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Generation of albino C57BL/6J mice by CRISPR embryo editing of the mouse tyrosinase locus

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Abstract:	After the arrival of the CRISPR/Cas9 genome editing technology, genetic engineering of model organisms has become much faster and more efficient. Development of genetically modified mouse models is also facilitated by the application of various CRISPR methodologies. Although, the very first studies utilized pronuclear injection of Cas9 mRNA and sgRNAs into the zygote stage embryos to create knockout and knockin mutations, the repertoire of techniques and collection of reagents for CRISPR editing have rapidly expanded. This presents researchers in the field with a versatility of choices for genetic engineering. However, there are not many comparative studies that analyzed the efficacy of gene editing when Cas9 and sgRNA/ssDNA oligos were transferred to the embryos by different methodologies. Here, we aimed to compare two different methods, electroporation and pronuclear injection (PNI). One of the recent developments gaining wide use in mouse models research, was the application of electroporation for introduction of Cas9/sgRNA ribonucleoprotein (RNP) complexes into zygote stage embryos. Here, we have used this technique to generate albino coat colored C57BL/6J mice by targeted inactivation of the mouse tyrosinase gene through indel or knockin mutations. We have also applied the PNI rotocol with the same set of reagents, to compare the efficiency of the two techniques in generation indel and knockin mutations. Although PNI results in a significantly higher efficiency for knockin mutations, it requires specialized equipment setup, and advanced training in embryo

	micromanipulation and microinjection. Therefore, for generation of simple gene knockouts by indel mutations, electroporation can be used.
Response to Reviewers:	A detailed response letter has been uploaded

We thank both reviewers for their constructive comments and recommendations that improved our manuscript. Below, you can find responses to specific comments. Changes that have been made in the manuscript to address the referee comments have been labeled in red font.

Reviewer 1:

The manuscript by Diril et. al. reports an electroporation-based method for generating gone knockout and knock-in mice models. There are multiple, more efficient methods of electroporation reported by other groups which are extensively used by multiple research groups. However, there are few methods of electroporation of mouse embryos using the gene pulser electroporator. This report will be of value to the mouse transgenic community.

The manuscript is written clearly and in simple language. There are some shortcomings in the manuscript that require further clarifications and actions.

1. The Figure 1A legend describes it as a Tyrosine gene locus, but in the figure a small part of the locus is shown, not the complete gene locus. Please represent the complete gene locus.

Figure 1A was modified according to the revievers' suggestions. The new figure represents the complete locus and a magnified inset of the edited region.

2. The figure 2 shows the genotyping data of the mutant locus. the data provided in this figure is limited to the PCR and restriction digestion analysis. The authors should provide the Sanger Sequencing data along with the chromatogram for the mutant alleles.

We have prepared a novel supplementary figure showing Sanger sequencing data for additional embryos.

3. The authors seem to have ignored a very important electroporation method used in mice in their discussion. The oviduct electroporation method of i-GONAD may be discussed in the context of their own results. Comparisons of the efficiency with the i-GONAD method may be discussed in the discussion section of the manuscript.

We thank the referee for pointing this out. In the revised manuscipt, we have added a discussion of the i-GONAD method.

Reviewer 2:

The following edits and additional details will significantly enhance the clarity, completeness, and scientific rigor of the manuscript.

Page 3:

Background Section: The problem statement is not included in lines 10-12. To enhance the clarity and purpose of the study, it is important to explicitly state the problem that this research aims to address. Including a concise problem statement will provide better context and relevance for the subsequent methods and results discussed.

We have modified the abstract section to include a concise problem statement.

Method Section: The description of the method in lines 15-16 is repeated and can be omitted to avoid redundancy. Streamlining this section will improve readability and focus on the unique aspects of the methodology.

The two sentences with repeated phrases were joined in a single sentence.

Comparative Analysis: There is a mention of a comparative analysis in lines 23-24 related to the use of PNI (Pronuclear Injection). It would be beneficial to expand on this analysis by providing more details on the parameters compared, the outcomes, and their implications. This will help readers understand the advantages and limitations of PNI in the context of the study.

We have modified the abstract section according to the reviewer's suggestions.

Page 5:

Gene Structure in Figures: In line 1, the figure should include the entire gene structure, not just exon 1. This will provide a more comprehensive understanding of the gene's architecture and its modifications. Including the full gene structure will also help in visualizing the context of exon 1 within the entire gene.

Figure 1A was modified according to the revievers' suggestions. The new figure represents the complete locus and a magnified inset of the edited region.

Page 6:
Embryo Viability Data: In lines 17-18, it is crucial to include data on embryo viability post-injection/electroporation and transfer. This information will provide insights into the efficiency and potential side effects of the CRISPR editing process on embryo survival, which is vital for evaluating the overall success and feasibility of the methodology used.

We have added additional info on embryo viability in the results section (2.5 Electroporation vs. PNI).

Page 7:
Efficiency of Homologous Recombination: There is a need to include data on the efficiency of homologous recombination. This information is essential to understand how effectively the introduced changes are incorporated into the genome. Additionally, in lines 11-13, it is mentioned that a frameshift mutation results in roughly two-thirds of the alleles generated after repair. It would be valuable to discuss whether the use of ssODN (single-stranded oligodeoxynucleotides) increases the incidence of indels (insertions or deletions). This discussion will provide a deeper understanding of the outcomes and efficiency of the CRISPR editing process.

We have added additional clarifications on efficiency comparison in results and discussion sections.

Discussion:
Comparative Analysis of Knockout Generation: From page 8, line 18 onwards, there is a lack of comparative analysis in the discussion regarding the generation of knockouts either by introducing indels or by providing the template ssODN. The study involves generating knockouts using both methods, but there is no calculation or experimental evidence presented on the percentage of indels generating knockout efficiency without ssODN versus the generation of knockouts using ssODN. Including this comparative data will strengthen the discussion by providing a clear evaluation of the methods used, their efficiency, and their impact on knockout generation.

We have added additional text for comparison of different methods for generation of knockouts.

Generation of albino C57BL/6J mice by CRISPR embryo editing of the mouse tyrosinase locus

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Generation of albino C57BL/6J mice by CRISPR embryo editing of the mouse tyrosinase locus

ABSTRACT

Background: After the arrival of the CRISPR/Cas9 genome editing technology, genetic engineering of model organisms has become much faster and more efficient. Development of genetically modified mouse models is also facilitated by the application of various CRISPR methodologies. Although, the very first studies utilized pronuclear injection of Cas9 mRNA and sgRNAs into the zygote stage embryos to create knockout and knockin mutations, the repertoire of techniques and collection of reagents for CRISPR editing have rapidly expanded. This presents researchers in the field with a versatility of choices for genetic engineering. However, there are not many comparative studies that analyzed the efficacy of gene editing when Cas9 and sgRNA/ssDNA oligos were transferred to the embryos by different methodologies. Here, we aimed to compare two different methods, electroporation and pronuclear injection (PNI).

Methods and Results: One of the recent developments gaining wide use in mouse models research, was the application of electroporation for introduction of Cas9/sgRNA ribonucleoprotein (RNP) complexes into zygote stage embryos. Here, we have used this technique to generate albino coat colored C57BL/6J mice by targeted inactivation of the mouse tyrosinase gene through indel or knockin mutations. We have also applied the PNI protocol with the same set of reagents, to compare the efficiency of the two techniques in generation indel and knockin mutations.

Conclusions: Although PNI results in a significantly higher efficiency for knockin mutations, it requires specialized equipment setup, and advanced training in embryo micromanipulation and microinjection. Therefore, for generation of simple gene knockouts by indel mutations, electroporation can be used.

KEYWORDS:

CRISPR, Cas9, tyrosinase, genetically modified mouse models, knockout, knockin, homology-directed repair, non-homologous end joining

1. INTRODUCTION

To elucidate the molecular mechanism and understand the etiology of many human diseases, one of the most successful approaches is the development of animal models that can precisely recapitulate the genetic alterations or mutations seen in the patients (McGonigle and Ruggeri 2014). Although each model organism has its own advantages and uses in biomedical research, mouse models constitute the golden standard due to their phylogenetic relatedness and physiological similarity to human, their ease of use in the laboratory and the availability of various techniques for their genetic manipulation (Perlman 2016).

Genetically engineered mice are used widely in biomedical research for studying gene function and human disease resulting from genetic alterations(Sharpless and DePinho 2006). They are of paramount importance and relevance for the study of rare genetic disorders (Wong *et al.* 2002). These mice provide a powerful tool to investigate the underlying molecular mechanisms, pathogenesis, and possible therapeutic strategies for these conditions. By introducing precise

genetic modifications that recapitulate specific disease-causing mutations found in humans, it is possible to replicate the genetic basis of rare diseases in mice. This approach allows for the thorough examination of disease development, organ or tissue-based pathologies, and gene-environment interactions that contribute to disease manifestation(Sun *et al.* 2014). The use of genetically modified mouse models also facilitates the identification and validation of potential disease biomarkers and therapeutic targets, accelerating the development of novel treatments(Sardiello *et al.* 2009).

Conventionally, genetically modified mouse models have been obtained by gene targeting in embryonic stem cells which are subsequently microinjected into blastocyst stage mouse embryos for production of germline chimeric mice (Capecchi, 1989). However, generating chimeric mice by this methodology is difficult as it requires advanced infrastructure and technical skills (Yang *et al.* 2014; Huijbers *et al.* 2015).

After the initial discovery in 2012 (Jinek *et al.* 2012), CRISPR/Cas9 genome engineering technology has been rapidly applied to develop mouse models with genetic manipulations. The very first landmark studies utilized pronuclear or intracytoplasmic microinjection of Cas9 mRNA and sgRNAs, either with or without repair templates (Wang *et al.* 2013; Yang *et al.* 2013). Although this increased the efficiency of gene editing and sped up the entire process of creating mouse models, specialized infrastructure and manpower are still a requirement.

To bypass specialized instrument requirements, three novel methodologies have recently been developed. In the so called CRISPR-EZ or CRISPR-EEZy methods, Cas9/sgRNA RNP complexes and short single-stranded oligonucleotides (ssODN) serving as DNA homology directed repair (HDR) templates with sizes up to 150 bps can be introduced to the zygote stage mouse embryos by a simple electroporation device (Qin *et al.* 2015; Tröder *et al.* 2018; Modzelewski *et al.* 2018). On the other hand, CRISPR-READI method uses adeno-associated virus (AAV) vectors for HDR donor delivery together with Cas9/sgRNA RNP electroporation into zygotes. This allows generation of larger (up to 4.9 kb) site-specific modifications in the mouse genome with upper limit set by AAV insert packaging (Chen *et al.* 2019). Finally, in the i-GONAD (Improved Genome editing via Oviductal Nucleic Acids Delivery) method, zygote stage embryos present in the oviducts of pregnant females are electroporated using tweezer-type electrodes. I-GONAD procedure is technically the least demanding method as it does not require isolation and ex vivo handling of embryos, and their subsequent transfer to recipient females (Takahashi *et al.* 2015; Gurumurthy *et al.* 2016, 2019; Ohtsuka *et al.* 2018).

Here, we have applied the electroporation protocol for engineering a knockout mutation in the *Tyr* locus of the C57BL/6 mice. Loss of function in the tyrosinase enzyme results in albino coat color(Kwon *et al.* 1987), which makes it possible to visually monitor the gene editing efficiency.

2. RESULTS

2.1. Targeting mouse *Tyr* locus and genotyping strategy

The mouse *Tyrosinase* gene locus stands as a basis in genetic research, with its historical significance and key role in understanding mammalian pigmentation genetics. Originally the *Tyr*

gene was identified due to its involvement in albinism. It encodes the key enzyme in melanin biosynthesis (Kwon et al. 1987; Bennett and Lamoreux 2003a). It has gained renewed significance through recent advancements in genome engineering technologies, particularly in the field of CRISPR-based studies. The discovery of CRISPR-Cas9 technology has facilitated precise genetic modifications, enabling researchers to utilize the Tyr locus as a framework for the comparison of various CRISPR based tools. Several studies have employed CRISPR-Cas9 to generate knockouts of the Tyrosinase gene, resulting in albino mice devoid of melanin production (Mizuno *et al.* 2014; Qin *et al.* 2015; Tröder *et al.* 2018).

We have used an sgRNA that targets the first coding exon of the mouse Tyr gene. This exon codes for the first 273 amino acids of the Tyrosinase enzyme and it is critical for its function. Introduction of a DNA double strand break (DSB) at this site (codons 78 and 79) by Cas9 nuclease and subsequent repair by NHEJ (Non-homologous end joining) introduces indel mutations and changes the HinFI restriction enzyme target sequence (GANTC). This results in destruction of the HinFI site, and a frameshift mutation results roughly in two-thirds of the alleles that are generated after repair (Modzelewski *et al.* 2018). Addition of a repair template in the form of an ssODN molecule enables homology directed in the site (knockin) and introduction of a premature STOP codon and a novel EcoRI restriction site. A premature STOP codon is introduced after amino acid 85 (Figure 1).

The presence of indel and knockin alleles can be screened and differentiated by restriction analysis of a PCR amplification product. A semi-nested PCR approach was used wherein primer pair 431-433 (251 bp amplicon in WT allele) and 432-433 (198 bp) were amplified in first and second PCR respectively. The products of the first PCR were 200-fold diluted and used as a template in the second PCR. This approach consistently yielded clear bands even when minute quantities of DNA template were used, such as from morulae stage embryos.

2.2. Embryo electroporation, transfer, and development of founder mice

To assess the efficiency of the genome editing by this strategy, we first cultured the embryos *ex vivo* and analyzed their genotypes as described above. To this end, zygote stage embryos isolated from super ovulated C57BL/6J female mice were electroporated with Cas9/sgRNA RNP complexes and ssODNs serving as HDR template, as explained in the methods section. After 72 hours incubation in the embryo culture medium, roughly half of the embryos developed to late morula/blastocyst stage. These embryos were collected and lysed in HotShot lysis solution(Truett *et al.* 2000). After addition of the neutralization buffer, 10-20% of the DNA lysate was used as a template in a nested PCR reaction to amplify the Tyr alleles we generated (Figure 2A).

Restriction digest analyses using HinFI and EcoRI enzymes was carried out to distinguish between indel and knockin alleles. PCR products from most embryos were not cut or partially cut by HinFI. This suggests the loss of HinFI restriction site, which can occur due to indel mutations or HDR mediated generation of the knockin allele. We observed EcoRI cleavage only in 10-20% of the samples. EcoRI digest was not complete in any of the embryos, suggesting genetic mosaicism

(Mehravar *et al.* 2019) with mixed indel/knockin alleles in the cells making up the embryo (Figure 2B).

After confirming the successful CRISPR editing in *ex vivo* cultured embryos, we proceeded to generation of genetically edited mice. After electroporation, the embryos were surgically transferred to the oviducts of pseudopregnant CD1 female mice. Three weeks after the embryo transfer, four pups were born. Two of the newborn pups (#1 and #2) were black, whereas the other two (#3 and #4) had albino coat color. Albino coat color can result from inactivation of both copies of the Tyr gene, which can be due to indel mutations or HDR-mediated knockin mutations, or a combination of these.

To genetically characterize the alleles, present in the founder mice, tail biopsies were taken, and genomic DNA was prepared. PCR amplification and restriction analysis showed that there was no genome editing in the black pups as their restriction pattern was identical to WT allele's. On the other hand, DNA fragments amplified from the albino pups were not cut by HinFI, suggesting a complete loss of the HinFI site by indel mutations. EcoRI digestion showed the presence of knockin allele in pup #4, however the proportion of this allele was low (Figure 3A and 3B).

2.3. Development of an albino C57BL/6J colony

To establish an albino C57BL/6J mouse colony, we used pup #3 (male) as the colony founder. Pup #4 did not survive until the weaning age. After reaching sexual maturity, we crossed this founder male with three WT female mice with C57BL/6J genetic background. 27 heterozygous pups were born, and their genotypes were analyzed as described earlier. All these pups were expected to be heterozygous. They would inherit a nonfunctional mutant allele from their albino father and a WT allele from their mothers. Although all pups would inherit a mutant allele, they are expected to have at least two, and potentially more, distinct mutant alleles due to versatility in repair of the DNA DSBs by NHEJ pathway(Maruyama *et al.* 2015; Paquet *et al.* 2016).

Amplified PCR products were separated by agarose gel electrophoresis. Image analysis showed a single DNA band that was running at the same size as WT, obtained from 18 of the pups (hereafter annotated as allele A or +/A). The remainder (9 pups) displayed a double band pattern with a secondary higher MW band (annotated as allele B or +/B) (Figure 3C).

The PCR products were cut with HinFI restriction enzyme. Unexpectedly, PCR products from samples with+/A genotype were fully cut with HinFI. Whereas the rest of the samples were partially cut, which would be expected from a mixed PCR product with WT and mutant alleles (Figure3D).

Complete digestion of the PCR product amplified from samples with +/A genotype could occur in two conditions. The mutation could inactivate the tyrosinase function without changing the HinFI recognition sequence. Or the mutation could cause a large deletion and mutant allele cannot be amplified. In the latter case, only the WT allele would be amplified. Restriction digestion of the PCR product from the founder mouse was not cut with HinFI (Figure 3D). This suggests the mutant allele

A is a large deletion, which cannot be amplified by the PCR primers. Our subsequent analysis below confirmed this conclusion.

2.4. Characterization of the mutant alleles

We intercrossed heterozygous mice with the same allele combinations with each other to obtain homozygous albino pups with A/A or B/B genotype. Roughly 25% of the pups from these crosses had albino coat color as expected from the Mendelian distribution of a non-pathogenic allele (data not shown). To characterize allele A, we designed a primer pair (520 and 521) and amplified a longer amplicon (525 bp) surrounding the target site (Figure 1A). Although the PCR yielded the expected size products from samples with WT and B/B genotype, there was no amplification in A/A samples (Figure 4A). This suggests that allele A is a larger deletion that cannot be amplified with selected primers.

Next, we used the PCR products from the albino pups with B/B genotype for Sanger sequencing. Detailed analysis of the results showed that, allele B had a 14 bp long sequence (GCCAGGACTCACGG) deleted and a 20 bp sequence (CCTATTATAAAACACAGAGG) inverted compared to the WT sequence (Figure 4B). This results in amino acid substitutions at codons 131 and 132 (from Arg-Glu to Leu-Leu) and conversion of the codon 133 to a STOP codon (Figure 4C). The protein product would be truncated from 533 to 132 amino acids. Therefore, allele B is expected to cause a complete loss of function of the *Tyr* gene.

2.5. Electoporation vs. PNI

Next, we decided to use PNI technique for transferring RNP complexes and ssODN fragments to the zygote stage embryos. Embryos were collected essentially as before. However, collected embryos were not treated with acid Tyrode's solution, as thinning of the zona is not required for injection. Embryos, that were successfully injected, were collected, and cultured for 72 hours until the blastocyst stage. Essentially, nearly half of the embryos were lysed during the PNI procedure and about half of the cultured embryos did not develop to mature blastocysts. Therefore, roughly 75% of the embryos were lost or degenerated during the whole procedure, bringing embryo viability to 25%. In comparison, less than 10% of the embryos are lost after the electroporation and half of these grow to blastocyst stage, giving 45% viability.

Genotyping of the recovered blastocysts by PCR and restriction analysis by HinFI showed that, *Tyr* locus was efficiently targeted in all embryos. EcoRI digestion of the PCR products showed that, 18 out of the 22 embryos had undergone homologous recombination and knockin mutation was inserted into the *Tyr* gene (Figure 5). Therefore PNI had an efficiency of 100% for indel mutations and 82% for HDR based knockin mutation. Although efficiency of indel mutations after electroporation was high (17 out of 18 embryos), knockin efficiency was significantly lower than PNI, with 4 out 18 embryos carrying the knockin allele (22%) (Figure 2). Higher efficiency of PNI is not only reflected in the percentage of edited embryos. Closer inspection of EcoRI restriction digest results (comparison of Figure 2B with Figure 5) suggests that, higher percentage of the PCR products are cut, suggesting a higher proportion of knockin alleles after PNI.

Therefore, PNI injection was found to be significantly more efficient than electroporation for introduction of knockin mutations.

3. DISCUSSION

The genes and loci determining the coat color in mice have been extensively studied by mouse geneticists (Dorothy C Bennett and Lamoreux 2003). Tyrosinase locus has been of special interest, as the absence of its gene product, the tyrosinase enzyme, causes a pigmentation defect and turns the coat color to albino. A tyrosinase mutation causing albinism in C57BL/6 mice spontaneously arose at Jackson Lab and it is widely used (Le Fur *et al.* 1996). ES cells derived from albino mice are available and often utilized in transgenic studies (Abu Alhaija *et al.* 2024). Thanks to the obvious phenotype that develops upon its knockout, *tyr* gene has been frequently exploited for development and optimization of CRISPR based gene editing protocols in mouse embryos (Chen *et al.* 2016; Modzelewski *et al.* 2018).

For development of the very first genetically engineered mouse models using CRISPR technology, Cas9 nuclease/sgRNA complexes, with or without repair templates, were injected into the pronucleus or cytoplasm of mouse zygotes(Yang *et al.* 2013, 2014). Subsequent studies developed electroporation protocols that do not require advanced microinjection setup (Qin *et al.* 2015; Gurumurthy *et al.* 2016, 2019; Chen *et al.* 2016; Tröder *et al.* 2018). Comparative studies that asses the efficiency of introducing the CRISPR/Cas9 system by microinjection and electroporation have been performed (Modzelewski *et al.* 2018; Le *et al.* 2021). These studies did not find an obvious difference in editing efficiency; however embryo viability was found to be higher after electroporation. In our experiments, we observed a significant increase in knockin mutation efficiency when PNI was used (~80%) in comparison to electroporation (~20%). This was also higher than obtained through the i-GONAD procedure (~50%) (Gurumurthy *et al.* 2019).

Another area that saw continuous improvement over the years, has been the efficiency of homologous recombination. Most research projects require introduction of tailored mutations into the gene locus (knockin) rather than a simple loss of gene function (knockout). Therefore, several studies explored ways to skew the repair mechanism of the DNA DSBs towards HDR at the expense of NHEJ. In one such study, use of asymmetric DNA repair templates that make use of Cas9/DNA interaction dynamics, was shown to increase the HDR efficiency significantly (Richardson *et al.* 2016). In another widely applied technique, inhibition of DNA ligase IV, a key enzyme in the NHEJ pathway, has been shown to dramatically increase the efficiency of HDR when PNI was used (Maruyama *et al.* 2015; Ma *et al.* 2016), however it had no effect on i-GONAD-mediated KI efficiency (Takabayashi *et al.* 2018).

Here we have generated a novel albino mouse model with C57BL/6J genetic background using CRISPR gene editing in zygote stage embryos. We have compared electroporation and pronuclear injection (PNI) as a means for delivery of CRISPR reagents. Our results suggest PNI is a superior approach for generation of knockin mutations. This is arguably due to the fact that, a higher concentration of ssODN molecules can be achieved thanks to direct transfer into the embryos by microinjection. Another advantage of the PNI technique is, it enables the use of Scr7 inhibitor to further tip the repair pathway towards HDR.

When the goal of gene editing is generation of a loss of function (knockout) allele, use of a single sgRNA to introduce indel mutations is the simplest approach. However, for screening of the newborn mice and subsequent genotyping of the established colony, it is advisable to target a restriction site. When this is not possible, an alternative approach could be using two sgRNAs flanking a critical exon or exons, and preferably causing a frameshift after deletion. WT and knockout alleles can be easily distinguished by a size shift of the PCR product on an agarose gel. Another caveat of indel mutations is, in one third of the cases there is no frameshift and therefore no guarantee of a knockout allele without extensive characterization of the phenotype. However, with increased HDR efficiency, PNI enables efficient introduction of knockout alleles by placing a premature STOP codon that will truncate the protein product, and addition of a novel restriction site that will make screening and genetic characterization of the newborn pups easier.

4. MATERIALS AND METHODS

4.1. Superovulation, isolation, manipulation, and culture of embryos

The research project involving experimental animals was approved by Izmir Biomedicine and Genome Center animal experimentation local ethics committee (IBG-HADYEK, protocol number:2020-017).

Pronuclear stage embryos were collected from four to six weeks old female mice from C57BL/6J strain. Mice were superovulated by intraperitoneal injection of 5 IU of Pregnant Mare Serum Gonadotropin (Folligon, MSD Animal Health) and 5 IU Human Chorion Gonadotropin (Chorulon, MSD Animal Health) 48 hours after the first injection. Females superovulated by hormone injections were put together with male mice (8-12 weeks old, C57BL6/j) in the same cage (one male with one female) for mating. Next morning (8-9 am), the female mice were euthanized for embryo (0.5 dpc) collection. Oviducts were isolated and placed in a 60-mm dish. Ampulla were cut and zygote stage embryos were collected under stereomicroscope. Cumulus cells were separated from the embryos by incubation at room temperature in a drop of hyaluronidase (Sigma, H3884) in Global-Hepes medium (300µg/ml, Global, LGTH-100). Embryos were transferred between three droplets of M2 medium by pipetting with a mouth pipette to remove the cumulus cells. Isolated and clarified embryos were cultured in Global medium (Global, LGGG-050) + BSA (4mg/ml, Sigma, A9647) in a CO2 incubator for 1-2 hours until CRISPR RNP electroporation was carried out.

Prior to electroporation, embryos were transferred to a droplet of Acid Tyrode's solution (Sigma, T1788) and incubated for 1-2 minutes. Thinning of the zona pellucida was visually monitored under the microscope. Embryos were sequentially transferred between three droplets of M2 media to wash.

4.2. Embryo culture

After electroporation, embryos were collected and transferred into droplets of Global medium (Global, LGGG-050) + BSA (4mg/ml, Sigma, A9647) overlaid paraffin oil (LifeGlobal Oils, LGPO-

100) and equilibrated in a CO2 incubator. Embryos were cultured for three days until morula/blastocyst stage. Developing embryos were identified and collected for polymerase chain reaction (PCR) genotyping.

4.3. sgRNA synthesis and purification

DNA fragment used as a template for in vitro transcription reaction was amplified by PCR as described below. sgRNAs were synthesized by HiScribe T7 High Yield RNA Synthesis Kit (NEB, E2040S) and purified by Monarch RNA Cleanup Kit (NEB, T2040L).

4.4. RNP complex assembly and electroporation

RNP complexes were assembled by incubating 80 pmol Cas9 nuclease (IDT, 1081059) and 100 pmol sgRNA in 10 µl of assembly buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM MgCl2, TCEP (Sigma, C4706), 2% glycerol (v/v)) for 10 min at 37°C. Embryos were washed by successive transfer between OPTIMEM droplets and transferred to RNP tube in 10 µl OPTIMEM. ssODN fragments for editing of the *Tyr* gene locus were added at a final concentration of 10 µM. 20 µl RNP-embryo mixture was transferred to the bottom of an electroporation cuvette (Bio-Rad, 1652089). Six pulses (30V, 3 ms pulse length and 100 ms interval) were applied to electroporate the embryos in a Bio-Rad Gene Pulser XCell device. Embryos were recovered by flushing the cuvettes with Global-HEPES media (Global, LGTH-100) and cultured or transferred to host females.

4.5. Pronuclear injection

For pronuclear injection, fertilized embryos with clearly distinguishable pronuclei were collected under the inverted microscope. Prior to injection, embryos were transferred through KSOM medium three times to remove cumulus complexes and hyaluronidase. Cas9 (0.6 µM), sgRNA (0.6 µM) and ssODN (0.4 µM) were mixed in 20 µl TE buffer and RNP/ssODN mix was microinjected into the male pronuclei. A positive pressure was maintained in the injection pipette throughout the procedure. Embryos were stabilized with a holding pipette, the pronuclear membrane was penetrated with the injection pipette filled with RNP/ssODN solution, swelling of the pronuclei was observed. Successfully injected embryos were collected and cultured until blastocyst stage. Lysed embryos were discarded.

4.6. Embryo transfer, pseudopregnant host mice

CD-1 female mice were used as recipient hosts for transferred embryos. They were mated with vasectomized male mice one day prior to embryo collection to synchronize pseudopregnancy. Plugged females were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Electroporated or microinjected embryos were surgically transferred into the oviducts entering through and incision to the infundibulum.

4.7. Primers and oligonucleotides

For in vitro transcription of sgRNAs, a DNA oligonucleotide template was first generated by a template fusion PCR using Q5 high-fidelity DNA polymerase (NEB, M0491) (Lin *et al.* 2014). The

two fused DNA fragments were synthetized by Eurofinns (EXTREmer oligos) and were comprised of the T7 promoter sequence followed by a 20-nucleotide long guide sequence (bold) targeting the *Tyr* gene

(5'GGATCCTAATACGACTCACTATAG**GGGTGGATGACCGTGAGTCC***GTTTTAGAGCTAGAA*)

, and the sgRNA scaffold sequence

(5'AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTAT*TTCTAGCTCTAAAAC*) with overlapping 3' sequences (italicized). Forward (5'GGATCCTAATACGACTCACTATAG) and reverse (5'AAAAAAGCACCGACTCGG) primers matching the templates were used for the amplification.

Sequence of the ssODN fragment used as a template for homology-directed repair editing of the *Tyr* gene has been published before (Modzelewski *et al.* 2018).

4.8. Genotyping PCR, cloning and Sanger sequence analysis

Genomic DNA lysates from embryos or mouse pups were prepared according to the HotSHOT lysis protocol (Truett *et al.* 2000). Briefly, entire embryos or tail biopsies were lysed by boiling in alkaline lysis solution (25mM NaOH, 0.2 mM EDTA) and neutralized by addition of 40 mM Tris-HCl to yield DNA lysates that were directly used as a template in genotyping PCR reactions.

For amplification of the *Tyr* locus, a semi-nested PCR strategy was followed (Chen *et al.* 2016). The first PCR amplification was performed using KD431-KD433 primer pair, whose product was 1:500 diluted and used as a template in a second PCR with KD432-KD433 primer pair. PCR products were digested with HinFI to determine Cas9 nuclease activity on the target *Tyr* sequence and by EcoRI, to determine the efficiency of knockin mutation incorporation.

For in depth characterization of the various alleles present in the founder mouse, an amplicon from the *Tyr* locus was amplified using KD485 and KD486 primers and cloned into pBluescript II SK(+) plasmid. Sanger sequencing of the resultant clones was performed using KD137 primer.

For characterization of the large deletion mutation (allele A), a longer amplicon from the *Tyr* locus was amplified by a single PCR reaction using primers KD520 and KD521.

Sequences of all primers and oligonucleotides used in this study and PCR amplification protocols were provided in the supplementary information.

5. FIGURE LEGENDS

Figure 1. CRISPR editing and genotyping strategy.

(A) Map of the mouse tyrosinase gene locus. Upper panel shows the locations of exons (protein coding: green boxes, non-coding: gray boxes). Lower panel shows a magnified view of the targeted region and the locations of (i) various primers (black arrows) used in PCR genotyping reactions, (ii) HinFI restriction sites and (iii) sgRNA targeted sequence (orange arrow). The HinFI site lost after CRISPR editing is displayed in red.

(B) DNA and amino acid sequences of WT and knockin tyrosinase alleles. The sequence of the HDR template is displayed on top. After Cas9/sgRNA induced DSB, repair of the locus by NHEJ results in indel mutations that destroy the HinFI site. On the other hand, HDR mediated editing replaces the HinFI with an EcoRI restriction site and results in a premature stop codon due to frameshift.

Figure 2. Results of gene editing in zygotes.

(A) After electroporation of zygote stage mouse embryos with Cas9/sgRNA complexes and HDR template targeting the *tyr* locus, they were cultured for 72 hours until they reached late morulae (M) or blastocyst (B) stage. A DNA fragment encircling the sgRNA targeted sequence was PCR amplified using a nested PCR protocol.

(B) Selected PCR products from (A) were digested with HinFI and EcoRI enzymes to analyze CRISPR targeting and HDR efficiency respectively. Asterisks denote the embryos carrying the knockin alleles.

Figure 3. Generation of albino BL6 mouse strain.

(A) Genotyping of founder mice after embryo transfer. #3 has an indel mutation that deletes HinFI site, and #4 has undergone HDR mediated knockin.

(B) Tyrosinase gene editing in the founder pups results in full albino coat color.

(C) Backcrossing of founder #3 with BL6 females resulted in heterozygous pups. Two different amplification patterns (alleles) were obtained. Allele A, which yields a single band, was later found to contain a large deletion that cannot be amplified in this PCR protocol. Allele B, which causes an inversion and deletion results in double bands.

(D) PCR products from the heterozygous pups with +/A pups can be efficiently cut by HinFI because only the WT copy can be amplified with the primer set. Products from +/B pups are partially cut.

Figure 4. Characterization of mutant alleles.

(A) DNA from albino mice with A/A (53-60) and B/B (61-66) were amplified using KD520-KD521 primer pair that yields a 525nt long amplicon. There is no amplification in A/A genotype. B/B genotype amplicon runs just below the WT amplicon due to shorter size.

(B) Sequencing of Allele B by Sanger shows the nucleotide changes in the tyrosinase locus.

(C) Allele B introduces an in frame STOP codon to the mouse Tyrosinase coding sequence and therefore results in a knockout allele.

Figure 5. Pronuclear injection increases HDR efficiency.

Cas9/sgRNA RNPs and ssODN fragments were delivered by pronuclear injection and the embryos were cultured for three days prior to genotyping. The HDR mediated knockin in Tyrosinase locus was remarkably increased in comparison to electroporation.

Figure S1. Sanger sequence analysis of knockin alleles.

Sequences of knockin alleles from selected embryos were determined by Sanger sequencing and aligned to the reference sequence. The two nucleotides (AT) inserted into the Tyr coding exon sequence were labelled red. The in frame STOP codon (TAA) that results due to the frameshift has been labelled blue.

1: Figure 3A, founder mouse #4

2: Figure 2B, embryo M16

3: Figure 2B, embryo B6

4: Figure 5, embryo #4

5: Figure 5, embryo #6

AUTHOR INFORMATION

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Author Contributions

M.K.D. conceived and designed the project, and acquired the funding. M.K.D., K.E., T.S. and G.O. performed the experiments and acquired the primary data. M.K.D. analyzed and interpreted the data. M.K.D. wrote the main manuscript text. All authors reviewed the manuscript.

Notes

The authors declare no competing financial interest.

The research project involving experimental animals was approved by Izmir Biomedicine and Genome Center animal experimentation local ethics committee (IBG-HADYEK, protocol number:2020-017).

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ABBREVIATIONS

AAV, adeno-associated virus; **CRISPR**, clustered regularly interspaced short palindromic repeats; **Cas9**, CRISPR associated protein 9; **DSB**, double strand break; **indel**, insertion/deletion; **HDR**, homology directed repair; **KO**, knockout; **KI**, knockin; **NHEJ**, non-homologous end joining; **PNI**, pronuclear injection; **RNP**, ribonucleoprotein; **sgRNA**, small guide RNA; **ssODN**, single-stranded oligodeoxynucleotides.

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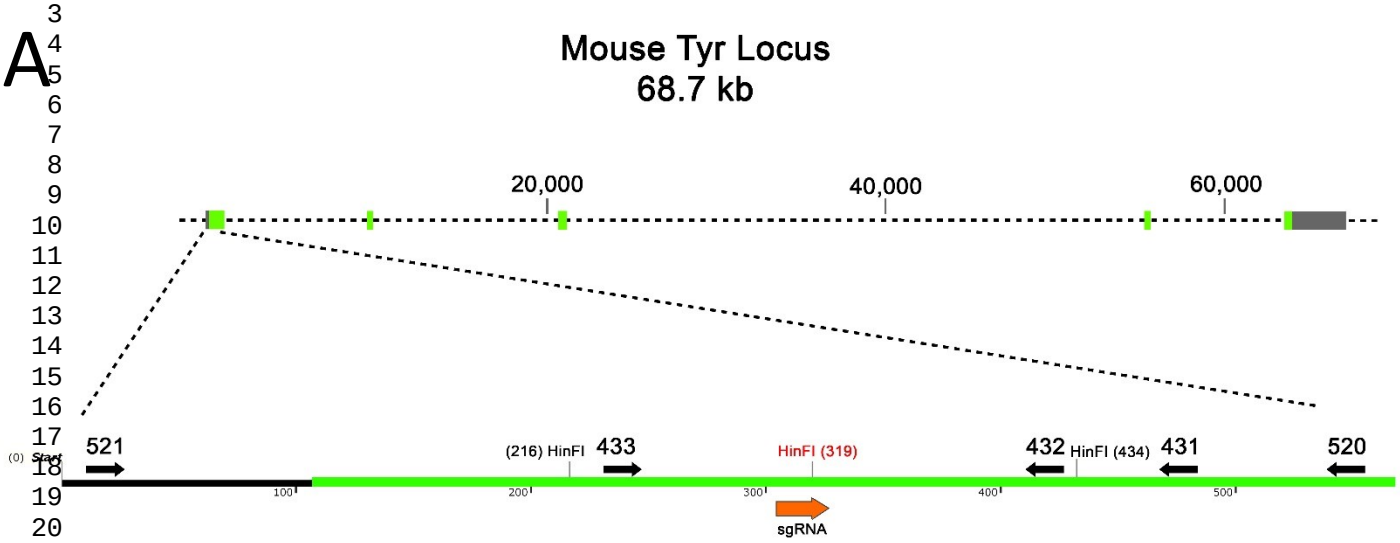
FIGURE 1 CRISPR editing and genotyping strategy

A



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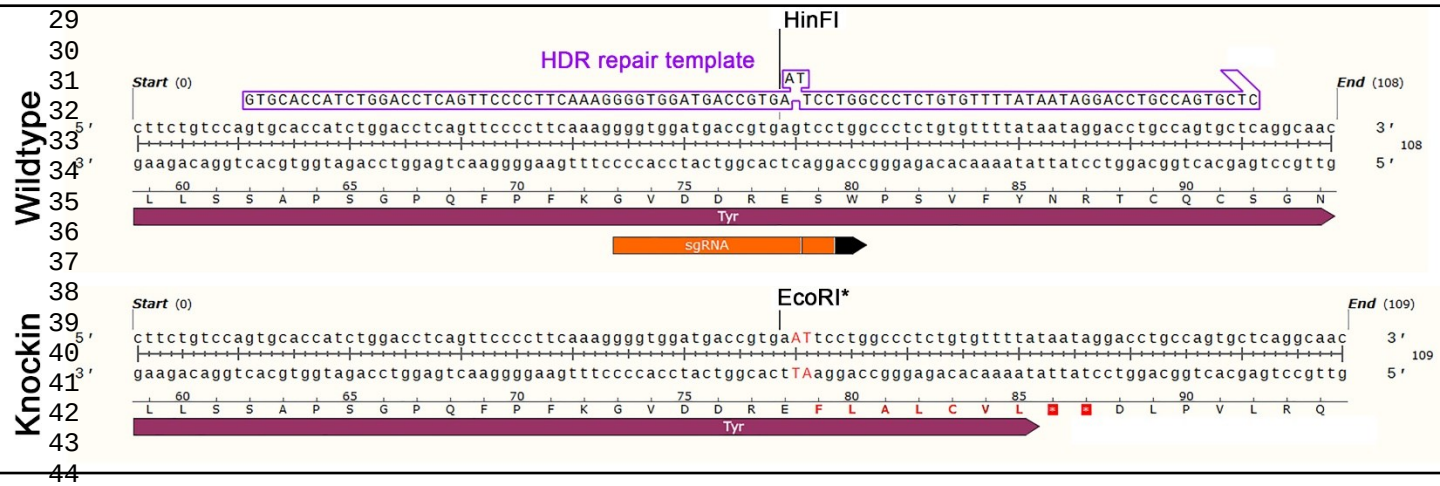
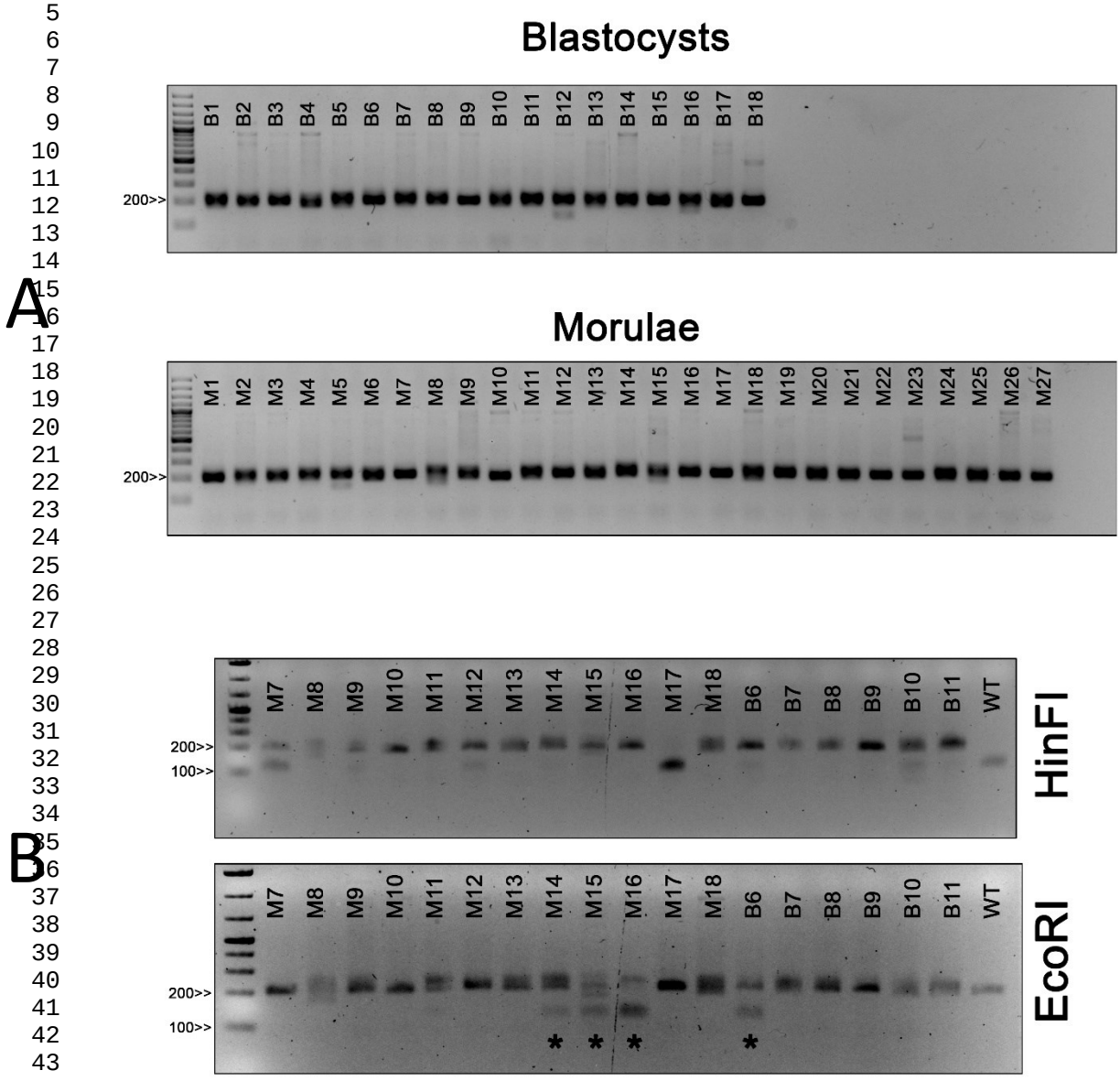


A) Map of the mouse tyrosinase gene locus. Upper panel shows the locations of exons (protein coding:green boxes, non-coding:gray boxes). Lower panel shows a magnified view of the targeted region and the locations of (i) various primers (black arrows) used in PCR genotyping reactions, (ii) HinFI restriction sites and (iii) sgRNA targeted sequence (orange arrow). The HinFI site lost after CRISPR editing is displayed in red.

B) DNA and amino acid sequences of WT and knockin tyrosinase alleles. The sequence of the HDR template is displayed on top. After Cas9/sgRNA induced DSB, repair of the locus by NHEJ results in indel mutations that destroy the HinFI site. On the other hand, HDR mediated editing replaces the HinFI with an EcoRI restriction site and results in a premature stop codon due to frameshift.

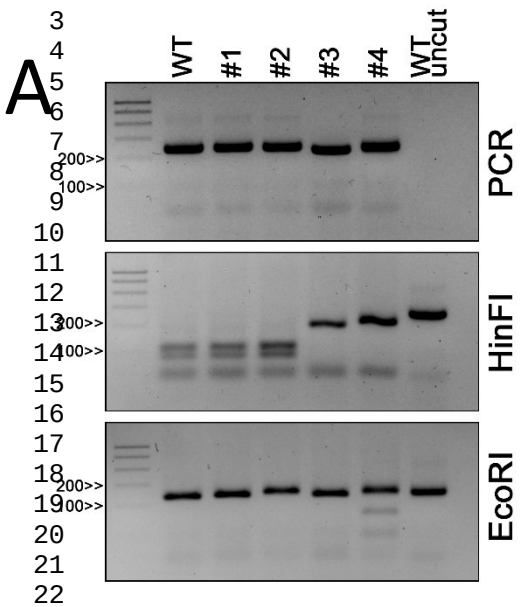
FIGURE 2 Results of gene editing in zygotes

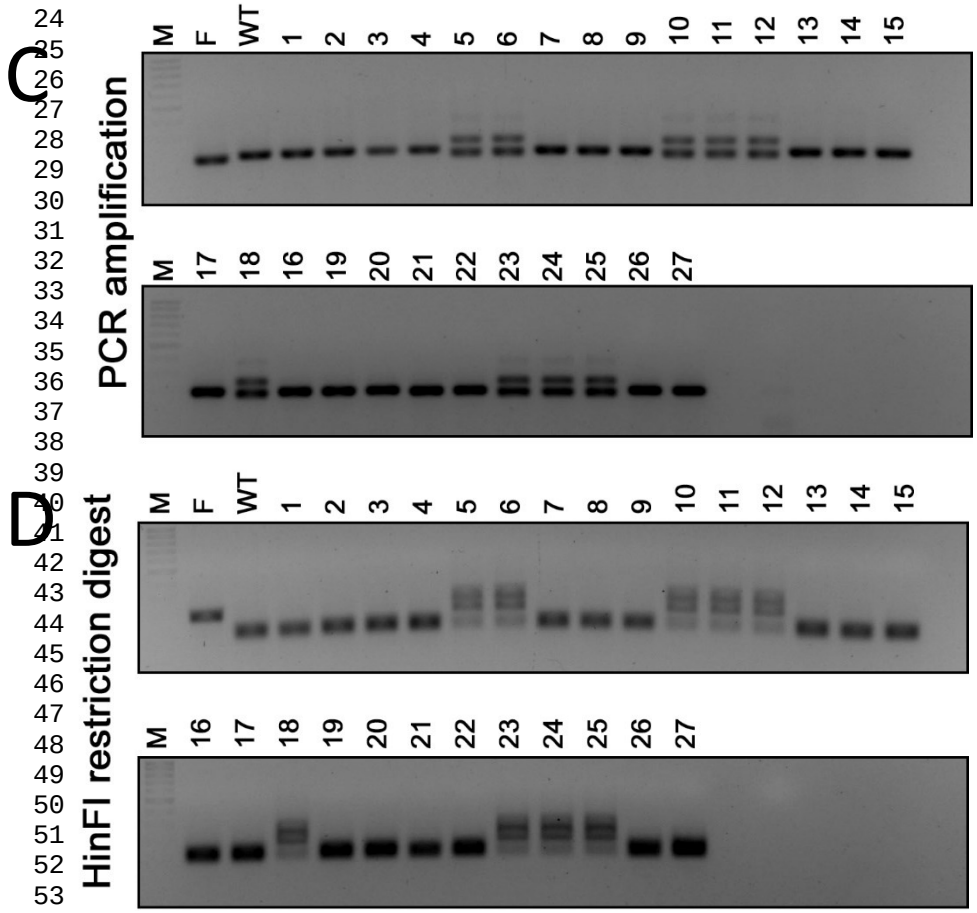


A) After electroporation of zygote stage mouse embryos with Cas9/sgRNA complexes and HDR template targeting the tyr locus, they were cultured for 72 hours until they reached late morula (M) or blastocyst (B) stage. A DNA fragment encircling the sgRNA targeted sequence was PCR amplified using a nested PCR protocol.

B) Selected PCR products from (A) were digested with HinFI and EcoRI enzymes to analyze CRISPR targeting and HDR efficiency respectively. Asterisks denote the embryos carrying the knockin alleles. Sequences of the knockin alleles from embryos M16 and B6 were verified by Sanger sequencing (Figure S1).

FIGURE 3 Generation of albino BL6 mouse strain





A) Genotyping of founder mice after embryo transfer. #3 has an indel mutation that deletes HinFI site, and #4 has undergone HDR mediated knockin (Figure S1).

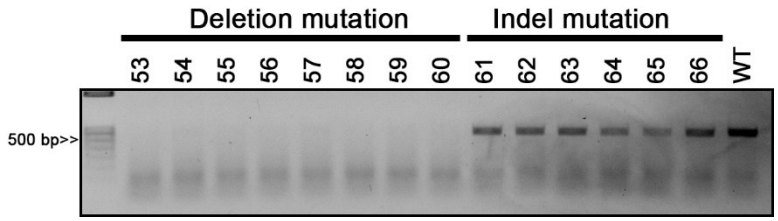
B) Tyrosinase gene editing in the founder pups results in full albino coat color

C) Backcrossing of founder #3 with BL6 females resulted in heterozygous pups. Two different amplification patterns (alleles) were obtained. Allele A, which yields a single band was later found to contain a large deletion that cannot be amplified in this PCR protocol. Allele B, which causes an inversion and deletion results in double bands.

D) PCR products from the heterozygous pups with +/A pups can be efficiently cut by HinFI because only the WT copy can be amplified with the primer set. Products from +/B pups are partially cut.

FIGURE 4 Characterization of mutant alleles

A



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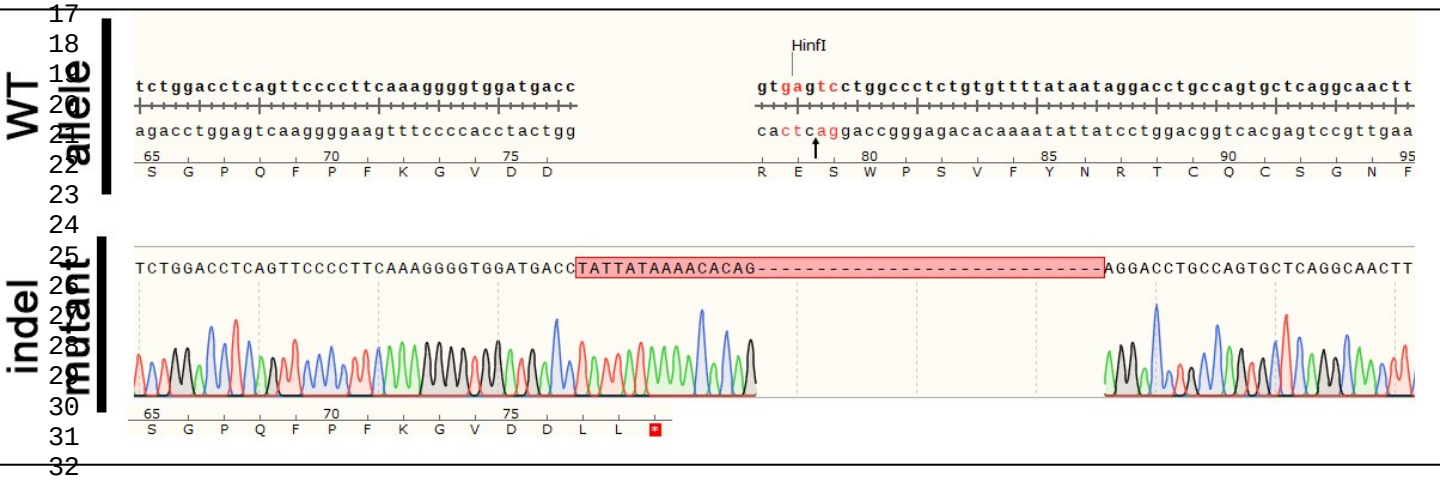

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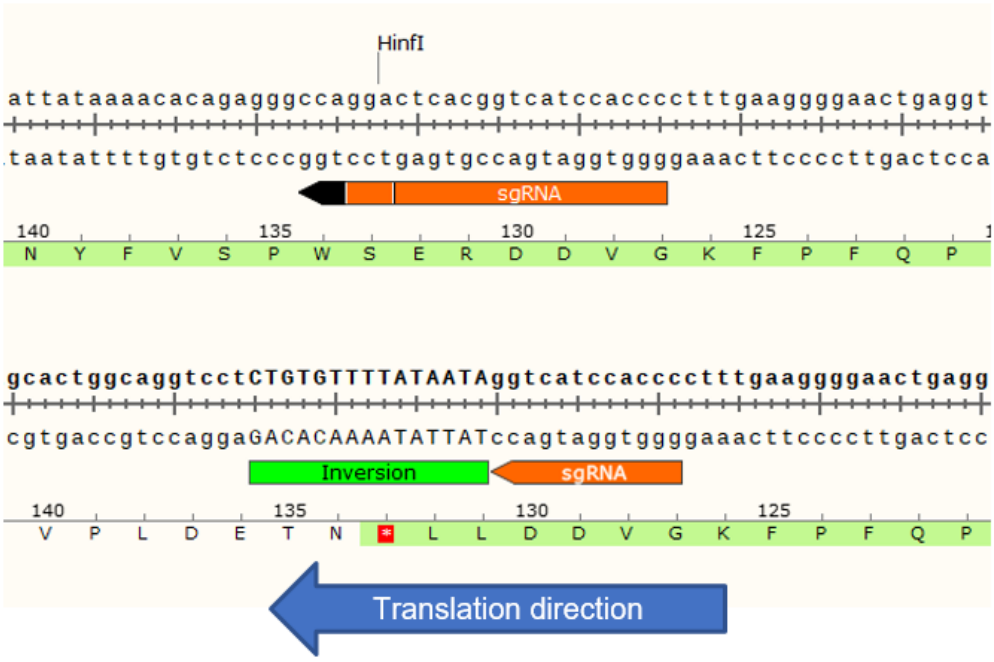
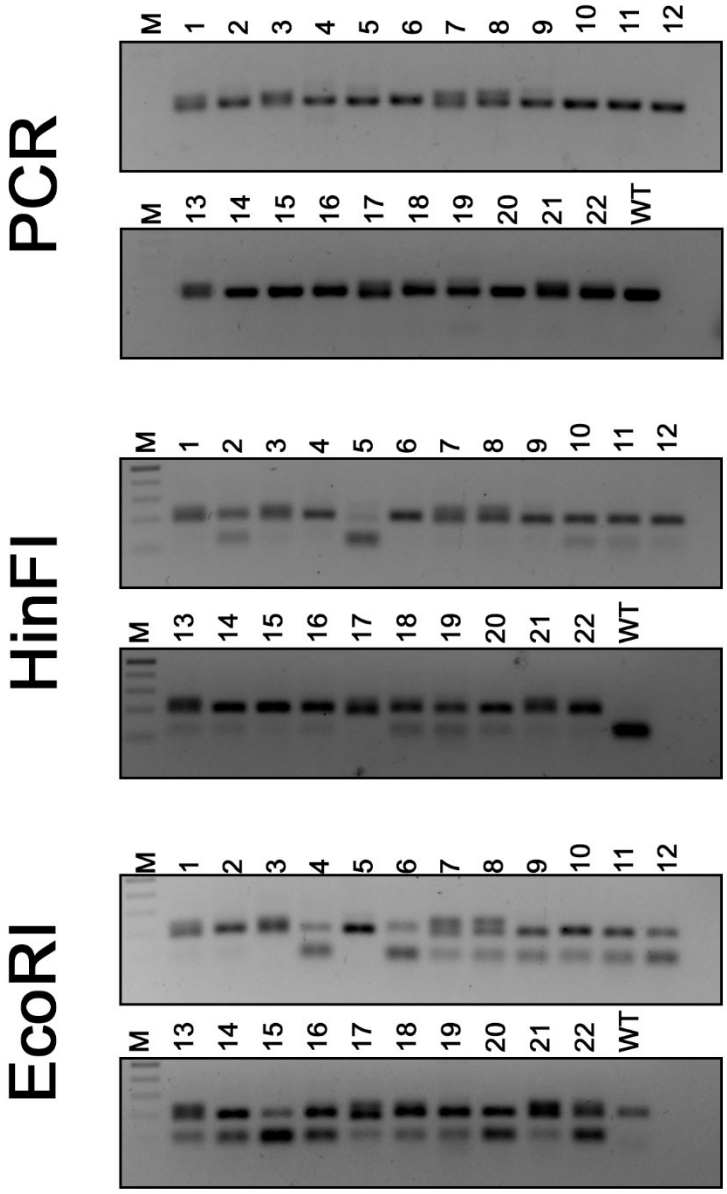


A) DNA from albino mice with A/A (53-60) and B/B (61-66) were amplified using KD520-KD521 primer pair that yields a 525nt long amplicon. There is no amplification in A/A genotype. B/B genotype amplicon runs just below the WT amplicon due to shorter size.

B) Sequencing of Allele B by Sanger shows the nucleotide changes in the tyrosinase locus

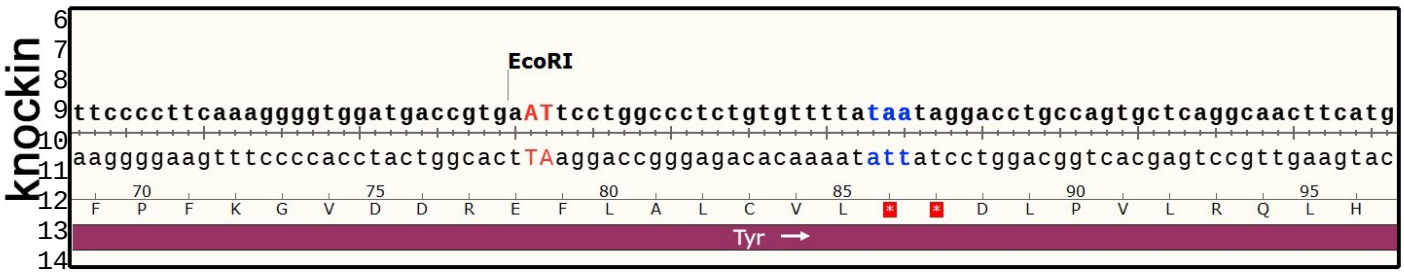
C) Allele B introduces an in frame STOP codon to the mouse Tyrosinase coding sequence and therefore results in a knockout allele

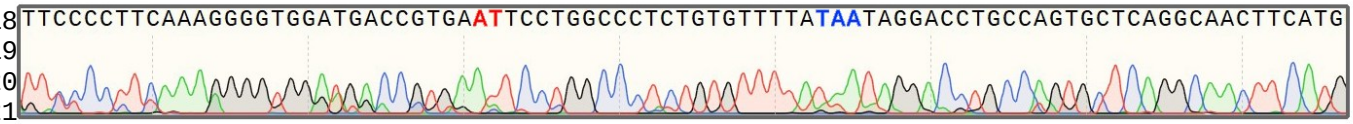
FIGURE 5 Pronuclear injection increases HDR efficiency

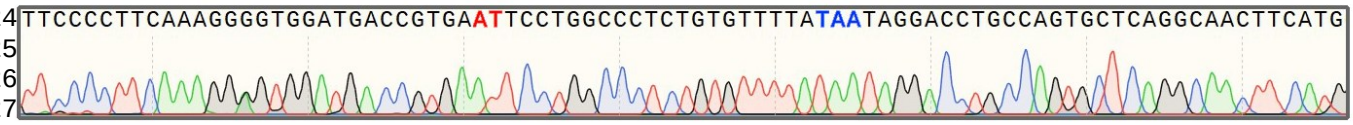


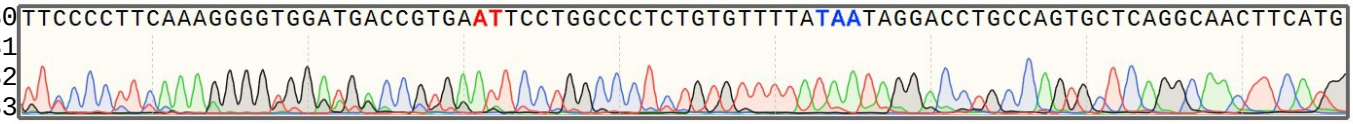
Cas9/sgRNA RNPs and ssODN fragments were delivered by pronuclear injection and the embryos were cultured for three days prior to genotyping. The HDR mediated knockin in Tyrosinase locus was remarkably increased in comparison to electroporation. Sequences of the knockin alleles from embryos 4 and 6 were verified by Sanger sequencing (Figure S1).

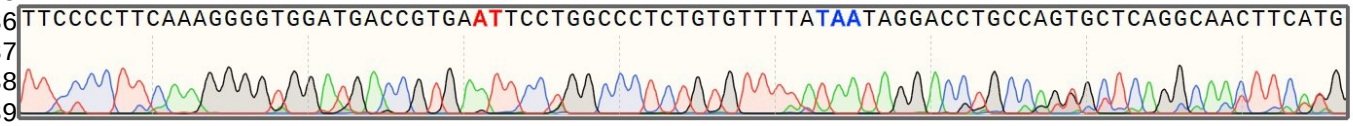
FIGURE S1 Sanger sequence analysis of knockin alleles

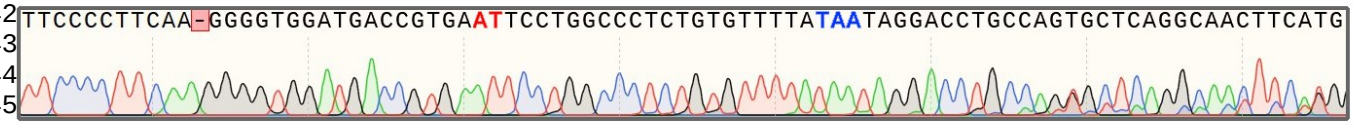












Sequences of knockin alleles from selected embryos were determined by Sanger sequencing and aligned to the reference sequence. The two nucleotides (AT) inserted into the *Tyr* coding exon sequence were labelled red. The in frame STOP codon (TAA) that results due to the frameshift has been labelled blue.

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2: Figure 2B, embryo M16
3: Figure 2B, embryo B6
4: Figure 5, embryo #4
5: Figure 5, embryo #6

Figure 1

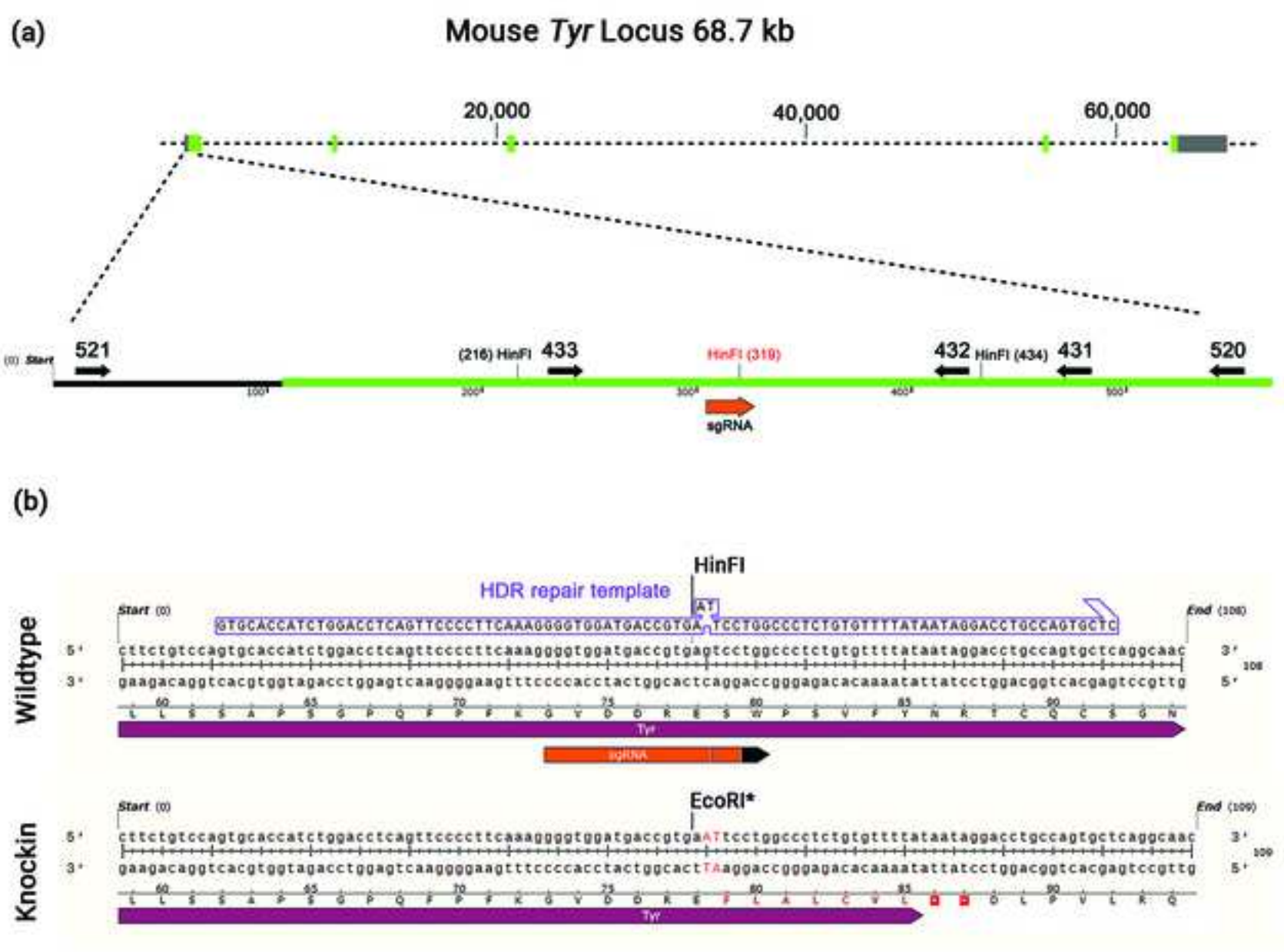

Figure 2

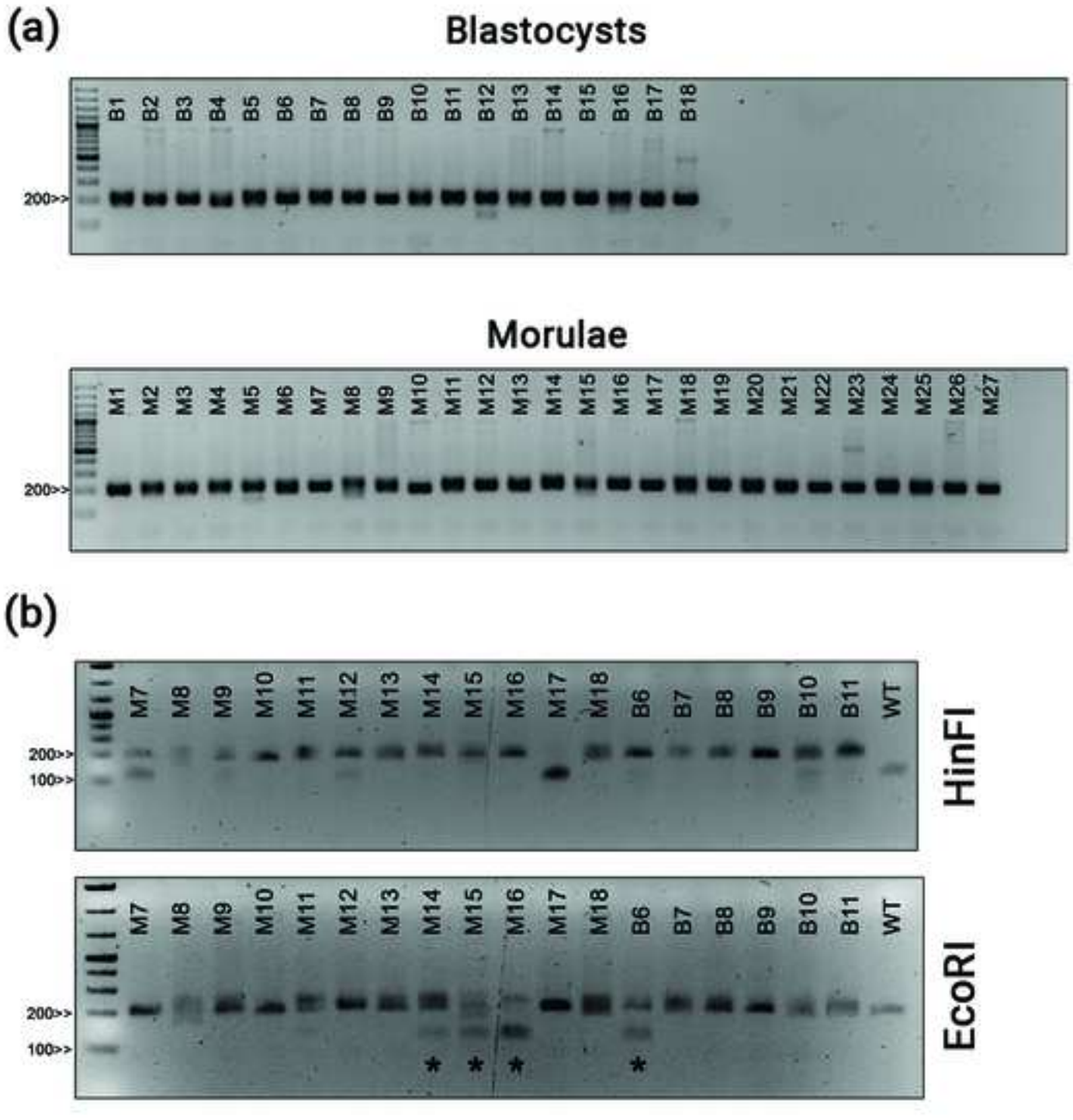

Figure 3

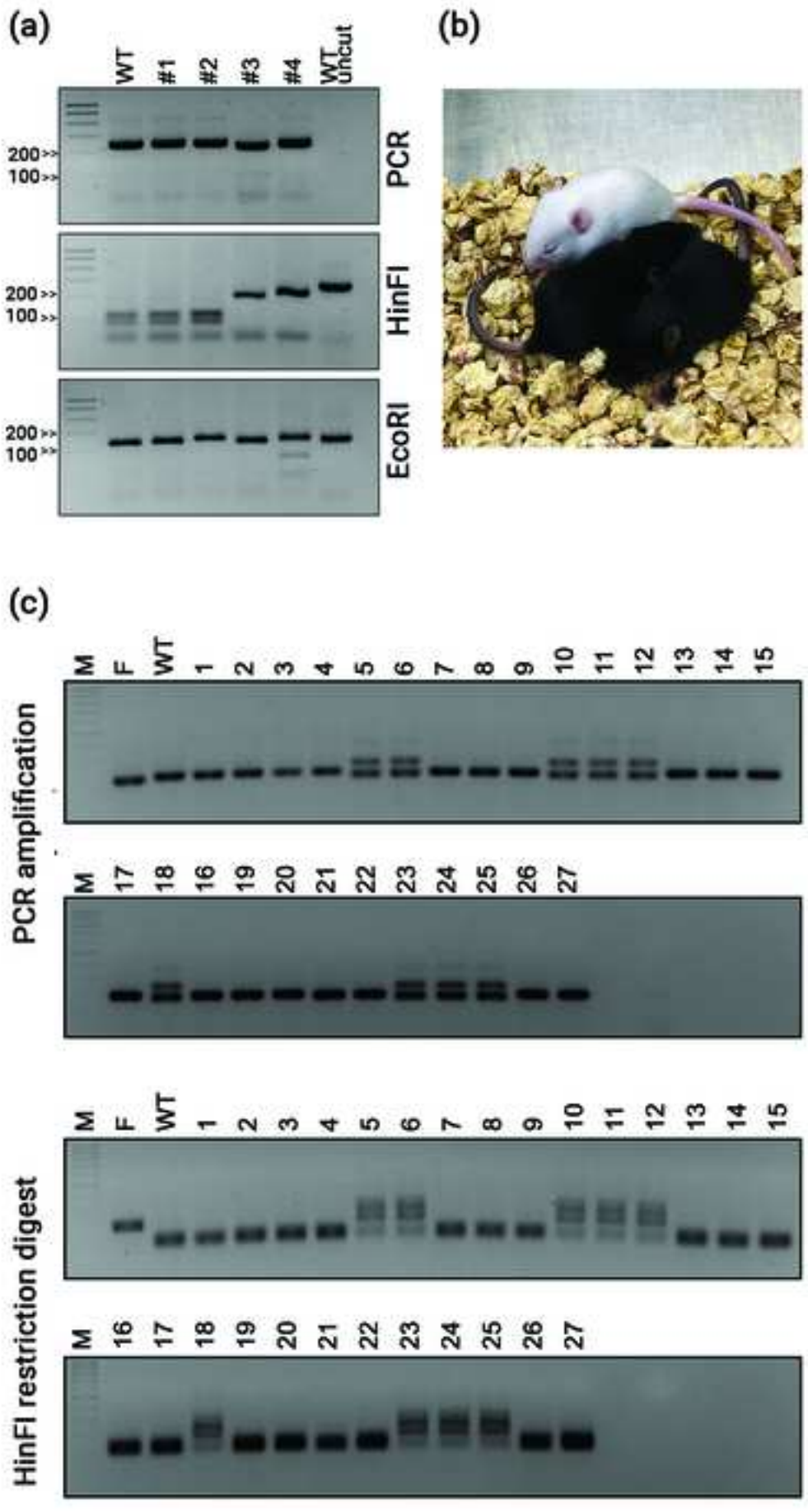

Figure 4

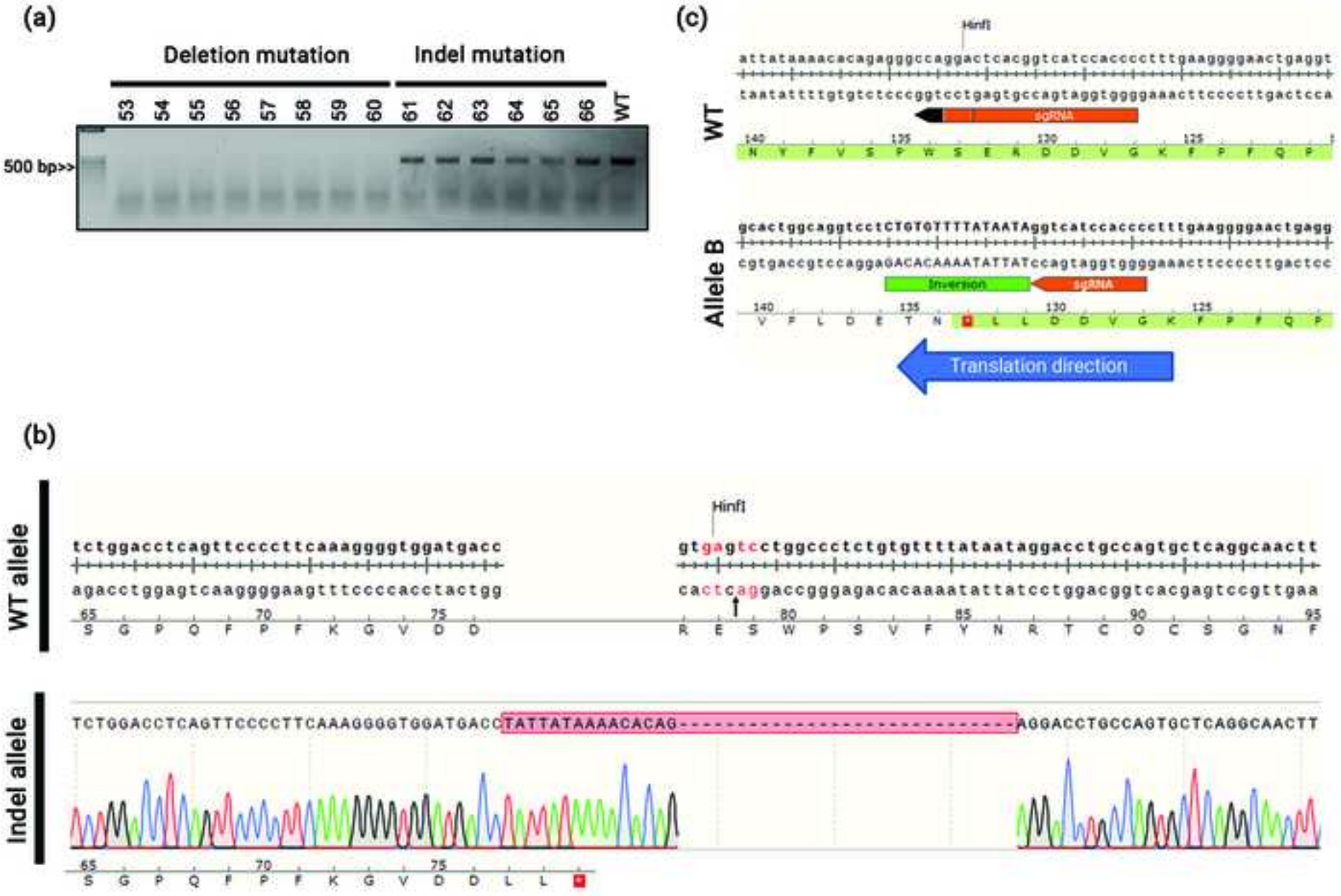

Figure 5

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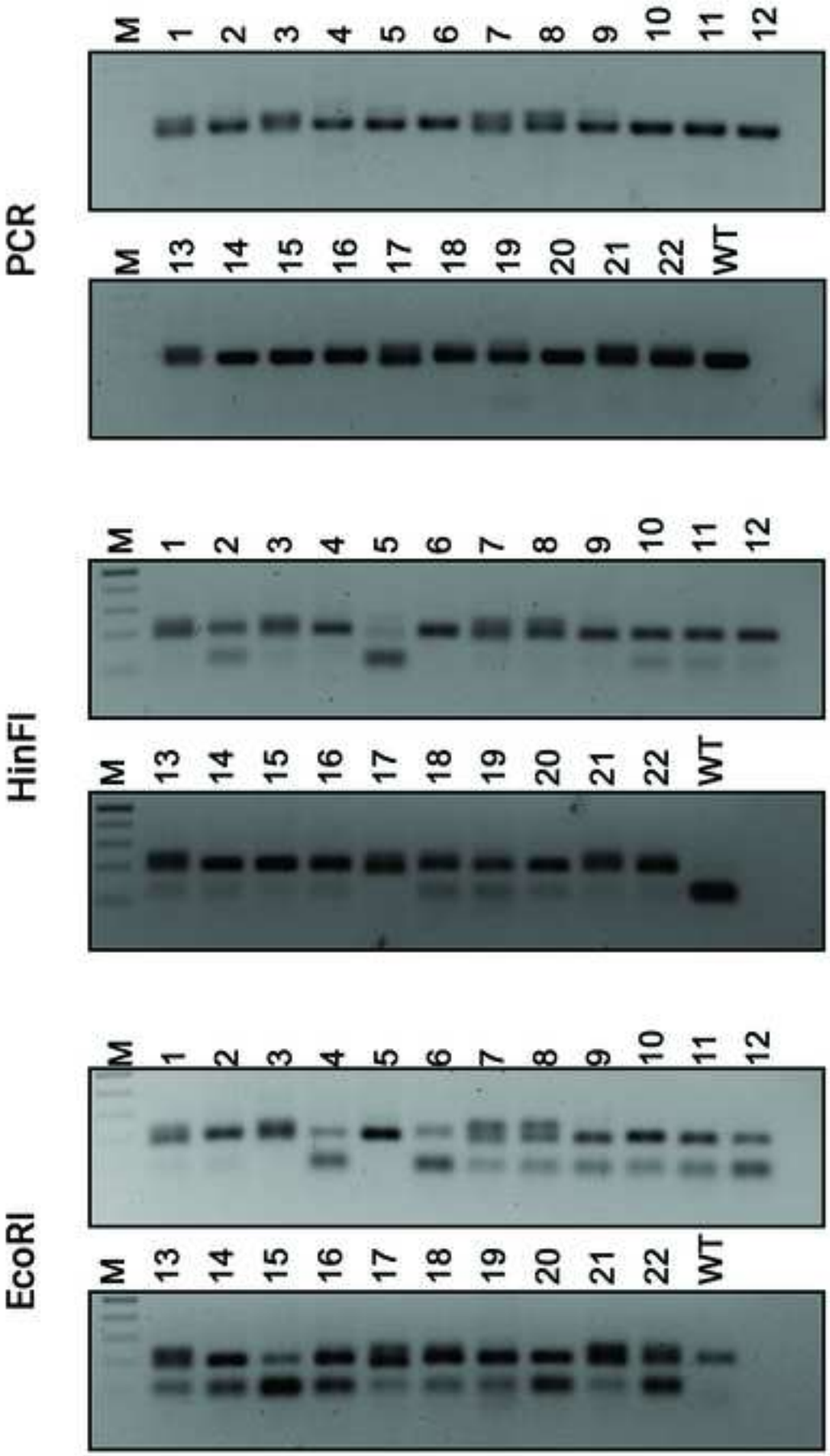

Figure S1

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