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TITLE:

Indel Detection Following CRISPR/Cas9 Mutagenesis Using High-resolution Melt Analysis in the Mosquito *Aedes aegypti*

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SUMMARY:

This article details a protocol for rapid identification of indels induced by CRISPR/Cas9 and selection of mutant lines in the mosquito *Aedes aegypti* using high-resolution melt analysis.

ABSTRACT:

Mosquito gene editing has become routine in several laboratories with the establishment of systems such as transcription-activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and homing endonucleases (HEs). More recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology has offered an easier and cheaper alternative for precision genome engineering. Following nuclease action, DNA repair pathways will fix the broken DNA ends, often introducing indels. These out-of-frame mutations are then used for understanding gene function in the target organisms. A drawback, however, is that mutant individuals carry no dominant marker, making identification and tracking of mutant alleles challenging, especially at scales needed for many experiments.

High-resolution melt analysis (HRMA) is a simple method to identify variations in nucleic acid sequences and utilizes PCR melting curves to detect such variations. This post-PCR analysis method uses fluorescent double-stranded DNA-binding dyes with instrumentation that has temperature ramp control data capture capability and is easily scaled to 96-well plate formats. Described here is a simple workflow using HRMA for the rapid detection of CRISPR/Cas9-induced indels and the establishment of mutant lines in the mosquito *Ae. aegypti*. Critically, all steps can be performed with a small amount of leg tissue and do not require sacrificing the organism, allowing genetic crosses or phenotyping assays to be performed after genotyping.

INTRODUCTION:

As vectors of pathogens such as dengue[1], Zika[2], and chikungunya[3] viruses, as well as malarial parasites[4], mosquitoes represent a significant public health threat to humans. For all these diseases, there is a substantial focus of transmission intervention on the control of mosquito vectors. Study of the genes important in, for example, permissiveness to pathogens, mosquito fitness, survivorship, reproduction, and resistance to insecticides is key for developing novel mosquito control strategies. For such purposes, genome editing in mosquitoes is becoming a common practice, especially with the development of technologies such as HEs, ZFNs, TALENs, and most recently, CRISPR with Cas9. The establishment of gene-edited strains typically involves backcrossing individuals carrying the desired mutations for a few generations to minimize off-target and founder (bottleneck) effects, followed by crossing heterozygous individuals to generate homozygous or trans-heterozygous lines. In the absence of a dominant marker, molecular genotyping is necessary in this process because, in many cases, no clear phenotypic traits can be detected for heterozygous mutants.

Although sequencing is the gold standard for genotypic characterization, performing this across hundreds, or possibly thousands of individuals, poses significant costs, labor, and time required to obtain results, which is especially critical for organisms with short lifespans such as mosquitoes. Commonly used alternatives are Surveyor nuclease assay[5] (SNA), T7E1 assay[6], and high resolution melt analysis (HRMA, reviewed in [7]). Both SNA and T7E1 use endonucleases that cleave only mismatched bases. When a mutated region of the heterozygous mutant genome is amplified, DNA fragments from mutant and wild-type alleles are annealed to make mismatched double-stranded DNA (dsDNA). SNA detects the presence of mismatches via digestion with a mismatch-specific endonuclease and simple agarose gel electrophoresis. Alternatively, HRMA uses the thermodynamic properties of dsDNA detected by dsDNA-binding fluorescent dyes, with the disassociation temperature of the dye varying based on the presence and type of mutation. HRMA has been used for the detection of single-nucleotide polymorphisms (SNPs)[8], mutant genotyping of zebra fish[9], microbiological applications[10], and plant genetic research[11], among others.

This paper describes HRMA, a simple method of molecular genotyping for mutant mosquitoes generated by CRISPR/Cas9 technology. The advantages of HRMA over alternative techniques include 1) flexibility, as it has been proven useful for various genes, a wide range of indel sizes, as well as the distinction between different indel sizes and heterozygous, homozygous, and trans-heterozygous differentiation[12-14], 2) cost, as it is based on commonly used PCR reagents, and 3) time-saving, as it can be performed in just a few hours. In addition, the protocol uses a small body part (a leg) as a source of DNA, allowing the mosquito to survive the genotyping process, permitting the establishment and maintenance of mutant lines.

PROTOCOL:

1. Scanning for single nucleotide polymorphisms (SNPs), HRMA primer design, and primer validation

1.1. SNP identification in wild-type laboratory colony mosquitoes

1.1.1. Select the target exon to disrupt proper polypeptide translation.

NOTE: The target should be close to the start codon or amongst key residues required for protein function. The shorter the exon (e.g., ≤200 bases), the more difficult it is to target and analyze. Avoid editing close to the boundaries of an exon, as this forces one of the HRMA primers to either cross an intron or be in an intron. This is undesirable because SNP rates tend to be much greater in those regions.

1.1.2. Primer design

1.1.2.1. Go to the NCBI Blast – Primer Blast website[15], copy and paste the chosen **exon** in the box at the top of the page | choose the **PCR product size** (ensure it encompasses most of the exon) | choose the **organism**.

1.1.2.2. Click on **Advanced parameters** | **Opt** (for PCR Product Tm) and add **60** (for an optimal temperature of 60 °C) | Get **Primers**. Keep the other parameters as default.

1.1.3. Obtain genomic DNA (gDNA) from the wild-type laboratory colony. Set aside 10 mosquitoes, anesthetize them with CO_2, and place them in a Petri dish on ice to keep them inactive. Set up 10 tubes containing 0.5 µL of the reagent for release of DNA from tissue and 20 µL of dilution buffer, both provided in the DNA release kit suggested in the **Table of Materials**.

1.1.4. Remove one leg from a single mosquito using forceps and place it in a corresponding tube of a diluted solution of the DNA-release reagent (from step 1.1.3), completely submerging the leg in the solution. Repeat this step with the remaining mosquitoes, wiping the forceps with 70% ethanol before proceeding to the next one.

1.1.5. Incubate the leg-containing solution at room temperature for 2–5 min, then at 98 °C for 2 min, and allow it to cool down while setting up the PCR reaction.

NOTE: Plates containing the released gDNA can be stored at -20 °C, and the protocol can be paused at this point.

1.1.6. Prepare 10 tubes containing 10 µL of 2x PCR Master Mix, primers to a 0.5 µM final concentration, and molecular grade water to a final volume of 20 µL, and transfer 1 µL of the diluted sample to each tube. Perform PCR following these cycling parameters: 98 °C 5 min, 40 cycles of 98 °C for 5 s, 60 °C 30 s, 72 °C 20 s per kb; final extension of 72 °C for 1 min.

1.1.7. Purify the PCR products with an enzyme to degrade the residual PCR primers and dephosphorylate excess dNTPs or any column clean-up kit. Proceed with sequencing the samples.

1.1.8. Analyze each electropherogram for the presence of double peaks/ambiguous bases, and adjust base calls manually in each sequence using the appropriate degenerate base code.

NOTE: This step must be performed before multiple sequence alignment as it is common for the base-calling software to select the more prominent peak as the "true" base call, giving the false impression of an absence of SNPs.

1.1.9. Perform a multiple sequence alignment using the alignment software, **SeqMan Pro**, listed in the **Table of Materials** or other open-source alignment software, such as ClustalW[16] or T-Coffee[17].

1.1.9.1. Open the alignment software | click on **Add Sequences** | select the desired sequences and click on **Add** | once all sequences are chosen, click on **Done**.

1.1.9.2. Click on **Assemble** to perform the alignment. To open the alignment, click on **Contig 1** and analyze the alignment and identify the SNPs (**Figure 1**).

NOTE: As an alternative to steps 1.1.3–1.1.6, PCR can be performed from isolated genomic DNA obtained from bulk samples (derived from >10 individuals). Sequenced amplicons can be analyzed directly for the presence of SNPs appearing as double peaks in the electropherogram, though rare SNPs will be more difficult to detect.

1.1.10. Design 3–5 single guide RNAs (sgRNAs), avoiding regions containing any SNP identified above, following the protocol described in [18].

1.2. HRMA primer design

1.2.1. Test the exon sequence for the possible formation of secondary structures during PCR using mFold[19].

1.2.1.1. Go to the UNAFold Web Server | click on **mFold** | on the dropdown menu, click on **Applications** | **DNA Folding Form**.

1.2.1.2. Enter the **sequence name** in the box and paste the **exon**.

1.2.1.3. Change the folding temperature to **60 °C**; on the **Ionic conditions**, change [Mg^{++}] to **1.5** and on Units switch to **mM**; click on **Fold DNA**.

1.2.1.4. On **Output**, below **Structure 1**, click on **pdf** to open the Circular Structure Plots.

1.2.2. Go to NCBI Blast – Primer Blast[15] for primer design.

1.2.3. Copy and paste the selected exon sequence determined by sequencing in step 1.1 (the sequence that contains the lowest number of SNPs or no SNPs) in the box at the top of the page.

1.2.4. Use the symbol **< >** to mark and exclude sequences that contain SNPs, the target site, and

regions with possible formation of secondary structure.

1.2.5. Select the **PCR product size** to be between 80 and 150 bp and choose the **organism**.

NOTE: Larger fragment sizes can be successfully used (~300 bp). However, longer amplicons may decrease sensitivity between sequences differing in one or just a few base pairs.

1.2.6. Click on **Get Primers**. Select 2–3 pairs of primers to be tested (ideally, primer sites are ≥20–50 bp away from any CRISPR target sites).

1.3. Primer validation

1.3.1. Perform a gradient PCR using gDNA from a single individual.

1.3.1.1. Prepare a master mix and remove one sample for the non-template control (NTC) in a separate tube. Add the template to the remaining master mix and aliquot into a 96-well plate.

1.3.1.2. Follow the cycling parameters: 98 °C 30 s, 34 cycles of 98 °C for 10 s, 55–65 °C 30 s, 72 °C 15 s; final extension of 72 °C for 10 min.

1.3.1.3. Generate thermal melt profiles following the parameters: denaturation step 95 °C for 1 min, annealing 60 °C for 1 min, melt curve detection between 75 °C and 95 °C in 0.2 °C increments, with a hold time of 10 s at each temperature.

NOTE: Only annealing temperatures with a single thermal melt profile should be used.

1.4. Proceed to generate the mutant lines with embryo injections as described in [12].

2. Preparation of genomic DNA from mosquito legs

2.1. Separate the G_1 mosquitoes by sex at the pupal stage so that they do not mate before genotyping, and make sure that age-matched, wild-type control mosquitoes will be available to be used for references and backcrosses. Sex-separate them likewise.

2.2. Gather the materials needed (**Figure 2A**) and prepare a 96 well PCR plate with 0.5 µL of the DNA-release reagent and 20 µL of dilution buffer (from the gDNA release kit) per individual to be genotyped (**Figure 2B**) and leave it on ice. Reserve two reactions for NTC.

2.3. Label a tray for mosquito vials (*Drosophila* vials) so each well on the 96-plate corresponds to the respective mosquito vial in the tray.

2.4. Anesthetize the G_1 mosquitos with CO_2 and place them in a glass Petri dish to keep them sedated (**Figure 2C,D**). Anesthetize and place 8 wild-type mosquitoes in a second Petri dish.

2.5. Wipe a pair of tweezers with 70% ethanol and remove one of the mosquito hind legs (**Figure 2E,F**).

2.6. Submerge the leg in the DNA-release reagent solution, place the mosquito in the corresponding vial, and close it with a sponge (**Figure 2G,H**).

2.7. Wipe the tweezers again with 70% ethanol and proceed with removing the leg from the next mosquito. Repeat steps 2.5–2.7 until the 96-well plate is completed.

NOTE: It is important to wipe the tweezers with 70% ethanol. This minimizes DNA cross-contamination.

2.8. Seal the plate with an optical PCR plate seal (**Figure 2I**) and incubate the 96-well plate containing the legs at room temperature (RT) for 2–5 min and then at 98 °C for 2 min. Allow the plate to cool down to RT while preparing the PCR mix.

NOTE: The entire process typically takes 3–4 h. If the mosquitoes must be kept in the vials for more time than the expected duration (especially ≥1 day), place a small piece of raisin (or source of sugar and water) with each mosquito to ensure the survival of the mosquitoes during extended incubations.

3. HRMA

3.1. Perform PCR.

3.1.1. Prepare a master mix containing the following components per each reaction: 10 µL of 2x buffer (from the gDNA release kit), 0.5 µM of each primer, 1 µL of EvaGreen dye, 0.4 µL of polymerase (from gDNA release kit), and complete to 19 µL with molecular-grade water.

3.1.2. Using a multichannel pipet, transfer 19 µL of the master mix into each well. Transfer 1 µL of the DNA release solution containing mosquito DNA prepared in section 2 to the plate (**Figure 3A**). Seal the plate with an optical PCR plate seal.

3.1.3. Perform the PCR following the cycling parameters: 98 °C for 5 min, 39 cycles of 98 °C for 10 s, the chosen annealing temperature (72 °C) for 30 s; final extension 72 °C for 2 min.

3.2. Generate thermal melt profiles following the parameters: denaturation step 95 °C for 1 min, annealing 60 °C for 1 min, melt curve detection between 75 °C and 95 °C in 0.2 °C increments, with a hold time of 10 s at each temperature. See the **Supplemental Material** and **Supplemental Figure S1–5** for a detailed description of the software setup for HRMA run using the CFX96 Real-Time System.

3.3. Examine the melt profiles (**Figure 3B**). Assign wild-type control to the reference cluster.

NOTE: The software (see the **Table of Materials**) automatically normalizes the data and designates clusters with colors for different melt curves.

3.4. Mark the different clusters with corresponding colors on the 96-well template (**Figure 3C**).

3.5. Select the individuals with curves of interest, remove them from the tubes, and backcross (**Figure 3D**). Blood-feed the mated females and collect G_2 eggs.

4. Sequence verification by Sanger sequencing

4.1. Purify the PCR product from the wells with selected mosquitoes (from the plate prepared in section 3.1), using an enzyme to degrade the residual PCR primers, and dephosphorylate excess dNTPs. Alternatively, use any column clean-up kit and proceed with sequencing the samples.

NOTE: Direct sequencing may be more challenging when the PCR product size is small (≤200 bp). In such cases, design primers to amplify larger fragments encompassing the target site (≥250 bp) and amplify by PCR using the DNA in the 96-well plate (step 2.2).

4.2. Analyze and identify the indels using trace viewer software (**Figure 4**). Alternatively, Poly Peak Parser software[20] can help detect the mutation in heterozygous individuals.

4.2.1. Go to the Poly Peak Parser website, select the sequence from the mutant individuals on the **Browse** menu, and copy and paste the reference sequence on the box.

NOTE: The alignment between the alternate allele and the reference will appear automatically on the right side of the screen.

REPRESENTATIVE RESULTS:

Mosquitoes containing mutations in the genes *AaeZIP11* (putative iron transporter[21]) and *myo-fem* (a female-biased myosin gene related to flight muscles[13]) were obtained using CRISPR/Cas9 technology, genotyped using HRMA, and sequence-verified (**Figure 5**). **Figure 5A** and **Figure 5C** show the normalized fluorescence intensity from the HRM curves from *AaeZIP11* and *myo-fem* mutant samples, respectively, along with wild-type controls. **Figure 4B** and **Figure 4D** show the magnification of the difference curves (from the *AaeZIP11* and *myo-fem* mutant samples, respectively) between the melt profiles of the different clusters assigned by the software after subtracting each curve from the wild-type reference. Heterozygous and homozygous mutant *AaeZIP11* individuals were placed in different clusters and are easily distinguished from the wild-type controls (**Figure 5B**). Heterozygous mutant *myo-fem* individuals are also distinct from the controls (**Figure 5D**). Note that two clusters within the wild-type controls were present in both cases, most likely due to the presence of SNPs in the target region (**Figure 5**), highlighting the need to use multiple control samples to avoid categorizing controls as mutants when SNPs cannot be avoided.

Figure 4A shows the sequence analysis of the *AaeZIP11* mutant. The electropherogram from

AaeZIP11 heterozygous mutants indicates the nucleotide position where the indel occurred. This is represented by a shift from single to double peaks as polymorphic positions will show both nucleotides concomitantly (**Figure 4A**). The number of base pairs deleted or inserted was calculated by counting the single peaks at the end of the run (**Figure 4B**) as one of the DNA strands will be shorter or longer than the other by the number of base pairs deleted or inserted, respectively. It is recommended to use sequence-verified gDNA from the mutants as a reference for the identification of the heterozygotes to help with HRMA analyses for subsequent generations. Manual assignment for the curves may be needed when similar curves are automatically assigned to different clusters. This can be done by comparing the temperature-shifted curves and difference curves. Manual adjustment of the software might be needed to properly assign samples to clusters. HRMA results from *AaeZIP11* and a mutant called *Aeflightin* show that individual sample analyses were needed to successfully categorize heterozygotes, homozygotes, and trans-heterozygotes. Initially, the automatic cluster assignment from the software could not make a proper distinction between the groups (**Figure 6A**, **Figure 6C**, **Figure 6E**, and **Figure 6G**). Each sample was then analyzed individually and assigned to the correct groups based on similarity to reference samples of heterozygotes, homozygotes, and trans-heterozygotes (previously verified by sequencing) (**Figure 6B**, **Figure 6D**, **Figure 6F**, and **Figure 6H**).

FIGURE AND TABLE LEGENDS:

Figure 1: SNP identification. Schematic representation of multiple sequence alignment of *AaeZIP11* fragment from wild-type. In red are the SNPs, and in green are the fragments free of SNPs; this SNP-free region is suggested for sgRNA and primer design. Abbreviations: SNP = single nucleotide polymorphism; sgRNA = single guide RNA; LVP = Liverpool strain.

Figure 2: **Experimental procedure for obtaining genomic DNA from mosquito legs for HRMA.** (**A**) Materials for obtaining genomic DNA including pipettes, tips, PCR plate, optical seal, reservoir, the dilution buffer, and DNA release solution. (**B**) PCR plate preparation containing DNA release reagent and dilution buffer. (**C**) Mosquito anesthesia with CO_2. (**D**) Wiping the tweezers with 70% ethanol to prevent contamination between samples. (**E**) Experimental setup including tweezers, rack for mosquito vials, Petri dish on top of an ice container with anesthetized mosquitoes, and the previously prepared PCR plate. (**F**) Removal of the mosquito leg. (**G**) Zoom view of the mosquito leg being submerged in the DNA release reagent solution. (**H**) Single mosquito placed in the vial. (**I**) Sealing the PCR plate containing the mosquito legs. Abbreviation: HRMA = high-resolution melt analysis.

Figure 3: Experimental procedure for HRM analyses. (**A**) Transferring the released gDNA to a 96-well plate containing the PCR mix. (**B**) Visual inspection of the difference curves. (**C**) Marking each sample on the 96-well template with the same color as its respective difference curve color. (**D**) Backcrossing the individuals from the same cluster. Abbreviations: HRM = high-resolution melt; gDNA = genomic DNA.

Figure 4: Sequence analysis of an *AaeZIP11* mutant. (**A**) Nucleotide alignment of the

*AaeZIP11*Δ56 mutant. Dashes highlighted in yellow are the deleted bases. In the box, the electropherogram transitions from single peaks to double peaks, depicting the position where the deletion occurred (arrow). (**B**) Electropherogram of the end of the sequencing run and the transition from double peaks to single peaks. Note that the number of single peaks represents the number of bases deleted (gray rectangle). Primer sequences are provided in the **Supplemental Table S1**.

Figure 5: HRMA of DNA extracted from mosquito legs. DNA was extracted from a single leg from *AaeZIP11* (**A** and **B**) and *myo-fem* (**C** and **D**) knockout *Ae. aegypti* mosquitoes and analyzed by HRMA. **A** and **C** denote the normalized fluorescence signals of the samples to relative values of 1.0 to 0. **B** and **D** denote the magnification of the curve differences by subtracting each curve from the wild-type reference (Liverpool strain). Abbreviations: HRMA = high-resolution melt analysis; LVP = Liverpool strain; RFU = relative fluorescence units. Primer sequences are provided in the **Supplemental Table S1**.

Figure 6: Examples of manual group assignments. (**A–D**) HRMA for *Aeflightin* mutants. (**A** and **C**) Melt curves (normalized linear scale curves and difference curves, respectively) are automatically grouped by the Precision Melt Analysis Software. (**B**) Normalization of differential curves altered manually. (**D**) After altering the normalization of the differential curves, each sample was assigned individually by the peak temperatures in the difference curves for corresponding positive controls (previously sequenced samples identified by Δ4 and Δ5 heterozygotes and Δ4Δ5 trans-heterozygotes), enabling the clear identification of the 3 groups. Red and brown arrows are added to highlight the peaks at different temperatures. (**E–H**) HRMA for *AaeZIP11* mutants. (**E** and **G**) Melt curves are automatically assigned by the software. (**F** and **H**) Mutants in the second heterozygous self-cross were appropriately assigned to the correct groups by the similarity of the normalized and difference curves to previously determined references (color-coded in curves). Heterozygotes asg, Homozygotes asg, and Trans-heterozygotes asg: assigned melt curves by Precision Melt Analysis Software. Abbreviations: HRMA = high-resolution melt analysis; LVP = Liverpool strain; RFU = relative fluorescence units. Primer sequences are provided in **Supplemental Table S1**.

Supplemental Material: Detailed protocol for setting up HRMA in the CFX96 Real-Time System (e.g., Bio-rad).

Supplemental Figure S1: Step-by-step instructions for setting up the cycling protocol on Bio-rad CFX Manager. See **Supplemental Material** 2.1–2.3.

Supplemental Figure S2: Step-by-step instructions for a plate setup on Bio-rad CFX Manager. See **Supplemental Material** 3.1–3.4.

Supplemental Figure S3: Step-by-step instructions for a plate setup on Bio-rad CFX Manager. See **Supplemental Material** 3.5–3.6.

Supplemental Figure S4: Step-by-step instructions for the run setup on Bio-rad CFX Manager. See

Supplemental Material 4.1.

Supplemental Figure S5: Step-by-step instructions for HRMA analysis on Bio-rad CFX Manager. See **Supplemental Material** 5.1–5.3. Abbreviation: HRMA = high-resolution melt analysis.

Supplemental Table S1: List of primers.

DISCUSSION:

High-resolution melt analysis offers a simple and fast solution for the identification of indels generated by CRISPR/Cas9 technology in the vector mosquito *Ae. aegypti*. It provides flexibility, enabling the genotyping of mosquitoes mutated for a wide range of genes from flight muscle to iron metabolism and more[13,14]. HRMA can be performed in just a few hours from sample collection to the final analyses. Additional time is required for primer design and will also depend on the time to receive any needed sequencing results (for identification of the SNPs), sequence analyses, and primer ordering.

The protocol presented here details the step-by-step methods for successful analyses. One of the most critical steps in this process is the proper design of the primers. HRMA is sufficiently sensitive that the presence of even a single SNP within the fragment amplified can result in a strong difference curve that could be misinterpreted as a mutation. Sequencing laboratory colony samples prior to CRISPR mutagenesis allows for the detection and avoidance of SNP-rich regions when designing the target; the HRMA primers will help to prevent this issue. Designing primers for small amplicons (70–100 bp) can also improve the sensitivity and reproducibility of the experiment as larger fragments can present several melt domains, decreasing the chance to distinguish variants[22]. A limitation of the technique is that completely avoiding SNPs might not be feasible depending on the gene; thus, sequencing and further use of multiple wild-type control individuals may aid the analyses.

Specifically for this protocol, it is important to keep genotyped mosquitoes alive so they can be backcrossed for the establishment of mutant lines. Removing one of the mosquito legs to extract gDNA may be the least invasive way of achieving that; however, the amount of gDNA recovered from this tissue is minimal. The kit suggested in the **Table of Materials** was sufficient for obtaining DNA from a mosquito leg and performing the PCR to complete HRMA.

HRMA can offer more flexibility in terms of detection of a broad range of indel sizes, varying from single-base mismatches to multiple base pairs (*AaeZIP11* had a 56 bp deletion) and surpassing methods such as the SNA or T7E1 (both enzyme mismatch cleavage assays) that are limited to single-base mismatch or small deletions (~20 bp)[5,23]. In addition, SNA does not detect homozygous mutants as the PCR products do not contain mismatches. Moreover, SNA results may also be masked by naturally occurring allelic polymorphisms when the nuclease-treated PCR products have a similar length to the mutant alleles. Neither of these limitations is applicable to HRMA. Ultimately, sequencing is necessary to identify the mutant DNA sequence. However, once a mutation in question is known, HRMA used for the identification of mutant individuals is much faster than direct sequencing. This method preserves valuable samples (more mosquitoes will

survive, the less they have to wait for their genotype results) and reduces the need for sequencing of large numbers of samples, lowering the total cost of the experiment.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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Figure

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Figure

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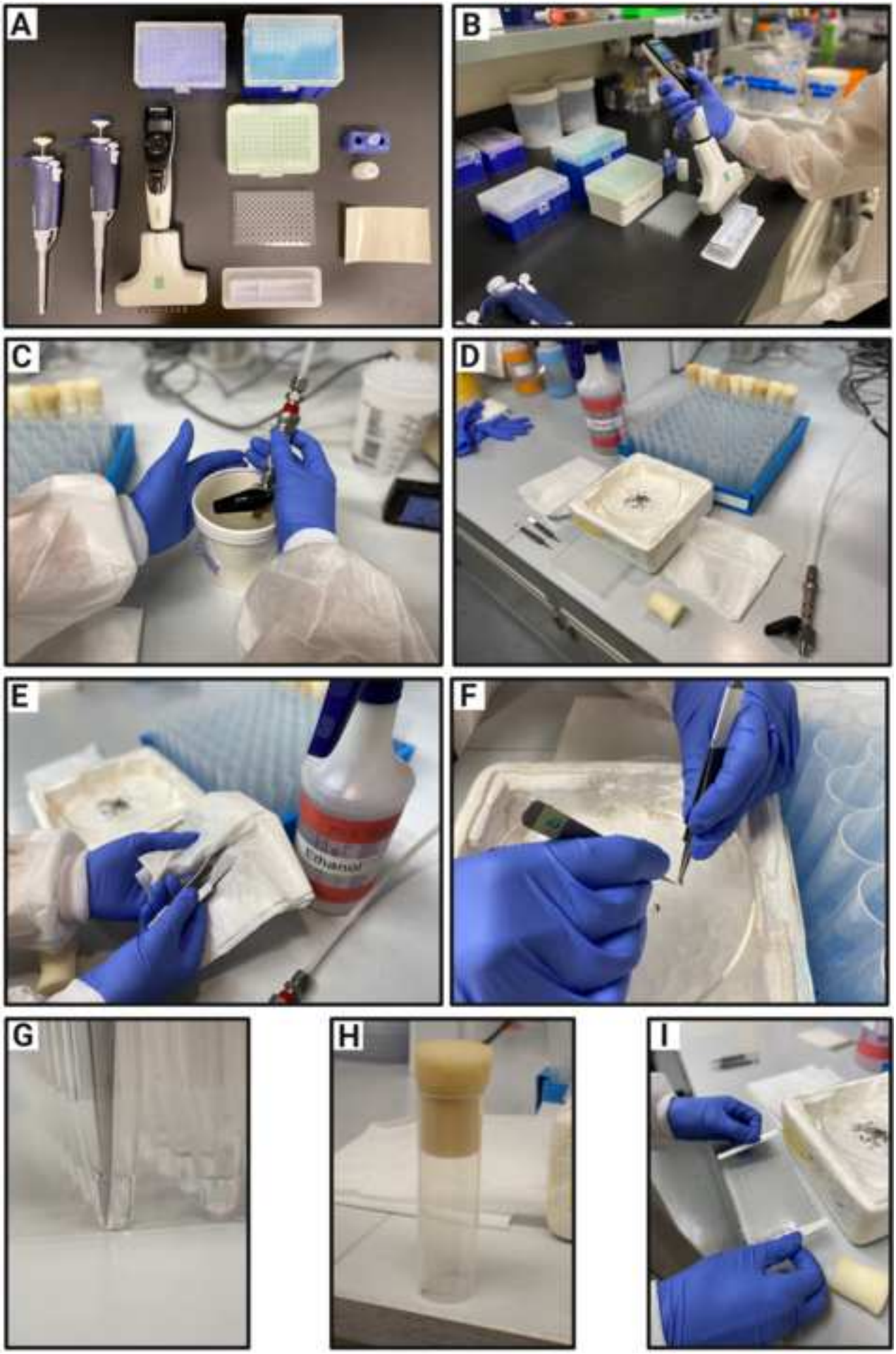

Figure

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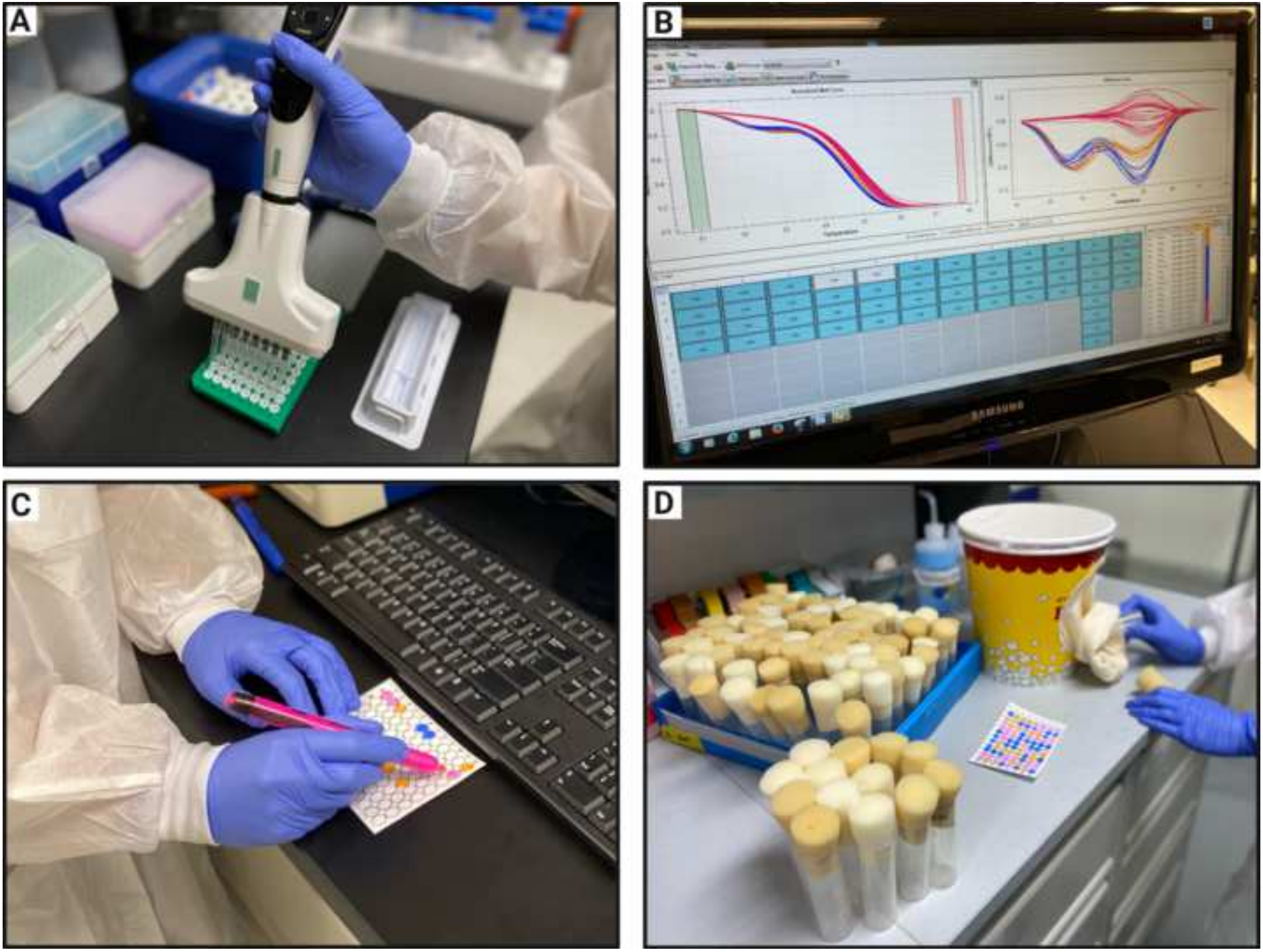

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Figure

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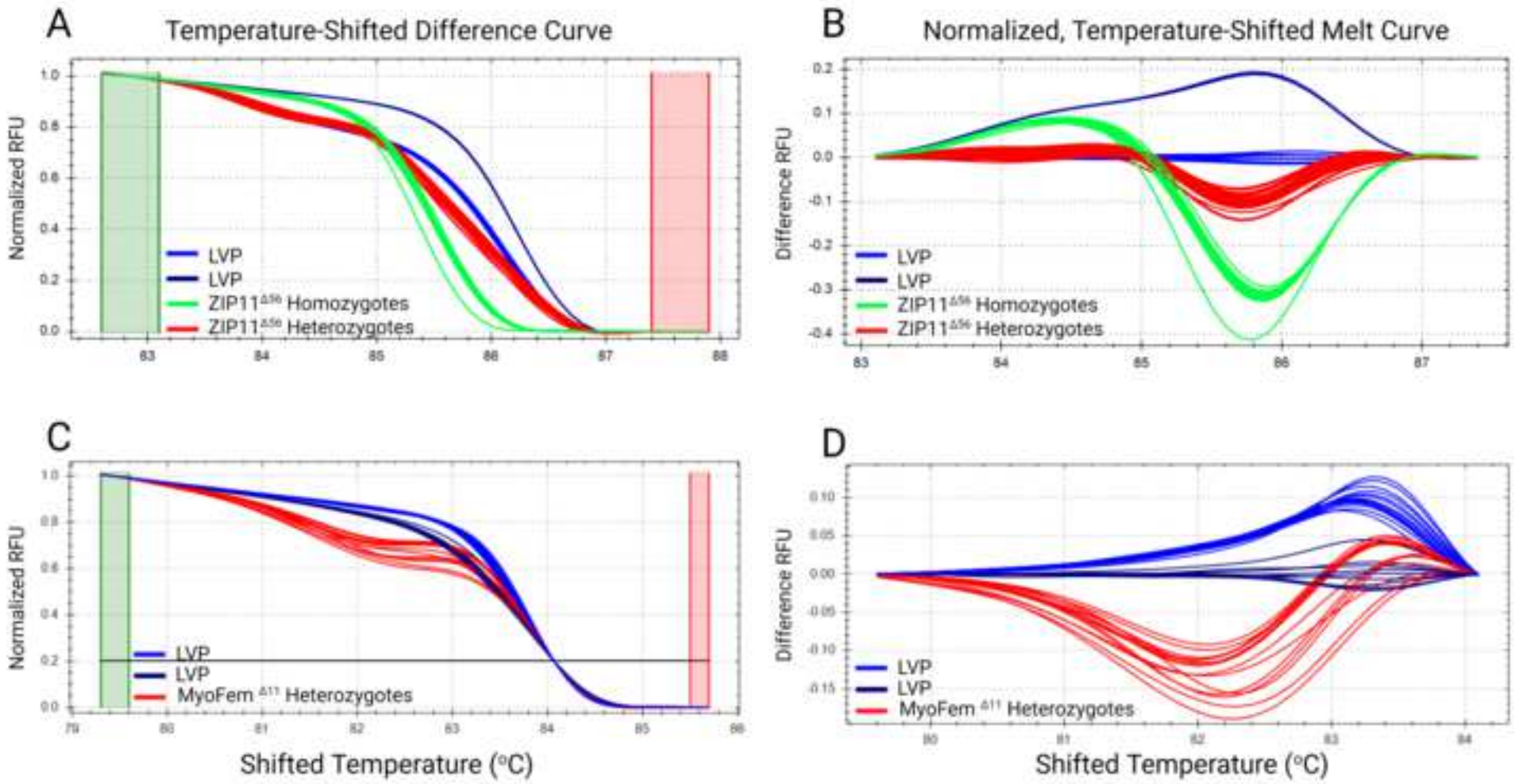

Figure

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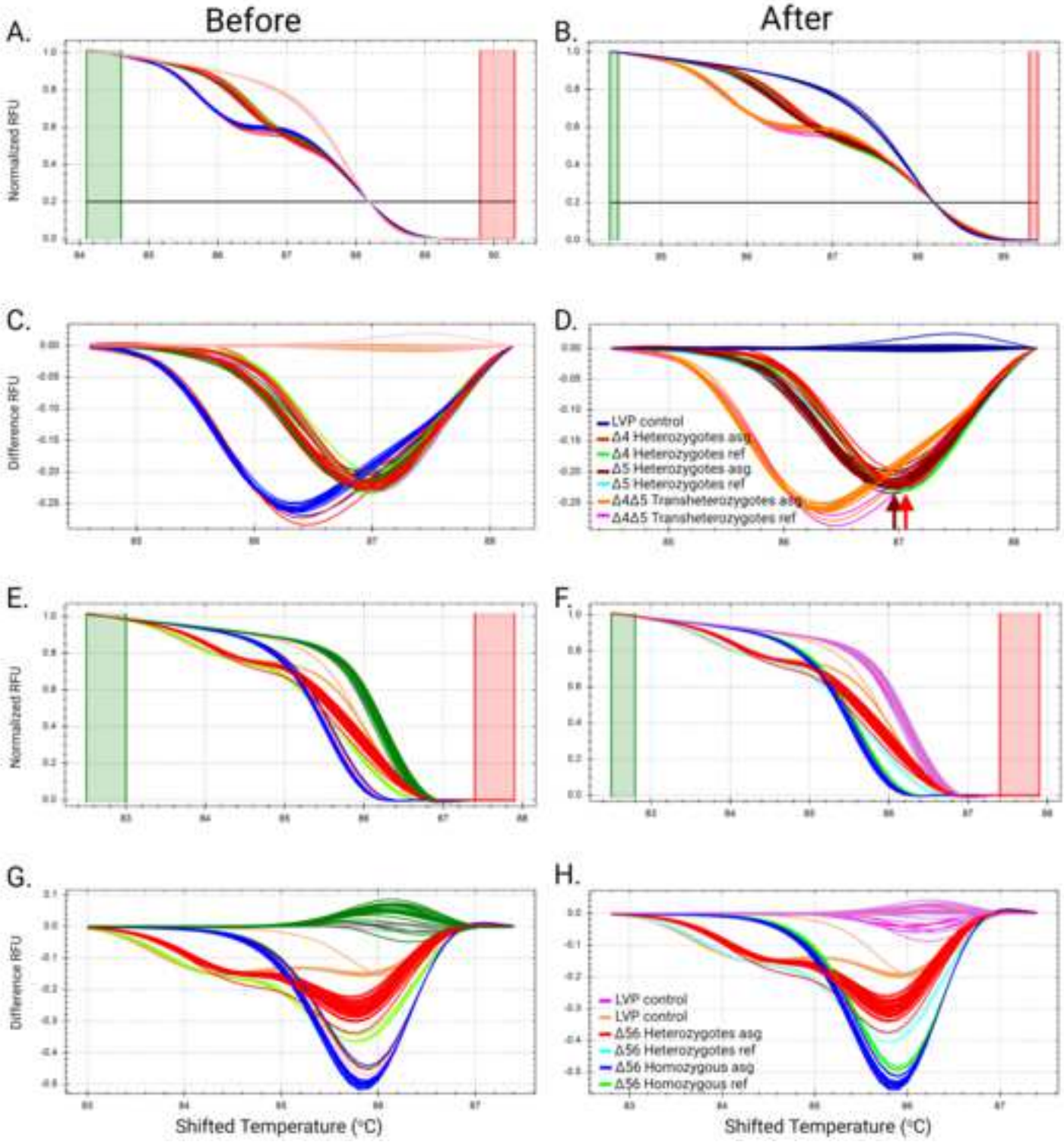

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Table of Materials

Materials list_revised R2.xlsx

Dear Editor,

We are resubmitting the research article entitled "**A protocol for indel detection following CRISPR/Cas9 mutagenesis using high resolution melt analysis in the mosquito *Aedes aegypti*.**" by Kojin et al to be considered for publication in JOVE. Please consider our revised version of the manuscript JoVE63008 for publication in this journal.

We would like to thank you and the reviewers for the comments and criticism, allowing us to improve the quality of the manuscript. Find below, marked in bold, our responses to each of the editorial and reviewer's comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We proofread the manuscript as suggested.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer: We revised the text as suggested

3. Please ensure that the Introduction includes all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Answer: We revised the introduction to make sure we made all points above clear.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Phire Animal Tissue kit, etc.

Answer: We removed the commercial language and used generic terms for all commercial products.

5. For SI units, please use standard abbreviations when the unit is preceded by a numeral

throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2

Answer: We revised the manuscript accordingly.

6. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Answer: We revised the manuscript accordingly.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Answer: We updated the protocol, adding more specific details, more specifically to the NCBI-Primer blast, mFold, and Poly peak parser websites as we believed those were missing more information on how to perform the analyses.

8. Line 89/137/140/250/251: Please include the links are references and cite the appropriate reference numbers.

Answer: We removed the links from the text and included them on the references, and included the citation numbers as requested.

9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Answer: We revise the highlighted steps necessary for the video to form a better narrative. We removed the highlight from steps 2.1, 2.3, and both notes from step 2, Preparation of genomic DNA from mosquito legs.

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Answer: We updated the discussion to better accommodate the points stated above.

11. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Answer: We fixed the bibliography.

12. Figure 5/6: Please specify the units of X and Y axis.

Answer: We added the X axis unit to Figure 5 legend (RFU: relative Fluorescence units) and added the Celsius symbol to the graphs on Figure 5 and 6 for the y axis unit.

13. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Answer: We updated the Material table accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript to the protocol for indel detection following CRISPR/Cas9 mutagenesis using HRMA in the mosquito Aedes aegypti where Bianca Kojin et al demonstrated that HRMA analysis provides the applicable extension for fast screening and marking out various mutated genotypes such homo-, hetero-, and trans-heterozygous in mosquitoes carrying with indel mutation by CRISPR/Cas9 system, by which time saving, cost down and importantly no need sacrifice of mosquitoes that are with no phenotypic traits were easily achieved and that benefits subsequent experimental performance, appreciating that this manuscript provides a significant and information-rich protocol in fast surveying genetic changes occurred within mosquitoes prior to a necessary with sequencing identification. In this protocol, the step by step description for detection a small deletion/insertion-created Ae. Aegypti is kindly detailed and comprehensive to follow. Altogether, this manuscript is highly suggested to be published in this journal due to not only this kind of genetic screening could be used widely for the research study but also technology-based interests to readers.

Major Concerns:

Here, few considerations as below proposed to this protocol might need to be addressed by the authors before it is published,

1. Not all steps are clear in the wider context. e.g. step 1: scanning for SNPs are from wild type

mosquito? Is step 1.1 same as step 1.4? Also step 1.2 was same as step 1.6? Step 1.2 seems like primers design of WT mosquito for PCR product?

Answer: We agreed that the differences between steps was not sufficiently clear, we updated the numbering and added information about which steps were regarding the wild-type mosquitoes.

Minor Concerns:
2. Lines 89-94: There is a newer version of Primer3 available at http://primer3.ut.ee. (for version 4.1.0). Which parameters are suggesting?

Answer: We updated the protocol and instead of using Primer 3 we suggest using NCBI-Primer blast which uses the primer 3 algorithm for primer design but in addition, it performs a blast search of the primer sequence to the chosen organism genome to exclude non-specific primer binding. This software reduces the number of steps spent on primer design while keeping high stringency and accuracy.

3. Lines 111-112. Can sample be storage at -20℃? Which step can be paused?

Answer: Yes it can and we added this note to the protocol.

4. Line 123: What size for small fragments?

Answer: Smaller than 80 bp. We included this information on the note.

5. Line 143: 1.1 is means the step 1.1?

Answer: Yes, we added a step before 1.1 on the protocol.

6. Line 319: please replace HRM to HRMA.

Answer: We fixed accordingly.

7. In Figure 5, is LVP means Liverpool strain as control in this protocol?

Answer: Yes, we added Liverpool strain specification to the figure legend

8. The authors should include which steps you can troubleshoot and how.

Answer: We decided to include this information in the discussion.

Reviewer #2:
Accept.

Reviewer #3:
I went through this very interesting article. Recently, CRISPR/Cas9 Technique has become a buzz in genome editing, not only in mosquitoes but also in other insects. In fact after target genome editing it remains a problem how to be comfortable either mutation or the gene has been verily edited or not. We see there's a lack of dominant marker and hence, it becomes difficult to detect the edited change. This article provides a handsome amount of information and I recommend to publish this article in this good journal.

I have also sent the attached copy of the manuscript. It took me to add a few queries and minor changes, overall it is well written and well presented.

Line 45 – malarial

Answer: Corrected as suggested

Line 100 - 1.4.4 Prepare 10 tubes containing 10 µl of 2x phire tissue direct PCR master Mix, if you are performing tissue direct PCR, you may add herein this heading:
"1.4 Genomic DNA (gDNA)extraction, PCR amplification and sequence analyses"

Answer: We updated the numbering on "1.1. SNPs identification in wild-type laboratory colony mosquitoes" and that step got incorporated on the step 1.1.3.

Line 105 - 20 µl of dilution buffer; dilution buffer can be explained in parentheses
If this solution coming from the Phire Animal Tissue Kit??

Answer: Yes the buffer is provided in the kit and the information was added to the protocol.

Line 108 - If this solution coming from the Phire Animal Tissue Kit??

Answer: We were referring to the solution from the previous step, we added which step (step 1.1.3) we were referring to in the protocol.

Line 123- Did you perform both the steps; means Sequencing of PCR product and cloning then sequence???
Is there any difference of quality of peaks and reproducibility??? Which one is simply the best one for which size of fragment???

Answer: As we mention in the note: "Alternatively, for small fragments (<80 base pairs - bp), the PCR product can be cloned into a standard cloning vector before sequencing

allowing the read of the entire length; however, it will add extra steps, which increase the overall procedure time."

We added the size of the fragment (<80 base pairs - bp) for clarification. And we never noted a difference in the quality of peaks and reproducibility.

Line 354 - Discussion section can be improved, incorporate few more references to cite and to justify the worth of work.
Comparison of HRMA with any other bioinformatics tool commonly used and show HRMA is better.....
Few points/annotations can be added for the mutants described AaeZIP11 and *myo-fem*

Answer: We updated the Discussion, comparing HRMA to other relevant alternative methods like sequencing, SNA, and T7E1. We added a brief description of both genes as well as the references.

Line 375 - myo-fem homozygotes are absent in Fig? Fig. 3??
Did this technique work well with double mutants?

Answer: Yes, we presented the data only for the *MyoFem* heterozygotes. And this technique can work for double mutants, however, each mutated gene will have to be analyzed by HRMA in separate experiments.

Supplemental Material

Detailed protocol for setting up HRMA in the CFX96 Real-Time System (Bio rad)

1. Turn on the CFX96 Real-Time System, push the button on the lid to open, place the plate inside, and push the front button to close.

2. Cycling protocol

2.1. Open the Bio-Rad CFX Manager, under **Select Run Type**, click on **User Defined**; under **Protocol** tab, click on **Create New** (**Supplemental Figure S1A,B**).

2.2. Double click on temperature and time for step 1 and change to 95 °C for 1 min. Double-click on temperature and then time for step 2 and change to 60 °C for 1 min (**Supplemental Figure S1C,D**). Select the default step 3 and click on **Delete Step**, select the default step 4 and click on **Delete Step** (**Supplemental Figure S1E,F**).

2.3. Click on **Insert Melt Curve** after step 2; double-click on the low- and then on the high-range temperature and change to 75 °C and 90 °C, respectively. Double-click on the time for the lower temperature and change to 10 s. Double-click on the decimal increment for the higher temperature and change to 0.2. Click in the **Sample Volume** box and change to the correct value (**Supplemental Figure S1G–I**).

3. Plate setup

3.1. Under the **Plate** tab, click on **Create New**. Click on **Select Fluorophores** and select the relevant colors via the check boxes and click on **OK** (**Supplemental Figure S2A–C**).

3.2. Click on **Experiment Settings** and under the **Targets** tab, go to the **New** box and type the target gene name. Click on **Add** and repeat for any other target genes that are present on the plate (**Supplemental Figure S2D–F**).

3.3. Click on the **Samples** Tab, go to the **New** box, and type the sample name. Click on **Add** and repeat for any other samples that are present on the plate; click on **OK** (**Supplemental Figure 2G,H**).

3.4. Click and drag to highlight cells in the same group or select cells individually by clicking once. Under **Set Sample Type**, click on what kind of sample this is and select the checkbox next to the fluorophores present in the selected samples under the **Load** header for **Target Name** (**Supplemental Figure S2I** and **Supplemental Figure S3A,B**).

3.5. Click on the dropdown and select the target gene. Click on **Sample Name** in the dropdown, and select the sample name (it will check off the load box automatically) (**Supplemental Figure S3C,D**).

3.6. For replicates, select the checkbox next to **Replicate #**. Under the **Load** header, change the number using the arrows on the **Replicate #** box. For replicate series, click on the **Replicate Series** button, change **Replicate Size** and **Starting Rep #**, select **Horizontal** or **Vertical**, click on **Apply**, and click on **OK** (**Supplemental Figure S3E–I**).

4. Run

4.1. Click on the **Start Run** tab and click on **Start Run**. Type the name of the file, check that the new file has a **.pcrd** extension, and click on **Save** (**Supplemental Figure S4A,B**).

5. Analysis

5.1. Open the Bio-Rad Precision Melt Analysis software, select **Create New Melt File**, and click on **OK** (**Supplemental Figure S5A**).

5.2. Navigate to the **.pcrd** file saved earlier and select it; click on **Open**. Type the name of the new file; check that the analysis file has a **.melt** extension and click on **Save** (**Supplemental Figure S5B,C**).

5.3. Note that the program will open to the **Precision Melt** tab, normalize the data, and assign colored clusters. Check the **Reference Cluster** dropdown and set it to the control group if not already done. Turn samples on or off by clicking individual wells, columns, or rows. Finally, visually inspect the differential curves (**Supplemental Figure S5D,E**).

Supplemental Figure S1

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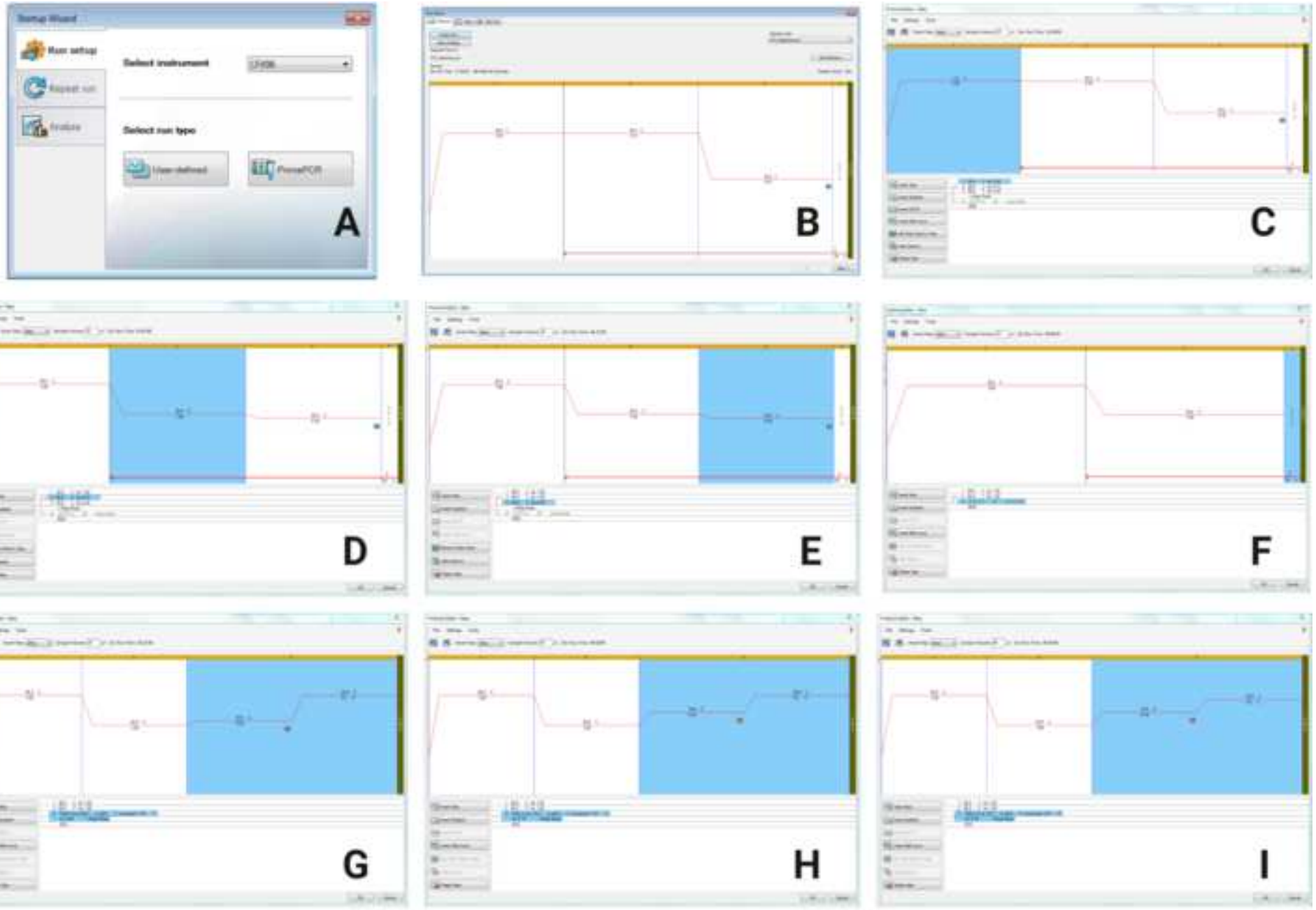

Supplemental Figure S2

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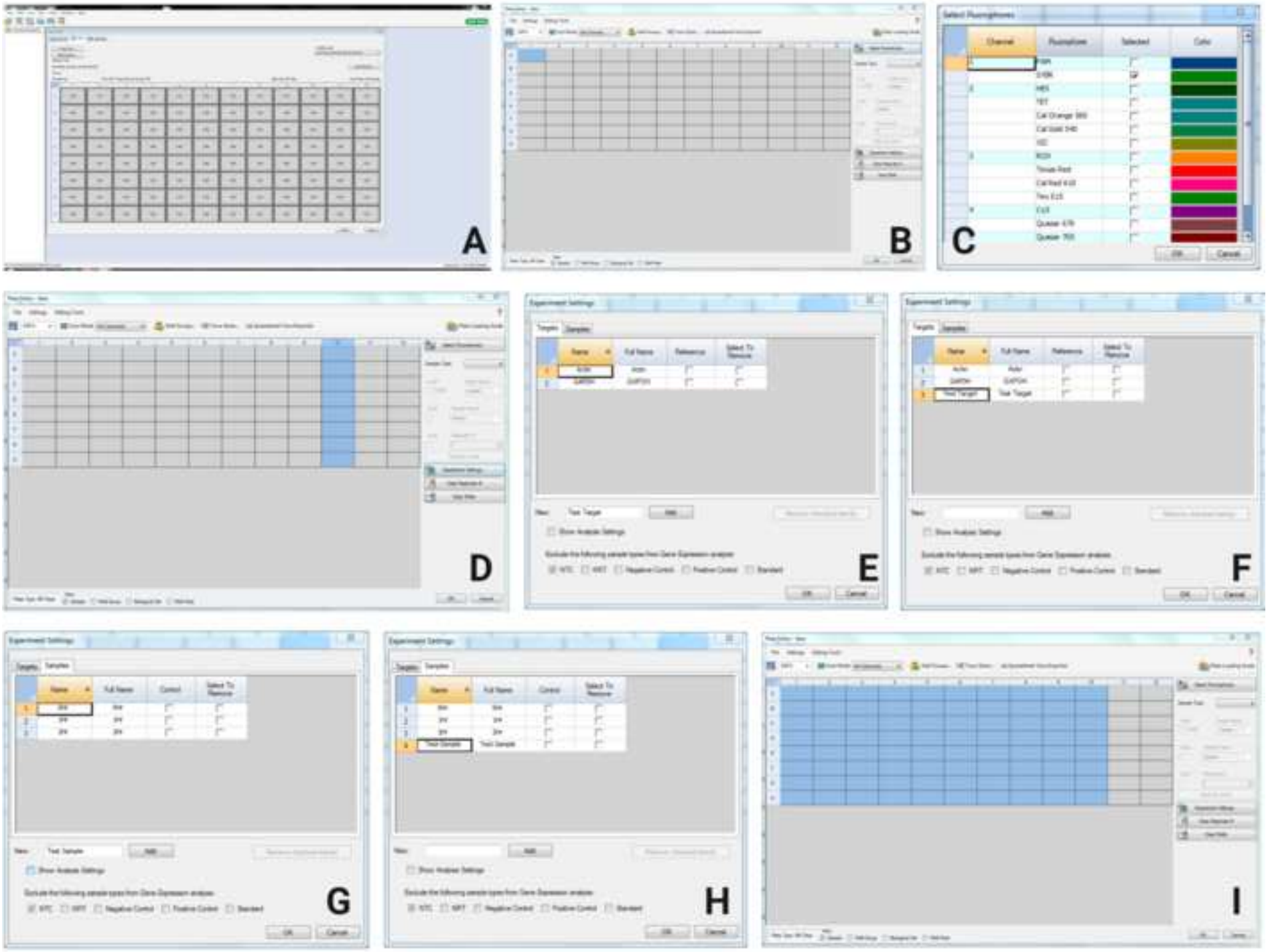

Supplemental Figure S3

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Supplemental Figure S4

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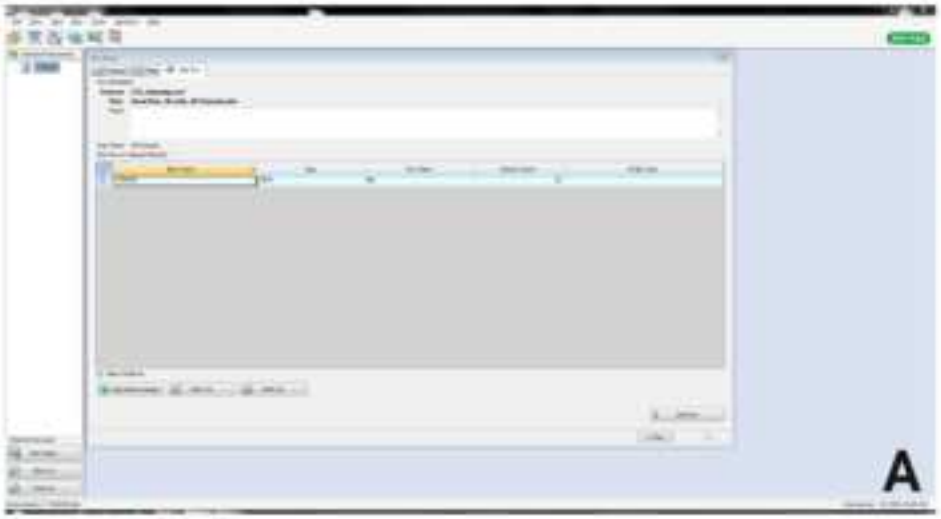

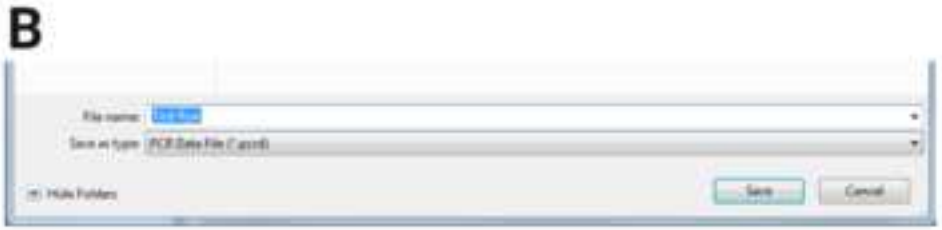

Supplemental Figure S5

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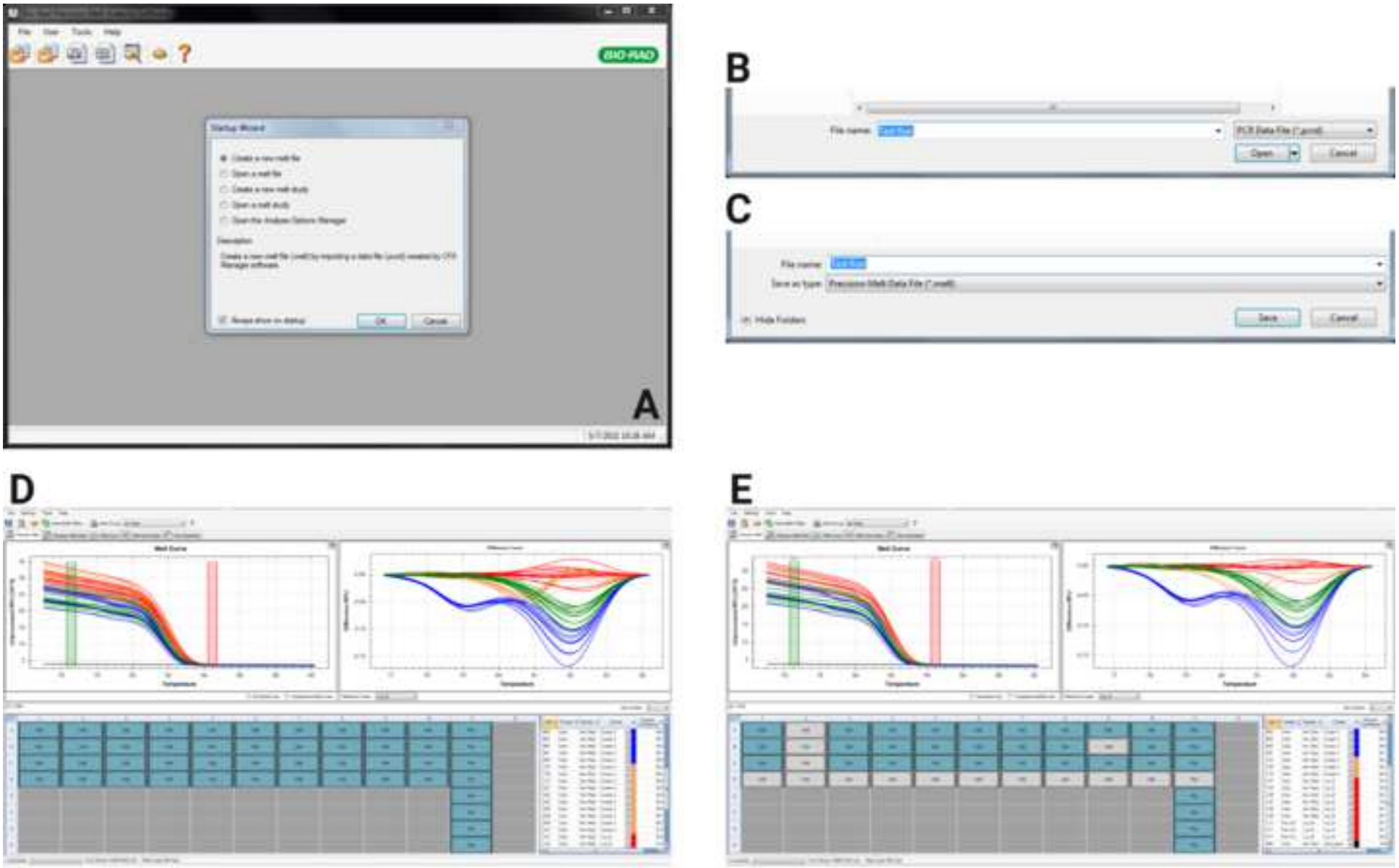

Supplemental Table S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Table S1.xlsx

Primer name	Sequence
Primer for Aeflightin (HRMA)	5' GTTCGACTACCAACTCACCG 3'
Primer for Aeflightin (HRMA)	5' TTCAGAAATAAGCGCTCGTG 3'
Primer for myo-fem (HRMA)	5' TATACTTACATAGATCAGCC 3'
Primer for myo-fem (HRMA)	5' TGCCGCAGACCAAGGATTTC 3'
Primer for ZIP11 (HRMA)	5' TTTGGCCGTCGGAGTGAGTTTT 3'
Primer for ZIP11 (HRMA)	5' GAATGATGCTGGCCACACTGAC 3'
Primer for ZIP11 (SNP screening)	5' GCTTATCCTGTCAATCCCACA 3'
Primer for ZIP11 (SNP screening)	5' CAGAATATCATCCGCCACAA 3'

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Author(s): Bianca Kojin, Hitoshi Tsujimoto, Emma Jakes, Sarah O'Leary, Zach Adelman

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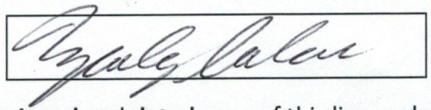
Signature:	
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