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Molecular Cloning of Selected Regions of Catalytic Domain of *CtsK* Gene in *Escherichia coli* – A Feasibility Study

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NDS, RC, PDSUW and HM conceptualized the study. Authors RC and HM involved in conducting the wet laboratory work. Authors NDS, HM, RC, PDSUW and LW analyzed the results. Author HM primarily involved in writing the manuscript. Authors NDS, RC and PDSUW conducted overall supervision of the entire research project. All authors read and approved the final manuscript.

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ABSTRACT

Cathepsin K (CatK), encoded by *CtsK* gene in human, is involved in bone remodeling through ossification. The objective of the work conducted here was to express catalytic domains of *CtsK* gene in bacterial expression system as an initial step, facilitating recombinant production of human CatK for downstream applications in pharmacology. Four healthy human blood samples were collected. Genomic DNA was extracted using FlexiGene® whole blood DNA extraction kit. Upon quantification of DNA through Nanodrop™ spectrophotometer, sufficient quantity and quality was observed. *CtsK* gene was amplified by Polymerase Chain Reaction (PCR) using two pairs of

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primers tagged with restriction endonuclease sites of *Sal1* and *HindIII* facilitating molecular cloning and visualized by Agarose Gel Electrophoresis (AGE). Two different bands of size 545bp and 265bp were observed. The bands were dissected and gel purified using Genaxxon™ gel purification kit and sequentially double digested by restriction enzymes; *SalI* and *HindIII*. Vector *PBS* was also subjected to sequential double digestion using same enzymes and visualized via AGE. Double digested insert of size 265bp and vector were ligated using T_4 DNA Ligase (all enzymes from Promega™). On another trail, ligation of the PCR product with band size 265bp to pGEM-T™ easy vector system (from Promega™) was also done and transformed to *Top10 Escherichia coli* competent cells for expression separately. Cells were grown in LB media in presence of XGAL, IPTG and Ampicillin and transformed cells were screened. In the restriction enzyme digestion and ligation setup, since the insert and vector were both double digested, it is confirmed that white colonies obtained were *Escherichia coli* cells were transformed with the desired recombinant vector and is therefore confirmatory. In the case of pGEM-T™ ligation, a colony PCR was done using the white colonies obtained and product size was confirmed via AGE. In conclusion, the objective of study was successfully achieved, by expressing a catalytic domain of *CtsK*. Developments and improvements could be made for expression of entire CatK gene and downstream production of the Cathepsin K protein for effective therapeutic purpose.

Keywords: Cathepsin K; CtsK; human; Escherichia coli; expression.

1. INTRODUCTION

Sri Lanka, been a developing country has shown a wide recruitment of a wide variety of knowledge, techniques and protocols associated with molecular biology and genetics in the past few decades especially in the diagnostics. However, it is evident that the majority of these technical recruitments have again, only been limited to diagnostics and not efficiently applied in local research and development. Molecular cloning of genes producing commercially important proteins and their recombinant production required for disease treatment for the healthy well-being of mankind is one such limitation that could be identified through comprehensive analysis of the local literature [1]; that is of deem necessity to attend. By been able to apply such techniques, thereby, developing molecular biology related research infrastructure, the need to import protein products such as pharmaceutical drugs at high cost could be avoided, which in turn would contribute to an improvement to the National economy.

The objective of this research was to conduct a feasibility study to investigate the possibility of molecular cloning and *in vitro* expression of selected catalytic domains of the gene responsible for the production of the human enzyme Cathepsin K, which is the *CtsK* gene, in the *Escherichia coli* bacterial expression system. Eventually the study shall be developed to cloning and expression of the entire *CtsK* gene, thereby facilitating Cathepsin K protein overexpression in the country. This way, an initial platform and a reliable workstation for human protein overexpression is thought to be established in the country for the first time in its history of research and development in molecular biology and human genetics.

Proteases are a group of proteinaceous enzymes that are involved in catalyzing the hydrolysis of amide bonds in bio molecules. Unlike the reversible changes such as phosphorylation and allosteric site changes that proteins undergo, proteolysis in general is considered to be an irreversible change [2]. Cysteine Proteases are a group of proteases that involve in typical metabolism including protein degradation and turnover, bone remodeling and pre-hormone processing [3]. Apart from the structural analysis, a variety of functional analysis of these proteinases have revealed, that in addition to their intracellular role in the process of recycling of proteins, they are also involved in other most usually occurring processes such as antigen presentation, bone remodeling, and prohormone activation [4]. Most remarkably, cysteine proteinases are involved and are suggested to involve in a variety of disease processes such as pulmonary emphysema, osteoporosis, alzheimer's disease, rheumatoid arthritis, and in cancer invasion and metastasis [5]. Cathepsin K (Cat K) is a member of the CA1 family of lysosomal cysteine proteases. This family comprises 11 human members and they are Cathepsins B, C, F, H, K, L, O, S, V, W, X which share a common papain-like structural fold and a conserved active site (Godat et al. 2004). All the enzymes of this class are synthesized in the form

of preproenzymes and are converted from the catalytically inactive zymogen to the active form in an acidic environment, in low pH conditions as found in the late endosome or lysosome. Until recent times cysteine Cathepsins were thought to exclusively function as nonspecific proteases in the lysosome. However, research has shown that Cathepsins are also secreted in the active form to acidic extracellular compartments in order to carry on the same function [6].

Cathepsin K (Cat K) is a human enzyme found in the osteoclasts of the human body. Osteoblasts are involved in the deposition of new bone tissue with along with a strong matrix of proteins in a process called the Ossification (Bossard et al. 1996). Osteoclasts are the cells in the body that is responsible for bone resorption. The extracellular matrix of the bone degrades during development along with the proteinaceous matrix and the calcium that make up the bone tissue. The degraded material is released to the blood stream and this process is completely regulated and controlled by homeostasis until they are recycled or removed from the body. This particular degrading process is catalyzed by the enzyme Cathepsin K. The degraded bone tissue is being replaced by Osteoblasts. This entire process of bone resorption and deposition is called “bone remodeling”. This is an important metabolic process that takes place in the bones or rather the skeletal system of the human body and takes place throughout the life time. This is the key activity in healing of fractured bones as well. This clearly illustrates the importance of the enzyme of study.

Cathepsin K (CatK) is also called cathepsin O, CTS02, CTSO, CTSO1, CTSO2, PKND, PYCD, Cathepsin K, encoded by the *CtsK* gene, (Galson and Roodman 2011) being highly responsible for cleaving and removing the organic matrix of the bone especially the type 1 collagen. However, CatK exhibits protease activity of both type I and type II collagenase activity that is predominantly expressed by osteoclasts. As far as the protease activity with regard to type 1 collagenase is concerned, it is secreted into the resorption lacunae below active osteoclasts resulting in type I collagen degradation. The major biochemical functions of this enzyme are collagen binding [4] cysteine- type endopeptidase activity [7], cysteine type peptidase, fibronectin binding (Stroup et al. 2001), proteoglycan binding and serine type binding [8].

On a global perspective, even though the protein sequence, gene sequence and the 3-dimensional structure of Cathepsin K has been completely studied and understood and is available in global databases such as the NCBI and UNIPROT, there is no published literature indicating molecular cloning and recombinant production of human CtsK to date. Despite having sound understanding on the therapeutic role that recombinant Cathepsin K could play in human health, this study could be considered as a feasibility study that would eventually fill in this research gap.

The immediate reason to improve and develop the national workstation for human protein recombinant production through Cathepsin K is that Sri Lanka has made a remarkable rise in diseases related to orthopedics including osteoporosis and osteoarthritis as a result of a variety of bone disorders. Eventually, this would turn out to be a national problem because orthopedic diseases take some considerably long time to completely recover. A recent local study reveals through clear statistics that the expected number of hip fracture cases and hip related orthopedic disorders in Sri Lanka would reach to nearly 70000 by 2040, as the population over 50 increases. The same study also suggests In addition, the same study also reveals that a sample of postmenopausal women from a single province in the country showed a 10% prevalence of vertebral fractures; characterized as menopausal induced or stimulating disorders. Such studies indicate the need to combat orthopedic disorders within the country and with the known physiological interaction of Cathepsin K in human as illustrated above; its recombinant production could be directed towards a tangible outcome as an efficient therapeutic to heal such fractures, since, the most basic therapeutic required here would be the need for a new stable protein matrix to be deposited on the bone to heal the fracture. Deposition could only be achieved if the existing bone matrix is completely removed. Therefore, the involvement of Cathepsin K is immense. Hence, this is strong evidence to show that studies with regard to this enzyme on a National perspective are of high importance. Simultaneously, this study could also be directed towards synthesis of a highly efficient inhibitor with minimum side effects to avoid unnecessary bone protein layer degradation as in cases like osteoporosis; both been clear long term achievements that could be expected through this initial study. Therefore this study could indeed considered as a feasibility study in Sri Lanka; which is a novel perspective in establishing a commercially important human

protein overexpression National workstation in the country to combat non communicable diseases.

2. RESEARCH METHODOLOGY

Approach to methodology: In brief the research methodology consisted of the following experimental workflow. DNA extraction and quantification, polymerase chain reaction (PCR) amplification of two selected catalytic domains of the *CtsK* gene, agarose gel electrophoresis (AGE) of the PCR products, gel band dissection and purification, double digestion of the two purified PCR products and the PBS vector separately by restriction endonuclease (RE) enzymes; *HindIII* and *SalI* respectively, ligation of the double digested PCR products and the vector using *T4 DNA Ligase*, bacterial transformation of the ligated product to competent *Top 10 E. coli* cells facilitating blue white screening and incubation of the bacterial plates. In addition, ligation of PCR amplicon to the pGEM-T vector and bacterial transformation to *Top 10 E. coli* was also done.

2.1 DNA Extraction

Four human blood samples were collected from four healthy individuals to extract whole genomic DNA. Ethical clearance for the use of blood in the study was obtained by the Ethics Review Committee of the Wayamba University of Sri Lanka. The FlexiGene^{TM} human whole blood DNA extraction kit from Qiagen^{TM} that consists of the buffers FG1, FG2, FG3 and Proteinase K was used for DNA extraction (see supplementary data "1" for detailed protocol). The DNA was checked for quality by running an AGE at 80V for 1 hour. In addition, the DNA was quantified using the Nanodrop^{TM} spectrophotometer to measure the A_{260}/A_{280} and the A_{260}/A_{230} purity indicator ratios (see supplementary data "2" for detailed protocol).

2.2 PCR

02 pairs of primers were selected from a previous study done by Donnarumma and colleagues [9] and the primers were verified by blasting them with the nucleotide sequence coding for the CtsK gene [>NG_011848.1 Homo sapiens cathepsin K (CTSK), RefSeqGene on chromosome 1)]. Primer Pair (PP) 1 as Forward Primer (FP) TTAATTCCATGGTTAGTTCCCC and Reverse Primer (RP) GGTCATGCCAGATTACATATGC to amplify a region of exon 2 of 545 base pairs and PP2 as FP; GTGTACCATCAGTACCTCGCAC and RP; CTTCCAAAGTGCATCGTTACAC to amplify a region of exon 5 of 265 base pairs. The primers were moderated to facilitate by double digestion by REs by tagging the forward primers with the flanking region of *SalI* RE (GTCGAC) and the reverse primers with the flanking region of *HindIII* RE (AAGCTT). In addition, a sitting sequence (ACGC) was added at the 5' end of each primer. Accordingly, the final PPs generated were as PP1- FP; 5'ACGCGTCGACTTAATTCCATGGTTAGTTCCCC3' and RP; 5'ACGCAAGCTTGGTCATGCCAGATTACATATGC3' and PP2- FP; 5'ACGCGTCGACGTGTACCATCAGTACCTCGCAC3' and RP; 5'ACGCAAGCTTCTTCCAAAGTGCATCGTTACAC3' (see supplementary data "3" for detailed protocol). PCR was conducted using PCR master mix incoperated with *Taq Taq) polymerase* (from Solis BioDyne^{TM}). Accordingly PCR was optimized for the PP1 and PP2 at conditions; 94^{o}C; initial denaturation, 94^{o}C denaturation, 55^{o}C; primer annealing, 72^{o}C; elongation, 72^{o}C; final elongation and 4^{o}C; final hold for 3 minutes, 30 seconds, 30 seconds, 40 seconds, 5 minutes and infinite time durations respectively(see supplementary data "4" for detailed protocol).

2.3 Age

The PCR products were analyzed on a 1.5% Tris Acetate Ethylenediaminetetraacetic acid (TAE) agarose gel. Slow run was conducted at 60 V for three hours duration with effective cooling. DNA ladders of 100bp and 1kb sizes from Solis BioDyne^{TM} was used for comparison of the resultant gel bands. The gel was visualized through a blue light transilluminator (Safe Imager™2.0 ^{TM}) and photographed.

2.4 Gel Band Dissection and Purification

The appropriate bands were carefully dissected and placed separately in sterile micro centrifuge tubes of known weight. The tubes with the gel fragment were weighed and the mass of the gel fragments were deduced. Genaxxon^{TM} Gel Purification Mini Prep Kit was used for gel band purification (see supplementary data "5" for detailed protocol). The DNA eluted through purification was quantified using the Nanodrop^{TM} spectrophotometer for quantity and quality.

2.5 Restriction Endonuclease Digestion (Double Digestion) of PCR Amplicons and *PBS* Vector

RE enzyme digestion was done on sequential basis first using RE *HindIII* and then by RE *SalI*. Both enzymes were obtained from Promega™, USA. Firstly, sequential Restriction Enzyme (RE) digestion of the two PCR products of sizes 545 base pairs, 265 base pairs and the vector *PBS* was done using the RE *HindIII*. The reaction mixtures were assembled accordingly (see supplementary data "6" for detailed protocol) and were incubated at 37°C for four hours. AGE was performed on the digested products, appropriate resultant bands were dissected and purified using the Genaxxon™ Gel Purification Mini Prep Kit and quantified. The second digestion using *SalI* RE was performed on the *HindIII* digested PCR amplicons and the vector. The reaction mixtures were assembled (see supplementary data "7" for detailed protocol) and were incubated at 37°C for four hours. AGE was performed on the digested products, appropriate resultant bands were dissected and purified using the Genaxxon™ Gel Purification Mini Prep Kit and quantified.

2.6 Ligation using T4 DNA Ligase

The double digested vector and the two PCR amplicons were subjected to ligation using *T4 DNA Ligase enzyme*, (from PromegaTM, USA) by assembling the reaction mixture accordingly (see supplementary data "8" for detailed protocol) and was incubated at 37°C for three hours. The recombinant plasmid was obtained this way.

2.7 Bacterial Transformation, Plating and Incubation

The recombinant plasmid was transformed to competent *Top 10 E. coli* cells (from Ceygen Biotech™, Sri Lanka) through the heat shock transformation method (see supplementary data "9" for detailed protocol). The bacterial cells were then cultured on culture plates containing 20 mL of Luria Bertani (LB) media along with 80 µL of 0.1M Isopropyl β- d-1-thiogalactopyranoside (IPTG) and 40 µL of 0.1M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 20 µL of antibiotic Ampicillin of concentration 100mg/mL. The culture plates were incubated overnight and transformants were observed by blue/white colony identification.

2.8 pGEM-T Ligation, Bacterial Transformation and Verification Protocol

In addition to the RE digestion and ligation experimental setup, ligation of the PCR amplicon with the pGEM-T vector system (from Promega, USA), was also done. Following PCR amplification, the 265bp amplicon was directly incubated with PCR master mix and *Taq* polymerase from (both from Ceygen Biotech™, Sri Lanka) at 72°C for 30 minutes to facilitate poly A' addition to 3' end of PCR amplicon(see supplementary data "10" for detailed protocol). This mixture was then ligated to the pGEM-T vector using the ligation buffer in the pGEM-T vector system and the reaction mixture was left to incubate in room temperature for one hour(see supplementary data "11" for detailed protocol) and was then taken forward towards bacterial transformation as illustrated in 2.7 above. The white colonies were selected and grid plated at the same growth conditions (see supplementary data "12" for detailed protocol). Each white colony that appeared after incubation of the grid plate was then dissolved in Phosphate Buffer Saline (PBS) and subjected to colony PCR (see supplementary data "13" for detailed protocol) and the PCR products were visualized through AGE to validate the correct band sizes.

3. RESULTS AND DISCUSSION

3.1 Genomic DNA Extraction and Quantification for Quality and Quantity

As illustrated in the method above, a commercial kit was used for the genomic DNA extraction process consisted of three buffers which was the FGI Buffer, FG2 Buffer and the FG3 Buffer along with protease. The FG1 Buffer is basically the washing buffer of which the key function is to break open the cell membrane of the cells allowing the DNA to leave out of the cell and disrupt the complex cellular arrangement so that further breakdown of the cell would be facilitated. The washing step performed with the FG1 buffer is highly important as it opens up the cell membrane and lysis of the complex biomolecules within the cell. Efficient inversion and a short time vortex was found to be key necessities that made maximum contact with of the cells with the buffer

and purify better. The key function of the FG2 Buffer along with the protease is involved in efficient breakdown and degradation of proteins that are associated with the DNA and the other proteins that are intact with the cell that might disturb the extraction process. Histone proteins are the most immediate proteins that are associated with DNA and that needs to be broken down. The FG3 Buffer is the resuspension buffer which is used to provide a medium for the extracted DNA to be hydrated and they provide a physical medium for the DNA to be transferred during other molecular approaches such as during the PCR. 100% Isopropanol was added in order to better pellet out the DNA from the other debris that are present in the solution because DNA does not dissolve in isopropanol due to differences in polarity. Therefore, this facilitates the separation of the DNA from other substances effectively. Increasing the volume of 100% Isopropanol added, increasing the number of times of isopropanol addition increased the DNA concentration and also the purity of the DNA. This is because the exposure of the DNA together with other organic compounds to isopropanol would facilitate in better combination of the organic compounds with isopropanol and make DNA a free entity, making it more pure. In addition, the concentration of DNA is increased because the higher the washing with isopropanol, the more DNA gets pelleted out and therefore is more effective. Addition of 70% Ethanol to facilitate the dissolution of all other organic compounds and make them separated from DNA in order to maintain a better purity in the DNA extracted. Similar to the washing done by isopropanol, increasing the number of times that both organic alcohols in total contribute upwards efficient separation of the DNA from other organic compounds. However, it should also be noted that the air dry steps indicated in the protocol were done in order to facilitate evaporation of all the organic volatile compounds such as ethanol. The inversion steps were performed to remove all the liquid components that are within the micro centrifuge tubes. Elution of the DNA extricated needs to be done depending on the yield of the DNA pellet that is seen. Elution of the DNA in an excess buffer might lead to dilution of the DNA and would be a difficult task in other downstream processes. Similarly, the incubation time needs to be increased if a lower amount of the elution buffer is used, in order to sufficiently dissolve the entire DNA completely in the minimum amount of the buffer. DNA extractions were carried out using several optimizations to the original protocol and the genomic DNA thus isolated was quantified for quality and quantity using a NanodropTM Spectrophotometer and also an agarose gels were run in order to determine the degradation/ intact nature of DNA.

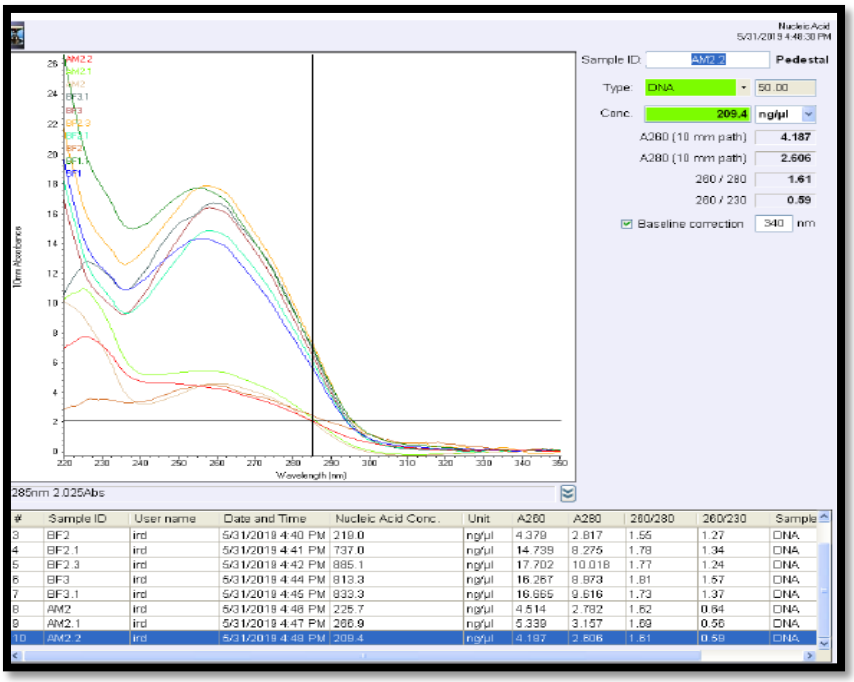


Fig. 1. DNA quantification data

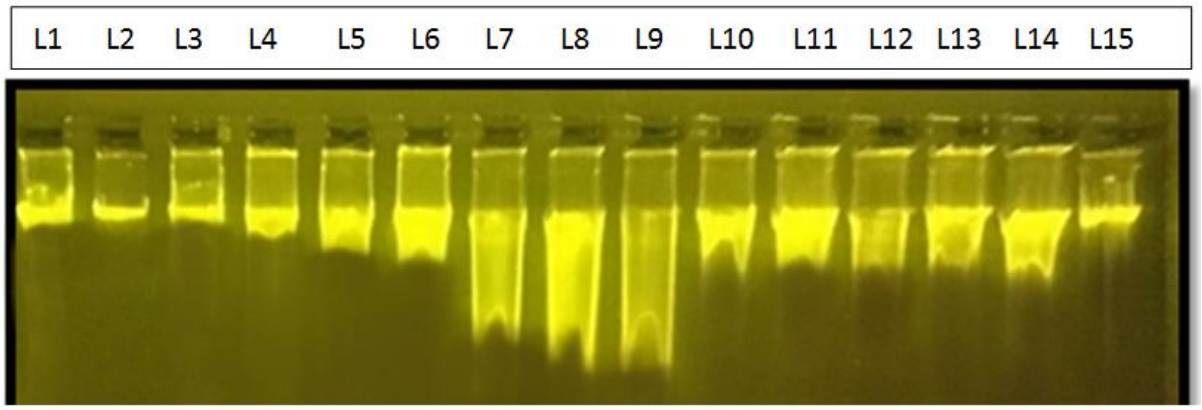


Fig. 2. Agarose gel image of the DNA extracted

The DNA samples were also subjected to an agarose gel electrophoresis. The dye used was the Safeview Nucleic Acid Dye™, which is a safer dye to be used in contrast to the typical Ethidium Bromide dye, which indicated records of having carcinogenic features. The gel as observed under a blue light transilluminator is shown below.

Gel Legend- L1to L15 contains 3 µL of genomic DNA with 1µL of the loading dye, bromophenol blue according to the gel, it is seen that that even though a few samples look degraded, in each band that is observed, a prominent sharp band is observed at the most top region (near the well) which indicated the DNA that is available without degradation. Actually, thus is the DNA that takes part in the PCR. It should also be noted the gel unit was placed on an ice filled tray while running the gel in order to avoid the distortion of the bands due to the heat that is generated when the gel experiences a constant voltage (75V) for a longer period of time (1 hour).

3.2 Primer Designing and Polymerase Chain Reaction for the Amplification of the *CtsK* Gene

Firstly, it is important to note that two pairs of primers that were used in a study done in 2007 by Donnarumma and colleagues were moderated with the addition of restriction endonuclease sites onto them. However, the validity of the primers were first verified by aligning the primers with the RefSeq sequence coding for the CtsK gene obtained by NCBI at accession number NG_011848.1. The sizes of the products formed were also verified sing the BLAST tool. The restriction sites of the RE enzymes *HindIII* and *SalI* were added on to the 5' end of the primer. After the addition of the RE site onto the primer a sitting sequence, also called a buffer region containing four arbitrary bases were added. This is to make sure that the restriction sites are not cleaved or destroyed during the process of restriction endonuclease digestion which might disturb the flanking region of the restriction enzyme causing failure in the digestion and causing erroneous results while cloning. Furthermore, the primers were tagged with restriction endonuclease sites in a way that double digestion could be facilitated. Therefore, the forward primer was tagged with the *SalI* RE enzyme and the reverse primer was tagged by the *HindIII* RE enzyme.

However, care was taken to moderate the primers by maintain a GC% of 50 and melting temperature variation between the two primers within the pairs not more than 5^0C to avoid failures in the amplification process or to hamper it. The arbitrary sequence was added in front of the restriction site in each primer very carefully by preserving all qualitative features that primers need to poccess. The use of Oligo Analyzer™ software was used to validate the formation of primer dimers, hairpin structures or loop structures and necessary precaution was taken to avoid them.

The annealing temperature of the primers was calculated in the following manner. Even though the values were deduced this way, the previous research article used indicated that the optimum annealing temperature that was used was 55°C. The increment of the annealing temperatures here as shown in the table below was thought to probably be due to the addition of the sitting sequence and the restriction endonuclease sites. However, the same annealing temperature used in tea per was used, since what is intended is that the original primer binds to the

complementary sequence in the template DNA, since the restriction endonuclease sites and the sitting sequences added are not within the interests of binding of any sought.

The PCR reactions were designed and conducted as per the protocol provided in chapter 3 and the products were visualized on a 1.5% agarose gel made with TAE buffer. The reason for using the TAE buffer has a specialty, that is the typically used TBE buffer has boric acid in it, and borate ions that would occur in the solution and hence the gel, would cause inhibition of the activity of restriction endonucleases, which in turn would hamper and stop the restriction enzyme digestion and as a result would inhibit cloning. The gel as observed under a blue light transilluminator is shown below. The gel was run at 60V for duration of 3 hours (slow running performed) with cooling by placing the electrophoresis unit on an ice tray.

Table 1. Primer specifications

Forward primer (5'>3')	**Reverse Primer (5'>3')**
C1 5'ACGCGTCGACTTAATTCCATGGTTAGTTCCCC3' Melting Temperature = 73.8^{0}C	C2 5'ACGCAAGCTTGGTCATGCCAGATTACATATGC3' Melting Temperature = 72.5^{0}C
Primer annealing temperature for Primers C1 and C2 = 73.8^{0}C + 72.5^{0}C = 146.3^{0}C / 2 = 73.15^{0}C - 5^{0}C = 68.15 ^{0}C	
C3 5'ACGCGTCGACGTGTACCATCAGTACCTCGCAC3' GC% = 59.38 Molecular Weight = 9730.4 gmol^{-1}	C4 5'ACGCAAGCTTCTTCCAAAGTGCATCGTTACAC3' GC% = 46.88 Molecular Weight = 9728.4 gmol^{-1}

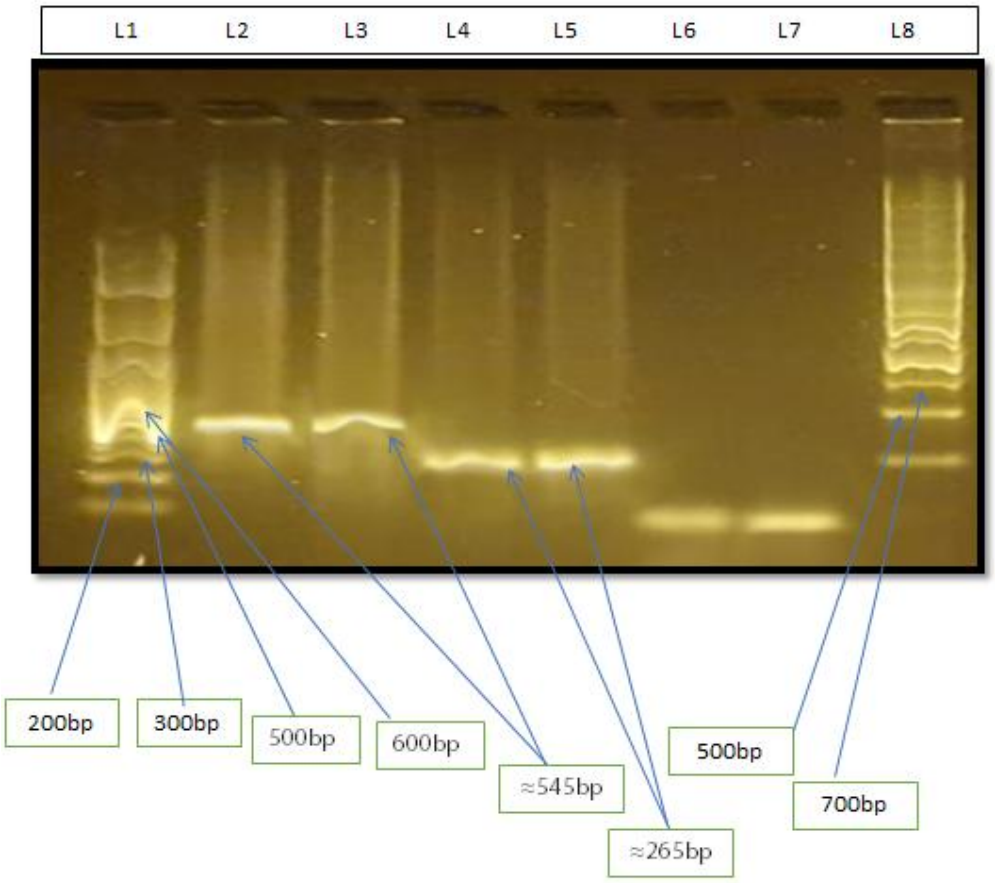


Fig. 3. PCR gel image
Gel legend

Table 2. Gel legend of Fig. 3

Lane (L)	Inclusion to the Well
L1	1µL of 100bp DNA ladder (Solis BioDyne™)
L2	3 µL of PCR reaction with C1 and C2 primers with 1 µL bromophenol blue
L3	3 µL of PCR reaction with C1 and C2 primers with 1 µL bromophenol blue
L4	3 µL of PCR reaction with C3 and C4 primers with 1 µL bromophenol blue
L5	3 µL of PCR reaction with C3 and C4 primers with 1 µL bromophenol blue
L6	3 µL of PCR reaction with C1 and C2 primers with 1 µL bromophenol blue Negative Control
L7	3 µL of PCR reaction with C3 and C4 primers with 1 µL bromophenol blue Negative Control
L8	1µL of 1kb DNA ladder (Solis BioDyne™)

- Note that the DNA samples used in the above PCR had a concentration of 366.7ng/ µL.
- Note that the C1 and the C2 primer pair generates a product of size 545bp while the C3 and the C4 primer pair generates a product o f size 265bp.
- Another trail of a PCR at same conditions but with two different DNA samples produced the following gel image.

Several precautions were taken to avoid any form of contamination that might occur. Personal protective ware was won in the first place and it was made sure that the PCR was conducted under a dedicated hood for PCR only. All new and sterile consumables were used at every new trail. An important consideration that needs to be highlighted was during and every PCR reaction that was conducted, water for injection was used as sterile, deionized, nuclease free water. A vial of water for injection contained 5 mL of the water and it was very safer to using rather than using from stock PCR water that every researcher in the laboratory uses, so that a probability for cross contamination might occur. However, water for injection was very cheap and as the name suggests its purpose in the medical field, it is highly sterile as well.

Upon visualization of the gel image the intended band sizes were obtained except for one lane in the second PCR trail which should most probably be due to a handling and manipulation error in preparing reaction mixtures for the PCR. The 100bp and the 1kb DNA ladders have been separated sufficiently well facilitating to deduce the size of the bands. The lanes to which the negative controls of the PCR reaction mixture was loaded, no band was observed except for very light primer dimers. This indicated that the PCR reaction has not been contaminated by any sought. In addition, no nonspecific band or any other attributes such as hairpin structures, loops or dimers were observed elsewhere. Since all these considerations were optimum the gel bands were considered for dissection and further manipulation.

3.3 Gel dissection and Gel Band Purification

Having identified the two bands as expected, one of 545bp and the other of 265bp, each band was dissected with a separate sterile surgical blade making sure that the sharp edges of the band were all within the excised fragment to make sure that the maximum yield is obtained. The indication as to why gel purification needs to be done is merely because all the unwanted complexes present within the DNA including, but not limited to agarose, TAE buffer components, staining dyes etc. need to be removed from been in contact with DNA. The protocol that was made available with the Genaxxon™ Gel Purification kit was followed to facilitate purification of the following gel bands that were excised. The bands of the gel diagram 2 above were used for gel purification. Based on the intensity of the bands obtained, the bands in lanes 2,3,4,6, 7 and 8 were used. Lane 5 was ignored because no amplification was noted. Lane 9 was ignored because the band that was generated was not very clear. Upon excision of the bands, the masses of the gel fragments obtained were as follows. Note that the mass of the empty micro centrifuge tube to which the dissected gel fragment was placed was 0.9873 g.

The gel bands were subjected to gel purification and eluted in the respective DNA elution buffer of the gel purification kit. Upon elution, the DNA samples in the six lanes above were quantified using Nanodrop™ spectrophotometry and the results obtained were as follows.

Table 3. Gel masses obtained for gel purification

Lane on gel	Total mass with the micro centrifuge tube and the gel fragment	Mass of the gel fragment
2 (Amplicon size 545bp)	1.1128g	0.1255g
3 (Amplicon size 545bp)	1.1274g	0.1401g
4 (Amplicon size 545bp)	1.1065g	0.1192g
6 (Amplicon size 265bp)	0.1338g	1.1211g
7 (Amplicon size 265bp)	0.1387g	1.1260g
8 (Amplicon size 265bp)	0.1284g	1.1157g

Table 4. Qaulity and quantity of DNA extracted

Corresponding gel lane	DNA concentration	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio
2	18.5ng/ µL	1.57	0.47
3	2.25ng/ µL	2.77	0.12
4	4.15ng/ µL	1.87	0.24
6	1.95ng/ µL	2.38	0.31
7	7.05ng/ µL	2.23	0.27
8	3.15ng/ µL	1.90	0.16

A few graphical illustrations are as follows.

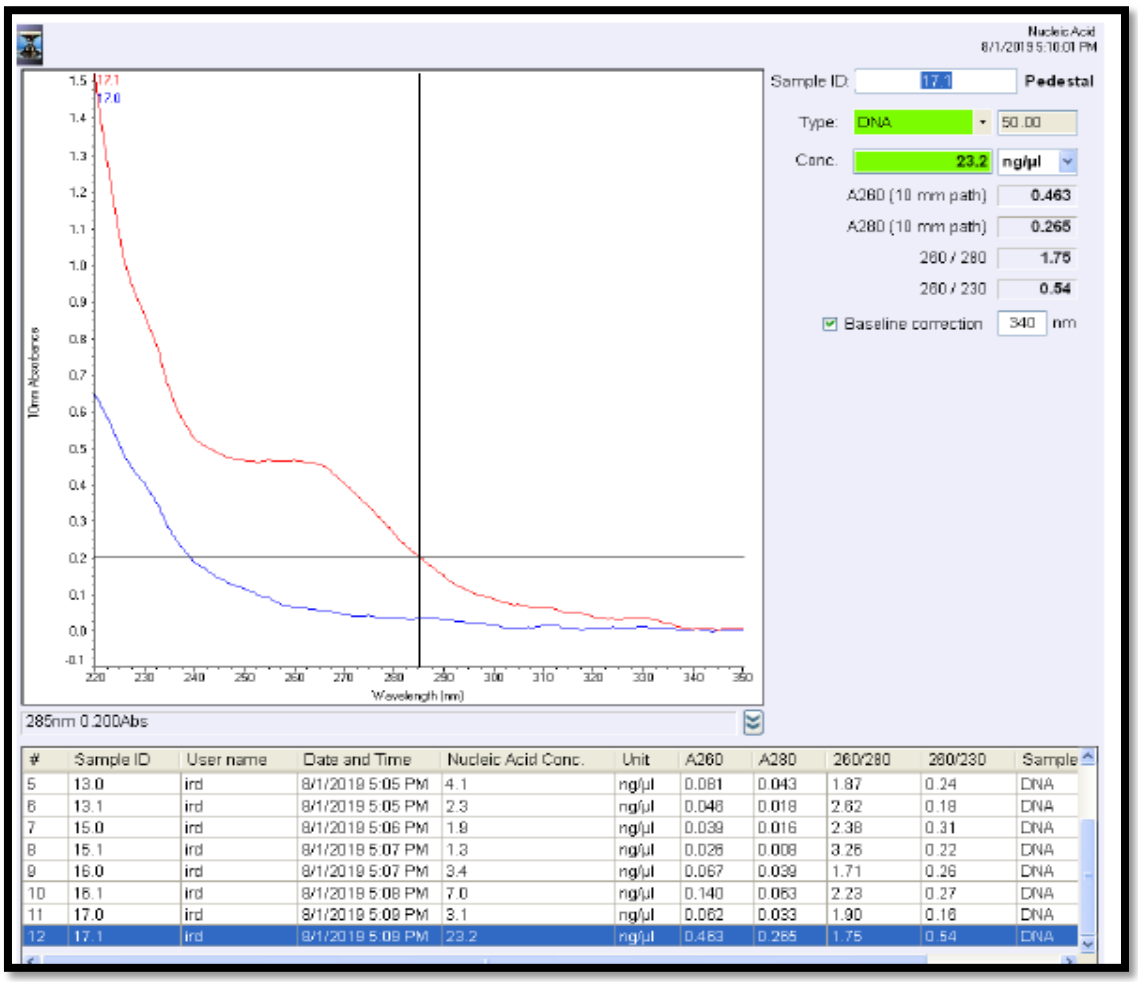
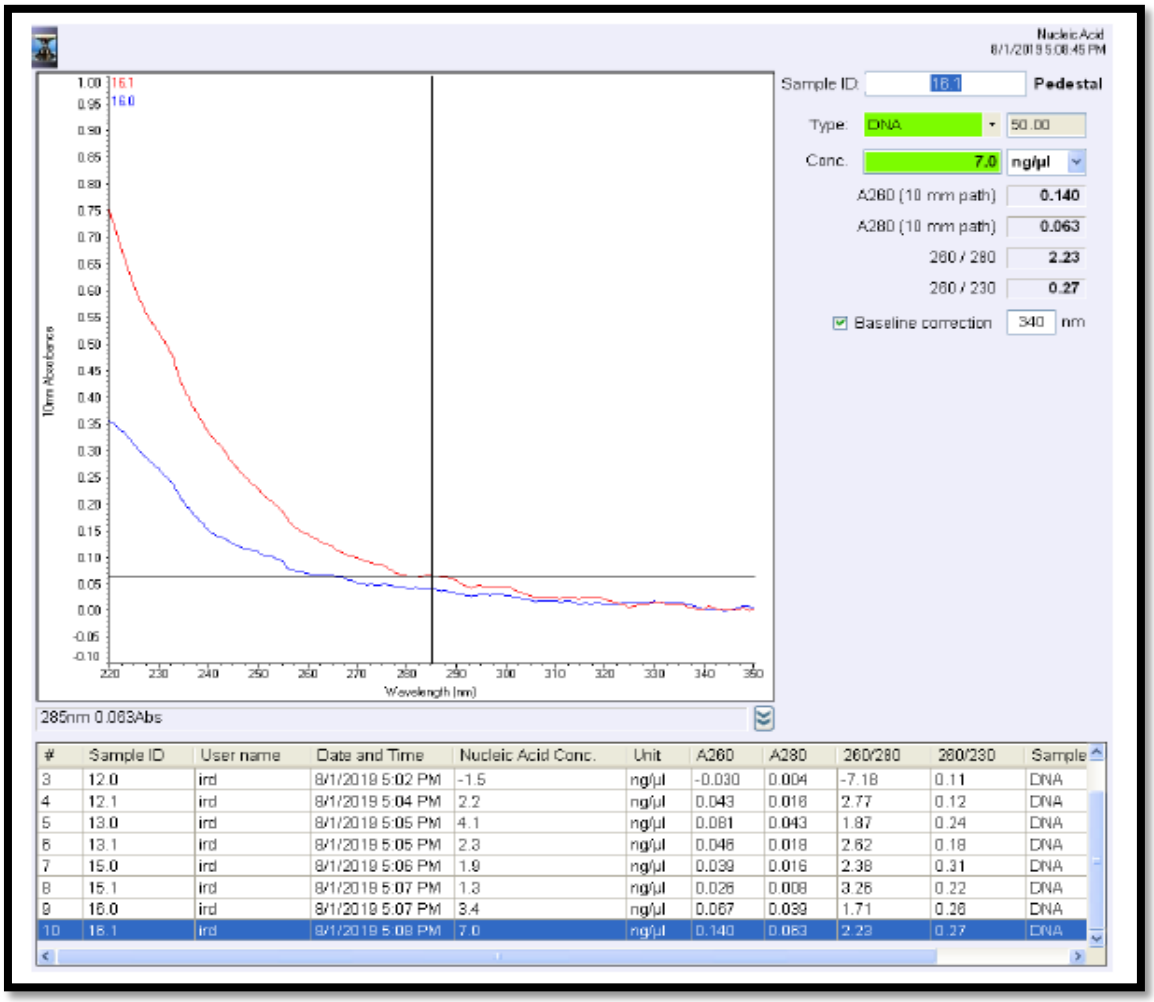


Fig. 4. DNA quantification data



#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample
3	12.0	ird	8/1/2019 5:02 PM	-1.5	ng/µl	-0.030	0.004	-7.18	0.11	DNA
4	12.1	ird	8/1/2019 5:04 PM	2.2	ng/µl	0.043	0.016	2.77	0.12	DNA
5	13.0	ird	8/1/2019 5:05 PM	4.1	ng/µl	0.081	0.043	1.87	0.24	DNA
6	13.1	ird	8/1/2019 5:05 PM	2.3	ng/µl	0.046	0.018	2.62	0.18	DNA
7	15.0	ird	8/1/2019 5:06 PM	1.9	ng/µl	0.039	0.016	2.38	0.31	DNA
8	15.1	ird	8/1/2019 5:07 PM	1.3	ng/µl	0.026	0.008	3.26	0.22	DNA
9	16.0	ird	8/1/2019 5:07 PM	3.4	ng/µl	0.067	0.039	1.71	0.26	DNA
10	16.1	ird	8/1/2019 5:08 PM	7.0	ng/µl	0.140	0.063	2.23	0.27	DNA

Fig. 5. DNA quantification data

As per the results obtained above, it is observed that the DNA concentration has dropped compared to the very initial DNA concentration that was present during the extraction process. This is quite obvious die to the various forms of manipulation that is done with various organic reagents in several downstream applications. However, upon recommendation of the kit, it was indicated that the purity of this DNA purified to be measured using the $A_{260}/_{280}$ ratio. In this perspective, it could be stated that the DNA has sufficient purity that could be used in further downstream applications including restriction enzyme digestion.

An important consideration that needs to on address under this section is that why the PCR reaction mixture cannot directly be used in restriction enzyme digestion, rather than having to run the PCR product on a gel and purify it which consumes some time and also reduces the net yield of the DNA relatively. The reason is that via a gel run and the exact band could be verified further and dissected appropriately. But in the case that a PCR is used directly, then PCR reaction failures and the presence of non-specific bands could be quite contradictory and further downstream processes wound not be accurate.

3.4 Sequential Restriction Enzyme Double Digestion of the Insert and the Vector

The gel purified bands of sixes 545bp and 265bp were then prepared to undergo restriction digestion along with the vector. Two enzymes were used in this regard as a confirmatory measure to make sure that self-binding of the vector or the insert does not take place to produce false positive results. The double digestion causes the formation of two different complementary sticky ends to generate in the vector and the DNA which in turn would only particularly facilitate the recombinant ligation of

the double digested insert and the double digested vector which could be expressed in the bacterial host.

The vector used in this study was the PBS vector in which it's multiple cloning sites containing the restriction endonuclease sites *HindIII* and SalI only occurring once and is resistant towards the antibiotic ampicillin. The size of the whole genome of the vector is 3204bp. The vector map is as shown below.

The sequential restriction endonuclease enzyme digestion in the study involved the digestion of the insert an the vector with RE, agarose gel electrophoresis to visualize the products of digestion, gel dissection and purification and ten digestion of the previously digested insert and the vector with the second RE and following the process again including agarose gel electrophoresis to visualize the products of digestion, gel dissection and purification.

However, an important consideration that needs to be considered is that double digestion of the product of interest could be facilitated at once rather than going through a sequential process as above, if both the enzymes show 100% efficiency in the same enzyme buffer conditions. However, in this regard the enzymes *SalI* and *HindIII* obtained from Promega™ USA did not show such efficiency. In the Promega MULTI-CORE Buffer™ which is a universal restriction endonuclease enzyme buffer, the two enzymes showed only 25% maximum efficiency. However, *HindIII* showed 100% enzyme efficiency in Buffer E™ FROM Promega™ while *SalI* showed 100% enzyme efficiency in Buffer D™ from Promega. Having this consideration the purified DNA and the vector *PBS* was first digested with the RE having the buffer conditions with lower ionic strength. In comparison of the ionic strengths of the buffers of the two enzymes, the following is outlined.

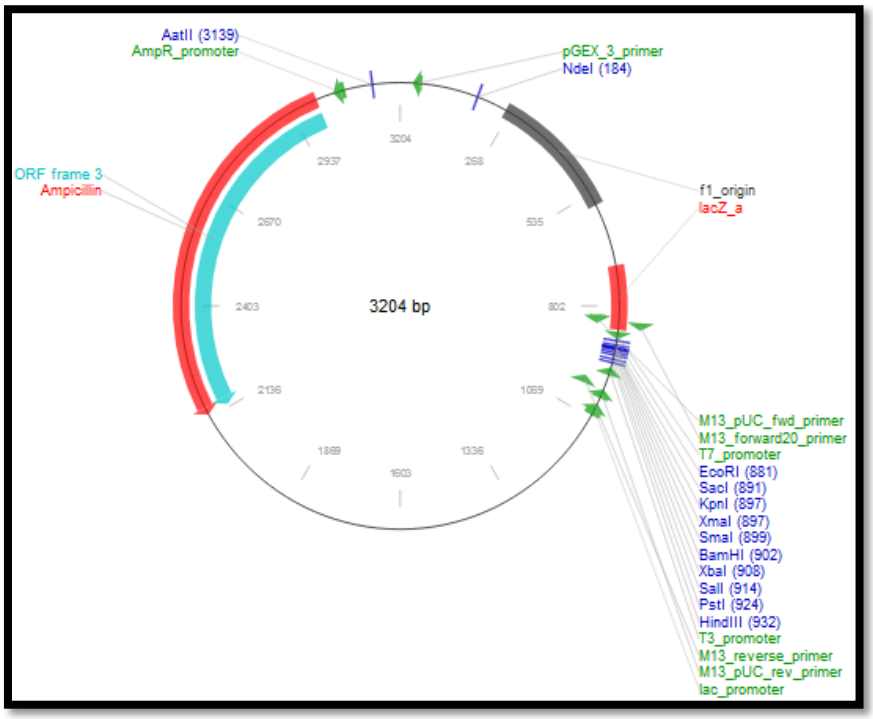


Fig. 6. Vector map of PBS

Table 5. Buffer composition of the buffers in the restriction enzymes

Buffer E ™ compatible with *HindIII* enzyme	Buffer D ™ compatible with *SalI* enzyme
60mM Tis HCl of pH 7.5	60mM Tis HCl of pH 7.5
1M NaCl	1.5 M NaCl
60mM $MgCl_2$	60mM $MgCl_2$
10mM DTT	10mM DTT

Accordingly the lower ionic strength it present in *HindIII* RE enzyme and therefore this enzyme was used first. This is a careful action taken to preserve enzyme features as much as possible, in order to avoid any loss of enzymatic feature during the reaction, which would impede the reaction. Upon setting up the reaction as illustrated in the methodology, the following gel image was obtained.

As per the gel image obtained above, the bands pertaining to the insert DNA in wells 2 and 2 of band sizes 545bp and 265 respectively are obtained. The band of 265bp is very clear and strong, while the band 545bp is less in intensity and probably should be sue to the low concentration of DNA. As far as the vector is concerned, two bands appeared in the gel, at the top and the bottom. However, the bottom band approximately in the 2500bp and in such cases it is mostly suggested that that the supercoiled, or the small segments of the circular DNA that did not linearize would appear this way this band which is a common indication in cases where the vector DNA is too high or the concentration of the RE been not sufficient for complete digestion of the vector. However, is more into non linearized circular DNA. The band at the top clearly lies between 3000-4000bp and is the correct indication. Accordingly the two gel bands corresponding to the 545bp band and the 256bp bands were excised separately which weighed 1.1249 g and 1.1747 g respectively and the upper band of the vector which weighed 1.1432g was also excised from the gel and subjected to the same gel purification protocol mentioned in the methodology. The results were as follows.

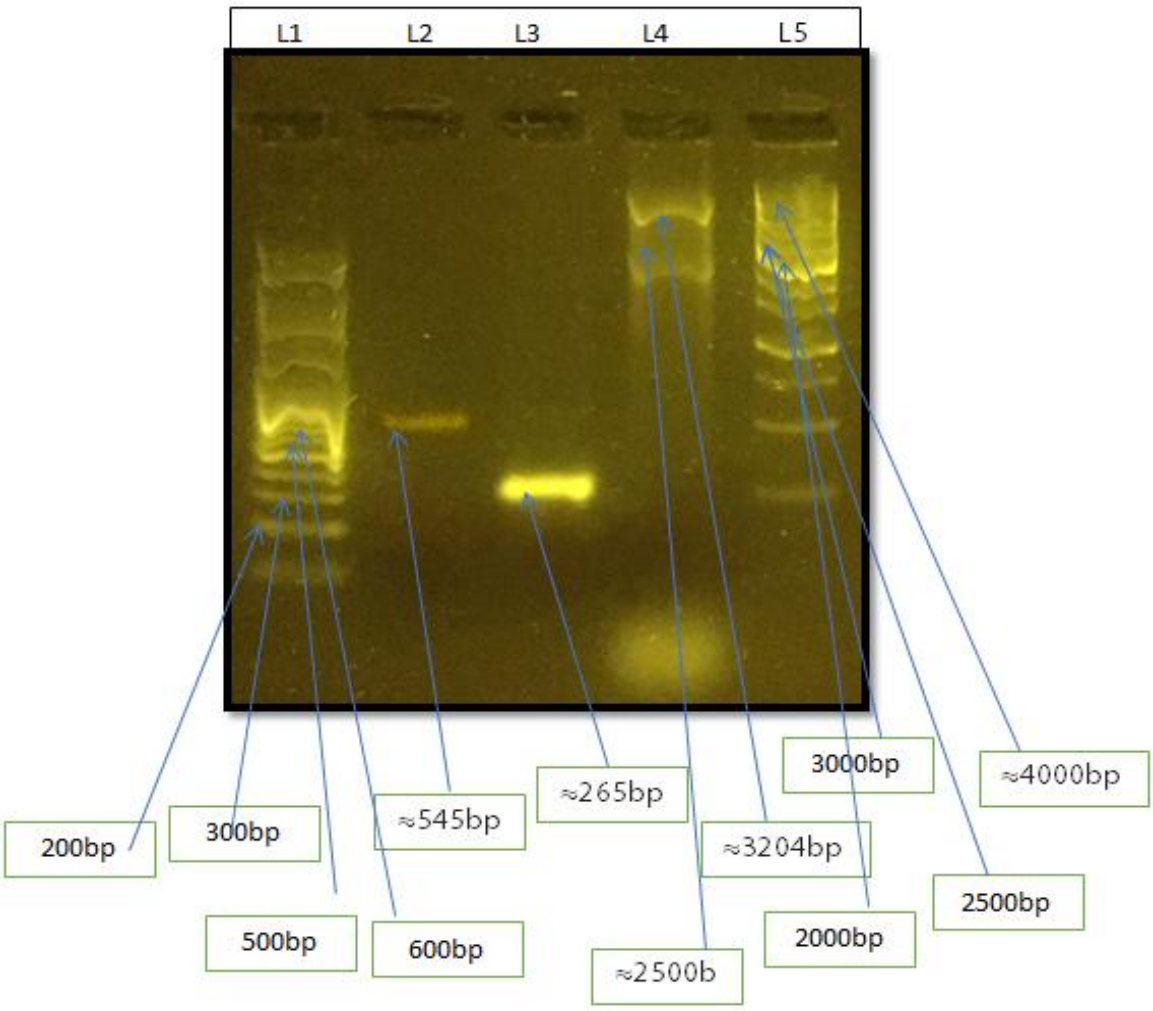


Fig. 7. Restriction digests using HindIII as visualized on a gel
Gel Legend

Table 6. Gel legend of Fig. 7

Lane (L)	Inclusion to the Well
L1	1uL 100bp ladder
L2	20uL of *HindIII* digested 265 Insert + 1 µL bromophenol blue
L3	20uL of *HindIII* digested 545 Insert + 1 µL bromophenol blue
L4	20uL of *HindIII* digested Vector + 1 µL bromophenol blue
L5	1uL 1kb ladder

Table 7. DNA quality and qauntity of restriction digests

Gel lane	DNA Concentration	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio
2	2.3ng/ µL	2.37	0.26
3	2.3ng/ µL	1.59	0.14
4	2.2ng/ µL	1.81	0.11

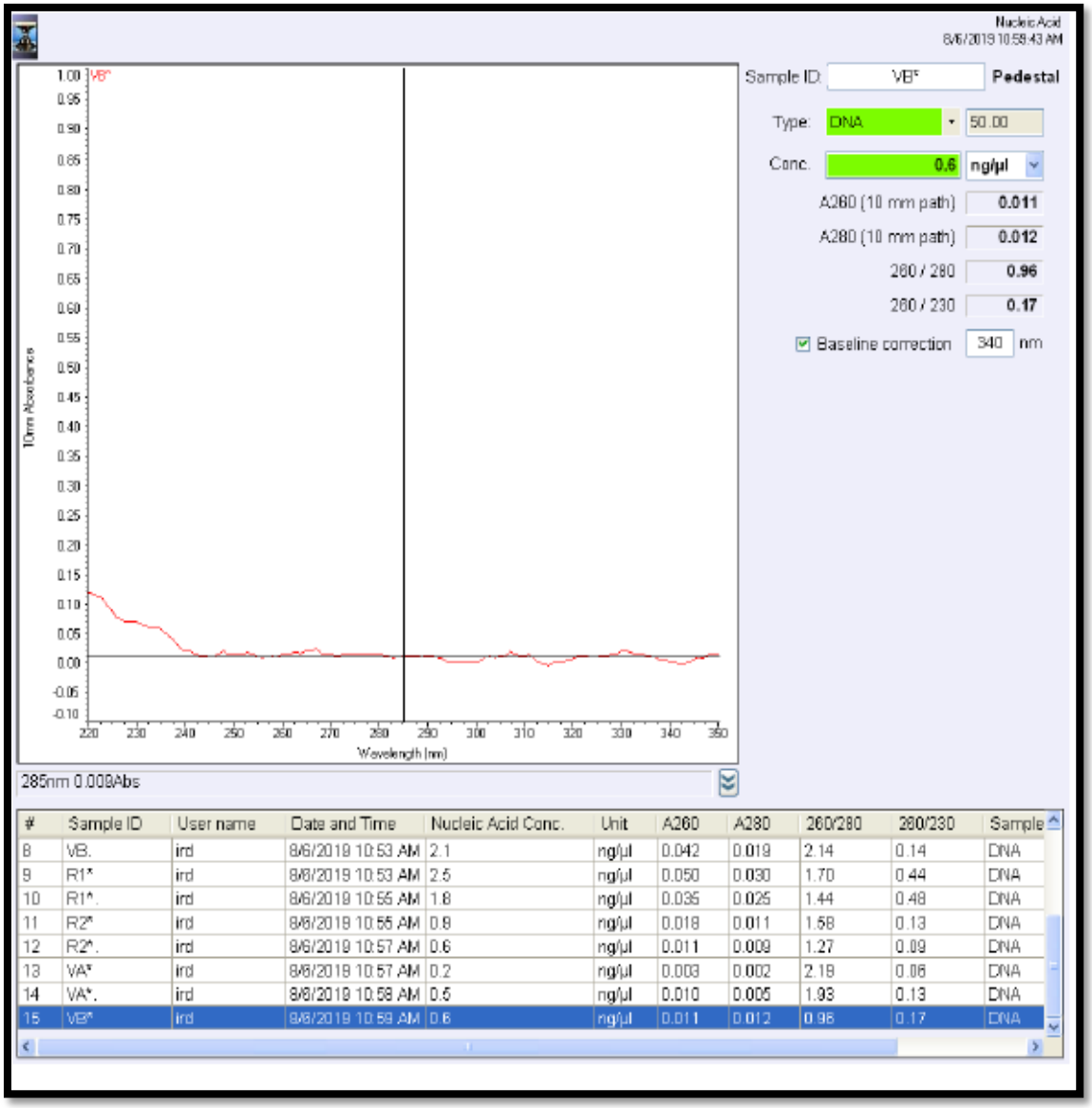


Fig. 8. DNA quantification data of the restriction digests

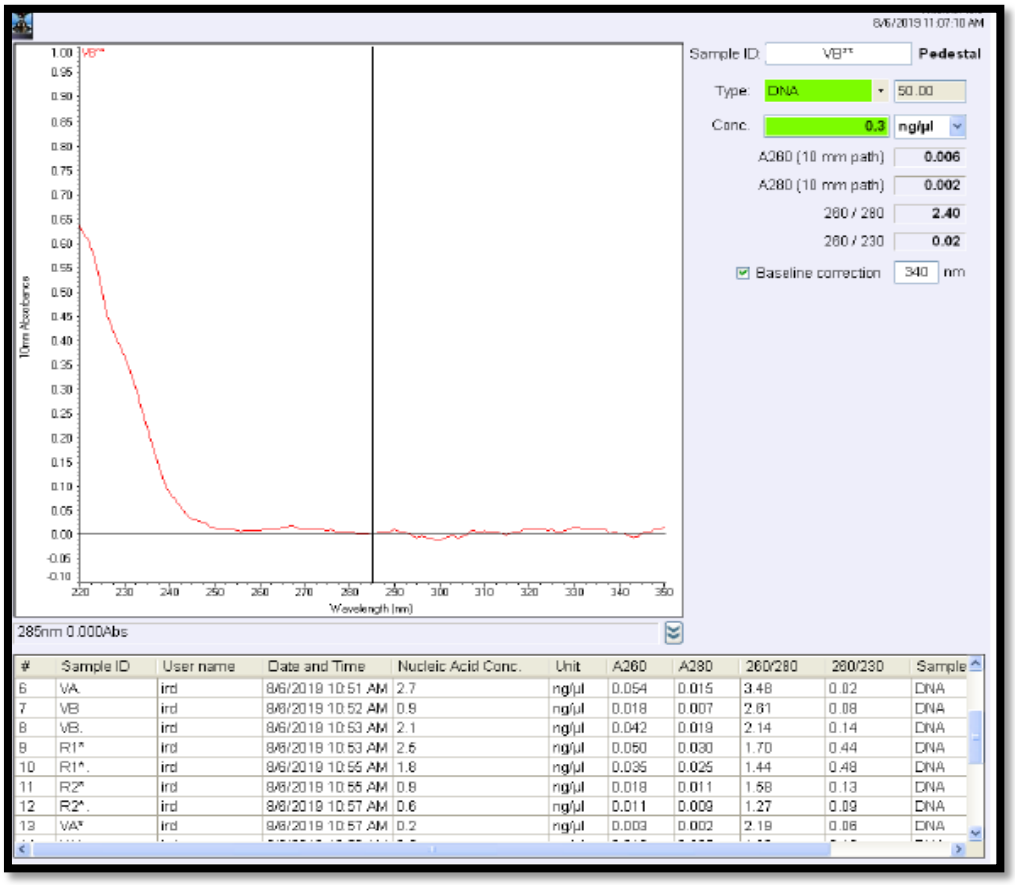


Fig. 9. DNA quantification data of the restriction digests

As it seems, in the data obtained the results are not highly satisfying in terms of both the quality and the quantity of the DNA and this probably should be due to the repetitive purification and the stress caused on this biomolecule by the application of a wide variety techniques and high frequency of exposure to chemicals. However with the available results the second sequence digestion using *SalI* RE was conducted on the already *HindIII* digested products above. Both enzymes *HindIII* and *SalI* were indicated by manufacturers that they show optimum activity for reaction at 37°C. Therefore, an incubation time of four hours was given to each digestion in a closed and dedicated incubator to avoid any form of cross contamination that is likely to occur. During the preparation of the reaction mixtures for restriction enzyme digestion, it should be noted that the restriction enzyme buffer was added as a 1/10t proportion of the total reaction volume. BSA in the reaction mixture aids in preserving the enzymatic properties for better function. The addition of the enzyme was done as per the concentration of DNA available. Upon digestion of these products with *SalI*, the following gel image was obtained.

Table 8. Gel legend for Fig. 10

Lane (L)	Inclusion to the Well
L1	1uL 100bp ladder
L2	20uL of double digested 545bp insert + 1uL bromophenol blue
L3	20uL of double digested 265bp insert + 1uL bromophenol blue
L4	20uL of double digested upper band of vector+ 1uL bromophenol blue
L5	20uL of double digested upper band of vector+ 1uL bromophenol blue
L6	20uL of only *HindIII* digested Vector + 1 µL bromophenol blue
L7	1uL 1kb ladder

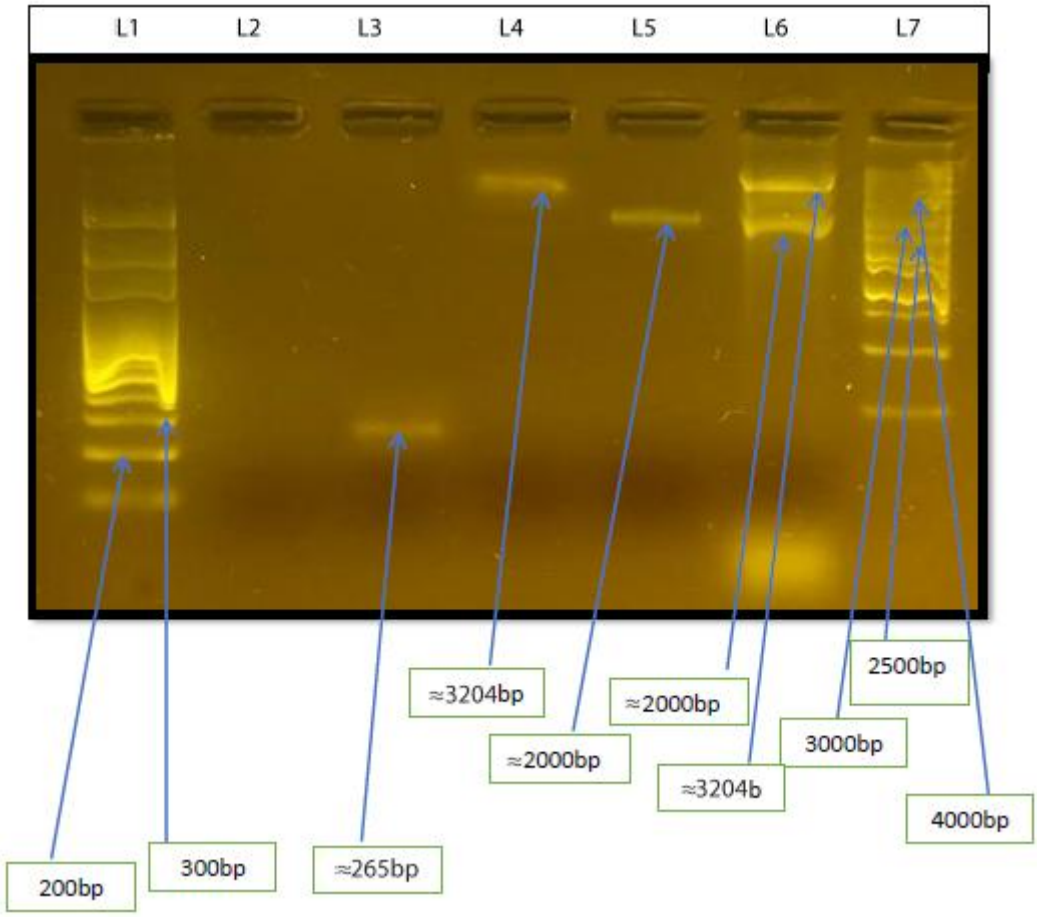


Fig. 10. Restriction digests using SalI as visualized on a gel
Gel Legend

As seen in the gel image very light bands were obtained from the double digestion enzymatic reaction process and related purification process. The major reason that could be identified immediately causing this is the poor concentration of DNA that yielded from the purification process of the gel bands. It should also be noted that during the restriction enzyme digestion here, no sterile water used as part of the reaction mixture because the presence of water could dilute the DNA in the mixture even more. The band that is expected at 545bp at lane 2 was not observed, which means that the reaction has not actively taken place; a key component lacking which most probably would be DNA. The top band received from the earlier digestion and of the vector and the lower band received from the earlier digestion of the vector, both were purified ad subjected to double digestion. Ad it seems both have provided bands, however, as illustrated earlier, the appropriate band would be the top one. Lane 6 that was loaded with only *HindIII* digested vector, another trail of the previous reaction, again confirmed that two bands would appear as the top one and the bottom one due to the existence of the vector in the form of different circular structures. It was analyzed earlier that in such cases, and is therefore important to obtain the upper band, However, it should be understood that in the case that the multiple cloning site of the vector contain two sites for the same restriction enzyme, then again, the occurrence of two such bands could be noted. However, it was identified that this vector *PBS* does not have such repeated sites for the same restriction enzyme. However, the bands were excised separately again and subjected to the same gel purification to elute the double digested DNA material into the elution buffer to proceed with ligation. Now that the double digested insert of product size 265bp and the double digested vector of 3204bp are available the study was proceeded towards T_4 DNA ligation.

The gel bands from the insert (265bp) and the vector weighed 1.398g and 1.2968g respectively. Upon purification, the results obtained are as follows.

Table 9. DNA quality and qauntity data

Corresponding gel lane	DNA Concentration	$A_{260}/_{280}$ Ratio	A_{260}/A_{230} Ratio
3	3.5 ng/ µL	2.06	0.04
4	5.0ng/ µL	1.86	0.16

A few graphical illustrations of the results are as follows

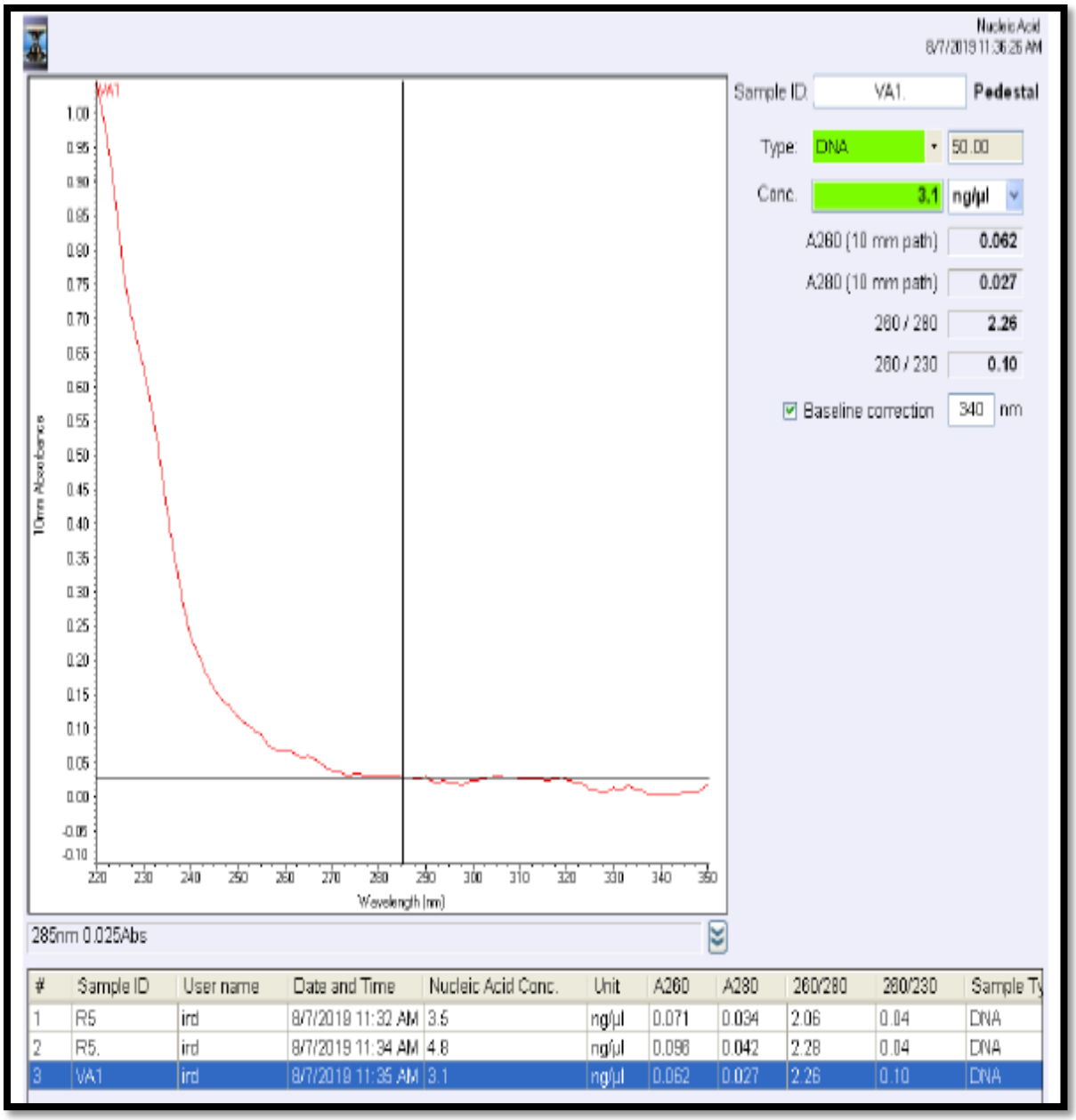


Fig. 11. DNA quantification data

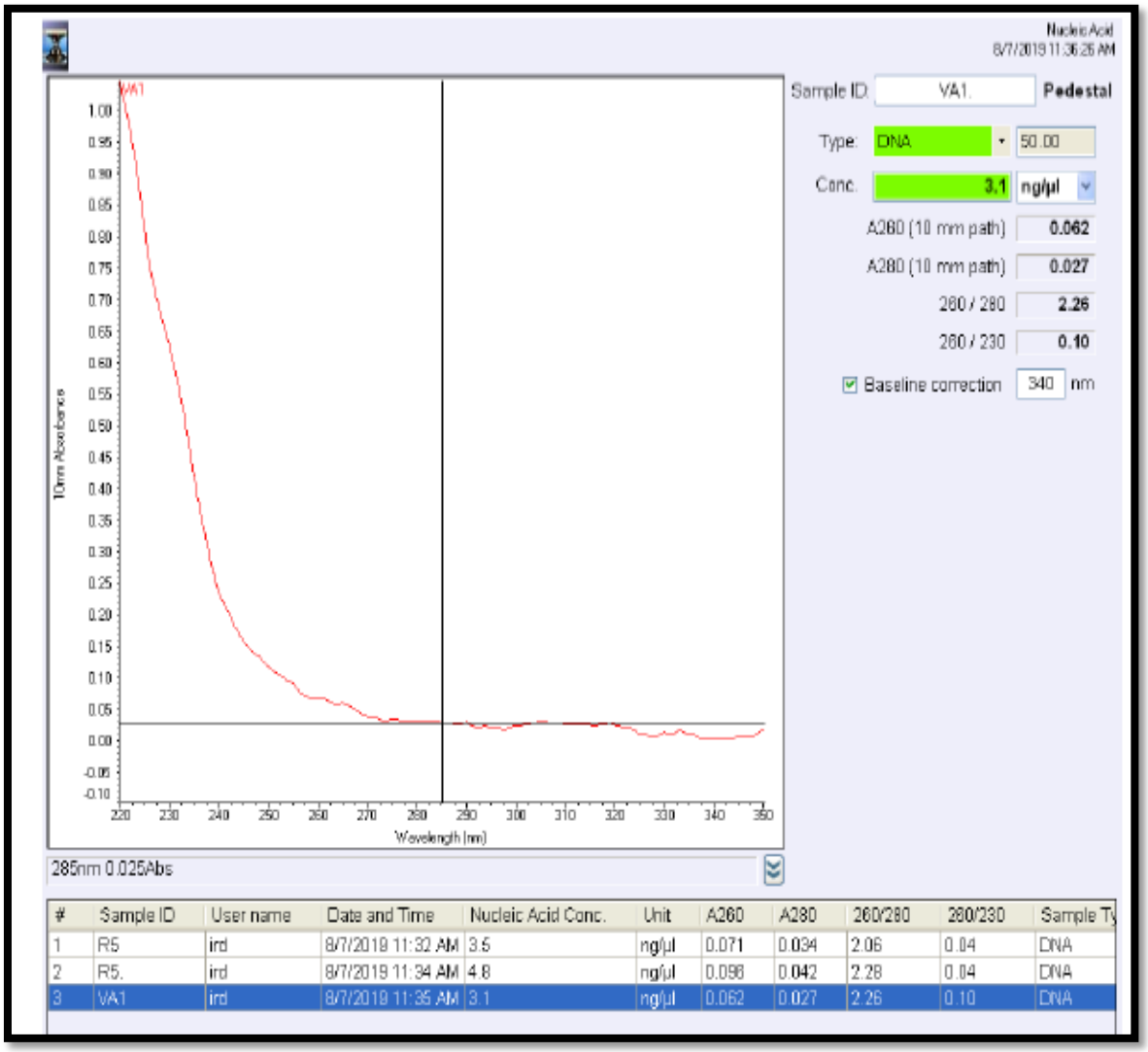


Fig. 12. DNA quantification data

The same consideration of failure to obtain a better yield of DNA with satisfying value is observed. However, the A_{260}/A_{280} ratios indicate some optimum value and some amount of DNA is available too, to proceed to ligation mentioned above.

3.5 Ligation of the Double Digested Vector and the Insert

The double digested vector and the insert of product size 265bp was brought together for ligation in the presence of T_4 DNA Ligase from Promega™. However, it has to be noted since the concentration of the DNA that yielded from the process of gel purification was low; ligation conditions had to be adjusted accordingly. The vector to insert ratio used generally is 3:1. Considering an example of vector concentration 100ng and insert of 265bp;

- [ng of Vector*kb of insert*molar ratio of insert/vector] / kb size of vector

Substituting;

- [100ng of Vector*0.265kb of insert*3 /1] / 3.204 = ng of insert

Therefore, the ng of insert needed would nearly be 24ng.

However, in this study too, the ligation was conducted at the vector to insert ratio used as 3:1.

- [ng of Vector*kb of insert*molar ratio of insert/vector] / kb size of vector

Substituting;

- [22ng of Vector*0.265kb of insert*3 /1] / 3.204 = 4.90ng of insert

Therefore, 4.90 ng of DNA was found in nearly 2 µL of insert DNA sample of initial concentration 2.3 ng/ µL was used. The ligation was carried out for three hours in a closed and a dedicated incubator facilitating an aseptic environment for the reaction to reach completion efficiently. However, it should be noted that only the insert of size 265bp was brought down to ligation and proceeded towards transformation since the 545bp product did not show the double digested band. Some important considerations in the use of restriction enzymes that were understood were as follows.

- The use of restriction enzymes that are compatible in one common buffer showing maximum activity is recommended to avoid unnecessary sequential digestion causing DNA loss as a result of gel purification.
- In the selection of restriction endonuclease enzymes, it is very important that two sites that are closely located within the multiple cloning sites are not selected to avoid any unnecessary digestion of the product of interests within the target site.
- The possibility of using the PCR product directly after verification in restriction enzyme digestion could be investigated for on the basis of commercial kits. For instance the GoTaq™ master mix from Promega™ could be used in restriction endonuclease digestion easily by simply adding the entire enzyme. Such recommendations need be given attention in order to avoid such unnecessary loss of DNA.

3.6 Poly A' Addition to 3' End of PCR Product and Ligation using the pGME-T Easy Vector System™

This method of ligation of the product on interest to the vector to facilitate expression into a bacterial expression system is easy and straight forward. By incubating the verified PCR product containing the amplimers of our study with a mixture of adenine bases at a higher temperature will incorporate the addition of more adenine basses to the 3'end other PCR product. Through this easy vector system, the addition of Adenine bases to the 3' end of the product is intended. Accordingly, with the provision the enzyme ligase and the ligase buffer, the Adenine overhangs attached to the product interacts and binds to a pre prepared vector that already have Thymine overhangs in it. This protocol saves a lot of time and also preserves more qualities of the initial sample provided. In the context of this study, the insert of product size 265bp was ligated to the pGME-T Easy Vector System™ through Adenine addition. However, unlike the confirmatory positive results observed when restriction enzyme double digested vectors and inserts of interest are expressed in bacterial hosts, this requires some other additional confirmations that follow in later parts.

3.7 Bacterial Transformation, Plating and Incubation and Grid Plating

Blue white screening is an extremely fast and a proven efficient technique for the identification of recombinant bacteria, which has an insert if a vector containing DNA of interest. It is based on the metabolic activity of β-galactosidase, which is an enzyme that is found in the *E. coli* expression system, which involves in cleaving the disaccharide lactose into its monosaccharide monomer units, which are glucose and galactose. The presence of lactose in the surrounding environment of the *E. coli* in the growth medium triggers the lacZ operon in *E. coli*. The operon is then involved in the production of β-galactosidase enzyme that metabolizes the lactose that is available for *E. coli*. Most plasmid vectors, including the *PBS* plasmid vector used in this study carry a short segment of lacZ gene that contains coding sequences for the first 146 amino acids of β-galactosidase. The host *E. coli* strains used are competent cells containing lacZΔM15 deletion mutation. In this study, the *Top 10 E.coli* cells were used. When the plasmid vector is taken up by such cells, due the biochemical mechanisms involved with α-complementation process, a functional β-galactosidase enzyme is produced, which is the key component in blue white screening. Commercially available plasmid vectors used in typical cloning processes are manipulated in such a way that this α-complementation process which serves as a marker for recombination to take place efficiently. A multiple cloning site (MCS) is present within the lacZ sequence in the plasmid vector. This sequence can be easily nicked by restriction endonuclease enzymes to insert the DNA of interest. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the α-complementation does not occur, therefore, a

functional β-galactosidase enzyme is not produced. If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the lacZ gene in the plasmid vector complements the lacZ deletion mutation in the host *E. coli* producing a functional enzyme. For screening the clones containing recombinant DNA, the chromogenic substrate X-gal was added to the LB media plate. The concept is that If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appeared in blue in color while the recombinant ones appear white. The desired recombinant colonies were picked and cultured again in a grid plate for better screening and segregation of the transformed cultures.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used along with X-gal for blue-white screening. In chemistry, IPTG is a non-metabolizable analog of galactose that induces the expression of lacZ gene. However, IPTG is not a substrate for β-galactosidase but only an inducer. It also has to be noted that the antibiotic ampicillin is been added to the culture plates in or to make sure that no other microbes that are unnecessary may grow and the *Top 10 E. coli* cells that do not have naturally have the ability of living in conditions where ampicillin is present, will be only able to live if the vector *PBS* with ampicillin resistance have been inserted to it and have started multiplying sufficiently well.

During the bacterial transformation processes, is illustrated in the methodology, it is important that to note that the heat shock is given appropriately. Competent cells are ruptured cells with openings on them, therefore during the heat shock the DNA is allowed to enter the bacterial cells and placing them immediately back in ice enables the ruptures in the cell membranes to close back making sure that the DNA molecules that entered the bacterial cells retain within.

An important consideration that needs to be addressed and given consideration is the time duration that needs to be given when the transformed cells are agitated in a shaking incubator. A longer incubation time with shaking makes sure that the cells come into good contact with the LB media and gets nourished while also allowing the transformed plasmids to become stable within the cells, so that the blue white screening yield to successful results.

A few limitations of the blue white screening are as follows.

- The blue-white technique is only a screening procedure and it is not a selection technique.
- The lacZ gene in the vector may sometimes be non-functional and may not produce β-galactosidase. The resulting colony will not be recombinant but still appear white.
- Even if a small sequence of foreign DNA may be inserted into MCS and change the reading frame of lacZ gene. This results in false positive white colonies.
- Small inserts within the reading frame of lacZ may produce uncertain light blue colonies as β-galactosidase is partially inactivated.

The results of the blue white screens obtained by ligation and cloning through the restriction endonuclease activity are as follows. The insert f product size 265bp was manipulated this way. It should be noted that the white colonies formed below are confirmatory due to the fact that the insert and the vector have been double digested and there is no chance at all by which self-annealing would occur due to the different sticky ends that are generated unlike in single digestion. The annealing defiantly should take place in between the double digested vector and the double digested insert and appears in white.

The results of the blue white screens obtained by ligation of the insert of product size 265bp and cloning through the pGME-T Easy Vector System™ is indicated as below.

Unlike the white colonies that were generated from restriction enzyme digestion, the white colony formed through this ligation needs a confirmatory step. As a result the white colonies that appeared here were streaked on a grid plate as illustrated in the methodology and made to grow again. The result of the grid plate is as follows.

Even though the grid plate was streaked with only white colonies, blue colonies also have shown growth to some extent it. This indicates that the within the white colonies streaked, there might have been a few hidden/ unseen blue colonies as well. That is why this type of ligation needs confirmation.

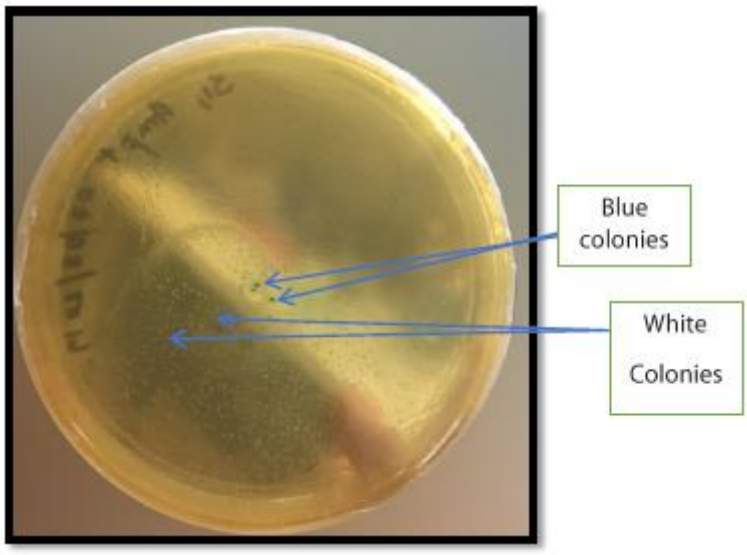


Fig. 13. LB culture plate indicating transformed and non-transformed cells

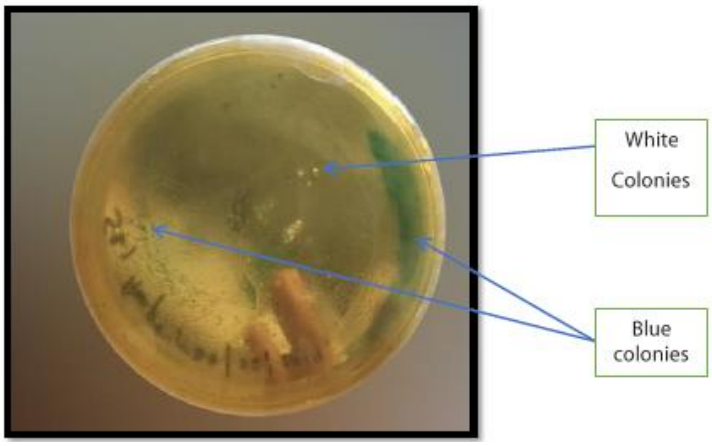


Fig. 14. LB culture plate indicating transformed and non-transformed cells

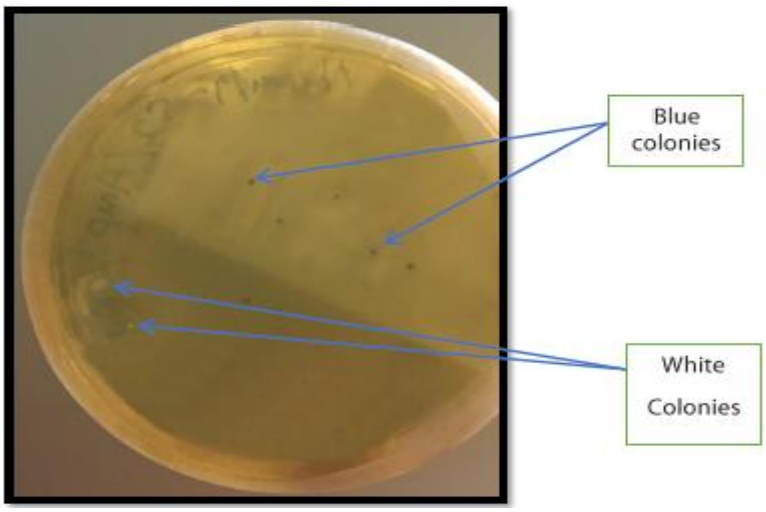


Fig. 15. LB culture plate indicating transformed and non-transformed cells

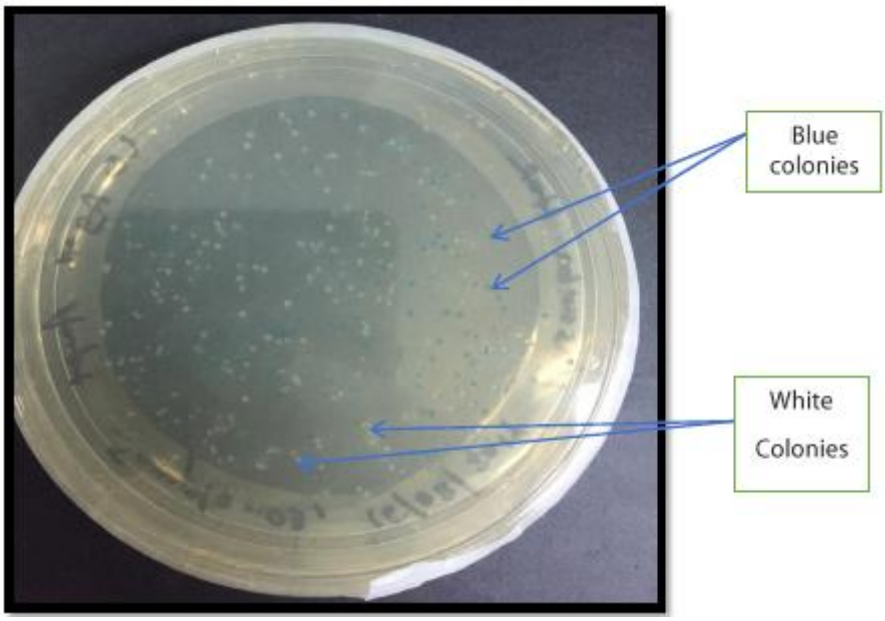


Fig. 16. LB culture plate indicating transformed and non-transformed cells

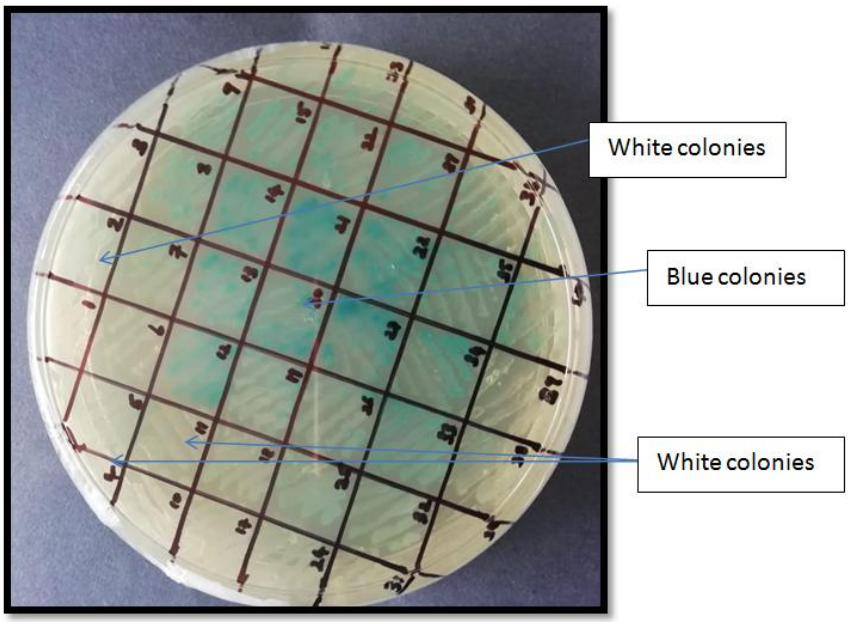


Fig. 17. LB grid culture plate indicating transformed and non-transformed cells

3.8 Colony PCR and Agarose Gel Electrophoresis

A colony PCR was one by considering the different white colonies that appeared in some selected grids on the grid plate above. It was made sure that the selected white colony was first dissolved in a volume of phosphate buffer solution as indicated in the methodology to make sure that the cell walls and membranes are broken down effectively and that the DNA comes into the solution. This is used as the DNA template. Upon visualization of the PCR products the result obtained is as follows.

Accordingly, it is seen that even though 16 white colonies from 16 different grids were selected and amplified with the use of the C3 and the C4 primers, only 8 colonies actually had the appropriate band of 265bp corresponding to the correct band size. It indicates that only 50% of the transformation has happened. Accordingly

lanes 2, 5, 7,8,9,14,16 and 17 generated the appropriate band. They correspond to the grids 11,26,24,01,03,38,30 and 05 on the grid plate. Therefore it is seen that unlike in the restriction digestion cloning, not that all white colonies in this ligation setup could have the truly positive result, meaning that the insert of interest is available. The limitations defined above are the major reason to indicate that the cells have not been transformed.

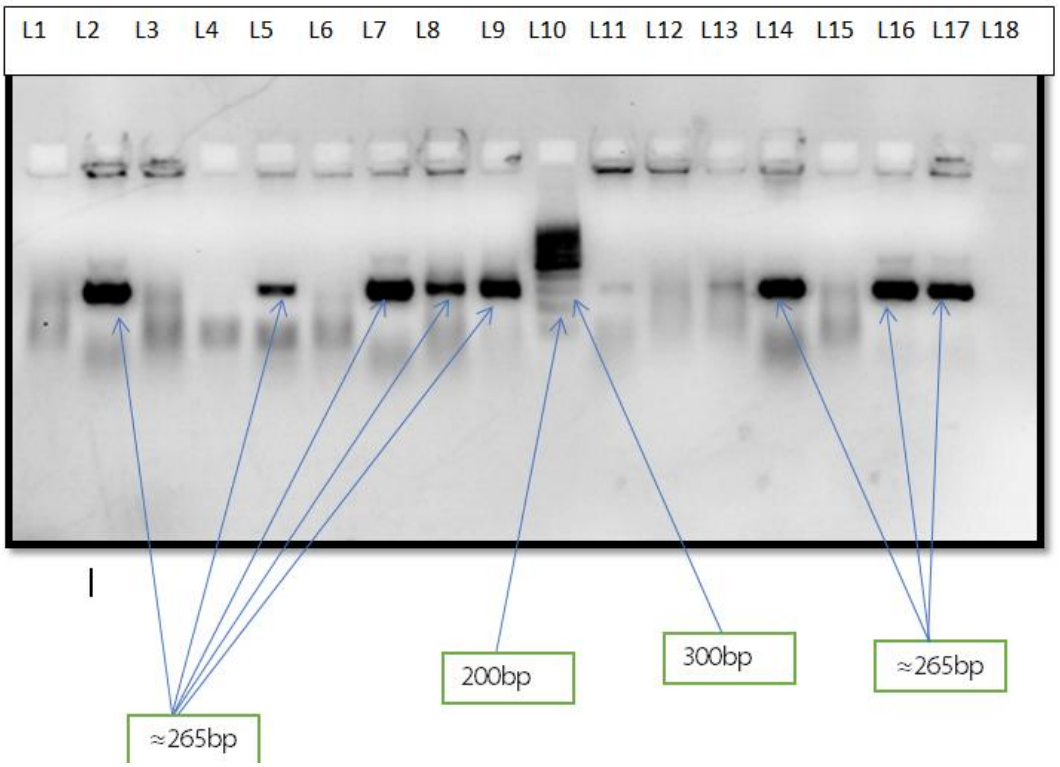


Fig. 18. Gel image of the colony PCR products
The gel legend

Table 10. Gel legend of Fig. 19

Lane (L)	Inclusion to the Well
L1	10 µL of PCR reaction from grid 06 with 1 µL bromophenol blue
L2	10 µL of PCR reaction from grid 11 with 1 µL bromophenol blue
L3	10 µL of PCR reaction from grid 18 with 1 µL bromophenol blue
L4	10 µL of PCR reaction from grid 19 with 1 µL bromophenol blue
L5	10 µL of PCR reaction from grid 26 with 1 µL bromophenol blue
L6	10 µL of PCR reaction from grid 32 with 1 µL bromophenol blue
L7	10 µL of PCR reaction from grid 24 with 1 µL bromophenol blue
L8	10 µL of PCR reaction from grid 01 with 1 µL bromophenol blue
L9	10 µL of PCR reaction from grid 03 with 1 µL bromophenol blue
L10	6 µL of 100bp DNA ladder (Ceygen Biotech,^{TM})
L11	10 µL of PCR reaction from grid 10 with 1 µL bromophenol blue
L12	10 µL of PCR reaction from grid 17 with 1 µL bromophenol blue
L13	10 µL of PCR reaction from grid 31 with 1 µL bromophenol blue
L14	10 µL of PCR reaction from grid 38 with 1 µL bromophenol blue
L15	10 µL of PCR reaction from grid 36 with 1 µL bromophenol blue
L16	10 µL of PCR reaction from grid 30 with 1 µL bromophenol blue
L17	10 µL of PCR reaction from grid 05 with 1 µL bromophenol blue
L18	10 µL of PCR reaction with 1 µL bromophenol blue as Negative Control

As such, a catalytic domain of 265bp was successfully cloned and expressed in the E.coli expression system, which was the major objective of the study.

4. CONCLUSION AND RECOMMENDATIONS

In conclusion it could be stated that the objective of the study which was to express a catalytic domain of the *CtsK* gene in bacterial expression system was successfully achieved. The feasibility study imitated towards expressing the entire CtsK gene in bacterial expression system in Sri Lanka is successful. Accordingly, with the availability of the protocol for this particular expression, gradual developments and improvements in the study could be made for the expression of the entire gene coding for Cathepsin K in bacteria, which in turn would facilitate the recombinant production of Cathepsin K and thereby, proceedings could be made towards considering further downstream applications of production and optimally purifying it along with the identification of efficient inhibitors and stimulators of the enzyme; along with incorporation of them within therapeutics. Therefore, it is strongly suggest that this initial work opens up a wide variety of further related potential research prospects in drug and pharmaceutical sciences with strong emphasis on orthopedic medicine. The outcome obtained from such research, especially, the production of recombinant Cathepsin K would be a timely tangible outcome of research centered around molecular biology that would clearly have a variety of beneficial effects on the global human population.

In addition, during the review of literature during the course of this study, it was noticed that a strong relationship exists in between a variety of cancer metastasis and the enzymatic activity of Cathepsin K in human. Therefore, on another branch of study, it is also suggested that the association and the precise biochemical pathway underlying the cancer metastasis activity of Cathepsin K in human to be studied in depth, which would be a novel area to explore, contributing towards the well been of mankind on a global platform.

ETHICAL CONSIDERATIONS

Ethical clearance for the entire study was obtained from the Wayamba University of Sri Lanka, Kuliyapitiya. (Application No: 201901HI01).

SUPPLEMENTARY MATERIALS

Supplementary material is available in this following link:
https://journalajb2t.com/index.php/AJB2T/libraryFiles/downloadPublic/5

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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