University of Nevada, Reno

Modifications to Molecular Biology Lab BCH 406: Expressing violacein in *E.coli* using Gateway Cloning

A Thesis submitted in partial fulfillment
Of the requirements of the degree of

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by

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Modifications to Molecular Biology Lab BCH 406

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Biochemistry, Ph.D., Veronica Zepeda

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Abstract

Biochemistry 406, a molecular biology lab, is a course offered at the University of Nevada, Reno that involves manipulating DNA, RNA, and proteins. Molecular biology is an evolving field; therefore, in order to create a relevant lab course for students, classroom lab modules must be updated. A modern molecular biology lab course can be created by adding and modifying to the current BCH 406 course. The current lab that involved cloning of *Chromobacterium violaceum* genes into *E. coli* has been modified to include a cloning technique known as Gateway cloning, which offers a new more efficient cloning tool. Gateway cloning gives students the ability to insert more DNA into the *E. coli* with more efficiency then previous methods in the course. This lab module engages students in relevant science techniques preparing them for science careers.

Acknowledgments

This project was truly a pleasure to work on because the wonderful people that helped make it possible and a more enjoyable experience. My lab partner Kim is one of the most considerate hard workers I know, and she definitely made all the days in the lab much easier. There are professors that simply give a student a senior thesis project, and then there are professors like Dr.Veronica Zepeda who are truly invested in their student's projects. Dr. Zepeda worked one on one with Kim and I every week to help keep us working towards completion of this project. Without here none of this would have been possible. Thank you Dr. Zepeda. Also thanks to Dr. Ian Wallace who was great to have around when Dr. Zepeda was gone and we needed help with an experiment or needed some plasmid for cloning.

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Introduction

Biochemistry 406 is the molecular biology undergraduate lab course at the University of Nevada, Reno. Molecular biology is a rapidly changing field; therefore, technology makes it so techniques that were once cutting edge in molecular biology are now obsolete. In order to prepare students for the biotechnology industry and beyond, they must be familiarized with the latest techniques. When students are working with applicable techniques to the real biotechnology field their engagement will be increased. Increased engagement increases performance and means that the students have a better background on techniques upon completion of an undergraduate degree (Stahelin, Forslund, Wink, & Cho, 2003). For this reason, BCH 406 lab modules must constantly be updated to keep student engagement high.

The BCH 406 lab module that was modified is the cloning lab that involves *Chromobacterium violaceum (C.vio)*. This bacteria has been selected as it provides visual cues that allow students to learn efficiently (Round & Lom, 2015). More specifically, *C.vio* contains the violacein operon which has 5 genes, VioA, VioB, VioC, VioD, and VioE, which can be manipulated to express a green or purple pigment by the cells. Seeing the colors gives students a tangible goal that makes grasping the actual chemical reactions taking place easier. This is important as it provides a good visualization and confirmation of the science.

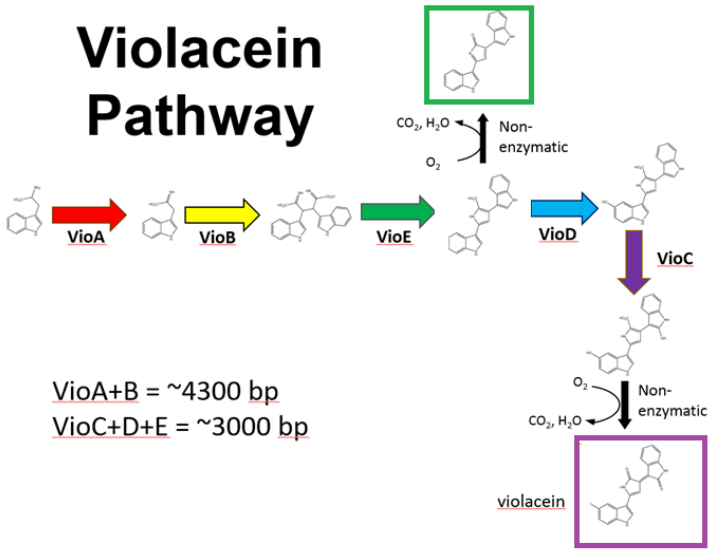

Figure 1: The Violacein pathway
Tryptophan (on the far left) is metabolized by the gene products of the violacein operon which works in the following order: VioA, VioB, VioE, VioD, and VioC. The product prodeoxyviolacein is seen outlined in green and violacein is outlined in purple. The green pigment was the final product in previous years of BCH 406 and the purple pigment is the final product of this years modified lab module. This image is from Dr. Veronica Zepeda.

Previously in BCH 406, Gibson assembly was used to express three genes of a tryptophan metabolite pathway VioA, VioB, and VioE. This resulted in the production of prodeoxyviolacein which creates a green pigment (Hoshino, 2011). To introduce new techniques the module was updated to use Gateway cloning which relies on a pENTR-dTOPO cloning reaction and then a LR recombinase reaction to insert genes of interest into *E.Coli*. This new technique offers students a useful skill allowing them to clone large amounts of DNA into bacteria efficiently. The reason Gateway cloning is so efficient lies in the engineering that has gone into the plasmids. A donor vector has recombination sites that flank the insert. Once the gene is inserted into the donor vector it can be moved to an expression vector. In this method the pENTR-dTOPO reaction moves our DNA of

interest which is either the VioAB or VioCDE genes into the donor vector using a topoisomerase reaction. During the LR-Clonase reaction recombination sites that flank a chloramphenicol resistance marker and a suicide gene, ccdB, on an expression vector will use the Att sites to effectively move the VioAB or VioCDE genes into the expression vector (Sasaki et al., 2004). No empty vectors will grow because of the presence of the suicide gene ccdB, which is a DNA gyrase inhibitor. If the recombination reaction does not occur the ccdB gene will be left in the expression vector and all cells that take up this plasmid will not be able to replicate their DNA. This will make it so only cells with the proper VioAB or VioCDE genes in the expression vector are grown (Miyazaki, 2010).

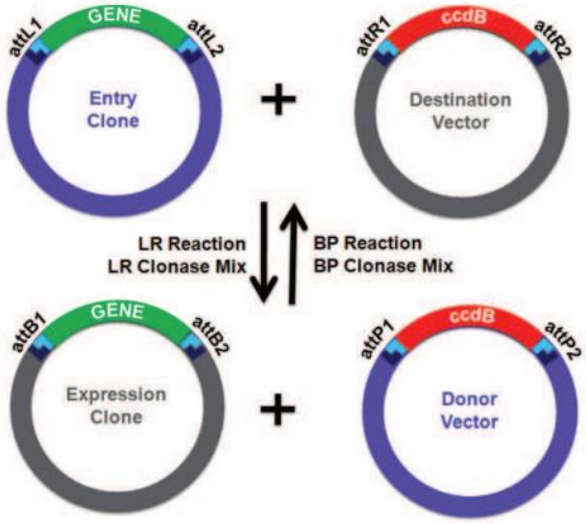

Figure 2: Basic schematic of Gateway Cloning
The entry clone represents pENTR with Vio insert and the Destination vector is pCOLA-2-DEST or pDEST-527. This method effectively uses a recombination reaction and the Att sites to insert the Vio insert into the destination vector effectively creating an expression clone. The donor vector will not only have the wrong antibiotic marker to grow but also contains the ccdB gene. Any empty ccdB Destination vectors will also be killed by the ccdB gene. Image from Fontes et al.

Furthermore, students will be cloning and expressing all 5 violacein genes VioA, B, E, D, and C in *E. Coli*. This will cause the expression of violacein which is a distinct purple color (Lee, Aswani, Han, Tomlin, & Dueber, 2013). This addition to the lab offers a more challenging procedure where 5 genes are now being expressed over 3 in previous years. Student perform better when challenged with complex tasks with a clear end goal (Round et al., 2015). In this case the goal is the expression of a purple pigment. A complex task will often take more time and longer projects simulate what real research is like (Farnham & Dube, 2015). For this reason, the time course of the module is remaining unchanged. The lab will take approximately 4 weeks as it did before which makes this module more closely resemble an actual laboratory research experience then some of the shorter modules in the course. This modification to the expression and cloning lab module introduces students in BCH 406 to exciting new lab techniques and can increase engagement and performance during the class and beyond.

Methods

Genomic DNA extraction of *Chromobacterium violaceum*

5 mL of lysogeny broth (LB) was used to start a cultures of *C. vio*. Cultures were grown for 24 to 48 hours until there was a purple hue. The first genomic DNA (gDNA) extraction protocol used was from the 2015 BCH 406 module. 5mL of *C.vio* culture was spun down and resuspended in 567 µl of buffer Tris-EDTA (TE). Sample was transferred to a microcentrifuge tube and 30 µl of 10% sodium dodecyl sulfate (SDS) and 10 mg/mL of proteinase K were added to the resuspended cells. After an hour incubation at 37ºC 100 µl of 5M NaCl was added and mixed in, and then 80 µl of Cetrimonium bromide (CTAB)/NaCl solution was added and mixed in. A 10 minute incubation at 65ºC was

then performed. An equal volume of chloroform/isoamyl alchohol (24:1) was then added and mixed. The sample was centrifuged for 5 minutes at maximum speed, and the supernatants were moved to a clean microcentrifuge tube. Phenol/chloroform/iroamyl alcohol (25:24:1) was added in equal volume to the sample and mixed. The sample was centrifuged for 5 minutes at maximum speed. Supernatant was moved to a new microcentrifuge tube and 0.6 volumes of cold isopropanol were added. Tubes were thoroughly mixed by inversion, and then samples were centrifuged at maximum speed for 10 minutes. The supernatant was then discarded and 500 µl of cold 70% EtOH was added. The sample was centrifuged for 5 minutes at the maximum speed, and the supernatant was discarded. Residual EtOH was allowed to evaporate off the sample until the pellet was completely dry. The pellet was resuspended in 100 µl of buffer TE. The gDNA was analyzed using a nanodrop spectrophotometer to determine purity and concentration.

Additionally, a DNeasy Blood and Tissue kit from Qiagen was used to extract gDNA and found to be more efficient and timely. For this experiment the gram-negative bacteria protocol in the provided manual was performed. For step 2 the optimal 56°C incubation time was found to be 45 minutes although the protocol suggests 1 to 3 hours (Qiagen, p.29). The sample was vortexed every 5 minutes during the incubation. Upon completeion of the protocol the sample was analyzed for concentration and purity. For both extractions gDNA samples were run on 40 ml 1% agarose gels with 0.75 µl of ethidium bromide until loading dye was at least half way down the gel. Gels were imaged using ImageLab software. A large band seen above the 10 kb mark on the loading ladder confirmed the presence of gDNA.

For the extraction from the 2015 lab protocol an RNase treatment was necessary if there was excessive smearing at lower molecular weight positions on the gel. This treatment was performed by adding 40 µl gDNA sample, 5 µl NEB Buffer 3 (100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT at pH 7.9), 1 µl RNase IF, and 4 µl ddH_2O. The sample was incubated at 37°C for 30 minutes and then incubated at 70°C for 20 minutes. The sample was run on a gel as before and smearing was decreased.

Cloning VioAB and VioCDE into pENTR

VioAB forward and reverse primers as well as VioCDE forward and reverse primers were designed to clone VioAB and VioCDE from genomic DNA of *Chromobacterium violaceum.* The forward primers also have a CACC added on the 5' for the dTOPO reaction with pENTR. PCR tubes were set up for VioAB with the following: 1 µl of genomic *Chromobacterium violaeceum* DNA with a concentration of approximately 75ng/µl, 1 µl of the forward primer, 1 µl of the reverse primer, 0.5 µl of dimethyl sulfoxide, 1 µl of dNTPs, 10 µl of Phusion HF buffer, ddH_2O to get reaction total to 50µl, and 0.5 µl of Phusion polymerase (NEB). Vio CDE was set up as follows: 75ng of genomic *Chromobacterium violaceum* DNA, 1 µl of the forward primer, 1 µl of the reverse primer, 1.0 µl of dimethyl sulfoxide, 1 µl of dNTPs, 10 µl of Phusion HF buffer, ddH_2O to get reaction total to 50 µl , and 0.5 µl of Phusion polymerase. A thermocycler was used to clone these genes with the following conditions for VioAB: 98°C for 30 seconds then a cycle of 30 times 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 135 seconds. Then 72 °C for 300 seconds to a hold at 4 °C. Vio CDE conditions were set up as follows: 98°C for 30 seconds then a cycle of 30 times 98°C for

10 seconds, 55°C for 20 seconds, and 72°C for 90 seconds. Then 72 °C for 300 seconds to a hold at 4 °C.

PCR products were run on 60ml 1% agarose gels with 0.75 µl of ethidium bromide. 6 well coombs were used and loading dye was allowed to run until approximately half way down the gel. Gel was imaged using ImageLab software. In order to obtain only the desired DNA a gel extraction was performed using the QIAquick PCR purification kit. Bands were excised for VioAB at approximately 4328 bp and for VioCDE at approximately 2996 bp. Elution of DNA was done with 30 µl 37ºC buffer EB, 10mM Tris-Cl ph 8.5 (Qiagen). Concentrations of PCR products were determined via a spectrophotometry.

A pENTR directional cloning kit was used to insert the VioAB and the VioCDE genes into their own pENTR vectors (Invitrogen). The reaction time recommended by Invitrogen is 5 minutes, but the reaction was allowed to proceed overnight at room temperature. 50 µl of DH-10-β chemically competent *E.Coli* were then transformed with 5.5 µl of pENTR-VioAB or pENTR-VioCDE. The transformation involved thawing DH-10-β chemically competent *E.Coli* ice and then adding the pENTR-VioAB. The reaction was incubated on ice for 10 minutes. The reaction was then heat shocked at 42 ºC for 30 seconds. The reaction was placed on ice for 2 minutes. 250µl of SOC outgrowth medium was added to the cells (New England Biolabs). The reaction was placed in a 37ºC shaking incubator for 45 minutes. The cells were plated on LB-kanamycin plates and allowed to grow overnight at 37ºC. 8 colonies of VioAB and 4 colonies of VioCDE were selected and liquid cultures were started in LB with kanamycin. Cultures were allowed to grow overnight in 37ºC shaking incubator. Qiaprep spin miniprep protocols were performed to

purify the pENTR VioAB and VioCDE plasmids (Qiagen). Elution was performed in 30 µl of 37 ºC buffer EB buffer, rather than the recommended 50 µl.

Confirmation of pENTR with VioAB and VioCDE inserts

To confirm that VioAB and VioCDE genes were inserted into the pENTR-dTOPO plasmids a restriction digest was performed. The digest for pENTR VioAB was set up for a total of 20 µl as follows: 2 µl of Cutsmart DNA buffer, 0.5 µl of EcoRI, 0.5 µl of AscI, 350 ng of plasmid DNA, and required amount ddH_2O to get reaction to a 20 µl total. The digest for pENTR VioCDE was set up for a total of 20 µl as follows: 2 µl of Cutsmart DNA buffer, 0.5 µl of EcoRV, 0.5 µl of Xmn1, 350 ng of plasmid DNA, and required amount ddH_2O to get reaction to a 20 µl total. Both digest reactions were incubated at 37ºC for 35 minutes. After incubation the digests were run on 40 ml 1% agarose gels with 0.75 µl of ethidium bromide until loading dye was at least half way down the gel. Gels were imaged using ImageLab software. pENTR VioAB bands at 6031 bp and 877 bp confirmed the VioAB insert in the correct orientation. pENTR VioCDE bands at 4660 bp and 916 bp confirmed the VioCDE insert in the correct orientation.

Constructing a Destination Vector with Spectinomycin Resistance

Two approaches to create a new destination vector with Spectinomycin resistance were taken. The first described was successful and the second approach described was unsuccessful. First, pCOLA-2-DEST's (Millipore) kanamycin resistance gene was removed via restriction digest which was setup as follows: 350 ng of pCOLA-2-DEST, 5 µl CutSmart DNA buffer, 1 µl Xmn1, 1µl Nhe1, and ddH_2O to reach a total of 20 µl for the reaction. Reaction was incubated at 37°C for 35 minutes. Digestion products were run on 60ml 1% 0.75 µl ethidium bromide agarose gels with 6 wells till half way across the

gel. The gel was imaged using ImageLab software. In order to obtain only the desired DNA a gel extraction was performed using Qiagen's QIAquick PCR purification kit. Bands were excised at 4026bp and the kanamycin resistance gene was seen at 925 bp. Elution of DNA was done with 30 µl of 37ºC buffer EB, 10mM Tris-Cl pH 8.5 (Qiagen).

The spectinomycin resistance gene with its promoter was obtained by PCR from the pCDF. The PCR was set up as follows: pCDF with a concentration of approximately 75 ng/µl, 1 µl of the forward primer, 1 µl of the reverse primer, 0.5 µl of dimethyl sulfoxide, 1 µl of dNTPs, 10 µl of Phusion HF buffer, ddH_2O to get reaction total to 50 µl, and 0.5 µl of Phusion polymerase. The reaction was run in a thermocycler with the following conditions: 98°C for 30 seconds then a cycle of 30 times 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. Then 72 °C for 300 seconds to a hold at 4 °C. PCR products were run on 60 ml 1% 0.75 µl ethidium bromide agarose gels with 6 wells until the dye front was half way across the gel. In order to obtain only the desired DNA a gel extraction was performed using the QIAquick PCR purification kit. The gel was imaged using ImageLab software. Bands were excised at 791 bp. Elution of DNA was done with 30 µl of 37ºC buffer EB, 10mM Tris-Cl ph 8.5 (Qiagen). To create sticky ends the purified Spectinomycin resistance gene was digested with 25 µl of SpecR, 2 µl of Cutsmart buffer, 2 µl of ddH_2O, 0.5 µl of Xmn1, and 0.5 µl of Nhe1. The digest was incubated at 37°C for 35 minutes. A Qiagen PCR purification kit was used to purify the plasmid. Sample was analyzed to obtain concentration and purity.

A Quick ligation kit and an Instant Sticky-end Ligase kit both from NEB were used to create the pCOLA-2-Dest plasmid with Spectinomycin resistance. In the Instant Sticky-end ligase reaction 4.5 µl of pCOLA-2-DEST digest, 0.5 µl of SpecR, and 5 µl of

master mix were mixed together. The Instant Sticky-end ligase reaction was incubated on ice for 20 minutes. For the Quick ligation kit 4.5 µl of pCOLA-2-DEST digest, 0.5 µl of SpecR, 5.0 µl of ddH_2O, 10 µl of ligation buffer, and 1 µl of T4 ligase were mixed and incubated at room temperature for 20 minutes. 2 separate transformations were then performed for both the Instant Sticky-end ligase reaction and the Quick ligation reactions as follows: 50 µl of DB3.1 chemically competent *E.Coli* with 4 ul of the reaction mixture. The transformation involved thawing DB3.1 on ice and then adding reaction mixture. The reaction was incubated on ice for 10 minutes. The reaction was then heat shocked at 42ºC for 30 seconds. The reaction was placed on ice for 2 minutes. 250µl of SOC outgrowth medium was added to the cells (New England Biolabs). The reaction was then placed in a 37ºC shaking incubator for 45 minutes. The cells were plated on LB chloramphenicol and spectinomycin plates and allowed to grow overnight at 33ºC. Liquid cultures were started of the pCOLA-2-DEST SpecR plasmid and miniprepped using the Qiagen protocol. Elution of DNA was done with 30 µl of 37ºC buffer EB, 10mM Tris-Cl ph 8.5.

The unsuccessful approach to creating a new destination vector was to PCR around pCOLA-2-DEST and exclude the kanamycin resistance gene. Conditions for this PCR are the same as the PCR for VioAB. A gel was set up just as VioAB for band excision. A band at 4315 bp confirmed the presence of the pCOLA-2-DEST with kanamycin removed. The spectinomycin resistance gene without its promoter was obtained from pMDC7 via PCR. The same PCR conditions and protocols were used as obtaining SpecR from pCDF. A band at 791 bp was excised. An Infusion Gibson Assembly kit was used to assemble pCOLA-2-DEST SpecR. 2 µl of enzyme mix, 1 µl of

SpecR, 1 µl of pCOLA-2-DEST backbone and 3 µl of ddH_2O were mixed and incubated at 50ºC. Cells were transformed with 2.5 µl of the Gibson reaction with chemically competent DB3.1 cells in the same way as the alternate pCOLA-2-DEST SpecR assembly above.

To confirm the presence of the pCOLA-2-DEST SpecR for both assembly methods a restriction digest was performed. Just as in the confirmation of pENTR AB and CDE 350 ng of plasmid was used. Instead of Cutsmart buffer, Buffer 3.1 was used, and the enzymes Bgl1 and EcoRV were used instead of the other restriction enzymes. A gel was prepared and run in the same manner as the pENTR Vio AB and CDE confirmations. Bands at 3949 bp and 1158 bp indicated the presence of pCOLA-2-DEST SpecR.

Inserting VioAB and VioCDE into Destination Vectors

VioAB and VioCDE were inserted into the Destination vectors via the Gateway Technologies Kit LR Recombination reaction (Life Technologies). The LR Clonase reaction was set up as follows: 2 µl of ddH_2O, 1.5 µl pENTR VioAB, 1 µl pDEST-527 (Addgene), and 1 µl LR-Clonase enzyme mix. The reaction was allowed to incubate overnight. 50 µl of NEB-5α chemically competent *E.Coli* were transformed with 5.5 µl of LR Clonase reaction mixture. Transformation was performed by incubation on ice for 10 minutes. The reaction was then heat shocked at 42 ºC for 30 seconds. The reaction was placed on ice for 2 minutes. 250 µl of SOC outgrowth medium was added to the cells (New England Biolabs). The reaction was then placed in a 37ºC shaking incubator for 45 minutes. The cells were plated on a LB ampicillin plate and allowed to grow overnight at 33ºC. Three liquid cultures of pDEST-527 VioAB were selected and grown in 5µl of LB

with ampicillin. Qiaprep spin minikit protocols were performed to purify the pDEST-527 VioAB plasmid (Qiagen). Elution was performed in 30µl of 37ºC buffer EB, 10mM Tris-Cl ph 8.5 buffer rather than the recommended 50µl.

The same procedure as above was used for the LR clonase reaction of pENTR VioCDE and pCOLA-2-DEST SpecR and another LR clonase reaction of pENTR VioCDE and pDEST-527. The only difference was the transformation with pCOLA-2-DEST SpecR was plated on LB containing spectinomycin rather than ampicillin.

Double Transformation of AB and CDE into *E.Coli*

Approximately 100 ng of each the pCOLA-2-DEST VioCDE with SpecR and pDEST 527 VioAB were added to 50 µl of NEB 5α cells. Transformation was performed by incubation on ice for 10 minutes. The reaction was then heat shocked at 42ºC for 30 seconds. The reaction was placed on ice for 2 minutes. 250 µl of SOC outgrowth medium was added to the cells (New England Biolabs). The reaction was then placed in a 37ºC shaking incubator for 45 minutes. The cells were plated on a LB ampicillin and spectinomycin plates and allowed to grow overnight at 33ºC. Additionally, another double transformation was performed in the same manner with pDEST 527 VioCDE and pCDF. Cultures were started in 5 mL of LB ampicillin and spectinomycin. 48 hours in a 37ºC shaking incubator was required to observe a purple pigment.

Table 1: Primers for PCR and for sequencing
These forward and reverse primers for PCR were created using A Plasmid Editor program (ApE). Primers were designed to have a minimum melting point of 50 °C. Sequencing primers were designed with the same criteria and are labeled seq.

Primer 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
AttR1 (+pCOLA) Forward seq	CGTCACAAGTTTGTACAAAAAA
AttR1 (+pCOLA) Reverse seq	TTTTTTGTACAAACTTGTGACG
ColA ori (+pCOLA) Forward seq	GGGTTATTGTCTCATGAGCG
Att R2 (+pCOLA) Reverse seq	TGGCAGCAGCCTAGGTTAAT
SpecR (+pCOLA) Forward seq	GAACCCCTATTTGTTTATTTTTC
SpecR (+pCOLA) Reverse seq	CGCTCATGAGACAATAACCC
pENTR Seq Reverse seq	ATGGCTCATAACACCCCTTG
pDEST Seq Forward 01 seq	CTAGAACTAGTGGATCCCCCATC

Results

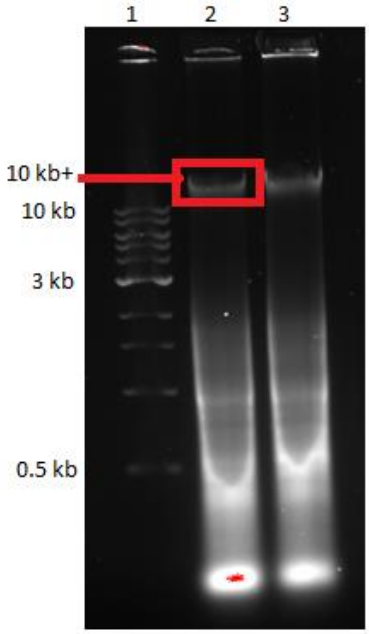

Figure 3: *Chromobacterium violaceum* gDNA Extraction 406 protocol
A 1% agarose gel with 3.5 µl 1kb ladder in lane 1. 5 µl of 2 separate gDNA samples were loaded into lanes 2 and 3 with 1 µl of loading dye in each. The band seen above 10 kb indicates gDNA and is seen in lanes 2 and 3. This gel was run on Janruary 25, 2016.

Table 2: *Chromobacterium violaceum* gDNA Extraction 406 protocol Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on January 1, 2016.

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

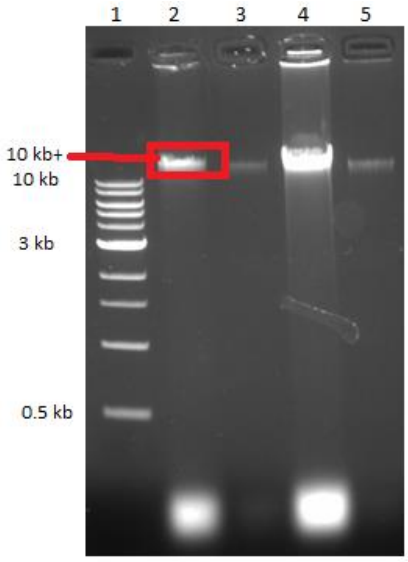

Figure 4: *Chromobacterium violaceum* gDNA Extraction 406 protocol RNase treated
The same gDNA samples from Figure 1 treated with RNase. A 1% agarose gel with 3.5 µl 1kb ladder in lane 1 was run. Lane 2 contained 5 µl of gDNA sample 1 with 1 µl of loading dye. Lane 3 contained a 10x dilution of sample 1 loading in the same manner. Lanes 4-5 were the same as lane 2-3 except with gDNA sample 2. The band highlighted in red is gDNA. gDNA was seen in lanes 2-4. This gel was run on February 2, 2016.

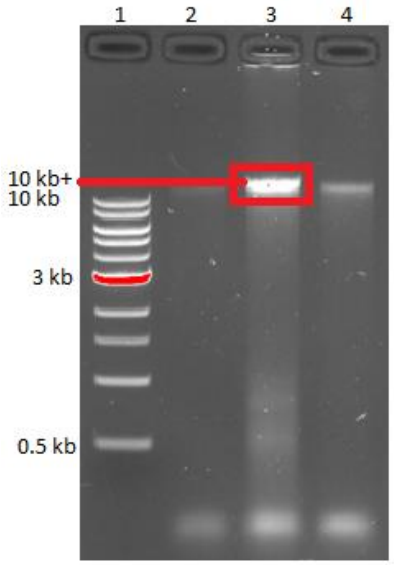

Figure 5: *Chromobacterium violaceum* gDNA Extraction DNeasy
A 1% agarose gel with 3.5 µl 1kb ladder in lane 1 was run. Lane 2 contained 5 µl of gDNA sample 2 with 1 µl of loading dye. Lane 3 contained gDNA sample 3 and lane 4 contained gDNA sample 4. Lane 3 and 4 both had the same amounts of gDNA and dye as lane 2. The band highlighted in red is gDNA and is seen in lanes 3 and 4. This gel was run on February 26, 2016.

Table 4: *Chromobacterium violaceum* gDNA Extraction DNeasy Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained 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

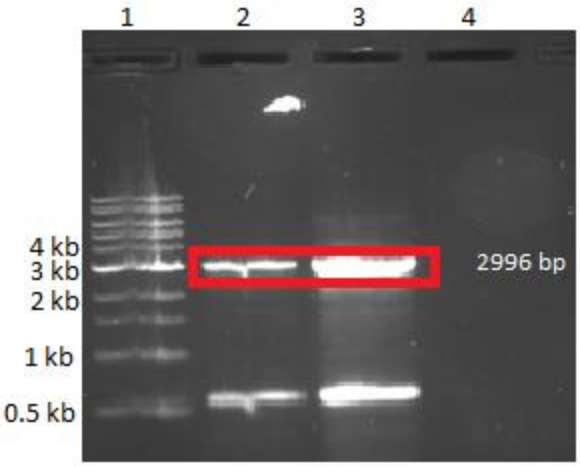

Figure 6: VioCDE PCR extraction agarose gel
A 1% agarose gel was loaded with 9 µl of 1 kb ladder in lane 1. Lane 2 and 3 both had 4 the same sample of 50 µl split between them with 10 µl of loading ladder. The band highlighted in red is VioCDE at 2996 bp. This gel was run on September 4, 2015.

Table 5: VioCDE PCR product purification Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on September 9. 2015 and January 12, 2016.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
Vio CDE (9/9)	34.4	1.74	0.10
Vio CDE (1/12)	42.6	2.06	0.07

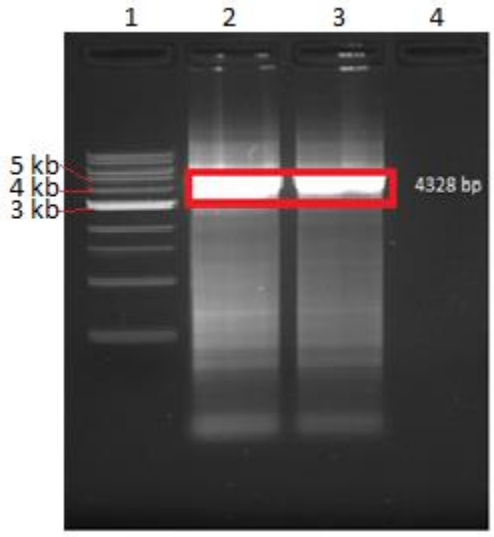

Figure 7: VioAB PCR extraction gel
A 1% agarose gel was loaded with 9 µl of 1 kb ladder. Lane 2 and 3 both contain 50 µl of 2 VioAB PCR reactions with 10 µl of loading dye. The band highlighted in red at 4328 bp is VioAB. This gel was run on September 9, 2015.

Table 6: VioAB PCR product extraction Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB (Qiagen). 2 µl of sample were used. Concentrations were obtained 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

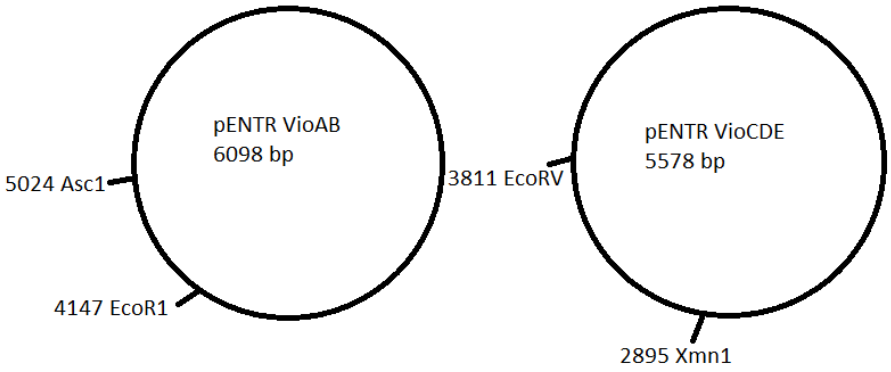

Figure 8: Plasmid maps for Confirmation of pENTR VioAB and VioCDE inserts
Restriction enzymes are shown to show location of cuts in the plasmid which were used to determine the desired band sizes for insert confirmation as discussed in the results below.

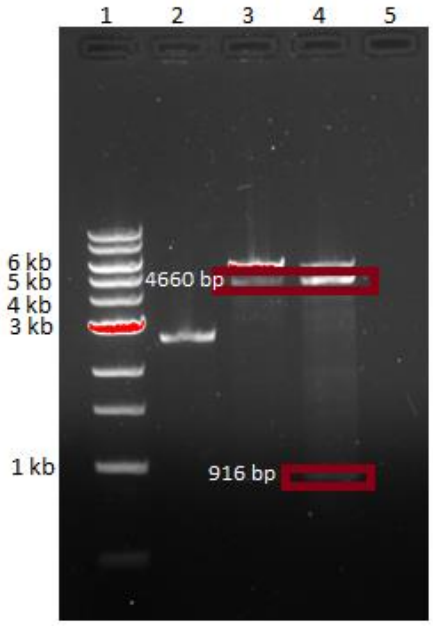

Figure 9: pENTR VioCDE Restriction Digest
A 1% agarose gel with 3.5 µl 1kb ladder in lane 1 was run. Lane 2 contained pENTR VioCDE digest 1. Lane 3 contained pENTR VioCDE digest 3 and lane 4 contained pENTR VioCDE digest 4. Lanes 2-4 were each loaded with 5 µl of digest and 1 µl of loading dye. Both lanes 3 and 4 had VioCDE inserted into the pENTR in the correct orientation as indicated by the gel bands highlighted in red. This gel was run on March 7, 2016.

Table 7: VioCDE pENTR Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained 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

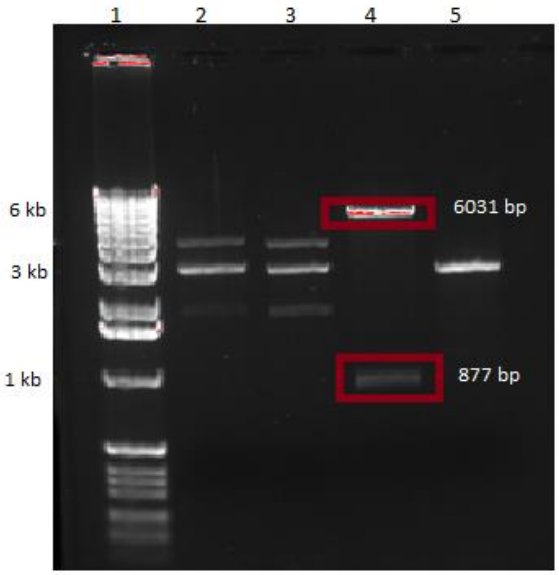

Figure 10: pENTR VioAB Restriction Digest
A 1% agarose gel with 3.5 µl 1kb ladder in lane 1 was run. Lane 2 contained pENTR VioAB digest B1. Lane 3 contained pENTR VioAB digest C1. Lane 4 contained pENTR VioAB digest C3. Lane 5 contained pENTR VioAB digest C4. Lanes 2-5 were each loaded with 5 µl of digest and 1 µl of loading dye. Lane 4 had VioAB inserted into the pENTR in the correct orientation as indicated by the gel bands highlighted in red. This gel was run on October 12, 2015.

Table 8: VioAB pENTR Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained 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

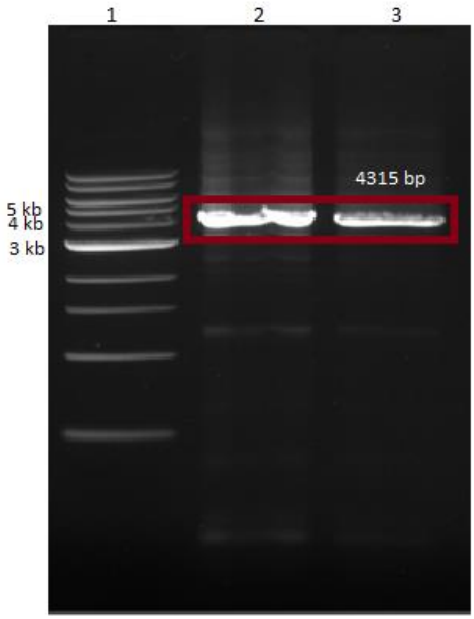

Figure 11: pCOLA backbone PCR Extraction Gel
A 1% agarose gel was loaded with 9 µl of 1 kb ladder. Lane 2 and 3 contain pCOLA backbone PCR samples from 2 separate PCR reactions. Both lanes contain 50 µl of sample and 10 µl of loading dye. The band highlighted in red is the pCOLA backbone. This gel was run on November 18, 2015.

Table 9: pCOLA PCR product purification Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on November 3, 2015 and November 18, 2015.

Sample	[DNA] ng/µl	$A_{260/280}$	$A_{260/230}$
pCOLA Backbone (11/3)	57.9	1.91	0.11
pCOLA Backbone (11/18)	22.7	1.90	0.16

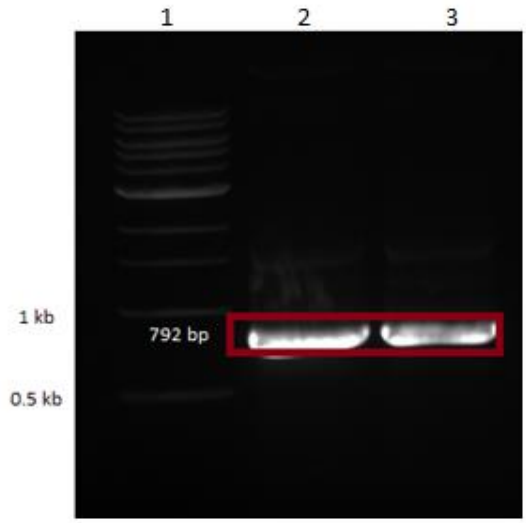

Figure 12: SpecR gene PCR from pMDC7 Extraction Gel
A 1% agarose gel was loaded with 9 µl of 1 kb ladder. Lane 2 and 3 both contained 2 PCR reaction samples of Spectinomycin resistance gene. Lane 2 and 3 were both loaded with 50 µl of sample and 10 µl of loading dye. The band highlighted in red is the SpecR gene. This gel was run on November 18, 2015.

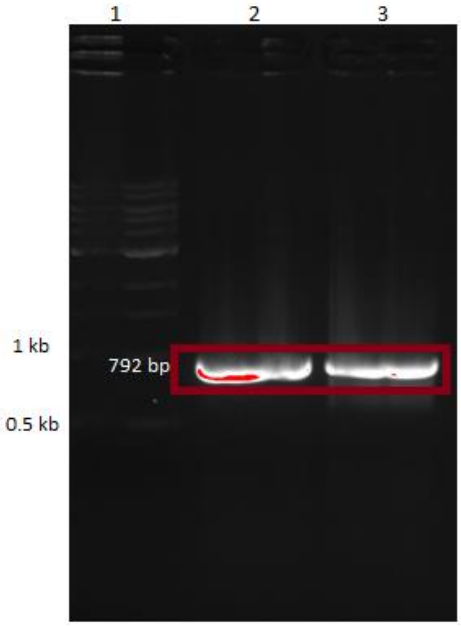

Figure 13: SpecR gene PCR from pCDF Extraction Gel
A 1% agarose gel was loaded with 9 µl of 1 kb ladder. Lane 2 and 3 both contained 2 PCR reaction samples of Spectinomycin resistance gene. Lane 2 and 3 were both loaded with 50 µl of sample and 10 µl of loading dye. The band highlighted in red is the SpecR gene. This gel was run on February 11, 2016.

Table 10: SpecR gene PCR product purification Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on November 3, 2015 and 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

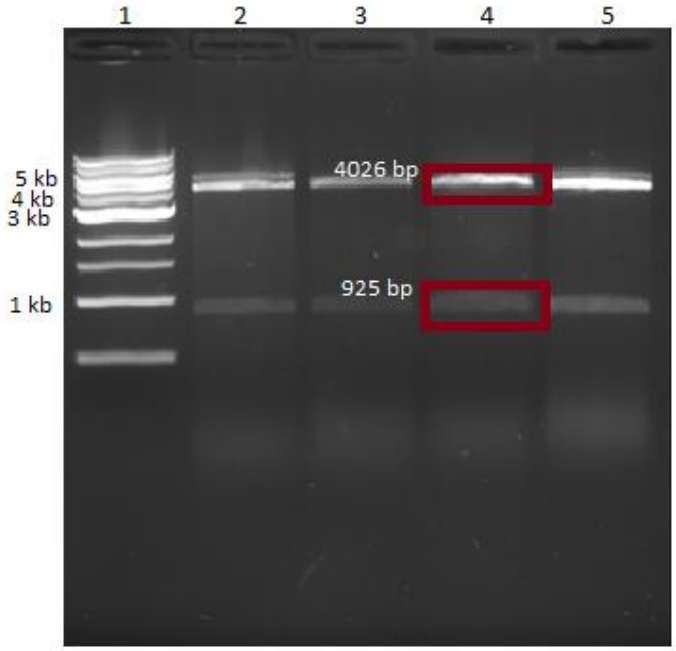

Figure 14: pCOLA Backbone restriction digest gel for extraction
A 1% agarose gel containing 9 µl of 1 kb loading ladder in lane 1 was run. Lanes 2-5 contained pCOLA digest 1-4 respectively. Lanes 2-4 each have 50 µl of digest sample with 10 µl of loading dye. The band at 4026 bp is pCOLA backbone and band at 925 bp is the kanamycin resistance gene. Lanes 2-5 show this. This gel was run on February 3, 2016.

Table 11: pCOLA Digest and extraction Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on February 4, 2016.

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

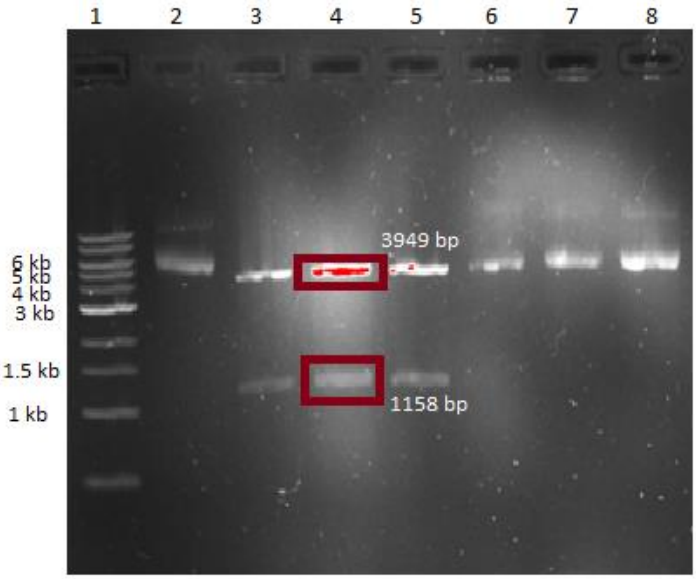

Figure 15: pCOLA Spectinomycin restriction digest
A 1 % agarose gel was loaded with 3.5 µl of loading ladder in lane 1 and run. Lane 2-9 contain pCOLA SpecR digest 1-7 respectively. Lane 2-9 were loading with 5 µl of sample and 1 µl of loading dye. The highlighted bands indicated pCOLA SpecR digested properly as seen in lane 4. This gel was run on February 29, 2016.

Table 12: pCOLA-Spec Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on February 2, 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

Table 13: pDEST 527-AB Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained on March 9, 2015.

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 14: pDEST 527 VioCDE
Data was recorded on Nanodrop 2000 which was blanked with buffer EB. 2 µl of sample were used. Concentrations were obtained 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 15: pCOLA-Spec CDE Nanodrop
Data was recorded on Nanodrop 2000 which was blanked with biffer EB. 2 µl of sample were used. Concentrations were obtained on March 4, 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

Genomic DNA extraction of *Chromobacterium violaceum*

Figure 3 is a *Chromobacterium violaceum (C.vio)* gDNA extraction that was performed using the protocol from the spring 2015 semester. A large band is indicative of genomic DNA (gDNA). The band in lanes 2 and 3 above 10 kb in this figure show a successful extraction was performed. The smearing is indicative of RNA contamination. Table 2 shows the concentrations of the gDNA obtained. This DNA was being used for the VioAB and VioCDE PCR reactions in which only 75 ng was necessary so this was an adequate amount of DNA. For nanodrop tables 260/280 ratios from around 1.8-2.0 indicated purity and 260/230 values of 2.0 and above indicate purity. These numbers are only relative and unless values are drastically far from these numbers the DNA was used. This gDNA had adequate purity to move forward. . To deal with RNA contamination an RNase treatment was performed seen in Figure 4 which shows the gDNA samples from Figure 3. Unsuccessful PCR of VioAB and VioCDE with the original untreated gDNA

showed that the RNA was interfering which meant this treatment was necessary to proceed. Compared to Figure 3 there is little to no smearing in the samples in lanes 2-5. This indicates that the RNA was successfully removed and the gDNA purified. As this protocol was time consuming an alternative approach was taken to the gDNA extraction. The Qiagen DNeasy Blood and Tissue kit was used for the extraction seen in Figure 5. As in figure 3 and 4 gDNA was successfully obtained which seen in the bands above 10 kb in lanes 2-5. There was no need to RNase treat this sample as it worked for the VioAB and CDE gene PCRs. The concentrations of gDNA samples are in Table 4. While not as concentrated as the original extraction protocol there was enough sample for 75 ng of gDNA in the VioAB and VioCDE PCR reactions. Some sort of phenol contamination was likely as seen in the low 260/230 ratio. This contamination did not interfere with PCR reactions.

Cloning VioAB and VioCDE into pENTR

Figure 6 is the VioCDE PCR and the band at 2996 bp is the VioCDE in lanes 2 and 3. An unknown band appeared at around 0.5 kb in lanes 2 and 3 which is likely due to the primers for the PCR annealing to an alternate site in the gDNA of *C.vio.* This did not interfere with the protocol as the VioCDE band was far enough away to extract without contamination. After extraction and purification, the concentrations of the VioCDE samples were obtained as seen in Table 5. These were adequate amounts of VioCDE as 4 µl of the insert DNA was needed and these concentrations would still give well over 100 ng for the pENTR reaction. Low 260/230 ratios are often seen in gel extractions due to contamination from the guanidine in Buffer QG that is part of the QIAquick Gel Extraction kit from Qiagen. Figure 7 is the VioAB PCR and the band at

4328 bp is the VioAB gene from *C.vio.* Table 6 shows the concentrations and purity of VioAB after the gel extraction. Low 260/230 values were seen, but as explained above the low 260/230 ratios were expected in the QIAquick gel extraction.

Confirmation of pENTR with VioAB and VioCDE inserts

Once VioCDE was cloned into pENTR it was important to determine whether or not it was inserted in the correct orientation. A restriction digest was setup using with an enzyme cut site that was located asymmetrically inside the VioCDE insert. This allows for a digestion in which the size of the bands can be predicted when run on a gel. Figure 8 is a plasmid map showing the overall size of the pENTR with the inserts and where the cut sites were located. This information was what allowed for the prediction of band sizes after digestion. Figure 9 is the gel of the VioCDE digest. Lanes 3 and 4 show a proper digestion which means that VioCDE was inserted into pENTR in the correct orientation. The small band above the band at 4660 bp in lanes 3 and 4 is due to a partial digestion which could have been avoided with a longer incubation of the digestion or new digestion enzymes. Lane 2 has a band around 2800 bp which indicates that the VioCDE insert went into the pENTR backwards. A band at a little over 3000 bp would indicated an empty pENTR with no VioCDE insert. Table 7 shows the concentrations and purity of the pENTR VioCDE. These were adequate amounts of DNA to perform the destination reaction as not a lot of DNA was needed; the purities of all 3 samples were acceptable to continue as well. Furthermore, Figure 10 is the pENTR VioAB digest reaction gel that was run to confirm the presence of VioAB in pENTR in the correct orientation. Just as with the pENTR VioCDE this digestion reaction was setup in a way that predictable band fragments would indicate that VioAB was inserted into pENTR correctly. Lane 4 shows

the digestion with the VioAB inserted into pENTR correctly. Lane 5 has a band at a little over 3000 bp which indicates a linear undigested pENTR. VioAB inserted into pENTR backwards would be seen in bands at approximately 5000 bp and 2000 bp. This is seen in lanes 2 and 3 which are partially digested backwards pENTR VioAB Table 8 shows the concentrations and purities for the pENTR VioAB after a miniprep. A high 260/280 in pENTR-VioAB B1 was unusual so it was not used. While high values of not usually indicative of contamination the other samples had more regular values making them better candidates to continue with. Additionally, a low concentration of DNA and a negative 260/280 value indicated that pENTR-VioAB B2 was unusable due to contamination.

Constructing a Destination Vector with Spectinomycin Resistance

In order to be able to select for 2 destination plasmids in *E.coli* destination vectors with 2 different antibiotic markers were needed. Additionally, these markers had to be different from the antibiotic resistance that the pENTR contained so selection could occur after the LR clonase reaction. pENTR has an KanR gene, pDEST-527 has AmpR gene, and pCOLA-2-DEST had a KanR gene. pCOLA-2-DEST was chosen to remove the KanR gene. Figure 11 is the pCOLA-2-DEST backbone. Backbone means that the KanR gene has been removed by cloning around it with a PCR reaction. A band at 4315 bp shows the backbone in lanes 2 and 3. If the KanR was not removed a band at around 5100 bp would have been seen. Just like VioAB and VioCDE the pCOLA backbone was gel extracted and concentrations and purities can be seen on Table 9. The extraction from 11/3 provided a better concentration for pCOLA backbone to be ligated with SpecR. The higher concentrated extract was always used for the Infusion Gibson reaction as it was

more likely the reaction would work with these samples. Figure 12 shows the PCR of the SpecR gene out of pMDC7. Its presence was seen in the band at 792 bp in lanes 2 and 3. Table 10 shows the concentration and purity of the SpecR in Figure 12 after a QIAquick gel extraction. Plenty of DNA was obtained to perform the Infusion Gibson reaction which required about 50 ng of DNA. Small colonies would grow after a transformation into chemically competent *E.Coli,* These colonies were theoretically pCOLA-2-DEST with SpecR as they were growing on spectinomycin plates; however, a restriction digest always showed the KanR gene still inserted into the plasmid. Another approach needed to be taken to create this second destination vector.

Continuing, in the second approach to create pCOLA-2-DEST SpecR the KanR was digested out of pCOLA-2-DEST while the SpecR was still obtained by PCR except from pCDF rather than pMDC7. Figure 13 is the SpecR gene obtained from the plasmid pCDF via a PCR reaction. The SpecR is seen at the band at 792 bp in lanes 2 and 3. Table 10 is the concentration and purity of the SpecR after an extraction. As always in a QIAquick gel extraction a low 260/230 was seen. Adequate amounts of DNA were obtained for the Quick ligation and Instant Sticky end ligation reactions. Rather than by PCR, the KanR was removed from pCOLA-2-DEST via restriction digest. This digest was run on a gel seen in figure 14 to purify the backbone from the KanR gene. The Band at 4026 bp was the pCOLA backbone and the band at 925 bp was the KanR gene. These bands can be seen in lanes 2-5 indicating successful ligation reactions. Lanes 2 and 3 were extracted as the 4026 bp band had the least amount of smearing nearby. Smearing likely indicated incomplete digestion. Table 11 is the concentrations and purity of the pCOLA backbone after the QIAquick gel extraction. pCOLA-2-DEST Backbone 1 did

not have enough DNA to perform ligation reaction so it was not used. Low 260/230 were expected and seen as with any gel extraction. Both the Instant sticky end ligase reaction and the quick ligation protocols yielded colonies on plates after transformation and incubation. While both grew only one was needed so, the quick ligation was chosen to be digested. This digest in Figure 15 shows whether or not the SpecR was successfully ligated into the pCOLA backbone. Bands at 3949 bp and 1158 bp indicated that the pCOLA SpecR plasmids was successfully assembled. This was seen in lanes 3-5. Table 12 is from a miniprep of the colonies that digested correctly in figure 15. This table shows that adequate amounts of DNA were obtained to perform an LR clonase reaction. While high values for 260/280 ratio are not normal pCOLA-Spec1 and 2 from table 12 were not selected to be used for the LR clonase reactions.

Inserting VioAB and VioCDE into Destination Vectors

The LR clonase reaction of pDEST-527 and pENTR VioAB yielded the plasmids pDEST-527 AB that grew on LB Ampicillin after inoculation. Cultures were started and miniprep results are seen in Table 13. pDEST-527 AB 1-4 all yielded significant amounts of DNA upwards to 100 ng/ µl which is a high enough concentration for the subsequent double transformation into *E.Coli*. The 260/280 and 260/230 values did not indicate any major impurities. The LR clonase reaction of pCOLA-2-DEST Spec with pENTR VioCDE was plated on LB spectinomycin after transformation. Only about 8 colonies grew and all were very small. Cultures of this were allowed to grow 48 hours to try and maximize the concentration. Table 15 shows the miniprep of this plasmid. It was common to see low concentrations of DNA in this plasmid such as pCOLA-Spec-VioCDE 1 at 3.3 ng/µl in Table 15. Concentrations of this plasmids were usually at the

highest around 30 ng/µl like pCOLA-SpecVio CDE 3 and this plasmid was better for the double transformation reaction in which more concentrated DNA was better.

Double Transformation of AB and CDE into *E.Coli*

Double transformations of pCOLA Spec VioCDE and pDEST-527 VioAB did not yield cultures when plated on LB Spectinomycin and ampicillin. pENTR VioCDE and pDEST-527 underwent a LR Clonase reaction to yield pDEST-527 VioCDE. Table 14 shows the minprep of this plasmid. 260/280 and 260/230 values all indicate few impurities in the DNA and concentrations were adequate to perform a double transformation reaction. A second plasmid pCDF from the 2011 UCSF IGEM team contained VioABE. When a double transformation was performed with pDEST-527 VioCDE and pCDF and these colonies were plated on LB spectinomycin and ampicillin cultures grew. After about 48 hours the cultures would look purple. Additionally, when liquid cultures were started a cloudy purple pigment was observed after 48 hours. The purple pigment was indicative that the violacein operon was properly expressed in *E.Coli* and the violacein product was expressed.

Discussion

The overall goal of this project was to create a modern replicable lab module that BCH 406 students could perform in the classroom. Students were able to create purple *E.coli* by expressing the violacein operon during April 2016. The objective of the lab was roughly met. . The main complications of this project were cloning of the VioAB gene and creating a second destination vector from pCOLA-2-DEST.

Complications

VioAB was not easily amplified by PCR out of the gDNA of *C.vio.* This is likely due to the large size of the gene which is 4328 bp or could be because the primers for the PCR were not annealing strongly. Originally, students in the BCH 406 class were supposed to clone both the VioAB and VioCDE pieces of the violacein operon. The reason to break it into two pieces is because of the large size of the whole operon being 7324 bp which makes PCR reactions difficult. With difficulties cloning VioAB the initial plan of cloning both VioAB and VioCDE was altered, and students would focus on cloning only VioCDE, which had a high PCR success rate. pDEST VioAB would be given to the students when the double transformation with pCOLA-2-DEST SpecR with VioCDE was ready to be performed.

The next challenge was the creation of the destination vector with an alternative antibiotic marker in order to get selection for both the VioAB and VioCDE inserts. pCOLA-2-DEST contains kanamycin resistance which is different from that of pDEST-527 which contains ampicillin resistance. The issue is the pENTR contains kanamycin resistance as well which means during the LR clonase reaction of pENTR VioCDE and pCOLA-2-DEST there would be no way to select for only the *E.coli* with properly recombined pCOLA-2-DEST VioCDE. pCOLA-2-DEST had to have the kanamycin resistance gene removed and a spectinomycin resistance gene added in its place. The first approach was to PCR around the kanamycin resistance gene of pCOLA-2-DEST and PCR out the spectinomycin gene of pMDC7. Then the pieces of DNA could be ligated together via a Gibson Assembly reaction. This approach did not work. An alternate approach of digesting around the kanamycin gene of pCOLA-2-DEST and then ligating the spectinomycin resistance gene in was more successful. The pCOLA-2-DEST SpecR

plasmid never grew as well as other plasmids though. Plasmid purifications of this were often around 10 ng/µl. During attempts to double transform pDEST VioCDE with pCOLA-2-DEST SpecR VioAB colonies never grew. This is likely due to the low concentration of the pCOLA-2-DEST plasmid. It is possible that when the pCOLA-2-DEST was digested and ligated with SpecR, off target areas of the pCOLA-2-DEST were effected. These unintended effects likely rendered the plasmid nonfunctional. Due to these complications the pCOLA-2-DEST SpecR was abandoned for use in the BCH 406 class. In the final form of the module that the students performed, they were given the plasmid pCDF which contained VioABE inserted by the 2011 UCSF IGEM team. The students then created the pDEST VioCDE plasmid which they double transformed with the pCDF to express the violacein operon and get a purple pigment.

Future Directions

In the future, the creation of a destination vector with spectinomycin gene would not only be useful to the BCH 406 class but to any research lab that was attempting to clone multiple genes into bacteria. It would offer a method to select for two different destination vectors. However, there is currently a way to clone multiple genes with a modified destination vector. Takefumi Sone et al. created a destination vector that allows for 3-4 genes to be inserted in parallel into a single destination vector for expression (Sone et al., 2008). This approach could be used by the BCH 406 class in the future to insert and express the 2 pieces VioAB and VioCDE in *E.Coli*. A different approach could also be not breaking the VioAB and VioCDE gene into 2 pieces. This could be achieved via a long range PCR reaction. KAPA Long Range HotStart DNA Polymerase is used when amplicons exceed 5 kb (Chua, Miller, & Kennedy, 2015). This makes the 7.3 kb

VioABCDE a perfect candidate for this approach. Only 1 destination vector would be needed with one gene segment.

Additionally, new lab modules with new technologies such as CRISPRi could be introduced to the class in the future. The same violacein pathway could be used in the addition of a new lab that introduces CRISPRi technology. CRISPR stands for clustered regularly interspaced short palindromic repeats, and it is a bacterial immune system that relies on guide RNA to destroy foreign DNA. CRISPR takes advantage of this system for genome editing including gene activation and repression. This time the violacein pathway would be expressed in *S. Cerevisiae* and both green and purple pigments will be created by manipulating the violacein pathway with a dCAS protein, a guide RNA and a gene activator CRISPRi is the latest technology that has massive potential in the field of gene editing and possibly gene therapy as it is much more accurate and reliable then other techniques such as transcription activator like receptors (TAL) and Zinc Fingers (Zalatan et al., 2015).

Conclusion

The cloning lab modification was successful as the students were able to use Gateway cloning to produce the violacein pigment from *C.vio* in *E.coli*. While complications arose with the pCOLA-2-DEST, the pCDF which contained VioABE was a good alternate for these genes. Continuing to modify the BCH 406 lab course is important for keeping the class up to date with modern techniques. The introduction of the modern Gateway cloning technique aides in students understanding techniques used in research labs. This makes students better candidates for any work beyond an

undergraduate education such as entering the biotechnology industry or graduate school research.

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