GENERATION OF SINGLE CHAIN VARIABLE FRAGMENTS (scFv) AGAINST GPI-ANCHORED PROTEINS USING A NOVEL CELL-BASED SELECTION SYSTEM

A PROJECT REPORT

Submitted by

LOKAVYA. M

in partial fulfillment for the award of the degree

of

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In

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KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

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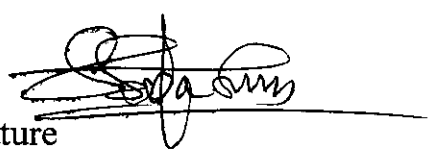
BONAFIDE CERTIFICATE

I certify that the project report, **GENERATION OF SINGLE CHAIN VARIABLE FRAGMENTS (scFv) AGAINST GPI-ANCHORED PROTEINS USING A NOVEL CELL-BASED SELECTION SYSTEM**, is the bonafide work done by **LOKAVYA. M**, who carried out the project work under my supervision.



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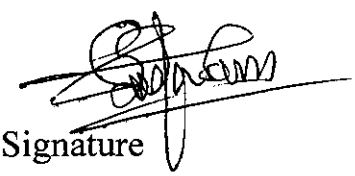

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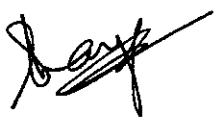
I certify that the project report, GENERATION OF SINGLE CHAIN VARIABLE FRAGMENTS (scFv) AGAINST GPI-ANCHORED PROTEINS USING A NOVEL CELL-BASED SELECTION SYSTEM, is the bonafide work done by LOKAVYA. M, under my supervision, during her project period.



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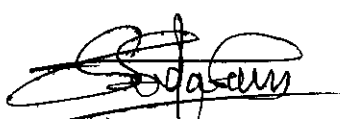
The report of the project work submitted by the above student in partial fulfillment for the award of Bachelor in Technology degree in Biotechnology, of Anna University was evaluated and confirmed to be a report of the work done by the above student. It was submitted for the viva voce examination on 20.04.2010 .



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LOKAVYA. M

ABSTRACT

ABSTRACT

Single chain variable Fragments (scFvs) have been recently reported as valuable alternatives over conventional monoclonal and polyclonal antibodies and have been used extensively for probing proteins, peptides, prions, nucleotide sequences etc. in the form of anti- and intrabodies. GPI (Glycosylphosphatidylinositol) anchored proteins are of sweeping significance in various facets of cellular physiology and provide a classic system for studies that include endocytic pathways and membrane organization. Generation of scFvs from the Tomlinson I + J library, applying the phage display technique and using these scFvs to tag the GPI anchored proteins- Folate Receptor (FR), Cyan fluorescent Protein fused to GPI (CFP-GPI) and the morphogen Hedgehog (Hh) in Drosophila - using fixed cells expressing the respective proteins, as selection systems, remains the prime focus of this project. The phage display library against the Folate Receptor gave a low yield at the end of six rounds of selection, which reduces possibility of encountering clones that are positive for scFvs of interest to 1 in 10^{+09}. The first round of selection against Hedgehog presented an output titer of very low magnitude, resulting in a low yield, suggesting possible inefficiencies in the selection methodology that was adopted. This was a result of extreme stringency in the selection protocol and hence has to be amended to incorporate the possibility of sensitive filtering of scFv phage particles, at least in round one. The screening procedure to identify clones positive for scFv against CFP-GPI from 96 colonies that were cultured from a similar phage display selection was carried out by random selection of 10 of the 96 colonies and subjected them to an array of primary and secondary screens and confirmed that one clone (F11) was positive for the target molecule, CFP-GPI and another (B9), a putative positive. To study the comprehensive behavior of the library at hand, when exposed to the ideal model of being selected against a purified form of the target protein in place of an array of different molecules (as in this case - a cell), purification of the Folate Biding Protein (FBP) from the FBP-GST-pGEX4T2 vector was attempted using a GST pull down affinity chromatography technique. The analysis of various fractions collected through this purification protocol showed that the bands obtained did not correspond to the expected values. Temperature and concentration parameters of this purification protocol need to be standardized.

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ABBREVIATIONS

BOI : Brother of Interference Hedgehog

CDR : Complementarity Determining region

CFP : Cyan Fluorescent Protein

C_H : Constant Heavy chain

CI : Cubitus Interruptus

C_L : Constant Light Chain

ER : Endoplasmic Reticulum

FR : Folate Receptor

GFP : Green Fluorescent Protein

GLI : Glioma-associated Oncogenes

GPI : Glycosylphosphatidylinositol

GSH : Glutathione

GST : Glutathione-S-Transferase

Hh : Hedgehog

Ig : Immunoglobulin

IG : Intergenic Region

IHOG : Interference Hedgehog

OD : Optical density

PFU : Plaque Forming Units

PI-PLC : Phosphatidylinositol Phospholipase-C

PTC : Patched

RF : Replicative Form

RT : Room Temperature

scFv : Single Chain Variable Fragment

SMO : Smoothed

V_H : Variable Heavy Chain

V_L : Variable Light Chain

INTRODUCTION

1. INTRODUCTION

The use of antibodies as a means of probing proteins, nucleotides and other molecules in order to study their physical and functional properties have been of importance for years. Advances in molecular biology and the exponential progresses attained by recombinant technology have led to the invention of a number of modern techniques, one of which is phage display library. With the production of single chain variable fragments (scFv), the generation of highly specific antibody fragments having strong affinities for its antigenic domain using phage display went on to become a much celebrated development in most areas of biological sciences. The project centers on generating such scFvs against one of the most significant class of membrane proteins- the GPI (Glycosylphosphatidylinositol) anchor. scFvs were attempted to be purified against three important GPI-anchored proteins, the significance of which is detailed in chapter 2. This chapter deals with an overview of the basic principles and components this project is built on.

1.1 ANTIBODIES

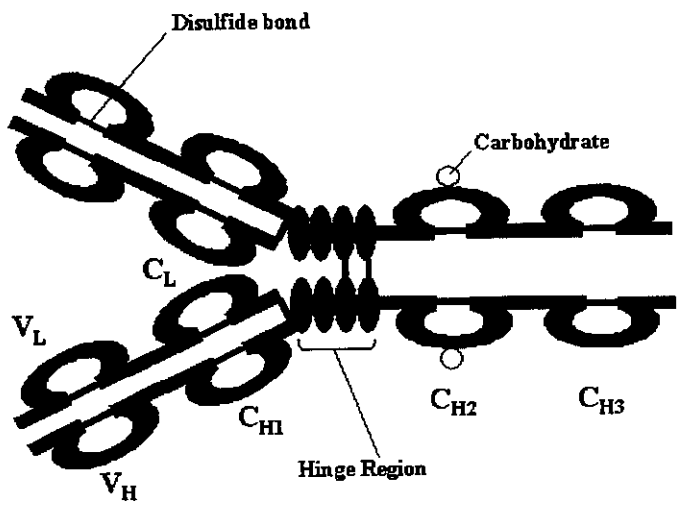
Antibodies or immunoglobulins are glycoprotein molecules produced by plasma cells as a result of an invasion by antibody generating (antigen) also known as an immunogen to provide protection from the se foreign bodies by neutralizing them (Mayer, Microbiology and Immunology Online Textbook, 2006). Immunoglobulins are highly specific to the antigen that triggers its generation and bind specifically to them and also to close relatives. Each antigen has a particular domain on itself that is recognized by the antibodies and the number of such antigenic determinants that an antibody can bind to determine its valency. Antibodies are also important in the vertebrate system due to its ability to perform an array of effector functions that include fixation of complement that results in the lysis of cells, releasing important biological molecules, the ability to bind to various cell types like lymphocytes, platelets, mast cells, basophils etc thereby activating their functions. They can also bind to certain placental trophoblasts which are then transported across the placenta imparting immunity to the fetus.

The property of immunoglobulins that is of prime importance in various research studies, apart from its functions, is the ability to bind specifically to its antigenic determinant. This property can

be attributed to the structure of the immunoglobulins which comprise of the same basic units found in differing combinations in the various classes of immunoglobulins (Igs) (Mayer, Microbiology and Immunology Online Textbook, 2006).

The basic structural units of the Ig molecule are as follows:

- Heavy chain
- Light chain
- Disulphide bond
- Variable region
- Constant region
- Disulphide bond
- Hinge region
- Oligosaccharides



Mayer, Microbiology and Immunology Online Textbook, 2006

FIGURE 1.1: IMMUNOGLOBULIN BASIC STRUCTURE

HEAVY AND LIGHT CHAINS

Different as the subclasses of Igs might be from one another, they are composed of a basic four chain structure (**Figure 1.1**) that consists of two heavy (**green**) and two light (**blue**) chains. The mammalian Ig heavy chains are of five types denoted by α, δ, ε, γ, and μ, each defining a different class of antibody, and range in size from 50 to 70 kD. The light chains are of two types- λ and κ- and are mostly 23 kD in size (Janeway CA, Jr et al, Immunology, 2001).

DISULPHIDE BOND

They are of two types: inter-chain disulphide and intra-chain disulphide bonds (**Figure 1.1, red**). The heavy and light chains are held together by inter-chain disulphide bonds and non-covalent interactions (Janeway CA, Jr et al, Immunology, 2001). Such bonds are also found within each polypeptide and these fall into the latter category.

VARIABLE AND CONSTANT REGION

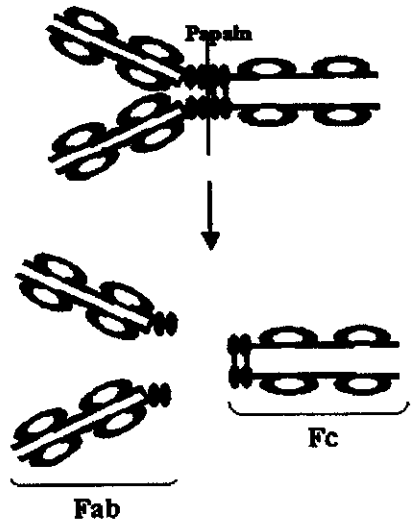
Both the heavy and light chain of the Ig structure can be sub-divided into light chain and heavy chain. In **Figure 1.1**, the light chain variable and constant chains are V_L and C_L, respectively. Similarly the heavy chain variable fragment is V_H and constant regions are C_{H1}, C_{H2}, and C_{H3} (Janeway CA, Jr et al, Immunology, 2001).

HINGE REGION

This is the flexible region found at the juncture of the arms of the Ig, which is responsible for the 'Y' shape of the molecule (**Figure 1.1**) (Janeway CA, Jr et al, Immunobiology, 2001).

OLIGOSACCHARIDES

In immunoglobulins, carbohydrates are attached to the C_{H2} domain in most classes (**Figure 1.1**) (Janeway CA, Jr et al, Immunobiology, 2001). Sometimes other domains might also have carbohydrates and in some structures more than one domain might have these molecules attached to them.



Mayer, Microbiology and Immunology Online Textbook, 2006

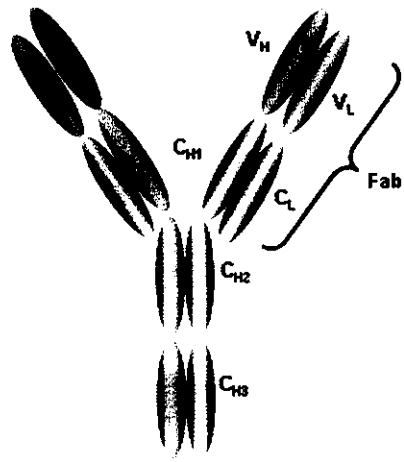
FIGURE 1.2: IMMUNOGLOBULIN FRAGMENTS

IMMUNOGLOBULIN FRAGMENTS

Proteolytic digestion, by a protease like papain, of the immunoglobulin structure yields two fragments known as the Fab and Fc regions (Mayer, Microbiology and Immunology Online Textbook, 2006) (**Figure 1.2**).

The Fab region yields two identical fragments that contain the light chain and the V_H and C_{H1} domains of the heavy chain, when lysed at the hinge region before the H-H inter-chain disulfide bond. The name "Fab" signifies the fact that this is the fragment that holds the antigen binding domains of the antibody. The binding site of the antibody is created by both V_H and V_L. An antibody is able to bind a particular antigenic determinant because it has a particular combination of V_H and V_L. The other fragment produced from proteolysis after the hinge region contains the remainder of the two heavy chains each containing a C_{H2} and C_{H3} domain. This fragment was called Fc as it could be easily crystallized.

1.2 SINGLE- CHAIN VARIABLE FRAGMENTS (scFv)



Manikandan et al, 2007

FIGURE 1.3: FAB SEGMENT

Single- chain variable fragments are fusion proteins that are derived from the variable fragments, Fv, of the Fab region in an antibody structure (**Figure 1.3**). scFvs are constructed using hybridoma technology, mouse Ig, sheep Ig and the human antibody repertoire by connecting the variable regions of heavy (V_H) and light (V_L) chains of immunoglobulins using a short linker peptide (Heng et al, 2006)(**Figure 1.4**).

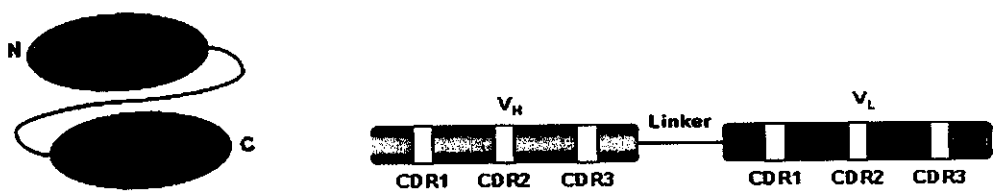


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FIGURE 1.4: SINGLE CHAIN VARIABLE FRAGMENT

The peptide linker mostly used to construct scFv is made up of a 15-residue sequence with repeats $(Gly_4Ser)_3$. The glycine moieties in the linker provide the molecule with a flexibility to move

approximately 35 to 40 Å between the carboxy terminal of the V_H and the amino terminus of the V_L chains (**Figure 1.5**). The serine, or threonine in some cases, is responsible for the solubility of these molecules (Heng et al, 2006). These proteins retain the specificity of the original immunoglobulin, probably in higher orders of magnitude as the redundant constant region is absent, despite the introduction of the linker. Disulphide bonds contribute to the stability and affinity of these molecules (Heng et al, 2006).

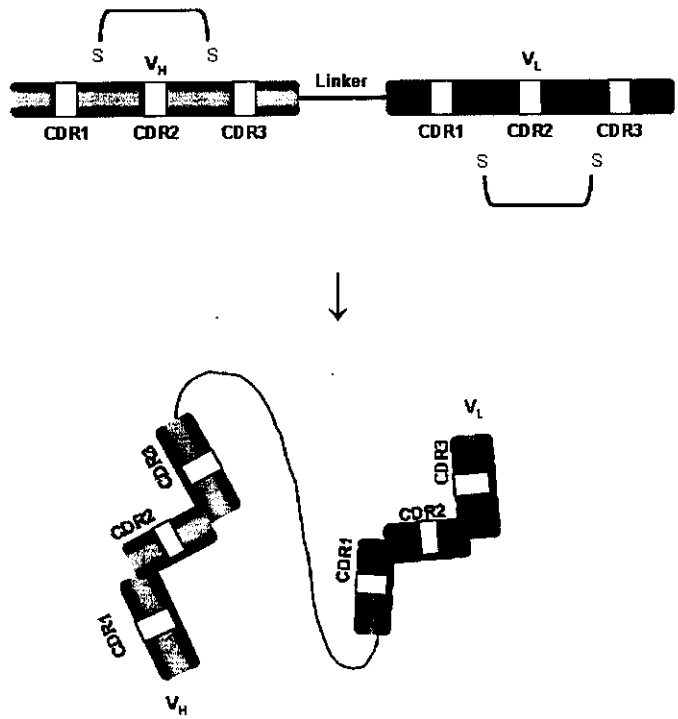

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FIGURE 1.5: DISUPHIDE BOND FLIEXIBILITY

scFvs can be generated from *E.coli* cells in a short term of time unlike the elaborate process of production of monoclonal antibodies which includes immunization of animals and hybridoma techniques. These molecules are the most important part of phage display, as it is highly convenient to express the antigen binding domain as a single peptide in place of a complex collection of various constant and variable domains. They find immense use in flow cytometry, immunohistochemistry and as antigen-binding domains in artificial T cell receptors. They are of key importance in drug targeting and therapy of cancer due to its high specificity and non-erroneous target identification.

1.3 THE GPI ANCHOR

GPI (Glycosylphosphatidylinositol) is a glycolipid that is attached to the C-terminus of a protein during posttranslational modification and is comprised of two components- the phosphatidylinositol group and a carbohydrate containing linker. The carbohydrate linker is composed of glucosamine and mannose and connects the phosphatidylinositol group to the C-terminus of a mature protein (**Figure 1.6**). Anchorage of protein to the cell membrane is done by the two fatty acids found within the hydrophobic region of phosphatidylinositol.

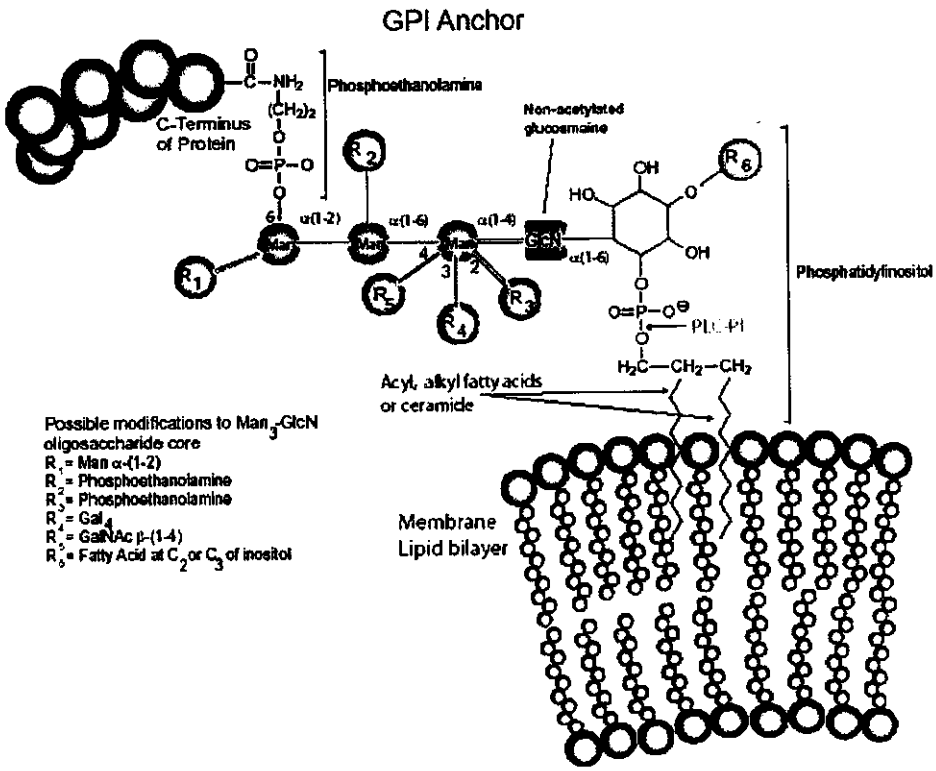


FIGURE 1.6: THE GPI ANCHOR

After translation, proteins that are to be sorted to the cell membrane are directed to the endoplasmic reticulum (ER) by a signal peptide. The C-terminus of this protein, which is composed of hydrophobic amino acids, stay inserted in the ER membrane. This hydrophobic residue is then

cleaved and replaced with the GPI-anchor. As the protein proceeds through the secretory pathway, it is transported to the Golgi apparatus in vesicles and ultimately to the membrane and extracellular space and stays there, attached to the exterior leaflet of the cell membrane. As the only connection these proteins have to the cell is the phosphoinositol group, lysis of this group by phospholipases will lead to the controlled release of the protein from the membrane.

The Glycosylphosphatidylinositol (GPI) anchors of proteins expressed on human erythrocytes and nucleated cells differ with respect to acylation of an inositol hydroxyl group, a structural feature that modulates their cleavability by PI-specific phospholipase C (PI-PLC) (Chen et al, 1998) (**Figure 1.7**).A characteristic of surface proteins that are membrane-anchored by Glycosylphosphatidylinositol (GPI) structures is that they can be cleaved from cells by PI-specific phospholipase C (PI-PLC) (**green**). GPI anchored proteins has a characteristic feature of being able to associate with glycosphingolipids and cholesterol to form detergent-resistant microdomains, or lipid rafts, in the outer leaflet of the plasma membrane .

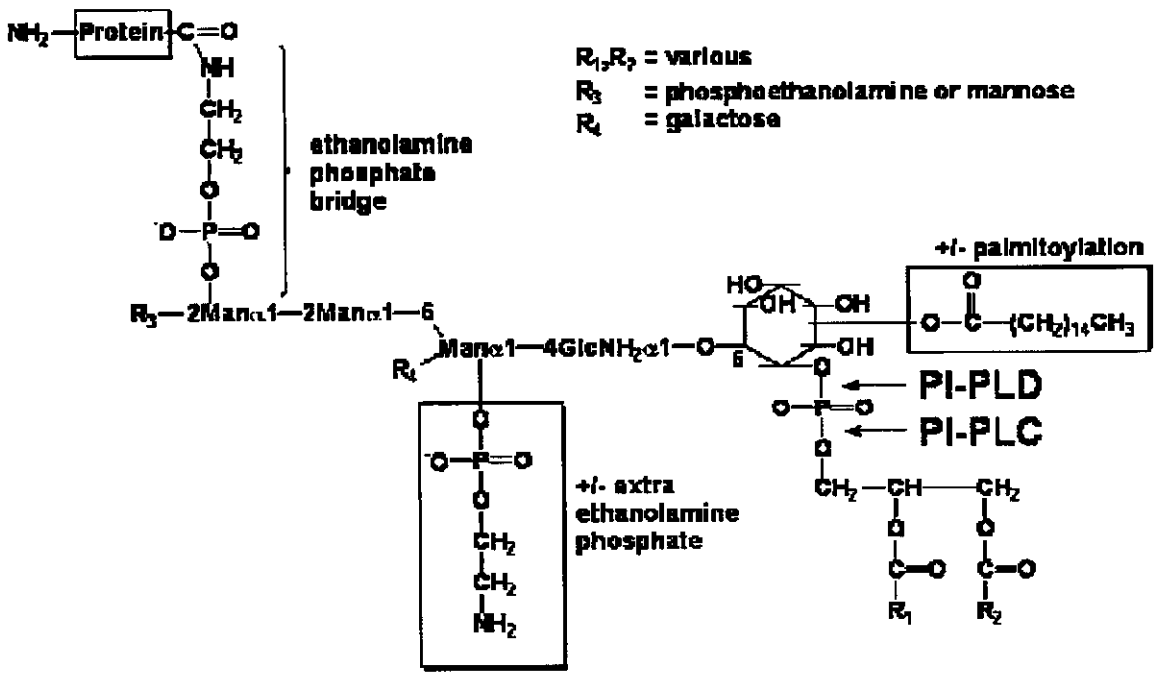


FIGURE 1.7: PI-SPECIFIC CLEAVABILITY BY PIPLC

OBJECTIVES

2. OBJECTIVES

The objectives of the project were as follows:

- To carry out a phage display selection to generate scFvs against GPI-anchored proteins-Folate Receptor and the Drosophila morphogen Hedgehog using a novel cell based selection system.
- To carry out primary and secondary screening of selected clones for positives against CFP-GPI
- To purify Folate Receptor using an FBP-GST-pGEX4T2 vector employing a Glutathione-S-Transferase pull down affinity chromatography.

LITERATURE REVIEW

3. LITERATURE REVIEW

The scFv molecules were chosen to be tagged against GPI proteins due to the colossal importance they hold in various facets of cellular physiology providing a classic system for studies that include endocytic pathways and membrane organization and it is therefore important to understand the functional relevance of these proteins in detail. This chapter deals with detailed descriptions of the three different GPI- anchored proteins that are targeted in this project- FR. Hh, CFP. It also sheds some light on diverse features of phage display library and the use of helper phage.

3.1 THE FOLATE RECEPTOR (FR)

Folates are inevitable for normal growth and maturation as they act as carriers of single carbons in a number of inter-conversion steps of the metabolic cycles of purine, thymidine, methionine, histidine and serine biosynthesis (Brzeziñska et al, 2000). Since eukaryotes are unable to synthesize folates, they depend on an external source for these molecules and acquire them through a complex pathway that utilizes membrane. It begins from the transport of these molecules into the cytoplasm across the cell membrane by means of molecules that binds to the target protein with high specificity and affinity. The most widely known mechanism by which folate delivery takes place is a carrier mediated or receptor initiated mechanisms, which function efficiently at physiological pH (Brzeziñska et al, 2000).

One such transport mechanisms is mediated by a form of folate binding protein that is anchored to the cell membrane by GPI proteins. There are three types of human FR: α and β isoforms, that are GPI-anchored and the γ isoform, which is a soluble protein. Though the expression of FR in normal cells is limited, epithelial malignancies lead to it's over expression. α and β isoforms of FR is found in placental tissues and all isoforms are expressed in normal tissue like lungs, kidney, etc. Buccal carcinoma cells (KB cells) are seen expressing FR- α and is often used as a diagnostic marker of other cancerous cell types and an attractive anti-cancer target (Sabharanjak et al, 2004).

Folate receptors are said to form clusters on the surface of the cell interacting uncoated membrane invaginations called caveolae, identified by the marker protein caveolin. This protein binds to cholesterol to form large protein complexes called raft organizers. Membrane trafficking, cell adhesion, etc. are processes undertaken by these microdomains or lipid rafts and are particularly useful for internalization and recycling of the FR (Brzeziñska et al, 2000).

Evidence proves that GPI-anchoring is the reason for certain special properties that are imparted to the FR- transporter system. These GPI-anchored receptors mediate the uptake of folate and enables cell survival in media containing trace amounts of folate. The replacement of the GPI-anchor in this system with transmembrane and cytoplasmic portions of LDL receptor led to the internalization of FR via clathrin-coated pits. However, the efficiency of folate uptake in the presence of GPI-anchor was found to be much larger than that seen in this system. Other mechanisms of regulation of transmembrane FR and the inhibitory effect of confluent state of cell culture on accumulation of folate as seen in the presence of native GPI-anchor largely varies from what is noticed in case of the replaced transmembrane protein. It could be inferred that the efficiency of folate absorption was considerably reduced as a result of the GPI-anchoring of folate receptor being replaced by a transmembrane sequence leading to different intercellular destinations of the FR trafficking (Sabharanjak et al, 2004).

These studies provide a baseline to establish the important relation between GPI-anchor and efficient folate delivery in cells, emphasizing on the physiological importance of investigating the FR-GPI system.

3.2 HEDGEHOG (Hh)

Hedgehog molecules are small signaling proteins that are secreted into the extracellular environment, where they can diffuse and bind to receptors on nearby cells and set off changes in gene expression in the target. Hh is synthesized as a 45 KDa precursor protein whose C-terminal domain (25 KDa) is autocatalytically cleaved and degraded, whereas the remaining 20 KDa N-terminal domain is covalently modified by cholesterol at the C terminus and palmitoylated at the N-terminus (Porter et al, 1996).

These molecules undergo a complex signaling pathway in which it first binds to a receptor protein, called Patched (PTC), on the membrane of cells that are ready to receive the signal. An inhibitory effect is triggered on the cytoplasmic protein called Smoothened (SMO) due to the PTC signal and this inhibition leads to the modification of the activity of the fly homologous of Glioma-associate Oncogene (GLI) called Cubitus Interruptus (CI). The CI can be modified in one of two ways to either form CIR or CIA, each of which enters the nucleus and bind to different genes and switching their functions on and off.(**Figure 3.1**- the inactive state, in which no Hedgehog signaling is present).

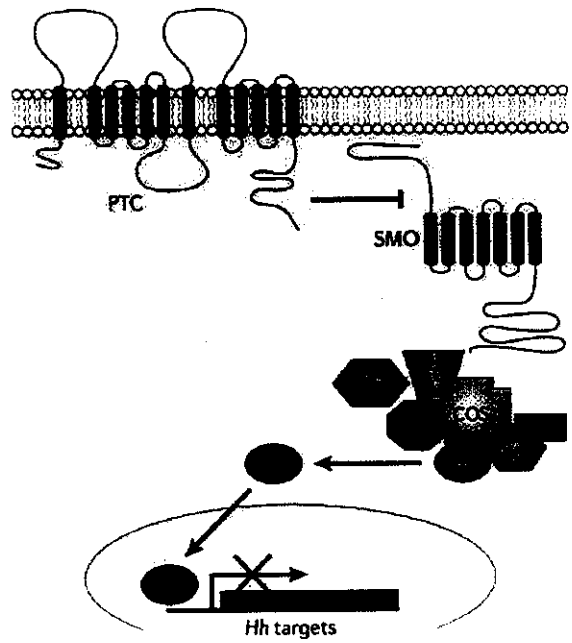


FIGURE 3.1: SIGNALLING IN ABSENCE OF HEDGEHOG

The result of the presence of Hedgehog (Hh) bound to the receptor is a set of Hedgehog target genes being switched on (**Figure 3.2**). Another set of proteins that facilitate Hh binding, with the names of Interference hedgehog (IHOG) and Brother of Interference hedgehog (BOI), are also shown here. The Drosophila hedgehog gene was identified as playing important roles in larval body development and in formation of adult appendages. Other important functions of Hedgehog (Hh) are cell-fate specification and tissue patterning during animal development by activation of distinct target genes in a concentration-dependent manner. These mechanisms by which the

concentration gradients of such morphogens are established have been the focus of much investigation (Ashe et al, 2006). There are various factors contributing to this behavior of morphogens like the amount of protein released, rate of capture and uptake of the released molecules by target cells, and the transport across developing tissues (Tabata et al, 2004).

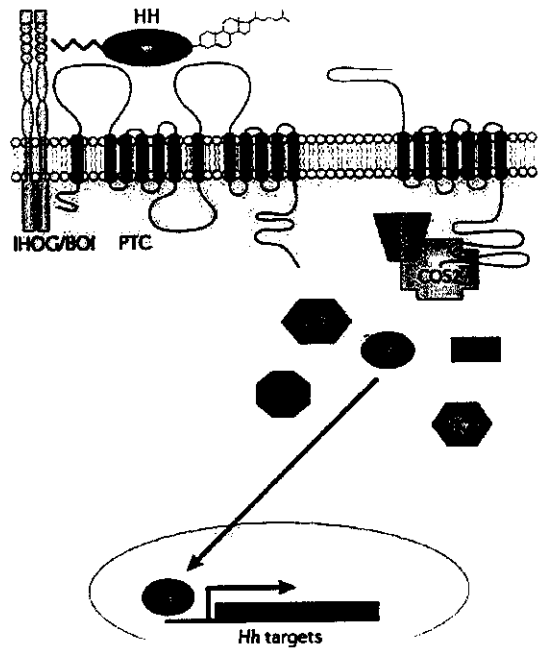


FIGURE 3.2: SIGNALLING IN PRESENCE OF HEDGEHOG

An ideal selection system of single chain variable fragments devised to efficiently tag these GPI proteins can be of high significance and will aid in elaborate studies pertaining to areas dealing with the transport and metabolic pathways of these molecules. The generation and purification of the specific scFv molecules against these proteins was attempted by use of phage display library.

3.3 THE GREEN FLUORESCENT PROTEIN (GFP)

The green fluorescent protein, derived from the jelly fish, is a protein made of 238 amino acids which expresses bright green color when exposed to blue light. The GFP sequence spontaneously forms the fluorophore p-hydroxybenzlyideneimidazoleinone due to the presence of residue 65-67 (ser-tyr-gly). The excitation spectrum of GFP fluorescence has a major excitation peak at a wavelength of 395 nm and a smaller maximum at 475 nm. The emission spectrum has a sharp maximum at 509 nm and a shoulder at 540 nm (Tsein et al, 1998). The crystal structure of GFP is a beta barrel structure that consists of an eleven-stranded β- barrel, threaded by an α-helix, running up along the axis of the cylinder (**Figure 3.3**). The chromophore is found in the alpha-helix that is seen close to the center of the cylinder. The formation of this chromophore is the result of specific cyclization reactions in the tri-peptide Ser65-Tyr66-Gly67 induced by the inward facing side chains of the barrel (Tsein et al, 1998).

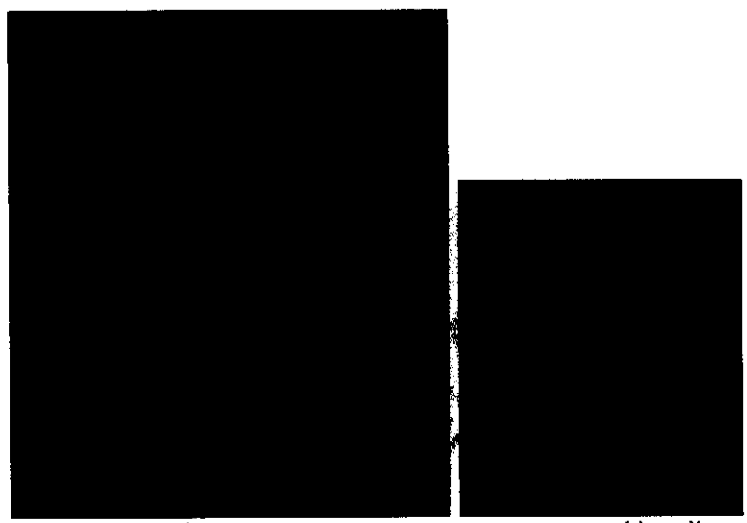


FIGURE 3.3: GREEN FLUORESCENT PROTEIN

The wild type GFP (wtGFP) and its derivatives express their colors due to the influence of the hydrogen bonding network and electron stacking interactions with these sidechains. Solvent

molecules are excluded from the core due to the tightly packed nature of the barrel thereby protecting the fluorescence of the chromophore by getting quenched by water (Tsein et al, 1998).

Extraction from other sources or small mutations in the existing GFP structure can lead to the formation a number of GFP variants. The currently known GFP variants may be divided into seven classes based on the distinctive component of their chromophores:

- Class 1: wild-type mixture of neutral phenol and anionic phenolate
- Class 2: phenolate anion
- Class 3: neutral phenol
- Class 4: phenolate anion with stacked π-electron system
- Class 5: indole
- Class 6: imidazole
- Class 7: phenyl.

Each class has a distinct set of excitation and emission wavelengths. Classes 1–4 are derived from polypeptides with Tyr at position 66, whereas classes 5–7 result from Trp, His, and Phe at that position (Tsein et al, 1998).

The focus of this discussion is limited to only the fifth class of GFP in which Tyr66 is substituted with Trp to form a new chromophore with an indole in place of a phenol/ phenolate. This fluorophore is termed the Cyan Fluorescent Protein (CFP) because of their blue-green or cyan emission (**Figure 3.4**) and has an excitation and emission wavelengths of 436 and 476 nm, intermediate between neutral phenol and anionic phenolate chromophores.

The increased bulk of the indole requires many additional mutations to restore reasonable brightness, but when such mutations are provided, the overall performance is found to be remarkably fair (Tsein et al, 1998). The study of CFP, tagged to the GPI-anchor, holds significance because of the fact that trafficking properties and other characteristic features of diffusion of these GPI proteins can then be easily visualized and followed in details that are otherwise less efficient. Live cell imaging to follow the protein dynamics in vivo is one of the most widely employed uses of CFP/CFP-tagged proteins

Crystallography structure expressed in cells

FIGURE 3.4: CYAN FLUORESCENT PROTEIN

3.4 M13 FILAMENTOUS PHAGE

Filamentous bacteriophages are genetically modified to provide single-stranded copies of fragments of DNA cloned in the other vectors. These single-stranded fragments are used mainly as templates for site-directed mutagenesis, sequencing of DNA fragments by the dideoxy chain-termination method, construction of subtractive cDNA libraries and synthesis of strand-specific probes. The most widely used vector for display of foreign peptides and proteins on the surface of bacteriophages are the M13 vectors (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001)

M13 is a member of a family of filamentous bacteriophages with single- stranded DNA genomes and are ~6400 bases in length (**Figure 3.5**). M13 bacteriophages can infect only male bacteria and do not terminate infection process with the lysis of the bacterial host cell. Instead they are released as and when the cells continue to grow and divide.

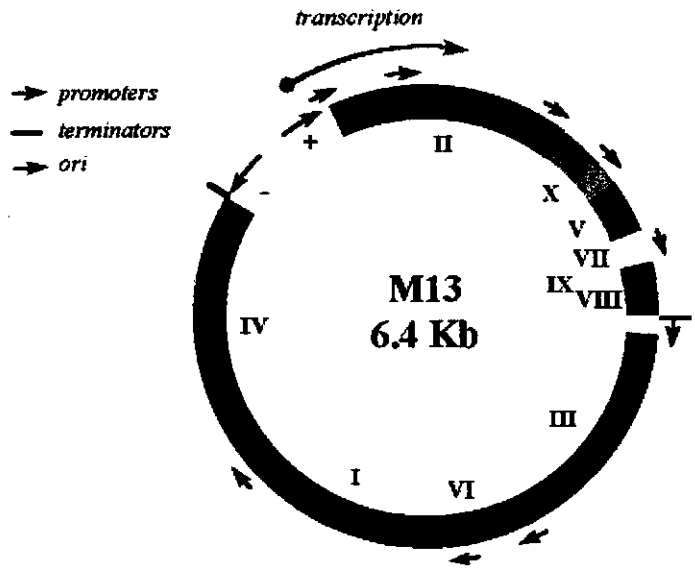
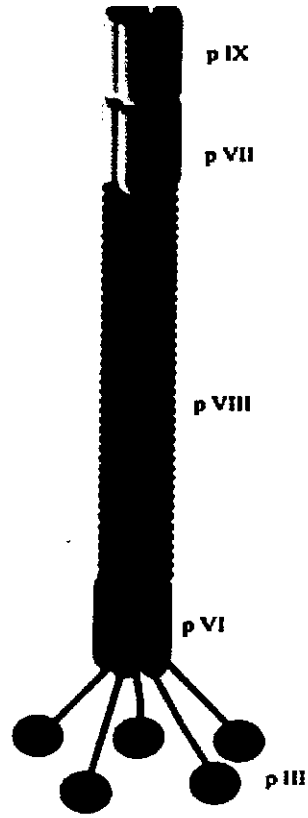


FIGURE 3.5: M13 VECTOR

During infection the bacteriophages genome is converted from single-stranded to a double-stranded circular form known as the replicative form (RF) which divides as a θ structure as in rolling circle mode of replication. They also act as the template for transcription of the M13 genes which are 11 in number. The viral gene products pII, pV and p X are responsible for replication of the viral genome whereas five virally coded transmembrane proteins- pIII, pVI, pVII, pVIII and pIX- make up the capsid (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001) (**Figure 3.6**).

The progeny particles are formed by an organized process in which the coat proteins are integrated into the bacterial membrane where they are assembled around the positive strand progeny DNA molecules and this protein-DNA complex is extruded from the cell as filamentous virus particle. The two processes of secretion and assembly of the filamentous phage takes place simultaneously in a coordinated manner. The most important elements of the M13 genome, around 508-bp, have been cloned into plasmids to form phagemids, which when infected by wild-type or recombinant

bacteriophages M13, single –stranded forms of the plasmid DNA are packaged into the viral progeny particles.



Arap et al, 2005

FIGURE 3.6: M13 FILAMENTUOS BACTERIOPHAGE

Since the genome of the recombinant filamentous bacteriophages are unstable, instead of using these genomes for cloning or long-term propagation of DNA segments, they are used to provide single-stranded copies of portions of DNA that have already been cloned into other vectors (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001).

M13 bacteriophages only infect bacterial strains that express sex pili encoded by an F factor. The virus adsorbs onto the bacterial surface through the interaction between the sex pilus and the minor viral coat protein pIII. As the rod shaped virus penetrates the pilus, pIII interacts with the host

proteins which mediate the removal of the major coat protein thereby releasing the viral genome into bacteria (**Figure 3.7**).

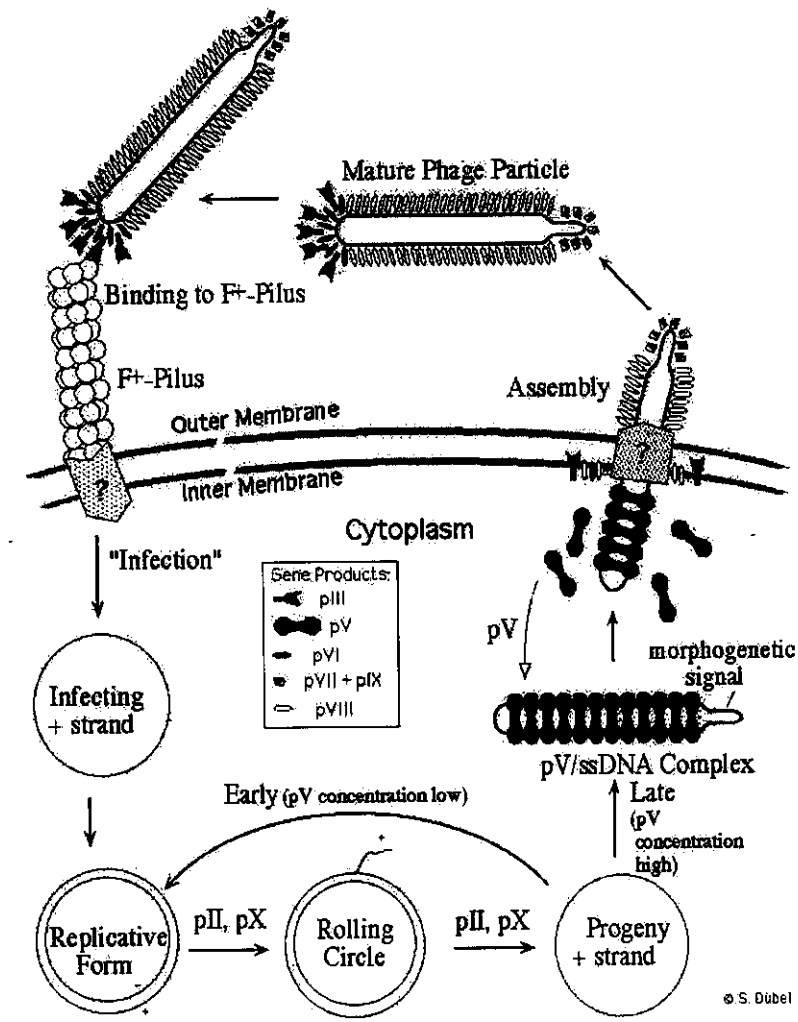


FIGURE 3.7: REPLICATION CYCLE OF M13 PHAGE

The infecting (+) - strand is then converted into the RF DNA and (-) –strand synthesis is initiated by an RNA primer. Transcription of viral genes begins at any one of the series of promoters in RF DNA and proceeds unidirectionally to one of the two terminators located downstream from genes VIII and IV (Figure 2.5). Amplification of viral genome begins when the protein product of gene II forms a nick at a specific location on the (+) –strand of RF DNA following which the DNA polymerase I adds nucleotides to the free 3'- hydroxyl terminal, progressively displacing the

original (+) -strand from the circular (-) –template (**Figure 3.7**). After replication, the displaced strand is cleaved by gene II protein generating a unit-length viral genome which is the circularized. These progeny strands, converted to closed circular RF DNA, now serve as templates for further rounds of replication. After 100-200 copies of RF DNA have been synthesized, translation of gene II mRNA is repressed by presence of adequate DNA-binding protein, which also performs the function of cooperatively binding to the (+) –strands preventing their conversion to RF DNA (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001). These replication steps are then followed by assembly and release of progeny phage particles.

This lays the basic principle of the phage display method of selection that is employed in order to comprehend the focus of this project. Various types of M13 filamentous phage have been used as the helper phage particle for these purposes. Due to its high efficiency in infection and replication, the helper virus that is used for experimental purposes in this case is the M13K07 recombinant M13 filamentous bacteriophages (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001).

3.5 M13K07 HELPER PHAGE

"The helper phage is commonly defined as a virus which helps a separate and unrelated defective virus to reproduce by infecting the same host cell that is already occupied by the defective virus and providing the proteins which the defective virus is missing and needs to complete its life cycle" (Biology Online.org). By defective virus, what is signified is that these viruses do not contain the entire complement of genes that is required in order to complete a cycle of infection, mainly due to deletion mutations, and therefore needs the "help" of another virus to provide the defective one with the gene functionalities which are otherwise absent. This virus used to help the defective virus complete its infectious cycle is called a helper phage (Mayer, Microbiology and Immunology Online Textbook, 2006).

In a system of defective and helper viruses, the role played by the former is very basic. The defective virus only needs to give out signals that allow a polymerase to replicate its genome and

for also packaging of its genome. In some cases, retroviruses have been seen to lose their viral functions and take up parts of the host cell sequences. To prove useful as a potential helper virus, these viruses should be closely related to the target defective virus and still retain its viral functions. However, there are cases in which defective virus can also use unrelated viruses as helper (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001).

In order to maximize the yield of single-stranded phagemid DNA packaging into the filamentous particles as a result of superinfection of phagemid- transformed cultures, a number of recombinant helper phages have been engineered. In an ideal case, the ratio of phagemid to helper genomes in the bacteriophages that has been released into the medium should be ~20:1. A few examples of recombinant M13 bacteriophages that are in use are R408, ExAssist, and VCSM13 etc (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001). The helper phage that has been used here in the phage display procedures is the M13K07 filamentous bacteriophages.

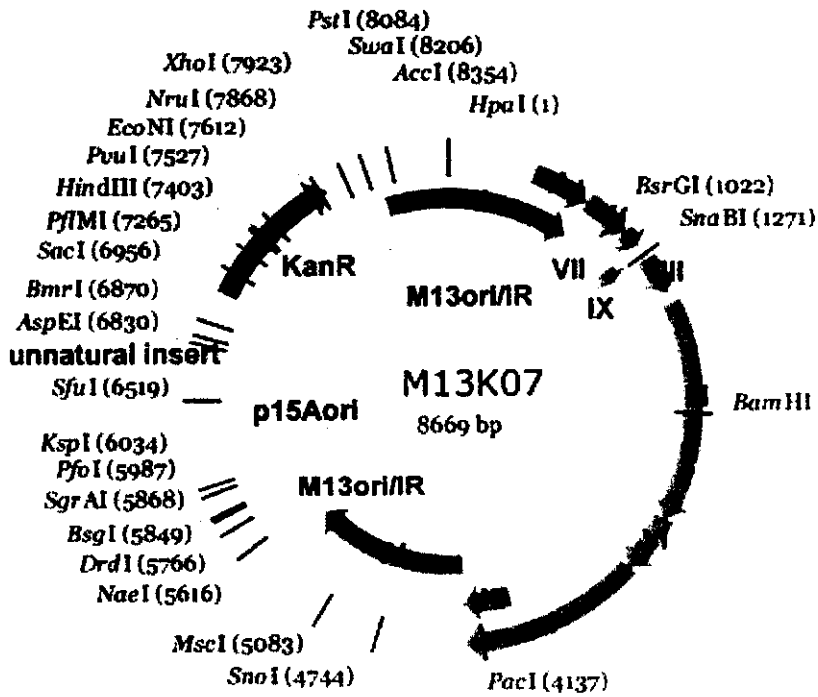


FIGURE 3.8: VECTOR MAP OF M13K07

M13K07 is a derivative of the M13 filamentous bacteriophages and carries the origin of replication derived from the plasmid p15A, the kanamycin-resistance gene from the transposon Tn903, and a mutated version of gene II in which the G residue at 6125 is replaced by a T residue (**Figure 3.8**).During infection of cells carrying phagemids by M13K07, the incoming single- stranded DNA of the helper bacteriophages is converted into a double-stranded form that employs the p15A origin to replicate (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001)

In the initial stages of replication of the incoming helper phage genome, there is very less interference from the resident phagemids, as accumulation of double- stranded DNA during this stage does not require any viral gene products. After a certain amount of time, the acquired pool of double-stranded M13k07 genomes express the proteins required to generate progeny single-stranded DNA. But the mutated gene II product encoded by M13K07 interacts less efficiently with the bacteriophage origin of replication on its own genome, due the lacZ sequence that is inserted in it, when compared to that cloned into the phage vector (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001). This affinity for one of the two origins results in the production of extra (+) –strands from the phagemid than from the helper phage and ensures that the virus particles produced from the cell holds a majority of single-stranded DNA derived from the phagemid. If M13K07 is grown in the absence of a phagemid vector, the mutant gene II protein interacts well enough with the disrupted origin of replication to produce sufficient amounts of bacteriophage for superinfection (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2001).

3.6 scFv AND PHAGE DISPLAY

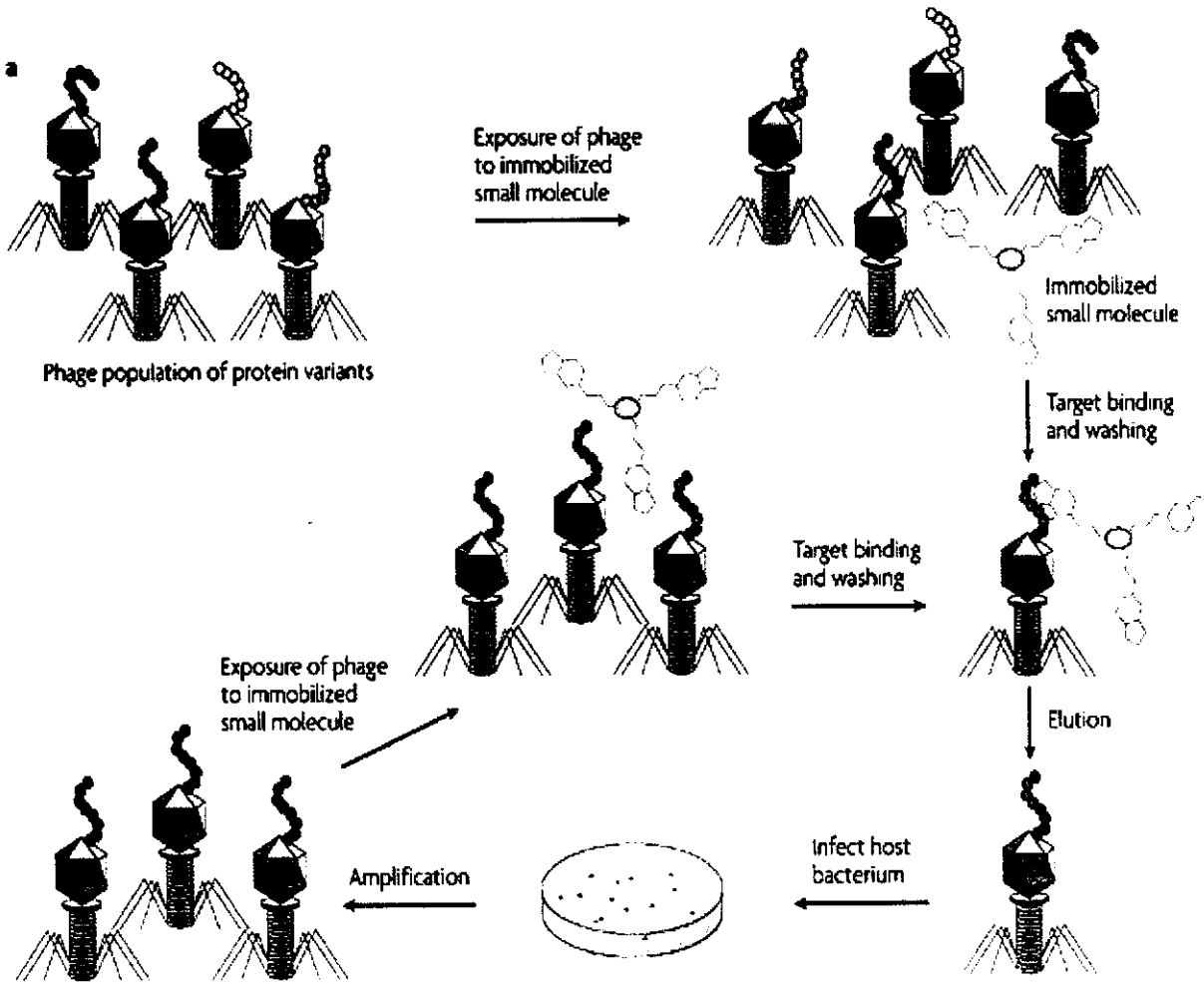
Monoclonal antibodies of reasonable specificity have been produced in the past years through tedious processes that calls for expertise and expensive equipments. Also, this specificity and affinity of the antibody so produced depends on the efficiency of the immunized animal's response to antigen invasion. To enhance the specificity and increase the affinity of candidate antibodies that are produced, various mutagenesis strategies have been experimented and proven useful and one such advance is the phage display technique of production of single chain variable fragments (Susi et al, 1997). A number of strategies can be engineered in phage display from focusing on the

complementarity determining region loops by substituting certain specific single residues within the region to expanding the target for random mutagenesis to the entire variable fragment sequence. It is practically not viable to generate all possible combinations of CDR residue mutants. Therefore, a range of methods were devised to select CDR loops that are thought to encompass the paratope, i.e., the domain on the antibody to which the antigen binds. Since the CDRs responsible for most of the binding specificity have been identified to be CDR-L3 and more importantly CDR-H3, these molecules have been extensively studied. Nevertheless, the exact mapping of the paratope is not as feasible as it may be thought because the original binding site involves multiple CDRs and also, it is not sure that such substitutions can improve binding. Therefore, deciding the components that make up the binding site, the ones to be enhanced and the other peripheral residues to be considered, is quite difficult (Barderas et al, 2008).

In phage display system, the minor coat protein (pIII) on the surface of the phage is used to fuse the functional fragments of the antibody. This antibody fragment that is inserted into the phage genome comprises the heavy and light chain variable, hypervariable antigen-binding, portions of antibody molecules bound by a linker made up of about 15 amino acids to form a single chain (scFv). Selection of clones expressing the particularfragment of interst can be done using large combinatorial scFv libararies (Susi et al, 1997). This selection of single chain variable fragments from combinatorial libraries, presented at the surface of phages allows a wide-ranging control of in vitro selection conditions. This gives scFvs one of its foremost advantages, in comparison with classical, animal-based approaches, of preserving the native conformation of target antigens. Lately, scFvs have seen to be used widely as intrabodies to target intracellular proteins, despite them being inactive in vivo due to misfolding induced by the reducing environment of the cytoplasm (Nizak et al, 2003)

Phage display (**Figure 3.9**) involves the fusion of peptides to the N-terminus of pIII or pVIII in the M13 phage vector. Even though large proteins cannot be displayed as fusion proteins without altering the function of the pVIII coat protein, multivalent forms of the polypeptide or antibody fragment can easily be displayed. A size specification of 6 residue (or less) inserts is usually followed in order to attain 100% translation of coat protein as fusion peptides, but if the size is

beyond 8 residues, only 40 % of phage are infectious and thus the overall efficiency decreases with increasing insert size.



Terstappen et al. 2007

FIGURE 3.9: PHAGE DISPLAY LIBRARY

However, pIII coat protein can be used to sport large fusion proteins, though a decrease in infectivity due to steric hindrance between the interacting N- terminus and F-pilus, when such proteins are displayed in a multivalent format. Such troubles of limited insert size while expressing the fusion protein engineered into the phage itself can be overcome by the usage of phagemids (Carmen et al, 2002).

Phagemids encode a signal sequence, the phage coat protein and an antibiotic resistance marker. The antibody fragment or polypeptide is cloned upstream of the gene III or gene VIII coat protein sequence and their expression is controlled by the use of a promoter such as lacZ. The relatively small size of these vectors means that they have higher transformation efficiencies than phage vectors, hence facilitating the construction of large repertoires or libraries of peptide or antibody fragments. Fusion protein expression in suppressor strains of E. coli such as TG1can be achieved by the incorporation of an amber stop codon between the displayed protein and the phage coat protein. Non-suppressor strains, such as HB2151, will not incorporate a glutamine at the amber codon, thereby resulting in production of only the antibody/polypeptide moiety. A phagemid cannot produce infective phage particles on its own and a helper phage such as M13KO7 or VCSM13 is required (Carmen et al, 2002).

The helper phage provides the genes which are essential for phage replication and assembly, including a wild-type copy of the coat protein used for display. Cells already containing the phagemid vector are superinfected with the helper phage. The pIII protein expression in the phagemid is prevented by repressing the lacZ promoter done by including glucose in the growth media, ultimately inhibiting superinfection. Once the helper phage genome is incorporated into the cell, the glucose is removed and phage production commences. The M13KO7 genome possesses a modified intergenic region (IG) causing it to be replicated and packaged less efficiently than the phagemid which carries the wild-type M13 IG. This ensures that the genotype (phagemid) and phenotype (pIII-scFv fusion) are linked in a single (phage) molecule, which is the key feature of phage display (Carmen et al, 2002).

3.7 TOMLINSON I & J LIBRARY

Libraries can be created from B cells of either non–immunized or immunized donors, providing the genetic information coding for the variable domains of an antibody (Carmen et al, 2002). Immunization can only generate antibodies against the particular antigen of the original immunogenic response. On the other hand non-immunized libraries have the advantage that they can be used against a wide range of antigens (Carmen et al, 2002). These libraries can be constructed by using basic molecular biology techniques like RT of mRNA and Polymerase Chain

Reaction (PCR) using germline-specific primers to amplify the heavy (V_H) and light (V_L) variable region gene segments from the cDNA template, and the use of restriction endonucleases to clone the engineered antibody segments into an appropriate phagemid display vector. These vectors can then be transformed into *E. coli* cells to express the antibody. Synthetic libraries can be created by artificially modifying the V_H and V_L genes and introducing manipulated Complementarity Determining Regions (CDRs) of variable loop lengths (Carmen et al, 2002).

The Tomlinson I+J libraries is made up of over 100 million different scFv fragments each, cloned into an ampicillin resistant phagemid vector and transformed into TG1 *E. coli* cells. The fragments that are inserted into the vector comprise a single polypeptide of the V_H and V_L antibody domains connected to each other by a flexible gly-ser linker. Production of these phagemids in large numbers can help select specific scFvs against target molecules attached to the surface of a matrix. The process of exposing the library to the target protein/s in called panning and at the end of each round of panning, the unbound molecules are washed away and the ones that are specifically bound to the protein of interest are eluted and amplified for subsequent rounds of selection. In order to ensure that a majority of the population of scFvs selected binds to the target molecule, at least 3 rounds of panning may be required. These monoclonal scFvs can then be screened for binding and utilized for further characterization and analysis of target molecules (de Wildt et al). Detection of scFvs can be done using immunostaining techniques. The Tomlinson I+J libraries have been artificially created based on a single human framework for V_H (V3-23/DP-47 and JH4b) and Vκ (O12/O2/DPK9 and Jκ1) with diverse side chain sequences incorporated at positions in the antigen binding site. The CDR3 of V_H was designed to be as short as possible and still form an antigen binding surface (de Wildt et al).

TOMLINSON I

Constructed in pIT2 (contains HIS6 and myc tags) (**Figure 3.10**). Diversified (DVT) side chains based mainly on those positions which are diverse in the primary repertoire (total of 18 residues - H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96). After selection, they can be matured by incorporating additional diversity based on somatic mutation.

Library size (with insert) : 1.47 x10 ^{8}

Percentage insert : 96%

TOMLINSON J

Constructed in pIT2 (contains HIS6 and myc tag) (**Figure 3.10**). NNK side chains based mainly on those positions which are diverse in the primary repertoire (total of 18 residues - H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96). After selection, they can be matured by incorporating additional diversity based on somatic mutation.

Library size (with insert) : 1.37 x 10^{8}

Percentage insert : 88%

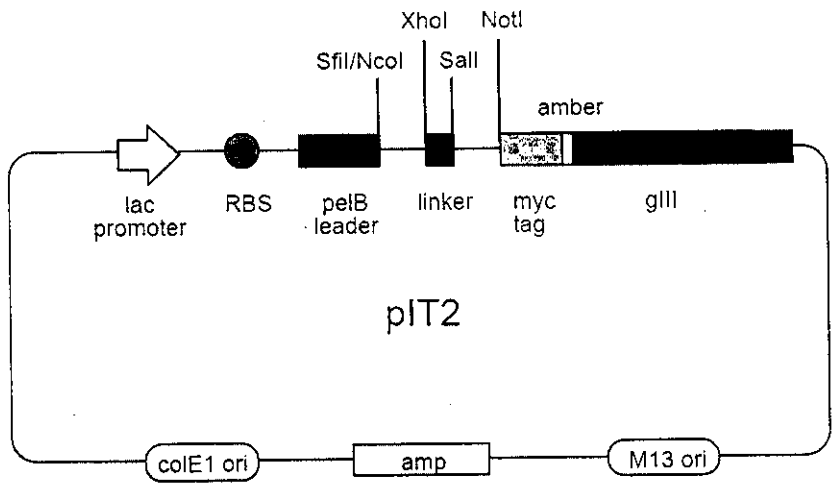


FIGURE 3.10: TOMLINSON I & J LIBRARY VECTOR

3.8 CELL SELECTION SYSTEMS

CHINESE HAMSTER OVARY CELL LINES:

- Trvb-1: These are stable cell lines that express Transferrin Receptor. They are used as pre-selection samples during the phage display selection of both Folate Receptor (FR) and GFP-GPIs. They are grown in single selection (geneticin) medium containing Fetal Calf Serum (FBS).

- IA2.2: These are stable cell lines expressing both Transferrin Receptor and Folate Receptor and differ from Trvb-1 cells by the one factor of expressing FR. They are hence grown in double selection (geneticin and hygromycin) medium containing FBS.

- cfrvb: These are stable cell lines expressing both transferrin receptors and CFP-GPI, the GFP derivative. They are also grown in double selection (geneticin and hygromycin) medium containing FBS. GG8, the cell line expressing GFP-GPI, has a myc tag fused to the GPI domain which interferes with immunostaining in later stages of selection. To overcome this, the relative cell line containing CFP-GPI (fused to his6 tag) is used for the assays.

Phage display and selection to identify scFvs against FR (and screening for positive clones against CFP-GPI) was performed in this system, where Trvb-1 cell lines serve as a preselection sample in which all scFvs specific to endogenous proteins that are common to Trvb-1 and IA2.2/cfrvb are filtered out. Those scFv phage molecules which do not adsorb on Trvb-1 should also include the ones specific for FR/CFP-GPI and when this is exposed to IA2.2/cfrvb, respectively, specific adsorption on to the FR-GPI/CFP-GPI takes place.

DROSOPHILA CELL LINES:

- S2R+: These are cell lines derived from drosophila embryos and exhibit a mixed expression system. They are subjected to DNA transfection (effectente transfection) to transiently express our protein of interest (hedgehog DNA in this case).Similar principle in phage display and selection is applied in this system as is mentioned above. The preselection sample in this case is untransfected S2R+ cells.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 CELL AND TISSUE CULTURE

PRINCIPLE

Cell culture is an important method by which cell lines are propagated and maintained under controlled conditions. Mammalian cells are isolated from tissue or explants in several ways to establish a primary cell line. These are then grown at 37°C in a 5% CO_2 incubator under varying culture conditions, as these variations determine the expression of particular phenotypes. The growth media, which include a carbon source such as glucose, an array of growth factors derived from animal blood components like calf serum, controlled pH conditions and other nutrients, differ from cell line to cell line.

Cells can either grow in suspension or adhere to a surface such as tissue culture plastic, which may be coated with extra cellular matrix components to improve adhesion and promote growth and differentiation. Cells are maintained for longer periods by transferring a small number of cells into a new culture vessel by a process called splitting. This can help culture the cells for longer by preventing senescence due to inappropriately high density.

MATERIALS AND REAGENTS

Ham F12 media (reconstituted); 1X PBS; 1X M1; 1X trypsin EDTA; Schneider's Medium (reconstituted) (**See appendix1**).

Dimethyl Sulphoxide (DMSO); tissue culture vessels; laminar air flow chamber.

PROCEDURE

CELL CULTURE OF CHINESE HAMSTER OVARY CELL LINES: Trvb-1 & IA2.2

- The freeze down of the cell lines obtained from liquid nitrogen storage (-196°C), is first thawed to 37°C.
- The cell suspension, 1 ml in volume, is immediately diluted using 9 ml of the reconstituted growth medium to remove traces of the cryopreservant, in this case Dimethly Sulfoxide (DMSO). The mixture is spun at 800 rpm for 10 minutes at R.T.
- The supernatant is discarded and the pellet is resuspended in 5 ml of reconstituted medium and transferred to a tissue culture flask and incubated for 24 hours in a 37°C CO_2 incubator.
- The cells would have adhered to the flask (T-25 flask, 5 ml) surface in this period but the medium is changed again to remove traces of DMSO. The flask is further incubated until cells are confluent enough for splitting.
- Once the flask reached 70-80% density, the medium is carefully removed and discarded.
- The adherent surface of the flask is washed with 5 ml of 1X PBS to remove any dead cell debris and FBS left in the flask. This washing step is repeated once.
- 1 ml of cold 1X Trypsin EDTA is added to the flask and it is incubated at 37°C for 3-4 minutes to cleave the cell-matrix adhesion in order to release the cells from the surface of the flask.
- The protease activity of trypsin is arrested immediately afterwards by adding 2 ml of reconstituted media due to the presence of anti-trypsin in FBS.
- The resulting suspension is called the seeding/plating inoculum. Ideally, 10% of fresh culture should constitute the inoculum and hence 0.5 ml of the seed is transferred to a flask and the volume is made up to 5 ml using reconstituted medium.
- The flask is incubated at 37°C for 2 days (until the cells are confluent enough for another round of splitting).

CELL CULTURE OF DROSOPHILA CELL LINES: S2R+ CELLS

- These cells are capable of doubling in culture within 12-18 hours and can ideally be maintained in cell densities ranging from 0.2 to 20 million per ml.
- High cell densities (10- 20 million cells/ml) will result in a significant number of floating cells but unlike CHO cells, these are healthy and can be used for assays and experiments since these cells do not require a matrix to propagate.
- In case of low cell densities, well-flattened, elongated cell morphology is noticed in the culture.
- Cells are seeded at 2 million/ml density and grown for 24 hours until they become 6 million/ml. Depending on the number of days the cells are meant to be cultured, cell seed densities can be varied.
- S2R+ cells are semi-adherent and hence do not require more than a few rounds of washing (using media) to separate them from the matrix.

CRYOPRESERVATION OF CELLS

Cryopreservation of cells that are cultured is done in the earlier passages using a freeze-down mixture that contains an appropriate cryopreservant. These freeze downs can then be thawed as needed.

- The media is removed from the flask and discarded.
- The adherent surface of the flask is washed with 5 ml 1X PBS thoroughly to remove any FBS, as it acts inhibitory to trypsin. Washing is repeated once.
- 1 ml of 1X trypsin EDTA is added to the flask and incubated at 37°C for 3-4 minutes. The trypsinisation reaction is arrested by addition of reconstituted medium.

- The suspension is then transferred to a centrifuge tube and spun at 800 rpm for 10 minutes. The supernatant is discarded.
- The pellet so obtained is resuspended in 2 ml of freeze-down mixture and transferred to sterile, labeled cryovials and kept in ice immediately as DMSO is toxic to cells at R.T.
- The vials are stored initially at -80°C and later kept in liquid nitrogen storage.

4.2 TRANSFECTION WITH LIPOFECTAMINE

PRINCIPLE

The manipulation of cells by the introduction of a foreign DNA, and sometimes RNAi constructs, to design the cells so as to express a gene or protein of interest is called transfection. Lipofectamine™ Transfection Reagent is a formulation that facilitates the transfection of nucleic acids into eukaryotic cells. It is a cationic lipid-based technique of gene delivery.

The complex of the nucleic acid and the lipid, called lipoplex, forming liposome-like compartments capable of carrying large cargo into the cell. The efficiency of delivery of gene is attributed to the ability of the liposome to integrate to endosomal membranes resulting in competent transfer of nucleic acid.

REAGENTS

Transfection mixture (serum-free cell culture media, with or without selection); Lipofectamine ™ Transfection Reagent; 1X PBS (**see appendix 1**); 35mm cover slip dishes

PROCEDURE

- 50µl of transfection medium is added each to two vials.

- To one of the tubes the DNA to be transfected is added and to the other, 3 times in volume of lipofectamine as the amount of DNA used is added.
- The tubes are incubated at R.T for 5 minutes.
- The contents of both the tubes are pooled to give 100µl of transfection mixture which should be incubated at R.T for 30 minutes.
- The cells, plated on 35mm cover-slip dishes, that reached a density of about 60-70 %, are prepared for transfection.
- The medium is removed from the dish and discarded.
- The dish is gently washed with fresh medium or 1X PBS.
- The transfection mixture is carefully added to the well and the dish is incubated at 37°C for 3 hours.
- The dish is gently washed with fresh medium or 1X PBS and filled with appropriate volume of reconstituted medium. The dish is left for incubation at 37°C for 48 hours after which it is examined for successful transfection.

4.3 TRANSFECTION WITH EFFECTENE

PRINCIPLE

Transfection using effectene reagent is a highly efficient way of introducing DNA into eukaryotic cells. The principle underlying this technique employs a non-liposomal approach in which the DNA is first condensed with an enhancer. The condensed DNA is the coated with micelles of cationic lipids to from a large complex which results in highly efficient transfections. The effectene method of transfection is widely practiced owing to its proficient success in most cases and almost negligible cytotoxicity.

REAGENTS

Effectene Transfection kit - EC buffer, Enhancer, Effectene; Cell culture media (Schneider's reconstituted media) **(see appendix 1)**; 6/12-well plates

PROCEDURE

- The EC buffer is taken in a vial at a volume of 75µl of promoter DNA and target DNA at an equal ratio, ideally at concentrations less than 1µg each.
- 4µl of the enhancer is added to this DNA mixture and mixed well and incubated at R.T for 5 minutes.
- The cationic lipid effectene is added next (5µl) and the complex is kept at R.T for 25-30 minutes after mixing well.
- The medium is removed from the dish and 400µl of fresh medium is added soon after, very gently.
- To the 75µl of reaction mixture, 325µl of fresh media is added and the solution is mixed thoroughly and added to the dish to make a total volume of 800µl.
- The dish is incubated for 48 hours to ensure high efficiency of transfection.

4.4 PHAGE DISPLAY AND SELECTION

MATERIALS AND REAGENTS

M9 minimal media; M9 minimal agar; 2X TY media; 2XTY agar (**see appendix 2**)

1X PBS; 1X M1 (**see appendix 1**)

20% glucose; 50mg/ml Kanamycin stock solution; 100mg/ml Ampicillin stock solution; 30% Polyethylene Glycol (PEG)8000- 2.5M NaCl; 2%PFA in 1XM1; 0.3% Tween20 -6% Skimmed Milk powder in 1X PBS (Tween-MPBS); 0.1% Tween20 in 1XPBS (TweenPBS); 1.4% Triethyamine in 1X PBS (TEA elution buffer); 1.5M Tris Hcl pH8.8; glycerol; 0.2% BSA in 1XM1 (M1BSA)

1:50 dilution of anti-his6 in M1 BSA; 1:200 dilution of anti-mouse in M1 BSA; 0.24 % Isopropyl β-D-thiogalactoside (IPTG) stock solution

Bioassay plates; 90mm Petri plates;1.5 ml vials; 96-well plates; 35 mm coverslip and non-coverslip dishes.

PROCEDURE

PREAPARTION OF TG1 STOCK CULTURE

- The M9 minimal agar is reconstituted after the temperature reaches around 45°C and is poured onto 90mm glass plates and left to set.
- A small amount (10µl) of the TG1 glycerol stock is streaked on the M9 minimal agar and incubated without shaking for more than 24 hours at 37°C.
- The M9 minimal medium is reconstituted and a single colony from the M9 plate is picked carefully and inoculated in the reconstituted medium which is then incubated in a shaker at 37°C till OD_{600} reaches 0.4.
- This culture is stored as stock culture for further use at 4°C for up to a month, after which a fresh stock culture is regenerated from step 1.

PRODUCTION OF LARGE QUANTITIES OF HELPER PHAGE

- 100ml of fresh TG1 culture is grown by using 1ml of mother culture as inoculum in the presence of 1% glucose till the OD_{600} reaches 0.4 (log phase).

- A 1:100 dilution, in 1X PBS, of the helper phage stock is made in order to obtain well separated phage particles. 10µl of this solution is added to 200µl of the log phase TG1 and the *E.coli* is kept for infection at 37°C without shaking for 30 minutes.
- After completion of infection, 50µl of the above solution is plated on a 2XTY plate containing a final concentration of 1%glucose, 50µg/ml kanamycin.
- A ten fold dilution of infected TG1 is prepared in fresh 2XTY and is plated on 2XTY agar plate containing 1%glucose, 50µg/ml kanamycin.
- A control plate of uninfected TG1 is plated on a kanamycin positive plate to ensure the Kan sensitivity of TG1 *E.coli*.
- All plates are incubated at 37°C without shaking overnight.
- A single colony, from the plate in which well separated colonies have formed, is picked and inoculated in 10ml of 2XTY and grown till OD_{600} reaches 0.4. This culture is centrifuged at 4000 rpm for 15 minutes and the pellet is resuspended in 1ml 2XTY.
- The resuspended cells are added to 500ml 2XTY broth in a 2 liter flask (no glucose, no antibiotics) and grown at 37°C in an incubator shaker for 1 hour. Add kanamycin to a final concentration of 50µg/ml and grow overnight shaking at 30°C.
- The overnight culture is centrifuged at 10,800g in a high speed centrifuge at 4°C for 15 minutes. 100ml of 30% PEG8000/NaCl is added to 400 ml of the supernatant and is mixed well before leaving on ice for 1 hour.
- This is then centrifuged at 10,800g at 4°C for 30 minutes to precipitate the phage particles.
- The supernatant is discarded and the centrifuge bottles are re-spun briefly to settle the precipitate adhered to the sides of the bottle. The pellet is resuspended in 4 ml 1XPBS.
- To the suspension, 2 ml of 30% PEG8000/NaCl is added, mixed well and left on ice for 20 minutes.
- The tubes are centrifuged at 3,300g for 30 minutes and the supernatant is drained. Any remaining dregs of PEG/NaCl are aspirated by respinning briefly.

- The pellet is resuspended in 4 ml of 1X PBS and transferred to 4 vials. These vials are centrifuged at 11,600g in a microcentrifuge to remove any bacterial debris. The supernatant from each of these vials are transferred to fresh vials.
- The resulting M13K07 helper phage solution can be stored at 4°C for short term usage but must be kept at -80°C (in 15% glycerol) for long term.
- The titer for the phage solution is determined by first making 100µl of the following serial dilutions in fresh 2XTY: 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}. These dilutions are set to infect with 100µl of log phase TG1 culture for 30 minutes without shaking at 37°C.
- The infected cells are plated on 2XTY plates containing 1% glucose and 50µg/ml kanamycin at 10µl and 100µl of each dilution and incubate without shaking at 37°C overnight. A control plate of uninfected TG1 on 2XTY plates containing 1% glucose and 50µg/ml kanamycin is also maintained. The ideal helper phage titer should lie between 10^{+13} and 10^{+16}.

GROWING THE LIBRARIES

- The library stocks (40-50µl) of I and J are added to separate flasks containing 100ml of 2XTY, 1% glucose and 100µg/ml ampicillin each.
- The library cultures are grown in an incubator shaker at 37°C for 2-4 hours until the OD_{600} reaches 0.4.
- To each of the flasks, 4 x 10^{+11} particles of M13K07 helper phage particles are added and kept for infection without shaking at 37°C for 30 minutes.
- Both the library cultures are centrifuged at 3,000g for 15 minutes and the pellets are resuspended in 200 ml 2XTY containing 100µg/ml ampicillin and 50µg/ml kanamycin and no glucose, each. The flasks are incubated shaking at 30°C overnight.
- The overnight cultures are centrifuged at 3,300g for 30 minutes to pellet down the cells.

- To 160ml of the supernatant, 40ml of 30%PEG8000/NaCl is added and mixed well and left on ice for 1 hour.
- The culture supernatants, after incubation on ice, are centrifuged at 3,300g for 30 minutes and the supernatant is discarded. The bottles are briefly spun again to settle the precipitate adhered to the sides of the bottle and to aspirate any remains of PEG/NaCl.
- The pellets are resuspended in 4ml 1X PBS and transferred into vials. The vials are centrifuged at 11,600g in a microcentrifuge for 10 minutes to remove bacterial debris. The supernatant is transferred to fresh vials for storage at 4°C until selection.
- Library titer is calculated by making 100µl of the following serial dilutions in fresh 2XTY: 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}. These dilutions are made to infect with 100µl of log phase TG1 culture for 30 minutes without shaking at 37°C.
- The infected cells are plated on 2XTY plates containing 1% glucose and 100µg/ml ampicillin at 10µl and 100µl of each dilution and incubate without shaking at 37°C overnight. A control plate of uninfected TG1 on 2XTY plates containing 1% glucose and 100µg/ml ampicillin is also maintained. The ideal library titer should lie between 10^{+12} and 10^{+14}.

SELECTION ON CELLS

- The 35mm Trvb-1 non-coverslip dish is washed twice with 1X M1 and fixed using 1ml of 2% PFA and incubated at R.T for 30 minutes.
- After washing with 1X M1 thrice, 0.5 ml of library is added to 1.5 ml of MPBS-tween solution and the mixture is added to the Trvb-1 dish and incubated at R.T for 1.5 hours with very gentle shaking.
- The dish is stood stationary for 30 minutes at R.T to allow strict binding and an IA2.2 dish is washed and fixed using 1ml 2% PFA for 30 minutes, simultaneously. (An additional

Trvb-1 pre-selection dish can be used prior to starting with IA2.2 selection, from second round of selection onwards.)

- The IA2.2 dish is washed thrice with 1X M1 and the solution from the Trvb-1 dish is transferred to the IA2.2 dish, which is then placed at R.T for 1.5 hours with very gentle shaking.
- The IA2.2 dish is brought to stationary and stood at R.T for 30 minutes.
- The library solution is removed from the IA2.2 dish and the dish is washed with 0.1% Tween PBS ten times (becomes twenty washes in all subsequent rounds of selection).
- 1ml of elution buffer is added to the IA2.2 dish and incubated standing at R.T for 10 minutes.
- After incubation, 500µl of the eluate is removed and added to 500µl of Tris Hcl (pH8.8) to buffer the pH of triethlyamine in the elution buffer, and the remaining 500µl of eluate is incubated in the dish for another 10 minutes.
- The eluate is completely removed from the IA2.2 dish and added to the vial containing Tris base and mixed well.
- 750µl of the eluted phage is added to 10ml of log phase TG1 culture and 3ml of the latter is added to IA2.2 dish. Both these infection mixtures are incubated at 37°C without shaking for 30 minutes.
- The infected TG1 from the dish is pooled with that in the tube and 100µl is removed from this solution to determine the output titer (by making serial dilutions of 10^{-1}, 10^{-2}, $10^{-3}$and plating these on 2XTY plates containing 1% glucose and 100µg/ml ampicillin and incubating at 37°C overnight). The remaining is centrifuged at 3500 rpm for 10 minutes to pellet down the cells.
- The pellet is resuspended in 2.5ml of fresh 2XTY media and plated onto 3 bioassay plates (833µl on each) containing 1% glucose and 100µg/ml ampicillin. These plates are incubated overnight at 30°C for 16 hours.

FURTHER ROUNDS OF SELECTION

- After overnight growth, the bacterial lawn formed on the bioassay plates is scraped off using 7ml of 2X TY containing 15% glycerol to loosen the cells and mix them thoroughly. 100µl of the scraped cells are inoculated in 100 ml of 2XTY containing 1% glucose and 100µg/ml ampicillin. The rest of the scraped cells are stored in -80°C.
- The inoculated bacteria is grown shaking at 37°C till the OD_{600} reaches 0.4.
- 10 ml of the log phase culture is infected with 5 x 10^{10} particles of M13K07 helper phage and kept without shaking at 37°C.
- The infected culture is centrifuged at 3500 rpm for 10 minutes and the pellet is resuspended in 50ml of 2XTY containing 100µg/ml ampicillin, 50µg/ml kanamycin and no glucose and grown overnight shaking at 30°C.
- The overnight culture is centrifuged at 3,300g for fifteen minutes.
- 10ml of 30%PEG8000/NaCl is added to 40ml of the supernatant and mixed well before leaving it on ice for 1 hour.
- The solution is then subject to centrifugation at 10,800g for 10 minutes and the supernatant is discarded.
- The bottle is spun again briefly to remove any remains of PEG/NaCl and the pellet is resuspended in 2ml 1X PBS and transferred to vials.
- Any bacterial debris remaining in the library solution is removed by spinning in a microcentrifuge at 11,600g for 10 minutes and the supernatant is transferred to fresh vials. 1ml of this library solution is used for the next round of selection.

NOTE:

The above selection protocol is against Folate Receptor (FR) expressed in the CHO cell line, IA2.2 where the control is Trvb-1

Selection for Hedgehog (Hh) in Drosophila cell line, S2R+, is done in the same manner where control cells are untransfected S2R+ cells and test cells are S2R+ cells that are transfected with the Hh DNA.

PRODUCTION OF SOLUBLE ANTIBODY FRAGMENTS

- The titer plates from the last round of selection are obtained from incubation before the individual colonies merge.

- Individual colonies are picked into 100 µl 2xTY (100 µg/ml ampicillin and 1 % glucose) in a 96 cell-well plate and grow shaking (250 rpm) overnight at 37°C.

- A 96 well transfer device 14 is used to aid in the transfer of a small inocula (about 2 µl) from this plate to a second 96 cell-well plate containing 200 µl 2xTY containing 100 µg/ml ampicillin and 0.1% glucose per well and is grown shaking (250 rpm) at 37°C until the OD_{600} is approximately 0.9 (about 3 hours). (A stock is made of the first plate, by adding glycerol to a final concentration of 15 % and storing at -80°C).

- Once OD 0.9 is reached, 25 µl 2xTY containing 100 µg/ml ampicillin and 9 mM IPTG (isopropyl β-D-thiogalactoside, final concentration 1 mM IPTG) is added and induction is begun by continuing shaking (250 rpm) at 30°C overnight.

- The overnight plates are centrifuged at 1,800 g for 10 min. 50 µl of the supernatant, taking care not to transfer any bacteria, is applied to target and control cells grown in 96 well glass bottom plates.

- After 1 hour the wells are washed gently with M1.

- Anti-scFv primary antibody (anti-his6) is applied for 1 hour, and then the secondary antibody (anti-mouse) is added for 1hr.

- The wells are imaged and the positives are recorded.

NOTE:

Once positives are obtained, those clones can be grown to log phase and, after making glycerol stocks, can be rechecked for specificity by performing the above assay on a larger scale where the 96 well plates are replaced by 35 mm coverslip dishes per positive clone.

IMAGING AND IMAGE PROCESSING

Wide field imaging was done using Nikon TE300 or Nikon TE2000-E, inverted microscope, using 60X objective 1.4NA or 20X objective 0.75NA. Mercury arc lamp (Ushio Inc.) was used to excite different fluorophores. Selective excitation was achieved using different excitation filters. CFP and the secondary anti-mouse fluorophore was visualized through cy5 and cy3 filters respectively. Images were collected using pre- cooled CCD camera (Princeton instruments), *via* Metamorph™ software (Universal imaging).

CHARACTERIZATION BY DOT BLOT:

1. 3µl of the scFv containing media was spotted on nitrocellulose and allowed to completely dry.
2. The membrane was then blocked with Milk-PBS for 3 hours.
3. After washing with PBS-Tween the primary antibody was applied overnight (4C, shaking).
4. After washing with PBS-Tween secondary antibody was applied for 3 hours.
5. After washing the blot was developed.

4.5 PURIFICATION OF RECOMBINANT FOLATE RECEPTOR USING GST PULL DOWN

PRINCIPLE

Glutathione-S-transferase (GST) is an enzyme found in abundance in cells, helping in cellular defense against electrophillic chemical compounds like toxic peroxides by 'transferring' glutathione (GSH), an antioxidant, to act on these toxic species. The high order affinity between glutathione and GST forms the basis of this affinity chromatography technique. The glutathione beads which are immobilized in a column or that in a batch separation process interacts with the GST in the sample. The GST would be tagged to the N terminus of the protein of interest to form a fusion protein which is retained in the column due its interaction with GSH. The strength of this interaction is such that employing this method of purification can yield up to 95% pure protein in only a single chromatographic step. The GST fusion protein is then eluted using a GSH containing elution buffer allowing the target protein to be collected in the flow through.

REAGENTS AND CONTRUCT DETAILS

LB broth; Glutathione elution buffer; Thrombin

0.24 % Isopropyl-beta-thio galactopyranoside (IPTG) stock solution; 1X PBS (see appendix 1); 20% Triton X100; Glutathione Sepharose 4B; 50 ml Falcon

Construct: pGEX4T2

PROCEDURE

- The *E.coli* cells are transformed with the plasmid vector, pGEX4T2, containing the sequence of GST-FBP (glutathione-S-Transferase-Folate Binding Protein) fusion protein, the expression of which can be induced by Isopropyl-beta-thio galactopyranoside (IPTG).

- A large scale culture (500 ml) is grown shaking at 37°C for 2-4 hours till the OD_{600} reaches 0.6-0.8.
- The expression of target protein is induced by adding IPTG to a final concentration of 1mM and the culture is kept for induction at 18°C overnight.
- The overnight culture is transferred to appropriate centrifuge bottles and centrifuged at 8000 rpm for 10 minutes at 4°C to sediment the cells.
- The supernatant is discarded and the pellet is placed on ice before resuspending in 50µl of ice cold 1X PBS per ml of culture.
- The suspended cells are disrupted in a sonicator for an appropriate amount of time in accordance with the volume of sonicate as insufficient sonication can prevent lysis of cells and oversonication can lead to co-purification of host proteins.
- 20% Triton X-100 is added to the sonicated cell suspension to a final concentration of 1% and mixed gently for 30 minutes to help the solubilization of the fusion protein.
- The solubilized suspension is centrifuged at 12000g for 10 minutes at 4°C and the supernatant is transferred into a fresh tube. A small aliquot of the supernatant and the cell debris pellet is saved for analysis by SDS-PAGE.
- The glutathione beads is a 75% slurry in ethanol and has to be equilibrated to 50% slurry.
- The bottle of Glutathione Sepharose 4B is gently mixed to resuspend the gel. For every 1ml of bed volume that is required for the purification, 1.33ml of original slurry is dispensed into a fresh flask. For a 5ml bed, 6.56ml of 75% slurry is taken into a fresh tube.
- The gel is sedimented by spinning the tube at 800 rpm for 10 minutes and the supernatant is carefully decanted.
- The pelleted beads are washed with about 50 ml of ice cold autoclaved water by inverting to mix. The gel is sedimented by spinning at 800 rpm for 10 minutes and the supernatant is decanted.

- The washing is repeated twice with 50ml of ice cold 1X PBS (approximately 1ml 1X PBS per 1.33ml of slurry) and sedimented to remove any traces of the 20% ethanol storage solution.
- Once washing is completed, for each 1.33ml of original slurry, 1ml of ice cold 1X PBS is added and mixed well to produce 50% slurry. This 50% equilibrated slurry can be stored at 4°C for up to a month.
- The bacterial lysate is added to the tube containing the equilibrated slurry and incubated with gentle agitation at 4°C for 1 hour to allow adsorption of fusion protein to the gel.
- The suspension is centrifuged at 800 rpm for 10 minutes to sediment the gel and the supernatant is discarded after saving a small aliquot of it for later analysis.
- The pelleted beads are then washed with 10 bed volumes of ice cold 1X PBS where 1 bed volume is 0.5 times the volume of the 50% glutathione slurry. The suspension is centrifuged at 800 rpm for 10 minutes and the wash is discarded.
- The washing steps are repeated twice and at each washing step, aliquots are collected to monitor loss of protein during washing.
- To the sedimented gel, 1 ml of elution buffer is added per ml of bed volume of Glutathione Sepharose 4B.
- The suspension is mixed well and incubated at 4°C for 10 minutes to liberate the fusion protein from the gel.
- The gel is sedimented by centrifuging at 800 rpm for 10 minutes and the supernatant is transferred to a fresh tube.
- The elution step is repeated twice more and the three eluates are pooled. An aliquot of eluate sample is collected for SDS-PAGE analysis.
- To the eluate collected, 50 cleavage units of Thrombin is added, mixed gently and incubated at 4°C for 16 hours.

- Once digestion is complete, glutathione can be removed by extensive dialysis against 2000 volumes of 1X PBS followed by removal of GST by batch or column purification using Glutathione Sepharose 4B. The purified protein of interest will be found in the flow-through.

RESULTS & DISCUSSIONS

5. RESULTS AND DISCUSSIONS

5.1PHAGE DISPLAY AND SELECTION AGAINST FOLATE RECEPTOR (FR)

The TG1 *E.coli* cells were grown in an M9 minimal media and stored as the parent culture at 4°C. This was used as inoculum to produce TG1 starter cultures through various stages in the generation of large quantities of helper phage and library as well as for calculating phage/library titers.

The helper phage prep was carried out and the following titer was obtained:

TABLE 5.1: HELPER PHAGE TITER

Dilution factor	1.00E-10		1.00E-11		1.00E-12		Average	No. of Phages (PFU/ml)
Frac. Plated (µl)	100	10	100	10	100	10		
No. of colonies	TMTC	400	TMTC	277	TMTC	60	1.00	
Estimation		4E+14		2.8E+15		1.2E+16	5.06E+15	5.06E+15

The helper phage titer was thus found to be 5.06 E+15 PFU/ml.

The phage titer value or the plaque forming units per ml (PFU/ml) is defined here as twice the number of colonies multiplied by the inverse of both the fraction plated (in ml) and the dilution factor.

Eg: For 10µl fraction of 1.00E-12 dilution, the titer estimation is:

$$2*60*(1/0.001)(1/1.00E-12)$$

$$= 1.2E+16$$

NOTE:

The factor of multiplication, 2, is used here as the volume plated on the 2XTY plates for the purpose of calculating titer includes two components in equal volume- phage dilution and fresh 2XTY. Therefore, since the essential volume plated comprises only half the original volume, the final value is multiplied by 2.

Using this helper phage solution, the libraries I & J were prepared. Their titer is as follows:

TABLE 5.2: LIBRARY TITER FOR I

Dilution factor	1.00E-08		1.00E-09		1.00E-10		Average	No. of Phages (PFU/ml)
Frac. Plated (µl)	100	10	100	10	100	10		
No. of colonies	600	13	100	14	450	14	1.00	
Estimation	6E+11	1.3E+11	1E+12	2.8E+15	9E+13	2.8E+13	2.04E+13	2.04E+13

Library titer value for Tomlinson I: 2.04E+13 PFU/ml

TABLE 5.3: LIBRARY TITER FOR J

Dilution factor	1.00E-08		1.00E-09		1.00E-10		Average	No. of Phages (PFU/ml)
Frac. Plated (µl)	100	10	100	10	100	10		
No. of colonies	TMTC	TMTC	450	37	220	14	1.00	
Estimation			4.5E+12	7.4E+12	4.4E+13	2.8E+13	2.10E+13	2.10E+13

Library titer value for Tomlinson J: 2.10E+13 PFU/ml

The library, having titer value as calculated as above, is now used for 1st round of selection. The output of this round is amplified and precipitated to serve as the input of the subsequent round and so on. Six such selections were performed and the enrichment after each round was monitored by tracking the yield (output phage particles/input phage particles) and graphically analyzing it at the end of each selection (**Figure 5.1**).

At the end of six rounds of selection, the yield obtained is relatively low. This was due to high stringency in selection right from round 1. The phage particles that are enhanced in the 1st round are the most important and a wrong amplification can lead to failure of the entire selection. Hence, a fair amount of phage particles are allowed to pass through the round 1 by decreasing stringency and by reducing washing steps. This is done in order to first "introduce" the library to the system, before beginning to subtract out those clones that are unnecessary.

TABLE 5.4: YIELD PER ROUND OF SELECTION

Round	1	2	3	4	5	6
Input	2.10E+13	8.12E+14	1.43E+15	4.93E+14	1.22E+17	8.00E+15
Output	1.31E+05	6.20E+06	3.28E+06	2.64E+06	6.30E+07	7.77E+07
Yield	6.2E-09	7.6E-09	2.3E-09	5.3E-09	5.2E-10	9.7E-09

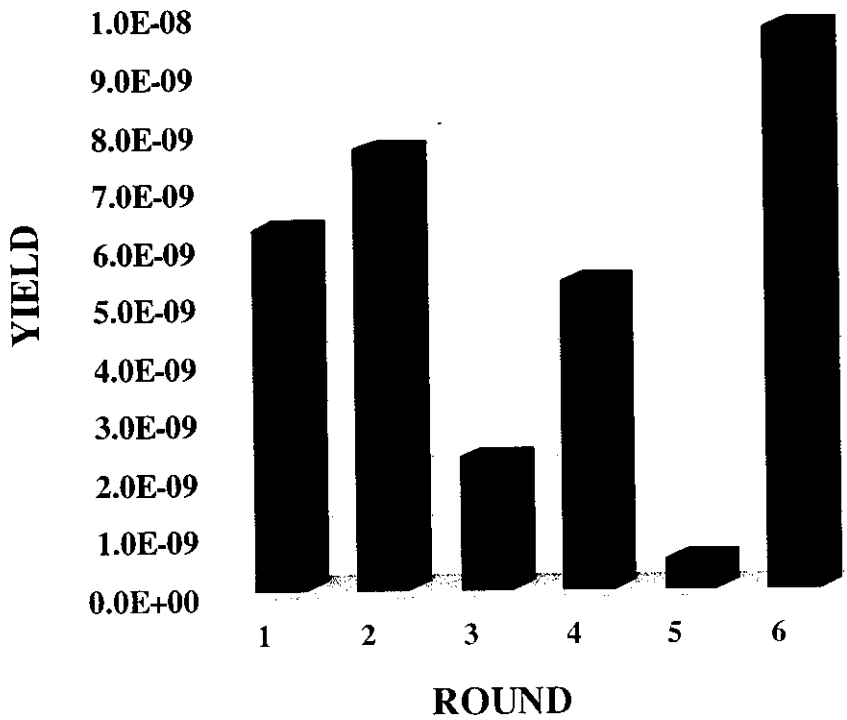


FIGURE 5.1: YIELD PER ROUND OF SELECTION

In this case, since two pre-selection dishes of Trvb-1 was used right in the first round, the system was too strict for the library to filter through and hence gave poor enhancement in the first round which became the trend followed in all subsequent rounds.

5.2 PHAGE DISPLAY AND SELECTION AGAINST HEDGEHOG (Hh)

The selection (round 1) done against Hedgehog gave too low a yield as is represented in the following table:

TABLE 5.5: OUTPUT TITER FOR I+J

Dilution factor	1.00E-01		1.00E-02		1.00E-03		Average	No. of Phages (PFU/ml)
Frac. Plated (µl)	100	10	100	10	100	10		
No. of colonies	28	0	3	0	0	0	1.00	
Estimation	3.29E+04		4.20E+04				4.06E+04	4.06E+04

The output titer from round 1 of section is: 4.06E+04 PFU/ml

This titer value is lower than what is expected (expected value is in the order of E+06) and hence holds no significance in proceeding to next round. New libraries with higher titer are to be used in the future in order to circumvent this hitch. Also, washing steps for first round can be comparatively lax in order to decrease stringency.

NOTE: In the output titer calculation, the final value (obtained from calculations employed for the input titer) is multiplied by a volume factor of 14 ml (which includes 10 ml of log phase TGI + 750 µl of library phage solution + 3 ml of infected TGI from selection dish).

The selection is to be repeated with lesser stringency and larger surface area of antigen in order to maximize binding capacity of the phage particle. When the yield is seen to plateau, due to saturation of the antigenic surfaces after a certain number of selections (ideally 4), the last round is chosen to pick colonies from. Subsequent rounds of screening, purification and characterization of the positive scFvs are performed.

The graphical representation of the yield obtained for selection against FR showed an unlikely trend in which the values were fluctuating at every round of panning. Instead, it is expected to steadily increase with every round, showing enrichment and finally, at the end of round 5, should give a yield in the range 10^{-4}. Also using a dish of test cells, IA2.2, before starting cycles of subtractive cell panning should lead to an increased yield (Yamashita et al, 2010).

5.3 PRIMARY AND SECONDARY SCREEN AGAINST CFP-GPI

Ten random clones were picked from a 96-well plate that contained clones obtained at the end of a phage display and selection round for scFv phage particles against the Green Fluorescent Protein derivative, Cyan Fluorescent Protein (by Aneesh Sathe). These clones were subjected to a round of primary screening using immunostaining. The murine primary antibody (anti-his), targeted against the his6-tag fused to the CFP domain can then be tagged using an anti-mouse secondary antibody and imaged using fluorescence microscopy.

The ten clones chosen were: A4, E5, G6, H8, E9, A10, B9, D7, F11 and C6, in order of testing (Table 5.7). The controls used for both the Trvb-1 and cfrvb cells (**Figure 5.2**) were: unprobed dishes testing auto florescence, dishes probed with anti-his6 and anti-mouse, dishes probed with anti-mouse alone (**Table 5.6**).

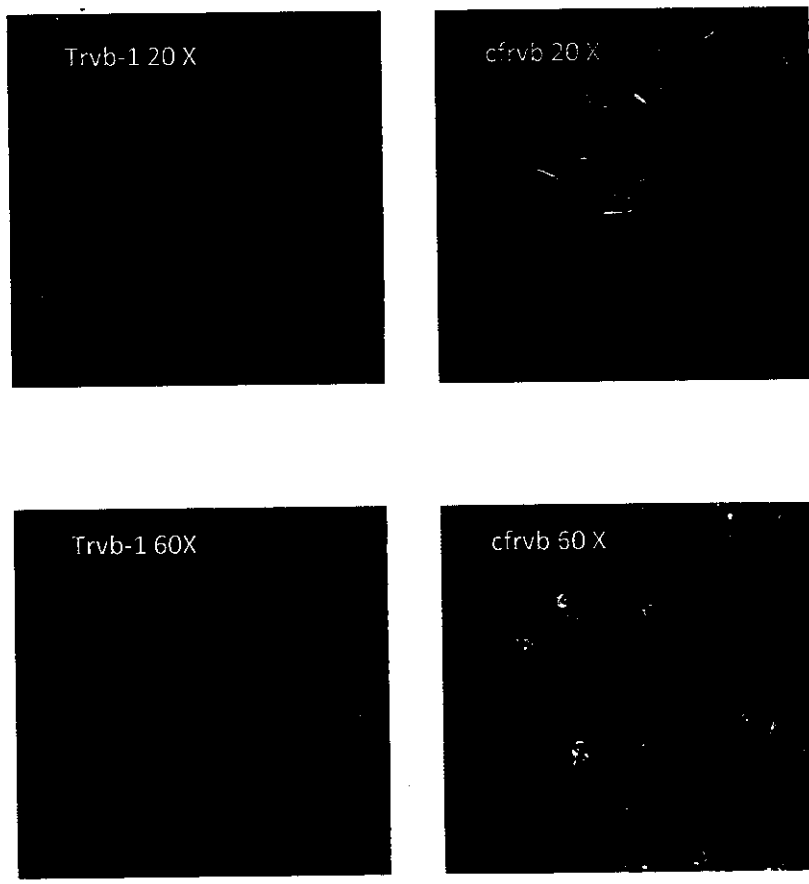
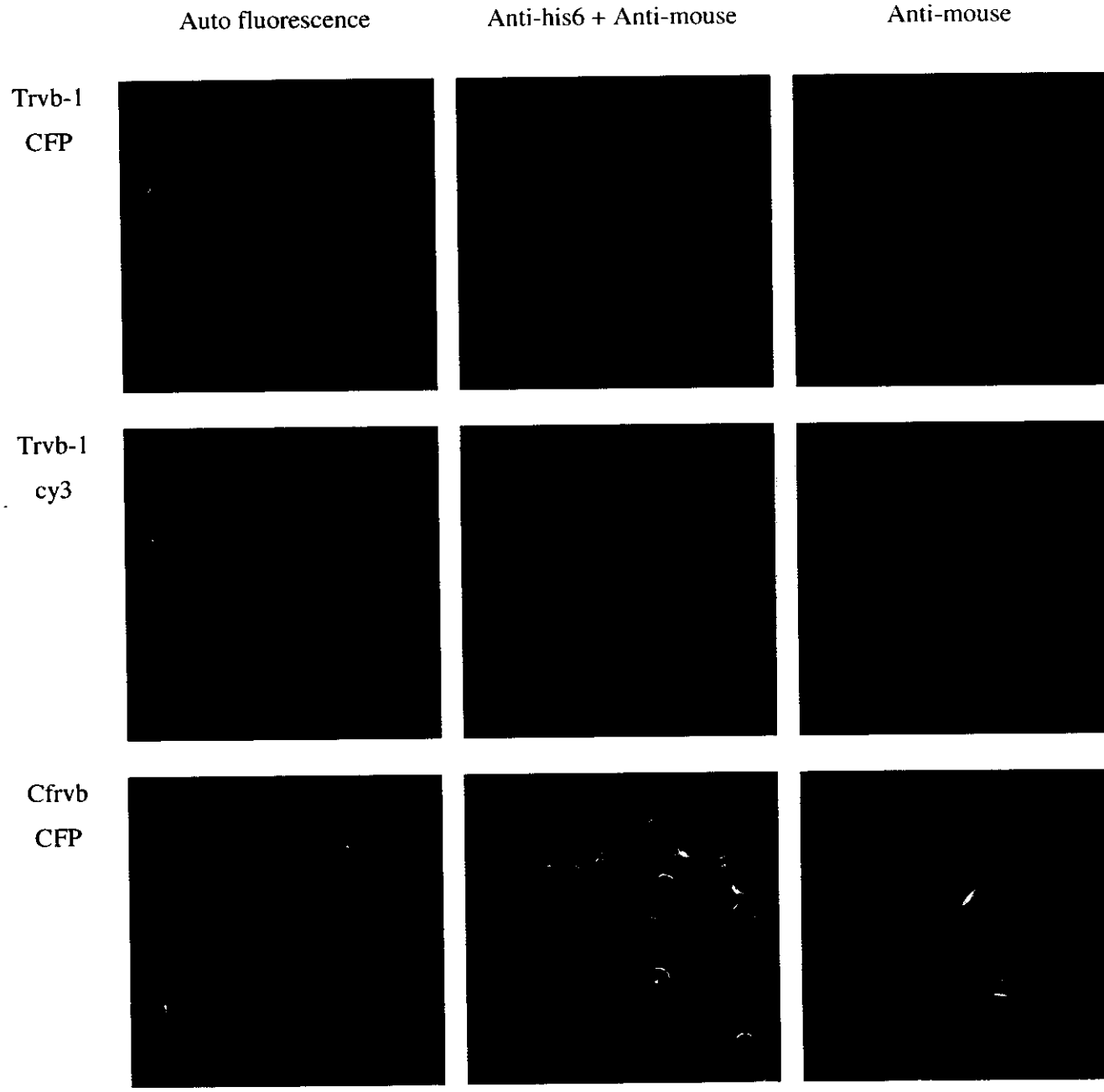


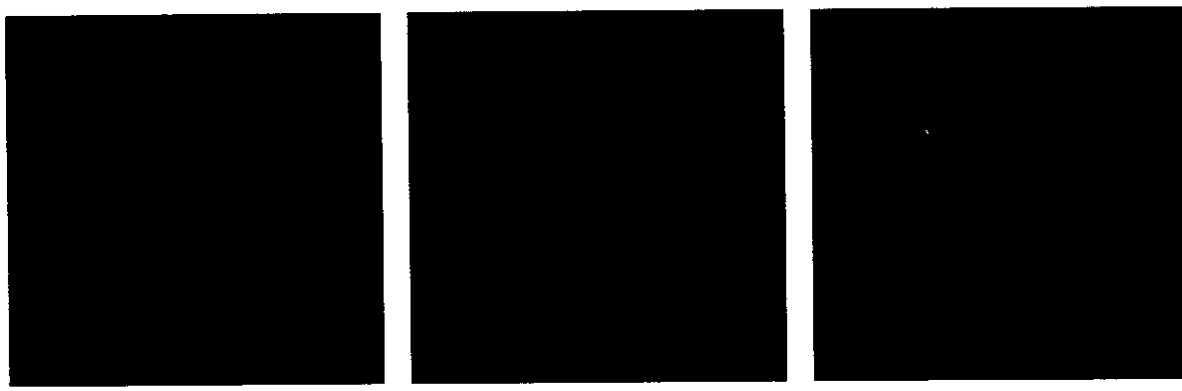
FIGURE 5.2: PHASE CONTRAST IMAGES

TABLE 5.6: SCREENING CONTROLS



Cfrvb

cy3



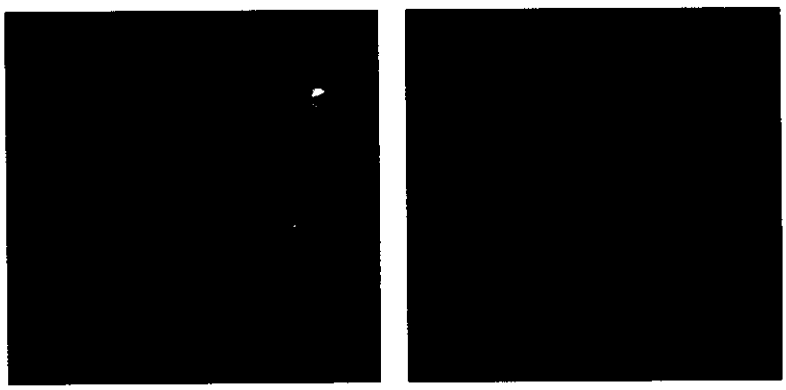
(images are not equally scaled and have been scaled in order to emphasize on the quantitative results that were obtained)

TABLE 5.7: PRIMARY SCREENING

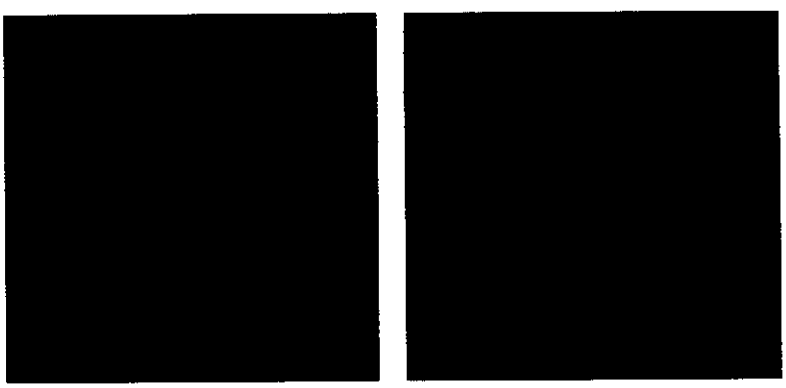
CFP channel cy3 channel

Clone A4

cfrvb

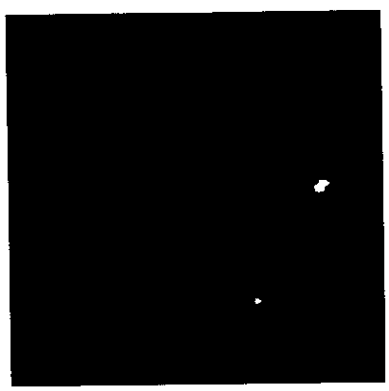


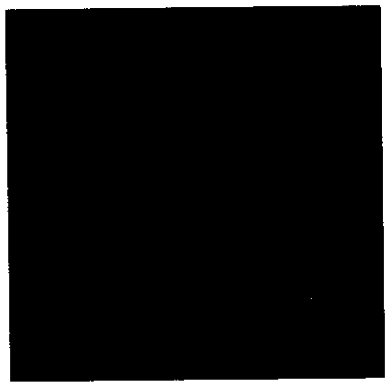
Trvb-1



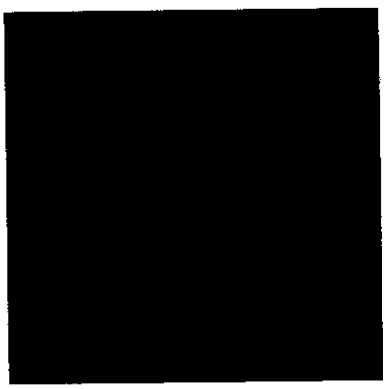
Clone A10

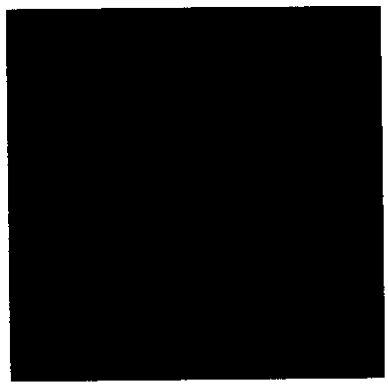
cfrvb





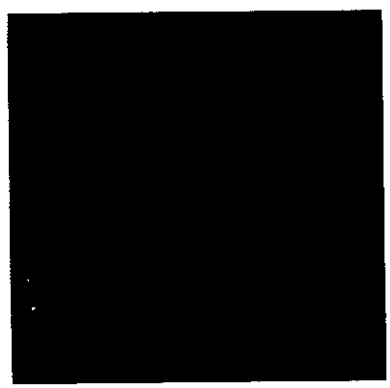
Trvb-1



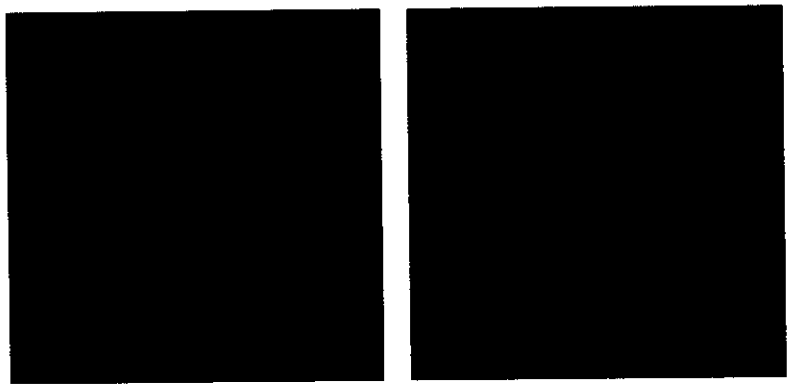


Clone B9

cfrvb

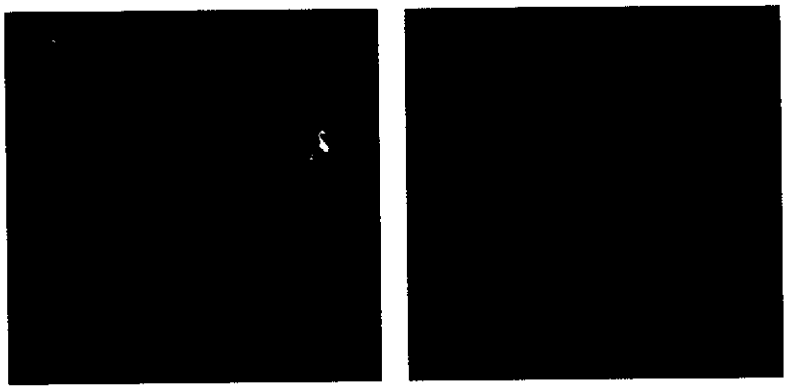


Trvb-1

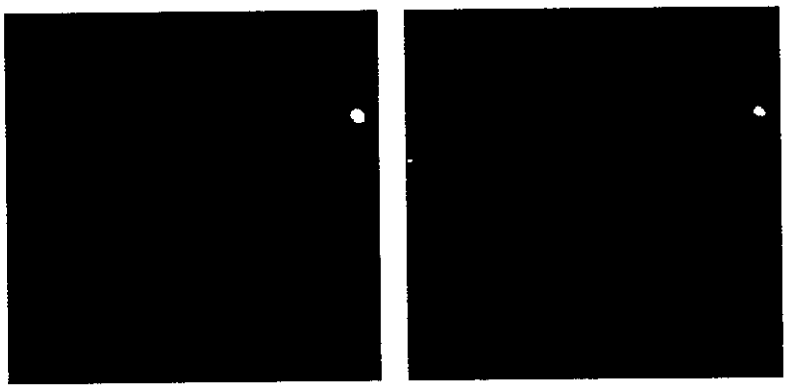


Clone C6

cfrvb

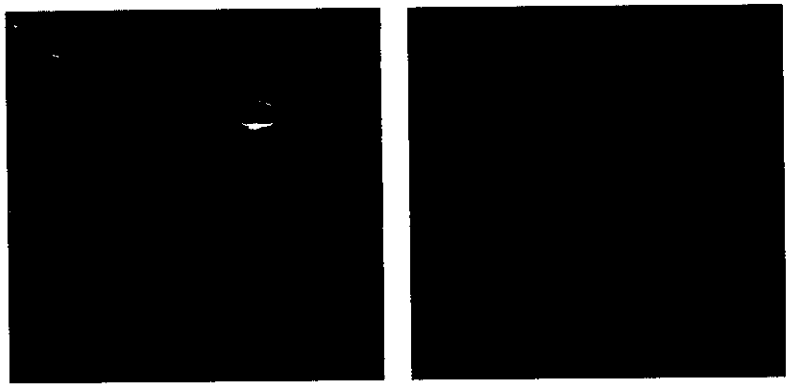


Trvb-1

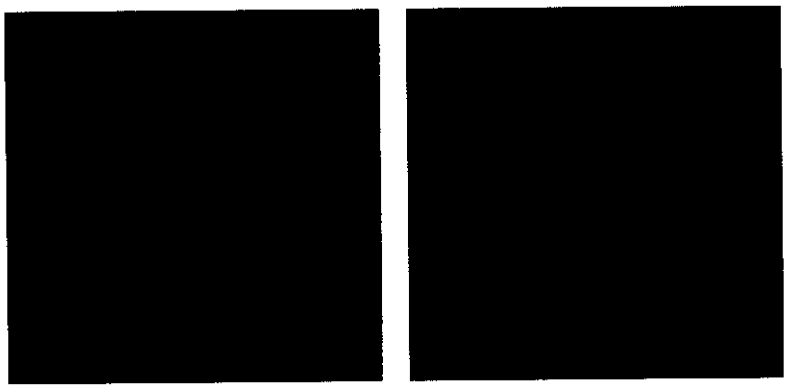


Clone D7

cfrvb

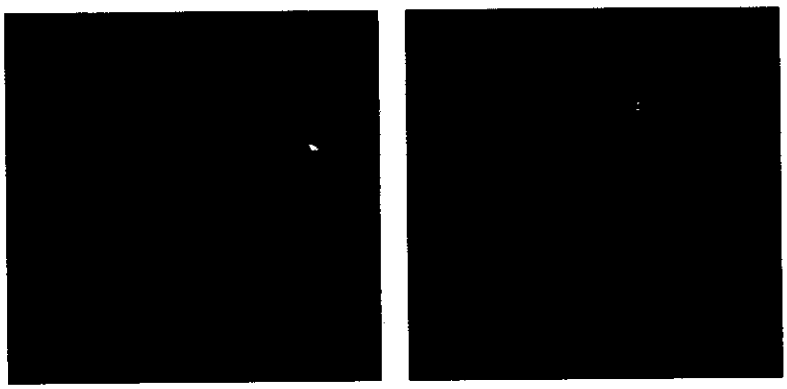


Trvb-1

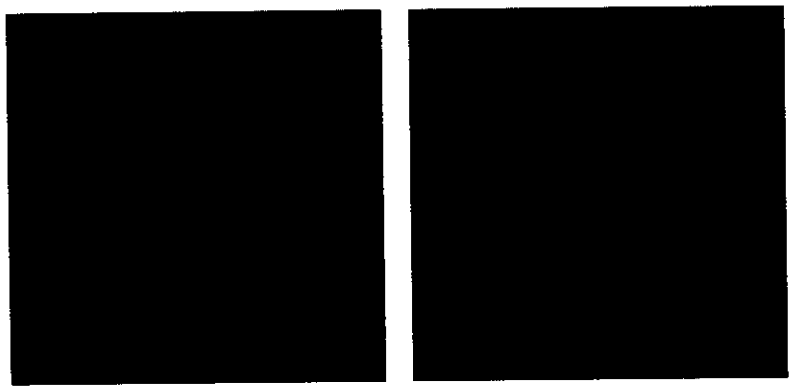


Clone E5

cfrvb

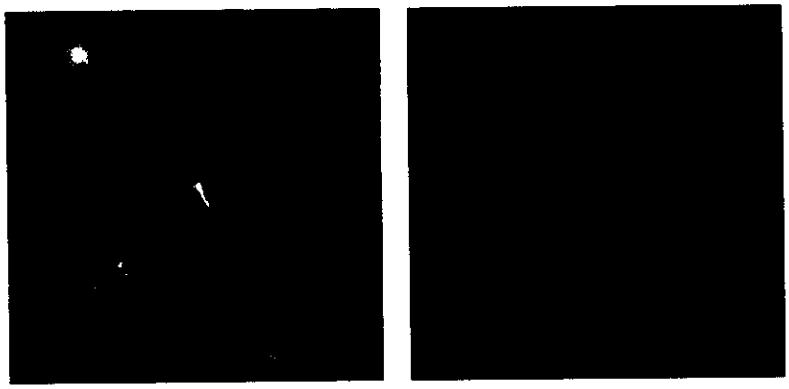


Trvb-1

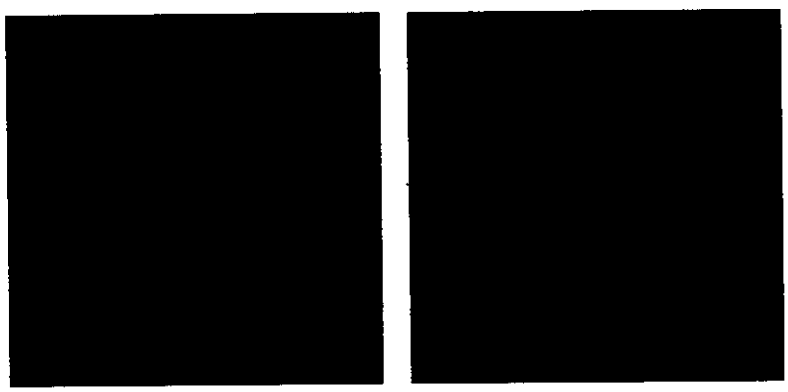


Clone E9

cfrvb

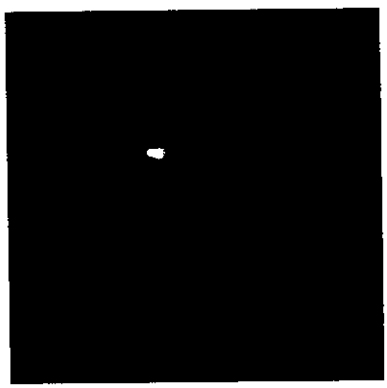


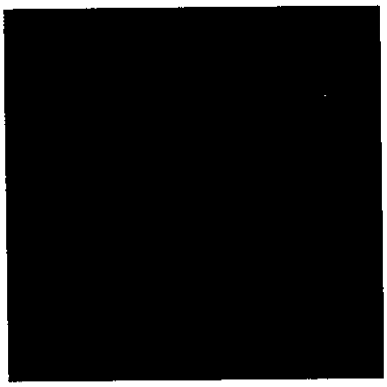
Trvb-1



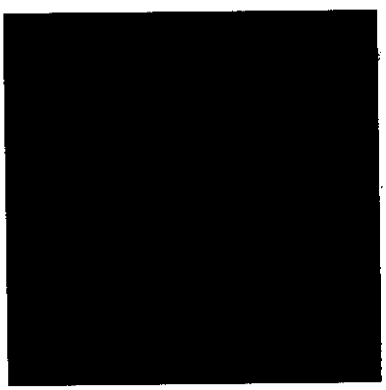
Clone F11

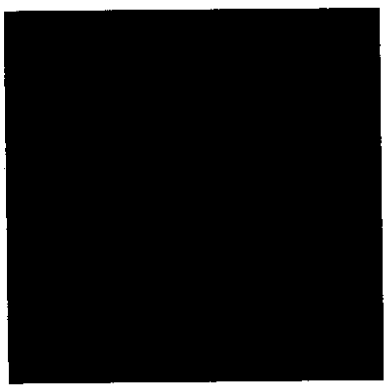
cfrvb





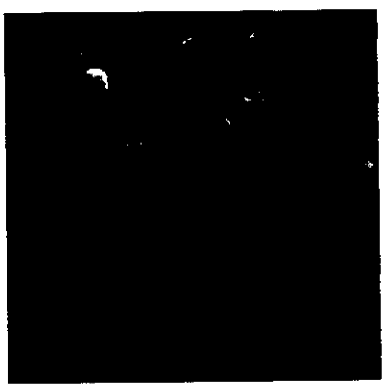
Trvb-1

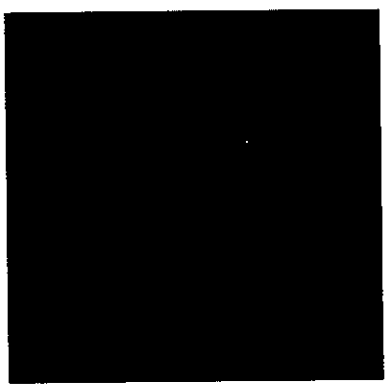


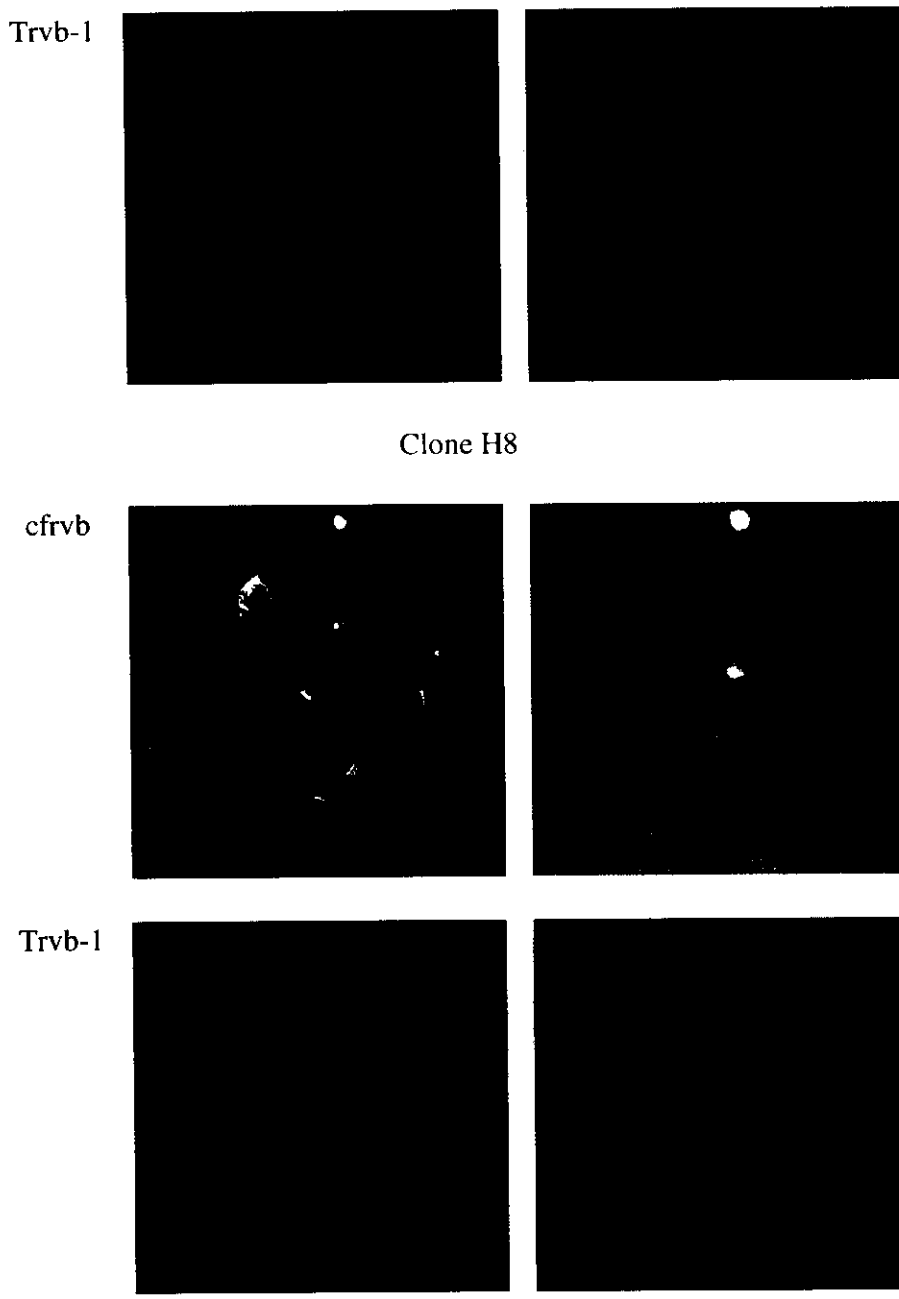


Clone G6

cfrvb





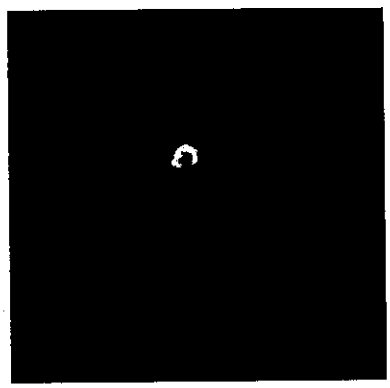
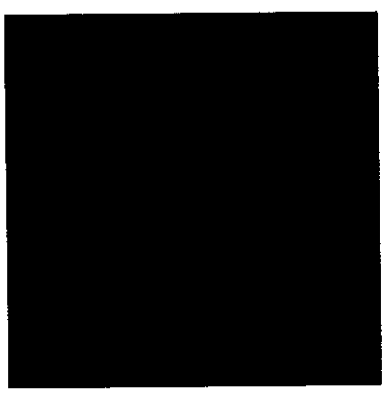
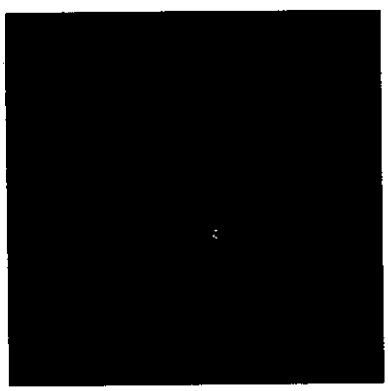


(images are not equally scaled and have been scaled in order to emphasize on the quantitative results that were obtained)

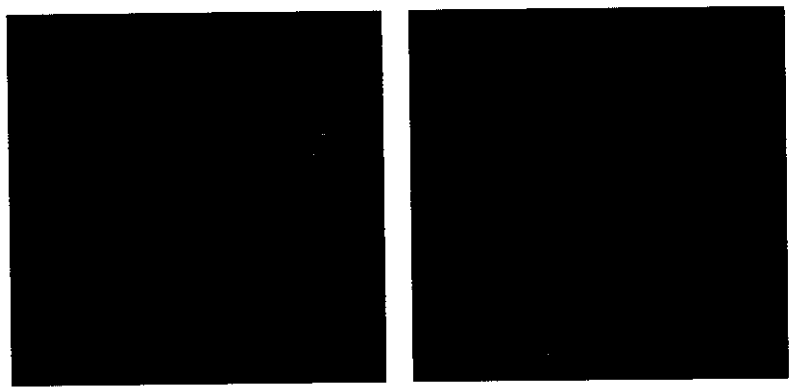
During the primary screen, 6 clones from the above depicted table were chosen for secondary screen due to significant signals in the cy3 channel- A10, B9, C6, E9, F11, H8 (Table 5.8). Those

clones that were not chosen either did not show significant signal in the cy3 channel or had intensities similar to that exhibited by their respective control dishes.

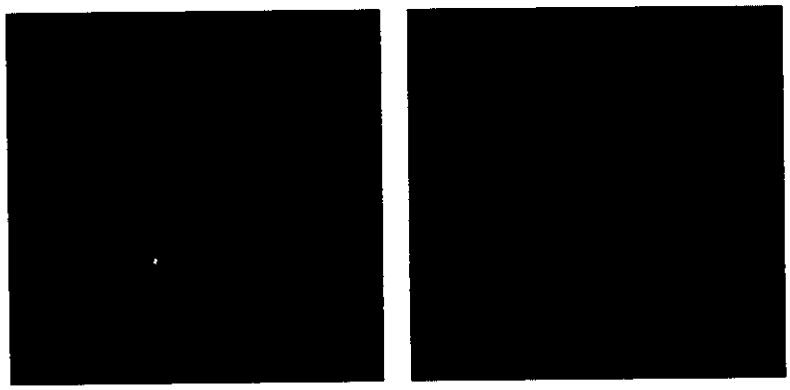
TABLE 5.8: SECONDARY SCREENING

	CFP channel	cy3 channel
Clone A10		
cfrvb		
Trvb-1		
Clone B9		

cfrvb



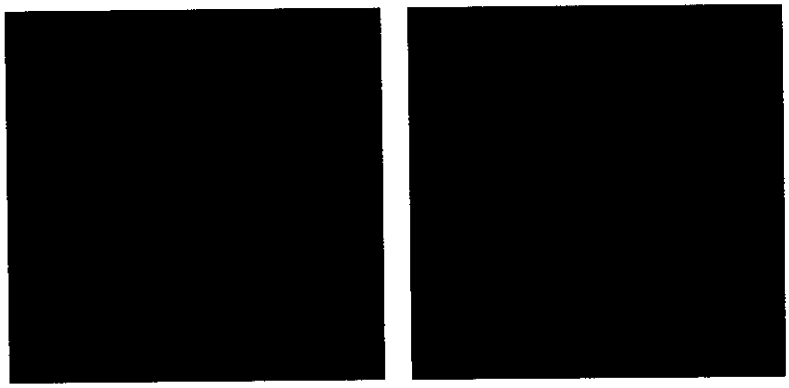
Trvb-1



Clone C6

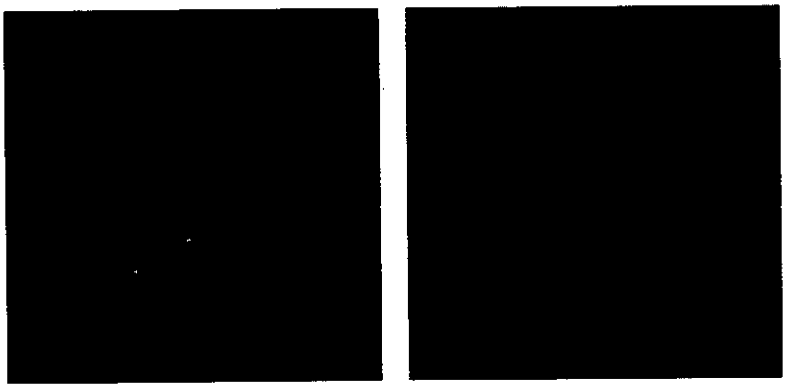
cfrvb

Trvb-1

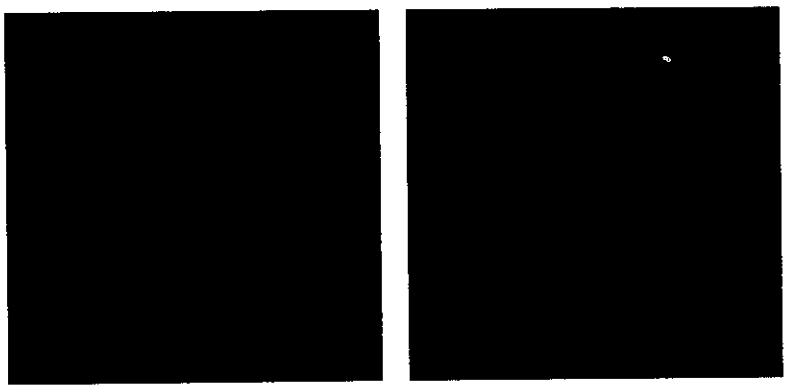


Clone E9

cfrvb

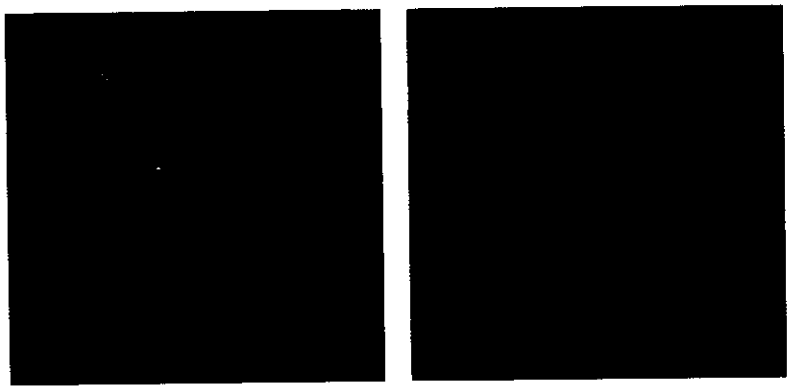


Trvb-1

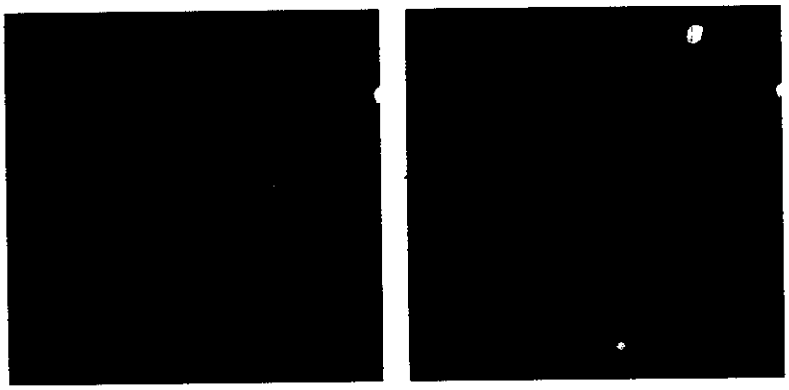


Clone F11

cfrvb

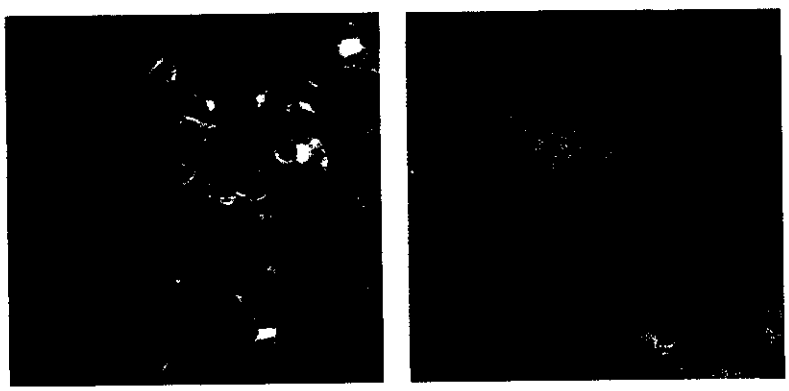


Trvb-1

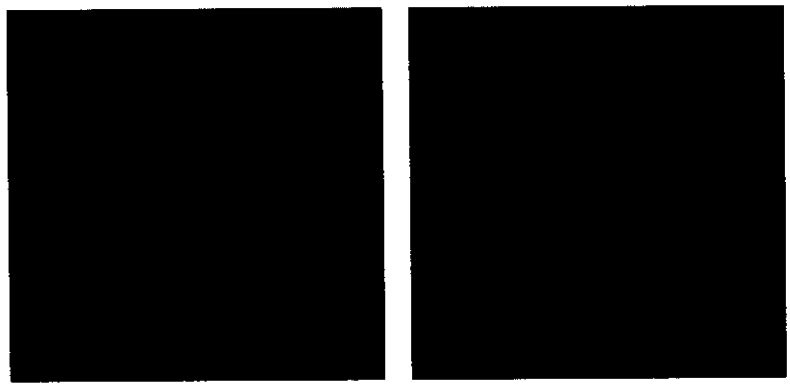


Clone H8

cfrvb

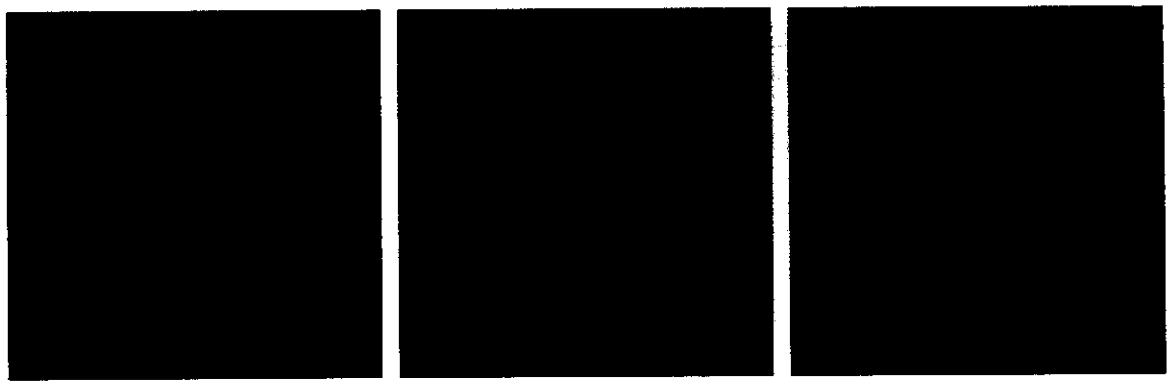


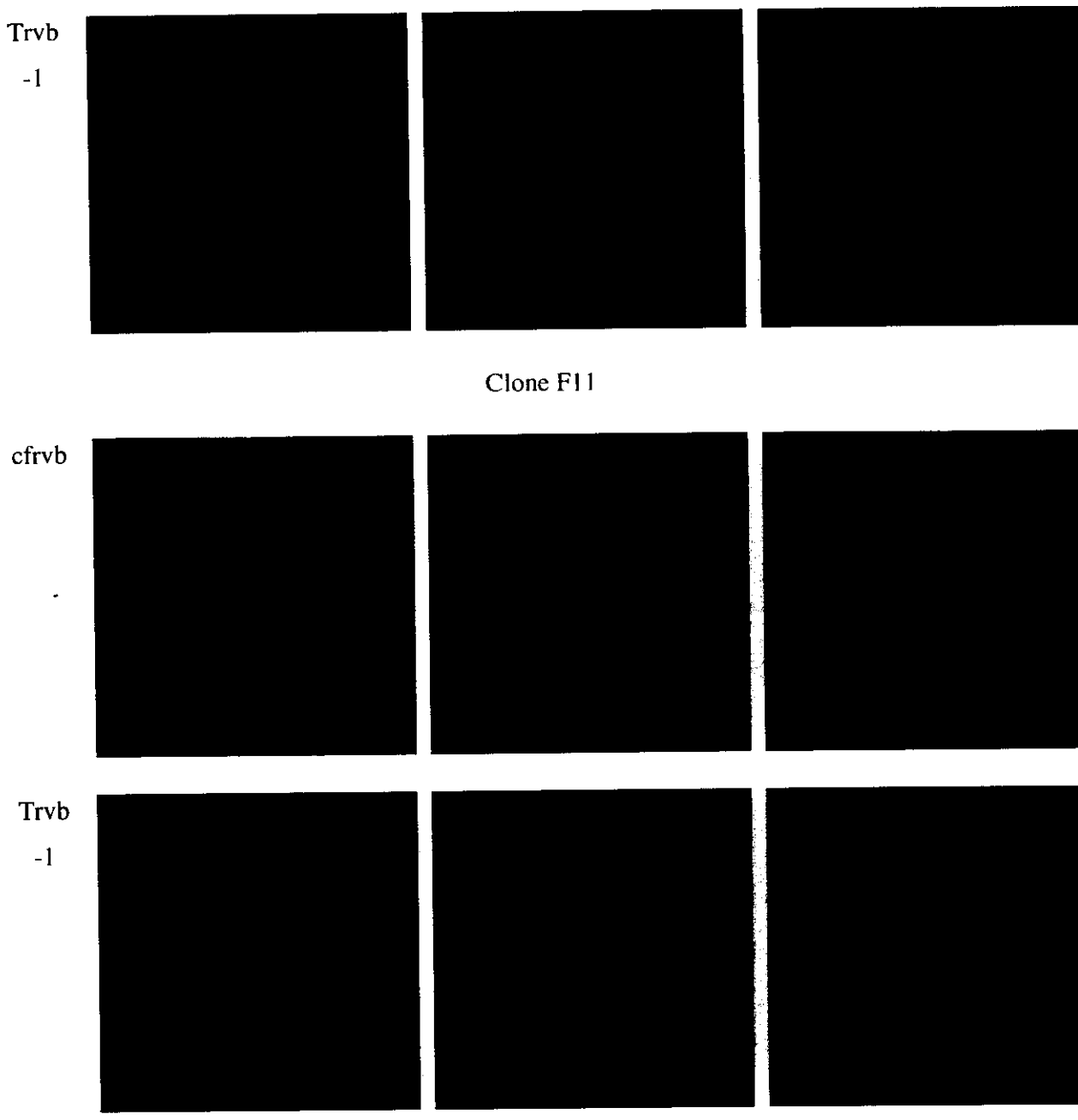
Trvb-1



Potential positives among these clones, B9 and F11, were then chosen and probed using a higher concentration of antibodies. The primary was used at a dilution of 1:150, when the original dilution is 1:50, and the secondary was used at 1:400 dilutions instead of the original 1:200 dilution. 60X color combine images of this high efficiency screening are shown in **Table 5.9.**

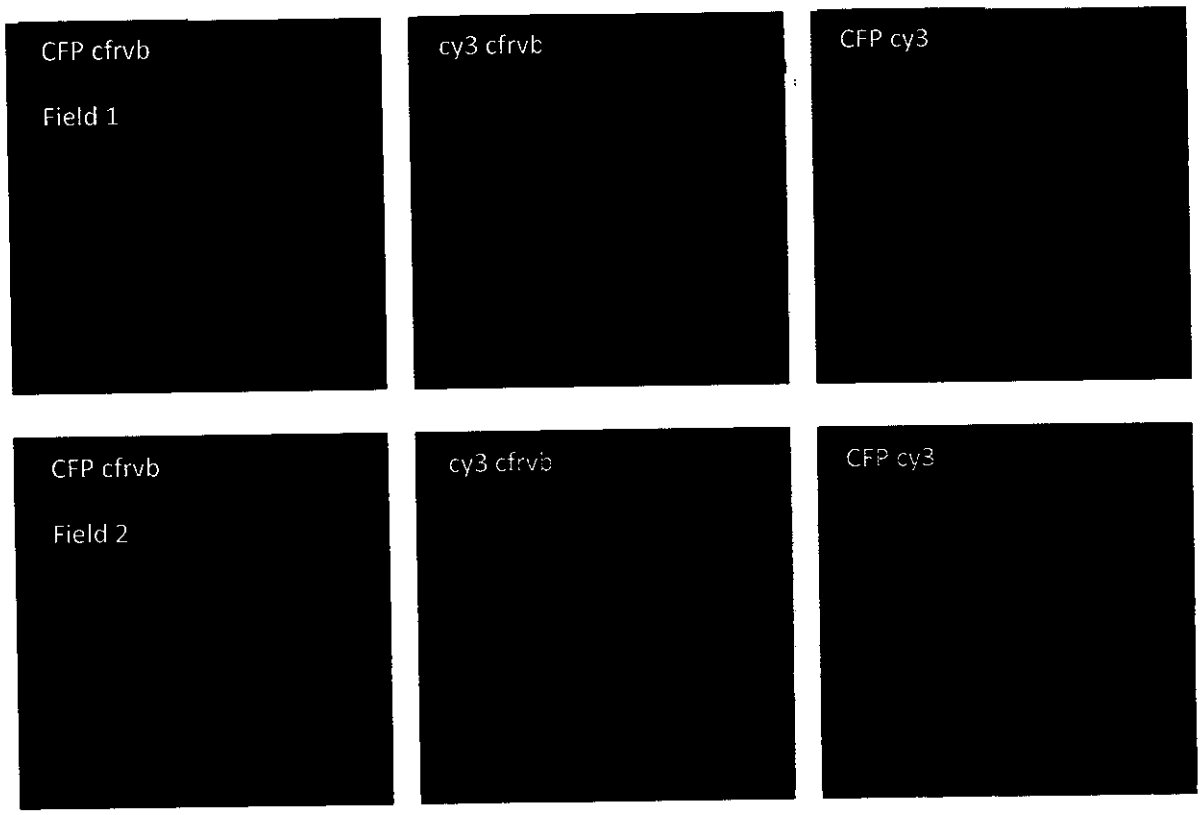
TABLE 5.9: 60X COLOR COMBINE IMAGES OF CLONE F11

	CFP channel	cy3 channel	Combined image
	Clone B9		
cfrvb			



(images are equally scaled)

Clone F11 was confirmed positive after the above rounds of screening. Images obtained from various fields are given in **Figure 5.3**.



(images are equally scaled)

FIGURE 5.3: 60X COLOR COMBINE IMAGES OF POSITIVE CLONE

The clone was to be amplified in order to harvest large amounts of the scFv molecules against CFP-GPI and analyzed in a dot blot. After confirmation of its specificity and antigen binding capacity, these scFv molecules are purified and characterized. The purified scFv can then be analyzed for its binding affinity towards and the number of binding sites per cell. Subsequent internalization and further processes can be confirmed using confocal microscopy (Uebeberg et al, 2010).

5.4 PURIFICATION OF FOLATE RECEPTOR

In view of the complexity and novelty of this cell-based assay, for which standardization and optimization strategies are still underway, it is necessary to establish the credibility of various aspects of the proposal. And hence, it was decided to check the library against the purified form of the protein alongside the selection on cells. This would help in the understanding of our experimental protocols in an ideal case thereby providing insight on the possible outcomes of the project.

The folate receptor was attempted to be purified using a GST pull down assay in which the Folate Binding Protein fused to a GST tag is engineered into a pGEX4T2 vector with an ampicillin resistance gene. The vector expresses the protein constitutively, though induction by IPTG can increase it many folds.

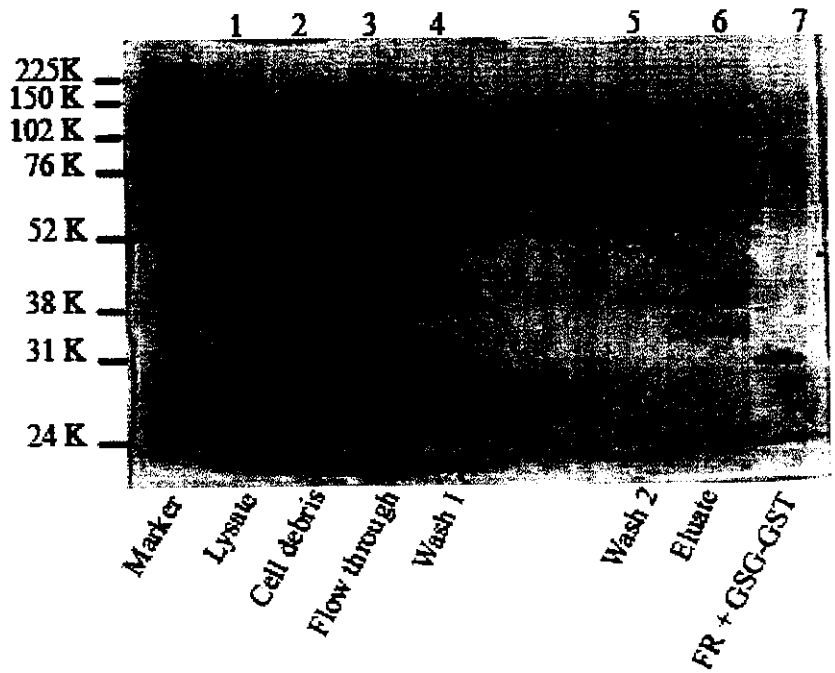


FIGURE 5.4: SDS-PAGE ANALYSIS

The entire protein prep was carried out at 4°C in view of the stability of the protein. Fractions were collected at every significant step for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis (**Figure 5.4**).

The samples in each lane are as follows:

Lane1: marker

Lane2: lysate of sonicate (supernatant)

Lane3: pellet of cellular debris obtained post sonication

Lane4: flow through from GST column, after adsorption of GST-FBP

Lane5: sample of first wash prior to elution

Lane8: sample of second wash prior to elution

Lane9: eluate pooled after 3 elution rounds

Lane10: sample after proteolytic digestion by thrombin

The band corresponding to the protein (~30 K) has not showed up on the gel. Lane9 should ideally show a band at 60 K which, after proteolysis, should have given rise to two fragments- the cleaved FBP and the GST bound to the glutathione (GSH).

The absence of purified protein might be due to inefficient activity of thrombin at the working temperatures that were employed. Temperature and concentration variations are to be made in future as an approach to optimize conditions for maximum protein yield. Sequencing of the plasmid to ensure presence of FBP gene is also to be done. Control experiments following the pattern of un-induced and induced bacteria should also be performed.

CONCLUSIONS

6. CONCLUSIONS

Inferring from the progress of the project work thus far, and bearing in mind the future directions to be followed up in each of the afore mentioned results, this project report can be summarized as follows:

- Importance of phage display library using single chain variable fragments (scFvs) and the various techniques involved in carrying out a full-fledged phage display selection was studied in detail.
- Selection was carried out for two GPI-anchor proteins- Folate Receptor, FR, (6 rounds) and Hedgehog, Hh, (1 round). However, this process is to be repeated with varied parameters as discussed in chapter 5, owing to the poor yield and therefore enrichment in the selection methodology adopted.
- Screening for positive clones containing scFvs specific for the GPI-anchored Cyan Fluorescent Protein was performed with ten clones, chosen randomly, and one of the clones, F11, was tested positive.
- Purification of Folate Binding Protein was attempted using a GST pull down chromatographic technique but did not result in yielding our protein of interest and therefore, optimization and standardization steps in this FBP purification module is underway.

APPENDICES

APPENDICES

APPENDIX 1

CELL AND TISSUE CULTURE MEDIA AND BUFFERS:

Ham F12 media

Added 10.49g of Ham F12 medium and 1.176g of sodium bicarbonate to 1000 ml of autoclaved water, the pH is set at 7.2, filter sterilized and stored at 4°C. Reconstituted media obtained by adding 90 ml of above media to 10 ml of Fetal Bovine Serum (FBS) and 1 ml of Penicillin-Streptomycin-Glutamine (PSG). Selection antibiotic is added to a final concentration of 200 µg/ml and 100 µg/ml of geneticin and hygromycin respectively, depending on the cell line used.

1X Phosphate buffer Saline (PBS)

Mixed the following components in the given composition and made up the volume with distilled water and the pH is set at 7.2. Filter sterilized and stored at room temperature.

NaCl	150mM
Na_2HPO_4	10mM
NaH_2PO_4	10mM

1X Medium 1 (M1)

Mixed the following components in the given composition and made up the volume with distilled water. The pH is set at 7.4, filter sterilized and stored at room temperature.

NaCl	140mM
HEPES	20mM
$CaCl_2.2H_2O$	1mM
$MgCl_2.6H_2O$	1mM
KCl	5mM

1X Trypsin EDTA

Mixed 0.05 grams/liter of Trypsin and 0.02grams/liter EDTA and made up the volume with 1X PBS, filter sterilized and stored at 4°C.

Schneider's Media

Schneider's Drosophila media is reconstituted with 7% FBS and 1 ml/100 ml of PSG.

APPENDIX 2

REAGENTS FOR PHAGE DISPLAY SELECTION:

M9 minimal broth

Added the following salts to 1000 ml of distilled water and autoclaved

Na_2HPO_4	6.3 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g

M9 minimal agar

Added the following salts to 1000 ml of distilled water and autoclaved

Na_2HPO_4	6.3 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g
Agar	15 g

Reconstituted the above solutions with filter sterilised stock solutions of the following components to the given final concentration

$MgSO_4$	1 mM
$CaCl_2$	5 µg/ml
Thiamine	0.1 mM
Glycerol	1 mg/ml

2XTY broth

Mixed 3.1 g of 2XTY media in 100 ml of distilled water and autoclaved.

2XTY agar

Mixed 3.1 g of 2XTY and 1.5 g of agar in 100 ml of distilled water and autoclaved.

APPENDIX 3

Luria Bertani broth (LB)

Added 2.5 g of LB broth to 100 ml of distilled water and autoclaved.

Elution Buffer

0.154 g of reduced glutathione dissolved in 50 ml of 50mM Tris HCl and pH is set at 8.0.

Thrombin

Dissolved 50 cleavage units of lyophilized bovine thrombin in 0.5 ml of 1X PBS pre chilled to 4°C and swirled gently to dissolve the thrombin.

Vector details

Plasmid Name: pGEX-4T2

Plasmid Type: Bacterial

Promoter: tac

Expression Level: High (activate with IPTG)

Plasmid Size: 4970

Sequencing Primer: pGEX5' Sequencing Primer Sequence GGGCTGGCAAGCCACGTTTGGTG

Protein Tags: GST

Bacterial Resistance: Ampicillin

Cleavage: thrombin or factor Xa protease sites to cleave protein from fusion. (AddGene Vector Database)

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