Molecular Cloning, Expression And Purification Studies With An ORF Of Mycobacterium Tuberculosis

Chiranjibi Chaudhary, Sudheer K Singh

Abstract: The study was initiated to develop a recombinant strain, for expression and production of large scale protein and to develop its purification protocol. The MRA_ORF-X was amplified from the genomic DNA of M. tuberculosis H37Ra. The amplicon was successfully cloned in a cloning vector pGEM-T Easy and transformed in cloning host DH5α. Recombinant clones were identified by blue-white screening and insert presence was confirmed by restriction digestion of plasmid isolated from white colonies. Expression vector pET32a was used for protein expression. The recombinant plasmid was transformed into expression host BL21 and protein expression was checked by SDS-PAGE. The desired protein was approximately 60 kDa in size, including tags. The purification protocol was established for purification from inclusion bodies. The purity of purified protein was assessed by SDS-PAGE gel run and presence of a single band at ~60 kDa suggested that the inclusion bodies were a good source of purified protein.

Key words: Mycobacterium tuberculosis, PCR, E.coli strains, Agarose gel, Digestive enzyme, Enzymes, SDS-PAGE, Sonication, Bradford test, Westerning blotting

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INTRODUCTION

Tuberculosis (TB) caused by Mycobacterium tuberculosis is a disease of great public concern globally as it is one of the leading causes of death. It causes a staggering burden of morbidity and mortality, and is responsible for an estimated 2 million deaths annually worldwide (**Dye et al. 1999**). New cases 9.2 million and 1.7 million death from tuberculosis occurred in 2002, of which 0.7 million cases and 0.2 million deaths were in HIV positive case. There are 2-3 million deaths every year and latent tuberculosis persists in over a billion individuals worldwide (**WHO. 2009**). This can be attributed to the human immunodeficiency virus (HIV) epidemics as well as demographic and socio-economic factors such as poverty and malnutrition, which have served to maintain the reservoir of potential infections. (**Bloom & Murray. 1998**) This alarming rise led the WHO to declare TB **'**a global emergency' in 1993**.** Presently, in the HIV infected patients it is leading cause of death in developing countries because of high incidence of dual infections and decrease in the immunity against both HIV as well as M. tuberculosis infection. In addition, the emergence of multi drug-resistant tuberculosis (MDR TB) is of great concern and represents 15 percent of all TB cases are acting as an exceptional global threat **(WHO. 2009, 2015).**

History of T.B

The TB disease history can be traced back to Egyptians in 2400 BC and possibly to much earlier times. For many centuries this tiny invader killed people worldwide without any insight into its causative agent and pathogenesis. Jean-Antoine Villemin in 1865 work on the pathogenesis of tuberculosis demonstrated the transmissibility of Mycobacterium tuberculosis infection. In 1882 Robert Koch identified the tubercle bacillus as etiologic agent of TB.

- *C.CHAUDHARY*
- *UNIVRSIY OF LUCKNOW, India*
- *CSIR-CDRI, LUCKNOW, India*
- *1994chiran@gmail.com*

Tuberculin skin test was developed by Clemens von Pirquet in 1907 and 3 years later he also demonstrated latent tuberculoses infection in asymptomatic children. After World War I, BCG vaccination came into existence. Further tuberculosis was controlled by the discovery of streptomycin in 1944 and isoniazid in 1952 (**Daniel, 2006**). Pulmonary TB was first time seen between 668-626 BC and found in extract; "The patient coughs frequently, his sputum is thick and sometimes contains blood. His breathing is like a flute. His skin is cold, but his feet are hot. He sweats greatly and his heart is much disturbed. When the disease is extremely grave, he suffers from diarrhoea" in the library of King Assurbanipal of Assyria. Sylvius, in the 17th century recorded the pathological changes in the TB patient lungs as well as anatomical description of TB (**Harms. 1997**). In 1720 Dr. Benjamin Marten first proposed that TB could be caused by "wonderfully minute living creatures" a truly revolutionary thought at the time. Then in 1882 Dr. Robert Koch described these bacteria and developed a successful staining technique, allowing him to visualize M. tuberculosis for the first time (**Daniel, 2006**). In 1921 Mycobacterium bovis Chalmette-Guerin (M. bovis BCG) was administered as a vaccine for the first time. By the 1940s chemotherapeutics were being developed and utilized against M. tuberculosis, ushering in a new era in the fight against T.B (**Orme. 1995**).

Global Burden of TB

According to World Health Organisation (WHO) 2007, The tubercle bacilli infect one third of the world's population, however only 5-10% persons will have active disease and other does not lead to disease which initially asymptomatic and experience latent infection. The WHO 2008 found 9.4 million cases of active TB globally, mostly in Asia (55%) and Africa (30%). In Asia, India and China most dominant about 35% of TB cases (**WHO. 2009**). Among infectious disease Tuberculosis is the major cause of mortality worldwide and approximately 2.5 million people are died every year. About 9.6 million people fell ill with TB in 2014, including 1.2 million people living with HIV. In 2014, 1.5 million people died from TB, including 0.4 million among people who were HIV-positive (**WHO. 2015**). Hence, TB is global hazards. M. tuberculosis replicates in resting

macrophages which is causing agent of tuberculosis. In recent year, due to increase in worldwide HIV epidemic the tuberculosis importance is also increased and different infection of cases are seen which multidrug-resistant strain of M. tuberculosis (**WHO. 2009,2015**).

OBJECTIVES

The approaches described in this dissertation involve PCR amplification of MRA_ORF-X from Mycobacterium tuberculosis H37Ra strain, its cloning, expression and purification

In the future MRA_ORF-X and the protein it encodes can be studied for their suitability as a candidate for drug targeting or as a vaccine candidate. Keeping in view of the above, the present investigation was conducted with following objectives:

- Cloning of "MRA_ORF-X" in the cloning vector pGEM-T Easy vector.
- Sub-cloning of "MRA_ORF-X" gene in pET32a expression vector.
- Construction of recombinant E. coli strain for protein expression.
- Transformation of E. coli and selection of protein expressing clones.
- Expression and purification of protein.
- Quantification of protein.

MATERIAL AND METHODS

Kits and Accessories: Plasmid isolation kit (Sigma) and Gel Elution Kit (MN) was used during study. Also, restriction enzymes and T4 DNA ligase were used from MBI-Fermentas. Taq DNA Polymerase and dNTPs were from Sigma.

CULTURE, VECTORS AND MEDIA

E. coli DH5α (Novagen) and E. coli BL21 (DE3) strains were used host cell for cloning and expression. T/A cloning vector pGEM-T Easy and expression vector pET32a were used for cloning and expression.The E. coli strains were cultured on LB agar and broth and Mycobacterium tuberculosis culture was inoculated in Sauton's medium. 40% Glycerol stock of cultures were prepared for long term preservation at -20°C. 400μL Glycerol was added to 600μL culture and mixed properly.

GENOMIC DNA ISOLATION

The genomic DNA is chromosomal DNA. Most of the organism contains gDNA in every cell. The gDNA encodes genome of an organism which is biological information of heredity passed from one generation to another. For the protein synthesis gDNA uses standard genetic code. The genomic DNA of Mycobacterium tuberculosis was isolated by Phenol: Chloroform: Isoamyl alcohol (25:24:1) method and desired Mtb ORF was PCR amplified using gene specific primers. For genomic DNA isolation following protocol was used.

Procedure: The cells are inoculated in 10 ml LB media containing Amp and kept o/n in a shaker at 180rpm and 37°C and pellet at 11000 rpm, added with 400 μL Tris-HCL. Again add glass beads, 30 μL lysozyme (50mg/ml) and vortex and incubated for 1 hrs at 37ºC. 10% of 70μL SDS and Proteinase K (20μL, 20gm/ml) added, vortex gently incubated in 65ºC 10-15 minutes. NaCl (100μL, 5M) and prewarmed at 65ºC CTAB/NaCl vortex and incubate at 65ºC 10-15 minutes. Phenol/chloroform 70μL is added twice and centrifuge at 11,000 rpm and 13,000 rpm for 15-20 minutes. To pricipate nucleic acid add 0.6 vol. of isopropanol and keep at -20ºC 30 minutes. Add milli-Q water (50μL) after air dried.

PCR (Polymerase Chain Reaction)

The polymerase chain reaction is an in vitro technique for the amplification of desired nucleotide fragment. It was developed by Kary B. Mullis (1983). This technique is based on thermal cycling consisting of repeated cycles of template denaturation, primer annealing and extension of DNA in presence of DNA polymerase. The reaction mixture contained DNA template, dNTPs, primers, Taq polymerase, ATP $MgCl_2$, DMSO, Taq buffer. These components enable selective and repeated amplification of template DNA. As PCR progresses, the DNA is itself used as a template for further amplification.

Procedure: The desired gene of Mycobacterium tuberculosis H37Ra was amplified by using reverse and forward primers.

Table No.1: *PCR reaction mixture composition*

S.N	Reaction components	Quantity
1	Genomic DNA	10 ng
2	Taq DNA polymerase	5 U
3	10x Taq DNA polymerase buffer	15 μL
4	10mM dNTP mix	0.5 mM
5	DMSO	7.5 μl
6	$MgCl_2$	2.5 mM
7	Forward primer	1 pico mole
8	Reverse primer	1 pico mole
9	Nuclease free water to make final volume	30μl

Table No.2: *PCR program for amplification*

S.N	Step	Temp. and time
1	Initial denaturation	92°C for 5 min
2	Denaturation	92° for 1 min
3	Annealing	58°C for 1 min
4	Extension	72°C for 1.50 min
5	Cycles	30 time
6	Final extension	72°C for 30 min
7	Hold at	4°C

GEL EXTRACTION

The amplified PCR product was run on agarose gel in TBE buffer and amplicon of desired size (~1.1 kb) was excised from the gel and eluted using NucleoSpin Gel extraction kit (MN).To extract the gel 3 buffers are used such as gel solubilisation buffer, wash buffer and elution buffer.

Procedures: PCR clean up (Nucleo Spin)

The PCR bands are excised using gel cutter and put inside the column and add 700μL solubilisation buffer to solubilise in incubator at 50ºC for 5-10 minutes. Again add 700μL wash buffer and centrifuge at 11,000xg for 30 sec. in both cases. Repeat for washing and centrifuge empty column to

remove wash buffer and put in the new vial for elution. The elution buffer (30µL) is added on column incubate at RT for 1 minute and centrifuge column at 11,000xg for 1 min to collect the DNA.

CLONING OF PCR AMPLIFIED PRODUCT INTO VECTOR (T/A CLONING)

T/A cloning is one of the most popular techniques of cloning of amplified PCR product. The Taq DNA polymerase lacks 5'-3' proof reading activity and is capable to adding adenosine (A) overhangs at the 3' end of PCR product. Such PCR product can cloned in linearized vector which have complementary 3' thiamine (T) overhangs. PCR product with (A) overhangs is complementary to the vector with T overhangs. They form H-bonds in the presence of DNA ligase.

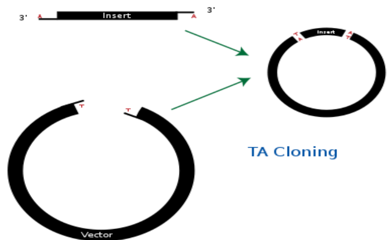

Fig 1: *T/A cloning*

The pGEM-T Easy PCR cloning kit was set up as per manufacture's (Promega) protocol. The vector contains numerous restriction sites within the MCS region.

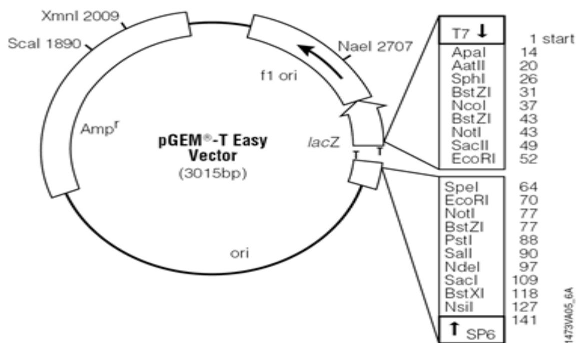

Fig 2: *Vector map of pGEM-T*

Table No. 3: *DNA ligation*

S.N.	Constituents	Quantity
1	DNA insert	300 ng
2	Vector pGEM-T Easy	40 ng
3	T4 DNA ligase	1U
4	Ligase buffer(2x)	5µl
5	Total	10µl

The reaction mixture was incubated at RT for 3 hrs. This mixture was used for transformation in competent cells of E. coli DH5α.

PREPARATION OF COMPETENT CELLS AND TRANSFORMATION

The competent cells possess more easily altered cell wall by which foreign DNA can pass through easily. They have been exposed to chemical to make them competent. In the $CaCl_2$ method, the competency is obtained by creating pores in bacterial cells by suspending them in calcium solution. DNA is then introduced into the host cell by heat shock.

Procedure

The E.coli DH5α was inoculated in LB media (5 mL) and placed in the shaker o/n at 37ºC, after the growth sub culture again in 5-10 mL LB medium and incubate for 4 hrs at 37º. Then, culture was centrifuged at 8500 rpm for 5 min to pellet the cells and discard the supernatant. The pellet was re-suspended using 0.1M $MgCl_2$ (1mL), incubated in ice foe 20 min and centrifuge at 8500rpm 5 min to pellet the cells, discard the supernatant. Again the pellet was re-suspended in 0.1M $CaCl_2$ (1mL) twice and incubated in ice for 10-15 min, centrifuge the suspension at 8500rpm for 5 min, again pellet was re-suspended in 0.1M $CaCl_2$ (200µL) incubated in ice for 5 min, cells become competent and the ligation mixture was added in competent cells put in ice for 5 minutes. After this, heat shock was given at 42ºC for 90 seconds and immediately placed in ice for 10 min. add 1mL media incubated for 45 min at 37ºC for culture. Culture (50µL) was inoculated in LB Agar plate supplemented with X-gal, IPTG and Amp by Spread Plate Method and plate was incubated o/n at 37ºC for growth

BLUE/WHITE SCREENING

Blue/white screening technique allows for rapid and convenient detection of recombinants DNA in a vector. The method is based on the principle of α-complementation of the β-galactosidase gene. 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 that are transformed with empty vector with an uninterrupted lacZ α (no insert) remain white.

Procedure: After o/n growth LB plates with blue and white colonies were observed. White clones were used for plasmid isolation and master plate preparation on LB agar plate. Culture was inoculated in 5mL LB broth with Amp and incubated at 37°C for o/n.

PLASMID ISOLATION BY ALKALINE LYSIS

Alkaline lysis is the method for isolating circular plasmid DNA from bacterial cells. The E. coli cells that contain the plasmid are lysed with alkali and plasmid DNA is extracted. The cell debris is precipitated using SDS and potassium acetate and pellet is removed. Isopropanol is then used to precipitate the DNA from the supernatant, the supernatant is removed and the DNA pellet was resuspended in TE buffer after washing with 70% ethanol.

Procedure: White clone from Blue/White Screening was inoculated in LB media (2-5mL) and put in shaker o/n at 37ºC for growth. Cells were pellet at 11,000rpm for 5 min and re-suspension solution (solution1) 300µL added vortex for 5-10 min. till the pellet mixed completely. Solution2 (lysis

buffer) 300µL added mix gently 1-3 min and add 450µL solution3 (isopropanol sol$^{n)}$ mixed gently and incubate at 4ºC for 10-20 min and centrifuged at 12,000rpm for 10 minutes. Transferred the supernatant into new eppendrof tube and discard the pellet. Equal amount of Phenol: Chloroform: Isoamyl alcohol was added to the supernatant mix vortex and centrifuged at 2,000rpm for 10 minutes. Two layers formed, upper layer was transferred in new vial and add isopropanol equal amount put at -20ºC 20 min. and centrifuged for 20 min. at 13,500rpm, discard supernatant. 70% ethanol added to pellet centrifuged 10 min. at 13,500rpm and pellet was air dried. Pellet of DNA was re-suspended in milliQ water and stored at -20ºC.

PLASMID ISOLATION USING KIT

The sigma mini prep kit was used for plasmid isolation. The protocol used is as follow;

Procedure: The confirmed clones were inoculated in 5 mL LB media with 5µL Amp and incubated o/n at 37°C on shaker and culture was centrifuged at 11,000 rpm for 1 min. The pellet was re-suspended using A1 250µL (resuspension buffer) vortex to mix and add A2 (lysis buffer) 250µL mix 1-3 min gently again add A3 300µL (neutralizing buffer) mixed 5-10 minutes and centrifuge 5 min at 11,000xg. Column was prepared with column preparation buffer and centrifuge 1 min at 11,000xg. The supernatant was loaded on column and centrifuged at 11000×g for 1min. Flow through was discarded. Wash buffer 750µL was added onto column centrifuge 11,000xg fir 1-2 min and again flow through was discarded. The column was centrifuged empty to remove residual wash buffer and placed in new 1.5mL eppendorf tube. Elution buffer 50µL added onto column and incubated for 1min at RT then centrifuged at 11000×g for 1 min to elude the DNA.

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a method of gel electrophoresis used to separate the DNA fragments. The DNA is separated on the bases of charge and size. Biomolecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. The dimension of the gel pores (gel concentration), size of DNA being electrophoresed, the voltage used, the ionic strength of the buffer and the concentration intercalating dye such as ethidium bromide if used during electrophoresis affect separation.

Procedure: Prepare 1% agarose gel, 0.25gm agarose was added in 25ml I X TBE buffer. Solution was boiled in microwave to ensure complete solubility. It was allowed to cool down to about 50ºC. Add 5 µl of Ethidium Bromide (EtBr) into the solution and mixed gently. Then, gel was poured in casting tray slowly to avoid air bubbles. Along with the tray, the gel was put in the electrophoresis tank containing 1X TBE. Comb was removed gently to avoid distortion of wells. Samples mixed with loading dye (6X) were loaded in wells and run at 80V for 30 to 45 min. Gels were visualized under UV light. The positive clones were selected on the basis of differences in mobilization compared to control plasmid DNA. These clones were further confirmed by restriction digestion.

SELECTION OF POSITIVE CLONES

Restriction digestion

This enzymatic technique is used for cleaving DNA molecules at specific sites. This ensures that all DNA fragments that contain restriction sites are digested and released.

***Table No. 4**: Restriction digestion reaction*

S.N.	Constituents	Volume
1	Plasmid DNA	18 µl
2	Restriction enzyme	3 µl
3	10X buffer	12µl
4	SDW	27µl
5	Total	60µl

The reaction mixture was incubated at 37°C for o/n.

CLONING IN EXPRESSION VECTOR

After confirmation, the insert was cloned in expression vector for expression of particular protein. The pET32a system are powerful tools developed for cloning and expression of recombinant protein in E. coli the pET32a carry N terminal His tag/thrombin/T7 tag configuration and an optional C terminal His tag. The target gene was cloned in pET32a with a T7 bacterio-phage promoter and expression was induced by IPTG.

***Table No. 5**: Vector digestion reaction*

S.N.	Constituents	volume(µL)
1	Vector	20 µl
2	HindIII enzyme	3 µl
3	10X buffer	12 µl
4	SDW	25µl
5	Total	60 µl

The reaction mixture was incubated at 37°C for overnight.

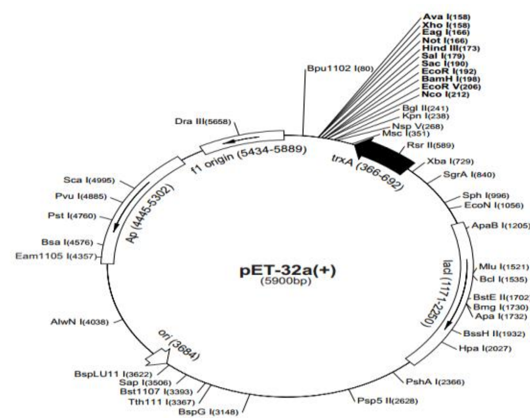

***Fig.3:** PET-32a*

ALKALINE PHOSPHATASE TREATMENT OF VECTOR

Fast alkaline phosphatase (Fast AP) is a type of alkaline phosphatase that catalyzes the removal of phosphate groups from the 5' end of DNA strands. This enzyme is frequently used in DNA sub-cloning, as DNA fragments that lack the 5' phosphate groups cannot self ligate. This

prevents recircularization of linearized vector DNA and improves the yield of vector containing the appropriate insert

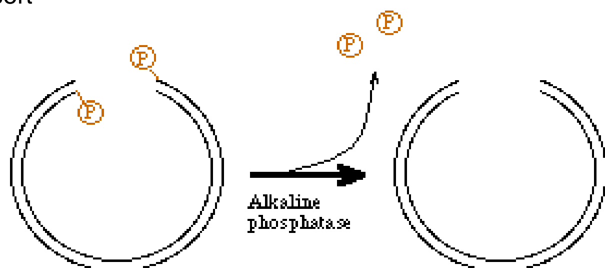

Fig. 4: *Fast AP treatment*

Table No. 6: *Alkaline Phosphatase Treatment*

S.N.	Constituents	Reaction mixture
1	Vector DNA	30 µl
2	Fast AP	2 µl
3	Fast AP buffer	3 µl
4	Water	5 µl
5	Total	40 µl

The reaction mixture was incubated at 37°C for 1.30 hours.

Table No. 7: *Ligation of DNA insert into pET32a*

S.N.	Constituents	Volume(µL)
1	Vector DNA	3 µl
2	Insert	9 µl
3	10X T4 DNA ligase Buffer	2 µl
4	T4 DNA Ligase	1 µl
5	Water	5 µl
6	Total	20 µl

The reaction mixture was incubated at 4°C for o/n.

Transformation in DH5α cells

Competent cells were prepared by $CaCl_2$ method. Ligation mixture was added to competent cells and incubated in ice for 15 min. Heat shock was given at 42°C for 90sec and then cells were immediately placed in ice for 10 min and add 1mL medium incubated at 37°C for 45min. Culture 50µL was inoculated by spread plate method on LB agar plate supplemented with Amp and plate was incubated at 37°C for o/n.

SCREENING AND SELECTION OF POSITIVE CLONES

Screening

Master plate was made from the clones and transferring them on LB plate supplemented with Amp. The plates were incubated for 7-8 hour at 37°C. The clones were inoculated in 5mL LB broth with Amp and incubated at 37°C for o/n.

Selection of positive clones

Plasmid of selected clones was isolated by the alkaline lyses. The positive clones were selected by running on agarose gel on the bases of mobility shift. The selected plasmid was checked by the restriction enzymes which in the insert and plasmid.

Table No. 8: *Restriction digestion reaction*

S.N	Constituents	Composition
1	DNA	18 µl
2	HindIII	3 µl
3	Buffer	12 U
4	SDW	17 µl
5	Total	60 µl

The reaction mixture was incubated at 37°C for o/n. The reaction mixture was run on agarose gel to observe release of fragments. The confirm clone was transformed into BL21 strain of E. coli.

TRANSFORMATION IN EXPRESSION HOST

Procedure: Competent cells were made from E. coli BL21strain. Confirmed plasmid was added in BL21 competent cells. Then, heat shock was given at 42°C for 90 sec and immediately placed in ice for 10 min. Add 1mL medium, incubated at 37°C for 45min. The culture was inoculated by spread plate method on LB agar plate supplemented with Amp and plate was incubated in invert position o/n at 37°C.

PROTEIN EXPESSION

Procedure: Clone was inoculated in 10mL LB medium with Amp and incubated at 37°C for o/n. Culture was sub-cultured in LB medium and allowed to grow for 4 h at 37°C. Uninduced culture was aliquoted in eppendorf tubes and IPTG was added in all culture and incubated at 37°C for another 4 h. Induced and uninduced cells were pelleted at 11000 rpm for 10 min**.** The pelleted cells were used to check the expression on SDS PAGE gel.

SDS PAGE

Sodium dodecyl sulfate Polyacrylamide gel electrophoresis uses electric field for separation of macromolecules. It is a very common method for separation of proteins by electrophoresis in which polyacrylamide gel is used as a support medium and sodium dodecyl sulfate (SDS) is used to denature the proteins. SDS is an anionic detergent, because of the negative charges on SDS it destroy the complex structure of proteins.

Procedure:

Gel preparation: Glasses were placed with spacer and clamped in casting stand. Resolving gel was poured in between the glasses, and allowed to polymerize. Afterwards, stacking gel was poured above resolving gel, and allowed to polymerize. After gel polymerization, clamps were removed and gel was moved to gel running chamber.

Sample preparation: Dye 1X 50µL was added to cells and mixed properly. Sample was heated at 95°C for 5-10 min. Sample was centrifuged at 13000 rpm for 5 min and 25µL supernatant was loaded on gel. Gel was run at 80 V in stacking gel and 100 V in resolving gel. After the completion of running, the gel was stained with Coomassie Brilliant Blue R for 2 hr. Gel was destained with destaining solution for o/n with frequent change of destaining solution. Bands were observed using white light trans-illuminator and image was taken using scanning device.

PROTEIN PURIFICATION

Protein purification is a series of processes intended to isolate one or a few proteins from a complex mixture. Separation of desired protein from the proteins complex is based on protein size, physico-chemical properties, binding affinity and biological activity.

Sonication: Positive clone was inoculated in 100 mL LB broth and induced by IPTG for expression. Culture was pelleted at 11,000 rpm for 5 min, and supernatant was discarded. Sonication buffer I was added and mixed properly and was sonicated by sonicator. Culture was centrifuged at 13,500 rpm for 5 min; supernatant was collected in a new tube. Then, pellet was mixed in sonication buffer II, centrifuged at 13500 rpm for 5 min and supernatant was collected in a new tube. Again, pellet was mixed in sonication buffer III, centrifuged at 13500 rpm for 5 min and supernatant was collected in a new tube and the pellet was mixed in sonication buffer IV again centrifuged at 13500 rpm for 5 min and supernatant was collected in new tube. At last, pellet was mixed in solubilisation buffer and kept for overnight.

Purification of insoluble fraction

The purification of natively folded protein from the expression systems is difficult because of the formation of insoluble protein aggregates called inclusion bodies. Affinity purification under denaturing conditions and then renaturation can yield natively folded protein. The reversible denaturation of proteins in imidazole containing solutions is used for purification. The denaturation of proteins ensures no secondary structure elements are favored.

Procedure: The protein purifying column was washed with 1mM Imidazole (10 mL) followed by 10 mL of 100 mM EDTA and charged with 10mL 100mM $NiSO_4$ and then again washed with 10mL SDW. The column Equilibrate with 10mL solubilization buffer and Sample was loaded on column, and loading was repeated. Column was washed with 10mL washing buffer and protein was eluted with 10mL elution buffer. Now, collect the protein in 10 eppendorf 1 ml each.

PROTEIN QUANTIFICATION

BRADFORD TEST

The Bradford assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. It is fairly accurate and samples that are out of range can be re tested within minutes.

Procedure: Samples 10µL were added in 250 µL of Bradford reagent and incubated in dark place at RT for 15 min. OD was determined at 595 nm.

DETECTION OF SPECIFIC PROTEIN

WESTERN BLOTTING

The western blotting also known as protein immunoblotting, is a technique used to detect and characterize a specific protein that has been previously separated based on size using gel electrophoresis. The immunoassay uses a membrane to transfer the protein which is made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. This technique exploits the inherent specificity of polyclonal or monoclonal antibodies.

Procedure: Cells lysate were mixed with sodium dodecyl sulphate (SDS) sample buffer and incubated in a boiled water bath for 10 min and samples were loaded on 12% SDS PAGE. The SDS gel is then placed into the transfer buffer and shaked on the rocker for 2-4 hrs. The protein bands in the gel were electro-transferred on the PVDF membrane at 0^0C for O/N with a constant voltage 25V in transfer buffer. The transfer membrane was washed with washing buffer and then blocked in blocking buffer for 5-6 hrs at RT and again washed the membrane with washing buffer. Add 1:3000 of primary antibody (Anti His) in the sol^n of 1X TBST and 1% dry milk. The membrane is put in the sol^n and keeps on the rocker for overnight at 4°C. The next day, again washed the membrane 2 to 3 times in 1 X TBST and added solution containing secondary antibody (HRP conjugated) of 1:5000 and put on rocker for 2-3 hrs in RT. The membrane is washed again and put on the solution containing TBST with DAB (3, 3'-diaminobenzidine) and one drop of hydrogen peroxide observed for colour development at RT which is completed in 5-10 min. At last the specific protein was detected as a band in the PVDF membrane.

RESULTS

AMPLIFICATION OF GENE

Genomic DNA was extracted from H37Ra strain of M. tuberculosis (Fig. 5a). DNA amplification was performed using specific primers for MRA_ORF-X. The PCR product was ~ 1.1 kb DNA segment (Fig. 5b)

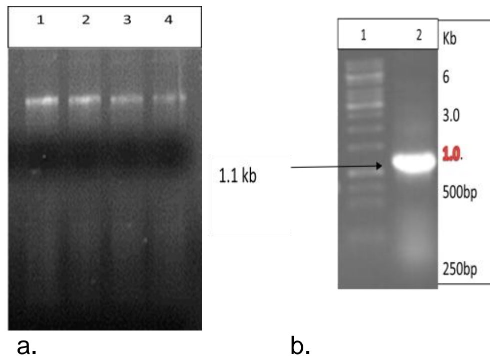

Fig 5.a: *Genomic DNA of H37Ra, b: PCR amplification of MRA_ORF-X from H37Ra, run on 1% agarose gel in TBE buffer. Lane 2 shows PCR product. Lane 1 is 1 kb DNA ladder.*

T/A CLONING

PCR product was cloned to pGEM-T Easy vector and plating on IXA plates led to blue and white colonies (Fig6). Recombinant (pGEM-T+X) and non-recombinant plasmid were isolated and electrophoresis was done in 1% agarose gel using 1 kb DNA ladder. The molecular weight of pGEM-T Easy vector was 3000 bp (control) and molecular weight of the inserted DNA was 1.1 kb. Recombinant and vector control showed differences in mobility due to presence of insert in the recombinant plasmid which led to lower mobility (Fig7).

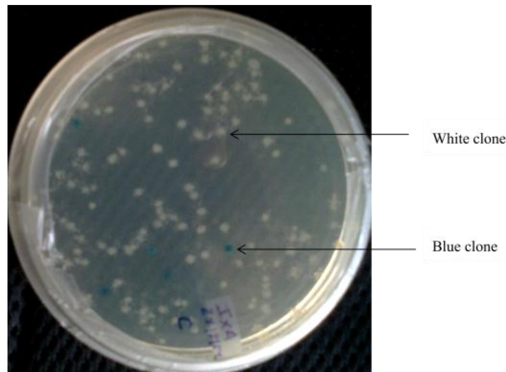

Fig 6: *Blue-White selection on IXA plate.*

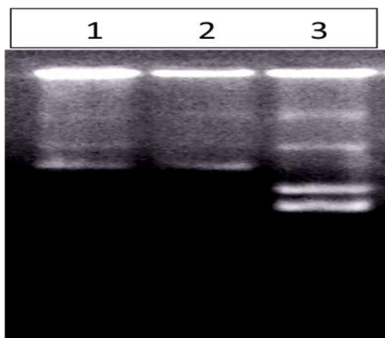

Fig 7: *Gel electrophoresis of T/A clones. Lane 1, 2 and 3 show clone 1, 2 and pGEM-T Easy (vector control) respectively.*

ANALYSIS OF RECOMBINANT CLONES

The gel run of recombinant clone1 digested with HindIII showed release of fragment of 1.1 kb along with another fragment of ~3.0 kb (Fig8). This showed the cloned insert was MRA_ORF-X.

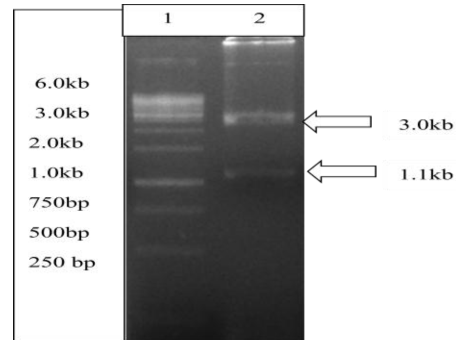

Fig 8: *Plasmid DNA of clone digested with HindIII run on 1% agarose gel in TBE buffer. Lane 2 shows digestion of clone 1. Lane 1 is a 1kb DNA ladder (Fermentas).*

CLONING IN EXPRESSION VECTOR

To carry out the ligation of insert in pET32a, HindIII digested pGEM-T Easy clone was ligated into HindIII digested pET32a vector (Fig9). The digestion of pET32a gave a single band of 5.9 kb. The band of 1.1 kb and 5.9 kb were gel eluted and used for ligation. The transformed E. coli DH5α cells showed whites colonies after overnight incubation of LB Agar plates with ampicillin (Fig10). The plasmid DNA isolated from clones showed lower mobility compared to pET32a vector control (Fig11).

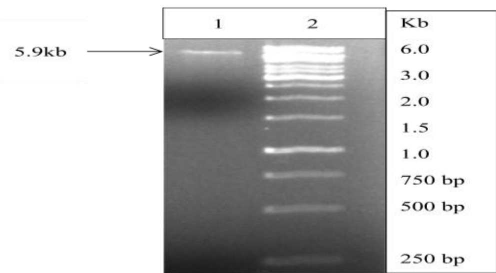

Fig 9: *Lane 1 shows vector pET32a digested with HindIII. Lane 2 is 1kb DNA ladder (Fermentas)*

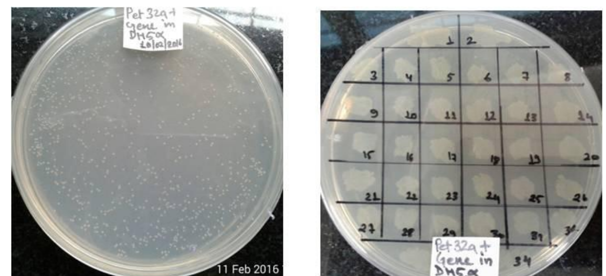

Fig 10: *LB Agar-Amp plate showing white colonies after transformation with pET-32a- MRA_ORF-X in DH5α and master plate respectively.*

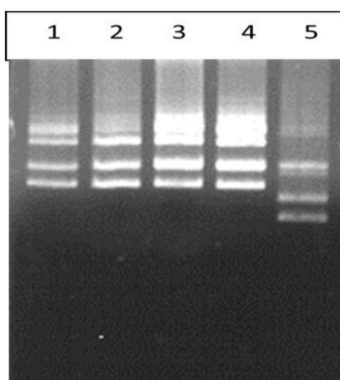

Fig 11: *Gel run of recombinant pET32a clones on 1% agarose gel in TBE buffer, showing mobility difference. Lane 1-4 is plasmid DNA from clones, lane 5 is pET32a vector control.*

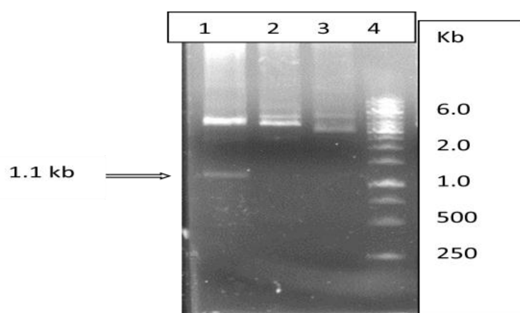

Fig 12: *Confirmation of MRA-ORF-X in expression vector. pET32a – MRA_ORF-X clone digested with HindIII and run on 1%agrose gel (TBE). The digested product in lane1 shows release of fragment. Lane 4 is 1 kb DNA ladder.*

PROTEIN EXPRESSION

Recombinant Plasmid of D1 clone consisting pET32a-X was transformed to expression host E. coli BL21 (DE3). The cells were grown on LB agar plate supplemented with Amp. Overnight incubation led to growth of numerous colonies. These were picked for preparation of master plate as shown in (Fig13). Clones 1 to 4 were selected for expression studies. Protein expression was observed only in the cultures induced with IPTG while no expression was observed in uninduced sample. The expressed protein was of approximately 60 kDa on SDS-PAGE (Fig14).

Fig 13: *Master plate of colonies of E. coli BL21 (DE3) strain transformed with recombinant plasmid pET32a-MRA_ORF-X.*

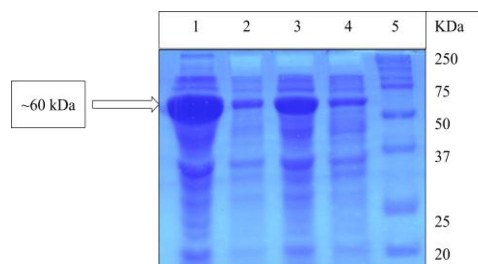

Fig 14: *SDS- PAGE gel for protein expression of clone 1 & 2. Lane 2, 4 shows the sample from uninduced and 1, 3 show induction with IPTG culture of clone 1, 2 respectively. Lane 5 is broad range pre-stained protein ladder (BIO-RAD).*

PROTEIN PURIFICATION

The supernatant obtained after sonication was quantified and details are provided in Table 1. No protein was observed in soluble fraction (lane 1, 2 and 3) and protein was observed in insoluble fraction (lane 4) (Fig15). So protein was purified from insoluble fraction and quantified (Table 9). Purified protein was eluted by 250 mM of imidazole in 10 fractions of 1ml. The eluted fraction 1, 2 and 3 showed a single band of 60 kDa (Fig16).

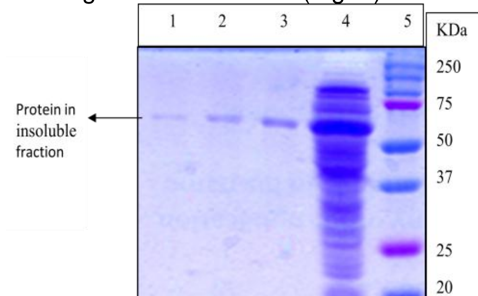

Fig 15: *SDS PAGE gel of soluble and insoluble fraction. Protein sample of insoluble fraction in lane 4 and Protein sample of supernatant obtained after sonication in lane 1, 2 and 3 respectively. Lane 5 is 250 kDa pre stained protein ladder (BIO-RAD).*

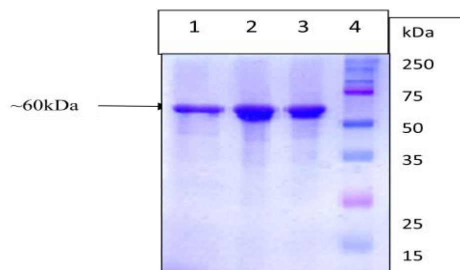

Fig 16: *SDS PAGE gel of eluted protein fraction. Lane 1, 2 & 3 show the purified protein from the insoluble fraction. Lane 4 is pre-stained protein ladder (BIO-RAD).*

BRADFORD ASSAY FOR PROTEIN ESTIMATION

The protein concentration was quantified by Bradford assay at various stages of purification. The average O.D. and corresponding protein concentrations are provided in table 9.

Fig 17: *Bardford assay. Well C is blank and well 1, 2, 3, 4, 5, 6, 7 and 8 are elution fractions.*

Table No. 9: *Protein estimation by using Bradford Method.*

Serial No.	Sample Purified Fraction	O.D at 595nm	Concentration of protein (in mg/ml)
1	Eluted fraction 1	0.413662	0.146584
2	Eluted fraction 2	0.842055	0.767443
3	Eluted fraction 3	0.477132	0.238569
4	Eluted fraction 4	0.349335	0.053357
5	Eluted fraction 5	0.329875	0.025357
6	Eluted fraction 6	0.399403	0.125919
7	Eluted fraction 7	0.321795	0.013443
8	Eluted fraction 8	0.316941	0.006409

WESTERN BLOTTTING

The presence of specific protein or the presence of antigen or specific antibody was visualised as a grey coloured band.

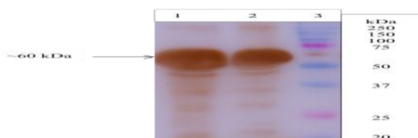

Fig 18: *Western blotting. Well 1 shows the eluted protein and well 2 is insoluble fraction. Well 3 is pre-stained protein ladder (BIO-RAD).*

DISCUSSION

The identification and characterization of proteins involved in Mycobacterium tuberculosis survival is important for developing strategies for its treatment. Biochemical characterization and activity assay development helps in identification of inhibitors against that protein. Also, some protein may be useful for eliciting immune response and can be used along with known vaccines to enhance their efficacy. The present study was an effort in that direction. In this study we did cloning and expression and developed purification protocol for obtaining ~60 kDa Mtb protein. The first step was PCR amplification of the gene. The gradient PCR suggested that 60^0C was optimum temperature with single band of good intensity. The presence of white colonies suggested disruption of β-galactosidase complementation due to presence of gene in pGEM-T Easy vector. The presence of insert gene was confirmed by gel mobility analysis as compared to control DNA isolated from blue colony. The result of digestion with restriction enzymes showed that the cloned insert was MRA_ORF-X. For the immunological studies as well as for assay development we need high amount of protein. So, it was necessary to over-express this protein in recombinant form in E. coli. For over-expression of MRA_ORF-X, pET32a expression system was used. Histidine tagged fusion proteins have affinity to Ni-NTA and can be purified under fully denaturing condition which is very convenient for purification of expressed protein. High level of expression was achieved at 1mm IPTG with overnight incubation. Purification analysis showed that purified protein was obtained when we used imidazole concentration at 250 mM. The current impetus for studying the Mycobacterium tuberculosis is needed to identify targets for development of new drugs. Biosynthetic pathways are important for the viability of the M. tuberculosis inside host. MRA_ORF-X studied in this work is annotated as a part of M. tuberculosis intermediary metabolism. Further studies are needed to study its importance in Mtb survival and to develop a bioassay for this protein. The present dissertation was an attempt to develop an expression system which can be used for large scale protein production.

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Appendix

Materials

ANTIBIOTICS
- Ampicillin (100mg/ml)

Antibiotics were filter sterilized by 0.22µm filter (Millipore) and stored at -20°C

IXA FOR 100 ML LB MEDIA
- 25µL of IPTG
- 100µL of 2% X gal (dissolved in DMF)
- 100µL of 100mg/mL amp^r

COMPOSITION OF LB BROTH MEDIA
- 10 gm Bacto Tryptone
- 5 gm Bacto Yeast Extract
- 5 gm NaCl
  Sterilized Distilled water 1 Litre
  Final pH adjusted to 7.2 ±0.2

COMPOSITION OF LB AGAR MEDIA
- 10 gm Bacto Tryptone
- 5 gm Bacto Yeast Extract
- 5 gm NaCl
- Sterilized Distilled water 1 Litre
- Final pH adjusted to 7.2 ±0.2
- ADD Agar 20gm

SOLUTIONS FOR PLASMID ISOLATION

SOLUTION 1
- 20Mm Tris HCl (pH 8.0)
- 10mM EDTA
- 50mM Glucose

SOLUTION 2
- 0.2 NaOH
- 1% SDS

SOLUTION 3
- 60mL CH_3COOK
- 11.5mL Glacial Acetic Acid
- 28.5mL SDW (To make the final volume 100mL)

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

AGAROSE GEL ELECTROPHORESIS
- 10X TBE BUFFER
- 890mM Tris
- 890mM Boric Acid
- 20mM EDTA (pH8.0)

DNA LOADING DYE
- 30% Glycerol
- 0.25% Bromophenol blue
- 0.25% Xylene Cyanol
- 100mM Tris buffer (pH8.0)
- 10mM EDTA

SDS PAGE

RESOLVING GEL BUFFER: 1.5M Tris HCl
- 72.6gm Tris dissolved in 250mL SDW
- Adjust pH 8.8 with 6N HCl
- Make final volume 400 mL with SDW and store at 4°C

STACKING GEL BUFFER: 0.5M Tris HCl
- 24gm Tris dissolved in 200mL SDW
- Adjust pH 6.8 with 6N HCl
- Make final volume 400 mL with SDW and store at 4°C

10% SDS
- 10gm SDS
- 100ml SDW
- Stored at room temperature

30%ACRYLAMIDE-BIS ACRILAMIDE
- 29.2gm acryl amide
- 0.8gm Bis-Acrylamide
- Make final volume 100 mL with SDW and store at 4°C

10% AMMONIUM PERSULFATE
- 0.2gm APS
- 2mL SDW
- Store at 4°C for up to 5 days

10X RUNNING BUFFER
- 250mM Tris
- 1.92M Glycine
- 1% SDS

STAINING SOLUTION
- 0.25% Coomassin blue
- 45% Methanol
- 10% Acetic acid
- 45% SDW

DESTAINING SOLUTION
- 45% Methanol
- 10% Acetic acid
- 45%SDW

SONICATION BUFFER

BUFFER 1
- 1mM PMSF
- 1mM EDTA
- 500mM NaCl
- 50mM Tris HCl (pH 8.0)
- 0.1mg/mL lysozyme

BUFFER 2
- 1mM PMSF
- 1mM EDTA
- 50mM Tris HCl (pH 8.0)
- 1% Triton X-100

BUFFER 3
- 1mM PMSF
- 1mM EDTA
- 50mM Tris HCl (pH 8.0)
- 1% Deoxycholate

BUFFER 4
- 1mM PMSF
- 1mM EDTA
- 50mM Tris HCl (pH 8.0)

SOLUBILIZING BUFFER
- 1mM PMSF
- 1mM EDTA
- 50mM Tris HCl (pH 8.0)
- 12.5mL Urea
- 1mM β-mercaptoethanol
- Make final volume 50mL with SDW

PURIFICATION OF INSOLUBLE FRACTION

EQUILIBRATION BUFFER
- 50mM Tris HCl (pH8.0)
- 2M Urea
- 1mM EDTA
- 1mM PMSF
- 1mM β-mercaptoethanol

WASH BUFFER
- 50mM Tris HCl (pH8.0)
- 2M Urea
- 1mM EDTA
- 1mM PMSF
- 1mM β-mercaptoethanol
- 5mM imidazole

ELUTION BUFFER
- 50mM Tris HCl (pH8.0)
- 2M Urea
- 1mM EDTA
- 1mM PMSF
- 1mM β-mercaptoethanol
- 200mM imidazole

WESTERN BLOTTTING

TRANSFER BUFFER (pH 8.3)
- 25 mM Tris HCl
- 192 mM Glycine
- 20% Methanol

TRIS BUFFERED SALINE-TWEEN20 (TBST) BUFFER/WASHING BUFFER
- 20 mM Tris pH 7.5
- 150 mM NaCl
- 0.1% Tween 20

BLOCKING BUFFER
- 1X TBST buffer
- 5% Dry milk powder

SOLUTION WITH 1^0 Ab and 2^0 Ab
- T BST buffer (10 ml)
- 0.1% milk powder
- 10µl Anti His (1^0 Ab) and Mice Ab (2^0 Ab)

COLOUR INDICATION SOLUTION
- 1X TBST buffer
- 0.05% DAB
- 0.01% H_2O_2

Abbreviations

APS Ammonium per Sulphate
dNTP Deoxyribo Nucleotide Triphosphate
EDTA Ethylene Diamine Tetra Acetic Acid
EtBr Ethidium Bromide
IPTG Isopropyl β D- Thiogalactopyranoside
Kbp Kilo base pair
Bp Base pair
KDa Kilo Dalton
LB Luria Bartani
LB Luria Bartani Agar
MQ MilliQ
mM Milli molar
M Molar
MCS Multiple Cloning Site
Ng Nanogram
Gm Gram
µL Micro litre
O/N Overnight
OD Optimal Density
SDS Sodium Dodecyl Sulphate
PAGE Poly Acrylamide GelElectrophoresis
PCR Polymerase Chain Reaction
PMSF Phenyl Methane SulphonylFluoride
Rm Revolution per minute
NA Deoxyribo Nucleic Acid
RT Room Temperature
IXA IPTG, X-gal, Ampicillin
SDW Sterile Distil Water
TB Tuberculosis
TBE Tris, Boric acid EDTA Buffer
TEMED N, N, N', N'-Tetramethylene Diamine
HIV Human immino Deficiency Virus
MDR Multi Drugs Resistant
XDR Extensively Drug Resistant
Mtb Mycobacterium tuberculosis
TB Tuberculosis
MAP Mycolyl arabinogalactan-peptidoglycan
IC Isochorismate
ICS Isochorismate Synthase
gDNA Genomic Deoxyribonucleic Acid
DBA 3'3-diaminobenzidine Tetra Hydrochloride
TBST Tris-buffered Saline-Tween 20
H_2O_2 Hydrogen Peroxide

S.N	CONSTITUENTS	CHEMICAL SUPPLIERS
1	Agar powder	Hi-Media
2	Agarose	Lonza
3	Acrylamide	Sigma Aldrich
4	APS	Sigma Aldrich
5	Ampicillin	Duchefa Biochemiel
6	Boric acid	SRL
7	Bromo Phenol blue	SRL
8	Bis acylamide	Sigma Aldrich
9	Bradford	Invitrogen
10	β mercepto ethanol	Sigma Aldrich
11	$CaCl_2$	SRL
12	Chloroform	Merck
13	Coomassien brilliant blue	SRL
14	Deoxycholate	Sigma Aldrich
15	DNA and Protein marker	Thermo Scientfic
16	EDTA	Gibco
17	Enzyme and Buffer	Takkara/Fermentas
18	EtBr	Sigma Aldrich
19	Glacial acetic acid	Fischer scientific
20	Glucose	Merck
21	Glycine	SRL
22	Isoamyl alcohol	SRL
23	Imidazole	Sigma Aldrich
24	LB	Hi-media
25	IPTG	Duchefa Biochemie
26	X gal	Duchefa Biochemie
27	NaOH	Merck
28	MgCl2	SRL

29	TEMED	Sigma Aldrich
30	Tris	SRL
31	HCl	Fischer Scientific
32	SDS	Sigma Aldrich
33	KoAc	SRL
34	Glycerol	Fischer Scientific
35	Methanol	Loba Chemie
36	PMSF	SRL
37	Phenol	SRL
38	Bis acrylamide	Sigma Aldrich
39	3, 3'-diaminobenzidine	Calbiochem
40	Hydrogen peroxide	Amresco