.

One of the simplest types of edits to achieve using CRISPR/Cas9 is insertion of a single nucleotide on both DNA strands, creating a novel homozygous alternative version of a gene (homoALT) [40]. For this reason, three of the four edited cells were constructed as such in this study.

The *MSTN* gene is a negative regulator of muscle growth, and animals (including humans) lacking functional myostatin protein have above-average muscle mass [41,42]. Consequently, disruption of this gene has been the target of genome editing in many animals, including the horse [2,12,43]. In both racing whippets and thoroughbred race-horses, naturally occurring variants of the *MSTN* gene affect racing performance [44,45]. In thoroughbred horses, where this phenomenon is well-characterized, the variant alters the amount and composition of muscle fiber types [46,47]. Thus, the *MSTN* gene is an obvious target for gene editing in racehorses, and it was important to show that our screening method would be able to detect *MSTN* gene editing.



Figure 1. Sanger sequencing detection of variants introduced using CRISPR/Cas9 into the horse *myostatin* gene. (**A**) Edited cell_1 (chr18: 66605476-66605554, exon3-intron2, 77-bp deletion, heterozygote); (**B**) Edited cell_2 (chr18: 66607750, exon2, 1-bp insertion, homozygote); (**C**) Edited cell_3 (chr18: 66607753, exon2, 1-bp insertion, homozygote); (**D**) Edited cell_4 (chr18: 66609936, exon1, 1-bp insertion, homozygote).

3.3. Genes Targeted for the Gene-Editing Test and Sequencing Summary

In this study, we targeted 52 genes containing 481 exons (as shown in Table 1) to screen as likely targets for the gene-editing detection test. In total, 1012 regions, including the SNVs incorporated for parent verification described below, were amplified using standard Illumina library preparation steps. The designed primer sequences are not disclosed as this test will be used for gene-doping control.

To validate the method, 120 samples, including 27 duplicate samples, were used (Table 4). Most of the genome-edited cell lines constructed in this study did not grow well, resulting in only a small number of edited cells being developed. To address this, the genomic DNA obtained from cultured edited cells was subjected to whole-genome amplification (WGA) to obtain enough DNA to achieve successful library preparation. Only the Edited Cell_1 line grew enough cells to allow for sufficient genomic DNA to be extracted for library preparation without WGA. It is not known whether this is because the edit made to Edited Cell_1 resulted in a heterozygous change rather than a homozygous change.

Table 4. DNA extraction conditions for 120 samples used for validation of the gene-editing test.

Sample	Condition	Number of Samples Used
Hair roots	Extracted/Purified	46
Hair roots	WGA, Extracted/Purified *	5 ***
Blood	Extracted/Purified	50 ****
Edited cell_1	Extracted/Purified	1
Edited cell_1	WGA, Extracted/Purified *	2
Edited cell_1	WGA **	1
Edited cell_2	WGA, Extracted/Purified *	4
Edited cell_2	WGA **	2
Edited cell_3	WGA, Extracted/Purified *	2
Edited cell_3	WGA **	1
Edited cell_4	WGA, Extracted/Purified *	4
Edited cell_4	WGA **	2

*: Amplified genomic DNA amplified was purified using the DNeasy Blood and Tissue Kit. **: Amplified genomic DNA was directly used for library preparation. ***: The five samples were duplicates of samples included in the group of 46. ****: Four of the fifty blood samples were taken from horses that were also typed using 46 hair root samples.

Details of the NGS output for the 120 samples are described in Table 5. On average, 1299 SNVs and 94 INDELs were detected in each sample when mapping to all chromosomes without filtering for quality, although these numbers varied widely across the group. A sufficient amount of FASTQ data and mapped reads were obtained from hair roots, blood (with the exception of one sample), and genome-edited cells both with and without WGA and purification. The sample with the smallest amount of FASTQ data was derived from blood that should be most suitable for NGS. No high-quality variant calls were made in the subsequent analysis, and it was assumed an error occurred in the library preparation of this sample.

	Mean	Standard Deviation	Maximum	Minimum
FASTQ data size	218,009,344	37,895,950	397,302,674	17,404,419
Mapped reads *	2,075,806	362,131	3,795,577	116,739
SNV **	1299	854	5345	388
INDEL **	94	56	319	30

Table 5. Summary of sequencing and variant calling in the gene-editing test.

*: The number of reads mapped after quality check. **: No filtrations.

Of the 481 targeted exons, 470 (97.7%) had greater than $50 \times$ read coverage, 478 exons (99.4%) were covered by more than 20 reads, and all but one exon (99.8%) averaged more than $10 \times$ coverage. The variation in coverage was attributed to variable PCR amplification efficiency of each primer (Figure 2). Whilst the coverage obtained in this study was sufficient to be fit for purpose, future iterations of this test could address this by altering the design of primers targeted at exons that showed low coverage in this instance.



Figure 2. Depth of sequencing coverage of 481 exons in 52 genes. While the coverage varied from exon to exon, over 97% (470 exons) were covered by more than 50 sequence-reads.

3.4. Detection of Artificial Mutations in the MSTN Gene Region

Using NGS and the analysis pipeline on the 120 samples, the 1-bp insertions (ho-moALT) introduced into Edited cells_2, 3, and 4 were easily detected (Figure 3B–D). However, the heterozygous 77-bp deletion (REF/ALT) introduced into Edited cell_1 was not detected (Figure 3A). The 77-bp deletion incorporated parts of exon 3 and intron 2 and was located 22-bp from the end of the PCR amplicon. The ends of the amplicon were excluded



from variable calling by GATK as this program performs a soft clip at the ends of reads, assuming this region is index or library sequence. Thus, large deletions near the ends of PCR amplicons will be difficult to detect using the analysis pipeline developed in this study.

Figure 3. Next-generation sequencing detection of variants introduced using CRISPR/Cas9 into the horse *myostatin* gene. Each variant was visualized using the Integrative Genomics Viewer. (**A**) Edited cell_1 (chr18: 66605476-66605554, exon3-intron2, 77-bp deletion, heterozygote, sequence-reads for the allele without the 77-bp deletion were detected but are out of the frame in the figure); (**B**) Edited cell_2 (chr18: 66607750, exon2, 1-bp insertion, homozygote); (**C**) Edited cell_3 (chr18: 66607753, exon2, 1-bp insertion, homozygote); (**D**) Edited cell_4 (chr18: 6660936, exon1, 1-bp insertion, homozygote).

However, all three homozygous, alternative, single-base-insertion alleles generated in the edited cells were detected by the proposed NGS sequencing and pipeline. CRISPR/Cas9 is particularly efficient at inserting single nucleotides on both DNA strands, creating a novel, homozygous, alternative version of a gene (homoALT) [40]. A single base insertion within a coding region will create a frameshift mutation that (depending on the position of the insertion within the coding sequence) is very likely to be destructive to the function of the mature protein. It is thus important that any gene-editing detection method will be able to detect these small but disruptive edits of gene sequence and function.

The use of CRISPR/Cas9 is not always 100% efficient and may introduce new alleles in the form of compound heterozygotes (ALT1/ALT2) [48]. Based on the results of this study, these should be detected by comparison with the reference sequence using the methods described. Therefore, artificially introduced homozygous alternative and compound heterozygous sequence changes should be identifiable when screening for gene-editing tests.

3.5. Establishment of Criteria for Detecting Artificially Edited Variants

An important part of screening for prohibited gene editing is to establish criteria to identify the most likely candidate genome-edited variants (i.e., homoALT or ALT1/ALT2).

These should differentiate between naturally occurring variants and those that are more likely to have been artificially introduced.

In this study, a total of 575 variable loci (492 SNVs and 83 INDELs) were detected in the targeted genes in the validation sample set of 120 samples, which included the gene-edited cells. Homozygous INDELs are likely to be preferred by someone wanting to simply knock out the function of a gene. Of the coding INDELs identified, ten were homoALTs and one was a compound heterozygous ALT1/ALT2. The remaining INDELs were heterozygous.

Among the INDELs, four homoALT mutations were excluded as candidates for gene editing because the particular variants had previously been reported in whole-genome sequence data from 101 thoroughbred horses [49]. One compound heterozygous INDEL (ALT1/ALT2) had also been previously observed in the thoroughbred population. Of the remaining six loci, three were excluded as gene-editing candidates because they were covered by extremely low sequence depth in the detected individuals. The remaining three loci were the specific homoALT single-nucleotide insertions introduced into the genome-edited cells (Edited cell_2, 3, and 4), which showed sufficient sequence depth.

Based on the above data, we proposed the following filtering criteria for identifying artificial editing candidates:

- (1) Selection of homoALT or ALT1/ALT2 caused by INDELs as artificial editing candidates;
- (2) Exclude variants that already exist in the thoroughbred population;
- (3) Exclude variants in regions with low sequence depth.

3.6. Casework Example

The gene-editing test and detection criteria established in this study were applied to 120 new samples (the casework sample set) collected for the standard parent verification process, which forms an important part of the registration of thoroughbreds in their Stud Book. A total of 508 variants were detected in this group of 120 samples. Those most likely to be gene-editing candidates (homoALT or ALT1/ALT2) constituted seven loci, of which six were already documented as present in the current thoroughbred population. The remaining loci had low coverage. Thus, it is assumed no gene editing was observed in the 120 samples using the developed gene-editing screening test under the described conditions.

3.7. Construction of a SNV Panel to Detect Parentage in Thoroughbred Horses

To create genetically modified animals, it is necessary to transplant edited eggs into surrogate mothers to carry to term [50,51]. Therefore, a simple way to identify a genetically engineered foal is to compare the foal's DNA with that of the mare that carried it. Therefore, we developed a SNV panel to confirm mother–child inheritance.

Primers were designed for amplicon sequencing of 147 loci used in the 2021 SNV comparison test run by the International Society for Animal Genetics (ISAG) [34,35]. These primers were included in the panel for the gene-editing test, and library preparation and sequencing by NGS were performed simultaneously. Three loci failed (chr2:134944, chr9:20379456, and chr18:34581176), leaving 144 SNVs genotyped on the 120 samples used for validating the gene-editing test.

Firstly, 29 trios (sire, dam and foal) were genotyped, and the inheritance of the SNVs by the foals investigated. Of the 144 SNV, 24 SNVs showed mis-inheritances among 27 trios (Table 6). The causes of these mis-inheritances were 1) insufficient coverage (i.e., depth < 50) in all or part of the analyzed individuals and 2) different depths of coverage across different alleles. The former is attributed to the difficulty of multiplexing PCR (1012 amplicons), whereas the latter may involve PCR amplification bias. Trio analysis of the remaining 120 SNV confirmed no mis-inheritances (Table 6). Therefore, these 120 SNVs were selected as the SNV parentage panel.

	144 \$	SNVs	120 SNVs			
	Consistent	Inconsistent	Consistent	Inconsistent		
Trio_1	142	2	120	0		
Trio_2	139	5	120	0		
Trio 3	142	2	120	0		
Trio 4	143	1	120	0		
Trio 5	143	1	120	0		
Trio 6	143	1	120	0		
Trio 7	141	3	120	0		
Trio 8	141	3	120	0		
Trio 9	143	1	120	0		
Trio 10	140	4	120	0		
Trio 11	140	4	120	0		
Trio 12	141	3	120	0		
Trio_13	141	3	120	0		
Trio 14	141	3	120	0		
Trio 15	143	1	120	0		
Trio 16	141	3	120	0		
Trio 17	144	0	120	0		
Trio 18	140	4	120	0		
Trio 19	141	3	120	0		
Trio_20	141	1	120	0		
Trio_20	143	0	120	0		
Trio_22	144	2	120	0		
Trio_22	142	2	120	0		
Trio_24	142	2	120	0		
Trio_24	142	2	120	0		
Trio_26	142	2	120	0		
Trio_27	142	2	120	0		
Trio_28	141	3	120	0		
Trio_20	141	3	120	0		
Pseudo-Trio 1	141	36	91	29		
Pseudo-Trio 2	100	34	>1 88	32		
Psoudo Trio 3	110	32	00	29		
Psoudo Trio 4	112	32	91	29		
Psoudo Trio 5	108	36	95	27		
Half pseudo Trio 1	100	21	90 10 2	18		
Half pseudo Trio 2	123	21	05	10		
Half pseudo Trio 2	110	34	95	23		
Half pseudo Trio 4	119	23	99 07	21		
Half pseudo Trio 5	110	20	97 105	23		
Darant Eagl 1	120	10	105	15		
Parent Foal 2	145	1	120	0		
Parent Foal 2	144	0	120	0		
Parent Foal 4	142	2 1	120	0		
Parent Foal 5	145	1	120	0		
Farent-Foal_5	144	U 10	120	U 11		
rseudo-parent-Foal_1	132	12	109	11		
Preudo-parent-Foal_2	128	16	108	12		
rseudo-parent-Foal_3	131	13	110	10		
rseudo-parent-Foal_4	132	12	110	10		
Pseudo-parent-Foal_5	136	8	114	6		

Table 6. Confirmation of inheritance for 144 and 120 SNVs.

Next, we evaluated the accuracy of the SNVs for detecting mis-inheritance using 1) five pseudo-trios (pseudo-sire, pseudo-dam, foal), 2) five half-pseudo-trios (sire, pseudo-dam, and foal; or pseudo-sire, dam, and foal), and 3) five pseudo parents (sire or dam)-foal (Table 6). Pseudo-trios, half-pseudo-trios, and pseudo-parent-foal were detected by 29.5, 20.4, and 9.8 SNVs on average using the 120 SNV panel. Table 7 shows the allele frequency, heterozygosity (He), and paternity exclusion (PE) values of the 120 SNVs. The combined

PE1 and PE2 values with 120 SNVs were 0.999999998 and 0.999997, respectively. Five new single parent–foal combinations were also evaluated and parentage confirmed using the selected SNV panel.

Table 7. Statistical summary of 120 SNVs in the gene-editing test.

SNV ID	Chromosome	Location	REF-Allele	ALT-Allele	REF Frequency	He	PE1	PE2
BIEC2-11336	1	24061861	С	Т	0.582	0.487	0.184	0.118
BIEC2-23891	1	58467370	T	С	0.847	0.259	0.113	0.034
BIEC2-34560	1	80153039	Т	C	0.520	0.499	0.187	0.125
BIEC2-34987	1	81012651	Т	G	0.724	0.399	0.160	0.080
BIEC42118	1	102064366	С	Т	0.255	0.380	0.154	0.072
BIEC2-60186	1	138352227	А	С	0.755	0.370	0.151	0.068
BIEC2-65145	1	153834277	С	Т	0.635	0.463	0.178	0.107
BIEC2-78523	1	166558525	G	А	0.592	0.483	0.183	0.117
BIEC2-459311	2	17808463	Т	С	0.740	0.385	0.156	0.074
BIEC2-476920	2	46942336	С	А	0.633	0.465	0.178	0.108
BIEC2-491394	2	78433836	А	G	0.571	0.490	0.185	0.120
BIEC2-502451	2	98905614	А	G	0.673	0.440	0.172	0.097
BIEC420894	2	120778620	Т	С	0.653	0.453	0.175	0.103
BIEC2-777914	3	38956274	Т	С	0.622	0.470	0.180	0.110
BIEC672139	3	86457199	Т	Ċ	0.694	0.425	0.167	0.090
BIEC2-798927	3	88082655	Ā	Ğ	0.633	0.465	0.178	0.108
BIEC2-799664	3	90095202	С	Ā	0.646	0.457	0.176	0.105
BIEC2-800511	3	91766696	Т	С	0.235	0.359	0.147	0.065
BIEC2-806771	3	101540587	Ĝ	Ă	0.816	0.300	0.127	0.045
BIEC 681989	3	104518656	Č	Т	0.592	0.483	0.183	0.117
BIEC2-811886	3	119599447	Ğ	Ă	0.327	0.440	0.172	0.097
BIEC707898	4	178566	Т	C	0.729	0.395	0.158	0.078
BIEC2-853347	4	21336785	Ġ	Ă	0.602	0.479	0.182	0.115
BIEC2-908630	5	45880924	C	Т	0.531	0.498	0.187	0.124
BIEC2-910827	5	53722932	Т	Ġ	0.604	0.478	0.182	0 114
BIEC2-914714	5	63272427	Ť	C	0.378	0.470	0.180	0.110
BIEC2-919835	5	73509875	G	Т	0.370	0.399	0.160	0.080
BIEC2-946446	6	32343233	A	Ġ	0.704	0.417	0.165	0.087
BIEC798010	6	45478480	A	G	0.398	0.479	0.182	0.115
BIEC811791	6	76708313	G	Т	0.622	0.470	0.180	0.110
BIEC819385	7	5215592	A	Ċ	0.480	0.499	0.187	0.125
BIEC2-1007607	7	80202186	C	Т	0.520	0.499	0.187	0.125
BIEC870244	8	17383321	Ğ	Ċ	0.653	0.453	0.175	0.103
BIEC871916	8	20028234	A	Т	0.551	0.495	0.186	0.122
BIEC2-1052417	8	57040628	C	Ť	0.714	0.408	0.162	0.083
BIEC2-1066033	8	94179121	G	Ť	0.520	0.499	0.187	0.125
BIEC2-1066179	8	95184881	G	Ă	0.735	0.390	0.157	0.076
BIEC2-1080866	9	25656223	Т	C	0.551	0.495	0.186	0.122
BIEC915102	9	27114849	Ċ	Т	0.501	0.500	0.188	0.122
BIEC86281	10	922571	C	Ť	0.439	0.493	0.186	0.121
BIEC2-95522	10	4751573	Ă	Ĝ	0.531	0.498	0.187	0.124
BIEC2-97679	10	7701310	G	A	0.643	0.459	0.177	0.105
BIEC2-119640	10	44453287	Т	C	0.673	0.440	0.172	0.097
BIEC2-121102	10	48342330	Ă	Ğ	0.735	0.390	0.157	0.076
BIEC2-123002	10	52573273	Т	C	0.867	0.230	0.102	0.026
BIEC2-126732	10	60049178	Ă	G	0.408	0.483	0.183	0.117
BIEC119158	10	65321527	A	Т	0.582	0.487	0.184	0.118
BIEC2-136591	10	7641341	Т	Ċ	0.502	0.500	0.187	0.125
BIEC136821	11	29398313	Ă	G	0.592	0.483	0.183	0.117
BIEC2-162245	11	57232386	Ċ	Т	0.653	0.453	0.175	0.103
BIEC154171	12	3878382	Ğ	Ť	0.337	0.447	0.173	0.100
BIEC155175	12	5186277	C	Ť	0.698	0.422	0.166	0.089
BIEC2-189666	12	22012560	G	A	0.550	0.495	0.186	0.122
BIEC2-214346	13	19184556	G	A	0.490	0.500	0.187	0.125
BIEC180122	13	35676903	A	G	0.450	0 447	0.173	0.120
BIEC183067	14	4666366	A	G	0.500	0 499	0.187	0.125
BIEC2-245923	14	14775426	C A	Т	0.704	0.417	0.165	0.087
BIEC204022	14	64551633	Ă	Ġ	0.813	0.305	0.129	0.046
212 220 1022	**	010010000	- 1	5	0.010	0.000	···/	0.010

Table 7. Cont.

BHC 2009 0.187 0.125 BHC 20095 14 8008377 C T 0.418 0.447 0.117 0.125 BHC 20095 14 8008377 C T 0.418 0.144 0.016 BHC 20026 15 52064101 T C 0.406 0.485 0.113 0.116 BHC 326437 16 18464099 A C 0.027 0.497 0.135 0.110 BHC 326437 16 3864430 C T 0.271 0.497 0.185 0.123 BHC 323649 16 3291549 A G 0.408 0.433 0.147 0.165 0.087 BHC 323549 16 42324495 C T 0.631 0.4433 0.175 0.125 BHC 3234411 16 8351410 G A 0.653 0.433 0.175 0.125 B	SNV ID	Chromosome	Location	REF-Allele	ALT-Allele	REF Frequency	He	PE1	PE2
BIEC2-270795 14 COMBAND T 0.418 0.434 0.134 0.135 BIEC2-27074 15 1201270 A G 0.433 0.444 0.018 BIEC2-310269 15 526661101 T C 0.403 0.448 0.118 0.116 BIEC2-302667 16 38960140 C T 0.571 0.409 0.185 0.120 BIEC2-32864 16 38960140 C T 0.571 0.409 0.182 0.113 BIEC2-38984 16 4232345 C T 0.402 0.447 0.182 0.013 BIEC2-348541 16 43253450 C T 0.404 0.433 0.169 0.047 0.152 0.125 <td>BIEC2-263616</td> <td>14</td> <td>69988182</td> <td>С</td> <td>А</td> <td>0.480</td> <td>0.499</td> <td>0.187</td> <td>0.125</td>	BIEC2-263616	14	69988182	С	А	0.480	0.499	0.187	0.125
BIEC2-27852 15 1201270 A C 0.776 0.348 0.144 0.061 BIEC2-370569 15 55661101 T C 0.406 0.445 0.183 0.116 BIEC2-326637 16 13960409 A C 0.627 0.470 0.180 0.110 BIEC2-326637 16 2596390 C T 0.571 0.490 0.187 0.120 BIEC2-306269 16 2596390 G A 0.233 0.339 0.147 0.065 BIEC2-342801 16 4233483 C T 0.662 0.433 0.167 0.132 BIEC2-342801 16 4233483 C T 0.663 0.433 0.167 0.123 BIEC2-344971 17 746644 G A 0.510 0.500 0.187 0.123 BIEC2-344571 17 786644 A C 0.445 0.147 0.103 BIEC2-349671 1	BIEC2-270795	14	83088387	С	Т	0.418	0.487	0.184	0.118
BIEC.247284 I5 #6661214 G A 0.033 0.448 0.178 0.0108 BIEC.2326637 16 1496069 A G 0.622 0.470 0.183 0.116 BIEC.2326637 16 3860140 C T 0.571 0.490 0.182 0.121 BIEC.230269 16 2989809 G A 0.233 0.335 0.147 0.063 BIEC.230269 16 2995809 G T 0.262 0.447 0.053 BIEC.230269 16 2995140 C T 0.264 0.437 0.162 0.175 BIEC.2346741 16 8905140 G A 0.641 0.447 0.167 0.125 BIEC.2346741 16 8905140 G A 0.631 0.560 0.187 0.125 BIEC.234670 17 17466634 G A G 0.443 0.167 0.125 BIEC.234670 18	BIEC2-278532	15	1201270	А	G	0.776	0.348	0.144	0.061
BIEC2-310269 15 52664101 T C 0.406 0.482 0.180 0.110 BIEC2-32687 16 13800430 C T 0.571 0.490 0.182 0.118 BIEC2-3262954 16 2598909 G A 0.235 0.339 0.147 0.065 BIEC2-3462851 16 2978909 G A 0.235 0.439 0.182 0.115 BIEC2-342881 16 42323483 C T 0.266 0.417 0.182 0.115 BIEC2-342881 16 42323483 C T 0.264 0.417 0.187 0.037 BIEC2-342841 16 42457380 C T 0.511 0.167 0.187 0.123 BIEC2-373671 17 7494943 G 0.453 0.147 0.187 0.125 BIEC2-373751 17 7696074 A G 0.429 0.490 0.187 0.125 BIEC2-373751	BIEC247284	15	48661214	G	А	0.633	0.465	0.178	0.108
BiFC2-6574 16 1496669 A G 0.622 0.470 0.188 0.120 BiFC2-328954 16 38960400 C T 0.571 0.490 0.185 0.120 BiFC2-328954 16 2983909 G A 0.235 0.339 0.147 0.065 BIFC2-328951 16 2924385 C T 0.602 0.479 0.182 0.115 BIFC2-326741 16 83054140 G A 0.644 0.433 0.169 0.094 BIFC2-366711 17 7.0464140 G A 0.6464 0.433 0.167 0.123 BIFC2-367911 17 7.056553 A G 0.660 0.450 0.157 0.123 BIFC2-307501 18 7.72056553 A G 0.465 0.158 0.158 BIFC2-407206 18 7.14215 A G 0.465 0.158 0.129 BIFC2-433906 19	BIEC2-310269	15	52664101	Т	С	0.406	0.482	0.183	0.116
BHC (226761) 16 386(430) C T 0.571 0.490 0.182 0.115 BUC2 (236) 16 2798300) G A 0.235 0.339 0.147 0.065 BUC2 (236) 16 36915139 A G 0.408 0.413 0.113 BUC2 (254) 16 36915139 A G 0.408 0.413 0.112 0.115 0.012 BUC2 (254) 16 83054140 G A 0.664 0.433 0.169 0.047 BUC2 (254) 16 83054140 G A 0.643 0.433 0.187 0.123 BUC2 (234) 17 7660174 A C 0.443 0.499 0.187 0.123 BUC2 (23150) 18 114365 C T 0.633 0.465 0.178 0.120 BUC2 (23150) 18 114365 C T 0.633 0.465 0.185 0.120 BUC2 (23150) <td>BIEC2-326637</td> <td>16</td> <td>1496069</td> <td>А</td> <td>G</td> <td>0.622</td> <td>0.470</td> <td>0.180</td> <td>0.110</td>	BIEC2-326637	16	1496069	А	G	0.622	0.470	0.180	0.110
BIBC2-328954 16 \$899011 A G 0.388 0.479 0.182 0.135 BIBC2-336269 16 2093090 G A G 0.488 0.183 0.117 BIBC2-342881 16 42232485 C T 0.296 0.479 0.182 0.115 BIBC2-346471 16 83257900 C T 0.544 0.437 0.187 0.125 BIBC2-346471 17 7246632 A 0.633 0.137 0.125 BIBC2-347671 17 7246632 A C 0.463 0.437 0.125 0.125 BIBC2-347671 17 7246525 A C 0.473 0.489 0.490 0.187 0.125 BIBC2-34757 19 19611080 A C 0.473 0.480 0.187 0.125 BIBC2-439057 19 19611080 A C 0.688 0.4470 0.180 0.180 BIBC2-439057	BIEC266761	16	3860430	С	Т	0.571	0.490	0.185	0.120
BIEC2-336269 16 2978390 G A G 0.433 0.147 0.062 BIEC2-342881 16 4223485 C T 0.602 0.479 0.182 0.117 BIEC2-342845 16 71914513 C T 0.642 0.479 0.182 0.017 BIEC2-356741 16 84357390 C T 0.541 0.497 0.187 0.123 BIEC2-3647451 17 72666174 A G 0.463 0.433 0.175 0.103 BIEC2-374571 17 72666174 A G 0.440 0.499 0.187 0.123 BIEC2-32730 18 11431565 C T 0.633 0.445 0.178 0.120 BIEC2-41515 18 56944269 G A 0.510 0.187 0.120 BIEC2-41515 18 56944269 G A 0.510 0.187 0.120 BIEC2-41515 18 569	BIEC2-328954	16	8598011	А	G	0.398	0.479	0.182	0.115
BIEC2-340595 16 36915439 A G 0.408 0.483 0.113 BIEC2-345801 16 47223485 C T 0.296 0.417 0.165 0.087 BIEC2-345441 16 8203740 C T 0.541 0.447 0.187 0.125 BIEC2-3664969 17 17649634 G A 0.510 0.500 0.187 0.125 BIEC2-374571 17 7866074 A G 0.430 0.499 0.187 0.125 BIEC2-370570 18 1143155 C T 0.633 0.465 0.175 0.108 BIEC2-407206 18 1143155 C T 0.633 0.465 0.125 BIEC2-415315 18 50494269 G A 0.510 0.500 0.185 0.125 BIEC2-4120906 19 42595105 C T 0.628 0.417 0.165 0.692 BIEC2-433766 20	BIEC2-336269	16	27983909	G	А	0.235	0.359	0.147	0.065
BIBC2-342881 16 42323485 C T 0.642 0.479 0.182 0.1182 BIBC2-366741 16 82054140 G A 0.684 0.433 0.169 0.087 BIBC2-366741 17 27036283 A G 0.653 0.453 0.175 0.123 BIBC2-376741 17 27036283 A G 0.653 0.453 0.178 0.123 BIBC2-37920 18 2190644 A C 0.745 0.380 0.154 0.072 BIBC2-4153157 18 15644155 A G 0.429 0.490 0.185 0.129 BIBC2-4153157 18 56944269 G A 0.510 0.500 0.187 0.125 BIBC2-439506 19 45488636 T C 0.422 0.470 0.180 0.122 BIBC2-349291 19 45488636 T C 0.231 0.412 0.161 0.082 <	BIEC2-340595	16	36915439	А	G	0.408	0.483	0.183	0.117
BIEC2-389001 16 71914513 C T 0.296 0.417 0.165 0.087 BIEC2-3649459 16 84257390 C T 0.541 0.497 0.187 0.125 BIEC2-3649459 17 17649634 G A 0.510 0.500 0.187 0.125 BIEC2-374571 17 72660174 A G 0.438 0.443 0.175 0.103 BIEC2-370573 18 1143135 C T 0.633 0.465 0.178 0.018 BIEC2-407206 18 1143135 C T 0.633 0.465 0.125 BIEC2-415315 18 56944269 G A 0.510 0.500 0.185 0.125 BIEC2-4207206 19 42595105 C T 0.688 0.430 0.169 0.110 0.080 0.187 0.125 BIEC2-432872 19 44848636 T C 0.428 0.444 0.161	BIEC2-342881	16	42323485	С	Т	0.602	0.479	0.182	0.115
BIEC.2-36/741 16 82054140 G A 0.684 0.433 0.169 0.094 BIEC.2-366699 17 17649634 G A 0.510 0.500 0.187 0.123 BIEC.2-366791 17 27036288 A G 0.653 0.453 0.178 0.103 BIEC.230734 17 2706641 A G 0.445 0.180 0.178 0.102 BIEC.2407206 18 1745136 C T 0.63 0.445 0.118 0.102 BIEC.2407206 18 17621415 A G 0.429 0.490 0.185 0.120 BIEC.240577 19 19614080 A G 0.296 0.417 0.168 0.087 BIEC.243820 19 442895105 C T 0.682 0.409 0.187 0.122 BIEC.2439078 20 3360077 T C 0.281 0.449 0.181 0.122 B	BIEC2-358061	16	71914513	С	Т	0.296	0.417	0.165	0.087
BIEC2-369495 16 84257390 C T 0.510 0.497 0.123 BIEC2-369699 17 1749624 G A 0.510 0.0430 0.493 0.175 0.103 BIEC2-374571 17 78660174 A G 0.480 0.499 0.187 0.123 BIEC2-370573 18 11431365 C T 0.633 0.465 0.178 0.108 BIEC2-410236 18 11431365 C T 0.633 0.465 0.178 0.108 BIEC2-410236 18 11431365 C T 0.633 0.465 0.178 0.102 BIEC2-402306 18 0.1493 0.168 0.120 0.187 0.125 BIEC2-412892 19 49488636 T C 0.648 0.449 0.186 0.122 BIEC2-530788 20 3360979 T C 0.449 0.185 0.122 BIEC2-5307846 22 201040	BIEC2-364741	16	83054140	G	А	0.684	0.433	0.169	0.094
BIEC2-369699 17 17464634 G A 0.500 0.507 0.125 BIEC232734 17 278660174 A G 0.653 0.433 0.175 0.125 BIEC239790 18 17431365 C T 0.633 0.465 0.178 0.108 BIEC2-415315 18 17621415 A G 0.429 0.400 0.185 0.125 BIEC2-415315 18 5694269 G A 0.500 0.167 0.015 0.087 BIEC2-439060 19 42595105 C T 0.682 0.430 0.169 0.092 BIEC2-439060 19 42595105 C T 0.622 0.470 0.168 0.125 BIEC2-439060 19 42595105 C T 0.622 0.470 0.185 0.125 BIEC2-439060 19 428595102 C T 0.622 0.470 0.185 0.125 BIEC2-430460	BIEC2-364945	16	84257390	С	Т	0.541	0.497	0.187	0.123
BIEC2-2374571 17 27036258 A G 0.653 0.453 0.475 0.103 BIEC2-230920 18 12190684 A C 0.748 0.138 0.175 0.103 BIEC2-230920 18 11431365 C T 0.633 0.465 0.178 0.108 BIEC2-407206 18 17621415 A G 0.490 0.485 0.120 BIEC2-405077 19 19961080 A G 0.296 0.417 0.165 0.087 BIEC2-405077 19 49848636 T C 0.688 0.430 0.169 0.019 BIEC2-350788 20 43848636 T C 0.281 0.440 0.110 0.082 BIEC2-530788 20 4386979 T C 0.494 0.118 0.112 BIEC2-533766 20 49037728 T C 0.495 0.186 0.122 BIEC2-84911 22 19064667	BIEC2-369699	17	17649634	G	А	0.510	0.500	0.187	0.125
BIEC232734 17 78660174 A G 0.49 0.187 0.127 BIEC2309020 18 11431365 C T 0.633 0.465 0.178 0.1078 BIEC2-407206 18 17621415 A G 0.429 0.490 0.185 0.120 BIEC2-415315 18 56944269 G A 0.510 0.500 0.187 0.125 BIEC2-439050 19 42595105 C T 0.688 0.430 0.169 0.092 BIEC2-439056 20 1638356 T C 0.480 0.499 0.187 0.125 BIEC2-439058 20 33600979 T C 0.480 0.499 0.187 0.125 BIEC2-537566 20 4041873 C T 0.551 0.490 0.185 0.122 BIEC2-537546 22 201740 C T 0.525 0.447 0.186 0.122 BIEC2-540491 <t< td=""><td>BIEC2-374571</td><td>17</td><td>27036258</td><td>А</td><td>G</td><td>0.653</td><td>0.453</td><td>0.175</td><td>0.103</td></t<>	BIEC2-374571	17	27036258	А	G	0.653	0.453	0.175	0.103
BIEC2-399020 18 2190684 A C 0.73 0.138 0.078 0.108 BIEC2-407206 18 17621415 A G 0.463 0.465 0.178 0.108 BIEC2-407206 18 17621415 A G 0.429 0.400 0.187 0.125 BIEC2-407577 19 19611080 A G 0.622 0.470 0.165 0.087 BIEC2-490500 19 42959105 C T 0.688 0.430 0.169 0.092 BIEC2-350788 20 36306979 T C 0.480 0.449 0.187 0.125 BIEC2-357566 20 40481673 C T 0.551 0.490 0.188 0.122 BIEC2-357566 22 2010440 C T 0.551 0.495 0.186 0.122 BIEC2-3675436 22 40979735 C G 0.551 0.497 0.186 0.122	BIEC322734	17	78660174	А	G	0.480	0.499	0.187	0.125
BIRC234530 18 11431365 C T 0.633 0.465 0.178 0.102 BIRC2-415315 18 56944269 G A 0.510 0.500 0.185 0.120 BIRC2-439560 19 44295105 C T 0.688 0.430 0.169 0.092 BIRC2-439060 19 4488636 T C 0.688 0.430 0.160 0.012 BIRC2-350788 20 3360079 T C 0.484 0.499 0.187 0.128 BIRC2-353766 20 49481673 C T 0.582 0.487 0.186 0.120 BIRC2-353766 20 4903728 T C 0.582 0.487 0.184 0.118 BIRC2-353766 20 49037728 T C 0.582 0.487 0.184 0.118 BIRC2-353746 22 101040 C T 0.592 0.447 0.165 0.087 BIRC2	BIEC2-390920	18	2190684	А	С	0.745	0.380	0.154	0.072
BIEC2-407206 18 17621415 A G 0.429 0.490 0.185 0.125 BIEC2-41315 18 56944269 G A 0.510 0.500 0.187 0.125 BIEC2-430977 19 19611080 A G 0.296 0.417 0.165 0.092 BIEC2-430906 19 42595105 C T 0.688 0.430 0.169 0.092 BIEC2-350788 20 33600979 T C 0.481 0.404 0.161 0.082 BIEC2-55766 20 40937728 T C 0.551 0.490 0.185 0.122 BIEC2-55766 22 40978756 T G 0.551 0.495 0.185 0.122 BIEC2-606469 23 4277770 G A 0.612 0.497 0.181 0.112 BIEC2-618284 23 1954053 G T 0.750 0.375 0.152 0.070 BIE	BIEC324530	18	11431365	С	Т	0.633	0.465	0.178	0.108
BIEC2-415315 18 56944269 G A 0.510 0.500 0.187 0.125 BIEC2-439060 19 42595105 C T 0.688 0.430 0.169 0.092 BIEC2-439292 19 44848036 T C 0.622 0.470 0.180 0.110 BIEC2-429292 19 44848036 T C 0.4281 0.404 0.161 0.082 BIEC2-530788 20 33600979 T C 0.480 0.499 0.187 0.120 BIEC2-535766 20 40937728 T C 0.551 0.495 0.186 0.122 BIEC2-549411 22 19064667 A G 0.398 0.479 0.182 0.118 BIEC2-54911 22 30797481 C T 0.296 0.417 0.165 0.037 BIEC2-64649 23 4277770 G A 0.612 0.477 0.181 0.113	BIEC2-407206	18	17621415	А	G	0.429	0.490	0.185	0.120
BIFEC2-430577 19 19611080 A G 0.296 0.417 0.165 0.0092 BIFEC2-4309060 19 4458636 T C 0.682 0.430 0.169 0.092 BIFEC2-43992 19 4458636 T C 0.622 0.470 0.180 0.110 BIFEC2-330788 20 33600979 T C 0.480 0.499 0.185 0.122 BIEC2-357566 20 40937728 T C 0.551 0.495 0.186 0.122 BIEC2-357564 22 2011040 C T 0.582 0.487 0.184 0.115 BIEC2-357565 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2-606469 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2-606469 23 1427770 G A 0.612 0.475 0.181 0.1123	BIEC2-415315	18	56944269	G	А	0.510	0.500	0.187	0.125
BIEC2-439060 19 4259105 C T 0.688 0.430 0.169 0.092 BIEC2-442992 19 4484863 T C 0.622 0.470 0.180 0.110 BIEC2-530788 20 33600979 T C 0.480 0.499 0.187 0.125 BIEC2-532766 20 40037728 T C 0.551 0.495 0.186 0.122 BIEC2-535746 22 2011040 C T 0.562 0.487 0.184 0.118 BIEC2-557366 20 49037728 T G 0.398 0.479 0.182 0.115 BIEC2-540411 22 19064667 A G 0.398 0.479 0.186 0.122 BIEC2-640849 23 1427770 G A 0.612 0.475 0.186 0.122 BIEC2-6408410 23 16413178 C G 0.512 0.070 BIEC2-64087 24 <t< td=""><td>BIEC2-430577</td><td>19</td><td>19611080</td><td>А</td><td>G</td><td>0.296</td><td>0.417</td><td>0.165</td><td>0.087</td></t<>	BIEC2-430577	19	19611080	А	G	0.296	0.417	0.165	0.087
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	BIEC2-439060	19	42595105	С	Т	0.688	0.430	0.169	0.092
BIEC431445 20 16883556 T C 0.281 0.404 0.161 0.082 BIEC2-530708 20 33600979 T C 0.450 0.499 0.187 0.123 BIEC2-535766 20 40437728 T C 0.551 0.495 0.186 0.122 BIEC2-535766 20 40037728 T C 0.551 0.495 0.184 0.118 BIEC2-535766 2 2011040 C T 0.560 0.477 0.185 0.122 BIEC439879 2 3079781 C G 0.398 0.479 0.181 0.113 BIEC439860 2 4079770 G A 0.61 0.495 0.186 0.122 BIEC2-618284 23 19540533 G T 0.700 0.375 0.132 0.070 BIEC52483 24 12615461 T C 0.29 0.333 0.145 0.0137 BIEC53483	BIEC2-442892	19	48488636	Т	С	0.622	0.470	0.180	0.110
BIEC2-530788 20 33600979 T C 0.480 0.499 0.187 0.125 BIEC2-535766 20 440481673 C T 0.571 0.490 0.185 0.120 BIEC2-535766 22 2011040 C T 0.582 0.487 0.184 0.112 BIEC2-585911 22 30797481 C T 0.296 0.417 0.165 0.087 BIEC298960 22 409787766 T G 0.551 0.495 0.186 0.122 BIEC298060 23 4277770 G A 0.612 0.475 0.181 0.113 BIEC2640649 23 12413178 C G 0.551 0.497 0.187 0.123 BIEC254383 24 12615461 T C 0.229 0.353 0.145 0.062 BIEC2546987 24 27556162 C T 0.694 0.425 0.167 0.090 BIEC25	BIEC431445	20	16383556	Т	С	0.281	0.404	0.161	0.082
BIEC2-532106 20 44481673 C T 0.571 0.490 0.185 0.122 BIEC2-535766 20 49037728 T C 0.551 0.495 0.186 0.122 BIEC2-575436 22 2011040 C T 0.582 0.487 0.184 0.118 BIEC2-384911 22 10964667 A G 0.398 0.479 0.182 0.118 BIEC2-364911 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2-06469 23 4277770 G A 0.612 0.475 0.181 0.113 BIEC2-61824 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC2-61824 23 19540553 G T 0.694 0.425 0.167 0.099 BIEC2-30897 24 1744264 C T 0.694 0.425 0.167 0.099 BIEC5	BIEC2-530788	20	33600979	Т	С	0.480	0.499	0.187	0.125
BIEC2-357566 20 49037728 T C 0.551 0.495 0.186 0.122 BIEC2-357546 22 2011040 C T 0.582 0.487 0.184 0.113 BIEC2-3584911 22 30797481 C T 0.296 0.417 0.165 0.087 BIEC290860 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2908610 23 16413178 C G 0.542 0.497 0.181 0.113 BIEC2630837 24 12615461 T C 0.694 0.425 0.167 0.090 BIEC23483 24 12615461 T C 0.296 0.417 0.165 0.087 BIEC230807 24 22556162 C T 0.694 0.425 0.167 0.090 BIEC531275 24 45741983 C T 0.296 0.417 0.165 0.087 BIEC54169	BIEC2-532106	20	40481673	С	Т	0.571	0.490	0.185	0.120
BIEC2-575436 22 201040 C T 0.582 0.487 0.184 0.115 BIEC2-584911 22 19064667 A G 0.398 0.479 0.182 0.115 BIEC49860 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2606469 23 427770 G A 0.612 0.475 0.181 0.113 BIEC2608410 23 16413178 C G 0.521 0.475 0.181 0.123 BIEC23483 24 12615461 T C 0.229 0.333 0.145 0.062 BIEC331275 24 2256162 C T 0.694 0.425 0.167 0.090 BIEC532060 24 2865489 A G 0.612 0.475 0.181 0.113 BIEC55737 25 1234028 G A 0.635 0.463 0.175 0.107 BIEC551695	BIEC2-535766	20	49037728	Т	С	0.551	0.495	0.186	0.122
BIEC2-584911 22 19064667 A G 0.398 0.479 0.182 0.115 BIEC499800 22 30797481 C T 0.296 0.417 0.165 0.087 BIEC499800 22 40787556 T G 0.551 0.495 0.186 0.113 BIEC260840 23 4277770 G A 0.612 0.475 0.181 0.113 BIEC2618284 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC253483 24 12615461 T C 0.296 0.417 0.165 0.087 BIEC2546987 24 27556162 C T 0.694 0.425 0.167 0.090 BIEC551692 24 2456489 A G 0.612 0.475 0.181 0.113 BIEC5516163 24 4571493 C T 0.694 0.425 0.167 0.004 BIEC5541693 <td>BIEC2-575436</td> <td>22</td> <td>2011040</td> <td>С</td> <td>Т</td> <td>0.582</td> <td>0.487</td> <td>0.184</td> <td>0.118</td>	BIEC2-575436	22	2011040	С	Т	0.582	0.487	0.184	0.118
BIEC493879 22 30797481 C T 0.296 0.417 0.165 0.087 BIEC2499860 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2-606469 23 16413178 C G 0.542 0.497 0.187 0.123 BIEC2-618284 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC2-36987 24 12615461 T C 0.229 0.353 0.145 0.062 BIEC531275 24 2255162 C T 0.694 0.425 0.167 0.090 BIEC531275 24 2755162 C T 0.694 0.425 0.167 0.909 BIEC555737 25 12340280 G A 0.635 0.463 0.175 0.103 BIEC554163 26 1937434 G A 0.469 0.493 0.186 0.121 BIEC25450 <td>BIEC2-584911</td> <td>22</td> <td>19064667</td> <td>А</td> <td>G</td> <td>0.398</td> <td>0.479</td> <td>0.182</td> <td>0.115</td>	BIEC2-584911	22	19064667	А	G	0.398	0.479	0.182	0.115
BIECC499860 22 40978756 T G 0.551 0.495 0.186 0.122 BIEC2-606469 23 4277770 G A 0.612 0.475 0.181 0.113 BIEC2608410 23 16413178 C G 0.542 0.497 0.187 0.123 BIEC2618284 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC263483 24 12615461 T C 0.229 0.353 0.145 0.062 BIEC331275 24 12654612 C T 0.694 0.425 0.167 0.090 BIEC5332060 24 28565489 A G 0.612 0.475 0.181 0.113 BIEC541693 24 45741983 C T 0.694 0.425 0.167 0.090 BIEC554813 25 12340280 G A 0.633 0.463 0.173 0.103 BIEC541693<	BIEC493879	22	30797481	С	Т	0.296	0.417	0.165	0.087
BIEC2-606469 23 427770 G A 0.612 0.475 0.181 0.113 BIEC2-618284 23 15413178 C G 0.542 0.497 0.187 0.123 BIEC2-618284 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC2-618284 23 12615461 T C 0.229 0.353 0.145 0.062 BIEC31275 24 17444264 C T 0.694 0.425 0.167 0.090 BIEC531275 24 28656489 A G 0.612 0.477 0.181 0.113 BIEC55737 25 12340280 G A 0.663 0.178 0.107 BIEC51695 26 1937434 G A 0.469 0.498 0.187 0.124 BIEC24651 26 19267701 T C 0.561 0.493 0.166 0.121 BIEC2465 26	BIEC499860	22	40978756	Т	G	0.551	0.495	0.186	0.122
BIECS08410 23 16413178 C G J 0.542 0.497 0.187 0.123 BIEC2-618284 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC5232483 24 12615461 T C 0.229 0.353 0.145 0.062 BIEC532060 24 27556162 C T 0.694 0.425 0.167 0.090 BIEC532060 24 28656489 A G 0.612 0.475 0.181 0.113 BIEC5532060 24 45741983 C T 0.694 0.425 0.167 0.090 BIEC554813 25 14083078 C G 0.347 0.433 0.175 0.103 BIEC25492543 26 1937434 G A 0.469 0.498 0.175 0.103 BIEC25705 26 42126216 A 0.663 0.447 0.173 0.244 BIEC254813 </td <td>BIEC2-606469</td> <td>23</td> <td>4277770</td> <td>G</td> <td>А</td> <td>0.612</td> <td>0.475</td> <td>0.181</td> <td>0.113</td>	BIEC2-606469	23	4277770	G	А	0.612	0.475	0.181	0.113
BIEC2-618284 23 19540553 G T 0.750 0.375 0.152 0.070 BIEC523483 24 12615461 T C 0.229 0.353 0.145 0.062 BIEC536987 24 1744264 C T 0.694 0.425 0.167 0.087 BIEC5302060 24 2856489 A G 0.612 0.475 0.181 0.113 BIEC541693 24 45741983 C T 0.694 0.425 0.167 0.000 BIEC554813 25 12340280 G A 0.635 0.463 0.178 0.107 BIEC581695 26 1937434 G A 0.469 0.498 0.187 0.124 BIEC5262453 26 42126216 A G 0.265 0.390 0.157 0.076 BIEC52708018 27 18560786 C T 0.724 0.399 0.160 0.080 BIEC2-708018<	BIEC508410	23	16413178	С	G	0.542	0.497	0.187	0.123
BIEC523483 24 12615461 T C 0.229 0.353 0.145 0.062 BIEC2-636987 24 17444264 C T 0.694 0.425 0.167 0.090 BIEC531205 24 27856162 C T 0.694 0.425 0.167 0.090 BIEC532060 24 28656489 A G 0.612 0.475 0.181 0.113 BIEC555737 25 12340280 G A 0.635 0.463 0.178 0.107 BIEC555737 25 12340280 G A 0.469 0.498 0.187 0.124 BIEC554813 25 14083078 C G 0.347 0.433 0.167 0.103 BIEC52465 26 19267701 T C 0.561 0.493 0.186 0.121 BIEC591695 26 42126216 A G 0.265 0.390 0.157 0.076 BIEC2-708018 <td>BIEC2-618284</td> <td>23</td> <td>19540553</td> <td>G</td> <td>Т</td> <td>0.750</td> <td>0.375</td> <td>0.152</td> <td>0.070</td>	BIEC2-618284	23	19540553	G	Т	0.750	0.375	0.152	0.070
BIEC2-636987 24 1744264 C T 0.694 0.425 0.167 0.090 BIEC531275 24 27556162 C T 0.296 0.417 0.165 0.087 BIEC532060 24 2856489 A G 0.612 0.475 0.181 0.113 BIEC532060 24 45741983 C T 0.694 0.425 0.167 0.090 BIEC554133 25 12340280 G A 0.635 0.463 0.178 0.107 BIEC54813 25 1937434 G A 0.469 0.498 0.187 0.124 BIEC54265 26 19267701 T C 0.561 0.493 0.186 0.121 BIEC562465 26 4216216 A G 0.265 0.390 0.157 0.076 BIEC2-798018 27 19561701 T A 0.663 0.447 0.173 0.108 BIEC2-721061	BIEC523483	24	12615461	Т	С	0.229	0.353	0.145	0.062
BIEC531275 24 27556162 C T 0.296 0.417 0.165 0.087 BIEC532060 24 28656489 A G 0.612 0.475 0.181 0.113 BIEC551693 24 45741983 C T 0.694 0.425 0.167 0.090 BIEC551737 25 12340280 G A 0.635 0.463 0.178 0.107 BIEC554813 25 14083078 C G 0.347 0.453 0.175 0.103 BIEC571705 26 19267701 T C 0.561 0.493 0.186 0.121 BIEC2-692543 26 30370457 G A 0.664 0.433 0.169 0.094 BIEC2-708018 27 19560786 C T 0.724 0.399 0.160 0.080 BIEC2-721061 27 39476969 G T 0.173 0.287 0.123 0.041 BIEC2-7253	BIEC2-636987	24	17444264	С	Т	0.694	0.425	0.167	0.090
BIEC532060 24 28656489 A G 0.612 0.475 0.181 0.113 BIEC541693 24 45741983 C T 0.694 0.425 0.167 0.090 BIEC555737 25 12340280 G A 0.635 0.463 0.178 0.103 BIEC554813 25 14083078 C G 0.347 0.453 0.175 0.103 BIEC551695 26 1937434 G A 0.469 0.493 0.186 0.121 BIEC2-692543 26 30370457 G A 0.664 0.433 0.169 0.094 BIEC562465 26 42126216 A G 0.265 0.390 0.157 0.076 BIEC2-708018 27 18560786 C T 0.724 0.399 0.160 0.080 BIEC2-721061 27 3903323 T C 0.367 0.465 0.178 0.108 BIEC2-721061	BIEC531275	24	27556162	С	Т	0.296	0.417	0.165	0.087
BIEC541693 24 45741983 C T 0.694 0.425 0.167 0.090 BIEC555737 25 12340280 G A 0.635 0.463 0.178 0.107 BIEC554133 25 14083078 C G 0.347 0.453 0.175 0.103 BIEC551695 26 1937434 G A 0.469 0.498 0.186 0.121 BIEC541693 26 19267701 T C 0.561 0.493 0.186 0.121 BIEC5465 26 42126216 A G 0.265 0.390 0.160 0.080 BIEC5405 26 42126216 A G 0.265 0.399 0.160 0.080 BIEC54058 27 19761701 T A 0.663 0.447 0.173 0.100 BIEC604433 27 3803323 T C 0.367 0.465 0.184 0.188 0.122	BIEC532060	24	28656489	А	G	0.612	0.475	0.181	0.113
BIEC555737 25 12340280 G A 0.635 0.463 0.178 0.107 BIEC554813 25 14083078 C G 0.347 0.453 0.175 0.103 BIEC55105 26 1937434 G A 0.469 0.493 0.186 0.121 BIEC571705 26 19267701 T C 0.561 0.493 0.160 0.094 BIEC5262465 26 42126216 A G 0.265 0.390 0.157 0.076 BIEC590986 27 19761701 T A 0.663 0.447 0.173 0.100 BIEC590986 27 19761701 T A 0.663 0.447 0.173 0.100 BIEC604433 27 39476969 G T 0.173 0.287 0.123 0.041 BIEC67400 28 5913256 G T 0.500 0.500 0.188 0.125 BIEC67700	BIEC541693	24	45741983	С	Т	0.694	0.425	0.167	0.090
BIEC5548132514083078CGG0.3470.4530.1750.103BIEC581695261937434GA0.4690.4980.1870.124BIEC5717052619267701TC0.5610.4930.1860.121BIEC2625432630370457GA0.6840.4330.1690.094BIEC5624652642126216AG0.2650.3900.1570.076BIEC5909862719761701TA0.6630.4470.1730.100BIEC2-721061273947699GT0.1730.2870.1230.041BIEC2725322285600796CT0.6040.4780.1820.114BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC2-737704283086784GT0.4180.4870.1840.118BIEC633181291367203GC0.3270.4400.1720.097BIEC2-7618512929285909TG0.3570.4590.1770.105BIEC2-7618512929285909TG0.5310.4980.1870.124BIEC6330792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.5310.4980.1870.124BIEC6386003012	BIEC555737	25	12340280	G	А	0.635	0.463	0.178	0.107
BIEC581695261937434GA0.4690.4980.1870.124BIEC5717052619267701TC0.5610.4930.1860.121BIEC26925432630370457GA0.6840.4330.1690.094BIEC562465264212616AG0.2650.3900.1570.076BIEC2-7080182718560786CT0.7240.3990.1600.080BIEC25909862719761701TA0.6630.4470.1730.100BIEC2-7210612738093323TC0.3670.4650.1780.108BIEC2-725322285600796CT0.6040.4780.1820.114BIEC2-734103282913256GT0.5000.5000.1880.125BIEC2-737704283865784GT0.4180.4470.1650.087BIEC2-731102913672303GC0.3270.4400.1720.097BIEC331812913672303GC0.3270.4400.1720.097BIEC633979291576506TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.3570.4590.1770.105BIEC2-7618512929285909TG0.5010.4980.1870.124BIEC6964803012032090 </td <td>BIEC554813</td> <td>25</td> <td>14083078</td> <td>С</td> <td>G</td> <td>0.347</td> <td>0.453</td> <td>0.175</td> <td>0.103</td>	BIEC554813	25	14083078	С	G	0.347	0.453	0.175	0.103
BIEC5717052619267701TC0.5610.4930.1860.121BIEC2-6925432630370457GA0.6840.4330.1690.094BIEC5624652642126216AG0.2650.3900.1570.076BIEC2509862719761701TA0.6630.4470.1730.100BIEC2710612738093323TC0.3670.4650.1780.108BIEC2723322285600796CT0.1730.2870.1230.041BIEC2-7341032821956151CT0.5000.5000.1880.121BIEC2-7377042823842936TC0.7040.4170.1650.087BIEC631812913672303GC0.3270.4400.1720.097BIEC631812929285909TG0.3570.4590.1770.105BIEC2-7618512929285909TG0.5310.4980.1870.124BIEC2-7816793307757606TG0.5310.4980.1870.124BIEC2-7816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.5310.4980.1870.124BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150<	BIEC581695	26	1937434	G	А	0.469	0.498	0.187	0.124
BIEC2-6925432630370457GA0.6840.4330.1690.094BIEC5624652642126216AG0.2650.3900.1570.076BIEC2-7080182718560786CT0.7240.3990.1600.080BIEC5909862719761701TA0.6630.4470.1730.100BIEC2-7210612738093323TC0.3670.4650.1780.108BIEC2-725322285600796GT0.6040.4780.1820.114BIEC607490285913256GT0.5000.5000.1880.125BIEC2-731032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.5310.4980.1820.124BIEC6964803012032090TG0.5310.4980.1870.124BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1020.125	BIEC571705	26	19267701	Т	С	0.561	0.493	0.186	0.121
BIEC5624652642126216AG0.2650.3900.1570.076BIEC2-7080182718560786CT0.7240.3990.1600.080BIEC5909862719761701TA0.6630.4470.1730.100BIEC2-7210612738093323TC0.3670.4650.1780.108BIEC2-7253222839476969GT0.1730.2870.1230.041BIEC207490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC6331812913672303GC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.5310.4980.1870.124BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-692543	26	30370457	G	А	0.684	0.433	0.169	0.094
BIEC2-7080182718560786CT0.7240.3990.1600.080BIEC5909862719761701TA0.6630.4470.1730.100BIEC2-7210612738093323TC0.3670.4650.1780.108BIEC6044332739476969GT0.1730.2870.1230.041BIEC2-725322285600796CT0.6040.4780.1820.114BIEC607490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC2-7377042823842936TC0.7040.4170.1650.087BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC562465	26	42126216	А	G	0.265	0.390	0.157	0.076
BIEC5909862719761701TA0.6630.4470.1730.100BIEC2-7210612738093323TC0.3670.4650.1780.108BIEC6044332739476969GT0.1730.2870.1230.041BIEC2-725322285600796CT0.6040.4780.1820.114BIEC607490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3570.4400.1720.095BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-708018	27	18560786	С	Т	0.724	0.399	0.160	0.080
BIEC2-7210612738093323TC0.3670.4650.1780.108BIEC6044332739476969GT0.1730.2870.1230.041BIEC2-725322285600796CT0.6040.4780.1820.114BIEC607490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC590986	27	19761701	Т	А	0.663	0.447	0.173	0.100
BIEC6044332739476969GT0.1730.2870.1230.041BIEC2-725322285600796CT0.6040.4780.1820.114BIEC607490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.102BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.4900.5000.1870.125BIEC2-838630311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-721061	27	38093323	Т	С	0.367	0.465	0.178	0.108
BIEC2-725322285600796CT0.6040.4780.1820.114BIEC607490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.102BIEC2-7618512929285099TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC604433	27	39476969	G	Т	0.173	0.287	0.123	0.041
BIEC607490285913256GT0.5000.5000.1880.125BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-725322	28	5600796	С	Т	0.604	0.478	0.182	0.114
BIEC2-7341032821956151CT0.4390.4930.1860.121BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC607490	28	5913256	G	Т	0.500	0.500	0.188	0.125
BIEC6170702823842936TC0.7040.4170.1650.087BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-734103	28	21956151	С	Т	0.439	0.493	0.186	0.121
BIEC2-7377042830865784GT0.4180.4870.1840.118BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC617070	28	23842936	Т	С	0.704	0.417	0.165	0.087
BIEC6331812913672303GC0.3270.4400.1720.097BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-737704	28	30865784	G	Т	0.418	0.487	0.184	0.118
BIEC6339792915765096TC0.3570.4590.1770.105BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC633181	29	13672303	G	С	0.327	0.440	0.172	0.097
BIEC2-7618512929285909TG0.8670.2300.1020.026BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC633979	29	15765096	Т	С	0.357	0.459	0.177	0.105
BIEC2-816793307757606TG0.5310.4980.1870.124BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-761851	29	29285909	Т	G	0.867	0.230	0.102	0.026
BIEC6868003012032090TG0.1530.2590.1130.034BIEC696480311930525TC0.4900.5000.1870.125BIEC2-8386303116325150CT0.5710.4900.1850.120	BIEC2-816793	30	7757606	Т	G	0.531	0.498	0.187	0.124
BIEC696480 31 1930525 T C 0.490 0.500 0.187 0.125 BIEC2-838630 31 16325150 C T 0.571 0.490 0.185 0.120	BIEC686800	30	12032090	Т	G	0.153	0.259	0.113	0.034
BIEC2-838630 31 16325150 C T 0.571 0.490 0.185 0.120	BIEC696480	31	1930525	Т	С	0.490	0.500	0.187	0.125
	BIEC2-838630	31	16325150	С	Т	0.571	0.490	0.185	0.120

Based on these results, the panel of 120 SNVs is capable of clearly identifying the nonbiological dams of thoroughbred foals. A similar method has been developed using maternally inherited mitochondrial DNA sequences [52]. Such approaches can indirectly

screen for genetically modified animals; however, it is not possible to directly identify the edited sequences. These approaches may also be useful to identify horses that have been produced by somatic cell cloning, another practice that is prohibited in thoroughbreds.

The development of this DNA profile panel is also important to verify the identity of the tested horses. Any horse that is found to be gene-edited will be required to be DNA profiled to ensure the sample was collected from the correct horse. Having the ability to run the identity analysis simultaneously with the gene-editing test enhances the integrity of the sample analysis. Currently, microsatellite analysis is used for thoroughbred parent verification. However, SNVs are being evaluated for this purpose so that once a final panel of SNVs is determined for standard parent verification across all laboratories this should be incorporated into the gene-editing screening process to allow for simultaneous screening and identification.

One of the reasons to use targeted resequencing to identify genome-edited horses instead of whole-genome resequencing is to reduce the cost of such testing. The gene-editing screening test combined with parentage verification using SNVs cost considerably less than WGR, but more than the currently used parentage verification using microsatellites. The two tests combined cost an amount similar to that of parentage verification using SNVs alone. Thus, once SNVs are confirmed for parentage verification, screening thoroughbreds for gene editing at the same time as parentage verification will be cost-efficient. This will benefit all stakeholders including the breeders, owners, racing authorities, and Stud Books in the racing and breeding industries and will also benefit horse welfare by discouraging prohibited and experimental gene editing.

4. Conclusions

The purpose of this study was to construct a screening tool using amplicon sequencing to detect genetically modified thoroughbred horses produced by the use of nonhomologous end-joining with CRISPR/Cas9 in embryos. When 52 gene candidates related to racing performance were targeted and sequenced, 97.7% of the target sequences were covered with sufficient sequence reads to use this method. As a proof of principle, four genetically modified cell lines were screened alongside normal samples. The screening test easily identified the cell lines containing homozygous single-nucleotide insertions, the most efficient product of CRISPR/Cas9 editing. Concurrently, the samples were also DNA profiled, which would allow confirmation of identity if evidence of gene editing were uncovered. It is likely in a real-life gene-editing detection scenario that Sanger sequencing may be necessary to confirm the mutation at that site.

The sequencing of PCR amplified amplicons illustrated that it is extremely difficult to uniformly amplify every targeted genomic region, and some regions may not be covered. Further, it will be difficult to differentiate between artificial and natural heterozygous variants. In this situation, further evidence of gene editing being carried out (for example, access to a laboratory with consumables for gene editing) may be required for successful prosecution. As such, identification of artificially introduced heterozygous variants would not be possible with the gene-editing test developed in this study. Nevertheless, this study is still an important step towards being able to identify genetically engineered thoroughbred racehorses.

Author Contributions: T.T. and A.O. conceived and designed the experiments; T.T. and A.O. performed the experiments and data analyses; K.N., K.H. and M.T. constructed genome-edited cells; N.T. and F.S. collected animal tissues; K.S., Y.T., M.K., T.I., H.K., K.-i.H. and S.-i.N. collected animal samples (hair and blood); N.A.H. provided critical comments and contributed to the discussion of the results, and T.T., A.O. and N.A.H. drafted the manuscript. All authors have reviewed and revised the manuscript and approved its submission. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Blood and hair sample collection in this study was approved by the Animal Care Committee of the Laboratory of Racing Chemistry (approval number 20-4, 13 February 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The primers used have not been listed, as this may prevent their use in actual gene-doping tests. Sequence information of the primers will be provided through a confidentiality agreement with the corresponding author.

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Conflicts of Interest: There are no competing interests, including patents, products in development, or marketed products, to declare in relation to this work.

References

- 1. Tozaki, T.; Hamilton, N.A. Control of gene doping in human and horse sports. *Gene Ther.* 2022, 29, 107–112. [CrossRef] [PubMed]
- Moro, L.N.; Viale, D.L.; Bastón, J.I.; Arnold, V.; Suvá, M.; Wiedenmann, E.; Olguín, M.; Miriuka, S.; Vichera, G. Generation of myostatin edited horse embryos using CRISPR/Cas9 technology and somatic cell nuclear transfer. *Sci. Rep.* 2020, 10, 15587. [CrossRef]
- Kim, D.E.; Lee, J.H.; Ji, K.B.; Park, K.S.; Kil, T.Y.; Koo, O.; Kim, M.K. Generation of genome-edited dogs by somatic cell nuclear transfer. *BMC Biotechnol.* 2022, 22, 19. [CrossRef] [PubMed]
- 4. Eghbalsaied, S.; Kues, W.A. Development of a transposon-based technology for transfection of day 0 chicken embryos. *Gene* **2020**, 730, 144318. [CrossRef] [PubMed]
- 5. Chandrashekran, A.; Casimir, C.; Dibb, N.; Readhead, C.; Winston, R. Generating transgenic mice by lentiviral transduction of spermatozoa followed by in vitro fertilization and embryo transfer. *Methods Mol. Biol.* **2016**, *1448*, 95–106. [PubMed]
- Zakhartchenko, V.; Flisikowska, T.; Li, S.; Richter, T.; Wieland, H.; Durkovic, M.; Rottmann, O.; Kessler, B.; Gungor, T.; Brem, G.; et al. Cell-mediated transgenesis in rabbits: Chimeric and nuclear transfer animals. *Biol. Reprod.* 2011, *84*, 229–237. [CrossRef]
- 7. Dickinson, D.J.; Ward, J.D.; Reiner, D.J.; Goldstein, B. Engineering the caenorhabditis elegans genome using Cas9-triggered homologous recombination. *Nat. Methods.* **2013**, *10*, 1028–1034. [CrossRef]
- Gu, B.; Posfai, E.; Gertsenstein, M.; Rossant, J. Efficient Generation of Large-fragment knock-in mouse models using 2-cell (2C)-homologous recombination (HR)-CRISPR. *Curr. Protoc. Mouse Biol.* 2020, 10, e67. [CrossRef]
- 9. Pavani, G.; Amendola, M. Targeted gene delivery: Where to land. Front. Genome Ed. 2021, 2, 609650. [CrossRef]
- Allen, F.; Crepaldi, L.; Alsinet, C.; Strong, A.J.; Kleshchevnikov, V.; De Angeli, P.; Páleníková, P.; Khodak, A.; Kiselev, V.; Kosicki, M.; et al. Predicting the mutations generated by repair of Cas9-induced double-strand breaks. *Nat. Biotechnol.* 2018, 37, 64–72. [CrossRef]
- Song, B.; Yang, S.; Hwang, G.H.; Yu, J.; Bae, S. Analysis of NHEJ-based DNA repair after CRISPR-mediated DNA cleavage. *Int. J. Mol. Sci.* 2021, 22, 6397. [CrossRef] [PubMed]
- 12. Proudfoot, C.; Carlson, D.F.; Huddart, R.; Long, C.R.; Pryor, J.H.; King, T.J.; Lillico, S.G.; Mileham, A.J.; McLaren, D.G.; Whitelaw, C.B.; et al. Genome edited sheep and cattle. *Transgenic Res.* **2015**, *24*, 147–153. [CrossRef] [PubMed]
- Mizuno-Iijima, S.; Ayabe, S.; Kato, K.; Matoba, S.; Ikeda, Y.; Dinh, T.T.H.; Le, H.T.; Suzuki, H.; Nakashima, K.; Hasegawa, Y.; et al. Efficient production of large deletions and gene fragment knock-in mice mediated by genome editing with Cas9-mouse Cdt1 in mouse zygotes. *Methods* 2021, 191, 23–31. [CrossRef] [PubMed]
- Tozaki, T.; Gamo, S.; Takasu, M.; Kikuchi, M.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Digital PCR detection of plasmid DNA administered to the skeletal muscle of a microminipig: A model case study for gene doping detection. *BMC Res. Notes* 2018, 11, 708. [CrossRef]
- 15. Tozaki, T.; Ohnuma, A.; Takasu, M.; Kikuchi, M.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Droplet digital PCR detection of the erythropoietin transgene from horse plasma and urine for gene-doping control. *Genes* **2019**, *10*, 243. [CrossRef]
- Tozaki, T.; Ohnuma, A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Microfluidic quantitative PCR detection of 12 transgenes from horse plasma for gene doping control. *Genes* 2020, 11, 457. [CrossRef]
- 17. Tozaki, T.; Ohnuma, A.; Iwai, S.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Robustness of digital PCR and real-time PCR in transgene detection for gene-doping control. *Anal. Chem.* **2021**, *93*, 7133–7139. [CrossRef]
- Tozaki, T.; Ohnuma, A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Robustness of digital PCR and real-time PCR against inhibitors in transgene detection for gene doping control in equestrian sports. *Drug Test. Anal.* 2021, 13, 1768–1775. [CrossRef]
- 19. Tozaki, T.; Ohnuma, A.; Hamilton, N.A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Low-copy transgene detection using nested digital polymerase chain reaction for gene-doping control. *Drug Test. Anal.* 2022, 14, 382–387. [CrossRef]

- 20. Cheung, H.W.; Wong, K.S.; Lin, V.Y.C.; Wan, T.S.M.; Ho, E.N.M. A duplex qPCR assay for human erythropoietin (EPO) transgene to control gene doping in horses. *Drug Test. Anal.* **2021**, *13*, 113–121. [CrossRef]
- Cheung, H.W.; Wong, K.S.; Lin, V.Y.C.; Farrington, A.F.; Bond, A.J.; Wan, T.S.M.; Ho, E.N.M. Optimization and implementation of four duplex quantitative polymerase chain reaction assays for gene doping control in horseracing. *Drug Test. Anal.* 2022; in press. [CrossRef] [PubMed]
- Haughan, J.; Jiang, Z.; Stefanovski, D.; Moss, K.L.; Ortved, K.F.; Robinson, M.A. Detection of intra-articular gene therapy in horses using quantitative real time PCR in synovial fluid and plasma. *Drug Test. Anal.* 2020, 12, 743–751. [CrossRef] [PubMed]
- 23. Jiang, Z.; Haughan, J.; Moss, K.L.; Stefanovski, D.; Ortved, K.F.; Robinson, M.A. A quantitative PCR screening method for adeno-associated viral vector 2-mediated gene doping. *Drug Test. Anal.* 2022, 14, 963–972. [CrossRef] [PubMed]
- 24. Shigemizu, D.; Asanomi, Y.; Akiyama, S.; Mitsumori, R.; Niida, S.; Ozaki, K. Whole-genome sequencing reveals novel ethnicity-specific rare variants associated with Alzheimer's disease. *Mol. Psychiatry* **2022**, *27*, 2554–2562. [CrossRef] [PubMed]
- 25. Jin, L.; Qu, K.; Hanif, Q.; Zhang, J.; Liu, J.; Chen, N.; Suolang, Q.; Lei, C.; Huang, B. Whole-genome sequencing of endangered dengchuan cattle reveals its genomic diversity and selection signatures. *Front. Genet.* **2022**, *13*, 833475. [CrossRef]
- Hack, Y.L.; Crabtree, E.E.; Avila, F.; Sutton, R.B.; Grahn, R.; Oh, A.; Gilger, B.; Bellone, R.R. Whole-genome sequencing identifies missense mutation in GRM6 as the likely cause of congenital stationary night blindness in a Tennessee Walking Horse. *Equine Vet.* J. 2021, 53, 316–323. [CrossRef]
- Tozaki, T.; Ohnuma, A.; Takasu, M.; Nakamura, K.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirora, K.; Tamura, N.; Kusano, K.; et al. Detection of non-targeted transgenes by whole-genome resequencing for gene-doping control. *Gene Ther.* 2021, 28, 199–205. [CrossRef]
- Tozaki, T.; Ohnuma, A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Simulated validation of intron-less transgene detection using DELLY for gene-doping control in horse sports. *Anim. Genet.* 2021, 52, 759–761. [CrossRef]
- Tozaki, T.; Ohnuma, A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Identification of processed pseudogenes in the genome of Thoroughbred horses: Possibility of gene-doping detection considering the presence of pseudogenes. *Anim. Genet.* 2022, 53, 183–192. [CrossRef]
- Maniego, J.; Pesko, B.; Hincks, P.; Taylor, P.; Stewart, G.; Proudman, C.; Scarth, J.; Ryder, E. Direct sequence confirmation of qPCR products for gene doping assay validation in horses. *Drug Test. Anal.* 2022, 14, 1017–1025. [CrossRef]
- 31. Maniego, J.; Pesko, B.; Habershon-Butcher, J.; Huggett, J.; Taylor, P.; Scarth, J.; Ryder, E. Screening for gene doping transgenes in horses via the use of massively parallel sequencing. *Gene. Ther.* **2022**, *29*, 236–246. [CrossRef] [PubMed]
- Metzger, J.; Gast, A.C.; Schrimpf, R.; Rau, J.; Eikelberg, D.; Beineke, A.; Hellige, M.; Distl, O. Whole-genome sequencing reveals a
 potential causal mutation for dwarfism in the Miniature Shetland pony. *Mamm. Genome* 2017, 28, 143–151. [CrossRef] [PubMed]
- Sinha, R.; Pradhan, S.; Banerjee, S.; Jahan, A.; Akhtar, S.; Pahari, A.; Raut, S.; Parakh, P.; Basu, S.; Srivastava, P.; et al. Whole-exome sequencing and variant spectrum in children with suspected inherited renal tubular disorder: The East India Tubulopathy Gene Study. *Pediatr. Nephrol.* 2022, 37, 1811–1836. [CrossRef] [PubMed]
- Morishita, A.; Iwama, H.; Fujihara, S.; Watanabe, M.; Fujita, K.; Tadokoro, T.; Ohura, K.; Chiyo, T.; Sakamoto, T.; Mimura, S.; et al. Targeted sequencing of cancer-associated genes in hepatocellular carcinoma using next-generation sequencing. *Oncol. Lett.* 2018, 15, 528–532. [CrossRef] [PubMed]
- 35. Hirota, K.; Kakoi, H.; Gawahara, H.; Hasegawa, T.; Tozaki, T. Construction and validation of parentage testing for thoroughbred horses by 53 single nucleotide polymorphisms. *J. Vet. Med. Sci.* **2010**, *72*, 719–726. [CrossRef]
- Holl, H.M.; Vanhnasy, J.; Everts, R.E.; Hoefs-Martin, K.; Cook, D.; Brooks, S.A.; Carpenter, M.L.; Bustamante, C.D.; Lafayette, C. Single nucleotide polymorphisms for DNA typing in the domestic horse. *Anim. Genet.* 2017, 48, 669–676. [CrossRef]
- Li, H.; Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009, 25, 1754–1760. [CrossRef]
- McLaren, W.; Gil, L.; Hunt, S.E.; Riat, H.S.; Ritchie, G.R.; Thormann, A.; Flicek, P.; Cunningham, F. The Ensembl Variant Effect Predictor. *Genome Biol.* 2016, 17, 122. [CrossRef]
- Tozaki, T.; Ohnuma, A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, I.; Hamilton, N.A.; Kusano, K.; Nagata, S. Whole-genome resequencing using genomic DNA extracted from horsehair roots for gene-doping control in horse sports. *J. Equine Sci.* 2020, *31*, 75–83. [CrossRef]
- Chakrabarti, A.M.; Henser-Brownhill, T.; Monserrat, J.; Poetsch, A.R.; Luscombe, N.M.; Scaffidi, P. Target-Specific Precision of CRISPR-Mediated Genome Editing. *Mol. Cell* 2019, 73, 699–713. [CrossRef]
- Grobet, L.; Poncelet, D.; Royo, L.J.; Brouwers, B.; Pirottin, D.; Michaux, C.; Ménissier, F.; Zanotti, M.; Dunner, S.; Georges, M. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mamm. Genome* 1998, 9, 210–213. [CrossRef] [PubMed]
- 42. Schuelke, M.; Wagner, K.R.; Stolz, L.E.; Hübner, C.; Riebel, T.; Kömen, W.; Braun, T.; Tobin, J.F.; Lee, S.J. Myostatin mutation associated with gross muscle hypertrophy in a child. *N. Engl. J. Med.* **2004**, *350*, 2682–2688. [CrossRef] [PubMed]
- 43. Wang, K.; Ouyang, H.; Xie, Z.; Yao, C.; Guo, N.; Li, M.; Jiao, H.; Pang, D. Efficient generation of myostatin mutations in pigs using the CRISPR/Cas9 System. *Sci. Rep.* 2015, *5*, 16623. [CrossRef] [PubMed]
- 44. Mosher, D.S.; Quignon, P.; Bustamante, C.D.; Sutter, N.B.; Mellersh, C.S.; Parker, H.G.; Ostrander, E.A. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. *PLoS Genet.* 2007, 3, e79. [CrossRef] [PubMed]

- Hill, E.W.; Gu, J.; Eivers, S.S.; Fonseca, R.G.; McGivney, B.A.; Govindarajan, P.; Orr, N.; Katz, L.M.; MacHugh, D.E. A sequence polymorphism in MSTN predicts sprinting ability and racing stamina in thoroughbred horses. *PLoS ONE* 2010, *5*, e8645. [CrossRef]
- 46. Rooney, M.F.; Hill, E.W.; Kelly, V.P.; Porter, R.K. The "speed gene" effect of myostatin arises in Thoroughbred horses due to a promoter proximal SINE insertion. *PLoS ONE* **2018**, *13*, e0205664. [CrossRef]
- 47. Miyata, H.; Itoh, R.; Sato, F.; Takebe, N.; Hada, T.; Tozaki, T. Effect of Myostatin SNP on muscle fiber properties in male Thoroughbred horses during training period. *J. Physiol. Sci.* **2018**, *68*, 639–646. [CrossRef]
- Edvardsen, R.B.; Leininger, S.; Kleppe, L.; Skaftnesmo, K.O.; Wargelius, A. Targeted mutagenesis in Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. *PLoS ONE* 2014, 9, e108622. [CrossRef]
- 49. Tozaki, T.; Ohnuma, A.; Kikuchi, M.; Ishige, T.; Kakoi, H.; Hirota, K.; Kusano, K.; Nagata, S. Rare and common variant discovery by whole-genome sequencing of 101 Thoroughbred racehorses. *Sci. Rep.* **2021**, *11*, 16057. [CrossRef]
- Galli, C.; Colleoni, S.; Duchi, R.; Lagutina, I.; Lazzari, G. Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim. Reprod. Sci.* 2007, *98*, 39–55. [CrossRef]
- Liu, Z.; Cai, Y.; Liao, Z.; Xu, Y.; Wang, Y.; Wang, Z.; Jiang, X.; Li, Y.; Lu, Y.; Nie, Y.; et al. Cloning of a gene-edited macaque monkey by somatic cell nuclear transfer. *Natl. Sci. Rev.* 2019, *6*, 101–108. [CrossRef] [PubMed]
- Maniego, J.; Pesko, B.; Habershon-Butcher, J.; Hincks, P.; Taylor, P.; Tozaki, T.; Ohnuma, A.; Stewart, G.; Proudman, C.; Ryder, E. Use of mitochondrial sequencing to detect gene doping in horses via gene editing and somatic cell nuclear transfer. *Drug Test. Anal.* 2022, 14, 1429–1437. [CrossRef] [PubMed]