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EXPRESSION, PURIFICATION AND CRYSTALLIZATION OF RECOMBINANT HUMAN RECEPTOR TYROSINE KINASE (RTK) USING BACTERIAL CELL LINES

By

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BONAFIDE CERTIFICATE

Certified that this project report **"EXPRESSION, PURIFICATION AND CRYSTALLIZATION OF RECOMBINANT HUMAN RECEPTOR TYROSINE KINASE (RTK) USING BACTERIAL CELL LINES"** is the bonafide work of **"B KIRTHIKAA"** who carried out the project work under my supervision.

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Certificate

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For Aurigene Discovery Technologies Limited,

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CERTIFICATE OF EVALUATION

COLLEGE : Kumaraguru College of Technology

BRANCH : Biotechnology

SEMESTER: Fourth Semester

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The report of the project work submitted by the above student in partial fulfilment for the award of Master of Technology degree in Biotechnology of Anna University was evaluated and confirmed to be the report of the work done by the above students and then evaluated.

(INTERNAL EXAMINER)

(EXTERNAL EXAMINER)

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B KIRTHIKAA

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LIST OF ABBREVATIONS

Kb	Kilobases
kDa	Kilodalton
LB	Luria-bertani (broth)
TB	Terrific broth
PAGE	Poly Acrylamide gel electrophoresis
SDS	Sodium Dodecyl Sulfate
AGE	Agarose gel electrophoresis
TBE	Tris borate/EDTA
TE	Tris/EDTA
IPTG	Iso Propyl - D- thio galactosidase
TEMED	N,N,N',N'-Tetramethylethylenediamine
PMSF	Phenylmethylsulphonyl fluoride
Ni-NTA	Nickel-Nitrilotriaceticacid
FPLC	Fast Performance Liquid Chromatography
GFC	Gel Filtration Chromatography
HGFR	Hepatocyte growth factor receptor
HGF	Hepatocyte growth factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
TK	Tyrosine Kinase
RTK	Receptor Tyrosine Kinase
STAT	Signal Transducers and Activators of Transcription

P13K	Phosphoinositide 3-kinases
MAPK	Mitogen-activated protein kinases
MET	Mesnchymal Epithelial Transition Factor
EDTA	Ethylene diamine tetra aceticacid
DNA	Deoxy ribonucleic acid
RNA	Ribonucleicacid
APS	Ammonium per sulphate
PEG	Poly ethylene glycol
PCR	Polymerase Chain Reaction
mg/ml	milligrams/millilitre
μg/μl	micrograms/microlitre

ABSTRACT

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The *Receptor Tyrosine Kinase (RTK)* gene codes for the protein Receptor Tyrosine Kinase which plays an important role in tumor formation by involving in various cascade reactions. The pET 28a and pACYC which contains the gene of interest (RTK and phosphatase) are the vectors for expression of proteins in bacterial cells.

The expression of receptor tyrosine kinase was optimized by varying the media (Luria Bertini broth, 2YT media and Terrific Broth), induction temperature (12°C, 18°C and 37°C) and the bacterial expression cell lines (BL 21 DE3 and BL 21 RIPL). The cloning of phosphatase gene into pACYC vector was carried out for co-expression of phosphatase along with receptor tyrosine kinase to produce unphosphorylated form of protein. The expression unphosphorylated Receptor Tyrosine Kianse (RTK) was done and purification of unphosphorylated Receptor Tyrosine Kianse (RTK) was carried out by using affinity chromatography, ion exchange chromatography and gel filtration chromatography techniques. The completely pure protein was obtained. Crystallization of purified unphosphorylated Receptor Tyrosine Kinase was carried out and small triangular crystals were obtained which will be used for further structural studies.

INTRODUCTION

1 INTRODUCTION

1.1 Recombinant DNA

Recombinant DNA is a form of synthetic DNA thereby combining DNA sequences that would not normally occur together (Jeremy M. Berg). Recombinant protein is a protein that is derived from a recombinant DNA. Through the use of recombinant DNA, genes that are identified as important can be amplified and isolated for use in other species or applications, where there may be some form of genetic illness or discrepancy, and provides a different approach to complex biological problem solving.(Nathan P. Kaplan *et al.*, 1980).

1.2 Expression of proteins and its regulation in bacterial cells

Expression of recombinant proteins can be approached by starting with a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing expression, and ending with cell lysis and SDS-PAGE analysis to verify the presence of the protein. With careful choice of host strains, vectors, and growth conditions, most recombinant proteins can be cloned and expressed at high levels in *E.coli*.

Expression of recombinant proteins encoded by vectors is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. Once the lac repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter. The transcripts produced are then translated into the recombinant protein.

Many proteins which may be used for medical treatment or for research are normally expressed at very low concentrations. Through recombinant DNA technology, a large quantity of proteins can be produced. This involves inserting the desired protein gene into an "expression vector" which must contain a promoter so that the protein can be expressed.

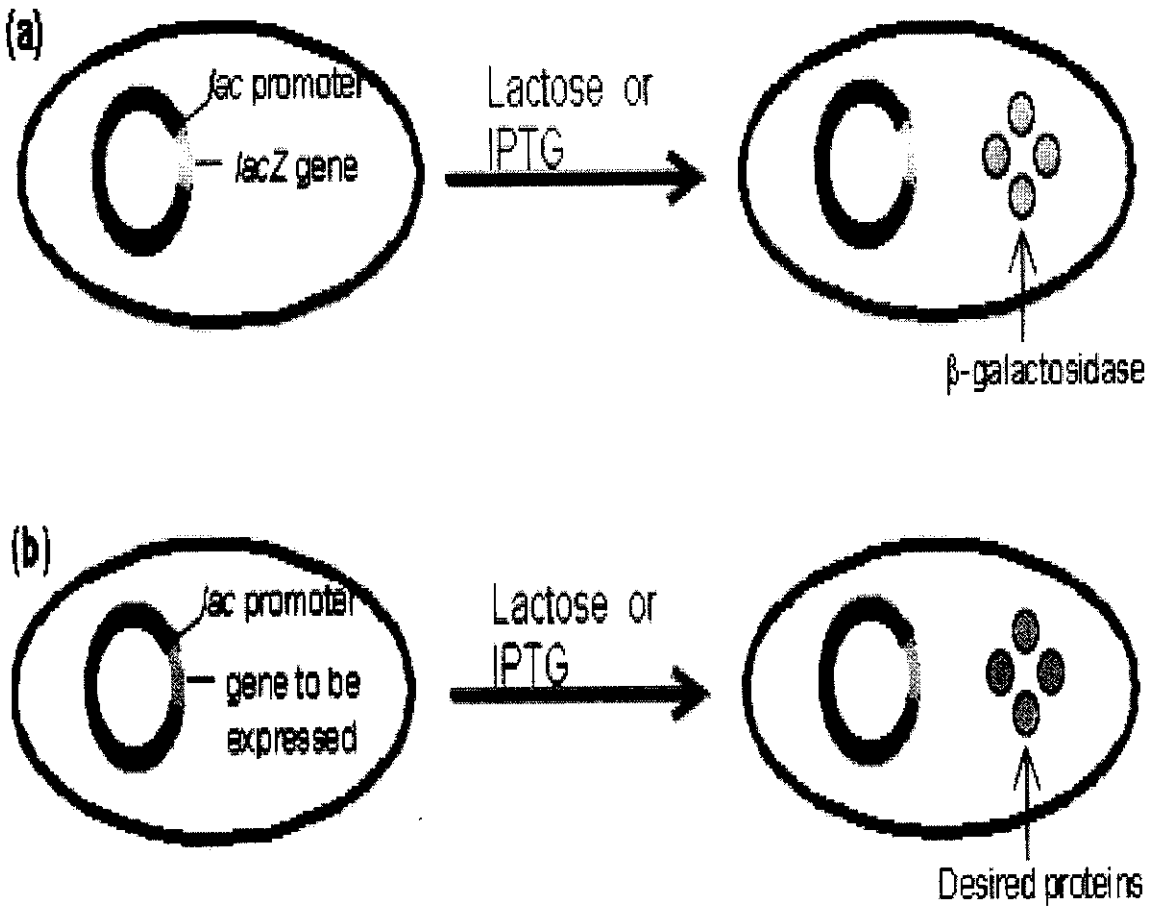


Figure 1: Production of recombinant proteins. **(a)** The expression vector contains the *lac* promoter and its neighboring *lacZ* gene encoding □-galactosidase. Lactose or its analog IPTG will stimulate the expression of □-galactosidase. **(b)** If *lacZ* is replaced by the gene encoding the protein of interest, lactose or IPTG will stimulate the expression of desired proteins.

1.3 Protein purification

Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column.

1.3.1 Affinity Chromatography

A common technique involves engineering a sequence of 6 to 8 histidines into the C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, as it has a ring structure similar to histidine or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. The protein of interest is now the major protein component in the eluted mixture, and can easily be separated from any minor unwanted contaminants by a second step of purification, such as size exclusion chromatography or RP-HPLC.

When the tags are not needed anymore, they can be cleaved off by a protease. This often involves engineering a protease cleavage site between the tag and the protein.

1.3.2 Ion Exchange Chromatography

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules.

Before the separation begins a buffer is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation. Ion exchange chromatography is a very powerful tool for use in protein purification.

1.3.3 Gel Filtration Chromatography

Gel Filtration Chromatography is a chromatographic method in which particles are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. (International Union of Pure and Applied Chemistry. "Size-exclusion chromatography (SEC)", Internet edition)

The underlying principle of GFC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This results in the separation of a solution of particles based on size. Provided that all the particles are loaded simultaneously or near simultaneously, particles of the same size should elute together. Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of this range and is where molecules are too large to be trapped in the stationary phase. The permeation limit defines the molecular weight at the lower end of the range of separation and is where molecules of a small enough size can penetrate into the pores of the stationary phase completely and all molecules below this molecular mass are so small that they elute as a single band.

1.4 Evaluating purification yield

The most general method to monitor the purification process is by running a SDS-PAGE at the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar molecular weight.

In order to evaluate the process of multistep purification, the amount of the specific protein has to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280 nm, however some reagents used during the purification process may interfere with the quantification. For example, imidazole (commonly used for purification of polyhistidine-tagged recombinant proteins) is an amino acid analogue and at low concentrations will interfere with the bicinchoninic acid (BCA) assay for total protein quantification. Impurities in

low-grade imidazole will also absorb at 280 nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

1.5 Protein Crystallization

Proteins, like many molecules, can be prompted to form crystals when placed in the appropriate conditions. In order to crystallize a protein, the purified protein undergoes slow precipitation from an aqueous solution. As a result, individual protein molecules align themselves in a repeating series of "unit cells" by adopting a consistent orientation. The importance of protein crystallization is that it serves as the basis for X-ray crystallography, wherein a crystallized protein is used to determine the protein's three-dimensional structure via X-ray diffraction. Protein crystallization is inherently difficult because of the fragile nature of protein crystals (Rhodes, 1993). Some factors that require consideration are protein purity, pH, and concentration of protein, temperature, and precipitants. In order for sufficient homogeneity, the protein should usually be at least 97% pure. The pH conditions are also very important, as different pH's can result in different packing orientations. Buffers, such as Tris-HCl, are often necessary for the maintenance of a particular pH (Branden and Tooze, 1999). Precipitants, such as ammonium sulfate or polyethylene glycol, are compounds that cause the protein to precipitate out of solution (Rhodes, 1993).

1.6 Crystallization methods

Two of the most commonly used methods for protein crystallization fall under the category of vapor diffusion. These are known as the hanging drop and sitting drop methods. Both entail a droplet containing purified protein, buffer, and precipitant being allowed to equilibrate with a larger

reservoir containing similar buffers and precipitants in higher concentrations. Initially, the droplet of protein solution contains an insufficient concentration of precipitant for crystallization, but as water vaporizes from the drop and transfers to the reservoir, the precipitant concentration increases to a level optimal for crystallization. Since the system is in equilibrium, these optimum conditions are maintained until the crystallization is complete (Rhodes, 1993; McRee and E.Duncan, 1993).

The hanging drop method differs from the sitting drop method in the vertical orientation of the protein solution drop within the system. It is important to mention that both methods require a closed system, that is, the system must be sealed off from the outside using an airtight container or high-vacuum grease between glass surfaces. (Rhodes, 1993; McRee and E.Duncan, 1993).

1.7 Protein Kinases

Sequencing of the human genome indicates there are >500 different protein kinase genes expressed in man (Manning G *et al.*,2002) Protein kinases are a large family of cell signaling mediators undergoing intensive research to identify inhibitors or modulators useful for medicine. As one strategy, small-molecule compounds that bind the active site with high affinity can be used to inhibit the enzyme activity. X-ray crystallography is a powerful method to reveal the structures of the kinase active sites, and thus aid in the design of high-affinity, selective inhibitors. However, a limitation still exists in the ability to produce purified kinases in amounts sufficient for crystallography. Furthermore, kinases exist in different conformation states as part of their normal regulation, and the ability to prepare crystals of

kinases in these various states also remains a limitation (Weiru Wang *et al.*,2006).

1.8 Tyrosine Kinase

A tyrosine kinase is an enzyme that can transfer a phosphate group from ATP to a tyrosine residue in a protein. Tyrosine kinases are a subgroup of the larger class of protein kinases. Phosphorylation of proteins by kinases is an important mechanism in signal transduction for regulation of enzyme activity.

The tyrosine kinases are divided into two groups:

- Those that are cytoplasmic proteins
- The transmembrane receptor-linked kinases

1.9 Receptor Tyrosine Kinase

Receptor tyrosine kinases (RTK) are the high affinity cell surface receptors for many polypeptide growth factors, cytokines and hormones. Of the ninety unique tyrosine kinase genes idenitified in the human genome, 58 encode receptor tyrosine kinase proteins.(Robinson D.R, *et al.,* 2000) Receptor tyrosine kinases have been shown to be not only key regulators of normal cellular processes but also to have a critical role in the development and progression of many types of cancer. (Zwick, E, *et al.,* 2001)

Most RTKs are single subunit receptors but some (e.g. the insulin receptor) exist as multimeric complexes. Each monomer has a single transmembrane spanning domain composed of 25-38 amino acids, an extracellular N-terminal region and an intracellular C-terminal region. The extracellular N-terminal region is composed of a very large protein domain which binds to

extracellular ligands e.g. a particular growth factor or hormone. The intracellular C-terminal region comprises domains responsible for the kinase activity of these receptors.

When a growth factor binds to the extracellular domain of an RTK, its dimerization is triggered with other adjacent RTKs. Dimerization leads to a rapid activation of the protein's cytoplasmic kinase domains, the first substrate for these domains being the receptor itself. The activated receptor as a result then becomes autophosphorylated on multiple specific intracellular tyrosine residues.

1.10 Receptor Tyrosine Kinase signaling pathway

RTK activation by its ligand TK induces kinase catalytic activity, which triggers transphosphorylation of the tyrosines Tyr 1234 and Tyr 1235. These two tyrosines engage various signal transducers, thus initiating a whole spectrum of biological activities driven by ligand tyrosine kinase, collectively knows as the invasive growth program. The receptor tyrosine kinase mediates a complex program known as invasive growth (Gentile A, *et al.,* 2008). RTK engagement activates multiple signal transduction pathways:

1. The RAS pathway mediates HGF-induced scattering and proliferation signals, which lead to branching morphogenesis (O'Brien L.E, *et al.,* 2004) Of note, HGF, differently from most mitogens, induces sustained RAS activation, and thus prolonged MAPK activity (Marshall, C. J ,1995)

2. The PI3K pathway is activated in two ways: PI3K can be either downstream of RAS, or it can be recruited directly through the multifunctional docking site. (Graziani A, *et al.,* 1991). PI3K activation also triggers a survival signal due to activation of the AKT pathway
3. The STAT pathway, together with the sustained MAPK activation, is necessary for the HGF-induced branching morphogenesis. MET activates the STAT3 transcription factor directly, through an SH2 domain
4. The beta catenin pathway, a key component of the Wnt signaling pathway, translocates into the nucleus following MET activation and participates in transcriptional regulation of numerous genes (Boccaccio C, *et al.,* 1998)
5. The Notch pathway, through transcriptional activation of Delta ligand. (Boccaccio C, *et al.,* 2006; Gude N.A, *et al.,* 2008)

MET pathway plays an important role in the development of cancer through:

1. activation of key oncogenic pathways (RAS, PI3K, STAT3, beta catenin);
2. angiogenesis (sprouting of new blood vessels from pre-existing ones to supply a tumor with nutrients);
3. scatter (cells dissociation due to metalloprotease production), which often leads to metastasis

1.11 OBJECTIVES

- To optimize the expression and purification of recombinant receptor tyrosine kinase (RTK)
- To express and purify recombinant receptor tyrosine kinase (RTK) in the active form (phosphorylated form)
- To clone the recombinant protein phosphatase which helps to produce inactive form of protein
- To co-express recombinant protein phosphatase and receptor tyrosine kinase (RTK)
- To purify recombinant receptor tyrosine kinase (RTK) in the inactive form (unphosphorylated form)
- To crystallize the receptor tyrosine kinase in the inactive form

REVIEW OF LITERATURE

2 REVIEW OF LITREATURE

Crystal structures have been reported for the extracellular tyrosine kinase (TK) Sema domain. It was shown that the serine protease-like TK β-chain alone binds to TK, and its crystal structure in complex with the Sema and PSI domain of the receptor was also reported. Mutation of Tyrosine Kinase residues in the area that constitutes the active site region in related serine proteases significantly impairs TK βbinding to TK. Key binding loops in this interface undergo conformational rearrangements upon maturation and explain the necessity of proteolytic cleavage for proper TK signaling. A crystallographic dimer interface between two TK β-chains brings two TK β receptor complexes together, suggesting a possible mechanism of tyrosine kinase receptor dimerization and activation by binding of Tyrosine Kinase (Stamos *et al.*, 2004)

Mutated form of the intracellular tyrosine kinase domain is also reported. (Cristiani *et al.*, 2005). Wild-type receptor tyrosine kinase activation requires phosphorylation of both Y1234 and Y1235 in the activation loop. Mapping the major phosphorylation sites in the kinase domain of a recombinant RTK protein was reported and further identification of the known residues Y1234 and Y1235 as well as a new phosphorylation site at Y1194 in the hinge region was done. Combining activating and silencing mutations at these sites, it was characterized in depth the mechanism of activation of wild-type and mutant receptor proteins. The Y1194F silencing mutation yielded an enzyme that could be activated to a similar extent as the wild type but with significantly slower activation kinetics, underlying the importance of this residue, which is conserved among different tyrosine kinase receptors.

There is a strong link between aberrant this tyrosine kinase activity and oncogenesis, which makes this kinase an important cancer drug target (Schiering *et al.,* 2003). Here it was reported that the crystal structures of an unphosphorylated kinase domain harboring a human cancer mutation. The structure follows the well established architecture of protein kinases. It adopts a unique, inhibitory conformation of the activation loop, a catalytically noncompetent orientation of helix aC, and reveals the complete C-terminal docking site.

Staurosporine is a potent, nonselective protein kinase inhibitor, the first of more than 50 alkaloids with an indolocarbazole subunit isolated to date (Wood *et al.*, 1997) Structures of staurosporine in complex with the Ser/Thr kinase domains as well as the tyrosine kinase domains have been reported (Prade *et al.,* 1997 and Zhu *et al.,*1999). They revealed a common binding mode in the adenosine cleft, induced conformational changes of the enzyme to accommodate the large compound through a complementary apolar interaction surface, and specific hydrogen-bonding interactions.

The conformational changes undergone by Ser/Thr and Tyr kinase domains as they turn on and off was reported (Lamers *et al.,* 1999). The structure of the kinase domain in the active state was reported, and two key regulatory elements within the domain, the activation loop and the aC helix was discussed. Several kinases that have been analyzed structurally in their inactive states were also discussed. On the basis of sequence and structure, these enzymes form a closely related superfamily that is distinct from the histidine kinases and other phosphotransfer enzymes.

Medulloblastomas are malignant brain tumors that arise by transformation of neural progenitor cells in the cerebellum in children. Treatment-related neurotoxicity has created a critical need to identify signaling molecules that can be targeted therapeutically to maximize tumor growth suppression and minimize collateral neurologic injury.

Tyrosine Kinase (TK) and its cell surface receptor Receptor Tyrosine Kinase (RTK) are highly expressed in human medulloblastomas, and elevated levels of RTK and TK mRNA predict an unfavorable prognosis for patients. This Tyrosine Kinase is neuroprotective for cerebellar granule cells and promotes growth of human medulloblastoma cells in culture and in murine xenografts. Modeling the ability of HGF was performed to induce medulloblastomas in mice using a version of the RCAS/*tv-a* system that allows gene transfer to cerebellar neural progenitors during their postnatal expansion phase when these cells are highly susceptible to transformation. Here, it was reported a high frequency of medulloblastoma formation in mice after postnatal expression of TK (Mandy *et al.,* 2008). These findings indicate a role for TK in medulloblastoma initiation and growth and show efficacy of TK-targeted therapy in a mouse model of endogenously arising tumors

On binding to the cell surface receptor tyrosine kinase (RTK) the Tyrosine Kinase (TK) stimulates mitogenesis, motogenesis, and morphogenesis in a wide range of cellular targets including, epithelial and endothelial cells, hematopoietic cells, neurons, melanocytes, and hepatocytes. These pleiotropic actions are fundamentally important during development,

homeostasis, and tissue regeneration. The TK signaling also contributes to oncogenesis and tumor progression in several human cancers and promotes aggressive cellular invasiveness that is strongly linked to tumor metastasis. This study indicates that tyrosine kinase oncogenic signaling supports at least three avenues of pathway selective anticancer drug development: antagonism of ligand/receptor interaction, inhibition of TK catalytic activity, and blockade of intracellular receptor/effector interactions (Benedetta and Donald, 2008)

Receptor tyrosine kinases (RTK) are often aberrantly activated in human malignancies and contribute to cancer development and progression. Specific receptor tyrosine kinase inhibitors have been shown to be clinically effective therapies in subsets of cancer patients with either hematologic or solid tumors. Activation of the tyrosine kinase / (TK)/RTK signaling pathway has been found to play a critical role in oncogenesis, cancer metastasis, and drug resistance. These observations have led to the development of agents that can effectively inhibit TK/RTK signaling through direct inhibition of the receptor (anti-RTK antibodies), through inactivation of its ligand TK (AMG102, L2G7), by interfering with TK binding to RTK (NK4), or by inhibiting RTK activity (PHA-665752 and SU11274). Moreover, the combination of anti-RTK therapeutic agents with either signal transduction inhibitors (ERBB family or mTOR inhibitors) or with cytotoxic chemotherapy has been evaluated in preclinical models. These studies provide insight into the rational development of combination therapeutic strategies that can be evaluated in clinical trials. This review discussed about the different strategies of RTK inhibition with a specific

focus on combination therapeutic approaches (Luca Toschi and Pasi Jänne, 2008)

The epidermal growth factor receptor (EGFR) kinase inhibitors gefitinib and erlotinib are effective treatments for lung cancers with *EGFR* activating mutations, but these tumors invariably develop drug resistance. Here, it was described that a gefitinib-sensitive lung cancer cell line that developed resistance to gefitinib as a result of focal amplification of the *HGFR* proto-oncogene. Inhibition of *HGFR* signaling in these cells restored their sensitivity to gefitinib. *HGFR* amplification was detected in 4 of 18 (22%) lung cancer specimens that had developed resistance to gefitinib or erlotinib. It was found that amplification of *HGFR* causes gefitinib resistance by driving ERBB3 (HER3)-dependent activation of PI3K, a pathway thought to be specific to EGFR/ERBB family receptors. Thus, it was propose that *HGFR* amplification may promote drug resistance in other ERBB-driven cancers as well (Jeffrey, *et al.*, 2007)

MATERIALS

3 MATERIALS

3.1 Medium Used

3.1.1 LB Medium (Luria – Bertani Medium)

Liquid media:

Per Liter:

The following were added to 950 ml of distilled water:

- Tryptone 10g
- Yeast Extract 5g
- NaCl 10g

- The pH is adjusted to 7.5 or 8.0 with 5N NaOH The volume is adjusted to 1liter with distilled water. Sterilize by autoclaving for 20minutes at 15psi
- The solid media is prepared by adding Bacto Agar of 15g/liter to the above composition just before autoclaving

3.1.2 TB Medium (Terrific Broth Medium)

For 1 liter:

- The following were added to 800ml distilled water
  - Tryptone 12g
  - Yeast Extract 24g
  - Glycerol 4ml
- The pH is adjusted to 7.5 or 8.0 with 5N NaOH. The volume adjusted to 900ml with distilled water
- Sterilized by autoclaving and allowed to cool to room temperature

- The volume adjusted to 1000ml with 100ml solution of [0.17M KH_2PO_4 and 0.72M K_2HPO_4 in 90ml of distilled water. Dissolved and adjusted the volume to 100ml with distilled water and sterilized by autoclaving]

3.1.3 2YT Medium

The following were added to 900ml distilled water

- Tryptone 16g
- Yeast Extract 10g
- NaCl 5g

- The pH is adjusted to 7.2 with 5N NaOH.
- The volume adjusted to 1 litre with distilled water
- Sterilized by autoclaving for 20minutes at 15psi

3.2 Antibiotics Used

3.2.1 Chloramphenicol

- Stock Concentration : 34 mg/ml
- Final Concentration : 34µg/ml and 17µg/ml

3.2.2 Kanamycin

- Stock Concentration : 50mg/ml
- Final Concentration : 50µg/ml and 25µg/ml

3.3 Vectors used

- pET28a vector
- pACYC vector

3.4 *E.coli* Strains used

- DH5α cells
- BL 21- DE3 cells
- BL 21- RIPL cells

3.5 Tris –HCl, 1M

121.1g of Tris base is dissolved in 800ml of distilled water. The pH is adjusted to the desired value by adding concentrated HCl. The solution should be allowed to cool before making final adjustments to pH

3.6 Lysozyme (10mg/ml)

The solid lysozyme at a concentration of 10mg/ml is dissolved in 10mM Tris – HCl (pH 8.0) immediately before use. The pH of the Tris solution should be 8.0

3.7 IPTG (1M)

0.238g of IPTG is dissolved in 1ml of distilled water to obtain the stock solution with 1M concentration and the final concentration is 0.5mM

3.8 Acrylamide Solution (45% w/v)

- Acrylamide 434g
- N,N' – Methylene bis acrylamide 16g
- Distilled water to 600ml

Heat the solution to 37°C to dissolve the chemicals

3.9 SDS Electrophoresis Buffer 5%

- Tris base 15.1g

- Glycine 72.0g
- SDS 5.0g
- Distilled water to 1000ml

3.10 4X SDS Gel Loading Buffer

- Tris – HCl, 100mM, pH 6.8
- SDS, 4% (w/v)
- Bromophenol blue, 0.2% (w/v)
- Glycerol, 20% (v/v)
- Dithiothreitol or -β-Mercaptoethanol, 200mM

3.11 SDS PAGE Composition

3.11.1 Resolving Gel (12%)

- Acrylamide / Bis-Acrylamide 2.0ml
- Tris, pH 8.8 1.3ml
- Distilled water 1.6ml
- SDS, 10% 50.0µl
- APS, 10% 50.0µl
- TEMED 4.0µl

3.11.2 Stacking Gel (5%)

- Acrylamide / Bis-Acrylamide 0.33 ml
- Tris, pH 6.8 0.25ml
- Distilled water 1.4ml
- SDS, 10% 20.0µl
- APS, 10% 20.0µl
- TEMED 2.0µl

3.12 Coomassie Blue Staining Solution

- Methanol 40ml
- Acetic acid 10 ml
- Distilled water 50ml
- Coomassie Brilliant Blue 250mg

3.13 Affinity Purification Buffers

3.13.1 Lysis Buffer

- Potassium phosphate, 100mM, pH 8.0
- NaCl, 250mM
- Igepal, 0.1%
- Glycerol, 5%
- Imidazole, 10mM
- PMSF, 2mM
- Lysozyme, 50 µg/ml

3.13.2 Equilibration buffer

- Potassium phosphate, 100mM, pH 8.0
- NaCl, 250mM
- Igepal, 0.1%
- Glycerol, 5%

3.13.3 Wash Buffer 1

- Potassium phosphate, 100mM, pH 8.0
- NaCl, 250mM

- Igepal, 0.1%
- Glycerol, 5%
- Imidazole, 25mM

3.13.4 Wash Buffer 2

- Tris, 50mM, pH 8.8
- NaCl, 150mM
- Imidazole, 25mM

3.13.5 Elution Buffer

- Tris, 50mM, pH 8.8
- NaCl, 150mM
- Imidazole, 250mM

3.14 Gel Filtration Buffer

- Tris, 20mM, pH 8.5
- NaCl, 100mM
- β- Mercaptoethanol, 14mM
- Glycerol, 5%

3.15 Ion Exchange Buffers

3.15.1 Buffer A

- Tris, 20mM, pH 8.5
- β- Mercaptoethanol, 14mM
- Glycerol, 5%

3.15.2 Buffer B

- Tris, 20mM, pH 8.5
- β- Mercaptoethanol, 14mM
- NaCl, 1.0M
- Glycerol, 5%

3.16 10X Bacteriophage T4 DNA Polymerase Buffer

- Tris – Acetate, 330mM, pH 8.0
- Potassium Acetate, 660mM
- Magnesium Acetate, 100mM
- Dithiothreitol, 5mM
- Bovine Serum Albumin, 1 mg/ml

3.17 50 XTAE Electrophoresis Buffer (Agarose Gel)

TAE (Tris/ Acetate / EDTA)

- Tris base 242.0g
- Glacial acetic acid 57.1ml
- Na_2 EDTA. $2H_2O$ 37.2g
- Distilled water to 1000ml

3.18 Agarose Gel loading buffer

- Glycerol, 50% (v/v)
- Na_2 EDTA. $2H_2O$, 10mM, pH 8.0
- Bromophenol blue, 0.2% (w/v)
- Xylene Cyanol, 0.25% (w/v)

3.19 Ethidium Bromide (10mg/ ml)

0.2g of Ethidium Bromide is dissolved in 20ml of distilled water

3.20 10 X Tris EDTA (TE), pH 8.0

- Tris- HCl, 100mM, pH 8.0
- Na_2 EDTA. $2H_2O$, 10mM

Solution should be sterilized by autoclaving for 20 minutes

3.21 Tris – Sucrose

- Tris – HCl, 50 mM, pH 8.0
- Sucrose, 10% (w/v)

The solution is sterilized by passing it through 0.22μm filter

3.22 Plasmid Extraction Buffers

3.22.1 Resuspension Buffer

- Ice – cold Tris – Sucrose solution, 50mM
- Lysozyme solution (10mg/ml)
- Na_2 EDTA. $2H_2O$, 0.25M
- RNAase (100μg/ml), pH 8.0

Lysozyme and EDTA – Breaks down the bacterial cell walls and punctures the outer membrane

Tris – Sucrose solution – Stabilizes the leaky spheroplasts

RNAase – Breaks the RNAs

3.22.2 Lysis Buffer

- NaOH, 0.2M
- Sodium Dodecyl Sulphate (SDS), 10%

NaOH (Alkaline condition) – Denatures the chromosomal and plasmid DNAs as well as proteins

Sodium Dodecyl Sulphate (SDS) – Solubilizes phospholipids and protein components of the cell membrane

3.22.3 Neutralization Buffer

- Concentrated Acetate, pH 5.0

Concentrated Acetate - It neutralizes lysate and renatures the plasmid

3.22.4 Wash Buffer

- Tris –HCl, 10mM
- NaCl, 50mM
- Na_2 EDTA. $2H_2O$, 0.1mM
- Ethanol, 70%

It is a high salt buffer. It removes contaminants such as salts, RNA and proteins

3.22.5 Elution Buffer

- TE buffer (Tris chloride, 10mM + EDTA, 1mM, pH 8.0)
- NaCl, 1.4M
- Ethanol, 15%

or Distilled water

EDTA inactivates nucleases by binding for metal ions required by these enzymes

It is a low salt buffer. Maximum elution between pH 7.0 and 8.5

3.23 Gel Extraction Buffers

3.23.1 Gel solubilization buffer

- Tris Acetate EDTA (TAE) or
- Tris Borate EDTA (TBE)

It contains high concentration of chaotrophic salt. It disrupts hydrogen bonding between sugars in the agarose polymer allowing the solubilization of gel slice. In addition, high salt concentration dissociates DNA binding proteins from the DNA fragments.

3.23.2 Wash Buffer

- Tris –HCl, 10mM
- NaCl, 50mM
- Na_2 EDTA. $2H_2O$, 0.1mM
- Ethanol, 70%

It is a high salt buffer. It removes contaminants such as salts, RNA and proteins

3.24 Restriction Digestion Buffers

3.24.1 Buffer 3 (pH 7.9)

- Tris – HCl, 50mM
- $MgCl_2$, 10mM
- NaCl, 100mM
- DTT, 1mM

3.25 10X T4 DNA Ligase Buffer

- Tris- HCl, 200mM
- Magnesium chloride, 50mM
- Dithiothreitol, 50 mM
- Bovine Serum Albumin, 50mg/ml

3.26 Crystallization Buffers

3.26.1 Well solution 1

- Diammonium hydrogen phosphate, 1.0M
- Sodium chloride, (0.1M – 0.2M)
- Sodium Citrate, 0.1M, (pH 4.75- pH 5.25)
- Glycerol, (0-10%)

3.26.2 Well solution 2

- PEG 5000 Monomethyl ether (6-10%)
- HEPES, 100mM, (pH 7.1 and pH 7.35)
- Isopropanol, 11%

METHODOLOGY

4 METHODOLOGY

The clone pET 28a with Receptor Tyrosine Kinase (RTK) was used for expression of protein, where pET 28a is an expression vector. The protein of interest receptor tyrosine kinase (RTK) is of molecular weight 36 kDa. The BL 21 RIPL competent cells were prepared for expression of RTK protein and the procedure is as follows:

4.1 Optimization of expression of Receptor Tyrosine Kinase

- The expression of receptor tyrosine kinase was carried out in different media like LB media, TB media and 2YT media
- The induction of protein was carried out at different temperatures such as 37° C, 18°C and 12°C
- The expression was also carried out by using different expression strains such as DE3 and RIPL cells
- The optimized expression of Receptor Tyrosine Kinase was obtained by using RIPL cells in TB media and induction temperature was optimized as 18°C
- The growth, induction and expression of Receptor Tyrosine Kinase is explained as follows:

4.2 BL 21- RIPL Competent Cell Preparation

Day 1:

- Desired cells (BL 21- RIPL) were streaked on LB chloramphenicol plate
- Incubated at 37°C for overnight

Day 2:

- A single colony from the plate was inoculated in 10ml LB broth with antibiotic
- Incubated at 37°C for overnight

Day 3:

- Inoculated 1% preinoculum (overnight culture) into 200ml LB media
- Allowed to grow till it reaches OD = 0.6 (approx. 1.5 – 2hrs)
- The cells were collected by centrifugation at 2700g for 10 minutes
- The pellet was thoroughly resuspended in 0.1M $MgCl_2$ and 0.1M $CaCl_2$ Incubated on ice for 10 minutes
- Centrifuged at 2700g for 10 minutes
- The pellet was resuspended in 0.1M $CaCl_2$ and incubated on ice for 30 minutes
- The 50% glycerol (0.6ml) was added to the resuspended cells
- The cells were aliquoted into prechilled tubes as 50µl each
- Stored at -80°C for further use

$$\text{Transformation efficiency} = \frac{\text{CFU (Colony Forming Units)}}{\text{Nanograms of DNA plated}} \times \frac{1 \times 10^3 \text{ng}}{1\mu\text{g}}$$

The transformation efficiency of BL 21 RIPL cells were found to be 0.2×10^6 CFU/ µg of DNA

4.3 Transformation of RIPL cells

The BL 21 RIPL competent cells were transformed with plasmid (pET 28a/ RTK) and the procedure is explained as follows:

- The plasmid (pET 28a/ RTK) of 1 µl was added to 50µl of compentent BL 21 RIPL cells
- Incubated in ice for 30 minutes
- Heat shock was given by incubating at 42°C for 90 seconds
- Transferred to ice immediately and incubated in ice for 10 minutes
- LB media (800µl) was added and incubated at 37°C for 1 hour
- Centrifuged at 10,000 rpm for 2 minutes and resuspended in 200µl of media
- The resuspended cells were plated in LB chloramphenicol and kanamycin and incubated at 37°C for overnight

4.4 Growth and Induction of BL 21 cells

- A single colony was picked from the transformed plate and inoculated into 10 ml TB containing chloramphenicol
- Incubated at 37°C for overnight
- From the overnight culture 5 ml was transferred into 500 ml of TB containing chloramphenicol
- Incubated at 37°C till the OD reaches 0.8
- The cells were now induced with 0.5mM IPTG and incubated at 18°C for 15 hours

4.5 Expression check for protein Receptor Tyrosine Kinase

- The cells were collected by centrifugation at 4,000 rpm for 20 minutes
- The pellet was resuspended with lysis buffer (20 ml per 1 litre of cells)
- The lysate was kept in rocker at 4°C for 30 minutes
- Lysis of cells was carried out by ultrasonication (15 seconds pulse; 30 seconds rest; 15 cycles)
- The lysed cells were centrifuged at 12,000 rpm for 1 hour
- The supernatant was collected for purification
- The supernatant and pellet were now checked for presence of protein of interest by running the SDS-PAGE and the SDS Polyacrylamide gel electrophoresis is done as follows:

4.6 SDS Polyacrylamide gel electrophoresis

One- dimensional gel electrophoresis under denaturing conditions (i.e. in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode.

4.6.1 Separating gel

- Glass plate sandwich of the electrophoresis apparatus is assembled using two clean glass plates and 0.75mm spacers
- Sandwich is locked to the casting stand
- Separating gel solution is prepared and applied to the sandwich along an edge of one of the spacers until the desired height of the solution between the glass plates is reached
- Isobutyl alcohol is added to the top of the gel as a layer and the gel is allowed to polymerize for 20 to 30minutes at room temperature

4.6.2 Stacking gel

- Isobutyl alcohol is poured off and washed completely with distilled water
- Stacking gel solution is prepared and poured using pipet into the sandwich
- Teflon comb is inserted into the layer of stacking gel solution
- Stacking gel is allowed to polymerize for 30 to 45 minutes at room temperature

4.6.3 Electrophoresis

- Protein sample is prepared by diluting with 1X SDS Sample loading buffer
- Teflon comb is carefully removed without tearing the edges of the polyacrylamide wells
- After the comb is removed the wells are rinsed with electrophoresis buffer.
- Sandwich is transferred to the chamber and filled up with electrophoresis buffer
- Power supply is connected at constant voltage of 180 volts and after the bromophenol blue reached the bottom of the separating gel, the power supply is disconnected

As the gel run is over it is removed and stained using Commasie Brilliant Blue staining solution. The gel is stained for 30 minutes to 1 hour and the gel is destained by using the destaining solution.

4.7 Affinity Purification of Receptor Tyrosine Kinase

The supernatant was affinity purified by using Nickel-NTA column and the protocol is described as follows:

- The column was washed with water and then equilibrated with one bed volume of equilibration buffer
- The supernatant obtained after centrifugation was added to the column
- The supernatant was incubated with nickel beads for 30 minutes at 4°C
- The supernatant was allowed to flow through the column
- The column was washed with one column volume of wash buffer 1 and subsequent wash with wash buffer 2 to remove nonspecific proteins
- The protein of interest was eluted with 5 ml elution buffer.
- The elutes were collected as 1 ml fractions
- The elutes were checked for the presence of proteins by using Bradford's assay explained in the section
- The presence of protein of interest was further checked by using SDS-PAGE
- The protocol adapted for SDS-PAGE is similar as explained in the section 4.6
- The SDS-PAGE gel is shown in Figure 7

4.8 Detection of proteins by Bradford's Assay

- Protein standards of known concentrations were prepared by using BSA

- The protein of 10µl was added to 200µl of Bradford's reagent and mixed well in 96 well plate
- The unknown protein was also prepared similarly
- The Bradford's reagent was used as blank
- Incubated for 5 to 45 minutes at room temperature for development of colour
- The colour change to blue indicates the presence of proteins
- The colour developed will be stable for 60 minutes

The absorbance was measured at 595nm to determine the concentration of protein

The concentration of receptor tyrosine kinase was nominal and the protein produced was in the phosphorylated form. The receptor tyrosine kinase can be crystallized and used as drug targets as unphosphorylated form only. To produce the unphosphorylated form of receptor tyrosine kinase, coexpression along with phosphatase should be carried out.
Hence, cloning of phosphatase into suitable vector was further performed.
The phosphatase was cloned from pET 28a vector into pACYC vector under T7 promoter region and the protocols were explained as follows

4.9 Cloning of Phosphatase into pACYC under T7 promoter

- The gene of interest phosphatase was cloned from pET 28a construct into pACYC under T7 promoter
- The phosphatase gene was cloned to produce unphosphorylated receptor tyrosine kinase by coexpressing both the genes together

4.10 Primer Designing

- The primer for cloning of Phosphatase was designed using Genetool software
- The forward primer was designed with Nde I restriction site and phosphatase gene specific regions
- The reverse primer was designed with Sal I site, phosphatase gene specific regions and stop codon

4.11 Polymerase Chain Reaction for Phosphatase

PCR for insert Phosphatase was carried out with gene specific primers and the reaction is as follows:

10X Thermopol buffer	5.0 µl
10mM dNTPs	1.0 µl
100 µM Forward primer (Nde I)	1.0µl
100 µM Reverse primer (Sal I)	1.0µl
DNA (Phosphatase/pET 28a)	0.5µl
Vent polymerase	1.0µl
Distilled water	40.5µl
Total volume	50.0µl

PCR cycles

Step 1: 1 cycle	Initial denaturation	95°C	4 minutes
Step 2: 30 cycles	Denaturation	95°C	30 seconds
	Annealing	65°C	30 seconds
	Extension	72°C	60 seconds
Step 3: 1 cycle	Final extension	72°C	10 minutes
Step 4:	Hold	4°C	

The amplification of insert phosphatase was checked by running agarose gel electrophoresis. The procedure for agarose gel electrophoresis is explained as follows:

4.12 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying and purifying DNA fragments.

The agarose gel electrophoresis is performed as follows:

4.12.1 Preparing the gel

- Adequate volume of electrophoresins buffer (1X TAE) is prepared to fill the electrophoresis tank and to prepare the gel (250ml for electrophoresis tank and 50ml for preparing the gel)
- Desired amount of agarose is added to the electrophoresis buffer. (0.8g of agarose is added as 0.8% gel is used for separation)
- Agarose is melted in a microwave oven and swirled for even mixing. The gel casting platform is sealed as it has open ends
- Agarose is cooled and ethidium bromide is added and poured in the platform and the comb is inserted
- Bubbles should not be trapped underneath the comb and all bubbles on the surface of the agarose should be removed before the gel sets

4.12.2 Loading and running the gel

- After the gel has hardened, the tape is removed from the open ends of the gel platform and gel comb is also taken out taking care not to tear the sample wells
- Electrophoresis buffer is added to the set gel until the buffer covers the gel to a depth of about 1mm

- DNA samples should be prepared in a volume that will not overflow the gel wells by addition of the appropriate amount of 6X loading buffer. Samples are typically loaded into the wells with a micropipette
- The leads are attached such that the DNA moves towards the anode or positive lead. The voltage is set to the desired level, to begin the electrophoresis. The progress of separation can be monitored by the migration of the dyes in the loading buffer
- Power supply is turned off when the bromophenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of the DNA fragments.
- DNA can be visualized by placing the gel on UV light source and can be photographed directly
- The agarose gel for PCR of Phosphatase is shown in Figure 8

4.13 Agarose gel extraction of amplified Phosphatase

The PCR amplified insert phosphatase was extracted from agarose gel and the protocol for gel extraction is described below:

- The DNA fragment of interest was excised from the agarose gel with a clean, sharp scalpel
- The gel slice was weighed
- Three gel volumes of the gel solubilization solution was added to the gel slice. In other words, for every 100 mg of agarose gel, 300µl of gel solubilization solution was added
- The gel mixture was incubated at 50-60 °C for 10 minutes, or until the gel slice was completely dissolved

- Vortexed briefly every 2-3 minutes during incubation to help dissolve the gel
- Preparation of the binding column can be completed while the agarose is being solubilized in previous step
- The binding column was placed into one of the provided 2 ml collection tubes
- 500µl of the column preparation solution was added to each binding column
- Centrifuged for 1 minute. The flow through liquid was discarded
- Once the gel slice was completely dissolved it should be made sure that the color of the mixture is yellow prior proceeding to the following step
- One gel volume of 100% isopropanol was added and mixed until it becomes homogenous
- The solubilized gel solution mixture was loaded into the binding column that was assembled in a 2 ml collection tube
- Centrifuged for 1 minute after loading the column each time. The flow-through liquid was discarded
- 700 µl of wash solution was added to the binding column
- Centrifuged for 1 minute. The flow through liquid was discarded
- Centrifuged again for 1 minute without any additional wash solution to remove excess ethanol
- The binding column was transferred to a fresh collection tube
- 50µl of elution solution was added to the center of the membrane and incubated for 1 minute

- Centrifuged for 1 minute. For efficient recovery of intact plasmid DNA, the elution solution was preheated to 65 °C prior to adding it to the membrane
- The gel eluted sample was checked by running agarose gel electrophoresis and the protocol for agarose gel electrophoresis is similar as explained in section 4.12
- The agarose gel for the eluted sample is shown in Figure 9

4.14 Restriction Digestion for PCR amplified Insert

The restriction digestion for PCR amplified insert (phosphatase) was done with restriction enzymes Nde I and Sal I and the reaction is given as follows:

Phosphatase (PCR amplified)	20.0µl
Buffer 3 (NEB buffer)	5.0µl
Nde I	1.0µl
Sal I	1.0µl
10 X BSA	0.5µl
Distilled water	22.5 µl
Total	50.0 µl

- The reaction mixture was incubated at 37°C for two hours and the DNA fragments were separated by running agarose gel electrophoresis
- The protocol for agarose gel electrophoresis is similar as explained in the section 4.12. The agarose gel picture is shown in Figure 10

4.15 Agarose gel extraction of digested Insert phosphatase

- The restriction digested insert phosphatase was extracted from agarose gel and the protocol for gel extraction is similar as explained in the section 4.13
- The gel eluted insert phosphatase was further used for setting up ligation reactions discussed in later sections

4.16 DH5α Competent Cell Preparation

- The DH5α competent cells were prepared for cloning of phosphatase into pACYC
- The procedure for competent cell preparation is similar as explained in section 4.2
- The efficiency of the competent cells was estimated as 0.3×10^6 CFU/ µg of DNA

4.17 Transformation of vector into DH5α cells and growth

- The vector pACYC for cloning was obtained from the construct pACYC/RXR
- The transformation of plasmid (pACYC/RXR) into DH5α competent cells was done and the protocol was similar as explained in section 4.3.
- The transformed cells were plated in LB plate containing chloramphenicol
- A single colony from the overnight incubated plate was inoculated in 10ml LB broth containing chloramphenicol
- Incubated at 37°C for overnight for extraction of plasmid

4.18 Extraction of plasmid from DH5α cells

- The plasmid pACYC/RXR was extracted from the overnight grown DH5α cells and the protocol adapted is as follows:
- 1 to 5 ml of an overnight recombinant cell culture was pelleted by centrifugation at 10,000 rpm for 1 minute. The supernatant was discarded
- The bacterial pellet was completely resuspended with 200µl of the Resuspension Solution
- Vortexed or pipetted up and down to thoroughly resuspend the cells until the solution becomes homogeneous as incomplete resuspension will result in poor recovery
- The resuspended cells were lysed by adding 200µl of the Lysis Buffer
- Immediately mix the contents by gentle inversion (6-8 times) until the mixture becomes clear and viscous. The lysis reaction should not exceed 5 minutes
- The cell debris was precipitated by adding 350µl of the neutralization/Binding Buffer. The contents were mixed by gently inverting the tube for 4-6 times.
- The cell debris were pelleted by centrifuging at 12,000 rpm for 10 minutes
- The binding column was prepared by adding 500µl of column preparation buffer and centrifuged at 10,000 rpm for 30 seconds to 1 minute. The flow-through liquid discarded
- The cleared lysate obtained after centrifugation was added to the prepared column and centrifuged at 10,000 rpm for 30 seconds to 1 minute. The flow-through liquid discarded

- The Wash Solution of 750µl was added to the column. Centrifuged at 10,000 rpm for 30 seconds to 1 minute. The column wash step removes residual salt and other contaminants introduced during the column load. The flow-through liquid was discarded
- Centrifuged again at maximum speed for 1 to 2 minutes without any additional Wash Solution to remove excess ethanol.
- The column was transferred to a fresh collection tube. 50µl of Elution Solution was added to the column and centrifuged at 10,000 rpm for 1 minute. The DNA present in the eluate can be for immediate use or can be stored at –20°C
- The size and quality of DNA may be determined by agarose gel electrophoresis
- The protocol for agarose gel electrophoresis is similar as explained in section 4.12
- The agarose gel for the plasmid (pACYC/RXR) extraction is shown in Figure 12

4.19 Restriction digestion for pACYC/RXR

The restriction digestion for pACYC/RXR was also carried out by using Nde I and Sal I and the reaction is given as follows:

Vector (pACYC/RXR)	20.0 µl
Buffer 3	5.0 µl
Nde I	1.0µl
Sal I	1.0 µl
BSA	0.5 µl
Distilled water	22.5 µl
Total	50.0 µl

- The reaction mixture was incubated at 37°C for two hours and separated by running agarose gel electrophoresis
- The procedure adapted for agarose gel electrophoresis is similar as explained in the section 4.12
- The agarose gel for the digested insert is shown in Figure 13

4.20 Agarose gel extraction of digested vector pACYC

- The restriction digested vector pACYC was extracted from agarose gel and the protocol for gel extraction is similar as explained in the section 4.13
- The gel eluted insert phosphatase was further used for setting up ligation reactions discussed in later section

4.21 Ligation of Insert and Vector

The ligation reaction was carried out in 1:1 and 1:2 ratios of vector and insert respectively. The vector control (VC)reaction was also performed to check the occurance of self ligation. The ligation reactions are given as follows:

	1:1	1:2	VC
Vector (pACYC)	5.0µl	5.0µl	5.0µl
Insert (phosphatase)	5.0µl	10.0µl	-----
T4 DNA ligase buffer	2.0µl	2.0µl	2.0µl
Ligase	1.0µl	1.0µl	1.0µl
Distilled water	7.0µl	2.0µl	12.0µl
Total	20.0µl	20.0µl	20.0µl

The above ligation reactions were incubated at 4°C for overnight

4.22 Transformation of ligated samples

- The overnight ligated samples were transformed into DH5α competent cells and the transformation was carried out as explained in section 4.3
- The transformed DH5α cells were plated in LB plate containing chloramphenicol and incubated at 37°C for overnight

Observation

- The vector control plate had no colonies and this indicates the absence of self ligation of vector
- Both the 1:1 and 1:2 ligation reaction plates had around 15 to 20 colonies
- From the overnight transformed plate with 1:1 ligation reaction, four colonies were selected randomly and each inoculated into 10ml LB media containing chloramphenicol
- Incubated at 37°C for overnight

4.23 Extraction of plasmid pACYC/Phosphatase from DH5α cells

- The plasmid pACYC/Phosphatase was extracted from overnight grown four colonies
- The plasmid extraction was carried out by adapting the protocol as explained in section 4.18
- The extracted plasmid was checked by running agarose gel electrophoresis and the protocol adapted is similar as explained in section 4.12
- The agarose gel picture is shown in Figure 15

4.24 PCR for clone (pACYC/ Phosphatase)

The PCR reaction was carried out by using primers specific to Phosphatase. This amplification was done to check the presence of the clone (pACYC/ Phosphatase)

The PCR reaction mixture was prepared as master mix for all the four colonies and the reaction is explained as follows:

10X Thermopol buffer	4.0 µl
10mM dNTPs	1.0 µl
100 µM Forward primer (Nde I)	2.0µl
100 µM Reverse primer (Sal I)	2.0µl
DNA (pET 28a/Phosphatase)	0.5µl
Vent polymerase	1.0µl
Distilled water	28.0µl
Total volume	38.0µl

- The master mix was aliquoted into 9.5µl each and 0.5µl of extracted plasmid was added
- The PCR cycles were similar as explained in section 4.11
- The amplified samples were checked in agarose gel electrophoresis as explained in section 4.12
- The agarose gel showing PCR amplification is given in Figure 16

4.25 Conformational digestion for clone (pACYC/ Phosphatase)

4.25.1 Restriction digestion using Nde I and Sal I

The restriction digestion was carried out by using Nde I and Sal I to conform the clone as pACYC/ Phosphatase

The restriction digestion reaction mixture was prepared as master mix for all the four plasmids and it is shown as follows:

Buffer 3	8.0µl
Nde I	2.0µl
Sal I	2.0µl
BSA	1.0µl
Distilled water	27.0µl
Total	40.0µl

- The master mix was aliquoted into 10µl each and 10µl of plasmid pACYC/ Phosphatase was added
- The reaction mixture was incubated at 37°C for two hours and separated by running agarose gel electrophoresis
- The protocol adapted for agarose gel electrophoresis is similar as explained in section 4.12
- The agarose gel picture showin restriction digestion is shown in Figure 17

4.25.2 Restriction digestion using Nde I and Xho I

The restriction digestion was carried out by using Nde I and Xho I to conform the clone as pACYC/ Phosphatase

The restriction digestion reaction mixture was prepared as master mix for all the first two plasmids and it is shown as follows:

Buffer 4	4.0µl
Nde I	1.0µl
Sal I	1.0µl
BSA	0.4µl
Distilled water	3.6µl
Total	10.0µl

- The master mix was aliquoted into 5µl each and 15µl of plasmid pACYC/ Phosphatasse was added
- The reaction mixture was incubated at 37°C for two hours and separated by running agarose gel electrophoresis
- The protocol adapted for agarose gel electrophoresis is similar as explained in section 4.12
- The agarose gel picture shown restriction digestion is shown in Figure 18

4.26 Sequence conformation

- The clones were sent for senquencing and the sequence conformed the clone as pACYC/ phosphatase. There was no mutations or errors in the clone produced
- Hence this clone pACYC/ phosphatase was used futher for producing unphosphorylated tyrosine kinase

4.27 Co- Transformation of Receptor Tyrosine Kinase and Phosphatase

- Transformation of both pET28a/ Receptor Tyrosine Kinase and pACYC/Phosphatase into BL21 RIPL cells was carried out

- The procedure for co-transformation adapted is similar as explained in the section 4.3

4.28 Colony PCR for Phosphatase

The Colony PCR is similar to normal PCR only one difference is that the entire colony is added instead of DNA as in normal PCR. This colony PCR was performed to check the presence of Phosphatase. From the co-transformed plate 18 colonies were chosen randomly and screened for the presence of phosphatase

Master mix was prepared for 18 colonies and one positive control (pACYC/Phosphatase). The reaction for colony PCR is given as follows:

10X Thermopol buffer	30.0 µl
10mM dNTPs	2.0 µl
100 µM Forward primer (Nde I)	2.5µl
100 µM Reverse primer (Sal I)	2.5µl
Deep Vent polymerase	3.0µl
Distilled water	220.0µl
Total volume	260.0µl

- The master mix was aliquoted into 13µl each and 2µl of co-transformed colony was added. The PCR cycles and conditions were similar as explained in section 4.11
- The PCR amplified samples were checked by running agarose gel electrophoresis. The agarose gel electrophoresis was performed as explained in section 4.12
- The agarose gel picture showing the amplification is given in Figure 19

- One single colony was found to be amplified indicating the presence of Phosphatase gene

4.29 Colony PCR for Receptor Tyrosine Kinase

- The colony containing Phosphatase gene was further checked for the presence of Receptor Tyrosine Kinase gene also for the coexpression of both the proteins
- This colony PCR was performed for one positive clone containing phosphatase, one negative clone and one positive control (pET28a/ Receptor Tyrosine Kinase)
- The master mix was prepared for two colonies and one control. The PCR reaction is shown as follows:

10X Thermopol buffer	4.5 µl
10mM dNTPs	0.5 µl
100 µM Forward primer (Nde I)	1.0µl
100 µM Reverse primer (Sal I)	1.0µl
Deep Vent polymerase	1.0µl
Distilled water	31.0µl
Total volume	39.0µl

- The master mix was aliquoted into 13µl each and 2µl of colony was added. The PCR cycles were similar as explained in section 4.11
- The PCR amplified samples were checked in agarose gel electrophoresis and the procedure for agarose gel electrophoresis is similar as explained in section 4.12
- The agarose gel showing amplification for receptor tyrosine kinase is shown in Figure 20

- The positive Phosphatase clone was observed to have Receptor Tyrosine Kinase gene also. Hence this clone containing both Receptor Tyrosine Kinase and Phosphatase was used for further Co-Expression

4.30 Preparation of Glycerol Stocks

- The glycerol stock was prepared for the clone containing both Receptor Tyrosine Kinase and Phosphatase
- The clone containing Receptor Tyrosine Kinase and Phosphatase was inoculated in 10ml Terrific broth containing Kanamycin and Chloramphenicol
- Incubated at 37°C for overnight
- From the overnight grown culture, 30% glycerol stocks were prepared
- The glycerol stock was aliquoted into 1ml each and stored at -80°C for further use

4.31 Co- Expression of Receptor Tyrosine Kinase and Phosphatase

- The optimized expression of Receptor Tyrosine Kinase was obtained by using TB media and RIPL cells and induction temperature was optimized as 18°C
- Hence the co-expression of Receptor Tyrosine Kinase and Phosphatase were also carried out under same conditions but optimization for antibiotic concentration was performed to increase the protein yield
- The growth, induction and co-expression of Receptor Tyrosine Kinase and Phosphatase is explained as follows:

4.32 Growth and Induction of BL 21 cells (Receptor Tyrosine Kinase / Phosphatase)

- The glycerol stock of 2µl was inoculated into 10 ml Terrific broth containing Kanamycin and chloramphenicol
- Incubated at 37°C for overnight
- The overnight grown culture was used to inoculate 500ml Terrific broth containing Kanamycin and Chloramphenicol
- The final concentration of kanamycin used was 50 µg/ml and the final concentration of chloramphenicol used was 34 µg/ml
- Incubated at 37°C till the OD reaches 0.8
- Induced with 0.5mM IPTG and the cells were incubated at 18°C for 15 hours
- The expression check of Receptor Tyrosine Kinase was carried out. The protocols adapted were similar as explained in sections 4.5
- The protein expression was checked in SDS-PAGE and the protocol adapted for SDS-PAGE is similar as explained in section 4.6
- The gel showing the expression is shown in the Figure 21
- The expression was not good and most of the protein was observed to be in the pellet
- Hence, further purification of supernatant containing Receptor Tyrosine kinase/ Phosphatase was not carried out
- Inorder to increase the protein expression, another batch with reduced kanamycin concentration was started and explained as follows:

4.33 Co-Expression in Reduced kanamycin condition

4.33.1 Growth and Induction of BL 21 RIPL cells

- The glycerol stock of 2µl was inoculated into 10 ml Terrific broth containing Kanamycin 50µg/ml and chloramphenicol 34µg/ml
- Incubated at 37°C for overnight
- The overnight grown culture was used to inoculate 1000ml Terrific broth containing Kanamycin and Chloramphenicol
- The final concentration of kanamycin used was 25 µg/ml and the final concentration of chloramphenicol used was 34 µg/ml
- Incubated at 37°C till the OD reaches 0.8
- Induced with 0.5mM IPTG and the cells were incubated at 18°C for 15 hours
- The expression check was carried out as described in the section 4.5
- The uninduced, induced, supernatant and pellet were checked in SDS PAGE
- The procedure adapted for SDS PAGE is similar as explained in the section 4.6
- The SDS PAGE gel showing expression under reduced Kanamycin is shown in the Figure 22

4.33.2 Affinity Purification of Co-Expressed Receptor Tyrosine Kinase

- The Nickel-NTA purification of co-expressed Receptor Tyrosine Kinase was carried out. The protocol adapted was similar as explained in section 4.7
- The elutions were checked in SDS PAGE and the protocol adapted for SDS electrophoresis is similar as explained in the section 4.6

- The gel picture showing the purified elution fractions is given in Figure 23
- The expression was observed to be good than with both antibiotics at high concentration
- But still most of the protein, Receptor Tyrosine Kinase was observed to be in the pellet
- Inorder to increase the yield of protein further another batch with reduced chloramphenicol concentration was carried out and it is explained as follows:

4.34 Co-Expression in reduced chloramphenicol condition

4.34.1 Growth and Induction of BL 21 RIPL cells

- The glycerol stock of 2µl was inoculated into 10 ml Terrific broth containing Kanamycin 50µg/ml and chloramphenicol 34µg/ml
- Incubated at 37°C for overnight
- The overnight grown culture was used to inoculate 1000ml Terrific broth containing Kanamycin and Chloramphenicol
- The final concentration of kanamycin used was 50 µg/ml and the final concentration of chloramphenicol used was 17 µg/ml
- Incubated at 37°C till the OD reaches 0.8
- Induced with 0.5mM IPTG and the cells were incubated at 18°C for 15 hours
- The expression check was carried out as described in the section 4.5
- The uninduced, induced, supernatant and pellet were cheked in SDS - PAGE

- The procedure adapted for SDS PAGE is similar as explained in the section 4.6
- The SDS PAGE gel showing expression under reduced Kanamycin is shown in the Figure 24

4.34.2 Affinity Purification of Co-Expressed Receptor Tyrosine Kinase

- The Nickel-NTA purification of co-expressed Receptor Tyrosine Kinase was carried out. The protocol adapted was similar as explained in section 4.7
- The elutions were checked in SDS PAGE and the protocol adapted for SDS electrophoresis is similar as explained in the section 4.6
- The gel picture showing the purified elution fractions is given in Figure 25
- The expression was observed to be good than with both antibiotics at high concentration and reduced kanamycin condition
- Considerable amount of protein was observed to be in supernatant also in this reduced chloramphenicol condition
- The affinity purification fractions were taken for further purification to obtain high concentration of pure protein

4.35 Desalting of Receptor Tyrosine Kinase

- The eluted fractions from affinity chromatography had salt concentration of 150mM
- To proceed further to the ion exchange chromatography the salt concentration in the sample should be minimal
- Hence, protein sample was diluted ten times with buffer A (i.e 5ml of protein sample was diluted to 50ml)

- The buffer for anion exchange chromatography should have pH above the pI of the protein of interest
- The isoelectric point, pI of tyrosine kinase was determined by using Expasy software
- The theoritical pI of tyrosine kinase with His tag is 7.7
- Hence the buffer A and buffer B were prepared with pH of 8.5

4.36 Purification of Receptor Tyrosine Kinase by Ion Exchange Chromatography

- The anion exchange chromatography was performed for purification of receptor tyrosine kinase and the procedure is explained as follows:
- The Hitrap Q (Amersham BioSciences) column was used for the purification and the entire purification was carried out at 4°C
- The column was connected to the peristalitic pump and equilibrated with buffer A of two bed volumes
- The diluted sample was now allowed to pass through the column for binding by pumping through peristalitic pump
- The protein bound column was now connected to AKTA FPLC (Amersham BioSciences) system for elution of protein.
- The FPLC system was connected to the computer terminal and the entire process can be monitored and controlled by unicorn software installed in it
- The pump wash was given to both pump A and pump B
- The column wash was done by passing two bed volumes of buffer A to remove the unbound proteins

- The elution of bound proteins was carried out by passing buffer B in varying concentration of salt
- The proteins get eluted at different salt concentrations of salt and it is based on the difference in pI of the proteins
- Elution was carried out by passing buffer B in linear gradient
- The UV absorbance of protein and the conductivity of the salt can be observed in the chromatogram as the protein elutes and passes through the detector
- Samples were collected as peaks were observed in the chromatogram
- The ion exchange chromatogram is given in Figure 26
- The fractions collected in ion exchange chromatography was checked in SDS-PAGE and the procedure for SDS-PAGE is similar as explained in section 4.6
- The SDS-PAGE for ion exchange fractions is given in Figure 27

4.37 Purification of Receptor Tyrosine Kinase by Gel Filtration Chromatography

- The protein fractions obtained from ion exchange chromatography was further purified by using gel filtration chromatography
- Gel filtration chromatography was performed to obtain homogenous protein
- The volume of ion exchange fraction was around 18ml and the fraction was concentrated to 500µl
- The concentration of protein sample was done by using 30kDa Amicon ultra centricon

- Gel filtration chromatography was done by using Superdex 75 column 10/300 dimension and the entire gel filtration chromatography was carried out at 4°C
- The S 75 column was connected to the FPLC system
- The FPLC system was connected to the computer terminal and the entire process can be monitored and controlled by unicorn software installed in it
- The pump wash was given to both pump A alone
- The column was equilibrated by passing one bed volume of gel filtration buffer
- The flow rate was maintained at 0.4ml/min and the maximum pressure was set at 1.4 MPa
- The sample injection valve was washed with buffer before injection of sample
- The concentrated 500µl of protein Receptor Tyrosine Kinase was injected into the column
- The proteins get eluted out of the column based on their difference in molecular weight
- The protein fractions were collected as peaks appeared in the chromatogram
- The gel filtration chromatogram is given in Figure 28
- The fractions collected in gel filtration chromatography was checked in SDS-PAGE and the procedure for SDS-PAGE is similar as explained in section 4.6
- The SDS-PAGE for ion exchange fractions is given in Figure 29

4.38 Determination of Receptor Tyrosine Kinase Concentration

- The Tyrosine Kinase obtained from gel filtration chromatography was concentrated to 150µl
- The concentration of sample was carried out by using 30kDa Amicon ultra centricon
- The final concentration of protein was estimated by performing Bradford's assay
- The procedure for the Bradford's assay is similar as explained in section 4.8
- The absorbance values obtained is given in Table 1
- The plot was drawn from Bradford's assay absorbance by using MS-EXCEL and is given in Figure 30
- The Bradford plot obtained and the slope were shown in Figure
- The final concentration of Receptor Tyrosine Kinase obtained was 18mg/ml
- The concentration of Receptor Tyrosine Kinase required to setup the crystallization was 16mg/ml for condition 1 and 10mg/ml for condition 2
- Hence the protein obtained was diluted to 10mg/ml and 16mg/ml
- The diluted Receptor Tyrosine Kinase with required concentration was aliquoted into 15µl each
- The aliquoted protein sample, receptor tyrosine kinase was freezed with liquid nitrogen and stored at -80°C for further crystallization

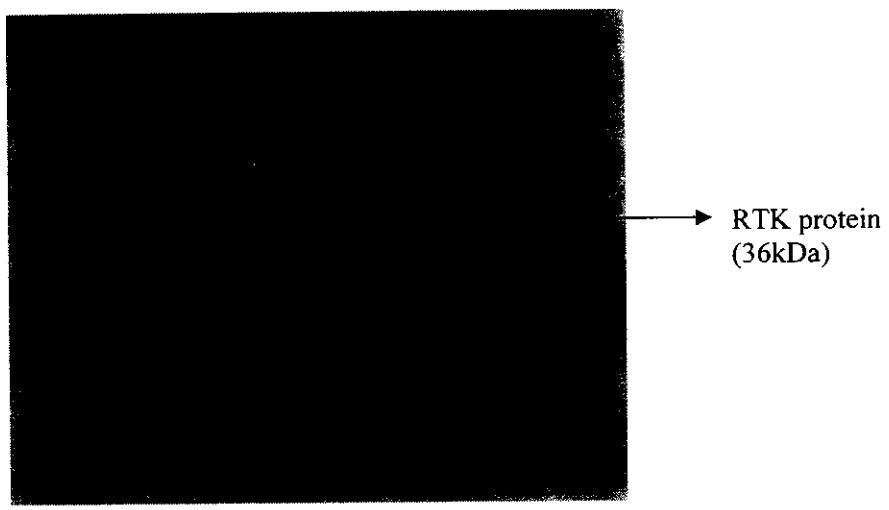
4.39 Crystallization of Receptor Tyrosine Kinase by hanging drop method

- The 24 well plate was used for setting up crystallization and the crystallization was carried out at 4°C
- The well solution was prepared for 1ml in the 24 well plate and the conditions were varied for each well solution
- A droplet (1 µl) of purified protein and a droplet (1µl) of well solution were added on the siliconized cover slip and mixed well
- The cover slip was inverted and sealed on the well containing respective well solution
- The sealing was done by using high vacuum grease
- The droplet containing purified protein and buffer was allowed to equilibrate with the large reservoir containing similar buffer at high concentration
- As water vaporizes from the drop and transfers to the well, precipitant concentration increases to a level optimal for crystallization
- This method requires a closed system and hence high vacuum grease is applied between glass surfaces
- The crystallization set up was done for 11 conditions with different buffers and the conditions were shown in Table 2
- The crystallization plate was incubated at 4 C allowing for the growth of the crystal
- The crystal growth was observed intermittently under light microscope for every 24 hours

RESULTS AND DISCUSSION

5 RESULTS AND DISCUSSION

5.1 Receptor Tyrosine Kinase expression in LB media using DE3 cells

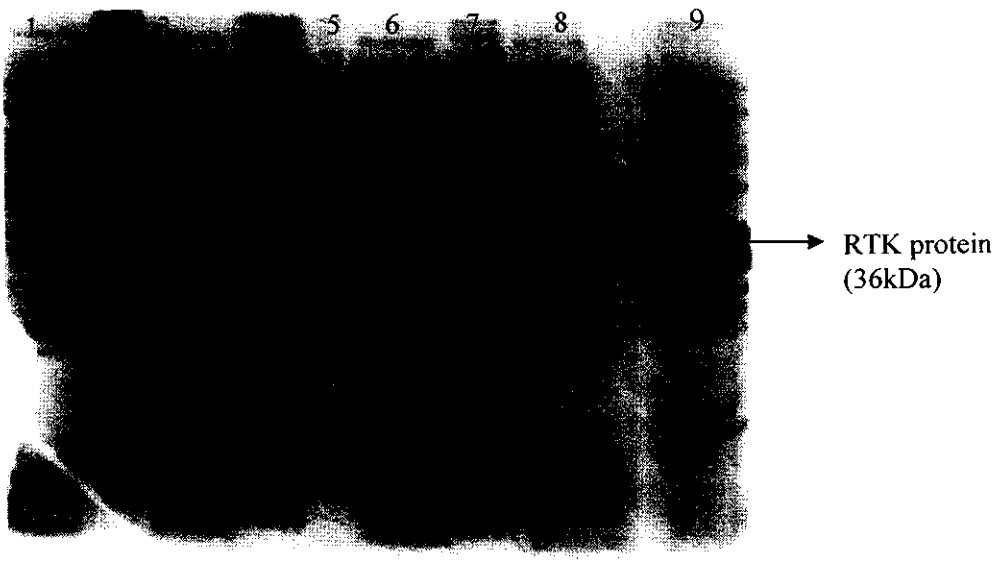


Lane 1: uninduced whole cells
Lane 2: uninduced supernatant
Lane 3: uninduced pellet
Lane 4: Protein marker
Lane 5: Induced 12 ° C whole cells
Lane 6: Induced 12 ° C supernatant
Lane 7: Induced 12 °C pellet
Lane 8: Induced 37 ° C whole cells
Lane 9: Induced 37 ° C supernatant
Lane 10: Induced 37°C pellet

Figure 2: Receptor Tyrosine Kinase expression in LB media using DE3 cells

The expression check was carried out in LB media under varying induction temperatures. The uniduced samples were also loaded for comparison with induced samples. The 12° C induction was observed to be better than 37°C induction as more amount of protein was observed to be coming out in supernatant. But still the amount of protein required to carry out purification was less. Hence expression of protein was carried out in other different media like 2YT media and TB media.

5.2 Receptor Tyrosine Kinase expression in 2YT media under different conditions



Lane 1: Supenatant DE3 cells at 12 °C

Lane 2: Pellet DE3 cells at 12 ° C

Lane 3: Supenatant RIPL cells at 12 ° C

Lane 4: Pellet RIPL cells at 12 ° C

Lane 5: Protein Marker

Lane 7: Pellet RIPL cells at 18 ° C

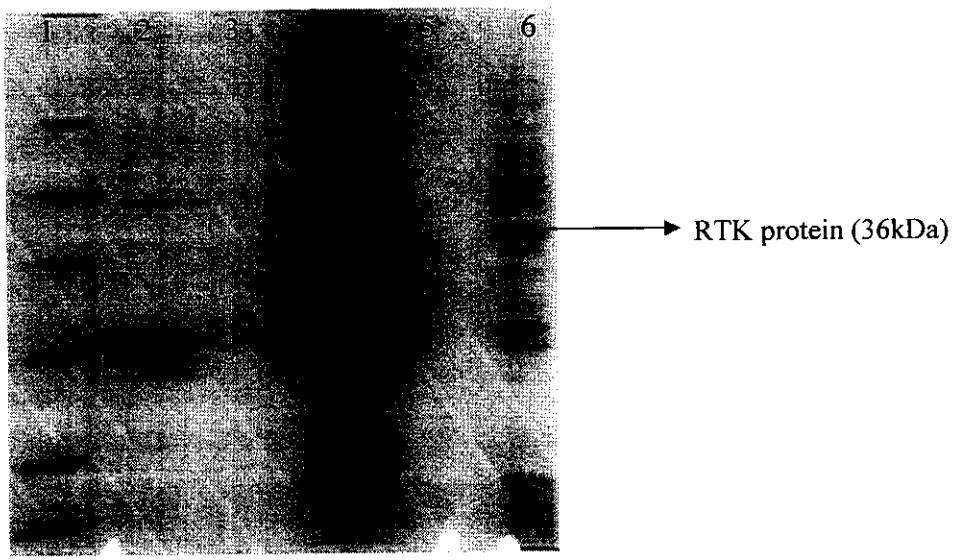
Lane 8: Supenatant DE3 cells at 18 ° C

Lane 9: Pellet DE3 cells at 18 ° C

Figure 3: Receptor Tyrosine Kinase expression in 2YT media under different conditions

The expression of protein RTK was better in 2YT media than the LB media. The RIPL cells expression for this protein was good than the DE3 cells. Most of the protein was observed in supernatant by using RIPL cells for expression. The expression was better at 18° C than at 12° C

5.3 Receptor Tyrosine Kinase expression in TB media in DE3 cells at 37 °C induction



Lane 1: Protein marker (1kb)

Lane 2: Induced 37 °C supernatant

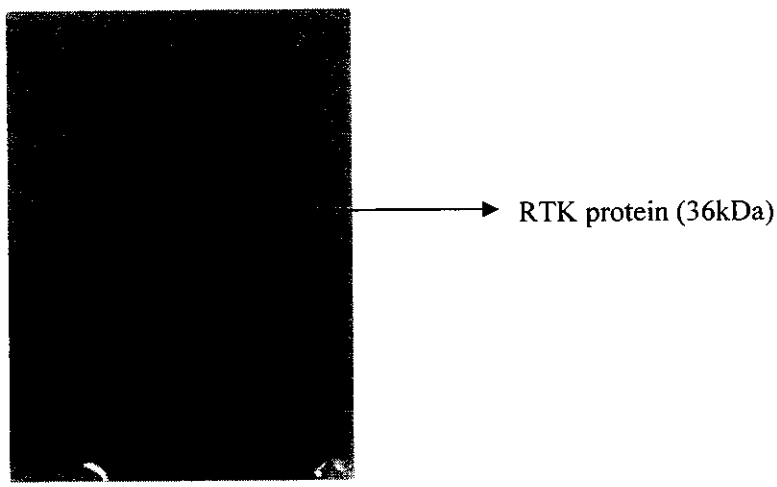
Lane 4: Induced 37 °C pellet

Lane 6: Induced 37 °C whole cells

Figure 4: Receptor Tyrosine Kinase expression in TB media in DE3 cells at 37 °C induction

The expression of RTK was observed to be good in Terrific broth media than in LB media and 2YT media. Most of the protein was observed to be in the pellet after centrifugation of sonicated sample. The pellet thus containing the protein of interest is known as inclusion bodies. The formation of inclusion bodies is much favoured at high temperatures. Hence, to bring the protein into the supernatant the induction temperature was reduced and the expression was checked under each conditions.

5.4 Receptor Tyrosine Kinase expression in TB media in DE3 cells at 18 °C induction



Lane 1: Protein marker

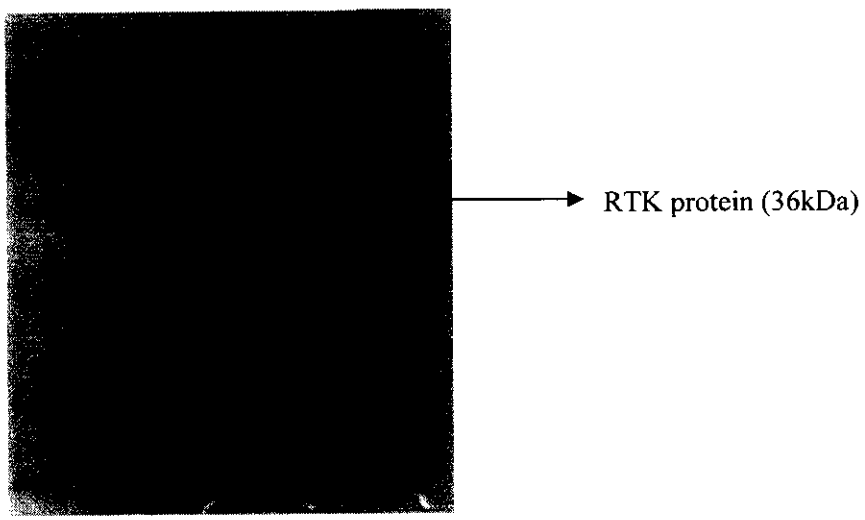
Lane 2: uninduced supernatant

Lane 3: Induced supermatant

Figure 5: Receptor Tyrosine Kinase expression in TB media in DE3 cells at 18 °C induction

The uninduced sample was loaded for comparison with induced sample. The expression of RTK at 18° C was good than at high temperature induction as less amount of protein was observed in the pellet. But the protein expression was less using DE3 cells. To increase the protein yield the expression was carried out in RIPL cells further using same TB media at 18 °C induction. The induction temperature was optimized as 18°C and the media for optimized expression was found in terrific broth

5.5 Expression check for Receptor Tyrosine Kinase in TB media in RIPL cells at 18 °C induction



Lane 1: Protein marker

Lane 2: Uninduced (Receptor Tyrosine Kinase)

Lane 3: Induced (Receptor Tyrosine Kinase)

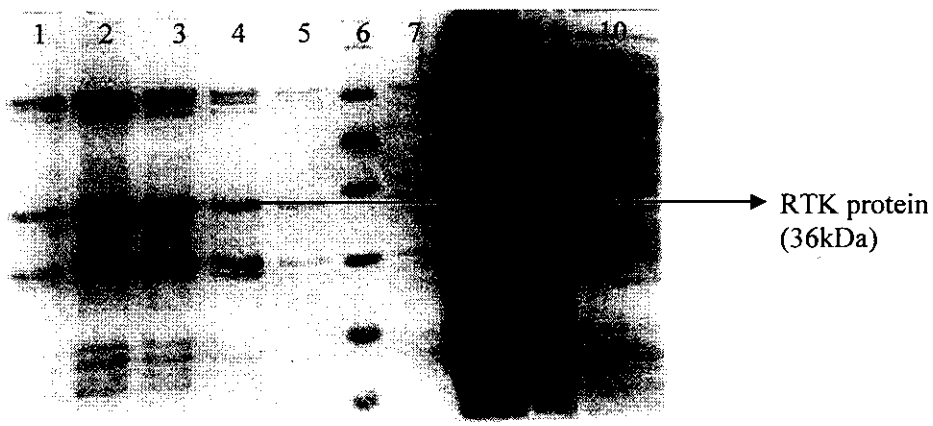
Lane 4: Supernatant

Lane 5: Pellet

Figure 6: Expression check for Receptor Tyrosine Kinase

The uninduced sample was loaded for comparison with induced sample. The expression was found to be better in TB media using RIPL cells at the induction temperature of 18°C. These conditions were used as optimized conditions for the expression of the protein, Receptor Tyrosine Kinase. The expression profile under these conditions was shown in Figure 6. Most of the protein was observed to be in the supernatant as the supernatant will be taken for further purification.

5.6 Nickel NTA purification of pET 28a/ Receptor Tyrosine kinase



Lanes 1 to 5& 7: Elution fractions

Lane 6: Protein marker

Lane 8: Nickel beads

Lane 9: Wash with 50mM imidazole

Lane 10: Wash with 25mM imidazole

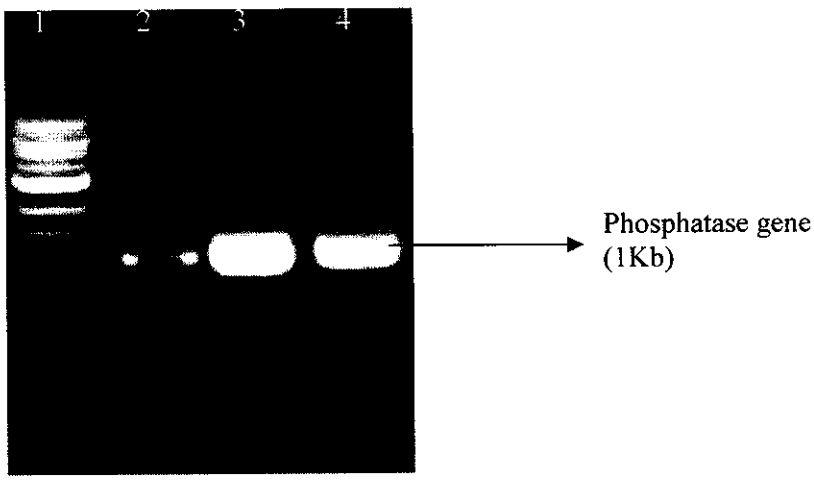
Figure 7: Nickel NTA purification of pET 28a/ Receptor Tyrosine kinase

The supernatant obtained at 18°C induction was taken for affinity purification. As the protein of interest is His tagged, it binds to the Nickel – NTA column. The unbound protein was washed by using 25mM imidazole and 50mM imidazole buffers. The nickel beads after elution were also loaded in the gel to check the presence of protein in the beads.

The phosphorylated receptor tyrosine kinase (36kDa) was obtained in elution fractions with 250mM imidazole shown in Figure 7

The unphosphorylated form of receptor tyrosine kinase was required for crystallization hence, further works were carried out to produce unphosphorylated receptor tyrosine kinase.

5.7 PCR for insert Phosphatase



Lane 1: DNA marker (1Kb)

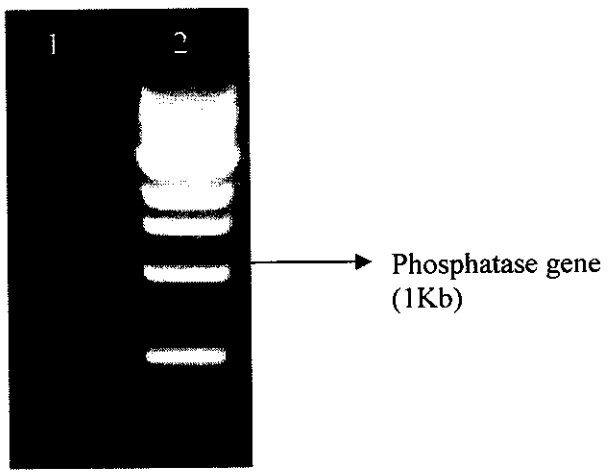
Lane 2: Phosphatase unamplified

Lanes 3 and 4: Phosphatase amplified

Figure 8: PCR for insert Phosphatase

The PCR amplification for the insert phosphatase was carried out with forward primer having Nde I site and reverse primer having Sal I site. The PCR amplified phosphatase was loaded in lanes 3 and 4 and the unamplified phosphatase was loaded in lane 2 for comparison. The amplification of phosphatase (1Kb) was observed clearly in Figure 8. The amplified phosphatase sample will be taken for further steps of cloning into pACYC.

5.8 Agarose gel elution of Phosphatase



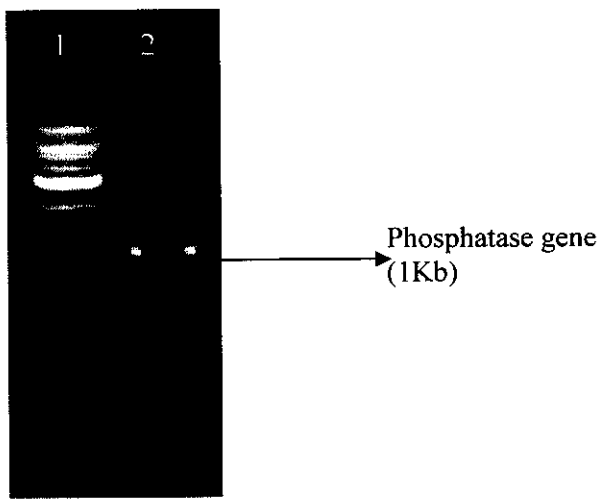
Lane 1: Gel eluted Phosphatase (1µl)

Lane 2: DNA marker (1Kb)

Figure 9: Agarose gel elution of Phosphatase

The PCR amplified phosphatase separated in agarose gel as shown in Figure 8 was extracted from the agarose gel and the presence of agarose gel eluted phosphatase was checked again in agarose gel by loading 1µl of the sample as shown in Figure 9. The concentration of gel eluted sample will be usually less than the initial concentration as loss of sample may occur during the gel extraction process. The gel extracted phosphatase was taken for further restriction digestion.

5.9 Restriction digestion of Phosphatase using Nde I and Sal I



Lane 1: DNA marker (1Kb)

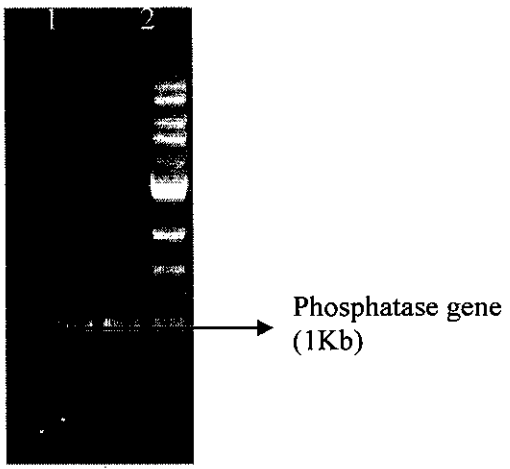
Lane 2: Phosphatase digested (Nde I and Sal I)

Figure 10: Restriction digestion of Phosphatase using Nde I and Sal I

The Nde I and Sal I digested phosphatase was separated from the vector by running agarose gel electrophoresis. The undigested samples were also loaded in agarose gel for comparison with digested samples. The difference in size between undigested and digested samples as shown in Figure 10 indicates that phosphatase was successfully digested.

The digested phosphatase (insert) was extracted from the agarose gel and further steps of cloning were carried out.

5.10 Agarose gel elution of digested phosphatase



Lane 1: Digested Phosphatase (Nde I and Sal I) gel eluted

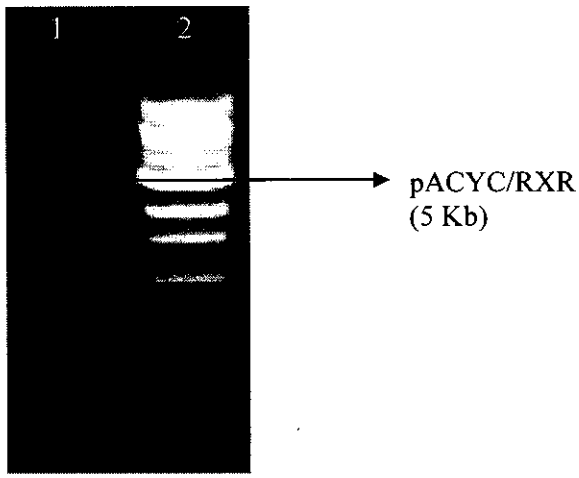
Lane 2: DNA marker (1Kb)

Figure 11: Agarose gel elution of digested phosphatase

The restriction digested phosphatase sample was separated from its initial vector pET28a by running agarose gel as shown in Figure 10. The digested insert phosphatase was extracted from the agarose gel and the agrarose gel extracted phosphatase concentration was checked in agarose gel again by loading 1µl of the sample. The gel lpicture is shown in the Figure 11.

The concentration of gel eluted sample will be usually less than the initial concentration as loss of sample may occur during the gel extraction process. The gel extracted digested phosphatase was used for the ligation reaction.

5.11 Plasmid (pACYC/RXR) extraction



Lane 1: pACYC/RXR (1μl)

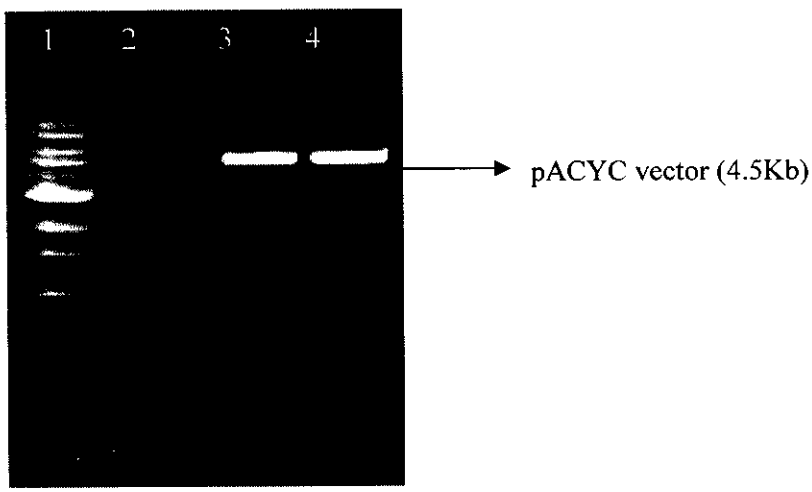
Lane 2: DNA marker (1Kb)

Figure 12: Plasmid (pACYC/RXR) extraction

The vector pACYC was obtained from the pACYC/RXR construct. Hence, the plasmid extraction from DH5α cells containing pACYC/RXR construct was carried out.

The vector pACYC/ RXR obtained from plasmid preparation was checked in agarose gel to determine the concentration of vector by comparison with the 1Kb ladder and the concentration of pACYC/ RXR was estimated as 80ng/μl. The gel picture showing concentration of the pACYC/RXR construct is shown in the Figure 12. The concentration of pACYC vector was sufficient to carry on with the cloning experiments.

5.12 Restriction digestion of pACYC/RXR using Nde I and Sal I



Lane 1: DNA marker (1Kb)

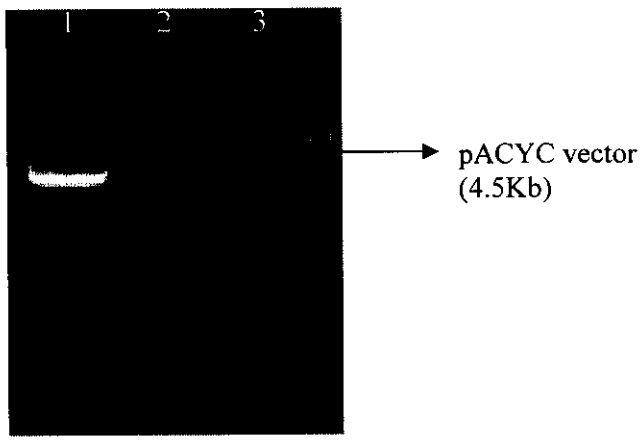
Lane 2: pACYC/RXR undigested

Lanes 3&4: pACYC/RXR digested (Nde I and Sal I)

Figure 13: Restriction digestion of pACYC/RXR using Nde I and Sal I

The Nde I and Sal I digested vector pACYC was separated by running agarose gel electrophoresis. The digested and the undigested samples were loaded in agarose gel for comparison. The difference in size between undigested and digested samples as shown in Figure 13 indicates that the vector pACYC / RXR was successfully digested and released the insert RXR. Now the vector pACYC can be gel eluted and can be used for further cloning

5.13 Agarose gel elution of digested vector pACYC



Lane 1: DNA marker (1Kb)

Lane 2: pACYC/RXR undigested

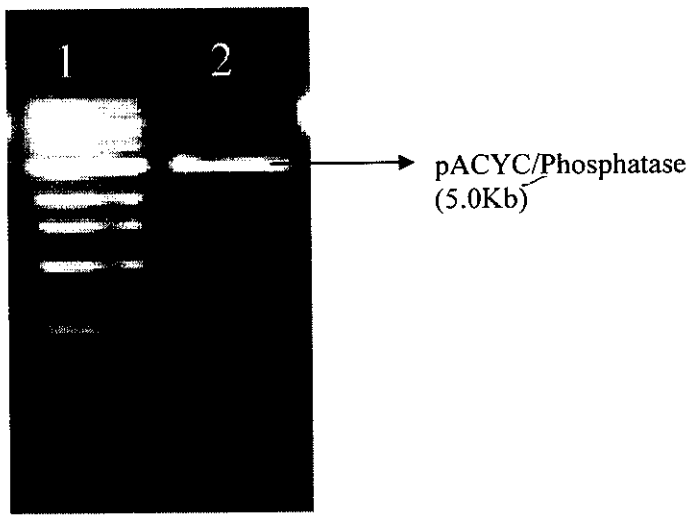
Lanes 3: Digested pACYC (Nde I and Sal I) after gel elution

Figure 14: Agarose gel elution of digested vector pACYC

The restriction digested pACYC/RXR sample was separated by running agarose gel as shown in Figure 13. The digested vector pACYC was extracted from the agarose gel and the agrarose gel extracted pACYC concentration was checked in agarose gel again by loading 1µl of the sample. The gel lpicture is shown in the Figure 14.

The concentration of gel eluted sample will be usually less than the initial concentration as loss of sample may occur during the gel extraction process. The gel extracted digested pACYC was used for the ligation reaction.

5.14 Plasmid (pACYC/Phosphatase) extraction



Lane 1: pACYC/Phosphatase (1µl)

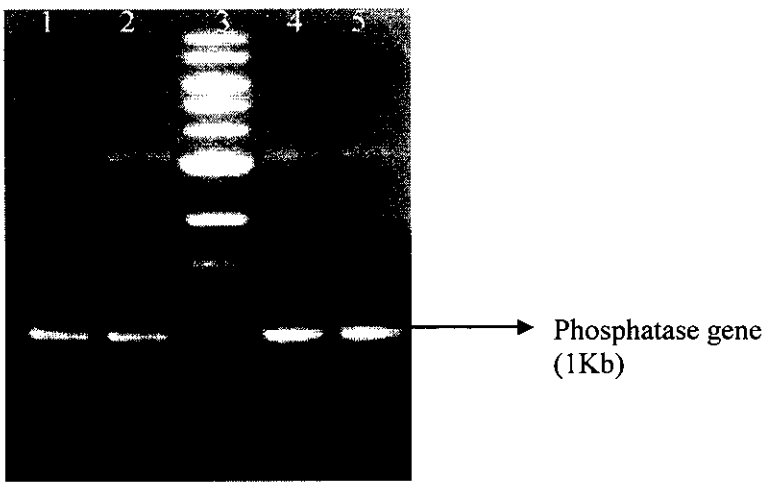
Lane 2: DNA marker (1Kb)

Figure 15: Plasmid (pACYC/Phosphatase) extraction

The desired clone pACYC/phosphatase was obtained by carrying out ligation of insert phosphatase with the vector pACYC. The plasmid pACYC/ Phosphatase was extracted from DH5α cells transformed with the ligated samples.

The plasmid pACYC/ Phosphatase obtained from plasmid preparation was checked in agarose gel by loading 1µl of the sample to determine the concentration of construct. The concentration of pACYC/ Phosphatase was estimated as 1µg/ µl by comparision with the 1Kb ladder loaded in parallel. The gel picture showing the concentration is given in figure 15.

5.15 PCR for pACYC/Phosphatase



Lane 1: Clone 1 (amplified)

Lane 2: Clone 2 (amplified)

Lane 3: DNA marker (1Kb)

Lane 4: Clone 3 (amplified)

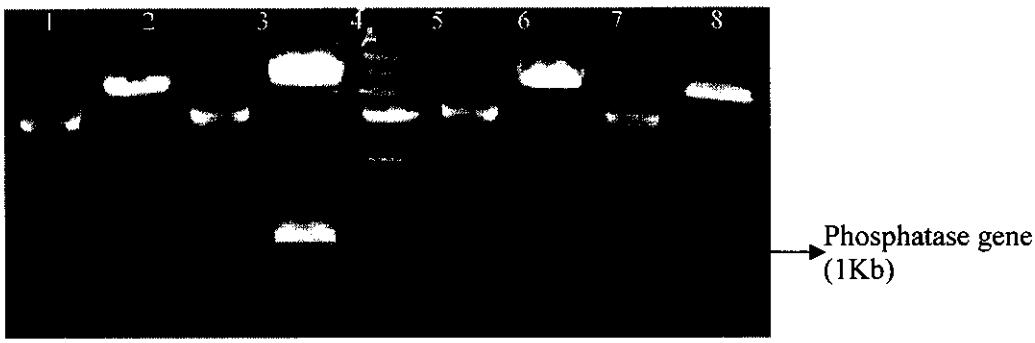
Lane 5: Clone 4 (amplified)

Figure 16: PCR for pACYC/Phosphatase

The presence of the clone pACYC/ phosphatase was checked by performing PCR amplification with Phosphatase specific primers. The amplified samples were loaded in the agarose gel to check the amplification of the sample.

The amplification of pACYC/ phosphatase (1Kb) was observed in all the four clones as shown in Figure 16. This conforms the presence of clone pACYC/ Phosphatase.

5.16 Restriction digestion of pACYC/Phosphatase using Nde I and Sal I



Lane 1: Clone 1 undigested

Lane 2: Clone 1 digested (Nde I/ Sal I)

Lane 3: Clone 2 undigested

Lane 4: Clone 2 digested (Nde I/ Sal I)

Lane 5: DNA marker (1Kb)

Lane 6: Clone 3 undigested

Lane 7: Clone 3 digested (Nde I/ Sal I)

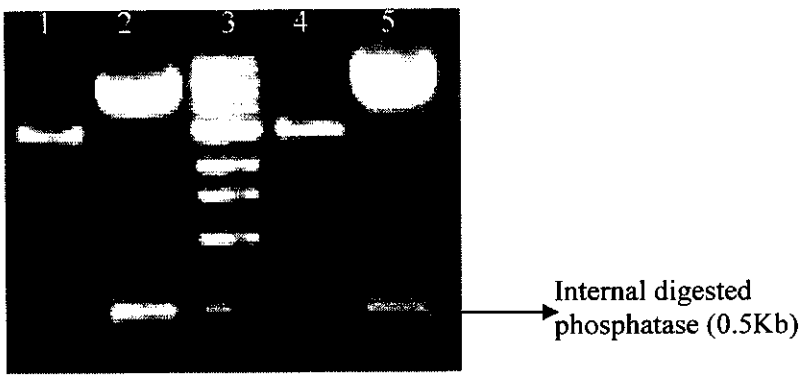
Lane 8: Clone 4 undigested

Lane 9: Clone 4 digested (Nde I/ Sal I)

Figure 17: Restriction digestion of pACYC/Phosphatase using Nde I and Sal I

The PCR amplified clones were conformed by digestion also. The digestion with Nde I and Sal I enzymes should release 1Kb insert. The release of insert phosphatase (1Kb) was observed in all the four clones as shown in Figure 17. This further conforms the clone as pACYC/ Phosphatase. The first two clones were taken for further steps.

5.17 Restriction digestion of pACYC/Phosphatase using Nde I and Xho I



Lane 1: Clone 1 undigested

Lane 2: Clone 1 digested (Nde I/ XhoI)

Lane 3: DNA marker (1Kb)

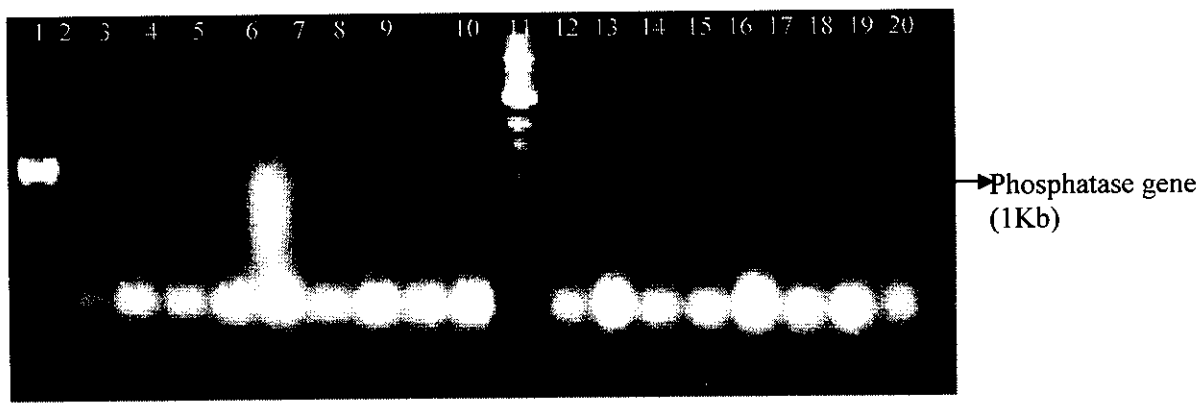
Lane 4: Clone 2 undigested

Lane 5: Clone 2 digested (Nde I/ XhoI)

Figure 18: Restriction digestion of pACYC/Phosphatase using Nde I and Xho I

The Nde I and Sal I digested clones 1 and 2 were further digested to check the presence of the clone. The insert phosphatase had an internal Xho I site at 503bp and hence the restriction digestion with Nde I and Xho I should release 0.5Kb fragment of insert to conform the insert as phosphatase. Insert release of 0.5Kb fragment was observed in both the clones as shown in Figure 18. This further conforms the clone as pACYC/ phosphatase.

5.18 Colony PCR for pACYC/Phosphatase



Lane 1: pACYC/Phosphatase positive control

Lanes 2 to 10: pACYC/Phosphatase clones 1 to 9

Lane 11: DNA marker (1Kb)

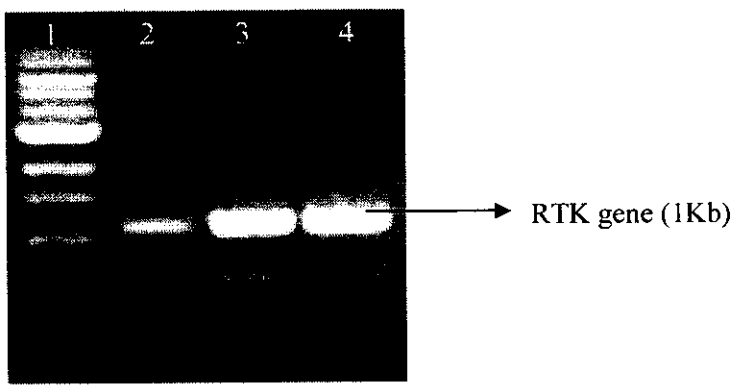
Lanes 12 to 20: pACYC/Phosphatase clones 10 to 18

Figure 19: Colony PCR for pACYC/Phosphatase

Both the RIPL cells and the pACYC vector were chloramphenicol resistance and this makes the selection of colony containing the clone pACYC/Phosphatase and pET28a/RTK difficult. Hence colonies were screened by performing colony PCR. This colony PCR was performed to check the presence of phosphotase gene. The PCR was carried out by using gene specific primers.

A faint band was observed in lane 14 (colony no. 12) of Figure 11 after amplification with phosphatase specific primers. This indicates the presence of phosphatase gene in that specific clone.

5.19 Colony PCR for pET28a/ Receptor Tyrosine kinase



Lane 1: DNA marker (1Kb)

Lane 2: Clone with Phosphatase positive

Lane 3: Clone with Phosphatase negative

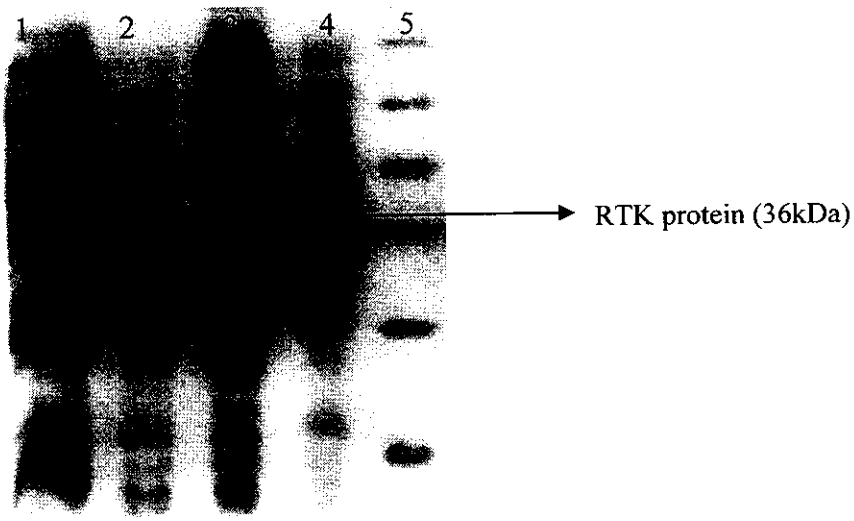
Lane 4: pET28a/ Tyrosine Kinase positive control

Figure 20: Colony PCR for pET28a/ Receptor Tyrosine kinase

The clone should have both the phosphotase gene and the Receptor Tyrosine Kinase gene as required for the co-expression to produce unphosphorylated form of Receptor Tyrosine Kinase. This colony PCR for RTK was performed with primers containing T7 promoter and gene specific region.

The phosphatase positive clone obtained in previous colony PCR amplification step was found to show amplification for receptor tyrosine kinase gene also, as shown in Figure 20. The phosphatase negative clone also found to have receptor tyrosine kinase gene. The amplification in phosphatase positive clone was observed to be less than that of negative clone.

5.20 Expression check of Receptor Tyrosine Kinase/ Phosphatase



Lane 1: Uninduced (Receptor Tyrosine Kinase /Phosphatase)

Lane 2: Induced (Receptor Tyrosine Kinase /Phosphatase)

Lane 3: Supernatant

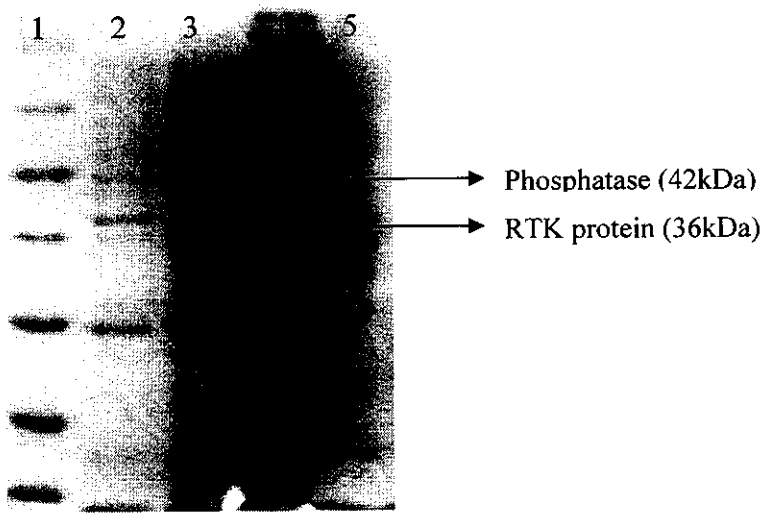
Lane 4: Pellet

Lane 5: Protein marker

Figure 21: Expression check of Receptor Tyrosine Kinase/ Phosphatase

The co-expression was carried out by using both antibiotics at high concentrations. The final concentration of kanamycin was 50μg/ml and the final concentration of chloramphenicol was 34μg/ml. The expression of receptor tyrosine kinase (36kDa) and phosphatase (42kDa) was not good under this condition and most of the protein was observed to be in the pellet. Hence further purification steps were not carried out for this batch. The co-expression was tried for varying concentrations of antibiotics to increase the yield of proteins.

5.21 Expression check under reduced kanamycin condition



Lane 1: Protein marker

Lane 2: Uninduced (Receptor Tyrosine Kinase /Phosphatase)

Lane 3: Induced (Receptor Tyrosine Kinase/ Phosphatase)

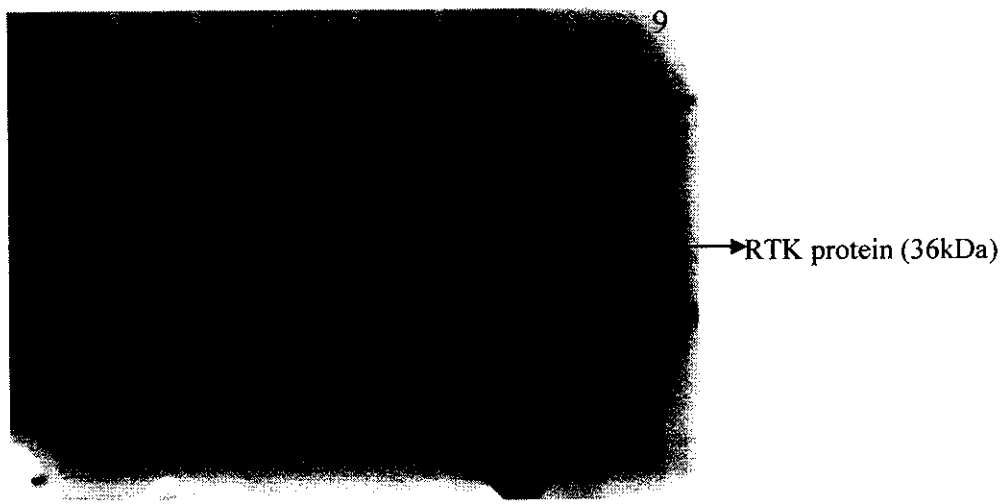
Lane 4: Supernatant

Lane 5: Pellet

Figure 22: Expression check under reduced kanamycin condition

The co-expression was tried under reduced kanamycin condition. The final concentration of kanamycin was 25μg/ml and the final concentration of chloramphenicol was 34μg/ml. The expression was found to be better in kanamycin reduced condition than with both the antibiotics at high concentration. The expression profile was shown in Figure 22. The expression of both the Receptor Tyrosine Kinase (36kDa) and the Phosphatase (42kDa) can be observed in the induced sample. The expression was good but still the amount of protein coming out in supernatant was less.

5.22 Ni-NTA purification of Receptor Tyrosine kinase/ Phosphatase under reduced kanamycin condition



Lane 1: Protein marker

Lane 2 to 6: 250mM Elution fractions

Lane 7: Flow through

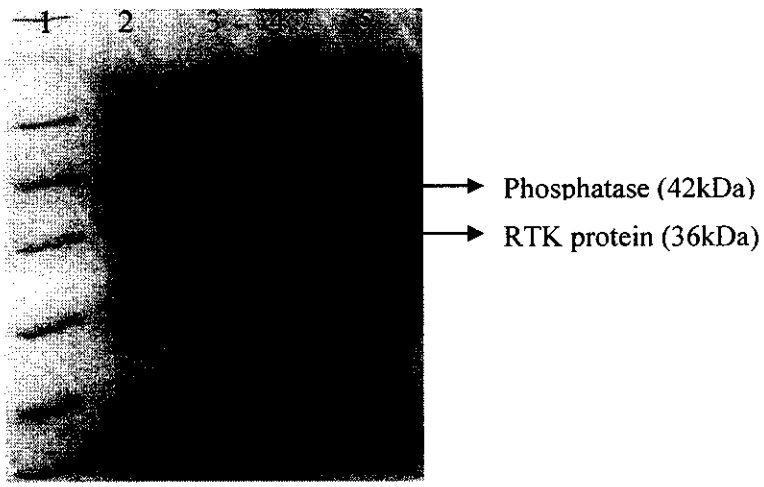
Lane 8: 25mM imidazole wash

Lane 9: 100mM imidazole wash

Figure 23: Ni-NTA purification of Receptor Tyrosine kinase/ Phosphatase under reduced kanamycin condition

The supernatant obtained under reduced kanamycin condition was taken for affinity purification. As the protein of interest is His tagged, it binds to the Nickel –NTA column. The unbound protein was washed by using 25mM imidazole and 100mM imidazole buffers. Minimal amount of protein was observed in the 250mM elution fractions. Most of the protein Receptor Tyrosine Kinase was observed to be coming out in 100mM imidazole wash as shown in Figure 23. As the protein in elution fractions was less further purification was not carried out for this batch.

5.23 Expression check under reduced chloramphenicol condition



Lane 1: Protein marker

Lane 2: Uninduced (Receptor Tyrosine Kinase/ Phosphtase)

Lane 3: Induced (Receptor Tyrosine Kinase/ Phosphtase)

Lane 4: Supernatant

Lane 5: Pellet

Figure 24: Expression check under reduced chloramphenicol condition

The co-expression was tried under reduced chloramphenicol condition. The final concentration of kanamycin was 50μg/ml and the final concentration of chloramphenicol was 17μg/ml.

The expression profile in Figure 24 indicates that expression of both receptor tyrosine kinase and phosphatase was higher than the kanamycin reduced condition and at high concentrations of both the antibiotics. Considerable amount of protein Receptor Tyrosine Kinase was observed in supernatant also under this chloramphenicol reduced condition.

5.24 Ni-NTA purification of Receptor Tyrosine Kinase/ Phosphatase

Lane 1 to 6: 250mM Elution fractions

Lane 7: Protein marker

Lane 8: Flow through 1

Lane 9: 25mMimidazole wash

Lane 10: 50mM imidazole wash

Figure 25: Ni-NTA purification of Receptor Tyrosine Kinase/ Phosphatase

The supernatant obtained under reduced chloramphenicol condition was taken for affinity purification. As the protein of interest is His tagged, it binds to the Nickel –NTA column. The unbound protein was washed by using 25mM imidazole and 50mM imidazole buffers

The 250mM elution fractions (1 to 6 fractions) were found to have good concentration of protein, Receptor Tyrosine Kinase (36kDa). Most of the non specific proteins were removed in 50mM imidazole wash. These elution fractions were taken for further purification steps.

5.25 Chromatogram for Ion Exchange Chromatography

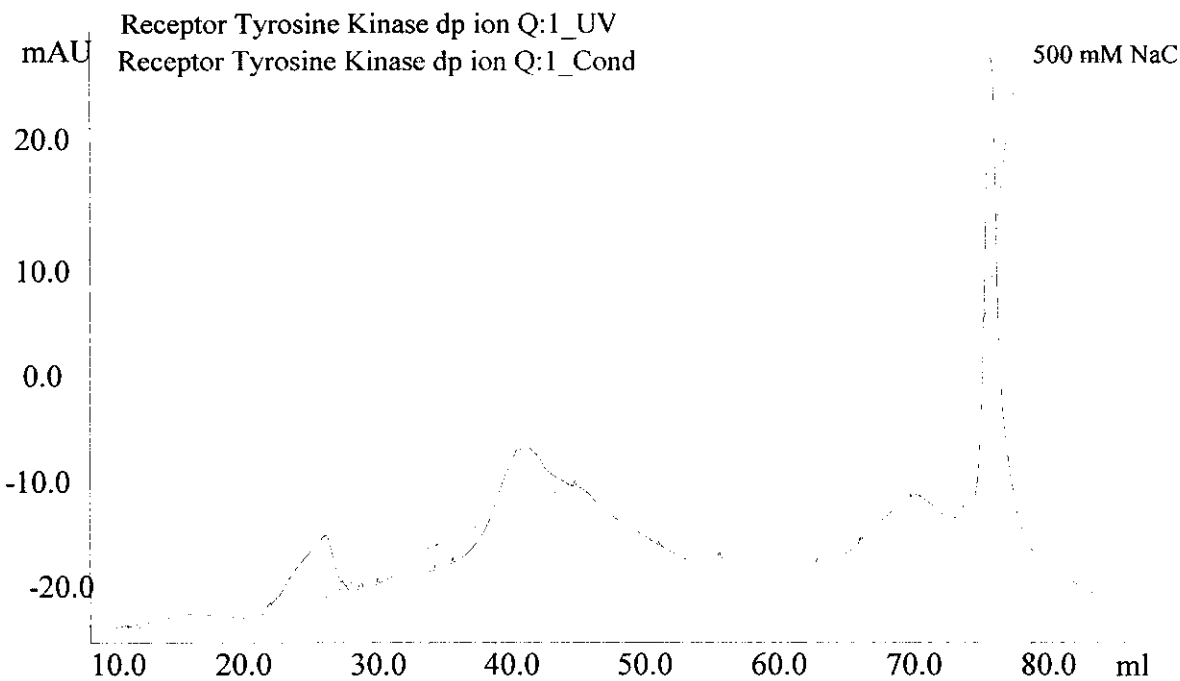
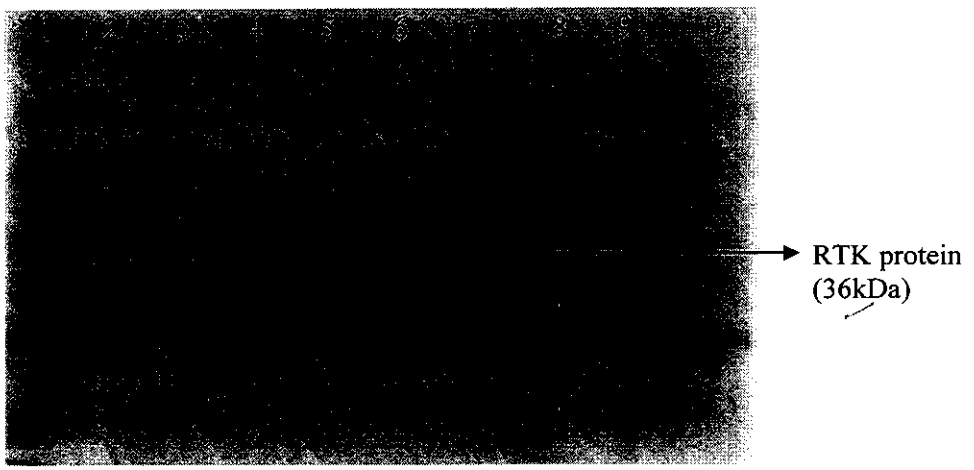


Figure 26: Chromatogram for Ion Exchange Chromatography

The elution fractions from the affinity chromatography were further purified by anion exchange chromatography. The ion exchange chromatography was carried out in linear gradient of salt concentration.

The UV absorbance and conductivity for proteins from the ion exchange column fractions were shown in Figure 26. The peaks were collected as individual fractions. The protein of interest Receptor Tyrosine Kinase eluted in 140 – 280mM salt concentration and around 18ml of fraction was obtained in this salt concentration and it was completely pure.

5.26 Ion exchange chromatography of Receptor Tyrosine Kinase/ Phosphatase



Lane 1: Protein marker

Lane 2 to10: Ion exchange fractions

Figure 27: Ion exchange chromatography of Receptor Tyrosine Kinase/ Phosphatase

The fractions collected from ion exchange chromatography were checked in the SDS PAGE to identify the fraction containing the protein of interest, Receptor Tyrosine Kinase (RTK)

The pure fractions were observed in 3rd, 4th and 5th fractions (lanes 4, 5 and 6) as shown in Figure 27.

The 25kDa protein which was present in equal amount as Receptor Tyrosine Kinase after Nickel purification also got separated in ion exchange chromatography and completely pure protein was obtained. The protein fractions from ion exchange chromatography were pooled together and concentrated. The concentrated protein fraction was taken for further purification to obtain homogenous protein

5.27Chromatogram for Gel filtration Chromatography

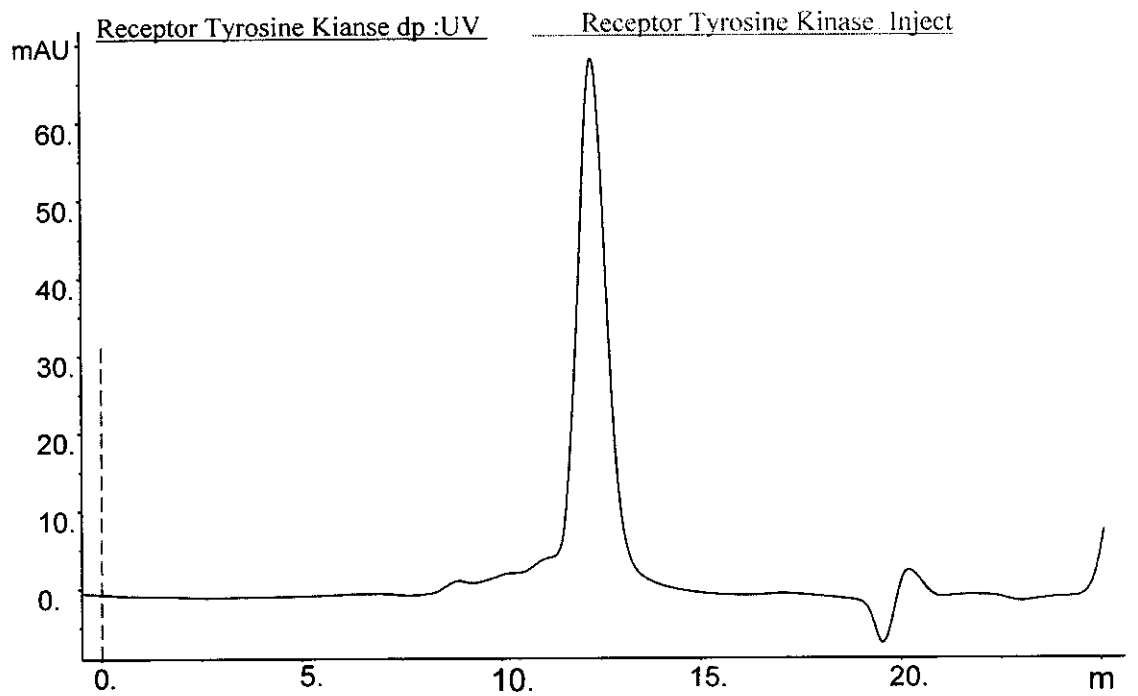
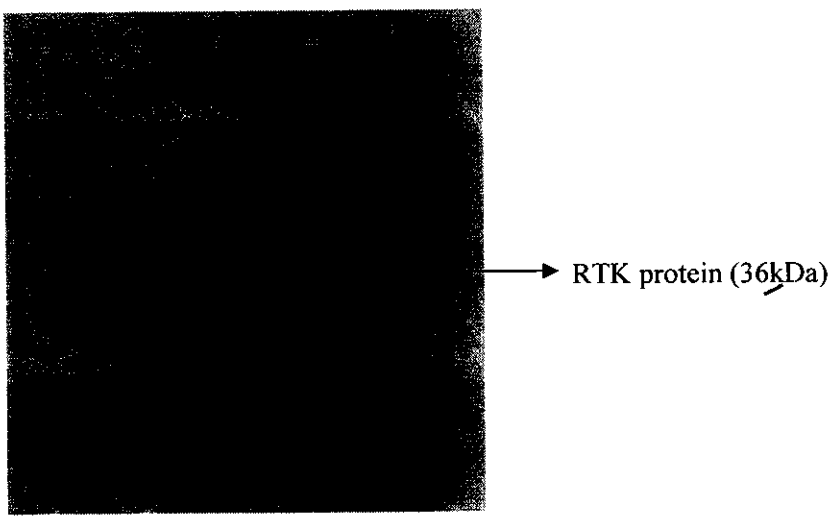


Figure 28: Chromatogram for Gel filtration Chromatography

The protein from the ion exchange chromatography was further purified by gel filtration chromatography using Superdex 75 column.

The UV absorbance for proteins from the gel filtration column fractions were shown in Figure 28. The peaks were collected as individual fractions. A single peak was observed at 12ml indicating the 35 KDa protein Tyrosine Kinase .The appearance of single peak itself shows the protein was completely pure.

5.28 Gel filtration chromatography of Tyrosine Kinase/ Phosphatase



Lane 1 to 4: Gel filtration fractions

Lane 5: Protein marker

Figure 29: Gel filtration chromatography of Tyrosine Kinase/ Phosphatase

The elution fractions of gel filtration chromatography were checked in SDS-PAGE to identify the fraction containing protein of interest, Receptor Tyrosine Kinase.

Completely pure protein, Tyrosine Kinase was observed in 2^{nd}, 3^{rd} and 4^{th} fractions of gel filtration chromatography as shown in Figure 29. The protein fractions were obtained as monomer also which is required and can be seen in the above gel picture. Each fraction volume was around 1ml.

These three fractions were concentrated using Amicon ultra Centricon (30kDa) and the concentrated fraction was used for further crystallization experiments.

5.29 Determination of Receptor Tyrosine Kinase concentration by Bradford's assay

Table 1: Determination of Receptor Tyrosine Kinase concentration by Bradford's assay

Concentration (μg/μl)	Absorbance at 595nm
Blank	0.0000
BSA 1.0	0.0502
BSA 2.0	0.0831
BSA 3.0	0.1387
BSA 4.0	0.1695
BSA 5.0	0.1878
Tyrosine Kinase (0.2μl)	0.1487
Tyrosine Kinase (0.2μl)	0.1470

The Bradford's reagent was used as blank and the Bovine Serum Albumin (BSA) was used as standards with range of known concentration. The absorbance was measured at wavelength of 595nm by using UV-Visible spectrophotometer. The absorbance of diluted sample of Receptor Tyrosine Kinase was measured as duplicates. The absorbance values were given in Table 1. From the absorbance obtained the unknown concentration of Receptor Tyrosine Kinase can be determined by plotting the graph between the absorbance and concentration.

5.30 Concentration of Receptor Tyrosine Kinase in Bradford assay

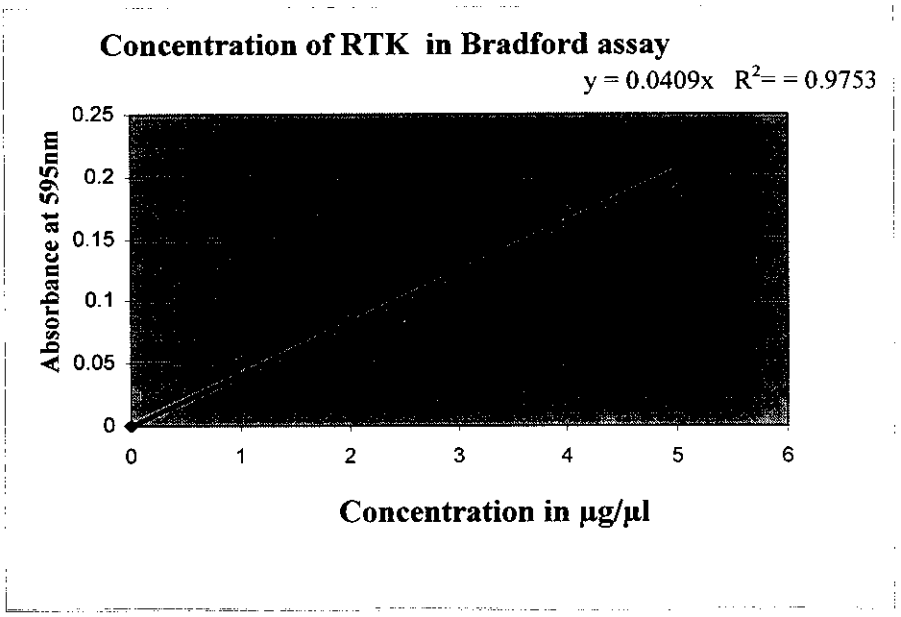


Figure 30: Concentration of Receptor Tyrosine Kinase in Bradford assay

The unknown concentration of Receptor Tyrosine Kinase was determined by plotting graph between the known concentration of BSA used and the absorbance measured at 595nm.The slope (y) and linear regression (R^2) were also calculated to determine the unknown concentration of Receptor Tyrosine Kinase. From the absorbance of Receptor Tyrosine Kinase and the slope obtained the unknown concentration was calculated and the final concentration was 18mg/ml. This protein fraction was used for setting up further crystallization.

5.31 Crystallization setup for Receptor Tyrosine Kinase

Table 2: Crystallization setup for Receptor Tyrosine Kinase

	1	2	3	4	5	6
A	1.0M (NH4)2HPO4 0.2M NaCl 0.1 M Sodium citrate, pH 4.75 7.5% Glycerol	1.0M (NH4)2HPO4 0.2M NaCl 0.1 M Sodium citrate, pH 5.0 7.5% Glycerol	1.0M (NH4)2HPO4 0.2M NaCl 0.1 M Sodium citrate, pH 5.25 7.5% Glycerol	1.0M (NH4)2HPO4 0.2M NaCl 0.1 M Sodium citrate, pH 5.0 5% Glycerol	1.0M (NH4)2HPO4 0.2M NaCl 0.1 M Sodium citrate, pH 5.0 0% Glycerol	1.0M (NH4)2HPO4 0.1M NaCl 0.1 M Sodium citrate, pH 5.0 7.5% Glycerol
B	6% PEG 5000 monomethyl ether 100mM HEPES pH7.1 11% Isopropanol	6% PEG 5000 monomethyl ether 100mM HEPES pH7.35 11% Isopropanol	8% PEG 5000 monomethyl ether 100mM HEPES pH7.1 11% Isopropanol	8% PEG 5000 monomethyl ether 100mM HEPES pH7.35 11% Isopropanol	10% PEG 5000 monomethyl ether 100mM HEPES pH7.35 11% Isopropanol	

The pure protein fraction obtained after several purification and concentration steps. The obtained completely pure protein was used for setting up crystallization by hanging drop method. The crystallization for unphosphorylated Receptor Tyrosine Kinase was done under above conditions.

The slight precipitation of proteins were observed in all the above conditions Small triangular crystal was observed in A-3 condition given above which will be further diffracted with X-rays for the structure determination of protein, Receptor Tyrosine Kinase in unphosphorylated form.

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