

## Production and Characterization of Recombinant Tropomyosin

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### Abstract

The aim of the research is cloning the human tropomyosin and produce the recombinant protein from them. Tropomyosin 4, tropomyosin 3 iso 2, tropomyosin 4 iso 2 and PJC20 plasmid vector were used. Competent cells were prepared by rapid and highly efficient method in order to transform the DNA for cloning. The prepared insert DNA from pcr product and plasmid vector DNA were digested with restriction enzymes such as BamHI or XhoI and NdeI followed by ligation step was carried out to produce the recombinant DNA. SDS-PAGE used to examine the expression of protein in the cell. The recombinant tropomyosin proteins were expected to characterize by using SDS-PAGE and western blotting technique and ELISA technique to check the reactivity of three different human tropomyosin isoforms with three different monoclonal antibodies. The simple method to produce the recombinant protein was described here. These proteins are useful in therapeutic replacement therapies and treatment of life threatening markers.

**Keywords** – tropomyosin, recombinant, isoforms, pcr, DNA

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Date of Submission: 21-07-2018

Date of acceptance: 12-08-2018

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### I. Introduction

The human bodies have several varieties of proteins. Those proteins play the different roles in the human bodies. Mainly the filamentous protein attached with actin fibres of muscle. In this case the Troponin regulates the interaction of both protein such as actin and myosin. That one of the proteins which are plays the role as to contraction of skeletal muscle is identified as Tropomyosin. But the compound helps to preventing muscles from building up where they are at rest. In the meantime this kind of protein act as block when the chemical process doing. That action produces muscle contraction by wrapping them self around chains of other protein present in muscle which is known as Actin. Another type of protein called as myosin, it should be able to bind with actin protein in order for muscles to contract. These whole structure help to structure of tropomyosin and functions. The role of tropomyosin is preventing muscle contraction it means sliding filament mechanism is happening to muscle contract. In the muscle cells it creates muscle fibres as bundle format. Presented proteins Actin and myosin arranged in alternating filaments. The myosin is play as movement protein which is produces the force behind muscle contraction by sliding back and forth along the actin filaments. (Nitanai et al ;2007)

Over the last few decades, the large area of molecular research studies distinguished multiple tropomyosin isoforms in cell tissue of mammalian. human four tropomyosin genes  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  are responsible for generating multiple different tropomyosin isoforms, formally known as TPM1, TPM2, TPM3 and TPM4. The research evidence says these tropomyosin isoforms are the major determinants for function of actin cytoskeleton, such as cell migration, intercellular vesicle movement, cell proliferation, cytokines and apoptosis. The in vivo and in vitro biochemical studies suggest that different types of tropomyosin isoforms have variance in their actin-binding properties and different actin-binding protein function. That's why they show the specification in actin microfilaments. (Lin et al;2008)

Tropomyosin isoforms are produce by the combination of different gene and alternative splicing. In the mammals, the four genes of tropomyosins are producing a large number of protein isoforms by alternative splicing. The history of tropomyosin says that tropomyosin belongs to two major classes; High molecular weight (HMW), containing 284-285 amino acids and Low molecular weight (LMW), contain 245-248 amino acids. The alternative promoters are generate this size difference which is leading to the inclusion of high molecular weight isoforms exon 1a and 2a or 2b or low molecular weight exon 1b at the N-terminus of the protein. Tropomyosin isoforms utilizing the alternative internal splicing with mutually exclusive of exons 6a or 6b and at the c-terminus exon 9a and 9d in the  $\beta$  tropomyosin gene, 9a, 9c and 9d in the  $\gamma$  tropomyosin gene and 9a, 9b, 9c and 9d in the  $\alpha$  tropomyosin gene to produce over the 40 tropomyosin isoforms (gunning et al; 2005)

DNA cloning helped to make recombinant DNA commonly used method for the development of recombinant is polymerase chain reaction. Under this study the organism which donates the DNA for the formation of recombinant is call donor organism. The donated DNA is call vector. Generally vectors are

deriving from bacterial plasmid. This vector DNA molecules have the capable of individually replicates with in a living host cell and these circular DNA molecules used as carriers to artificially carry the foreign DNA fragments in to the another cells. The vector DNA with their inserts are called recombinant DNA (miller,1999).

The development of recombinant DNA technologies are biochemically useful to produce the particular proteins for therapeutic use in replacement therapies and treatment of life threatening markers. (pandyshivanand). Tropomyosin recombinant DNA is one of them, plays the role in cancer, allergies and muscle disease. These procedures help to produce the functional protein from the therapeutic gene (Liras, 2008)

## **II. Material And Methods**

### **2.1Preparation of LB –broth medium**

0.5g yeast extract, 1g bactopectone, 0.5g Nacl were weighed and dissolved in to the distilled water. Then the pH of the solution was measured and adjusted to pH 7.5 with the help of con NaOH and con HCL. Then distilled water was added to make up to 100 ml and placed in to the autoclave for 2 hours. The starting temperature was 121 °C. After autoclave reached 70°C, LB broth was allowed to cool to 55°C.

### **2.2Preparation of LB liquid media and agar plates with Ampicillin**

LB-broth was prepared similarly as described above. Then 100µl ampicilin was added in to the 100 ml LB agar medium. Poured the thin layer of LB agar amp solution into petri dishes and swirled in a circular motion to distribute agar on bottom completely. Then allowed to cool.

### **2.3Growth of Escherichia coli bacteria cells on agar plates. (XL-1, BL-21)**

LB agar plates (without ampicilin) were prepared. 1µl XL-1 was transferred on LB agar plate and it was speared on the plate. Likewise 1µl BL-21 was transferred on another LB agar plate and spread by the loop. Then the plates were kept in the 37°C incubator for overnight for grow the bacteria cells.

### **2.4 Prepare the starter culture of the cell**

One colony of XL-1 was selected from LB agar plate by using a sterile pipette tip. Then dropped the tip in the 1ml LB solution (no ampicilin). Likewise one colony of BL-21 was selected from LB agar plate by sterile pipette tip then dropped the tip in 1ml LB solution and were placed in the 37°C shaken incubator for overnight.

### **2.5Make the own XL-1 and BL-21 Escherichia coli competent bacteria cells**

Media A (LB broth supplemented with 10mM MgSO<sub>4</sub> 7 H<sub>2</sub>O and 0.2% glucose) was prepared. 1ml glucose and 500µl mgSO<sub>4</sub> .7 H<sub>2</sub>O were added in to the 50 ml LB broth and mixed well. Then transferred 1ml media A in to the 1ml microcentrifuge tube and kept in ice. Then 1ml of XL-1 overnight starter culture was incubated with media A culture. The allowed to grow with aeration in media A in 37°C shaken incubator.

Likewise another media A culture incubated with 1ml BL-21 overnight starter culture. Then grown with aeration in medium A in 37°C shaken incubator for 4 to 5 hours.

After cells grown, the both 50 ml culture transferred in to two 50 ml tubes. Then they were kept in ice for 10 minutes. Then centrifuged at 1500g for 10 minutes. Again kept in ice (4°C). After that, 500 µl of medium A pre cooled on ice was added and resuspended gently. Then 2ml storage solution B [36% glycerine, 12% Peg (MW7500), 12mM MgSO<sub>4</sub>.7H<sub>2</sub>O added to LB-broth (pH 7.0) and sterilized by filtration] was added and mixed well. The XL-1 competent cells were divided in aliquots of 250µl each in 12 eppendorf tubes. Likewise BL-21 competent cells were divided in aliquots of 250µl each in another 12 eppendorf tubes. Then stored at -80°C until use it. (Nishimura,1990)

### **2.6 Prepare the PJC20 plasmid vector DNA for cloning.**

competent cell XL-1 and BL-21 were taken from the -80°C refrigerator. Transferred 125µl of XL-1 cell in to fresh sterile 1.5 ml microcentrifuge tube. Then transferred 125µl of BL-21 cell in new sterile 1.5 ml microcentrifuge tube. Kept the four tubes and purified PJC20 and SUMO in ice. 1µl of PJC20 was transferred in to 125µl of XL-1 and BL-21 cells. Likewise 1µl SUMO was transferred in to 125µl another XL-1 and BL-21 cells. Then kept the four tubes in ice for 15-30 minutes. Then heat shock was given at 42.5°C for 90 second. Again kept in ice for 2 minutes. Taken out from ice and 900µl sterile LB-broth was added in each tube. then incubated for 1 hour at 37°C. Then 100µl PJC20 XL-1 was transferred on LB agar amp plate and spread over on it with the help of loop. And 100µl PJC20 BL-21 was transferred on LB agar amp plates and spread over on it with loop. (low concentration) Likewise the same step was done for SUMO XL-1 and SUMO BL-21 (low concentration)

Then the four tubes were centrifuged for 30 second. Supernatant was removed. Then pellet was resuspended and transferred on another four LB agar amp plates. (high concentration). placed the eight plates in

the 37°C incubator for overnight. Here SUMO was used as control. Transformation frequency was calculated by counting the colony on the plates.

### 2.7 Growth of the Bacterial culture with introduced plasmid DNA

Picked two colonies from each LB agar amp plates of PJC20 XL-1 and PJC20 BL-21 by using sterile pipette tip. Dropped the tip in the 1ml LB ampicillin solution and swirled. Then each tube was split in to half and 500µl LB amp solution was added in each tube. Incubated these bacteria culture at 37°C in a shaken incubator for 12-18 hours. Then kept in -80°C.

### 2.8 Extract plasmid DNA from Bacteria culture by using mini prep protocol

The grown 2 ml bacteria culture was taken out from the shaken incubator. Centrifuged at 5000g for 1 minute. The supernatant was removed. Then 250µl P1 buffer was added and vortex. And resuspended the pellet by pipetting up and down. 250µl P2 was added and mixed well. And 350µl N3 buffer was added and mixed well. Then the tube was centrifuged at 17000 g for 10 minutes. The supernatant was poured in to the silica column and spun at 17000 g for 1 minute to bind the DNA with column. Removed waste. 750µl PE was added to the column. Centrifuged at 17000 g for 1 minute and removed the supernatant. Then the silica column was placed in the 1.5 ml microcentrifuge tube. 50µl pure water was added on silica membrane. Left for 1 minute to elute DNA. Then centrifuged for 1 minute. Column was removed. Then final DNA was kept in -20°C ice.

### 2.9 Estimate the plasmid vector DNA concentration by Agarose Gel Electrophoresis

0.5% Agarose powder was measured and poured in to the conical flask. And added 50ml TAE. Then the flask was heated in microwave for 1-3 minutes until agarose completely dissolved. Every 10 second the flask was taken out and swirled well. Agarose solution was allowed to cool down for 15 minutes. After that 2µl 10mg/ml ethidium bromide was added and mixed well. Agarose was poured in to a gel tray with the well comb and allowed to set the agarose gel for 15-20 minutes until it solidified. Then the comb was removed carefully. Filled the gel box with 10xTAE until the gel was covered. Then each sample was carefully loaded in to the wells. And 2 µl plus DNA ladder also loaded. Set up the voltage 80V and current 100 mA and switched on the power supply for 20 minutes for 20 minutes. After 20 minutes the vector DNA bands were visualized under UV light.

### 2.10. Generate insert DNA from PCR product ( amplify insert DNA from a template by pcr)

Reagents	Tube 1	Tube 2	Tube 3
10x thermopol reaction buffer	5µl	5µl	5µl
dNTP	1.5µl	1.5µl	1.5µl
Forward primer	1.5µl RMM098	1.5µl RMM112	1.5µl RMM114
Reverse primer	1.5µl T7R	1.5µl RMM113	1.5µl RMM115
DNA tag polymerase	0.2µl	0.2µl	0.2µl
Template	0.5µl Tpm4	0.5µl Tpm3 iso2	0.5µl Tpm4 iso2
water	40µl	40µl	40µl

Three pcr tubes were prepared as shown in the above table. After prepared, spun the tubes then placed in pcr thermocycle for the pcr reaction. Initial denature the sample at 94°C for 2 minutes. Denature at 94°C for 20 second. Anneal the primers at 50°C for 2 minutes. Extend at 72°C for 1 minute. Final extend at 72°C for 4 minutes.

These steps were repeated 30 times. Then kept the three samples at 4°C. Run the 3 samples in agarose gel electrophoresis to check whether the pcr product work or not.

### 2.11 Purify the PCR products

The prepared pcr tubes were taken from ice. 150µl binding buffer was added in to each tube. After that 50µl isopropanol was added. Transferred each sample in to silica spin column. Centrifuged at 8000 g for 1 minute. Filtrate was discarded. Then spin column was washed with 750µl washing buffer. Then again centrifuged at 8000g for 1 minute. Filtrate was discarded. Spin columns were replaced in 1.5 ml collection tubes. Then 30µl pure water was added and spun at 8000 g for 1 minute. After that 20µl pure water was added and spun at 13000g for 1 minute. Purified pcr products were kept in ice.

### 2.12 Digest the insert DNA and vector DNA with restriction enzyme

NdeI, bamHI and XhoI restriction enzymes were used for the digestion. Following reaction components were prepared according to the table.

**Insert DNA digestion**

1	2	3
5µl buffer 4	5µl buffer 4	5µl buffer 4
1µl NdeI	1µl NdeI	1µl NdeI
1µl BamHI	1µl BamHI	1µl BamHI
43µl per product 1	43µl per product 2	43µl per product 3

**Vector DNA digestion**

XL-1 PJC20 (1)	XL-1 PJC20 (2)
5µl buffer 4	5µl buffer 4
1µl NdeI	1µl NdeI
1µl XhoI	1µl BamHI
43µl XL-1 PJC20	43µl XL-1 PJC20

5 tubes were spun quickly. And incubated at 37°C for 1 hour.

**2.13 Gel purification**

The all digested sample were run in the agarose gel electrophoresis and Visualized under uv light .then by using visualization specks and blade, the DNA bands were cut down in the dark room under uvlight.and put it in to the 1.5 ml tubes.

**2.14 Gel clean up procedure**

DNA band in gel slices were weighed out. And 3 volumes of binding buffer were added for every 1 volume of the gel slice. Then, incubated at 50°C for 10 minutes. After that 1x original gel slice volume of isopropanol was added and mixed by repeated pipetting. Added up to 800µl sample to the spin column in supplied collection tube. Centrifuged at 8000g for 1 minute and the filtrate were discarded. 750µl diluted wash buffer was added. Centrifuged at 8000g for 1 minute. Filtrate was discarded.centrifuged again at 8000g for 1 minute. Placed the spin column in new 2 ml collection tube. And 30µl pure water was added. It was allowed for 1 minute. And centrifuged at 8000g for 1 minute. Then again 20µl pure water was added .allowed to settle down. Centrifuged at 13000g for 1 minute. Then kept in ice. After purified the digested insert and vector DNA, run the gel electrophoresis to estimate the concentration of DNA bands.

**2.15 Ligation of insert and vector DNA**

Ligation 1	Ligation 2	Ligation 3
3µl digested pcr product 1	3µl digested pcr product 2	3µl digested pcr product 3
14µl digested XL2 PJC20	14µl digested XL1 PJC20	14µl digested XL2 PJC20
2µl ligation buffer	2µl ligation buffer	2µl ligation buffer
1µl DNA ligase	1µl DNA ligase	1µl DNA ligase

According to the gel picture, insert DNA has 5 times high intensity than vector DNA.so 5 times more volumes of digested vector DNA was used for the ligation. 5 tubes of digested samples were vortex followed by ligation as shown in the table. Then three ligation mixes were centrifuged for 60 sec at 8000g. And placed in thermo cycle for 30 minutes at 20°C.

**2.16 Transformation of recombinant DNA**

Transformation of 10µl ligated mix (recombinant DNA) in to Escherichia coli XL-1 competent cell was done similarly as described above in the methodology.

**2.17 Growth of bacterial cell with introduced recombinant DNA**

Selected single colonies were marked on the agar plate.and Picked each marked transformed colony by using sterilized pipette tip. Dropped the tip in 1ml LB amp solution. Placed in the shaken incubator to grow the cell for 4 -5 hours.

**2.18 PCR colony screen.**

40µl 10x pcr buffer ,8µl dNTPs,8µl T7 forward primer,8µl T7R,1µl DNA tag polymerase,335µl water were used to prepare the 400µl pcr reaction mixture .Then the pcr mixture was vortexed . 24µl of mixture was transferred in to each 16 sterilized tubes. By using sterile tip, each colony which already marked was lightly touched and gently pipette up and down to mix the cells with pcr reaction components. XL-1 PJC20 vector colony was mixed in the 16<sup>th</sup> tube as blank. Then the 16 samples were placed in the thermo cycle for 2 hours for the pcrreaction.Then kept the 16 samples at 4°C. Run the 16 samples in agarose gel electrophoresis to check whether the insertion in vector is good or not.

### **2.19 Growth of XI-1 bacterial culture with introduced recombinant DNA**

With the help of the gel picture, grown cell tubes were selected from the shaken incubator. Then split each tube in to half in another 6 fresh tubes. Then 1ml LB amp solution was added in to each 12 tubes which contained 500µl cell. Placed in the 37°C shaken incubator for 12-18 hours to grow the cell.

### **2.20 Purification of recombinant DNA**

Same mini-prep protocol was used to purify the DNA and run the gel electrophoresis to check whether the purified sample has the DNA or not.

### **2.21 Mini digestion with the restriction enzyme for the ligated DNA**

BamHI, NdeI and XhoI restriction enzymes were used for the mini digestion. 6 reaction components were prepared ( 0.5µl NdeI, 0.5µl BamHI or XhoI, 8µl DNA and 1µl fast digested buffer) and mixed gently by pipetting. Incubated at 37°C for 30 minutes. Digestion was verified by running the sample of the reaction mix on an agarose gel.

### **2.22 DNA sequencing**

After mini digestion, 15µl each DNA samples were transferred in to new fresh tubes and placed in speed vacuum to dry the DNA for 30 minutes. Then they were sent for the DNA sequencing.

### **2.23 Transformation of Recombinant DNA in to Escherichia coli BL-21 competent cell**

2 µl each recombinant DNA samples were added in to each 75µl of BL-21 competent cell. Then kept the three tubes in ice for 15-30 minutes. Then heat shock was given at 42.5°C for 90 second. Again kept in ice for 2 minutes. Taken out from ice and 900µl sterile LB-broth was added in each tube. Then incubated for 1 hour at 37°C. After 1 hour incubation three tubes were centrifuged for 30 second. Supernatant was removed. Then pellet was resuspended. The fresh LB agar amp plates were taken out from fridge. Each pellet was transferred on each LB agar amp plate and spread over on it with the help of loop. (High concentration) .placed the plates in the 37°C incubator for overnight.

### **2.24 Growth of BL-21 bacterial cell with introduced recombinant DNA**

Picked the single colony from each plate by using sterilized pipette tips. Incubated in 1ml LB amp solution in sterile 1.5 ml tubes in shaken incubator at 37°C until it grow 4 -5 hours.

### **2.25 Screening the cells for protein expression with IPTG promoter.**

After cells grown, 500µl each cell mixture was added in to each 500µl LB amp solution ( used as control) and 500µ each cell mixture was added in to 500µl LB amp+IPTG ( 15ml LB-broth +15µl ampicillin +30µl IPTG)

All the samples were kept in the shaken incubator at 30°C for overnight to harvest the cell. The after overnight incubation 125µ harvested cells were transferred in to fresh tubes and they were labelled carefully. Then they were centrifuged for 1 minute at 8000 g. Supernatant was discarded and pellet was resuspended. After that 20µl pure water and 20µl 4xSDS loading buffer were added in each tube. Heated at 95°C for 4 minutes.

### **2.26 Set up the SDS PAGE gel electrophoresis to analyse the expression of protein.**

Preparing the resolving gel, 1.8 ml acrylamide, 1.0 ml resolving buffer, 1.2 ml water, 25µl APS was added in to the small beaker. And 7µ TEMED was added at last and swirled the solution gently and immediately resolving gel was pipette in to the gap between the glass slides. Iso propanol was added to level up the separating gel. Waited for 15-20 minutes to set the gel.

Preparing the stacking gel : 535µl acrylamide, 500µl stacking buffer, 950µl water ,15µl APS and at last 5µl TEMED were added in to the small beaker .then pipette between two glass slides until a overflow. Well forming comb was inserted between the two glass slides without trapping air under the teeth. Waited for 30 -60 minutes for the gelate. After complete gelation of stacking gel, the comb was taken out. Then glass slides were taken out of the casting frame and they were set in the cell buffer dam. The running buffer was poured in to the inner chamber until buffer surface reached required level. 10µl of each 22 samples and 5µl SDS PAGE marker was loaded in to the wells. Set up the voltage at 200v and current 80mA and switched on the power supply. Gel was allowed to run 30-35 minutes. Then was staining with coomassie blue. After the required staining time, analysed the protein bands and gel was let it to dry.

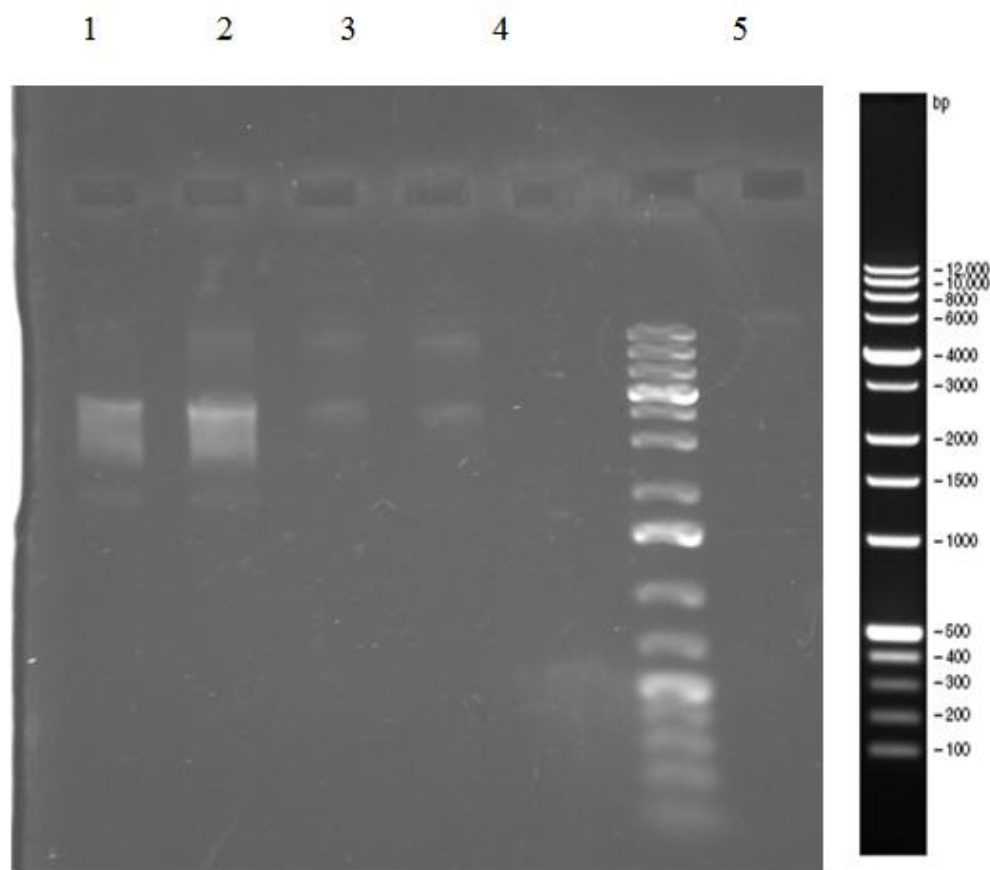
## **III. Results And Discussion**

This section describes the result of cloning of human tropomyosin and production of recombinant protein. The purpose of making the competent bacteria cell to take up the plasmid DNA. A rapid and highly

efficient method was used for the preparation of competent *Escherichia coli* cells. While prepared the *Escherichia coli* XL-1 and BL-21 competent cells, used PEG (*polyethylene glycol*),  $Mg^{2+}$  and glucose for the preparation and storage of competent bacteria cells. Transformation efficiency stimulates by the  $Mg^{2+}$  and the glucose in the medium, increase the growth rate and enhancing the transformation efficiency. (Nishimura et al ;1990).

while grow the *Escherichia coli* XL-1 and BL-21 competent cells on LB agar plates, the plates were prepared without ampicilin .because the bacteria cells are not antibiotic resistance.ampicilin prevent bacteria from growing. Competent bacteria cell become resistance to ampicilin when do the transformation (when the bacterial cell absorb the DNA). The plasmid DNA (PJC20) normally containing the resistance to the ampicilin . Transformation was done for PJC20plasmid DNA in to the competent bacteria XL-1 cell, which is sensitive to the ampicilin.. Then bacterial cells were spreaded over the LB agar plate that contains ampicilin. The ampicilin give the selectivity pressure because the competent XL-1 bacteria which have absorbed the PJC20 plasmid DNA only will grow on the agar palte.XL-1 cell is 10 times high efficiency than BL-21 to absorb the DNA. It has high efficiency for the DNA transformation.

Transformation was done by the heat shock method. It is the process on introduce the foreign DNA in to the bacterial cell. DNA will bind to the cell surface by up taking the DNA across the membrane in to the cytoplasm. *Bacillus subtilis*, *streptococcus Pneumoniae* and *Haemophilus influenza* organism have the ability to competent naturally during the growth. *Escherichia coli* take up the plasmid DNA only in the high concentration of divalent cations.(Weston et al;1981)



**Figure 1: agarose gel electrophoresis with PJC20 pasmid DNA.**

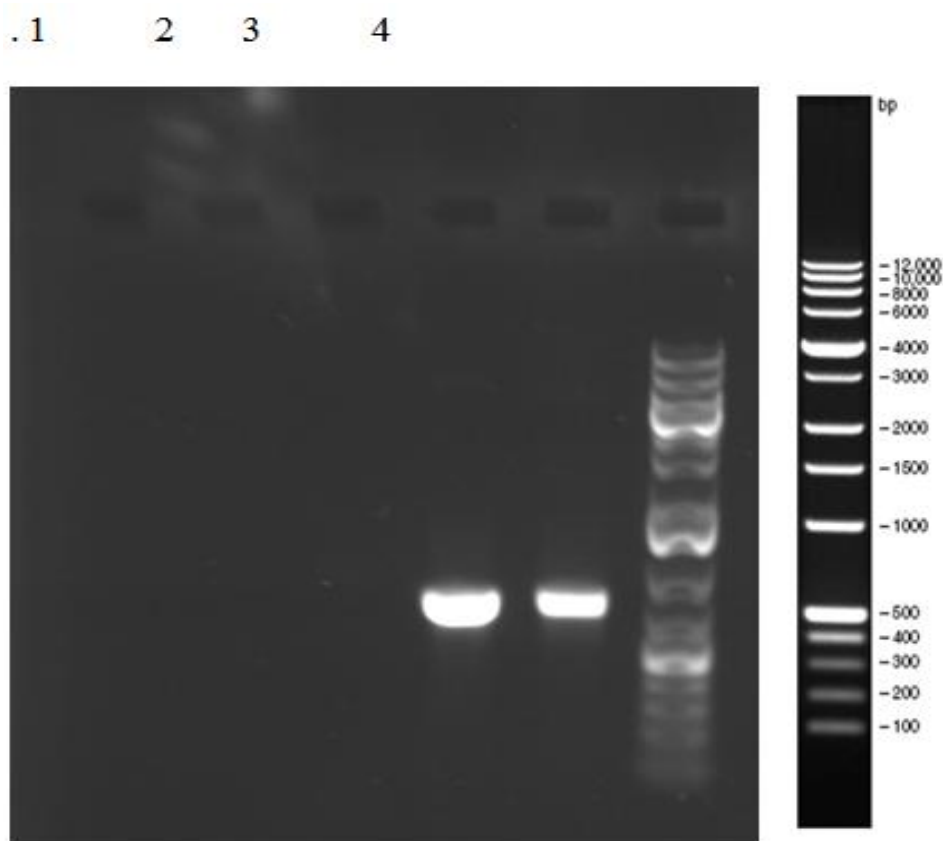
- 1) 4µl PJC20 plasmid vector DNA in *Escherichia coli* XL-1 competent cell
- 2) 4µl PJC20 plasmid vector DNA in *Escherichia coli* XL-1 competent cell
- 3) 4µl PJC20 plasmid vector DNA in *Escherichia coli* BL-21 competent cell
- 4) 4µl PJC20 plasmid vector DNA in *Escherichia coli* BL-21 competent cell
- 5) 2µl Plus DNA ladder

Figure 1 shows the results of the prepared PJC20 and SUMO plasmid vectors. SUMO was taken as control. Bands were analysed under uvligh , they were appeared in the expected range of 2000-3000 bp.

In the agarose gel electrophoresis ethidium bromide was used. Because it is a fluorescent dye. It is used for the staining of nucleic acid. It will bind with the DNA and allow visualizing the clear bands.While

generating the insert DNA from pcr product, 3 pair of primers were used. RMM098 and T7R for tropomyosin 4 long chain, RMM112 and RMM113 for tropomyosin 3 iso short chain and RMM114 and RMM115 for tropomyosin 4 iso 2 short chain. Polymerase chain reaction produces large number of copies of a gene. This is very important to have enough starting DNA.pcr has three major steps: denaturation, annealing and extension. During the denaturation at 94°C the double stand of DNA melt by disturbing the hydrogen bond between the complement bases of DNA structure and yield single strand DNA. In the annealing stage at 50°C primers bind to their complementary DNA then begins the DNA synthesis by DNA polymerase. In the extension step at 72°C , by adding the dNTPs DNA polymerase synthesis the new strand DNA to the DNA template strand from 5'-3' direction ( pandeyshivanad.)

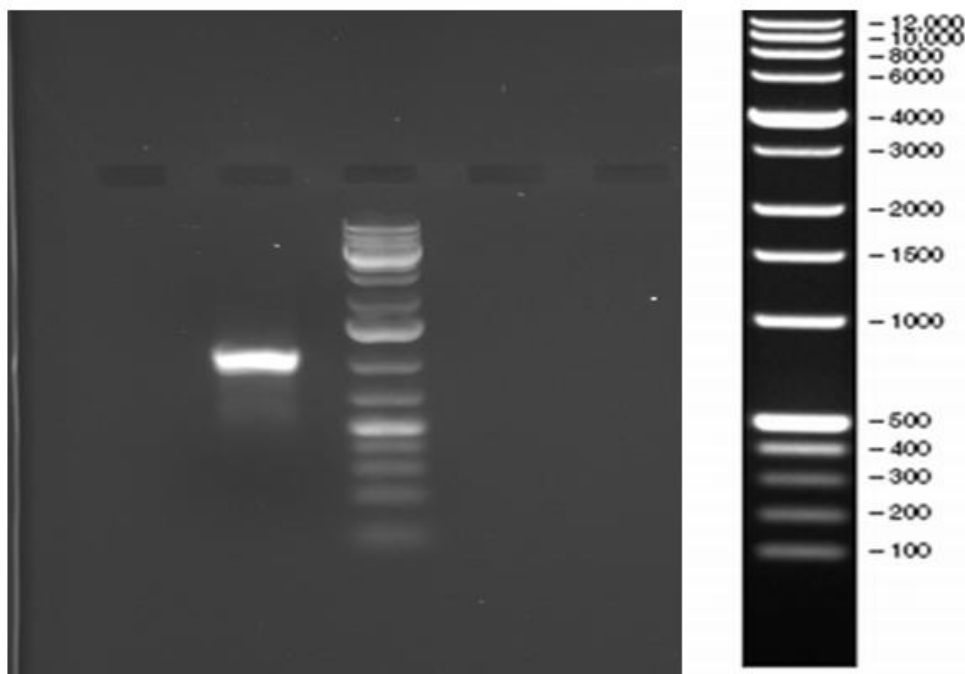
By using the bio informatics method,targeted human tropomyosin 3 and human tropomyosin 4 DNA sequence and protein sequence were designed. Variant results of DNA sequence of tropomyosin were obtained. The identity was checked in the bio edit by translating the nucleotide to amino acid. The result is all the sequence was identical. The primers for the pcr reaction for long tropomyosin 4 also designed.



**Figure 2: Gel electrophoresis of pcr products.(insertion DNA) .**

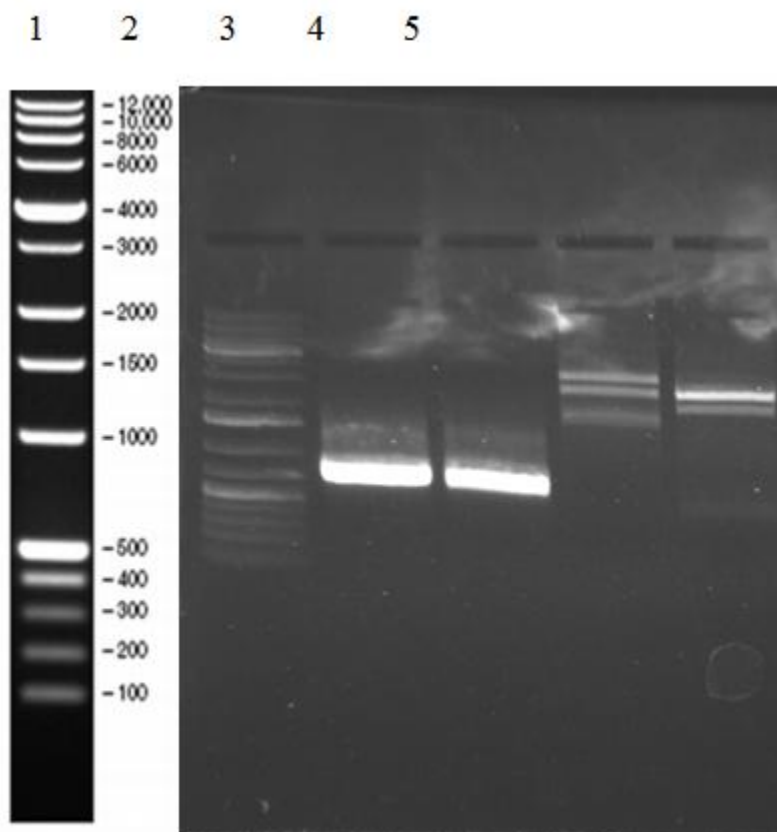
- 1) 4µl pcr product 1 (tropomyosin 4) .
- 2) 4µl pcr product 2 (tropomyosin3 iso 2) .
- 3) 4µl pcr product 3 ( tropomayosin 4 iso2).
- 4) 2µl plus DNA ladder.

The figure 2 shows in the results of generation of insert DNA from pcrproducts. The bright bands were appeared for tropomyosin4 iso 2 and tropomyosin 3 iso 2 in the expected range 750-1000 bp.Tropomyosin 4 bands of long chain was not achieved in this particular experiment because of unknown reason ,may be the error of pipetting accuracy.



**Figure 3: gel electrophoresis for pcr product 1 (insert DNA tropomyosin 4)**

The figure 3 shows the clear bands for repeated tropomyosin 4 from pcr product. These results say the insertion DNA is standard to carry on the cloning process.



**Figure 4: Gel electrophoresis of digested insert DNA samples and vector DNA samples.**

- 1) 2µl plus DNA ladder
- 2) Digested pcr product 2 ( tropomyosin3 iso 2 digested with NdeI and XhoI).
- 3) Digested pcr product 3 ( tropomyosin 4 iso 2 with NdeI and BamHI).



4) Digested PJC20 vector DNA with NdeI and XhoI .

5) Digested PJC20 vector DNA with NdeI and BamHI..

This image shows two clear bands for pcr products 2 and 3 in the expected range of 750-1000bp.

1 2 3 4 5

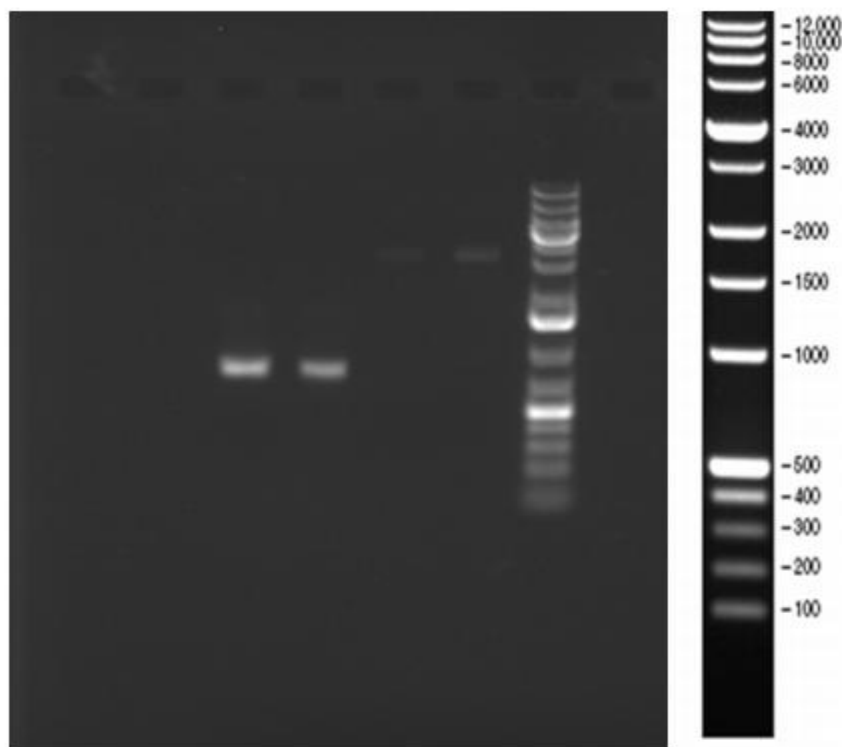


Figure 5: gel electrophoresis after clean up the DNA bands

1) 4µl Purified pcr product 2 (tropomyosin3 iso 2 digested with NdeI and Xhoi).

2) 4µl purified pcr product 3 (tropomyosin 4 iso 2 with NdeI and BamHI).

3) 4µl purified PJC20 vector DNA digested with NdeI and XhoI .

4) 4µl purified PJC20 vector DNA digested with NdeI and BamHI..

5) 2µl plus DNA ladder.

Figure 5 shows 2 clear bands in the expected range of 750-1000 bp for digested insertion DNAs. And two faint bands in the range of 2000-3000bp for the digested vector DNAs.

Digest the DNA with restriction enzyme was the most careful step. Selecting the restriction enzyme that cut with sticky ends of primers are very important. BamHI, NdeI and XhoI were used in my experiments. With the help of restriction enzyme map, the restriction sites of BamHI, NdeI and XhoI were found.

BamHI NdeI XhoI

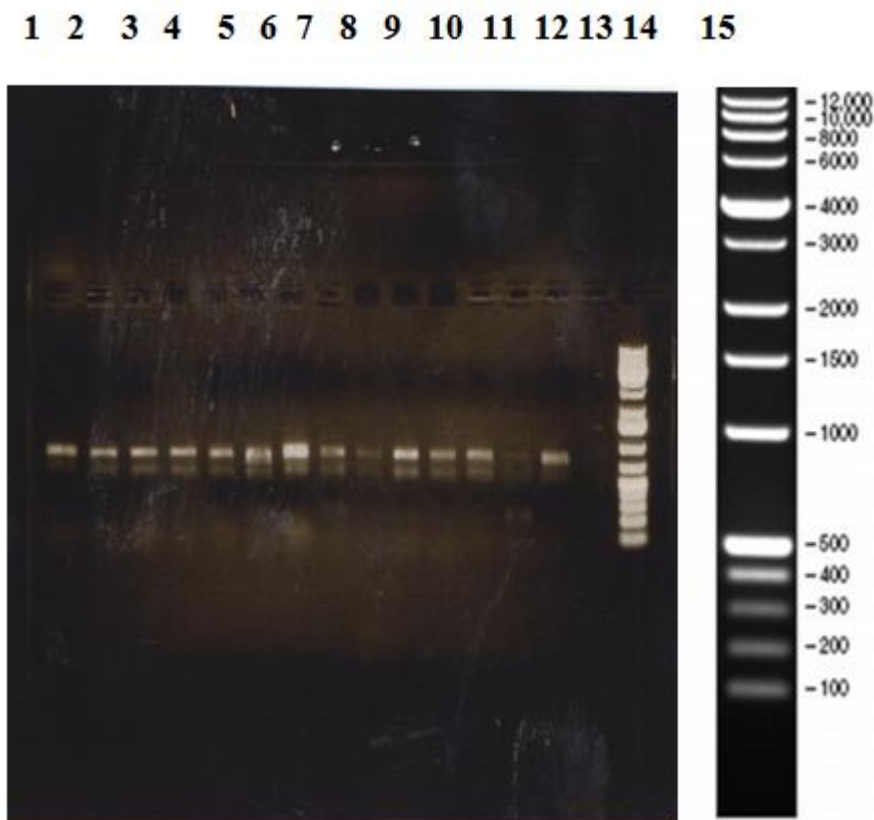
5'GGATCC3'      5'CATATG3'      5'CTCGAG3'

3'CCTAGG5'      3'GTATAC5'      3'GAGCTC5'

Then choose which sequence is found the least amount of time in the tropomyosin gene. For tropomyosin 4, NdeI and BamHI. For tropomyosin 3 iso 2, NdeI and XhoI and for tropomyosin 4 iso 2, NdeI and BamHI.

For the cloning the insert DNA and plasmid should be cut with the same restriction enzyme. Restriction enzymes are known as bacterial enzyme. It will recognize the specific 4-8 base pair sequence. This is called restriction sites and it will cleave double strand DNA at this site. The restriction site sequence is similar to each DNA strand when it is read in the 5' - 3' direction. (Lodish et al; 2000)

Restriction enzyme cut within the sequence of targeted gene. But in a pair of staggered cuts between the G and A nucleotide. This staggered cut will leave a pair of identical single strand "sticky ends". These ends are known as sticky. Because these sticky ends can make hydrogen bonds and stick to a complementary sequence. When the insert and vector DNA cut with the same restriction enzyme, both will make fragments with same complementary sticky ends. So sticky ends of the vector will bind to the sticky end of the insert fragment, when we do the ligation. (Miller et al; 1999).



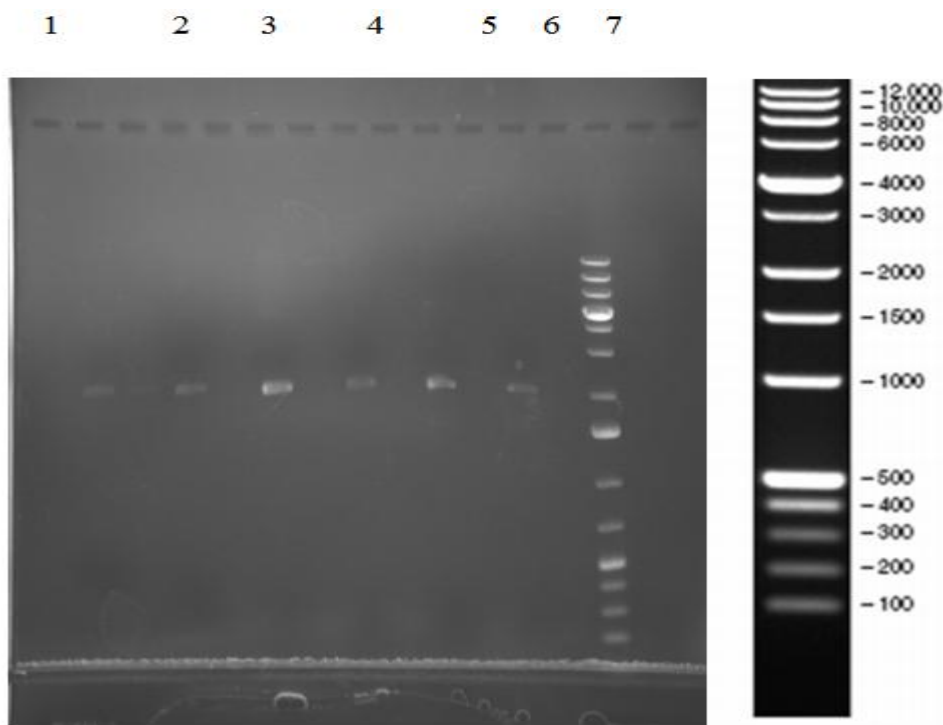
**Figure6 : Gel electrophoresis after pcr colony screening**

Wells(1-4)- ligated pcr product 1( tpomyosin 4 inserted in to PJC20 vector DNA),  
 Wells(5-9) - ligated pcr product 2( tropomyosin 3 iso 2 inserted in to PJC20 vector DNA),  
 Wells(10-14)-ligated pcr product 3( tropomyosin 4 iso 2 inserted in to PJC20 vector DNA).  
 Well 15 - plus DNA ladder

Figure 6 shows, excellent band for each sample in the range 750-1000bp.

In the ligation step, when we mix the insert and vector DNA which are digested with the same restriction enzyme, the sticky ends of the both DNA will bind with each other and produce recombinant DNA. The digestion with plasmid DNA is converted to the linear DNA with sticky ends. With the use of restriction enzymes the insert DNA also produce the fragment with same sticky ends. Then the DNA ligase enzyme will catalyze the formation of 3'-5' by creates the phosphodiester bonds covalently between the short restriction fragments. T4 ligation buffer containg ATP and Mg<sup>2+</sup>.it is very important for the ligase activity. (Miller et al;1999).

In order to check the efficiency of the insertion, pcr colony screening was done.T7 and T7R products were used in the PCR screening. PJC20 is T7 based plasmid vector. While the pcr screening T7 and T7R will bind to the vector .not with the gene. And pcr will amplify the inserted gene. It will not amplify the control vector.the expected band for empty vector was 170bp. The size means, bit of vector between the restriction sites. Expected bands for inserted tropomyosin gene were (170+750) bp.The results in the figure6 suggested the insertion has happened efficiently.



**Figure 7: gel electrophoresis after extract the recombinant DNA from bacteria culture using mini prep protocol.**

Well 1 and 2 - purified inserted tropomyosin4 in PJC20 vector DNA.

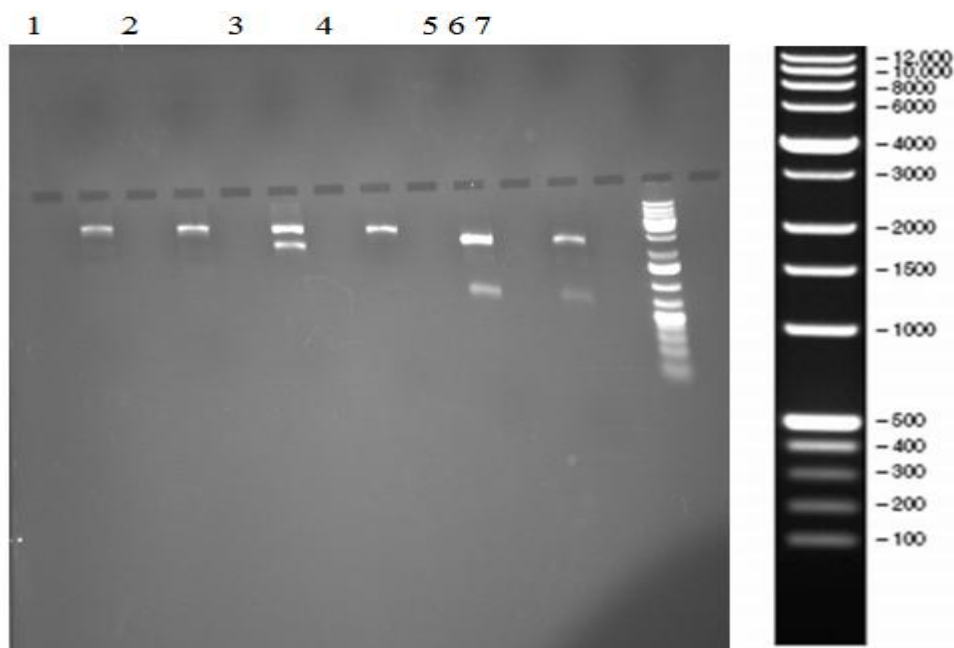
Well 3 and 4 -purified inserted tropomyosin 3 iso 2 in PJC20 vector DNA

Well 5 and 6 - purified inserted tropomyosin 4 iso 2 in PJC20 vector DNA.

Well 7- plus DNA ladder.

Figure 7 shows the gel picture of purified recombinant DNA from bacteria cell using mini prep protocol. Clear bands were appeared at 1500bp. So this result suggests the mini prep has worked. It has DNA.

Mini digestion was done with fast digestion buffer and with the same restriction enzymes for the confirmation the insertion. Double bands were expected for each sample. Band for insert DNA in the range of 750-1000bp and band for vector DNA in the range of 2000-3000bp.



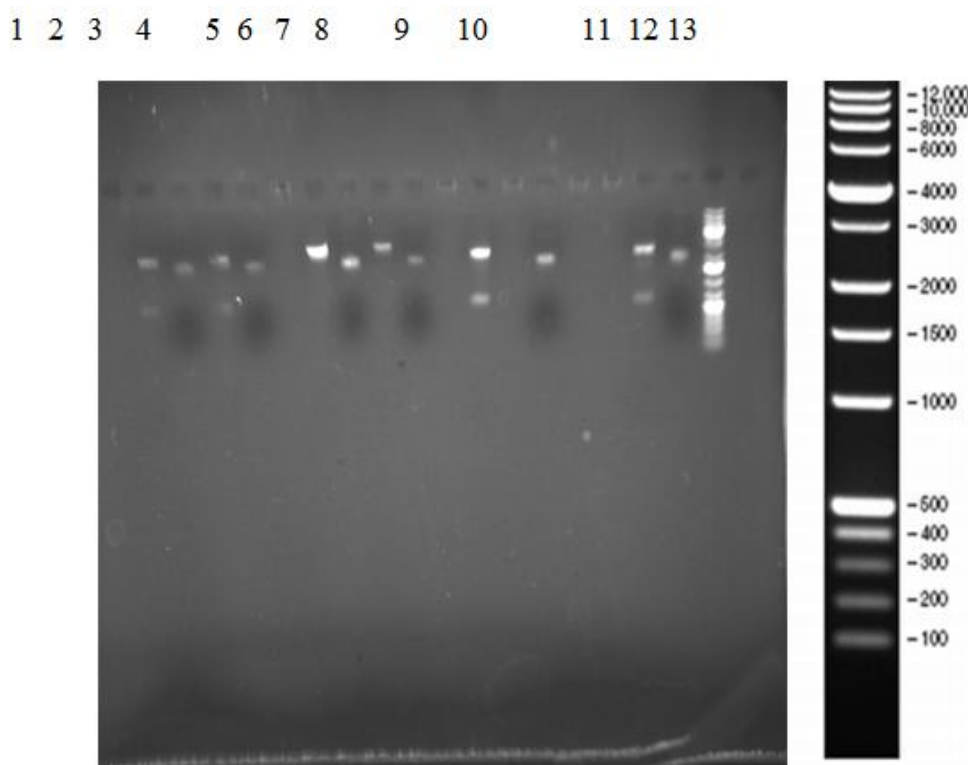
**Figure 8: Gel electrophoresis after the mini digestion**

Wells (1 and 2)-4µl of digested tropomyosin 4,  
 well(3 and 4)- 4µl of tropomyosin 3 iso 2 ,  
 well(5 and 6)- 4µl of digested tropomyosin 4 iso 2 and  
 well7 -2µl ladder.

Figure 8 shows the results of the mini digestion. According to the gel picture expected double band was appeared only for tropomyosin 4 iso 2 recombinant DNA. The results says insertion of tropomyosin 4 iso 2 in to the vector is good. And single bands were appeared for tropomyosin 4 and tropomyosin 3 iso 2. It means insertion is not good. The reason for the unexpected result is unknown, may human error with mini digestion.

Mini digestion was repeated again with trouble shooting and run the gel with cut DNA and uncut DNA(empty circular vector). Figure 9 shows the results of gel picture.

The expected bands for digested insert DNA in the range of 750-1000bp. Digested vector DNA in the range of 2000-3000 and control uncut vector lower than cut vector bands.



**Figure 9: gel electrophoresis of cut DNA and un cut DNA**

Well (1 and 3) –cut DNA of tropomyosin 4  
 Well (2and 4)- uncut DNA of tropomyosin 4  
 Well (5 and 7)-cut DNA of tropomyosin 3 iso 2  
 Well (6 and 8)-uncut DNA of tropomyosin3 iso 2  
 Well (9 and11)- cut DNA of tropomyosin 4 iso 2  
 Well (12 and 13)-uncut DNA of tropomyosin 4 iso 2  
 Well 14- plus DNA ladder

Expectation was double band for cut DNA and single band for uncut DNA. For tropomyosin4 and tropomyosin 4 iso2 bands were appeared.inthe range of 750-1000bp and 2000-3000bp. But no double band for tropomyosin3 iso 2 cut DNA.but the cut DNA bandwas higher than the uncut DNA band.

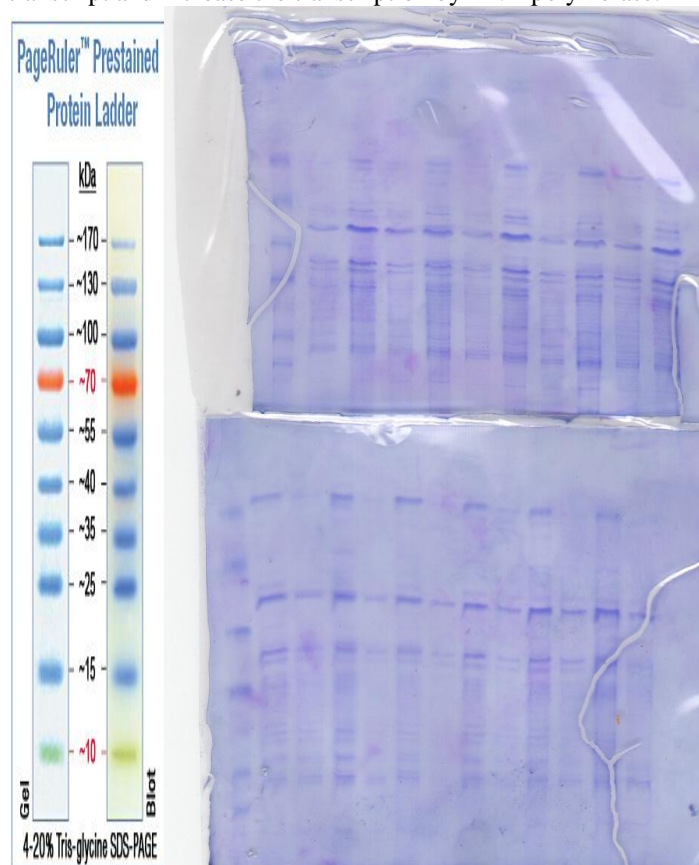
After done the transformation of recombinant DNA in to the BL-21 competent cell. Harvested the bacteria culture and run the sds page gel to analyse the expression of the protein with the help of IPTG.i expected the band in the range of 32-40 KD.

In the result expected bands for tropomyosin 4and tropomyosin4 iso 2 were appeared. But for cut tropomyosin 3 iso 2, only one band was appeared in the range of 2000-3000bps. That is digested vector. No bands appeared for insert DNA. When compared the cut and uncut vector, cut DNA band was little bit higher than uncut DNA band. It suggest insertion has happened. But the restriction enzymes may not cut the DNA very well.

SDS PAGE electrophoresis used to analysis protein expression of the cell. The recombinant DNA transformed in to Escherichia coli BL-21 competent cell. Because to make recombinant protein, the cell should have RNA polymerase. RNA polymerase present in BL-21 bacteria cell. But not present in XL-1 cell. So the recombinant DNA transformed in to BL-21 to produce protein. T7/lac promoter in the vector will use RNA polymerase. (Clancy and brown, 2008)

Meaning of expressing a gene is producing protein. The genes in the recombinant DNA encoded for protein molecules. Through the transcription and translational step, the recombinant DNA produce recombinant protein. In the transcription, when RNA polymerase attach with double strand DNA, the gene will activate and unwind. It is termed promoter. One of the strands of DNA served as template by RNA polymerase and manufacture of RNA stand 5'-3'.that complements the DNA strand. This is call messenger RNA. All the genetic information of the DNA is copied in to mRNA. In the translation step mRNA carries all the information to ribosome from nucleus. The ribosome binds to the start of the mRNA and read the codon for the amino acid. Transfer RNA carrying the amino acid for the codon. Here mRNA use as a template to produce the amino acid chain. This chain of amino acid will form protein. ( miller et al;1999)

For the protein expression IPTG was used. T7 RNA polymerase actively initiate the transcription from T7 lac promoter. Normally the Escherichia coli express the protein. It is known as lac repressor. It is a DNA binding protein. When lactose is absence in the medium, the lac repressor will bind to the lac promoter sequence and prevent the RNA polymerase from binding to the promoter. Lac promoter helps to initiate the transcription by RNA polymerase. So lac repressor allows the transcription only in the present of lactose. But if we add the lactose to the cell, the cell will eat lactose and drop the gene then cell will turn off. So we add IPTG inducer. It is look same like lactose. So the lac repressor binds with IPTG instead of lac promoter and allows the lac promoter to initiate the transcript and increase the transcription by RNA polymerase.



**Figure 10: SDS PAGE gel electrophoresis for the protein expression**

In the figure 10,SDS PAGE gel shows the results of the expressed tropomyosin in Escherichia coli BL-21 bacteria cell in the expected range of 32-40KDa.

The dried recombinant DNA sample was sent to design the sequence. We got back the sequence 5 out of 6. When checked the designed sequence of recombinant DNA in bio edit and blast.. It showed same sequence for three different tropomyosin isoforms and these sequence not matched with either tropomyosin3 or tropomyosin 4. It was matched with tropomyosin1 gene. The possible reason must be contamination with empty plasmid vector.

#### IV. Conclusion And Future Prospect

My research study has about production of recombinant protein by cloning the human tropomyosin. By performing the experiment, I could not produce the desired recombinant protein within my study period. It does not mean, transformed the recombinant DNA into Escherichia coli BL-21 cell is not producing protein. The reason, protein was expressed but that is different from the desired protein. The designed sequence for recombinant tropomyosin 3 and 4 not matched with the original sequence. By repeating the experiment, can get better results and when they matched with original human tropomyosin, required future work will be producing the large scale of recombinant protein and characterize them by SDS-PAGE and western blotting technique and then use the three different monoclonal antibodies to check the reactivity and specificity of different tropomyosin isoforms.

If the research is successful, it will specially use full in clinical diagnostics and molecular genetics.. Recombinant proteins mostly benefit than non-recombinant proteins. The recombinant tropomyosin particularly can be used in replacing the abnormal and deficient protein, interfering with specific organisms in order to control the complex progression, treating the autoimmune disease and cancer, delivering the therapeutic protein into the specific site to blocking some unwanted functions in muscle disease and will be help to the researches who are doing research in genetic field.

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Glanista Tharmaratnam "Production And Characterization Of Recombinant Tropomyosin."  
IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 13.4 (2018): 44-57.