Non-Destructive Larval Genotyping of *Danio rerio* for Mitochondrial DNA Genetics

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Abstract

The rapid advancement of nuclear and mitochondrial genomic editing tools has created an urgent need for efficient, non-lethal larval genotyping methods in zebrafish (*Danio rerio*) research. This study optimizes and validates a non-destructive proteinase K digestion method for mitochondrial DNA genotyping while characterizing its impact on larval survival and gene expression. Using optimized protocol parameters, we demonstrate successful amplification of different mitochondrial genetic loci with consistently high sensitivity. Molecular validation through PCR, restriction fragment length polymorphism analysis, and Sanger sequencing confirmed the specificity and reliability of the extracted DNA, while RNA sequencing analysis revealed no significant transcriptional differences between genotyped and unshaken control larvae, indicating minimal physiological impact. The method successfully detected C-to-T base edits in the *mt-tl1* gene introduced using the FusX TALE Base editor system, demonstrating its applicability to gene editing studies. Both 48-well and optimized 96-well formats were used, enabling this approach to be deployed at scale. This optimized method enables researchers to correlate genotypes with phenotypes in longitudinal studies while maintaining specimen viability. It is particularly valuable for investigating early-onset mitochondrial diseases and utilizes standard laboratory equipment and reagents, facilitating widespread adoption in zebrafish research while adhering to ethical principles in reducing animal mortality.

Introduction

Zebrafish (*Danio rerio*) are the most widely used aquatic model system in scientific research, transcending their initial role in developmental biology to become instrumental in unraveling the genetic and biochemical intricacies of various cellular processes. This small vertebrate boasts an impressive array of advantages as a model organism, including rapid reproductive cycles, high offspring yield, external embryonic development, transparent embryos, swift organ formation, and straightforward husbandry requirements.[1–5] The versatility of zebrafish has led to groundbreaking insights across a spectrum of biological processes and human diseases. From hematological and cardiovascular disorders to neurological conditions and cancer, zebrafish models have significantly contributed to our understanding of these complex systems.[6–8] Zebrafish shine particularly bright in the realm of mitochondrial research. Their mitochondrial genome shares remarkable similarities with humans, with 100% gene synteny and approximately 65% sequence identity at the nucleotide level. This genetic kinship extends to codon usage, strand-specific nucleotide bias, and gene order, making zebrafish an ideal vertebrate model for studying human mitochondrial disorders.[9–11] Since mitochondrial DNA (mtDNA) is extra-nuclearly segregated, it has different requirements for genotype to phenotype correlation. The complex genetics underlying mtDNA disorders, particularly their variable penetrance and diverse clinical presentations, can be attributed to the presence of either homoplasmy or heteroplasmy of mtDNA type within cells. The severity and progression of mitochondrial diseases are directly influenced by the ratio of mutated to normal (wild type) mtDNA within a cell.

Recent years have witnessed significant advancement in the mitochondrial DNA (mtDNA) editing toolbox such as DdCBEs and TALEDs to engineer precise edits across different species enabling to model pathogenic mutations.[12–17] Notably, DddA-derived cytosine base editors (DdCBEs) have achieved editing efficiencies at targeted mtDNA loci, enabling accurate modeling of pathogenic mutations. Additionally, engineered variants such as DddA6 and DddA11 have expanded the editing scope by targeting previously inaccessible sequence contexts, while monomeric DdCBE (mDdCBE) constructs facilitate broader applicability and simplified delivery.[13,18] These developments collectively enhance the utility of zebrafish as a robust vertebrate model for mitochondrial genetic research. The advent of these mitochondrial genomic editing tools underscores the critical need for rapid and efficient larval genotyping in zebrafish research. Traditional approaches to live larval genotyping have relied on microsurgical fin biopsy performed in water droplets,[19] while alternative methods involve the complete lysis of embryos for cohort genotype determination[20] or an automated genotyping device.[21] However, these conventional techniques present significant limitations: fin microsurgery is labor-intensive and frequently lethal, while embryonic lysis precludes the survival of genotyped larvae for subsequent analysis or breeding. Non-destructive enzyme genotyping presents a scalable solution that overcomes these constraints by employing standard laboratory reagents and materials. This technique facilitates DNA extraction through controlled proteinase K digestion of epithelial cells, yielding genetic

material that can be PCR amplified while preserving larval viability.[22] This protocol enables greater efficiency in husbandry and high-throughput assays, enabling genotype determination within days of fertilization. Notably, this non-lethal approach proves particularly valuable for investigating early-onset lethal phenotypes, where rapid genotyping of living specimens is essential for understanding disease progression.

In this study, we employed and refined a non-destructive genotyping protocol for the mitochondrial genome and characterized the impact of these assays on larvae through survival and transcriptomics data. We also optimized assay conditions for differential amplification and universal genotyping applications. In testing and troubleshooting this non-destructive assay, we note advantages and disadvantages to the proteinase K method as well as demonstrating successful amplification of mitochondrially encoded genes alongside nuclear genes. RNAseq and subsequent analysis showed that the proteinase K method is amenable to differential amplification. Finally, modifications to the previously published enzyme protocol have enabled stronger survival.

Materials and Methods

Zebrafish handling and husbandry

All adult zebrafish and embryos were maintained according to the guidelines established by Mayo Clinic Institutional Animal Care and Use Committee (IACUC number: A34513-13-R16) and The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC number: AUP-2023-00200).

Enzyme genotyping

Wild-type and mutant transgenic lines, *mt-tl1*[14] were used to generate larvae for non-destructive genotyping.[22] Three days post-fertilization (dpf) larvae were rinsed three times in embryo media followed by three washes in tricaine buffer (30 mM Tris-HCl in 0.4% tricaine solution). Using a p200 pipette tip trimmed to accommodate larval width, individual larvae were aspirated in 40 µL of ProK genotyping buffer (25 µg/mL Proteinase K in tricaine buffer) and placed in 96-well and 48-well plates. For 48-well plates, droplets were dispensed centrally within each well to minimize contact between larvae and well walls during agitation. Proteinase K concentration and incubation time can be adjusted based on larval age and condition to optimize survival rates. The plate was incubated in a heated shaker at 37°C with 500 rpm shaking for 20 minutes. After incubation, 30 µL of the liquid around the fish was carefully pipetted (avoiding larval aspiration) into 15 µL of lysis buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.3% Tween-20, 0.3% NP-40, 1 mM EDTA) in 0.2 mL strip tubes and pipetted up and down ~10 times. The solution was then incubated at 98°C for 5 minutes to extract DNA. Following media collection, wells were quickly replenished with embryo media using a Pasteur pipette, and larvae were transferred to 24-well plates containing fresh embryo media.

103 To prevent tail damage during the shaking procedure, plates can be pre-treated with a protein-rich
104 blocking solution. If extensive tail damage is observed within shaken larvae, plates can be
105 incubated with 5% non-fat dry milk (NFDM) solution prepared in embryo media for 5 minutes,
106 followed by three washes with embryo media prior to genotyping (Fig. 1A).

107 **Survival assay**

108 Larvae collected after enzymatic genotyping were transferred in individual wells of 24-well plate.
109 Unshaken age-matched zebrafish larvae obtained from the same clutch were used as controls. Live
110 counts for larvae from each group were recorded at 24 hours post-genotyping, and morphological
111 defects were examined pre- and post-procedure.

112 **PCR amplification and Restriction Fragment Length Polymorphism**

113 To investigate the sensitivity of the protocol for mitochondrial DNA genotyping, we prioritized
114 protein-coding genes from each of the respiratory chain complex, transfer RNA and ribosomal
115 RNA genes. We targeted six mitochondrial genetic loci with distinct functional roles. Four of them
116 were protein coding genes: mitochondrially encoded NADH:ubiquinone oxidoreductase core
117 subunit 1 (*mt-nd1*), mitochondrially encoded cytochrome b (*mt-cytb*), mitochondrially encoded
118 ATP synthase membrane subunit 6 (*mt-atp6*) and mitochondrially encoded cytochrome c oxidase
119 subunit 1 (*mt-co1*). Remaining two were non-coding genes: mitochondrially encoded tRNA-Leu
120 (*mt-tl1*) and mitochondrially encoded 12S rRNA (*mt-rnr1*) (Fig. 1B). PCR amplification was
121 performed for each of the six genes using their respective primers mentioned in Supplementary
122 Table 1. PCR consisted of 10 µL of 2X OneTaq master mix with standard buffer (NEB, USA), 1 µL
123 of 10 µM forward primer, 1 µL of 10 µM reverse primer and 8 µL of template. Samples were run
124 in a thermal cycler with the following PCR conditions: (1) 95°C, 4 min; (2) 95°C, 30 sec; (3) 60°C,
125 30 sec; (4) 72°C, 30 s; (5) go to step 2 30 × ; (6) 72°C, 4 min; and (7) 12°C, hold. PCR amplicons
126 were run on 1% agarose gel and analyzed.

127 Five to eight microliters of the PCR amplicon (depending on intensity of the amplicon on the gel)
128 were used as the substrate for restriction fragment length polymorphism (RFLP) in 20 µL reaction
129 solution (2 µL of the 10X corresponding buffer and 5 U of restriction enzyme and remaining
130 volume with nuclease free water). PCR amplicons for genetic loci *mt-nd1*, *mt-cytb*, *mt-tl1,* and *mt-*
131 *rnr1* were surveyed for restriction digestion by SpeI, AvaI, SacI and HindIII, respectively. Subset
132 of the PCR amplicons were then submitted to Sanger sequencing at GENEWIZ (Azenta, USA) to
133 confirm the sequence specificity. Chromatograms files (.ab1) were then uploaded to the EditR web
134 server to estimate the heteroplasmy in the mtDNA mutants.

135 **RNA extraction and sample preparation**

136 To assess for any differential transcriptional signatures in the proteinase K-shaken larvae to those
137 unshaken, we performed whole body RNA sequencing for the two groups. Adult zebrafish were

incrossed and non-destructive genotyping was performed on larvae aged 3 dpf. Larvae were subjected to the protocol for the non-destructive enzymatic proteinase K conditions and were then allowed to recover in embryo water until 6 dpf. Six larvae were pooled, and RNA isolation was performed using Qiagen RNeasy Mini kit as per manufacturer's instructions. An additional DNAse treatment step was performed before the final wash and elution. RNA concentration was measured using a spectrophotometer.

RNA sequencing and analyses

RNA library preparations and sequencing reactions were performed at GENEWIZ (Azenta USA). For transcriptome sequencing, RNA was isolated by pooling six individual larvae from each condition. RNA quantification was performed using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA), and RNA integrity was assessed using the Agilent TapeStation (Agilent Technologies, USA). RNA sequencing libraries were constructed following the NEBNext Ultra II RNA Library Prep Kit for Illumina protocol (NEB, USA). Briefly, mRNA was enriched using oligo(dT) beads, fragmented for 15 minutes at 94°C, and then reverse transcribed into cDNA. The cDNA was end-repaired, adenylated at the 3' ends, and ligated with universal adapters, followed by index addition and PCR-based library amplification. The sequencing library was validated on the Agilent TapeStation (Agilent Technologies, USA) and quantified using both the Qubit 2.0 Fluorometer and quantitative PCR (KAPA Biosystems, USA). The sequencing libraries were then multiplexed, clustered onto a flow cell, and loaded onto the Illumina HiSeqX instrument according to the manufacturer's instructions. Sequencing was performed using a 2x150 bp Paired-End (PE) configuration. Image analysis and base calling were carried out with HiSeq Control Software (HCS). The raw sequence data (.bcl files) generated from the Illumina HiSeqX platform were converted into fastq files and demultiplexed using Illumina's bcl2fastq 2.20 software with one mismatch allowed for index sequence identification. The raw fastq data was uploaded to the Basepair cloud bioinformatics platform (https://www.basepairtech.com/). Filtered reads were aligned to the zebrafish reference genome (Zv10 version) using STAR. Raw and normalized gene and transcript counts were calculated. Differential expression analysis was performed using DESeq2, with a threshold of p-adjusted <0.05 for identifying differentially expressed genes between the genotyped and unshaken control larvae.

Results

Survival rates of enzyme-genotyped zebrafish larvae support high-throughput screening

Non-destructive enzyme genotyping of zebrafish larvae demonstrates high survival rates and compatibility with high-throughput screening. Larvae shaken in 96-well plates without milk pretreatment had a median survival rate of 75%, while 48-well larvae had a median of 94%, compared to 100% in unshaken controls (Fig. 1C). Most larval deaths occurred within 24 hours post-shaking, primarily due to tail fin damage, with no observed differences in survival between undamaged shaken and unshaken fish after this period. Key survival-enhancing steps included

175 rapidly replacing embryo water after DNA collection and promptly removing larvae from the
176 heated shaker. These parameters can be further adapted to accommodate different larval ages and
177 sensitive phenotypes. Importantly, no morphological changes were observed in surviving
178 genotyped larvae compared to unshaken controls, suggesting the method's minimal physiological
179 impact.

180 **Mitigating characteristic tail fin damage among ProK-genotyped larvae via 48-well plates**
181 **and milk solution pretreatment**

182 Initial attempts using 96-well plates for ProK genotyping resulted in high mortality rates that
183 undermined the purpose of pre-experimental genotyping. Despite optimizing ProK concentration,
184 incubation time, and post-genotyping rescue protocols, improvements in survival were only
185 moderate. Many larvae exhibited fatal damage, including partial to complete tail fin tearing. We
186 attributed this damage pattern to tail fin adhesion to the well walls during heated shaking,
187 exacerbated by ProK-induced weakening of fin structural integrity. We determined this damage
188 pattern likely resulted from tail fin adhesion to well walls during heated shaking, exacerbated by
189 ProK-induced weakening of fin structural integrity. Two effective mitigation strategies were thus
190 developed. First, utilization of 48-well plates enabled placement of larvae in centered droplets
191 without wall contact, significantly increasing survival and reducing tail fin damage. However, the
192 efficacy of this approach may vary depending on the strength of the hydrophobic coating applied
193 by different manufacturers to maintain protective droplet formation. Alternatively, pretreating 96-
194 well plates with 5% non-fat dry milk (NFDM) solution created a protective proteinaceous film that
195 reduced larval tail fin adhesion during shaking (Fig. 1A). We observed decreased tail fin damage
196 and improved survival rates among genotyped larvae in the 96-well plate treated with 5% NFDM
197 in embryo media (Supplementary Fig. 1).

198 **High sensitivity of amplification across different mitochondrial genetic loci**

199 Non-destructive enzyme genotyping demonstrates high amplification sensitivity across diverse
200 mitochondrial genes encoding critical cellular machinery. Amplification sensitivity was assessed
201 for *mt-nd1*, *mt-cytb*, *mt-rnr1*, and *mt-tl1* by calculating the percentage of successfully amplified
202 samples across these genes. For each gene-well combination, we conducted three experimental
203 trials, with each trial containing 20 larvae. Thus, we analyzed a total of 60 larvae for each gene-
204 well combination, resulting in 120 total samples per gene across both formats. Consistently high
205 amplification rates were observed across all four genes in both formats. All experimental sets
206 exhibited over 80% sensitivity (Fig. 1D), demonstrating robust performance of this non-destructive
207 genotyping method across diverse mitochondrial genes.

208

209 **Specificity of mitochondrial DNA amplification verified by PCR and RFLP analysis**

210 We validated the specificity of our non-destructive enzyme genotyping method through
211 comprehensive molecular analysis of four mitochondrial genes. PCR amplification and restriction
212 fragment length polymorphism (RFLP) analysis were performed on wild-type zebrafish larval
213 DNA samples from 96-well and 48-well plates, with all samples referenced against Quick-Load
214 Purple 50 bp DNA Ladder (NEB, USA). For *mt-nd1*, we observed a 350 bp PCR amplicon with
215 SpeI restriction digest yielding 236 bp and 114 bp fragments (Fig. 2A, B). The *mt-cytb* gene
216 generated a 460 bp PCR amplicon with AvaI restriction digest yielding 317 bp and 143 bp
217 fragments (Fig. 2D, E). Amplification of *mt-rnr1* showed a 395 bp PCR amplicon with HindIII
218 restriction digest producing 253 bp and 142 bp fragments (Fig. 2G, H). The *mt-tl1* gene produced
219 a 363 bp PCR product (Fig. 3A, B) with SacI restriction digest creating 186 bp and 177 bp
220 fragments. PCR amplification of *mt-atp6* produced a 369 bp amplicon (Supplementary Fig. 2A),
221 while *mt-co1* generated a 494 bp PCR product (Supplementary Fig. 2B). Sanger sequencing
222 confirmed the presence of restriction enzyme sites for the first four genes (Fig. 2C, F, I, 3E), and
223 all samples selected for gel visualization represented the median amplification range within their
224 respective cohorts.

225 **Genotyping of mtDNA mutant**

226 We demonstrated the utility of our non-destructive genotyping approach for gene editing by
227 introducing a C-to-T base substitution in zebrafish *mt-tl1* mutants.[14] Three dpf *mt-tl1* zebrafish
228 mutants were genotyped non-destructively, enabling subsequent longitudinal studies (Fig. 3C).
229 The engineered C-to-T transition at position m.C3744 (C10 on the forward strand) resulted in the
230 loss of the SacI restriction site, thereby facilitating the identification of larvae with mtDNA
231 variations. RFLP analysis demonstrated the loss of the SacI restriction site in mutant larvae, with
232 digested products of 186 bp and 177 bp, and an undigested product of 363 bp. Wild-type controls
233 from both 96-well and 48-well cohorts showed only digested products of 186 bp and 177 bp (Fig.
234 3D). RFLP analysis was confirmed by Sanger sequencing where sequencing files (.ab1) were
235 assessed using EditR software to quantify the level of editing in the mutant larvae. Sanger
236 sequencing confirmed the C-to-T edit, revealing over 40% mtDNA mutant heteroplasmy levels at
237 the target locus (Fig. 3E).

238 **Transcriptomic signature in non-destructively genotyped zebrafish larvae**

239 To examine the genome-wide transcriptional changes in zebrafish larvae following non-destructive
240 genotyping, we performed strand-specific paired-end RNA sequencing to identify a candidate
241 differential expression profile. Sequence data were processed using the Dseq2 pipeline on the
242 Basepair cloud bioinformatics platform (https://www.basepairtech.com/). For both Proteinase K-
243 shaken larvae and wild-type controls, we generated between 34 and 39 million reads. Reads below
244 a Phred score of Q30 were discarded, and the remaining high-quality reads were aligned to the
245 Zv11 zebrafish reference genome, with approximately 78% mapping uniquely. These uniquely

mapped reads were then assembled to quantify transcript levels across the genome. No genes met the criteria of a $\log_2$ fold change of ≥ 1 or ≤ -1 with an adjusted p-value < 0.05 in the dataset.

Discussion

In this study, we optimized and validated a non-destructive enzyme genotyping method for efficient molecular analysis of zebrafish mitochondrial DNA while maintaining high survival rates. Building upon the non-destructive genotyping protocol originally established by Zhang et al.,[22] we demonstrated its utility specifically for mitochondrial DNA genetics and adapted it for high-throughput applications, allowing simultaneous processing of up to 96 larvae. Our approach addresses a significant challenge in zebrafish research—the ability to collect sufficient DNA for reliable genotyping from larvae without sacrificing the animal or compromising its development, which is inherently important for correlating genotype to phenotype in mitochondrial genetics. The optimized protocol achieves high amplification sensitivity across diverse mitochondrial genes while preserving larval viability.

The protocol provides a balanced approach for mtDNA genotyping that minimizes stress to the larvae. Notably, the high survival rates observed, particularly in 48-well plates (94%), demonstrated the method's suitability for high-throughput screening applications. Our technical improvements, particularly the implementation of 48-well plates and milk pretreatment steps, significantly mitigated tail fin damage—a principal cause of larval mortality in our earlier approaches. These modifications addressed the critical issue of tail fins adhering to well walls during heated shaking, which when combined with ProK-induced weakening of fin structural integrity, frequently resulted in fatal damage including torn tail fins. The 48-well plates allowed for centered droplet placement that prevented wall contact, while the milk pretreatment created a proteinaceous coating that reduced the adhesive properties of the well surfaces, preventing tail fins from sticking to the walls during agitation. Together, these adjustments substantially improved survival rates, enabling effective non-destructive genotyping without compromising experimental viability. The absence of morphological changes in surviving larvae, supported by our mRNA transcriptomics data showing no significant changes in gene expression, further confirmed the minimal physiological impact of the protocol on mitochondrial homeostasis. These findings are particularly valuable for longitudinal studies and subsequent phenotypic analyses that require the same individual to be monitored over time. Several technical considerations emerged during out optimization process. We found that rapid replenishment of embryo water post-DNA collection and prompt removal from the heated shaker significantly improved larvae survival. These insights highlight the importance of minimizing exposure to Proteinase K solutions and elevated temperatures. Additionally, our observation that most larval mortality occurred within 24 hours of shaking due to tail fin damage suggests areas for further protocol refinement. The DNA collected through this non-destructive method was found to be stable for long periods comparable to conventionally collected fin clip DNA, with successful amplification and sequencing more than 6 months post-collection.

Our results demonstrated consistent amplification success (>80% sensitivity) across four distinct mitochondrial genes—*mt-nd1*, *mt-cytb*, *mt-rnr1*, and *mt-tl1*—involved in mitochondrial homeostasis. The robust performance across these diverse genetic loci suggested that our method could reliably detect a range of mitochondrial sequences, which is particularly important for studies examining mitochondrial genetics and function in zebrafish disease models. The molecular validation through PCR, RFLP analysis, and Sanger sequencing confirmed the specificity and reliability of the genotyping method. The precise restriction enzyme digestion patterns obtained for each mitochondrial gene demonstrated the specificity of the extracted DNA. This methodological validation is crucial for ensuring the accuracy of downstream genetic analyses and interpretations. Additionally, we successfully amplified two other important mitochondrial genes—*mt-atp6* and *mt-co1*—conducting only PCR amplification for these loci with strong amplification throughout. Significantly, we successfully applied our non-destructive genotyping method to detect gene editing changes in the *mt-tl1* mutants established earlier using the FusX TALE Base Editor system.[14] The detection of C-to-T base edits with over 40% mtDNA mutant heteroplasmy levels underscored the protocol's sensitivity for identifying genetic modifications. This capacity is particularly pertinent for mitochondrial disorders, where early detection of heteroplasmic variations is essential for elucidating pathogenic mechanisms.

By obviating the need for destructive sampling, the protocol preserves the integrity of the larval model, thereby facilitating downstream analyses that correlate precise genotypes with phenotypic outcomes. By preserving larval integrity, this method enables longitudinal analyses that are crucial for determining the germline inheritance of mitochondrial variations, an aspect that remains underexplored in current zebrafish models. Moreover, the ability to correlate specific mtDNA mutations with metabolic dysfunction and disease progression opens avenues for more detailed phenotypic and metabolic assays. This continuous tracking across developmental stages not only enhances the robustness of genotype–phenotype correlations but also paves the way for translational applications in modeling mitochondrial diseases with early onset lethality. Moreover, the high-throughput nature of the protocol facilitates the screening of large sample sizes, enhancing statistical power when investigating rare mitochondrial variants and their associated phenotypes.

In conclusion, the optimization of the non-destructive enzyme genotyping method provides a valuable tool for zebrafish researchers, enabling efficient molecular characterization of both nuclear and mitochondrial genomes while maintaining animal viability. This approach not only enhances the ethical aspects of zebrafish research by reducing animal mortality but also expands the experimental possibilities for developmental, genetic, and disease modeling studies, particularly for conditions with early onset lethality.

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Authors' Contributions

The idea was conceived by A.S and S.C.E. The article was written by K.S.M, S.R.H, A.S., and S.C.E. Experiments were executed by K.S.M., S.R.H., M.M., and A.S. with experimental guidance from S.C.E. Data analysis was completed by K.S.M, S.R.H., and A.S. The article was reviewed and edited by K.S.M, A.S, and S.C.E.

Data Availability

Raw sequencing data has been uploaded on NCBI SRA (SRA ID: PRJNA1243465).

Author Disclosure Statement

The authors declare that there are no conflicts of interest related to the work reported in this manuscript.

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Figures

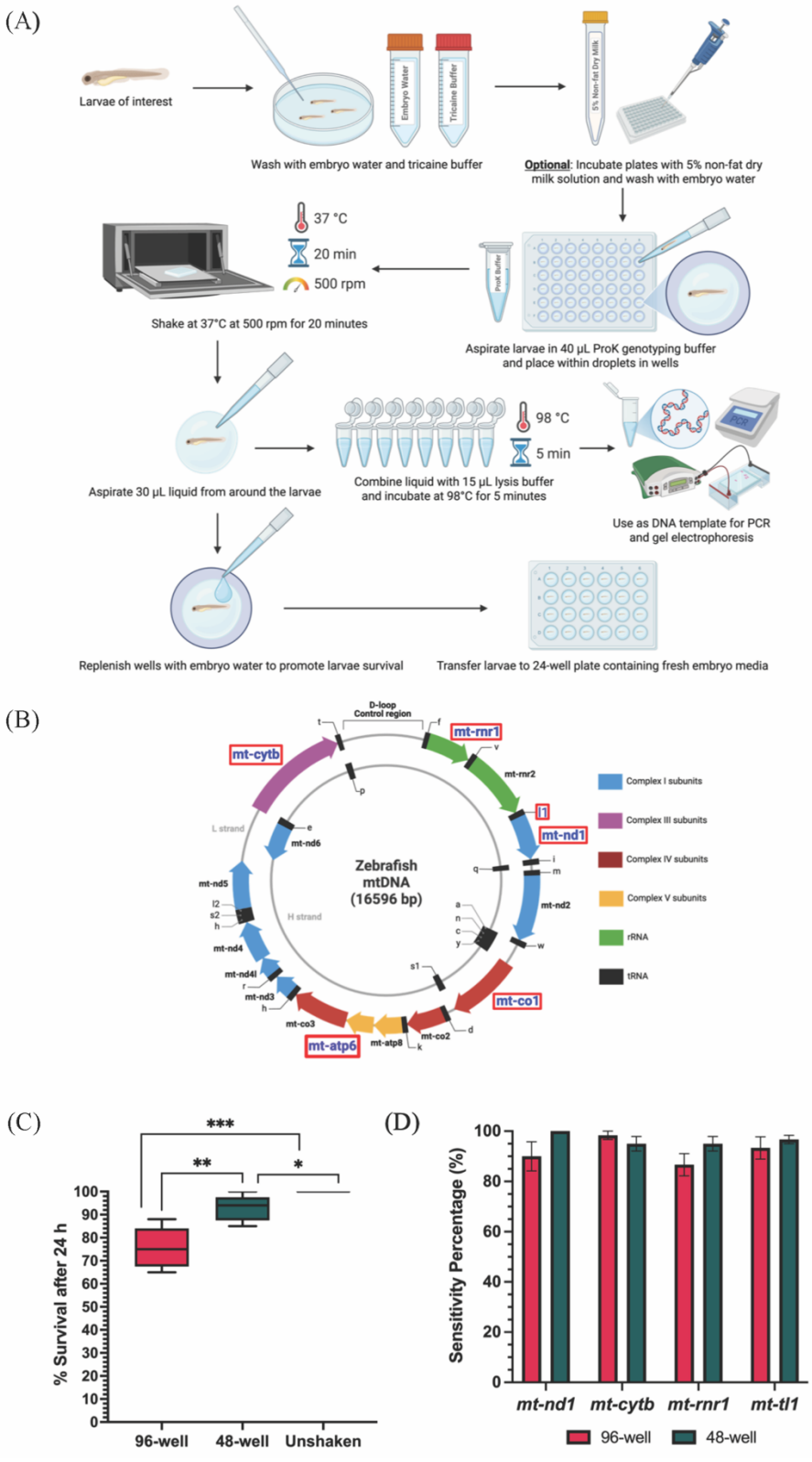
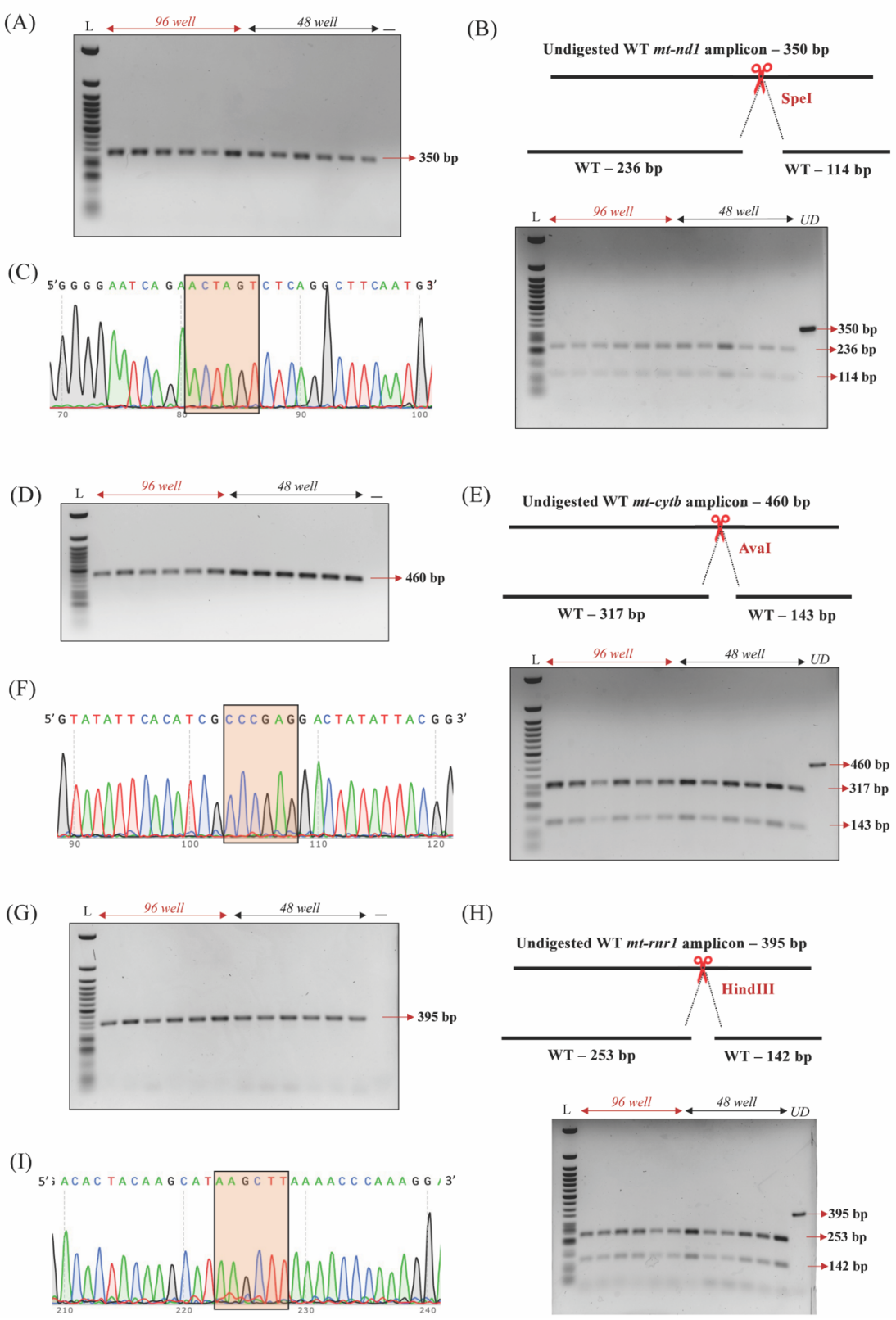

(A)
Larvae of interest
Embryo Water
Tricaine Buffer
Wash with embryo water and tricaine buffer
5% Non-fat Dry Milk
Optional: Incubate plates with 5% non-fat dry milk solution and wash with embryo water
37 °C
20 min
500 rpm
Shake at 37°C at 500 rpm for 20 minutes
ProK Buffer
Aspirate larvae in 40 µL ProK genotyping buffer and place within droplets in wells
Aspirate 30 µL liquid from around the larvae
98 °C
5 min
Combine liquid with 15 µL lysis buffer and incubate at 98°C for 5 minutes
PCR
Use as DNA template for PCR and gel electrophoresis
Replenish wells with embryo water to promote larvae survival
Transfer larvae to 24-well plate containing fresh embryo media
(B)
D-loop Control region
mt-rnr1
mt-rnr2
mt-cytb
mt-nd1
mt-nd2
mt-co1
mt-atp6
mt-atp8
mt-co2
mt-co3
mt-nd3
mt-nd4l
mt-nd4
mt-nd5
mt-nd6
L strand
H strand
Zebrafish mtDNA (16596 bp)
Complex I subunits
Complex III subunits
Complex IV subunits
Complex V subunits
rRNA
tRNA
(C)
% Survival after 24 h
96-well
48-well
Unshaken
(D)
Sensitivity Percentage (%)
mt-nd1
mt-cytb
mt-rnr1
mt-tl1
96-well
48-well

Figure 1: Non-Destructive Enzyme Genotyping Workflow and Validation of Survival and Sensitivity. (A) Schematic of non-destructive enzyme genotyping protocol. **(B)** Schematic of the zebrafish mitochondrial genome showing genes targeted for non-destructive genotyping (highlighted in red boxes) **(C)** Box-and-whisker plot comparing survival percentages of larvae 24-hour post genotyping in 96-well, 48-well, and unshaken control groups. Whiskers represent maximum and minimum values. p-Values were determined by Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p<0.001$). (N=100 technical replicates across 5 experiments). **(D)** Sensitivity of the protocol is represented by the percentage of successfully amplified samples across four mitochondrial genes (N=60 technical replicates across 3 experiments). No statistically significant difference was observed between the well formats with respect to the amplicon sensitivity. Error bars are represented as standard error of mean. Figure 1 A and B was created using Biorender.com



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409 **Figure 2: Specificity of Mitochondrial DNA Amplification. (A)** Gel electrophoresis of the
410 mitochondrial gene *mt-nd1*, showing amplification of the 350 bp band. **(B)** Screening of *mt-nd1*

411 amplicon by RFLP. PCR amplicons from wild type larvae show the presence of uncut product
412 (350 bp) and expected 236 and 114 bp digested bands. UD = undigested PCR product. **(C)**
413 Representative chromatogram of the *mt-nd1* amplicon depicting the restriction enzyme recognition
414 site. **(D)** Agarose gel depicting amplification of the mitochondrial gene *mt-cytb*, with the expected
415 460 bp fragment. **(E)** RFLP analysis of the *mt-cytb* PCR product, showing the intact 460 bp band
416 and the predicted 317 and 143 bp digestion fragments. UD = undigested PCR product. **(F)**
417 Representative electropherogram of the *mt-cytb* amplicon, with the restriction enzyme recognition
418 site indicated. **(G)** Visualization of *mt-rnr1* amplification, showing a discrete 395 bp band. **(H)**
419 Restriction digest of the *mt-rnr1* PCR product, displaying both the undigested 395 bp fragment
420 and the expected 253 and 142 bp cleavage products. UD = undigested PCR product. **(I)** Sanger
421 sequencing trace of the *mt-rnr1* amplicon, highlighting the enzyme cut site.

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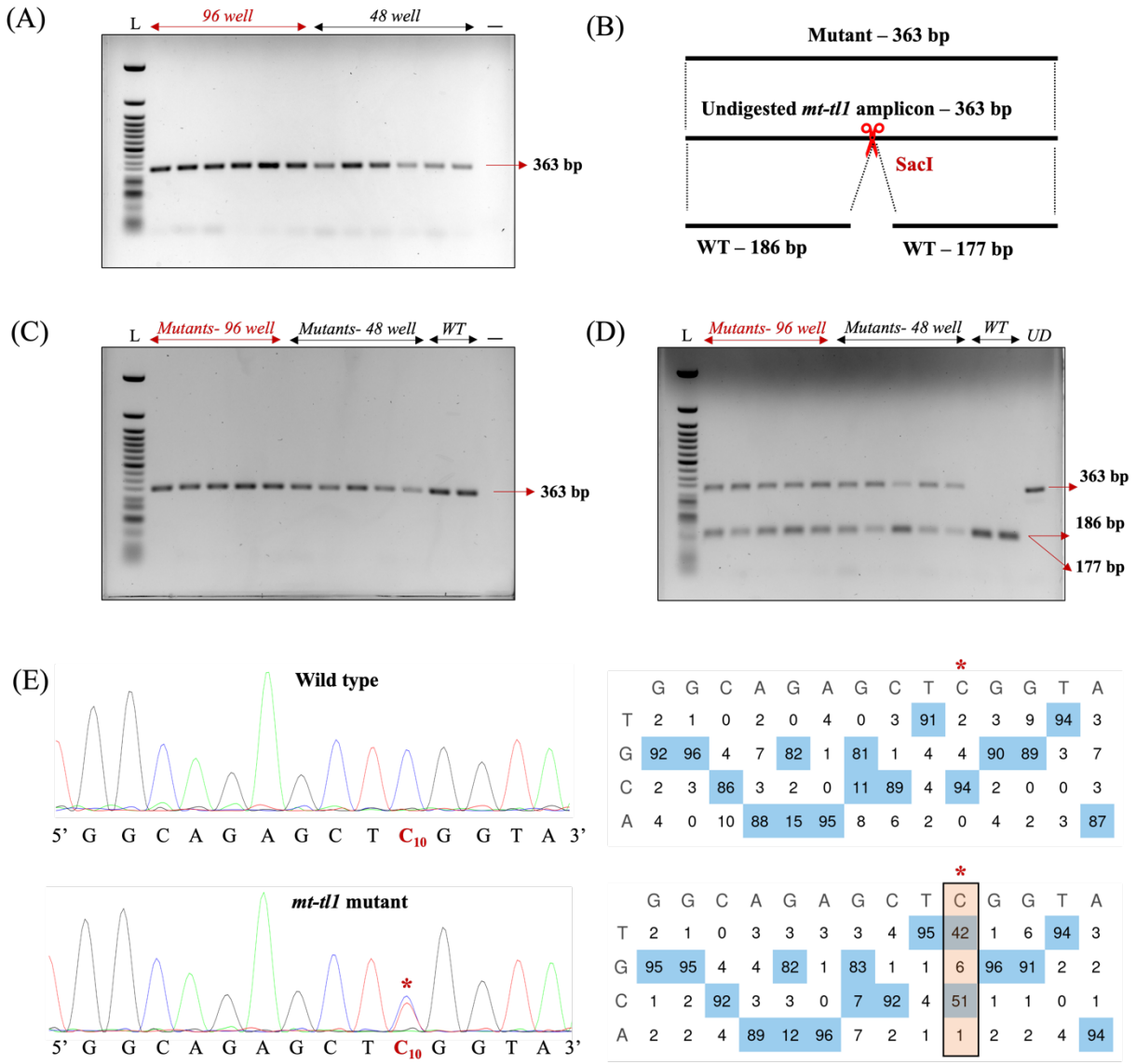

	G	G	C	A	G	A	G	C	T	C*	G	G	T	A
T	2	1	0	2	0	4	0	3	91	2	3	9	94	3
G	92	96	4	7	82	1	81	1	4	4	90	89	3	7
C	2	3	86	3	2	0	11	89	4	94	2	0	0	3
A	4	0	10	88	15	95	8	6	2	0	4	2	3	87

	G	G	C	A	G	A	G	C	T	C*	G	G	T	A
T	2	1	0	3	3	3	3	4	95	42	1	6	94	3
G	95	95	4	4	82	1	83	1	1	6	96	91	2	2
C	1	2	92	3	3	0	7	92	4	51	1	1	0	1
A	2	2	4	89	12	96	7	2	1	1	2	2	4	94

Figure 3: Screening for mtDNA mutants using non-destructive genotyping in *mt-tl1* locus. **(A)** PCR amplification of the *mt-tl1* loci from wild-type larvae resolved on a 1% agarose gel to confirm product integrity. **(B)** Schematic of the SacI recognition site within the amplicon (363 bp) and the expected fragment sizes (186 and 177 bp) for both unedited and edited alleles. **(C)** PCR products from *mt-tl1* mutants are shown on an agarose gel, demonstrating successful amplification. **(D)** RFLP analysis reveals that C-to-T conversion at the target cytosine leading to loss of SacI recognition site: control larvae yield the expected 186 bp and 177 bp fragments, whereas edited samples display a single undigested band (each lane represents individual sample DNA; UD = undigested PCR product). **(E)** Sanger sequencing chromatograms from control and mutant larvae. Asterisk (*) denotes the site of edit with corresponding editing percentage (C-to-T). Chromatograms and editing table plot were obtained using EditR.

436 ## Supplementary Materials

437 **Supplementary Table 1:** List of oligonucleotides used in this study

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
mt-nd1	CAGGAAGACACATGACTTCTA	AGTTGGTCGTATCGGAATCG
mt-cytb	GCAATACACTACACCTCAGACAT	ATCCGGTTTCGTGGAGAAATAG
mt-rnr1	GGTTAAACGAGAGGCTCTAGT	ATTCCTTCACAGGGTAAGCTG
mt-tl1	GTTCAGACCGGAGTAATCCA	GGAGTTGTATATAGCCTAACAC
mt-atp6	ACCGTCCAAACATGATTGACT	TGCTGTGAGGTTTGCAGTAAG
mt-co1	GGAAACTGACTTGTGCCACT	ACTTCTGGGTGGCCAAAGAA

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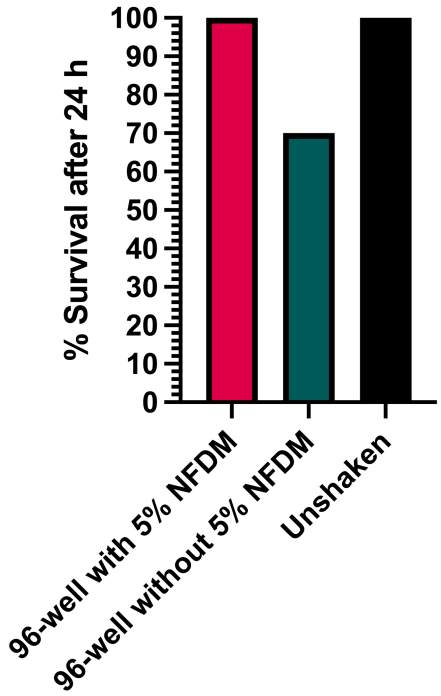

Supplementary Figure 1: Effect of 5% non-fat dry milk (NFDM) solution on larval survival post genotyping. Survival percentage of wild type larvae at 24 hours post genotyping in 96-well plate treated with NFDM, 96-well plate without 5% NFDM and unshaken control group. Bars represents survival percentage from a single independent genotyping experiment with 20 larvae per condition.

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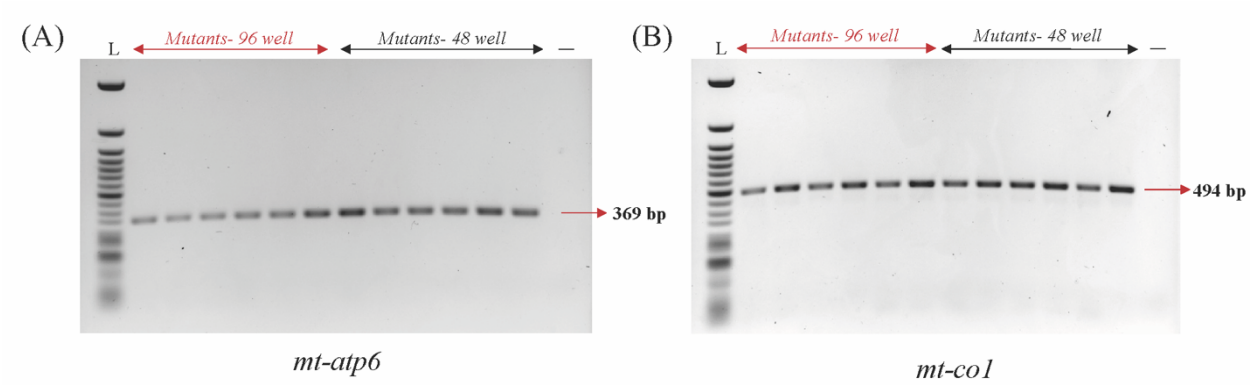

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449 **Supplementary Figure 2: Mitochondrial DNA Amplification for zebrafish *mt-apt6* and *mt-***
450 ***co1*. (A)** Gel electrophoresis of the mitochondrial gene *mt-atp6*, showing amplification of the
451 369 bp band. **(B)** Gel electrophoresis of the mitochondrial gene *mt-co1*, showing amplification of
452 the 494 bp band.