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TITLE:

Digital-Droplet PCR to Detect Indels Mutations in Genetically Modified Anopheline Mosquito Populations

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KEYWORDS:

polymorphism, DNA sequence, non-homologous end-joining, ddPCR, mutagenesis, DNA extraction

SUMMARY:

This protocol provides the steps from DNA extraction to experimental set-up for digital droplet PCR (ddPCR), including analysis for the identification and quantification of non-homologous end-joining (NHEJ) events at target sites following gRNA-induced Cas9 cleavage and DNA repair. Other uses of this method include applications such as polymorphism detection and gene-editing variant verification.

ABSTRACT:

Recent advances in mosquito genomics and genetic engineering technologies have fostered a need for quick and efficient methods for detecting targeted DNA sequence variation on a large scale. Specifically, detecting insertions and deletions (indels) at gene-edited sites generated by CRISPR guide RNA (gRNA)/Cas9-mediated non-homologous end-joining (NHEJ) is important for assessing the fidelity of the mutagenesis and the frequency of unintended changes. We describe here a protocol for digital-droplet PCR (ddPCR) that is well-suited for high-throughput NHEJ analysis. While this method does not produce data that identifies individual sequence variation (single nucleotide polymorphism [SNPs]), it provides a quantitative estimate of the sequence variation within a population. Additionally, with appropriate resources, this protocol can be implemented in a field-site laboratory setting more easily than next-generation or Sanger

sequencing. ddPCR also has a faster turn-around time for results than either of those methods, which allows a more quick and complete analysis of genetic variation in wild populations during field trials of genetically-engineered organisms.

INTRODUCTION:

Gene drives have immense potential to control insect populations of medical and agricultural relevance[1–5]. For example, gene-drive systems based on CRISPR Cas nucleases and guide RNAs (gRNAs) can be used to modify vector mosquito populations by introducing traits that confer refractoriness to malaria parasites leading to reduced transmission and less disease[1,4,5]. The gene-drive system copies itself and the associated trait from one homologous chromosome to another in the pre-meiotic germ cells, and this ensures that the majority of the offspring inherit the drive and create the potential for long-lasting and sustainable population modification in the field. However, one disadvantage of the Cas/gRNA-based methods is the possibility of generating insertion and deletion (indel) mutations through non-homologous end-joining (NHEJ) DNA repair, resulting in the generation of drive-resistant alleles, which when accumulated to a high enough frequency in the population, can stop the drive system from spreading[1-4]. This protocol details a high-throughput and reliable method that can determine the prevalence and relative quantity of indel mutations, at both the population and individual level, during Cas/gRNA-based gene drive.

Next-generation sequencing (NGS) methods provide unparalleled sequencing resolution. However, the cost and technical requirements associated with NGS prohibit routine testing and limit its use as a high-throughput method to assess indels[6–8]. Traditional PCR quantification methods have long been used as the standard evaluation procedure for genome indels; however, these methods are labor-intensive, take a long time to procure data, and have a high degree of variability. Droplet Digital PCR (ddPCR) has been proven to be more sensitive at detecting mutations than Sanger sequencing in some applications and has a lower detection limit than NGS in others[6–9]. Moreover, the cost to assess a sample set and turn-around time for obtaining results is less expensive and faster, respectively, for ddPCR than either Sanger sequencing or NGS[9]. Using a dual-probe system, this Drop-Off assay identifies NHEJ alleles based on the absence of wild-type (WT) sequence at the gRNA-directed target Cas9 cut site. In this assay, a short amplicon including the predicted cut site of the Cas/gRNA-based system is amplified with a specific primer pair. One fluorescent probe is designed to bind to a conserved region of the amplicon and another fluorescent probe recognizes the WT sequence of the cut site. In the presence of an NHEJ allele, the latter will not bind to the amplicon.

The use of ddPCR provides the ability to design primers to target deletions, single base-pair differences and insertions, which will allow for NHEJ profiling in mosquito population analyses[9]. Given these attractive features, we created a protocol for ddPCR for high-throughput detection of indels generated from a Cas/gRNA-based gene-drive system in mosquitoes.

PROTOCOL:

1. DNA extraction

1.1 Prepare EDTA/Nuclei Lysis Buffer (EDTA/NLS) with the ratio of 500 µL of NLS and 120 µL of EDTA per sample. Scale-up for multiple samples. Chill the mixture on ice.

NOTE: The solution will turn cloudy in 2–5 min when chilled depending on the volume.

1.2 Homogenize the mosquito sample using a mechanical homogenizer for 10–15 s in a 1.5 mL microcentrifuge tube filled with 600 µL of chilled EDTA/NLS; mix thoroughly.

1.3 Add 17.5 µL of 20 mg/mL of Proteinase K to the tube and mix thoroughly.

1.4 Incubate overnight at 55 °C. Alternately, incubate the sample at 55 °C for 3 h with shaking and vortex the sample every 1 h.

1.5 Add 200 µL of Protein Precipitation Solution to the room temperature sample and vortex vigorously for 20 s.

1.6 Chill the sample for 5 min on ice.

1.7 Centrifuge the sample to pellet proteins at 15,890 RCF for 4 min.

1.8 Carefully aspirate the supernatant which contains the DNA and transfer it to a clean 1.5 mL microcentrifuge tube containing 600 µL of isopropanol.

1.9 Gently mix the solution by inverting the tube 5–10 times. Centrifuge for 1 min at 15,890 RCF. Carefully decant the supernatant preserving.

1.10 Add 600 µL of room-temperature 70% ethanol. Wash the pelleted DNA by gently inverting the tube.

1.11 Centrifuge for 1 min at 15,890 RCF. Carefully remove the supernatant by aspiration using a glass pipette tip.

1.12 Invert the tube onto clean absorbent paper and air-dry the pellet for 10–15 min.

1.13 Resuspend the DNA with PCR-grade water. Use 20 µL per individual mosquito sample or 100 µL for 10 pooled mosquitoes.

NOTE: DNA extraction methods for mosquito samples using a commercially-available kit (see **Table of Materials**) are adapted from the manufacturer's Isolating Genomic DNA from Tissue Culture Cells and Animal Tissue protocol.

2. ddPCR reactions and droplet generation preparation

2.1 Quantify DNA using a fluorometer.

NOTE: For the drop-off assay, it is recommended to use a range of 3,000–30,000 haploid genome copies per reaction, which is designed to detect NHEJ events with a HEX-labeling probe that binds to a WT sequence of the targeted cut site and will not anneal (drop-off) if there is a deletion or insertion at the target site, indicating the presence of an NHEJ variant.

2.2 Calculate the copy number using the haploid genome weight and concentration of DNA in the extract. This is done by multiplying the concentration of the extracted DNA by the volume used to obtain the total DNA mass, then dividing it by the haploid genome weight. Ensure that the volume added is between 1–10 µL. Dilute as necessary to be in the recommended haploid genome copy range.

NOTE: One *Anopheles gambiae* haploid genome is estimated to be 0.27 pg per adult mosquito[10].

2.3 Design primers and probes. Design forward and reverse oligonucleotide primers with a Primer Melting Temperature (Tm) in the range of 55–60 °C that flank the 5'- and 3'-ends of the gRNA target site producing an amplicon of 150–400 bp.

2.3.1 HEX (Hexachloro-fluorescein)-labeled probe for NHEJ detection: Design an oligonucleotide of ~15–20 bp in length complementary to the target site and add the HEX-probe to the 5'-end.

2.3.2 FAM (6-carboxyfluorescein)-labeled probe for reference WT: Design an oligonucleotide ~15–20 bp in length complementary to a conserved genome site distant (about 25 bp) from the target site and add the FAM-probe to the 5'-end.

3. PCR reaction preparation

3.1 Prepare 25 µL of the ddPCR Sample Mix with the following components: ddPCR supermix for probes (no UTP): 12 µL, forward and reverse primers (10 µM): 1 µL each, HEX/FAM probes (10 µM): 0.625 µL each, DNA: 1–5 µL (3,750–37,500 haploid genome copies) and water: up to 25 µL.

3.2 Thoroughly mix the reactions by vortexing or reflux pipetting (up and down) (20x).

NOTE: If the reactions are in a 96-well plate, pipette the entire volume up and down 20 times rather than vortexing to avoid bubble formation.

3.3 Briefly centrifuge the samples to settle the mixture at the bottom of the tube or well.

NOTE: Ensure the reactions are at room temperature for the droplet generation. Prepare 1x of ddPCR mix for extra/unused wells in each cartridge (each cartridge has 8 wells).

4. Droplet generation

4.1 Using a 50 µL multichannel pipette, load 20 µL of the ddPCR sample mix into the middle row of the cartridge (**Figure 1A**, top).

4.2 Load 70 µL of the oil into the bottom row. Load 20 µL of 1x ddPCR supermix into unused wells.

NOTE: Do not introduce bubbles.

4.3 Place the gasket touching only the edges, avoiding the center concaved area (**Figure 1B**).

4.4 Place the plate securely in the droplet generator and close the cover to start the run.

4.5 Using the multichannel pipette, transfer 40 µL of the emersion mix from the top row of the cartridge (**Figure 1A**, bottom) into the 96-well plate.

4.5.1. Draw liquid sample for 3–5 s at a 45–30° angle. Expel the mixture slowly for over 3 s at a 45° angle into the side of the well, allowing it to drip down the side. It is okay to go to the second stop (complete expulsion) of the pipette to expel all the liquid.

4.6 Using foil heat seals, seal the plates for 5 s at 180 °C.

5. PCR

5.1 Place the sealed plate into the thermocycler **(Figure 1C)** and set the recommended PCR conditions if following the NHEJ Drop-Off guidelines as follows:

5.1.1 Initial denaturation at 95 °C for 10 min.

5.1.2 Set 40 cycles of 94 °C for 30 s to denature, 55 °C for 1 min to anneal, and 60 °C for 2 min to extend.

5.1.3 Hold at 98 °C for 10 min.

5.1.4 Hold at 4 °C.

NOTE: The annealing temperature for specific primers and probe sets may be optimized using a thermal gradient. Use a ramp rate of 2 °C/s for all the steps. PCR conditions should be adjusted depending on each experimental design and set-up.

6. Droplet reading

6.1 Place the plate securely in the droplet reader with well A-1 at the top left (smoothed corner, the other three are edged) (**Figure 1D**).

6.2 Set up the plate in the program: Designating FAM as the known reference channel and HEX as the unknown one (**Figure 2A**).

6.3 Run the Droplet reading experiment as direct quantification. After the run finishes, change the Experiment type to **Drop-Off** (DOF) for the analysis (**Figure 2A**).

7. Analysis

7.1 Designate the correct experimental parameters (**Figure 2A**): **Sample Information**, **SuperMix**, **Target Name** (WT or NHEJ), **Target Type** (Ref or Unknown), **Signal Ch1** (HEX or FAM), **Signal Ch2** (HEX or FAM), and manually set the threshold for droplet count (recommend above 10,000 for reliable results). The software will perform most of the analysis with the designated parameter.

7.2 Check the droplet count in the **Droplet** tab; ensure that all are above 10,000 (**Figure 2B**).

7.3 Check 1 D amplitude for efficient signal separation from negatives.

7.4 In the **Plate Editor**, highlight the entire plate and set the **Experiment Type** to **Drop off**.

7.5 Set the WT target as a **Reference**, designating channel one for FAM and channel two for HEX.

7.6 Set NHEJ target as unknown, designating channel one for FAM and channel two as none.

7.7 In the 2D amplitude tab, set the **Cluster Thresholds** with the graph tools for each sample. Consider the tail associated with the WT cluster; this is normal for NHEJ assays (**Figure 3A**).

NOTE: Under the **Ratio** tab, click on the **Gear Icon** from the top-right of the graph. Select the **Fractional Abundance**. The graph will now plot a point corresponding to the percentage of NHEJ events (**Figure 3B**).

REPRESENTATIVE RESULTS:

An application of this procedure appears in Carballar-Lejarazú et al.[9]. The ddPCR Drop-off assay utilizes two fluorescent probes to discern WT and indel sequences: A FAM probe binds to a conserved sequence within the amplicon, whereas the HEX probe targets the WT sequence of the targeted site (**Figure 4A**). In the presence of an indel, the HEX probe will not bind. Representative results can be found in Figure 2, Table 1, and Table 2 of Carballar-Lejarazú et al.[9]. Using this protocol, ddPCR has been proven to detect a wide variety of CRISPR-Cas9 induced NHEJ events and quantify the NHEJ frequency in an individual or pooled sample. Fifteen different pooled samples of 10 mosquitoes each contained various NHEJ alleles (Table 2 of Carballar-Lejarazú et al.[9]). These were analyzed with ddPCR using the protocol and parameters presented here. Results from Table 1[9] show that all 15 samples carried 100% indel alleles as identified by the Drop-off assay (**Figure 4B**). In another experiment, 11 pooled samples of WT mosquitoes and

NHEJ mosquitoes with different NHEJ percentages (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) were examined with this ddPCR protocol, and the results (Figure 2; Carballar- Lejarazú et al.[9]) showed that the identified percentage is close to the compared technique of Indel Detection by Amplicon Analysis (**Figure 4C**).

FIGURE LEGENDS:

Figure 1: Experimental set-up and procedure. (**A**) Cartridge preparation for Droplet generation. (Top) Samples are filled in the middle row of the cartridge, while oil is filled in the bottom row. (Bottom) Top row filled with emulsified droplets after droplet generation. (**B**) Droplet generator with a cartridge filled with sample and covered by a gasket in place. (**C**) 96-well plate covered with foil seal in a Thermo-Cycler. (**D**) Droplet Reader with 96-well-plate in place with a metal cover latched over the plate to secure it.

Figure 2: **Droplet reading.** (**A**) Software interface for droplet reading. Orange boxes show wells with samples. Gray boxes are empty wells. Experimental parameters are set up in the **Edit Tools** panel (right-hand side). Each sample can be edited by clicking on the respective sample box. Select **Drop Off** (DOF) for **Experimental Type**. In sample information, fill in the appropriate information for the sample's **Name** and **Type**, as well as **SuperMix**. Choose the **Basic Drop-Off** for the **Assay Information**. For the WT sample, choose WT for the **Target Name**, Ref for **Target Type**, and both FAM and HEX for **Signal Ch1** and **Ch2**, respectively. For NHEJ samples, fill in the appropriate name for the **Target Name**, choose Unknown for the **Target Type**, and choose FAM for **Signal Ch1**. Leave **Signal Ch2** at None. (**B**) Droplet count results for multiple samples.

Figure 3: Drop-Off assay analysis. (**A**) Cluster 2D plot for the droplet count of the WT and NHEJ alleles. In the 2D Amplitude tab, all droplets are unclassified by default. In this figure, colors are manually assigned for distinguishing. The orange dots cluster are WT allele counts obtained by binding of both FAM and HEX probes at the reference sequence and target site sequence, respectively. Blue dots represent scores of droplets that have FAM binding to the reference sequence but no HEX binding at the target site sequence (hence drop-off of HEX). Gray dots are empty droplets that don't have either FAM or HEX binding. (**B**) Ratio/Abundance graphs of NHEJ events. Under the **Ratio** tab, select **Fractional Abundance** for a graph with the correspondent percentage of NHEJ events.

Figure 4: Application of Drop-Off assay with ddPCR for non-homologous end-joining identification and quantification in the transgenic *Anopheles stephensi* line, AsMCRkh1. (**A**) Schematic presentation of the ddPCR Drop-Off assay to detect mutations at a targeted DNA site with a dual-probe system. An amplicon of 150–400 bp is amplified with the forward and reverse primers. A FAM-labeled probe is designed to bind to a conserved sequence of the amplicon, whereas a HEX-labeled probe is designed to bind to the WT gRNA targeted site. (**B**) Detection of various types of indels with ddPCR. Fifteen pools of 10 AsMCRkh1 mosquitoes each containing various types of indel, including insertion, deletion, and substitution, were analyzed with the ddPCR Drop-Off assay. Details of mutations and sequences can be found in Table 2 and Table S3 of Carballar *et al*.[9]. (**C**) Quantification of NHEJ in mixed samples of AsMCRkh1 and WT mosquitoes with various ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10) using ddPCR and a

compared technique of Indel Detection by Amplicon Analysis[9]. Images adapted from Carballar-Lejarazú et al. *Biotechniques*. 68(4):172–179 (2020)[9].

Table 1: Sequences of primers and probes.

DISCUSSION:

Digital-droplet PCR is an efficient method to determine the presence of indel alleles resulting from NHEJ events in a Cas/gRNA-based gene-drive system and allows for quantification of the frequency of these alleles in individuals or populations. Some steps of the protocol need to be followed with special care to achieve reliable results. Firstly, the genomic DNA extraction needs to be performed carefully to ensure high quality and sufficient quantity. A good extraction will allow accurate determination of the haploid genome copies per reaction. In our experience, a commercially-available kit (see **Table of Materials**) provided consistently high-quality DNA extractions. However, extractions of individual mosquitoes can prove to be particularly challenging as the DNA pellet becomes hard to visualize and can easily be sucked up with the supernatant if not careful. Secondly, primers and probes must be designed carefully. Before completing the ddPCR experiment, ensure that the designed primers result in a single PCR product by first performing a traditional PCR and visualizing a single product via gel electrophoresis. The reference FAM probe also must be designed so that it is complementary to a highly-conserved sequence. This will ensure accurate detections of WT alleles throughout a diverse population. The primer/probe combinations for each unique experiment will have different thermocycler conditions, and it is recommended to optimize those conditions using a thermal gradient.

Other methods for identifying indels exist, such as Sanger sequencing or NGS. Sanger sequencing is limited because it has a lower limit of detection and low discovery power to identify novel variants. Sanger sequencing also is labor-intensive and is not high-throughput. Compared to Sanger sequencing, NGS does not have the same limitations of low sensitivity, discovery power, and throughput. Another benefit of NGS is its ability to detect a variety of mutations from SNPs to rearrangements. However, NGS is a more costly and time-consuming method in the application of determining Cas9/gRNA-associated indels because there is only one target region of interest, and it is best suited for larger genome-wide analyses. Compared to the aforementioned methods, ddPCR is high throughput and has a quick turn-around time. If the ddPCR materials and instruments are available in-house, 96 samples can be processed within 1–2 days, making it well suited for quick analysis of large trials of Cas9/gRNA -modified organisms.

While many benefits exist for ddPCR there are also limitations. Firstly, the ddPCR equipment is not frequently available in an independent laboratory environment. ddPCR equipment may be available communally at larger research institutions, but this does not allow for ease of data generation and analysis outside of the institution. Secondly, unlike the alternatives, ddPCR does not provide the individual unique sequences of identified indel mutations. Digital-droplet PCR will provide the frequency of indel mutations within a population, but without the sequence, one cannot determine whether the indels present are more likely to conserve or inhibit the function of the gene of interest. The ddPCR method is perhaps suited best to analyze wild populations

after a field release trial of a Cas9/gRNA-based drive organism because it can efficiently determine the frequency of introduction of the transgene into the native population and the generation of indels within the population in close to real-time. Due to the ddPCR quick turn-around time, it would be feasible to perform sampling and analyze the population in a field trial region weekly if materials were available locally. The start-up costs to purchase, import, and set up the ddPCR equipment would be high in remote laboratories but the benefits of being able to assess rigorously a wild population as it is undergoing modification from a drive system would justify the costs.

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DISCLOSURES:

The authors have no disclosures.

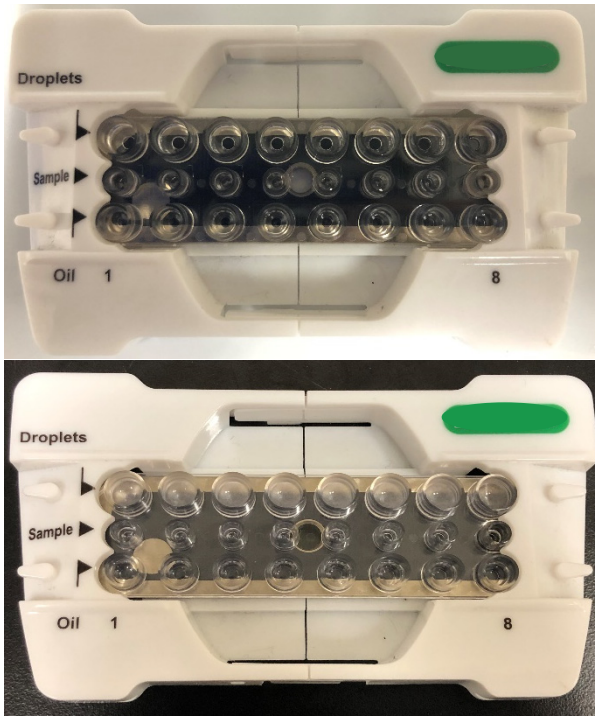
REFERENCES:

1. Gantz V. M. et al. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences of the United States of America*. **112** (49), E6736–743 (2015)
2. Hammond, A. et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology*. **34**, 78–83 (2016).
3. Courtier-Orgogozo V., Morizot B., Boëte C. Agricultural pest control with CRISPR-based gene drive: time for public debate: Should we use gene drive for pest control? *EMBO Reports*. **18** (6), 878–880 (2017).
4. Carballar-Lejarazú, R. et al. Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae. Proceedings of the National Academy of Sciences of the United States of America*. **117** (37), 22805–22814 (2020).
5. Adolfi, A. et al. Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications*. **11**, 5553 (2020).
6. Wang, Z. et al. Comparison of droplet digital PCR and direct Sanger sequencing for the detection of the *BRAF*^{V600E} mutation in papillary thyroid carcinoma. *Journal of Clinical Laboratory Analysis*. **33** (6), e22902 (2019).
7. Dong, L., Wang, S., Fu, B., Wang, J. Evaluation of droplet digital PCR and next generation sequencing for characterizing DNA reference material for *KRAS* mutation detection. *Scientific Reports*. **8**(1), 9650 (2018).
8. Bell, C. C., Magor, G. W., Gillinder, K. R., Perkins, A. C. A high-throughput screening strategy for detecting CRISPR_Cas9 induced mutations using next-generation sequencing. *BMC Genomics*. **15** (1), 1002 (2014).
9. Carballar-Lejarazú, R., Kelsey, A., Pham, T. B., Bennett, E. P., James, A. A. Digital droplet PCR and IDAA for the detection of CRISPR indel edits in the malaria species *Anopheles stephensi*. *Biotechniques*. **68** (4), 172–179 (2020).

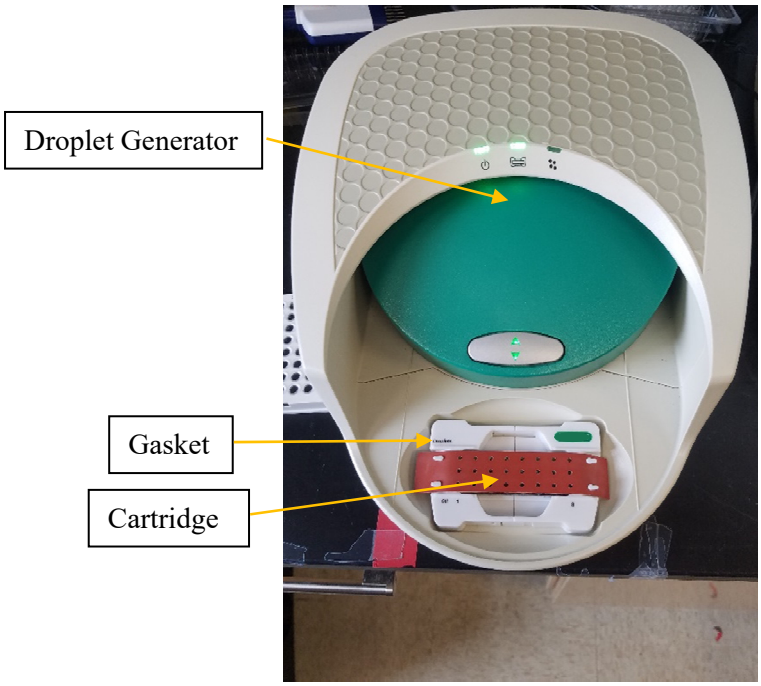
10. Holt, R. A. et al. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science*. **298** (5591), 129–149 (2002).

Figure 1

A.



B.



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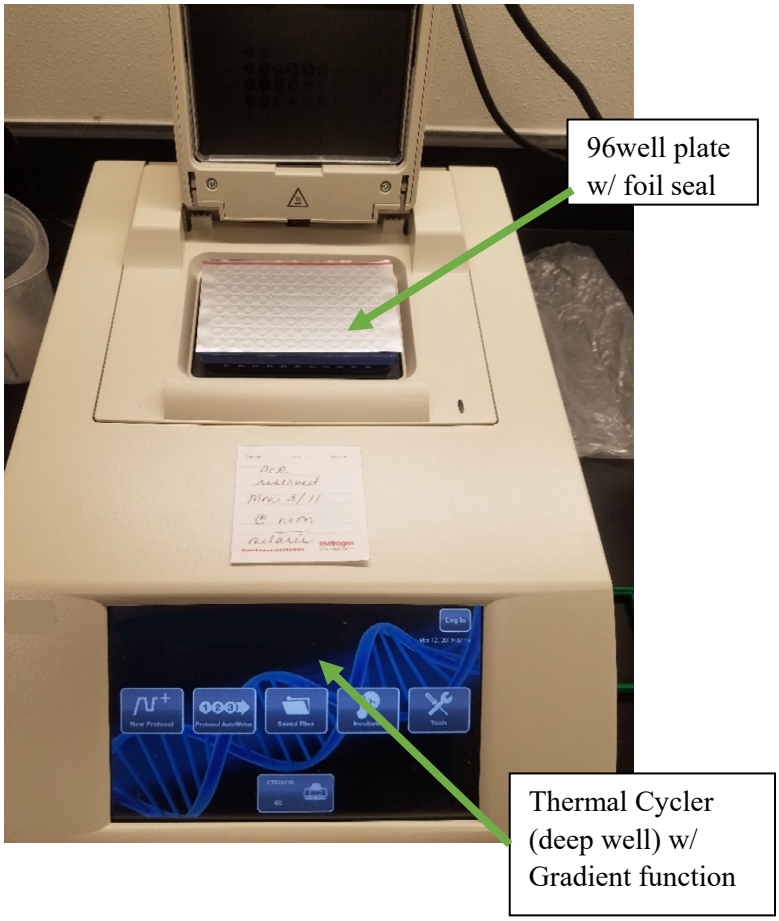

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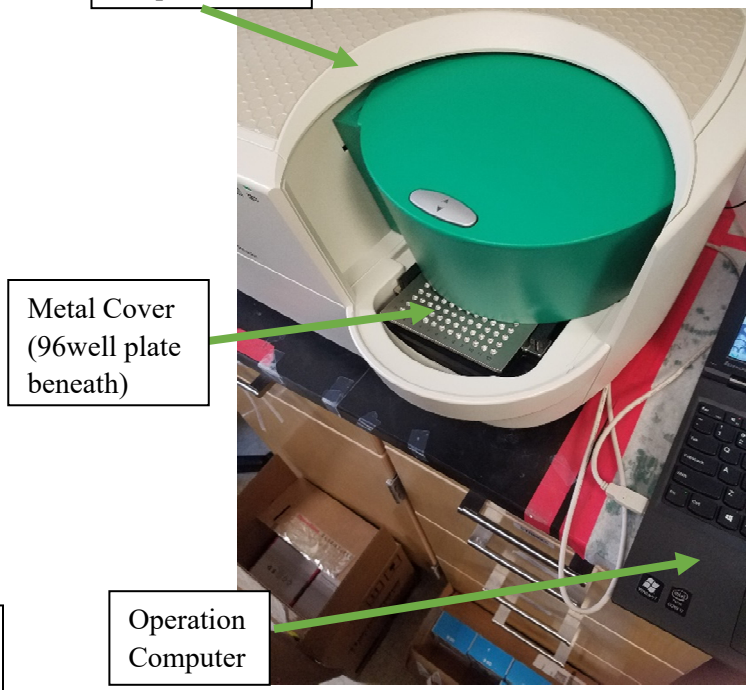
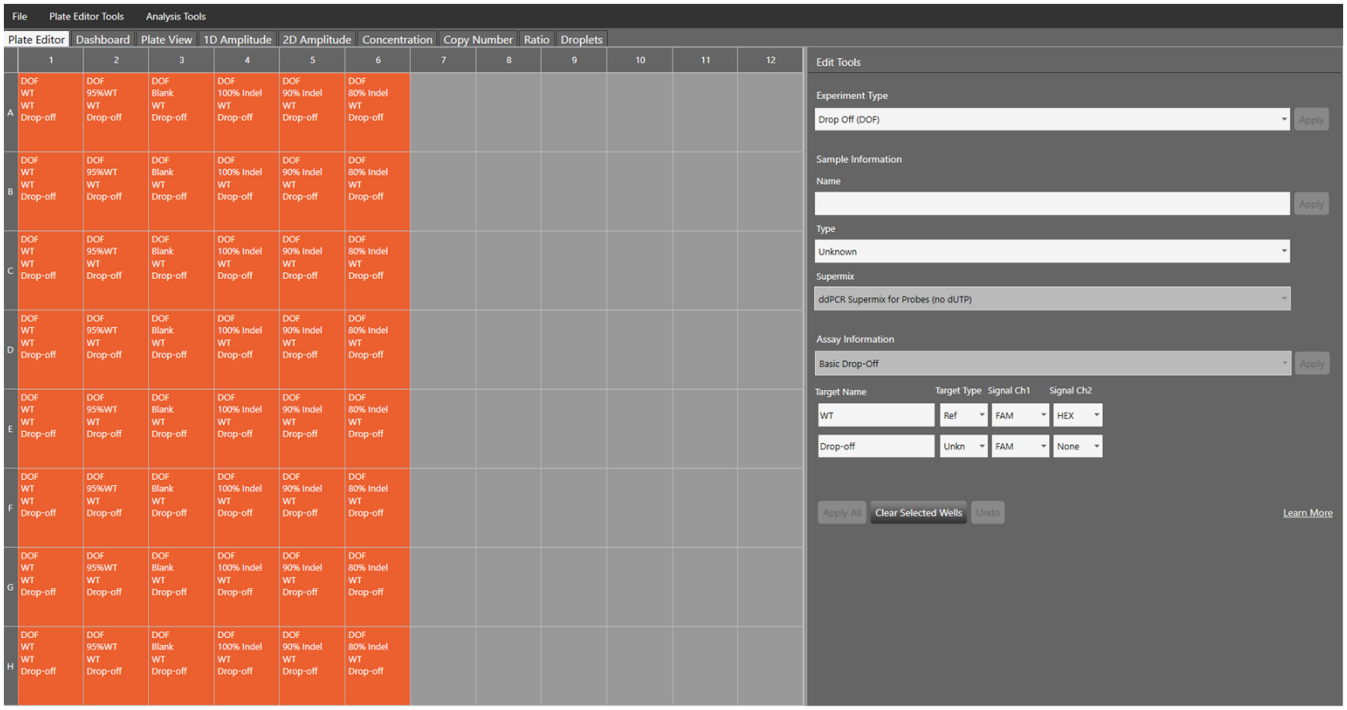
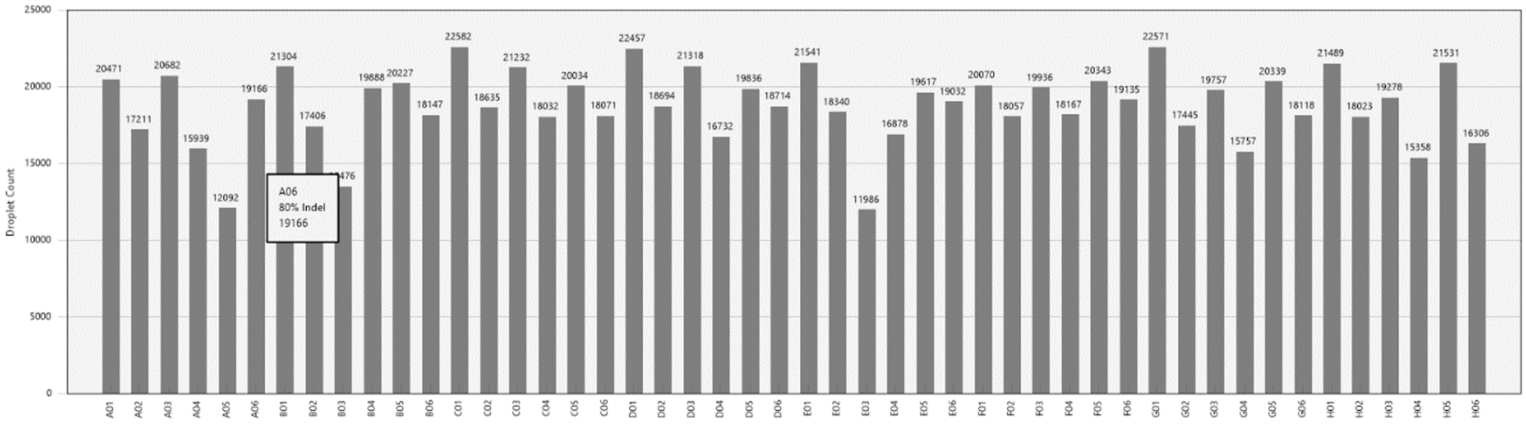


Figure 2

A. Plate Editor



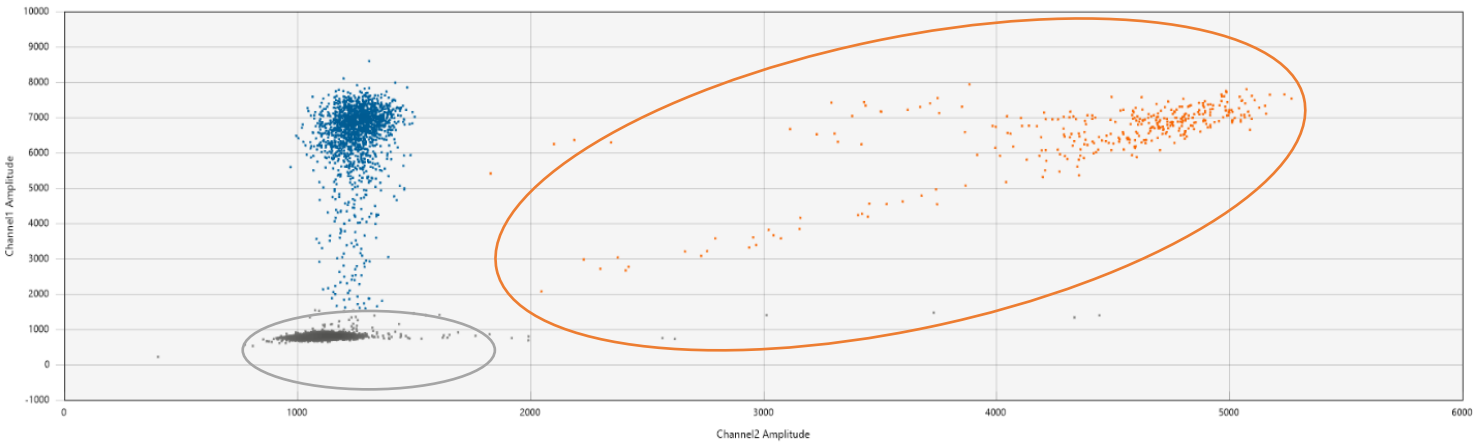
B. Droplet Count



10,000

Figure 3

A. 2D Plot



B. Ratio/Abundance

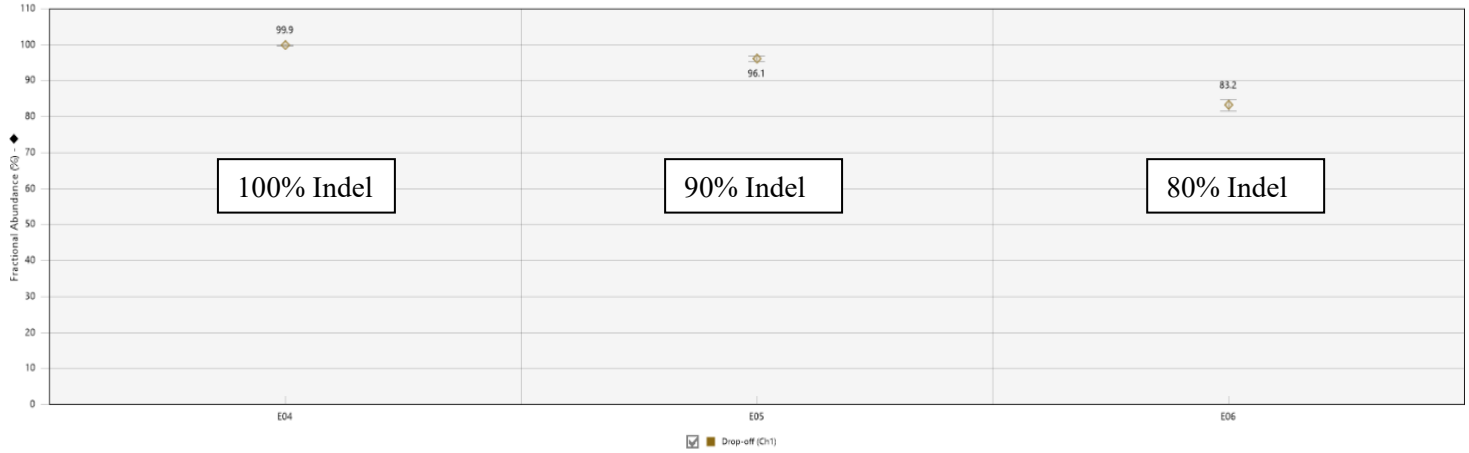
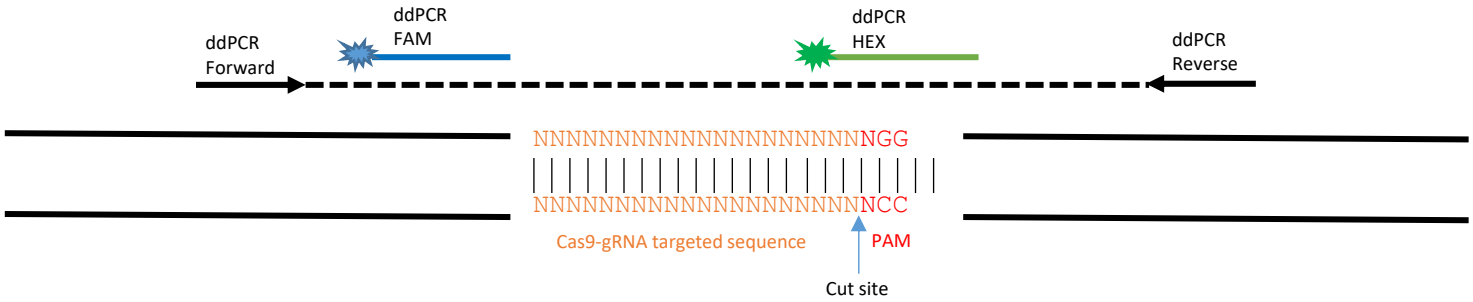


Figure 4

Figure 4

A.



B.

	A	B	C	Average
Indel-2	99.9	100	100	99.97
Indel-3	100	100	100	100.00
Indel-4	100	100	100	100.00
Indel-5	100	100	100	100.00
Indel-6	100	100	100	100.00
Indel-7	100	100	100	100.00
Indel-8	100	100	100	100.00
Indel-9	100	100	100	100.00
Indel-10	100	100	100	100.00
Indel-11	99.9	100	100	99.97
Indel-12	100	100	100	100.00
Indel-13	99.9	99.8	99.7	99.80
Indel-14	100	100	100	100.00
Indel-15	99.9	100	100	99.97

C.

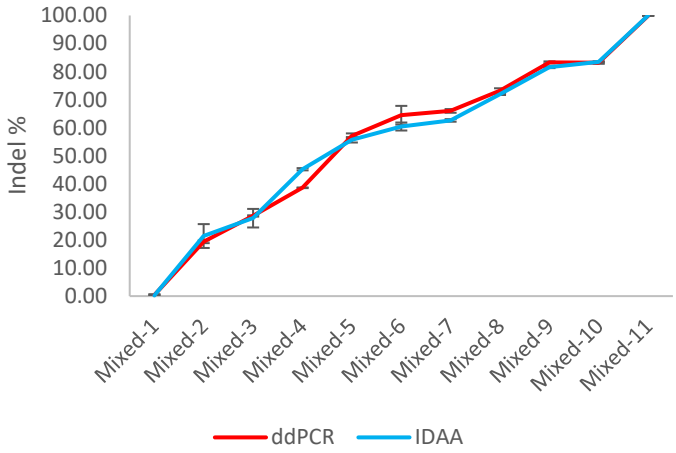


Table. Sequences of primers and probes used in this protocol and the experiment reported in the Representa

Primer/Probe	Sequence (5' » 3')
ddPCR Forward Primer	ATGATCAAATGTCGACCG
ddPCR Reverse Primer	ACCGTACTGGTTGAACA
ddPCR HEX Probe (BHQ1)	[HEX]-TTCTACGGGCAGGGC-[BHQ1]
ddPCR FAM Probe (BHQ1)	[6FAM]-CCACGTGGGATCGAAGG-[BHQ1]

HEX: Hexachloro-fluorescein, FAM: 6-carboxyfluorescein, BHQ: Black Hole Quencher

ative Results.

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: checked.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Answer: Used find function and changed one “should” phrase, could not find any others

3. 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? 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: reviewed.

4. How many mosquitos were sampled in one experiment?
Answer: The number of mosquitoes sampled in each experiment varied depend on the experimental design and setup. Individual or multiple mosquitoes per sample can be analyzed. For the experiment discussed in Representative Result paragraph 1, there were 10 mosquitoes per sample and there were 26 samples.

5. 2.1 How do you perform DNA quantification?
Answer: Detail has been added. DNA quantification is done using a Nanodrop.

6. Please provide sequences of the primers and probes used in the study. Please include this as a separate file in .xlsx format.
Answer: A table of primers and probes used in the study has been provided.

7. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Answer: made one change in step 2 to meet this requirement, otherwise appears to be all good

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.
Answer: made one change in step 2 to meet this requirement, otherwise appears to be all good

9. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Answer: a section has been highlighted for filming.

10. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data.
Answer: figure 4 has been added to demonstrate the date in the Representative Results.

11. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.
Answer: the figures from the Representative Results have been discussed and all the figures showing the experimental setup are referenced in the Protocol.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Answer: Approval to use previously-published images is included in the submission materials.

13. Figure 1: Please remove the commercial terms from the images.
Answer: all commercial terms from the images have been removed.

14. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.
Answer: Have removed the embedded table and an .xls file has been generated.

15. Please upload each Figure individually to your Editorial Manager account. Please ensure all panels of one figure are combine into a single image file.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors describe in detail the protocol on detection of indel mutations generated during the NHEJ-mediated DNA repair, when the CRISPR/Cas gene editing approach is used. The protocol is Droplet Digital PCR as a basis, and a mosquito genome as an in vivo example.

Major Concerns:
n/a

Minor Concerns:
1) The protocol is well-described. The information on Proteinase K is missing.
Answer: Information is added into the Table of Material

2) Line 107. How many times the solution is mixed by inversion.
Answer: Change has been added (5-10 times…)

3) Line 117. "About" 20 could be simply "20", to be more precise.

Answer: Change has been added. Removed “about”

4) Line 167. Why water cannot be used for the "unused wells"? It would be cheaper.
Answer: Water or TE are NOT sufficient substitutes for the Buffer control reagent. The Buffer control reagent has the proper viscosity to allow all of the reagent-containing wells to produce the proper number of droplets.

5) Line 255. "tradition" PCR is "traditional?"
Answer: Change has been added

6) Table of Materials. Ethanol 200% Proof. Is it correct? Is it really 200%? The website suggests "Ethanol 200 Proof. 100%"
Answer: Change has been made to “Ethanol 200 Proof”.

Reviewer #2:
Manuscript Summary:
The manuscript describes a protocol for high-throughput detection of CRISPR/Cas9-mediated non-homologous end-joining (NHEJ) events in the genetically engineered mosquitos. All the steps listed in the procedure are well explained with critical steps highlighted, which could yield the expected outcomes and are applicable to the associated fields for NHEJ analysis.

Major Concerns:
Although the manuscript has given sufficient introduction for the protocol, the overall idea for experimental design is not sufficiently described. It would be better to provide a schematic diagram regarding the usage of HEX-labeled probe and FAM-labeled probe for NHEJ detection.
Answer: A schematic for the Drop-off assay with ddPCR has been added as Figure 4.

Minor Concerns:
The authors claim that "Compared to next-generation of Sanger sequencing, ddPCR also has faster turn-around time for results". If the turn-around time is used for comparison, the time for DNA extraction should also be considered. Therefore, for L95, "1.4 Incubate overnight at 55°C", I believe there is a quicker way to do this.
Answer: Alternate protocol for DNA extraction that is shorter (3h incubation vs Overnight incubation) has been added to the protocol.

Reviewer #3:
Manuscript Summary:
in this manuscript, the authors outline a protocol for detecting indels in genetically modified mosquitoes using the CRISPR/Cas9 methodology. This is a unique approach because it somewhat relies on a negative test result. My overall assessment is that the paper is very well written, presented, and the logic is good. I recommend accepting the paper with some considerations/minor adjustments that can help others to replicate but also improve results somewhat.

Major Concerns:

My main concern is not really for the author (more for the journal) but i could not find a way to view the video component of this paper. Fortunately, the paper was prepared well enough that i didnt need the video to assess it but digital PCR is a challenge for new users. If the video is available i would like to see it but if it is at a similar quality as the text, i suspect it is fine. Really the value in video for this paper is set up of the assay itself which is time consuming and difficult for new users. I just want to make sure the video clearly shows the set up of the experiment.
We understand and appreciate the comment, but the journal protocols make the video images available after the text is approved.

ddPCR parameters need to be adjusted. While they clearly produce distinct clusters on chart, there are a lot of outliers. The clusters i think can be made more compact by reducing cycle number to 30 or 35 (max). Also the extension time is very long for such a small product. 100 to 400 bp should only need 30 sec (probably less with this chemistry) extension. Also 30 sec for annealing should be adequate. I would recommend playing with these parameters to try and reduce the extraneous hits.
Answer: A statement was added clarifying that “PCR conditions should be adjusted depend on each experimental design and set-up.” The PCR parameters described in this protocol were used to generate the data reported in the Representative Results.

Also, for the water in the mix, to make up volume, i use half water and half PVP-40 at 10% to further offset inhibitors. I used chip-based tech so not sure how this will translate but usually i get a few hits that are not true when i only use water. When i add PVP, it helps a lot to eliminate those non-target hits.
Answer: Thank you for the recommendation. However, the usage of PVP-40 in ddPCR samples has not been validated so we cannot suggest it in this protocol. We will try it out, and, if effective, we will report this technique in future publication.

Minor Concerns:
Line 88: How long to chill on ice? (min. and max. time)
Answer: we usually prepare the mixture before we start processing the samples and put it on ice while we get ready to homogenize the mosquitoes. We haven’t calculated the time but 2-5 min or until the solution is cloudy.

Line 107: 1min needs a space, saw this in other spots, maybe use find/replace option
Answer: Change has been added.

Line 118: "10 mosquitoes pool" should be "10 pooled mosquitoes".
Answer: Change has been added.

Line 134 to 136: Should read "Primer and probe design" and "Forward and reverse primers were designed with melting temperature…" and "produces" not "produce"
Answer: The Author Guideline states that the protocol section should be written in imperative tense as if telling someone how to do the technique.

Line 161 to 229: Yellow is a beautiful color but maybe cut back the highlighting here.
Answer: Highlighted lines are for indication of essential steps for the video.

Anthony A. James

From: Sarah Mayes <s.mayes@future-science.com>
Sent: Friday, June 4, 2021 4:39 AM
To: Anthony A. James
Cc: rcarball; Thai Binh T. Pham
Subject: RE: request for permission to reuse materials

Hello AAJ,

Thanks for coming back to me on this, I have good news for you. We are happy for you to use the figure as long as the original BioTechniques manuscript in cited.

Hope this helps.

Thanks

Sarah

From: Anthony A. James <aajames@uci.edu>
Sent: 03 June 2021 16:56
To: Sarah Mayes <s.mayes@future-science.com>
Cc: rcarball <rcarball@uci.edu>; Thai Binh T. Pham <thaibp@uci.edu>
Subject: RE: request for permission to reuse materials

Hello Dr. Mayes:

I hope you had an enjoyable break.

We are awaiting to hear back from you on this request. The *JoVE* article is accepted, but we cannot move ahead with it until we hear from you.

Thank you for your attention to this request.

Best,

AAJ

From: Anthony A. James
Sent: Thursday, May 27, 2021 1:49 PM
To: smayes@biotechniques.com
Cc: rcarball <rcarball@uci.edu>; Thai Binh T. Pham <thaibp@uci.edu>
Subject: request for permission to reuse materials

Dr. Sarah Mayes,
Publisher of Biotechniques

Dear Dr. Mayes:

My colleagues and I are preparing a manuscript for the *Journal of Visual Experimentation* (*JoVE*) and it includes work that was featured in our *Biotechniques* Open Access Report: Carballar-Lejarazú *et al*., (2020) Digital droplet PCR and IDAA for the detection of CRISPR indel edits in the malaria species *Anopheles stephensi*. *Biotechniques*. **68**(4):172-179PMID:32040336. As you may know, although *JoVE* publications emphasize visual presentations of techniques, they also ask for a paragraph or two describing 'representative' results. We have developed Figure 4 (copy attached) to show this and portions of it were derived from the *Biotechniques* report. Specifically, Figure 4A and 4B are modified from Figure 1 and Table 1 of the *Biotechniques* contribution, respectively, and 4C is a copied from Figure with modifications to the coloring and X and Y axes labelling.

We are requesting permission to use this material in the *JoVE* submission.

Thanks you for our response to this request.

Sincerely,

Dr. Anthony A. James
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and Molecular Biology & Biochemistry
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