# Optimizing Viral Detection: Creating Fluorescent Chikungunya Virus Infectious Clones

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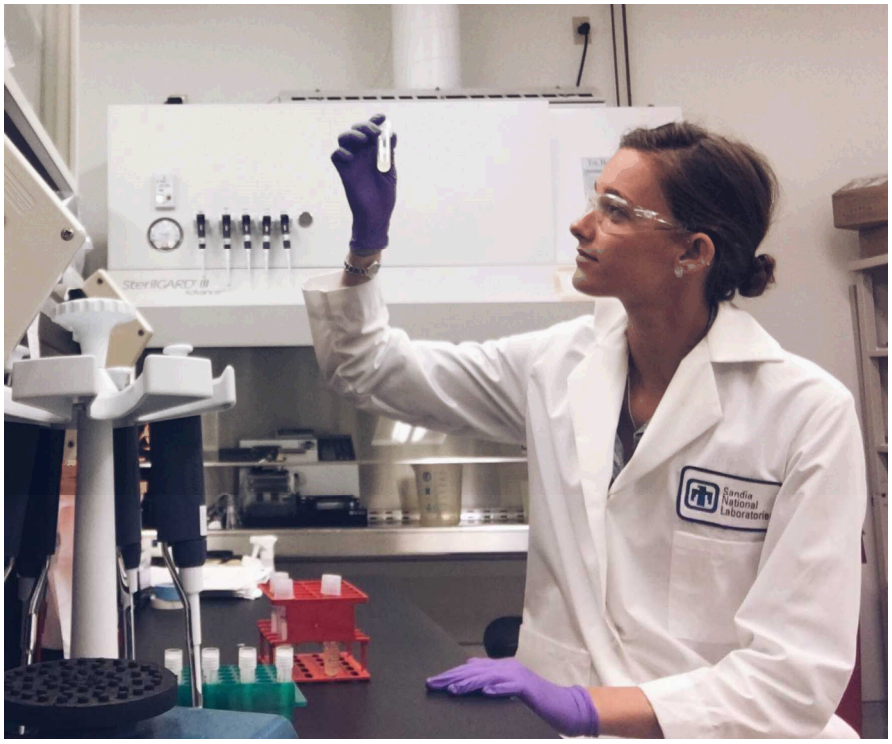
**Abstract:** Human infection with Chikungunya virus (CHIKV) results in debilitating joint pain and arthritis that can persist for months or even years. CHIKV is a re-emerging virus responsible for large epidemics in Asia, Africa, Europe, and most recently South and Central America. Yet, CHIKV has no specific treatment or vaccine. Therefore, steps towards eradicating the virus are important to public safety. Tools to facilitate drug screens are especially important when searching for viable treatments. Therefore, our lab sought to create reporter CHIKV encoding fluorescent markers, to detect infection in cells. Cloning was used to develop a CHIKV infectious clone encoding GFP. Creation of this tool required use of PCR, Gibson assembly, and the CRISPR Cas9 system. We are also working to create a CHIKV expressing mCherry. Next steps include determining the efficiency of these infectious clones compared to wildtype virus and performing drug screen studies. I also made contributions to other projects including development of a CRISPR Cas9 guide RNA library, a bioinformatics project using new software, and CRISPR Cas9 expressing cell line, that will be used in Zika virus studies. Finally, I assisted in development of a market survey focused on biodefense technologies for DHS.



## Infectious clones, and other studies

*Chikungunya Infectious Clones*

The bulk of my time at Sandia was spent working on creating an infectious clone of chikungunya virus (CHIKV), labelled with Green Fluorescent Protein (GFP). CHIKV is a positive sense RNA alphavirus that is considered a class C priority pathogen (4). CHIKV has no treatment or cure, and is transmitted by mosquito vectors (1). The CHIKV infectious clone I worked with was a DNA plasmid, which acts as a template for the RNA genome of the virus. GFP specifically was inserted in the nsP3 gene of CHIKV, a non-structural protein. The labelled infectious clone then could be used in transfection studies when RNA is transcribed from the plasmid and inserted into cells. There are many practical applications of creating a fluorescent virus, including work on drug screens for CHIKV.

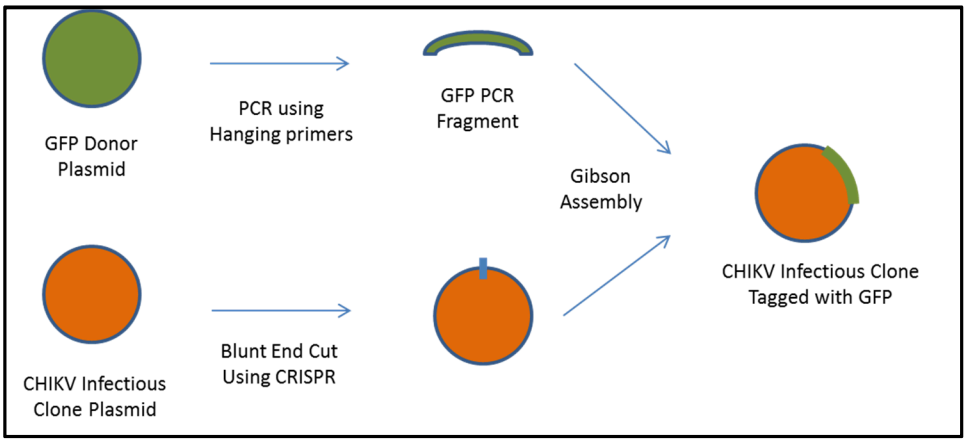



Figure 1. Simplified schematic of adding GFP to the CHIKV Infectious Clone

The cloning project had many independent steps that came together to create the final product. I first used PCR to create copies of GFP, but with "hanging" primer ends. Therefore, the PCR product had additional sequences on the end that matched the CHIKV plasmid. I used gel electrophoresis to verify that the fragment had been amplified, and then cleaned up the PCR product.

In order to ligate GFP into the CHIKV plasmid, I used the RNA-guided CRISPR Cas9 system, a revolutionary technology in biological research that enables precise and efficient gene editing for a variety of cell types and organisms. In order to utilize this system, I first employed PCR to create the guide RNA. The PCR product was the DNA sequence template that RNA was transcribed from afterwards. CRISPR works by using guide RNA to scan the genome for a matching sequence, and creating a double strand cut in the DNA at the site of the matching RNA sequence (2). The PCR product matched the sequence of the CHIKV plasmid, precisely where we wanted to cut the plasmid and ligate in GFP following the nsP3 gene. I then verified amplification and fragment size using gel electrophoresis.

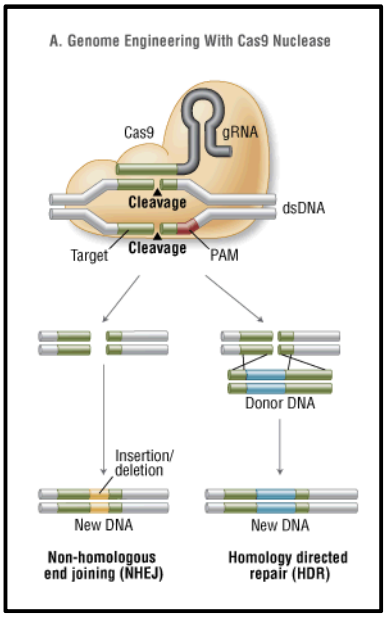



Figure 2. Diagram of how the CRISPR Cas9 System Works (6)

After creating the DNA template for the guide RNA, I used a transcription kit to make the RNA strand. Working with RNA was a novel process for me, and it was interesting the see the similarities and differences to working with DNA. Gel electrophoresis verified that there was amplification of the

product. After cleaning up the product, the concentration of the guide RNA was low, so this step was repeated to improve efficacy and increase the RNA concentration.

The CRSIPR Cas9 proteins and guide RNA were complexed together, and then this complex was added to the CHIKV plasmid so CRISPR could make the double stranded cut. I ran a low melt gel to verify that CRISPR did indeed cut the template, and I extracted the product from the gel to move forward. I completed the gel extraction with a kit, but the concentration of cut plasmid was again low. To account for this in the next step we used a higher volume of plasmid to complete the ligation.

In the previous steps, I was assembling pieces to put the infectious clone together. I amplified the GFP fragment, with hanging primer ends that matched the plasmid vector. I assembled the CRISPR Cas9 system, including the guide RNA and Cas9 protein complex. I then used this complex to cut the plasmid vector in the correct location to facilitate ligation to the GFP sequence. Finally, Gibson assembly was used as the toolkit to put all of the pieces together. The kit had all the necessary enzymes to ligate the GFP into the cut vector. Exonucleases "chewed" back a single strand of the DNA from the vector to expose the sequences that match the hanging primer ends on the PCR-engineered GFP. DNA polymerase filled in the gaps in the DNA, and ligase joined GFP and the vector (3).

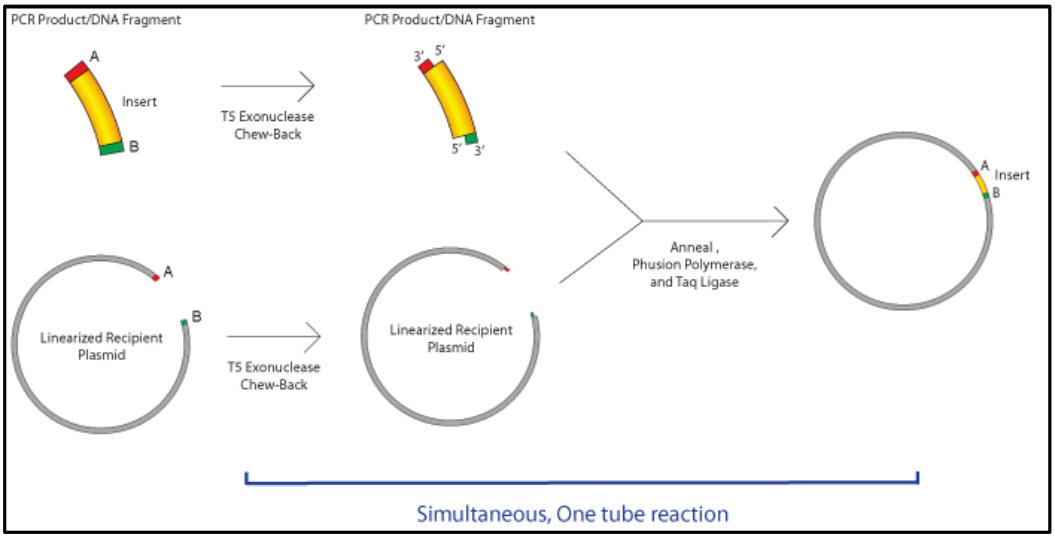



Figure 3. Diagram of Gibson Assembly Protocol (5)

After this step, I had created the CHIKV-GFP infectious clone. But, there were still procedures to both increase the plasmid concentration, and insure cloning accuracy. Firstly, I transformed the plasmid into *E. coli*. After waiting 24 hours for the *E. coli* to grow, I selected colonies to mini-prep. I picked the colonies and grew them in LB media with carbenicillin. The plasmid had a selection marker for carbenicillin resistance so only colonies that obtained the plasmid could grow on the plate. Again after 24 hours, I used a mini-prep kit to extract the plasmid DNA from the *E. coli*. In order to insure that the correct plasmid was prepared, I ran a restriction enzyme digest using BSTEII. If the infectious clone plasmid had GFP, the gel had a different band pattern than the infectious clone without GFP. Two of the seven mini-preps matched the correct banding pattern, and we sent them for sequencing.

The infectious clone sequence showed that the cloning was successful. Therefore, we transcribed RNA from the DNA plasmid to use for transfection. The infectious clone had a promoter sequence for transcription of the positive sense strand that was transfected into cells. After 48 hours, the virus induced expression of green fluorescence and cell death. The infectious clone was ultimately successful.

After successfully creating the CHIKV-GFP infectious clone, and creating live infectious virus from the clone, we initiated a similar process to clone CHIKV with mCherry, a protein that fluoresces red. The beginning process was very similar to the GFP infectious clone. I used PCR to amplify the mCherry fragment from a donor plasmid with the "hanging primers." After verifying the amplification via gel electrophoresis, I moved on to preparing the vector. I started with the CHIKV-GFP infectious clone, which had restriction sites around the GFP. The idea was that the GFP fragment could be cut out, and the mCherry fragment could be ligated in with Gibson assembly. After completing the digest of the vector, and the Gibson assembly to ligate mCherry, *E. coli* were transformed with the plasmid. With the first attempt, the negative control plate had more colonies than the mCherry plate. This was a

counterintuitive result, but we went ahead with extracting the plasmid DNA. Yet, the restriction digest showed that the plasmids were really just CHIKV-GFP, suggesting that the plasmid re-ligated or the enzyme did not cut to completion. On the second attempt, during the restriction digest of CHIKV-GFP, we used CIP to dephosphorylate the linearized plasmid which prevents re-ligation. Yet again, the transformation yielded a paradoxical result where the negative control plate had a higher amount of colonies. Given these results it seemed that the initial enzyme was not cutting the CHIKV-GFP plasmid to completion. If this were true, it was still likely that some of the plasmids could have been cut and ligated correctly. Therefore, we decided to do colony PCR, where I picked 48 colonies from the mCherry plate and ran a PCR reaction of the plasmid DNA using the mCherry primers. If the Gibson assembly and the transformation were successful, the mCherry fragment would amplify. If not, nothing would amplify. I found after completing the process that 5 of the 48 colonies had mCherry. I grew up the colonies and extracted the plasmid DNA to send of for sequencing to confirm the result.

Overall, both the fluorescent infectious clones will be very useful for future lab experiments. Dr. Harmon and Dr. Saada are screening small molecule inhibitors for treatments effective against CHIKV. From 40,000 chemicals, around 300 have been selected for further testing. In addition to small molecules, gene editing tools such as the CRISPR Cas9 system are also being explored as potential therapeutics. One of the traditional ways to screen for a drug's effectiveness is to use antibody staining. Yet, this method is expensive, time consuming, and can lead to loss of cells. Use of fluorescent viruses, is an easy and direct method to determine viral titer on live cells, therefore, the CHIKV-GFP and CHIKV-mCherry can be used to screen a multitude of therapeutic avenues to determine anti-viral efficacy by measuring a decrease in fluorescent signal.

*CRISPR Library*

Per my request, I wanted to start learning some bioinformatics during my time at Sandia. I started teaching myself python in order to have a better skillset when looking at bioinformatics problems. One of the projects involving bioinformatics under Dr. Negrete was CRISPR library design. CRISPR is becoming an increasingly powerful tool to use in viral studies. CRISPR can knock in or knock out genes that are responsible for virus entry and infection (2). I used the CRISPR library designer (CLD) software to identify potential guide RNA sequences that target a specific group of genes that could be involved in virus entry. Dr. Negrete is interested in membrane proteins, and I used a list of 1,800 proteins to get a handle on how the software works. Overall, it was a good learning experience for me to begin looking at bioinformatics.

*BioWatch Market Survey*

My mentor, Dr. Harmon, is a consultant for Department of Homeland Security due to her expertise in microbiology. She is a part of a project focused on screening and detecting biological agents for national security reasons. I was interested in her work specifically for DHS, so I was added to the project to help with construction of a market survey database of biodefense related technologies. I went through a database of technologies to find vendor and product websites to be added to the database. It was exciting for me to do something specifically for DHS as I am interested in the agency. I was also happy to help make the database more functional for future applications.

*SaCas9 Expressing Cell Line*

Another cloning project I began will eventually lead to a CRISPR Cas9 expressing cell line. Again, CRISPR systems are being examined as a potential anti-viral tool. In this project, Zika virus is the target. By creating a cell line that expresses Cas9 proteins, guide RNAs can be created and inserted into cells that will target a specific sequence in the virus.

There are variations of Cas9 from different bacterial strains. SaCas9, from *S. aureus,* is supposed to be most effective for use in the cell line. SaCas9 was amplified using PCR from a donor plasmid. Unlike bacteria, mammalian cells do not naturally express Cas9. Therefore, the DNA that encodes the Cas9 proteins has to be incorporated in the cellular DNA. Therefore, a lentivirus vector was used to transport Cas9 DNA into the cells. The lenti-vector plasmid we specifically used originally expressed spyCas9 proteins from *S. pyogenes.* I digested the vector plasmid to remove spyCas9 using restriction enzymes (8). Then, I used Gibson assembly to ligate SaCas9 with the lentiviral plasmid. After Gibson assembly, the plasmid was transformed into *E. coli.* I mini-prepped the plasmid DNA and we sent off four potential colonies for sequencing. When the sequences come back, RNA will be transcribed from the DNA plasmid, and transfected into cells.

**Impact of Internship on My Career**

Coming into Sandia, I thought I would be working under Dr. Kelly Williams in a bioinformatics project. Yet, due to my previous BSL2 experience and lack of computer experience, they decided to switch me to Dr. Brooke Harmon's group. Ultimately, I believe this switch was extremely valuable for me as my previous lab experience made me a better fit for the virology projects. Over the course of ten weeks, I helped with various projects and learned many new techniques. Overall, the lab focused on viruses that are a national security risk. Therefore, I got first-hand experience in biodefense related research. I learned many useful laboratory techniques including, standard cloning techniques (PCR, restriction digests, gel electrophoresis, ligation) and novel cloning techniques such as Gibson cloning and CRISPR-Cas9 gene editing. I also learned how to do mammalian tissue culture and I got a brief introduction to bioinformatics tools such as python and the CLD. Most of all, I learned how to think through a problem and come up with solutions and controls to troubleshoot my issues. The projects I contributed to should result in peer-reviewed publications with the next year and presentations at local

and international conferences. I also learned a lot about the projects that are ongoing at Sandia, such as the wet lab research on viruses, bacteria, and novel genome editing techniques and the other National security missions related to biodefense such as the work that is being done for DHS. I will be presenting a poster at an internal Sandia symposium and I hope to gain a lot of useful feedback

In addition to my lab activities, I attended the Bay Area Virology Symposium with Dr. Harmon and Dr. Negrete and it was very informative. I interacted with local scientists and learned more about virology. Also a part of the symposium, they included a panel discussion on Zika virus. I thought it was amazing to see how they could bring together scientists from various backgrounds to collaborate and help solve a pressing issue. In addition to the scientific material presented, there was a talk that shed light on how problems are being handled from a public health perspective. It was interesting to hear from a diversity of backgrounds coming together to address not only Zika virus, but other concerning viruses as well. Overall, the symposium was extremely beneficial to me as a great introduction to the types of problems faced in virology and bio-defense.

Sandia also put together many intern events that were geared towards graduating college students. One of these talks was "How to Get into Grad School," presented by the Berkeley Engineering department. I was very happy to get insight on the graduate admissions process, as I am looking into masters' programs in forensic science and bio-defense. There was useful information presented about writing outstanding personal statements, emailing professors, and choosing the right program. I am glad that I could attend such events to help me with my future decisions and applications.

Taking this internship to Sandia National Laboratories was a risky decision for me. I left my biosciences technician job of two years at the USDA because I wanted more experience in forensics and bio-defense, the field I see a future in. Not only did I learn applicable skills that I can take with me into my future research, but I solidified my desire to pursue forensics and bio-defense as a career.

**Acknowledgments**

I want to express my thanks to Dr. Brooke Harmon and her team for all of their help this summer. I appreciate Dr. Edwin Saada for all the time and effort he put in to help me with my project. I also want to thank Sandia for providing me a truly invaluable experience to my education and my future.

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