Raising and characterization of polyclonal antibodies against the arginine transporter AAP3 from *Leishmania donovani*

Master thesis in Pharmacy

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Throughout this year I have grown immensely and learned a lot. Though there has been though times, I would without a doubt do it all again.

ABBREVIATIONS

aa	Amino acid
AAP	Amino acid permease
AP	Alkaline Phosphatase
ARG	Arginase
BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	Base pair
CL	Cutaneous leishmaniasis
CAT	cationic amino acid transporter
Cter	C-terminal
DNA	Deoxyribonucleic acid
DSLR	Digital single-lens reflex
EDTA	Ethylenediaminetetraacetic acid
FT	Flow Through
FT-BE	Flow Through Buffer Elution
I	Induced
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
g	gravity of acceleration (~9,8 m/s^2)
g	gram
h	hour
hs	hours
kb	kilobase
kDa	kilodalton
LB	Luria Broth
LPG	Lipophosphoglycan
M	Molar
mg	Milligram
min	Minutes
ML	Mucocutaneous leishmaniasis
mL	Milliliter
mM	MilliMolar

NBT	nitroblue tetrazolium
Ni-NTA	Nickel-nitrilotriacetic acid
NO	Nitric oxide
Nter	N-terminal
O.D.	Optical density
ORF	Open Reading Frame
PAGE	Polyacrylamid Gel Electrophoresis
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase chain reaction
pg	Picogram (10^{-12}g)
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
sec	Seconds
SLA	Soluble *Leishmania amazonensis*
S.O.B	Super Optimal Broth
S.O.C	Super Optimal Broth with Catabolic repressor
TAE	Tris-acetate-EDTA
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween 20
T7RNAP	T7RNA polymerase
U	unit
UI	uninduced
µL	microliter
UV	Ultraviolet
v	volume
V	volts
VL	Visceral leishmaniasis

ABSTRACT

Leishmania is an intracellular protozoa causing the tropical disease leishmaniasis. The disease is spread by female sandflies from the *Phlebotomus* and the *Lutzomyia* genera, and can cause diverse manifestations ranging from asymptomatic disease to lethal visceral leishmaniasis. The amino acid permease (AAP) transporter is conserved in the different *Leishmania* species, and is important for *Leishmania* survival and virulence. This makes the investigation on this protein important for development of new drug and vaccines.

The aim of this study was to successfully express and purify the LdAAP3 (*Leishmania donovani* amino acid permease 3) transporter protein in *E. coli* and use this recombinant antigen to produce polyclonal antibodies in rabbit.

The LdAAP3 gene was cloned into a pET TOPO vector, for expression of recombinant protein in *E. coli*, and purified by affinity chromatography as His-tags were attached to the N-terminal (Nter) end of the target protein. The purified recombinant protein was quantified and injected in rabbits to raise polyclonal antibodies. Sera obtained from the injected rabbits were tested for their binding affinity to recombinant LdAAP3 protein or lysated *Leishmania amazonensis* by using Western blots and ELISA.

The results from the expression were analyzed on different SDS-PAGE and Western blots, and revealed a successful expression of the LdAAP3 protein. Comparing the binding of antibodies from pre-immunized and immunized sera to LdAAP3 recombinant protein revealed a successful production of anti-LdAAP3 antibodies. ELISA experiments confirm that anti-LdAAP3 antibodies have binding affinity to epitopes of *Leishmania amazonensis*. Throughout the experiments a binding pattern was obtained by the observation of repeated protein bands often found at sizes around 56 kDa, 45 kDa, 38 kDa, 32 kDa, 26 kDa and 12 kDa on SDS-PAGE and Western blots incubated with sera from infected subjects.

In conclusion, the successful production of the LdAAP3 recombinant protein, lead to the production of polyclonal anti-LdAAP3 antibodies, which could be used for future localization of the AAP3 protein in *Leishmania* cells by imaging techniques such as confocal or electron microscopy. The LdAAP3 recombinant proteins also enabled detection of antibodies from infected subjects by binding to the epitopes on the recombinant protein. This gave a binding pattern that was characteristic for infected subjects. To be able to use this method for further diagnostics, experiments will have to be done comparing the binding specificity and avidity, and testing the similarities of similar genus like *Crithidia* and *Leptomonas,* which also produce AAP transporters.

ABSTRAKT

Leishmania er en intracellulær protozo som fører til sykdommen leishmaniasis. Sykdommen spres med sandfluer av typen *Phlebotomus* og *Lutzomyia*. Den kan føre til ulike manifestasjoner fra asymptomatisk til dødelig visceral sykdom. Aminosyrepermeasene (AAP) er konservert i de ulike *Leishmania*-stammene, og er viktig for *Leishmania* sin overlevelse og virulens. Derfor har dette proteinet potensialle for utvikling av nye medisiner og vaksiner.

Målet til denne studien var å uttryke og rense LdAAP3 (*Leishmania donovani* amino acid permease 3) transporter proteinet i *E.*coli, og bruke dette rekombinante proteinet til å produsere polyklonale antistoffer i kaniner.

LdAAP3 genet ble klonet i pET TOPO vektoren for ekspresjon av recombinant protein i *E.coli*, og renset ved hjelp av affinitets kromatografi siden proteinet inneholdt polyhistidine-tags. Det rensede rekombinante proteinet ble kvantifisert og kaniner ble immuniset med proteinet for å lage polyklonale antistoffer. Ved hjelp av Western blot og ELISA ble sera fra de inokulerte kaninene undersøkt for sin binding til det rekombinante LdAAP3 proteinet eller lysert *Leishmania amazonensis.*

Resultatene fra ekspresjonen ble analysert ved SDS-PAGE og Western blot. LdAAP3 proteinet ble uttrykt og renset. ELISA og Western blot analyser viste at antistoffer hadde blitt produsert når man sammenlignet serum fra før og etter immunisering i kaninene. ELISA eksperimenter bekreftet at anti-LdAAP3 antistoffer har bindingsaffinitet til epitoper på *Leishmania amazonensis.* Når Western blot ble inkubert med sera fra personer med leishmaniasis fant man proteinbånd ved rundt 56 kDa, 45 kDa, 38 kDa, 26 kDa og 12 kDa.

For å konkludere, det rekombinante proteinet LdAAP3 ble produsert og ble brukt til å produsere polyklonale LdAAP3 antistoffer i kaniner. Antistoffer kan potensielt brukes til lokalisering av AAP3-proteinet i *Leishmania* parasitten. LdAAP3 proteinet ble også brukt til detektering av antistoffer hos personer med leishmaniainfeksjonen. For at metoden skal kunne brukes i diagnostikk vil eksperimenter måtte bli utført for å undersøke spesifisitet, sensitivitet og vurdere kryssreaktivitet til andre mikrober som *Crithidia* og *Leptomonas,* som også produserer AAP transportører.

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1. INTRODUCTION

1.1 The obligatory intracellular protozoa, Leishmania

1.1.1 Epidemiology

Leishmaniasis is a tropical disease caused by protozoan parasites of the genus *Leishmania*, and it spreads by the bite of specific sandflies. It occurs in diverse areas, ranging from rain forests to desserts and cities, and can be found on every continent aside from the Antarctic and Australia. Evaluating the disease burden of leishmaniasis can be challenging due to lack of reliable data on incidence, duration, severity, and due to its clinical and epidemiological diversity (1). Each year up to 20.000-40.000 people die because of leishmaniasis. Over 12 million people in the world are believed to be infected, and about 350 million people are at risk of infection (2). Leishmaniasis exists in three main clinical manifestations; visceral, mucocutaneous and cutaneous. There are an estimated 0.2-0.4 million visceral leishmaniasis (VL) cases, with more than 90% of these occurring in India, Bangladesh, Sudan, Ethiopia and Brazil (Figure 1). There are an estimated 0.7-1.2 million cutaneous leishmaniasis (CL) cases occurring each year. Afghanistan, Algeria, Brazil, Costa Rica, Ethiopia, Iran, North Sudan, Peru and Syria are the ten countries with the highest estimated case counts of CL (3). Southern European countries are also affected, and popular destinations such as Greece, Italy, Spain, Portugal, Cyprus, France, Turkey and Bulgaria are a few of the destinations countries with reported *Leishmania* incidences (Figure 1 and 2) (4). Children and elderly with reduced immune response and people using drugs suppressing the immune system are more susceptible to getting infected. The increase in travelling and the increase in migration from high endemic areas may increase the incidence in European countries (5). Leishmaniasis remains a disease of developing countries, and lack of investment and funding for research, in addition to its complicated biology makes it challenging to develop vaccines and medication with less side effects (6).

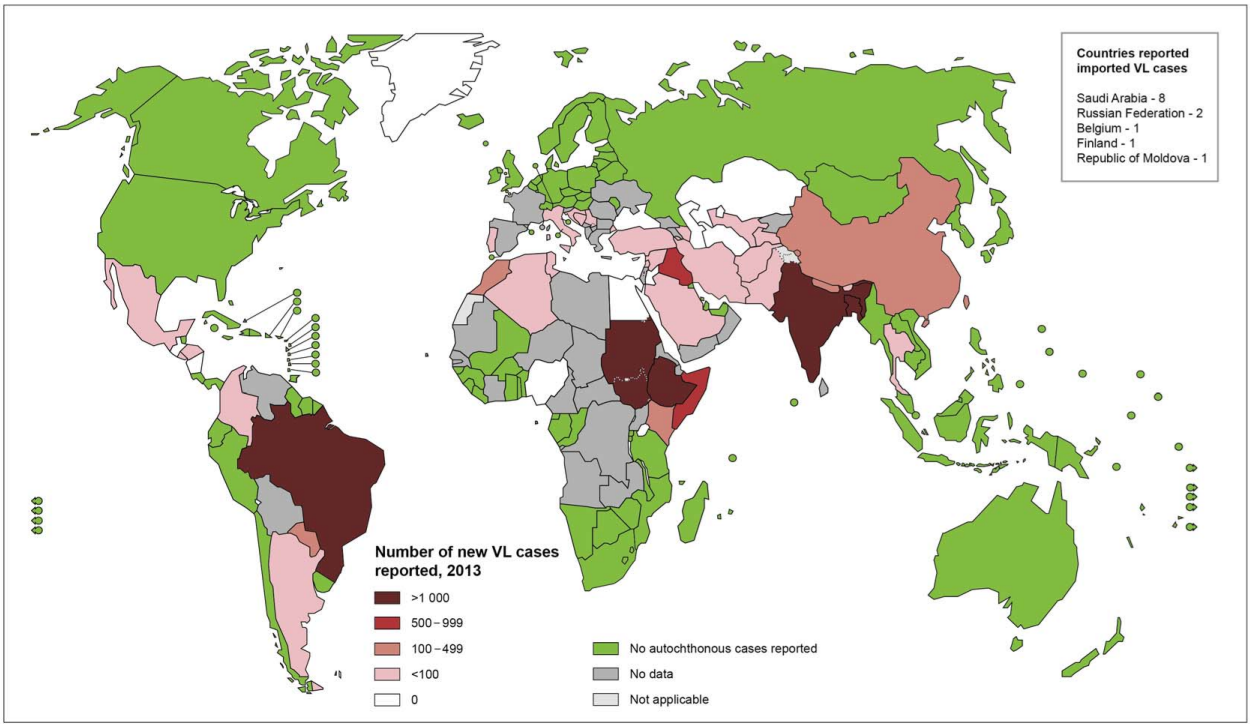


Figure 1: *Status of the endemicity of visceral leishmaniasis worldwide, 2013 (7).*

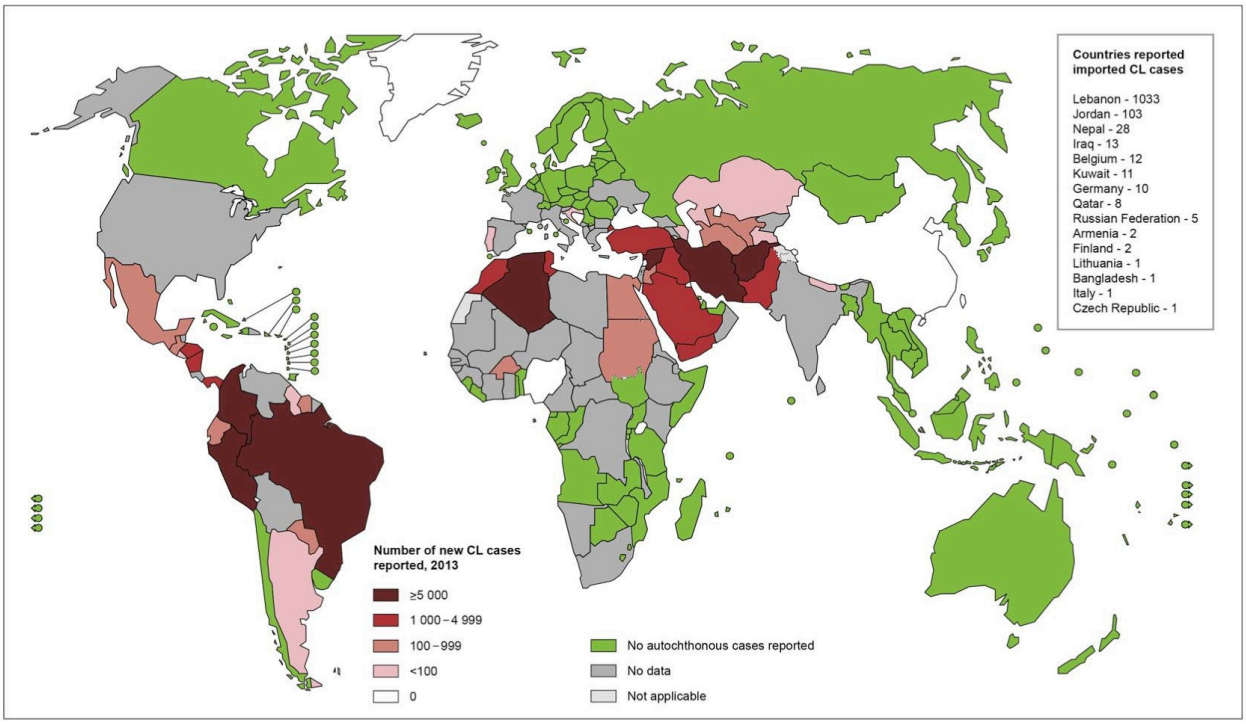
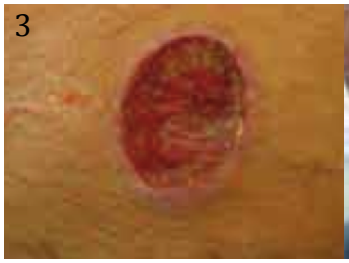
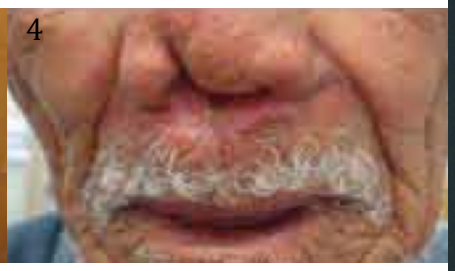


Figure 2: *Status of the endemicity of cutaneous leishmaniasis worldwide, 2013 (7).*

1.1.2 Pathology

Manifestation of leishmaniasis varies from asymptomatic to critical illness. The three main forms are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL). The incubation time and severity of the disease will vary from different species of *Leishmania*, the health status of the infected subject, genetic variation and immune system of the infected host. CL normally occurs in areas of the body exposed to the sandflies, such as the neck, limbs and face. It generally starts off as a papule, and progress to a nodule and can become a dry or wet ulcer (Figure 3). Self-healing can occur without treatment. ML occurs in mucosal tissue, often in the nasal cartilage leading to nasal collapse (Figure 4). VL affects the inner organs especially the spleen and liver. Symptoms include fever, anorexia, weakness, hepatosplenomegaly, and is lethal if not treated (Figure 5) (1, 4, 8). Because *Leishmania* infect and proliferate in immune cells, co-infections tend to occur, especially in subjects with HIV (9, 10).





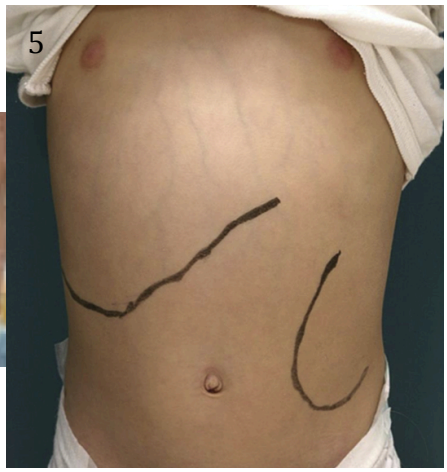


Figure 3: *CL on a leg. Figure taken from (11).*
Figure 4: *ML in the mucosa of the nose. Figure taken from (11).*
Figure 5: *VL of a 20 month old girl showing hepatosplenomegaly. Figure taken from (4).*

1.1.3 Diagnosis

Various laboratory techniques can be used to diagnose leishmaniasis, and the specific species can be detected by isoenzyme analysis(12). Asymptomatic leishmaniasis is not well defined, but can be confirmed by a serological tests or polymerase chain reaction (PCR) test (13). Microscopic examination of *Leishmania* from tissue aspirates or biopsies from bone marrow, spleen, skin, lymph nodes and even in the blood can be used for diagnosis of the disease(14). Diagnosing VL can be particularly challenging because of the similarity of its clinical features compared to other diseases such as malaria, typhoid and tuberculosis, which are prevalent in the endemic areas for VL. Some of the techniques used are light microscopic

examination of relevant tissue, detection of parasite DNA in tissue using PCR or immunodiagnosis by detection of parasite antigen. Direct agglutination tests (DAT) are used by Médecins Sans Frontières (MSF) for diagnosis of VL, although agglutination is not observed before the next day (15, 16).

1.1.4 Treatment

Treatment of leishmaniasis infection will depend on the species of the infecting parasite, the innate host-immune response, geographical location, and the different types of diseases. Although the same species, regional difference can lead to different responsiveness to a medication. Dosing regiments will also vary regionally. The treatment options may vary from local therapy, encompassing topical and intralesional injections, to systemic therapy, including the most commonly used pentavalent antimonials, amphotericine B, and miltefosine (8, 17). With some exceptions, the treatment are expensive, have long duration of use and can generate many side effects (17). Liposomal amphotericin B is first line treatment for VL in Europe and the United States and shows relatively low toxicity. It is also the chosen treatment for Leishmania/HIV co-infected patients (18). The anti-cancer drug miltefosine, is the first effective oral drug for VL, but is also teratogenic (16). It has been used for over a decade, however the efficacy of miltefosine has declined, indicating that the parasite has developed resistance mechanisms (17). The main problem of treatment is the access and cost for both the medications and hospitalization. Poor people living in endemic areas are most affected, and is one of the reasons why finding a treatment with short treatment duration and easy administration is desired (8, 19).

Using liposomal amphotericin B with either miltefosine or paramomycine, or paramomycine combined with miltefosine has proved to give an enhanced effect. Combination therapy gives increased treatment efficacy, reduced treatment time and cost, shows less toxicity, is better tolerated, and may prevent drug resistance (16, 17, 20). The different *Leishmania* species show different sensitivity to a drug, which results in different drug efficacy. Resistance to the different drugs against leishmaniasis seem to occur, which further implicates the need of new drugs(21).

1.1.5. The Leishmania organism

Leishmania is an obligatory intracellular protozoa of the Kinetoplastida order and the Trypanosomatidae family (2, 17). As of today, there is still no universal agreement on how to classify the different species of *Leishmania* (22). About 53 *Leishmania* species has been described, whereas 20 of those species are known to be pathogenic for humans (16, 22). *L. donovani* and *L. infantum* (also called *L.chagasi*) are the species known to cause VL. CL can be caused by numerous different species, which can vary from different geographical regions. ML can be caused by *L. braziliensis, L. panamensis,* and *L. major* (17). It is important to note that the disease outcome of a specific species can vary in different patients. *L. donovani* infections could result in an aggressive VL infection but could also lead to an asymptomatic infection (13). It is normal to divide the different *Leishmania* species into Old World or New World (Figure 6). It is a poorly defined division, based on the sandflie species and geographical origin.

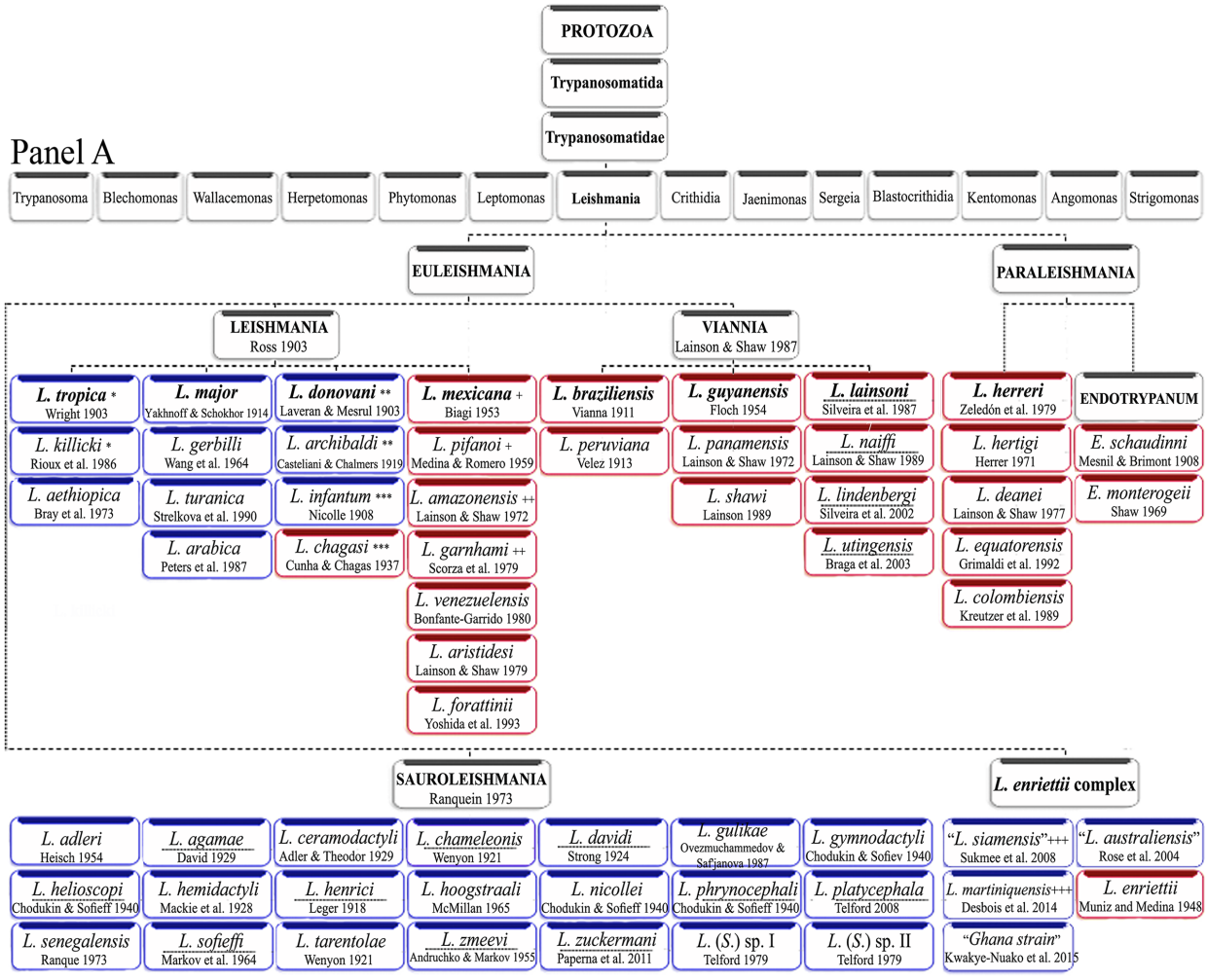


Figure 6*: Classification of Leishmania species, The blue boxes indicate Old World species and the red boxes indicate New World species. Figure taken from Akhoundi et al. (22).*

The unicellular *Leishmania* parasite differentiates between two main life cycle stages; the flagellated extracellular promastigote stage, which is motile and present in the sandfly vector, and the intracellular amastigote stage, which are nonflagellated and present within mammalian host cells and adapting to the hydrolytic environment of the lysosome (8, 23). The promastigotes reside in basic pH (about 7) with a mean temperature of 26℃, while the amastigotes reside in an environment with acidic pH of about 4.5-5.5 and temperatures between 33-37℃. As soon as amastigotes reaches an environment in neutral pH it will start differentiating to promastigotes (24). Asexual growth is typical for *Leishmania* parasites, although sexual recombination also seems to occur. Sexual recombination does not happen in vitro (25). The *Leishmania* parasite also contains kinetoplast, which are DNA-containing regions sitting in a large mitochondrion (26). Kinetoplastids contain glycosomes, which are peroxisome-like organelles (27).

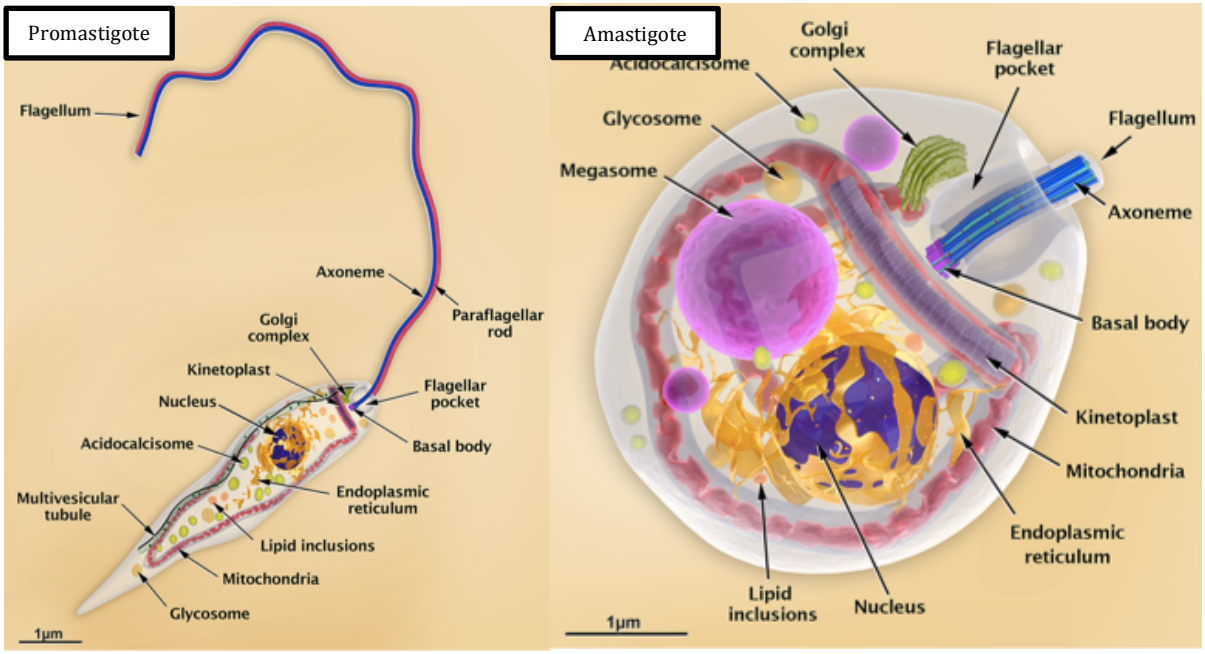


Figure 7: *Contents of promastigotes and amastigotes Leishmania. Figure from PLOS Pathogens (28).*

1.1.6 Sandflies

Sandflies are the known vector of *Leishmania*, with an estimation of about 30 species of phlebotomine sandflies that are able to transmit infection to humans (16). Over 800 sandfly species have been described, however, less than 10% have been confirmed to be vectors of *Leishmania* (29). The sandflies can be classified as Old and New World sandflies based on a phenetic approach, classifying the sandflies by their similarity (22). Specific sandflies transmit only a certain species of the parasite, *Phlebotomus* sandflies of the Old World and

Lutzomyia of the New World species of *Leishmania*, and will infect the host with different presentations and progress (8, 26). The sandfly has to take a new blood meal for it to spread the infection, limiting the spread to female sandflies, who need blood for their reproduction (30). Some sandflies only have one blood meal during their entire life span. The infectiveness of the vectors may vary depending on time of blood meal excretion and the amount of lipophosphoglycan (LPG) on the parasites membrane enabling the parasite to bind to the mid-gut of the vector (31).

The complexity of the infection is further enhanced due to different variables in the host. Stress, host-immune system, general health status, age, sex, differences in the genome, may play an important role when fighting off the infection. Geographical and epidemiological differences will also play a role. People with low-grade housing, located near the habitat of infected vectors have increased risk of infection (13). Many species serve as reservoirs, such as humans, dogs, rodents, bats and lions. (32).

1.1.7 The Leishmania life cycle

The life cycle of the *Leishmania* parasite (Figure 8) starts with a bite of a female sandfly from an infected host. The parasite is transmitted to the sandfly from the blood of the infected host and will differentiate from the amastigotes to the non-infective procyclic promastigote stage due to changes in pH and temperature (33). The promastigotes differentiate from proliferating procyclic promastigotes to motile nectomonad promastigotes which are normally located in the anterior part of the midgut, before the parasites develop into leptomonad, haptomonad and finally the infective and non-dividing metacyclic promastigotes (34). A new blood meal must take place to spread the infection, and when this happens the sandfly will regurgitate metacylcic promastigotes into the host. Once the parasite is in the host cells of the innate immune system, they will phagocytize the metacylcic promastigotes through receptor-mediated binding. Inside the phagolysosome the parasite will differentiate into the amastigotes form and start proliferating until the cell membrane is disrupted and amastigotes are released into the blood stream (Figure 8) (8, 14, 33).

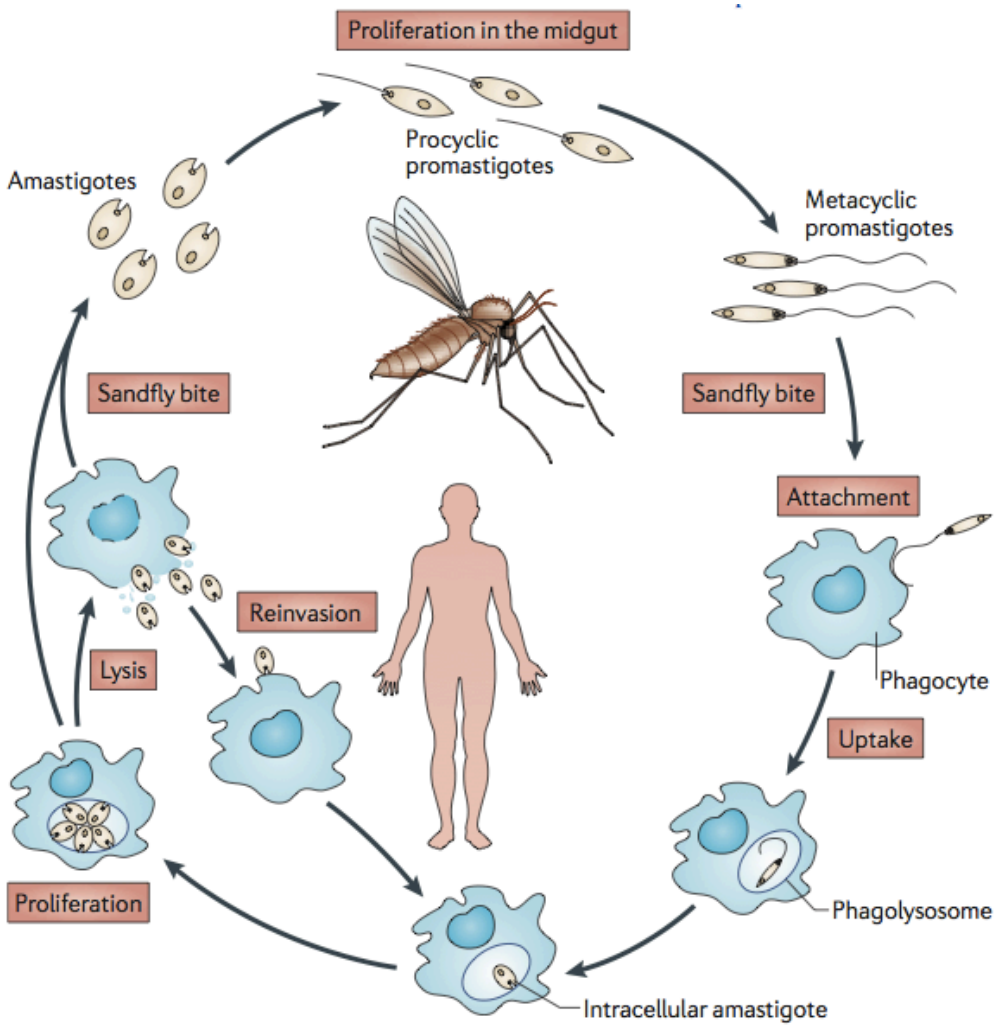


Figure 8: *The life cycle of Leishmania parasites. An infected female sandfly bites the host and transmits the parasite into the blood stream, where it enters the host cells of the innate immune system and differentiates into amastigotes. There it may proliferate until it disrupts the host cell membrane and can invade other cells. Figure taken from Kaye et al. (33).*

1.2 The Immune response and mechanisms behind arginase and L-arginine availability

1.2.1 Immune response

Leishmania parasites are found primary in the macrophages where they are engulfed and proliferate intracellularly (2). Macrophages have many defense mechanisms, among them is the oxidative burst induced by phagocytosis, vesicular acidification and induction of inducible nitric oxide synthase (iNOS) (27). To clear out the *Leishmania* infection we are dependent on the macrophages ability to produce nitric oxide (NO) by producing iNOS and other oxidative intermediates (2, 8).

Macrophages are activated by cytokines, like interferon-gamma (IFN-γ) and interleukin-12 (IL-12), (Figure 9) (27). Interleukin-1 (IL-1), tumor necrosis factor-alfa (TNF-α), interferon-alfa (IFN-α) and interferon-beta (IFN-β) are cytokines also know to activate macrophages and induce upregulation of iNOS. Production of iNOS will catalyze L-arginine

to generate NO, which is toxic for the *Leishmania* parasites. Dendritic cells producing IL-12 promotes protective immunity by stimulating Th1 cells and NK cells. The alternative macrophage activation leads to parasite survival. Cytokines produced by Th2, Treg and other macrophages produce cytokines, such as IL-4, IL-13, IL-10, and TGF-β favors parasite survival by inhibiting parasiticidal activity of infected cells (2).

The proliferation and survival of the parasite inside the host is dependent on the balance between the factors in the host and parasite that control the activation or deactivation of the macrophages defense mechanism (2, 27). When the internal pool of arginine is not sufficient to sustain NO production, the macrophages will increase the expression of the arginine transporter CAT2B(35).

Neutrophils are phagocytes able to uptake *Leishmania* in the early stages of infection. It has been proposed that they might function as "Trojan horse´s" for the parasite. *Leishmania* may induce apoptosis of the neutrophils rather than necrosis, thereby entering the macrophages without inducing reactive oxygen species (2).

The disease pathology of the different species of *Leishmania* is variable. It is therefore very challenging and nearly impossible to generalize the infection mechanism to all hosts and strains (2).

1.2.2 The importance of arginase and L-arginine

Crithida, *Leptomonas* and *Blastocrithidia* are other genus aside from *Leishmania* that seem to express arginase (Figure 6) (36). Arginase also called L-arginine amidinohydrolase is a trimeric metalloenzyme that generates L-ornithine and urea by catalyzing the hydrolysis of the amino acid L-arginine. L-arginine is important in several metabolic and physiological pathways and is a precursor for protein synthesis, NO, urea, ornithine, citruline, proline, glutamate, agmatine creatinine and polyamines (27). *Leishmania* parasites depend on external supply of L-arginine since it is an essential amino acid for *Leishmania* (37, 38).

Polyamine synthesis is essential for the cell replication and viability of the *Leishmania* parasite, and is dependent on the production of ornithine, which again can be generated from L-arginine by arginase (39, 40). Arginase can be found in the glycosome of both the promastigotes and the amastigotes, indicating that the arginine needs to be transported from the outside of the macrophages, through the parasitophorous vacuolum, through the *Leishmania* and finally through the glycosome in order to be used as a substrate for arginase (27, 41).

In mammalian cells there are two arginase genes that code for arginase-I and arginase-II which differ in their relativities and distribution. Arginase-I is mostly found in the cytosol and expressed in the hepatocytes. Arginase-II is expressed in macrophages, intestines, mammary glands, kidneys and brain, and are localized in the mitochondria (40, 42). The *Leishmania* and human produced arginase seem to be quite similar. The main important differences are two non-conserved amino acids rendering a different channel like structure and a cleft containing a different charge (43). Because the arginase produced in the parasite and the host are so similar, screening for specific inhibitors to only target parasite arginase has not been successful (41). Being able to exploit those differences to inhibit only the *Leishmania* arginase would be a good target for a future chemotherapy (27).

It has been shown that in *L. major*, *L. amazonensis* and *L. mexicana* arginase knockout parasites lack polyamines. This confirms the importance of arginase for production of polyamines and the survival of *Leishmania (Figure 9)*. An increase in NO production also occurred with *L. mexicana* arginase knockout parasites, which indicates the importance of L-arginine availability. This was not the case for *L. major*, which further underlines the differences among *Leishmania* species (27).

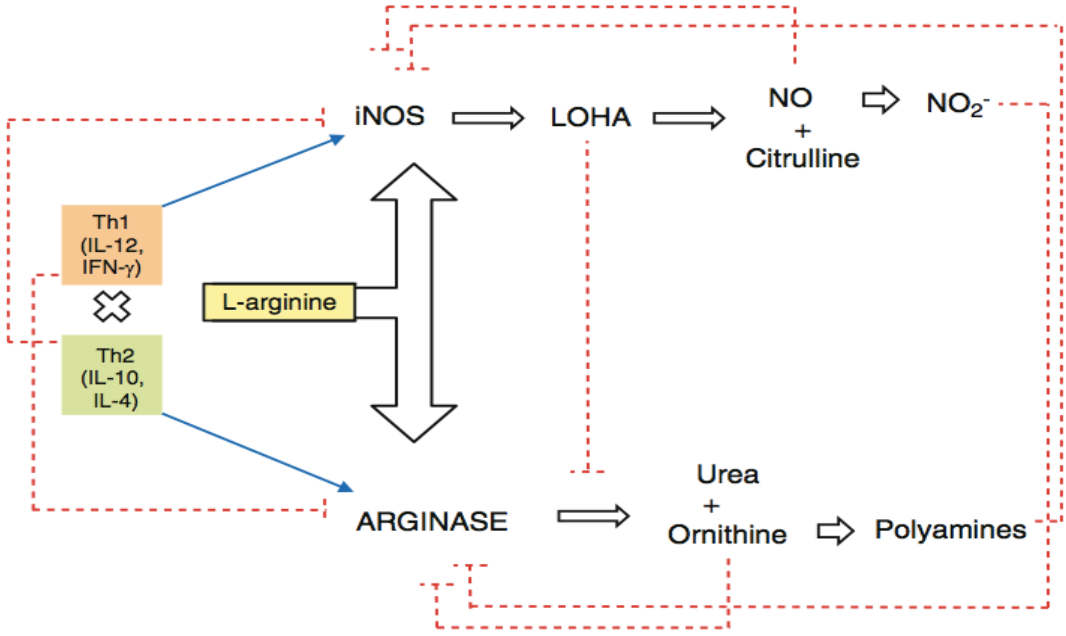


Figure 9: *How Th1 and Th2 immune response can regulate iNOS and arginase. Th1 response activates inflammatory reactions and may lead to macrophage activation and antibody opsonization that will further enable the fight against the parasite. Th2 response will enable the humoral response and often exhibit anti-inflammatory reactions, but will not be effective against the* Leishmania *parasite since it generally is located inside macrophages(42). L-arginine is a substrate for both arginase and iNOS, the* Leishmania *parasite and the macrophage will therefore compete for the L-arginine for their survival (27). Blue arrows represent stimulatory routes, while red dots represent inhibitory routes. Figure taken from da Silva et al. (2014).*

1.2.3 The L-arginine transporter

In mammalian cells L-arginine is transported through cationic amino acid transporters called CATs. In total there are six CAT family members called CAT1, CAT2A, CAT2B, CAT3, CAT4 and CAT14, which carries L-arginine, L-lysine and L-ornithine. When responding to cytokines produced during Th1 and Th2 immune responses the CAT2B is responsible for the transportation of arginine into the macrophages. When this transporter is blocked iNOS and arginase levels are undetected underlining the importance of this transporter but also suggesting that the concentrations of arginine inside the cell will have an impact on the amino acid metabolism by iNOS and arginase. An increase in CAT2B expression and a decrease of iNOS expression is detected during a Th2 response in macrophages infected with *Leishmania*. This will lead to increased levels of arginine available for parasite to utilize for growth and replication, which also underlines the importance of an arginine transporter to the parasite (27).

The AAP3 (amino acid permease3) contains 480 amino acids and 11 trans-membrane domains, and is a highly specific arginine transporter (37, 44). Permeases are responsible for amino acid uptake driven by membrane potential created by H^+-pumps. To date, AAP genes has been cloned from plants, mammals and protists, and has been characterized for yeast and protozoa (37). *L. donovani* contains eight AAP (amino acid permease) genes and is able to sense decrease in arginine levels and respond by increasing arginine uptake (37).

Similar to the *L. major* and *L. infantum*, the *L. donovani* genome has two identical copies of LdAAP3. These *L. donovani* copies show a 93% similarity to the *L. amazonensis* copies with 98% similarity of the ORF (open reading frame). The two identical LdAAP3 (*Leishmania donovani* AAP3) copies are arranged in tandem on chromosome 31. The 5´UTRs (untranslated region) of the LdAAP3 copies are identical while the 3´UTR copies are different, which give rise to two different transcripts (35, 37).

Leishmania parasites exhibit polycistronic transcription and therefore lack the control mechanism of gene expression at the transcription level. However, *Leishmania* can control gene expression at the mRNA maturation level and also by changing mRNA half-lives in different conditions (35). This is a possible explanation for the presence of two copies of the AAP3 gene. Each copy could be regulated differently according to the current environment of the parasite. The mRNA transcripts of the two different copies seem to have different stability, and will therefore differ in how much and how long it can be translated. The stability of the mRNA can be regulated by the external and intracellular amino acid

concentration. In this way, it is postulated that *Leishmania* can regulate the arginine transport as needed (35).

Though activity of the AAP is registered at a broad pH range, it is more active at pH 5 than pH 7. The transporter may therefore be more active when inside the macrophages (37). Localization of the LdAAP3 using green fluorescence protein has localized the LdAAP3 on the surface membranes of promastigotes (37).

The arginase reaction has been localized in the glycosome, although some authors suggest that the glycosomal location is not essential for arginine function in promastigotes (40). Da Silva et al (2012), on the other hand, confirm how the lack of arginase and incorrect localization may reduce infection and parasite proliferation. They also showed that the arginase remains in the glycosome during macrophage infection (41). The results from their study done for *L. amazonensis* indicate the importance of the proper compartmentalization of the arginase in the glycosome for enzyme activity and parasite infectivity.

According to Shaked et al (2006) it is likely that the glycosome functions as an intracellular pool for arginine, which means it has to be transported through the glycosomal membrane, which again implies the need of another arginine transporter (37). The transporter that leads the arginine to the glycosome still remains unknown (Figure 10).

The importance of the glycosomal compartmentalization of the arginase for parasite infectivity, and the differences between the parasite and host transporter makes further investigations into the arginase transporters relevant. New treatments against leishmaniasis may reside by the inhibition of arginine trafficking to the parasite and by blocking the arginase import to the glycosome (38).

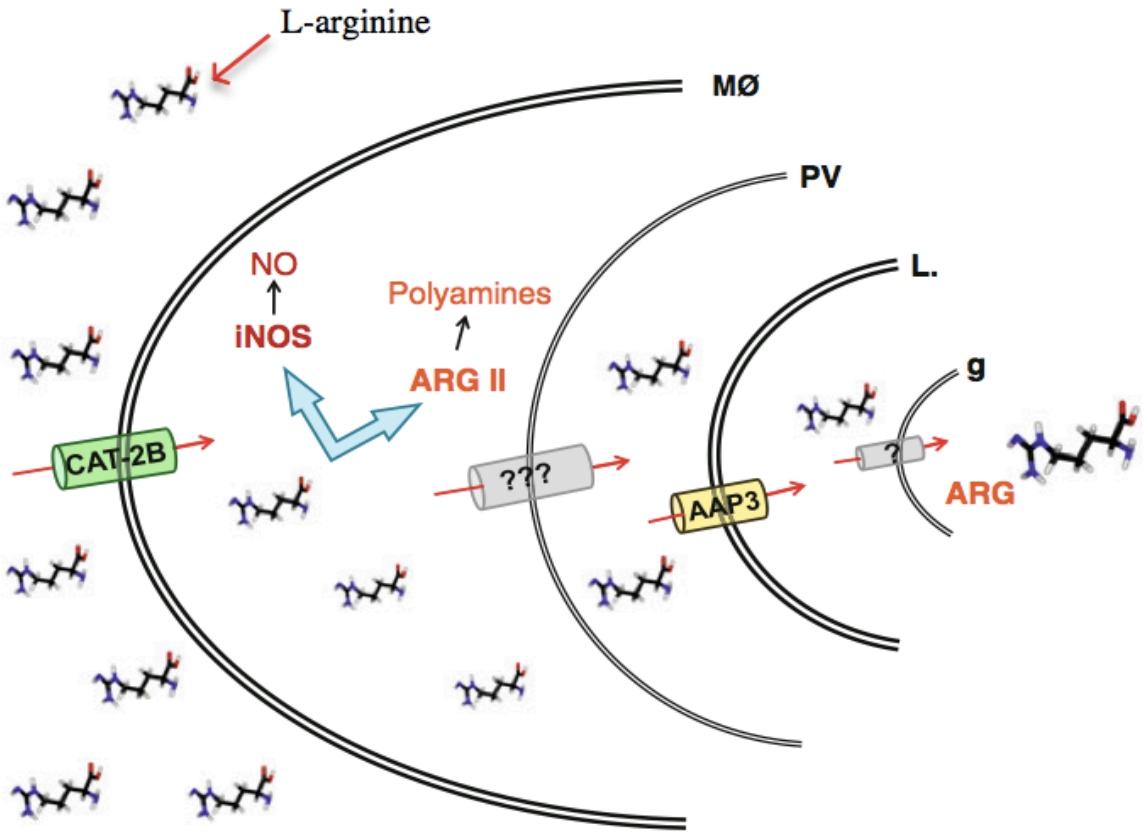


Figure 10: *"L-arginine traffic through macrophages infected with Leishmania. Arginine is taken up by macrophage (Mø) through CAT-2B membrane transporter. In the cytoplasm of the Mø the amino acid is metabolized by Mø's arginase (ARG II) or iNOS, or is taken up by parasitophorous vacuolum (PV) through an unknown mechanism. In order to reach Leishmania's arginase(ARG), L-arginine must be taken up by the parasite through the AAP3 transporter and then must reach the glycosome (g) by unknown mechanisms". This text and figure is taken from da Silva et al. (27).*

1.3 The expression of recombinant proteins in *E. coli*

To obtain a desired quality of a recombinant protein choosing an appropriate method and system is critical and there are numerous expression systems to choose from. Knowing the characteristics and intended application of the recombinant protein may increase the success of the expression. This may lead to a better selection of expression system and enable the production of sufficient proteins (45).

Protein misfolding, poor expression, the lack of posttranslational modifications or inappropriate modifications can occur when the wrong expression host is chosen. Aspects one must consider when selecting the appropriate expression system include posttranslational modifications desired on the protein, destination of the protein, the proteins intended use, and it's mass and number of disulfide bonds. However, there are still may circumstances that needs to be optimized which makes expression approaches time consuming and challenging (45).

Expressing prokaryotic and eukaryotic recombinant proteins in *Escherichia coli* (*E. coli)* is a commonly used technique, and allows the production of a wide variety of proteins from a variety of organisms (46, 47). It offers rapid and inexpensive expression of recombinant proteins.

Expression in an *E. coli* strain that can co-express tRNAs for rare codons can be preferable if the protein contains a high number of rare *E. coli* codons. When the recombinant protein contains increased amount of disulfide bonds, it may be preferable to choose an *E. coli* strain with a more oxidizing cytoplasmic environment to stimulate proper folding. When dealing with toxic proteins choosing an *E. coli* strain containing vectors that allow a tight regulation of the expression system is favorable. Membrane-bound protein expression seem to be favored in mutant strains like C41(DE3), C43(DE3) (47, 48) and Lemo21 (DE3) (49).

1.3.1 Inclusion body formation

Recombinant proteins expressed in *E. coli* can be directed to the periplasm, to the cytoplasm or be secreted from the bacteria cell. High expression levels can often result in aggregation of the recombinant protein forming inclusion bodies (45). Expression under high temperatures, high inducer concentration and strong promoter systems may result in higher translation rates of the recombinant protein. This may overwork the quality control system of the bacteria host resulting in completely or partially misfolded aggregates of the protein, forming inclusion bodies. Factors like reduced environment in the bacterial cytosol, lack of post-translational machinery and chaperones may increase the chance of protein aggregation and inclusion body formation (50). Although inclusion bodies require comprehensive processing to isolate, solubilize, refold and purify the protein to produce bioactive proteins, it may also offer some advantages. Inclusion bodies are often resistant to proteolysis and are easy to concentrate using centrifugation and thereby minimalizing contamination of other proteins when purifying (45, 50).

1.3.2 Protein Solubility

Several steps can be improved to maximize the formation of properly folded soluble proteins. Lowering the expression temperature to 15-30 ℃ can slow the rate of transcription, translation and refolding which allows the host to properly fold the proteins. It can also decrease the heat shock protease activity, which may lessen the protein degradation (45).

Co-expressing the recombinant protein with molecular chaperones can promote protein solubility, but has to be tested individually for each protein due to the chaperones

protein specificity. Fusing the recombinant protein to a soluble fusion tag to the N-terminus or C-terminus part of the protein may also increase the solubility (45).

1.3.3 Codon usage

Most amino acids can be encoded by more than one codon. Each organism has its own bias when it comes to using the 61 available amino acid codons (51). The genes of parasites use codons that may be rarely used in *E. coli* (52). It is important to consider the bias for codon usage in *E. coli*, which is mirrored by the abundance of the tRNA pools. Expressing a protein using several of these rare codons can lead to misincorporation of amino acids, premature termination of translation and frameshifts in translation, which may lead to the failure of expressing the recombinant protein with the desired property (45).

1.3.4 Toxic proteins

Many of the genes expressed in *E. coli* interfere with the survival of the *E. coli*. These expressed proteins can be toxic for the *E. coli* and cause maintenance defects during growth or even cell death (53). Toxic proteins can therefore be challenging to express, but can usually be overcome.

One way to circumvent the toxicity is by tight regulation of the transcription and translation of the toxic genes. Tightly regulated inducible expression systems have been developed for *E. coli* and Yeast cell (*Pichia Pastoris*). This will allow a more controlled regulation of the recombinant protein by using inducible promoters, transcription terminators, modifications of the coding sequence, and by optimizing plasmid copy number. If the recombinant proteins toxicity is host cell specific, it is wise to choose a more compatible expression host (45, 53).

Manipulating the copy numbers of the vectors may also increase the level of expression of toxic proteins. To keep the *E. coli* stable while maintaining the highly toxic gene, a low-copy number of the vector is favorable. To be able to express high quantities of the recombinant protein, an increase in vectors prior to induction is favorable. Therefore, using a system with a low-copy number of the vector, that can be increased before induction would be optimal. This copy number can be controlled by expression using thermo sensitive alleles (53).

1.3.5 Expressing toxic membrane proteins

E. coli BL21(DE3) is a commonly used host for overexpression of proteins. This strain has the gene of the target protein located on a plasmid, which is under the control of a T7 promoter. The T7 promoter is activated by the T7RNAP (T7 RNA polymerase) with its gene located on the chromosome of the bacteria. The expression of the T7RNAP is induced by the non-titratable IPTG (Isopropyl β-D-1-thiogalactopyranoside) which induces the lacUV5 promoter that is located in front of the T7RNAP gene (49).

Lemo21(DE3) is a BL21(DE3) strain supplied with another plasmid containing a gene for an inhibitor of the T7RNAP, called T7 lysozyme (Figure 11). This gene is under the control of the well-titratable rhamnose promoter. Using this strain allows us to modulate the amount recombinant protein expressed by inhibiting the T7RNAP as needed using different concentrations of rhamnose, which will induce the production of T7 lysozyme inhibitor. The amount of rhamonse that is optimal for production of a certain protein needs to be individually tested, as the proteins are different (49).

Membrane protein expression occurs naturally in low abundance. Because of this, isolating sufficient amounts of recombinant membrane proteins needs to be obtained from overexpressing systems, which often tends to be toxic for the host. The Lemo21(DE3) strain is suggested to be a well-suited host for the production of recombinant membrane proteins (49).

Although using the Lemo21(DE3) strain for production of toxic recombinant proteins, leaky protein expression can still affect the system and cause toxicity. A small amount of the T7RNAP coming from a single copy of the gene is enough to produce high transcription levels of the target protein (53). Leaky genes of the lacUV5 promoter may lead to the production of T7RNAP before induction, and may cause cell death to the host if the target protein is highly toxic (54, 55).

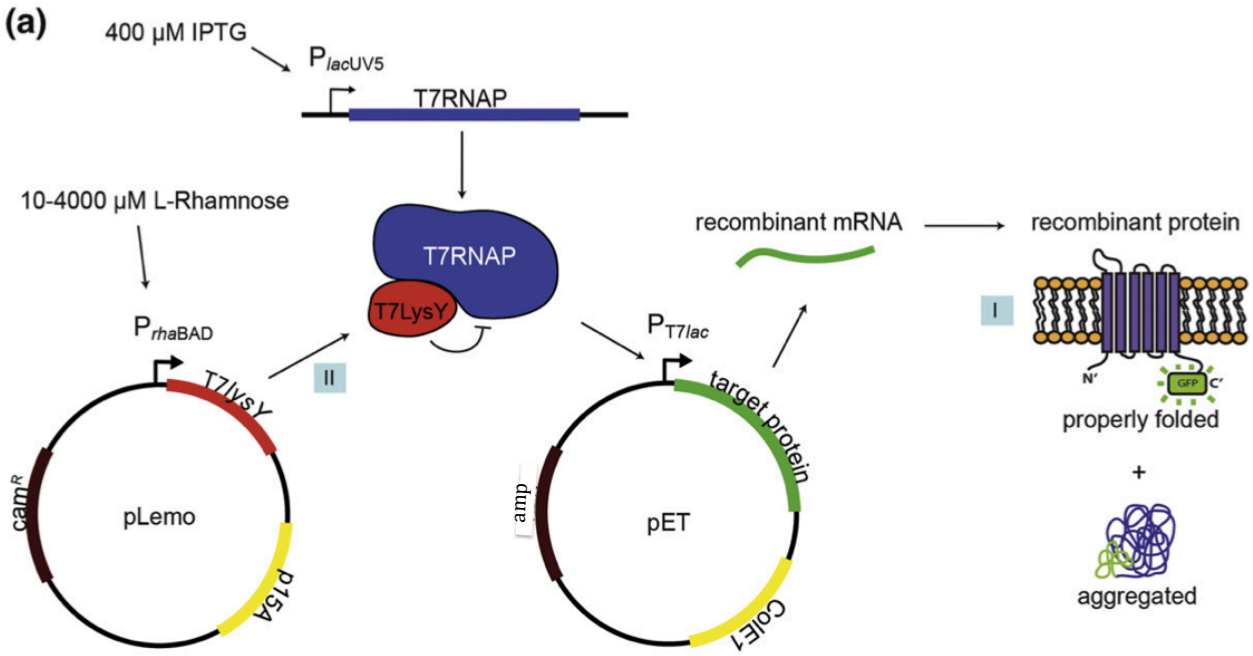


Figure 11: *Lemo21(DE3) strain and the expression of a target membrane protein. The gene for the target protein is located on the pET plasmid. The pLemo plasmid contains the T7lysozyme gene. When T7lyzosyme is expressed it can bind and inhibit the T7RNAP which may lead to less expression of the target protein (49).*

1.3.6 Tagging recombinant proteins

When expressing recombinant proteins, tags, also called fusion proteins, can be used to improve solubility, for protein detection and quantification, and to enable affinity purification. During the cloning step the fusion proteins can be added to the N-terminal (Nter) or C-terminal (Cter) end of the protein of interest. Placing the tag on the Nter can be an advantage because of the translation initiation site and because of the endoproteases that cut at or close to the Cter of their recognition sites (56).

Affinity tags are important during protein purification, and allow target protein to bind on an affinity matrix containing immobilized metal ions. The His-tag contains six or more consecutive histidine residues and is one of the most used purification tags. The histidine residues have affinity for Ni^{2+} (nickel) or Co^{2+} (cobalt) metal ions that normally are immobilized on beads or resins for purification. To immobilize the metal ions on the beads or the resin linkages such as NI-NTA (Ni(II)-nitrilotriacetic acid) or Co^{2+}-carboxymethyl-aspartate can be used. This system is called immobilized metal affinity chromatography (IMAC) (56).

IMAC is relatively cheap and has high binding capacities at about 5-40mg of His-tagged protein per mLof media. The nickel metal ion can often be stripped from the resin when EDTA buffers are used. Cobalt is more specific for His-tags and has minimal metal leakage (56). Because the His-tag can bind to the IMAC resins without any special protein conformation, purification during denaturing conditions is possible. The binding to the IMAC resins may also be enhanced because of the increased exposure of the His-tags in denatured condition (56).

The histidine imidazole ring on the His-tag bind to the immobilized metal, and can be eluted using buffers containing imidazole (100-300 mM) or low pH (4.5 – 6). The concentrations of imidazole needed may vary from different proteins. Low concentrations of imidazole (5-20 mM) should be added to the loading and washing buffers to get rid of endogenous protein that exhibit weak binding to the IMAC media (56).

His-tags are small in size and may have little impact on the structure of the folded protein. Though the tag can be removed by introducing a protease cleavage site, keeping the tag allows protein detection by using His-tag-specific antibodies (56).

1.4 Aims of the study

This work was part of a larger project for the characterization of the arginine transporter AAP3 from *Leishmania*, where the ultimate goal is development of new drugs and vaccines. Availability of specific antibodies is an important tool for studying function and effects of components of organisms. The main aim of this study was therefore to raise antibodies against the arginine transporter (LdAAP3) from *Leishmania donovani*.

The specific aims of this study:

- To optimize the production of recombinant LdAAP3 in *E.coli*
- To establish a method for purification of the recombinant protein
- To produce and purify recombinant protein in a quantity that enabled immunization of rabbits
- Immunization of rabbits with the recombinant protein
- Characterize binding patterns from sera of infected and non-infected individuals using the LdAAP3 recombinant protein

2. MATERIALS AND METHODS

2.1 Strains and vectors

2.1.1 Champion pET151 Directional TOPO Expression Kit

This kit comes with reagents, One shot TOP10 chemically competent *E. coli,* BL21 Star (DE3) One Shot Chemically competent *E.col,* and pET 151/D-TOPO vector. To clone and express LdAAP3 ORF, Nter and Cter genes the pET Directional TOPO system was chosen. The system utilizes a highly efficient 5 min cloning strategy to directionally clone a blunt-end PCR product into a vector for high level, T7-regulated expression in *E. coli* (57).

2.1.2. Strains

Leishmania (Leishmania) amazonensis (MHOM/BR/1973/2269)

L. (L.) donovani (MHOM/IN/80/DD8)

Escherichia coli One Shot TOP10 Chemically Competent (ThermoFisher Scientific, USA) was used for efficient transformation of all plasmid DNA used in this work.

E. coli BL21 Star(DE3) (ThermoFisher, USA) was used for expression and induction of Nter construct.

E. coli Lemo21(DE3) (NewEngland BioLabs, USA) was used for expression and induction of Cter and ORF construct.

2.1.3 Vectors

The LdAAP3 gene was cloned into the **pGEM-T easy** vector (Promega, Madison, WI, USA). This construct was obtained from Professor Lucile M. Floeter-Winter and her group at the University of São Paulo (USP), Brazil(41).

The **pET151/D-TOPO** (ThermoFisher, USA), vector allows expression of recombinant protein with Nter tag containing the V5 epitope and a 6xHis tag. It contains ampicillin base resistance gene as a selection marker, and a T7 promoter.

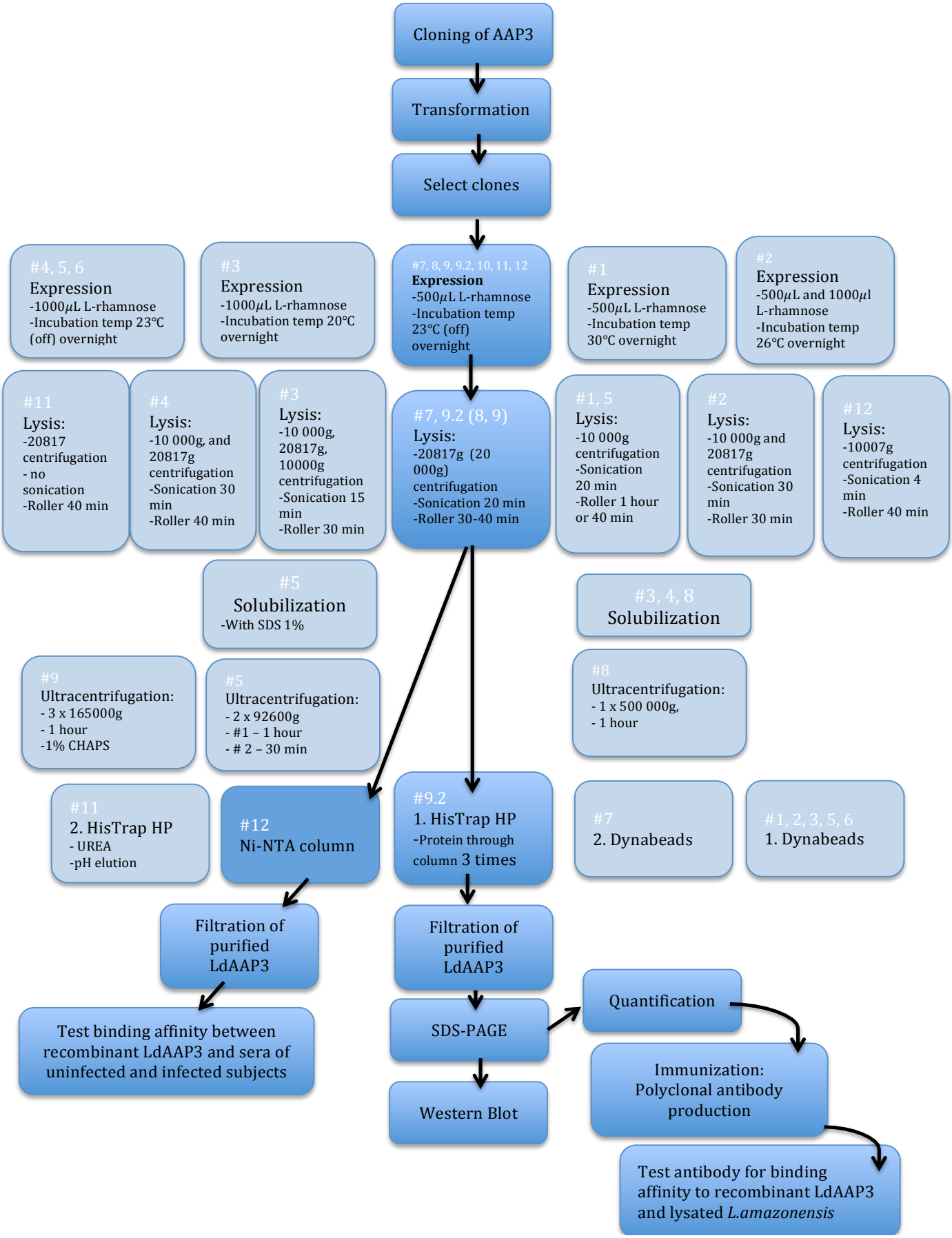


Figure 12: *Flowchart demonstrating the workflow steps conducted during this thesis. The protocol number marked in white can be found in several parts of this thesis. It is meant to give an overview over the different combinations of steps done throughout the whole proses from cloning, transformation, expression and so on. The light blue boxes show the different attempts made. The dark blue boxes indicate steps chosen to move forward with, completing the expression and purification of LdAAP3 recombinant protein and the testing of the recombinant protein and antibodies produced from the recombinant LdAAP3 protein.*

2.2 DNA Replication and Purification

2.2.1 Polymerase Chain Reaction (PCR)

The PCR mix reaction (final volume of 50 µL) included: 1X Pfx50 PCR buffer mix, 0.3 mM of dNTP mix, 0.3 µM of each of forward and reverse primer, 5 units of Pfx50 DNA polymerase (Invitrogen) and 100 pg of DNA. PCR conditions were 1 cycle at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing at 52ºC-60ºC (the temperature given for each individual reaction) for 1 min and extension at 72ºC for 1min, followed by a final extension at 72ºC for 7 min on Veriti thermal cycler (Life technologies, USA)(58).

2.2.2. Agarose gel preparation and gel electrophoresis

1% agarose (SeaKem LE, ME, USA) was melted in TAE-buffer (40mM tris-acetate, 1mM EDTA, pH 8,0). When the mix was slightly cooled, 3μL GelRed (Invitrogen, USA) (1:10,000) was added. The mix was poured onto a horizontal gel Electrophoresis system (Life Technologies, USA) and left to set for 1h. 10 μL of PCR samples were mixed in 2 μL loading buffer and loaded on the gel (6x8,5cm) and run at 80V for 1h using TAE as running buffer. Gels were visualized using UVIProChemi (Uvitec, Cambridge, UK) set at 300nm (59, 60).

2.2.3 Purification of PCR product

QIAquick PCR Purification Kit (Qiagen, Germany) was used for purification of double stranded PCR products (100bp-10bp). The principle of this technique is that DNA from the PCR products adsorbs to the silica membrane when high salt concentrations are added, while the contaminants pass through the column. DNA is released from the silica in low salt concentrations. Adsorption to the silica is improved at pH $\leq$7,5. This column provides high yields of pure nucleic acid, giving good substrates for further ligation and transformation processes (61).

5 volumes of buffer PB with 100% ethanol was added to 1 volume of PCR product and loaded onto a QIAquick column with a 2mL collection tube. This was centrifuged for 30 sec at 17900 x g, at room temperature (RT). 0.75mL buffer PE was added to the column for washing, then centrifuged for 30 sec, at 17900 x g, at RT. The centrifugation was repeated for 1 min to remove residual wash buffer. The QIAquick column was placed into a clean 1.5mL microcentrifugation tube, before 50μL buffer EB (10mM Tris-Cl, pH 8,5) was added for elution. The column was then centrifuged for 1 min (61).

2.2.4 Cloning

When using QIAprep Miniprep (Qiagen, Germany) for lysis of *E coli,* the bacterial cells were lysed with alkaline, enabling the adsorption of DNA onto silica. This silica membrane has a selective adsorption to plasmid DNA in high salt buffer solutions, and elutes the DNA in low salt buffer solutions. Elution efficiency is maximized when pH is between 7.0 and 8.5 (62).

The recovered PCR products from the pGEM-T easy vector were cloned directly into pET151/D-TOPO (Invitrogen, USA) according to the manufacturers instructions. Ligated products were then transformed to *E. coli* TOP10 chemically competent cells (Invitrogen). Positive colonies were isolated from LB-agar plates containing ampicillin (100μg/mL) and then by MiniPrep kit (Qiagen, Germany), according to the manufacturers instruction.

2.2.5 Transformation into competent cells

Transformation protocol into *E. coli* competent cells was based on the protocol described by Chung and Miler (1988), with some modifications. *E. coli* TOP10 competent cells (Invitrogen, USA) were used to propagate the DNA. Cells were thawed on ice and mixed with 3 µL of pET TOPO construction. Tubes were swirled gently and incubated on ice for 30 min. After incubation, cells were heat-shocked (Eppendorf Thermomixer Comfort, Germany) at 42°C for 30 sec and then immediately transferred on ice for 5 min. Then, 800 µL of the SOC medium was added to the cells and incubated at 37°C for 1h with shaking (311DS Labnet) at 250 rpm. For positive selection, 100 µL of transformation mixtures were plated on LB agar plates containing ampicillin (100 µg/mL) and incubated at 37°C (Termaks, Norway).

2.2.6 Plasmid purification

Plasmid purification was performed according to the QIAprep instruction kit. *E. coli* was grown overnight in 1 mL LB medium, pelleted by centrifugation, and resuspended in 250μL buffer P1, containing RNase A. 250 μL buffer P2, and 350 μL buffer N3 (Qiagen, Germany) was added before centrifugation for 10 min at 17900 x g. The supernatant from the centrifugation was loaded onto the QIAprep spin column and further centrifuged for 30 sec. The flow through was discarded. 0.5mL buffer PB was added to the column for washing, and centrifuged for 30 sec. The flow through was discarded before applying 0.75mL buffer PE and centrifuged for another 30 sec. The flow through was discarded before a final centrifugation for 1 min, to remove residual wash buffer. The QIAprep column was then

placed in a clean 1,5mL microcentrifuge tube, 50 μL Buffer EB (10mM Tris-Cl, pH8,5) was added for elution, before the last centrifugation for 1 min (62).

2.2.7 Sequencing

The constructs were confirmed if they were cloned in frame by BigDye v.3.1 Sequencing (ThermoFisher, UK), performed using the Applied Biosystem 3730XL Analyzer at "Seksjon for laboratoriediagnostikk" at the "Senter for medisinsk genetikk og molekylærmedisin" (MGM), Haukeland University Hospital, Bergen.

Sequencing analysis were performed using Sequencing Scanner Software (Applied Biosystem by ThermoFischer) and GenBank of *Leishmania* by TriTryp Database (www.tritryp.org).

2.3 Expression of LdAAP3 ORF, Nter and Cter recombinant protein

Sequencing verified successful cloning regarding size of the inserts and correct reading frames. The plasmid containing the entire 1440 bp open reading frame (ORF) was called pET151-ORF, the plasmid containing 311 bp from the 5' end of the ORF was called pET151-Nter, and the plasmid containing the last 453 bp of the ORF was called pET151-Cter.

Small scale expression was performed to determine the IPTG induction, incubation time and the solubility. After the standardization of the settings, big scale expression was performed.

2.3.1 Expression and transformation of LdAAP3 Nter recombinant protein

Expression of LdAAP3 Nter was performed using *E. coli* BL21 Star(DE3) competent cells (ThermoFisher, USA). 3 µL of the pET151-Nter plasmid was added to a vial (50μL) containing *E. coli* BL21 Star(DE3) One Shot (ThermoFisher, USA). Contents were gently mixed, incubated on ice for 30 min, heated at 42℃ for 30 sec, then incubated on ice for 2 min. 900 µL LB-media was added to the mix incubated for 1 h, at 37℃ with 200 rpm on shaker, Shaker Control System, (Dublin, Ireland). 100 µL was spread on an agar plate containing ampicillin (100 µg/mL). The agar plate was incubated at 37℃ for 18 hs.

Colonies were recovered into 3 mL of LB with ampicillin (100 µg/mL), incubated overnight at 37ºC, with 250 rpm shaking.

The overnight culture was diluted 1:50 in fresh LB with ampicillin (100 µg/mL) to OD_{600} 0.6-0.8 (Eppendorf BioPhotometer, Germany) using 8,5 mm cuvette. 1 mL of uninduced culture was removed, centrifuged at 9300 x g, for 3 min at RT. The pellet was

frozen for later gel analysis. IPTG (1 mM) was added for induction of expression of recombinant protein. 1 mL aliquot was removed, centrifuged at 9300 x g, for 3 min at RT, and froze the pellet for gel analysis. Aliquots were removed every 1 h of incubation time to standardize the induction incubation time (1 to 6 hs).

After the induction, the bacteria culture was centrifuged at 4500 x g, for 15 min, at 4ºC, the pellet was eluted into lysis buffer (20 mM sodium phosphate pH 8, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF and 1 mg/ mL lysozyme) and sonicated on Misonic Sonicator 3000, (Newton,USA), for 30 min (30 sec on, 90 sec off, 15W). The lysate was clarified by centrifugation (20817 x g, 15 min, at 4ºC). Since LdAAP3 Nter was in inclusion bodies, the pellet of lysate was solubilized using solubilization buffer (20 mM sodium phosphate pH 8, 500 mM NaCl, 8 M urea, 5 mM imidazole) and incubated at room temperature, stirring, overnight. The solubilized solution was centrifuged at 20817 x g, 30 min, at 4ºC and the supernatant was further used in Dynabeads His-Tag purification.

2.3.2 Expression and transformation of LdAAP3 ORF and Cter recombinant proteins

4 µLof the pET151-ORF or pET-Cter plasmid was added to an *E. coli* Lemo21(DE3) vial, mixed gently, incubated on ice for 30 min, heated at 42℃ for 10 sec, then incubated on ice for 5 min. Added 250 µL S.O.C and incubated for 1 h, in 37℃ with 200 rpm on shaker. 100µL was spread on agar plate containing ampicillin (100 µg/mL), chloramphenicol (30 µg/mL) and L-rhamnose (500 µM). The agar plate was incubated at 37℃ for 18 hs.

Colonies were recovered into 3 mL of LB with ampicillin (100 µg/mL) and chloramphenicol (30 µg/mL), incubated overnight at 37ºC, with 250 rpm on shaker.

The overnight culture was diluted 1:50 into fresh LB with ampicillin (100 µg/mL), chloramphenicol (30 µg/mL) and L-rhamnose in different concentrations (0 to 2000 µM), using 200rpm, at 30-37℃, for about 3h, until OD reached OD_{600} 0.3-0.6. 1 mL of uninduced culture (UI) was removed, centrifuged at 9300 x g, for 3 min at RT, and froze the pellet for gel analysis.

When standardizing incubation time after induction with IPTG, 1 mL of uninduced culture containing 2000 μM L-rhamnose was saved for gel analysis. IPTG (400 µM) was then added to all aliquots with different amount of L-rhamnose, for induction of expression of recombinant protein. 1 mL aliquot was removed, centrifuged at 9300 x g, for 3 min at RT, and froze the pellet for gel analysis. Aliquots were removed from incubation after 4hs, 18hs

and 24hs, to standardize the induction incubation time (4 to 24hs) and the L-rhamnose concentration.

When not standardizing incubation time after induction, aliquots from the first incubation for 3 hs were added IPTG (400 μM), incubated on shaker for 4h at 200rpm, at 20-30℃. The culture was again diluted 1:50 into fresh LB with ampicillin (100 μg/mL), chloramphenicol (30 μg/mL) and L-rhamnose in different concentrations (0 to 2000 μM), using 200rpm, at 27-30℃, and incubated for 16-18hs. 1 mL aliquot was removed, centrifuged at 9300 x g, for 3 min at RT, and froze the pellet for gel analysis. The rest of the culture was centrifuged at 3220 x g, at 4℃, for 15min.

The pellet was eluted into lysis buffer (4 different lysis buffers described in Appendix E were tested), incubated for 15-60 min at RT, 9rpm, and sonicated using Misonic Sonicator (Newton, USA), for 30 min (30 sec on, 90 sec off, 15W). The lysate was clarified by centrifugation (20817 x g, 15 min, at 4ºC). 1 mL aliquot was removed, centrifuged at 9300 x g, for 3 min at RT, and froze the pellet for gel analysis. The rest of the supernatant was saved for further purification steps.

All aliquots, pellets and supernatants, saved for gel analysis were stored at -20℃, and thawed prior to gel analysis. Pellets were resuspended in 150μl - 500μl Milli-Q water before applying to gel.

2.3.3 Lysation using Ni-NTA Superflow Cartridge

When using Ni-NTA Superflow Cartridge column for purification a different lysis protocol was followed and was done according to the manual from Qiagen, Ni-NTA Superflow Cartridge Handbook (63).

5mL of NPI-10 buffer was added to each gram of wet pellet, lysozyme (0.1mg/mL) and Triton (0,1%) was added, then incubated on Echo Therm, at 30℃ using a Stuart rotator (Stone, United Kingdom), for 15 min. Sonicated in two sets with the same settings; 10 sec on, 10 sec off, W12, 6 rounds (2 min), on ice to minimize the heat.

To pellet cellular debris and spin down unbroken cells and heavy nuclei, the sonicated solution was centrifuged at 4℃, 10 007 x g for 30 min on Sorvall RC 5C Plus (Delaware, USA), using a Sorvall SS-34 rotor. Pellets were saved for later gel analysis while proceeding with the supernatant to the 2.4.5 Ni-NTA Superflow Cartridge column purification step.

2.3.4 Ultra Centrifugation steps

In the following section, different centrifugation attempts are listed along with the protocol number. Lysated solutions prior to these centrifugations were taken right after the sonication.

- ***Protocol #5****:* Lysate was centrifuged using 10000 x g, in 4℃, for 15 min and continued with supernatant with 1% SDS, then incubated on Stuart, Rotator (Stone, United Kingdom), for 1h, 9rpm, and 40 degrees incline. The lysate supernatant was then ultracentrifuged at 92600 x g, 4℃, for 1h. 4 mL solubilization buffer (20mM Sodium Phosphate, 300mM NaCl) and 1mM PMSF was added to the pellets, and set on the rotator for 1h, RT at 30 degrees incline. Solubilized pellet was then ultracentrifuged at 92600 x g, in 4℃, for 30 min.
- ***Protocol #8*****:** Lysated solution was centrifuged at 20000 x g, in 4℃, for 15 min. Supernatant was further used for ultracentrifugation at 500 000 x g, at 4℃, for 1h.
- ***Protocol #9*****:** The lysate was at 20000 x g, in 4℃, for 15 min. Supernatant was then ultracentrifuged at 165 000 x g, in 4℃, for 1h. Pellet was resuspended into 20 mL lysis buffer and 1mM PMSF, and then passed through a syringe (21G) five times. The resuspended pellet was then ultracentrifuged with the same settings for 1h. The pellet was then resuspended into 15 mL lysis buffer, 1mM PMSF and CHAPS (1%), and incubated on the roller with 9rpm, 40^0 incline, for 1h, then set on ultracentrifugation with the same settings for another hour.

2.4 Purification

2.4.1 Immobilized Metal Affinity Chromatography (IMAC):

The mechanism of adsorption of a protein when using IMAC is based on an interaction between an immobilized metal ion (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}) and an electron donor group located on the surface of the protein to be purified. These electron-donor atoms like N, S, and O can be found in for example histidine, cysteine and tryptophan and form chelates when bound to a metal compound (64).

2.4.2 Dynabeads His-Tag Isolation and Pulldown

Dynabeads are magnetic beads used to purify small volumes of different target molecules. The beads are coupled with IMAC chemistry, and are bound to a metal chelator with affinity to histidine residues due to two out of six coordination sites on cobalt (Co^{2+}) that are

unoccupied. When the His-tagged protein produced from *E. coli* occupy these two remaining coordination sites, a covalent bond is generated. Dynabeads are superparamagnetic and loose all their magnetic remanence once the magnetic field is removed. Beads at a size of 1μm in diameter are suited for purification of proteins and nucleic acids (65).

Because of the size, the beads have limited binding capacity and surface area. Increasing the volume of the Dynabeads may help overcome low binding capacities, but may as well lead to increased nonspecific adsorption and sample loss due to dilution of the sample with His-tag (66).

During small scale production purification was done using Dynabeads His-Tag kit (Life Technologies, Oslo, Norway), according to manufacturer's instructions (67). All fractions of purification steps were collected for gel analysis (flow through, washing 1, washing 2, washing 3 and eluted). Buffers were also changed from the manufacturers instructions to contain increasing imidazole concentrations, which can be seen in Appendix E.

2.4.3 IMAC columns

Both the HisTrap HP and Ni-NTA superflow cartidge use the IMAC system with Ni^{2+} as the immobilized metal ion and have great affinity for histidine residues (68). Ni^{2+} shows high metal ion stability, and is less susceptible to transfer its metal ion to the protein (69).

2.4.4 His-Trap HP column

His-Trap columns enabled purification of bigger amounts of sample at once. The purification protocol was done according to the HisTrap™ HP manual from GE Healthcare Life Sciences (70). In addition to this manual we used washing buffers with increasing imidazole concentration.

The column was washed with 10 mL Milli-Q water, then equilibrated with 10 mL 1HisTrap HP-binding buffer. Lysated protein solution was passed through the column 1-3 times before 10 mL of washing buffer 1 was passed through the column, followed by 10 mL of washing buffer 2, and then 10 mL of washing buffer 3 and finally 10 mL of elution buffer. The solution that passed through the column was saved for subsequent gel analysis.

Elution of the protein by pH differences was attempted by using the 2HisTrap HP-buffers. When pH is lowered to 4.0 the imidazole on the histidine residue will be protonated, and will not be able to bind to the metal ion resulting in elution from the column (64, 71, 72). We started washing the column with 10 mL water, at a speed of 30 drops per min. Then

passed through 10 mL 2HisTrap HP-lysis buffer, before passing through the lysated recombinant protein solution three times to make sure the proteins were able to bind to the column. 10 mL 2HisTrap HP-washing buffer 1 was then passed through, followed by 10 mL 2HisTrap HP-washing buffer2, and finally 10 mL 2HisTrap HP-elution buffer. The flow through was saved for gel analysis.

2.4.5 Ni-NTA Superflow Cartridge column

The purification was done according to the manual from Qiagen, Ni-NTA Superflow Cartridge Handbook (63).

The Ni-NTA Superflow Cartridge column (Qiagen, Hilden, Germany) was equilibrated with 10 mL NPI-10 buffer counting 30 drops a min. Bacterial lysate containing the recombinant protein obtained from the 2.3.3 lysation using the Ni-NTA Superflow Cartridge was passed through the column with the same flow rate. 10 mL of NPI-20 buffer was then passed through the column, and then 10 mL of NPI-250 buffer, and finally 10 mL of Buffer E (elution buffer from denaturizing conditions) was added as a final step to see if this could elute proteins that might be left on the column. Samples from the flow through were saved for gel analysis.

2.4.6 Filtration

To concentrate and purify the purified solution further, the Amicon Ultra-15 centrifugal filter (Merck, Germany) was used, with a 3 molecular weight cut-off. The solution from purification step containing the LdAAP3 recombinant protein was added to the Amicon filter, centrifuged at 2330 x g, 23℃, for 30 min.

2.4.7 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamid Gel Electrophoresis) is a technique used to separate molecules according to their molecular weight. Separation is generated as the proteins move through a sieving gel matrix in response to an electric field. Gel pore size is adjusted by the ratio between the acrylamide monomer and the cross-linker. The gel commonly consist of a stacking gel (4%) with larger pores on the top, and a resolving gel with an increasing gradient (until 12%) and smaller pores towards the bottom. The higher the percentage gives a higher polymer-to-water ratio, which gives smaller pore sizes (73). The protein is heated on 70℃ for 10 min, to denature the protein. Sample buffer added to the protein solution will keep the protein denatured, and the reducing agent will remove the

secondary and tertiary structure(74). SDS is a negatively charged detergent that will denaturize the protein, bind to it and give it a negative charge.

Protein fractions were analyzed by SDS-PAGE, using 4-12% Nu-PAGE Bis-Tris gel (Invitrogen, USA). 5 µL sample buffer (Invitrogen, USA), 2 µL reducing agent (Invitrogen, USA), was added per 10-35 µL of sample The mixture was heated at 70℃, for 10 min, then put on ice for 2 min before applying 20-40 µL of the contents per well on the gel.

4-10 µL of ladder SeeBlue Plus2 Pre-Stained Protein (Life Technologies, CA, USA) was used as a ladder. Precision Plus Protein ^{TM} Unstained Standards (BioRad, CA, USA) was used as a ladder when using ImageJ for quantification.

Running settings were 80V for 30 min, then 150V for 1 h. The gels were stained with Coomassie Blue (0.1%) mixed with acetic acid (10%) and ethanol (30%), for 1-2 hs. Acetic acid(10%) and ethanol (30%) was used for destaining overnight.

Alternatively, the gel was transferred to nitrocellulose membrane for Western blot analysis.

2.5 Quantification of protein

2.5.1 ImageJ

After purifying the expressed LdAAP3 recombinant proteins they were further used for production of anti-LdAAP3 polyclonal antibodies by immunization of rabbits. Because BioGenes GmbH (Germany) wanted the protein in a concentration of 100-200μg/500μl, we had to quantify the target protein. For this we chose to use ImageJ (75). This allowed an estimate of the amount LdAAP3 recombinant proteins in a band per well on the SDS-PAGE, and was done by comparing the density/intensity of the bands. An image of the SDS-PAGE gel was taken with a DSLR camera and uploaded in ImageJ. Image was set on grey-scale at 32-bit, with auto brightness/contrast. Rectangular lanes were drawn for each well using the **Rectangular** option on the toolbar.

By pressing “command” and “3” a profile plot showed the relative density of the different bands in each rectangular lane. The peak of interest on the profile plot was defined using the **Straight** option on the toolbar and marked using the **Wand** tool (Figure 23)

Each marked peak gave a value shown in the Results Window. These arbitrary numbers were used to calculate the amount protein (Table 6 and 7).

2.5.2 Quantification calculations

The equations below were used to calculate the amount of the LdAAP3 recombinant protein in the band of interest compared to the ladder band with known amount. Precision Plus Protein Standards, Unstained from BioRad (CA,USA) had a band at 50 kDa with a known amount of 750ng.

- **Calculating amount protein to ng:**

$$\frac{\frac{Arbitrary\ number\ of\ unknown\ sample}{Total\ amount\ sample\ added\ (\mu L)}}{\frac{Arbitrary\ number\ of\ ladder}{Total\ amount\ ladder\ added(\mu L)}} * 750ng = X\ ng$$

- **Converting ng to μg:**

$$\frac{X\ ng}{1000} = X\ \mu g$$

- **Calculating μg/μl:**

$$\frac{X\ \mu g}{Amount\ of\ recombinant\ sample\ added\ (\mu l)} = X\ \mu g/\mu l$$

- **Calculating amount protein per 500 μl:**

$$\frac{X\ \mu g/\mu l}{500} = X\ \mu g/500\mu l$$

2.6 Raising anti-LdAAP3 polyclonal antibodies

After the purification steps, the LdAAP3 protein solution was loaded on an SDS-PAGE. By comparing Western blots and SDS-PAGE with the same aliquots, two bands at the size of 32 kD (N1) and 26 kDa (N2) were chosen for immunization. These bands were cut out from the gel and shipped to BioGenes GmbH in Germany for immunization in 4 different rabbits. Two rabbits were immunized with gel slices from bands at the size of about 30-32 kDa, called N1. Two other rabbits were immunized with gel slices from bands at the size of about 26 kDa, called N2. Pre-serum was obtained before the first immunization. The immunization was then boosted twice to further enhance the immune response, before the rabbit was bleed rendering the blood from which the sera containing anti-LdAAP3 polyclonal antibodies were obtained.

2.7 Protein Blotting

This procedure was used to confirm the LdAAP3 recombinant His-Tagged proteins produced from *E. coli* Lemo21 containing the pET TOPO151-ORF plasmid, and to detect antibodies produced against the LdAAP3 recombinant protein from sera of *Leishmania spp.* infected animals and humans.

2.7.1 WesternBreeze Chromogenic Immunodetection System

This system contains a secondary antibody conjugated with Alkaline Phosphatase (AP). The enzyme (AP) converts several substrates to a colored precipitate giving a colorimetric detection of the matching antibodies. AP catalyzes BCIP (bromochloroindyl phosphate) and NBT (nitro blue tetrazolium) to produce a stable purple precipitate, visualizing the protein on the Western blot (76).

2.7.2 Blotting LdAAP3 recombinant protein on nitrocellulose membrane

Foam pads, filter papers and Nitrocellulose membrane, Amersham Hybond ECL 0.2μm (GE Healthcare), were soaked in transfer-buffers for 10 min. A sandwich of gel, nitrocellulose membrane, filter papers and foam pads were set in a chamber, X Cell II (Life Technologies, CA, USA), and filled with NuPAGE transfer buffer (CA, USA). Fridge-cold Milli-Q water was added to the two outer chambers of the device. The transfer was performed for 90 min at 40V, and placed in a cold room at 4℃. The blotted nitrocellulose membrane paper was removed from the devise. The membrane was blocked with blocking buffer for 1h, at RT, on the Belly Dancer (Stovall, USA).

2.7.3 Detecting the His-Tagged LdAAP3 recombinant proteins

After blocking the blotted nitrocellulose membrane for 1h, TBST (Tris-Buffered Saline with Tween 20) was added three times, and then placed on The Belly Dancer (Stovall, USA), at RT, for 5 min. TBST was discarded, and then AP conjugated HisG Epitope Tag primary antibody (mouse/IgG2a, Thermo Scientific, USA) was added at a dilution of 1:2000 and incubated on The Belly Dancer (Stovall, USA), for 2 hs at RT.

The solution was discarded before TBST was added to the membrane, and incubated for 5 min. This was repeated three times before adding TBS once and alkaline phosphatase buffer twice, with 5 min incubations. The alkaline phosphatase buffer was then discarded, 15 mL of Chromogen containing BCIP (bromochloroindyl phosphate) and NBT(nitro blue tetrazolium) was added, (Invitrogen, Carlsbad, CA, USA), until clear bands started showing. Washing with Milli-Q water was done for 10 min, before letting the membrane dry.

2.7.4 Testing sera from Leishmania infected and non-infected individuals

LdAAP3 recombinant protein blotted on nitrocellulose membrane from step 2.7.2 was cut into strips. The strips were washed 3-4 times in 4,5 mL CryoTube vials (Thermo Scientific, Denmark) with PBST (Phosphate Buffered Saline with Tween 20), with 5 min incubation and 60rpm on Orbital Shaker (Biosan, Latvia). The PBST was discarded, blocking buffer and different amounts of sera from infected test subjects was added to each tube, and was incubated overnight, 2 hs or 1h.

The solution containing sera and blocking buffer was discarded. The membranes were washed 3-4 times with PBST. Another washing step with PBS was added in later attempts. The secondary antibody was added to blocking buffer in a 1:5000 ratio, and added to each tube with strips and incubated for 1h, while covered with aluminum foil. Then the strips were washed 3-4 times with PBST, then once with PBS in later attempts. Strips were scanned on the Odyssey scanner (Lincoln, NE, USA) set on a channel of 700nm.

2.7.5 Dot-Blot

Five strips of nitrocellulose membrane were cut to fit into 4,5 mL CryoTube vials (Thermo Scientific, Denmark). 3μl of a 50:50 ratio mix of NPI-20 and NPI-250 flow through containing recombinant protein, in five different dilutions, was added to five strips of nitrocellulose paper. Two lines were marked on each strip for negative control. 3 μl of MilliQ-water was added between these marked lines, and let dry for 15 min.

The strips were blocked with TBS+5%BSA, incubated for 1h on the Belly Dancer (Stovall, USA), in RT. Thereafter, the strips were washed four times with TBST, on the Belly Dancer (Stovall, USA), at RT for 5 min between each wash, and then washed once with TBS. TBS was discarded. TBS+5%BSA and primary antibody, serum from rabbit injected with *E. coli* produced LdAAP3 recombinant protein, was added in different dilutions, then incubated for 1 at RT on the Belly Dancer (Stovall, USA).

TBST was added three times for washing, and once with TBS. Secondary antibody conjugated with alkaline phosphatase (Thermo Scientific, USA) was added and incubated for 1h, at RT, on the Belly Dancer (Stovall, USA), before washing three times with TBST and once with TBS.

BCIP/NBT was added to the strips and incubated at RT, on the Belly Dancer (Stovall, USA), until bands showed. Finally the strips were washed with Milli-Q water for 10 min and dried in RT.

2.7.6 Testing anti-LdAAP3 polyclonal antibodies against E. coli produced LdAAP3 proteins

These nitrocellulose membrane strips were produced following the Ni-NTA lysis and purification protocols. These membrane strips were blocked for 1h and cut into strips containing ladder, SeeBlue Plus2 Pre-Stained Protein, as a marker.

The strips were washed by adding 20 mL TBST 3 times, and once with TBS, with 5 min incubation, at RT, using the Belly Dancer (Stovall, USA). Each strip was placed into a CryoTube vial (Thermo Scientific, Denmark). Blocking buffer and sera from rabbit injected with the LdAAP3 recombinant protein was then added in different dilutions, and incubated for 2 hs on the Belly Dancer (Stovall, USA) at RT. After 2h the fluid was discarded, the strips were then washed four times with TBST, then washed 1 time with TBS. 2 mL secondary antibody conjugated with alkaline phosphatase (Thermo Scientific, USA) was added to the strips and incubated for 1h, on the Belly Dancer (Stovall, USA) at RT, then washed four times with TBST, and once with TBS. The strips were then washed two times with alkaline phosphatase buffer, with 5 min incubation, at RT, using the Belly Dancer (Stovall, USA). The buffer was discarded, before BCIP/NBT was added. The strips were incubated in BCIP/NBT until clear bands started showing. Finally the strips were washed with Milli-Q water for 10 min and dried.

2.7.7 Production of Soluble Leishmania antigen (SLA)

Leishmania amazonensis (MHOM/BR/73/M2269) was kept in M199 supplemented with L-glutamine, Hanks salts, 10% fetal bovine serum, 5 ppm hemin, 100 μM adenine, 50 U/mL penicillin, 50 μg/mL streptomycin, 40 mM HEPES and 12 mM sodium bicarbonate, at 26°C, until stationary phase. $2.5x10^8$/mL of parasites were harvested and centrifuged at 800 x g, for 10 min, at RT. The supernatant was removed and the pellet was washed with Dulbecco PBS (pH 7.4) and centrifuged again. This step was done three times. After resuspending the pellet in Dulbecco PBS, the parasites were lysated by repeated freeze and thaw; frozen in liquid nitrogen for 5 min and thawed in RT, respectively. This step was also done three times. The protein concentration was determined by the Direct Detect infrared spectrometer (Merck Millipore, Germany) using the direct detect assay-free green cards and the software NIST-BSA standard.

2.7.8 Testing SLA and recombinant LdAAP3 against anti-LdAAP3 antibody using ELISA

Anti-LdAAP3 polyclonal antibodies produced in rabbits (Biogenes GmbH, Germany) were evaluated by ELISA. Flat-bottomed microtest plates (Sarstedt, USA) were coated with 10 ug/mL of either the purified recombinant protein or a soluble *Leishmania amazonensis* antigen, overnight, at 4°C. The antigens were diluted in 0.5 M carbonate buffer (pH 9.5). After the overnight incubation, the plates were washed with TBS with 0.1% Tween-20 and blocked with 100 μL of 5% BSA (bovine serum albumin) in TBS (tris-buffered saline) with 0.1% Tween-20, for 1 h at RT. Thereafter, the blocking-buffer was removed and 100 μL pre-immune or immune anti-AAP3 polyclonal antibodies were added at various dilutions (1:100-1:1600 for pre-immune, and 1:100-1:52000 for immune), for 1 h on a Thermo Shaker (Biosan, Latvia) at 250 rpm at room-temperature. After washing with TBS with 0.1% Tween-20, 100 μL of goat anti-rabbit IgG-Horseradish peroxidase (GE Healthcare, UK) was added at 1:2000 dilution in TBS with 0.1% Tween-20 and 0.1% BSA for 1h on a shaker (250 rpm) at RT. After washing, 100 μL of 1-step ultra 3,3´,5,5´-tetramethylbenzidine (Thermo Scientific, USA) substrate was added to each well. The reaction was stopped with 100 μL of 2M sulfuric acid. The plates were read at 450 nm on a Synergi H1 Hybrid reader (BioTek instruments, USA). Absorbance values were read and background subtracted.

3. RESULTS

3.1 DNA replication and purification

The gene encoding the AAP3 from *L. donovani*, cloned into the pGEM-T easy vector, was obtained from the research group of Professor Lucile Floeter-Winter, USP, Brazil.

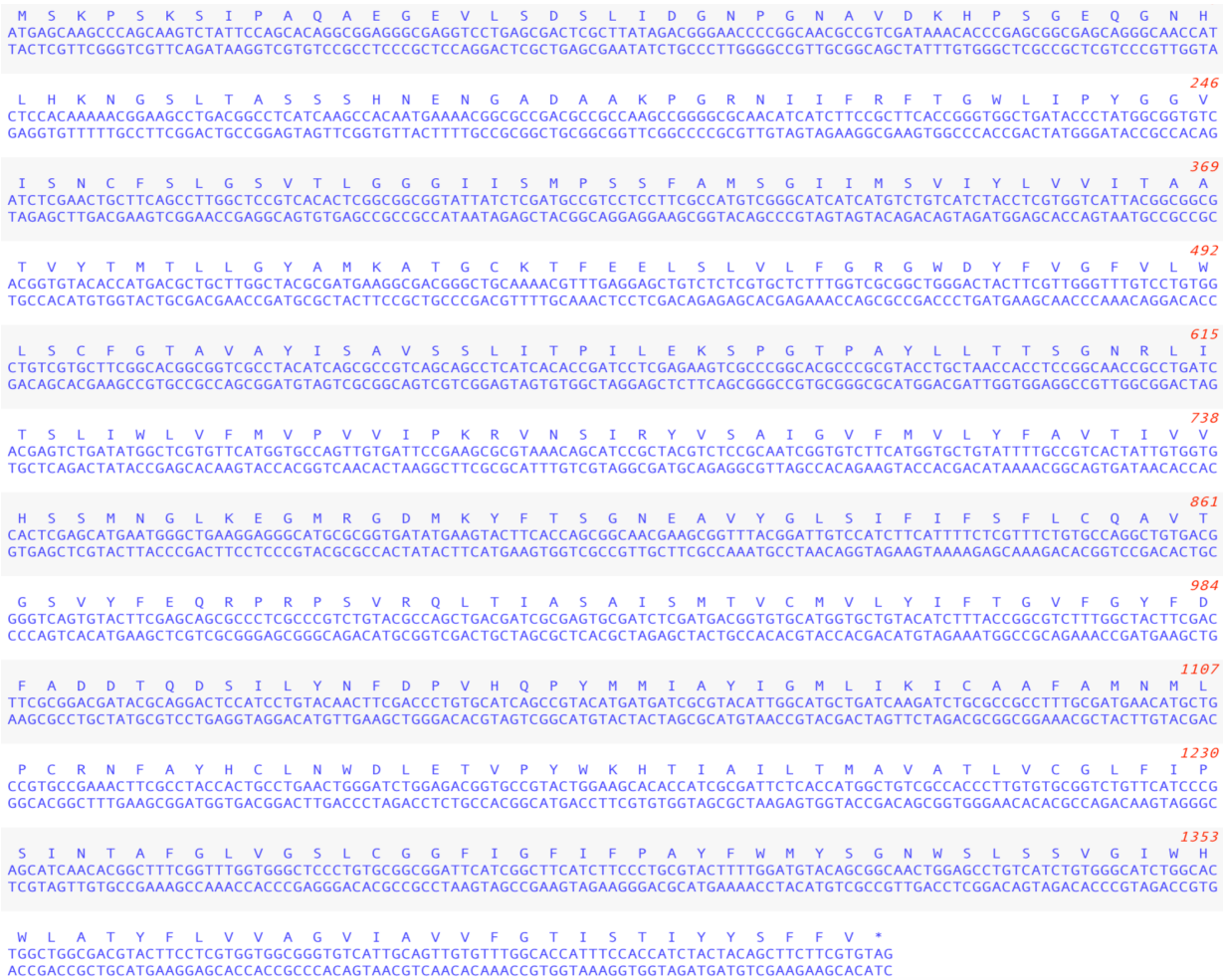


Figure 13: *The ORF (open reading frame) of the LdAAP3 gene and the deduced amino acid sequence.Image obtained from MacVector software (MacVector Inc, NC, USA)*

Analysis of the deduced amino acid sequence indicated that the protein contained 11 transmembrane spanning domains (Figure 15). It was therefore determined to perform the expression in *E. coli* using three different approaches; cloning of the N-terminal part (Nter), cloning of the C-terminal part (Cter) and cloning of the entire ORF, respectively.

3.1.1 PCR and gel electrophoresis

The forward primers were designed including four base sequences (CACC) underlined. The following primers were designed:

LdAAP3_F: 5´CACCATGAGCAAGCCTAACGAGC 3´

LdAAP3_R: 5´CTACACGAAGAAGCTGTAGTAGATCGTCG 3´

LdAAP3_NT_R: 5´ CTAGACGACGGCATCGAGATGATACC 3´

LdAAP3_CT_F: 5´ CACCGACGATACGCAGGACTC 3´

The primer pairs (LdAAP3_F, LdAAP3_NT_R), were used for the Nter-, (LdAAP3_CT_F, LdAAP3_R) were used for Cter, and (LdAAP3_F, LdAAP3_R) were used for ORF-segments.

The PCR products were run on a 1% agarose gel, stained with GelRed and photographed under UV-light. The results from the gel electrophoresis allow us to visualize the size of the PCR products. The picture (Figure 14) revealed bands corresponding to fragments of expected size, confirming a successful cloning.

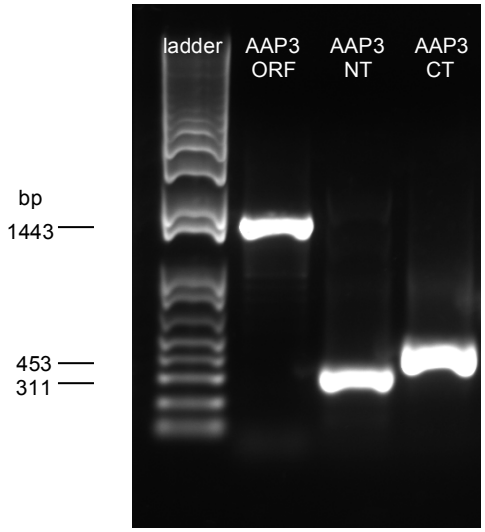


Figure 14: *Electrophoresis in 1% agarose gel stained with GelRed (Invitrogen), representing PCR amplification of LdAAP3 open reading frame (ORF), LdAAP3 Nter and LdAAP3 Cter. 1kb Invitrogen ladder. GelRed fluorescent stain starts emission once it is bound to the DNA. Because they are fluorescent they will absorb visible UV light and transmit the energy as visible light (77).*

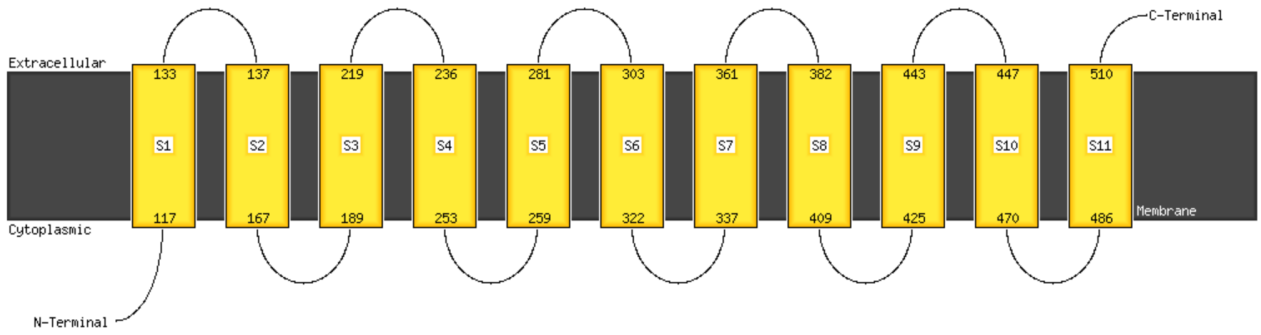


Figure 15*: Transmembrane helix prediction of the LdAAP3 amino acid sequence. Nter has 138aa giving a size at about 14,6 kDa and one transmembrane domain. Cter has 183aa giving a size of about 20.7 kDa and two transmembrane domains. The whole ORF has 514aa giving a size at about 56,2 kDa with 11 transmembrane domains. Figure is obtained from the Phyre2 web portal for protein modeling, prediction and analysis (78).*

3.1.2 Cloning and transformation of the ORF, Nter and Cter plasmid into competent cells

Purified DNA from the PCR products of the pGEM-T easy vector were cloned into the pET151/D-TOPO expression plasmid (Invitrogen, USA). This plasmid was transformed into *E. coli* One Shot TOP10 Chemically Competent (Invitrogen, USA) for general cloning of blunt-end PCR products. Single colonies were picked, inoculated for growth in LB medium with ampicillin, and processed for isolation of plasmid DNA. The resulting plasmids were designated pET151-ORF, pET151-Nter and pET151-Cter, respectively.

The purified plasmids were then transformed into the *E. coli* BL21 Star(DE3) and *E. coli* Lemo21(DE3), which contain the proper genetic elements for expression of recombinant proteins.

The plasmid pET151-Nter turned out not to be stable in *E. coli* BL21 Star(DE3). After some trials and errors it was determined to terminate the work with both pET151-Nter and pET151-Cter, and focus entirely on pET151-ORF.

3.2 Expression of the LdAAP3 ORF recombinant protein

3.2.1 Standardization of L-rhamnose concentration and incubation time after induction

E. coli Lemo21 allows tunable expression by varying the amount of L-rhamnose added. To determine an optimal concentration and incubation time a pilot experiment was done with different incubation times and concentrations of L-rhamnose added.

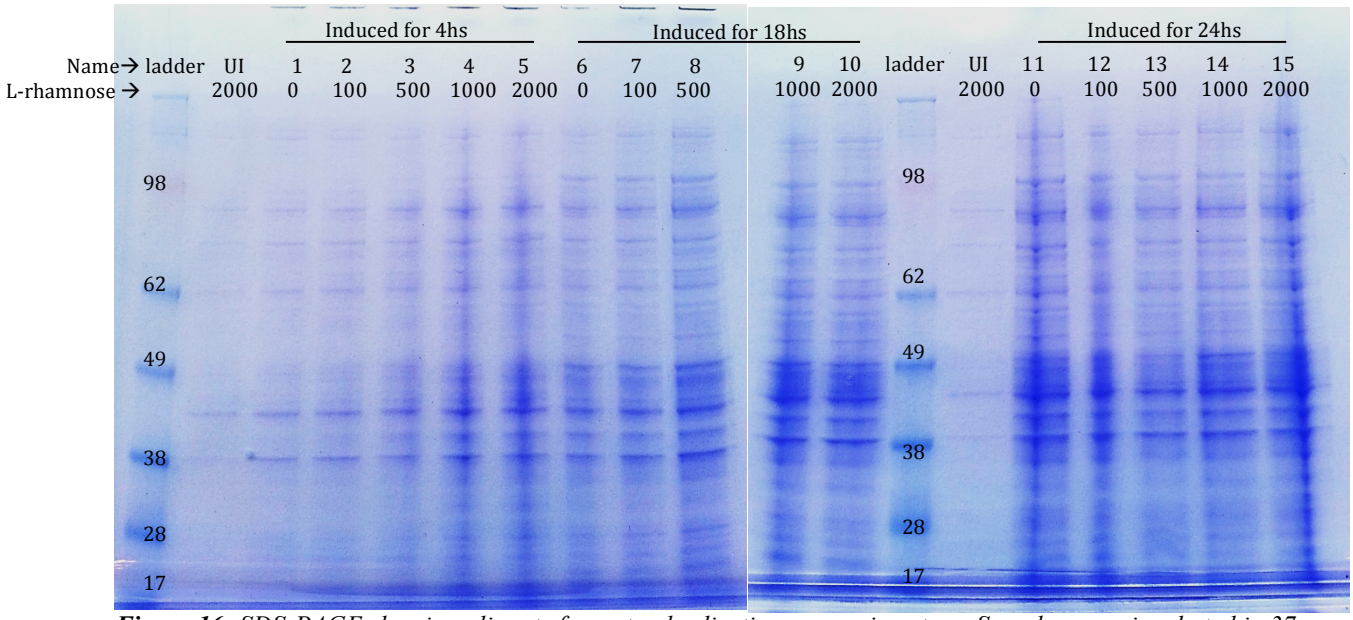


Figure 16: *SDS-PAGE showing aliquots from standardization expression steps. Samples were incubated in 37 °C, for 4, 18 and 24 hs containing concentrations of 0, 100, 500, 1000 and 2000 µM of L-rhamnose. Uninduced aliquots were taken after 3h incubation with L-rhamnose and were not added IPTG. 400µM IPTG was added to the rest of the aliquots before incubation for 4, 18 or 24h.*

The uninduced (UI) aliquots showed quite weak bands. Aliquot 1, 6 and 11 were all induced with 400μM IPTG, but was not added L-rhamnose. Band 1 and 6 seems to be weaker than the according bands with different amounts of L-rhamnose but same incubation time. Bands from the 24-hs induction seem to be quite similar in band intensity. When comparing all the bands together, it seems the bands get stronger the longer the incubation time, which may seem logical because the *E. coli* has more time to produce more protein. The bands also get stronger with higher concentrations of L-rhamnose. Since there were no major differences in band intensity from the 18 to the 24h incubation we decided to continue with the 18h incubation. Lane number 8-10 seemed to have stronger bands, so we decided to continue with 500 and 1000 μM of L-rhamnose in our protocols.

3.2.2 Combinations of temperature, incubation time and L-rhamnose concentration

Less expression of the recombinant protein may lead to more production of protein of interest, because of less toxicity and stress for the *E. coli*. Attempts were done to find the best combination of temperature, incubation time and concentration of L-rhamnose that would give good conditions for ORF production, and enable us to produce the LdAAP3 recombinant protein properly folded and unfragmented.

When analyzing the different gels obtained from several experiments it seems that expression using lower temperature (around 20℃) gives more protein production. Comparing gels when using 500 or 1000 μM L-rhamnose, the difference does not seem to be of major importance for production and purification of the LdAAP3 recombinant protein, and show relatively similar bands.

Table 1: *Quick overview of the different combinations of temperature, incubation time and L-rhamnose concentration used during expression steps.*

Concentration of L-rhamnose in μM	**First incubation:** Expression until OD_{600} was between 0.3 and 0.6	**Second incubation:** After 4h of induction with IPTG added	**Third incubation:** Overnight incubation
500 Small scale	**Protocol #1:** 30°C, 3h, 250rpm	30°C, 4 h, 250rpm	30°C, 16-18hs, 250rpm
	Protocol #2: 30°C, 3h, 250 rpm	27,4°C, 4 h, 200rpm	26°C, 16-18hs, 200rpm
1000 Small scale	**Protocol #2:** 30°C, 3h, 250 rpm	27,4°C, 4 h, 200rpm	26°C, 16-18hs, 200rpm
	Protocol #3: 30°C, 2h, 250 rpm	30°C, for 1h and 30 min, 250rpm . Then 20°C for 2h and 30 min.	20°C, 16-18hs
500 Big scale	**Protocol # 7,8,9,9.2,10,11:** 37°C, 2 h, 200rpm	23°C, 4 h, 200rpm	23°C, 16-18hs, 200rpm
	Protocol #12: 37°C, 1h and 30min, 200rpm	24°C, 4 h, 200rpm	24°C, 16-18hs, 200rpm
1000 Big scale	**Protocol # 4,5,6:** 37°C, 2 h, 200rpm	23°C, 4 h, 200rpm	23°C, 16-18hs, 200rpm

3.2.3 Lysis and Centrifugation

In order to purify the LdAAP3 recombinant protein expressed in *E. coli* Lemo21, lysation steps were necessary. Lysation could break up the cell wall and cell membrane of the *E. coli* and free the LdAAP3 recombinant protein from the *E. coli.* Changes during lysation were done to find the optimum amount of pellet, buffers and settings that allowed good lysation yielding good solutions for purification, which is shown in Table 2. After lysis the solution containing lysated *E. coli* Lemo21 went through several centrifugation steps in attempts to direct more of the LdAAP3 recombinant protein to the pellet, some explained under 2.3.4 Ultra Centrifugation steps.

Lysis was verified by pipetting up and down, yielding a slimy consistence, with a visible line from the pipet tip to the solution due to destruction of the cell wall and liberation of DNA (79). Sonication showed to be successful by testing with a pipet to see that the fluidity had increased, due to cells and DNA being sheared.

When comparing the gels (all images not shown) it seems that centrifugation steps helps further separation of the LdAAP3 recombinant protein bands and unwanted bands that may come from intact cells, nuclei and other heavy organelles. Shorter sonication also seemed to benefit the purification.

Table 2: *Overview of the different changes made during lysis. Pellets from the end of the expression step were used. Changes were done to amount pellets used, amount buffer added for each pellet, different incubation time, and different sonication and centrifugation settings. For small scale production one pellet was obtained from 10 mL of centrifuged overnight solution containing E. coli Lemo21 and the pET TOPO 151 plasmid containing the LdAAP3 ORF sequence. For big scale production one pellet was obtained from 50 mL centrifuged overnight solution.*

Pellet	Amount buffer	Roller/ shaker	Sonication	Centrifugation
Small scale: Protocol #1 1 pellet taken from 10mL centrifuged overnight solution.	30mL lysis buffer1	Shaker: 1h, 30℃, 60rpm.	20 min, 30sec on, 90 sec off, level 5, 15W.	10 000xg, 4℃, 15 min. Protein assumed in supernatant.
Small scale: Protocol #2 1 pellet taken from 10mL centrifuged overnight solution. (OD:0.31)	10mL lysis buffer1	Roller: 30min, 8rpm, RT. After 30 min added 20μL DNase(50U/ mL)	30 min, 30sec on, 90 sec off, level 5, 15W.	#1. 10 000xg, 4℃, 15 min. Protein assumed in supernatant. #2. 20817xg, 4℃, 30 minutes. Protein assumed in pellet.
Small scale: Protocol #3 1 pellet taken from 10mL centrifuged overnight solution. (OD: 0,309)	10mL lysis buffer1 with	Roller: 30min, 8rpm, RT.	15 min, 30sec on, 90 sec off, level 5, 15W.	#1. 10 000xg, 4℃, 15 min. Protein assumed in supernatant. #2. 20817xg, 4℃, 45 min. Protein assumed in pellet. #3 10 000xg, 20℃, 30 min.
Big scale: Protocol #4 10 pellets from 50mL centrifuged overnight solution. (OD:0.538)	50mL lysis buffer1	Roller: 40min, RT, 9rpm, 40degrees incline.	30 min, 30sec on, 90 sec off, level 4,5, 12W.	#1. 10 000xg, 4℃, 15 min. Protein assumed in supernatant. #2. 20817xg, 4℃, 45 min. Protein assumed in pellet.
Big scale: Protocol #5 10 pellets from 50mL centrifuged overnight solution. (OD: 0.541)	50mL lysis buffer1	Roller: 40min, RT, 9rpm, 40 degrees incline.	20 min, 30sec on, 90 sec off, level 4,5, 12W.	#1. 10 000xg, 4℃, 15 min. Protein assumed in supernatant. #2. 92600xg x 2 protein assumed in pellet.
Big scale: Protocol #7 1 pellet from 50mL centrifuged overnight solution. (OD: 0.547)	10mL lysis buffer1	Roller: 40min, RT, 9rpm, 40 degrees incline.	20 min, 30sec on, 90 sec off, level 4,5, 12W.	20817xg, 4℃, 15 min. Protein assumed in supernatant.

Big scale: Protocol #8 1 pellet from 50mL centrifuged overnight solution. (OD: 0.547)	10mL lysis buffer1	Roller: 40min, RT, 9rpm, 40 degrees incline.	20 min, 30sec on, 90 sec off, level 4,5, 12W.	#1. 20 000xg, 4℃, 15 min. Protein assumed in supernatant. #2. 500 000xg (ultra) Protein assumed in pellet.
Big scale: Protocol #9 2 pellets from 50mL centrifuged overnight solution. (OD: 0.547)	20mL lysis buffer1. (CHAPS 1% added to lysis buffer to dissolve pellet before last centrifugation)	Roller: 30min, RT, 9rpm, 40 degrees incline.	20 min, 30sec on, 90 sec off, level 4,5, 12W.	#1. 20 000xg, 4℃, 15 min. Protein assumed in supernatant. #2. 165000xg x2 (ultra) Protein assumed in pellet then in supernatant after last centrifugation.
Big scale: Protocol #9,2 5 pellets from 50mL centrifuged overnight solution. (OD: 0.547)	15mL lysis buffer1	Roller: 40min, RT, 9rpm, 40 degrees incline.	20 min, 30sec on, 90 sec off, level 4,5, 12W.	20817xg, 4℃, 15 min. Protein assumed in supernatant.
Big scale: Protocol #11 2 pellets from 50mL centrifuged overnight solution. (OD: 0.502)	15mL lysis buffer2. Added 1mMEDTA, 10 μM TLCK, 2 μg/mL Leupeptin, 1 μg/mL aprotinin.	Roller: 40min, RT, 9rpm, 40 degrees incline.	No sonication because lysis was so fluid.	20817xg, 4℃, 15 min. Protein assumed in supernatant.
Big scale: Protocol #12 20 pellets from 50mL centrifuged overnight solution. (OD: 0.393)	34mL NPI-10. Lysozyme (0,1mg/mL) and Triton (0,1%).	Roller: 15 min, 30℃, 9 rpm.	2 rounds (2 min in total per round) with 10s on, 10s off, level 4,5.	10007xg, 30 min, 4℃. Protein assumed in supernatant.

3.2.4 Results from SDS-PAGE analysis

During the expression and purification, aliquots of the samples containing *E. coli* Lemo21 and LdAAP3 recombinant protein were collected for analysis using SDS-PAGE.

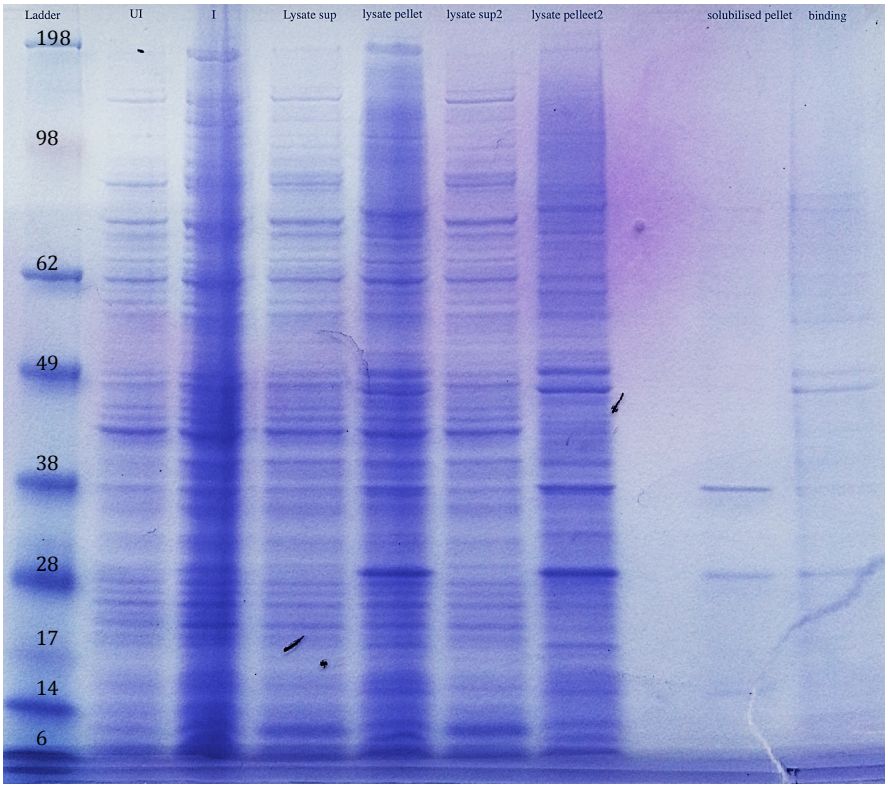


Figure 17: *SDS-PAGE showing that the LdAAP3 recombinant protein may be in both pellet and supernatant. This gel is from protocol* ***#3*** *using 1000 µM L-rhamnose, 20℃ overnight, 1 pellet from expression step resuspended in 10 mL lysis buffer, 15min sonication, centrifuged at 10 000 x g, 20817 x g and 10 000 x g, purification using 1Dynabeads buffers. Lysate supernatant and Lysate pellet are aliquots taken from the first centrifugation round. Lysate sup2 and lysate pellet2 are obtained from the lysate supernatant centrifuged for the second time.*

Table 3: *Content of the aliquots from Figure 17. Each sample lane was added 5 µL sample buffer, 2 µL reducing agent, 3 µL water and 10 µL protein sample, and gave a total of 20 µL applied to each well. 7 µL Ladder was added.*

Name: Gel from protocol #3	**Aliquots on the SDS-PAGE obtained from**
Ladder	SeeBlue Plus2 Pre-Stained Protein (Life Technologies, CA, USA)
UI: Un-Induced	Undiluted sample of the *E. coli* solution after 3 h incubation.
I: Induced	Undiluted sample of the induced overnight *E. coli* sample from overnight incubation.
Lysate Supernatant	1:5 dilution of centrifuged and sonicated *E. coli* lysate obtained from one pellet from 50 mL *E. coli* sample solubilized in 10mL lysis buffer
Lysate Pellet	1:5 dilution of centrifuged and sonicated *E. coli* lysate obtained from one pellet from 50 mL *E. coli* sample solubilized in 10mL lysis buffer
Lysate Supernatant2	Supernatant obtained from 20817 x g centrifugation of lysate centrifuged at 10 000x g
Lysate pellet2	Pellet obtained from 20817 x g centrifugation of lysate centrifuged at 10 000x g

Solubilized Pellet	Pellet taken from centrifuged solution of 4 pellets, from 8mL of centrifuged supernatant, solubilized in 4 mL solubilization buffer
Binding buffer	1:8 dilution of supernatant from 4 mL centrifuged solubilized solution.

Amongst other bands we could see strong bands on the SDS-PAGE from aliquots obtained from the pellets, and weaker bands from aliquots obtained from the supernatant, at sizes around 38 kDa and 28 kDa (Figure 17). Bands at about 56 kDa also appear on the gel. The stronger bands in the pellet may be due to higher concentrations of the pellet sample compared to supernatant sample. The SDS-PAGE with the washing and eluted aliquots (image not shown) showed bands at around 38 kDa and 28 kDa. These bands were weaker than the bands on the binding well from figure 16, but were also cleaner.

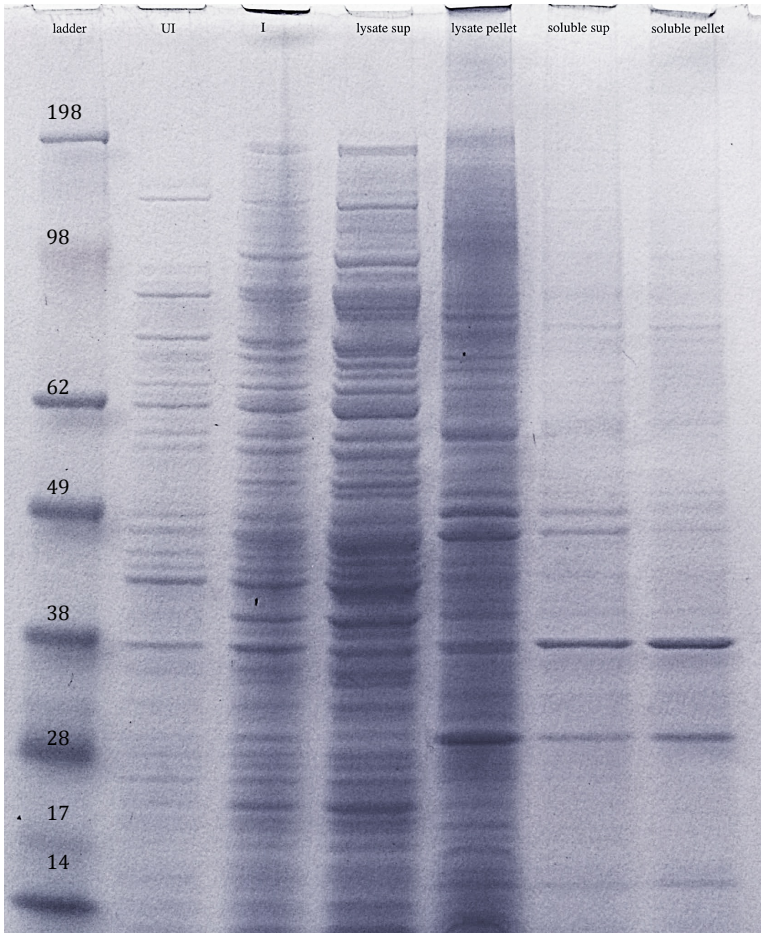


Figure 18: *SDS-PAGE from protocol* ***#4*** *using 1000 µM L-rhamnose, 23℃ overnight, 10 pellets from expression step resuspended in 50mL lysis buffer, 30min sonication, centrifuged at 10000 x g and 20817 x g, without any purification step. Lysate supernatant and lysate pellet were the aliquots from the first centrifugation round. Soluble supernatant and soluble pellet is taken from the lysate supernatant centrifuged for the second time at 20817 x g.*

Table 4: *Content of the aliquots from Figure 18. Each sample lane was added 5 µL sample buffer, 2µL reducing agent, 3µL water and 10 µL protein sample, and gave a total of 20 µL applied to each well. 7 µL Ladder was added.*

Name: Gel from protocol #4	**Aliquots on the SDS-PAGE obtained from**
Ladder	SeeBlue Plus2 Pre-Stained Protein (Life Technologies, CA, USA)
UI: Un-Induced	Undiluted sample of the *E. coli* solution after 3 h incubation.
I: Induced	Undiluted sample of the induced overnight *E. coli* sample from overnight incubation.
Lysate Supernatant	1:30 dilution of centrifuged and sonicated *E. coli* lysate obtained from 10 pellets from 500 ml *E. coli* sample solubilized in 50 mL lysis buffer
Lysate Pellet	1:30 dilution of centrifuged and sonicated *E. coli* lysate obtained from 10 pellets from 500 ml *E. coli* sample solubilized in 50 mL lysis buffer
Solubilized Supernatant	Sample taken after centrifugation of supernatant from lysate step
Solubilized Pellet	Sample taken after centrifugation and solubilization of pellet from supernatant lysate step

Bands at the size around 38 and 28 kDa can again be seen in aliquots from both the supernatant and the pellet in figure 17. Bands at these sizes occur in most of the SDS-PAGE gels. Weaker bands at around 56 kDa can be seen after the first centrifugation in aliquots until lysate pellet, but is lost after the second centrifugation (Figure 18). By looking at this gel it may seem as though the whole LdAAP3 protein is not produced in large amounts. Small amounts can be seen on the gel, and stronger bands of fragments at 38kDa and 28 kDa seem to occur, which may indicate complication during expression yielding only fragments of the recombinant protein. Protein obtained from pellet and inclusion bodies are often undesirable because of problems occurring in attempts of renaturing the denatured proteins (80, 81).

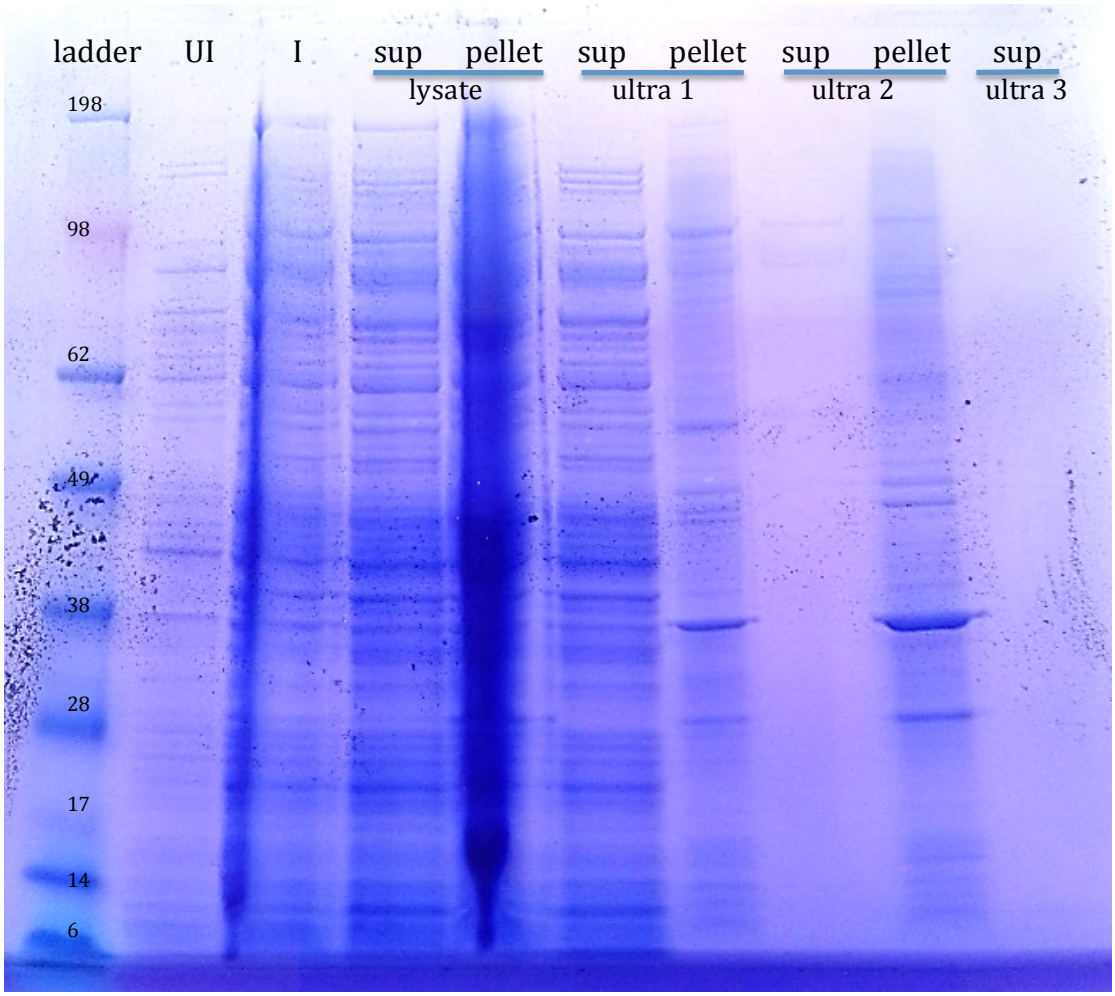


Figure 18: *SDS-PAGE from protocol* ***#9*** *with 500 µM L-rhamnose, 23*℃ *overnight, 2 pellets from expression step resuspended in 20mL lysis buffer and 1%CHAPS, 20min sonication, centrifuged at 20 000 x g– then 3x 165000 x g, no purification. Lysate sup and lysate pellet were taken from the fist centrifugation with 20 000 x g. Ultra 1 sup and pellet were aliquots taken from pellet from the first ultra centrifugation (165 000 x g) resuspended in lysis buffer and centrifuged at 165000 x g. Ultra 2 sup and pellet are taken from pellet from the second centrifugation (165000 x g) resuspended in lysis buffer and 1% CHAPS and centrifuged at 165000 x g. Ultra sup 3 is supernatant from the last centrifugation.*

When performing ultracentrifugations, the solution gets more purified. One centrifugation at 40 000 x g may be enough since pellet from ultra 1 and ultra 2 seem to be similar. Continuing with the pellet from ultracentrifugation 1 may have been wise to do since we have relatively clean bands, at around the size of 56 kDa, 38 kDa and 28 kDa. Because we wanted to use this purified protein for polyclonal antibody production we wanted to see if we could purify the protein in the supernatant to avoid the difficulties of renaturation and refolding processes(82). Ultracentrifugation steps were therefore not continued.

3.2.5 Codon usage

The amino acid sequence of the *Leishamnia donovani* arginine transporter (LdAAP3) gene, obtained from NCBI, USA, was monitored for rare codons in the *E. coli* using the Rare Codon Calculator provided by the Molecular Biology Institute at the University of California, Los Angeles(83). The rare codons obtained from this analysis coded for the amino acids proline, icoleucine, leucin, and arginine. The ratio from Table 5 represents the abundance of the specific codon relative to the other codons that code for the same amino acid.

Each nucleotide sequence was translated using a translator program from ExPASy Bioinformatics Resource Portal (Switzerland), to see if these fragments could match the sizes of the fragmentations occurring of the recombinant LdAAP3 protein.

Note that the fragments sizes starting from the Cter end do not contain the His-tag, because the His-tag is on the Nter end, and will therefore unlikely show as a band on the Western blots, but could show as bands on the SDS-PAGE and on the nitrocellulose membranes blotted with LdAAP3 recombinant protein incubated with infected sera.

Table 5: *The sizes of the proteins generated if translation were terminated due to the presence of rare codons.*

Rare codon	**Ratio**	**Size of proteins from N-terminal end (kDa)**	**Size of proteins from C-terminal end (kDa)**
CCC(Pro)	0.10	4,24	52,04
ATA(Ile)	0,07	0,07	50,23
CCC(Pro)	0.10	6,45	49,83
ATA(Ile)	0,07	11,84	44,46
CCC(Pro)	0.10	11,94	44,34
CCC(Pro)	0.10	23,74	32,54
CCC(Pro)	0.10	23,94	32,28
CTA(Leu)	0,03	24,40	31,84
ATA(Ile)	0,07	25,72	30,58
CGA(Arg)	0,05	44,12	12,22

3.3 Purification

Purification protocols were performed using Dynabeads His-tag isolation or His-trap HP or Ni-NTA columns. The LdAAP3 recombinant protein was often lost before elution steps during purification. Different attempts like changing imidazole concentrations in the buffers and eluting using pH were tested to see if more of the target protein would be obtained.

3.3.1 Imidazole concentrations

How high the concentration of the imidazole before the protein loos their binding affinity to the immobilized metal ion seems to vary between the different purification methods. In protocol #3 (Figure 17) using 1DynaBeads solution, weak bands were seen in the eluted aliquot using 300mM of imidazole (image not shown). During protocol #9.2 (Figure 24) using HisTrap column with 1HisTrap buffers, almost all of the proteins loos their binding affinity to the column when washing 2 buffer was added containing 75mM imidazole. When using the Ni-NTA column the target protein loos their binding affinity to the column when using NPI-250 buffer containing 250mM imidazole (Figure 25). A lot of bands were seen in the eluted aliquots from protocol #7, when using 2DynaBeads solutions with 300mM imidazole (image not shown). The different protocols cannot be directly compared to each other.

3.3.2 Denaturation conditions

In case the LdAAP3 recombinant protein did not elute from the column during purification, buffer E (8M UREA, 100mM Tris-base, 100mM NaH_2HPO_4) from the Ni-NTA Superflow buffers with denaturing conditions was used as the final flow through solution during protocol #12 (Figure 25). If you look at Figure 25, you could see a weak band in the FT-BE well of the SDS-PAGE at sizes about 56 kDa. When looking at the Western blot, from Figure 25, of this aliquot it did not show any proteins. Using denaturing buffers seem to elute the remaining LdAAP3 protein. This may indicate that some of the LdAAP3 recombinant proteins have strong binding affinity to the column.

During protocol #11, denaturing conditions using 2HisTrap buffers with decreasing pH was tried out (Figure20). Most of the proteins seemed to let go of the column at pH 6.3 when using 2HisTrap – washing buffer 1, though weak bands can be seen under washing buffer 2 and eluted 1 (Figure 20). Elution by acidic pH was not continued because target proteins were not obtained during pH elution. In the same protocol many protease inhibitors were added to see if this could help stop the fragmentation of the ORF. We did not see

remarkable changes in the bands and still had fragments compared to other attempts without the addition of these inhibitors. This can further support the theory that the fragments obtained are not due to degradation of the ORF LdAAP3 recombinant protein, but may rather be due to problems during translation of the whole LdAAP3 ORF, yielding problems in purifying the whole LdAAP3 recombinant protein.

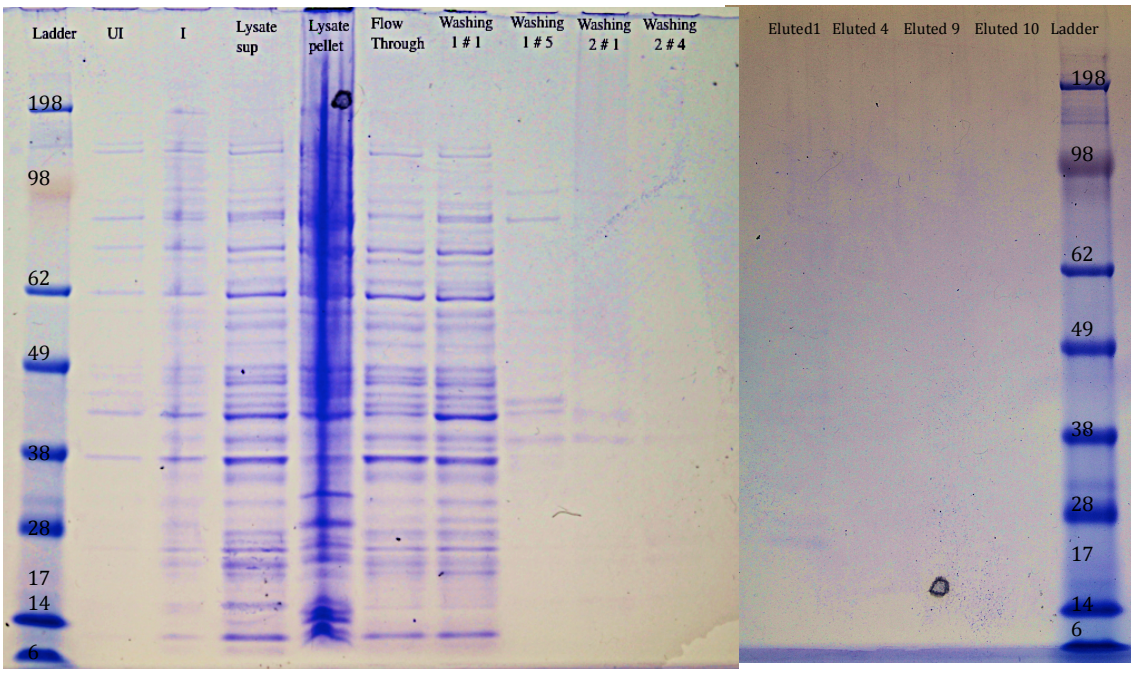


Figure 20: *This gel is from protocol* ***#11*** *with 500 µM L-rhamnose, 23*℃ *overnight, 2 pellets from expression step resuspended in 15mL lysis buffer and protease inhibitors, no sonication, centrifuged at 20817 x g, purification with His-Trap column using 2HisTrap buffers with denaturing conditions and pH elution.*

3.3.3 Testing SDS-PAGE limits

Because other purification steps were not completely successful, SDS-PAGE gels were used to separate the proteins further. Small amounts of the LdAAP3 recombinant protein seemed to be obtained. Filtration of the purified solution was performed to increase the concentration. To further increase the amount target protein obtained per well, the maximum amount of sample loaded in each well was tested. Increasing amounts of concentrated washing 3 flow through from protocol #9.2 was added. 5 μL sample buffer and 2 μL reducing agent was added per well. From this experiment we chose to continue with 35 μL sample per well.

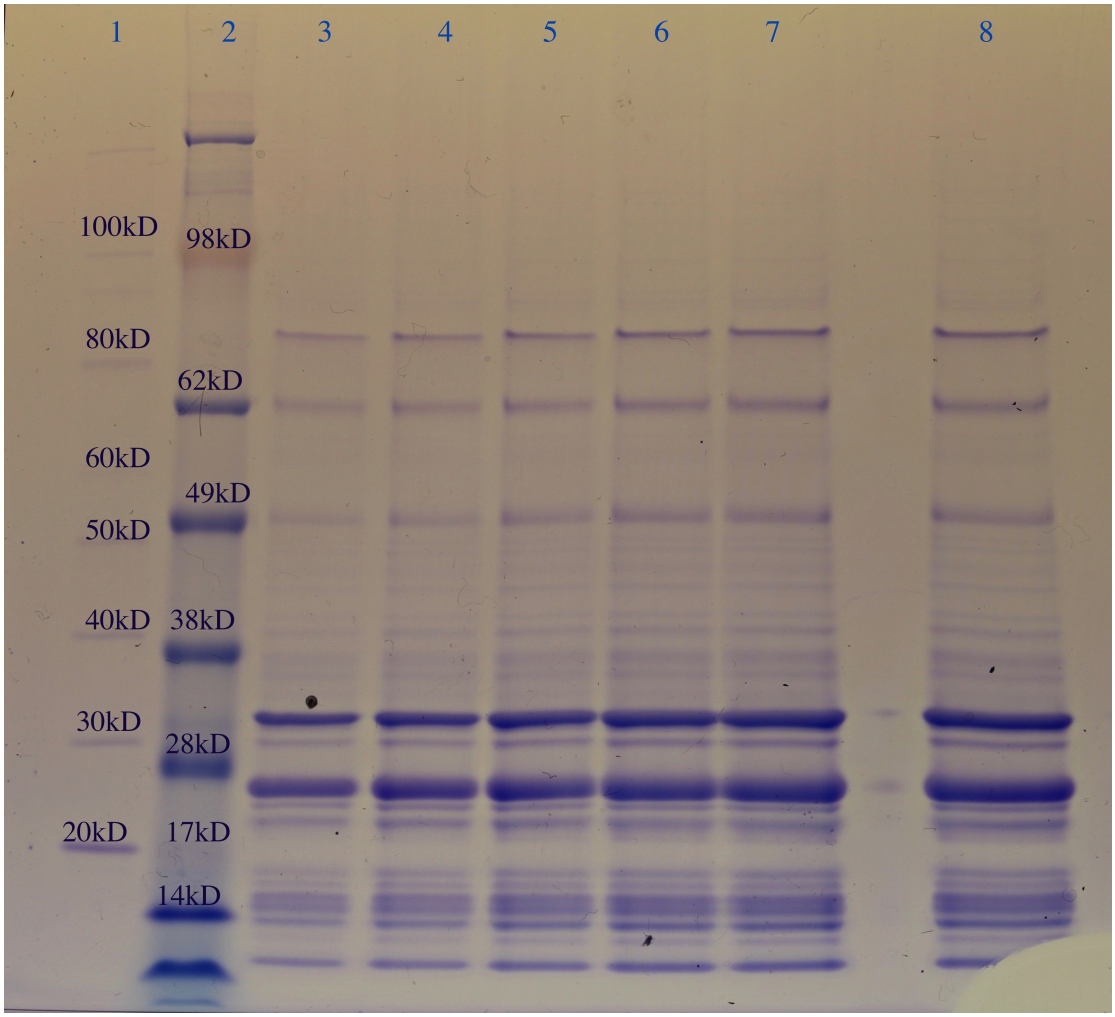


Figure 21: SDS-PAGE with wells containing increasing amount of washing 3 flow through aliqotes from protocol #9.2. Each well was added 5 µL more sample than the latter. Lane 1: un-stained ladder. Lane 2: pre-stained ladder. Lane 3: 10 µL sample in a total of 20 µL. Lane 4: 13 µL sample in a total of 20 µL. Lane 5: 18 µL sample in a total of 25 µL. Lane 6: 23 µL sample in a total of 30 µL. Lane 7: 28 µL sapmle in a total of 35 µL. Lane 8: 33 µL sample in a total of 40 µL.

3.4 Quantification of protein

Before immunization of the rabbits for production of polyclonal antibodies against the LdAAP3 recombinant protein, we had to quantify the protein. After running SDS-PAGE and Western blots it was decided to use concentrated aliquot washing 3 from protocol #9,2 because it had relatively few bands on the gel and contained the LdAAP3 recombinant protein confirmed by His-tag binding on the Western blot (Figure 24). Dilutions of the concentrated washing 3 solution were added on a SDS-PAGE to find a dilution with the optimal amount of proteins. Calculations were done for target LdAAP3 recombinant proteins found in bands at around 30-32kDa, called N1 and around 26kDa, called N2.

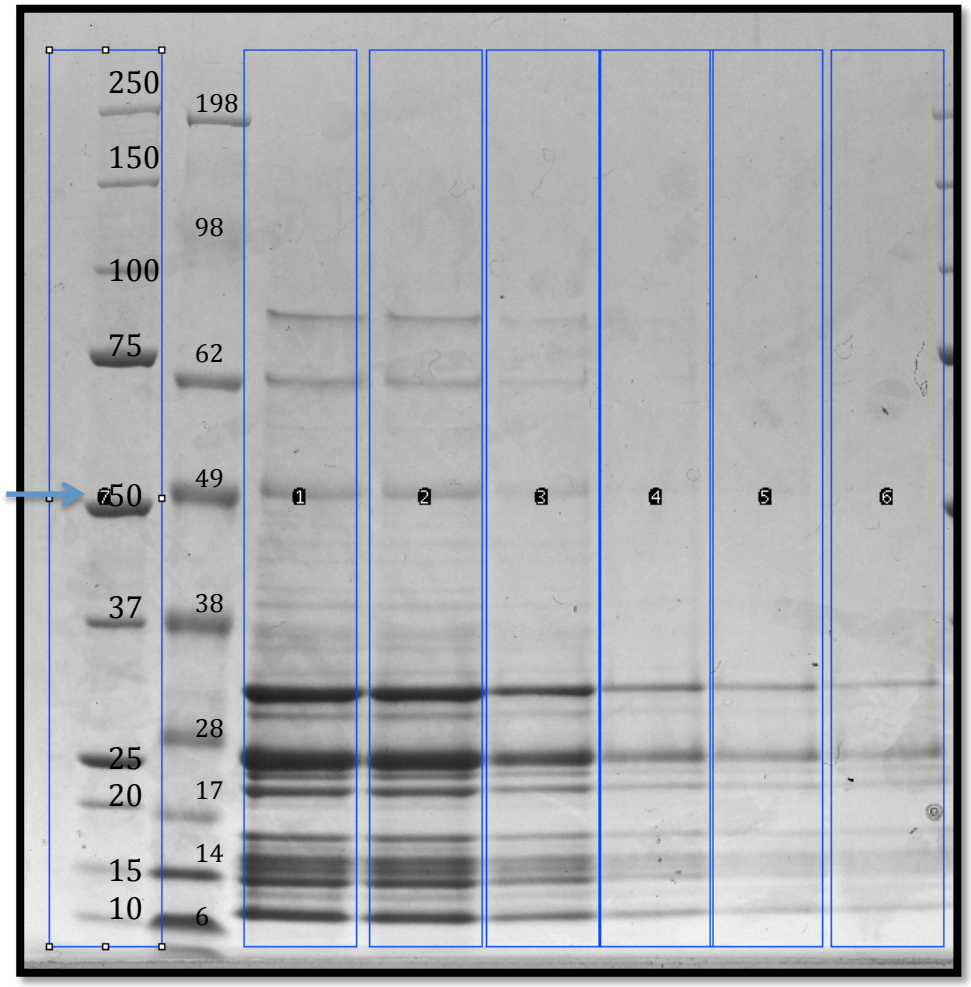


Figure 22: *SDS-PAGE with two different ladders and decreasing amounts of concentrated washing 3 samples containing LdAAP3 recombinant protein. Rectangles are drawn for quantification using ImageJ. Dilutions in the following lanes from 1-6 was: 1:1.5, 1:2, 1:5, 1:10, 1:20, 1:30 respectively, in a total amount of 35* µL. *7* µL ladder was added.

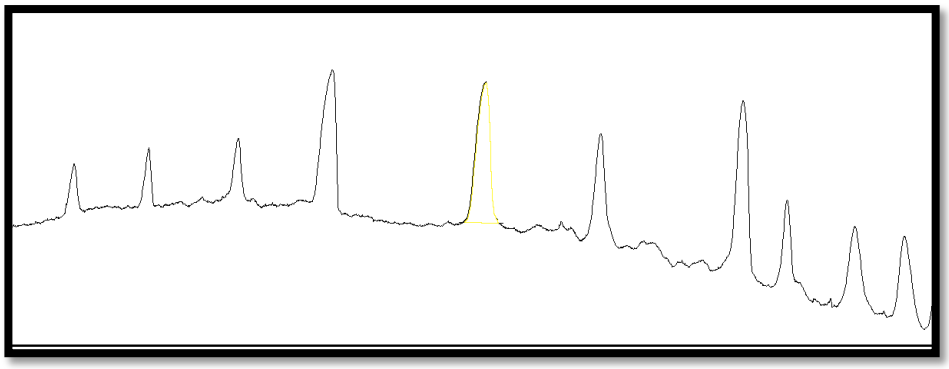


Figure 23: *Shows the profile plot of the marked ladder(#7) from Figure 21. The yellow peak indicates the fifth band on the ladder, the peak of interest, which had a size of 50kDa and known amount of 750ng.*

Because the sample is not completely purified, the gel consists of many bands. We had to choose the band of interest by counting the peaks against the bands from the gel, and controlling by comparing the peak intensity with the band intensities as best possible. Each

peak on the profile plot from Figure 23 represents one band in one marked rectangle. We have seven marked rectangles. A higher peak means darker bands, and wider peaks gives bands with bigger size range on the gel.

In each rectangle we are only interested in the bands at size around 32 kDa and 26 kDa. For each profile plot from a single rectangle the two peaks representing these two bands were marked, and showed two areas/arbitrary values. When marking the peak from the profile plot it turned yellow and its arbitrary values came up in the Results Window. These values are used to calculate the amount of protein (equations found in methods section 2.5.1).

Table 6: *Values of peaks chosen for N1 bands and their calculations. Arbitrary number obtained from ImageJ using Figure 22.*

Name: N1	**Area/arbitrary number**	**Amount protein in ng**	**Amount protein in μg per 500μl**
1	7594,50	430,75	9,23
2	7346,50	416,69	11,91
3	4732,11	268,40	19,17
4	2311,34	131,10	18,73
5	1294,21	73,41	20,97
6	773,26	43,86	18,74
7 standard/ladder	3778,234	-	-

Table 7*: Values of peaks chosen for N2 bands and their calculations. Arbitrary number obtained from ImageJ using Figure 22.*

Name: N2	**Area/arbitrary number**	**Amount protein to ng**	**Amount protein in μg per 500μl**
1	12779,35	724,84	15,53
2	12321,57	698,87	19,97
3	8316,67	471,72	33,69
4	4286,55	243,13	34,73
5	3187,38	180,79	51,65
6	2002,01	113,55	48,53
7 Ladder (50 kDa)	3778,234	-	-

3.5 Raising anti-LdAAP3 polyclonal antibodies

For an optimal immunization regime BioGenes GmbH (Germany) recommended to prepare purified LdAAP3 protein in a concentration of 100-200μg/500μL. Due to low protein concentrations obtained of the LdAAP3 recombinant protein, it was decided to use the highest dilution (1:1.5). 23,33μL solution of purified LdAAP3 recombinant protein from washing 3 aliquots obtained from protocol #9.2 was loaded per well in a total of 35μL with buffer added. The gel bands at size 26 kDa (N1) and 30-32 kDa (N2) were cut out of the gel, and were sent to BioGenes GmbH in Germany for immunization in rabbits.

3.6 Protein blotting

3.6.1 Detecting the His-Tagged LdAAP3 recombinant proteins

Western blots were performed in an attempt to localize the His-tagged *E. coli* Lemo 21 LdAAP3 recombinant proteins in order to decide which aliquot to continue with. The decision was based on purity of the solution and whether it contained the His-tagged LdAAP3 recombinant proteins.

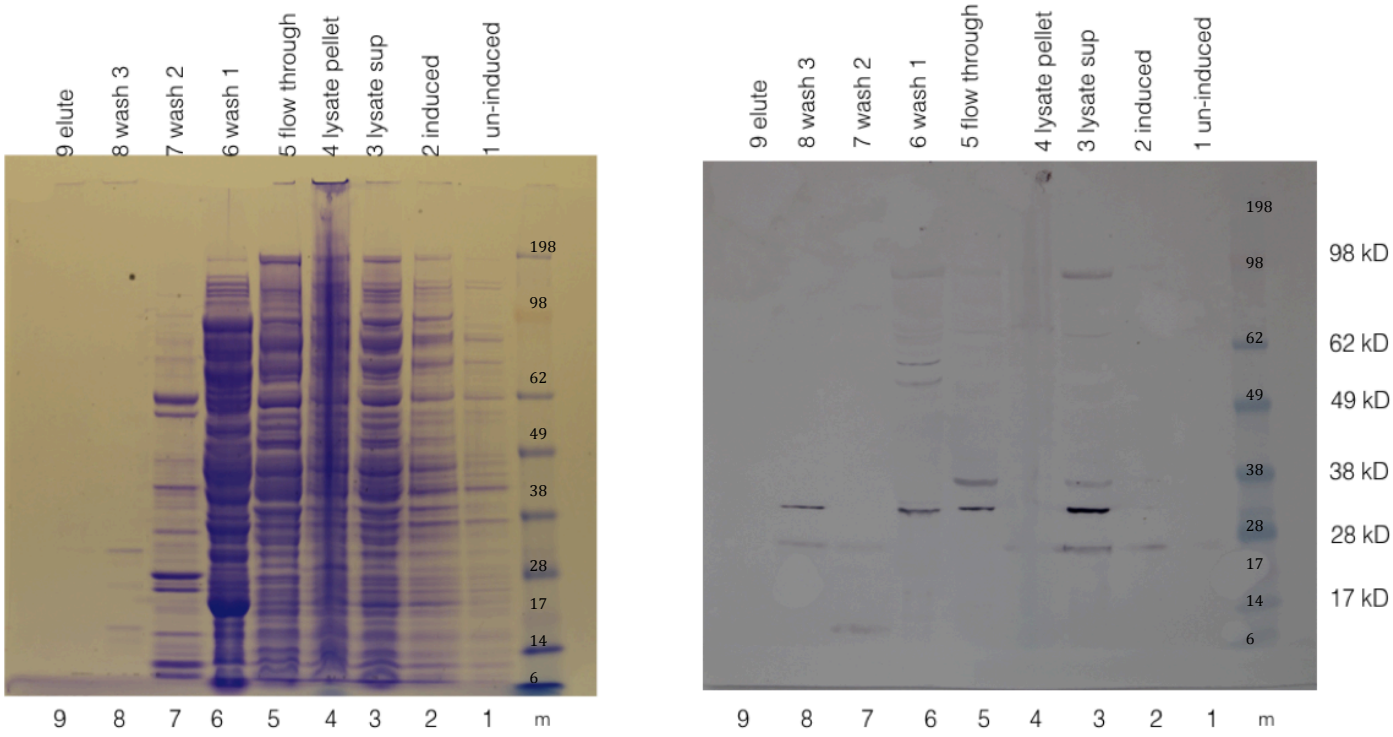


Figure 24: *The SDS-PAGE on the left and a Western blot to the right; following the protocol* ***#9,2*** *using 500 µM L-rhamnose, 23℃ overnight, 5 pellets from expression step resuspended in 15mL lysis buffer, 20min sonication, centrifuged at 20817 x g, and purification using 1HisTrap.*

Figure 24 shows a Coomassie stained SDS-PAGE compared with a Western blot where the same aliquots were applied, which enables localization of the LdAAP3 proteins containing the His-tag. His-tags cut off from the protein may result in the LdAAP3 recombinant protein not showing on the nitrocellulose membrane.

We had difficulty revealing the whole ORF protein (56 kDa) with the Western blot. Proteins at the size of 56 kDa could be found weakly in aliquot number 6 (Wash 1) on the Western blot from Figure 24. Stronger bands of smaller fragments at the size 38 kDa and above and below 28 kDa are repeatedly seen. Because aliquot number 6 (wash 1) contained a lot of other proteins, we decided to use aliquot number 8 (wash 3) with fragments at the size of about 32 kDa and 26 kDa for polyclonal antibody production.

3.6.2 Blotting LdAAP3 recombinant protein on nitrocellulose membrane

Production of more LdAAP3 recombinant protein was made following protocol #12 and using the Ni-NTA Superflow column. The nitrocellulose strips were blotted with an equal mix (50:50) of lysated sample from NPI-20 flow through and NPI-250 flow through. A 1:2000 ratio of AP conjugated HisG Epitope Tag primary antibody, (mouse/IgG2a, Thermo Scientific, USA), was used to visualize the His-Tagged proteins.

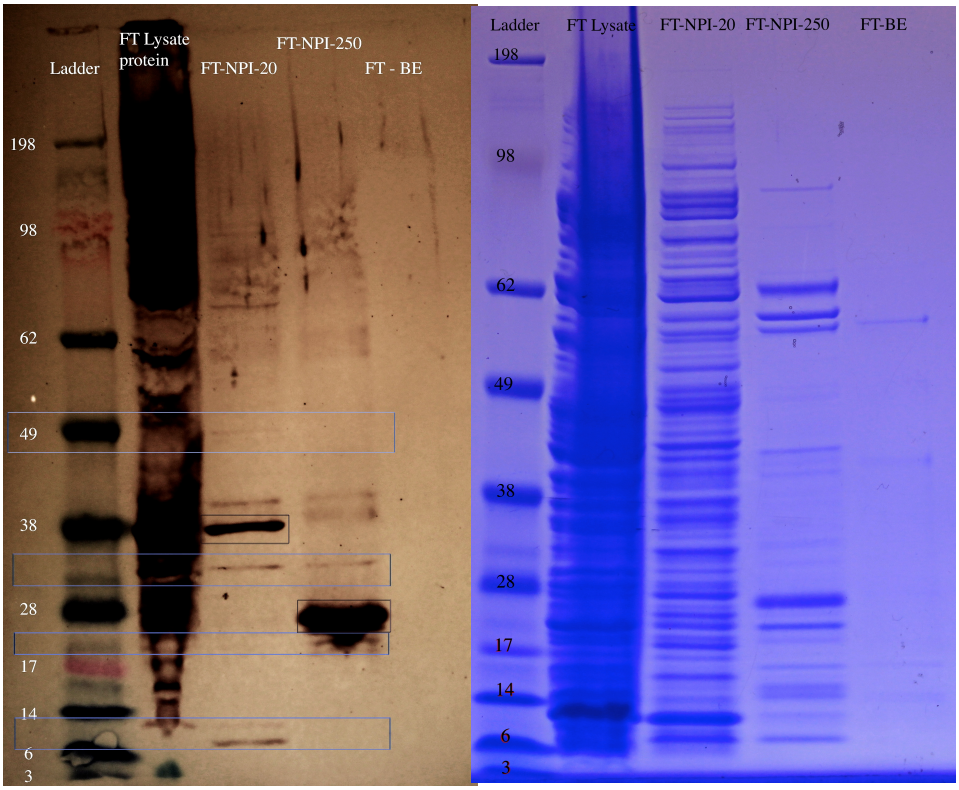


Figure 25: *Western blot (left) and SDS-PAGE (right) from protocol #12, using 500 µM L-rhamnose, 24°C overnight, 20 pellets from expression step resuspended in 34mL NPI-10 buffer, 4 min sonication, centrifuged at 10007 x g, and purification with Ni-NTA column. 20 µl of each sample was loaded per well.*

The new column and buffers used seemed to be more successful in purifying the ORF LdAAP3 recombinant protein. When looking at the aliquot containing FT-NPI-20 and FT-NPI-250 on the SDS-PAGE to the right, bands at the size of about 56 kDa can be seen (Figure 25). The FT-NPI-250 was relatively purified, indicating a successful production of the recombinant protein found at a band at about 56 kDa that most likely is the whole ORF LdAAP3 recombinant protein, in a relatively purified state. These bands were not verified by AP conjugated antibody binding to the His-tag and are not seen on the Western blot (to the left). Several bands on the Western blot can be seen. Strong bands at the size of about 38 kDa and 28 kDa is also seen here.

3.6.3 Testing sera from Leishmania infected and non-infected individuals

Using the nitrocellulose membrane strips blotted with the LdAAP3 recombinant protein from the lysated sample of the flow through NPI-20 and NPI-250, sera from different types of *Leishmania* infections and different hosts were tested. This was done to see if antibody produced from infected subjects could bind to the LdAAP3 recombinant protein that was blotted on the nitrocellulose membrane.

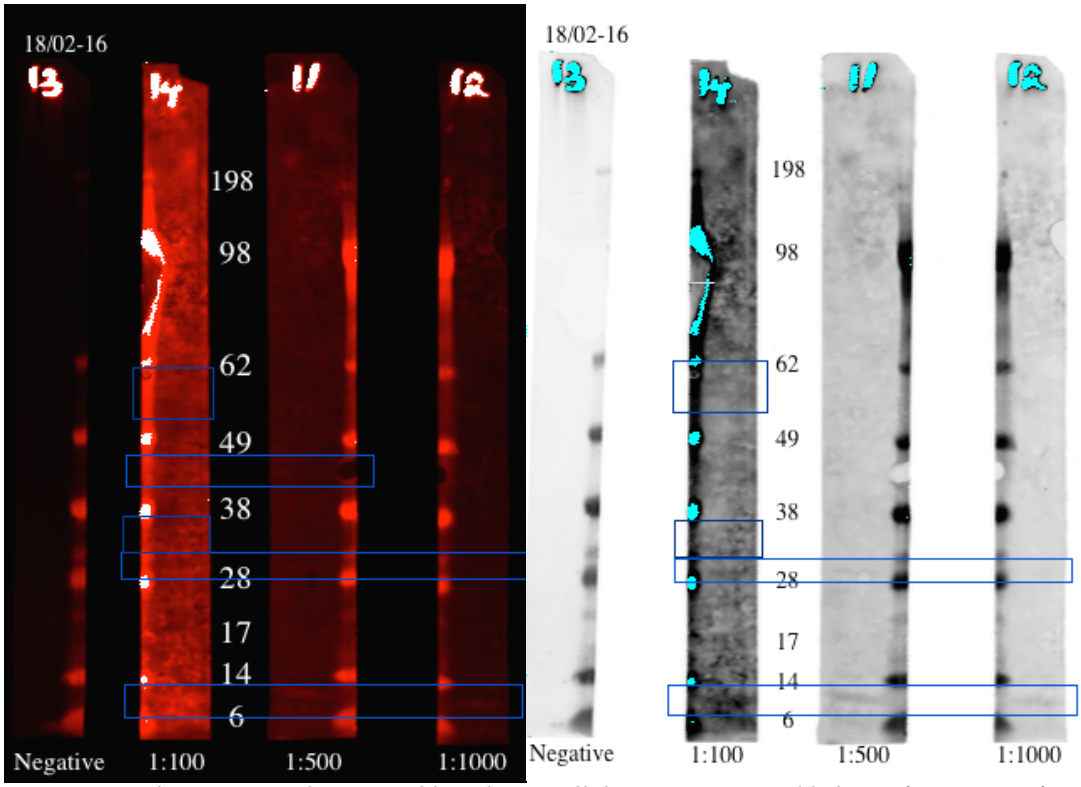


Figure 26 : *The NPI-20 and NPI-250 blotted nitrocellulose strips were added sera from mice infected with L.amazonesis and incubated overnight, Donkey anti-mouse was used as the secondary antibody, Odyssey blocking buffer in a total of 2 mL was used. Strips were washed three times with PBST. Marked boxes indicate bands occurring at the size of 56 kDa, 45 kDa, 32 kDa, 30 kDa and 12 kDa.*

1:100, 1:500 and 1:1000 dilutions of sera in 2 ml blocking buffer were tested in this protocol. No serum was applied to strips number 13, and was the negative control against the contents of the buffers in the protocol. Strip 13 (negative control) showed no bands or background. Dilution 1:100 showed many bands, but with a lot of background. Strips 11 and 12 displayed less background, but weak bands.

Bands can be found at around 56 kDa, 45 kDa, 32 kDa, 28 kDa, and around 12 kDa. These band sizes can be found on the Western blot and SDS-PAGE from Figure 25, and indicate that the proteins produced from LdAAP3 recombinant expression seem to have epitopes that match the antibodies generated in *Leishmania amazonensis* infected mice.

These strips were incubated with sera overnight in 4°C. Overnight incubation gives time for albumin, other proteins and antibody with little affinity, to bind to the strips. Shorter incubation and more washing steps may help decrease these unspecific bindings, and were attempted.

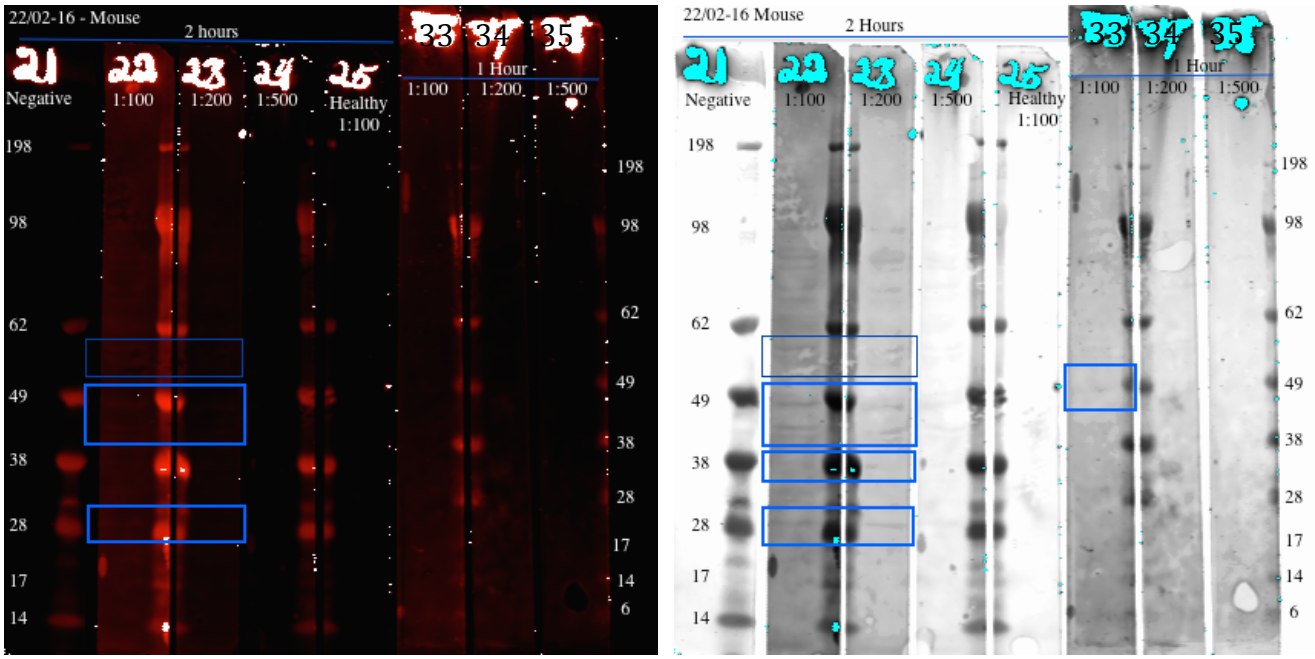
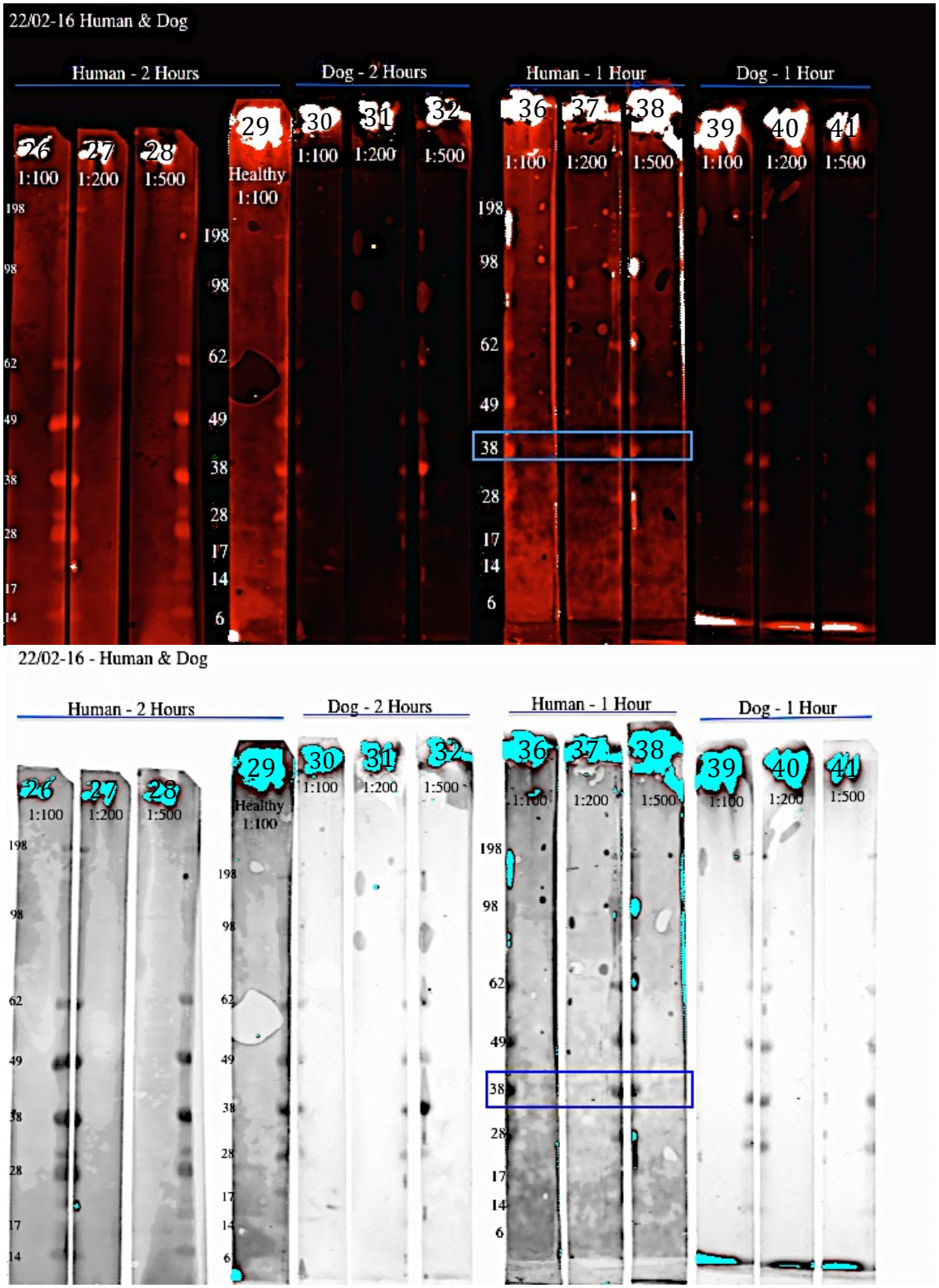


Figure 27: *The strips were added sera from mice infected with L.amazonesis and healthy mice (BALB/c, 40 days, male) as negative controls and incubated for 1 and 2 hs. Donkey anti-mouse was used as the secondary antibody, Blocking buffer with 0.1% Nonfat-dry milk was added in a total of 3 mL. Strips were washed four times with PBST and once with PBS. Strip 21(negative) was not added sera. Trip 25 was added sera from healthy mouse in a dilution of 1:100. Strips 22-24 from the 2h incubation contained dilutions of 1:100, 1:200 and 1:500 respectively. Strips from 33-35 from the 1h incubation contained dilutions of 1:100, 1:200 and 1:500 respectively.*

1h incubations seems to be too little due to almost no bands occurring on the strips. A weak band could be seen in the black and white image at around 49 kDa on strips number 33. For the strips incubated for 2 hs the bands show at sizes around 56 kDa, 49 kDa, 38 kDa and 30

kDa. Dilution 1:200 and incubation for 2h seem to obtain nice bands with less background. Negative and healthy controls did not show any bands, which may indicate that the bands showing on the strips incubated with infected sera can be due to the *Leishmania* infection. Antibodies generated from infection seem to match the proteins blotted on the nitrocellulose membrane.



22/02-16 - Human & Dog
Human - 2 Hours
Dog - 2 Hours
Human - 1 Hour
Dog - 1 Hour

Figure 28: *These strips were incubated with human sera infected with L.infantum chagasi and dog sera infected with L. donovani, and incubated for 1 and 2 hs. Goat anti-human IgG was used as the secondary antibody for the strips incubated with human sera. Blocking buffer with 0.1% Nonfat-dry milk in 3mL solutions was used. The strips were washed four times with PBST and once with PBS. For the 2h incubation, strip 29 was incubated with sera from healthy human in a dilution of 1:100. Strips 26-28 contained dilutions of 1:100, 1:200 and 1:500 respectively. For the 1h incubation with human sera, strips 36-38 contained dilutions of 1:100, 1:200 and 1:500 respectively.*

3 different incubations were done for samples added with human sera. The first incubation was with sera from a patient infected with *L. infantum*. The second incubation was with goat anti-human IgG peroxidase, which binds to the primary antibody from infected human sera. The third incubation was with anti-goat IRD 680 (Odyssey-Licore), which binds to the secondary antibody produced in and is revealed in the Odyssey scanner.

The bands from the 1h incubation with human sera were better than the 2h incubation and showed slightly weak bands around 38 kDa for strip 36 and 37. Wrong secondary antibodies were used for the dog samples and are the reason for the empty bands.

Several attempts were done for human sera. In these attempts (image not shown) the strip incubated for 1h with 1:100 dilution of sera from healthy human showed bands around 62 kDa and again below 28 kDa. Sera from infected human diluted in 1:200 showed bands in 17, 14 and a band in the middle of 62 and 49 kDa.

3.6.4 Dot-Blot

This protocol was done to get an indication of what dilutions to use when testing the sera from LdAAP3 recombinant protein immunized rabbit against the LdAAP3 Lemo21 produced recombinant protein.

Figure 29: *Shows dots containing sera produce from rabbit before and after injection of the LdAAP3 recombinant protein, and the Lemo 21 produced LdAAP3 recombinant protein. AP-conjugated secondary antibody was added for detection. Beneath the dots are marked controls containing Milli-Q water. LdAAP3 produced recombinant protein bind to the sera from rabbit produced from the LdAAP3protein, which again will bind to the AP conjugated secondary antibody. Dilutions: 1 – 1:500, 2 – 1:1000, 3 – 1:2000, 4 – 1:4000, 5 Pre-immune 1:1000.*

Strips number five containing sera from rabbit before injection of the LdAAP3 protein show a lighter band indicating that there probably is no antibody produced in this sera that is able to bind to the secondary antibody conjugated with alkaline phosphatase. A colorimetric detection is seen in purple when the LdAAP3 antibody from rabbit, bound to the LdAAP3 protein, is added BCIP/NBT. The dilutions on the other hand were not as easy to part from each other although strip number 4 is slightly lighter than the rest. But it is clear that the rabbit immunized with LdAAP3 recombinant protein produces antibody that binds to the LdAAP3 recombinant protein.

3.6.5 Testing anti-LdAAP3 polyclonal antibodies against E. coli produced LdAAP3 proteins

Nitrocellulose membrane blotted with LdAAP3 recombinant purified solution from the Ni-NTA lysis and purification step was used and incubated with sera from rabbit immunized with recombinant LdAAP3 protein. Rabbit number 71 and 72 were injected with proteins at the size of around 32kDa, called N1. Rabbit number 73 and 74 were injected with proteins at the size of about 26 kDa, called N2. AP conjugated HisTag secondary antibody was used for visualization of the His-tag.

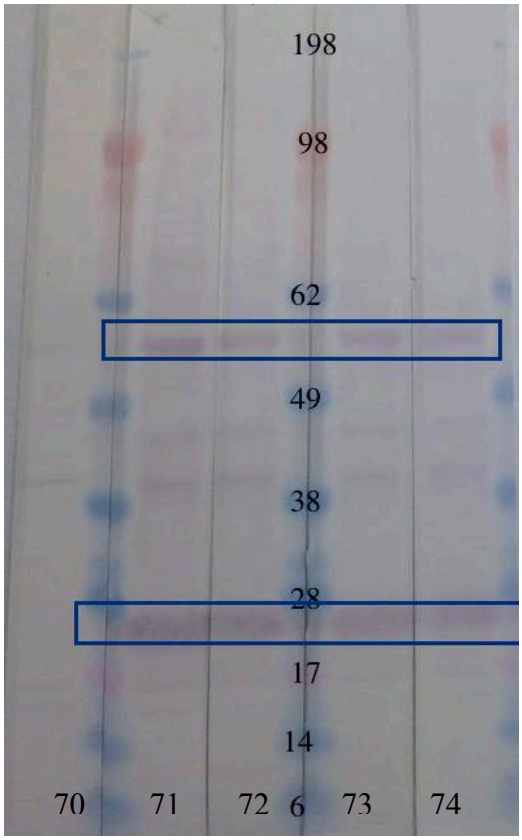


Figure 30: *LdAAP3 blotted nitrocellulose strips were incubated with sera from rabbit number 73 of N2 in different dilutions. Sera incubated on strip 70 was obtained before immunization with LdAAP3 recombinant protein and functioned as a control. Dilution ratios: 70 – 1:1000, 71 – 1:2000, 72 – 1:4000, 73 – 1:5000, 74 – 1: 6000.*

When comparing the ratio used in the Dot-blot and sera obtained from rabbit 73, it was decided to continue with 1:4000 ratio when testing the sera from all four rabbits.

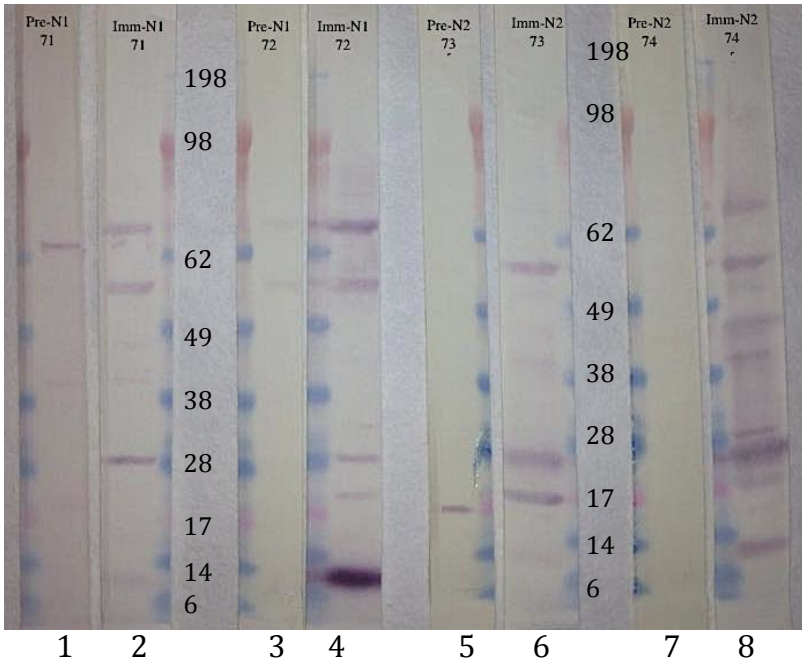


Figure 31: *LdAAP3 blotted nitrocellulose strips incubated with sera from pre-immune and immunized rabbit number 70, 71, 72, 73 and 74, in a dilution of 1:4000 of sera and blocking buffer (TBS + 5% BSA).*

Detection of antibodies are produced by colorimetric detection when adding BCIP/NBT to the AP conjugated secondary anti-rabbit antibody that could bind to the primary antibody from the sera of the LdAAP3 injected rabbit, which again could bind to the recombinant LdAAP3 protein blotted on the nitrocellulose membrane.

Clear bands can be seen at around 56 kDa and around 26kDa (Figure 30). On Figure 31, clear and strong bands also occur at around 56 kDa along with other strong bands at the size of about 32 kDa and 26 kDa. Rabbits immunized with N1 (26 kDa) and N2 (32 kDa) proteins seem to detect bands at bigger and smaller band sizes than the size of the protein they were immunized with. Several bands can also be found above 56 kDa which may be due to conjugation. Strip number 8 (immunized N2 rabbit number 74) had many bands, and may indicate a strong immune response. Pre-immune strips from rabbit 71, 72 and 73 showed some bands, which may be due to *E. coli* proteins.

3.6.6 Testing SLA and recombinant LdAAP3 against anti-LdAAP3 antibody using ELISA

Purified recombinant protein and soluble *Leishmania amazonensis* antigen was tested, and visualized by goat anti-rabbit IgG-Horseradish peroxidase (GE Healthcare, UK) secondary antibody in a ratio of 1:2000.

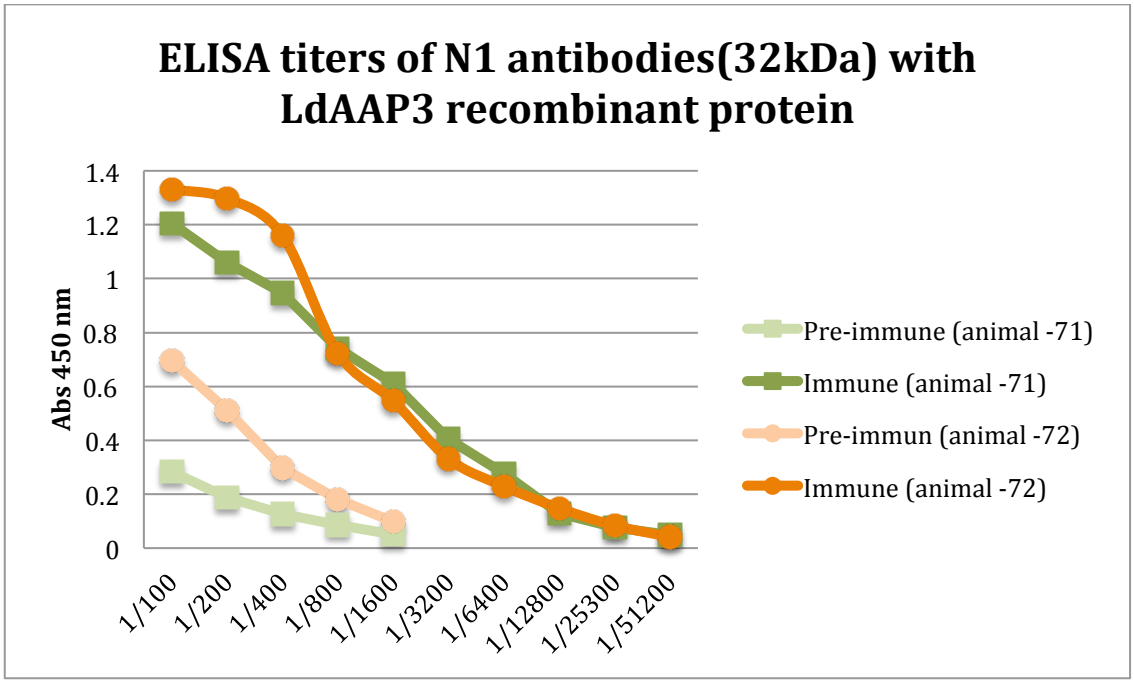


***Figure 32**: Wells were coated with purified recombinant LdAAP3 protein before anti-LdAAP3 polyclonal antibodies were added as primary antibody, and goat anti-mouse Horseradish peroxidase (GE Healthcare, UK) was used as secondary antibody to be able to measure the absorbance.*

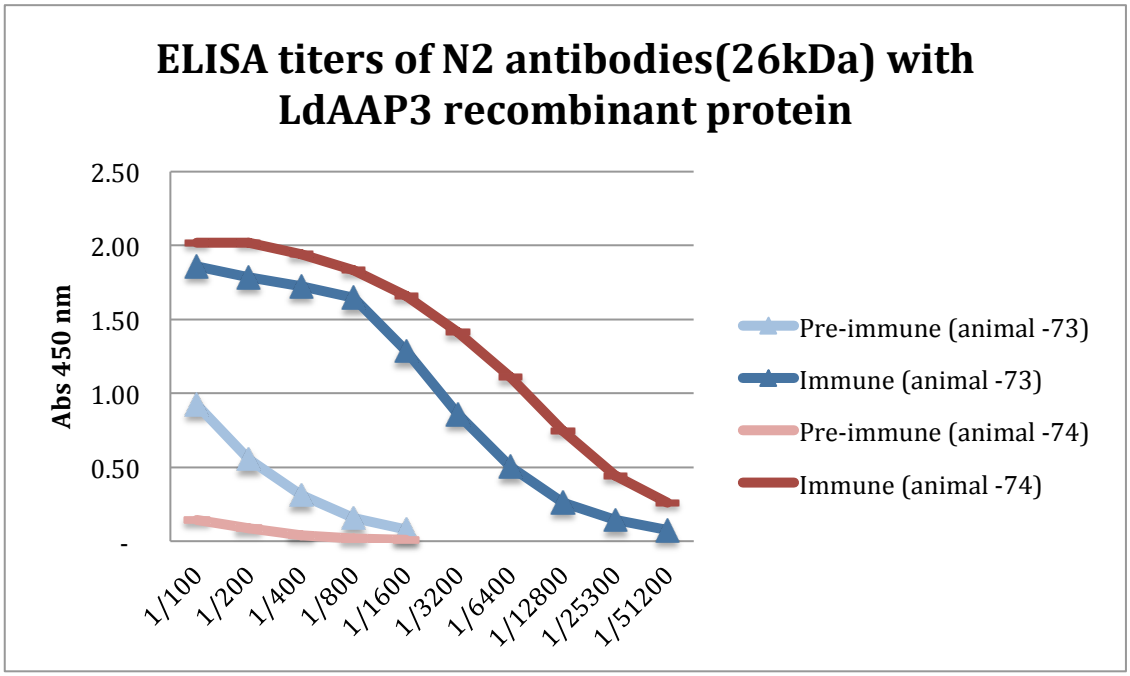


***Figure 33:** Wells were coated with purified recombinant LdAAP3 protein before anti-LdAAP3 polyclonal N2 antibodies were added as primary antigen, and Horseradish peroxidase (GE Healthcare, UK) was used as secondary antigen to be able to measure the absorbance.*

Looking at the graphs showing the N1 and N2 titers when using purified recombinant LdAAP3 protein as the antigen, a clear difference can be seen between the pre-immune and immune sera. This indicates that the animals have produced antibodies against the recombinant LdAAP3 recombinant protein, and that there is some specificity for the antigen.

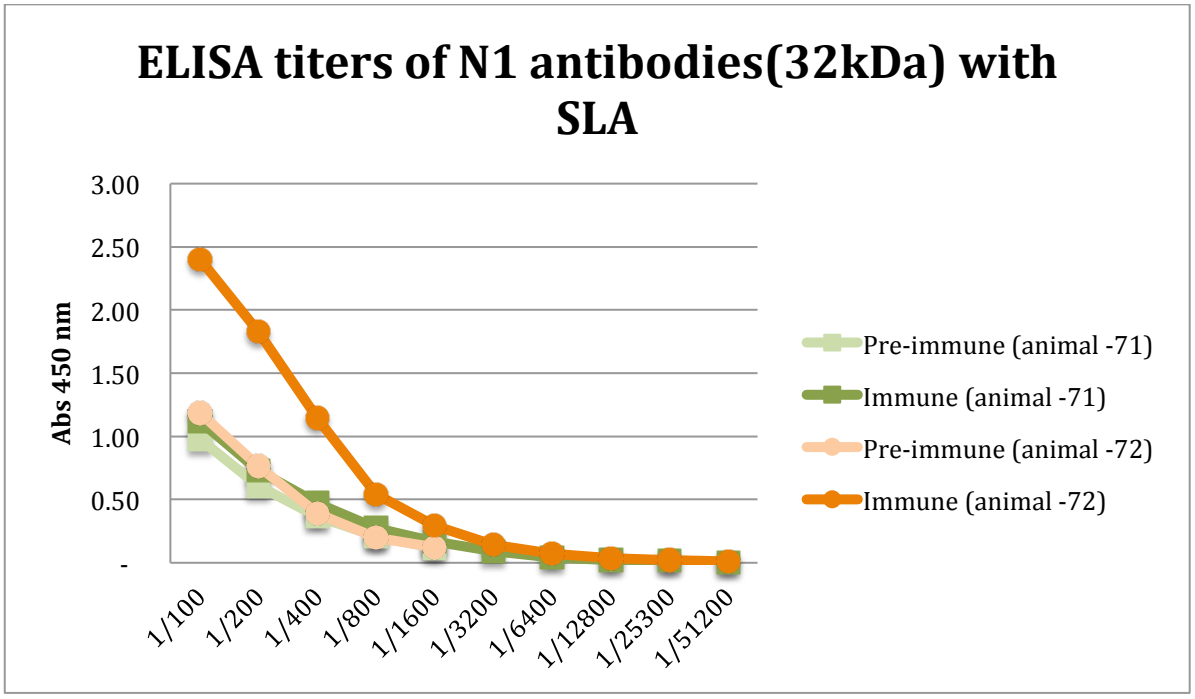


Figure 34: *Wells were coated with soluble L. Amazonensis antigen (SLA) before anti-LdAAP3 polyclonal N1 antibodies were added as primary antigen, and Horseradish peroxidase (GE Healthcare, UK) was used as secondary antigen to be able to measure the absorbance.*

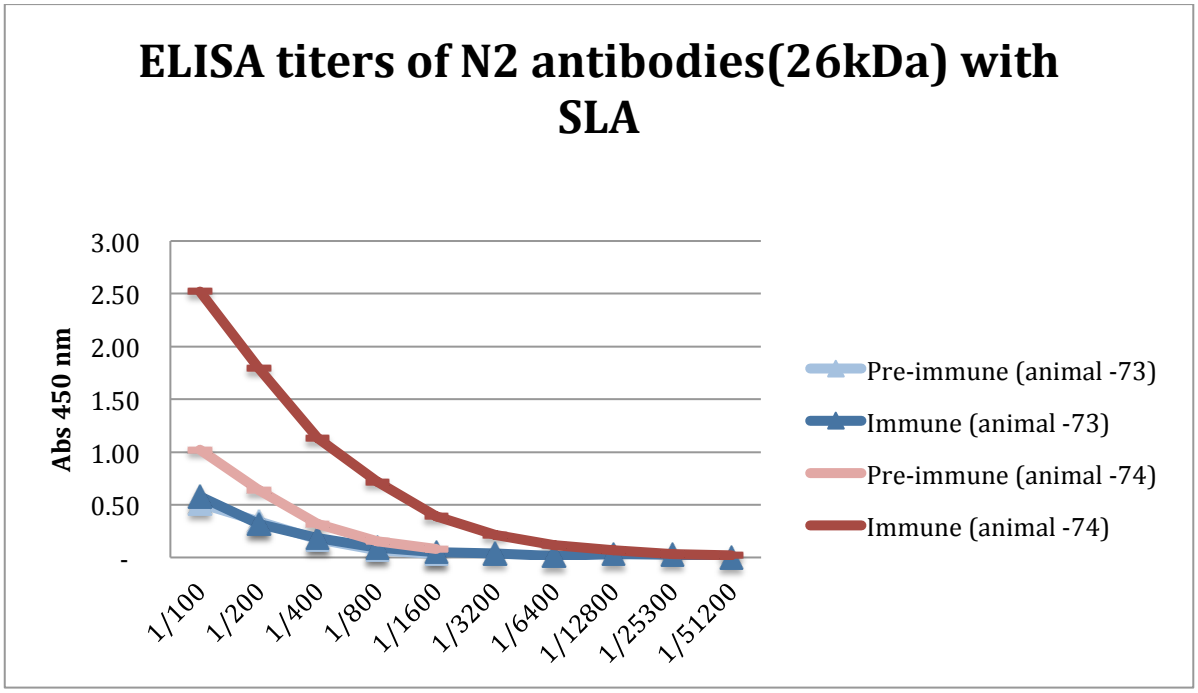


Figure 35: *Wells were coated with soluble Leishmania amazonensis antigen (SLA) before anti-LdAAP3 polyclonal N2 antibodies were added as primary antigen, and Horseradish peroxidase (GE Healthcare, UK) was used as secondary antigen to be able to measure the absorbance.*

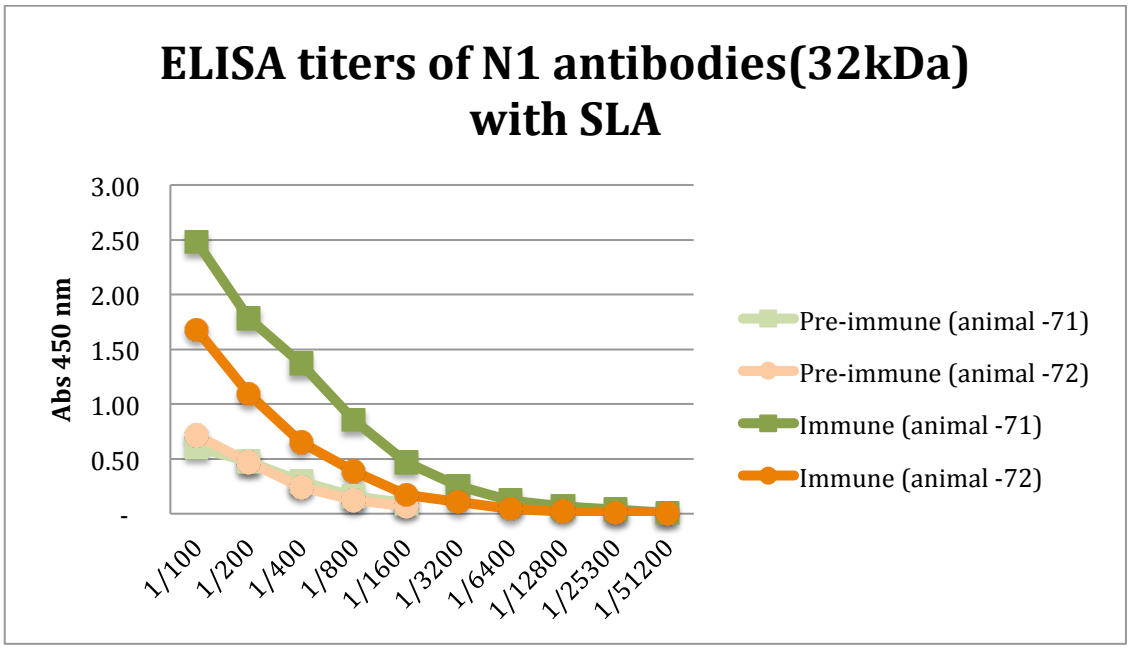


Figure 36: *Wells were coated with soluble L. Amazonensis antigen (SLA) before anti-LdAAP3 polyclonal N1 antibodies were added as primary antigen, and Horseradish peroxidase (GE Healthcare, UK) was used as secondary antigen to be able to measure the absorbance.*

When testing N1 and N2 serum against soluble *Leishmania amazonensis* antigen the results show that antigen in the serum had low affinity for *L. amazonensis*. For N1, only animal 72 produced antibodies. For N2, only animal 74 produced antibodies. In general, sera from N1 animals showed increased titers compared with the pre-immune animals. Animal 71 had higher titers than animal 72. Titers for N2 animals showed high affinity for animal 74, but low affinity for animal 73.

4. DISCUSSION

4.1 Amplification of the LdAAP3 ORF

Overexpressing membrane proteins can be challenging as each protein behaves in an individual and unpredictable way. Though choosing a specific host and plasmid with the desired qualities can give a positive advantage, it is difficult to foresee the amount of protein production or whether the recombinant protein will be soluble, or maintain its proper conformation by correctly folding (84). In earlier studies, overexpression of 300 membrane proteins were conducted in *E. coli*, but did not show any correlation between protein specific parameters, i.e. size, hydrophobicity or number of transmembrane helices, and the expressability. Generally, smaller proteins tend to be better expressed than larger proteins (85).

Because the whole LdAAP3 protein consists of 11 transmembrane domains (37), and is assumed to be toxic for the *E. coli,* we chose to use competent *E. coli* Lemo21(DE3) for transformation and expression for the Cter LdAAP3 protein and the ORF LdAAP3 protein, with 2 and 11 transmembrane domains respectively.

Although the Lemo21 strain is a host more suited for toxic proteins, it utilizes lac promoters, which are known to be leaky (46). This means that the transcriptional control of the operon is not completely effective and there can be some basal transcription without the addition of inducer because the promoter is never completely off (86). This can be observed in Figure 18, where expression of proteins at a size of 38 kDa occurs in the un-induced aliquots. This leak could lead to incomplete repression of the protein expression, which could further lead to plasmid instability or loss. Because of this, the culture could be overgrown by *E. coli* that no longer render the plasmid, or not grow at all. Titrating the amount L-rhamnose added to an optimal level of T7 lysozyme production to inhibit enough of the T7RNAP is therefore essential when producing toxic proteins. Using a more tightly regulated promoter or adding 1% glucose to suppress the induction of lac promoter in the presence of lactose could help eliminate these problems and give a better regulation of expression (46, 54, 87, 88). Fine tuning expression kinetics can be done by lowering the amount IPTG added (89), and shortening induction periods (85).

4.2 Expressing the LdAAP3 ORF recombinant protein with 11 transmembrane domains

E. coli is a much used host for expression of recombinant proteins. The main reason for this is its well-known genetics, transformation efficiency, low cost and cultivation simplicity (53). The gene of interest is cloned in an expression vector, transformed into a host where production of the gene of interest is induced, the host cell is then lysated to free the translated gene, which then can be purified. Although the theory is simple, expressing a pure unfragmented recombinant protein is complicated. The number of choices ranging from which vector and host to use to methods of purification and different concentrations to use, are immense and finding the perfect combination at once is difficult and therefore often requires a great deal of trial and error (84). Incorrect translations, different protein folding, degradation, and premature termination may give us different variants of our target protein (64). Possible explanations to the challenge we meet during expression are explained in the following.

4.2.1 Toxic genes

To productively utilize the host for expression, knowing the genetics of the bacterium is an important factor, especially when expressing toxic proteins. In the very beginning of this project expression of the ORF was attempted using *E. coli* Bl21(DE3) Star without success. When analyzing the nucleotide sequence of the *L. donovani* AAP3 protein, we saw that it contained 11 transmembrane domains (Figure 15). We concluded that it might be a toxic protein for the expression vector and therefore chose *E. coli* Lemo21 as the host. To find out whether the recombinant protein is toxic for the *E. coli,* we could have monitored and compared the cell growth of the recombinant strain and an empty-vector.

4.2.2 Plasmid stability

A central problem when expressing highly toxic genes is the instability of the plasmid during the growth phase. Cells without the pET151/D-TOPO plasmid inserted can rapidly take over the culture because of their physiological advantages (49). This can explain the low production of our LdAAP3 recombinant protein leading to challenges during purification. Adding expression of genes that could lead to cell death in plasmid-free cells could aid the survival of the cells containing plasmid. Harboring the lysozyme gene may sometimes not be enough to prevent toxicity, and the plasmid carrying the toxic gene cannot be maintained in the host. This can be avoided by growing *E. coli* without the T7 gene, to then induce the host in mid-log growth face by delivering the T7 by infection (53).

Another attempt to avoid this toxicity is to rout the expression of the recombinant protein to the extracytoplasmic space by fusing a leader peptide to the protein(84). Adding a lac promoter downstream of the cloned gene could lead to less expression of the toxic protein due to less expression of T7RNAP. Introducing more T7 promoters in the host plasmid would lead to competition with the T7 promoter in front of the toxic gene, which could lead to decreased basal expression.

4.2.3 Copy number of plasmid

Manipulating the plasmid-copy number before induction can be critical for *E. coli* survival, and should have been done for our experiments. Lowering the plasmid number before induction may enhance *E. coli* survival prior to the expression. Regulating the expression of genes controlling expression of vector can do this. Increasing the plasmid-copy number by thermal control, or using T7-based expression vectors that increases their plasmid copy number when arabinose is added may increase the expression of the recombinant protein. (53). The best expression system for toxic proteins would be completely repressed until induction, with a titreable protein production (46).

4.2.4 Resistance markers

In our case the ampicillin and chloramphenicol resistance markers are sitting on two different plasmids, containing genes for the T7 promoter and T7 lysozyme respectively (Figure 11).

Chloramphenicol is important to maintain a selective pressure to keep the plasmid coding the T7lysozyme inside the Lemo21 *E. coli* (49). The resistance mechanism of *E. coli* to ampicillin and chloramphenicol, though not completely the same, is to enzymatically degrade the antibiotic. After a couple of hs, the antibiotics can be depleted and growth of *E. coli* without these markers could occur (84). Ampicillin concentration at 100μg/mL is often used, as done for our experiments. Too high levels of antibiotics can lead to less production of the recombinant protein due to energy being focused on getting rid of the antibiotics (53). Chloramphenicol binds reversibly to the ribosome and obstructs the protein synthesis in the *E. coli*. Using chloramphenicol as a resistance marker may thus affect the production of recombinant protein. To test for plasmid stability and how well the resistance markers have worked, it could have been wise to do a plasmid stability test. This could give us a better indication on whether the bacteria we use for expression actually contains the plasmids of interest. When picking colonies on the agar plate we made sure to get small and isolated colonies. Colonies with plasmids where mutations have occurred, tend to form bigger

colonies even though IPTG is added. This can be explained by the excess energy for growth when recombinant protein is not expressed. Small colonies on the other hand, often contain plasmid, and do not grow into bigger colonies because energy is used for production when IPTG is added.

Using elevated levels of antibiotics or using tetracycline as a resistant marker, with efflux active resistance mechanism, may have increased the chances of maintaining the plasmid inside the host (87).

4.2.5 Host selection

For expression of soluble proteins larger than 10-50 kDa, yeast or baculovirus can be more preferable host compared to *E. coli* (45). Using an *E. coli* host can be particularly challenging due to its limited capacity for posttranslational modifications allowing proper folding of the protein (45). However, researchers have experienced modest success when using yeast or baculovirus as hosts (90).

Due to challenges during production of the ORF LdAAP3 in *E. coli,* it may have been better to use yeast with improved folding and posttranslational modifications that may allow better expression (45). Shaked-Mishan et al. (2006) along with other scientist groups has been successful in expressing LdAAP3 in *Saccharomyces cervisiae* yeast (35, 37).

4.2.6 Standardization of L-rhamnose concentration and incubation time after induction

Producing the ORF protein does not benefit the *E. coli* host, and its production will probably be harmful for the bacterial cells. When adding L-rhamnose the rhamnose promoter will be activated and promote synthesis of T7lysozyme, which is an inhibitor of T7RNAP(49). Less expression of T7RNAP can lead to less activation of the T7promoter that sits right in front of the ORF sequence. The result is less recombinant protein expressed, which can presumably lead to increased cell survival. Lowering the expression by changing the concentrations of L-rhamnose and lowering the temperatures could aid the challenge regarding the toxicity. During our experiments we used 0-2000 μM L-rhamnose.

When testing optimal L-rhamnose concentration and incubation time the un-induced (UI) bands were week, as expected (Figure 16). This could be explained by the lack of inducer IPTG, but we cannot exclude that this could be due to a shorter incubation time than the rest of the aliquots. The best samples to compare would be UI (2000μM) and induced aliquot number 5 (2000μM), incubated for 3 and 4 hs respectively. An increase in band

intensity for induced aliquot 5 can be seen in Figure 16, confirming the effects of the inducer. Further, the effect of T7 lysozyme could be seen in aliquots 1, 6 and 11 (Figure 16), which do not contain L-rhamnose. Band 1 and 6 show lower band intensities. This may indicate less expression without L-rhamnose, which can be explained by the toxicity of the protein. It could seem that after enough amount of time of incubation a limit in the expression is reached. This can be observed for the 24h incubated aliquots, where the bands seem to be equally strong despite the different amounts of L-rhamnose added (Figure 16). Because of this we decided to continue with induction for 16-18h. The band intensity seem to increase with higher levels of L-rhamnose and longer incubation time. During our experiments we chose to use 500μM and 1000 μM of L-rhamnose, without seeing any big difference in the expression of the recombinant LdAAP3 protein.

The same test could have been repeated with different temperatures to see the effects of temperature in different L-rhamnose concentrations. Although we did small scale-production, we could also have done micro-scale production. This would allow us to test a high number of culture conditions and save time while doing so. We concluded that adding IPTG induces the production of protein and that increasing the levels of lysozyme with L-rhamnose affects the expression in a positive way.

4.2.7 Temperature

Changes in temperature ranging from 20 to 30 ℃, were done to se if it could affect the production of the LdAAP3 recombinant protein. Lower protein concentrations, and slower rates of production, could improve transcription and translation and favor proper folding of the proteins (85, 89). It can decrease aggregation, giving us more of the protein in the supernatant (84). Lower temperatures during growth of *E. coli* generally give improved biological activity and higher yields of the recombinant protein (91, 92). Using too low temperature might reduce synthesis and result in to little protein yields (84), but lowering the temperature during expression has also shown to improve the solubility of the proteins (93). This occurs because hydrophobic interactions that form inclusion bodies are temperature dependent (93). Higher temperature means higher entropy and more inclusion body formation. These temperature changes may also affect L-rhamnose and therefore also affect how much the T7RNAP may be inhibited (49). Optimal expression temperature will vary and depend on the protein, amount L-rhamnose added, and incubation time.

Although we did just a few expressions with higher temperature than 24℃, when running an SDS-PAGE at 37℃ (images not shown), the bands seem to be slightly weaker. Experiments using a lot higher and a lot lower temperatures could have been conducted to compare the effects of temperature utterly.

It would have been interesting to add a GFP (green fluorescent protein) gene in the vector to be able to use flow cytometry to get a more reliable estimation of the LdAAP3 production (49). This would allow us to count the amount of cells that are able to express our protein, making it easier to analyze the results when changing temperature, incubation time and L-rhamnose concentration (49).

4.2.8 Lysis and Centrifugation

Though sonication normally does not break up primary structure of proteins, it may lead to unfolding and enhanced aggregation because of the heat generated and mechanical forces produced (94). Long sonication may result in more cells lysated generating more free organelles, nuclei and lipids in the solution. This crowded solution may affect the purification by sterically standing in the way for the His-tagged proteins to bind when performing IMAC, which may be an explanation to our problems during purification.

During centrifugation, cell organelles normally are found in the pellet, while soluble proteins remain in the supernatant. Knowing that the LdAAP3 protein is an 11 domain transmembrane protein, it is likely that this protein will aggregate and precipitate from aqueous solutions when centrifuged due the size and its hydrophobic regions that may be exposed when the protein is free from the cell membrane (95). Some of the protein may still be attached to some lipids from the cell membrane, which also may lead to accumulation and aggregation. In our experiments we found the LdAAP3 recombinant protein both in the supernatant and in the pellet. Whether our protein is in the pellet due to inclusion body aggregation, or because of its size and that it is bound to the cell membrane is hard to determine. Though if only in the pellet, the protein might be integrated in the cell membrane retaining its natural folding. Refolding the inclusion bodies on the other hand, could affect the proteins activity and conformation. To avoid this problem, continuing with the protein in the supernatant was chosen, although more of our protein seemed to be in the pellet (93, 96).

4.2.9 Inclusion bodies formation

The environment in the *E. coli* host is different from the environment in the *Leishmania* parasite. The recombinant protein can be affected by this and cause protein instability and

aggregation. Unbalance in aggregation and solubilization of the protein can lead to inclusion body formations (84).

During high levels of expression the cytosol can become crowded, and can saturate the quality control mechanism of the cell leading to misfolding.

Incorrect disulfide bond formation can lead to the generation of inclusion bodies. The AAP3 protein contains 10 cystin amino acids that may be bale to generate disulfide bindings, and lead to formation of inclusion bodies. Increasing the solubility may aid this problem. This can be done by co-expressing chaperon molecules together with the recombinant protein (85, 91) or fusing the recombinant Cter or Nter part of the protein with a soluble fusion tag. Molecular chaperones utilize cycles between ATP binding and hydrolysis to facilitate the folding of the protein (97). Optimization still has to be done, as no single tag appears to work for all recombinant proteins (45, 56).

4.2.10 Results from SDS-PAGE analysis

SDS-PAGE was used to give us an indication on how the different changes in temperatures, incubation time, centrifugation and L-rhamnose concentration could effect the production of the LdAAP3 recombinant protein. No matter what changes we made, fragmentation of the LdAAP3 recombinant protein seem to occur and we were not successful in completely purifying the protein using IMAC. When comparing all the different gels and trials with changes in the protocol, the SDS-PAGE from protocol #12 (Figure 25) using Ni-NTA buffers and columns seem to be most successful. This experiment yielded a relatively purified solution (FT-NPI-250) containing bands at the size of about 56 kDa and 26 kDa. When comparing this protocol with similar protocols with same temperature and amount L-rhamnose added the major difference seem to be the sonication time. Sonication with Ni-NTA protocol was used for 4 min compared to other protocols with 15-30 min sonication. This could imply that production of the ORF LdAAP3 protein may be occurring in the different protocols but the lysation has been conducted for too long rendering a crowded solution, making it difficult for the His-tag proteins to bind to the IMAC.

All aliquots should have had as similar concentrations and conditions as possible, with preferably only one difference from each other, when comparing them. For instance comparing the band intensity of the uninduced bands, with 3h induction, would naturally have weaker bands than the induced bands with overnight incubation. The uninduced sample should have been incubated using the same settings for as long time as the induced aliquots to get a more accurate observation on how the IPTG affected the induced aliquots.

Growing a culture with an empty vector inserted and using the same conditions as the culture with the plasmid would have been possible to do in order to compare the differences between the two cultures. This may also allow us to eliminate some of the bands from other protein production in *E. coli* to the LdAAP3 recombinant protein bands.

4.2.11 Codon usage

When expressing genes in another host like *E. coli* significant differences in codon usage between the two organisms can interfere with the translation because one or more tRNAs could be rare or lacking and lead to fragmentation (51). tRNA depletion can lead to improper translation leading to wrong construction of the different amino acid or even termination of translation that could lead to the production of fragments. This seems to occur for our recombinant proteins. Changes like these may also affect the proteins activity. To avoid this problem it may help to increase the availability of underrepresented tRNAs (84, 98). This can be done by overexpressing genes encoding the rare tRNAs (99).

When analyzing the rare codons of *E. coli* and the size of the protein fragmentations it may have produced, we could see some fragments matching with the sizes of the bands found when running several test of our recombinant protein, using nitrocellulose LdAAP3 recombinant protein blotted membranes. When comparing the Western blot from Figure 24 and 25, bands at the size of about 26 kDa and 12 kDa occur. The same sizes could be found in Table 5 showing fragment sizes starting from the Nter. Finding these bands on the Western blot can be logical because the Nter contains the His-tag.

When comparing the bands obtained from LdAAP3 recombinant protein blotted nitrocellulose strips incubated with sera from infected subjects, bands at around 49, 45, 32, 30 and 12 kDa seem to reoccur. These protein sizes are also found in Table 5 showing the protein fragment sizes starting from the Cter end. Bands at the size of 31 kDa and 12 kDa can be found from LdAAP3 blotted nitrocellulose strips incubated with sera from immunized rabbit. These sizes can also be found in Table 5 showing fragments generated from the Cter end. The Cter end does not contain His-tag but can be detected by antibody affinity against epitopes.

The LdAAP3 blotted nitrocellulose strips incubated with sera from immunized rabbit also showed bands at the size of around 26 kDa and 30 kDa (figure 30). Summed together these two fragments gives the whole LdAAP3 size at about 56kDa.

In theory codon bias could affect the expression and lead to fragmentation (51), but optimizing codon depletion has also shown not to be able to improve the expressions (49). It is therefore not easy to confidently conclude that fragmentations occur due to codon bias.

4.3 Purification

Protein purification is a time elaborate procedure with no straightforward recipes to follow, as the different proteins will need to be treated uniquely. Main issues that are frequently met are protein degradation, protein insolubility, problems in preserving the protein structure, and protein aggregation. The plasmids used for this expression contain genes for 6x histidine residues, which allow purification of the recombinant protein by affinity chromatography.

After the expression and lysis of the LdAAP3 recombinant protein expressed in *E. coli*, we hoped that the target protein had been properly transcribed, folded, was free from the *E. coli* and that histidine residues attached were exposed on the surface of the molecule and not hidden in the conformational folding of the protein, to allow purification of the protein. After running SDS-PAGE and Western blots it was clear that we were not able to purify the lysated solution to obtain a solution with only our His-tagged recombinant protein. Several attempts were pursued with different conditions, but all attempts failed in yielding a completely purified protein in solution.

Our main problem was loosing the protein in early washing steps, rendering empty aliquots in the eluted solution, and unpurified aliquots in the washing steps where our recombinant proteins remain together with other proteins.

4.3.1 Imidazole concentrations and denaturation conditions

Different imidazole concentrations were tested to see when the protein would release their binding to the column or beads. Although the protein seemed to release their binding at different imidazole concentrations it was not possible to conclude that it was only because of the imidazole concentrations. Not only was there a difference when performing purifications with Dynabeads and columns, but also a difference in using different experimental conditions.

If the protein has the histidine residue hidden in the conformational folding of the protein, it would hinder the binding to the IMAC resins. Attempts to denature the lysated solution during purifications were done in an attempt to improve the purification, without any further success (figure 20). Proteins were still lost during early washing steps, and eluted

solutions showed empty bands. The most successful elution was from protocol #12 (Figure 25) using FT-NPI-250 buffer containing 250 mM imidazole.

4.3.2 Binding to the IMAC

The Dynabeads and the columns use Co^{2+} and Ni^{2+} based resins respectively, and it is said that Co^{2+} has a higher specificity for the His-tagged proteins compared to Ni^{2+} (68). The buffers used during purification varied in their concentrations of sodium phosphate, sodium chloride and imidazole. In addition, the amount of lysated sample varied rendering different concentrations of the proteins from the lysated *E. coli*. As mentioned earlier, doing a flow cytometric detection on these samples would have been interesting and might have given a more conclusive result when measuring the effects of the amount of our recombinant protein in the different aliquots from the purification step(49).

It is not easy to point out why the purification was unsuccessful, and there are several possible explanations. Low access of the LdAAP3 His-tagged proteins to the immobilized Ni^{2+}-chelated ligands could be one reason. This could be due to low affinities between the metal ion and our His-tagged LdAAP3 protein, or even low concentrations of the protein, which lead to reduced levels of adsorption to the column. Then again one must take into account the whole solution. If the *E. coli* solutions from the expression steps contains a lot of *E. coli* that has lost the recombinant plasmid, there will be a higher ratio of proteins produced that is not the LdAAP3 recombinant protein. This may lead to a crowded solution, making it difficult for the His-tagged proteins to bind to the IMAC resin. To long sonication may also lead to a more crowded solution, which seem to be the case during our experiments.

The conformation and the surface accessibility of the His-tag of the recombinant protein are also important for the binding affinity to the metal ion resin. Interaction between the aromatic group of the ligand on the IMAC resins and the hydrophobic side chains of the His-tagged protein may cause steric hindrance due to hydrophobic pi stacking interactions, and may lead to lower binding affinity (69).

Degradation and fragmentation is a common problem in the expression of the recombinant protein. Attempts in the purification process could be done to improve the extraction of the whole LdAAP3 recombinant protein by doing a two-step purification. This implies two tags cloned into the plasmid vector and could be placed at the Nter and the Cter. Only proteins with both tags will be purified (100).

It is not common for bacteria cells to have histidine residues on their own proteins. Though this should result in low contamination of bacterial proteins during purification,

many of the host cell proteins bind, though weak, to the IMAC resin. These bindings can be due to host cell proteins with metal ion binding sites or the potential clustering on the surface of the protein that could mimic a His-tag. These proteins can be removed by adding a low concentration of imidazole that will compete for these binding sites (64).

Although this was attempted, we still obtained a lot of other proteins. This can be explained by high concentrations of *E. coli* without our plasmid producing other proteins. The problem we experience during expression and purification may consequently be because of too high concentrations of *E. coli* growth without the plasmid. Attempts in lowering the growth and improving the selection of *E. coli* with plasmids seem to be important in future attempts.

4.3.3 IMAC limitations:

Although a popular method for protein purification, the IMAC system also has several limitations. All chelating agents that could strip the resin of the metal ions should be avoided, and was avoided during our experiments. Acidic pH could inhibit the binding between the resin and the target protein, which explains the method of elution using pH, which also failed to purify the target protein when tried out in this thesis (Figure 20). Metal ion transfer and leakage is some of the weaknesses when using IMAC. Having proteins with too strong affinity to the metal ion may remove the metal ions from the IMAC resin, resulting in protein loss due to proteins passing through the column with no binding. Zn^{2+} immobilized ions are claimed to be better than Cu^{2+} and Ni^{2+} because of their lower binding affinity to the host proteins (64). Attempts using column with Zn^{2+} could have been done.

Cleavage of protein backbones and destruction of amino acid side chains can be caused by the IMAC complex. Amino acids like histidine found in the target protein can be vulnerable to metal-catalyzed oxidation reactions, which could lead to protein damage. Site-specific cleavage can be initiated by metal ions sitting on the IMAC complex and cause fragmentation of the target protein (64).

4.4 Quantification of protein

Most quantification methods determine only the total protein content in a solution (101, 102). Because we were not able to purify the LdAAP3 recombinant protein, we did not have a purified solution that easily could be used for quantification. We therefore chose to run a SDS-PAGE, stain the gel with Coomassie blue. Thereafter we used ImageJ to analyze the

bands on the gel, to get an approximation of the quantity of our recombinant *E. coli* proteins. It allows us to compare band intensities(75). This method is semi-quantitative and several issues have to be considered when the analysis is performed.

One of the main problems using this method relies on the different affinity of the proteins for Coomassie. It may not be appropriate to quantify the unknown *E. coli* protein bands based on an unstained ladder, since Coomassie may have different affinity for the two types of protein(103). Because of this a measured density of 1000 for a ladder band and 1000 for an *E. coli* band does not mean the *E. coli* band contains the same amount protein as the ladder. A true concentration estimate is therefore not likely to be achieved. Further, when measuring band intensities saturation will eventually be achieved where differences will not be detected (75). ImageJ will then not be able to measure the difference between the two bands. This is why we made a serial dilution of the sample to see where this limit may be reached. If bands from the highest dilution are used for quantification, it may not give a good estimation. In addition to this, background also complicates the measurement further. In Figure 23, the peaks are not touching the baseline. This is due to background.

Comparing densities of bands of different molecular weights may also be problematic and was done for our experiments. Using a known ladder with the same molecular weight as the unknown is preferable when using this method. This will allow more accurate density comparisons between the unknown and known bands.

ImageJ simply measures the band intensities on the image. As long as the Coomassie binding has equal affinity to the various proteins, then ImageJ should be able to give a reliable measurement. But since we do not know the affinity to the different proteins, it is not easy to estimate how good this quantification can be.

4.5 Raising anti-LdAAP3 polyclonal antibodies

When raising antibodies sensitivity and selectivity of the antibody against the antigen is not guaranteed (104). Most antigen surfaces will be able to bind several possible antibodies. Any part of a surface that is accessible on an antigen can be a potential target of an antibody(105). Since polyclonal antibodies were produced it is likely that the antibodies will bind to several epitopes on the surface of the antigen. Because we wanted to raise anti-LdAAP3 polyclonal antibodies for localization of AAP3 proteins in *Leishmania* parasites, producing the whole ORF LdAAP3 recombinant protein was not vital, though preferred. The fragments of LdAAP3 recombinant protein obtained from the expression and purification was

good enough to produce antibodies able to bind to the LdAAP3 protein in *Leishmania amazonensis* parasites.

Sera obtained from rabbit immunization in Germany were not purified, which means that the sera contain several antibodies. Testing sensitivity and specificity requires positive and negative tests and subjects with and without infection. By purifying the antibodies over a protein A or G column, leaving us with only IgG, could have increased specificity and sensitivity further. A larger experiment should have been done to be able to conclude the specificity or sensitivity of the antibodies generated from infected subjects against LdAAP3 antigens.

4.6 Protein blotting

Western blotting was performed to detect where the LdAAP3 recombinant proteins were localized throughout the expression and purification. For detection of His-tagged proteins, alkaline phosphatase conjugated His-tag antibodies were used. A weakness when using this method is that it only detects His-tagged proteins, and not the LdAAP3 proteins specifically.

4.6.1 Detecting the His-Tagged LdAAP3 recombinant proteins

His-tag detection allowed us to choose the bands we knew had His-tag on, making us more confident about the choice of protein to cut out from the gel for immunization of the rabbit. The Western blots using AP conjugated antibodies for His-tag detection generally showed us strong bands of the repeated fragments of the LdAAP3 recombinant protein occurring at a size of about 32 kDa and 26 kDa. It also showed weak bands at a size of 56 kDa (figure 24). This may indicate that the production of the whole ORF LdAAP3 recombinant protein is modest, and that the problem may be during the expression of the recombinant protein and not necessarily due to fragmentations of the whole ORF LdAAP3. Looking at Figure 20, showing the results from protocol #11, may further support this. Protease inhibitors were added during this protocol, but did not improve the yield of the whole ORF LdAAP3 recombinant protein. Improving the expression protocol by lowering temperature, decreasing plasmid number, and adding co-expression of chaperones may have aided the expression.

4.6.2 Blotting LdAAP3 recombinant protein on nitrocellulose membrane

Using Ni-NTA buffers and column gave us a more successful purification. Although not shown on the Western blot (Figure 25) we could see bands at around 56 kDa on the SDS-

PAGE, on FT-NPI-250 aliquots. The band at the size of 56 kDa is repeatedly seen when using sera from infected subjects (figure 25, 26, 29 and 30). This aliquot from FT-NPI-250 had few other bands on the gel and also had a His-tag verification of the protein at a size of about 26 kDa on the Western blot. When choosing aliquots to be used as antigen to be blotted on the nitrocellulose paper, we chose to mix NPI-20 and NPI-250 together. This was done because both aliquots showed strong bands in different places on the Western blot, giving us two arias (26 kDa and 38 kDa) where we could expect antibody binding to the epitope/antigen, and be able to use this as a marker when comparing with infected sera. Mixing these two aliquots and not using the most purified aliquot alone could increase the unspecific binding on the nitrocellulose membrane due to the increased sites of antigen that different antibodies from sera could bind to (76).

4.6.3 Testing sera from Leishmania infected and non-infected individuals

Optimal detection methods that are suitable for field applications are lacking for leishmaniasis. Lack of specialized skills, laboratory equipment and easy detection methods limit the ease of detection in endemic areas (106). Antibodies detected by Western blotting have been done for *L.donovani,* where samples of whole parasite were blotted onto a nitrocellulose membrane and tested with serum from patients (14, 15). The experiments we performed using sera from infected individuals were meant to explore whether infected subjects could be detected by using blotted nitrocellulose papers containing the LdAAP3 recombinant antigen as detection marker.

Sera from mice infected with *L. amazonensis*, and sera from human infected with *L. infantum chagasi* were tested for binding against the LdAAP3 recombinant protein produced.

Negative and healthy controls of mice sera did not reveal any bands on the blotted nitrocellulose strips, indicating that the bands we got from the infected sera may come from antibodies generated from infection (Figure 27). Bands showing at the same sizes as the bands from earlier Western blots with His-tag detection may indicate that the antibodies generated in infected subject are selective for antigen from LdAAP3 recombinant protein.

Not only did the antibodies from infected sera bind at the same position as the His-tagged protein bands from the Western blot (38 and 28 kDa) (Figure 25), but it also bind to several sites on the LdAAP3 recombinant blotted nitrocellulose membrane, at sizes around 56, 49, 38, 28 and 12 kDa (Figure 26 and 27). Bands at around 56 kDa that are shown on several SDS-PAGE (Figure 18-20) and not on Western blots seem to occur on the strips blotted with LdAAP3 recombinant protein solution incubated with infected sera. Because this

binding is not detected by His-tag affinity during Western Blots but rather detected during antibody-epitope binding, it can be that the His-tag residues are cut off. This can also explain the challenges we met when purifying the recombinant solution using Nter His-tag affinity. Adding His-tag on the Cter protein could have been tested.

The LdAAP3 nitrocellulose strips incubated with human sera with *L. infantum* infection showed a band at about 38 kDa. The person used as healthy human control was chosen because he had never had any symptoms indicating infection with *Leishmania*. This does not have to mean that he was not infected, since the disease could be asymptomatic. A problem with the protocol for human sera was the use of 3 three layers of antibodies (human, goat anti human and anti goat IRDye 680), which could lead to more unspecific binding and produce irregular results. This may also be due to the binding to *E. coli* proteins that may be on the blotted nitrocellulose membrane, as the recombinant proteins were expressed in *E. coli.* Several control tests should have been conducted to be able to conclude further.

From the experiments we conducted we could see the same bands at the same sizes appeared repeatedly. Sera from *L. amazonensis* infected mice, seem to bind to the *L. donovani* AAP3 recombinant protein. This could indicate affinity between the antibody from infected sera and the LdAAP3 recombinant antigen, which may further support the fact that the AAP3 protein is conserved between the different species of *Leishmania*. However, similar epitopes could generate affinities for the same antibody and generate cross-reactions that can occur with distantly related organisms (107, 108). Because of this, diagnostic accuracy may decrease and be less reliable. Cross reaction with *Trypanosoma cruzi* has been confirmed and is one of the setbacks when using detection by antibodies, which have led to significant false positive reactions, which further complicates the analysis (109). This is the fact for the immnochromatographic strip test using the rK39 recombinant antigen, which gave positive results in patients with malaria, tuberculosis or typhoid fever (15, 16). This underlines the importance of generating antibodies specific for *Leishmania* parasite. Several tests could therefore have been performed, to analyze the antibody for cross reactivity.

To improve the comparison between infected and uninfected sera it would have been better to use the same subjects before and after infection. This is possible for animal subjects. Human subjects should have been obtained from non-endemic areas.

It must be noted that the sera is not purified. This could increase the background and give false positive reactions due to the nonspecific antibody binding. Another weakness was the usage of BSA or dry milk, which both contained bovine IgG. A lot of secondary antibodies, such as anti-goat Ig, can react strongly with bovine IgG. The secondary antibody

used on human sera was anti goat IRDye 680 (Odyssey-Licor). This could explain the increased background (110).

4.6.4 Testing anti-LdAAP3 polyclonal antibodies against E. coli produced LdAAP3 proteins

The affinity of the polyclonal antibodies produced in rabbit injected with our recombinant LdAAP3 protein was tested against the recombinant LdAAP3 protein blotted on a nitrocellulose membrane. Again we could detect bands with a similar kDa sizes as mentioned earlier, showing bands at the sizes around 38 and 28 kDa, further supporting our other results indicating that these bindings are not random and a cause to unspecific bindings, but on the contrary indicate binding between the recombinant protein and antibodies with affinity to its epitope.

Several bands were detected on the Western blot in figure 29, but clear bands could be detected at 56 kDa and 26 kDa. This could again indicate the successful production of the whole LdAAP3 recombinant protein detected by antibody binding to epitope, which may indicate that the His-tag is hidden in the conformational folding of the LdAAP3 protein.

Although the rabbits were immunized with either 32 kDa or 26 kDa size purified recombinant LdAAP3 protein, the antibodies produced seem to detect bands at several sizes. This can be explained by the generation of epitopes and antibody affinity. Antibodies produced from immunized rabbits may recognize different epitopes on the blotted target protein. Epitopes in the beginning of the 56 kDa protein may be the same as the epitopes from the 26 kDa protein. The antibodies produced from injection of the 26 kDa size proteins may therefore be able to detect bands in other sizes.

A few bands on the pre-immune strips also occurred (Figure 31). Because the bands do not occur at the same size, it most likely cannot be due to the bacterial production of its own alkaline phosphatase proteins which might react with the AP conjugated His-tag antibody used for detection. A possible explanation could be antibody production in the rabbit from earlier exposure to *E. coli*, which now recognizes some epitopes on the LdAAP3 membrane blotted nitrocellulose membrane. This can be explained because the LdAAP3 recombinant solution blotted on the nitrocellulose membrane was produced in *E. coli.* Proteins from the *E. coli* would therefore most likely be on the membrane.

4.6.5 Testing SLA and recombinant LdAAP3 against anti-LdAAP3 antibody using ELISA

The LdAAP3 recombinant protein and soluble lysated antigens (SLA) from *Leishmania amazonensis* were tested against the polyclonal antibodies produced. The polyclonal

antibodies produced from the LdAAP3 recombinant protein will consist of a mixture of different antibodies produced by many different B cells. The same results occurred showing clear differences in titer levels of pre-immune and immune rabbit, indicating the successful production of polyclonal antibodies against the recombinant protein.

Titers of SLA showed increased titers of animal 71, 72 and 74. Indicating that there is affinity between sera from immunized recombinant protein and antigens produced from lysated *Leishmania*. Where these bindings occur is hard to determine. We can therefore not determine whether these interactions are between the AAP3 protein in *Leishmania* or simply interactions with other antigens with similar epitopes. This must be further interpreted in different analysis like using confocal microscopy.

4.7 Limitations of the study

The antibodies produced from LdAAP3 recombinant protein have been characterized to some extent, but need further characterization to be able to determine their specificity. This is important for the further use of the antibodies for localization of the AAP3 protein. Several tests should have been conducted with other *Trypanosomatidae*.

Another limitation of this study is the small study population of infected subjects tested against the recombinant LdAAP3 antigen. *Leishmania* infection is relatively uncommon in Norway, and experiments were performed during a short stay in the endemic country Brazil. Only sera from two human, two mouse and two dogs were obtained and tested. Additionally, more proper control groups should have been used. The same subject pre and post of infection could have been used for animal subjects. Human control groups should have been obtained from non-endemic areas.

Testing the polyclonal antibody against several species was limited to *L. amazonensis* due to time-limitations. However, testing the antibody against other species has been initiated.

4.8 Conclusion

- The production of recombinant LdAAP3 protein in *E. coli* was optimized, enabling the expression of two fragments of the whole LdAAP3 recombinant protein at a size of 32 kDa and 26 kDa.
- The two proteins were successfully purified by separation using SDS-PAGE, and shipped to BioGenes in Germany for polyclonal antibody production in rabbits.

- The polyclonal antibodies produced in rabbit were tested against the recombinant LdAAP3 protein and antigen/epitopes produced from lysated *L. amazonensis*. Binding affinities between the antigen and antibodies occurred, which implies the successful production of antibodies.
- Binding patterns seem to occur when testing sera of infected and non-infected individuals using the LdAAP3 recombinant protein. Further studies are needed to characterize the antibodies.

4.9 Further research

Arginine is important for *Leishmania* survival and infectivity. The parasite needs arginase to utilize arginine. Arginase is located inside the glycosome of the *Leishmania* parasite. The main goal of this project was to produce antibodies that could be used to localize the AAP3 transmembrane protein.

The antibodies produced from the LdAAP3 recombinant proteins were tested for their binding affinity to *Leishmania* antigens. They could potentially therefore be used further for the localization of the AAP3 transporter in *Leishmania* using imaging techniques, such as confocal or electron microscopy. It would be of interest to investigate if the AAP3 located to the cell-membrane is the same as the transporter hypothesized to be located to the glycosome. After localization studies, it would be of interest to analyze the transporter protein and block its functions. If the transporter sitting on the glycosome is similar to the AAP3 transporter on the *Leishmania* membrane, which we know is different from the arginine transporter of the macrophages (CAT2B), it could be a target for a new drug against leishmaniasis.

Not only does the drug need affinity for the AAP3 transporter, but it also has to be carried to its activation site. Because the drug would have to go through several membranes, this could be a challenging task. But there are several techniques possible to utilize to reach this goal. Designing a drug with several layers with different markers may eventually lead it to its activation site.

The antibodies that we have produced might also be possible to use for localization of the parasite in the body, both during the infection process and after establishment of the infection. This may allow treatment of specific organs or tissues. Again an obstacle would be to enable the antibodies to reach their binding site.

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APPENDIX

A Kits used during the project

Kit name	Supplier	Catalog/Lot number
QIAquick PCR Purification Kit	Qiagen 40724 Hilden, Germany	Cat: 28104
QIAprep Miniprep Handbook	Qiagen, Germany	Cat: 1071266
BigDye Direct Cycle Sequencing Kit	Life Technologies. USA, 5791 Van Allen Way, Carlsbad, CA 92008	-
Dynabeads His-Tag Isolation & Pulldown	Life Technologies. Norway, Oslo	**Ref:** 10103D (2 ml) Lot: 167525800
HisTrap™ HP, 5 x 1ml	GE Healthcare Life Sciences. SE-751 84 Uppsala, Sweden	17-5247-01 Lot: 10236714
NuPAGE 4-12% Bis-Tris Gel (1.0mmX10well)	Life Technologies. USA, 5791 Van Allen Way, Carlsbad, CA 92008	**Ref**: NP0321BOX LOT: 15090471
NuPAGE 4-12% Bis-Tris Gel¨(1.5mmX15well)	Life Technologies. USA, 5791 Van Allen Way, Carlsbad, CA 92008	Ref: NP0336BOX Lot: 15081811
WesternBreeze Chromogenic Immunodetection System (for detection of Mouse Primary Antibodies)	Invitrogen. USA, Carlsbad, CA 92008, 760 603 7200	Cat: WB7103 Lot: 1089126
Champion pET151 Directional TOPO Expression Kit	Invitrogen Life technologies. USA, 5791 Van Allen Way, Carlsbad, CA 92008	K151-01
PureLink Quick Plasmid Miniprep Kit	Invitrogen Life technologies USA, 5791 Van Allen Way, Carlsbad, CA 92008	Cat: K2100-10
Ni-NTA Superflow Cartridge	Qiagen 40724 Hilden, Germany	Lot: 151052475

B Instruments

Photometer

Eppendorf BioPhotometer, with 8,5mm cuvette (Hamburg, Germany).

Sonicator

Misonix, Sonicator 3000 Ultrasonic Liquid processor (Newton, CT, USA)

Centrifuge

1. Centrifuge 5810 R (Ependorf, Hamburg, Germany)
2. Centrifuge 5430 R (Eppendorf, Hamburg, Germany)
3. Centrifuge 5415 D (Eppendorf, Hamburg, Germany)
4. Ultracentrifuge, Beckman, USA
5. Sorvall RC 5C Plus, Dupont, (Wilmington, Delaware, USA), using a Sorvall SS-34 rotor.

Shaker

1. HT INFORS minitron (Infors AG, Bottmingen/Basel, Switzerland).
2. Shaker Control System, 311DS Labnet (Dublin, Ireland).
3. Orbital Shaker, OS-20, Biosan (Riga, Latvia)

Roller

1. Stuart, Rotator SB3 (Stone, United Kingdom).
2. The Belly Dancer, Stovall (Greensboro, NC, USA).

PowerBank

1. PowerNPac 300, Bio-Rad (USA).
2. PowerPac Basic, Bio-Rad (USA).

Thermomixer

Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany)

Incubator

1. Termaks, Type: B8420, Labolytic AS, (Bergen, Norway).
2. Echo Therm, Torrey Pines Scientific (San Marcos, CA, USA).

Vortex

Labinco vortex mixer (Breda, The Netherlands).

Chamber for Western blot

X Cell II, Blot Module, InVitrogen, Life Technologies (Carlsbad, CA, USA)

Scanner

LI-COR Inc, Odyssey, CLx Infrared Imaging System (Lincoln, NE, USA).

C **Computer software**

Word

Word 2011 (Microsoft Corporation, Santa Rosa, California, USA) was used to write and edit the thesis.

Excel

Excel 2011 (Microsoft Corporation, Santa Rosa, CA, USA) was used to calculate results from ImageJ.

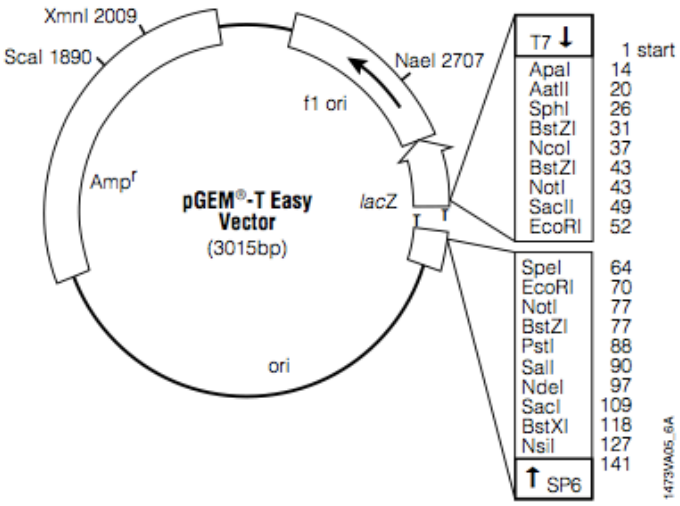
ImageJ:

ImageJ, version 2.0.0-rc-43/1.50e was used to estimate amount protein.

D Vectors:

- **pGEM-T easy**

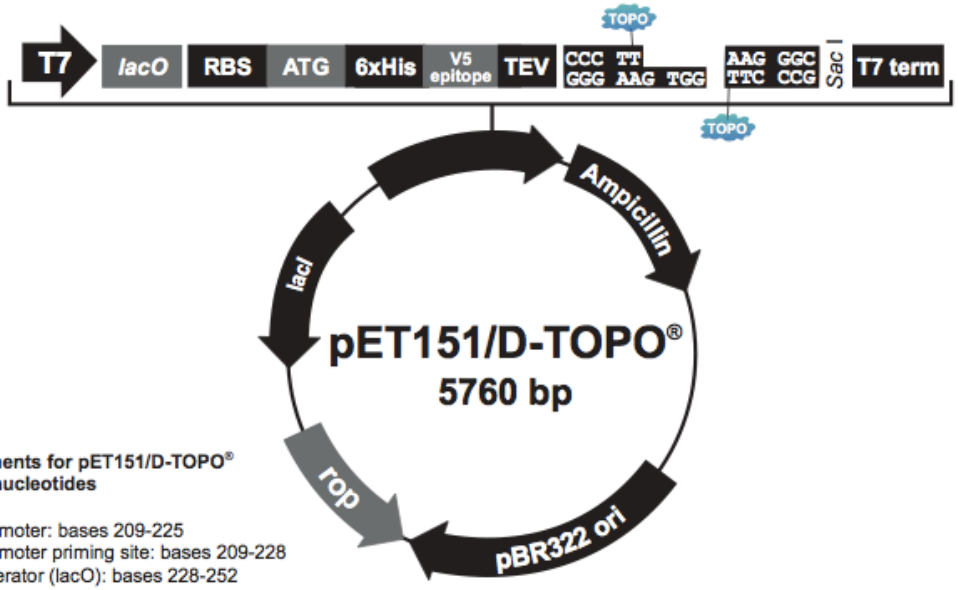
5.D. pGEM®-T Easy Vector Map and Sequence Reference Points



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

- **pET151/D-TOPO**



Comments for pET151/D-TOPO®
5760 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-289
Initiation ATG: bases 297-299
Polyhistidine (6xHis) region: bases 300-317
V5 epitope: bases 318-359
TEV recognition site: bases 360-380
TOPO® cloning site (directional): bases 387-400
T7 reverse priming site: bases 455-474
T7 transcription termination region: bases 416-544
bla promoter: bases 849-947
Ampicillin (*bla*) resistance gene: bases 948-1808
pBR322 origin: bases 1953-2626
ROP ORF: bases 2997-3188 (complementary strand)
lacI ORF: bases 4500-5612 (complementary strand)

E Contents of solutions used

Buffers for lysis	
Solution	**Ingredients**
Lysis buffer 1 Adjust to 50 mL solution with Milli-Q water (pH 8.0)	20 mM Sodium Phosphate pH 8.0 (1 mL, from 1M stock) 500 mM NaCl (5 mL, from 5M stock) 5 mM imidazole (10 μL, from 5M stock) 1 mM PMSF (add only at time of use) 0.2 mg/ml lysozyme (add only at time of use)
Lysis buffer 2 Adjust to 50 mL solution with Milli-Q water (pH 8.0) Filter with 0.2 μm.	5 mL of 1M NaH_2HPO_4(1M) 500 μL Tris (100%) 40 mL UREA (10M)
1M Sodium phosphate buffer (pH 8) Filter with 0.2 μm.	6,8 mL of 1M NaH_2HPO_4 (12g to 100 mL water) 93,2 mL of 1M Na_2HPO_4(14,2g to 100 mL water)

Solubilization buffer	
Solution	**Ingredients**
Solubilization buffer Adjust to 40 mL solution with Milli-Q water (pH 8.0)	20mM Sodium Phosphate pH 8 (0,8 mL, from 1M stock) 300mM NaCl (2,4 mL, from 5M stock) 1mM PMSF

Buffers for Dynabeads purification	
Solution	**Ingredients**
1Dynabeads – binding buffer Adjust to 10 mL solution with Milli-Q water	100 mM Sodium Phosphate pH 8 (1 mL, from 1M stock) 600 mM NaCl (1,2 mL, from 5M stock) 5 mM imidazole (10 μL, from 5M stock) 0,2% Tween-20 (20 μL))
1Dynabeads–washing buffer1 Adjust to 10 mL solution with Milli-Q water (pH 8.0)	100 mM Sodium Phosphate pH 8 (1 mL, from 1M stock) 600 mM NaCl (1,2 mL, from 5M stock) 5 mM imidazole (10 μL, from 5M stock)
1Dynabeads–washing buffer2 Adjust to 10 mL solution with Milli-Q water (pH 8.0)	100 mM Sodium Phosphate pH 8 (1 mL, from 1M stock) 600 mM NaCl (1,2 mL, from 5M stock) 30mM imidazole (60 μL, from 5M stock)
1Dynabeads–washing buffer3 Adjust to 10 mL solution with Milli-Q water (pH 8.0)	100mM Sodium Phosphate pH 8 (1 mL, from 1M stock) 600mM NaCl (1,2 mL, from 5M stock) 75mM imidazole (150 μL, from 5M stock)
1Dynabeads – elution buffer Adjust to 10 mL solution with Milli-Q water (pH 8.0)	50mM Sodium Phosphate pH 8 (1 mL, from 1M stock. 300mM NaCl (1,2 mL, from 5M stock) 0,10% Tween-20 (10 μL.) 300mM imidazole (600 μL, from 5M stock)
2Dynabeads – 1Xbinding/wash buffer Adjust to 10 mL solution with Milli-Q water	50mM Sodium Phosphate pH 8 (0,5 mL, from 1M stock) 300mM NaCl (0,6mL, from 5M stock) 0,01% Tween-20 (1μL)
2Dynabeads–elution buffer Adjust to 10 mL solution with Milli-Q water (pH 8.0)	50mM Sodium Phosphate pH 8 (0,5 mL, from 1M stock) 300mM NaCl (0,6mL, from 5M stock) 0,01% Tween-20 (1μL) 300mM imidazole (600μL, from 5M stock)

HisTrap HP purification	
Solution	**Ingredients**
1HisTrap HP – binding buffer Adjust to 50 mL solution with Milli-Q water (pH 8.0)	20mM Sodium Phosphate (1000 μL, from 1M stock) 500mM NaCl (5000 μL, from 5M stock) 5mM imidazole (50 μL, from 5M stock)
1HisTrap HP – washing buffer 1 Adjust to 50 mL solution with Milli-Q water (pH 8.0)	20mM Sodium Phosphate (1000 μL, from 1M stock) 500mM NaCl (5000 μL, from 5M stock) 20mM imidazole (200 μL, from 5M stock
1HisTrap HP – washing buffer 2 Adjust to 50 mL solution with Milli-Q water (pH 8.0)	20mM Sodium Phosphate (1000 μL, from 1M stock) 500mM NaCl (5000 μL, from 5M stock) 75mM imidazole (750 μL, from 5M stock)
1HisTrap HP – washing buffer 3 Adjust to 50 mL solution with Milli-Q water (pH 8.0)	20mM Sodium Phosphate (1000 μL, from 1M stock) 500mM NaCl (5000 μL, from 5M stock) 200mM imidazole (2000 μL, from 5M stock)
1HisTrap HP – elution buffer Adjust to 50 mL solution with Milli-Q water (pH 8.0)	20mM Sodium Phosphate (1000 μL, from 1M stock) 500mM NaCl (5000 μL, from 5M stock) 500mM imidazole (5000 μL, from 5M stock)
2HisTrap HP – lysis buffer Adjust to 50 mL solution with Milli-Q water (pH 8.0)	1M NaH_2HPO_4 (5 mL) Tris (500 μL) } Stock lysis buffer 10M Urea (40 mL) Took out 15ml of stock lysis buffer and added: 1mM EDTA (30 μL from 0,5M stock) 1mM PMSF (150 μL from 100mM stock) 10 μM TLCK (15 μL from 10mM stock) 2 μg/mL Leupeptin (30 μL from 1mg/ml stock) 1 μg/mL Aprotinin (15 μL from 1mg/ml stock) 0,2 mg/mL Lysozyme (30 μL from 100mg/ml stock)

2HisTrap HP – washing buffer 1 Adjust to 50 mL solution with Milli-Q water (pH 6.3)	1M NaH_2HPO_4 (5 mL) Tris (500 μL) 10M Urea (40 mL)
2HisTrap HP – washing buffer 2 Adjust to 50 mL solution with Milli-Q water (pH 5.3)	1M NaH_2HPO_4 (5 mL) Tris (500 μL) 10M Urea (40 mL)
2HisTrap HP – elution buffer Adjust to 50 mL solution with Milli-Q water (pH 4.0)	1M NaH_2HPO_4 (5 mL) Tris (500 μL) 10M Urea (40 mL)

Buffers for Ni-NTA Superflow	
Solution	**Ingredients**
NPI-10 (Binding/lysis buffer for native conditions) Adjust to 1L solution with Milli-Q water (pH 8.0) Filter before use with 0,2**µm**	50mM NaH_2HPO_4 (6.9g, MW: 137.99g/mol) 300mM NaCl (17.54g, MW: 58.44g/mol) 5mM imidazole (0,34g, MW 68,08 g/mol)
NPI-20 (Wash buffer for native conditions) Adjust to 1L solution with Milli-Q water (pH 8.0) Filter before use with 0,2**µm**	50mM NaH_2HPO_4 (6.9g, MW: 137.99g/mol) 300mM NaCl (17.54g, MW: 58.44g/mol) 20mM imidazole (01,36g, MW 68,08 g/mol)
NPI-250 (Elution buffer for native conditions) Adjust to 1L solution with Milli-Q water (pH 8.0) Filter before use with 0,2**µm**	50mM NaH_2HPO_4 (6.9g, MW: 137.99g/mol) 300mM NaCl (17.54g, MW: 58.44g/mol) 250mM imidazole (17.0g, MW 68,08 g/mol)
Buffer E (denaturating elution buffer) Adjust to 1L solution with Milli-Q water (pH 4.5) Filter before use with 0,2**µm**	8M urea (480.5g, MW: 60.06g/mol) 100mM NaH_2HPO_4 (13.80g, MW: 137.99g/mol) 100mM Tris-base (12.10g, MW: 121.11 g/mol)

Buffers for Western blot	
Solution	**Ingredients**
TBS 1x Adjust to 1L solution with Milli-Q water (pH 7.5) Filter with 0,45**µm**	50mM Tris-Cl (6,05g) 150mM NaCl (8,7g) Solution is stable at 4℃ for 3 months
TBST	0,1% Tween20 (100 μL, from 100% stock) 100mL TBS solution
Transfer buffer Adjust to 1L solution with Milli-Q water	Transfer buffer 20X 100mL Methanol
Blocking-buffer	50ml TBS solution 5% BSA (2,5g)
Dilution buffer	10ml TBST 5% BSA (0,5g)
Alkaline phosohatase buffer (pH 9.5) Adjust to 50mL solution with Milli-Q water Filter with 0,2**µm**	100mM diethanolamine/TRIS (5000***µ***L, from 1M stock) 100mM NaCl(1000***µ***L, from 5M stock) 5mM $MgCl_2$ (250***µ***L, from 1M stock)

Buffers for Odyssey	
Solution	**Ingredients**
PBS 1x Adjust to 1L with Milli-Q water Adjust pH to 7,2 -7,4 with NaOH/HCl. Auoclavate	137mM (8g) 2.7mM KCl (200mg) 10mM Na_2PO_4O (1,44g) 2mM KH_2PO_4O (240mg)
PBST	0,1% Tween20 (100 μL, from 100% stock) 100ml PBS solution
Blocking buffer Adjust with Milli-Q water to a total amount of 500mL	Nonfat-Dried milk (0.1%) To 0.2x of PBS Added 0.2% Tween20