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Presentation of the CNRS

This work was made at Center for molecular Biophysique CNRS in Orléans (France) in Patrick Midoux's team. The Centre National de la Recherche Scientifique (National Center for scientific Research, CNRS) is a government-funded research organization created in 1939, under the administrative authority of France's Ministry of Research. The CNRS had grouped various state agencies of fundamental and applied researches. It coordinates also researches at national level. The CNRS is the largest French public research institution and is at the first rank at European level.

The CNRS has following missions:

- To evaluate and carry out all researches capable of advancing knowledge and bringing social, cultural, and economic benefits for society
- To contribute to the application and promotion of research results.
- To develop scientific information
- To support research training
- To participate in the analysis of the national and international scientific climate and its potential for evolution in order to develop a national policy of research.

CNRS research fields

The CNRS carried out research in all major fields of knowledge, through its nine institutes:

- Institute of Chemistry (INC)
- Institute of Ecology and Environment (INEE)
- Institute of Physics (INP)
- Institute of Biological Sciences (INSB)
- Institute for Humanities and Social Sciences (INSHS)

- Institute for Mathematical Sciences (INSMI)
- Institute of Information and Engineering Sciences and Technologies (INST2I) and two national institutes:
- National Institute of Nuclear and Particle Physics (IN2P3)
- National Institute for Earth Sciences and Astronomy (INSU)

The CNRS possess 98 proper laboratories, named Proper Research Units (UPR) and 1250 associated research laboratories, named Mixed Research Units (UMR), partnered with higher educations institutions (Universities) or other research organizations, foundations or industries.

Interdisciplinary research

CNRS encourages collaborations between specialists from different disciplines in particular with the university thus opening up new fields of enquiry to meet social and economic needs. CNRS develops interdisciplinary programs which bring together various CNRS departments as well as other research institutions and industries.

Interdisciplinary research is undertaken in the following domains:

- Life and its social implications
- Information, communication and knowledge
- Environment, energy and sustainable development
- Nanosciences, nanotechnologies, materials
- Astroparticles: from particles to the Universe

This work was done in the Center for Molecular Biophysics (CBM) founded in 1967 by Professor Charles Sadron and now directed by Dr Jean-Claude Beloeil. It is a Proper Research Unit (UPR) of the CNRS (UPR CNRS 4301). This laboratory is located on the CNRS campus in Orleans-la Source. It is a unique interdisciplinary laboratory in France, which assembles in a same place chemists, biologists and physicians, with researches focused on the understanding of the specific properties of biomacromolecules. The CBM is a key participant in the development of biophysics in France and Europe, and it is the largest research laboratory in the Centre Region in France.
The CBM is structured in 4 departments with group 22 research teams, supported by an administrative and technical staff.

The 4 research departments are:

- Synthesis, structure and dynamics of biologically interesting molecules
- Imagery, spectroscopy and chemistry of living organisms
- Nucleic acids and proteins, structure and interactions
- New therapeutic targets and vectorology: molecular and cellular approaches.

Researchers at the CBM, working at the physics-chemistry-biology interface, seek insight into the structure, dynamics and interactions of biomacromolecules, at different levels: *in vitro* and *in silico*, but also *in vivo*, as it is at this level that the challenge for the coming years lies. This approach entails searching for the causes of macromolecular dysfunctions which trigger the development of certain diseases.

Center of Molecular Biophysics

The Patrick Midoux's team research activity concerns "Gene transfer by synthetic vectors". The researchers develop synthetic vectors to introduce nucleic acids (DNA and RNA) into human cells for gene therapy applications. They have designed polymers and cationic lipids containing histidine residues that acquire fusiogenic properties in endosomes to improve the delivery of acid nucleic into the cytosol. They have also set up a nucleic sequence that mediates the nuclear import of plasmid DNA in the cell nucleus. They also study the intracellular trafficking of vectorized DNA by cell imaging. The team is composed of 12 persons. The team leader Dr Patrick Midoux (INSERM Research Director), Dr Chantal Pichon (Professor at the University of Orleans), Dr Jean Marc Malinge (INSERM senior scientist), Dr Jean Pierre Gomez, (Assistant Professor at the University of Orleans), Julie Lodewick (PostDoc), Ludivine Billet (PostDoc), Federico Perche (PhD student), Anthony Delalande (PhD student), Dr Cristine Gonçalves (CNRS Engineer), Loïc Lebègue (CNRS technician), Thomas Thibault (Master Student), Aida Duarte (Master Student).

Resumo

O objectivo deste trabalho consiste em produzir e purificar a proteína E3-14.7K e a proteína intracelular FIP-1, com o fim de estudar as suas interacções moleculares, a relação com os microtubulos e a sua respectiva implicação na apoptose. Para este efeito, produziu-se e purificou-se cinco proteínas recombinantes: GST-FIP-1, E3-14.7K, proteínas homologas fluorescentes nomeadamente: EGFP - FIP-1, Cherry-FIP-1 e E3-Cherry. A construção do plasmídeo pGST-FIP-1 foi realizada clonando o gene FIP-1 e inserindo-o no vector pGEX-6P-2, com fim de purificar a proteína GST-FIP-1 através de cromatografia afinidade com GST Bind™ Kits (Novagen). Através desta técnica, a proteína foi purificada com 95% de pureza. Todas as outras proteínas, contendo a 6 His-tag, foram purificados por cromatografia de afinidade utilizando His.Bind® Purification Kit (Novagen) baseado na immobilized metal affinity chromatography (IMAC) em condições nativas. Todas estas proteínas foram expressas em *Escherichia coli* Arctic Express™ competent cells. Neste trabalho observou-se que a proteína E3-14.7K forma habitualmente corpos de inclusão, no entanto foi purificada com sucesso, em cerca de 91% de pureza. A sua extracção foi realizada em presença de uma concentração de 0.5 M de ureia. Na purificação das proteínas fluorescentes (EGFP-FIP-1, E3-Cherry e Cherry-FIP-1) não foi possível remover todos os contaminantes presentes, no entanto conseguiu-se obter um espectro de emissão de fluorescência razoável. Estas proteínas têm um particular interesse, para o estudo das suas interacções, no tráfico intracelular através da microscopia confocal, o que consiste numa inovação na investigação, especialmente em relação à apoptose.

Palavras Chave: Protein-protein interactions; adenovirus; E3-14.7K; FIP-1; production and purification.

Abstract

The aim of this study was to produce and purify the E3-14.7K adenovirus protein and its intracellular FIP-1 protein, in order to study their molecular interactions, their relationship with microtubules, and respective implication in apoptosis cycle. For this purpose, we have produced and purified five recombinants proteins: GST-FIP-1, E3-14.7K, and their fluorescent counterparts: EGFP-FIP-1, Cherry-FIP-1 and E3-Cherry, fused with EGFP or Cherry labels. We have constructed the pGST-FIP-1 plasmid by cloning the FIP-1 gene into the pGEX-6P-2 vector, in order to purify GST-FIP-1 protein by affinity chromatography with, GST Bind™ Kits (Novagen). By this technique, this protein was purified with 95% of purity. All the other proteins, containing a 6 His-tag, were purified by affinity chromatography using His.Bind® Purification Kit (Novagen) based on an immobilized metal affinity chromatography (IMAC) method in native conditions. All these proteins were expressed in *Escherichia coli* Arctic Express™ competent cells. Typically it was observed that E3-14.7K protein forms inclusion bodies but the protein was successfully purified with 91% of purity when the extraction was performed in the presence of 0.5 M urea. The purification of the fluorescent proteins (EGFP-FIP-1, E3-Cherry and Cherry-FIP-1) failed to remove all contaminants, however their fluorescent properties exhibited a good emission spectrum. These fluorescent proteins will be of particular interest for studying their intracellular trafficking and interactions by confocal microscopy. These will constitute innovative tools for future researches, especially concerning to the apoptosis cycle.

Keywords: Protein-protein interactions; adenovirus; E3-14.7K; FIP-1; production and purification.

Abbreviations used throughout this work

Ads - Adenovirus

DISC - Death inducing signalling complex

OD - Optical Density

GFP- Green fluorescent protein

GST - Glutathione S-transferase

HC - Heavy chains

IMAC - Immobilized metal affinity chromatography

IPTG - Isopropyl-I-thio-ß-D-galactopyranoside

LB - Luria Broth

LBS – Laemmli buffer solution

MBP - Maltose binding protein

Mini-Prep - Mini preparation of plasmids by alkaline lysis

mRFP1 - Monomeric red fluorescent proteins

NF-kB - Nuclear factor kappa B

NI-NTA - Nickel chelated nitrilotriacetic acid

PLA2 - Phospholipase A2

PVDF - Polyvinylidene difluoride membrane

Rpm - Rounds per minute

SDS-PAGE - Sodium dodecyl sulfate poly acrylamide gel electrophoresis

TCTEL 1 - Human homologue of murine Tctex 1

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Preface

In this work, we have produced the cellular FIP-1 protein and its exogenous ligand the viral E3-14.7K protein, and their fluorescent variants EGFP-FIP-1, Cherry-FIP-1 and E3-Cherry.

After production we have studied the interactions between the E3-14.7K and protein FIP-1.

The presentation of this work is divided into five chapters, which include an introduction, a materials and methods section, chapters on results and discussion, and finally conclusion and future perspectives.

The general introduction analyzes the actual knowledge concerning the proteins FIP-1 and E3-14.7K, and particularly the molecular interactions between these two proteins. The results obtained concerning this interaction are expected to be used for better understanding in the intracellular transport of molecules between the nucleus and cytoplasm.

Chapter I

Introduction

1 - Introduction

Adenovirus (Ads) are non-enveloped virus that contain more than 20 genes dedicated to control various aspects of the innate or acquired immune responses of the infected host [Horwitz, 2001]. During the past 20 years, Ads vectors have been the focus of considerable interest because of their potential applications as delivery vehicles for gene therapy [Alemany *et al.,* 2000; Bramson *et al.,* 1995; Chuah *et al.,* 2003; Curiel 2000, Hitt *et al.,* 2000; Liu *et al.,* 2002; Sadeghi *et al.,* 2005; St George, 2003]. This choice is based on the fact that: (i) many human and animal adenovirus are non-pathogenic for their natural hosts, (ii) a variety of both proliferating and quiescent cell types, such as epithelial cells, fibroblasts, hepatocytes, endothelial cells and stromal cells, can be infected with Ads, (iii) Ads vectors can be grown to very high titers that offers a means to infect a large number of target cells, and (iv) replication-competent.In general different types of Ads vectors have been developed, which are based on modifications of the virus genome using conventional molecular biology techniques [Volpers and Kochanek, 2004]. Their design was specially fitted for reduced immunogenicity, systemic and repeated delivery [Bangari *et al.,* 2006]. Other types of viral vectors including retroviral and lentiviral vectors have been also widely developed. Although to date, viral vectors are the best vehicles to introduce genes into cells. However, there are still serious immunogenicity concerns associated with the use of viral vectors. Specifically, a limited insert-size and a higher product cost. All of these points are increasingly making the non viral gene delivery reagents, the vectors of choice.

The aim of the present work concerns the investigations of molecular interactions between the endogenous FIP-1 protein and the E3-14.7K adenoviral protein. This behaviour is visualized during the early life cycle of human adenoviruses and it plays several roles during the infectious cycle comprising the inhibition of the TNF-α mediated apoptosis [Gooding *et al.*, 1988; Horton *et al.*, 1991]. Also, this protein has been shown to have a role in the cytoplasmic transport of other molecules and vesicles during the infectious cycle. E3-14.7K interacts with the FIP-1 protein, a cytosolic protein of the small GTPase family, which has been shown to interact with a low molecular component of dynein called TCTEL (named also TcTex-1) [Williams *et al.,* 2005]. The paradigm of a viral protein or its receptor binding to TCTEL *or* other components of dynein has been reported recently for other viruses such as herpes

simplex, poliovirus and rabies virus. However, a full understanding of the reason for this common association awaits further studies.

The main objective of this thesis was to produce FIP-1 and E3-14.7K recombinant proteins as well as several chimera of FIP or E3 proteins fused with fluorescent proteins such as EGFP and mCherry. These recombinant proteins will be further used to investigate FIP-1/E3 interactions as well as their interactions with microtubules both *in vitro* by immunoprecipitation methods and in cultured cells by confocal microscopy.

1.1 - E3-14.7K protein

Typically the adenovirus express several proteins which down regulate the host immune response. Protein coded by early region 3 (E3) genetic segments are expressed early in the life cycle of human adenoviruses to protect the virus from the antiviral response of host cells. Among the seven E3 proteins, E3-14.7KDa protein (E3-14.7K) inhibits the cell death, mediated by TNF-α and FasL receptors [Krajcsi *et al.*, 1996; Chen *et al.*, 1998]. The mechanism by which this protein exerts its anti-TNF-α effect remains incompletely understood.

The E3-14.7K is a glycoprotein encoded by various serotypes of adenovirus [Horton *et al.*, 1990]. This protein varies in size from 122 amino acids in Ad40 to 136 amino acids in Ad3. However, its sequence is largely conserved between different subgroups (Figure 1).

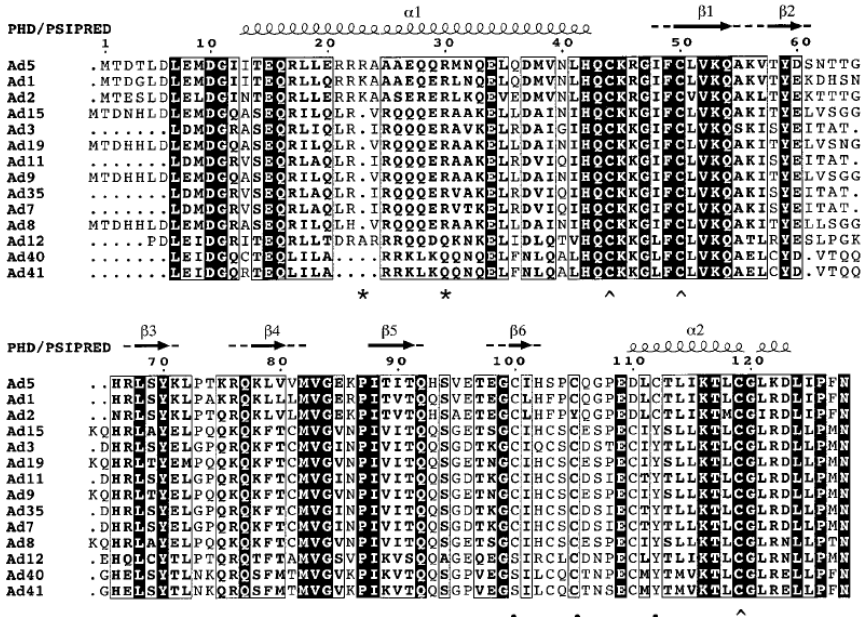


Figure 1 - Sequence alignments of 14.7K proteins from various human adenovirus serotypes [Kim and Foster, 2002]

Normally, E3-14.7K is localized in the cytoplasm, in discrete foci near the plasma membrane and in the nucleus as demonstrated by immunofluorescence [Li *et al.*, 1997, 1998]. Biophysical studies with a purified maltose binding protein (MBP) tagged E3-14.7K recombinant protein, indicate that in the C-terminal two third of the protein is highly resistant to proteases and adopts a tertiary structure composed of five β-strand and one α-helice [Kim and Foster, 2002]. This region appears to be highly structured and allows E3-14.7K binding to its cellular protein effectors (FIP-1 and FLICE = FADD, such as interleukin-1-β-converting enzyme). These authors have also demonstrated that (i) E3-14.7K binds zinc with a 1:1 stoichiometry on three invariants cysteines residues located in the C-terminal domain and (ii) the MBP-E3-14.7K protein exists in a higher structure order, probably a monomer, than the natural protein [Lichtenstein *et al.*, 2004].

In general, wild type and Ads mutants were used to demonstrate that Ads infection makes cells sensitive to TNF-mediated cytolysis while conversely the resistance to cell death is conferred by expression of E3-14.7K. It protects Ads infected cells against cytolysis and apoptosis induced by TNF-α, when expressed independently by virus infection [Gooding *et al.*, 1988] or by transfection with a bovine papillomavirus vector [Horton *et al.*, 1991]. The mechanism by which this protein exerts its anti-TNF-α effect remains completely unknown. However, it appears that E3-14.7K does not induce down regulation of the TNF-α receptor [Horton *et al.*, 1991]. In a recent study, it was demonstrated that E3-14.7K inhibits internalization of TNF-α receptor ligand induced by DISC (death inducing signalling complex) formation [Schneider-Brachert *et al.*, 2006]. This endocytosis inhibition acts by a failure in the coordinated temporal and spatial assembly of the effectors molecules Rab5 and dynamin 2, though caspase-8 inhibition [Chen *et al.*, 1998].

The anti-TNF-α effect of E3-14.7K has been also demonstrated *in vivo*. A recombinant vaccine virus (VV) producing TNF-α and coexpressing E3-14.7K exhibits an increased virulence by reversing the attenuating effect of TNF-α on VV pathogenicity [Tufariello *et al.*, 1994].

Also, E3-14.7K appears as an immunoregulatory protein reducing the inflammatory reaction. This mechanism occurs through the inhibition of the expression of inflammatory cytokines such as IL-6 and IL-12, probably by the inhibition of the nuclear factor (NF-κB) transcriptional activity induced by the TNF-α receptor signalling cascade. Recently, E3-14.7K was found to specifically inhibit p50 homodimer DNA

binding [Carmody *et al.*, 2006]. In addition, E3-14.7K inhibits the TNF-α induced secretion of the phospholipase A2 (PLA2) and thus the release of arachidonic acid, a process with could have effects on the inflammatory response [Krajcsi *et al.*, 1996]. This effect is indirect, as E3-14.7K does not inhibit the enzymatic activity of PLA2. This anti-inflammatory effect was also found *in vivo*. Indeed, E3-14.7K has been showed to protect BALB/c mice against lipopolysaccharide induced acute hepatitis [Gantzer *et al.*, 2002].

Four mammalian proteins have been identified to interact with E3-14.7K. They were named as FIPs for Fourteen.7K-Interacting Proteins. These proteins are: FIP-1 (RagA); FIP-2 (Optineurim); FIP-3 (NEMO, IKKY) and FIP-4 (AIF). For instance, FIP-1 is involved in the cytoplasmic transport of molecules or vesicles during the virus infection cycle.

1.2 - FIP-1 protein

The FIP-1 protein is a member of a new family of low-molecular weight GTPases, and belongs to the Ras superfamily. It was first discovered in rat and human cells and was named RagA [Schurmann *et al.*, 1995; Sekiguchi *et al.*, 2001]. This molecule was isolated by comparing its sequence with sequences common to a variety of GTPases. It has a functional homologue in *Saccharomyces cerevisiae* called Gtr1p protein [Hirose *et al.*, 1998].

Typically, FIP-1 is ubiquitously expressed in various tissues as skeletal muscle, heart and brain but the highest levels are found in adrenal gland [Schurmann *et al.*, 1995]. In human cells, FIP-1 is associated with several phosphorylated proteins after stimulation with TNF-α, suggesting that is an intermediate of the TNF-α signalling pathway [Li *et al.*, 1997]. Sequence analysis of RagA (FIP-1) revealed five to six GTP-binding domains, where three domains (PM1, PM2, PM3) are localized in the N-terminal region [Schurmann *et al.*, 1995]. The C-terminal region, unusually large compared to the others Ras-related GTP binding protein, is implicated for its interaction with E3- 14.7K [Li *et al.*, 1997]. In human cells infected with adenovirus, FIP-1 was localized with E3-14.7K in the cytoplasm, especially near the nuclear membrane and in discrete foci near or within the plasma membrane [Li *et al.*, 1997].

It has been showed with its functional *Saccharomyces cerevisiae* Gtr1p protein homologue, that the localization is dependent on the nucleotide form (GTP or GDP), that is associated with the protein [Hirose *et al.*, 1998] (Figure 2). This hypothesis was also reinforced by the observation that FIP-1 is able to interact with the microtubule network through the formation of a ternary complex between TCTEL1 (light-chain component of the dynein located at the negative ends of microtubules) and the viral protein E3-14.7K [Lukashok *et al.*, 2000]. Therefore, FIP-1 could play a role in the transcytoplasmic transport of organelles, viruses and signal transduction molecules along microtubules.

The FIP-1 protein was also demonstrated to have a crucial role as a signalling molecule. In yeast, Gtr1p (FIP-1) appears to be involved in the Ran/Gsp 1 GTPase pathway [Hirose *et al.*, 1998]. By its interaction with Gtr2p (homologous of Rag C and D), Gtr1p regulates negatively this pathway [Nakashima *et al.*, 1999]. Recently, it was also demonstrated that Gtr1p is involved in epigenetic control of gene expression, by repressing nitrogen catabolic repressed genes in the TOR signaling pathway [Seckiguchi *et al.*, 2008]. Furthermore, RagA (FIP-1) might regulate directly Nop 132, a

nucleolar protein, involved in post-transcriptional events, such as ribosome RNA processing and /or assembly [Sekiguchi *et al.*, 2004]. So, it appears that FIP-1 is a multi functional G protein with functions in both cytoplasm and nucleus.

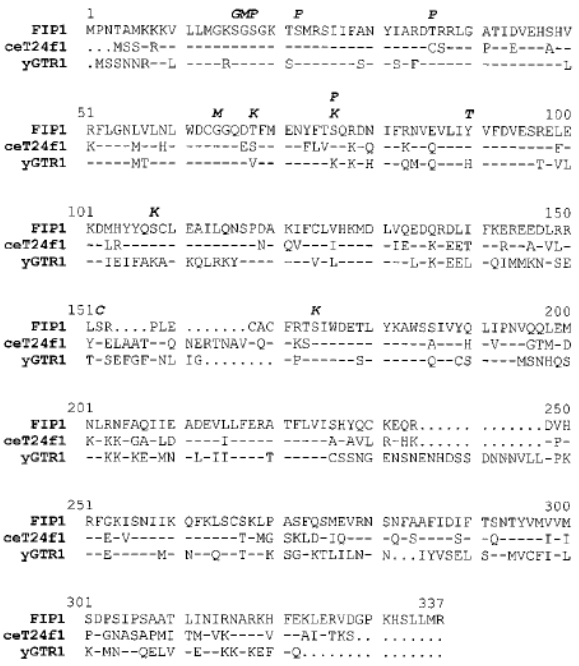


Figure 2 – Sequence comparison of FIP-1 to *S. cerevisae* GTP-Binding protein GTR1 (yGTR1) and a *C. elegans* putative protein (ceT24F1) proposed from genomic sequencing. Dashes indicate identical amino acids, and dots indicate gaps in the alignment. Putative posttranslational modification sites are marked above the residues: M, myristoylation; P, protein kinase C phosphorylation site; C, cyclic AMP- and cyclic GMP- dependent protein phosphorylation site; K, casein kinase II phosphorylation site G, glycosaminoglycan attachment site; T, tyrosine kinase phosphorylation site [from Li *et al.*, 1997].

1.3 - Motor Proteins

Molecular motors are involved in a variety of biological movements, such as mitosis, axoplasmic transport and secretion. Motor proteins exhibit extensive homology in their mechanochemical domains. Specifically they drive the transport of membrane organelles, protein complexes and nucleic acids/proteins complexes. Structurally, molecular motors are consisted of two functional parts: a motor domain (also called the head) that reversibly binds to the cytoskeleton and converts the chemical energy into motion; and the second part often referred as the tail, that interacts with cargo directly or through accessory light chains [Karcher *et al.*,2002].

The cytoskeleton is a complex network made of three cytosolic filaments: microtubules, intermediate filaments and microfilaments. This structure maintains the cell morphology and is required for cytoplasmic transports. The cytoskeleton is highly

dynamic and undergoes constant reorganization under several conditions as the cell response to external stimuli, cell migration over the extracellular matrix or cell division. This network also serves to transport a variety of cargos where network made with microtubules and actin microfilaments is implicated. The transport is mediated by motor protein complexes, namely kinesins, dyneins, and myosins which represent the three major classes of molecular motor that translocate along cytoskeletal elements.

Typically, kinesins and myosins have a motor domain that contains both ATPase and filament-binding sites attached to a α-helical lever arm through a neck region [Rayment, 1996]. Cytoplasmic dynein is a multimeric complex consisting of two identical heavy chains containing the minus end-directed motor domains, and several associated chains (light intermediate chain, intermediate chain, roadblock, Tctex1 and LC8) (Figure - 3). In contrast to the other cytoskeletal motors, dynein complexes exhibit a greater molecular complexity compared to kinesin complexes. As an example, the 1.9 MDa outer dynein arm is a large multimeric protein which contains 13 different polypeptide components whilst the 360 kDa kinesin contains tetramer subunits [King, 2000]. Also, cytoplasmic dyneins appear to be homodimers of 530 kDa heavy chains (HC), which form two globular heads containing an ATPase and microtubule motor domain, a stem which allows dimerization between HC and also binding of the intermediate and light chain domain. The N-terminal stem domain is also involved in cargo attachment [King SM; 2000]. Dynein is part of the molecular motor system, which is located at the negative ends of microtubules near the nuclear membrane.

As mentioned before, microtubules have been shown to be involved in many transport processes including the locomotion of adenovirus, herpes simplex virus, and cytoplasmic organelles such as endosomes from the plasma membrane to the nuclear membrane [Suomalainen *et al.*, 1999; Whittaker and Helenius, 1998]**.** By fluorescence studies, it was demonstrated that the microtubule network and the microtubule molecular motors are important in the transport of the Ads particles from the endocytic vesicle across the cytoplasm to the nucleus during the process of viral entry. [Döhner *et al.*, 2005; Greber and Way, 2006]. In the transport of the Ads particles, the cytoplasmic dynein constituted the primary mode of association of Ads with microtubules [Kelkar *et al*, 2004].

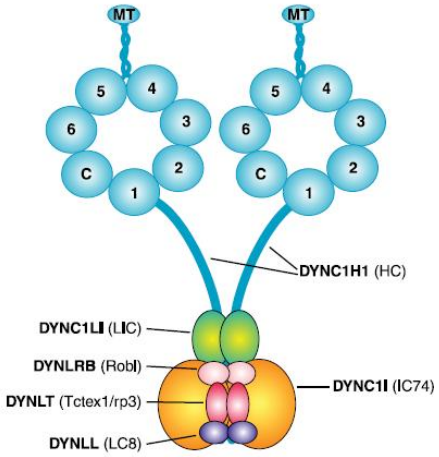


Figure 3 - Structural model for the association of the cytoplasmic dynein complex subunits.
The core of the cytoplasmic dynein complex is made of two DYNC1H1 (HC) heavy chains that bind to microtubules (MT), two DYNC1I intermediate chains (IC74) and two DYNC1LI light intermediate chains (LIC) that bind on the heavy chain. Three dimers of the three light chain families: DYNLT, the Tctex1 light chains; DYNLRB, the Roadblock light chains; and DYNLL, the LC8 light chains, bind to the intermediate chain dimers. [adapted from K. Kevin Pfister *et al.*, 2006]

Among the components of the Dynein, TCTEL 1 (human homologue of murine Tctex1) is able to bind FIP-1.

1.4 - Interaction between the E3-14.7K and FIP-1 protein

Several FIPs: FIP-1, FIP-2, FIP-3 and FIP-4 have been identified to date [Horwitz, 2001; 2004]. The interactions of each of the four FIPs with E3-14.7K proteins are presented in figure 4. The functions of these proteins have been elucidated, each of them act on specific signalling pathways. FIP-1 has been shown to interact with E3-14.7K [Li *et al.*, 1997]. As described above, FIP-1 is able to interact with a low molecular component of dynein TCTEL1 [Watanable *et al.*, 1996; Lukashok *et al.*, 2000]. Also, deletion mapping studies of FIP-1 have revealed that the first 34 aminoacid residues at the N-terminal sequence containing the GTP-binding domain and the 43 aminoacid residues at C-terminal extremity are required for interaction with E3-14.7K [Li *et al.*, 1997]. The overlap region between FIP-1 and E3-14.7k is tenth of total length of the protein sequence. Therefore, GTP binding seems to be required to maintain a correct conformation of the protein facilitating the interaction of the C- terminus with E3-14.7K. E3-14.7K determinants for recognition of FIP-1 are located between the 31 and 128 aminoacid residues. This overlap region is composed of the two-third of the protein, which implies the secondary structures constituted by the six β strands and the second α-helix [Kim and Foster, 2002]. The significance of FIP-1/TCTEL/E3-14.7K

complex remains subject to assumptions. This complex may be involved either in mediating signals initiated by E3-14.7K to TNF-α induced cell death or in the transport of E3-14.7K to its acting site [Lukashok *et al.*, 2000].

Although adenoviruses use dynein to migrate in the cytoplasm during the early stages of infection, E3 proteins are not present at this time. In fact, they are synthesized only when the viral DNA enters the nucleus and the virus is completely uncoated. The paradigm of a viral protein or components of dynein has been reported recently also for other viruses such as herpes simplex, poliovirus and rabies virus [Mebatsion 2001; Mueller *et al.*, 2002; Ye *et al.*, 2000]. A full understanding of the reason for this common association awaits for further studies.

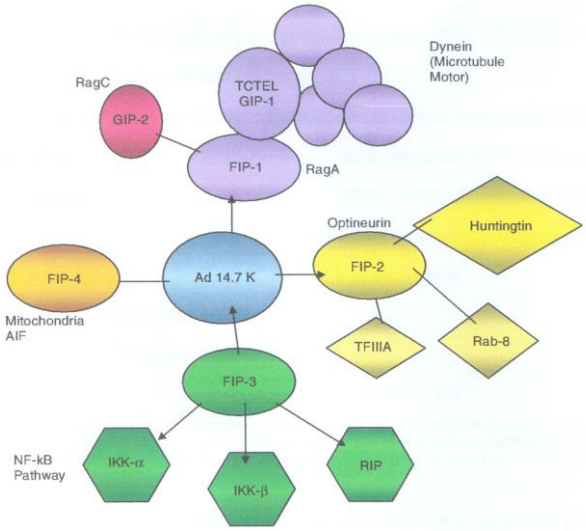


Figure 4 – Typical functions of FIPs and their interactions with other cellular signalling molecules. [Horwitz, 2004]

1.5 - Fluorescent Proteins as tools for investigation of protein-protein interactions

Fluorescent proteins provide the ability to visualize, track, and quantify molecules and events in fixed or living cells. Today, several fluorescent proteins are available and offer suitable tools for numerous applications, from locating proteins of interest or monitoring gene expression in order to observed specific interactions between molecules with defined regions of a living cell [Patterson GH, 2007]. The Green Fluorescent Protein (GFP) was discovered in 1961 in the hydrozoan *Aequorea Victoria* jellyfish and its gene was cloned in 1992. GFP has revolutionized many areas of cell biology and biotechnology, because it provides direct genetic encoding of strong visible fluorescence. The significant potential of this molecular probe was reached

several years later with the development of fusion proteins containing this fluorescent tag. This can be achieved easily as the target was easily fused to the gene of the target protein at both N-and C-terminal [Kobayashi *et al.*, 2008].

Wild type GFP protein fluoresces in the lower green portion of the visible spectrum. The GFP has two excitation peaks, the major one at 395 nm (in the long UV range) and a smaller one at 475 nm (blue). The emission peaks were founded at 509 nm (green). The crystal structure of GFP was solved in 1996. Specifically, it has a barrel cylindric structure constituted with to 11-straind β-barrels and a short segment of α-helix which runs through the interior of the barrel (Figure 5).

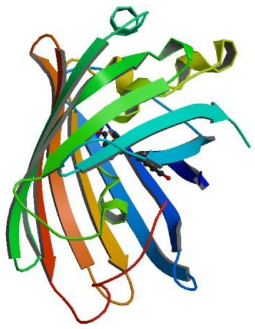


Figure 5 - GFP protein crystal structure.
(Information Portal to Biological Macromolecular Structures: http://www.rcsb.org)

In the central portion of this α-helix, the light-producing p-hydroxybenzylidene-imidozolidinone chromophore consists of a cyclised tripeptide, composed of serine 65, tyrosine 66 and glycine 67 residues. The formation of a fluorescent protein requires proper folding into the barrel structure, followed by cyclization of the three amino acids making up the chromophore and oxidation/deshydration reactions to produce a functional fluorescent molecule (Figure 6).

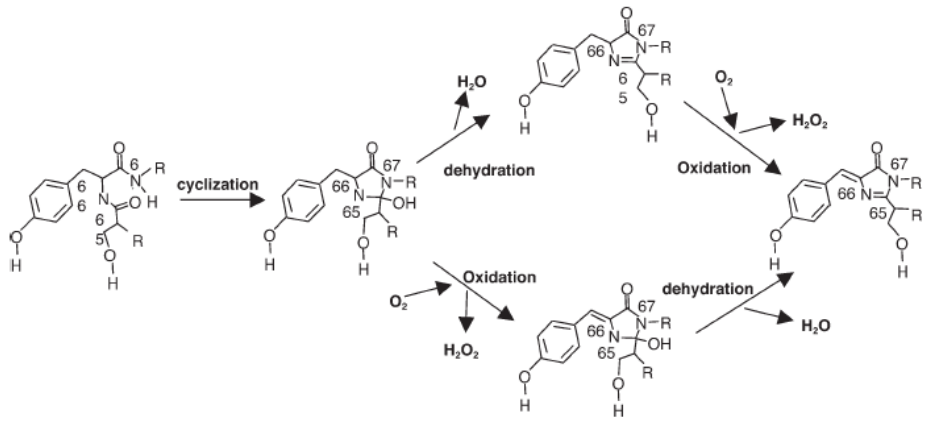


Figure 6 - Formation of the Aequorea Victoria GFP chromophore. The chromophore of GFP consists of S65, Y66 and G67. These residues undergo a cyclization reaction between S65 and G67 to form a five-membered ring. Oxidation and dehydration reactions follow to extend the π-bonding system of the aromatic Y66 and produce a fluorescent molecule. [From Patterson, 2007].

In recent years, several different colour forms of GFP have been genetically produced. Mutants with blue, cyan, green and yellow emissions are now available, but none with emission maxima longer than 529 nm [Geoffrey *et al.*, 2000]. Fluorescent proteins provide a good self-indicate of their folding, since the unfolded proteins generally do not fluoresce, and mutations to residues surrounding the tripeptide fluorophore can dramatically alter the fluorescence properties. So the protein will fluoresce if is in the inative state.

Red fluorescent protein

The development of red fluorescent proteins have been performed since these molecules had spectral properties that are ideal for double and triple labelled fluorescent experiments, particularly for confocal microscopy imaging. The DsRed (*Discosoma* sp.) fluorescent protein was derived from *Discosoma striata*. This protein has an excitation and emission maxima at 558 and 583 nm, respectively. Also, biophysical and X-ray crystallographic studies revealed that DsRed forms a stable tetramer, and that each monomer is structurally very similar to GFP [Brooke *et al.*, 2002]. The overall DsRed structure is similar to GFP, and consists of a β-barrel with an α-helix in the center containing the chromophore. The formation of the DsRed fluorescent chromophore is proposed to be similar to GFP. DsRed was engineered as mRFP1, the first true monomeric red fluorescent protein [Campbell *et al.*, 2004]. The mRFP1 contains 33 amino acid mutations but its structure is very stable. While the maturation process of its chromophore part occurs much faster, is less bright and its photostability is lower than DsRed. However mRFP1 properties remains comparable to EGFP ones. In 2004, further

engineering of mRFP1 led to the mFruits fluorescent proteins family that covers from the yellow (mHoneydew) to dark-red part (mPlum) of the visible spectrum [Shaner *et al.*, 2004]. Among mFruits, mCherry is the promising red fluorescent protein in terms of photostability, maturation, and tolerance for tagging. The mCherry protein is considered to be superior to the mRFP1. Indeed the excitation and emission maxima of the native mCherry protein are 587 and 610 nm, respectively. The red fluorescent of mCherry is extremely relevant for fluorescence spectroscopy because of its wide separation with green fluorescent protein providing excellent conditions to investigate protein-protein interactions inside the cells. [Bin Wu *et al.*, 2008].

Table 1 - Properties of novel fluorescent protein varians [Shaner *et al.*, 2004]

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)
DsRed	558	583
T1	555	584
Dimer2	552	579
mRFP1	584	607
mHoneydew	487/504	537/562
mBanana	540	553
mOrange	548	562
dTomato	554	581
tdTomato	554	581
mTangerine	568	585
mStrawberry	574	596
mCherry	587	610

Chapter II

Material and Methods

2 - Materials and Methods

2.1 - GST-FIP-1

GST fusion protein was constructed by inserting a gene or a gene fragment into the multiple cloning site of the pGEX-6P-2 vector (GE Healthcare, Bio-Sciences AB, Orsay, France) (Figure 1). This vector contained the glutathione-S-transferase (GST) gene, under the control of the Ptac promoter, which is induced by the lactose analogue isopropyl β-D-thiogalactoside (IPTG). The pGEX vector possesses also the internal *lac*Iq gene. The *lac*Iq gene product is a repressor protein that binds to the operator region of the *tac* promoter. This protein prevents expression of the protein of interest until induction by IPTG and thus maintaining tight control over expression of the insert. The GST tag is constituted by a large protein tag (26 kDa) which is easily recognized by the glutathione. Recombinant protein with a GST tag can be purified in a single-step procedure by using the glutathione matrix on the affinity chromatography with mild elution and non denaturing conditions [Terpe, 2002; Waugh, 2005].

2.1.1 - Construction of pGST-FIP-1 plasmid

The FIP-1 cDNA sequence was extracted from the pEGFP-FIP-1 plasmid previously constructed in the laboratory from the pEGFP-C1 vector (BD Biosciences Clontech, Ozyme, ST. Quentin en Yvelines, France) (Figure 2). After linearization of pEGFP-FIP with XhoI and BamHI restriction enzymes (Promega, Charbonnieres, France), the FIP-1 cDNA sequence was inserted between the XhoI and BamHI sites of the pGEX-6P-2.

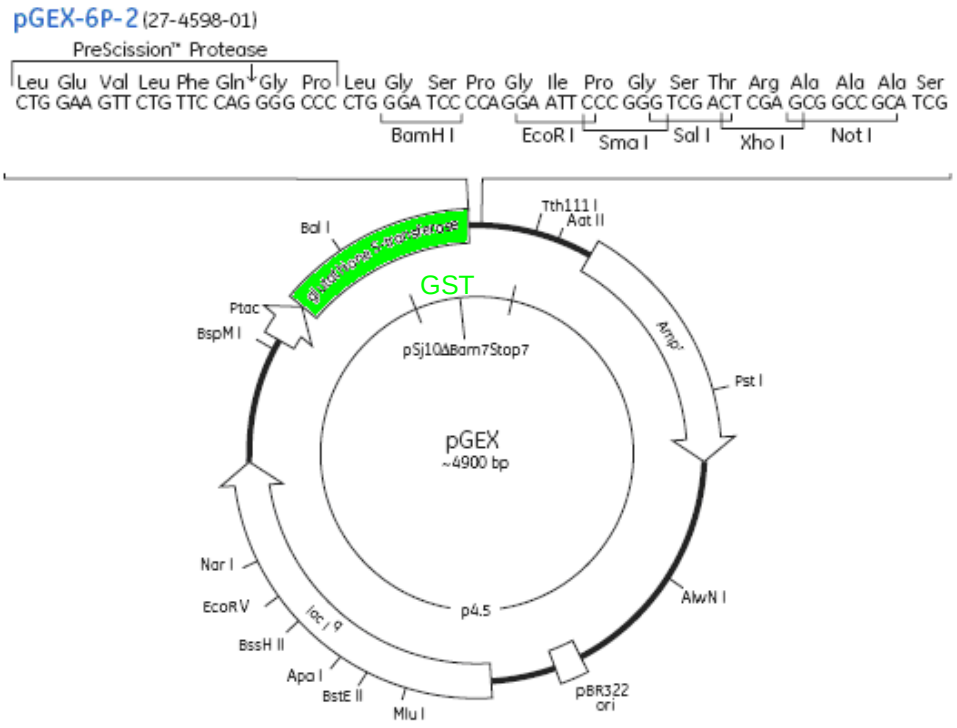


Figure 1- Map of the pGEX-6P-2 vector (from : GE Healthcare, Bio-Sciences AB)

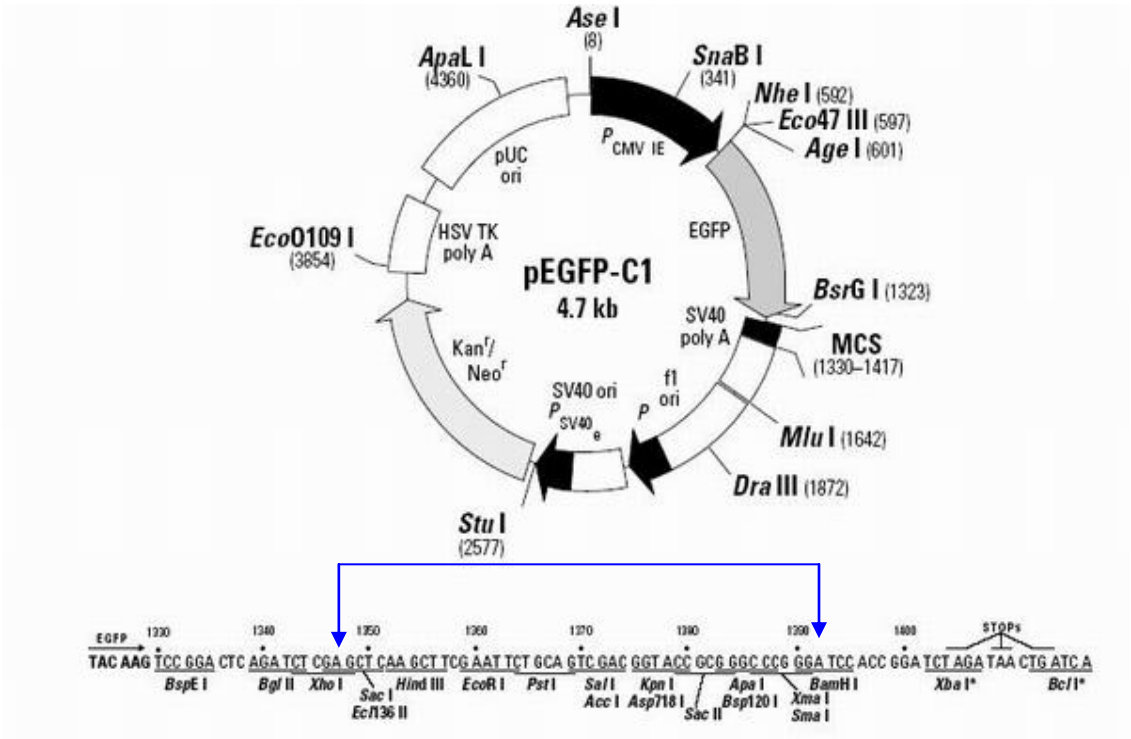


Figure 2 – Map of the pEGFP-C1 vector (from : BD Biosciences Clontech)

Subsequently, pGEX-6P-2 and pEGFP-FIP-1 vectors were digested at 37°C for 2 hours in the presence of XhoI and BamHI restriction enzymes. The quality of cloned DNA was analyzed by electrophoresis in a 0.6% agarose gel with TBE as running buffer (see annex 1). The size of the digested bands was determined from the double-stranded DNA of 250 to 10000 base pairs by the marker bands Bench Top 1Kb DNA ladder (Promega, Charbonnières, France) used as control double-stranded DNA size. One band of 900 pb for FIP-1 and one at 4900 pb for pGEX-6P-2 were expected. Next, the FIP-1 DNA fragment was excised from the agarose gel and purified according to the QIAEX II gel Extraction procedure MACHEREY-NAGEL (QIAGEN, Courtaboeuf, France) (see annex 2). The purified FIP-1 DNA fragment was inserted into pGEX-6P-2 by using the T4 DNA ligase in the ligase Buffer (Promega). The ligation was conducted overnight at 16°C at pGEX/FIP-1 DNA ratios of 100:50; 100:150 or 300:50 (ng vector / ng insert).

2.1.2 - Bacteria transformation

Bacteria were then transformed with the pGST-FIP-1 plasmid (see annex 3). The DH5α *Escherichia coli* bacterial strain (Invitrogen, Cergy Pontoise, France) are competent host cells for the production of pGST-FIP-1 plasmid (Figure 3). The expression of the recombinant protein is usually performed using strains of *E. coli* as host cells. In this work, this method is widely used because *E. coli* cells are well characterized at a genetic level; are available for an increasingly large number of cloning vectors; easy and cheap to cultivate and presents a high reproducibility and abundance for the target proteins produced. Typically, DNA is cloned into the open reading frame (ORF) in the direction of the initiation codon (ORI). The vector should have the following characteristics: an origin of replication, a promoter region, a multiple cloning site (Polylinker), an antibiotic-resistance gene and an ATG initiator. Also, a sign of the termination codon (the termination codon) should be present in the vector or added in the insert. After construction, the expression vector containing the coding sequence of the protein of interest is used for bacteria transformation. The pGST-FIP-1 plasmid contained the ampicillin resistance gene. This gene confers resistance by encoding to the β-lactamase enzyme that degrades the antibiotic. Typically, transformation is a process that consists in the introduction of DNA into cells by specific treatments, such as heat shock. Immediately, non-selective medium was added and the cells were incubated for 60 minutes at 37ºC in the presence of ampicillin (100 µg/ml). Four colonies were observed and isolated. Then, 500 µl of each colonies were inoculated in 2 ml of LB medium

supplemented with 2 µl ampicillin at 100 mg/ml and the cells were grown overnight at 37°C under vigorous shaking (approx. 300 rpm). The quality of the plasmid in the transformed bacteria was analyzed after extraction by electrophoresis in a 0.6% agarose gel.

2.1.3 - Plasmid extraction from *Escherichia coli* - Mini-Prep procedure

The pGST-FIP-1 plasmid was extracted from the cells using the alkaline lysis chemical method. Alkaline lysis was performed with a SDS solution to denature proteins and to dissolve lipids and in the presence of NaOH to denature the entire DNA (chromosomal and plasmid). After neutralization with a (3.0 M) potassium acetate solution, the plasmid DNA (cccDNA: covalently closed circular DNA) was quickly renatured because its chains cannot be separated due to super coiled structure. In contrast, the genomic DNA consisting of long chains remained denatured and precipitated with proteins (see annex 4).

2.1.4 - Determination of the concentration and the purity of pDNA

The quality of the purified plasmid was checked by electrophoresis in a 0.6% agarose gel. The pDNA quantity and its purity was determined from the absorbance at 260 nm and 280 nm. The absorbance at 260 nm (A_{260}) is proportional to the amount of nucleic acids whereas at 280 nm (A_{280}) is proportional to the amount of protein and phenol. The purity degree is calculated from the ratio A_{260}/A_{280} which should be between 1.8 and 2.0. The amount of pDNA was calculated from A_{260} = 1 for 50 µg/ml.

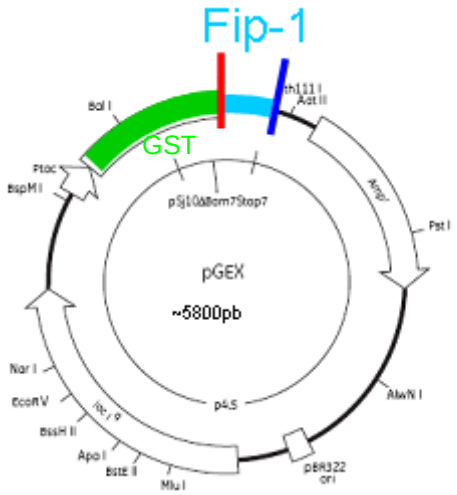


Figure 3 – Map of the plasmid pGEX-6P2 / FIP-1, modified from GE Healthcare, Bio-Sciences AB.

2.1.5 - Production of pGST-FIP-1 plasmid –Mega Prep procedure

A 2 ml preculture of DH5α *E. coli* transformed cells with pGST-FIP-1 was carried out overnight at 37°C under shaking (225 rpm), from the previously tested colonies, in LB medium supplemented with 2 µl ampicillin at 100 mg/ml. The next day, a second culture was performed overnight at 37°C under shaking (225 rpm) by introducing 500 µl of the previous preculture into 500 ml LB medium supplemented by similar concentration of antibiotics.

2.1.6 - Purification of Plasmid pGST-FIP-1

The purification of the plasmid was made according to the protocol of the EndoFree Plasmid Purification Kit (QIAGEN, Courtaboeuf, France) (see annex 5). This kit was based on the binding of plasmid DNA on an anion-exchange resin, whereas impurities (such as RNA, proteins, etc) were removed by medium salt conditions. Plasmid DNA was eluted with higher-salt buffers, then concentrated and desalted by isopropanol precipitation. The purification degree of the plasmid was checked by electrophoresis in a 0.6 %. agarose gel. Finally, the quality and quantity of pDNA were determined from absorbance measurement at 260 and 280 nm as described above. Purified pDNA was next stored at -20°C before further experiments.

2.2 - Production of proteins

2.2.1 - Transformation in Arctic Express™ competent cells (STRATAGENE®)

The GST-FIP-1 fusion protein was produced by transformation of Arctic Express™ competent cells (STRATAGENE®, Agilent Technologies, Massy, France) with the pGST-FIP-1 plasmid. This bacterial strain is a cold adapted *E. coli* strain optimized to increase protein yield by facilitating proper protein folding. This result in an increase production of a soluble active protein. In this work, the following transformation protocol was used: first, by the competent cells were thawed on ice with careful swirl. For each transformation, 100 µl of competent cells were transferred into a prechilled 14-ml BD falcon polypropylene round-bottom tube. Then, 2 µl of diluted β-mercaptoethanol (one volume of XL10-Gold β-mercaptoethanol added to 9 volumes of water) were added to each of the 100 µl aliquots of competent cells and gently mixed. The cells were incubated on ice for 10 minutes and slowly swirled every 2 minutes. Subsequently, one to 50 ng of plasmid DNA containing the gene of interest were added

to each tube with cells. After swirling, the mixture was incubated on ice for 30 minutes. Then 900 µl of preheated (42°C) SOC medium was added in the tube and the cells were incubated at 37°C for 1 hour under shaking at 225 rpm. A heat shock was applied for 20 seconds at 42°C and then the cells were placed on ice during 2 minutes. One hundred µl of transformed cells were spread on LB agar plates containing 100 µg/ml ampicilline for the selection of transformed bacteria which will appear as colonies upon overnight incubation at 37°C.

2.2.2 - Expression of GST-FIP-1 protein

A 2 ml preculture of Arctic Express^{TM} cells transformed with pGST-FIP-1 plasmid was started from isolated colonies coming from transformation plates and grown overnight at 37°C under shaking (225 rpm) in LB medium supplemented with ampicillin (100 µg/ml). The day after, a second preculture was done overnight in 20 ml LB with 200 µl of the first one, this preculture was supplemented with ampicillin (100 µg/ml). The third day, a sub culture was done for 3 hours at 30°C under shaking (225 rpm) by mixing 20 ml of the second preculture into 1 litre of LB medium without antibiotic. After that, a 1 ml culture medium sample was removed and the absorbance at 600 nm was measured with a spectrophotometer (CARY 50 Scan – VARIAN). A 10 µl sample was denatured and prepared according to the protocol described in annex for SDS PAGE. When the absorbance at 600 nm was between 0.6 and 0.8, isopropyl-I-thio-ß-D-galactopyranoside (IPTG) was introduced in the culture medium at a final concentration of 0.5 mM, in order to induce GST-FIP-1 expression. Bacteria were then incubated for 24 hours at 13°C under shaking (225 rpm). IPTG allows the expression of a gene under the control of promoter-T7 by removing inhibition of the lactose operator. At the end of the induction period, 1 ml of the medium culture was removed to check the cell culture growth by the absorbance measure at 600 nm. Subsequently, the sample was denatured and submitted to a 12% SDS PAGE (see annex 6). After growing, the bacterial culture was centrifuged (BECKMAN-AVANTI J2-21, Labcare Ltd Buckinghamshire, England) at 7000 rpm, during 15 min at 4°C in Nalgene ® tubes. The bacterial pellet was weighted and stored at -20°C until protein purification.

2.2.3 - Purification of the GST-FIP-1 protein

The GST tag is a 26 kDa protein that can be used to purify and detect proteins of interest. This tag allows protein purification under non-denaturing conditions by affinity chromatography using GST.bind ™ resin (Novagen, Nottingham, United Kingdom) in which glutathione is coupled to sepharose matrix [Terpe, 2006]. The structure of glutathione is complementary to the glutathione S-transferase binding site, which allows a high affinity for binding. This provides a rapid and single-step method for purification of recombinant GST-proteins. After loading the affinity column with the protein extract from bacteria lysates and washing, the GST-fusion protein was eluted with 10 mM reduced glutathione, under mild and non denaturing conditions.

2.2.4 – Preparation of the bacterial lysate

The bacterial pellet (about 8.6 g) was lysed with Bug Buster Reagent (Novagen) at 5 ml/g cell pellet supplemented with Benzonase® Nuclease (Novagen) (1 µl/ml), rLysozyme™ (SIGMA, Saint Quentin Fallavier, France) (1KU/ml) and 100 µl of protease inhibitor cocktail (SIGMA). The mixture was incubated for 20 min at room temperature on a shaking platform at a slow speed. Insoluble debris were removed by centrifugation at 16,000g for 20 min at 4°C. The supernatant was transferred into a tube and centrifuged under same conditions. Ten µl of the pellet and 10 µl of the supernatant were collected and further subjected to electrophoresis in a 12% SDS-PAGE gel.

2.2.5 - Affinity Chromatography

The purification process was performed at room temperature and monitored by absorbance at 280 nm. Nearly, 2.5 ml of suspended resin (1 ml resin can purified up to 5-8 mg GST fusion protein) corresponding to 1.25 ml of settled resin or 1 volume unit, was transferred on a small polypropylene column. The resin was then washed with 5 volumes of GST bind/wash Buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3). The supernatant of bacterial lysate (~ 25 ml) was deposed on the top of the column and the flow rate was adjusted to 10 volumes per hour (417 µl /min). After loading, the column was washed with 10 volumes of GST Bind /Wash Buffer. The protein of interest was then eluted with the GST Elution Buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8) and fractions (~ 1 ml) were collected. To check the purity of the eluted protein, each fraction containing the target protein was

then submitted to electrophoresis in a 12% SDS-PAGE gel. The positive fractions were stored at -20ºC.

2.3 - E3 - 14.7K

The production of E3-14.7K protein was performed by using the prokaryote pET 28a-Ad5 E3-14.7K vector kindly given by Dr Ronald Rooke from Transgen SA (Strasbourg, France) [Gantzer et al., 2002]. As shown in figure 4, the E3-14.7 K DNA sequence has been inserted between NdeI and EcoRI of pET28a (+) vector. This construction inserted a His-tag at the N-terminus of E3-14.7K. Arctic Express^{TM} bacteria were transformed with the PET 28a-Ad5 E3-14.7K plasmid as described above. The bacteria culture growth was performed with minor changes, such as, LB medium was supplemented with Gentamycin (20 µg/ ml) and Kanamycin (50 µg/ml).

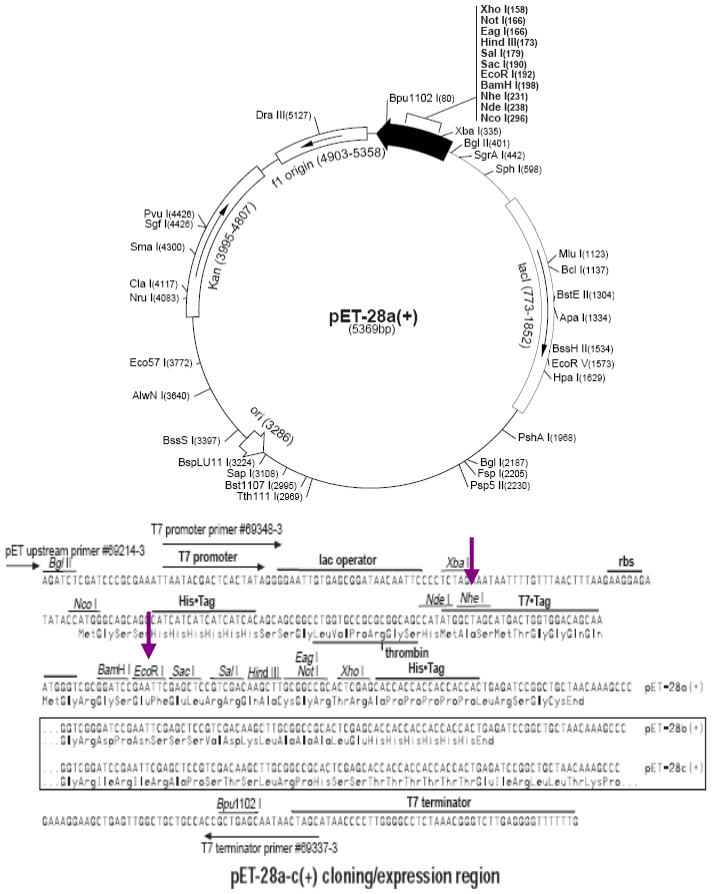


Figure 4 – Map representation of the vector pET28a (+), (from: Novagen)

2.3.1 - Purification of the E3 - 14.7K protein

The purification was performed with a His·Bind® Purification Kit (Novagen®) (Nottingham, United Kingdom) based on an immobilized metal affinity chromatography (IMAC) [Porath *et al.*, 1975]. This allows the purification of a protein of interest from a biochemical mixture due to a highly specific interaction between a Ni-NTA (nickel chelated nitrilotriacetic acid) resin and the 6 histidines tag of the recombinant protein. The protein of interest bound to the Ni-NTA resin is eluted with increasing concentrations of imidazole in the buffer.

2.3.2 - Preparation of the bacterial lysate

Preparation of the bacterial lysate is a critical step. The conditions were optimized in order to maximize cell lysis and to increase the amount of the recombinant protein, while minimizing protein oxidation, proteolysis and sample contamination with genomic DNA. The bacterial pellet was lysed for 20 min with Yeast protein Extraction Reagent Y-PER (PIERCE) (5 ml/g of cell pellet) supplemented with 1mM PMSF (phenylmethanesulphonylfluoride) (SIGMA), 0.1mM DNase I (SIGMA) and RNase A (Macherey-Nagel), 100 µl Protease inhibitor cockail (SIGMA) and 10 mM $MgCl_2$. After the sonication (Vibra cell 75186, BioBlock Scientific, Illkirch, France) according to protocol 10 x 10 pulses (of 5 seconds with a 2 seconds delay between pulses), the lysate was centrifuged at 20,000g for 15 min at 4°C. The first supernatant was removed and the pellet was submitted to a second lysis using the same solutions but in the presence of 0.5 M urea to facilitate the solubilisation of the proteins. After centrifugation at 20,000g for 15 min at 4°C, 10 µl of the pellet and 10 µl of the supernatant were collected and further submitted to a 14% SDS-PAGE.

2.3.3 - Affinity chromatography

The purification was performed at room temperature and monitored by absorbance at 280 nm. Normally, 2.5 ml of suspended His Bind resin allowing purification of up to 20 mg of His tagged protein (1.25 ml of settled resin corresponding also to 1 volume unit of resin). Therefore, the appropriate resin volume was transferred on a small polypropylene column. Firstly, the resin was washed with 3 volumes of sterile deionised water, follow by 5 volumes of charge buffer to charge the NTA resin with Ni^{2+} ions and finally 3 volumes of binding buffer to equilibrate the column with 5 mM of imidazole. After this procedure, the bacterial lysate (~ 25 ml) was deposed on

the top of the column and the flow rate was adjusted to approximately 10 volumes per hour (208 µl/min), for an efficient purification. Indeed, if the flow rate is too fast, more impurities will contaminate the eluted fractions. After loading, the column was washed once with 20 volumes (25 ml) of binding buffer and then with 20 volumes of wash buffer containing 30 mM of imidazole. The protein E3 -14.7K was then eluted sequentially with 12 volumes (15 ml) of the elute buffer solutions containing 100 mM and then 200 mM imidazole in non denaturing conditions. The eluted protein was collected in fractions with a volume of ~ 1 ml.

2.4 – Fluorescent Proteins

2.4.1 - EGFP-FIP-1

The EGFP-FIP protein was produced by using the expression prokaryotic vector, previously constructed by introducing the EGFP-FIP-1 or FIP-1-EGFP sequence in the vpTrc6HisA vector (Invitrogen, Cergy Pontoise, France) (figure 5). This vector contained a 6 histidine tag. The recombinant EGFP-FIP protein was expressed in Arctic Express^{TM} competent cells with a culture protocol similar to that used for the E3 -14.7K protein except that ampicillin (100 µg / ml) was used instead of kanamycin and gentamycin.

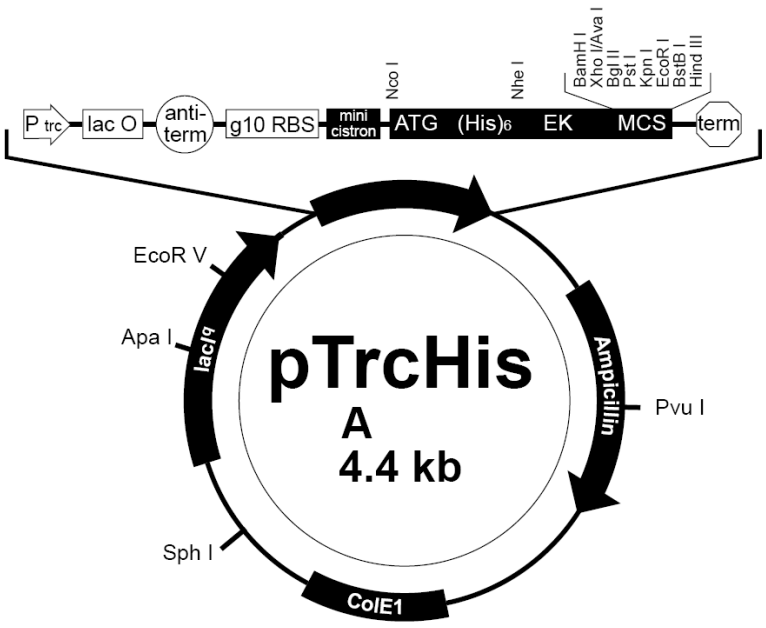


Figure 5 – Map representation of the vector pTrcHis A, (from: Invitrogen)

2.4.2 - Purification of EGFP-FIP-1 protein

The bacterial lysate was prepared as described for E3-14.7K and the purification was performed by chromatography with the His·Bind Resin as described above.

2.4.3 - Cherry-FIP-1

The pET28a-FIP-1 plasmid (previously constructed in the laboratory) was digested at 37°C for 2 hours with BamHI and XhoI restriction endonucleases. Then, the cDNA of Cherry protein was extracted from the pmCherry-C1 plasmid (BD Biosciences Clontech, Ozyme, St. Quentin en Yvelines, France) with NheI and BamHI restriction enzymes (Figure 7). After this procedure, the Cherry cDNA fragment was excised from the gel according to the protocol for DNA extraction from agarose gels, namely, QIAEX II gel Extraction procedure (MACHEREY-NAGEL, QIAGEN Courtaboeuf, France). The FIP-1/mCherry plasmid was constructed by insertion of the Cherry cDNA sequence between the NheI and BamHI sites of pET-28a-FIP-1 (Figure 6). The quality of the construction and the effectiveness of plasmid integration in DH5α competent cells was tested according to the protocol and the following steps: ligation, transformation, colonies screening, extraction of the plasmid DNA by the mega prep procedure and final plasmid purification, as described above for pGST-FIP-1.

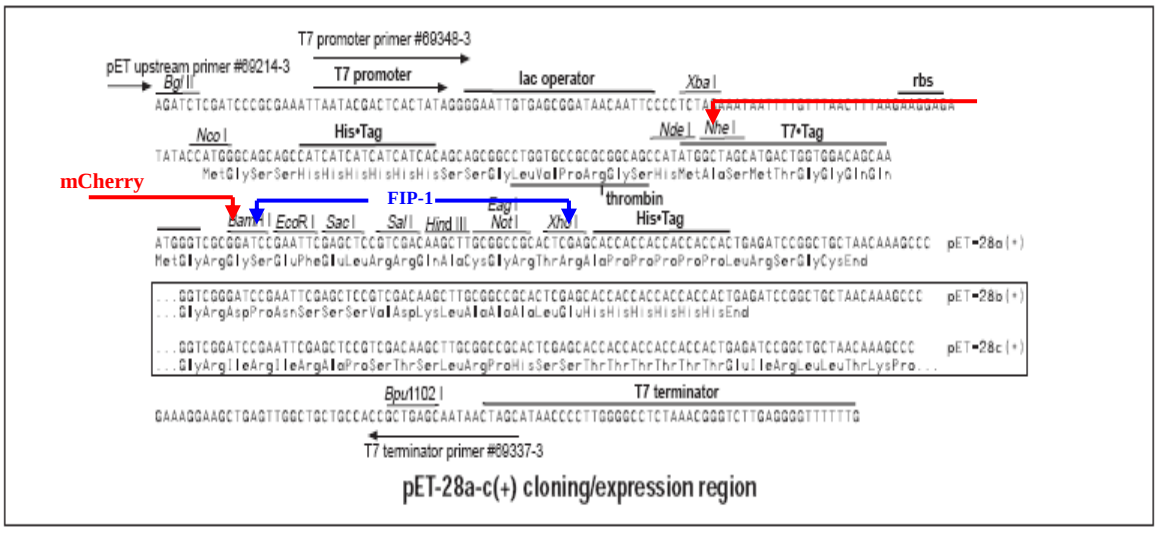


Figure 6 – Map of the cloning expression region of pET28a- FIP-1 Vector, (from: Novagen)

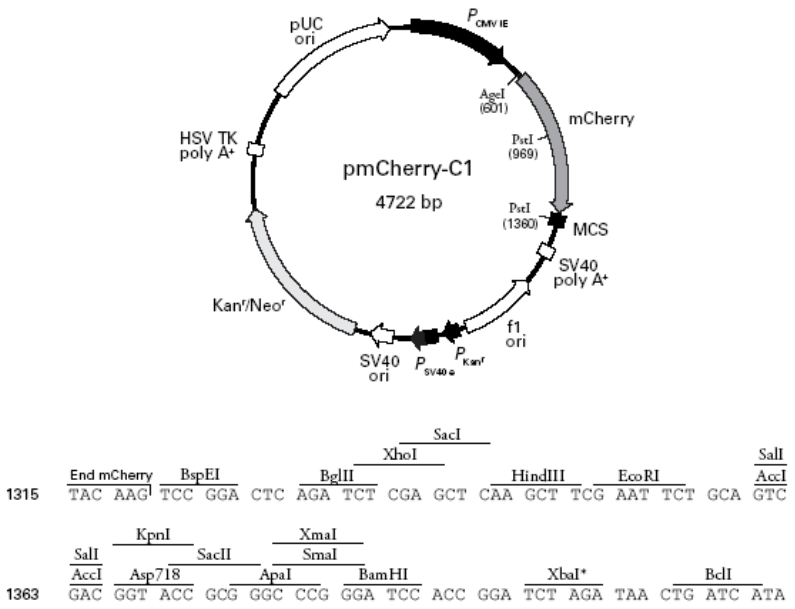


Figure 7 – Map representation of the vector pmCherry-C1, (from: BD Biosciences).

2.4.4 - E3-Cherry

The pET28a-Ad5 E3-14.7 K vector was linearized at 37ºC for 2 hours with EcoRI restriction enzyme. Then, the linear plasmid was treated for 15 min at room temperature with the Klenow fragment (Promega, Charbonnières, France). This fragment is extremely useful for the synthesis of double-stranded DNA from single-stranded templates, filling in recessed 3' ends of DNA fragments or digesting away protruding 3' overhangs. In our case the Klenow fragment transformed a 5' overhangs in blunt termination to allow digestion with HindIII.

The pmCherry-C1 was linearized with NcoI and also treated with the Klenow fragment to generate a blunt extremity (Figure 8). After this treatment, mCherry cDNA was extracted by digestion with HindIII. The blunt extremity allowed ligation between the extremity of pET28a-Ad5 E3 - 14.7K linearized by EcoRI and the mCherry fragment cut with NcoI. Next, the plasmid was closed by ligation overnight at 4°C of its HindIII and blunt extremity's. The quality of the construction was checked as described for the other plasmids applied in this work.

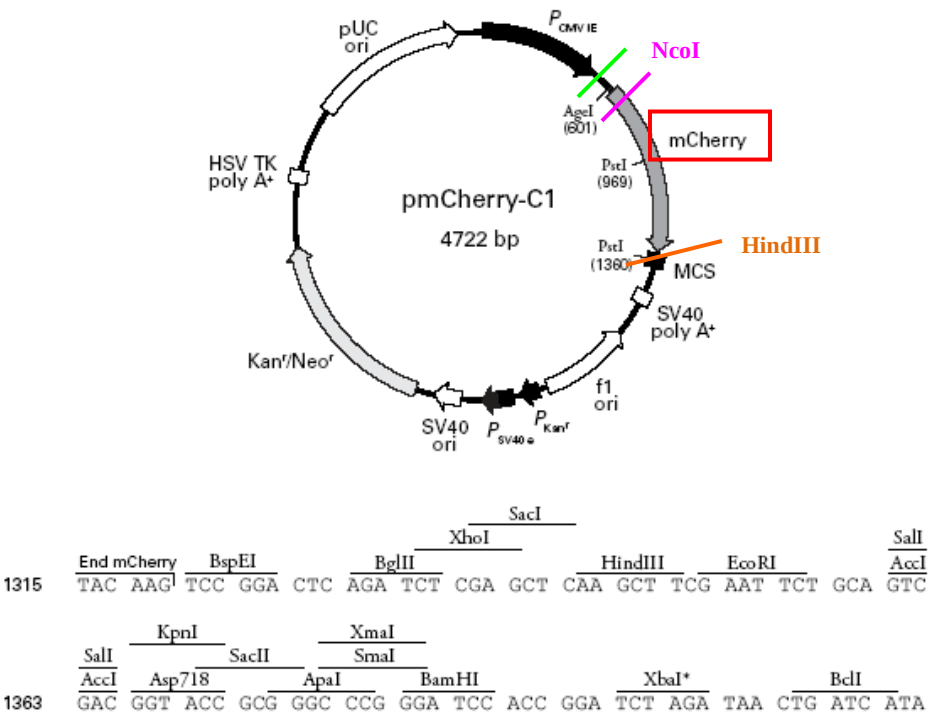


Figure 8 – Map representation of the vector pmCherry-C1, modified vector from(BD Biosciences Clontech).

2.4.5 - Protein production

Cherry-FIP-1 and E3-Cherry proteins were expressed after transformation of Arctic Express™ competent cells by using the protocol similar to that used for GST-FIP-1, except that the selection was performed with Kanamycin (50 µg / ml).

2.4.6 - Purification of the Cherry-FIP-1 and E3-Cherry proteins

The purification was performed with a His·Bind® Purification Kit (Novagen®) as previously described for E3-14.7K and EGFP-FIP-1.

2.5 - Western Blot Protocol

Western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or purified protein. First, native or denatured proteins are separated by gel electrophoresis. The proteins are then transferred to a membrane (typically a Polyvinylidene Difluoride Membrane, PVDF), and subsequently are detected using specific antibodies.

2.5.1 - Tank-blotting procedure

Cut 8 pieces of filter paper (BIORAD, Marnes la coquette, France) and a piece of PVDF membrane (BIORAD, Marnes la coquette, France) to the same size as the gel. The PVDF membrane was incubated for 10 min in pure methanol until the membrane became transparent. Filter paper was soaked in a semi-dry-transfer buffer (see annex 7). The SDS-PAGE gel was removed and placed in the crystallizer containing electrophoresis tank-blotting transfer buffer. Then the SDS-PAGE was sandwiched on the fiber pad between two sets of 4 filter paper sheets. The sandwich was put in the tank filled with blotting transfer buffer. The time of transfer is dependent on the size of the proteins and the percentage of the SDS-PAGE gel used. The transfer was carried out for 1 hour at 100 V and 0.35 A, under agitation. After that, the orientation of the membrane on the gel was marked.

2.5.2 - Protein revelation

All the following steps were carried out at room temperature. The PVDF membrane was washed twice for 10 min in TBS buffer and then incubated for 1 hour in the blocking buffer. The membrane was washed twice for 10 min in TBS-Tween/Triton buffer and then twice for 10 min in TBS buffer. The membrane was incubated for 1 hour with Penta-His-HRP conjugated antibodies (QIAGEN, Courtaboeuf, France) diluted (1/1500 µl) in blocking buffer. The membrane was washed for 10 min in TBS-Tween/Triton buffer and then for 10 min in TBS buffer. Then, the membrane was incubated for 15 minutes in the substrate solution (30 mg 4-1-chloronaphtol 4C1N dissolved in 10 ml methanol was added to 40 ml of 1M Tris-NaCl, pH 8 and immediately supplemented with 100 µl H_2O_2 before use). The colour reaction was stopped by washing the membrane twice with distilled water. Finally, the membrane was dried.

Chapter III

Results and Discussion

3 - Results and Discussion

3.1 - GST-FIP-1 protein

3.1.1 - Construction of pGST-FIP-1 plasmid

In order to check the previously constructed pGST-FIP-1 **plasmid**, the digestion of pGST-FIP-1 with XhoI and BamHI lead to the two expected bands (figure 9). Indeed, one band at 900 pb for the FIP-1 insert (blue arrow) and one at 4900pb for pGEX-6-P2 were observed after electrophoresis in a 0.6% agarose gel. The migration of the full length pGST-FIP-1 plasmid was at about 5800bp (red arrow, b), approximately 1000pb above the migration of the pGEX-6P-2 vector. This reveals that the pGST-FIP-1 plasmid was successfully constructed.

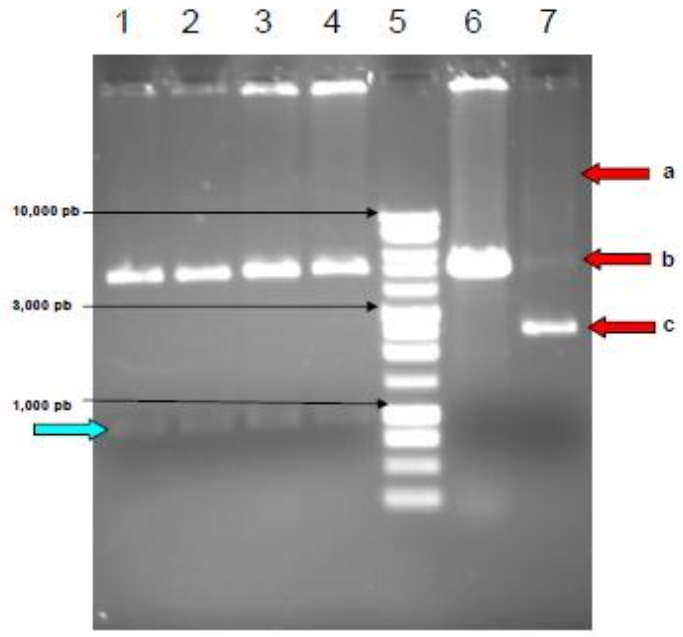


Figure 9 – Electrophoresis of pGST-FIP-1 in a 0.6% agarose gel. Lines 1, 2, 3 and 4 correspond to the digestion by XhoI and BamHI of the plasmid isolated from the tested colonies. Line 5 corresponds to nucleic acid size Marker. Line 6 is the result of the digestion of pGEX-6P-2 by XhoI and BamHI. Line 7 corresponds to the non digested pGEX-6P-2 vector under its (a) relached, (b) linearized and (c) super coiled forms.

In addition, the spectrophotometric analysis of pGST-FIP-1 gave 1.62 for the ratio A_{260}/A_{280} which indicated that the plasmid was in a high purity forms, with a final yield of 84.2 % in table 2. Typically, from a culture volume of 500 ml, we obtained pGST-FIP-1 at a final concentration of 421 µg/ml, usually obtained for a low copy plasmid (annex 5).

Table 2 - Concentration and purity of pGST-FIP-1

Absorbance	
λ_{260nm}	0.0421
λ_{280nm}	0.0259
$\lambda_{260}/\lambda_{280}$	1.62
Final Yield	84.2%
Final concentration	421µg/ml

3.1.2 - Production and purification of GST-FIP-1

Although a wide variety of *E. coli* host strains can be used for cloning and protein expression [Baneyx, 1999; Jonasson *et al.*, 2002]. In this work, we used the Arctic Express^{TM} (Stratagene) competent cells which are specially engineered strains maximizing expression of full-length fusion proteins. Indeed, the bacterial strain applied in these experiments is a cold adapted *E. coli* strain, optimized to increase protein yield by facilitating proper protein folding. This results in an increased production of soluble potentially active protein. Resumidly, the bacterial strains used for plasmid stock or expression are summarized in Table 3.

Table 3- Bacterial strains used for plasmid stock and protein production

Proteins	**Expression vector used**	**tag**	**Plasmid stock strain host**	**Expression host bacteria**
GST-FIP-1	pGEX-6P-2/pEGFP-c1	GST -tag	*E. coli* DH5α	*Arctic Express^{TM} competent cells.*
EGFP-FIP-1	pTrc6HisA	6-His -tag	*E. coli* DH5α	*Arctic Express^{TM} competent cells*
E3-14,7K	pET-28a(+)	6-His -tag	*E. coli* DH5α	*Arctic Express^{TM} competent cells*
E3-Cherry	pET-28a(+)/pmCherry-c1	6-His -tag	*E. coli* DH5α	*Arctic Express^{TM} competent cells*
FIP-1-Cherry	pmCherry-c1	6-His -tag	*E. coli* DH5α	*Arctic Express^{TM} competent cells*

After transformation of Arctic Express^{TM} competent cells with pGST-FIP-1, the expression of the GST-FIP-1 protein was induced at 30ºC by 0.5 mM IPTG. After the induction, bacteria were cultured at 13 °C for 24 h and proteins were extracted. The 12% SDS-PAGE gel showed high quantity of a band at about 62 kDa, which was in accordance with the standard molecular weight of GST-FIP-1 (Figure 10). The purification of GST-FIP-1 was performed by affinity chromatography using GST.bind ^{TM} resin (Novagen). The protein was eluted with the GST Elution Buffer. The 12% SDS PAGE showed that GST-FIP-1 with a 62 kDa molecular weight was obtained with a high degree of purity (Figure 11).

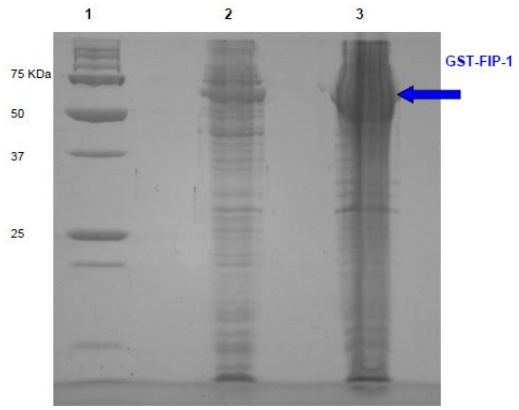


Figure 10 - Electrophoresis in 12% SDS-PAGE gel of protein extract from pGST-FIP-1 transformed bacteria. Line 1: Precision Plus Protein Standards Marker; Lines 2 and 3: protein extract profiles obtained after 24h induction with IPTG (line 3) and compared with non induced culture (line 2). Gel was stained with Coomassie blue.

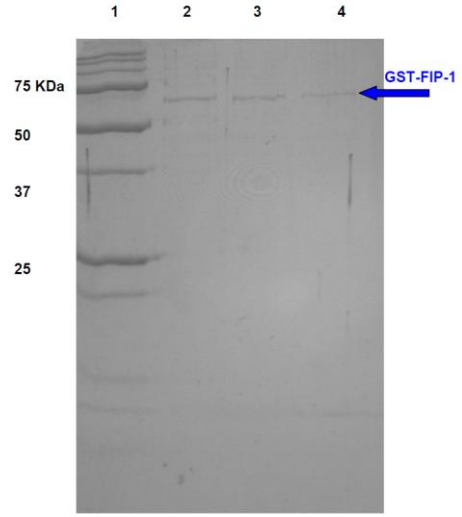


Figure 11- Electrophoresis in 12% SDS-PAGE gel of GST-FIP-1. 1: Precision Plus Protein Standards Marker; 2, 3 and 4: purified fractions of GST-FIP-1 protein. Gel was stained with Coomassie blue.

3.2 - Production and purification of E3 - 14.7K

The production of E3-14.7K protein was performed with Arctic Express ™ competent cells previously transformed in the laboratory with the pET 28a-Ad5 E3 - 14.7K plasmid. The E3-14.7K expression was induced by 1 mM IPTG at 30ºC and the culture was performed at 13 ° C during 24h**.** After protein extraction, the electrophoresis showed an increase after 24 h of a band at 15.3 kDa molecular weight (figure 12, line 2), in accordance with the standard molecular weight of E3-14.7 kDa plus the 6-His tag (600 kDa). The protein purification was done in non-denaturing conditions in order to keep the protein in its native conformation. Although E3-14.7K was contained mostly in inclusion bodies indicating that protein extraction will be better under denaturating conditions, we found that the supernatant contained enough amount of the protein to perform extraction under non-denaturing conditions.

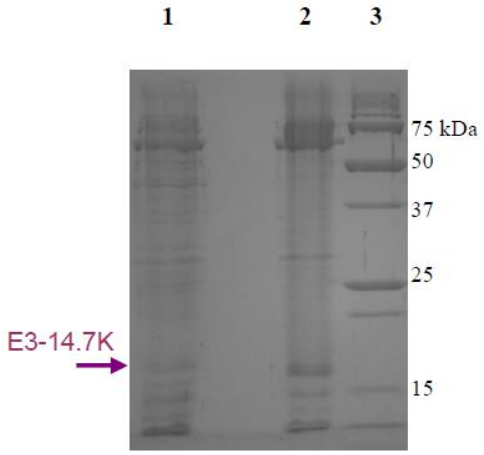


Figure 12 - Electrophoresis in a 14% SDS-PAGE of a protein extract from the pET 28a-Ad5E3 14.7K transformed bacteria. Lines 1 and 2: protein extract profiles obtained after 24h induction with IPTG (line 2) and compared with non induced culture (line 1). Line 3: Precision Plus Protein Standards Marker. Gel was stained with Coomassie blue.

The purification of E3-14.7K was performed by affinity chromatography using a Ni-NTA column with an elution performed in the presence of imidazole. The absorbance at 280 nm of the solution that passed through the column was measured continuously (Figure 13). The chromatogram obtained revealed that the absorbance increased after the protein extract was loaded onto the column (a to b). During the washing steps with binding buffer containing 30 mM of imidazole (b to c) to discard unspecific proteins, the absorbance decreased. The first elution with the elute buffer containing 100 mM of imidazole released a small peak (c to d). At this stage, only contaminants with a low affinity to Ni-NTA resin were removed. Conversely, with the second elute buffer containing 200 mM imidazole, a significant peak was obtained (d to e), that probably correspond to the protein of interest. As shown in Figure 14, the elution with 100 mM of imidazole lead to fractions of E3-14.7K protein slightly contaminated at 10 kDa and approximately at 57 kDa, these two bands corresponds to the co-expressed Cpn10 and Cpn60 chaperonins in this specific ArcticExpress ™ bacterial strain. In contrast, the elution at 200 mM of imidazole lead to a pure fractions of E3-14.7K protein, with a molecular weight of 15.3 kDa.

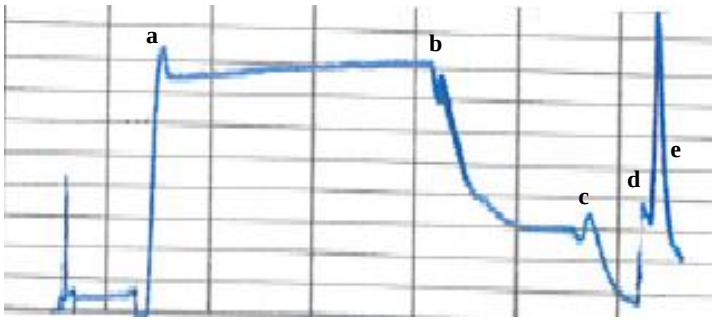


Figure 13 – Representative chromatogram of E3 14.7K purification. From a to b the column was loaded with the protein extract in the Binding Buffer. From b to c the column was washed with binding buffer containing 30 mM imidazole. Then elution was performed from c to d in a binding buffer containing 100 mM imidazole and from d to e with 200 mM imidazole.

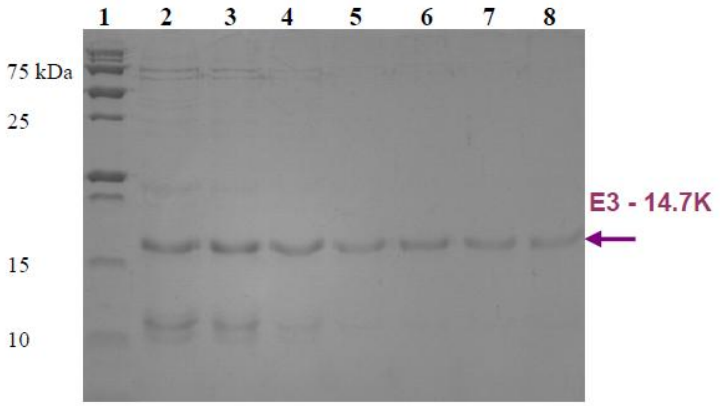


Figure 14 - Electrophoresis in a 14% SDS-PAGE gel from the Ni-NTA column elution profile. Line 1: Precision Plus Protein Standards Marker. Lines 2, 3 and 4: elutions with 100 mM Imidazole. Lines 5, 6, 7 and 8: elution with 200 mM Imidazole. Gel was stained with Coomassie blue.

In order to analyze the purity degree of the protein fractions, the absorbance was measured at 280 nm, as this parameter is a commonly used index for the quantification of protein concentration [Simonian, 2002]. A specific spectrum was obtained (Figure 15).

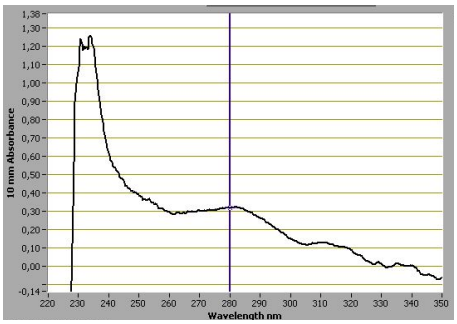


Figure 15 – Absorbance spectrum of E3-14.7K protein, obtained from the elution with 200 mM imidazole in the affinity adsorbent, using a NanoDrop spectrophotometer (Nanodrop Technologies USA)

The absorbance spectrum obtained showed a peak at 280 nm, corresponding to the E3-14.7K protein, however the reduced size reveals that the concentration of the protein obtained is quite low.

3.3 - Production and purification of EGFP-FIP-1

After transformation of Arctic Express^{TM} competent cells with pEGFP-FIP-1 plasmid, the expression of EGFP-FIP protein was induced by 0.5 mM IPTG at 30ºC and bacteria were cultured at 13 °C for 24h. The result of the SDS-PAGE gel (Figure 16) showed an enhanced band nearly at the molecular weight of 64kDa, which was in accordance with the molecular weight of the EGFP-FIP-1 protein. The purification of the protein was performed by affinity chromatography using a Ni-NTA column. A 100 mM imidazole concentration allowed substantial elution of a 64 kDa protein, unfortunately with contaminants, as revealed by the SDS-PAGE (Figure 17, line 2).

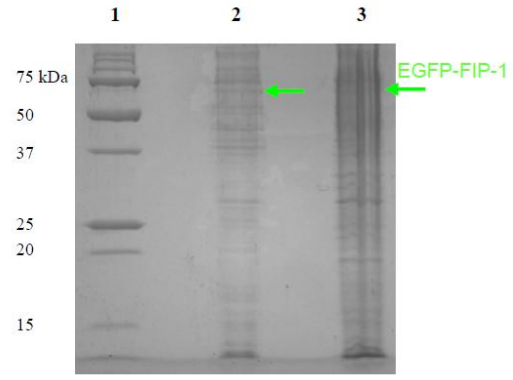


Figure 16 - Electrophoresis in 12% SDS-PAGE gel of protein extract from pEGFP-FIP-1 transformed bacteria. Line 1: Precision Plus Protein Standards Marker. Lines 2 and 3: protein profiles after 24h induction with IPTG (line 3) and compared to non induced culture (line 2). Gel was stained with Coomassie blue.

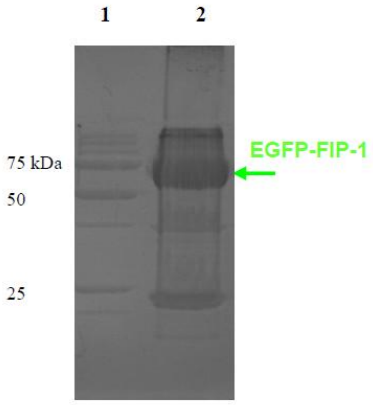


Figure 17 – Electrophoresis in a 12% SDS-PAGE gel of EGFP-FIP-1 after elution in 100 mM imidazole. Line 1: Precision Plus Protein Standards Marker. Line 2: elution in 100mM Imidazole. Gel was stained with Coomassie blue.

3.4 - Production and purification of E3-Cherry protein

After transformation of Arctic Express™ competent cells with pCherry-E3 plasmid, the expression of the E3-Cherry protein was induced by 1mM IPTG at 30ºC. After induction, bacteria were cultured at 13 °C for 24h. The results obtained by the SDS-PAGE (Figure 18) show that a protein of 42.7 kDa was produced, corresponding to the theoretical value of the expected protein.

According to Hendrix and other workers [2008] we have first assayed protein content in the bacterial pellet after 24h induction with 1mM IPTG. This was performed to analyze whether the protein was soluble or contained in inclusion bodies. The majority of the protein was found in the pellet extracts, therefore the extraction was conducted in the presence of 0.5 M urea, as identically found for E3-14.7K. In the production trials the results showed that after the induction with IPTG a progressive increase in E3-Cherry expression was achieved (Figure 18, line 3, red arrow).

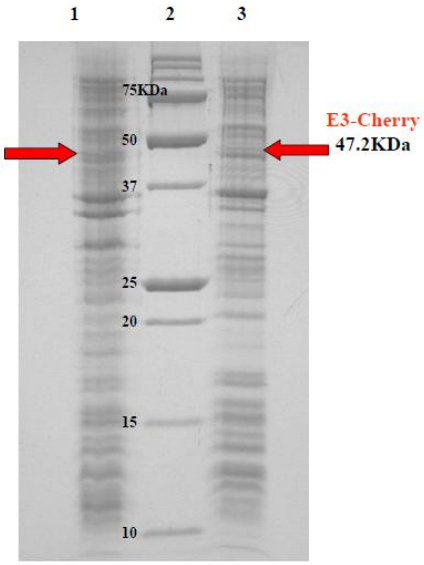


Figure 18 - Electrophoresis in a 14% SDS-PAGE gel of protein extract from the pE3-mCherry transformed bacteria. Lines 1 and 3: protein profiles after 24h IPTG induction (line 3) and of non induced culture (line 1). Line 2: Precision Plus Protein Standards Marker. Gel was stained with Coomassie blue.

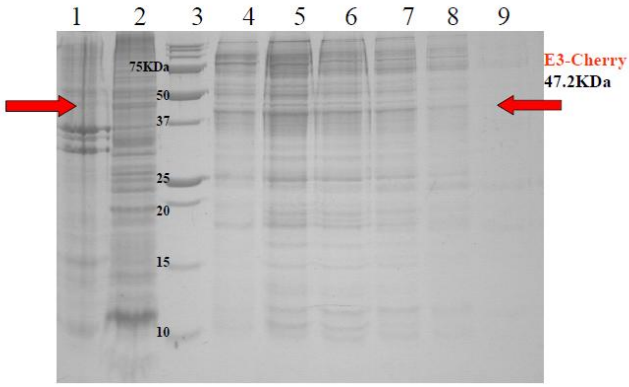


Figure 19 - Electrophoresis in a 14% SDS-PAGE gel of E3/mCherry protein. 1:pellet; 2:Supernatant ; 3: Precision Plus Protein Standards Marker ; 4 :5 :6 :7 and 8 : elutions of the purified E3-Cherry protein in 50 mM Imidazole; 9: elutions purified E3-Cherry protein in 100 mM Imidazole.

After the recombinant production, a suitable purification was performed by affinity chromatography using a Ni-NTA column. The protein of interest was eluted with 50 mM imidazole (Figure 19). A further increase in the imidazole elution, (100 mM), did not promote an additional recovery of any E3-Cherry protein (Figure 19 line 9). Also, the gel indicated that E3-Cherry protein was not in a high purity degree due to the presence of several contaminating proteins with distinct molecular weights.

To assess the fluorescence properties of E3-Cherry, the fluorescence emission spectrum was measured between 587 and 800 nm, upon 587 nm excitation. As showed in figure 20, the fluorescence spectrum exhibited a maximum emission at 610 nm (Figure 20), a native propertie of the target protein accordingly to Nathan and co-workers (2004).

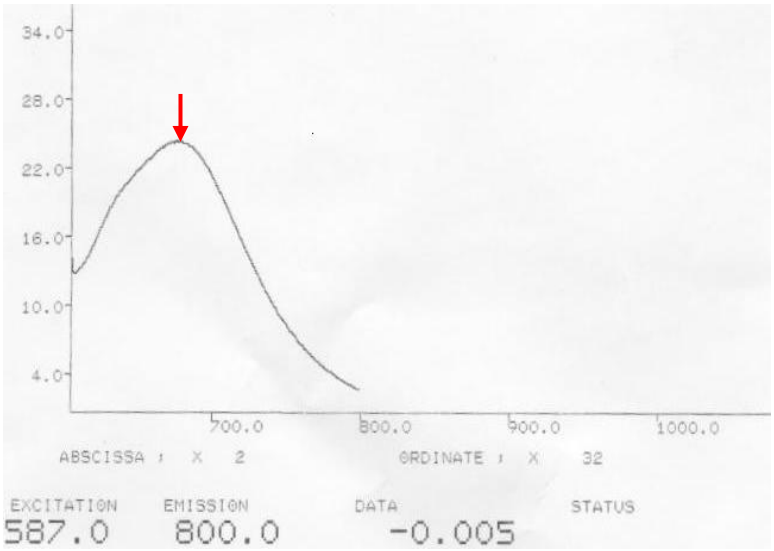


Figure 20 - Fluorescence emission spectrum of E3-Cherry protein. The excitation step was performed at the wavelength of 587 nm.

3.5 - Expression and purification of Cherry - FIP-1 protein

The expression of Cherry-FIP-1 protein in the transformed bacteria after IPTG induction shows that the protein extract contained an enhanced quantity of a protein with a molecular weight of 60 kDa in accordance with the theoretical molecular weight of Cherry-FIP-1 (Figure 21). The electrophoresis gel of the eluted fractions obtained from the Ni-NTA column showed a band of about 60 kDa. Also it was observed, that many contaminant proteins were eliminated by washing the column with 30 mM imidazole. The FIP-1-Cherry protein was obtained by a elution step at 50 mM of imidazole. Nevertheless, the positive fractions still contains a lower percentage of contaminants proteins (Figure 22).

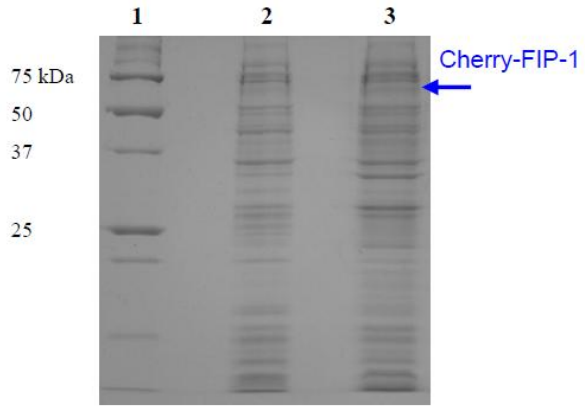


Figure 21 - Electrophoresis in a 12% SDS-PAGE gel of the protein extract from FIP-1-Cherry transformed bacteria. Line 1: Precision Plus Protein Standards Marker. Line 2 and 3: protein profiles after 24h induction with IPTG (line 3) and after a non induced culture (line 2). Gel was stained with Coomassie blue.

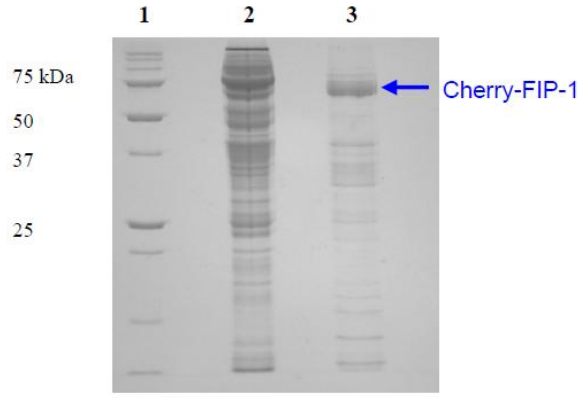


Figure 22 - Electrophoresis in a 12% SDS-PAGE gel of FIP-1-Cherry protein purified from a Ni-NTA column. Line 1: Precision Plus Protein Standards Marker. Line 2: elution with 30 mM Imidazole. Line 3: elution with 50 mM Imidazole. Gel was stained with Coomassie blue.

To confirm the presence of Cherry-FIP-1 protein in the IMAC step a fluorescence spectrum was performed. The fluorescence emission spectrum of Cherry-FIP-1 upon excitation 587 nm showed an emission centred at 610 nm (Figure 23, blue arrow) that reveals the fluorescence typical propertie of Cherry-FIP-1.

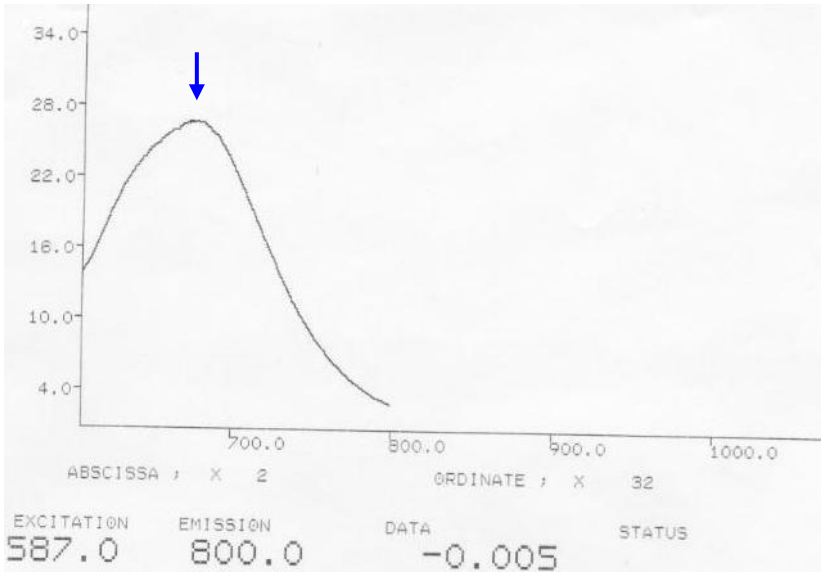


Figure 23 - Fluorescence Emission spectrum of FIP-1-Cherry. Excitation wavelength: 587nm.

In addition, western blotting analysis using specific antibodies against the 6 his sequence was performed for EGFP-FIP-1 and E3-14.7K fractions obtained from the purifications. As shown in the Figure 25, only one band at 64 kDa (line 1; arrow) was revealed indicating that the 64 kDa protein eluted from the Ni-NTA column at 100 mM imidazole (Figure 24) corresponded to EGFP-FIP-1. In contrast, the western blot revealed two bands for E3-14.7K protein (line 3), one at ~30 kDa (arrow a) and the second near the 15.3 kDa (arrow b). Since these proteins possessed the 6 histidine tag, they corresponded to E3-14.7K proteins. The protein at 30 kDa, while appears to twice the molecular weight of E3-14.7K could correspond to the E3-14.7K dimmer. This is in agreement with the results of Kim and Foster demonstrating that E3-14.7K adopts a stable multimeric structure in solution [Kim and Foster, 2002].

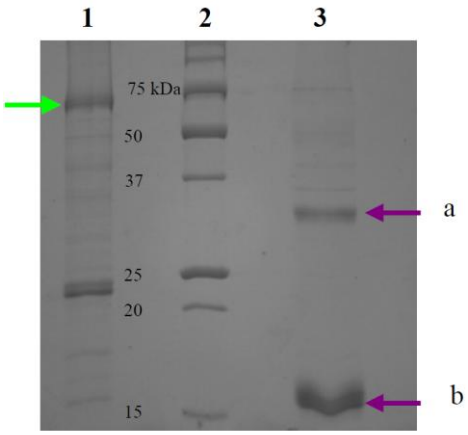


Figure 24 - 14% SDS-PAGE gel electrophoresis of purified EGFP-FIP-1 and E3 14.7K proteins. Line 1: EGFP-FIP-1 after elution in 100 mM imidazole. Line 2: Precision Plus Protein Standards Marker. Line 3: E3-14.7K elution with 200 mM Imidazole. Gel was stained with Coomassie blue.

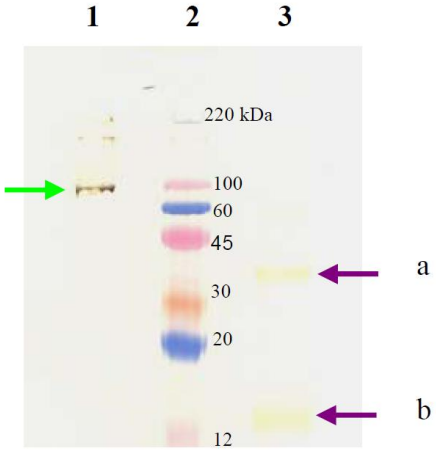


Figure 25 - Western blot of purified EGFP-FIP-1 and E3 14.7K proteins. Line 1 represents EGFP-FIP-1. Line 2 ColorBurst™ Electrophoresis Marker. Line 3 represents E3 14.7K. Arrows indicate (a) E3-14.7K dimer and (b) E3-14.7K monomer.

Table 4- Global purification parameters of the different protein produced

Proteins	Absorbance (280nm) After purification	Purity %	Concentration mg/ml	Yield %
GST-FIP-1	0,24	95%	0,32	48%
E3 – 14.7K	(100mM Imidazole) 0,36 (200mM Imidazole) 0,21	(100mM Imidazole) 65% (200mM Imidazole) 91%	(100mM Imidazole) 2,06 (200mM Imidazole) 1,20	51,5% 30%
EGFP-FIP-1	1,36	64%	1,89	47,25%
Cherry-FIP-1	0,84	67%	0,85	42,5%
E3-Cherry	0,93	n.d.	1,17	n.d.

n.d. - not determined

As previously reported by Kim and Foster [2002], E3-14.7K is a larger insoluble protein. So, these authors used a MBP (Maltose binding protein) fusion tag strategy to produce and purify this protein. In addition, different strategies can be used to promote protein solubilisation such as reduction of the rate of protein synthesis, growth of the bacteria culture at low temperature or over expression of intracellular chaperones [Jonasson *et al.*, 2002]. The latter strategy helps the protein folding with intracellular molecular chaperones. For this purpose, the best characterized ones are the GroEL-GroES chaperones [Baneyx, 1999]. In our study, we have used a new bacterial strain - the Arctic Express™ competent cells - developed by Stratagene®. This could adapted *E.coli* strain to increase protein yield by promoting proper protein folding, resulting in an increased production of soluble and potentially active protein. For this reason, the strain specifically expresses the cold adapted chaperones Cpn 60 and Cpn 10, which have respectively 74% and 54% amino acid identity to the *E. Coli* GroEL and GroES chaperonins. A higher protein refolding activity at lowers temperatures, such as 4ºC and 12°C, is allowed by these chaperonins. Therefore in this work, the bacterial growth is carried out at 13°C. This strategy proved to be effective because we have obtained a general protein yield around 50% in Table 4 and a final concentration close to 1 mg/ml. The protein expression after 3 hours IPTG induction was found similar for all proteins produced. The exception was for the GST-FIP-1 protein, while has required 5 hours of induction due to the weakness of the tac promoter. Although this promoter is better than the lac promoter in terms of efficiency, it is not so strong than the T7-inductible system of pET vectors [Baneyx, 1999; Terpe, 2006].

In the expression of a fusion protein, the choice of the N- and C-terminal positions of the reporter proteins represents an important consideration, because the position can dramatically influence solubility, expression and the activity of the fusion protein [Graslund *et al.*, 2008]. In EGFP-FIP-1, EGFP was to put at the N-terminal of FIP-1, in the opposite side of the 6 His tag, in order to facilitate the subsequent purification step using Ni-NTA column.

The purity degree of each protein was assessed from SDS-PAGE by using the program Image Quant 5.1 Software (Amersham Biosciences). For GST-FIP-1 a 95% degree of purity was obtained by GST-chromatography affinity purification (Table 3). For E3-14.7K, a purity degree of 91% was also obtained by Ni-NTA chromatography affinity purification (IMAC). This technique has the advantage of a strong, specific binding, mild elution conditions and the ability to control selectivity by including low concentrations of imidazole in the chromatographic buffers. A denaturant reagent such as urea has been required to extract E3-14.7K from bacteria due to the propensity of this protein to form aggregates. Indeed, Kim and Foster [2002] used 6M urea and a refolding procedure to purify E3-14.7K. Knowing that oligomeric and unfolded proteins are scarce below 1M urea, in this work we used 0.5 M urea to extract and solubilise the protein. We have successfully purified E3-14.7K protein in non denaturing conditions.
In comparison to E3-14.7K protein, the purity degree reached for EGFP-FIP-1 and FIP-1-Cherry was 64% and 67%, respectively. For these proteins, it should be emphasised that typically were eluted with a low concentration of imidazole (100 mM and 50 mM, compared to 200 mM for E3-14.7K) resulting in the co-elution of other proteins. Indeed, for E3-Cherry, IMAC lead to a very low level of purification. Therefore, other strategies including gel filtration should by applied to purify this fusion protein. In terms of quantity, higher concentrations of EGFP-FIP-1, E3-Cherry protein and the E3-14.7K were obtained.

Chapter IV

Conclusion and Perspectives

4 - Conclusion and Perspectives

The production of recombinant proteins is essential for biotechnology industry and also supports expanded areas of basic and biomedical researches, including structural Genomics and Proteomics. The production in bacteria still represents the convenient production system. In this study, we have produced and purified five recombinant proteins. One of the limitations in the production of recombinant proteins in bacteria is the formation of insoluble protein aggregates, known as inclusion bodies. From a molecular point of view, inclusion bodies are considered to be formed by unspecific hydrophobic interactions between disorderly deposited polypeptides and results in the acquisition of new β-sheet structures compared with the native conformation, even for β-sheet-rich proteins.

In this work, two proteins (GST-FIP-1 and E3-14.7K) were purified with a high degree of purity. Remarkably, E3-14.7K that forms inclusion bodies was purified with 91% of purity using 0.5 M urea protein extraction. These proteins can be used for biophysical and biological studies. The other proteins were purified with a weak level of purity and still contained contaminant proteins, especially proteins fused with EGFP or Cherry. For these proteins, further purification procedures must be applied before further biological studies. Size exclusion chromatography (SEC) could be performed after the purification step by affinity chromatography with Ni-NTA column. An ion exchange chromatography either before or after SEC could be also performed.

The production and purification of the E3-14.7K and FIP-1 proteins will allow further studies concerning their interactions and their effects on apoptosis. The interactions could be studied both *in vitro* by several techniques: immunoprecipitation, Surface Plasmon resonance (Biacore) and in cultured cells: microinjection, FRAP (fluorescence recovery after photobleaching) and FRET (fluorescence resonance energy transfer).

The relationship between these proteins and apoptosis are not yet fully understood. These research approaches have not been used until now and appears so as a great interest at the research level.

Chapter V

Bibliography

Alemany R, Balagué C, Curiel DT. (2000) "Replicative adenovirus for cancer therapy". Nature Biotechnol.; 18 (7): 723-727.

Baneyx, F. (1999) "Recombinant Protein Expression In Escherichia Coli". Curr Opin Biotechnol 10, 411-421.

Bi Wu, Yan Chen, And Joachim D. Müller. (2009) "Fluorescence Fluctuation Spectroscopy Of Mcherry In Living Cells". Biophysical J Volume 96, 2391-2404.

Brooke J. Bevis And Benjamim S.Glick. (2002) "Rapidly Maturing Variants Of The Discosoma Red Fluorescent Protein (Dsred)". Nature Biotechnology Volume 20, 11:1159.

Carmody, R.J., Maguschak, K., And Chen, Y.H. (2006) "A Novel Mechanism Of Nuclear Factor-Kappab Regulation By Adenoviral Protein 14.7k". Immunology 117, 188-195.

Chen, P., Tian, J., Kovesdi, I., And Bruder, J.T. (1998) "Interaction Of The Adenovirus 14.7-Kda Protein With Flice Inhibits Fas Ligand-Induced Apoptosis". J Biol Chem 273, 5815-5820.

David T. Curriel. (2000) "The Development of Conditionally Replicative Adenovirus For Cancer Therapy". Clinical Cancer Research 6, 3395-3399.

Gantzer, M., Spitz, E., Accard, N., And Rooke, R. (2002) "Constitutive Expression Of The Adenovirus E3-14.7k Protein Does Not Prolong Adenovirus Vector Dna Persistence But Protects Mice Against Lipopolysaccharide-Induced Acute Hepatitis". Hum Gene Ther 13, 921-933.

Geoffrey S. Baird, David A. Zacharias, and Roger Y. Tsien. (2000) "Biochemistry, Mutagenesis, And Oligomerization Of Dsred, A Red Fluorescent Protein From Coral". Pnas, Vol. 97, No (22) 11984-11989.

George H. Patterson (2007) "Fluorescent Proteins for Cell Biology". From: Methods In Molecular Biology, Reporter Genes: A Pratical Guide Vol. 411:47-80.

Gooding, L.R., Elmore, L.W., Tollefson, A.E., Brady, H.A., and Wold, W.S. (1988) "A 14,700 Mw Protein from the E3 Region Of Adenovirus Inhibits Cytolysis By Tumor Necrosis Factor". Cell 53, 341-346.

Graslund, S., Nordlund, P., Weigelt, J., Hallberg, B.M., Bray, J., Gileadi, O., Knapp, S., Oppermann, U., Arrowsmith, C., Hui, R., Ming, J., Dhe-Paganon, S., Park, H.W., Savchenko, A., Yee, A., Edwards, A., Vincentelli, R., Cambillau, C., Kim, R., Kim, S.H., Rao, Z., Shi, Y., Terwilliger, T.C., Kim, C.Y., Hung, L.W., Waldo, G.S., Peleg, Y., Albeck, S., Unger, T., Dym, O., Prilusky, J., Sussman, J.L., Stevens, R.C., Lesley, S.A., Wilson, I.A., Joachimiak, A., Collart, F., Dementieva, I., Donnelly, M.I., Eschenfeldt, W.H., Kim, Y., Stols, L., Wu, R., Zhou, M., Burley, S.K., Emtage, J.S., Sauder, J.M., Thompson, D., Bain, K., Luz, J., Gheyi, T., Zhang, F., Atwell, S., Almo, S.C., Bonanno, J.B., Fiser, A., Swaminathan, S., Studier, F.W., Chance, M.R., Sali, A., Acton, T.B., Xiao, R., Zhao, L., Ma, L.C., Hunt, J.F., Tong, L., Cunningham, K., Inouye, M., Anderson, S., Janjua, H., Shastry, R., Ho, C.K., Wang, D., Wang, H., Jiang,

M., Montelione, G.T., Stuart, D.I., Owens, R.J., Daenke, S., Schutz, A., Heinemann, U., Yokoyama, S., Bussow, K., And Gunsalus, K.C. (2008). "Protein Production and Purification". Nat Methods 5, 135-146.

Greber, U.F., And Way, M. (2006). "A Superhighway to Virus Infection". Cell 124, 741-754.

Harrison A, King Sm. (2000). "The Molecular Anatomy of Dynein". Essays Biochem, 35:75-87.

Dinesh S; Bangari And Suresh K. Mittal. (2006) "Current Strategies and Future Directions For Eluding Adenoviral Vector Immunity". Curr Gene Ther, 6 (2): 215-226

Hirose, E., Nakashima, N., Sekiguchi, T., and Nishimoto, T. (1998). "Raga Is A Functional Homologue Of S. Cerevisiae Gtr1p Involved In The Ran/Gsp1-Gtpase Pathway". J Cell Sci, 111 (Pt 1), 11-21.

Hitt Mm, Graham Fl. (2000) "Adenovirus Vectors for Human Gene Therapy". Department Of Pathology and Molecular Medicine, Mcmaster University, Hamilton, Ontario, Canada". Adv Virus Res, 55:479-505.

Horton, T.M., Ranheim, T.S., Aquino, L., Kusher, D.I., Saha, S.K., Ware, C.F., Wold, W.S., And Gooding, L.R. (1991). "Adenovirus E3 14.7k Protein Functions In The Absence Of Other Adenovirus Proteins To Protect Transfected Cells From Tumor Necrosis Factor Cytolysis". J Virol 65, 2629-2639.

Horton, T.M., Tollefson, A.E., Wold, W.S., and Gooding, L.R. (1990). "A Protein Serologically And Functionally Related To The Group C E3 14,700-Kilodalton Protein Is Found In Multiple Adenovirus Serotypes". J Virol 64, 1250-1255.

Horwitz, M.S. (2001) "Adenovirus Immunoregulatory Genes And Their Cellular Targets". Virology 279, 1-8.

Horwitz, M.S. (2004). "Function of Adenovirus E3 Proteins and their interactions with immunoregulatory Cell Proteins". J Gene Med 6 Suppl 1, S172-183.

Ja St George. (2003) "Gene Therapy Progress And Prospects: Adenoviral Vectors". Gene Therapy 10, 1135-1141.

Jelle Hendrix, Cristina Flors, Peter Dedecker, Johan Hofkens, and Yves Engelborghs. (2008) "Dark States In Monomeric Red Fluorescent Proteins Studied By Fluorescence Correlation And Single Molecule Spectroscopy". Biophysical J 3844 94, 4103-4113.

Jonasson, P., Liljeqvist, S., Nygren, P.A., and Stahl, S. (2002) "Genetic Design For Facilitated Production And Recovery Of Recombinant Proteins In Escherichia Coli". Biotechnol Appl Biochem 35, 91-105.

Jonathan L Bramson, Frank L Graham and Jack Gauldie. (1995) "The use of Adenoviral vectors for gene therapy and gene transfer in vivo ". Current Opinion In Biotecnology , 6:590-595.

Karcher Rl, Deacon Sw, Gelfand Vi. (2002) " Motor-Cargo Interactions: The Key To Transport Specificity". Trends Cell Biol; 12(1):21-7.

Katinga Döhner, Claus –Henning Nagel And Beate Sodeik; (2005) "Viral Stop-And-Go Along Microtubules: Taking A Ride With Dynein And Kinesins". Trends in Microbiology Vol. 13 No.7: 320-7.

Kelkar Sa, Pfister Kk, Crystal Rg, Leopold Pl. (2004) "Cytoplasmic Dynein Mediates Adenovirus Binding To Microtubules". J Virol; 78(18):10122-32.

Kim, H.J., and Foster, M.P. (2002) "Characterization of Ad5 E3-14.7k, an Adenoviral Inhibitor of Apoptosis: Structure, Oligomeric State, And Metal Binding". Protein Sci 11, 1117-1128.

King, S.M. (2000) "AAA domains and organization of the dynein motor unit". J Cell Science 113, 2521-2526.

Krajcsi, P., Dimitrov, T., Hermiston, T.W., Tollefson, A.E., Ranheim, T.S., Vande Pol, S.B., Stephenson, A.H., And Wold, W.S. (1996) "The Adenovirus E3-14.7k Protein And The E3-10.4k/14.5k Complex Of Proteins, Which Independently Inhibit Tumor Necrosis Factor (Tnf)-Induced Apoptosis, Also Independently Inhibit Tnf-Induced Release Of Arachidonic Acid". J Virol 70, 4904-4913.

Li, Y., Kang, J., And Horwitz, M.S. (1997) "Interaction Of An Adenovirus 14.7-Kilodalton Protein Inhibitor Of Tumor Necrosis Factor Alpha Cytolysis With A New Member Of The Gtpase Superfamily Of Signal Transducers". J Virol 71, 1576-1582.

Li, Y., Kang, J., And Horwitz, M.S. (1998) "Interaction Of An Adenovirus E3 14.7-Kilodalton Protein With A Novel Tumor Necrosis Factor Alpha-Inducible Cellular Protein Containing Leucine Zipper Domains". Mol Cell Biol 18, 1601-1610.

Lichtenstein, D.L., Toth, K., Doronin, K., Tollefson, A.E., and Wold, W.S. (2004). "Functions And Mechanisms Of Action Of The Adenovirus E3 Proteins". Int Rev Immunol 23, 75-111.

Liu Y, Huang H, Saxena A, Xiang J. (2002) "Intratumoral Coinjection Of Two Adenoviral Vectors Expressing Functional Interleukin-18 And Inducible Protein-10: Respectively, Synergizes To Facilitate Regression Of Established Tumors". Cancer Gene Ther; 9:533-542.

Lukashok, S.A., Tarassishin, L., Li, Y., And Horwitz, M.S. (2000) "An Adenovirus Inhibitor Of Tumor Necrosis Factor Alpha-Induced Apoptosis Complexes With Dynein And A Small Gtpase". J Virol 74, 4705-4709.

Maarit Suomalainen, Michael Y. Nakano, Stephan Keller, Karin Boucke , Robert P. Stidwill, And Urs F. Greber. (1999) "Microtubule-Dependent Plus-And Minus End-Directed Motilities Are Competing Process For Nuclear Targeting Of Adenovirus". The J of Cell Biology, Volume 14**,** 657-672.

Mebatsion T. (2001) “Extensive Attenuation Of Rabies Virus By Simultaneously Modifying The Dynein Light Chain Binding Site In The P Protein And Replacing Arg333 In The G Protein”. J Virol; 75(23):11496-502.

Mueller, S., Cao, X., Welker, R., And Wimmer, E. (2002) "Interaction of the Poliovirus Receptor Cd155 with the Dynein Light Chain Tctex-1 and Its Implication for Poliovirus Pathogenesis". J Biol Chem 277, 7897-7904.

Nakashima N, Noguchi E, Nishimoto T. (1999) “Saccharomyces Cerevisiae Putative G Protein, Gtr1p, Which Forms Complexes With Itself And A Novel Protein Designated As Gtr2p, Negatively Regulates The Ran/Gsp1p G Protein Cycle Through Gtr2p”. Genetics; 152(3): 853-67.

Nathan C Shaner, Robert E Campbell, Paul A Steinbach, Ben N G Giepmans, Amy E Palmer & Roger Y Tsien. (2004) "Improved Monomeric Red, Orange and Yellow Fluorescent Proteins Derived From Discosoma Sp. Red Fluorescent Protein". Nature Biotechnology Volume 22 Number 12 (10) 1038-1037.

Porath J, Carlsson J, Olsson I, Belfrage G. (1975) “Metal Chelate Affinity Chromatography, a New Approach To Protein Fractionation”. Nature 18;258 (5536): 598-9.

Rayment I. (1996) " Kinesin and myosin: molecular motors with similar engines". Structure 4, 501-504.

Schneider-Brachert, W., Tchikov, V., Merkel, O., Jakob, M., Hallas, C., Kruse, M.L., Groitl, P., Lehn, A., Hildt, E., Held-Feindt, J., Dobner, T., Kabelitz, D., Kronke, M., And Schutze, S. (2006) "Inhibition Of Tnf Receptor 1 Internalization By Adenovirus 14.7k As A Novel Immune Escape Mechanism". J Clin Invest 116, 2901-2913.

Schurmann, A., Brauers, A., Massmann, S., Becker, W., and Joost, H.G. (1995). "Cloning Of A Novel Family Of Mammalian Gtp-Binding Proteins (Raga, Ragbs, Ragb1) With Remote Similarity To The Ras-Related Gtpases". J Biol Chem 270, 28982-28988.

Sekiguchi T, Hayashi N, Wang Y, Kobayashi H. (2008) “Genetic Evidence That Ras-Like Gtpases, Gtr1p, And Gtr2p, Are Involved In Epigenetic Control Of Gene Expression In Saccharomyces Cerevisiae”. Biochem Biophys Res Commun; 11; 368(3):748-54.

Sekiguchi, T., Hirose, E., Nakashima, N., Ii, M., and Nishimoto, T. (2001) "Novel G Proteins, Rag C and Rag D, Interact With Gtp-Binding Proteins, Rag A and Rag B". J Biol Chem 276, 7246-7257.

Sekiguchi, T., Todaka, Y., Wang, Y., Hirose, E., Nakashima, N., and Nishimoto, T. (2004) "A Novel Human Nucleolar Protein, Nop132, Binds To The G Proteins, Rrag A/C/D". J Biol Chem 279, 8343-8350.

Simonian, MH. (2002) "Spectrophotometric Determination of Protein Concentration". Curr Protoc Cell Biol Appendix 3, A.3B.1-A.3B.7.

Terpe, K. (2006) "Overview of Bacterial Expression Systems for Heterologous Protein Production: From Molecular and Biochemical Fundamentals to Commercial Systems". Appl Microbiol Biotechnol 72, 211-222.

Tufariello, J., Cho, S., And Horwitz, M.S. (1994) "The Adenovirus E3 14.7-Kilodalton Protein Which Inhibits Cytolysis By Tumor Necrosis Factor Increases The Virulence Of Vaccinia Virus In A Murine Pneumonia Model". J Virol 68, 453-462.

Ventura, S., and Villaverde, A. (2006) "Protein Quality In Bacterial Inclusion Bodies". Trends Biotechnol 24, 179-185.

Volpers, C., and Kochanek, S. (2004) "Adenoviral vectors for gene transfer and therapy". J Gen Med 6, S164-S171.

Watanabe TK, Fujiwara T, Shimizu F, Okuno S, Suzuki M, Takahashi E, Nakamura Y, Hirai Y. (1996) "Cloning, expression, and mapping of TCTEL1, a putative human homologue of murine Tcte1, to 6q". Cytogenet Cell Genet; 73(1-2):153-6.

Williams JC, Xie H, and Hendrickson WA. (2005) "Crystal Structure of Dynein Light Chain TcTex-1". J Biol Chem 280, 21981-21986.

Xiaokun Shu, Nathan C. Shaner, Corine A. Yarbrough, Roger Y. Tsien, and S. James Remington. (2006) "Novel chromophores and Buried Charges Control Color in mFruits". Biochemistry 45 (32), 9639-9647.

Ye, G. J., Vaughan, K. T. & Roizman, B. (2000) "The herpes simplex virus 1 UL34 protein interacts with a cytoplasmic dynein intermediate chain and targets nuclear membrane". Journal of Virology 74: 1355-1363.

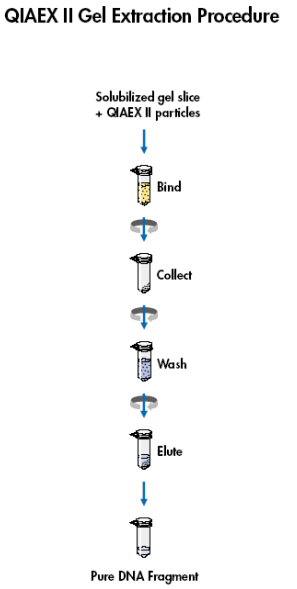
Annex:

Annex 1- Agarose gel electrophoresis

1- Weight 0.6 g of agarose and add 100 ml of 1X TAE buffer into the Erlenmeyer flask
2- Boil the mixture in a microwave oven (at middle power) until the agarose melts completely. Swirl the flask several times while boiling.
3- Weight the flask again and if necessary add hot distilled water to restore the initial weight
4- Cool slightly
5- Add 10% TBE (10mg/ml) to the gel and mix
6- Place the comb
7- Solidify the gel for about 30-40 min.
8- Immerse the gel into the desired electrophoresis buffer and load samples.
9- Deposit the samples

The loading buffer was the nucleic acid size marker of the Bench top 1kb DNA ladder (Promega). The migration is running for 1h30 to 2h at 80V

Annex 2- Protocol for DNA extraction from agarose gels (MACHEREY-NAGEL)



(From: QIAGEN)

1-Excision of DNA fragment and Solublilisation of gel slice

Excise carefully the gel slice containing the DNA fragment to minimize the gel volume. Incubate the sample at 50°C until the gel slices are dissolved 5 at 10 min.

2-DNA binding

Place a NucleoSpin ® extract II column into a collection tube. Centrifuge for 1 min at 11,000 x g. Discard flow-through and place the NucleoSpin® extract II column back into the collection tube.

3- Washing of silica membrane

Add 600µl Buffer NT3. Centrifuge for 1 min at 11, 000 x g. Discard flow-through and place the NucleoSpin ® Extract II column back into the collection tube.

4- Drying of silica membrane

Centrifuge for 2 min at 11,000 x g to remove Buffer NT3 quantitatively. Residual ethanol from Buffer NT3 might inhibit subsequent reactions and has to be removed in this step. In addition to centrifugation, total removal can be achieved by incubation of NucleoSpin ® extract II columns for 2-5 min at 70°C prior to elution.

5- DNA elution

Place the NucleoSpin ® Extract II Column into a clean 1.5 ml microcentrifuge tube. Add 15-50µl Elution Buffer NE and incubate at room temperature for 1 min to increase the yield of eluted DNA. Centrifuge for 1 min at 11,000 x g.

Annex 3- DH5 α bacterial transformation

The bacterial strain used was the Subcloning Efficiency DH5α Competent Cells (Invitrogen). They are recommended for routine subcloning into plasmid vectors and not suitable for the generation of cDNA libraries.

1. Throw on ice one tube of DH5α cells.
2. Gently mix cells with the pipette tip and aliquot 50 µL of cells for each transformation into 1.5 ml microcentrifuge tube
3. Add 5 µl (1-10ng) of DNA to the cells and mix gently.
4. Incubate tubes on ice for 30 minutes
5. Heat cells for 30 seconds in a 42°C water bath without shaking
6. Place tubes on ice for 2 minutes
7. Add 250 µl of pre-warmed SOC medium
8. Incubate tubes at 37°C for 1 hour at 225 rpm
9. Spread 150 µl from each transformation on pre-warmed selective plates. Plate 100 µl on an LB plate containing 100 µg/ml ampicillin.
10. Incubate plates overnight at 37°C.

Annex 4- Extraction of the plasmid DNA from *Escherichia coli* - Mini-Prep procedure

1. 1.5 ml of bacterial culture is placed on a tube
2. Centrifuge for 1 min at 10 000 rpm
3. Remove the supernatant
4. Add 100 µl of the solution I and mix by vortexing
5. Add 200 µl of the solution II and mix by vortexing
6. Place tube on ice for 5 min to lyse the cell
7. Add 150 µl of the solution III
8. Centrifuge for 5 min at 14500 rpm
9. Collect the supernatant and clean tube
10. Add 1ml ethanol (100%) to homogenize the solution and to precipitate the protein
11. Place the tube on ice for 2 min
12. Centrifuge for 5 min and remove supernatant
13. Add 500 µl of ethanol (70%)
14. Centrifuge for 5 min, 14500 rpm
15. Remove supernatant
16. Incubate tube at 65°C for 5 min
17. Add 50 µl of TE solution
18. Incubate tube at 37°C for 5 min

Solutions used for extraction of the plasmic DNA from *Escherichia coli* - Mini-Prep procedure

Product	Composition
solution I	50mM Glucose 25mM Tris –HCl 10mM EDTA , pH 8.0
solution II	0.4 M NaOH 2% SDS
solution III	3M potassium acetate 5M acetic acid , pH 8.0
TE Buffer	10mM Tris.Cl, pH 8.0 1mM EDTA

Annex 5- Purification of Plasmid pGEX-6P-2 / FIP-1

EndoFree Plasmid Purification Kit (QIAGEN).

A) Bacterial culture, harvesting and lysis

1- Pellet 500 ml or 2.5 litres (Low copy) overnight LB bacterial culture at 6000 x g for 15 min at 4°C.

2- Resuspend homogeneously the bacterial pellet in 50 ml of Buffer P1.

3- Add 50 ml of Buffer P2, mix thoroughly by vigorously inverting 4-6 times, and incubate at room temperature for 5 min.

4- Add 50 ml of chilled Buffer P3, mix thoroughly by vigorously inverting 4-6 times.

B) Bacterial lysate clearing

5- Pour lysate into the QIAfilter Cartridge and incubate for 10 min. Switch on the vacuum source. After all liquid has been pulled through, switch off the vacuum source. Keep the QIAfilter Cartridge attached. Add 50 ml of Buffer FWB2 to the QIAfilter Cartrige and gently stir the precipitate with a sterile spatula. Switch on the vacuum source until the liquid has been pulled through completely.

6- Add 12.5 ml of Buffer ER to the filtered lysate, mix by inverting the tube approximately 10 times, and incubate on ice for 30 min.

C) Binding, washing and elution of plasmid DNA on QIAGEN-tip

7- Equilibrate a QIAGEN-tip 2500 by applying 35 ml of Buffer QBT and allow the column to empty by gravity flow.

8- Apply the filtered lysate from step 6 to the QIAGEN-tip and allow it to enter into the resin by gravity flow.

9- Wash the QIAGEN-tip with 200ml of Buffer QC.

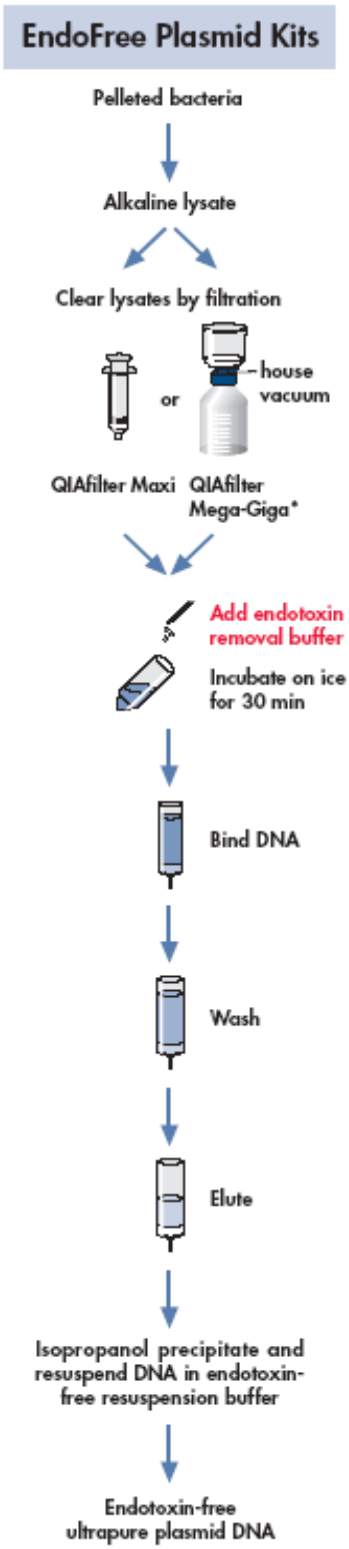
10- Elute DNA with 35 ml of Buffer QN.

D) Precipitation, washing and solubilisation of plasmid DNA

11- Precipitate DNA by adding 24.5 ml of room-temperature isopropanol to the eluted DNA and mix. Centrifuge at 17,000 x g for 30 min at 4°C. Carefully decant supernatant.

12- Wash DNA pellet with 7 ml of endotoxin-free room-temperature 70% ethanol and centrifuge at 17,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.

13- Air-dry the pellet for 5-10 min, and solubilise the DNA in a suitable volume of endotoxin-free Buffer TE.


EndoFree Plasmid Kits
Pelleted bacteria
Alkaline lysate
Clear lysates by filtration
or
house vacuum
QIAfilter Maxi
QIAfilter Mega-Giga*
Add endotoxin removal buffer
Incubate on ice for 30 min
Bind DNA
Wash
Elute
Isopropanol precipitate and resuspend DNA in endotoxin-free resuspension buffer
Endotoxin-free ultrapure plasmid DNA

(From: QIAGEN)

Solutions used for EndoFree Plasmid Purification Kit (QIAGEN).

Product	Composition
Buffer P1 (Resuspension buffer)	50mM Tris.Cl, pH8.0 10mM EDTA 100µg/ml RNase A
Buffer P2 (Lysis buffer)	200mM NaOH 1% SDS (w/v)
Buffer P3 (neutralization buffer)	3.0M potassium acetate, pH5.5
Buffer FWB2 (QIAfilter wash buffer)	1M potassium acetate, pH 5.0
Buffer QBT (equilibration buffer)	750 mM NaCl 50mM MOPS, pH7.0 15% isopropanol (v/v) 0.15% Triton® X-100 (v/v)
Buffer QC (wash buffer)	1.0M NaCl 50mM MOPS, pH7.0 15% isopropanol (v/v)
Buffer QN (elution buffer)	1.6 M NaCl 50 mM MOPS, pH7.0 15% isopropanol (v/v)
TE	10mM Tris-Cl, pH8.0 1mM EDTA

Solutions for agarose gel electrophoresis

TBE Buffer

Product	Volume (ml)	Concentration
Tris	3.23 g	89 mM
Boric Acid	1.65 g	89 mM
EDTA	0.279 g	2.5 mM

Annex 6- SDS-PAGE gel electrophoresis

Solutions for the SDS-PAGE gel electrophoresis

Resolving gel solution	12%	14%
Acyl bis-Acrylamide (40%)	1.65 ml	1.94 ml
Resolving gel solution 3X	2.2 ml	2.2 ml
Ultrapure water	1.65 ml	1.36 ml
TEMED	5 µl	5 µl
APS (10%)	50 µl	50 ml
Total volume	5.555 ml	5.555 ml

Stacking gel soluction	12%	14%
Acyl bis-Acrylamide (40%)	0.21 ml	0.21 ml
Stacking gel solution 3X	0.33 ml	0.33 ml
Ultrapure water	1.070 ml	1.070 ml
TEMED	2 µl	2 µl
APS (10%)	15 µl	15 µl
Total volume	1.627 ml	1.627 ml

Resolving gel solution 3X

Product	Volume (ml)	Concentration
1.5M Tris-HCl, pH 8.8	38.5	1.155 mM
SDS (10%)	1.5	0.3 %
H_2O ultrapure	10	

Stacking gel solution 3X

Product	Volume (ml)	Concentration
0.5M Tris-HCl, pH 6.8	37.5	375 mM
SDS (10%)	1.5	0.3 %
ultrapure H_2O	11	

Running buffer10X, pH 8.3

Product	Volume (ml)/weight
Tris base	30.3 g
Glycine	144 g
SDS	10 g

Protein denaturation

Add 5 µl of 2X LBS (Laemmli Buffer System) to 10 µl of sample (proteins) and heat for 3 minutes at 85 ° C.

Deposit the samples in the gel electrophoresis.

The migration is realized at 200V, 1.85 A. The time depends on the % of the gel.

After migration the gel is washed 2 times for 5 minutes with bi-distillate water. The protein was stained for 1h with Coomassie blue (Sigma).

Coomassie Blue solution

Product	Volume (ml)/weight	Concentration
Acetic Acid 100%	30 ml	10%
Ethanol	150 ml	10%
H_2O	300 ml	
Coomassie blue	0.75 g	

Annex 7- Western Blot (QIAexpress® Detection and Assay Handbook)

Solutions for western transfer:

Tank-blotting transfer buffer

Product	Concentration
Tris base	25 mM
Glycine	150 mM
Methanol	20 %
Should be at pH 8.3 without adjust	

TBS buffer

Product	Concentration
Tris-Cl, pH 7.5	10 mM
NaCl	150 mM

TBS- Tween / Triton buffer

Product	Concentration
Tris-Cl, pH 7.5	20 mM
NaCl	500 mM
Tween 20	0.05 %(v/v)
Triton X-100	0.2 %(v/v)

Blocking solution

Product	Concentration
BSA	3 % (w/v)
TBS 1x	