

MOLECULAR DISSECTION OF KIF 15

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JUNE 2006

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Date of submission: 8 May 2006

Date of defence examination: 8 June 2006

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JUNE 2006

KİF 15'İN MOLEKÜLER DİSEKSİYONU

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Tezin Enstitüye Verildiği Tarih : 8 Mayıs 2006
Tezin Savunulduğu Tarih : 8 Haziran 2006

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HAZİRAN 2006

ACKNOWLEDGEMENTS

I am grateful to my supervisor Assoc. Prof. Dr. Arzu Karabay Korkmaz for her invaluable help, guidance, and confidence throughout my study.

I would like to thank Ayşegül Yıldız and Şirin Korulu for their all kind of support during this long process. I would also like to thank to all CYTO members for their morale support.

I especially thank to Güney Akbalık for her invaluable help during my hard times.

I would like to express my sincere thanks to all my friends in MOBGAM for their endless help during my study and for their warm friendship.

I wish to express my appreciation to Assoc. Prof. Gianluca Gallo, Prof. Dr. Peter Baas and Dr.Robert Loudon, for having shared their experiences with me during my study in USA, and I also want to thank to all Gallo's Lab. members for their wonderful friendship that made me feel like home.

I would also like to thank Murat Şenol for his endless patience, sensibility and encouragement.

Finally, I am especially grateful to my family, without their endless encouragement, support and love it would not be possible to finish this thesis.

May, 2006

Ayşegül DİLSİZOĞLU

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ABBREVIATIONS

AAA	: ATPases Associated With Various Cellular Activities
ADP	: Adenosine Diphosphate
ATP	: Adenosine Threephosphate
C_c	: Critical Concentration
cDNA	: Complementary Deoxyribonucleic Acid.
DNA	: Deoxyribonucleic Acid
DNAase	: Deoxyribonuclease
DEPC	: Diethyl Pyrocarbonate
EDTA	: Ethylenediaminetetraacetic Acid
ELC	: Essential Light Chains
F-Actin	: Filamentous Actin
G-Actin	: Globular Actin
GDP	: Guanosinediphosphate
GFP	: Green Fluorescent Protein
GTP	: Guanosinethreephosphate
IPTG	: Isopropyl-[Beta]-D-Thiogalactopyranoside
KHC	: Kinesin Heavy Chain
KIF	: Kinesin-Like Protein
KLC	: Kinesin Light Chain
LB	: Luria Broth
MAPs	: Microtubule-Associated Proteins
mRNA	: Messenger-Ribonucleic Acid
MTOC	: Microtubule Organizing Center
MTs	: Microtubules
MARK	: Microtubule-Affinity-Regulating-Kinase
MCS	: Multiple Cloning Site
NF	: Neurofilament
O/N	: Over Night
PEG	: Polyethylene Glycol
RT	: Room Temperature
RLC	: Regulatory Light Chains
RT-PCR	: Reverse Transcription Polymerase Chain Reaction
RNA	: Ribonucleic Acid
rRNA	: Ribosomal Ribonucleic Acid
RNAase	: Ribonuclease
TAE	: Tris-Acetate-EDTA
γ-TuRC	: γ-Tubulin Ring Complex

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LIST OF SYMBOLS

bp	: Base pair
kb	: Kilobase
kDa	: Kilodalton
MOPS	: 3-(N-morpholino)propanesulfonic acid
PIPES	: 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid
T _m	: Melting Temperature
Tris	: Hydroxymethyl aminomethane
UV	: Ultraviolet
X-Gal	: 5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside

MOLECULAR DISSECTION OF KIF15

SUMMARY

Kif15, member of kinesin motor protein family, opposes to the forces generated by other motors that move microtubules (MTs) to maintain the separation of spindle poles in mitotic cells. Kif15 motor domain interacts with MTs, while its tail domain interacts with other MTs or actin filaments, depending on the phase of mitosis.

Kif15 is also found in neurons, but there its function is still a mystery. Many neurons are born far from their ultimate destinations and have to migrate to their target places. When neurons arrive to their target places, the forces generated by other motors that affect migration, have to be neutralized. It is thought that Kif15 crosslinks MTs and microfilaments (MFs), and as a result they oppose the capacity of other motors to generate independent MT movements.

The aim of the project is to specifically identify the domains of Kif15 that interact with the above mentioned cytoskeletal elements, and ultimately to understand the molecular mechanisms of neuronal migration and neuronal diseases like Lissencephaly caused by abnormalities during migration.

For this reason, an adult rat was dissected and whole brain was obtained. RNA isolation was performed from the whole brain. cDNA was synthesized by using the total RNA as a template. Then, functional domains (motor, myosin interacting and tail domains) of Kif 15 was amplified by using sequence specific primers. Sequences of the clones were verified and then, these domains were subcloned into fluorescent expression vectors in order to be used in cell culture studies.

Ongoing experiments include transfection of HeLa and primary neurons by GFP constructs, immunostaining and fluorescent microscopy studies.

KIF15'İN MOLEKÜLER DİSEKSİYONU

ÖZET

Kinezin motor protein ailesinin bir üyesi olan Kif15, mitotik hücrelerde, diğer motor proteinler tarafından yaratılan kuvvetlere karşı koyarak mitoz mekiğinin stabilitesini sağlar. Kif15'in motor bölgesi mikrotübüllerle etkileşirken, kuyruk kısmı -mitozun evresine bağlı olarak- mikrotübüller veya aktin filamentleriyle etkileşir.

Kif15 nöronlarda da bulunur ama buradaki fonksiyonu halen bilinmemektedir. Organizmanın gelişimi sırasında merkezi sinir sisteminden menşe alan nöronların görevlerini yerine getirebilmeleri için hedef yerlerine ulaşmaları, yani göç etmeleri gerekmektedir. Nöronlar hedef yerlerine geldiklerinde, nöronal göçte etkili diğer motor proteinlerinin yarattığı kuvvetler nötralize edilmelidir. Kif15'in de motor kısmıyla mikrotübüllere bağlanıp, kuyruk kısmıyla da diğer mikrotübüller ve/veya aktin filamentleriyle etkileşerek bu ağın stabilizasyonunu sağladığı ve böylece nöronal göçün durdurulmasında önemli rol oynadığı düşünülmektedir.

Bu projenin amacı, Kif 15'in özellikle yukarıda bahsedilen sitoskelet elemanlarıyla etkileşen bölgelerini ayrı ayrı tanımlamak, ve sonunda nöronal göçün ve bu sırada meydana gelen anormallikler sonrası oluşan Lissencephaly gibi nöronal hastalıkların moleküler mekanizmasını anlamaktır.

Bu amaçla ergin bir sıçan disekte edildi ve beyin bölgesi alındı. Bu beyin kullanılarak total RNA izolasyonu yapıldı. Elde edilen RNA'dan cDNA sentezlendi ve diziye özel primerler kullanılarak Kif15'in özelleşmiş fonksiyonu olan bölgeleri (motor, myosinle etkileştiği düşünülen bölge ve kuyruk bölgeleri) klonlandı. Klonlama vektöründeki alt bölgeler dizileri doğrulandıktan sonra, hücre kültürü çalışmalarında kullanılmak üzere floresan ekspresyon vektörlerine aktarıldı.

Şu an sürdürülmekte olan deneyler, HeLa ve primer nöron hücrelerinin bu vektörlerle transfeksiyonunu, immunositokimyasal boyama ve floresan mikroskobu çalışmalarını içermektedir.

1. INTRODUCTION

1.1. Cytoskeleton

Cells have to organize themselves and interact with their environment. The ability of cells to adopt a variety of shapes and to carry out coordinated and directed movements depends on the cytoskeleton, a complex network of protein filaments that extend throughout the cytoplasm [1,2].

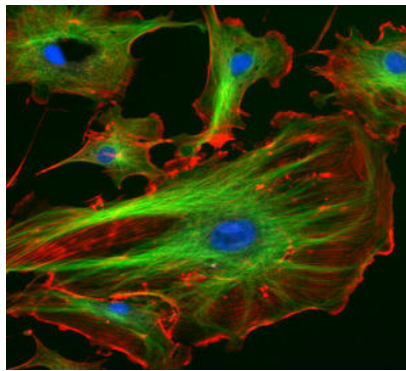


Figure 1.1: A eukaryotic cell stained for visualizing its cytoskeleton. Blue color indicates the nucleus, red color indicates the actin filaments and green color indicates the microtubules.

The cytoskeleton pulls chromosomes apart in mitosis and meiosis, splits the dividing cell into two, guides the intracellular traffic of organelles, supports the fragile plasma membrane and provides mechanical strength. In addition, it enables some cells swim or crawl across surfaces and it is responsible for contraction in muscle cells and also it provides the machinery needed for extension of axon and dendrites in neurons [1-3].

These diverse activities of the cytoskeleton depend on three principal types of protein filaments: actin filaments, microtubules and intermediate filaments. Each type of filament is formed from a different protein monomer and can be built into a variety of structures according to its associated proteins. Some of the associated proteins link filaments to one another or to other cell components, such as the plasma membrane.

In addition to the cell shape and movements, the cytoskeleton controls, directly or via regulatory proteins, several cellular functions, comprising the decision of a cell to enter the cell cycle or the apoptotic death pathway [1,3,4].

1.1.1. Microfilaments (Actin Filaments)

The actin cytoskeleton is a dynamic structure that participates in cellular functions including the maintenance of cell polarity and morphology, intracellular trafficking of organelles, cell motility, and cell division [5,7].

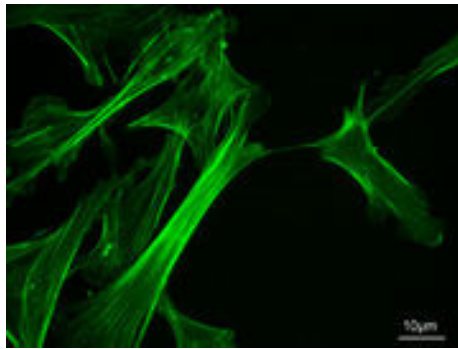


Figure 1.2: Actin filaments stained with green fluorescent proteins.

Actin subunit is a single globular polypeptide chain termed “G-actin” which has an ATP binding site at the centre of the molecule. ATP is hydrolyzed immediately after the molecule is incorporated into an actin filament. The ADP is trapped in the actin filament until it depolymerizes. Then an exchange can occur. This monomer dimerizes or forms a trimer and serves as nucleation site for further growth of the actin protofilament. As a result, the actin subunits assemble head-to-tail to generate filaments with a distinct structural polarity termed “F-actin” [1, 6, 8].

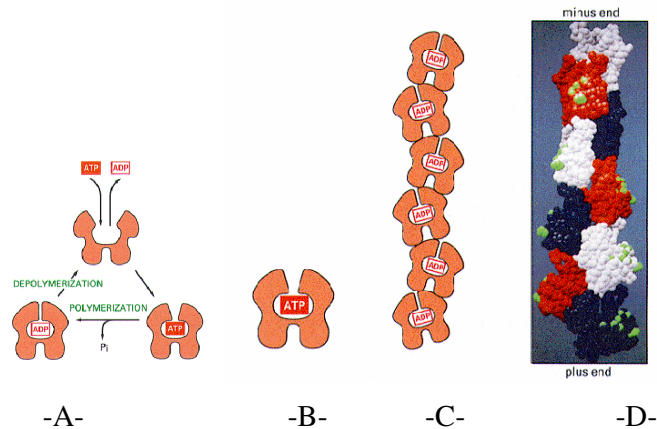


Figure 1.3: A) shows actin polymerization and depolymerization B) shows globular actin, G-actin C) shows filamentous actin, F-actin D) shows the 3D structure of F-actin

G-actin forms F-actin (the filament) in the presence of ATP, Mg^{+2} and K^{+} . The concentration of G-actin is also critical. Above the critical concentration of G-actin, the molecules will polymerize. Below the critical concentration, the actin filaments will depolymerize. ATP hydrolysis is not required for polymerization, but it is required to promote depolymerization (when it is converted to ADP). Growth and polymerization are more rapid at the plus end.

Actin filaments consist of two parallel protofilaments that twist around each other in a right-handed helix. In living cells they are cross-linked and bundled together via different accessory proteins [1, 2, 6].

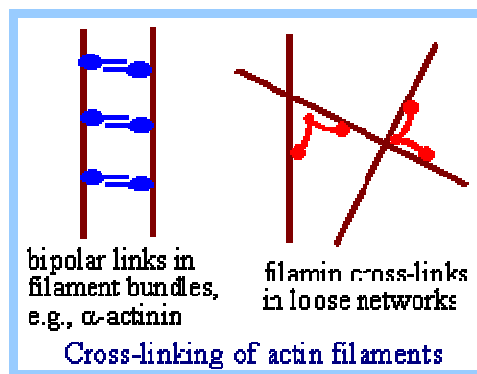


Figure 1.4: Cross-linking of actin filaments via different accessory proteins.

1.1.2. Intermediate Filaments

Although all eukaryotic cells contain microtubules and microfilaments, intermediate filaments are found in only some of them. These filaments have mechanical properties that are especially useful to animals that have soft-bodies and no exoskeleton, such as nematodes and vertebrates. Intermediate filaments are particularly prominent in the cytoplasm of the cells that are subjected to mechanical stress. Thus their major function is thought to be to impart physical strength to cells and tissues.

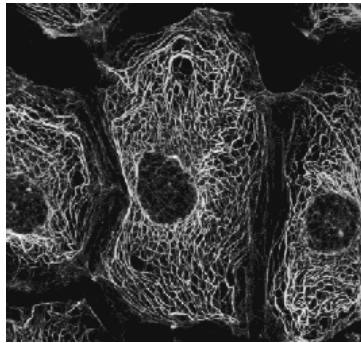


Figure 1.5: The intermediate filaments of keratin in epithelial cells stained with a fluorescent stain.

Intermediate filament proteins form parallel homo or heterodimers with a highly conserved coiled-coil core domain and nonhelical tails and heads. A tetramer is formed by antiparallel, staggered side-by-side aggregation of two identical dimers. Since tetrameric subunit is made up of two dimers pointing in opposite direction, its two ends are the same. Thus, the assembled intermediate filament lacks the overall structural polarity that is critical for both microtubule and microfilaments. Tetramers aggregate end to end, forming a protofilament; pairs of protofilaments then laterally associate into a protofibril. Lateral association of four protofibrils form a cylindrical intermediate filament in 10 nm thickness [1,9].

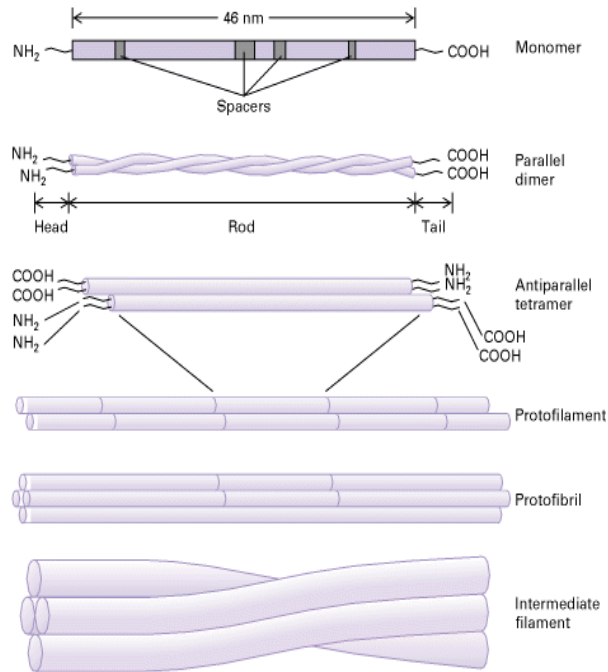


Figure 1.6: Levels of organization and assembly of intermediate filaments

There are several types of intermediate filaments, each constructed from one or more proteins characteristic for it. For example, keratins are found in epithelial cells and also form hair and nails, nuclear lamins form a meshwork that stabilizes the inner membrane of the nuclear envelope, neurofilaments strengthen the long axons of neurons, vimentins provide mechanical strength to muscle (and other) cells [11].

Although for many years they were considered as stable cytoskeletal elements, many researches are being made to show their dynamic and motile properties[10,11].

1.1.3. Microtubules

Microtubules are filamentous intracellular structures that are responsible for various kinds of movements in all eukaryotic cells. They are involved in nucleic and cell division, organization of intracellular structure, and intracellular transport, as well as ciliary and flagellar motility [1,2].

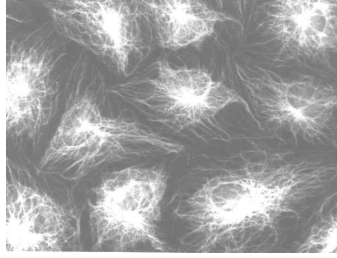


Figure 1.7: This micrograph shows cells in culture immunolabeled with fluorescein-labeled antibodies for tubulin

Microtubules are made up of α,β -tubulin heterodimers. This heterodimer does not come apart, once formed. The α and β tubulins which are about 55 kDa MW are homologous, but not identical. Each has a nucleotide binding site. α -tubulin has a bound molecule of GTP, that does not hydrolyze. β -tubulin may have bound GTP or GDP. Under certain conditions, β -tubulin can hydrolyze its bound GTP to GDP plus Pi, release Pi, and exchange the GDP for GTP [12, 14].

γ -Tubulin, which is homologous to α and β tubulins, nucleates microtubule assembly within the centrosome. Several (12-14) copies of γ -tubulin associate in a complex with other proteins called "grips" (gamma ring proteins) [1].

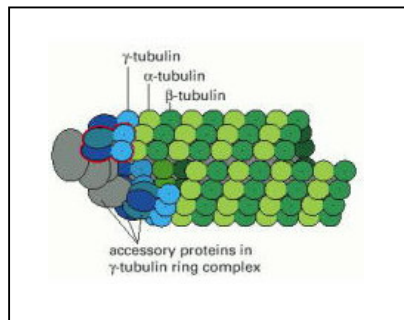


Figure 1.8: γ -Tubulin ring complex

When intracellular conditions favor assembly, tubulin heterodimers assemble into linear protofilaments. Staggered assembly of 13 protofilaments yields a helical arrangement of tubulin heterodimers in the cylinder wall (24 nm in diameter).

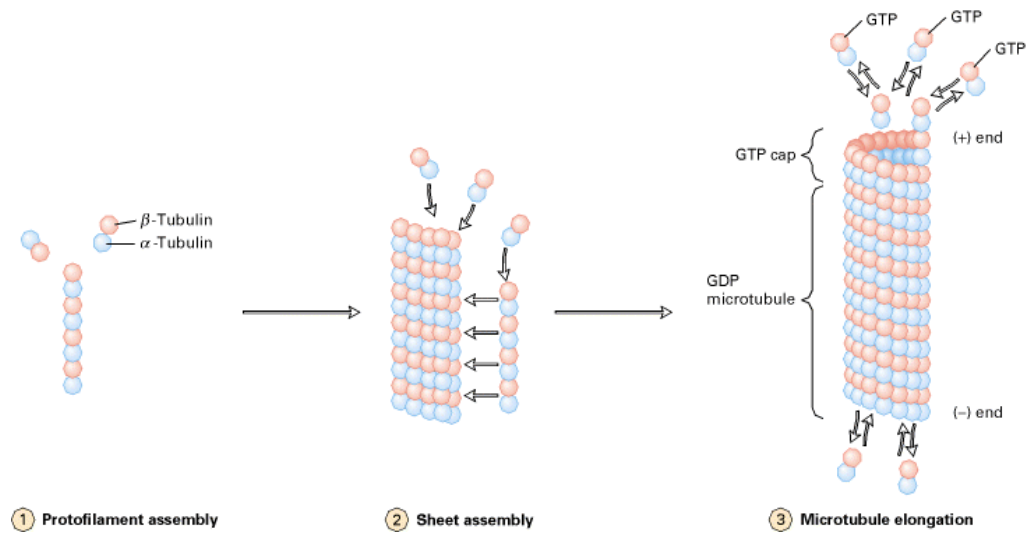


Figure 1.9: Assembly of microtubules

Microtubules are polar structures. Their minus end grows slower compared to the plus end, and the minus end is terminated by α tubulin subunits, while the plus end is terminated by β subunits. In addition, microtubules may vary in their rate of assembly and disassembly, and show dynamic instability [1,2].

If the free tubulin concentration in solution is between the critical values (the critical concentration (C_c) is the concentration of dimeric $\alpha\beta$ -tubulin in equilibrium with microtubules.), a single microtubule end may undergo transitions between a growing state and a shrinking state [1]. A growing microtubule has GTP cap which nucleates growing. If nucleotide hydrolysis occurs more rapidly than subunit addition, this cap is lost and microtubule starts to shrink. This event is called “catastrophe”. Meanwhile, GTP containing subunits can still be added to the shrinking end, and form a new cap, then microtubule growth resumes. This stage is called “rescue” [2,13,15].

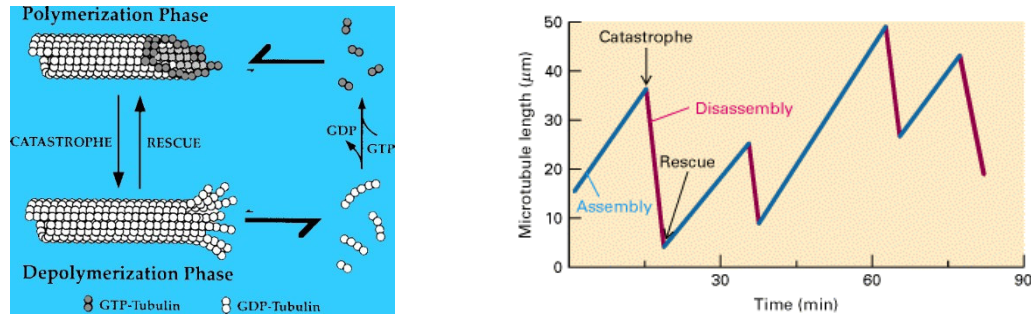


Figure 1.10: Shows polymerization and depolymerization of microtubules, and microtubule length changing via time intervals. (McEwen Lab.)

Assembly and disassembly each proceeds at uniform rates, but there is a large difference between the rate of assembly and that of disassembly (see fig.1.10). During periods of growth, the microtubule elongates at a rate of $1\mu\text{m}/\text{min}$. Abrupt transitions to the shrinkage stage (catastrophe) and to the elongation stage (rescue) are observed. The microtubule shortens much more rapidly ($7\mu\text{m}/\text{min}$) than it elongates [1,16].

In mitotic cells, microtubules are nucleated at the centrosome at their minus ends by microtubule organizing centre (MTOC), so the plus ends point outward and grow toward the cell periphery [17].

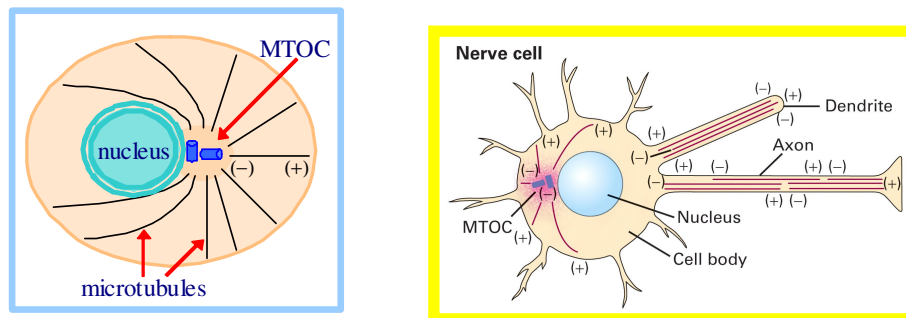


Figure 1.11: Microtubule organization in mitotic and neuronal cells

In neuronal cells, microtubules are found in cell body just like in their orientation in mitotic cells, but in axon and dendrites it lays without an MTOC. In axons their minus end points cell body and their plus end points outward. In dendrites, the opposite orientation is also possible [2,17].

1.2. Accessory Proteins of Cytoskeleton

Once a cytoskeletal filament is formed by nucleation and elongation from the subunit pool, its stability and mechanical properties are often altered by a set of proteins that bind along the sides of the polymer. Different filament-associated proteins use their binding energy to either lower or raise the free energy of the polymer state, and they thereby either stabilize or destabilize the polymer, respectively [1,18].

1.2.1. Microtubule Associated Proteins (MAPs)

Microtubule-associated proteins interact with the microtubules of the cellular cytoskeleton. MAPs bind to the tubulin subunits that make up microtubules to regulate their stability [19].

The numerous identified MAPs are classified into two categories: Type I (including MAP1 proteins) and type II (including MAP2, MAP4 and tau proteins) [1]. The MAPs which have been identified in many different cell types, have a wide range of function. These include both stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in the cell [2].

Within the cell, MAPs bind directly to the tubulin monomers of microtubules. This binding can occur with either polymerized or depolymerized tubulin, and in most cases, this leads to the stabilization of microtubule structure, further encouraging polymerization [1,20]. Usually, it is the C-terminal domain of the MAP that interacts with tubulin, while the N-terminal domain can bind with cellular vesicles, intermediate filaments or other microtubules. MAP-microtubule binding is regulated through MAP phosphorylation. This is accomplished through the function of the microtubule-affinity-regulating-kinase (MARK) protein.

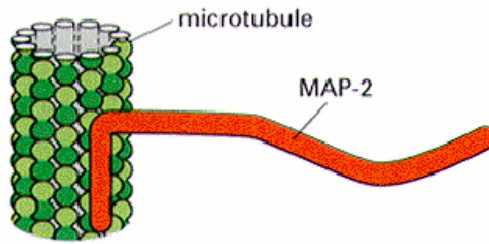


Figure 1.12 : MAP-2, a microtubule associated protein

Phosphorylation of the MAP by the MARK causes the MAP to detach from any bound microtubules. This detachment is usually associated with the destabilization of the microtubule causing it to fall apart. In this way, the stabilization of microtubules by MAPs is regulated within the cell through phosphorylation [21].

1.2.2. Molecular Motors

Molecular motors or motor proteins are divided into 3 groups: Myosins, dyneins and kinesins. Myosins are actin-based motor proteins, kinesins and dyneins are microtubule-based motor proteins. Motor proteins are able to recognize the microtubule polarity. Therefore, organization of the rails combined with the specific motor determines the direction of transport [23].

They all have a globular motor domain which interacts with microtubules and microfilaments. They move along these polarized cytoskeletal “highways” by using the mechanical energy converted from ATP hydrolysis and they carry their ‘cargoes’ or bind to other cytoskeletal elements via their tail domains [22,27].

1.2.2.1. Myosins

The myosins are large family of motor proteins that move along actin filaments by using ATP hydrolysis. The first identified motor protein was a skeletal muscle myosin (conventional myosin or myosin-II) which is responsible for generating the force for a muscle contraction. Since then, there has been extensive characterization of these myosins and much is known about their function [24].

Myosins are composed of two light and two heavy chains. Each of the heavy chains has a mostly conserved globular head domain at its N-terminus that contains the

force generating machinery. Each myosin head binds and hydrolyses ATP. They use the revealed energy in order to walk towards the positive end of the actin filaments. (Myosin VI is an exception since it moves towards the minus end). Head domain is followed by a long amino acid sequence that forms an extended coiled coil that mediates heavy chain dimerization. There are two types of light chains: essential light chains (ELC) and regulatory light chains (RLC). The overall activity and polymerization can be modulated by phosphorylation on the light chains and the C-terminus of the heavy chains respectively. C-terminus shows diversity among different myosin families [25].

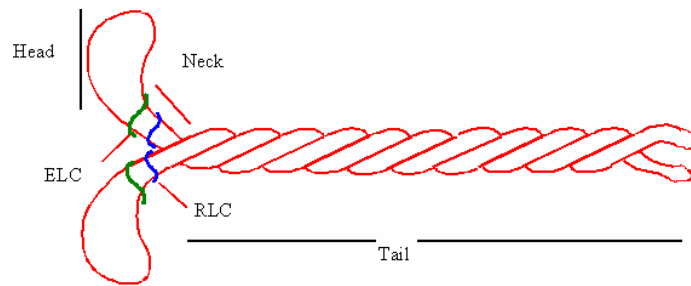


Figure 1.13: Structure of myosin II.

It was initially thought that myosins were only found in muscles, but then many others were discovered in different cell types. This reveals that myosins are not only responsible for generating force for contraction, they also have some other functions. For example myosin II is required for not only for contraction, but also for cytokinesis and forward translocation of the body of a cell during cell migration, myosin I generally involves in intracellular organization and myosin V is involved in vesicle and organelle transport [1,26].

1.2.2.2. Dyneins

Dynein is a minus-end-directed microtubule motor that has diverse cellular processes. Dyneins contain one to three heavy chains. Each heavy chain consists of a C-terminal globular head together with two elongated flexible structures called the stalk and the N-terminal tail (the cargo-binding domain). Dyneins are categorized into two major classes, axonemal and cytoplasmic [28].

The axonemal dynein produces the bending motions that propagate along cilia and flagella. They are localized in the outer doublet microtubules in the axoneme and

form projections called dynein arms, each of which is composed of multiple dynein molecules [1].

Cytoplasmic dynein is an AAA (ATPases associated with various cellular activities) type molecular motor. Cytoplasmic dynein and its activator dynactin are essential in higher eukaryotes [27]. It moves along a single microtubule in multiple steps which are accompanied with multiple ATP hydrolysis [27,28]. This processive sliding is crucial for many fundamental cellular processes, including nuclear migration, organization of the mitotic spindle, chromosome separation during mitosis, and the positioning and function of many intracellular organelles. Especially in neurons, they carry out many crucial processes like; retrograde axonal transport, neurotrophic factor signaling, neurofilament transport, mRNA localization, neuronal migration, and protein recycling and degradation. Mutations in either dynein or dynactin lead to motor neuron degeneration and its loss [28].

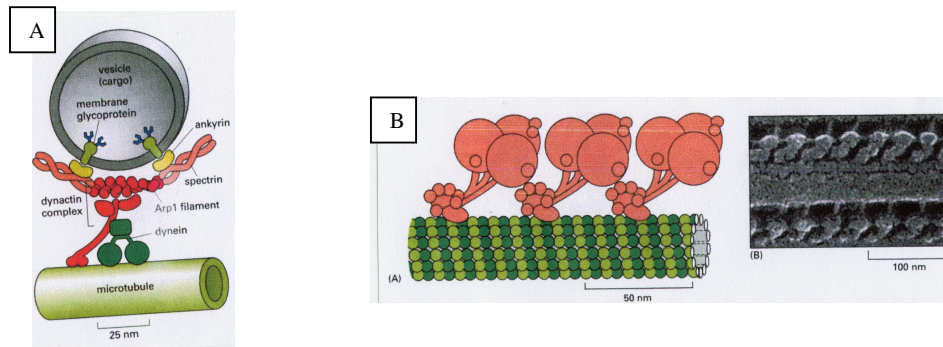


Figure 1.14: A) Structure of a cytoplasmic dynein and dynactin complex B) Dynein movement on microtubules

1.2.2.3. Kinesins

Kinesins are a large family of positive and negative end directed motor proteins present in all eukaryotic phyla [28]. The kinesin superfamily proteins (KIFs) are microtubule based proteins that have been shown to have a wide range of functions, including the transport of different cargoes (vesicles, organelles, protein complexes, chromosomes), and the regulation of microtubule dynamics in an ATP-dependent manner in both mitotic and neuronal cells[33]. In addition, they participate in polarized transport in epithelial cells and KIFs also play significant roles in neuronal development [29,30].

Kinesins are mainly made up of 2 heavy chains (KHC) and 2 light chains (KLC). Two identical polypeptides (KHC) dimerize to form a coiled-coil stalk, with a cargo binding tail at one end and twin globular heads at the other [41]. Each head is a catalytically active ATPase that attaches to microtubules with nucleotide-dependent affinity. Short (15 amino acid) polypeptide segments, called neck-linkers join each head to the stalk [31].

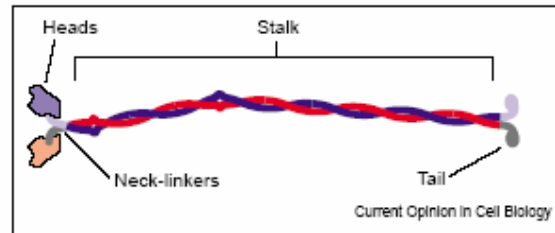


Figure 1.15: Structure of kinesin [33]

Kinesins use microtubules and microfilaments as railways. They bind to these cytoskeletal elements by their motor domains. They can also interact with many different cargoes and other cytoskeletal elements [36]. It has been shown that, kinesins can bind to a cargo by their tail domain directly, or this interaction can be mediated via adaptor/scaffolding proteins. In addition they can bind to other cytoskeletal elements [22].

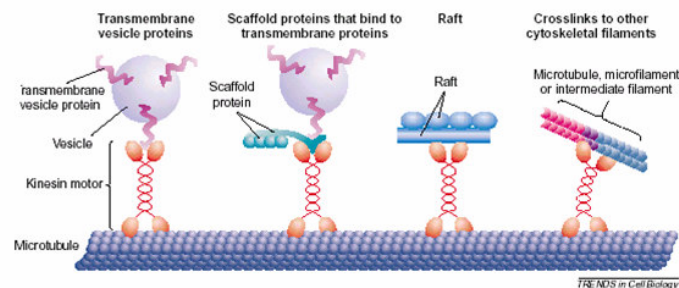


Figure 1.16: Different interaction ways of kinesins with different cargoes

There are 3 major types of kinesin superfamily proteins which have been identified according to the position of the motor domain: NH_2 -terminal motor domain type (N-type), middle motor domain type (M-type), and COOH -terminal motor domain type (C-type) [32, 37].

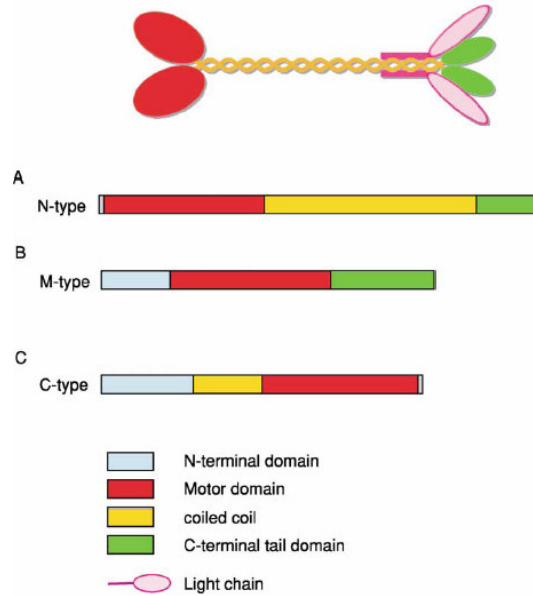


Figure 1.17: Domain structures of typical members of the kinesin superfamily. A) N-type kinesin Bar diagram of the kinesin heavy chain B) M-type kinesin C) C-type kinesin

Kinesins walk on microtubules or microfilaments by ATP hydrolysis. ATP binding to the leading head triggers docking of the neck linker onto the catalytic core and initiates movement towards the plus end. The trailing head is pulled forward, releasing P_i . Thus leading and trailing heads change their positions. Upon binding to the microtubule, the leading head releases its ADP, resulting in a conformational change that results in the hydrolysis of ATP by the trailing head. Zippering of the neck linker is critical to this process, with the linker docked to the catalytic core only when the core has bound ATP or ADP- P_i [34,38,39].

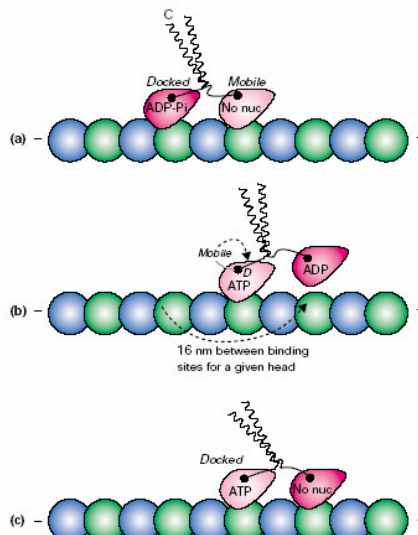


Figure 1.18: A schematic model illustrating processive movement by the plus-end directed motor kinesin. The two kinesin heads are indicated with light pink and dark pink and the α - and β -tubulin subunits from a single microtubule protofilament are indicated in blue and green [34].

Kinesins are not always active in cells. They only become active when they are needed [41]. The mechanism by which this could occur is still unclear, but there are many hypothesis about this situation. One possible mechanism is that kinesin becomes active when a cargo binds to a tail domain. This releases the self-inhibition and allows motor-cargo complex to bind to and move on microtubules(Fig.19-a). On the other hand, in other models it is thought that cargo binding might not lead a direct activation. Additional changes might be needed such as phosphorylation of kinesin (Fig 19-b), pH changes (Fig 19-c) or any other cellular factors (Fig 19-d) can trigger this activation [36, 45].

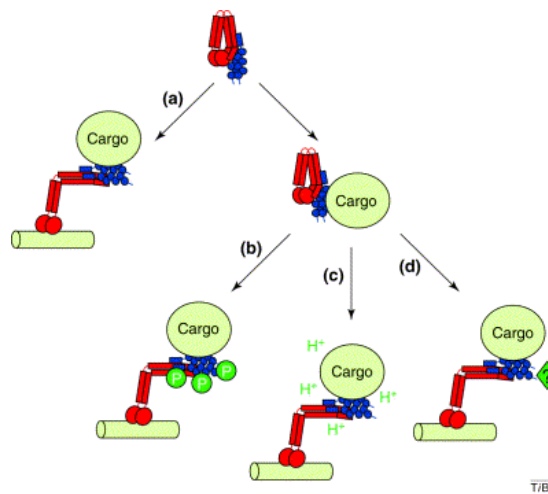


Figure 1.19: Possible ways of kinesin activation [45]

In addition how kinesins choose their specific cargoes and how can they leave them to the right place is still a mystery [42].

There are several ways by which motor proteins may be regulated: regulation of affinity to the cargo, to the cytoskeleton, or regulation of protein levels in the cell. Often more than one level of regulation is imposed on a single motor protein [43].

Kinesin light and heavy chains can be regulated by phosphorylation, changing affinity for the kinesin receptor, the microtubule, or altering the efficient hydrolysis of ATP. Accessory factor binding to the complex can also be modulated, which in turn can affect kinesin activity[40, 44]. The affinity of kinectin for kinesin can also be regulated. Finally, the binding of microtubule-associated proteins to the microtubule can be regulated by phosphorylation, which alters the ability of kinesin to travel along the microtubule path [52].

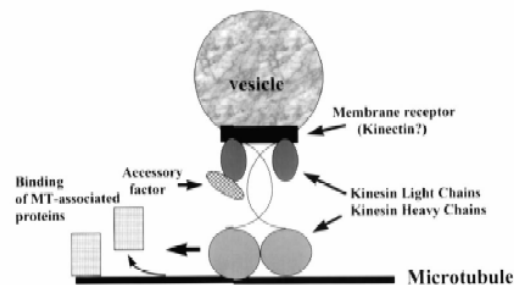


Figure 1.20: Possible targets for regulation of kinesin dynamics [40].

1.2.2.4. KIF 15

Kif15 is a member of N-type kinesin super family. Hence, it has all the general properties of these kinesins. In addition, since it has some crucial and intriguing roles in both mitotic and neuronal cells, it has become popular recently.

Kif 15 is mainly made up of 2 heavy and 2 light chains like all other kinesins. Heavy chain is made up of a catalytic motor domain, neck linker, neck, stalk (coilI and coil II) domains, and a tail domain [45].

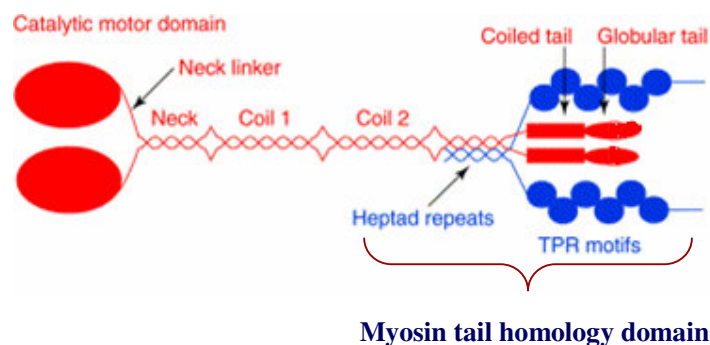


Figure 1.21: Functional domains of Kif15

In mitotic cells, Kif 15 interacts with microtubules and microfilaments according to the phase of mitosis. In interphase, it interacts with actin filaments (microfilaments), but not microtubules but during mitosis, from prophase to early anaphase it interacts with microtubules. Finally, Kif 15 enrichment has been observed in cleavage furrow during cytokinesis with actin filaments.

Since its homologues crosslink microtubules by binding on them with their motor domain and interact with other microtubules with their tail domain, it was shown that Kif15 act just like its homologues in mitotic cells [46].

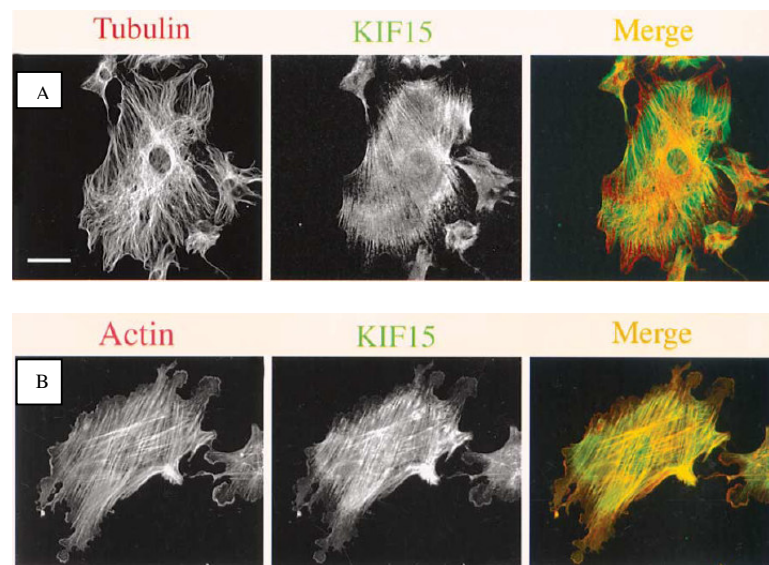


Figure 1.22: A) No co-localization of Kif 15 with microtubules in interphase B)Co-localization of microtubules with actin filaments in interphase

Note: Red indicates the tubulin and actin, respectively, green indicates Kif15 yellow indicates the co-localization.

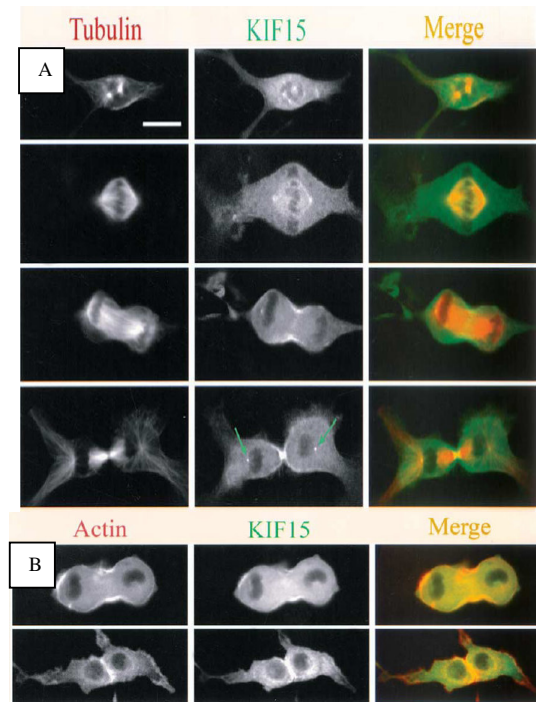


Figure 1.23: A) Kif 15 co-localization with microtubules from metaphase to late anaphase B)Kif 15 co-localization with actin filaments during cytokinesis

Kif 15 is also actively expressed in postmitotic neurons and it has been shown that Kif 15 enrichment was high in critical points where microtubules form bundles, like dendrites or stalled growth cones [46].

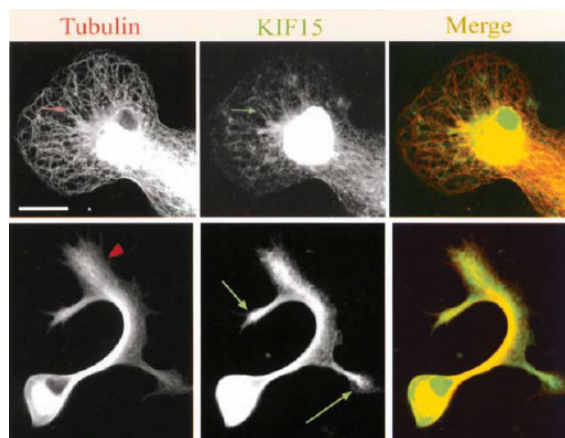


Figure 1.24: Kif15 enrichment in knots of microtubules in neuronal cells

This results revealed the possibility that Kif15 could be an important molecule for neuronal migration.

Cell migration occurs widely at all stages of embryonic development. Migration of neurons during mammalian brain development is essential for the proper functioning of the central nervous system [47].

One of the remarkable features of the mammalian brain is the laminar structure of its neurons. The adult cerebral and cerebellar cortex consist of six and three neuronal layers, respectively. This intricate laminar construction is achieved by the highly ordered migration of postmitotic neurons, because neurons are usually generated in sites different from where they eventually reside, they migrate and find their resting places. Then, they become mature here and send out their axons and dendrites.

Neurons migrate following three steps and they are ordered into architectonic patterns at the end of migration. These three steps are:

- 1- Leading edge extension: proceeds at the extremity of the axon and directed by microfilaments.
- 2- Nucleokinesis (nuclear translocation): is a process dependent on the microtubule network. Nucleus is carried in a unison with the cell.
- 3- Retraction of the trailing process [47,49]

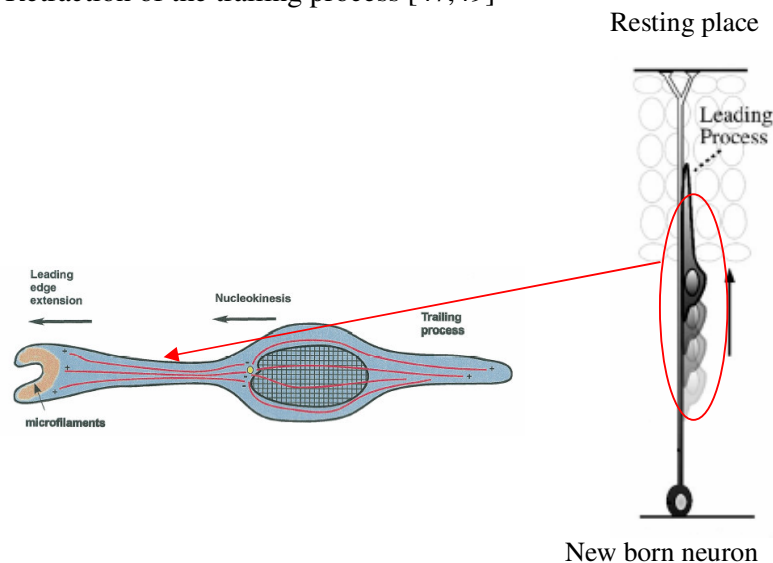


Figure 1.25: Neuronal migration steps

If the migrating neurons can not achieve to find their final destinations or if they can not stop where they have to stay, abnormal neuronal development occurs and different kinds of neurodegenerative diseases are observed [50].

As a result, Kif 15 is thought to crosslink microtubules by interacting with its both motor and tail domain as it does in mitotic cells and it could play an important role in ending the neuronal migration by using this feature.

1.3. Aim of the study

Motor proteins (kinesins, dyneins and myosins) are responsible for cytoskeleton-based transport and motor function, which have impact on all dynamic aspects of cell behavior including cell division, the generation and maintenance of cell polarity, cell signalling, and cell motility. There is an advanced balance between these forces in organizing the microtubule and microfilament dynamics. Thus, understanding the regulatory mechanisms that control motor function is important for attempts to define the basic cellular processes failing in numerous disease states including cancer, birth defects, deafness, respiratory disorders and neurodegenerative diseases.

Kinesins are positive and negative end directed motor proteins found in all eukaryotic organisms which undertake crucial roles in both mitotic and neuronal cells. They were once believed to exclusively transport membrane organelles, besides the force generation function. However, it is now known that they can also move many different cargoes such as cytosolic components, protein complexes, nucleic acids etc.

Kinesins structurally consist of two functional parts: a motor domain that reversibly binds to the cytoskeleton and converts chemical energy into motion by ATP hydrolysis, and a tail domain that interacts with cargo directly or through an adaptor.

Kif 15 is a member of kinesin motor family whose homologues are believed to crosslink and immobilize spindle microtubules. This is achieved by crosslinking neighboring microtubules and by exerting forces that oppose the compressive forces that would otherwise collapse the bipolar spindle to a monastral array.

Kif 15 interacts with different cytoskeletal elements based on the phase of the mitosis. For example, it localizes to spindle poles and microtubules from prometaphase to early anaphase but then to the actin-based cleavage furrow during

cytokinesis. Moreover, Kif15 is localized to actin bundles in interphase. This switch mechanism between these cytoskeletal elements is still a mystery.

On the other hand, Kif 15 is not only found in actively dividing mitotic cells, but also it is expressed in terminally postmitotic neurons. Kif 15 enrichment has been observed in microtubule bundles that occupy stalled growth cones and dendrites. It has been also observed that migratory neurons contain high amounts of Kif 15 comparing to other neurons.

In migrating neurons, there is a cage-like configuration of microtubules around the nucleus linked to the microtubule array in leading process which enables the cell to move in unison as it migrates. Since microtubules have the ability to move independently from one another, this movement is regulated by motor proteins in all of these cases. Hence, it is thought that Kif 15 plays a crucial role in neuronal migration. It achieves this by binding to the microtubules with its motor domain while it interacts with other microtubules with its tail domain. Thus, it is thought that it has an affect on ending the neuronal migration. This is of great importance for the proper development of the nervous system because abnormalities observed in neuronal migration causes diseases like Lissencephaly.

Considering Kif15's crucial role in both mitotic cells and neuronal cells, this protein was selected for the dissection on its functional domains. Understanding the interaction of Kif15's motor and tail domain with other cytoskeletal elements will give us a clue about the complex mechanisms of neuronal migration and shed light on unknown features of mitosis. In addition, since Kif15 can represent other Kifs which have similar functions, any information gained for Kif15 will be useful for many others. Finally, since motor proteins are important in intracellular transport, cell division, nucleokinesis, cellular and ciliary motility, cellular morphogenesis and neuronal migration, defects in motors can cause many diseases in both mitotic and neuronal cells, like Charcot-Marie-Tooth disease, Alzheimer and cancer [51].

In order to understand all these puzzled mechanisms and ultimately to put some input in curing these diseases, investigating all motor proteins and the filament network of cytoskeleton is necessary. Thus, molecular dissection of Kif15 for understanding the

interactions of its functional domains with other cytoskeletal elements will be a step for these purposes.

In this study, it was aimed to reveal the cytoskeletal elements interacting with Kif 15's functional domains (motor, tail, myosin interacting etc. domains) by using cloning, cell culture and staining techniques. By this way, some information about how and when motor domain binds to the microtubules or microfilaments could be obtained. More importantly, since the tail domain is specific to all kinesins and it can both carry different cargoes and interacts with other cytoskeletal elements, valuable informations could be obtained about tail interactions.

1.3.1. Rat (*Rattus norvegicus*) Kif15 full sequence and functional domains

Kif15 was dissected, in order to understand the functions of its functionally active domains. Kif15's full sequence and the domains which were decided to be dissected is indicated in the table below.

Table 1.1: Full sequence of Kif15 and its functional domains

		MOTOR F
atggctcctggctgcaaatctgagttacggaatgtgacaaattctcattctaac		cagccaagtaacgaagatgatgccatcaaagttt
tgtgcggattcgccctgctgaagagggcgctaggtcagctgatggagagcagagcctctgctgtgtgctctccagactgctctc		
cggctgcactccaacccagacccaagacctttgtatfcgattatgttgaggcatggacaccactcaggagtctgtattttcaacagt		
gctaaaagcattgtggagtctgcatgagtggttacaacgggaccatcttgcataatgggcagactggctctgggaagacattactatg		
atgggaccatcggactctgataatttttcataatctaagaggagtaattccacgaagtttgaatattgttttccttaattgatcgtgaaaa		
agaaaaggctggagctgggaagagtttcttgaagtgtcctcattgaggtctacaatgagcagatatacagctgctggactctg		
catcgctggactgtatttaagagagcacataaagaaggcgctctcgtggttggtgagtgaggcaggtggtggcctcagctgctg		
aagcctaccaggtcttatctagaggctggaggaacaggcgctgtagcatcaacatctatgaacagagaatcttcaggtctcatgcagt		
ctttacaatcacaatagaatcaatggagaaaagcagtgaggctgtgaacattcgacacctctactcaacctgggtggacttagcaggat		
ctgagaggcaaaaagacacccatgcagaaggatgaggtgaaggaggcaggtacataaaccgatcactgagctgcctgggcc		
aagtcactactgccctgtgatgtggcaatgggaacagaggcatgtctgctaccgggactccaaactfaccttctacttgggact		
cccttggtggcaatgccccaaacagccataattgcaaatgttcacaggatccaggtgtttggggaaacactgtcaacacttaatttgc		
tcaaagagccaagctgattaaaaacaaggctgtgtaaatgaagacaccaaggaaatgtgagccagctac		
		MOTOR R
aagctgaagtgaagagactcagagaacaactgtctcagtttacgtctgggcagctgacaccaggaagctcattggccagagataa		aagctgaagtgaagagactcagagaacaactgtctcagtttacgtctgggcagctgacaccaggaagctcattggccagagataa
agagaaggctaattacatggaatatttctggaagcaatgttattctttaagaaatctgaacaggaaaagaagctctagtagaaaaaatt		
acccaactagaagacctcacctcaaaaaggaaaaattattcagcttaataaaatgattgtgaagtccgagaggaccaaactcatgcg		
tctggaaaggcgccagaaggaggcgaggaagcttctccggaggagcaggatgcctgctgcagaactgagggatgagat		
ccggacgcttcgagagcaagtggagcaccatcccagactcgcgaagtatgctatggagaatcattccctcagggaagagaatagaa		
aactgaaattattagcacctgtgaaaagagcccatgaactcgtgccaggtattgctcactagaacaggctttttctgaagtgtcta		
gtacagagacaaatgacataggctgcaaggacttctcccaaagcaataaaagaaccaagtttctcacaagcaccgagaagttaa		
agtacaactcctgcaaatcagacagagctgaataattcaaagcaagagtatgaagagttcaaagagtttaactcgaagaagcagcta		
gaattagaatcagagcttcagctccttgcataaagcaaacctcaatcttgaacacctttggaggcaacaaaagtctgcaaacgccagg		
aagtttctcagctgaataaaatacatgctgaacactcaagataattaccactccaaccaaggcttatcagctttgtctagactagtacca		
aagtcaagccctgaggtggggagctttggcttctgcgctcacagagtgtccagataatgatattgaatgagccagtgccacctg		
agatgagtgaacaagctctggaggccatttctgaggagcttgaactgtgcaggaacaactgagtgcttctcaagtcaag		

Table 1.2: Full sequence of Kif15 and its functional domains (continue)

MYO F	
ctggatgaagaagaacataaaaacctgaaa cttcaacagaatgttgacagactagaacaccattctacacaaatgcaggagctgtt ctcatcagaaagaagcgattggagcaacaacagcaggactacctcacacagctcagcgaccttgagaagcagctccaggatgcg cagactaagaatgacttttgaaatgtgaggtacatgacctgcgaattgttcttaattctgctgacaagaactttcttggtgaaactgga gtatagcacattcaagagagtgcaagagaagaactaagccagctttctgacagacatgtgcaggttcagcttcagctggacaatgcc agattagaaaatgaaaaacttcttgagagccaagcctgtctacaggattcctacgataacttacaagaagtaataaattcgaagtgtac caactttcaaaaaacctccaaaactgcaaacaagagaatgaaactttaaagtctgatctgcataacttggtggagcttttgaggcagaa aaagagcgcaacaacaattgtcactacagtttgaagaagataaagaaaacagctctaagaaaactttaaggctcttgaaactgtac gtcaggaaaaacaggaagagatggccagatgtgaaaagcagatggcaaaggtacaggaactagaagaagcttgctgtgctga aaatgttgcagctgtctggagaagtctaggagctgtgacaaggaactgtcaccaacctcatgaacaaatccaggagttaagaacat cagctggtgaaaaatcagaagccatagacacctgaaacaagagctgcaagacataagttgcaaatacactgctgctgtggtgaca aagaagagagcaaggagttgatcaggagacaggaagtggatatcctggaactgaaagaaactcttagactgagaattctctcagag gacatcgagaggggatgtgtgtgaagatctggccatgccactgacagctgaacatgctcacagaggcctccaaaagcactct ggactactgcagctgtcccaggaagagctgaccaagaaggaggtctgatccaggaaacttcagcacaagt	
TAIL F	
taaaccaggagaaggaggaagtggagcagaagaagagtgagtacaactgaagatgaagcagct tgagagcacgtgatgggtctt gtccttgagtatccacagagtc aaaaacaccacccattttcaaaactcatttgcaaaactcttgaaacacaagaacaagagatag aagacggaaggatcctaagatgtctttgcaacacctctgcacaaagctaaacgaagacagagaagtcaaaaacgctgagatcctca ggatgaaggatcagttgtgtgaaatgaaaatctgcgcctggagtctcagcagttgagagagagaacctggctcctgcagactcagc tggatgatataagaggcagggggagagcagcagtcagagtcgtccagacagccaacagctgaagaatgaatatgaaggaggaga ttatcagagagagacttgctaaaaataaacttattgaagaaatgctaaaaatgaaaacagatctagagggaagtccagagtgcaactgac agcaaggagaagtctgccacagaatgagcaggaagtgtgagcgaaccagaactttgagtcagagcatttcaggaaggagc aattgcggtcaaaagtgaagaaatgtatgaagagagagagagaacttgccaggaaatggaatgctgagaaaacaactagagtttc ttgctgaggaaaatgggaaattgataggtcatcaaacctacatcagaagattcaatatgtagtgcgactgaagaaggaaaatatcag gcttgctgaggagacagaaaaattgcg tgctgaaaatgtattttgaaagaaaggaaaaaagaatga	
MYO R	TAIL R
nnn: motordomain: 1139 nnn: taildomain: 790bp nnn: myosin tail homology domain.1897bp	

MATERIALS AND METHODS

2.1. Materials

2.1.1. Lab Equipments

The following equipments mentioned in the table 2.1. were used during the project

Table 2.1: Laboratory equipments used in project

Centrifuges	Beckman coulter, Avanti J-30I, Microfuge 18, Beckman Coulter, Memmert, Sigma Biolab 6K15
Centrifuge rotors	MLA 80, Beckman Coulter, JLA-16250, Beckman Coulter, 70Ti, Beckman Coulter, TS-5.1-500, Beckamn Coulter
DNA sequencer	Applied Biosciences 3100-Avant
Autoclave	2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik. NuveOT 4060 Vertical Steam Sterilizer, Nuve
Magnetic stirrer	Labworld Standard Unit
Transilluminator	Biolab
PCR machines	Applied Biosystems, Gene Amp ^R , PCR system 2700 Techne TC-412/ TC-312
Deep freezes and refrigerators	Heto Polar Bear 4410 ultra freezer, JOUAN, Nordic A/S, catalog# 003431, 2031 Deep freezer, Arçelik, Deep freezer, Uğur, 1061 M refrigerator, Arçelik
Vortexing machine	Reax Top, product# 541-10000, Heidolph2.2
Shaker	Certomat ^R SII B-Braun Biotech International

Table 2.2: Laboratory equipments used in Project continue

Incubator	EN400, Nüve
Microwave	MD582, Arçelik
Electrophoresis equipments	EC 120 Mini Vertical Gel System, Thermo EC
Power supply	EC 250- 90, E-C Apparatus
Ice machine	AF 10, Scotsman
pH meter	MP 220, Metler Toledo International Inc, Inolab pH level 1, order# 1A10-1113, Wissenschaftlich-Technische Werkstätten, GmbH & Co KG
Balances	Precise BJ 610C, order# 160-9423-050, Presice Instruments AG Dietikon
Pipettes	0.1-2.5 µl, 500-5000 µl, Mettler Toledo, 10 µl, 20 µl, 200 µl, 1000 µl, Gilson
Pipette tips	1-1000 µl, 1-200 µl, Axygen, 1-10 µl, Molecular Bioproducts
Laminar flow cabinet	Ozge
Pure water systems	USF Elga UHQ-PS-MK3, Elga Labwater
Spectrophotometers	DU530 Life Science UV/Vis, Beckman, UV-1601, Shimadzu. Corporation, Lambda 25 UV/Vis, Perkin Elmer
Spectrophotometer cuvettes	Delta
Test tubes	1.5 ml tubes, Eppendorf
Water bath	Memmert
Gel documentation system	UVIpro GAS7000, UVitech Limited

2.1.2 Chemicals and enzymes

Chemicals and enzymes mentioned in table 2.3. were used during the project

Table 2.3: Chemicals and enzymes

EcoRI restriction enzyme SmaI restriction enzyme KpnI restriction enzyme PstI restriction enzyme MassRuler™ DNA Ladder (Mix, 80bp-10Kb) Fast Ruler High Range DNA Ladder Fast Ruler Middle Range DNA Ladder 10 mM dNTP mix Taq DNA polymerase 10 X Taq DNA polymerase Buffer (with MgCl ₂) 10 X Taq DNA polymerase Buffer (without MgCl ₂) 10X Y ⁺ Tango buffer PEG 4000 T4 DNA Ligase Buffer T4 Ligase	Fermentas
Control mouse beta actin primer pair	BD
pEGFP-C1 vector pEGFP-C2 vector	Clonthech
M13 Forward Primer M13 Reverse Primer	Invitrogen
6X Loading dye Benchtop 1kb DNA ladder	Promega
Tryptone Yeast Extract	LABM
IPTG Ampicillin Kanamycin XGal	Sigma
PIPES	BDH Biochemical
KCl MgSO ₄ NaCl ₂	Riedel-deHaen
Glycerol	Carlo Erba Reagenti

2.1.3 Buffers

2.1.3.1. TAE Buffer (50x)

50x TAE buffer stock was prepared by using the content and amount which was mentioned in table 2.4. During the agarose gel electrophoresis 1x TAE buffer was used by diluting the 50x buffer stock.

Table 2.4: Preparation of 50x TAE buffer

Content	Amount
Tris base (40 mM)	242 g
Glacial acetic acid (20mM)	57.1 ml
0.5M EDTA (pH 8.0) (1mM)	100 ml
H ₂ O	up to 1 ml

2.1.3.2. Buffers of restriction and ligation enzymes

Buffers of restriction and ligation enzymes were purchased together with the enzymes. Suitable buffers were selected according to the recommendation of Fermentas Double Digest web page. (www.fermentas.com/doubledigest/index.html)

2.1.3.3. 10x MOPS Buffer

10x MOPS buffer was prepared by using the chemicals below in table 2.5.

Table 2.5: Preparation of MOPS buffer

Content	Final concentration
MOPS	0.2 M
Sodium acetate	50 mM
EDTA	5 mM

Note: The buffer is adjusted to pH 7.0 with 1M NaOH and sterilized by autoclaving.

2.1.3.4. RNA denaturing buffer

RNA denaturing buffer was prepared by following the constructions mentioned in table 2.6. in order to be used in RNA electrophoresis.

Table 2.6: Preparation of the RNA denaturing buffer

Content	Amount
100% deionized formamide	10ml
40% formaldehyde	3.5ml
10x MOPS buffer	1.5ml

2.1.4 Special reagents and kits

Reagents and kits (see table 2.7.) were used for RNA isolation, cDNA synthesis, gel extraction, TA cloning, plasmid isolation respectively during the study.

Table 2.7: Special reagents and kits used in project

Pure RNA Tissue Kit, Roche
RevertAid TM H Minus First Strand cDNA Synthesis Kit, Fermentas
Agarose Gel DNA Extraction Kit, Roche and Qiagen
InsTA TM PCR Cloning Kit, Fermentas
High Pure Plasmid Isolation Kit for small-scale (mini) preparations, Roche

2.1.5 Bacterial strains

Escherichia coli (*E.coli*) strain XL1 Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44relA1 lac* [F' *proAB lacIqZAM15 Tn10* (Tetr)]], Novagen

2.1.6 Bacterial Culture Media

2.1.6.1. Preparation of LB and LB-Agar Plates

For preparing 1 L of LB, following contents (see table 2.8.) were measured and resolved in dH₂O, after completeing the volume to 1 L, mixture was autoclaved for 15 minutes (min.). This LB was used for overnight growth of the bacteria (overnight culture) with including related antibiotic.

Table 2.8: LB media contents

Content	Volume-gram (g)/L
Tryptone	10
Yeast Extract	5
NaCl	10

For preparing LB-Agar plates with antibiotics, LB medium was prepared as mentioned above, in addition to this mixture, 15 g agar for 1 L was added and after mixing with stirrer it was autoclaved for 15 min. Before pouring the plates, the bottle which contains the LB-agar was cooled enough to hold by hand, related antibiotic was added (1µl /1ml), then he plates were poured and stored at 4° C.

2.1.6.2. Preparation of SOC Medium

SOC medium was used to cultivate *E.coli* for 1 hour after temperature shock during transformation. Following contents (see table 2.9.) were measured and distilled water was added to a final volume of 100 ml, and it was sterilized by autocaving at 120°C for 15 min.

Table 2.9: Content of SOC medium

Content	Amount,g
Tryptone	2
Yeast Extract	5
NaCl ₂	0.058
KCl	0.0186
MgCl ₂	0.095
MgSO ₄	0.024
Glucose	0.36

2.1.6.3. Preparation of Ampicillin and Kanamycin Stocks

For preparing ampicillin or kanamycine stocks (100 µg/ml), 1gr from kan/amp was taken and resolved in 10 ml dH₂O, after filter sterilization they were aliquoted and kept at -20° C.

2.1.7. Preparation of IPTG/Xgal

IPTG/Xgal was used in the last step of transformation which was performed following the TA cloning ligation, in order to determine the colonies carrying the desired inserts.

1.25 g of IPTG and 1g of XGal were measured and di-methyl-formamide was added to a final volume of 25 ml. IPTG/XGal was aliquoted and kept at -20° C.

2.1.8. TA Cloning vector

pRZ57R/T cloning vector provided by InsTATM PCR Cloning Kit, Fermentas was used as a cloning vector. (see figure 2.1.)

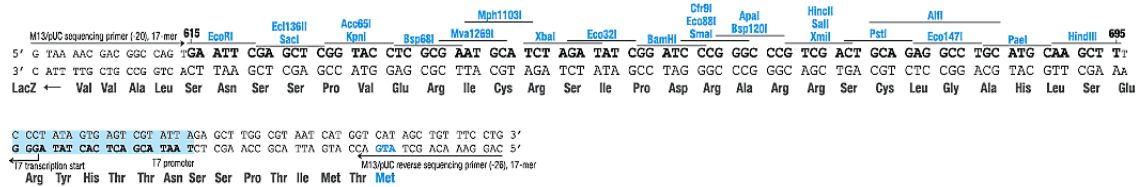
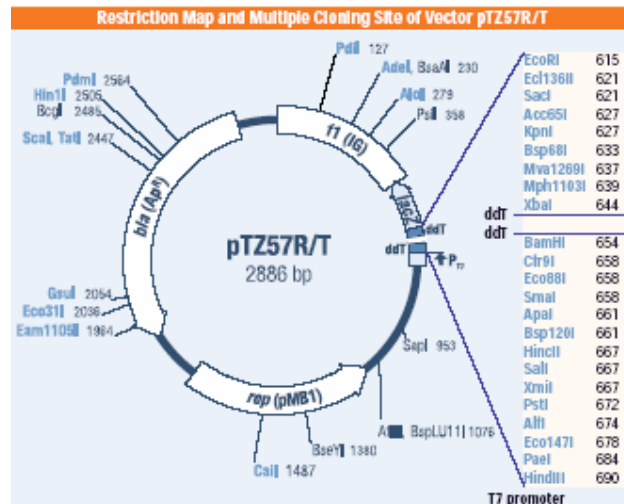


Figure 2.1: Vector map of pTZ5R/T cloning vector. Source:www.fermentas.com

2.1.9 Expression vectors

pEGFP-C1 and pEGFP-C2 were used as expression vectors. (see Figure 2.2 and 2.3.)

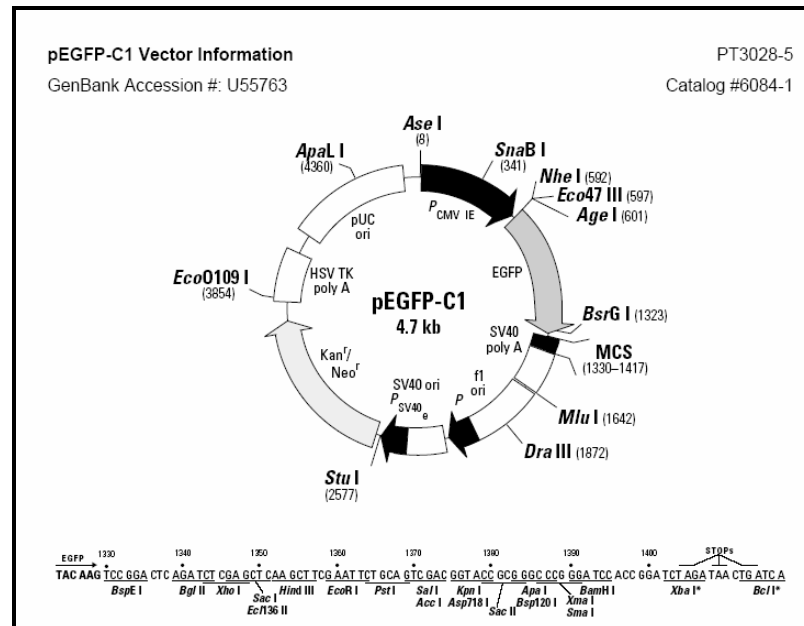


Figure 2.2: Vector map of pEGFP-C1. Source: www.fermentas.com

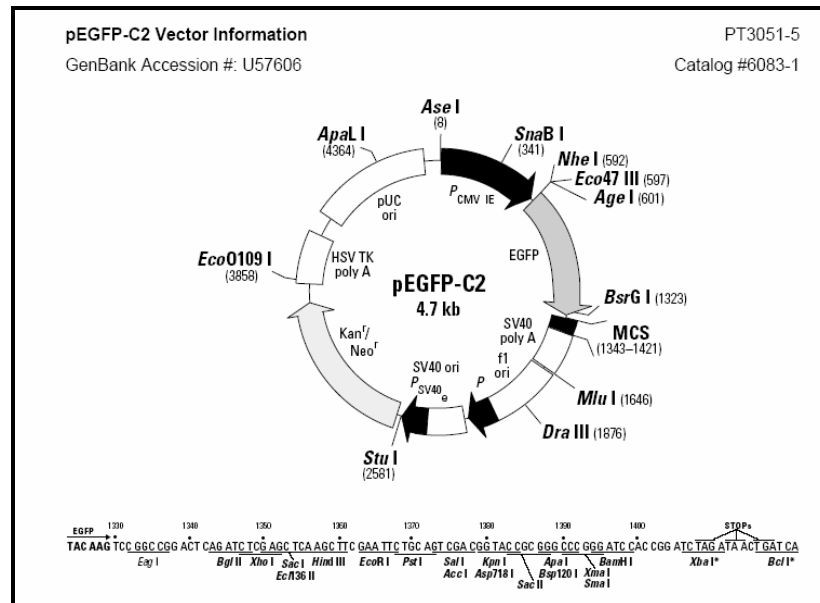


Figure 2.3: Vector map of pEGFP-C2. Source: www.fermentas.com

2.2 METHODS

2.2.1 RNA Isolation

RNA isolation was performed using Roche, High Pure RNA Tissue Kit. The principle of this isolation is as follows: As a prerequisite for the reverse transcription polymerase chain reaction (RT-PCR), intact RNA must be isolated from tissue. Tissue samples are homogenized in the presence of a strong denaturing buffer to inactivate RNases and to ensure the isolation of intact RNA. After the addition of ethanol, RNA binds selectively to a glass fiber fleece in the presence of chaotropic salts. Residual contaminating DNA is digested by DNaseI and applied directly on the glass fiber fleece. The binding process is specific for nucleic acids, which are purified from salts, proteins, and cellular components by repeated washing and centrifugation steps. The bound RNA is eluted in water.

The protocol is as followed:

- Adult rat was sacrificed and brain was taken out by using fine forceps.

- Amounts of lysis/binding buffer was chosen according to the disruption and homogenization method, 400 μ l lysis/binding buffer was added into a nuclease-free 1.5 ml microcentrifuge tube containing the tissue.
- Disruption and homogenization was performed with the help of a nuclease-free stick. In order to obtain an optimum and homogeneous mixture, this lysate was passed 30 times through a gauge needle fitted to a syringe.
- Lysate was centrifuged for 2 min. at maximum speed in a microcentrifuge and only the collected supernatant was used for subsequent steps.
- 200 μ l (0.5 volumes) absolute ethanol was added to the lysate supernatant and mixed well.
- The high pure filter tube was combined with the collection tube and the entire sample was pipetted in the upper reservoir (maximal volume 700 μ l).
- The tube was centrifuged 30 seconds (sec.). at maximal speed ($13,000 \times g$) in a standard table top microcentrifuge.
- After this centrifugation step, dryness of the glass fleece was checked. Since it must be dry; if it looked wet, the centrifugation time must have been increased.
- The filter tube was removed from the collection tube, and the flowthrough liquid was discarded. The filter tube was recombined with the used collection tube.
- For each isolation, 90 μ l DNase incubation buffer was put into a sterile 1.5 ml reaction tube, and 10 μ l DNase I working solution was added and mixed. This solution was pipetted to the upper reservoir of the filter tube and incubated for 1 hour at room temperature.
- 500 μ l wash buffer I was added to the upper reservoir of the filter tube and centrifuged 15 s at $8,000 \times g$.
- The filter tube was removed from the collection tube and the flowthrough liquid was discarded. The filter tube was combined with the used collection tube again.
- 500 μ l wash buffer II was added to the upper reservoir of the filter tube and centrifuged 15 s at $8,000 \times g$ the flowthrough was discarded.
- The filter tube combined with the used collection tube.

- 300 μ l wash buffer II was added to the upper reservoir of the filter tube and centrifuged 2 min at full speed (approx $13,000 \times g$).
- The column was removed carefully from the collection tube in order to prevent the any possible contact of the column with the flowthrough which will result in carryover of ethanol.
- The filter tube was inserted into a nuclease free, sterile 1.5 ml microcentrifuge tube.
- 100 μ l elution buffer was added to the upper reservoir of the filter tube and the tube was centrifuged for 1 min at $8,000 \times g$.

Note: This step was repeated (double elution was performed) in order to obtain denser RNA.

- Isolated RNA was aliquoted into nuclease free, sterile 0.6 ml microcentrifuge tubes and stored at -80°C for later analysis.

Note: Gloves were changed frequently during the experiment and all steps were performed on ice.

2.2.2 Agarose-Formaldehyde electrophoresis for RNA

RNA electrophoresis under denaturing conditions in 2.2 M formaldehyde is performed according to Maniatis et al., (1982) using MOPS buffer system. RNA under these conditions is fully denatured and migrates according to the $\log_{10}$ of its molecular weight.

In order to check if the RNA isolation was performed successfully, 1% agarose-formaldehyde gel was prepared as follows:

0.5 g of agarose was melted in 37 ml H_2O , it was cooled to the hand hot. Then, 5ml 10x MOPS buffer and 8.75 ml 40% formaldehyde was added.

The sample RNA was prepared by adding up to 25 mg of RNA in a maximum of 5 μ l sterile H_2O , to 15 μ l of RNA denaturation buffer. 1 μ l 10mg/ml ethidium bromide was added to help visualization of RNA after electrophoresis. Electrophoresis was performed with 1x MOPS. The sample was placed into a well on the agarose gel and the gel was run at 70 mV for 30 min.

RNA within agarose gels are only visible when stained with ethidium bromide and can then be visualized under UV light. The gel was placed onto an UV illuminator that emits UV light at 302 nm and photographed with a camera connected to a computer. Image files were saved with UVIPhotoMW Version 99.05 for Windows 95 & 98, UVItect Ltd. and subsequently analyzed.

Note: Since formaldehyde is extremely toxic, gloves were used while handling.

2.2.3 cDNA Synthesis

To obtain cDNA from the RNA that we isolated, Fermentas, RevertAid™ H Minus First Strand cDNA Synthesis Kit was used. By using this kit, first strand cDNA was synthesized from our adult rat total RNA. The Kit uses RevertAid™ H Minus M-MuLV Reverse Transcriptase which, due to the lack of RNaseH activity, does not degrade RNA in DNA-RNA hybrids during the first strand cDNA synthesis. This allows more RNA templates to be completely reverse transcribed into total cDNAs. The cDNA, synthesized by using this system, can be used as a template in the polymerase chain reaction (PCR) or it can be used as a template for second strand synthesis. In this experiment, we used it for the first purpose:

- The following reaction mixture mentioned in table 2.10. was prepared in a tube on ice:

Table 2.10: cDNA synthesis reaction

Content	Concentration	Amount
template RNA (total RNA)	10 ng-5 µg	10µl
primer (oligo(dT)18 primer)	(0.5 µg/µl)	1µl
DEPC-treated water	-	1µl
Total reaction mixture	12µl	

- The mixture was gently mixed and spined down for 3-5sec. in a microcentrifuge.
- The mixture was incubated at 70°C for 5 min, then chilled on ice and the drops were collected by brief centrifugation.

- The tube was placed on ice and the following components mentioned in table 2.11. were added in it in the indicated order

Table 2.11: cDNA synthesis reaction continue

5x reaction buffer	4µl
RiboLock™ Ribonuclease Inhibitor (20u/µl)	1µl
10mM dNTP mix	2µl

- The mixture was mixed gently and drops were collected by brief centrifugation.
- The mixture was incubated at 37°C for 5min
- Add RevertAid™ H Minus M-MuLV Reverse Transcriptase (200u/µl) was added (1µl) .Then the final volume would be 20µl.
- The mixture was incubated at 42°C for 60min
- The reaction was stopped by heating at 70°C for 10min.
- cDNA was stored at -20°C

After cDNA synthesis, PCR reactions were set in order to check if we successfully performed the cDNA synthesis process. By using the beta-actin commercial primers, different concentrations of the cDNA obtained was tried to determine the optimum amount of cDNA which will be used in further steps.

2.2.4 Primer Design:

In order to clone desired domains of Kif 15, seven primers were designed for motor domain, myosin tail homology domain and tail domain respectively. During primer synthesis followings were considered:

- Melting temperatures of the primers were selected as close as possible in order to facilitate the optimum annealing temperature selection.
- GC content of the primers were taken into consideration and primers that have GC content more than 60% were not chosen.
- Melting temperature, hairpin and self dimer formation were checked by using IDT Scitools oligo analyzer.

(www.idtdna.com/analyser/Applications/OligoAnalyzer)

Although some hairpin and self dimer formations were observed, the sequences where primers will be synthesized were chosen as suitable as possible by considering

the points above. Primers synthesized by considering these points were mentioned in table 2.12.

Table 2.12: Kif15 sequence specific primers list

Primer	Sequence	Lenght	Tm°C	Location (bp)
MotorF	CAGCCAAGTAACGAAGATGATG	22	62	55
MotorR	CTGCCCAGACGTAAACTGAGAC	22	66	1194
Myo F	CTTCAACAGAATGTTGACAGACTAGAACAC	30	64.6	2227
Myo R	CGCAATTTTCTGTCTCCTCAG	22	65	4121
Tail F	TGGAGCACTGATGGGCTCTGCTCCTGAG	28	71.7	3382
Tail R	TCATTCTTTTTTCCTTTCTTTCAAAAATACATTTTCATCA	40	69.1	4192

2.2.5 PCR

The polymerase chain reaction is a technique for quickly "cloning" a particular piece of DNA in the test tube. So, unlimited copies of a single DNA molecule can be amplified bu using this technique. After deciding the primers which restricts the sequence we want to amplify, related components were added and some different PCR programs were performed. For initial denaturation, where two strands of DNA were separated, 94°C was used. Same temperature was used for also denaturation step. Annealing temperatures, where primers bind to the complementary regions of the DNA, were changed according to the primers melting temperatures used. Finally, elongation and final elongation steps where suitable bases are arranged in an order, 72°C was used.

PCR reactions were performed by using the cDNA which synthesized from adult rat brain and primers designed as mentioned before. 25 µl total reactions were prepared. PCR conditions (see table 2.13, 2.15, 2.17.)) and programs (see table 2.14, 2.16,2.18.) by which desired results were obtained are as followed.

Table 2.13: PCR reaction set up for motor domain

Content	Amount
Template (Motor DNA after gel Extraction)	0.5 μ l
Primer-1: Motor F (25 μ g/ml)	0.5 μ l
Primer-2: Motor R (25 μ g/ml)	0.5 μ l
dNTP mix (10 mM each)	0.5 μ l
10X Taq Buffer	2.5 μ l
Taq DNA Polymerase (5u/ μ l)	1 μ l
H ₂ O	19.5 μ l
Total reaction:	25 μ l

Table 2.14: PCR run program for motor domain

Initial Denaturation	94	1 min.									
Denaturation	94	30 sec.	94	30 sec.	94	30 sec.	94	30 sec.	94	30 sec.	} 10 cycles each
Annealing	54	1 min.	55	1 min.	56	1 min	57	1 min.	58	1 min.	
Elongation	72	2 min.	72	2 min	72	2 min	72	2 min.	72	2 min.	
Final Elongation	72	10 min.									
Note: All values are °C.											

Table 2.15: PCR reaction set up for myosin tail homology domain

Content	Amount
Template (adult rat cDNA- 3.96 μ g/ml)	0.5 μ l
Myo F (25 μ g/ml)	0.5 μ l
Myo R (25 μ g/ml)	0.5 μ l
dNTP mix (10 mM each)	0.5 μ l
10X Taq Buffer	2.5 μ l
Taq DNA Polymerase	0.5 μ l
H ₂ O	20 μ l
Total reaction:	25 μ l

Table 2.16: PCR run program used for myosin tail homology domain

Initial Denaturation	94°C	30 sec	40 cycles
Denaturation	94°C	1 min	
Annealing	55°C	1 min	
Elongation	72°C	2 min	
Final Elongation	72°C	5min	

Table 2.17: PCR reaction setup for tail domain

Content	Amount
Template (adult rat cDNA- 3.96µg/ml)	0.5µl
Tail F (25µg/ml)	0.5µl
Tail R (25µg/ml)	0.5µl
dNTP mix (10 mM each)-Qiagen	0.5µl
10X Taq Buffer-Roche	2.5µl
Taq DNA Polymerase-Roche	1µl
H ₂ O	19.5µl
Total reaction:	25µl

Table 2.18: PCR run program used for tail domain

Initial Denaturation	94°C	30 sec	40 cycles
Denaturation	94°C	1 min	
Annealing	60°C	1 min	
Elongation	72°C	2 min	
Final Elongation	72°C	5min	

2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis is the most commonly used system which is employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel. The principle in agarose gel is the migration of negatively charged DNA, by electric current through a matrix of agarose. Since small molecules migrate faster than large ones, it will be possible to

get information about DNA in the cases indicated above. In this project, 1% low melting agarose gels were used.

To prepare 1% agarose gel, 0.4 g of low melting point:

- Agarose was dissolved in 40 ml (small gel) 1x TAE (Tris-acetate-EDTA) buffer.
- The agarose was solubilized in a microwave oven until the agarose was completely dissolved.
- Gel was cooled to $\leq 45^{\circ}\text{C}$ and ethidium bromide was added to a final concentration of 0.5 $\mu\text{g/ml}$ and mixed through gentle swirling.
- The agarose gel was then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel.
- The gel was solidified for about 20 min. and then placed into an electrophoresis tank, where the gel was covered by 1x TAE buffer used to make the gel.

The DNA was mixed with loading dye and the sample was placed into a well on the agarose gel. As fragment size control, suitable marker which was selected according to the expected DNA fragment size, was used. Electrophoretic separation was performed by constant current at 100 mV for 20-30 min.

DNA and RNA fragments were visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis and can then be visualized under UV light, or the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. So, the gel was placed onto an UV illuminator that emits UV light at 302 nm and photographed with a camera connected to a computer. Image files were saved with UVIPhotoMW Version 99.05 for Windows 95 & 98, UVIttec Ltd. and analyzed. The size of the DNA was determined by comparing fragments formed the suitable DNA marker.

2.2.7 Gel Extraction

For isolation of DNA fragments from agarose gel, Qiagen MinElute Gel Extraction which is designed to purify DNA of 70 bp to 4 kb from standard or low-melt agarose gels in TAE or TBE buffer was used. Gel extraction is necessary for isolating DNA after PCR reactions and restriction reactions in cloning process. Since DNA is extracted from agarose gel, it should be cut as thin as possible in order to obtain DNA in sufficient concentration. Following steps were followed for extraction of DNA from 1% agarose gel:

- DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose.
- The gel slice in a colorless tube was weighed. 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl) was added.
- Tube was incubated at 50°C for 10 min. To help dissolve gel, tube was mixed by inverting in every 2–3 min. during the incubation. Finally, gel was solubilized completely and the colour of the mixture was checked.
- After the gel slice has dissolved completely, the color of the mixture was checked (it should be like QG buffer color).
- 1 gel volume of isopropanol was added to the sample and mixed by inverting the tube several times.
- A MinElute column was placed in a provided 2 ml collection tube in a suitable rack.
- To bind DNA, the sample was applied to the MinElute column and centrifuged for 1 min. at 14.000 rpm.
- The flow-through was discarded and the MinElute column was placed back in the same collection tube.
- 500 µl of Buffer QG was applied to the spin column and centrifuged for 1 min. at 14.000 rpm.
- The flow-through was discarded and the MinElute column was placed back in the same collection tube.
- To wash, 750 µl of Buffer PE was added to the MinElute column and centrifuged for 1 min at 14.000 rpm. This step was carried out twice.
- The flow-through was discarded and the MinElute column was centrifuged for an additional 2 min. at 14.000 rpm.

- The MinElute column was placed into a clean 1.5 ml microcentrifuge tube.
- To elute DNA, double elution was performed by adding 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the membrane, and then centrifuged for 1 min at 14,000 rpm.

Note: In some cases, 30 µl of EB was used in order to have more concentrated DNA.

2.2.8 TA cloning

TA cloning is used for cloning of PCR products with 3'-dA overhangs into cloning vectors which are previously cut and ready to use. InsTA™ PCR Cloning Kit, Fermentas was used for TA cloning. In order to put PCR products into cloning vector pTZ57R/T, first ligation reaction was set considering the manufacturer's recommendations. Recommended amounts were reduced in order to extend the kit's usage period.

Ligation reactions were set both for motor and tail domains as follows:

2.2.8.1. Ligation For TA Cloning

Following ligation reactions mentioned in table 2.19 were used for both motor and tail domains:

Table 2.19: Ligation reaction for TA cloning

Content	Amount
Plasmid Vector (pTZ57R/T)-0.165µg	1.5 µl
PCR fragment (Motor-Tail)	8 µl
10X ligation Bufer	1.5 µl
10X PEG 4000	1.5 µl
T4 DNA Ligase (5u/µl)	0.5 µl
H ₂ O	2µl
Total Reaction:	15 µl

2.2.8.2. Transformation for TA cloning:

- The competent cells were taken from -80°C and thawed on ice. 2-3 μl of purified plasmid DNA or 15 μl of ligation mixture was added to 25 μl of competent cells and the eppendorf tube containing the cells was incubated on ice for 30 min.
- Then, heat shock was done by putting the cells in waterbath at 42°C for 40-45 sec. and they were then immediately incubated on ice for 2 minutes.
- 80 μl of SOC medium was added to competent cells and the eppendorf tube was vigorously shaken at 37°C for 1 hour.
- The cells were taken and 20 μl of IPTG/X-Gal solution was added to each tube. Then, the cells were spreaded onto LB plate containing ampicillin since the pTZ57R/T plasmid has ampicillin resistance. The plates were incubated at 37°C overnight.
- The following day plates were taken from 37°C incubator and white colonies were counted. Plates were kept at 4°C until their reuse.
- Colonies were streaked into a new ampicillin resistant plate in order to perform colony PCR.

In transformation for TA cloning, we used IPTG/X-Gal for blue white screening. The principle of this screening method is as follows.

The plasmid used (pTZ57R/T here) contains a gene called LacZ. The LacZ gene codes for an enzyme called beta-galactosidase. This enzyme is made up of two fragments, alpha and omega. When the two fragments are associated they form a functional enzyme. Normally, beta-galactosidase metabolizes galactose producing two products, lactose and glucose. Beta-galactosidase converts other substrates such as X-Gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside) into a colored product. X-Gal is a colorless modified galactose sugar, however, when it is metabolized by beta-galactosidase the products are a bright blue.

In order for the gene to be actively transcribed from the DNA and for the enzyme to be produced, an activator called IPTG (isopropyl-[beta]-D-thiogalactopyranoside) must be added. Both X-Gal and IPTG are delivered to the bacteria through the growth medium (generally a supplemented and enriched bacto-agar blend).

Within the LacZ gene there are multiple cloning sites where the plasmid may be cut and DNA may be added. This produces a plasmid with foreign DNA located within the LacZ gene. When transcription of the gene is activated by IPTG the foreign DNA that has been inserted is transcribed as well. When the gene is later translated into the enzyme the inserted DNA is translated as well. Because of its location within the enzyme the foreign DNA's translated protein product disrupts activity and function of the enzyme. The disrupted enzyme activity is observed as a white bacterial colony. (If the enzyme is functioning fully each colony is a bright blue color.) Very small inserts of foreign DNA may lead to light blue colonies.

2.2.9. Colony PCR

After transformation for TA cloning, by the help of the blue-white screening, white colonies which are candidates for carrying desired inserts were chosen. Colony PCR was performed to be sure of selecting right colonies which are carrying inserts. Since some white colonies that are expected to have insert are sometimes empty, colony PCR is essential.

- 25 µl of sterile H₂O was used for each colony which was taken from the replica plate by using sterile tips.
- Tubes which contain water and a piece of colony were incubated at 99 ° C for 5 min.
- Cells were centrifuged at maximal speed for 1 min(optional)
- Following PCRs mentioned in table 2.20. were performed for motor and tail domain respectively:

Table 2.20: Colony PCR reactions for motor and tail domains

	Motor Domain	Tail Domain
Template+H ₂ O	20.8 µl	20 µl
dNTP mix	0.5 µl	0.5 µl
10X PCR buffer	2.5 µl	2.5 µl
Primer 1	(motor F) 0.5 µl	(tail F) 0.5 µl
Primer 2	(motor R) 0.5 µl	(tail R) 0.5 µl
Taq Polymerase	0.2 µl	1 µl
Total Reaction	25 µl	25 µl

2.2.10. Overnight culture preparation:

After identification of the right colonies which contains the inserts by colony PCR, these colonies were taken into the 5 ml LB medium containing 5 µl suitable antibiotic (ampicillin or kanamycin) which was added in order to make selection media. Then they were incubated in 37 °C shaker overnight.

2.2.11. Small scale plasmid DNA preparation (mini-prep)

Plasmid preparation was performed using Roche, High Pure Plasmid Isolation Kit for small-scale (mini) preparations, following instructions of the manufacturer. The principle of this purification is to release plasmid DNA from bacteria by alkaline lysis and to remove all the RNA in the lysate by RNase. Then, since the plasmid DNA has the ability to bind selectively to glass fiber fleece in a centrifuge tube in the presence of a chaotropic salt (guanidine HCl), DNA remains bound. After washing steps performed to remove contaminating bacterial components, low salt elution removes the DNA from the glass fiber fleece.

The protocol of High Pure Plasmid Isolation Kit, Roche is as follows:

- Overnight culture was prepared: A single bacterial colony was picked and inoculated into 5 ml LB media (with 5 µl of ampicillin) containing Falcon tube, and grown overnight with vigorous shaking (200 rpm) at 37°C.
- The next day, 2 ml of the culture was put into an eppendorf tube and centrifugated for 1 minute at 9000 rpm. The supernatants were discarded.
- The bacterial pellet was resuspended in 250 µl of suspension buffer by vortexing the eppendorf tube.
- To lyse the cells, 250 µl lysis buffer was added (contains NaOH), mixed by inverting the tube 6 times and incubated at room temperature for up to 5 min.
- Lysis was stopped by addition of 350 µl ice-cold binding buffer. Tube was again inverted 6 times and incubated on ice for up to 5 min.

- The mixture was centrifuged for 10 min. at 14.000 x g and the supernatant was transferred to a filter tube. Chromosomal DNA was precipitated with cellular debris during centrifugation and this supernatant contains the plasmid DNA.
- Again centrifugation for 1 minute at maximum speed was performed. Plasmid DNA is bound to the glass fibers pre-packed in the filter tube. Supernatant was discarded from the collection tube.
- To wash the cells, 700 µl of wash buffer was added to the filter tube and centrifuged at maximum speed for 1 minute. Supernatant from collection tube was discarded. This step was done twice.
- Tube was centrifuged at 14.000 rpm for additional 2 min. with adding nothing.
- To elute the DNA, 100 µl elution buffer was added, and the DNA solution was obtained by centrifugation for 1 minute at full speed (double elution was done).

2.2.12 DNA Sequencing

2.2.12.1. PCR for Sequencing:

The purpose of the sequencing is to determine the order of the nucleotides of a gene. The sequencing method is based on the use of modified nucleotides, so called 2', 3'-dideoxy analogs. These special nucleotides lack a hydroxyl residue at the 3' position of the deoxyribose. When DNA polymerase adds nucleotides into a DNA chain through its 5' triphosphate groups, the absence of a hydroxyl group on a dideoxy analog avoids the formation of a phosphodiester bond with an adjacent nucleotide, leading to a stop in elongation. As the concentration of the analogs is very low, termination happens just occasionally. With four different analogous nucleotides in four separate reactions, numerous fragments corresponding to every base position will be synthesized. As fluorescently labelled primers are used, newly synthesized DNA fragments are marked and the sequence can be detected by excitation with a laser and detection with photodiodes in a sequencing machine.

The plasmids with inserts were verified by DNA sequencing using Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence reaction was prepared in a sterile PCR tube by adding the compounds shown below (see table 2.21). Same compounds were used for both motor and tail domains.

Table 2.21: PCR set up for sequencing

Contents	Volume, μ l
Big dye reaction mix	2
5X sequence mixture	2
Template DNA	1
Primer M13	0.5
dH ₂ O	4.5
Total reaction volume	10

The sequence reactions were performed using a thermal cycler with the following program mentioned in table 2.22.

Table 2.22: PCR program for sequencing

	Temperature	Time
Initial Denaturation	95 °C	5 min.
Denaturation	95 °C	45 sec.
Annealing	55 °C	45 sec.
Elongation	60 °C	4 min.
Final Elongation	4 °C	-

2.2.12.2. PCR Purification For Sequencing:

- PCR tubes were taken and short spin was performed.
- PCR products were transformed into 1.5 ml eppendorf tubes.
- 2 μ l, 3M NaAC and 50 μ l cold ethanol (95%) was added to each tube and incubated on ice for 30 min.
- Tubes were centrifuged at 14.000 rpm for 20 min.
- After discarding the supernatant by pipetting, 250 μ l cold ethanol (70%) was added into each tube and centrifuged at 14.000 rpm for 20 min.
- After discarding the supernatant with the help of a pipette, tubes were put into a 95°C incubator for drying.
- 20 μ l formamide was used for elution.

- Tubes were kept at 4°C till use.
- Denaturation was performed at 95 for 2-3 min. and samples were put into the sequencer.

2.2.13. Alignment of sequences

Nucleotide alignments were made both with BLAST tool, (available at <http://www.ncbi.nlm.nih.gov/BLAST/>) and ClustalW program (available at www.ebi.ac.uk/clustalw/) in order to compare the sequences we had after sequencing with the original sequences.

2.2.14. DNA cleavage with restriction endonucleases

Restriction endonucleases are enzymes that cleave the sugar-phosphate backbone of DNA. These enzymes cut both strands of duplex DNA within a stretch of just a few bases. A large majority of restriction enzymes have been isolated from bacteria, where they appear to serve a host-defense role. The idea is that foreign DNA, for example from an infecting virus, will be chopped up and inactivated ("restricted") within the bacterium by the restriction enzyme. Restriction endonucleases recognize a specific nucleotide sequence, and cut DNA wherever this specific sequence is found. Usually the palindromic restriction sites have a length of 4 to 8 base pairs. The purified restriction endonucleases are commercially available, and are used to generate DNA fragments for cloning experiments. Therefore, restriction endonucleases are a major tool in recombinant DNA technology.

From the data obtained from sequence analyses which were performed for motor and tail domain, it was understood that, tail domain had integrated into pTZ57R/T cloning vector in right orientation, but motor domain was integrated into cloning vector in reverse orientation. Since these domains had to be cloned into GFP expression vectors, suitable vectors and enzymes were selected in order to subclone these domains into GFP expression vectors considering their orientation. Two different restriction conditions were used for two different domains of Kif 15 (motor-tail). These enzymes were chosen according to the followings:

- They have to cut both T/A and expression vectors;

- Enzyme cutting sites in the vectors should be same for tail domain and they should be reverse for motor domain since the orientation of motor domain is reverse in the cloning vector. So, the enzyme which cuts 5' restriction site on T/A vector should be in 3' restriction site on expression vector;
- Restriction endonucleases should cut the DNA at the most suitable points which does not allow shifting of the sequence.
- Restriction endonucleases should not cut inserts (motor and tail domains.)

Considering all these circumstances, pEGFP-C1 was found suitable for motor domain and pEGFP-C2 was found suitable for tail domain.

2.2.14.1. Restriction for motor domain

Restriction enzymes and contents were selected as shown in the table 2.23.

Table 2.23: Restriction of pTZ57R/T-Motor domain and pEGFP-C1 with KpnI/PstI

Contents	Volume, μ l	Contents	Volume, μ l
pTZ57R/T-Motor	20	pEGFP-C1	15
10X Y ⁺ Tango buffer	2.5	10X Y ⁺ Tango buffer	2
KpnI	1	KpnI	1
PstI	1	PstI	1
dH ₂ O	0.5	dH ₂ O	1
Total reaction	25	Total reaction	20

Reaction mixtures were put in 37°C waterbath for 4 hours.

2.2.14.2. Restriction for tail domain

Since two different temperatures are suitable for maximum restriction efficiency for these two enzymes (30°C for SmaI and 37°C for EcoRI), following restriction (see table 2.24.) mixtures and conditions were used for maximum efficiency.

Table 2.24: Restriction of pTZ57R/T-Tail domain and pEGFP-C2 with SmaI/EcoRI

Content	Volume, μ l	2 hours at 30°C	Add	Content	Volume, μ l	2 hours at 37°C
pTZ57R/T-Tail	20			10X Y ⁺ buffer	3.5	
10X Y ⁺ buffer	2.5			EcoRI	1	
SmaI	1			dH ₂ O	0.5	
dH ₂ O	1.5			Total Reaction:	30	
Total Reaction:	25					

Content	Volume, μ l	2 hours at 30°C	Add	Content	Volume, μ l	2 hours at 37°C
pEGFP-C2	15			10X Y ⁺ buffer	3	
10X Y ⁺ buffer	2			EcoRI	1	
SmaI	1			dH ₂ O	1	
dH ₂ O	2			Total Reaction:	25	
Total Reaction:	20					

2.2.15. Ligation

Ligation reactions were performed in order to subclone inserts found in cloning vectors (pTZ57R/T-Motor, pTZ57R/T-Tail) into expression vectors (pEGFP-C1 and pEGFP-C2). Following the restriction reactions, related domains were gel purified and considering the brightness of the fragments observed on gel, their concentrations were estimated and ligation reactions were set.

2.2.15.1. Ligation For Motor Domain:

Two different ligation conditions were tried as mentioned in table 2.25.

Table 2.25: Ligation reaction for subcloning of motor domain into GFP

Content	1 st condition/volume- μ l	2 nd condition/volume- μ l
pEGFP-C1	1	3
Insert-Motor	7	10
PEG 4000	1	1
T4 DNA Ligase buffer	1.5	1.5
T4 DNA Ligase	0.5	0.5
H ₂ O	4	4
Total Reaction	15	20

2.2.15.2. Ligation For Tail Domain:

Two different conditions were tried as mentioned in table 2.26.

Table 2.26: Ligation reaction for subcloning of tail domain into GFP

Content	1 st condition/volume- μ l	2 nd condition/volume- μ l
pEGFP-C2	1	3
Insert-Tail	7	10
PEG 4000	1.5	2
T4 DNA Ligase buffer	1.5	2
T4 DNA Ligase	1	1
H ₂ O	3	2
Total Reaction	15	20

2.2.16. Competent Cell Preparation-CaCl₂ Method:

In order to carry out transformation, competent cells must be used because normal *E.coli* cells are not competent and they do not have the ability to take foreign DNA from environment. For this reason, some physiologically healthy cells growing under optimal conditions are suddenly chilled and resuspended in a “starvation medium” consisting of a dilute solution of calcium chloride. Since cells that are undergoing very rapid growth are made competent more easily than cells in other stages of growth, they should be taken at this stage.

Although the exact mechanism is still somewhat unknown, calcium ions are thought to open some hydrophilic channels in the membrane and disrupting the cell wall integrity of bacteria, which allows the passage of the DNA. When these competent cells are mixed with plasmid DNA, the DNA attaches to the exposed portions of the cell membrane. If a “heat shock” is given to these cells, the DNA is absorbed into the cell's cytoplasm. The transformed cells are then allowed to grow in a rich media like LB broth or SOC to allow time for resynthesis of the cell wall and expression of any genes on the newly acquired plasmid DNA. Finally, these cells are plated on agar plates which only transformed cells can grow on.

To introduce plasmid DNA or recombinant plasmid DNA into bacteria, the bacteria had to be made competent for this purpose. We homemade competent cells according to the following protocol:

E. Coli - XL1 Blue cells were taken from a glycerol stock culture by scraping with a tip and it was directly put in a falcon containing 5ml LB medium and incubated overnight at 37°C in a rotatory shaker.

- The following day, 100 ml LB medium was inoculated with 5 ml of overnight culture solution and was incubated at 37°C in a rotatory shaker. Cell density was measured several times by a spectrophotometer at OD₆₀₀ to control if they reached approximalety 0,6. When the OD₆₀₀ reached to 0,56 the bacteria were transferred to 50 ml prechilled sterile ultracentrifuge tubes and incubated on ice for 10 min.
- The cells were spun down at 1600 x g for 7 min. at 4°C, the supernatant was discarded.
- Each bacterial pellet was resuspended in 10 ml ice-cold CaCl₂ and centrifuged for 5 min. at 1600 x g at 4°C, the supernatant was discarded.
- Each bacterial pellet was resuspended in 10 ml ice-cold CaCl₂ and they were incubated on ice for 30 min.
- Centrifugation was performed again at 1600 x g for 5 min. at 4°C
- Each pellet was resuspended completeley in 2 ml of CaCl₂.
- The competent cells were distributed into prechilled sterile microfuge tubes each contains 40 µl and they were stored at – 80°C.

For 50 ml CaCl₂ solution following contents with mentioned concentrations and amounts in table 2.27. were used.

Table 2.27: CaCl₂ Solution Preparation:

Contents	Concentration	Amount
CaCl ₂	60 mM	0.33 g
PIPES	10mM	0.15 g
Glycerol	15%	7.5 ml
H ₂ O	X	up to 50 ml

2.2.17. Transformation of competent cells

Transformation is the term used to describe the introduction of plasmid DNA into bacteria. As mentioned above, competent cells were used for transformation. Although transformation can be performed by an electrical or a chemical method, we used the chemical method which consists of a heat shock to introduce the DNA into the host as follows;

- The competent cells were taken from -80°C and thawed on ice. 2-3 μl of purified plasmid DNA or 10-15 μl of ligation mixture was added to 20-25 μl of competent cells and the eppendorf tube containing the cells was incubated on ice for 30 min.
- Then, heat shock was done by putting the cells in waterbath at 42°C for 40-45 sec. and they were then immediately incubated on ice for 2 minutes.
- 80 μl of SOC medium was added to competent cells and the eppendorf tube was vigorously shaken at 37°C for 1 hour.
- The cells were then spreaded onto LB plate containing the appropriate antibiotic. The plates were incubated at 37°C overnight.

2.2.18. Confirmation Restriction

After transformation, colonies grown on plates was checked by colony PCR to find out which colonies were carrying the inserts. The colonies with inserts were transferred to 5 ml LB culture with kanamycine. Following day, plasmid preparation was carried out for pEGFP-C1/Motor domain and pEGFP-C2/Tail domain. Confirmation restriction is another way to confirm the plasmid if it has the insert or not. For this reason, couple of enzymes, usually the enzymes used for restriction reaction, are used. Therefore, we expect two clear fragments of vector alone and insert.

Confirmation reaction was carried out with the same enzymes which were used for digesting the desired domains from pTZ57R/T vector and expression vectors.

Following conditions (shown in table 2.28 and 2.29.) were used:

Table 2.28: Confirmation restriction for motor domain

Content	Volume- μ l
pEGFP-C1/Motor domain	5
KpnI	0.5
PstI	0.5
10 X Y ⁺ Tango Buffer	1
dH ₂ O	3
Total reaction	10

Table 2.29: Confirmation restriction for tail domain

Content	Volume- μ l		
pEGFP-C1/Tail domain	5	→ Add	EcoRI
SmaI	0.5		0.5
10 X Y ⁺ Tango Buffer	1		10 X Y ⁺ Tango Buffer
dH ₂ O	3.5		2
			dH ₂ O
			2.5
			Total reaction
			15

2.2.19. Glycerol Stock Preparation:

In order to keep our constructs safely for long time to use in further studies, glycerol stocks have to be made. Glycerol is used in order to minimize the damage which can be revealed during freezing and thawing of the cells. Four glycerol stocks, (final concentration of 20%), were prepared for plasmids which are carrying desired domains.(pTZ57R/T-Motor, pTZ57R/T-Tail, pEGFP-C1/Motor, pEGFP-C2/Tail). For this purpose;

800 μ l cells (XL1 Blue, carrying related plasmid with desired insert)

200 μ l glycerol (100%) were mixed together and kept at -80° C

2.2.20. Determination of nucleic acid concentration

Recovery, purity and concentration of nucleic acids were determined by spectrophotometric analysis and mostly estimation from the gel.

The ratio of absorbance at (A_{260}) is used for DNA. For the measurement, we used a spectrophotometer from Shimadzu. The DNA was diluted 1:1000 in distilled water and transferred to a quartz cuvette. The absorption was at wavelength of 260 nm. An optical density (OD) of 260 nm of 1.0 is equivalent to 50 $\mu\text{g/ml}$ DNA. The formula used to calculate the concentration (C) is as follows:

$$C = OD_{260} \times \text{dilution factor} \times \text{equivalent} = x \mu\text{g/ml}$$

3. RESULTS

3.1. Adult Rat Brain Dissection



Figure 3.1: An adult rat

An adult rat (see figure 3.1.) was taken under anesthesia by using a piece of cotton treated with ethanol. By the help of scissors, skin above the head was cut directly from the nape towards the forebrain. The skin was removed gently till the whole skull was seen clearly. Since the top of the skull has to be removed in order to reach the brain, a scissors tip was used to get just inside then the skull was snipped gently by using the tip of forceps in order to reveal the whole brain. (see figure 3.2.). Then the thin membranes (meninges) covering the brain was removed by forceps and brain was very carefully removed from skull cavity by cutting the connections of brain to spinal cord and cranial nerves.

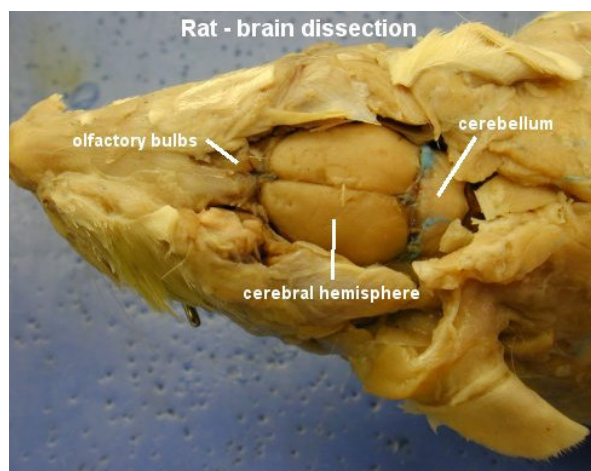


Figure 3.2: An adult rat brain and its parts.

3.2. RNA isolation from adult rat brain tissue

Since RNAases are found throughout environment, (hands, laboratory benches, distilled water, chemicals), precautions must be taken to avoid problems with RNAase contamination. For this reason, RNA isolation was performed in a highly sterile environment where diethyl pyrocarbonate (DEPC) treated instruments and RNAase free tips were used during the procedure.

Since the brain tissue amount needed for RNA isolation procedure is very small (10 mg maximum according to the High Pure RNA Tissue Kit, Roche) a sterile razor blade was used for scrapping the needed amount from the brain and DEPC treated plastic sticks, sterile needles were used in homogenization of the brain.

RNA was eluted by the elution buffer and gel electrophoresis was performed in order to see the result of the isolation procedure.

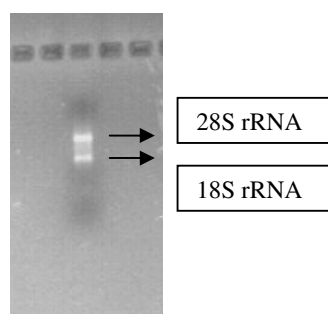


Figure 3.3: Gel electrophoresis result of RNA isolation from adult rat tissue. Arrows indicate the 28S rRNA and 18S rRNA

3.3. cDNA synthesis

cDNA was synthesized by using Fermentas, RevertAid TM H Minus First Strand cDNA Synthesis Kit. Total RNA isolated from adult rat brain was used as a template for this synthesis. Since the cDNAs obtained from this procedure could be used as templates in PCR reactions, a control PCR reaction with commercially available beta-actin primer was performed in order to check if the cDNA was successfully synthesized.

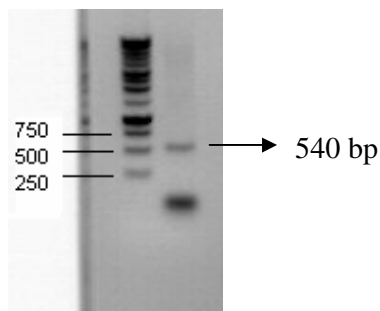


Figure 3.4: Control of DNA purity by PCR reaction

It was also important to determine the optimum amount of cDNA which will be used in PCR reactions. Thus, cDNA concentration was also calculated by using the spectrophotometer as follows:

$$\text{Concentration (C)} = \text{OD } 260\text{nm} \times \text{dilution factor} \times \text{constant (33 } \mu\text{g/ml)} = X \mu\text{g/ml}$$

$$C = 0.0012 \times 100 \times 33 = 3.96 \mu\text{g/ml}$$

(Note: $1A_{260}$ of ssDNA: $33\mu\text{g/ml}$)

In addition, three different amounts of cDNA were used as templates and the most suitable amount was chosen.

Table 3.1: Control PCR reactions after cDNA synthesis

Content	1 st condition	2 nd condition	3 rd condition
Template (adult rat cDNA)	0.2 μ l	0.5 μ l	1 μ l
Primer: Control mouse β -actin*	1 μ l	1 μ l	1 μ l
dNTP mix (10 mM each)-	0.5 μ l	0.5 μ l	0.5 μ l
10X Taq buffer-Roche	2.5 μ l	2.5 μ l	2.5 μ l
Taq DNA polymerase-Roche	0.5 μ l	0.5 μ l	0.5 μ l
H ₂ O	20.3 μ l	20 μ l	19.5 μ l
Total reaction:	25 μ l	25 μ l	25 μ l

*Commercially available mouse beta-actin primer pair, which contains both forward and reverse primers and amplifies a 540 bp DNA fragment was used.

Table 3.2: PCR program used for control PCR reactions after cDNA synthesis

Initial Denaturation	94°C	1 min	30 cycles
Denaturation	94°C	30 sec	
Annealing	55°C	1 min	
Elongation	72°C	2 min	
Final Elongation	72°C	5min	

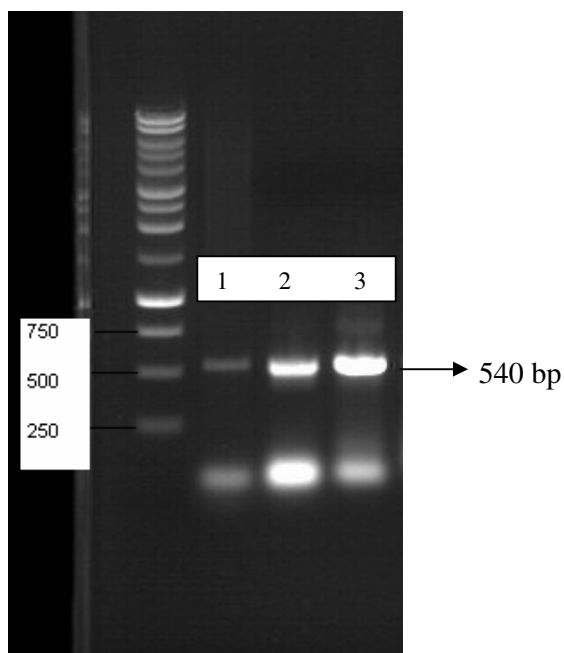


Figure 3.5: Gel electrophoresis showing the PCR results of different concentrations of cDNA templates, 0.2µl, 0.5µl, 1µl respectively.

According to these results, the optimum amount of beginning template was chosen as 0.5µl.

3.4. Cloning of motor, myosin tail homology and tail domains of Kif15

3.4.1. PCR for motor domain

Motor domain was amplified by gradient PCR which was based on gradual increase of the annealing temperatures, from adult rat cDNA. Amounts were used as mentioned in the methods section and annealing temperatures were started from 54°C and increased 1°C in each 10 cycles till 58°C.

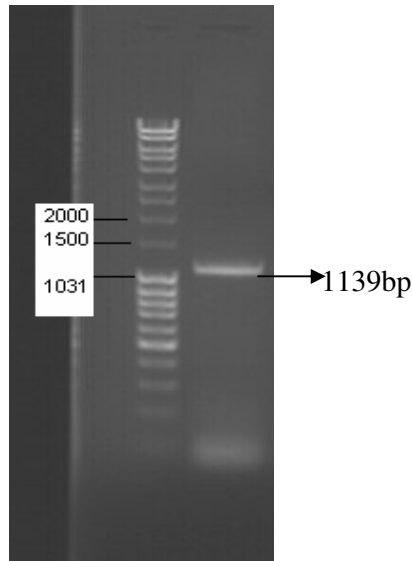


Figure 3.6: 1% gel electrophoresis showing the result of PCR for motor domain.

3.4.2. PCR for myosin tail homology domain

Myosin tail homology domain (myo) which is thought to have an interaction with myosin II and is the largest domain of Kif 15 which was chosen for amplification, was successfully amplified by using a standard PCR program as mentioned in the Methods section from cDNA.

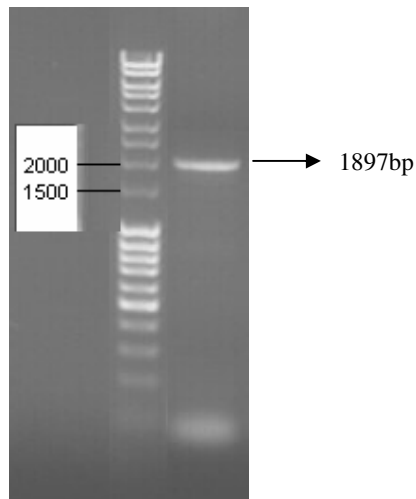


Figure 3.7: PCR results for myosin tail homology domain

Following PCRs, DNA fragments were cut out from the gel as clean as possible, (avoiding to cut excessive than needed). Gel extraction was performed using the Qiagen MinElute Gel Extraction Kit for motor and myosin tail homology domain. After the gel extraction, 1 % agarose gel was used to check the concentration of

DNA at the end of the process. The concentrations were also checked by spectrophotometer.

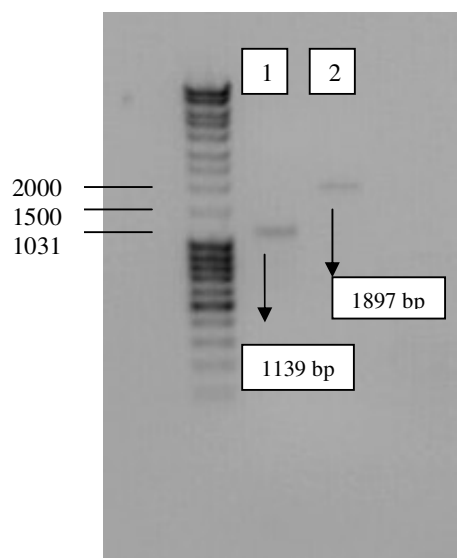


Figure 3.8: 1st lane: Gel extraction result for motor domain, 2nd lane: Gel extraction result for myosin tail homology domain

Motor and myosin tail homology domain DNA concentrations were calculated as follows:

$$C = OD_{260nm} \times \text{dilution factor} \times \text{constant (50 } \mu\text{g/ml)} = X \mu\text{g/ml}$$

For motor domain: $A_{260} = 0.006$

$$C = 0.006 \times 1000 \times 50 = 300 \mu\text{g/ml}$$

For myosin tail homology domain: $A_{260} = 0.015$

$$C = 0.015 \times 1000 \times 50 = 750 \mu\text{g/ml}$$

3.4.3. TA cloning for motor domain

After amplification of motor domain by gradient PCR, this PCR product was ligated into pTZ57R/T cloning vector using the InsTATM PCR Cloning Kit (Fermentas). Suitable conditions were created by following the manufacturer's recommendations as mentioned in the Methods section. Following overnight room temperature ligation, transformation was carried out. By the help of the blue white screening, white colonies were picked and they were scanned with colony PCR in order to check if they had inserts or not. Same set of PCR conditions which was used to amplify motor domain from cDNA was used in the colony PCR reaction.

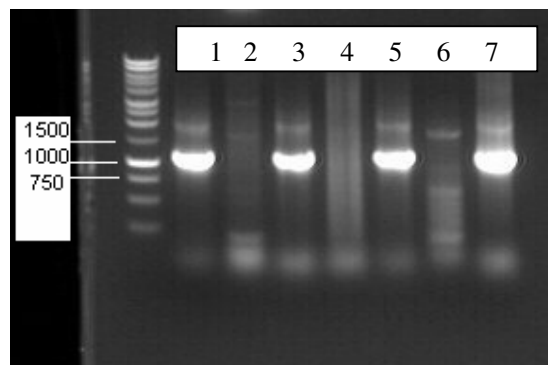


Figure 3.9: Colony PCR results for motor domain. PCR products found in lane 1,3,5 and 7 show that these colonies contain the insert.

After confirmation of the presence of the insert in these colonies, 5 ml LB-ampicillin overnight culture was prepared with colony number 1. The following day, 2 mls of this culture including pTZ57R/T-Motor domain construct, was used for plasmid isolation as recommended in Roche, High Pure Plasmid Isolation Kit. Glycerol stock was prepared as final concentration would be 20% as mentioned in the Methods section.

Presence of the insert was also confirmed by sequencing with M13F commercially available primer. First sequencing PCR was carried out for pTZ57R/T-Motor domain construct and then PCR purification for sequencing was performed. The sequencing result was checked by using ClustalW alignment program and, 100% similarity was observed.

Alignment of the motor domain in pTZ57R/T cloning vector and original motor sequence of Kif 15 was mentioned in table 3.3.

Table 3.3: Alignment results of motor domain in pTZ57R/T cloning vector

orjkif15	-----CAGCCAAGTAACGAAGATGATGCCATCAAAGTTTTGT	60
Kif15	-----CAGCCAAGTAATGAAGTGATGCCATCAAAGTTTTGT	60

orjkif15	GCGGATTCGCCCTGCTGAAGAGGGCGCTAGGTCAGCTGATGGAGAGCAGAGCCTCTGCTT	120
Kif15	GCGGATTCGCCCTGCTGAAGAGGGCGCTAGGTCAGCTGATGGAGAGCATAGCCTCTGCTT	120

orjkif15	GTCTGTGCTCTCCCAGACTGCTCTCCGGCTGCACCTCAACCCAGACCCCAAGACCTTTGT	180
Kif15	GTCTGTGCTCTCCCAGACTGCTCTCCGGCTGCACCTCAACCCAGACCCCAAGACCTTTGT	180

orjkif15	ATTGATTATGTTGCAGGCATGGACACCACTCAGGAGTCTGTATTTTCAACAGTGGCTAA	240
kif15	ATTGATTATGTTGCAGGCATGGACACCACTCAGGAGTCTGTATTTTCAACAGTGGCTAA	240

orjkif15	AAGCATTGTGGAGTCTTGCAATGAGTGGTTACAACGGGACCATCTTTGCATATGGGCAGAC	300
Kif15	AAGCATTGTGGAGTCTTGCAATGAGTGGTTACAACGGGACCATCTTTGCATATGGGCAGAC	300

orjkif15	TGGCTCTGGGAAGACATTTACTATGATGGGACCATCGGACTCTGATAATTTTCTCATAA	360
Kif15	TGGCTCTGGGAAGACATTTACTATGATGGGACCATCGGACTCTGATAATTTTCTCATAA	360

orjkif15	TCTAAGAGGAGTAATTCACGAAGTTTGAATATTTGTTTTCCTTAATTGATCGTGAAAA	420
Kif15	TCTAAGAGGAGTAATTCACGAAGTTTGAATATTTGTTTTCCTTAATTGATCGTGAAAA	420

orjkif15	TTCCAGGTCTCATGCAGTCTTTACAATCACAATAGAATCAATGGAGAAAAGCAGTGAGGC	704
Kif15	TTCCAGGTCTCATGCAGTCTTTACAATCACAATAGAATCAATGGAGAAAAGCAGTGAGGC	704

orjkif15	TGTGAACATTTCGGACCTCCTTACTCAACCTGGTGGACTTAGCAGGATCTGAGAGGCAAAA	764
kif15	TGTGAACATTTCGGACCTCCTTACTCAACCTGGTGGACTTAGCAGGATCTGAGAGGCAAAA	764

orjkif15	AGACACCCATGCAGAAGGGATGAGGTTGAAGGAGGCAGGTAACATAAACCGATCACTGAG	824
Kif15	AGACACCCATGCAGAAGGGATGAGGTTGAAGGAGGCAGGTAACATAAACCGATCACTGAG	824

orjkif15	CTGCCTGGGCCAAGTCATCACTGCCCTTGTTGATGTGGGCAATGGGAAACAGAGGCATGT	884
Kif15	CTGCCTGGGCCAAGTCATCACTGCCCTTGTTGATGTGGGCAATGGGAAACAGAGGCATGT	884

orjkif15	CTGCTACCGGGACTCCAACTTACCTTCTTACTTCGGGACTCCCTTGGTGGAATGCCAA	944
Kif15	CTGCTACCGGGACTCCAACTTACCTTCTTACTTCGGGACTCCCTTGGTGGAATGCCAA	944

orjkif15	AACAGCCATAATTGCAAATGTTTCATCCAGGATCCAGGTGTTTTGGGGAAACACTGTCAAC	1004
kif15	AACAGCCATAATTGCAAATGTTTCATCCAGGATCCAGGTGTTTTGGGGAAACACTGTCAAC	1004

orjkif15	ACTTAATTTTGCTCAAAGAGCCAAGCTGATTAATAACAAGGCTGTGGTAAATGAAGACAC	1064
kif15	ACTTAATTTTGCTCAAAGAGCCAAGCTGATTAATAACAAGGCTGTGGTAAATGAAGACAC	1064

orjkif15	CCAAGGAAATGTGAGCCAGCTACAAGCTGAAGTGAAGAGACTCAGAGAACAACCTGTCTCA	1124
kif15	CCAAGGAAATGTGAGCCAGCTACAAGCTGAAGTGAAGAGACTCAGAGAACAACCTGTCTCA	1124

3.4.4. PCR for tail domain

Tail domain was amplified from cDNA which was synthesised from total RNA isolated from adult rat brain by using sequence specific primers Tail F and Tail R (see Materials and Methods section) Standard PCR was used in amplification as its conditions were mentioned in the Methods section.

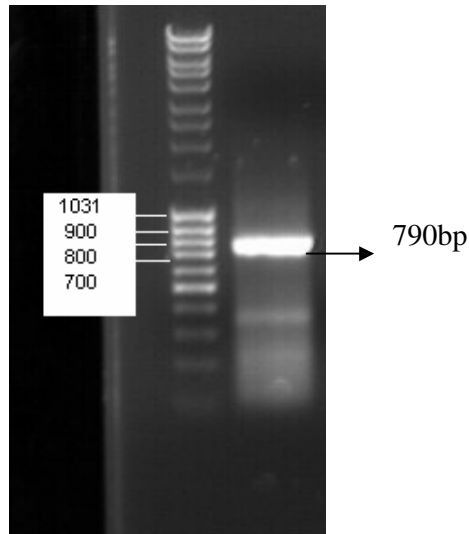


Figure 3.10: PCR result of tail domain.

After PCR, the PCR product was carefully cut out from the gel and following the instructions of Qiagen MinElute Gel Extraction Kit, gel extraction was carried out. Eluted tail DNA was run on 1% agarose gel in order to observe the extraction efficiency.

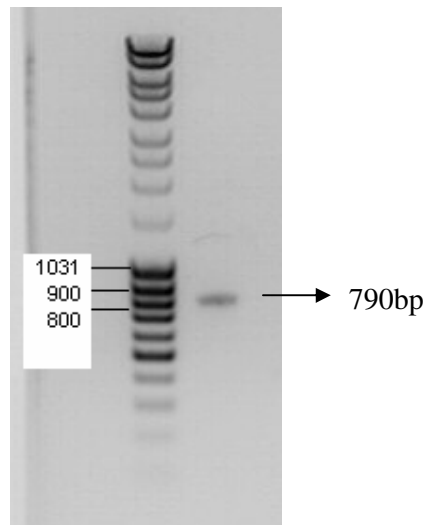


Figure 3.11: 1% agarose gel photograph showing the tail domain of Kif15 after gel extraction.

3.4.5. TA cloning for tail domain.

Following the gel extraction, tail domain was ligated into pTZ57R/T cloning vector by using the InsTATM PCR Cloning Kit (Fermentas). After transformation, 5 white and 2 blue colonies were observed on plate. Based on the blue white screening

principle, 5 white colonies were selected and colony PCR was performed to check if these colonies had the insert. Same set of PCR conditions which was used to amplify tail domain from cDNA was used in colony PCR reaction.

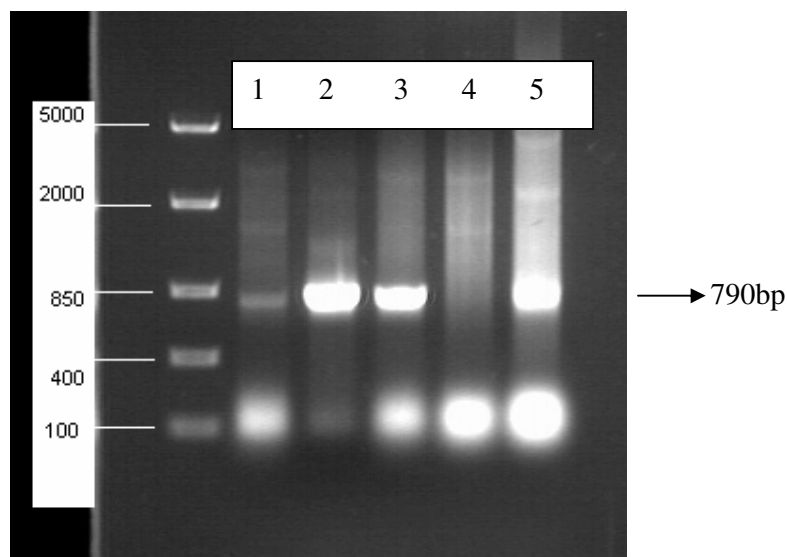


Figure 3.12: Colony PCR results for tail domain. PCR products in lanes 2,3 and 5 shows that these colonies contain the insert

Colony number 2 was taken to a 5 ml overnight LB-ampicillin culture and the following day plasmid was isolated by using High Pure Plasmid Isolation Kit (Roche) following the instructions mentioned in methods section. In addition, same culture was used to prepare the glycerol stock as final concentration would be 20%.

Although the insert's presence was confirmed by colony PCR, it was also confirmed by sequencing with M13F primer. Thus, sequence PCR then purification of PCR product were carried out for pTZ57R/T -Tail domain construct.

Sequence result was checked by using ClustalW alignment program. Tail domain's sequence in pTZ57R/T cloning vector was compared with the original Kif15 tail domain sequence and 100% similarity was observed. (see table 3.4.)

Table 3.4: Sequence alignment of tail domain in pTZ57R/T

kif15	-----GTGATGGGCTCTGCTCCTGAGTATCCACAGAGTCCAAAAACACCACCCCAT	60
orjkif15	-----GTGATGGGCTCTGCTCCTGAGTATCCACAGAGTCCAAAAACACCACCCCAT	60

kif15	TTTCAAACCTCATTGGCAAACTCTTGGAAACACAAGAACAAGAGATAGAAGACGGAAGG	120
orjkif15	TTTCAAACCTCATTGGCAAACTCTTGGAAACACAAGAACAAGAGATAGAAGACGGAAGG	120

kif15	GCATCTAAGATGTCTTTGCAACACCTCGTCACAAAGCTAAACGAAGACAGAGAAGTCAAA	180
orjkif15	GCATCTAAGATGTCTTTGCAACACCTCGTCACAAAGCTAAACGAAGACAGAGAAGTCAAA	180

kif15	AACGCTGAAATCCTCAGGATGAAGGATCAGTTGTGTGAAATGGAAAATCTGCGCCTGGAG	240
orjkif15	AACGCTGAGATCCTCAGGATGAAGGATCAGTTGTGTGAAATGGAAAATCTGCGCCTGGAG	240

kif15	TCTCAGCAGTTGAGAGAGAGAACCTGGCTCCTGCAGACTCAGCTGGATGATGAAGAGG	300
orjkif15	TCTCAGCAGTTGAGAGAGAGAACCTGGCTCCTGCAGACTCAGCTGGATGATGAAGAGG	300

kif15	CAGGGGAGAGCAGCAGTCAGAGTCGTCAGACAGCCAACAGCTGAAGAATGAATATGAA	360
orjkif15	CAGGGGAGAGCAGCAGTCAGAGTCGTCAGACAGCCAACAGCTGAAGAATGAATATGAA	360

kif15	GAGGAGATTATCAGAGAGAGACTTGCTAAAAATAAACTTATTGAAGAAATGCTAAAAATG	420
orjkif15	GAGGAGATTATCAGAGAGAGACTTGCTAAAAATAAACTTATTGAAGAAATGCTAAAAATG	420

kif15	AAAACAGATCTAGAGGAAGTCCAGAGTGCACCTTGACAGCAAGGAGAAGTTCTGCCACAGA	480
orjkif15	AAAACAGATCTAGAGGAAGTCCAGAGTGCACCTTGACAGCAAGGAGAAGTTCTGCCACAGA	480

kif15	ATGAGCGAGGAAGTTGAGCGAACCAGAACTTGGAGTCCAGAGCATTTCAGGAAAAGGAG	540
orjkif15	ATGAGCGAGGAAGTTGAGCGAACCAGAACTTGGAGTCCAGAGCATTTCAGGAAAAGGAG	540

kif15	CAATTGCGGTCAAAGTTGGAAGAAATGTATGAAGAGAGAGAGAGAACTTGCCAGGAAATG	600
orjkif15	CAATTGCGGTCAAAGTTGGAAGAAATGTATGAAGAGAGAGAGAGAACTTGCCAGGAAATG	600

kif15	GAAATGCTGAGAAAAACAACCTAGAGTTTCTTGCTGAGGAAAATGGGAAATTGATAGTTCAT	660
orjkif15	GAAATGCTGAGAAAAACAACCTAGAGTTTCTTGCTGAGGAAAATGGGAAATTGATAGTTCAT	660

kif15	CAAAACCTACATCAGAAGATTCAATATGTAGTGCGACTGAAGAAGGAAAAATATCAGGCTT	720
orjkif15	CAAAACCTACATCAGAAGATTCAATATGTAGTGCGACTGAAGAAGGAAAAATATCAGGCTT	720

kif15	GCTGAGGAGACAGAAAAATTGCGTGCTGAAAATGTATTTTGAAGAAAGGAAAAAGAA	780
orjkif15	GCTGAGGAGACAGAAAAATTGCGTGCTGAAAATGTATTTTGAAGAAAGGAAAAAGAA	780

kif15	TGA	
orjkif15	TGA	

After completing the sequence alignments, it was observed that motor domain was inserted into pTZ57R/T cloning vector in reverse orientation while tail domain was inserted in straight orientation. This information is quite important not only for deciding the enzymes which will be used in the restriction reaction, but also for deciding the suitable expression vectors. If an insert is in reverse orientation, it has to be converted into straight position in expression vector in order to be expressed.

3.5. Subcloning of Kif15 motor and tail domains into GFP expression vectors

3.5.1. Subcloning of motor domain

Restriction for motor domain and the expression vector chosen, was performed with KpnI and PstI restriction enzymes. pEGFP-C1 was chosen as the expression vector suitable for motor domain to be inserted. These enzymes and the expression vector were chosen by considering the following points:

- ✓ These enzymes were not cutting the motor domain.
- ✓ Since this domain located in reverse orientation in cloning vector, the cutting sites of these enzymes had to be reverse in expression vector. Only then, it would be possible to straighten the orientation. (See pTZ57R/T and pEGFP-C1 restriction maps)
- ✓ These enzymes both cut the cloning and expression vector which is favorable in recognition of the cut ends in ligation.
- ✓ pEGFP-C1 had the suitable frame that prevents shifting of the motor domain sequence when inserted.

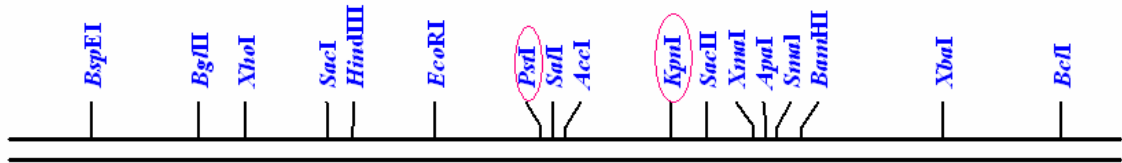


Figure 3.13: pEGFP-C1 expression vector, multiple cloning site (MCS)

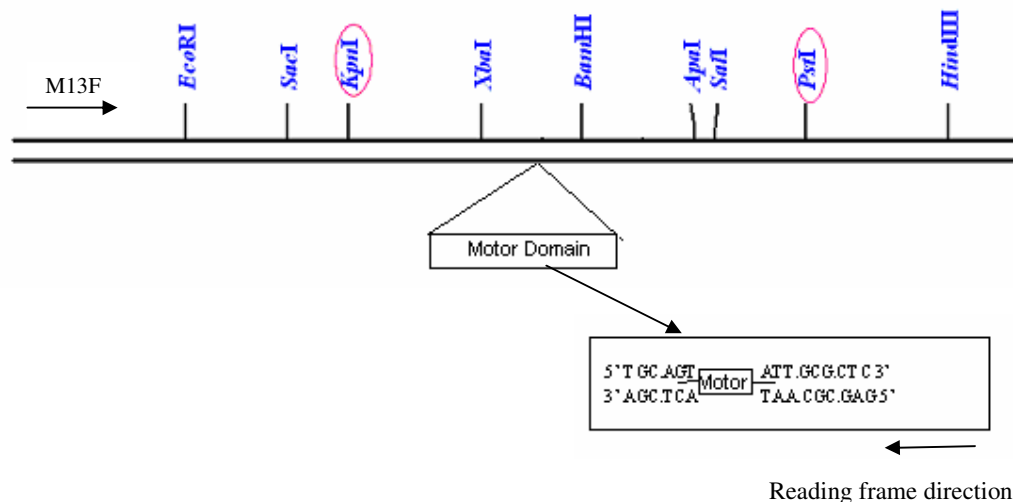


Figure 3.14: pTZ57R/T-Motor domain construct

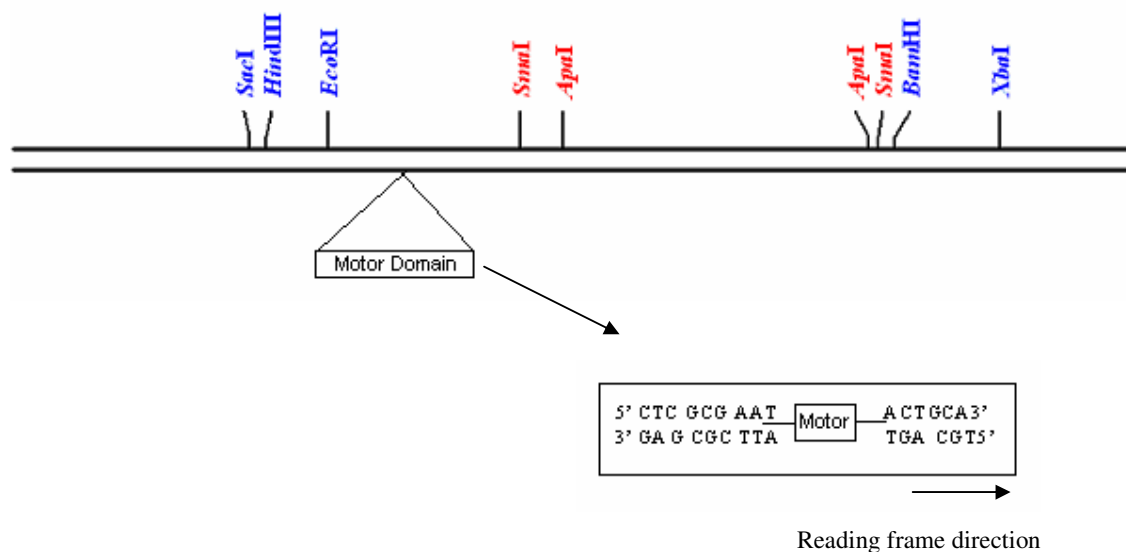


Figure 3.15: pEGFP-C1/Motor domain construct

By considering these, pTZ57R/T-Motor construct and pEGFP-C1 expression vector was cut with PstI and KpnI enzymes.

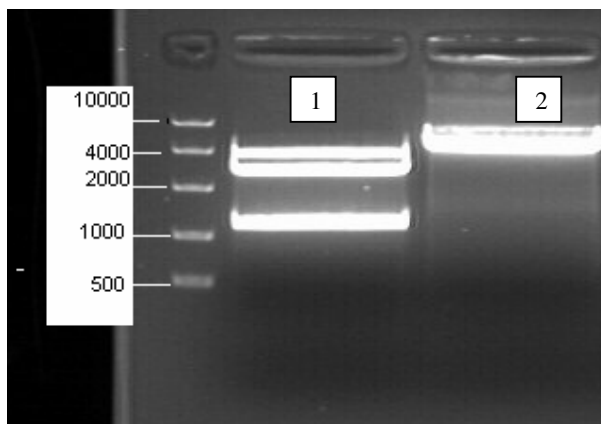


Figure 3.16: 1st lane: Gel electrophoresis showing the restriction result of pTZ57R/T-Motor construct
2nd lane: Gel electrophoresis showing the restriction result of pEGFP-C1 expression vector.

After restriction, DNA fragments were cut out from the gel and gel extraction was performed by using Qiagen MinElute Gel Extraction Kit. In order to check DNA concentrations following gel extraction, 1% agarose gel was used. This is important for setting the ligation reaction.

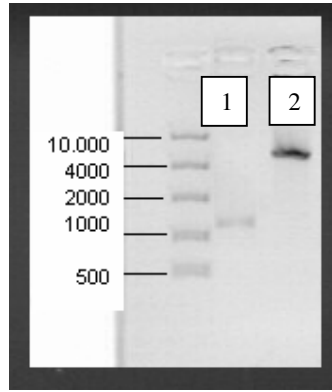


Figure 3.17: 1st lane: Gel extraction result of pTZ57R/T-Motor construct, 2nd lane: Gel extraction result of pEGFP-C1 expression vector

According to these results, concentrations were calculated and ligation reaction was set as mentioned in the Methods section. Following ligation, transformation was carried out and colonies grown on LB-agar plate were checked to find out if they had the insert or not. For this purpose, colony PCR was performed.

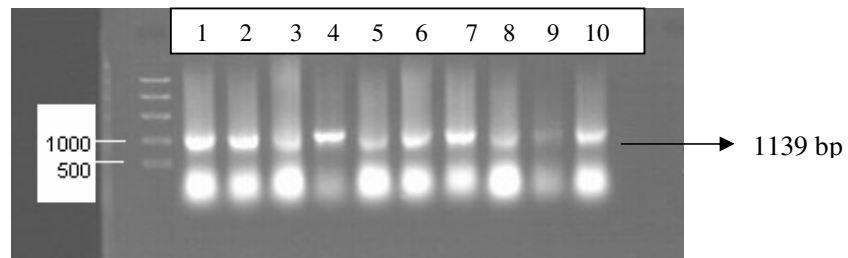


Figure 3.18: Colony PCR results for motor domain.

From the gel, it was observed that most colonies had the insert. Following the colony PCR, colony number 4, which is indicated in the 4th lane, was picked for 5ml overnight LB –kanamycine culture. The next day, this culture was used for plasmid isolation. Following the High Pure Plasmid Isolation Kit (Roche) instructions, plasmid isolation was performed and glycerol stock was prepared as mentioned before.

Following plasmid preparation, confirmation restriction was carried out with the same enzymes used for restriction reaction.

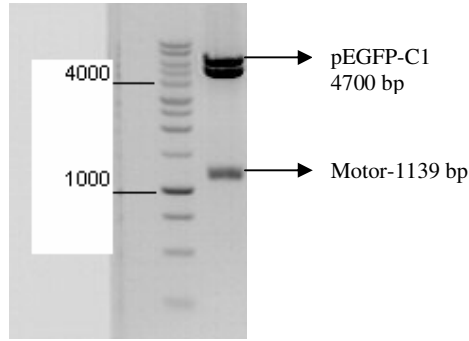


Figure 3.19: 1% agarose gel showing the confirmation restriction result

In addition, pEGFP-C1/Motor construct was checked by sequencing with commercially available pEGFP-C primer. ClustalW program was used for alignment and 100% similarity was observed between the motor domain in expression vector and the original motor sequence. (see table 3.5.)

Table 3.5: Alignment results for motor domain in pEGFP-C1

seq	-----CAGCCAAGTAACGAAGGTG	120
orj	-----CAGCCAAGTAACGAAGATG	19

seq	ATGCCATCAAAGTTTTTGTGCGGATTGCGCCCTGCTGAAGAGGGCGCTAGGTCAGCTGATG	180
orj	ATGCCATCAAAGTTTTTGTGCGGATTGCGCCCTGCTGAAGAGGGCGCTAGGTCAGCTGATG	79

seq	GAGAGCAGAGCCTCTGCTTGTCTGTGCTCTCCAGACTGCTCTCCGGCTGCACTCCAACC	240
orj	GAGAGCAGAGCCTCTGCTTGTCTGTGCTCTCCAGACTGCTCTCCGGCTGCACTCCAACC	139

seq	CAGACCCAAGACCTTTGTATTGATTATGTTGCAGGCATGGACACCACTCAGGAGTCTG	300
orj	CAGACCCAAGACCTTTGTATTGATTATGTTGCAGGCATGGACACCACTCAGGAGTCTG	199

seq	TATTTTCAACAGTGGCTAAAAGCATTGTGGAGTCTTGCATGAGTGGTTACAACGGGACCA	360
orj	TATTTTCAACAGTGGCTAAAAGCATTGTGGAGTCTTGCATGAGTGGTTACAACGGGACCA	259

seq	TCTTGCATATGGGCAGACTGGCTCTGGGAAGACATTTACTATGATGGGACCATCGGACT	420
orj	TCTTGCATATGGGCAGACTGGCTCTGGGAAGACATTTACTATGATGGGACCATCGGACT	319

seq	CTGATAATTTTCTCATAATCTAAGAGGAGTAATTCCACGAAGTTTTGAATATTTGTTTT	480
orj	CTGATAATTTTCTCATAATCTAAGAGGAGTAATTCCACGAAGTTTTGAATATTTGTTTT	379

seq	CCTTAATTGATCGTGAAAAAGAAAAGGCTGGAGCTGGGAAGAGTTTCCTTTGTAAGTGCT	540
orj	CCTTAATTGATCGTGAAAAAGAAAAGGCTGGAGCTGGGAAGAGTTTCCTTTGTAAGTGCT	439

seq	CCTTCATTGAGGTCTACAATGAGCAGATATACGACCTGCTGGACTCTGCATCGGTGCGAC	599
orj	CCTTCATTGAGGTCTACAATGAGCAGATATACGACCTGCTGGACTCTGCATCGGTGCGAC	499

seq	TGTATTTTAANAGAGCACATAAAAGAAAGGGCGTCCTCCTTGGGTTGGTGCAATGGAAGC	659
orj	TGTATTTTAAGAGAGCACATAAAAGAAAGGGCGTCCTCCTTGGGTTGGTGCAATGGAAGC	553

seq	AGGTGGTGGGCCCCAGCTGCTGAAAGCCTACCAGGTCTTATCTAGAGGCTGGAGGAACA	719
orj	AGGTGGTGGGCCCCAGCTGCTGAAAGCCTACCAGGTCTTATCTAGAGGCTGGAGGAACA	610

3.5.2. Subcloning of Tail Domain

Restriction for tail domain and pEGFP-C1 expression vector was carried out with EcoRI and SmaI restriction enzymes. These enzymes and the expression vector were chosen by considering the following points:

- ✓ Tail sequence did not include these enzyme's restriction sites.
- ✓ Since tail domain located in straight orientation in cloning vector, the enzyme cutting sites were chosen exactly in the same orientation with the expression vector. (See Figure 3.20. and 3.21.)
- ✓ These enzymes both cut the cloning and expression vector which is favorable in recognition of the cut ends in ligation.
- ✓ pEGFP-C2 had the suitable frame that prevents shifting of the tail domain sequence when inserted (See Figure 3.22.).

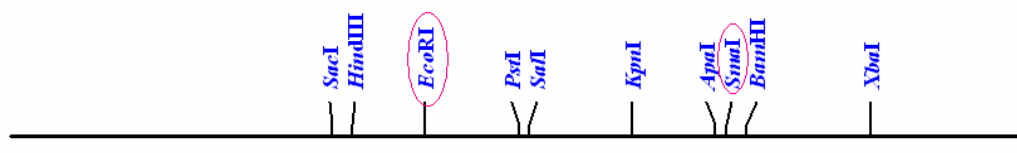


Figure 3.20: pEGFP-C2 expression vector MCS

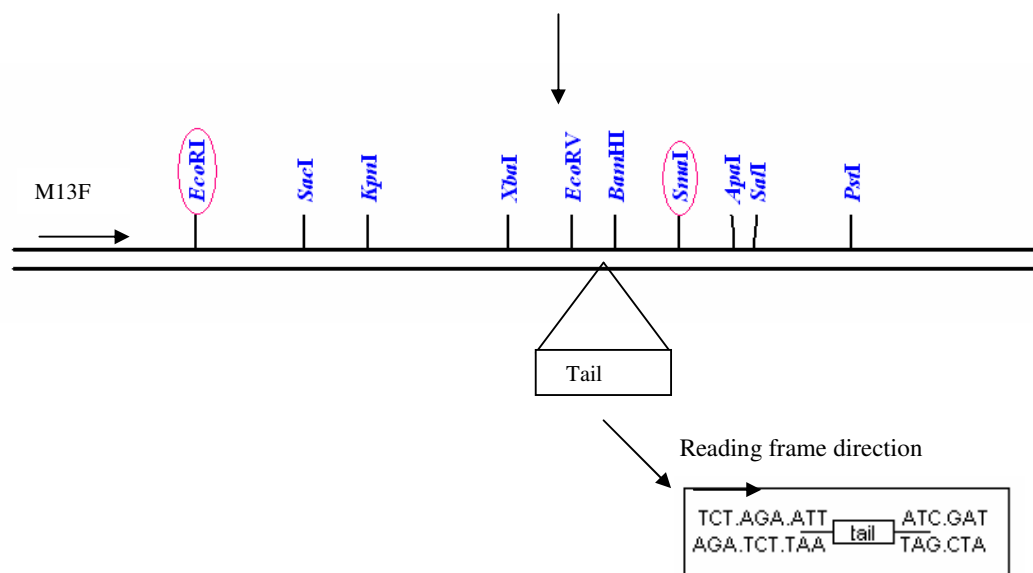


Figure 3.21: pTZ57R/T cloning vector MCS

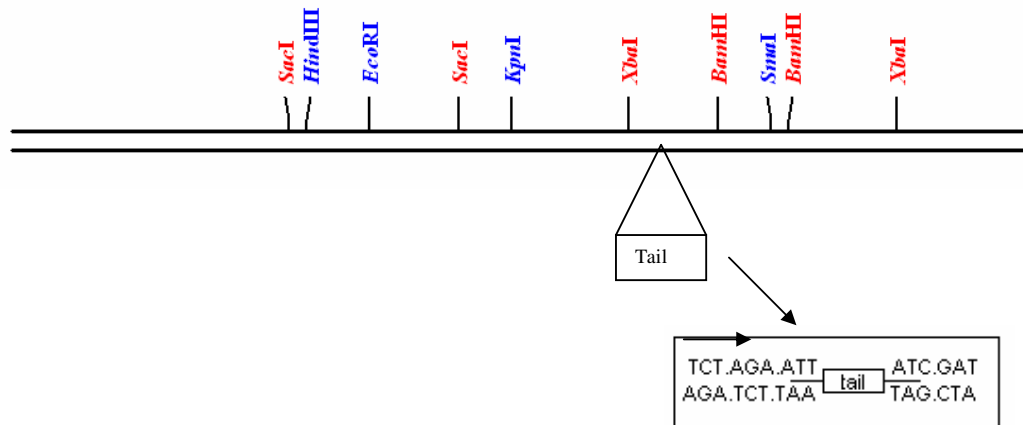


Figure 3.22: pPEGFP-C2/Tail construct

By considering these, pTZ57R/T-Tail construct and pEGFP-C2 expression vector were cut with EcoRI and SmaI enzymes.

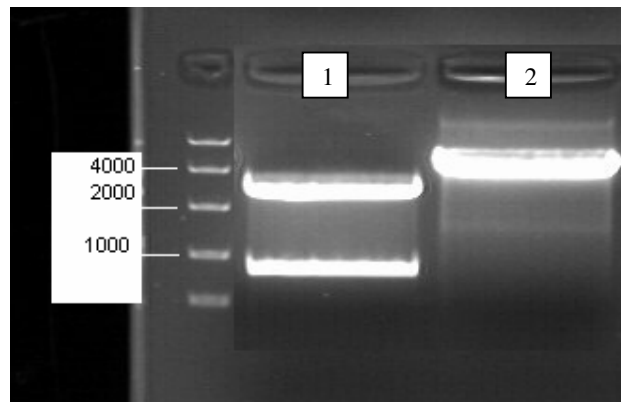


Figure 3.23: 1st lane: the restriction reaction result for pTZ57R/T-Tail construct, 2nd lane: pEGFP-C2 expression vector

These DNAs were cut out from the gel and gel extraction was carried out.

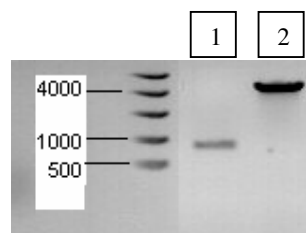


Figure 3.24: 1st lane: Gel extraction results for tail domain, 2nd lane: Gel extraction results for pEGFP-C2 expression vector

After the restriction and gel extraction processes, different ligation reactions were set considering the DNA concentration of the vector and the insert. Thus, different insert to vector ratios were carried out as mentioned in methods section in order to find the most suitable condition. Following ligation, transformation was performed and colonies observed on LB-agar plates were checked with colony PCR.

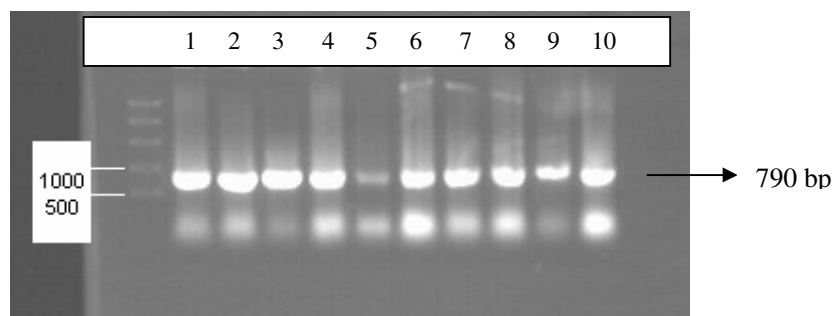


Figure 3.25: Colony PCR results of the tail domain

Since, all DNA fragments observed in the gel were in expected size, it was concluded that all colonies had the insert.

Colony 3 was selected for overnight LB-kanamycine culture. The following day plasmid was isolated by using the High Pure Plasmid Isolation Kit (Roche) and glycerol stock for pEGFP-C2/Tail construct was prepared.

After plasmid isolation, pEGFP-C2/Tail construct was cut with SmaI and EcoRI for confirmation.

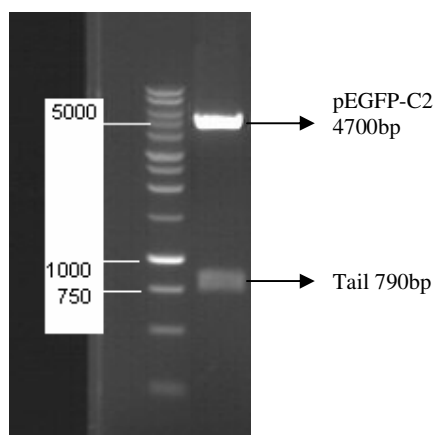


Figure 3.26: Confirmation restriction result for tail domain.

In addition to this confirmation, pEGFP-C2/Tail construct was also confirmed with sequencing with commercially available pEGFP-C primer, to show the existence of insert in the expression vector.

After alignment with original Kif15 tail domain sequence, by using ClustalW program, 100% similarity was observed. (see table 3.6.)

Table 3.6: Alignment of tail domain in pEGFP-C2

tail	TCAGCTCAAGCTTCGAATTCGAGCTCGGTACCTCGCGAATGCATCTAGCTGGAGCACGAG	120
orj	-----GCTGGAGCACGAG	11

tail	TGATGGGCTCTGCTCCTGAGTATCCACAGAGTCCAAAAACACCACCCCATTTTCAAATC	180
orj	TGATGGGCTCTGCTCCTGAGTATCCACAGAGTCCAAAAACACCACCCCATTTTCAAATC	71

tail	ATTGGCAAACTCTTGGAACACAAGAACAAGAGATAGAAGACGGAAGGGCATCTAAGA	240
orj	ATTGGCAAACTCTTGGAACACAAGAACAAGAGATAGAAGACGGAAGGGCATCTAAGA	131

tail	TGTCTTTGCAACACCTCGTCACAAAGCTAAACGAAGACAGAGAAGTCAAAAACGCTGAGA	300
orj	TGTCTTTGCAACACCTCGTCACAAAGCTAAACGAAGACAGAGAAGTCAAAAACGCTGAGA	191

tail	TCCTCAGGATGAAGGATCAGTTGTGTGAAATGGAAATCTGCGCCTGGAGTCTCAGCAGT	360
orj	TCCTCAGGATGAAGGATCAGTTGTGTGAAATGGAAATCTGCGCCTGGAGTCTCAGCAGT	251

tail	TGAGAGAGAGAACCTGGCTCCTGCAGACTCAGCTGGATGATATGAAGAGGCAGGGGGAGA	420
orj	TGAGAGAGAGAACCTGGCTCCTGCAGACTCAGCTGGATGATATGAAGAGGCAGGGGGAGA	311

tail	GCAGCAGTCAGAGTCGTCCAGACAGCCAACAGCTGAAGAATGAATATGAAGAGGAGATTA	480
orj	GCAGCAGTCAGAGTCGTCCAGACAGCCAACAGCTGAAGAATGAATATGAAGAGGAGATTA	371

tail	TCAGAGAGAGACTTGCTAAAAATAAACTTATTGAAGAAATGCTAAAAATGAAAACAGATC	540
orj	TCAGAGAGAGACTTGCTAAAAATAAACTTATTGAAGAAATGCTAAAAATGAAAACAGATC	431

tail	TAGAGGAAGTCCAGAGTGCACCTTGACAGCAAGGAGAAGTTCTGCCACAGAATGAGCGAG	600
orj	TAGAGGAAGTCCAGAGTGCACCTTGACAGCAAGGAGAAGTTCTGCCACAGAATGAGCGAG	490

tail	GAAGTTGAGCGAACCAGAACTTTGGGAGTCCAGAGCATTTCAGGAAAAGGGAGCAATTGC	660
orj	GAAGTTGAGCGAACCAGAACTTTGGCAGTCCAGAGCATTTCAGGAAAAGGGAGCAATTGC	548

tail	GGTCAAAGTTTGGAAGAAATGTNTGAAGAGAGAGAGAGAACTTGCCAGGAAATGGAAATG	720
orj	GGTCAAAGTTTGGAAGAAATGTATGAAGAGAGAGAGAGAACTTGCCAGGAAATGGAAATG	607

tail	CTGAAAAACAACCTAGAGTTTCTTGCTGAGG-----	751
orj	CTGAAAAACAACCTAGAGTTTCTTGCTGAGGAAATGGGAAATGATAGGTCATCAAAAC	667

4. DISCUSSION

4.1. RNA isolation from adult rat brain tissue

RNA isolation from adult rat brain was performed twice, since there were no clear RNA observed on the gel after the first trial. (see figure 4.1.) The possible reasons could be RNA degradation, unsatisfactory cell disruption or inadequate starting amount of brain tissue.

Undesirable RNAses readily found in the environment could cause RNA degradation. In addition, since cell disruption and homogenization are the first and one of the most critical steps affecting yield and quality of the isolated RNA, unsatisfactory cell disruption and homogenization could lead to this problem. Another possibility could be the inadequate tissue amount. Since the brain tissue amount needed for RNA isolation was very little, maybe the needed amount was not decided properly by estimation.

After the second trial, two clear RNA fragments were observed on the gel. (see figure 4.1.) Although the yield of the RNA did not seem to be perfect, since it was intact, this RNA was used for cDNA synthesis and satisfactory results were obtained in the control PCR reaction.

Spectrophotometric RNA concentration measurement was not performed because the integrity of the RNA could not be understood by measuring the concentration of the RNA. Hence, based on the spectrophotometric analysis, the result could have been fallacious, and since degraded RNA could not be used for cDNA synthesis, the gel result was taken into consideration.

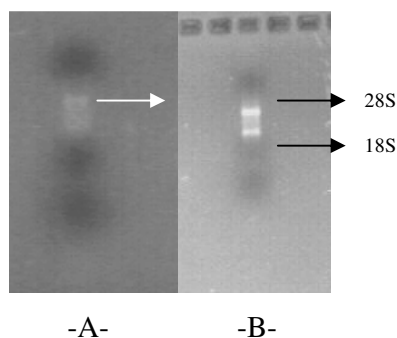


Figure 4.1: A) Result of the RNA isolation after first trial. Arrow indicates the slight RNA fragments which should have been clear and intact B) Result of the RNA isolation after second trial. Arrows indicate the 18S and 28S rRNAs

4.2. Cloning of Motor Domain

In order to amplify motor domain from the cDNA; the following PCR program (program no:1, see table 4.1.) with the amounts previously mentioned in the Methods section was performed and the PCR result mentioned in figure 4.2. was obtained.

Table 4.1: PCR run program for motor domain No:1

Program No:1			40 cycles
Initial Denaturation	94°C	30 sec	
Denaturation	94°C	1 min	
Annealing	55°C	1 min	
Elongation	72°C	2 min	
Final Elongation	72°C	5 min	

Note: Melting temperatures (T_m) for motor domain's forward and reverse primers were 62°C and 66°C respectively.

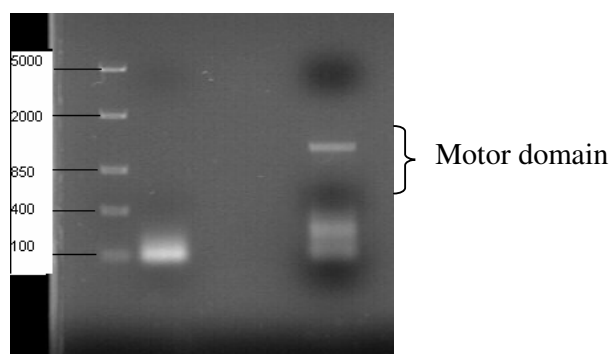


Figure 4.2: Agarose gel photograph showing the result of motor domain PCR from the cDNA.

After this PCR although there were some unspecific products (most probably primer dimers), since the motor domain DNA was clearly observed in the correct size on the gel, gel extraction was performed for this fragment. Following the gel extraction, eluted DNA was run on a 1% agarose gel as shown in figure 4.3.

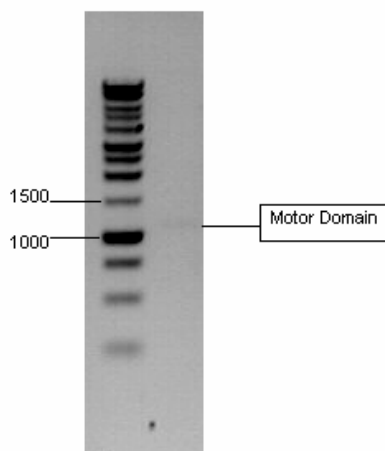


Figure 4.3: Gel extraction result for motor domain.

DNA observed on the gel after gel extraction was slightly visible. Although Roche High Pure Plasmid Isolation Kit was used for plasmid preparation and every step of the process was followed carefully, generally it was observed that the DNA concentration after gel extraction was always a lot lower than the concentration at the beginning. Since the beginning concentration of the DNA obtained after PCR amplification is low itself, this gel extraction result was reasonable. In addition, since the contribution of excessive gel pieces is highly possible and it directly effects the yield and quality of the DNA obtained after the process, this could also be a reason for unsatisfactory gel extraction result.

In order to optimize the PCR reaction, several different PCRs were performed by changing the conditions PCR reactions. Especially annealing temperatures were changed. Since the optimum amount of cDNA used as a template in these reactions was decided by control PCR reaction mentioned in the results section, this amount was not changed. In addition, no significant changes were carried out for other PCR contents.

Considering the T_m values of the primers designed for motor domain, the cDNA synthesized from the whole rat brain RNA was used as a template and annealing temperature of the programs was changed from 56°C to 62°C by increasing 1°C in each PCR in order to find the optimum annealing temperature. The reason why we increase the annealing temperatures is that higher temperatures minimize nonspecific primer annealing and increase the amount of specific products produced while reducing the amount of primer-dimer formation.

No satisfactory results were obtained from these trials. Since there were no DNA fragments, even slight, and only DNA observed on gels were primer-dimers, it was thought that the reason of these results could be the primers that could not anneal to the desired sequences. Thus, in order to find the optimum annealing temperature, firstly a touch-down PCR was performed with annealing temperatures starting from 60°C and decreased to 54°C and since no results were obtained after touch-down PCR, secondly, a new PCR program (program no:2) in which annealing temperatures were gradually increased from 54°C to 58°C considering the same idea behind increasing the annealing temperatures 1°C in each PCR, while searching for the suitable annealing temperature was performed for motor domain as shown in table 4.2. By using this program PCR result mentioned in figure 4.4. was obtained.

Table 4.2: PCR run program for motor domain No:2

Program No: 2										
Initial Denaturation	94	1 min.								
Denaturation	94	30 sec.	94	30 sec.	94	30 sec.	94	30 sec.	94	30 sec.
Annealing	54	1 min.	55	1 min.	56	1 min	57	1 min.	58	1 min.
Elongation	72	2 min.	72	2 min	72	2 min	72	2 min.	72	2 min.
Final Elongation	72	10 min.								
Note: All values are °C.										

} 10 cycles
each

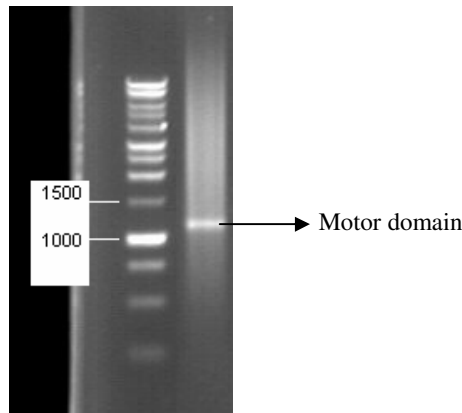


Figure 4.4: Agarose gel showing the PCR result

PCR product in expected size was obtained but a DNA smear was also observed on gel. The reasons of such smear could be inappropriate template amount or impurity. Since the DNA fragment in expected size was clearly observed in spite of the smear, this fragment was cut carefully and gel purified in order to be used as a template in a new reaction. Hence, if the problem was purity we would have had a purer template for next reactions. In addition, since the gel result (see figure 4.4.) proved that the fragment observed on gel was certainly our interested domain and since it was once amplified from cDNA, by using the same primers we could increase the annealing probability of primers and better results could be obtained than PCR results which cDNA was used as a template. Moreover, the DNA which was amplified from cDNA and gel purified (see fig. 4.3.) was also used as a template in a new reaction. Thus 2 new PCRs by using 2 different gel extracted DNAs as templates were performed by using program 2 as shown in figure 4.5.

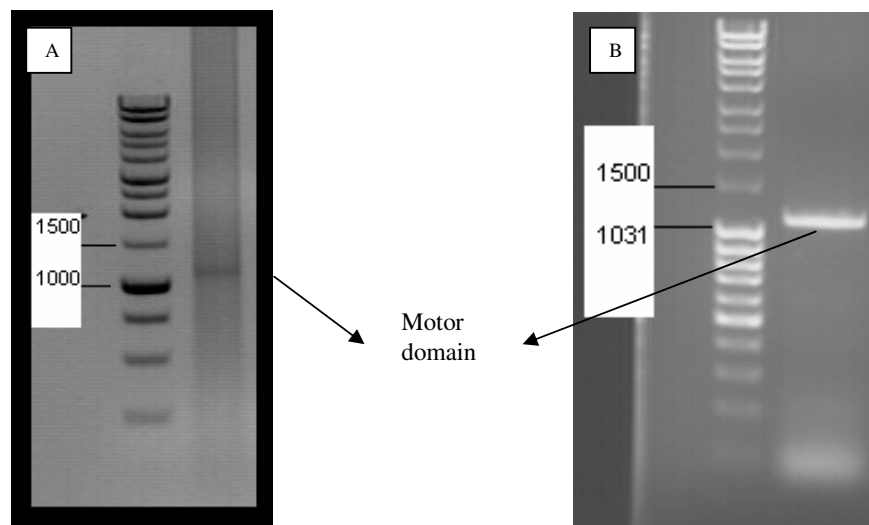


Figure 4.5: A) PCR result, where DNA extracted from the gel in Figure 4.4 was used as a template.
B) PCR result, where DNA in Figure 4.3. was used as a template

After checking the PCR products on gel, a slight DNA fragment was observed for the first reaction and a clear DNA fragment was observed for the second reaction. Thus, the second one was gel purified (see figure 4.6.) and used in TA cloning reactions.

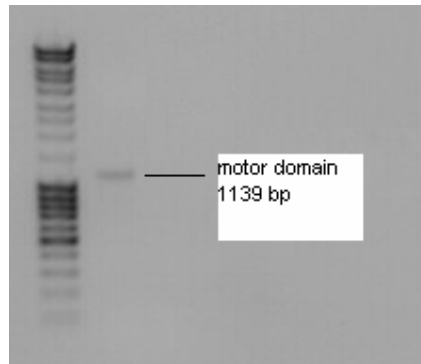


Figure 4.6: Gel extraction result for second reaction

4.3. Subcloning of motor and tail domain into GFP vectors

After deciding the suitable enzymes and vectors that should be used for subcloning, related GFP vectors and pTZ57R/T constructs were digested with the enzymes mentioned in the methods section.

Digestion reactions were run on the agarose gel in order to cut out the insert and GFP vector for further applications (gel extraction and then ligation).

Several restriction and ligations were performed in order to obtain desired results.

4.3.1. Optimization for subcloning of motor domain into GFP vector

In the first trial, appropriate vector (pEGFP-C1) and insert (pTZ57R/T-Motor construct) DNAs were cut with KpnI and PstI enzymes. Following digestion, the results were checked on gel as shown in figure 4.7.

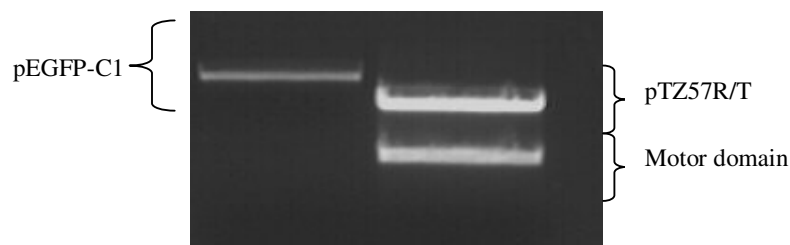


Figure 4.7: Gel electrophoresis showing the result of digestion reaction for motor domain

After digestion, gel extraction was performed as shown in figure 4.8.

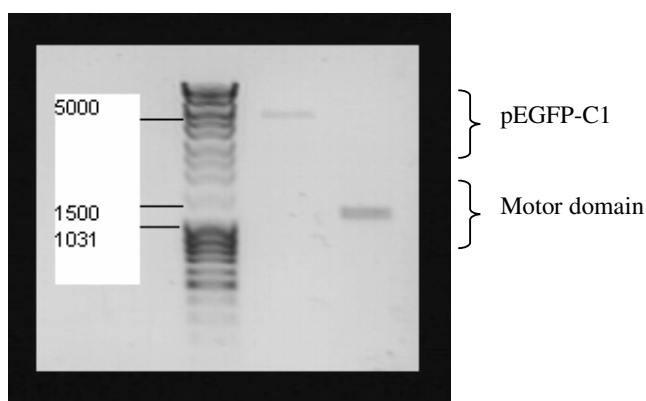


Figure 4.8: Gel extraction result for motor domain.

Since the vector DNA concentration observed on gel was insufficient for ligation reaction, more vector was digested.(see figure 4.9.).Following this step gel extraction was performed as mentioned in figure 4.10.

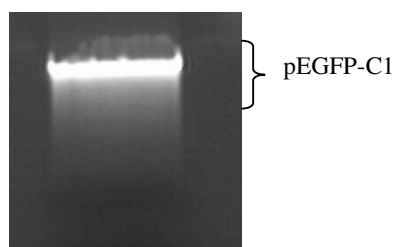


Figure 4.9: Restriction result for pEGFP-C1 expression vector.

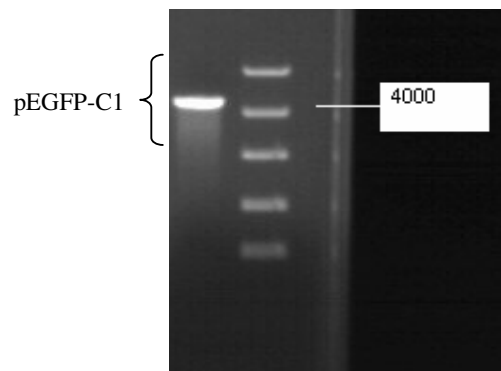


Figure 4.10: Gel extraction result for pEGFP-C1 expression vector.

After the gel extraction for pPEGFP-C1 vector, since the concentration observed was sufficient on gel, 3 different ligation reactions were set as mentioned below in table 4.3.

Table 4.3: 3 Different ligation reactions for motor domain

1 st ligation reaction		2 nd ligation reaction		3 rd ligation reaction	
Content	Amount- μl	Content	Amount- μl	Content	Amount- μl
pEGFP-C1 (100ng)	5	pEGFP-C1	1	pEGFP-C1	2
Motor domain (25ng)	12	Motor domain	5	Motor domain	8
10X ligation buffer	2	10X ligation buffer	1	↓ *	
T4 ligase	1	T4 ligase	0.5		
		dH ₂ O	2.5	10X ligation buffer	2
				T4 ligase	3
				PEG	2
				dH ₂ O	3
Total reaction	20	Total reaction	10	Total reaction	20
↓		↓		↓	
Incubated overnight (O/N) at room temperature (RT)				Incubated O/N at 4°C	

*Insert and vector were mixed and put at 65°C for 5 min. Then they were put on ice for 10 min.

- ✓ In the first ligation reaction, insert amount was selected as almost 3 three fold excess of the vector's amount as usually recommended in ligation reactions.
- ✓ In the second reaction, 5 five fold excess insert was added to the mixture, in order to increase the possibility of integration.
- ✓ In the third reaction, 4 fold excess insert was used. In addition PEG was used. Because PEG is a volume excluder, hence your DNA and enzyme are concentrated into a smaller volume than you set up and this increases the ligation efficiency. The reason of the incubation of the vector and insert at 65°C is to facilitate the denaturation of the helices and putting them on ice after denaturation is to increase the possibility of the integration.

Since no satisfactory results were obtained after transformations performed for these ligations, this time it was decided to digest more motor construct in order to increase the concentration obtained after gel extraction. Result after restriction is shown below in figure 4.11.

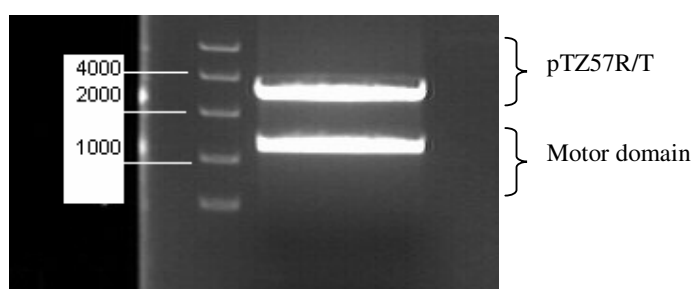


Figure 4.11: Agarose gel electrophoresis showing the restriction result of the construct.

After restriction, motor domain was cut out from the gel and gel extraction was performed as shown in figure 4.12.

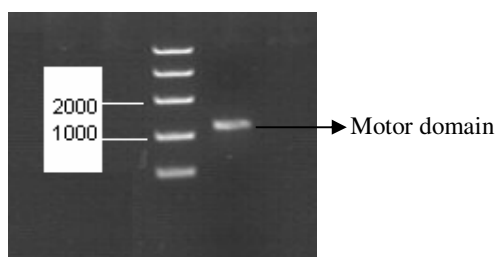


Figure 4.12: Agarose gel electrophoresis showing the gel extraction result of the motor domain

Following this digestion and gel extraction, motor DNA obtained from this gel extraction and pEGFP-C1 vector mentioned in figure 10 were used in different ligation reactions as shown in table 4.4.

Table 4.4: 5 Different ligation reactions for motor domain

1 st ligation reaction		2 nd ligation reaction	
Content	Amount- µl	Content	Amount- µl
pEGFP-C1	2	pEGFP-C1	2
Motor domain	8	Motor domain	8
	*		*
10X ligation buffer	1.5	10X ligation buffer	2
T4 ligase	1	T4 ligase	1.5
PEG	0.5	PEG	2
dH ₂ O	2	dH ₂ O	4.5
Total reaction	15	Total reaction	20

↓
Incubated O/N at 16°C

↓
Incubated O/N at 4°C

*Insert and vector were mixed and put at 65°C for 5 min. Then they were put on ice for 10 min.

	3 rd ligation reaction	4 th ligation reaction	5 th ligation reaction
Content	Amount- µl	Amount- µl	Amount- µl
pEGFP-C1	1	2	1
Motor domain	7	9	7
10X ligation buffer	1	1.5	1.5
T4 ligase	1	1	2
PEG	-	1.5	1.5
dH ₂ O	-	-	2
Total reaction	10	15	15

↓
O/N- RT

↓
O/N -4°C

↓
O/N-RT

In the first and second reactions vector to insert ratios were kept constant and ligase and PEG concentrations were increased. In addition, the ligation reactions were performed at different temperatures.

In the other ligation reactions, 7 and 2,5 fold excess inserts were put into the reaction with or without PEG and the amount of enzyme in 5th reaction was increased twice. These changes were performed in order to find the optimum conditions for ligation reaction. In addition, since there are different temperatures recommended for ligation reactions in the literature, the temperatures widely used in these reactions were selected, and room temperature, 4°C, 16°C were used.

No satisfactory results were obtained from these ligations. Since many different ligation reaction conditions and different vector to insert ratios were tried, it was thought that the digestion should be repeated. Since we used the same enzymes to cut out the insert from cloning vector and to open up the expression vector, it was thought that there were not any problems about the enzymes and buffers used. Thus, the digestions were repeated (especially to be sure of the complete digestion of the vector) as mentioned in the methods section and desired results were obtained.

4.3.2. Optimization for subcloning of tail domain into GFP vector

Appropriate amounts of pTZ57R/T-Tail construct and the pEGFP-C2 vector were digested with EcoRI and SmaI enzymes.(see figure 4.13.). After digestion, gel extraction was performed for both insert and expression vector and the results of extraction were checked on gel as shown in figure 4.14.

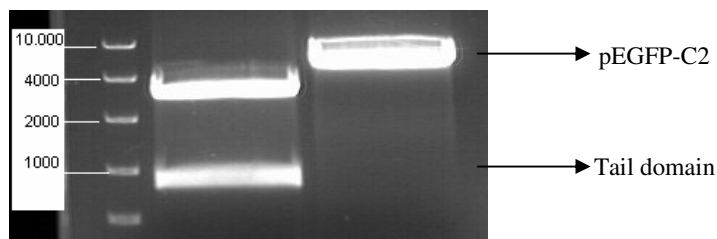


Figure 4.13: Restriction results for tail domain.

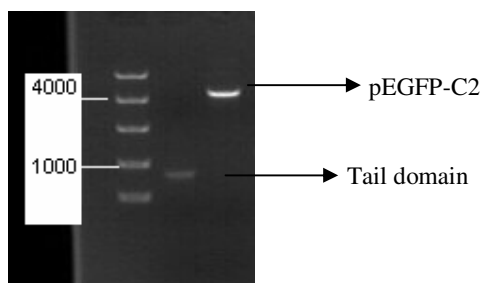
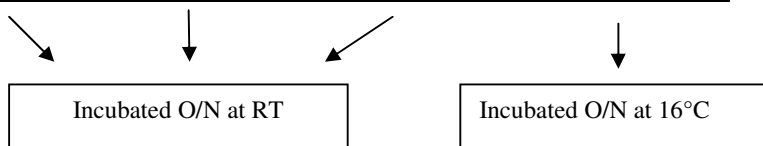


Figure 4.14: Agarose gel electrophoresis showing the result of the gel extraction after restriction.

According to the concentrations of the insert and vector observed on the gel after the gel extraction, different ligation reactions were performed as mentioned below in table 4.5.

Table 4.5: 4 different ligation reactions for tail domain

	1 st ligation reaction	2 nd ligation reaction	3 rd ligation reaction	4 th ligation reaction
Content	Amount- µl	Amount- µl	Amount- µl	Amount- µl
pEGFP-C2	1	2	1	2
Tail domain	6	6	7	8
10X ligation buffer	1	1.5	1.5	2
PEG 4000	1	1	2	1.5
T4 DNA ligase	1	1.5	2	3
dH ₂ O	-	3	1.5	3.5
Total reaction:	10	15	15	20



In the first trial, since the DNA concentration of the insert was lower than the vector, 6 fold excess insert was used for the reaction. Since there were no results, 3,4 and 7 fold excess inserts were also used for following reactions.

PEG 4000 was used to increase the efficiency of the ligation reaction by increasing the possibility of recognition of the restricted ends of the tail and the pEGFP-C2 to each other. Since SmaI creates blunt ends and EcoRI creates sticky ends, PEG 4000 was used in every reaction in different amounts. In addition, based on the fact that increasing amount of ligase enzyme has positive effects on ligation reaction, enzyme amount was increased gradually. After these ligations, since no satisfactory results were obtained, the following ligation reactions mentioned in table 4.6. were carried out.

Table 4.6: 2 different ligation reactions for tail domain

1 st ligation reaction		2 nd ligation reaction	
Content	Amount- µl	Content	Amount- µl
pEGFP-C2	2	pEGFP-C2	2
Tail domain	6	Tail domain	8
↓*		↓*	
10X ligation buffer	2	10X ligation buffer	2
T4 ligase	2	T4 ligase	3
PEG	2	PEG	2
dH ₂ O	6	dH ₂ O	3
Total reaction	20	Total reaction	20
↓		↓	
Incubated O/N at 4°C		Incubated O/N at RT	

*Insert and vector was mixed and put at 65°C for 5 min.
Then they were put on ice for 10 min.

In this procedure, the principle is to increase the possibility of the integration of the insert into the expression vector, as mentioned in optimization for motor domain. For this reason, two different ligation reactions were performed. 3 or 4 fold excess insert was used in two different reactions respectively. In addition the enzyme amount was increased and the incubation temperature was changed. These reactions did not give satisfactory results. Finally, ligation reactions mentioned in the methods section worked properly and it was seen that 1:7 and 1:3,5 vector to insert ratios worked best for tail domain.

5. CONCLUSION

Since Kif 15 is a motor protein whose functions are crucial for both neuronal and non-neuronal cells, in this study, we aimed to dissect this motor protein into its functional domains in order to understand the interactions of these domains with other cytoskeletal elements. To achieve our goal, first of all we successfully isolated whole RNA from adult rat brain. Then, cDNA was successfully synthesized from RNA and functional domains of Kif15 (motor, myosin interacting and tail domains) were cloned from this cDNA by using sequence specific primers. These PCR products were ligated into cloning vectors in order to carry out further processes. After checking the insert existence by sequencing method, finally these domains were successfully subcloned into GFP vectors in order to track in cell culture studies.

Since Kif 15 functions to maintain the spindle poles during mitosis in mitotic cells, and it is thought that it uses a similar mechanism in ending the neuronal migration, both cell types will be used in cultures. For non-neuronal cell culture studies, HeLa cells will be used. For neuronal cell culture studies, adult rat hippocampus cells and will be used. In these cultures, Kif 15 and other cytoskeletal elements (MT, MF, other motors etc.) will be analyzed stained by using immunostaining techniques. Then, it will be possible to reveal the interactions of Kif 15's functional domains with other cytoskeletal elements.

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RESUME

Ayşegül DİLSİZOĞLU was born in Kayseri in 1981. After getting her high school degree from TED Kayseri Collage in 1998, she continued her undergraduate degree at Marmara University, Faculty of Arts and Science, Department of Biology. She had her Bachelor degree in 2003. She has continued to her graduate studies at Advanced Technologies in Molecular Biology – Genetics and Biotechnology program. She is still pursuing her studies in the same department.