University of Nevada, Reno

Designing a Gateway Cloning Lab Showcasing the Pigment from *Chromobacterium violaceum* for Molecular Biology Lab Classes

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelors of Science in Biochemistry and Molecular Biology and the Honors Program

by

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We recommend that the thesis
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Abstract

Laboratory courses are an integral part of a science based education at any university, and it is important to ensure that the concepts students learn in these laboratory classes are modern and scientifically relevant. For this reason, laboratory modules are periodically modified to keep their learning concepts in line with the curriculum being taught in the accompanying lecture classes. In this case, a cloning module in the molecular biology lab class will be modified to utilize Gateway cloning in the place of Gibson Assembly cloning. The new protocol will involve extracting genomic DNA from the bacterium *Chromobacterium violaceum*, cloning the violacein operon using polymerase chain reaction, and placing the cloned operon into *E. coli* using Gateway cloning methods. The final product produces *E. coli* cells that express the entire five gene violacein pathway, and as a result produce the violet pigment that is characteristic of *Chromobacterium violaceum.* This type of modification to the current molecular biology lab curriculum will result in students who graduate with a deeper knowledge about current laboratory techniques, which will help them to achieve a more successful career in science.

Acknowledgments

I would like to express my sincerest appreciation for Dr. Veronica Zepeda, my thesis advisor, for mentoring me throughout this project. She worked side by side with us almost every day for the last nine months to complete this protocol for the BCH 406 lab, and provided endless advice and guidance to keep the project on track. I have learned so much about cloning, molecular biology, and research in general throughout this entire process. This thesis could not have happened without her, and I could not think of anyone that would have been a better mentor.

I would also like to thank Tanner DeGiovanni, my partner on this project, for being an awesome partner and making me laugh every day.

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Introduction

Undergraduate level laboratory classes are of vast importance, particularly in the sciences, and due to the rate at which technology changes the way science works, it is important that those lab classes teach relevant material. Right now, scientists find themselves in the midst of a "worldwide climate of curriculum reform" (Anderson & Schönborn, 2008), wherein many professors are beginning to implement laboratory modules that directly reflect current scientific research in an educational way. The push is not to teach students the information on the basest level, but to consider what kind of skills an undergraduate program would like its graduates to possess (Anderson & Schönborn, 2008). Most importantly, the program should showcase the interdisciplinary nature of biochemistry and molecular biology (Boyer, 2003). For instance, the natural product violacein, extracted from the bacterium *C. violaceum* has a variety of possible uses in medicine as an antitoumarant, an enzyme modulator, an antibacterial agent, or many others, which makes it a perfect target for molecular biology labs (Durán et al., 2007). Additionally, the traditional purple pigment, as well as a spectrum of other pigments produced by the violacein operon, makes it a great candidate for cloning type reactions, because verification of a successful protocol can simply be seen with the naked eye (Lee, Aswani, Han, Tomlin, & Dueber, 2013).

Figure 1 shows a sketch of the violacein pathway beginning with a tryptophan derivative that is metabolized by each of the five genes in the violacein pathway – Vio A, B, C, D, and E (Zalatan et al., 2015). When only the Vio A, B, and E genes are active in the pathway, *C. violaceum* produces prodeoxyviolacein and a dark green pigment, whereas if all five of the genes are active, the bacterium produces violacein and a rich

violet pigment. Turning on either the Vio D or C genes in tandem with Vio A, B, and E can produce two additional pigments known as deoxyviolacein and proviolacein, but only the green and violet will be explored for the purposes of this laboratory module (Zalatan et al., 2015).

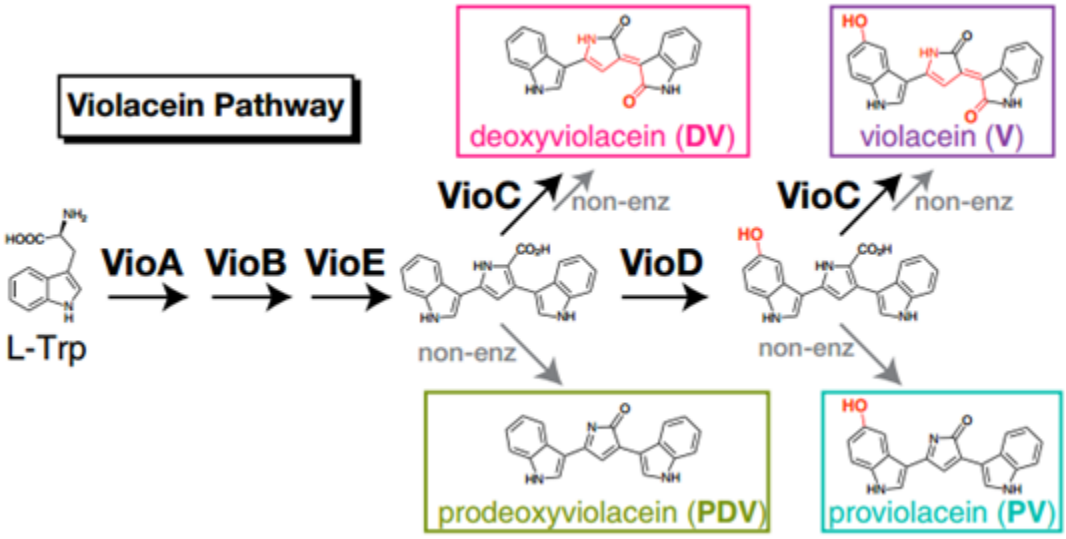

Figure 1. Violacein Operon Pathway. Image edited from Zalatan et al. (2015) shows the metabolic pathway of the violacein operon, and its various end products. The colored boxes around each end product indicate the color expressed by cells when the pathway moves primarily in that direction.

These qualities of violacein and the overall enzymatic pathway are exactly what scientists should be looking to implement into their laboratory course curriculums, because a topic that is socially relevant, easy to manipulate, and versatile, is also interesting to students in the field. This project hopes to modify the existing violacein pathway cloning protocol in the molecular biology lab class here at the University of Nevada, Reno. The existing laboratory protocol involves Gibson assembly cloning and will be modified to incorporate Gateway cloning, as it is considered to have “high speed, accuracy, and reliability” (Alberti, Gitler, & Lindquist, 2007). With Gibson Assembly, an

enzymatic reaction involving nuclease, polymerase, and ligase can seamlessly clone multiple gene pieces into a plasmid using a single reaction (Gibson & Christopher, 2011). By simply adding single stranded overhangs to double stranded DNA pieces, hundreds of kilobases of DNA can be put together in any array (Gibson & Christopher, 2011). However, this method does not quite have the efficiency of Gateway cloning, which is why this lab protocol will seek to replace Gibson assembly with Gateway cloning.

Gateway cloning is such a unique method in molecular biology, because the final product of its LR-clonase reaction selects only for plasmids that have taken up the gene of interest in the correct orientation (Alberti et al., 2007). Destination vectors designed specifically for these LR-clonase reactions contain a ccdB gene as well as a chloramphenicol resistance gene, and both of these features are flanked by att sites (Alberti et al., 2007). The ccdB gene is otherwise known as the death gene, because when it is fully expressed, it traps DNA gyrase and as a result it kills the host cell (Miyazaki, 2010). To initiate the reaction, a designated amount of entry vector with the gene of interest is combined with some amount of destination vector, which the gene of interest will move into. An LR-clonase enzyme causes a recombination event to occur between the two vectors, and as a result the gene of interest ends up in the destination vector, while the ccdB gene and the chloramphenicol resistance gene both end up in the entry vector (Alberti et al., 2007). When the cells are plated on the antibiotic resistance of the destination vector used in the LR-clonase reaction, everything that does not contain both the destination vector and the gene of interest will die. The cell colonies that grow on the final plate are selected for so specifically, that every living cell should contain the gene of interest in the correct orientation (Alberti et al., 2007).

Moving from the well-known Gibson assembly cloning methods also reveals that modern cloning technology can be done more than one way. Incorporating a modern technique like Gateway cloning into a classroom laboratory setting will show the students that modern molecular biology is within the grasp of their knowledge (Cong et al., 2013). This laboratory protocol will bring cutting edge science face to face with students in the hopes of inspiring them to continue on in the field, and conduct further research in areas that the molecular biology lab class may have interested them in.

Methods

Genomic DNA (gDNA) Extraction

CTAB Protocol

To extract the violacein operon, single colonies of *Chromobacterium violaceum* (*C. violaceum*) were each inoculated in 5mL of lysogeny broth (LB) and grown for 24 hours in a shaking incubator at 37°C. After the incubation period, cells were spun down at 3500rpm for 10 minutes. The LB was decanted off the cells, and the cells were resuspended in 567µl of TE buffer and vortexed to mix. The resuspension was then transferred to a microcentrifuge tube where 30µl of 10% SDS and 6µl of 10mg/mL proteinase K were added. The solution was then mixed by vortexing and incubated at 37°C for one hour. After incubation, 100µl of 5M NaCl was added and solution was mixed by vortexing. Next, 80µl of CTAB/NaCl solution was added and mixture was votexed before being incubated at 65°C for 10 minutes. Equal volumes of chlororform/isoamyl alcohol (24:1) were added to the microcentrifuge tube, and the resulting solution was centrifuged for 5 minutes at 14,000rpm. The supernatant was

transferred to a clean microcentrifuge tube where 0.6 volumes of cold isopropanol were added. To mix, the tube was inverted several times. The mixture was then centrifuged for 10 minutes at 14,000rpm. After centrifugation, the supernatant was removed from the solution, and 500µl of cold 70% ethanol was added. Again, the solution was centrifuged for 5 minutes at 14,000rpm before the supernatant was removed. The remaining ethanol was then evaporated off in a fume hood, and the dry pellet was resuspended in 100µl TE buffer. Resulting samples of *C. violaceum* gDNA were analyzed using a NanoDrop spectrophotometer to determine concentration and purity.

To determine the quality of gDNA obtained during the extraction, the samples were loaded on a 40mL 1% agarose gel prepared with a ten-well comb and 0.75µl Ethidium Bromide (EtBr). Next, 3.5µl of Quick-load 1KB DNA ladder (NEB) was loaded into the first lane. The remaining lanes were each loaded with 5µl of *C. violaceum* gDNA combined with 1µl 6x loading dye. The gel was run at 100V for 35 to 45 minutes, or until the dye front had moved halfway through the gel and was imaged using ImageLab software. To confirm the presence of gDNA, a band would appear above the 10,000bp marker of the loading ladder. If bright bands and smearing appeared around and below the 500bp marker, an RNase treatment was performed on the gDNA samples to further purify them. To set up the RNase treatment 40µl of *C. violaceum* gDNA was added to 5µl of buffer 3, 1µl RNase If, and 4µl of sterile water. The reaction was incubated at 37°C for 30 minutes, and then the RNase was heat inactivated by incubating at 70°C for 20 minutes. After treatment, the purified samples were analyzed using a NanoDrop instrument to determine the new concentration and purity values. RNase

treated samples were then run on a 40mL 1% agarose gel prepared with a ten-well comb as indicated above.

DNeasy Blood and Tissue Kit Protocol

A Qiagen DNeasy Blood and Tissue Kit was used to extract gDNA from *C. violaceum* in a more efficient manner. The protocol for gram-negative bacteria was followed exactly as described in the handbook provided. The purified gDNA samples were analyzed with a NanoDrop spectrophotometer to determine concentration and purity values, and the samples were run on a 40mL 1% agarose gel prepared with a ten-well comb and 0.75µl EtBr. The gel was loaded and run in the same fashion as mentioned in CTAB gDNA extraction protocol above.

Polymerase Chain Reaction (PCR) for VioAB and VioCDE Genes

The A and B genes from the violacein operon were isolated by PCR, using the purified gDNA as a template. The reaction was set up by combining 10µl 5x Phusion Reaction Buffer with 1µl 10mM dNTP mix, 1µl 10x VioAB forward primer, 1µl 10x VioAB reverse primer, approximately 75ng *C. violaceum* gDNA, 0.5µl DMSO, 0.5µl Phusion DNA Polymerase, and enough sterile water to fill to 50µl total volume. This sample was then placed in a thermal cycler that ran at 98°C for 30 seconds, before doing thirty cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 135 seconds. It was then held for final extension at 72°C for five minutes before finally holding at 4°C.

The C, D, and E genes from the violacein operon were isolated by PCR. The reaction was set up by combining 10µl 5x Phusion Reaction Buffer with 1µl 10mM dNTP mix, 1µl 10x VioCDE forward primer, 1µl 10x VioCDE reverse primer, approximately 75ng *C. violaceum* gDNA, 1µl DMSO, 0.5µl Phusion DNA Polymerase,

and sterile water to 50µl total volume. This sample was then placed in a thermal cycler that ran at 98°C for 30 seconds, before doing thirty cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 90 seconds. It was then held for final extension at 72°C for five minutes before finally holding at 4°C.

Analysis and Purification of VioAB and VioCDE PCR Products

The PCR products for VioAB and VioCDE were analyzed using gel electrophoresis to check the length of DNA produced by the PCR reactions. A 60mL 1% agarose gel was prepared using a six well comb and 0.75µl EtBr. To each 50µl PCR reaction, 10µl of 6x loading dye was added. The entire resulting 60µl sample was then loaded into one lane of the prepared gel alongside 9µl of 1KB loading ladder for comparison. The gel was run at 100V for 35 to 45 minutes, or until the dye front had moved halfway through the gel, and it was imaged using ImageLab software. A band at 2996bp for the VioCDE PCR product indicated correct cloning of all three genes from the violacein operon. A band at 4328bp for the VioAB PCR product indicated correct cloning of both genes from the violacein operon. Bands appearing in the appropriate places on the agarose gel were excised to further purify the DNA samples. The excised bands were then solubilized and purified using Qiagen QIAquick Gel Extraction Kit protocol except that here 30µl of warm EB was used to elute instead of the 50µl indicated in the protocol. Purified samples of VioAB and VioCDE DNA were analyzed using a NanoDrop instrument to determine the concentration and purity.

Inserting VioAB and VioCDE into an Entry Vector with a pENTR-dTOPO Reaction

Four microliters of purified insert DNA, either VioAB or VioCDE PCR product, were added to 1µl of salt solution and 0.5µl of TOPO vector from the pENTR Directional TOPO (pENTR-d-TOPO) Cloning Kit purchased. Reaction was incubated at room temperature for 16 to 24 hours before the entire 5.5µl reaction was transferred to a vial with 50µl of NEB-5α chemically competent *E. coli* cells for transformation. The cells were incubated on ice for 20 minutes before being heat shocked at 42°C in a heat block for exactly 30 seconds. Immediately following the heat shock, the cells were placed back on ice for two minutes before 250µl of super optimal broth with catabolite repression (SOC) media was added to the solution. The *E. coli* were then placed in a shaking incubator at 37°C for one hour to complete the transformation. After incubation, all pENTR reactions were plated on LB plates with Kanamycin (Kan). The plates were grown at 37ºC overnight in an incubator before single colonies from each plate were selected and inoculated in 5mL of LB-Kan. These inoculated cultures were grown for 24 hours in a shaking incubator. The cultures were purified using Qiagen QIAprep Spin Miniprep Kit and protocol except 30µl of Buffer EB was used to elute instead of the indicated 50µl. Purified pENTR plasmids were analyzed using a NanoDrop spectrophotometer to determine the concentration and purity of each sample. The pENTR-d-TOPO reaction was performed once for the VioAB insert and once for the VioCDE insert.

Verifying pENTR-VioAB and pENTR-VioCDE are in the Correct Orientation

To initially verify that VioAB was inserted into the pENTR plasmid correctly, a restriction enzyme digest was performed on the purified plasmid samples. The digest was set up by combining 2µl 10x Cutsmart Buffer, 0.5µl EcoRI, 0.5µl AscI, and approximately 350ng of pENTR-VioAB DNA, up to 17µl. If needed, sterile water was added to fill to 20µl total reaction volume. Digests were incubated at 37ºC for 35 minutes. To initially verify that VioCDE was inserted into the pENTR plasmid correctly, a restriction enzyme digest was performed on the purified plasmid samples. The digest was set up by combining 2µl 10x Cutsmart Buffer, 0.5µl EcoRV, 0.5µl XmnI, and approximately 350ng of pENTR-VioCDE DNA, up to 17µl. If needed, sterile water was added to fill to 20µl total reaction volume. Digests were incubated at 37ºC for 35 minutes.

To check the results of the pENTR digests, a 40mL 1% agarose gel was prepared using a ten well comb and 0.75µl EtBr. The first lane was loaded with 3.5µl 1KB loading ladder. The remaining lanes were each loaded with 5µl of pENTR-VioAB or pENTR-VioCDE digest combined with 1µl 6x loading dye. The gel was run at 100V for 35-45 minutes, or until the dye front had moved halfway through the gel. Figure 2 shows where the enzymes cut on each of the pENTR plasmids. For pENTR-VioAB digests, bands appearing at 6031bp and 877bp indicated that the VioAB DNA was inserted in the correct orientation. For pENTR-VioCDE digests, bands appearing at 4660bp and 916bp indicated that the VioCDE DNA was inserted in the correct orientation.

If the results of the digestions indicated that the insert DNA was inserted into pENTR correctly, approximately 500ng of purified plasmid was added to 1µl of pENTR-

Seq Reverse primer and sent for sequencing at the Nevada Genomics Center to verify that it had been inserted in the correct orientation.

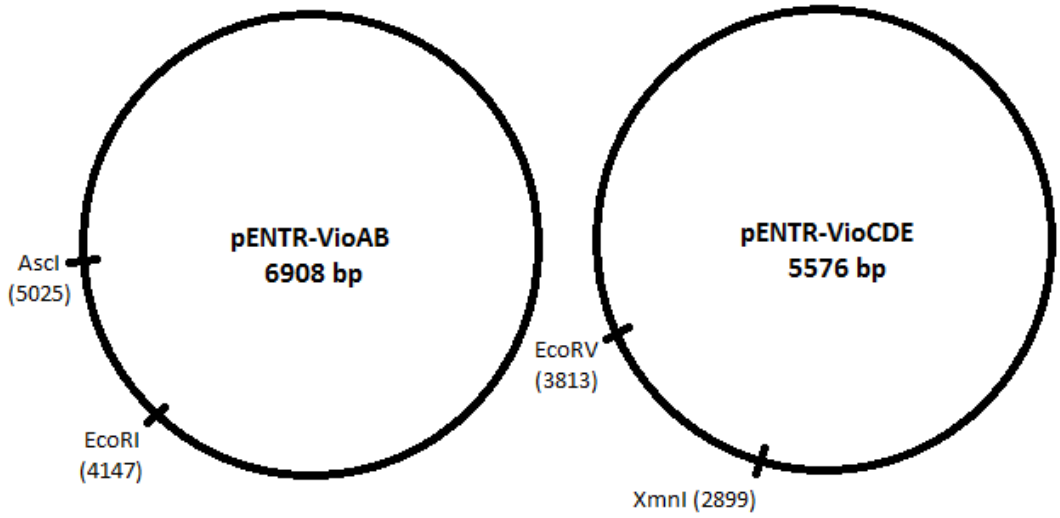

Figure 2. Plasmid Maps of pENTR-VioAB and pENTR-VioCDE. A depiction of the two pENTR plasmids with their inserts being either VioAB (left) or VioCDE (right) as well as the restriction enzyme cut sites for each plasmid. Proportions are not to scale.

LR-Clonase Reaction

The LR-Clonase reaction used in Gateway Cloning was set up by combining 1.5µl entry vector, in this case either pENTR-VioAB or pENTR-VioCDE, 3µl destination vector and 1µl of LR-Clonase enzyme mix. Here, pENTR-VioAB was combined with the destination vector pCOLA-2-DEST-Spec^{R} and pENTR-VioCDE was combined with the destination vector pDEST527. Additionally, the LR-clonase reactions were also perfomed with pENTR-VioAB and pDEST 527, as well as with pENTR-VioCDE and pCOLA-2-DEST. The LR-Clonase reactions were incubated at room temperature for 16 to 24 hours. After incubation, all 5.5µl of the reaction were added to a 50µl vial of NEB-5α chemically competent *E. coli* cells for transformation. The cells were then incubated

on ice for 20 minutes before being heat shocked at 42°C in a heat block for exactly 30 seconds. Immediately following the heat shock, the cells were placed back on ice for two minutes before 250µl SOC media was added to the solution. The *E. coli* were then placed in a shaking incubator at 37°C for one hour to complete the transformation. After this final incubation, all LR-Clonase reactions involving pDEST527 were plated on LB with ampicillin (Amp), and all LR-Clonase reactions involving pCOLA-2-DEST-Spec^{R} were plated on LB with spectinomycin (Spec). The plates were grown at 37ºC overnight in an incubator before single colonies from each plate were selected and inoculated. All pDEST527 colonies were inoculated in 5mL of LB-Amp, and all pCOLA-2-DEST-Spec^{R} colonies were inoculated in 5mL of LB-Spec. Inoculated cultures were grown for 24 hours in a shaking incubator. The cultures were purified using Qiagen QIAprep Spin Miniprep Kit and protocol except 30µl of Buffer EB was used to elute instead of the indicated 50µl. The purified plasmids were analyzed using a NanoDrop instrument to determine the concentration and purity of each sample. To verify that the VioAB or VioCDE genes were inserted correctly, 500ng of purified plasmids were added to 1µl of pDEST-Seq Forward 01 primer and sent for sequencing at the Nevada Genomics Center.

Double Transforming Destination Vectors

In order to make *E. coli* cells express the entire violacein operon, a double transformation using both destination vectors from the LR-Clonase reaction was performed. Approximately 100ng each of pDEST527-VioAB and pCOLA-2-DEST-Spec^{R}-VioCDE were added to a 50µl vial of NEB-5α chemically competent *E. coli* cells. The cells were then incubated on ice for 20 minutes before being heat shocked at 42°C in a heat block for exactly 30 seconds. Immediately following the heat shock, the cells were

placed back on ice for two minutes before 250µl SOC media was added to the solution. The *E. coli* were then placed in a shaking incubator at 37°C for one hour to complete the transformation. After incubation, the cells were plated on LB-Spec+Amp plates and incubated at 37°C overnight. Growth of purple colonies on the plate indicates that the double transformation was a success.

A second double transformation was also performed in the same fashion using approximately 100ng each of pCDF from the 2012 UCSF iGEM team and pDEST527-VioCDE. The transformation followed the same protocol as above, the cells were still plated on LB-Spec+Amp, and plates were incubated at 37°C overnight. Growth of purple colonies on the plate indicates that this double transformation was also a success.

Constructing a New Destination Vector Containing Spectinomycin Resistance Using Gibson Assembly

To insert $Spec^R$ into pCOLA-2-DEST in another way, both fragments were amplified by PCR before being ligated using Gibson Assembly. To isolate the $Spec^R$ gene from pMDC7, 10µl 5x Phusion Reaction Buffer, 1µl 10mM dNTP mix, 1µl 10x SpecR (+pCOLA promoter) Forward primer, 1µl 10x SpecR (+pCOLA promoter) Reverse primer, approximately 75ng pMDC7 plasmid DNA, 0.5µl Phusion DNA Polymerase, and enough sterile water to fill to 50µl total volume. This sample was then placed in a thermal cycler that ran at 98°C for 30 seconds, before doing thirty cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. It was then held for final extension at 72°C for five minutes before finally holding at 4°C.

To isolate pCOLA-2-DEST without Kan^R, a PCR reaction was set up by combining 10µl 5x Phusion Reaction Buffer, 1µl 10mM dNTP mix, 1µl 10x pCOLA

(+SpecR) Forward primer, 1µl 10x pCOLA (+SpecR) Reverse primer, approximately 75ng pCOLA-2-DEST plasmid DNA, 0.5µl Phusion DNA Polymerase, and enough sterile water to fill to 50µl total volume. This sample was then placed in a thermal cycler that ran at 98°C for 30 seconds, before doing thirty cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 135 seconds. It was then held for final extension at 72°C for five minutes before finally holding at 4°C.

The PCR products for $Spec^R$ and pCOLA-2-DEST without Kan^R were analyzed using gel electrophoresis. A 60mL 1% agarose gel was prepared using a six well comb and 0.75µl EtBr. To each 50µl PCR reaction, 10µl of 6x loading dye was added. The entire resulting 60µl sample was then loaded into one lane of the prepared gel alongside 9µl of 1KB loading ladder for comparison. The gel was run at 100V for 35 to 45 minutes, or until the dye front had moved halfway through the gel, and it was imaged using ImageLab software. A band at 792bp for the $Spec^R$ PCR product indicated correct isolation of the $Spec^R$ gene from pMDC7. A band at 4315bp for pCOLA-2-DEST without Kan^R PCR product indicated correct isolation of the destination vector's backbone. The bands appearing in the appropriate places on the agarose gel were excised to further purify the DNA samples. The excised bands were then solubilized and purified using a Qiagen QIAquick Gel Extraction Kit protocol except that here, 30µl of warm EB was used to elute instead of the 50µl indicated in the protocol. Purified samples of $Spec^R$ and pCOLA-2-DEST without Kan^R were analyzed using a NanoDrop spectrophotometer to determine concentration and purity.

To ligate the PCR products together, a Gibson Assembly reaction was set up by combining 2µl $Spec^R$ PCR product, 3µl pCOLA-2-DEST PCR product, and 5µl 2x

Gibson Assembly Master Mix. The reaction was incubated at 50°C for 20 minutes before 4µl of the Gibson Assembly ligation reaction was transferred to a vial with 50µl of NEB-5α chemically competent *E. coli* cells for transformation. The cells were incubated on ice for 20 minutes before being heat shocked at 42°C in a heat block for exactly 30 seconds. Immediately following the heat shock, the cells were placed back on ice for two minutes before 250µl of SOC media was added to the solution. The *E. coli* were then placed in a shaking incubator at 37°C for one hour before being plated on LB-Spec media.

Constructing a New Destination Vector Containing Spectinomycin Resistance Using Ligation Reactions

In order to create a destination vector with spectinomycin resistance ($Spec^R$), two plasmids were used. To isolate the $Spec^R$ gene with its promoter from pCDF, a PCR reaction was set up. The reaction was set up by combining 10µl 5x Phusion Reaction Buffer, 1µl 10mM dNTP mix, 1µl 10x SpecR (+XmnI) Forward primer, 1µl 10x $Spec^R$ (+Nhe1) Reverse primer, approximately 75ng pCDF DNA, 0.5µl Phusion DNA Polymerase, and enough sterile water to fill to 50µl total volume. This sample was then placed in a thermal cycler that ran at 98°C for 30 seconds, before doing thirty cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. It was then held for final extension at 72°C for five minutes before finally holding at 4°C. These PCR products containing $Spec^R$ with its promoter were analyzed using gel electrophoresis. To do so, a 60mL 1% agarose gel was prepared using a six well comb and 0.75µl EtBr. To each 50µl PCR reaction, 10µl of 6x loading dye was added. The entire resulting 60µl sample was then loaded into one lane of the prepared gel alongside 9µl of 1KB loading ladder for comparison. The gel was run at 100V for 35 to 45 minutes, or until the dye

front had moved halfway through the gel, and it was imaged using ImageLab software. A band at 791bp indicated that the $Spec^R$ gene with its promoter was successfully isolated. Bands appearing in the appropriate places on the agarose gel were excised to further purify the DNA samples. The excised bands were then solubilized and purified using a Qiagen QIAquick Gel Extraction Kit protocol except that here, 30µl of warm EB was used to elute instead of the 50µl indicated in the protocol. Purified samples of $Spec^R$ with its promoter were analyzed using a NanoDrop instrument to determine concentration and purity. To give $Spec^R$ with its promoter sticky ends for ligation, the ends of the PCR product were digested. The digestion was set up by combining 3µl 10x Cutsmart Buffer, 0.5µl NheI, 0.5µl XmnI, and approximately 350ng of $Spec^R$ with its promoter DNA, up to 26µl. If needed, sterile water was added to fill to 30µl total reaction volume. The digests were incubated at 37ºC for 35 minutes before being purified with the Qiagen QIAquick PCR Purification Kit and protocol, except 30µl Buffer EB was used to elute instead of the 50µl indicated in the protocol.

The kanamycin resistance gene (Kan^R) was digested out of an existing destination vector, pCOLA-2-DEST, in order to prepare it for the insertion of $Spec^R$ with its promoter. The digestion was set up by combining 5µl 10x Cutsmart Buffer, 1µl NheI, 1µl XmnI, and approximately 350ng of pCOLA-2-DEST plasmid DNA, up to 43µl. If needed, sterile water was added to fill to 50µl of total reaction volume. The digests were incubated at 37ºC for 35 minutes. To check the results of the digests, a 60mL 1% agarose gel was prepared using a six well comb and 0.75µl EtBr. The first lane was loaded with 9µl 1KB loading ladder. The remaining lanes were each loaded with 50µl of pCOLA-2-DEST digest combined with 10µl 6x loading dye. The gel was run at 100V for 35-45

minutes, or until the dye front had moved halfway through the gel. Bands appearing at 4026bp and 925bp indicated that Kan^R was successfully digested out of the pCOLA-2-DEST plasmid. Bands appearing at 4206bp on the agarose gel were excised to further purify the DNA samples. The excised bands were then solubilized and purified using Qiagen QIAquick Gel Extraction Kit protocol except that here, 30µl of warm EB was used to elute instead of the 50µl indicated in the protocol. Purified samples of pCOLA-2-DEST without Kan^R were analyzed using a NanoDrop instrument to determine the concentration and purity values.

Two ligation reactions were set up to insert $Spec^R$ with its promoter into pCOLA-2-DEST without Kan^R. The first reaction was set up using the NEB Quick Ligation Protocol which combined 4.5µl of pCOLA-2-DEST without Kan^R, 0.5µl of $Spec^R$ with its promoter, 5µl of sterile water, 10µl 2x Quick Ligation Buffer, and 1µl T4 DNA Ligase in a microcentrifuge tube. This reaction was incubated at room temperature for 30 minutes. The second reaction was set up using the NEB Instant Sticky End Ligase Master mix protocol and combined 4.5µl of pCOLA-2-DEST without Kan^R, 0.5µl of $Spec^R$ with its promoter, and 5µl of Instant Sticky End Ligase Master Mix in a microcentrifuge tube. This reaction was incubated on ice for 30 minutes.

After the incubation times, 4µl of the Quick ligation reaction was transferred to a vial with 50µl of NEB-5α chemically competent *E. coli* cells, and 4µl of the Instant Sticky End ligation reaction was transferred to a second vial with 50µl of NEB-5α chemically competent *E. coli* cells for transformation. The cells were incubated on ice for 20 minutes before being heat shocked at 42°C in a heat block for exactly 30 seconds. Immediately following the heat shock, the cells were placed back on ice for two minutes

before 250µl of super optimal broth with catabolite repression (SOC) media was added to the solution. The *E. coli* were then placed in a shaking incubator at 37°C for one hour to complete the transformation.

After transformation, Quick, Instant Sticky End, and Gibson Assembly ligation reactions were plated on LB plates with spectinomycin (Spec) and chloramphenicol (Chlor). The plates were grown at 37ºC overnight in an incubator before single colonies from each plate were selected and inoculated in 5mL of LB-Spec+Chlor. Inoculated cultures were grown for 24 hours in a shaking incubator. Cultures were purified using a Qiagen QIAprep Spin Miniprep Kit and protocol except 30µl of Buffer EB was used to elute instead of the indicated 50µl. Purified pCOLA-2-DEST-Spec^{R} plasmids were analyzed using a NanoDrop instrument to determine the concentration and purity of each sample.

Analyzing pCOLA-2-DEST-Spec^{R} Plasmids

To check if the pCOLA-2-DEST-Spec^{R} plasmid was ligated correctly, the plasmids created using Quick Ligation, Instant Sticky End Ligation, and Gibson Assembly underwent restriction enzyme digestions. The digest was set up by combining 2µl 10x Cutsmart Buffer, 0.5µl EcoRV-HF, 0.5µl BglI, and approximately 350ng of pCOLA-2DEST, up to 17µl. If needed, sterile water was added to fill to a 20µl total reaction volume. The digests were incubated at 37ºC for 35 minutes. To check the results of the digests, a 40mL 1% agarose gel was prepared using a ten well comb and 0.75µl EtBr. The first lane was loaded with 3.5µl 1KB loading ladder. The remaining lanes were each loaded with 5µl of pCOLA-2-DEST-Spec^{R} digest combined with 1µl 6x loading dye. The gel was run at 100V for 35-45 minutes, or until the dye front had moved

halfway through the gel. Bands appearing at 3949bp and 1158bp indicated that the $Spec^R$ gene was inserted into the plasmid correctly. If a pCOLA-2-DEST-$Spec^R$ plasmid sample appeared to digest correctly, approximately 200ng of that sample was sent for sequencing with 1µl of one of six sequencing primers designed to show almost the entire sequence of this new plasmid. The sequencing primers used were AttR1 (+pCOLA) Forward, AttR1 (+pCOLA) Reverse, ColA Ori (+pCOLA) Forward, AttR2 (+pCOLA) Reverse, $Spec^R$ (+pCOLA) Seq Forward, and $Spec^R$ (+pCOLA) Seq Reverse.

Table 1. Primers used for PCR. List of primers, used in PCR reactions, and their sequences.

Name	Sequence
Vio AB Forward	CACCATGAAGCATTCTTCCGATATCTGCATTGTCG
Vio AB Reverse	TCAGGCCTCTCTAGAAAGCTTTCCACA
Vio CDE Forward	CACCATGAAAAGAGCAATCATAGTCGGAGGC
Vio CDE Reverse	CTAGCGCTTGGCGGCGAAGAC
SpecR (+pCOLA promoter) Forward	ATCCGCTCATGAATTAATTCTTAGAAAAACTCATCGAGCATCAAATG
SpecR (+pCOLA promoter) Reverse	AATATTGAAAAAGGAAGAGTATGAGCCATATTCAACGGGA
pCOLA (+SpecR) Forward	GCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTGAAGCA
pCOLA (+SpecR) Reverse	CCAAGGTAGTCGGCAAATAAGAATTAATTCATGAGCGGATACATATTTG
SpecR (+XmnI) Forward	TGCGAATTAATTCTATTTGCCGACTACCTTGGTGATCTC
SpecR (+NheI) Reverse	CCGAGTGAGCTAGCTATTTGTTTATTTT

Table 2. Primers used for sequencing. List of primers, used in sequencing plasmids at the Nevada Genomics Center, and their sequences.

Name	Sequence
AttR1 (+pCOLA) Forward	CGTCACAAGTTTGTACAAAAAA
AttR1 (+pCOLA) Reverse	TTTTTTGTACAAACTTGTGACG
ColA Ori (+pCOLA) Forward	GGGTTATTGTCTCATGAGCG
Att R2 (+pCOLA) Reverse	TGGCAGCAGCCTAGGTTAAT
SpecR (+pCOLA) Seq Forward	GAACCCCTATTTGTTTATTTTTC
SpecR (+pCOLA) Seq Reverse	CGCTCATGAGACAATAACCC
pENTR Seq Reverse	ATGGCTCATAACACCCCTTG
pDEST Seq Forward 01	CTAGAACTAGTGGATCCCCCATC

Results

Genomic DNA (gDNA) Extractions

Four different samples of genomic DNA (gDNA) were extracted from liquid cultures of *Chromobacterium violaceum* (*C. violaceum*) using two different methods. The first two samples, samples 1 and 2, were extracted using the CTAB protocol, and the Nanodrop values for these are shown in table 3. Both samples had very high concentrations at 1675ng/µl and 1227.3ng/µl, and the purities were fairly pure according to the given $A_{260/280}$ and $A_{260/230}$ values. A ratio of about 1.8 for $A_{260/280}$, and a ratio between 2.0 and 2.2 for $A_{260/230}$ would be considered ideal. Here with $A_{260/280}$ values of 2.18 and 2.20 and $A_{260/230}$ values of 2.14 and 2.36, for samples 1 and 2 respectively, it can be concluded that the samples had decent purity. The $A_{260/280}$ values are not too far outside the expected ratio, and the $A_{260/230}$ values are both almost perfect. These two samples were run on a gel, shown in figure 3, to check that gDNA was in fact present, as well as to check the purity of the two samples. The gel on the left shows a bright band above 10,000bp which indicates the presence of gDNA; however, the bright band and smearing below 500bp indicates a significant amount of RNA contamination was present

in each sample. To further purify samples 1 and 2 they underwent an RNase treatment and were run on a gel again, also shown in figure 3. The gel image on the right shows significantly less RNA contamination for each gDNA sample. The samples were diluted to 10x to lower the gDNA concentrations for use in PCR.

Samples 3 and 4 of the *C. violaceum* gDNA were extacted using the Qiagen DNeasy Blood and Tissue Kit and protocol, and these samples also underwent NanoDrop analysis as shown in table 4. The concentrations for each sample, at 177.7ng/µl and 50.0ng/µl, were high enough to use in PCR. The $A_{260/280}$ values of 2.13 and 2.02 and $A_{260/230}$ values of 0.69 and 0.35, for samples 3 and 4 respectively, indicate that the samples are fairly pure. Here, the $A_{260/230}$ values were somewhat low due to a high level of guanidine in one of the buffers used in this kit. These gDNA samples were also run on a gel, shown in figure 4, and the bright band above 10,000bp indicates the presence of gDNA in each sample. The significantly lower amount of RNA contamination below 500bp indicated an RNase treatment was not necessary with this protocol.

Analysis of PCR Reactions for VioAB and VioCDE

Using the gDNA samples extracted with the Qiagen DNeasy Blood and Tissue Kit and protocol, PCR reactions were performed to amplify the VioAB genes and the VioCDE genes. Once the reactions were complete, the PCR samples were run on a gel, shown in figure 5, to check for the correct length of amplified DNA. The gel image on the left shows the VioAB amplification, wherein bands appearing at 4328bp indicated that both the A and B genes from the violacein operon had successfully been amplified. The gel image on the right shows the VioCDE amplification, wherein bands appearing at 2996bp indicated that the C, D, and E genes from the violacein operon had all

successfully been amplified. The bands in the appropriate places were excised and purified using the Qiagen QIAquick Gel Extraction protocol. These purified samples underwent NanoDrop analysis, shown in tables 5 and 6, to determine their concentrations and purities. Since 4µl of DNA was used in each of the pENTR-d-TOPO reactions for VioAB and VioCDE, DNA concentrations around 20ng/µl from the gel extraction were adequate to move forward with the pENTR-d-TOPO reactions. The $A_{260/280}$ and $A_{260/230}$ values for both VioAB and VioCDE DNA samples were in the appropriate ranges to indicate purity from a gel extraction. The $A_{260/230}$ values for gel extraction are always expected to be low, because there are high concentrations of guanidine in buffer QG which is used to solubilize the agarose gel for purification.

Verifying pENTR-VioAB and pENTR-VioCDE are in the Correct Orientation

After pENTR-d-TOPO cloning reactions were performed for both the VioAB and the VioCDE DNA samples, the plasmids were prepped for digestion to check the orientation of the insert DNA. After the Qiagen QIAprep Miniprep was performed on liquid cultures of pENTR-VioAB and pENTR-VioCDE, the samples were analyzed using a NanoDrop instrument. The concentration and purity values for pENTR-VioAB plasmids were recorded in table 7, and the values for pENTR-VioCDE plasmids were recorded in table 8. Concentrations of at least 20ng/µl for each pENTR plasmid were adequate to perform the digest since 350ng of DNA was required to visualize the digest pieces on a gel. The $A_{260/280}$ and $A_{260/230}$ values for all of the pENTR plasmid samples were in the appropriate ranges to indicate sufficient purity levels.

Once plasmids were prepped, restriction enzyme digestions were set up on each sample of pENTR plasmid. The digests were run on agarose gels, and the gels were

imaged. Figure 6 shows the digest results of the pENTR-VioAB samples. In lanes 2 and 3 a band at around 3000bp indicates a linear cut piece of pENTR-VioAB, and the bands around 5000bp and 2000bp suggest that the plasmid was inserted backwards and was only partially digested. In lane 5, a band at around 3000bp also indicates a linear piece of plasmid, but the plasmid was not completely cut into two pieces of DNA. Lane 4 shows bands at 6031bp and 877bp which indicates that the VioAB DNA was inserted into the pENTR plasmid in the correct orientation to move forward with the LR-Clonase reaction. To better visualize the cut sites for this digestion, reference figure 2 in the methods section. Figure 7 shows the digest results of pENTR-VioCDE. Lane 2 shows a band around 2400bp which indicates that the pENTR plasmid took up no insert DNA and is therefore empty. Lanes 3 and 4 show bands around 4660bp and 916bp which indicate that the pENTR plasmid took up the VioCDE insert DNA in the appropriate orientation. In lane 3 the band at 916bp is faint, likely due to partial digestion or a lower DNA concentration than shown in lane 4. The bands appearing at around 6000bp in lanes 3 and 4 are likely undigested pENTR-VioCDE plasmid. For a better visualization of the cut sites for this digestion, reference figure 2 in the methods section.

LR-Clonase Reactions for VioAB and VioCDE

LR-clonase reactions were then performed on the correct pENTR-VioAB and pENTR-VioCDE plasmids to move the insert DNA into a destination vector. The LR-clonase reactions were performed with one of two destination vectors – pDEST 527 or pCOLA-2-DEST-Spec^{R}. Each pENTR plasmid underwent an LR-clonase reaction with both destination vectors. Colonies grew on both plates that used pDEST 527 as a destination vector, but only grew on the pCOLA-2-DEST-Spec^{R} plate when pENTR-

VioCDE was used as the entry vector, indicating a problem with the pCOLA-2-DEST-Spec^R plasmid. Colonies were selected for pDEST 527-VioAB, pDEST 527-VioCDE, and pCOLA-2-DEST-Spec^R-VioCDE and the resulting cultures were prepped using Qiagen QIAprep miniprep protocol. The purified samples of each plasmid were analyzed using a NanoDrop instrument, and concentration and purity values were recorded in table 9 for pDEST 527-VioAB, in table 10 for pDEST 527-VioCDE, and in table 11 for pCOLA-2-DEST-Spec^R-VioCDE. About 100ng of DNA from each destination vector was required to move forward with the double transformation reaction, so the concentrations were adequate to use 1-2µl of purified plasmid.

Double Transforming into *E. coli*

The pDEST 527-VioAB plasmid was double transformed with pCOLA-2-DEST-Spec^R-VioCDE, while pDEST 527-VioCDE was double transformed with pCDF. The presence of purple colonies on the double transformation plates indicates that the double transformations were successful, and that all five genes in the violacein operon were expressed appropriately. Purple colonies only grew on the plate with cells that were double transformed with pDEST 527-VioCDE and pCDF. This double transformation plate can be seen in figure 11, where the original *C. violaceum* plate was also provided for color comparison.

Constructing a New Destination Vector with Spectinomycin Resistance

To construct a new destination vector, an existing destianation vector, pCOLA-2-DEST, was manipulated to remove its resistance to kanamycin (Kan^R) while inserting a resistance to spectinomycin (Spec^R) from another plasmid. To do so, the pCOLA-2-DEST plasmid was cloned using a PCR protocol that excluded Kan^R for use in a Gibson

assembly reaction. Additionally, the pCOLA-2-DEST plasmid was digested to remove Kan^R in preparation for a ligation reaction. Both the PCR product and the digest product were run on gels, which were imaged and are shown in figure 8. The gel image on the left shows the results of the PCR reaction, wherein a band at 4315bp indicates that the entire pCOLA-2-DEST backbone without Kan^R was amplified correctly. The gel image on the right shows the results of the digestion, wherein a band at 925bp indicated Kan^R, and a band at 4026bp indicated that the pCOLA-2-DEST backbone had been separated from the Kan^R gene. The bands at 4315bp in the PCR gel and the bands at 4026bp in the digest gel were extracted and purified using Qiagen QIAquick gel extraction protocol. The purified samples were analyzed using a NanoDrop instrument. The results of the analysis are shown in table 12 for the pCOLA-2-DEST PCR products and in table 13 for the pCOLA-2-DEST digest products. The gel extract concentrations were much higher for the PCR products than for the digest products, but since a small amount of DNA is required to perform Gibson assembly or ligation reactions, it was enough to move forward. Once again the $A_{260/230}$ values were low because of the high levels of guanidine in buffer QG, but the $A_{260/280}$ values were right within the desired range for purity.

$Spec^R$ was isolated from two different plasmids, pMDC7 and pCDF, using the same PCR conditions, with different primers. The $Spec^R$ gene isolated from pMDC7 used primers with overhangs to the pCOLA-2-DEST PCR product for use in Gibson assembly, whereas the $Spec^R$ gene isolated from pCDF used primers that annealed to the restriction digest sites of the pCOLA-2-DEST digest product for use in ligation reactions. After the PCR reactions were complete both $Spec^R$ genes were run on gels, which were imaged and are shown in figure 9. The gel image on the left shows the PCR product from pMDC7

and the gel image on the right shows the PCR product from PCDF. Both gel images show a band at 792bp which indicates that the $Spec^R$ gene was correctly amplified. Bands appearing at 792bp on both gels were excised and purified using Qiagen QIAquick gel extraction protocol. Purified samples were analyzed using a NanoDrop instrument, and the concentration and purity values for each sample are shown in table 14. The concentrations for all of the $Spec^R$ samples were really high, and the purity values were right in the expected range. As always with gel extraction, the $A_{260/230}$ values were lower than might be expected.

Ligating the New Vector Construction

After isolating each of the pieces, Gibson assembly and ligation reactions were perfomed to create a pCOLA-2-DEST plasmid with $Spec^R$. The pCOLA-2-DEST PCR product was used with the $Spec^R$ isolated from pMDC7 to perform a Gibson assembly reaction. The pCOLA-2-DEST digest product was used with the $Spec^R$ isolated from pCDF to perform a Quick ligation reaction and an Instant Sticky End Ligase ligation reaction. Once the Gibson assembly and ligation reactions had been transformed, colonies were picked and grown in LB-Spec liquid cultures. These cultures were prepped using Qiagen QIAprep miniprep protocol, and purified samples were analyzed for concentration and purity using a NanoDrop instrument. The concentration and purity values for all pCOLA-2-DEST-$Spec^R$ plasmids can be found in table 15. The concentrations for each of the samples were in the correct range to be able to perform a restriction enzyme digest in the next step. The $A_{260/280}$ values for all the samples were around the desired range, but the $A_{260/230}$ values were all a bit higher than expected. This likely indicates some contamination from phenols found in the Qiagen QIAprep miniprep

buffers. After digesting the pCOLA-2-DEST-Spec^R plasmid samples, the digests were run on a gel, which was imaged and can be found in figure 10. Bands at 3949bp and 1158bp as seen in lanes 3, 4, and 5 indicate that the Spec^R gene was ligated into the pCOLA-2-DEST plasmid in the correct orientation. Lanes 2, 5, 6, 7, and 8 all show a band slightly larger than 3949bp and no band at 1158bp which indicates that the pCOLA-2-DEST backbone did not take up the Spec^R gene. Correctly oriented plasmid was retransformed and used in the LR-clonase reactions with pENTR-VioAB and pENTR-VioCDE.

Table 3. *C. violaceum* gDNA from CTAB Protocol. Two samples of gDNA, extracted using CTAB gDNA extraction protocol, were analyzed for concentration and purity values using a NanoDrop instrument. The samples were purified on January 25, 2015.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
C. violaceum gDNA 1	1675.0	2.18	2.14
C. violaceum gDNA 2	1227.3	2.20	2.36

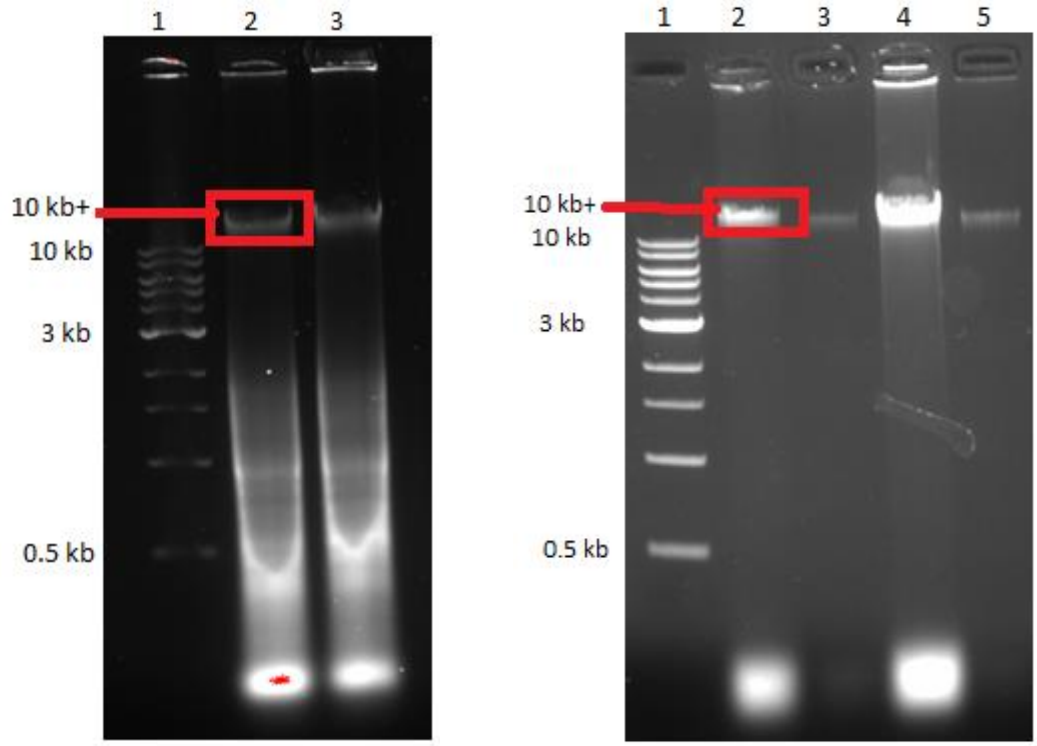

Figure 3. CTAB gDNA Extraction. Images taken of 1% agarose gels loaded with gDNA extracted from *C. violaceum* using CTAB protocol. On left, 5µl gDNA and 1µl 6x loading dye were loaded into lanes 2 and 3 next to 3.5µL 1KB loading ladder loaded in lane 1. On right, 5µl of RNase treated gDNA samples with 1µl 6x loading dye were loaded into lanes 2 and 4 next to 3.5µl 1KB loading dye loaded in lane 1. Lanes 3 and 5 were loaded with 10x dilutions of each RNase treated gDNA sample in the same fashion. The gel on the left was run on January 25, 2016 and the gel on the right was run on February 1, 2016.

Table 4. *C. violaceum* gDNA from DNeasy Protocol. Two samples of gDNA, extracted using DNeasy Blood and Tissue kit and protocol, were analyzed for concentration and purity values using a NanoDrop instrument. The samples were purified on February 26, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
C. violaceum gDNA 3	117.7	2.13	0.69
C. violaceum gDNA 4	50.0	2.02	0.35

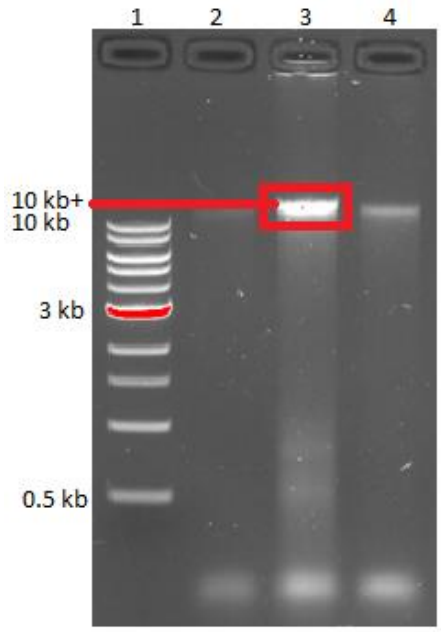

Figure 4. DNeasy gDNA Extraction. Image taken of a 1% agarose gel loaded with gDNA extracted from *C. violaceum* using DNeasy Blood and Tissue Kit and protocol. 5µl gDNA and 1µl 6x loading dye were loaded into lanes 2 and 3 next to 3.5µL of 1KB loading ladder in lane 1. The gel was run on February 26, 2016.

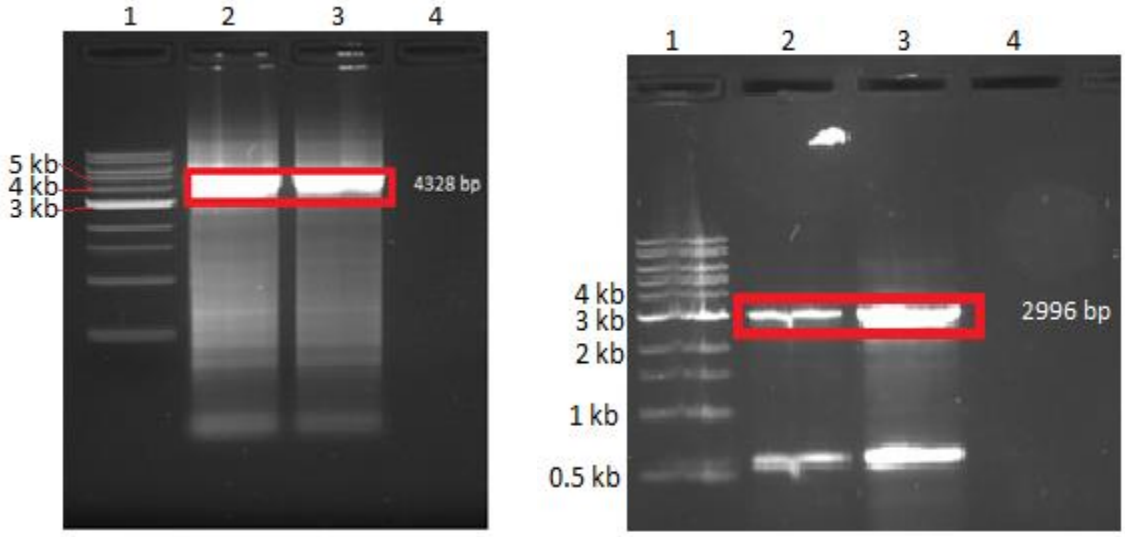

Figure 5. VioAB and VioCDE PCR Products. Images taken of 1% agarose gels loaded with VioAB and VioCDE PCR products. On the left, 50µl of Vio AB PCR sample and 10µl of 6x loading dye were loaded into lanes 2 and 3 next to 9µL of 1KB loading ladder in lane 1. On the right, 50µl of Vio CDE PCR sample and 10µl of 6x loading dye were loaded into lanes 2 and 3 next to 9µL of 1KB loading ladder in lane 1. The gel on the left was run on September 9, 2015 and the gel on the right was run on September 4, 2015.

Table 5. VioAB Gel Extraction. Gel slices extracted at 4328 bp were purified using Qiagen QIAquick Gel Extraction Kit and protocol, and the resulting samples were analyzed to determine concentration and purity values using a NanoDrop instrument. The samples were extracted on September 9, 2015.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
Vio AB-B	16.1	1.88	0.11
Vio AB-C	39.0	2.0	0.18

Table 6. VioCDE Gel Extraction. Gel slices extracted at 2996 bp were purified using Qiagen QIAquick Gel Extraction Kit and protocol, and the samples were analyzed to determine concentration and purity values using a NanoDrop instrument. Sample 1 was extracted on September 9, 2015, while sample 2 was extracted on January 12, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
Vio CDE 1	34.4	1.74	0.10
Vio CDE 2	42.6	2.06	0.07

Table 7. pENTR-VioAB Miniprep. Cultures grown from pENTR-VioAB colonies were purified using Qiagen QIAprep Miniprep Kit and protocol. The plasmid samples were analyzed for concentration and purity using a NanoDrop instrument. The samples were purified on October 9, 2015.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pENTR-Vio AB B1	24.0	4.99	2.48
pENTR-Vio AB B2	9.3	-2.95	2.06
pENTR-Vio AB C3	45.3	2.88	2.16
pENTR-Vio AB C4	30.9	3.15	1.22

Table 8. pENTR-VioCDE Miniprep. Cultures grown from pENTR-VioCDE colonies were purified using Qiagen QIAprep Miniprep Kit and protocol. The plasmid samples were analyzed for concentration and purity using a NanoDrop instrument. The samples were purified on March 4, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pENTR-Vio CDE 1	44.7	2.03	3.23
pENTR-Vio CDE 3	84.3	1.95	2.72
pENTR-Vio CDE 4	39.6	1.88	2.64

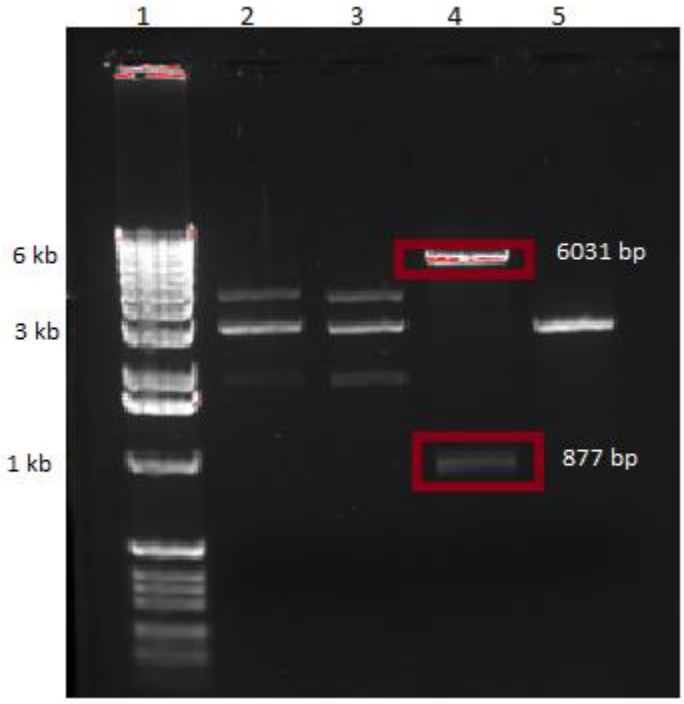

Figure 6. pENTR-VioAB Digest Results. Image of 1% agarose gel with samples of pENTR-VioAB that were digested using EcoR1 and Asc1. 5µl of digest was loaded with 1µl of 6x loading dye into lanes 2, 3, and 4 to check the orientation of the VioAB insert. Lane 1 was loaded with 3.5µl of 1KB loading ladder for comparison. The gel was run on October 12, 2015.

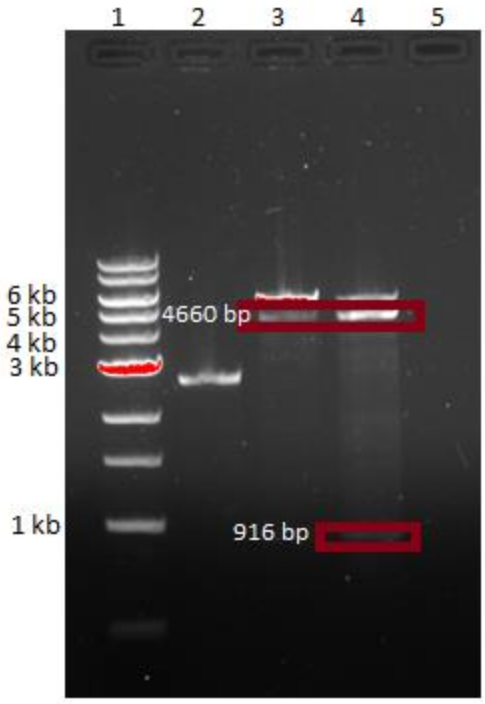

Figure 7. pENTR-VioCDE Digest Results. Image of a 1% agarose gel with samples of pENTR-VioCDE that were digested using EcoRV and Xmn1. 5µl of digest was loaded with 1µl of 6x loading dye into lanes 2, 3, and 4 to check the orientation of the VioCDE insert. Lane 1 was loaded with 3.5µl of 1KB loading ladder for comparison. The gel was run on March 7, 2016.

Table 9. pDEST 527-VioAB Miniprep. Cultures grown from pDEST 527-VioAB colonies were purified using Qiagen QIAprep Miniprep Kit and protocol. The purified plasmid samples were analyzed for concentration and purity using a NanoDrop instrument. These samples were purified on March 9, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pDEST 527-Vio AB 1	67.2	1.90	2.64
pDEST 527-Vio AB 2	113.9	1.88	1.88
pDEST 527-Vio AB 3	82.1	1.94	2.56
pDEST 527-Vio AB 4	66.4	1.92	2.67

Table 10. pDEST 527-VioCDE Miniprep. Cultures grown from pDEST 527-VioCDE colonies were purified using Qiagen QIAprep Miniprep Kit and protocol. The purified plasmid samples were analyzed for concentration and purity using a NanoDrop instrument. These samples were purified on March 10, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pDEST 527-Vio CDE 1	95.6	1.87	1.91
pDEST 527-Vio CDE 2	53.3	1.88	2.42
pDEST 527-Vio CDE 3	71	1.90	2.16

Table 11. pCOLA-Spec^{R}-VioCDE Miniprep. Cultures grown from pCOLA-Spec^{R}-VioCDE colonies were purified using Qiagen QIAprep Miniprep Kit and protocol. The plasmid samples were analyzed for concentration and purity using a NanoDrop instrument. These samples were purified on March 14, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pCOLA-Spec^{R}-Vio CDE 1	3.3	2.89	3.72
pCOLA-Spec^{R}-Vio CDE 2	15.9	2.19	1.05
pCOLA-Spec^{R}-Vio CDE 3	31.8	1.81	1.56

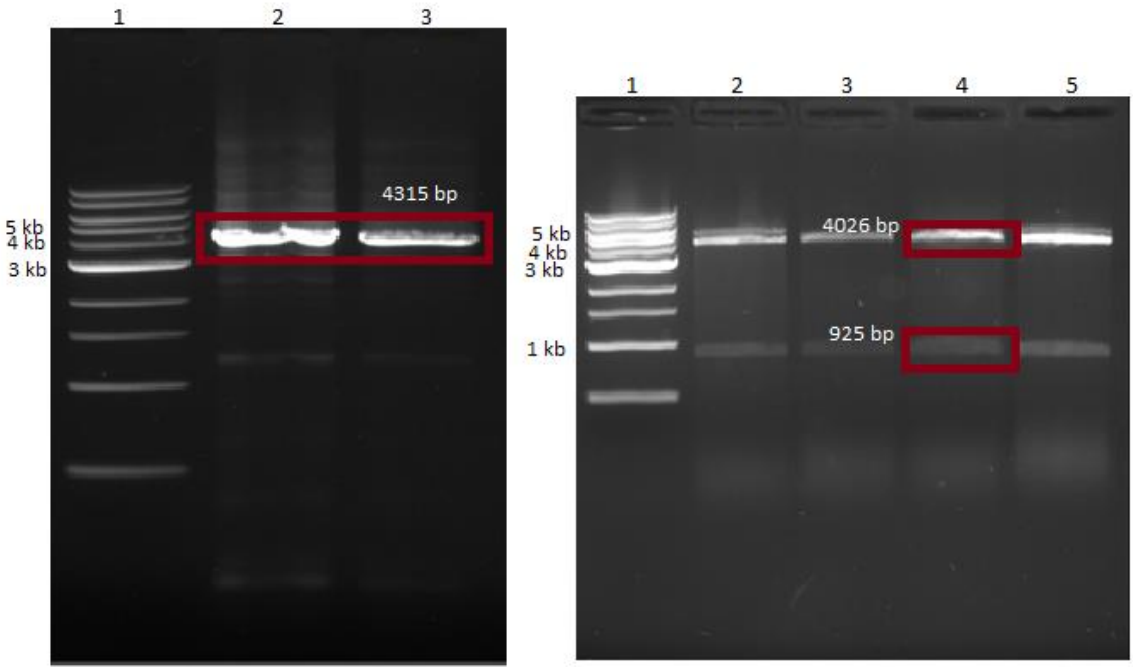

Figure 8. pCOLA-2-DEST PCR and Digest Results. Images taken of 1% agarose gels loaded with pCOLA-2-DEST PCR and digestion products. On the left, 50µl of pCOLA-2-DEST PCR sample and 10µl of 6x loading dye were loaded into lanes 2 and 3 next to 9µL of 1KB loading ladder in lane 1. On the right, 50µl pCOLA-2-DEST, digested using EcoRV-HF and Bgl1, and 10µl 6x loading dye were loaded into lanes 2 and 3 next to 9µL 1KB loading ladder in lane 1. The gel on the left was run on November 18, 2015 and the gel on the right was run on February 3, 2016.

Table 12. pCOLA-2-DEST Gel Extraction from PCR Gel. Gel slices extracted at 4315bp were purified using Qiagen QIAquick Gel Extraction Kit and protocol, and the samples were analyzed to determine concentration and purity values using a NanoDrop instrument. Sample 1 was extracted on November 3, 2015, while sample 2 was extracted on November 18, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pCOLA-2-DEST Backbone 1	57.9	1.91	0.11
pCOLA-2-DEST Backbone 2	22.7	1.90	0.16

Table 13. pCOLA-2-DEST Gel Extraction from Digest Gel. Gel slices extracted at 4315bp were purified using Qiagen QIAquick Gel Extraction Kit and protocol, and the samples were analyzed to determine concentration and purity using a NanoDrop instrument. The samples were extracted on February 4, 2015.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pCOLA-2-DEST Backbone 1	2.6	2.80	0.01
pCOLA-2-DEST Backbone 2	11.3	1.86	0.03

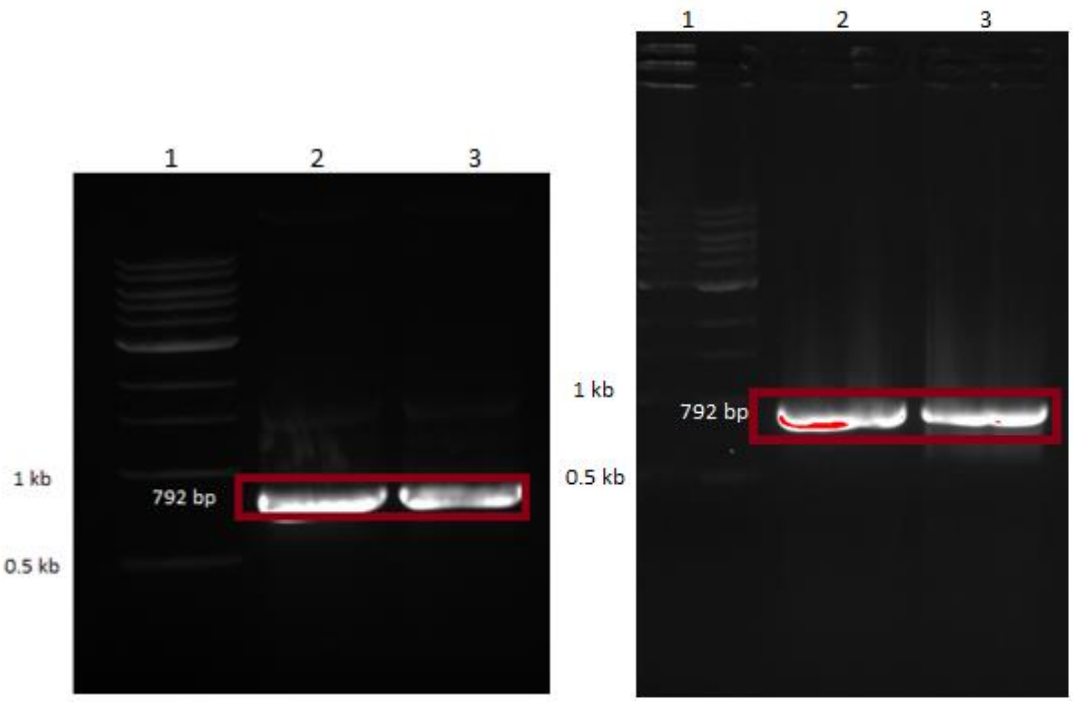

Figure 9. Spec^R PCR Results from pMDC7 and pCDF. Images taken of 1% agarose gels loaded with Spec^R PCR products from both pMDC7 and pCDF. On the left, 50µl of Spec^R PCR sample from pMDC7 and 10µl of 6x loading dye were loaded into lanes 2 and 3 next to 9µL of 1KB loading ladder in lane 1. On the right, 50µl of Spec^R PCR sample from pCDF and 10µl of 6x loading dye were loaded into lanes 2 and 3 next to 9µL of 1KB loading ladder in lane 1. The gel on the left was run on November 18, 2015 and the gel on the right was run on February 11, 2016.

Table 14. Spec^R Gel Extraction from pMDC7 and pCDF PCR Gels. Gel slices extracted at 792bp were purified using Qiagen QIAquick Gel Extraction Kit and protocol, and the samples were analyzed to determine the concentration and purity values using a NanoDrop instrument. The sample from pMDC7 was extracted on November 3, 2015, while the samples from pCDF were extracted on February 11, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
Spec^R pMDC7	57.9	1.91	0.11
Spec^R pCDF 1	44.5	1.96	0.05
Spec^R pCDF 2	44.9	1.99	0.07

Table 15. pCOLA-2-DEST-Spec^R Miniprep. The liquid cultures grown from pCOLA-2-DEST-Spec^R colonies were purified using Qiagen QIAprep Miniprep Kit and protocol. The plasmid samples were then analyzed for concentration and purity using a NanoDrop instrument. The samples were purified on February 29, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pCOLA-Spec^R 1	34.1	2.18	4.46
pCOLA-Spec^R 2	24.3	2.12	3.91
pCOLA-Spec^R 3	32.7	2.05	3.00
pCOLA-Spec^R 4	24.8	2.03	2.84

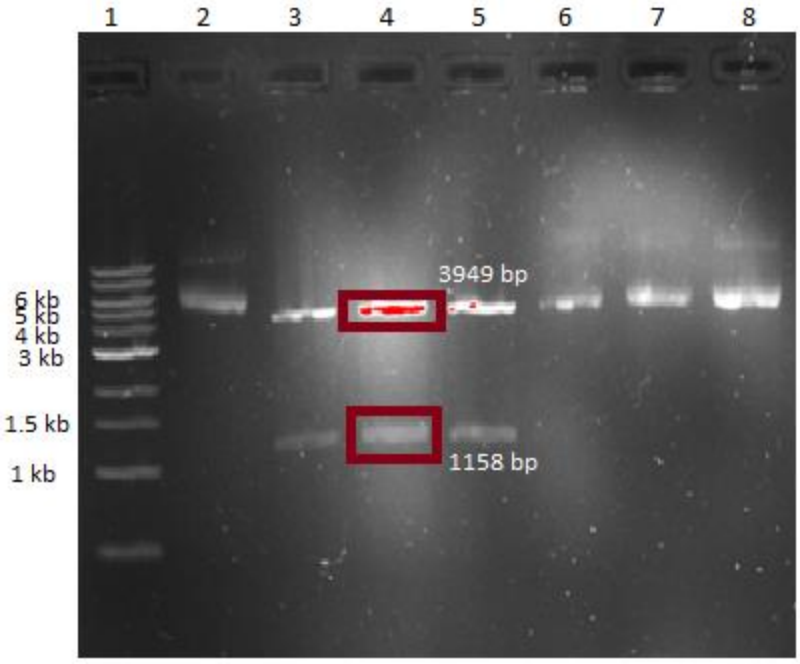

Figure 10. pCOLA-2-DEST-Spec^R Digest Results. Image taken of a 1% agarose gel loaded with purified pCOLA-2-DEST-Spec^R plasmid that had been digested with EcoRV and Bgl1 to screen for plasmids with the Spec^R insert in the correct orientation. In lanes 2 through 8, 5µl of digest sample and 1µl of 6x loading dye were loaded. In lane 1, 9µL of 1KB loading ladder was loaded for comparison. The gel was run on February 29, 2016.

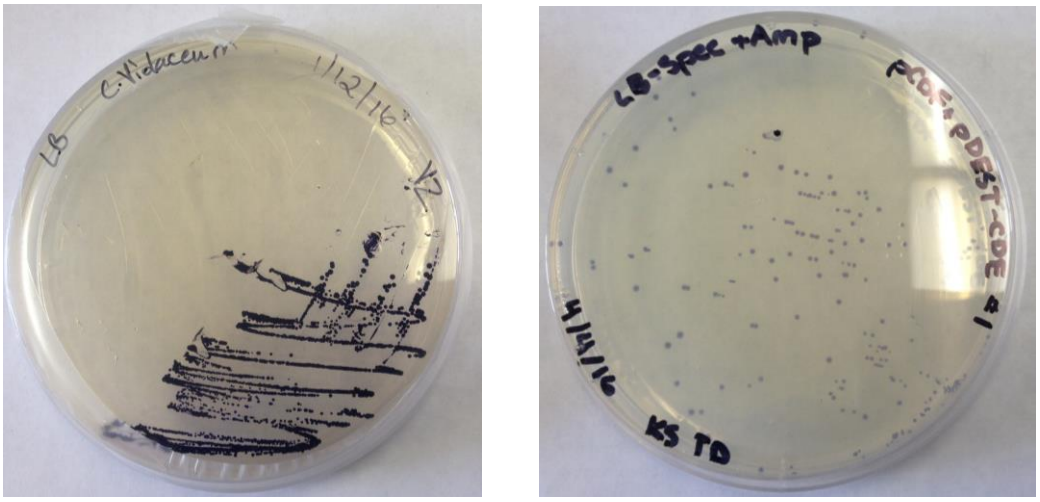

Figure 11. *C. violaceum* and Double Transformation Plates. The image on the left was taken of the original *C. violaceum* plate used to perform the gDNA extraction protocols. The image on the right was taken of the final double transformation plate with *E. coli* containing the pCDF and pDEST 527-VioCDE plasmids.

Discussion

Complications Overall

The growth of purple colonies on the pCDF and pDEST 257-VioCDE double transformation plate indicates that the goal of this laboratory module was achieved. The purpose was to use Gateway cloning to insert all five genes from the violacein operon into *E. coli* through double transformation, resulting in visibly purple cell growth. However, the modification to use pCDF instead of the newly constructed pCOLA-2-DEST-Spec^{R} plasmid means that overall the laboratory module can still be improved.

The reason a double transformation reaction with two destination vectors was selected for this laboratory protocol is due to the sheer size of the violacein operon. All five genes together are a total of 7,324bp in length which is very large and difficult to insert into a single plasmid at one time in a reproducible manner. Separating the A and B genes from the C, D, and E genes simply resulted from the orientation in which the genes exist within the operon. The next challenge was trying to insert the two different pieces of gene into the entry vector, pENTR-dTOPO. The pENTR plasmid is on its own a 2,576bp plasmid, and logistically speaking, the smaller the gene of interest is to be inserted into pENTR, the more likely it will insert in the correct orientation. With VioCDE, there was an almost fifty percent chance of the gene inserting into pENTR in the correct orientation over all the pENTR-VioCDE digestions performed here. However, with VioAB which was much larger, the gene of interest was taken up by pENTR only once in all the digestions and transformations performed for this module.

The initial intention for this laboratory module was for the students to work with all five of the violacein genes and perform the entire protocol on their own. After having

more than a 75 percent failure rate with VioAB, it was determined that the students should work only with VioCDE which had an almost 90 percent success rate throughout all the steps. VioAB was difficult to PCR, and even using the same protocol for multiple reactions, there was not a good success rate. Additionally, the pENTR-VioAB plasmid did not grow very reliably, and therefore only a few colonies could be digested to check orientation. Even after digesting the colonies that did grow, the VioAB insert was rarely inserted in the correct orientation. It was then decided that VioAB should be placed inside of a destination vector and provided to the students for the double transformation reaction.

Complications with the pCOLA-2-DEST-Spec^{R} Vector

The decision to develop a new destination vector, rather than purchasing one as with pDEST 527, stems from the fact that the pENTR plasmid has kanamycin resistance. Many of the entry vectors on the market contain the kanamycin resistance marker, which is not in itself a problem, but it proved to be a problem when trying to do a double transformation with Gateway cloning. To perform the LR-clonase reaction, the pENTR plasmid must have a different antibiotic resistance marker than the destination vector taking up the gene of interest. If the entry and destination vectors had the same resistance, there would be no way to select for cell growth on the plates grown from the LR-clonase reactions. Beyond that, in order to know that the cells growing on the final plate actually contained both plasmids – and all five violacein genes – the two different destination vectors have to be different. Specifically, they each have to have a unique antibiotic resistance marker, both different from kanamycin found in pENTR, and each one has to have a unique origin of replication, different from the other destination vector. Without

these differences, it would be impossible to determine whether or not the *E. coli* colonies growing on the plate actually took up all five genes.

Even though the correct colonies should turn purple, and this could indicate a correct double transformation, the other selection markers are really vital to ensuring that the lab actually works as expected. Additionally, there is no way to prep the purple *E. coli* colonies and sequence them since they do not contain a single pure plasmid. pDEST 527 was purchased through the AddGene website, but all the other destination vectors available for purchase had either an ampicillin resistance or a kanamycin resistance which would not work for the second destination vector. This led to the conclusion that a new destination vector would need to be constructed, and this construction would include spectinomycin resistance to get around the existing problem.

The fact that none of the double transformations with pCOLA-2-DEST-Spec^R grew, indicates that there is something inherently wrong with the plasmid, and it does not work appropriately as a destination vector. Sequencing results showed that the pCOLA-2-DEST backbone was present as should be expected. Additionally, it verified that the Spec^R gene was present in the pCOLA-2-DEST-Spec^R and it appeared to be in tact. The results provided no definite conclusions as to why the plasmid construct did not work as it theoretically should have.

Throughout working with the pCOLA-2-DEST plasmid, it was discovered that even the original plasmid was slow growing and difficult to manipulate. Even though many of the plates and cell cultures were allowed to grow much longer than the 24 hour period indicated in the methods, cell growth often still did not occur. When cell growth did occur, there was usually very little, and plates only grew up to six colonies at a time.

This low amount of growth made the plasmid difficult to manipulate once the ligation reactions were performed. Theoretically, since the pCOLA-2-DEST-Spec^{R} plasmid construction grew on a plate with spectinomycin resistance, the vector should have been constructed correctly. Additionally, since no modification should have occurred with the plasmid outside of the Kan^{R} gene, there is no reason why the destination sites should not work properly.

To get around the problems with the pCOLA-2-DEST-Spec^{R} construction, the plasmid pCDF was instead provided to the students for the double transformation. The pCDF plasmid used was created by the 2012 UCSF iGEM team and already has the A, B, and E genes from violacein inserted into it. As a result this plasmid shows the green pigment produced by prodeoxyviolacein through the violacein pathway as indicated in figure 1. Double transforming this plasmid with the pDEST 527-VioCDE plasmid did result in *E. coli* that expressed the purple pigment characteristic of *C. violaceum*, which was the primary goal of this cloning module.

Future Directions

A different destination vector may be a better candidate for modification than pCOLA-2-DEST. For instance, the pDEST 527 plasmid always grew well and prepped well which would probably make it easier to manipulate. Since the spectinomycin genes from both pCDF and pMDC7 worked well with this PCR protocol and with the Qiagen QIAquick gel extraction protocol, they could still be useful in inserting a new antibiotic resistance gene into a destination vector as done here. Additionally, if students could perform the laboratory protocol from start to finish with all five genes of the violacein operon, they would probably maximize their learning of molecular biology and cloning.

With the amount of time allotted for this project, it was impossible to complete the module as originally planned. Yahata et al. (2005) suggests an alternative to double transforming with Gateway cloning. Instead he talks of modifying a single destination vector to take up multiple pieces of cDNA. This would require much more research, but it is a valid suggestion for inserting the entire violacein operon into a single Gateway vector without needing to construct a new plasmid altogether (Yahata et al., 2005).

Also, a new laboratory protocol could be incorporated that also utilizes the violacein operon's variability. The new protocol would be similar to one already published that uses CRISPRi to express particular genes in the violacein pathway in the hopes of creating various colored strains of yeast (Zalatan et al., 2015). These different strains of yeast could be manipulated using CRISPRi to express or repress certain genes in the violacein operon which results in the various colors seen in figure 1 (Zalatan et al., 2015). Encompassing the unique method of genome engineering through CRISPRi into a laboratory setting with students will show them that modern molecular biology is rapidly changing, and that molecular biology now contains much more than restriction enzyme digests and cloning.

Conclusion

The creation of new laboratory protocols for undergraduate teaching labs is vital to the education of biochemistry and molecular biology students. With the growth of bioinformatics making it more possible to access basic information about proteins and DNA, it has become easier to incorporate labs that reach further into the field of molecular biology (Boyer, 2003). This new lab protocol was designed to teach undergraduate students about cutting edge molecular biology by introducing Gateway

cloning. Through the use of Gateway cloning and other molecular biology techniques, students were able to successfully transform regular *E. coli* cells into vibrant, violet colonies by transforming all five genes of the violacein operon into the cells themselves.

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