Recombinant expression and bioinformatic analysis of *Plasmodium falciparum* lactate dehydrogenase and heat shock protein 70-1

by

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Specialised degree in Biochemistry

Submitted in fulfilment of the academic requirements for the

degree of Master of Science in Biochemistry

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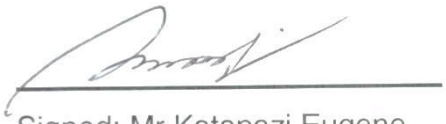
4-05-2017

PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biochemistry, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa from January 2014 to November 2016, under the supervision of Professor J. P. D. Goldring. The research was financially supported by NRF.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.



Signed: Mr Katapazi Eugene

Date: 04/05/2017

As the candidate's supervisor I agree to the submission of this dissertation.

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Signed: Prof J. P. D. Goldring

Date: 04/05/2017

DECLARATION OF PLAGIARISM

I, Katapazi Eugene, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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Abstract

Malaria remains a serious human health problem and the disease is particularly prevalent in developing countries. Malaria is caused by a parasite of the genus *Plasmodium*. Diagnosis of malaria is required before any treatment or intervention. The gold standard for malaria diagnosis is microscopy. Rapid diagnostic tests (RDT) have been used at point-of-care because of their relative ease of use. Correct and accurate diagnosis of malaria is a prerequisite as a point-of-care intervention aimed at eradication, detection of an asymptomatic reservoir, quantification of parasite load and tracking of drug resistance to malaria. Therefore, RDTs that have high sensitivity and specificity are required.

Plasmodium LDH (*P*LDH) is one of the three proteins in current use as antibody targets in RDTs to detect human malaria. The other two proteins are *Plasmodium falciparum* histidine rich protein II (*Pf*HRPII) and aldolase. Of the three proteins, HRPII is the most widely used protein in RDTs in sub-Saharan Africa. One of the requirements in improving the current RDTs is to improve specificity and sensitivity. *Plasmodium falciparum* heat shock protein 70-1 (*Pf*HSP70-1) has been found to be immunogenic in infected humans, abundantly expressed in the asexual stages and thought to be a potential diagnostic target for malaria. Conditions were optimized for the recombinant expression of *Pf*LDH and *Pf*HSP70-1 in different growth media, temperatures, concentrations of IPTG, times of induction, stages at which IPTG is introduced, use of a single colony as starting material or a dilution of an overnight culture to inoculate fresh media. Terrific Broth was found to be a better growth medium than Lysogeny Broth and does not require induction with IPTG. Use of a single starting colony was found to be better than dilution of an overnight culture as it saves time. Inducing at the stationary phase of bacterial growth yields more soluble protein than at mid-log phase. Expressing at lower temperatures lower than 37°C produces more soluble protein than growth at 37°C.

Methods of lysing the host bacterial cells expressing *Pf*LDH and *Pf*HSP70-1 by freezing and thawing, sonication, lysozyme digestion and a combination of these methods were compared and optimized. The combination of freeze and thaw followed by repeated sonication was found to be optimal for lysing the *E. coli* host cells.

Both proteins were affinity purified using an affinity Talon® resin and proteins were eluted with 150 mM imidazole. The purification protocol was monitored by separating the proteins on a 12.5% sodium dodecyl sulphate polyacrylamide electrophoresis gel. Purifying recombinant protein at 4°C produced higher yields of recombinant protein. The identity of the recombinant protein was confirmed by probing a western blot with anti-His-tag antibodies against each protein. The anti-His-tag antibodies detected both *Pf*LDH and *Pf*HSP70-1.

Preliminary experiments on *Pf*LDH enzyme found that the recombinant enzyme was active. *In silico* studies were done on *Pf*LDH and *Pf*HSP70-1 to identify potential immunogenic peptides to be used in the production of antibodies in chickens using a prediction program Predict7™. Potential post-translational modifications that may affect the activity of the native protein were evaluated. There is evidence that some post-translational modifications may affect the activity of proteins such as *Pf*LDH and *Pf*HSP70-1. Potential lysine acetylation, phosphorylation, glycosylation and proteolytic sites were identified on both *Pf*LDH and *Pf*HSP70-1.

This research generated soluble *Pf*LDH which can be used to produce antibodies in chickens and can be evaluated to detect malaria in blood.

ACKNOWLEDGMENTS

I would like to thank my supervisor Professor J. P. D. Goldring for offering me the opportunity to do my Msc. For the relentless number of hours of supervision, guidance, criticism and correction of my work. Thank you for imparting so many skills in me.

I would like to extend my gratitude to Professor T. H. T. Coetzer, Professor C. Niesler, Dr M. Simelane, Dr R. Hewer, Dr Che Pillay and Mr E. Dzomba for the advice, encouragement and assistance throughout my entire period of studies. You were always available when I needed help.

My colleague Dr R. Krausse for being such a pillar in our research group – giving advice every time I needed it. Kelvin Addicott, Faiaz Shaik, Mark Hutton, Mlondi Shezi, Abdulmalik Salmane , Kajal Fowdar, Sinothile Khuzwayo, Sheldon Sookai and Zainab Baig for being great inspiration and a wonderful group to work with. The funny games we enjoyed together as a group are memorable, especially bowling!

To colleagues in the other research groups Murtala Issa, Laurees Eissen, Alex Delport, Amukalani Murivate, Mphindile and Faith for the laughter and assistance you gave me.

To Jesse and Yegan for the technical assistance you offered me. The support staff Charmaine, Natalie, Tanya and the cleaners for making my work run smooth and for your friendliness.

To my Mum and Dad for believing in me and allowing me to follow my heart. You taught me the importance of hard work and value of being honest. My brothers and sisters, especially Paul for the laughter, friendship and brotherliness we share.

I dedicate this dissertation to my late grandmother for having sacrificed everything in her life to give me a decent education, notwithstanding her humble background, and my wife Nachilima Namwala Katapazi for being such a pillar of faith, strength, belief encouragement and guidance. You are a very strong woman. Thank you for the prayers you prayed for me.

I would like to thank the South African National Research Foundation, Medical Research Council and the University of KwaZulu-Natal for their financial support throughout this program.

Finally I thank the Almighty God for many gifts that He has endowed me with, especially the gift of Jesus Christ, time and health, without which it would have been impossible to complete this task.

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LIST OF ABBREVIATIONS AND SYMBOLS

18S rRNA	Small eukaryotic ribosomal subunit component
ACT	Artemisinin-based combination therapy
Anti-His-tag	Anti histidine tagged
$APAD^+$	Acetylpyridine adenine dinucleotide
APADH	Acetylpyridine adenine dinucleotide hydrogenate
AuNP	Gold nano-particles
AV	Autophagic vesicles
BamHI	Bacillus amyloliquefaciens type II restriction endonuclease
BC	Before Christ
BCP	Benzothiocarboxypurine
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CDC	Centre for Disease Control
CoxIII	Cytochrome oxidase 3
CRT	Chloroquine resistant transporter
DAPI	4', 6-Diamidino-2-phenylindole
DDT	Dichloro-diphenyl-trichloroethane
DHFR	Dihydrofolate reductase
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
DHPS	Dihydopteroate synthase
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EtBr	Ethidium bromide

FAD	Flavin adenine dinucleotide
FCA	Freud's complete adjuvant
FIA	Freud's incomplete adjuvant
FIND	Foundation for Innovative New Diagnostics
GFP	Green fluorescent protein
His_6	6 Histidine residues
HNB	Hydroxyl naphthol blue
HRPO	Horse radish peroxidase
HSA	Human serum albumin
HSP	Heat shock protein family
IFA	Indirect immunofluorescent antibody assay
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IRS	Indoor residual spraying
KAP1	Kruppel associated box (KRAB)-associated protein 1
kKDa	Kilo Dalton
LAMP	Loop mediated isothermal amplification
LB	Lysogenyauria Brothertani media
LOD	Limit of detection
MAPS	Multiple antigenic peptides
MDR	Multidrug resistance p-glycoprotein pump
MSP-1	Merozoite surface protein
NCBI	National Centre for Biotechnology Information
OmpT	Outer membrane protease
PAIL	Prediction of Acetylation on Internal Lysine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol

*Pf*EMPI	*Plasmodium falciparum* erythrocyte membrane protein 1
*Pf*HRPII	*Plasmodium falciparum* histidine rich protein 2
*Pf*HSP70-1	*Plasmodium falciparum* heat shock protein 70-1
*Pf*LDH	*Plasmodium falciparum* lactate dehydrogenase
*Pf*RH5	*Plasmodium falciparum* reticulocyte binding protein homologue 1
*P*LDH	*Plasmodium* lactate dehydrogenase
PMT	Phosphoethanolamine N-transferase
QBC	Quantitative buffy coat
RBC	Red blood cell
RDT	Rapid diagnostic test
RIG	Arginine, Iisoleucine and Glycine
RTS, S/AS02	Pre-erythrocytic malaria vaccine based on circumsporozoite surface protein of *Plasmodium falciparum* fused to HBsAg, incorporating a new adjuvant AS02
ScFv	Single-chain variable fragement
SE-HPLC	Size exclusion high performance liquid chromatography
SELEX	Systematic evolution of ligands by exponential enrichment
Ss rRNA	Small subunit ribosomal RNA
STET	Salt (NaCl), Tris-HCl, EDTA and Triton Xx-100
SYBR	Cyanine dye used to stain nucleic acids
TAE	Tris, acetic acid and EDTA buffer
TB	Terrific broth media
TBST	Tris buffered saline with Tween 20

TEMED	N,N,N’,N’- tetramethylethylenediamine
ULK1	unc-51-like kinase 1
WHO	World Health Organization

Chapter 1
Introduction and literature review

1.1 History of malaria

Malaria is an ancient disease infecting mankind. Symptoms of malaria have been described in an Egyptian papyri from 1570 BC, a Chinese document from 2700 BC and clay tablets from Mesopotamia from 200 BC (Cox, 2010). For over 2500 years the idea that malaria fevers were caused by unpleasant vapour rising from swamps persisted, and thus probably led to the naming of the disease as malaria, which in Italian translates as bad air (mal - bad, aria - air). Intensive studies into malaria were observed in the 19th century (Cox, 2010).

Marchiafava and Bignami using eosin-based staining and an oil immersion microscope, observed amoeboid movement of blood in patients suspected of malaria, as these organisms invaded red blood cells, grew within the cells and produced daughter cells (Marchiafava and Bignami, 1894). Camillo Golgi between 1885-56, observed erythrocytic stages of the malaria parasite, and differentiated between the tertian (48 hrs periodicity) and quartan (72 hr periodicity) malaria (Golgi, 1886). Marchiafava and Golgi further described mild spring malaria (benign tertian) and severe summer-autumn (malignant tertian) malaria. The French scientist, Charles Louis Alphonse Laveran, observed that the symptoms characteristic of malaria coincided with the presence of infected red blood cells (Laveran, 1893).

In 1897, Ronald Ross discovered that avian malaria parasite *Plasmodium relictum* was transmitted by *Culicine* mosquitoes and suggested that human malaria parasites may also be transmitted by mosquitoes. In 1889 while in Sierra Leone, he demonstrated that human malaria parasites were indeed transmitted by anopheline mosquitoes (Bynum and Ovary, 1998). Ross later discovered spores in the salivary glands of the *anopheline* mosquito and found the developmental stages of human malaria parasites in *anopheline* mosquitoes (Ross, 1923; Bynum and Ovary, 1998). After working with avian malaria, Ross concluded that mosquitoes fed on infected birds and took up male and female gametocytes which fertilized in the mosquito's gut. In the mosquito gut rod-like structures were produced that invaded the mosquito's salivary glands and these rod like structures were then injected into the new host, as the infected mosquito is feeding (Ross, 1898; Manson, 1898). Bignami and Grassi produced evidence that female anopheles mosquitoes would transmit malaria and described the whole-blood mosquito life cycle of *P. vivax*, *P. falciparum* and *P. malariae* (Grassi, 1899).

1.2 Overview of malaria

Malaria is a disease caused by a eukaryotic parasite of the genus *Plasmodium* (Cox, 2010; Miller *et al.*, 1994; Bannister *et al.*, 2000). Malaria transmission occurs globally and an estimated 3.3 billion people are at risk of being infected with malaria and 1.2 billion are at high risk (WHO, 2014). An estimated 198 million cases of malaria occurred globally in 2013 and 584 000 deaths were recorded. About 90% of the deaths occurred in the African region and mostly in children under the age of five years (WHO, 2014; Black *et al.*, 2003). The number of malaria cases seems to be on the increase due to increased transmission risks in areas where malaria control has declined, increases in the prevalence of drug resistant strains of parasites, and in a few cases, international travel to endemic regions and migration of people from endemic regions (Tangpukdee *et al.*, 2009; Pasvol *et al.*, 2005; Cui *et al.*, 2015; Sinha *et al*., 2014). Adults in endemic regions develop natural immunity against malaria. Though they may be prone to infection, they do not show symptoms of malaria but they are reservoirs of malaria (Miller *et al.*, 1994; Hafalla *et al.*, 2011).

Though slow, the development of a vaccine against malaria has reached an advanced stage (Schwartz *et al.*, 2012; Mordmüller *et al*., 2017). The strategies in vaccine development include multi-peptide vaccines such as RTS,S/AS02 (Bojang *et al.*, 2001; Snounou *et al*., 2005; Alonso *et al.*, 2005; Aponte *et al.*, 2007), multi-stage vaccines using chimeric proteins (Thiesen *et al.*, 2014), erythrocyte stage vaccine that uses Merozoite surface protein-1 (Moss *et al.*, 2012; Cavangh *et al.*, 2014; Fong *et al.*, 2015; De Silva *et al.*, 2016) and another that uses *Plasmodium falciparum* Recticulocyte Binding Protein homologue 5 (*Pf*RH5) (Wright *et al.*, 2014). Other vaccines being developed include subunit protein and sporozoite vaccines (Nuussenzweig *et al*., 1967; Nussenzweig *et al.*, 1989; Friesen *et al.*, 2010; Calvo-Calle *et al.*, 2005), anti cyto-adhesion vaccines (Magowan *et al.*, 1988; Bull *et al.*, 1998) and placental malaria vaccines (Salanti *et al.*, 2003; Salanti *et al.*, 2004; Viebig *et al.*, 2005). Even when a vaccine for malaria has been discovered, the accurate diagnosis and treatment with anti-malarials still remain an important part of malaria treatment and eradication of the disease (Moody 2002; Mouatcho and Goldring, 2013).

1.3 The life cycle of malaria parasite

The life cycle of malaria in the human host begins when the infected female *anopheles* mosquito injects sporozoites in the skin of the host during a blood meal (Baldacci and Menard, 2004) and during feeding the female *anopheles* mosquitoes releases the sporozoites along with their saliva anti-coagulants as part of their feeding process (Miller *et*

al., 1994). Only a fraction of the sporozoites released in the host survive the hostile environment in the blood, skin and lymphatic system (Kebaier *et al.*, 2009), and successfully invade the hepatocytes. The sporozoites possess an apical complex machinery involved in host cell invasion (Florens *et al.*, 2002). Approximately 10-100 sporozoites are injected per mosquito bite (Kappe *et al.*, 2010; Baldacci and Menard, 2004) in the exoerythrocytic or liver stage. The sporozoites in the lymphatic system move by gliding motion and finally invade the hepatocyte within 60 minutes (Frischknecht *et al.*, 2004; Amino *et al.*, 2007; Kebaier *et al.*, 2009). However, in *P. vivax* and *P. ovale* the sporozoites can be transformed into a dormant form known as hypnozoite for up to six months (Krotoski *et al.*, 1985; Fujioka and Aikiwa, 2002). The schizonts, with time, divide to occupy the entire cytoplasm of hepatocytes causing them to rapture and release the thousands of merozoites in the blood stream of the host (Miller *et al.*, 1994; Fujioka and Aikawa, 2002) as shown in Figure 1.1 step 1-4.

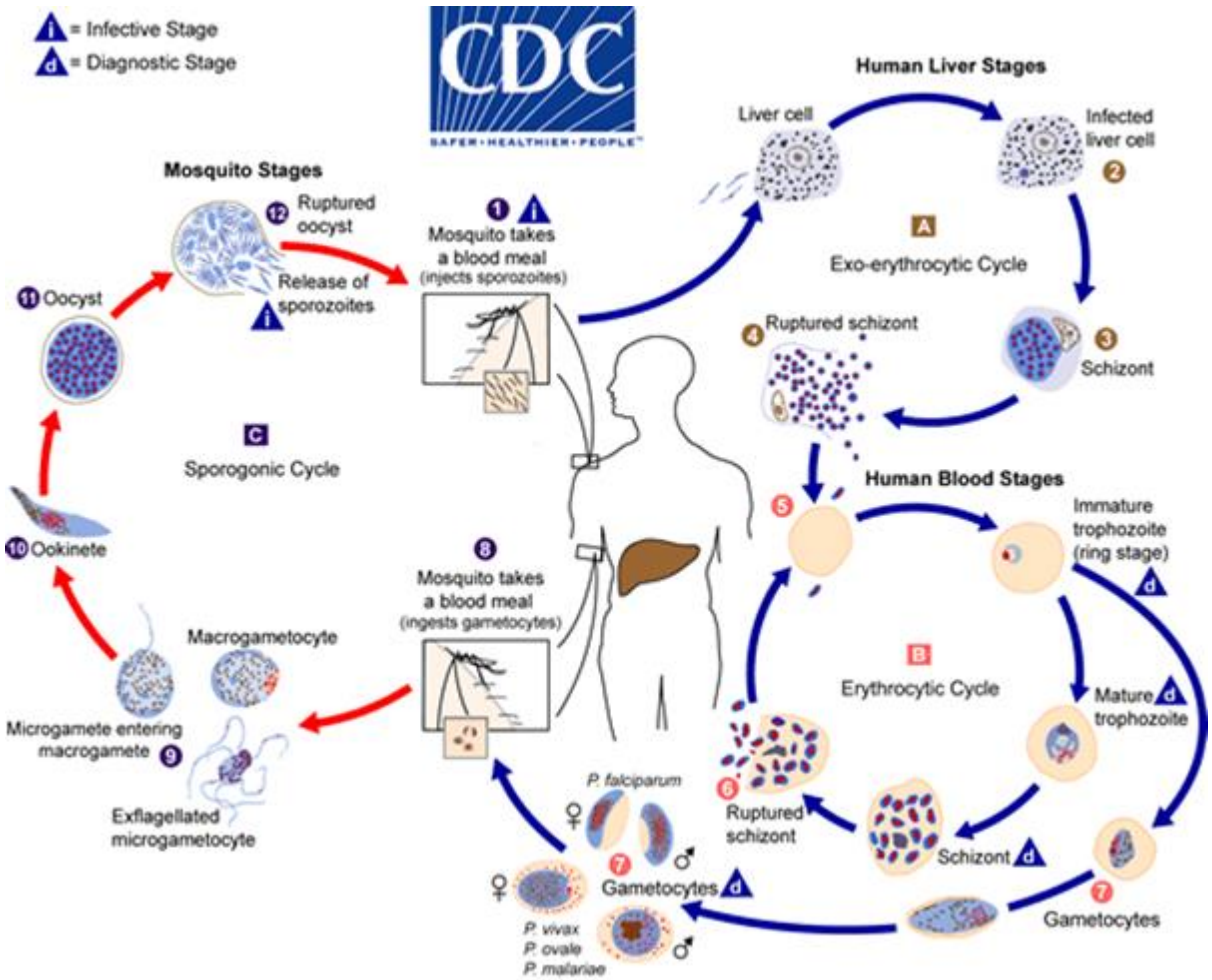


Figure 1. 1 The life cycle of a malaria parasite in the mosquito and human host. The liver stage or exoerythrocytic, erythrocytic or blood stage and the mosquito stages are shown here. The stage at which the

diagnostic targets (d triangles) are found and the infective stage (I triangles) are shown. Taken from CDC 2016 (https://www.cdc.gov/malaria/about/biology/).

The erythrocytic or blood stage begins when the released merozoites invade uninfected red blood cells. Within a few minutes of their release from the hepatocytes or host RBCs the merozoites recognise new target RBCs and parasite entry begins which lasts about 27.6 s after primary contact (Gilson and Crabb, 2008). The entry phase can be divided into two distinct phases. After invasion, an echinocytosis phases commences where a dramatic dehydration-type morphology is adopted by the infected RBC every 36 seconds. The infected RBC recover after a 5-11 minute period. At this time, the merozoites would have assumed the amoeboid-like state and is apparently free in the cytoplasm (Gilson and Crabb, 2008). The infected RBCs, now in the ring stage, then develop into the trophozoite stage at which point haemozoin begins to appear and finally they develop into the schizont stage after which the infected RBCs rapture and reinfection of new target RBCs occurs as shown in Figure 1.1, section B6 and 5. The erythrocytic stage has a cycle that ranges from 28-72 hrs depending on the infecting species of the malaria parasite (Cowman and Crabb, 2006; Aikawa *et al*., 1978; Lee *et al*., 2013; Murray *et al.*, 2008). This stage is responsible for the symptoms associated with malaria and is targeted for diagnosis as shown in Figure 1.1 blue triangle with the letter ”d” (Antia *et al*., 2008; Murray *et al*., 2008; Fong *et al*., 2015; Stansic *et al*., 2015; Piper *et al.*,1999; Lee *et al*., 2014; Sessions *et al*., 1997). The period of time from infection to appearance of symptoms is known as the incubation period and ranges from 12-40 days for other species and 6-12 months in *P. vivax*. The erythrocytic cycle repeats several times, after 3-10 cycles of red blood cycle reinfection, male and female gametocytes are formed at which point female *anopheles* mosquitoes can ingest the gametocytes.

Table 1.1 The approximate number of parasites per stage that are used to infect or are ingested by the host and vector, respectively.

Infection stage	Approximate number of parasites Per stage
Infected mosquito bite	10 - 100
Schizont liver stage rupture	10 000 - 30 000
Merozoite formed per infecting merozoite	14 - 32
Gametocyte ingested per blood	

meal of mosiquito	10 - 1000

The data was taken from Antia *et al.*, 2008; Smith and Craig, 2005; Fujioka and Aikowa, 2002; Baldacci and Menard, 2004; Kappe *et al.*, 2010; Miller *et al.*, 1994.

The gametocytes mature into gametes in the gut of mosquitoes (Fujioka and Aikowa, 2002).The parasite densities associated with each stage are shown Table 1.1.

During a blood meal, female *anopheles* mosquitoes ingest the gametocytes (MacCallum, 1898) into the mid-gut lumen of mosquitoes where the male gamete, which is flagellated (Microgamete), fuses with the female gamete which is non-motile (Macrogamete) to form a zygote (Kappe *et al.*, 2010). The zygote develops into an Ookinete that traverses the mid-gut epithelial cells. The Ookinete then becomes sessile and transforms into Oocysts. The Oocysts are the only parasitic developmental stage that grows extracellulary and the Ookinete nucleus inside the Oocyst divides (sporogony) resulting in the formation of sporozoites. The sporozoites are then released into the mosquito body cavity and invades the salivary glands (Hillyer *et al.*, 2007).

1.4 Species of malaria that infect humans

There are five species of malaria parasites that infect humans: *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and the recently discovered and formerly thought to only infect macaques *Plasmodium knowlesi* (Moutaucho and Goldring, 2013; Van den Eede *et al*., 2009; Van den Eede *et al*., 2010; Lee *et al.*, 2009; Singh & Daneshvar, 2010; Suh *et al.*, 2004; Tangpukdee *et al.*, 2009). A summary of the life cycles in the exoerythrocytic (liver) and erythrocytic (blood) stages are shown in Table 1.2.

1.5 Malaria endemic regions

All forms of human malaria are transmitted by female *anopheles* mosquitoes (Ross, 1923; Bynum, 1998; Baldacci and Menard, 2004; Kiszewski *et al.*, 2004), and malaria endemic region encompass all the areas in which these species of mosquitoes are prevalent.

The areas most affected are the tropical regions that include sub-Saharan Africa, East Asia and part of South America. Most of the developed North American and European countries have managed to eradicate the infectious species of mosquitoes and are thus regarded as non-transmitting regions. Figure 1.2 shows the regional distribution of *anopheles* mosquitoes and the prevalent species.

1.6 Vector control of malaria

In the mid-20th century, North America and most of Europe eliminated the malaria vector by spraying agricultural crops with an insecticide dichloro-diphenyl-trichloroethane (DDT) (Shiff, 2002). DDT was initially developed as a public insecticide prior to its widespread agricultural use and recognition as an environmental pollutant (Curtis & Lines, 2000)

Table 1.2 The data of the life cycle of the five human infecting species

	Plasmodium species				
	P. falciparum	***P. vivax***	***P. ovale***	***P. malariae***	***P. knowlesi***
Exo-erythrocytic phase (days)	5-7	6-8	9	14-16	No data
Erythrocytic cycle (hours)	48	48	50	72	24
Incubation period (days)	9-14	12-17; sometimes 6-12 months	16-18; sometimes more	18 – 40; or more	No data
Sporogony (days)	9-10	8-10	12-14	14-16	10-12
Hypnozoites (Dormant stage)	No	Yes	Yes	No	No
Maximum number of merozoites /RBC	32	24	20	14	16
Merozoites released/infected hepatocyte	30 000	10 000	15 000	10 000	No data

Type of RBC they infect	RBCs of all ages	Prefer young RBCs	Prefers young RBCs	Aging RBCs	No data available
Blood stream parasitaemias/µl	250 000	About 50 000	about 50 000	10 000	No data available

The table is summary of the life cycles in the exo-erythrocytic stage (liver) and erythrocytic stage (blood) of the five human infecting species. The data of the life cycle of the five human infecting species was collected from: Lee *et al*, 2009; Murray *et al.*, 2008; Aly *et al.*, 2008; Fujioka and Aikawa, 2002; Cox, 2010; D'Alessandro *et al.*, 2013.

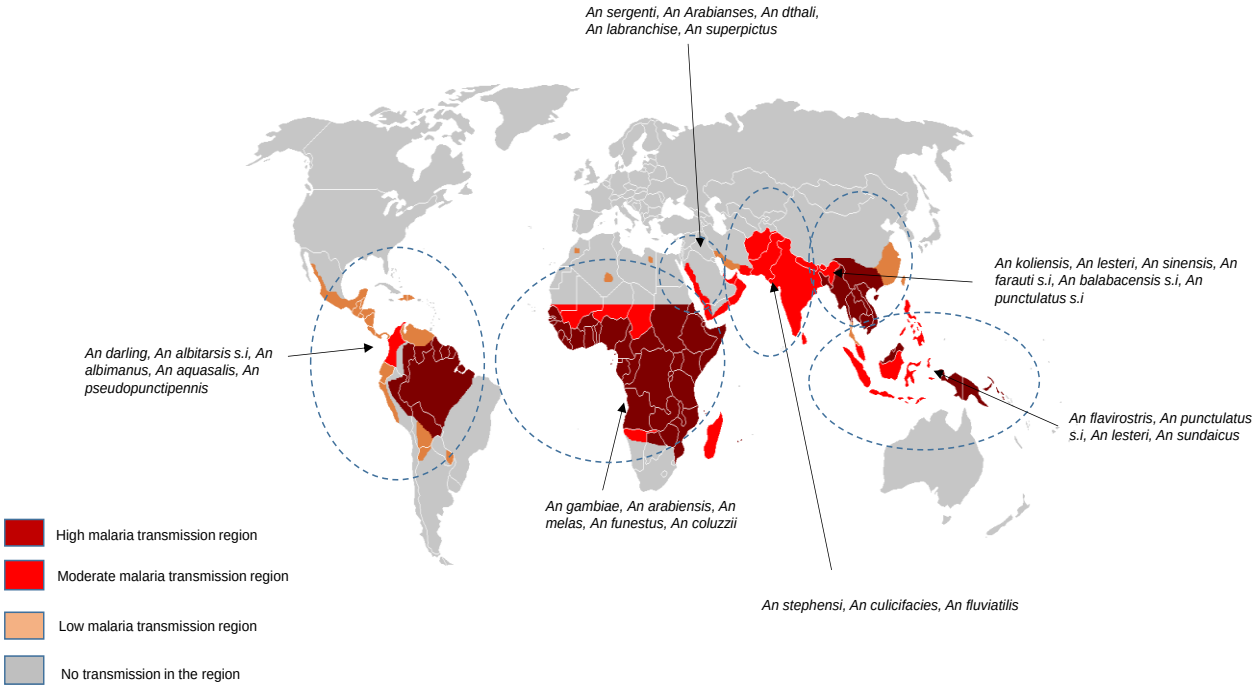


Figure 1. 2 Regional distribution of anopheles mosquito vector. The information was taken from the following sources: White, 1974; Fanello *et al.*, 2002; Choi *et al.*, 2013; Soleimani-Ahmadi *et al.*, 2015; Hay *et al*., 2010, Daneshvar *et al*., 2010; Abdoon & Alshahran, 2003; Kiszewski *et al*., 2004; Sinka *et al*., 2012. The key to the colours of regions is shown in the left bottom panel.

Insecticide classes include organochloride (DDT, hexachlorocyclohexane (HCH), dieldrin) (Goodman *et al.*, 1999, Goodman *et al*., 2001), organophosphates (malathion, temephose), carbamites (proposur, carbaryl), pyrethroids (synthetic compounds that resemble active constituents of flowers), insect regulator (diflubenzuron, methoprene, pyripoxyfen). Most of these classes have a serious impact on the environment (Arrow *et al.*, 2003). Around 1966 the emergence of resistance by *anopheles* mosquitoes to DDT became evident as 15 species were found to be resistant (WHO expert committee on insecticide, 1974). Also the environmental pollution of DDT was known, and as such, activists forced most governments in developed countries to discontinue its use (Shiff, 2002). Other methods included draining of the marshes, so that mosquitoes would have less breeding places.

Indoor residual spraying (IRS) was introduced to destroy the mosquito vectors that entered homes and it reduced mortality rates (Pluess *et al.*, 2010). Insect-treated mosquito nets were

also introduced and reduced the malaria mortality rate by about 55% in children under five years of age in sub-Saharan Africa, and reduced childhood death from other causes that are exacerbated by malaria (Lengeler *et al.*, 2004). In Gambia, Alonso *et al*, (1991) reported an overall childhood mortality reduction by 60% when the use of insecticide-treated mosquito nets was combined with malaria chemoprophylaxis. But, to be effective, insecticide-treated mosquito nets need to be used properly and must contain insecticides that are effective against killing mosquitoes. With the prevalence of insecticide-resistant mosquitoes, this method may become ineffective and thereby increase the re-emergence of malaria.

Other vector control measures introduced include House Siting and construction, by building houses far from mosquito breeding sites was demonstrated. There was a reduction of malaria cases (Trape *et al.*, 1992). Zooprophylaxis was used, where animals were used to divert mosquitoes from biting humans, however in some cases the opposite happened (Hewitt *et al.*, 1994; Booma and Rowland, 1995; Mouche, 1998). The introduction of repellants, aerosols and fumigation reduced mosquito bites (Snow *et al.*, 1987; Lindsay *et al.*, 1998). Environmental and biological management of breeding areas, such as the use of petroleum oil for larvicide, draining of pools, salinating of coastal marshes reduced breeding of mosquitoes (Romi *et al.*, 1993; Karch *et al.,* 1993).

1.7 Treatment of malaria and the development of drug resistance

The earliest recorded treatment of malaria came from the use of the Cinchona tree by Native Americans around 1934, which contains quinine. In China, the Chinese herb Quinghaosu (Artemisinin) was used to reduce fevers associated with malaria as early as 340 AD as recorded in the Zhou Hou Bei-ji Fang handbook (Klayman, 1985). In 1934, a German, Hans Andersag discovered Resochin, which later was named chloroquine. Because of World War II, chloroquine was not recognised until 1946. During the 1960s and 1970s chloroquine began losing its effectiveness against *P. falciparum* malaria in Asia, and later in Africa around the 1980s. This was caused by genetically-mediated chloroquine resistance appearing in the parasite. Because of poor diagnostic techniques that existed in that era, symptoms similar to malaria were treated as malaria, until proven otherwise (Perkins and Bell, 2008; Okiro and Snow, 2010), which lead to the overuse of chloroquine by many clinicians. Pyrimethamine, doxycycline, quinine and primaquine have been used to treat malaria.

Malaria treatment is dependent on the geographical location of the infection and the type of infecting species. Species of importance such as *P. vivax* and *P. ovale* have hypnozoites that are reservoirs of patient infections as shown in Table 1.2 and Table 1.3.

Table 1.3 Antimalarial drugs used and their targeted stages, mutation positions in corresponding resistant allele's and the regions where resistance in the drugs has been reported.

Drug name	**Organism targeted**	**Mutations in corresponding resistant alleles**	**Malaria stage targeted**	**Region showing resistance**
Chloroquine	*P. falciparum, P. vivax, P. ovale, P. marlariae*	CRT, MDR	Gametocyte in blood stage	Aisia, Africa, central and South America
Mefloquine	*P. falciparum, P. ovivax, P. ovale*	DHFR, Cytochrome b	Schizont in blood stage	
Halofantrine	*P. falciparum*	DHFR, Cytochrome b	Blood stage	
Lumefantrine	*P. falciparum*	DHFR, Cytochrome b	Blood stage	
Pyrimethamine	*P. falciparum, P. vivax*	DHFR, Cytochrome b	Blood stage by stopping DNA synthesis in parasites	
Cycloguanil	*P. falciparum*	DHFR, Cytochrome b	Schizonts in blood stage in children	
Chlorocycloguanil	*P. falciparum*	DHFR, Cytochrome b	Schizonts in blood stage in children	
Atovaquone	*P. falciparum*	DHFR, Cytochrome b	Asexual and sexual blood stage. Used for prophylaxis	
Sulfonamide (Fancidar)	*P. falciparum*	DHPS	Schizonts and asexual blood stages. Also used for	

			chemoprophylaxis	
Quinine	*Drug resistant P. falciparum*	DHFR	Blood stage	Thai border, Myanmar
Artemisinin (Qinghaosu)	*P. falciparum, P. vivax, P. ovale, P. marlariae, P. knowlesi*		Asexual and gametocytes in blood stage.	Asia, Africa
Amodiaquine	*P. falciparum*		Blood stage	
Clindamycin	*P. falciparum*		Schizonts in blood stage, especially in pregnant women	
Tetracycline	*P. falciparum*		Blood stage	
Doxycycline	Drug resistant *P. falciparum*		Blood stage	
Primaquone	*P. vivax, P. Ovale, P. falciparum* gametocytes		Liver hypnozoites, schizont and gametocytes in blood stage	
Sulfones	P. falciparum	DHPS	Schizont and asexual blood stage, chemoprophilaxis	
Rifampicin	*P. falciparum, P. vivax, P. ovale, P. marlariae*		Liver and blood stage	
Fosmidomycin	*P. falciparum, P. vivax, P. ovale, P. marlariae*		Liver and blood stage	

A summary of antimalarial drugs used and their targeted species, the targeted stage, mutation positions in corresponding resistant allele's and the regions where resistance in the drugs has been reported. Chloroquine resistance transporter, Multidrug resistance p-glycoprotein pump, Dihydrofolate reductase, Dihydropteroate synthase. Data was adapted from Bruce-Chwatt, 1981; Kappe *et a*l., 2010; Lallo *et al.*, 2007; Suh *et al.*, 2004; Peterson *et al.*, 1998; Kublin *et al*., 2003; D'Alessandro *et al.*, 2013.

Drug resistance in malaria has been associated with protein transporters such as the Chloroquine resistance transporter (CDR), Multidrug resistance p-glycoprotein pump (MDR), dihydrofolate reductase (DHFR), dihydropteroate synthase (DHPS), cytochrome b of different species against different antimalarials as shown in Table 1.3 (Bruce-Chwatt, 1981; Kappe *et al*., 2010; Lallo *et al.*, 2007; Suh *et al.*, 2004; Peterson *et al.*, 1998; Kublin *et al*., 2003; D'Alessandro *et al.*, 2013). Table 1.3 shows the different types of drugs used to treat malaria, the type of *Plasmodium species* they target, the stage targeted and resistance that has been reported on the drug with its corresponding mutation in the resistant allele.

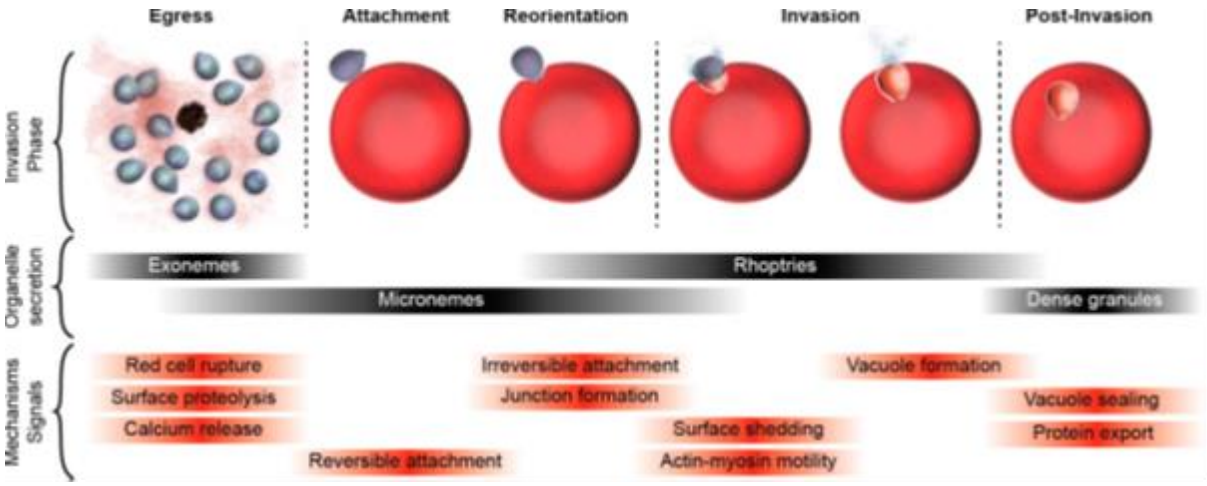


Figure 1.3 A time course of merozoite invasion of the erythrocyte. A cellular overview is given with associated timing of organelle secretion and key mechanistic or signalling steps listed below. After apical reorientation, the merozoite establishes a tight junction that is marked by RON4 and AMA1. The tight junction is ultimately connected to the actomyosin motor, although the exact nature of this has yet to be established. As the tight junction moves across the merozoite surface, proteins are shed into the supernatant through the activity of proteases such as ROM4, ROM1, SUB1, and SUB2. The parasitophorous vacuole and membrane are formed primarily from the rhoptries, although some red blood cell membrane components are included, which expel their contents, forming the space into which the parasite can move under the action of the actomyosin motor. Once the tight junction reaches the posterior end of the parasite, the membranes seal by an as yet unknown mechanism. Adapted from Cowman *et al*, 2012.

To combat widespread resistance against malaria drugs, the WHO recommended the use of Artemisinin-based combination treatment (ACT). ACT has reduced the incidence of *P. falciparum* (Nosten *et al.*, 2000).

As a result of high prevalence of malaria drug resistance to antimalarial treatment, there is a great need for diagnostic testing that is fast, accurate, reliable and that can be used to monitor treatment (Gupta *et al.*, 2015).

1.8 Diagnosis of malaria in the human host

Malaria diagnosis mostly targets the erythrocytic (blood) stage of parasite growth (Bannister *el al.*, 2000; Parra *et al.*, 1991; Calderwood *et al.*, 2007; Beere & Green, 2001; Desakorn *et al.*, 2005; Howard *et al.*, 1986). The erythrocytic stage begins with the merozoites either from the exoerythrocytic stage or from another erythrocytic stage infecting fresh red blood cells as shown in Figure 1. 3.

This stage has high levels of parasitaemia (Table 1.2) and is associated with the symptoms of malaria (Miller *et al*., 2002; Antia *et al*., 2008; Miller *et al*., 1994). The parasite changes its morphology as it changes from one stage to another as depicted in Figure 1.4. These stages can be identified using a microscope and appropriate stain (Moody, 2002). As the ring stage develops and grow asexually, haemozoin begins to appear. The gametocytes of the parasite are found in the peripheral blood for several days after their release (Fujioka and Aikowa, 2002). Other methods of diagnosis make use of the presence of specific proteins released during the erythrocytic stage, associated with the parasite as described later.

1.9 How to measure the performance of diagnostic tests

Most malaria endemic countries are poor. In these countries the choice of the diagnostic method used is often based on the available skilled labour, presence or absence of electricity, easy use and interpretation of results (Wongsrhrichanalai *et al.*, 2007). A criterion was developed to compare the performance of different tests. The Gold standard being microscopy (WHO, 1999; Mouatcho and Goldring, 2013, Murray *et al.*, 2008). The criteria use specificity, sensitivity and limit of detection (Bharti *et al*., 2008, Murray *et al*., 2008). Specificity is how accurate a method is in identifying the infecting specie of *Plasmodium*. Sensitivity refers to the ability by the test method to detect infection out of a total number of positive and negative samples. Limit of detection (LOD) refers to minimum number of parasites per microliter that can be detected by the test (Bharti *et al.*, 2008, Murray *et al.*, 2008).

1.10 Methods of malaria diagnosis

Diagnosis of malaria in the blood of patients involves identification of *Plasmodial* DNA, proteins and/or antigens and antigen products in the blood (Conroy *et al.*, 2009). Some

methods were developed in which malaria infections were identified in the urine and saliva of patients (Buppan *et al.*, 2010; Nwakanama *et al.*, 2009; Sutherland and Hallet, 2009).

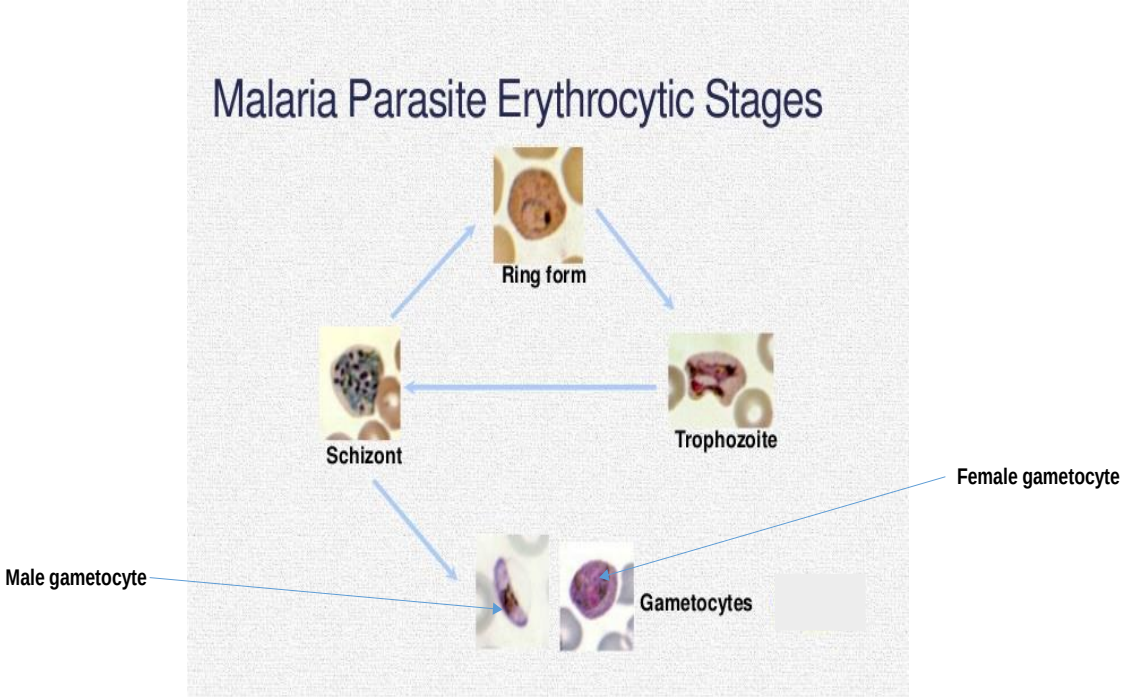


Figure 1.4 Erythrocytic stages of malaria parasite development. The data was described by Cowman *et al.*, 2012; Bannister *et al.*, 2003; Bannister *et al.*, 2000.The ring stage lasts about 0-5 hr, early trophozoite 10-20 hr, late trophozoite 10-20 hr, schizont > 40 hr. After several cycles of erythrocytic development male and female gametocytes are formed. The rupture of schizonts releases new merozoites and haemozoin.

1.10.1 Clinical diagnosis

During the erythrocytic stage of malaria, patients develop clinical symptoms of malaria such as fever, headaches, chills and sweating that are associated with the rupture of the schizonts (Miller *et al.*, 1994; Antia *et al.*, 2008). The parasitaemia, erythrocytic cycle and the incubation time of each infecting species has been detailed in Table 1.2. Other symptoms manifested during malaria include acute respiratory distress, jaundice, renal failure, coma and vomiting (Das, 2008; Wilairatana *et al.*, 2013). Most tropical diseases, especially the mosquito vector borne diseases such as dengue share common symptoms like fever (Kallander *et al*., 2004) which leads to mis-diagnosis of malaria (Perkins and Bell, 2008; Gwer *et al*., 2007). Presumptive diagnosis is reported to have reduced mortality (Kidane and Morrow, 2000), but can also lead to development of drug resistant strains of parasite due to overuse of antimalarials (Murray *et al.,* 2008).

1.10.2 Diagnosis of malaria with microscopy

In 1880, Charles Laveran visualised the protozoan parasite that causes malaria in a blood smear under a microscope (Sutherland and Hallet, 2009; Cox, 2010; Laveran, 1881). In 1891, Dimitri Romanowsky accidentally discovered that methylene blue eosin stains the parasite nucleus red and cytoplasm blue (Garnham, 1966) which became the basis of staining blood of infected patients in thin and thick films of patient blood (Sutherland and Hallet, 2009). Microscopy makes use of the fact that the mature RBCs do not contain a nucleus (Moody, 2002; Suh *et al.*, 2004). The eosin dye, therefore, stains the parasite DNA making the parasite visible. Thin blood films of parasitised blood are fixed by methanol and stained with diluted Giemsa or Wright's stain (Moody, 2002). Because the parasite is fixed in a monolayer of peripheral blood cells, identification of parasite morphology is possible, and parasites can be enumerated and the species of malaria identified. Thick blood films have many layers of RBC on a small surface stained with Field's or Wrights stain without fixation. This method has greater sensitivity than a thin film, and can detect 50 parasites/µl of blood. It is used for detecting low numbers of parasites and the reappearance of circulating parasites during recrudescence or relapse as observed in *P. vivax* and *P. ovale* infections (Moody *et al.*, 2002).

1.10.3 Detecting malaria with fluorescence microscopy

Fluorescence microscopy uses fluorescent dyes that have an affinity for nucleic acid molecules in the parasite nucleus. Commonly used dyes include acridine orange (AO) (Gaye *et al*., 1999; Kawamoto *et al*., 1991a, Kawamoto *et al*., 1991b; Lowe *et al*., 1996) and AO in the quantitative buffy coat system (QBC) (Baird *et al*., 1992; Clendeman *et al*., 1995, Craig and Sharp, 1997), Benzothiocarboxypurine (BCP) (Makler *et al*., 1991; Cooke *et al*., 1992) and Rhodamine-123 (Srinavasan *et al*., 2000). When stained with dyes, the nucleus fluoresces strongly under UV light at an appropriate wavelength.

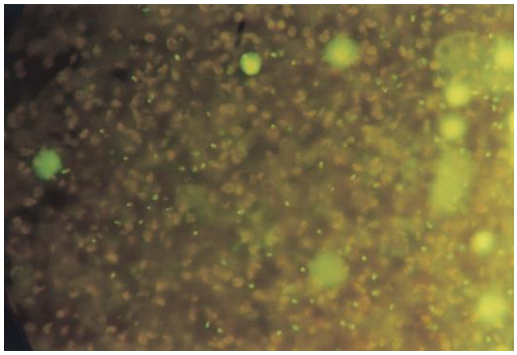


Figure1.5 Trophozoites of *P. falciparum* stained with AO in the QBC fluorescence method. The figure was taken from Moody, 2002.

Rodamine123 is used to access the viability state of parasites, because it is taken up only by membranes of living cells (Srinavasan *et al*., 2000; Makler *et al.*, 1991). Another fluorescent method is the QBC which uses acridine orange after white blood cells (WBC) and platelets have been separated (Baird *et al*., 1992), leaving the parasite to settle below the layer of cells as shown in Figure 1.5. Alternatively BCP is applied directly to lysed blood suspension or to unfixed dry blood film and stains the nucleic acid of living *P. falciparum* parasite as shown in Figure 1.6. A gold nanoparticle-based fluorescence immunoassay for malaria antigen detection has been developed (Guirgis *et al.*, 2011) and a method based on fluorescence spectra of a set of blood plasma biomolecules such as tyrosine, tryptophan, nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) and red blood cells-associated porphyrin is under development (Masilamani *et al.*, 2014).

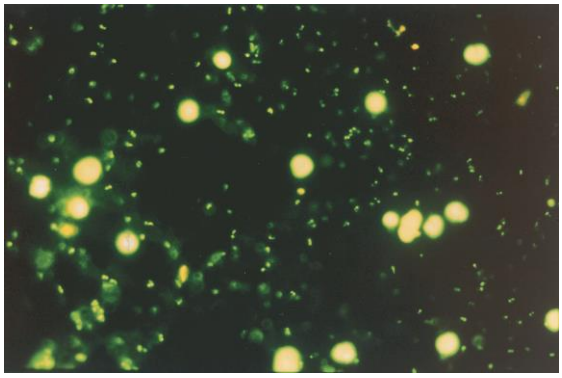


Figure 1.6 Trophozoites of *P. falciparum* stained with benzothiocarboxypurine (BCP) in the fluorescence method. The figure was taken from Moody, 2002.

1.10.4 Colorimetric detection of malaria in patients using immunomagnetic or nano-particle capture

Antibodies against a biomarker for malaria such as parasite LDH have been couple to magnetic beads and used to measure enzyme activity, (Markwalter *et al.*, 2015) or HRPII interacton with gold nan-particles (Gulka *et al.*, 2015). In the reaction, *P*LDH in the infected lysed blood sample oxidises lactate to pyruvate, while reducing the cofactor acetylpyridine adenine dinucleotide ($APAD^{+)}$ to acetylpyridine adenine dinucleotide hydrogenate (APADH). The APADH in turn reduces a yellow tetrazolium dye nitro-blue tetrazolium (NBT), to a blue compound (diformazan) with the assistance of phenazine ethosulfate (PES). The reaction is measured at 650 nm (Makler *et al.*, 2004; Makler and Hinrichs, 1993; Makler *et al*., 1998). Limit of detection (LOD) is as low as 21 parasites/µl. Advantages of *P*LDH is that *P*LDH is conserved across all five species of *Plasmodium* known to infect humans (Makler *et al*.,

2004), *P*LDH activity is lost within 24 hrs post treatment and, therefore, can be used in the management of malaria and measuring drug susceptibility (Markwalter *et al*., 2015). Another magneto-colorimetric method uses the detection of malaria pigment hemozoin in the intraerythrocytic stage (Orban *et al.*, 2016). Malaria parasites break down haemoglobin in the food vacuole as a source of amino acids. This releases free haematin a toxic substance which inhibits parasite enzyme activity and releases free radicals (Bannister *et al.*, 2000; Olszewsk *et al.,* 2011). The parasite, therefore, polymerises haematin to haemozoin, which begins to appear in the late ring stage as a brown pigment (Dossert *et al.*, 2009). The LOD of 50 parasites/µl was obtained which exceeded that of RDTs and competes with the threshold achievable by light microscopic observation of blood smears. Another potential colorimetric method uses Cu^{2+} peroxidase-catalytic activity, which can catalyse H_2O_2-mediated oxidation of peroxidase substrate and obtain the oxidation product colour change (Zheng *et al.*, 2016; Xianyu *et al.*, 2013). This method has the potential to detect many malarial proteins that interact with Cu^{2+}, such as HRPII proteins.

1.10.5 Detecting malaria with the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) amplifies and detects specific sequences of malaria parasite nucleic acid (DNA or RNA) in whole blood lysates. A number of PCR assays have been developed. Some methods use sequences coding for small subunit ribosomal RNA (ssrRNA) (Snounou *et al*., 1993), 18S rRNA (Echeverry *et al*., 2016; Kawamoto *et al*., 1996; Gunderson *et al*., 1987; Water, 1994), sequences coding for specific genes such as dihydrofolate reductase-thymidylate synthase (DHFR-TS) (Wataya *et al*., 1993; Wataya *et al*., 1991), direct PCR combined with a rapid readout system, nucleic acid lateral flow immunoassay (Mens *et al*., 2012), amplified *Plasmodium* DNA probed with a specific DNA probe (Barket *et al*., 1992). A single-step direct-PCR uses the cytochrome oxidase III gene (COX-III) to detect microscopic and submicroscopic malaria infections in humans (Echeverry *et al.*, 2016). Some methods use species-specific fluorescein such as 4',6-diamidino-2-phenylindole (DAPI) or radio labelled probes for detection of *P. vivax* DNA on a microscope that uses a rechargeable battery (Sethabutr *et al.*, 1992). PCR has high sensitivity with a LOD of < 5 parasites/µl of blood and is species specific (Snounou *et al*., 1993). However, PCR requires highly skilled labour, electricity, very expensive equipment and reagents. Investigations showed that disappearance of parasite *P. falciparum* using PCR was achieved for a median 144 hrs compared to 66 hrs for microscopy (Kain *et al.*, 1994) and that the DNA of non-viable parasites may be detected from circulating blood (Srinavasan *et al.*, 2000).

1.10.6 Diagnosis of malaria with loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) was described as a novel DNA amplification method by Notomi *et al*, 2000.LAMP is an isothermic nucleic acid amplification using four different primers and a polymerase to identify six distinct regions on the target gene. Intensive amplification leads to accumulation of magnesium pyrophosphate, which turns the solution turbid, and this turbidity is visible to the naked eye or can be measured using a turbidity meter (Notomi *et al*., 2000). Poon *et al*, (2006) first reported the use of LAMP for the diagnosis of clinical malaria caused by *P. falciparum*.Han *et al*., (2007) designed primers that target 18S rRNA gene in a genus- and species-specific LAMP method that detected four *Plasmodium* species. Goto *et al*, 2009 introduced the concept of pre-addition of hydroxylnaphthol blue (HNB) as a cheap and easily visible dye in the reaction mixture that does not inhibit the amplification of target nucleic acid. The use of 18S rRNA as a target of *P. falciparum* in whole blood samples for the diagnosis of clinical malaria has been used recently (Mohon *et al.*, 2014; Gupta *et al.*, 2016). This method has also been used to detect other parasitic diseases such as visceral leishmaniasis (Khan *et al.*, 2012). LAMP has been reported to have detected low-density malaria parasites in Zanzibar (Cooke *et al.*, 2015). Two LAMP assays have been developed for use in detecting *P. knowlesi* which is difficult to identify with a microscope and RDTs (Jeremiah *et al.*, 2014). One of the problems associated with LAMP is DNA contamination (Cooke *et al*., 2015).

1.10.7 DNA aptamers used to diagnose malaria

DNA aptamers are specific oligonucleotides, peptides or chemical molecules that bind specifically to target DNA. DNA aptamers specific for a malaria biomarker can be used to diagnose malaria (Godonoga *et al*., 2016). Some DNA aptamers can be tethered with a capture enzyme where the product can be measured colorimetrically (Dirkzwager *et al*., 2015). . Jain *et al.,* (2016a) used a novel ssDNA developed through systematic evolution of ligands by exponential enrichment (SELEX) to detect *Pf*LDH. Aptamers have several advantages over antibodies such as, ease of chemical synthesis, thermal stability and reduced cost (Cho *et al*., 2009). Jain *et al.,* (2016b) developed a 90 mer ssDNA aptamer against *Pf*LDH.

1.10.8 Antibody based diagnosis of malaria

Enzyme-linked immunosorbent assay (ELISA) is based on the use of specific antibodies to capture target antigens in solution that is accompanied by a colour change. Antigens specific for malaria parasites have been used in ELISA (Wongsrhrichanalai *et al.*, 2007; Spencer *et al.*, 1979; Inoue *et al.*, 2013; D'Allessandro *et al*., 2013; Sousa *et al.*, 2014; Piper *et al.*,

1999). ELISA can also be used to detect gametocytes in the asexual blood stage (D'Allessandro *et al.*, 2013). Antibodies can be used to detect malaria antigens using an indirect immunofluorescence antibody assay (IFA) (Sulzer *et al.*, 1969; Wongsrhrichanali *et al.*, 2007). Another test, the Latex agglutination assay is based on specific agglutination of sensitive polystyrene particles that contain carboxylic acid with antigen or antibody molecules in the presence of corresponding antibody or antigen, respectively, in human plasma (Polpanich *et al*., 2007).

Malaria antigens can be detected in the serum of infected blood using ELISA or IFA based on the presence of antibodies against malaria proteins like MSP-1 (She *et al.*, 2007; Doderer *et al*., 2007). These antibodies in serum may persist in blood for longer periods (Makler *et al.*, 1998) and as such may not detect a current infection.

1.10.9 Flow cytometry

Flow cytometry uses fluorescent dyes such as Hoechst 33258 to detect malaria parasites and monitor antimalarial drug sensitivity (Woodrow *et al.*, 2015; Van-Vianen *et al*, 1993; Hanscheid, 1999). A laser flow cytometry system may detect the malaria pigment hemozoin in monocytes (Grobusch *et al.*, 2003; Wongchotigul *et al.*, 2004; Shapiro & Mandy, 2007) or detect stained *P. falciparum* DNA at different stages using SYBR Green I (Izumiyama *et al.*, 2009). Hemozoin within the phagocytes can be detected by depolarisation of laser light as cells pass through a flow cytometer (Padial *et al.*, 2005). Though flow cytometry offers automated counts of parasitaemias, this is offset by low sensitivity due to background noise (Janse & Van-Vianen, 1994).

1.11 Rapid diagnostic tests

The WHO recognised the urgent need for a new, simple, quick, accurate and cost effective diagnostic test for the detection of malaria parasites to overcome the deficiencies of using microscopy (WHO, 2000). This led to the increase in the use of RDTs for malaria detection, as affected regions lack electricity and skilled manpower required to do microscopic examinations (Bell *et al*., 2006; Tangpukdee *et al*., 2009). The use of RDT is quick, requires basic skills and does not require electricity. Although most RDTs target *P. falciparum* specific proteins such as histidine rich protein II (HRPII) (Leow *et al.*, 2014; Verma *et al.*, 2014; Laurent *et al*., 2010; Ho *et al*., 2014), other RDTs target lactate dehydrogenase (LDH) (Shin *et al.*, 2013; Otsuki *et al.*, 2015; Miao *et al.*, 2013; Brown *et al.*, 2004) or both HRPII and LDH (Bharti *et al.*, 2008). Other proteins targeted as antigens include aldolase (Wongsrhrichanalai *et a*l., 2007), phosphoethanolamine N-methyltransferase (PMT) (Pessi *et al*., 2004) and heat shock protein 70-1. Recently a new RDT for detecting *P. knowlesi* has

been developed (McCutchan *et al.*, 2008). Many RDTs on the market are used to diagnose *P. falciparum* only (Lee *et al.*, 2008; Park *et al.*, 2006; Kim *et al.*, 2008).

1.11.1 How RDTs work

RDTs use a chromatographic nitrocellulose membrane strip as a solid phase upon which a lysed infected blood sample moves by capillary action. This strip is fixed with antibodies at a capture point coupled to colloidal gold-nanoparticles against a *Plasmodium* antigen epitope. To capture the antigen in the infected blood, the antibodies on strip bind to the antigen and produce a colour change as shown in Figure 1.7.

A second capture point with non-specific antibodies conjugated with colloidal gold nan-particles against a protein such as human serum albumin (HSA) are fixed at a point further than the capture point to act as a control that shows the test works (Makler *et al.,* 1998; Murray *et al.*, 2008) as shown in Figure 1.7.

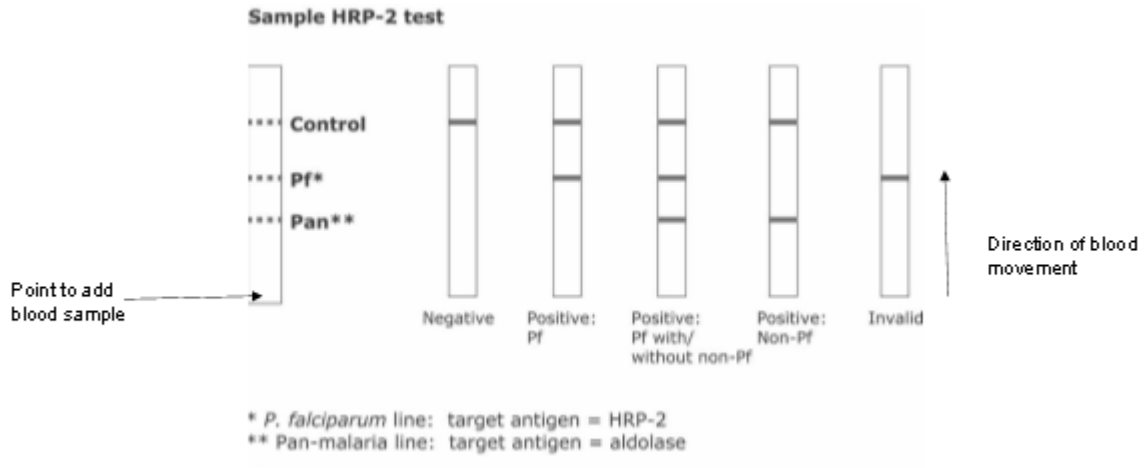


Figure 1.7 Sample test line configurations on commercial RDTs that detect malaria and their result interpretation. Taken from Wongsrhrichanalai *et al*., 2007.

There are more than 86 commercially available RDTs for malaria (Tangpukdee *et al.*, 2009). Figure 1.7 also shows how blood with mixed infection samples are electrophoresed and interpreted on an HRPII/aldolase RDT (Wongsrhrichanalai *et al.*, 2007).

1.11.2 Why RDTs need to be improved

Rapid diagnostic tests have shown problems that relate to the device itself and problems associated with the antigen/antibody targeted for detection. In order to improve performance of RDTs, problems associated with transport, storage and durability of antibodies used in RDT have to be understood.

1.11.3 False negative or invalid results caused by high temperature and humidity

RDTs can be damaged by extreme temperatures of over 40°C and humidity (Jorgenesen *et al*., 2006; Chiodini *et al*., 2007). Most RDTs are used in tropical regions where temperatures are over 40°C (Jorgensen *et al.*, 2006). LDH based RDT were found to be more thermal stable than HRPII based RDTs (Chiodini *et al.*, 2007). This may lead to the test showing a positive result without a confirmatory test line, making the result a false negative or invalid test (Wongsrhrichanalai *et al.*, 2007). The need to transport RDTs to field clinics using refrigerated transport was recommended (Wongsrhrichanalai *et al.*, 2007).

1.11.4 False positive test due to device

Approved buffers sometimes runout or are lost in the field. Therefore, use of distilled water or tap water as a substitute of approved buffers has shown to yield false positive results (Gillet *et al*., 2010). Now RDTs supply buffers in each box containing RDTs.

1.11.5 Antigen based diagnostic problems

A RDT may give false results if there are factors that interfere with antibodies to detect corresponding epitopes in the antigen.

1.11.6 False negative results

Gene deletion and single nucleotide polymorphism (SNP) in the DNA of *P. falciparum* HRPII has been reported in Peru and caused false negative results in HRPII-based RDT tests (Gamboa *et al.*, 2010; Baker *et al.*, 2010; Lee *et al.*, 2006a, Solano *et al.*, 2015; Okoth *et al.*, 2015). LDH has also shown to have SNPs in some isolates of *P. vivax* (Shin *et al.*, 2013; Cicek *et al.*, 2013), though no false negative has been reported as a result. Low parasite densities can also cause a false negative result (Palmer *et al.*, 1998; Lee *et al.*, 2007).

The prozone effect caused by excessive HRPII antigen concentration has resulted in false negative tests in *P. falciparum* infected blood samples (Gillet *et al.*, 2009; Ho *et al.*, 2014; Ho *et al.*, 2015). LDH RDTs were not affected by the prozone effect.

1.11.7 False positive results

HRPII persists in blood for upto 28 days after parasite clearance. Therefore HRPII-based RDTs may test positive in the absence of viable parasites (Iqbal *et al.*, 2004; Tjitra *et al.*, 2001; Vakharia 1997; Kodisinghe *et al.,* 1997; Humar *et al.*, 1997). LDH and aldolase antigens persist less than 2-7days (Iqbal *et al.*, 2004; Ashley *et al.*, 2009; Murray *et al.*, 2008; Markwalter *et al*., 2015) and can, therefore, be used to monitor the treatment of malaria. LDH and aldolase are produced during gametocyte stages of the asexual erythrocytic stage and, as such, low levels of these antigens may be present if the gametocytes are not cleared in the blood of infected humans (Vakharia *et al.*, 1997; Farcas *et al.*, 2003; Tjitra *et al.*, 2001; Murray *et al.*, 2008).

1.11.8 Rheumatoid factor

Rheumatoid factor can react with the antibodies employed in RDTs and give false positive results for RDT (Mishra *et al.*, 1999; Jelinek *et al*., 2001; Karbwang *et al*., 1996, Sing *et al.*, 2000, Iqbal *et al.*, 2000; WHO, 2000). Rheumatoid factors are autoantibodies against the F_c region of IgG and were found to react with the mouse IgG isotype monoclonal antibodies often used in RDTs (Iqbal *et al.*, 2000). The Fc portion of lgG molecules is made of a heavy chain at the carboxyl termini involved in the activation of complement by binding effector molecules or immune Fc receptors (Delves *et al*., 2006). Producing antibodies in chicken can circumvent this problem as chicken antibodies are in form of lgY.

Other cross reaction were observed in LDH and aldolase based-RDTs in which *P. vivax*-specific LDH based RDT cross reacted with and detected *P. knowlesi* (Kawai *et al.*, 2009; McCutchan *et al.*, 2008).

1.12 Quality assurance of RDTs

In 2008, the WHO recommended a series of tests for RDTs to improve the quality of RDTs and delivery of quality product (Wongsrhrichanalai *et al.*, 2007). Among the initiatives outlined were guidelines for testing RDTs, standardising manufacturing process and distribution (Wongsrhrichanalai *et al.*, 2007) and evidence of good manufacturing practise (Bell *et al.*, 2006). The WHO, working together with Foundation for Innovated New Diagnostics (FIND), has established and offers a testing program to evaluate performance of RDTs (WHO, 2009, 2010, 2011b; Mouatcho & Goldring, 2013). Positive controls have been developed (Murray *et al.*, 2008; Lon *et al.*, 2005) to ensure RDT sensitivity is maintained after delivery to the field (Lon *et al.*, 2005; McMorrow *et al.*, 2008).

1.13 Why improve malaria RDT?

The limit of detection (LOD) of current RDTs is about 50-100 parasites/µl (Tangpedkee *et al*., 2009). Therefore, there is a possible need to look at alternative proteins so that detection of malaria can be improved. This requires RDTs to detect <100 parasites/µl that act as reservoirs (Hans *et al*., 2010; Miller *et al*., 1994; Hafalla *et al*., 2011). Identifying other parasite antigens can be an added advantage. If these alternative antigen can be used to detect viable parasite, clear in peripheral blood within a short period and are present in higher concentration in the intraerythrocytic stage they, therefore, can be potential diagnostic targets.

1.13.1 *Pf*LDH as target antigen in malaria RDTs

Plasmodium LDH (*P*LDH) is a metabolic protein and is associated with a viable parasite (Lang-Unnasch and Murphy, 1998; Vaidya and Mather, 2009; Brown *et al.*, 2004). *P*LDH-based RDTs reverted back to negative within 2-7 days after clearance of infection (Iqbal *et al.*, 2004; Murray *et al.*, 2008; Markwalter *et al*., 2015). The presence of *P*LDH can be used to measure a current infection. *P*LDH was found not to produce the prozone (Gillet *et al.*, 2009; Martin *et al.*, 2009). Aldolashas little antigenic variation and single nucleotide polymorphism, and has been found to show no false negative results (Lee *et al.*, 2006b). *P*LDH has a conserved catalytic residue involved in enzyme activity and shares > 90% amino acid identity among *Plasmodia* species (Brown *et al.*, 2004; Turgut-Balik *et al.*, 2004). Because *P*LDH is a conserved enzyme across all species and all malarial *P*LDH sequences share common epitopes (Hurdayal *et al*., 2010) it can, therefore, be used to detect pan-specific malaria (Makler and Hinrichin, 1993; Piper *et al.*, 1999; Brown *et al.*, 2004). Differences in the *P. falciparum, P. knwolesi* and *P. vivax* LDH amino acid sequences are present that can allowto differentiate between the among thesespecies (Hurdayal *et al.*, 2010; McCutchan *et al.*, 2008; Piper *et al.*, 2011). *P*LDH-based RDTs does not detect low parasitaemias (Ashley *et al.*, 2009, Abba *et al.*, 2011, Kattenburg *et al.*, 2011, Hendrisken *et al.*, 2011; Hopkins *et al.,* 2008; Heutmekers *et al.*, 2012).

1.13.2 *Pf*HSP70-1 as target antigen in malaria RDTs

*Pf*HSP70-1, a member of the heat shock protein family (HSPs), is induced in cells exposed to sublethal heat shock. In 1984 Hugh Pelham suggested that the ability of HSP70 to enhance the recovery of stressed cells was mediated by its ability to catalyse the assembly of damaged ribosomal proteins (Pelham *et al*., 1984). Subsequently, research revealed that such chaperoning function was characteristic of HSP70 proteins and that it was essential for HSP70 mediated protection against stress (Sharma, 1992; Josh *et al*., 1992) that cause protein denaturation as well as many of the newly discovered house-keeping roles of

constitutively expressed HSP70 in non-stressed cells. Heat shock is not the only stimulus that can induce and alter the synthesis of HSPs. Exposure of cells to amino acid analogues (Kelly and Schlesinger, 1978; Li and Laszlo, 1985), glucose analogues (Pouyssegur *et al*., 1977), heavy metals (Levison *et al.,* 1980), protein kinase C stimulator (Ding *et a*l., 1996), Ca^{2+} increasing agents (Ding *et al.,* 1986), ischemia, sodium arsenate (Johnson *et al*., 1980), microbial infections, nitric oxide, hormones and antibiotics induce the expression of HSPs. HSPs in malaria parasites play important roles in parasite growth, development and survival and are, therefore, important targets for diagnosis, anti-malarial vaccines and drug targets (Archaya *et al*., 2007; Guirgis *et al*., 2012; Dat *et al*., 2000; Shonai *et al*., 2007; Calderwood *et al*., 2007; Kumar *et al*., 1990). *Pf*Hsp70-1 was found to be abundantly expressed in the asexual stages at 37^{o}C and is also induced and translocated to the nucleus upon heat shock at 41^{o}C (Beere and Green, 2001; Calderwood *et al.*, 2007; Bindu *et al.*, 1992; Singh, 2004). Studies have shown the effect of various body temperatures (close to malaria fever) on the *P. falciparum* viability, growth and expression of the *Pf*HSP70-1 gene (Bindu *et al*., 1992). *Pf*HSP70-1 is abundantly available as soluble cytosolic proteins during the intra-erythrocytic stage of parasite development after fever (Bindu *et al.*, 1992; Bottger *et al.*, 2012; Beere and Green, 2001, Ardeshir *et al.*, 1987; Calderwood *et al.*, 2007; Pavithra *et al*., 2004) and, therefore, can be used as an alternative to HRPII, *P*LDH and aldolase in RDTs. Previously, *Pf*HSP70-1 was detected by antibodies in infected blood during the intra-erythrocytic stage (Ardeshir *et al.*, 1987; Zhang *et al.,* 2001). Other studies have shown that *Pf*HSP70-1 is a good target for detection in blood samples during the intra-erythrocytic stage (Kumar *et al.*, 1990; Tatu *et al.*, 2007; Dat *et al.*, 2000; Haldar *et al.*, 2007). The *P. falciparum* heat shock protein is well conserved across all plasmodia species and was found to be immunogenic in infected humans (Mattei *et al.*, 1989) and experimentally infected monkeys (Gysin *et al.*, 1982). Patients' sera and T-cells collected from malaria endemic areas showed increased activity to *Pf*HSP70-1 in individuals already exposed to malaria (Duboi *et al.*, 1987). *Pf*HSP 70-1 is produced by sporozoite (Tsuji *et al.*, 1994), hepatic (Renia *et al.*, 1990) and gametocyte stages.

1.14 Aims and objectives of the current study

RDTs currently in use have shortcomings. These shortcomings include the lack of thermal stability, lower sensitivity caused by high limit of detection and cross reactivity with rheumatoid factor (Mishra *et al.*, 1999; Jelinek *et al.*, 2001; Karbwang *et al.*, 1996; Sing *et al.*, 2000; Iqbal *et al.*, 2000; WHO, 2000).

The first aim of this study was to optimise the expression and purification of *Pf*LDH and *Pf*HSP70-1 to produce soluble proteins for use in producing polyclonal and peptide-specific

antibodies in chicken and test the antibodies in detecting the expressed protein and the native protein in ELISA, western blots and dip stick format. The antibodies produced in chicken do not cross react with rheumatoid factors as they lack the Fc portion to which rheumatoid factors bind.

*Pf*HSP70-1 was reported in literature as being expressed in higher amounts during the intraerythrocytic stage. The second aim was, therefore, to assess whether the use of *Pf*HSP70-1 would improve the LOD below 50 parasites/µl and compare it with that of *Pf*LDH. The objectives were:

1) To optimise the recombinant expression and purification of *Pf*LDH and *Pf*HSP70-1.
2) To identify immunogenic peptides with higher surface probability in order to produce anti-peptide antibodies in chicken.
3) To identify possible post-translation modifications that may affect the activity of *Pf*LDH and *Pf*HSP70-1 in the native protein.

Chapter 2

Materials and Methods

This chapter describes the techniques used in this study. The techniques include biological, biochemical, bioinformatical and immunochemical methods. The chapter also lists the reagents used in the experiments and the source of the reagents.

2.1 Materials

2.1.1 Equipment

Beckman Coulter™ Allegra™ X-22R centrifuge, Beckman Coulter™ Avanti™ J-26XPI centrifuge were purchased from Beckman Coulter (California, USA). Labnet Spectrafuge™ 16M Brushless Laboratory Microcentrifuge was purchased from Labnet International Inc (New Jersey, USA). Shimadzu UV-1800 UV spectrophometer, Shimadzu CPS- Controller were purchased from (Japan). MRC Benchtop shaker incubator 300 x 400 mm, 250 rpm, 70°C and MRC Refrigerated shaker incubator 960 x 600 mm, 250 rpm, 0-60°C were purchased from MRC (London, UK). Another orbital shaking incubator was purchased from New Brunswick Scientific (New Jersey, USA). Small volume shaker Labnet™ Pro 30 Laboratory reciprocal shaker was purchased from Labnet (New Jersey, USA). A Virosonic™ cell disruptor using a rod was purchased from VirTis (New York, USA) and a Shalom Laboratory Supplies c.c Utrasonic cell disruptor that uses water was purchased from advanced Labs (Durban, South Africa). Mini Protean II™ vertical SDS-PAGE unit, Poly Prep® system for electrophoresing polyacrylamide gels. DNR Bio-imaging system MiniBis Pro was purchased from Bio-Imaging Systems (USA) for gel imaging and the Vacutec G-BOX Syngene system was purchased from Syngene (Maryland, USA) for western and chemiluminescence imaging. The pH meter microprocessor was purchased from Hanna

Instruments (Durban, South Africa). Magnetic stirrer Veep was purchased from Fisher Scientific (USA) and the Corning hot plate stirrer PC-351 was purchased from Sigma-Aldrich (Steinheim, Germany). An Edward 5A.C. pump was purchased from GEC Machines Ltd. (Newcastle, UK) and Micro tube peristaltic pump MP-3 was purchased from Rikakikai Co. Ltd (Tokyo, Japan), NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

2.1.2 Reagents

Unstained molecular weight marker with sizes ranging from: 116-14.4 kDa, dithiothreitol (DTT) molecular biology grade, restriction enzymes, 10 mM dNTP mix, DNA MassRuler^{TM} were all purchased from Fermentas (Vilinius, Lithuania). Tween-20, yeast extract, Rabbit albumin, kanamycin, penicillin, Ponceau S, Coomassie Brilliant Blue R-250, Coomassie Brilliant Blue G-250, bisacrylamide, acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue, 4-chloro-naphthol, ethidium bromide (EtBr), triton X-100, β-mercaptoethanol, isopropyl β-D-1- thiogalactopyranoside (IPTG), tryptone, Freud's complete adjuvant (FCA), Freud's incomplete adjuvant (FIA), sodium azide, ammonium persulphate, kanamycin sulphate analytical grade, ampicillin analytical grade, were all purchased from Sigma- Aldrich Fluka (Steinheim, Germany). Sodium chloride, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, potassium chloride, agar bacteriological, D^+ glucose, sodium dihydrogen orthophosphate, hydrogen peroxide, sucrose, all types of polyethylene glycol, glycine, were all purchased from Merck (Germany). Agarose for DNA gel electrophoresis, agarose for protein gel electrophoresis were purchased from Whiteman Scientific (Rockland, USA). Dimethyl sulfoxide (DMSO) was purchased from Capital Laboratory supplies (New Germany, RSA). Ninety-six well culture plates and immunotubes were from NUNC (Roskilde, Denmark). The anti-M13 monoclonal antibody was from Thermo Scientific (Waltham, MA, USA) and the goat anti-mouse-HRPO secondary antibody was from Jackson Immunochemicals (Pennsylvania, USA).

2.2 Molecular biology techniques

These are the methods that were used to clone, transform, express and isolate the recombinant proteins used in this study. The expression vector expressing recombinant *Plasmodium falciparum* lactate dehydrogenase (r*Pf*LDH) was a kind gift from Professor R.L. Brady, University of Bristol, UK. Recombinant *Plasmodium falciparum* heat shock protein 70-1 (*rPf*Hsp70-1) was kindly provided by Professor A. Shonhai, University of Venda, SA.

2. 2. 1 Expression plasmids and plasmid evaluation

2.2.1.1 The pKK233-3 plasmid

The vector map shows the cleavage sites for inserting the desired coding gene sequences and the ampicillin resistance marker in Figure 2.1. *Pf*LDH sequence was inserted between the *Pst*I and *Eco*RI restriction sites. The size of the vector without an insert is 4.6 kb.

2.2.1.2 The pQE30 plasmid

The plasmid used for the expression of His_6 recombinant *Plasmodium falciparum* Heat shock protein 70-1 (His_6-*rPf*Hsp70-1) was pQE30 which was used to transform the *Escherichia coli* BL21 (DE3) expression host. The pQE30 plasmid was co-transformed with the RIG plasmid. The RIG plasmid codes tRNA for rare codons during protein synthesis in *E. coli* namely: Arg, Ile and Gly and thus the name RIG (Baca and Hol, 2000). The plasmid vector map is shown in Figure 2.2.

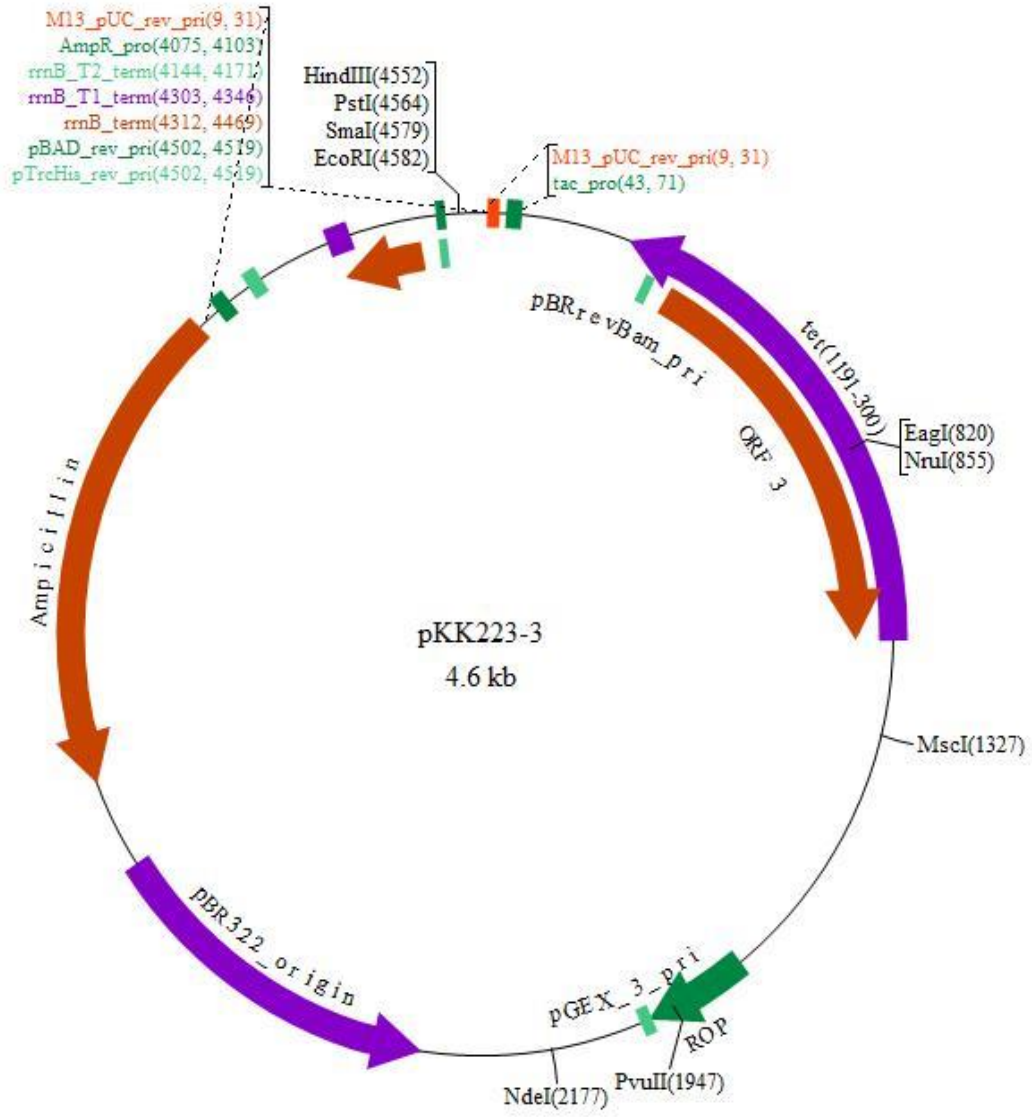


Figure 2.1 pKK233-3 vector map expressing *Pf*LDH.

The pKK233-3 plasmid used for the expression of His_6 recombinant *Plasmodium falciparum* lactate dehydrogenase (His_6-*rPf*LDH) which was transformed into an *Escherichia coli* BL21 (DE3) expression host. Restriction and endonuclease sites and position of the ampicillin resistance gene are shown.

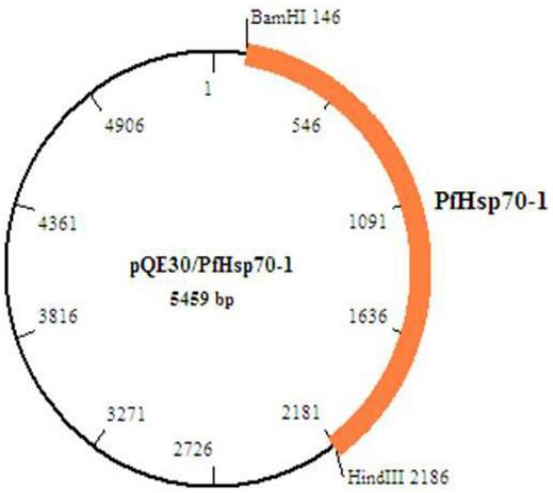


Figure 2.2 Map of the pQE30/*Pf*HSP70-1 plasmid. Restriction maps of pQE30/*Pf*HSP70-1 indicating the *Bam*HI and *Hind*III restriction sites and the *Pf*HSP70-1 gene insert.

*Pf*HSP70-1 was inserted between *Bam*HI and *Hind*III sites in pQE30 plasmid as shown in Figure 2.2.The size of the plasmid vector without insert is 5459 base pairs.

2.2.1.3 Agarose gel electrophoresis

A 1% (w/v) agarose gel (0.3 g agarose was dissolved in 30 ml TAE buffer (2 M Tris, 0.95 M glacial acetic acid, 50 mM EDTA, pH 8.0)) was prepared and heated in a microwave until the agarose was melted. 2 µl of ethidium bromide (1% (w/v)) was added. The solution was poured into a casting tray with a dimension of (7 x 6.5 cm) and left to solidify at RT. A volume of 5 µl sample was mixed with 1 µl of loading dye (0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in TAE buffer). A volume of 3 µl of DNA ladder and 12 µl of sample were used to load the lanes. The gels were electrophoresed at 70V for about 45 min in TAE buffer containing ethidium bromide. Gels were visualised under UV light and images of gels were taken using VersaDoc^{TM} imaging system. Sizes of the unknown bands were calculated from the log of relative distance travelled from start point to the band of DNA and compared to the molecular mass standards.

2.2.1.4 Plasmid isolation

A volume of 5 ml of an overnight culture (16 hrs) grown in LB media supplemented with 10 µl of 100 µg/ml ampicillin with 200 rpm shaking at 37°C was centrifuged at 6800xg, 4°C, 5 min and the supernatant was discarded. The plasmid was isolated from the pellet following the instructions in the GeneJET miniprep kit.

2.2.1.5 Restriction digestion

Restriction digestion was performed on plasmid DNA. A single restriction and double restriction digestion was done. The A_{280}/A_{230} ratio was determined to establish the purity of DNA.

A single restriction digest of plasmid DNA was used. The reaction mixture of 0.5µl of restriction endonuclease, 2 µl of 10 x digestion buffer and milliQ water to a final volume of 50 µl. For double digestion using *Bam*HI and *Hind*III, the following reaction mixture was used; 1 µg of plasmid DNA, the amount of restriction enzyme was chosen according to the selected buffer chosen using the Fermentas restriction endonuclease buffers and the mixture was made up to 50 µl using milliQ water. The reaction was done at 37°C for 2 hrs.

2.2.2 Expression of recombinant proteins in various growth media

From a glycerol stock a 3-way streak on an LB agar plate [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% NaCl, 0.2% (w/v) glucose, and 1.5% (w/v) bacto-agar] was done and incubated overnight at 37°C. A single colony was picked and inoculated into 5 ml of either fresh LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and 0.2% (w/v) glucose] or TB [1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.23% (w/v) KH_2PO_4, 1.25% (w/v) K_2HPO_4] broth supplemented with 50 µg/ml ampicillin for *Pf*LDH and 100 µg/ml ampicillin for *Pf*HSP70-1 or 34 µg/ml kanamycin for *Pf*HSP70-1 and left to incubate overnight at 37°C, with shaking at 200 rpm. These cultures were used to inoculate large volumes of either LB or TB growth media at 1:10 or 1:100 dilutions of overnight cultures or using a single starting colony supplemented with the appropriate antibiotic. Cultures were left to incubate at 37°C or 25°C, with shaking at 200 rpm until OD_{600} of 0.5-0.6 for mid-log phase induction or 0.7 for induction after stationary phase. A concentration of 0.3 mM IPTG was used for *Pf*LDH and 1 mM for *Pf*HSP70-1. IPTG Induced expression was allowed to continue for 4 hrs after addition of IPTG. TB media was not induced because it is self-inducing. Cultures grown in TB were left to incubate for 8 hrs. The cultures were then centrifuged at 4000xg, 4°C, 10 min and the pellet was resuspended in 4% original volume with appropriate buffer PBS pH 8.0 [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 1.02% (w/v) Na_2HPO4, 0.02% (w/v) KH_2PO_4] or 10 mM imidazole lysing buffer [0.6% (w/v) NaH_2PO4, 1.753% (w/v) NaCl, 0.068% (w/v) imidazole, and 0.02% NaN_3] as required.

2.2.3 Lysis of bacterial cells

Lysis of the bacterial cells was accomplished through freeze and thaw, lysozyme digestion, sonication or a combination of these methods. For freezing and thawing, liquid

nitrogen was used to freeze the bacterial pellet and cells were thawed at 37°C. The steps were repeated three times. Lysozyme samples were prepared by addition of STET buffer [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 5% (v/v) Triton X-100] to the freshly prepared lysozyme solution (10 mg of lysozyme dissolved in 1 ml in dH_2O). The mixture was vortexed and put in a water bath at 37°C for 30 min. Samples for small volumes were lysed 4 x 30 s with resting on ice using a box sonicator. Large sample volumes were lysed 4 x 30 s on ice at 80 watts, 6 rms using a rod sonicator.

2.2.4 Affinity purification of *Pf*LDH and *Pf*HSP70-1 on Talon® (Co^{2+}) resin

The insert in pKK233-3/*Pf*LDH and pQE30/*Pf*HSP70-1 plasmids are expressed as proteins with an N-terminal poly-histidine (His_6) tag. Poly-histidine has a high affinity for Co^{2+} immobilised on resin. The polyhistidine-tagged recombinant proteins selectively bind to the column during purification and are eluted with a high concentration of imidazole in the elution buffer in a step wise method. Low concentrations of imidazole were incorporated in the wash buffer to reduce the binding of contaminating proteins with low affinity for the Talon resin.

A 10 ml of the clarified sample (from section 2. 2. 3) was added to the Talon® resin. The resin was rocked gently at RT (25°C) or at 4°C overnight to allow the protein to bind to the resin. The resin was centrifuge at 700xg for 5 min and the supernatant was carefully removed. The resin was washed with 10 bed volumes of 10 mM imidazole wash buffer [10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, 0.02% NaN_3], centrifuged at 700xg for 5 min and the supernatant discarded. The resin was resupended in one column volume of 10 mM imidazole wash buffer and added to a column. The column was washed with 10 mM imidazole until A_{280} of <0.02 was reached. The polyhistidine-tagged proteins were eluted by adding 5 ml of 150 mM imidazole buffer (150 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl, 0.02% NaN_3), 250 mM imidazole buffer (250 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, 0.02% NaN_3) or gradient of 10 mM to 250 mM imidazole. Eluents were analysed by absorbance at A_{280} nm. Samples from each step of the purification protocol were evaluated by a 12.5% SDS-PAGE gel and a western blot. The Talon® resin was regenerated according to manufacturer's instruction.

2.2.5 Concentrating proteins by dialysis against polyethylene glycol 20 000 (PEG_{20000})

A dialysis bag (snake skin) with a molecular weight cut-off of 3, 500 Da was briefly soaked in distilled water. Excess water and bubbles were squeezed out, and one end was clamped. The protein sample solution was poured into the bag and the other end clamped. The sample was surrounded by PEG_{20000} (Mr 20 000) at 4°C. Wet PEG_{20000} was changed

from time to time until the proteins reached the desired concentration. High concentration of imidazole were removed from the protein by dialysis against dialysis buffer [PBS, 10% (v/v) glycerol, 5 mM EDTA, 10 mM imidazole, 10 mm DTT, and pH 8.0]. The buffer was changed after 2 hr twice and was left overnight at 4°C with a magnetic stirrer.

2.2.6 Concentrating protein using SDS/KCl precipitation

5% SDS (10 µl) was added to the sample (100 µl) in 1.5 ml tube. The solution was mixed by inverting the tube and 3 M KCl (10 µl) was added. The mixture was inverted again and centrifuged at 12,000xg, 2 min, RT, the supernatant was discarded and the precipitate resuspended in stacking gel buffer and reducing treatment buffer (Hejazi *et al*., 2013; Reynolds and Tanford, 1970; Laemmli, 1970).

2.3 Biochemical methods

Biochemical methods were used to analyse and characterise the recombinant and native proteins.

2.3.1 Bradford method to measure protein concentration

This method was performed according to (Bradford, 1976) without modifications. A standard curve was obtained by preparing BSA standards from 0-80 µg. Each standard was made up in triplicate with dH_2O up to 100 µl and 900 µl of Bradford (0.09 mg/ml) reagent was added and incubated in the dark for 15 minutes. Absorbance was measured at 595 nm (A_{595}) and a graph plotted. The samples whose concentration was not known were prepared the same way as the standard and protein concentration determined from the standard curve in Figure 2. 3.

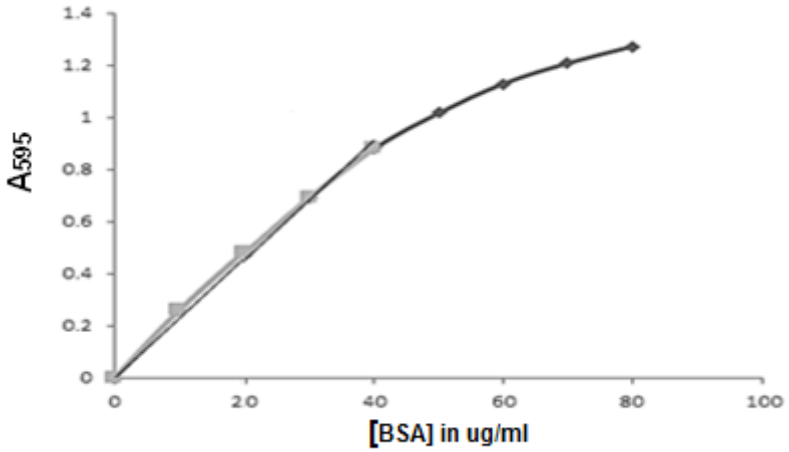


Figure 2. 3 Bradford standard curve to measure protein concentration. All values are the average of triplicate readings. The area of the graph where there is a straight line was used to measure protein concentration.

2.3.2 A_{280} determination of protein concentration

Samples were prepared to appropriate dilutions before measuring absorbance at 280 nm using a quartz cuvette in a Shimadzu UV-1800 spectrophotometer.

2.3.3 Measuring A_{280}/A_{260} and A_{260}/A_{230} using a Nano-drop spectrophotometer

Samples were measured according to the manufacturer's instructions of the NanoDrop 2000 spectrophotometer. The measurements were done at 280, 260 and 230 nm to determine the purity of proteins and the amount of contaminating nucleic acids.

2.3.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was used as described by (Laemmli, 1970). The gel consists of a lattice made by cross linking of acrylamide with N, N'-methylenebisacrylamide (bisacrylamide). The cross linking creates a sieve that separates proteins according to size as smaller proteins are less impeded through the lattice. According to Chart (1994) the size of the sieve can be altered by changing the ratio of acrylamide to biscacrylamide to make different percentage gels. The proteins that contain disulphide bonds are reduced by β-mercaptoethanol in the reducing buffer (Walker and Rapley, 2002). Sodium dodecyl sulphate (SDS), an anionic detergent, is used to further denature the proteins and covers the protein in an overall negative charge so that in an electric field proteins are separated only based on size and not charge as they migrate towards a positive electrode. The samples are also boiled for 5 minutes to denature the proteins.

The appropriate separating and stacking gels were prepared according to Table 2.1. Solution A [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]; Solution B [1.5 M Tris-HCl, pH 8.8]; Solution C [500 mM Tris-HCl, pH 6.8]; Solution D [10% (w/v) SDS); Solution E [10% (m/v) ammonium persulfate]. Gels were set in a Bio-Rad Mini PROTEAN II® vertical slab apparatus. Samples were prepared in 2 x reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.1% bromophenol blue, pH 6.8] in a ratio of 1:1, boiled for 5 min and loaded in the lanes. The gel was then placed in an electrophoresis tank filled with tank buffer [250 mM Tris-HCl, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3] and electrophoresed at 20 mA per gel until the bromophenol blue was 0.5 cm

from the bottom of the gel. The gel was stained in coomassie brilliant blue R-250 [125% (m/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid] for at least 4 hr and destained in destain solution I [50% (v/v) methanol, 10% (v/v) acetic acid] until bands were clearly seen. The gels were left in destain solution II [7% (v/v) acetic acid, 5% (v/v) methanol] to swell back to the original size. The size of the protein bands were determined by extrapolation from a graph of log molecular weight of the molecular weight standards electrophoresed on the same gel against relative distance travelled by the protein (Rf values).

Table 2. 1 The ingredients to make different percentage acrylamide electrophoresis gels.

	Running gel %					**Stacking gel %**	
	15*	**12.5***	**10***	**7.5***	**5****	**4***	**3****
A	0.5	0.42	0.33	0.25	0.17	0.13	0.1
B	0.25	0.25	0.25	0.25	0.25	0	0
C	0	0	0	0	0	0.25	0.25
D	0.01	0.01	0.01	0.01	0.01	0.01	0.01
E	0.005	0.005	0.005	0.005	0.005	0.005	0.005
dH_2O	0.23	0.32	0.4	0.48	0.57	0.6	0.64
TEMED	0.0005	0.0005	0.0005	0.0005	0.0005	0.002	0.002

***refers to % gel to be prepared**

% Acrylamide	Separation range (kDa)
6	60 - 300
8	40 - 100
10	20 - 70
12	20 - 60
14	10 - 40

2.3.5 Western blot

Western blot is done after completion of SDS-PAGE technique described in section 2. 3. 4. The SDS-PAGE gel was soaked in blotting buffer [0.6% (w/v) Tris, 1.44% (w/v) glycine, 20% (v/v) methanol]. Electrophoretic transfer to nitrocellulose was carried out overnight at 40 mA. The nitrocellulose membrane was stained with Ponceau S [0.1% (m/v) Ponceau S, 1% (v/v) glacial acetic acid] to mark the molecular weight markers and removed with distilled

water containing a little NaOH. The gel was stained with Coomassie Brilliant Blue R-250 to determine the efficiency of the blotting. The nitrocellulose membrane was rinsed with TBST [0.24% (w/v) Tris, 1.17% (w/v) NaCl, and 0.05% (v/v) Tween-20] for 5 minutes. The membrane was blocked with 5% (m/v) Elite low fat milk in TBST for 1 hr. The membrane was washed in TBST 3 x 5 minutes and incubated with primary antibody 1:2000 in 0.5% BSA-TBST for 2 hrs. The membrane was washed in TBST 3 x 5 minutes. Secondary antibodies were prepared in 0.5% BSA-TBST at a dilution of 1:6000 and was incubated for 1 hr followed by 3 x 5 min washes in TBST. The nitrocellulose membrane was then immersed in substrate solution (4-chloro-1-naphthol (2 ml), TBS (8 ml) and 30% H_2O_2 (4 µl)) and the colour was allowed to develop in the dark for 10 - 30 minutes. The membrane was allowed to dry before the picture was taken using a Vacutec G-Box Syngene system.

2.4 Bioinformatics

Algorithms were used *in silico* to predict the peptide epitopes for recombinant *Pf*HSP70-1 and the effect of certain post-translational modifications on the activity of the native proteins as described in the section below.

2.4.1 Predict7™

Predict7™ was used to predict immunogenic and soluble peptides in the primary structure of proteins in order to raise antibodies against these peptides in chickens (Carmenes *et al*., 1989). Graphs were generated based on four parameters; antigenicity (Kolaskar and Tongaona, 1990), surface probability (Emini *et al.*, 1985), flexibility (Karplus and Shulz, 1985) and hydrophilicity (Parker *et al.*, 1986). Peptides with potential immunogenicity should be soluble and situated on the surface of proteins for easy interaction with antibodies. However, not all predicted peptides are necessarily immunogenic.
The National Centre for Biotechnology Information (NCBI) (www.ncbi.org/) or plasmoDB (www.plasmodb.org/) were used to obtain protein sequences.

2.4.2 Sequence alignments

The alignment of the primary structure of proteins and coding DNA were done using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalW/). The protein sequences were either obtained from PlasmoDB (www.plasmodb.org/) or the national centre for biotechnology information (NCBI) (www.ncbi.org/).

2.4.3 The 3D crystal structure of *Pf*LDH

The 3D structure of *Pf*LDH was viewed using SwisPDB viewer software at spdv.vital-it.ch/disclaim.html#.

2.4.4 Prediction of acetylation, phosphorylation, glycosylation and proteolytic sites

The prediction of potential acetylation of *Pf*LDH and *Pf*HSP70-1 were done using PAIL (bdmpail.biocuckoo.org/predicitions.php/) and NetAcet (www.cbs.dtu.dk/services/NetAcet/).
The prediction of potential phosphorylation sites on *Pf*LDH and *Pf*HSP70-1 was done using Kinasephos (www.kinasephos.mbc.nctu.edu.tw/predict.php/).
The prediction of potential 'O' glycosylation sites on *Pf*LDH and *Pf*HSP70-1 was done using DictyOGlyc (www.cbs.dtu.dk/services/DictyOGlyc/) and 'N' glycosylation sites were done using (www.cbs.dtu.dk/services/NetNGlyc/).
The prediction of potential proteolytic sites on *Pf*LDH and *Pf*HSP70-1 was done using NetChop (www.cbs.dtu.dk/services/NetChop/).

Chapter 3

Recombinant expression of *Pf*LDH and *Pf*HSP70-1

3.1 Introduction

In order to study and characterise a protein of interest, the protein has to be available in sufficient quantities. Proteins can be recombinantly expressed in different expression systems including prokaryotic *Escherichia coli* (Baneyx, 1999) and the *Bacillus* genus, eukaryotic (*Saccharomyces cerevisiae, Pichia pastoris*), human (*HeLa*) and insect cell (*Luciferase vectors*) systems and cell free systems (Yin *et al.*, 2007). The most commonly used expression system is gram negative *E. coli* bacteria (Baneyx, 1999). Malaria rapid diagnostic tests (RDTs) in current use are based on three soluble malaria parasite proteins. The *Plasmodium falciparum* histidine rich protein II (*Pf*HRP-II), and the metabolic parasite proteins *Plasmodium* lactate dehydrogenase (*P*LDH), and aldolase (Murray *et al.*, 2008).

Malaria infecting humans is caused by five malaria species namely: *P. malariae*, *P. ovale*, *P. vivax*, *P. falciparum* and a monkey parasite *P. knowlesi* has emerged causing zoonotic malaria in humans in South East Asia (Anderios, 2009; Daneshvar *et al.*, 2009; Lee *et al.*, 2009; Sabbatani *et al.*, 2010; Van den Eede *et al.*, 2010; Van Hellemond *et al.*, 2009; Collin, 2012). Most RDTs currently use *Pf*HRPII as the antigen but this protein is only expressed by *P*. *falciparum* and can only be used to detect *Plasmodium falciparum* malaria. Problems that stem from the use of *Pf*HRPII as the antigen include patients testing positive for malaria even after parasite clearance. This is because the protein persists in blood after parasite clearance (Murray *et al.*, 2008; Iqbal *et al.*, 2004; Tjitra *et al.*, 2001; Kodisinghe *et al.*, 1997; Bell *et al.*, 2005). False positive RDTs for malaria may be due to the presence of rheumatoid factor in patients (Iqbal *et al.*, 2000). Another problem is false negatives which can be due to deletion of the *Pf*HRPII gene as reported by Gamboa *et al.,* in 2010 in Peru and other parts of the world (Koita *et al.*, 2012). Single nucleotide polymorphisms (SNPs) in parasite DNA have been reported to affect the detection of *P. falciparum* malaria infections (Baker *et al.*, 2010; Lee *et al.*, 2006a).

Because of possible co-infection of *P. falciparum* with other species of malaria, there is a need for RDTs that can detect other forms of malaria, so that drugs can be prescribed

that target each infecting species to avoid relapses (Murray *et al.*, 2008; Chuangchaiya *et al.*, 2009) and the development of drug resistant strains (Price *et al.*, 2001; Price *et al*., 2006; Van Vugt *et al.*, 1998; Van Vugt *et al*., 1999; Van Vugt *et al*., 2000). *Plasmodium* LDH and *Plasmodium* aldolase enzymes are found in all *Plasmodium species* (Murray *et al.*, 2008). *P*LDH and aldolase are involved in metabolism, and are, therefore, associated with living parasites (Murray *et al.*, 2008; Iqba *et al.*, 2004). The proteins have a short half-life and can be used to detect current infection and monitor the progress of treatment. Ashley *et al,* 2009 reported that some *P*LDH-based RDTs revert back to negative within 2-7 days post drug clearance thus confirming the rapid clearance of *Pf*LDH from blood. All the RDTs developed to date have limited specificity, sensitivity and limits of detection (Moody, 2002; Moutacho and Goldring, 2007).

Malaria has similar symptoms to other tropical diseases such as Dengue and, therefore, incorrect treatment can be administered (Gwer *et al.*, 2007; Kallander *et al.*, 2004). The most vulnerable to malaria are children (Black et al., 2003; WHO 2014). Often children with malaria-like symptoms who end up testing negative for malaria still get treated for malaria. This led the CDC to outline a requirement for diagnosis before treatment (Lallo *et al.*, 2007). Confidence in malaria RDTs is still low among clinicians who still prescribe presumptive treatment of malaria when the RDT test is negative (Bisoffi *et al.*, 2009). The WHO, together with the Foundation for Innovated New Diagnostics (FIND), has established and offers a testing programme to evaluate the performance of RDTs in order to improve confidence in their use among clinicians (WHO, 2008; WHO, 2009; WHO, 2010; WHO, 2010b). Other measures taken include the buying of RDTs from manufacturers with evidence of good manufacturing practice as well as “in field testing” of RDTs. As a method of testing RDTs in the field a positive control was included. This would entail the use of recombinant antigens or peptides reconstituted in uninfected blood (Murray *et al.*, 2008; Lon *et al.*, 2005) or dried blood spots (Versteeg and Men, 2009). This format was found to work better for *Pf*HRPII-based tests than *P*LDH-based and aldolase-based tests and this was thought to be due to the stability of the *Pf*HRPII antigen.

*Pf*HSP70-1, a member of the heat shock protein family (HSPs), is induced in malaria parasite exposed to sublethal heat shock. In 1984 Hugh Pelham suggested that the ability of HSP70 to enhance the recovery of stressed cells was mediated by its ability to catalyse the assembly of damaged ribosomal proteins (Pelham *et al.*, 1984). Subsequently, research revealed that chaperoning was characteristic of HSP70 proteins and that it was essential for the HSP70-mediated protection against stresses that cause protein denaturation and other newly discovered house-keeping roles of constitutively expressed HSP70 in non-stressed cells. Heat shock is not the only stimulus that can induce and change the synthesis of HSPs.

Exposure of cells to amino acid analogues (Kelly and Schlesinger, 1978; Li and Laszlo, 1985), glucose analogues (Pouyssengur *et a*l., 1977), heavy metals (Levison *et al.,* 1980), protein kinase C stimulator (Ding *et al*., 1986), Ca^{2+} increasing agents (Ding *et al.,* 1986), Ischemia, sodium arsenate (Johnston *et al.*, 1980), microbial infections, nitric oxide, hormones and antibiotics induce the expression of HSPs. HSPs in malarial parasites play important roles in growth, development and survival and HSPs are, therefore, potential targets for anti-malarial vaccines and drugs. *Pf*Hsp70-1 has been found to be abundantly expressed in the asexual stages and is induced and translocated to the nucleus upon heat shock at 41°C. Studies have shown the effect of various body temperatures (close to malaria fever) on the *P. falciparum* viability, growth and expression of *Pf*HSP70-1 proteins (Bindu *et al*., 1994). *Pf*HSP70-1 is abundantly available as a soluble cytosolic protein during the intra-erythrocytic stage of parasite development that is induced by heat shock in the form of febrile episodes and has potential as a malaria diagnostic target.

For this study, two parasite proteins were selected for study. The first protein *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) is in use as a RDT target and the second protein is *Plasmodium falciparum* heat shock protein 70-1 (*Pf*HSP70-1). *Pf*LDH is a conserved glycolytic metabolic enzyme, making it a good target to detect and measure current infection (Lee *et al*., 2006b; Murray, 2008). *Pf*HSP70-1 is also highly conserved across all *Plasmodial species* and was found to be immunogenic in infected humans (Mattei *et al.*, 1989) and experimentally infected monkeys (Gysin *et al.*, 1982). *Pf*HS70-1 is abundantly expressed during fever and has diagnostic potential. These proteins were recombinantly expressed in *E. coli* BL21 (DE3) host cells and were characterised using SDS-PAGE and western blotting.

3.2 Results

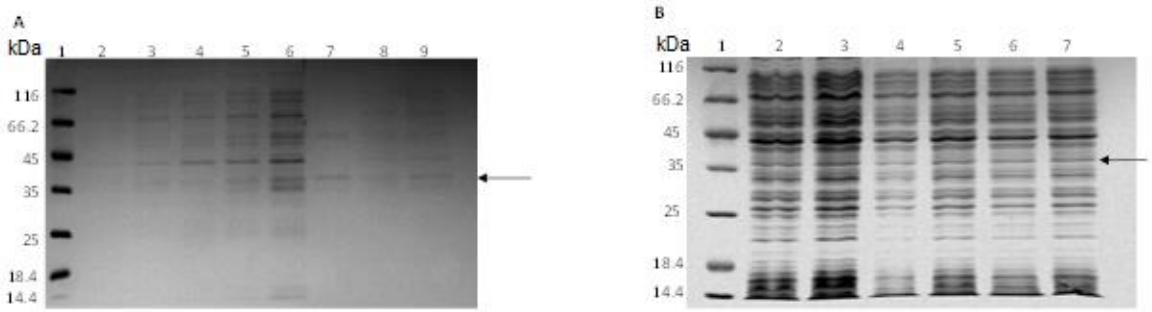


Figure 3.1 Expression of *Pf*LDH in Lysogeny Broth and Terrific Broth media at (A) 30°C and (B) 37°C. Recombinant *Pf*LDH was expressed at 30°C and 37°C in LB (panel **A)** and TB (panel **B**). The samples were

analysed on 12.5% reducing SDS-PAGE gels, stained with Coomassie Brilliant Blue R-250. Panel **A** lane 1: molecular weight marker, non-induced *Pf*LDH lane 2 and 3 at 30°C, lane 4 and 5 , at 37°C; induced *Pf*LDH lane 6 and 7 at 30°C, lane 8 and 9 at 37°C. Panel **B** lane 1: molecular weight markers, lane 2, 3 and 4: samples of *Pf*LDH incubated at 30°C, lane 5, 6 and 7: samples of *Pf*LDH incubated at 37°C. The experiment was repeated three times, an example experiment is shown. The arrow shows the expected size of *Pf*LDH.

3.2.1 Comparison of media used to express *Pf*LDH

Recombinant protein expression was evaluated in different growth media to determine which media promoted high amounts of soluble recombinant protein.

The media compared were Terrific Broth (TB) and Lysogeny Broth (LB). Terrific broth contains glycerol and phosphates and is said to be self-inducing (Li *et al*., 2011).

The expression of *Pf*LDH appeared to be higher in TB than LB media. The expression was marginally better at 37°C compared to 30°C in TB as shown by the intensity of a 36 kDa band on the gels above shown by the arrow (figure 3.1). Lower expression was observed in LB media.

3.2.2 Growth of *E. coli* host cells in Terrific and Lysogeny Broth media

Figure 3. 2 shows that *E. coli* host cells initially grow faster in TB media than in LB media. All cells reach stationary phase at approximately 4 hrs after inoculation. The growth of *E .coli* host cells was also observed after inoculation with a single colony. Figure 3.3 shows similar growth patterns for cells from a single starting colony grown in TB and LB media. All cells reach stationary phase at approximately 4 hrs after inoculation at an OD_{600} of 0.6. It is suggested that a single colony can be used to inoculate media for expression of *Pf*LDH as this can save time.

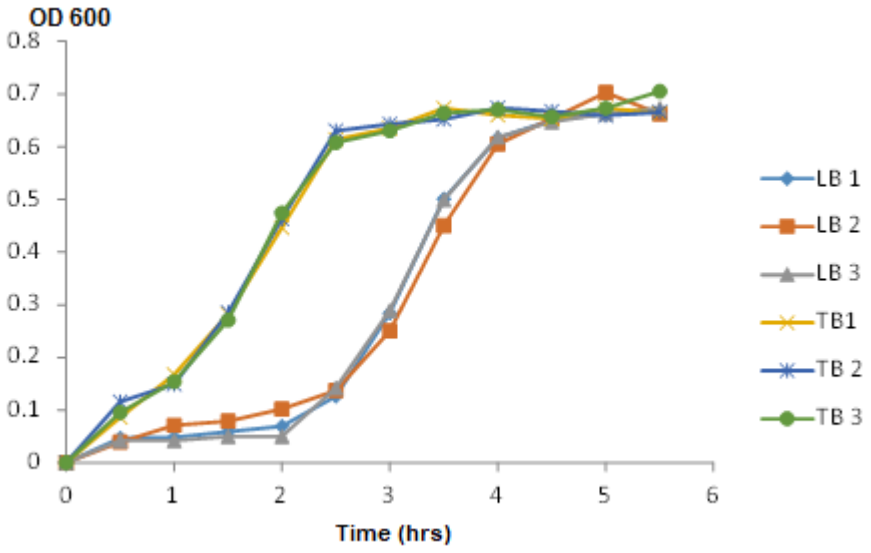


Figure 3.2 Growth of *E. coli* host cells grown in Terrifc Broth and Lysogeny Broth inoculated from overnight cultures at 1: 100 dilution. *E. coli* host cells growing in TB and LB media at 37°C. LB media and protein expression was induced with 0.3 mM IPTG at OD_{600} of 0.5-0.6.Triplicate cultures are shown.

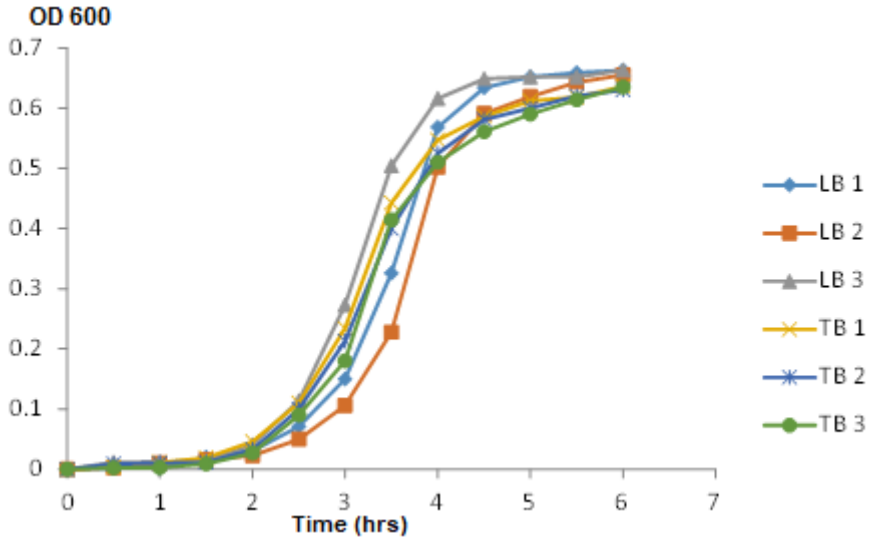


Figure 3.3 Growth of *E. coli* host cells grown in Terrifc and Lysogeny Broth media from a single starting colony. *E. coli* host cells growing in TB and LB media at 37°C. Triplicate cultures are shown.

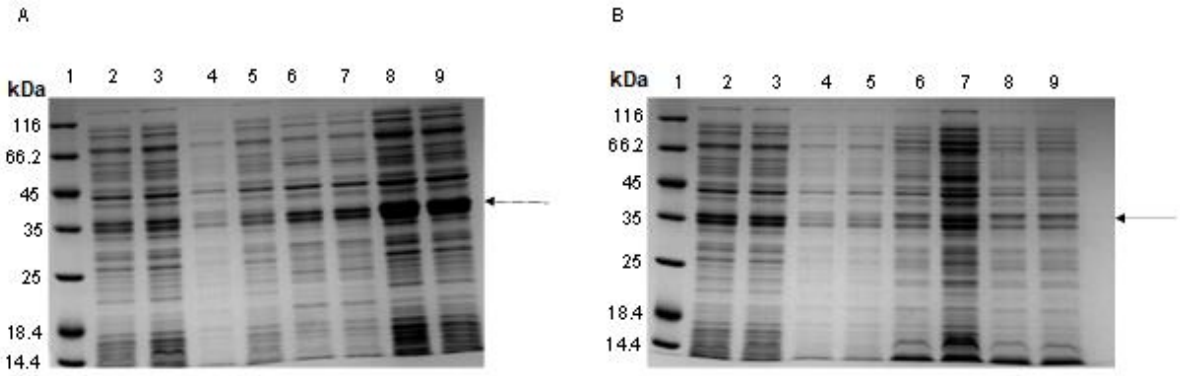


Figure 3.4 Expression of *Pf*LDH comparing inoculating with a single starting colony and from an overnight culture. Expression of recombinant *Pf*LDH in TB media was monitored on a reducing 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Panel **A** are individual colonies taken from the same LB agar plate, lane 1: Molecular weight marker, Lane 2, 3, 4, 5, 6, 7, 8 and 9: are different colonies containing

6XHisr*Pf*LDH insert grown on the same agar plate. **B** is a 1:100 dilution of an overnight culture. Lane 1: Molecular weight maker, lane 2, 3, 4, 5, 6, 7, 8 and 9: are *Pf*LDH samples of different 1:100 dilution. The experiment was repeated three times, an example experiment is shown. The arrow indicates the 36 kDa *Pf*LDH band.

Cells in TB appear to grow faster and do not require induction, and constant checking of ODs that may contaminate the cell culture (Li *et al.*, 2011). So growing cells in TB media was chosen.

Result in Figure 3.4 suggests the use of single starting colony appears to express more *P*LDH than an overnight starting culture. It should be noted that the protein profile for each colony with the same plasmid insert does not always yield the same amount of protein as shown by the variation in the intensity of the *Pf*LDH protein band of individual samples grown in TB media. The cells were normalised in each lane.

3.2.3 Comparison of methods for lysing *Pf*LDH *E. coli* cells

The methods of lysing bacterial host cells were compared to determine which methods released more proteins. Lysozyme digestion, freezing and thawing, sonication, freezing and thawing followed by sonication and a combination of all the methods were compared. Results in Figure 3.5 shows that similar amounts of the 36 kDa *Pf*LDH protein were observed in the sample from freezing and thawing followed by sonication Figure 3.5 lane 2 and 3 in A and B. There were more protein bands and a larger concentration of the 36 kDa *Pf*LDH protein in the supernatant of the combination of freeze and thaw followed by sonication and the combination of all three methods.

Figure 3.6 shows that after the first round of freeze and thaw followed by sonication, there is more protein in the pellet than in the supernatant. To see if more protein could be released after the first round of freezing and thawing followed by sonication, freezing and thawing followed by sonication step was repeated.

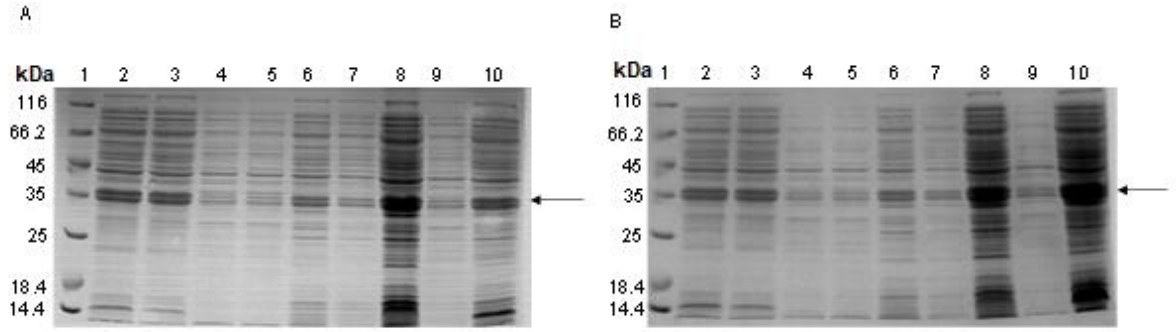


Figure 3.5 Comparing methods of lysing *E. coli* cells. The effectiveness of the lysing methods was evaluated on a reducing 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Panel **A** is the supernatant and **B** is the pellet: lane 1: molecular weight maker, lane 2 and 3: samples from lysozyme digestion, lane 4 and 5: samples from freezing and thawing, lane 6 and 7: samples from sonication, lane 8 and 9: samples from freeze and thaw followed by sonication, lane 10: samples from all methods combined. The experiment was repeated three times, a sample experiment is shown. The arrow indicates the 36 kDa band of *Pf*LDH.

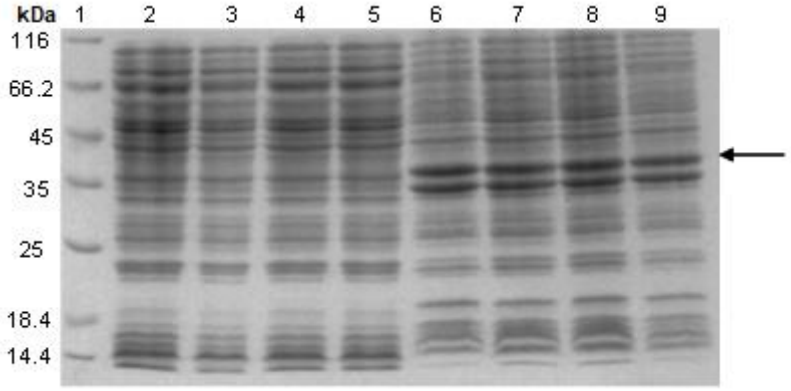


Figure 3.6 Evaluating the amount of recombinant *Pf*LDH in the soluble fraction after lysing using a combination of freeze and thaw followed by sonication. The recombinant *Pf*LDH was frozen and thawed four times and sonicated four times. The amount of protein in the supernatant and pellet was evaluated using a reducing 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker, lane 2, 3, 4 and 5: supernatants (soluble fraction) after lysis, lane 6, 7, 8 and 9: pellets that remained after removing each supernatant respectively. The experiment was repeated three times, a sample experiment is shown. The arrow indicates the 36 kDa band of *Pf*LDH.

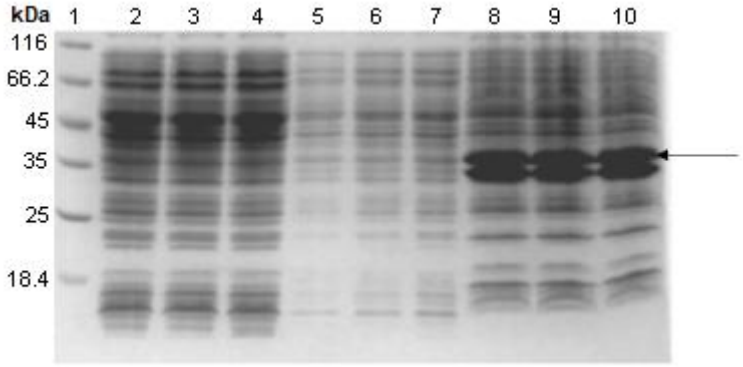


Figure 3.7 Evaluating the amount of recombinant *Pf*LDH which remains in the insoluble fraction after two rounds of lysis using a combination of freeze and thawing followed by sonication. The recombinant *Pf*LDH was frozen and thawed X4 followed by sonication, the first supernatant was collected. The processes was repeated and the second supernatant collected. The amount of supernatant and pellet that was obtained was evaluated using a reducing 12.5% SDS-PAGE gel, stained with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker, lane 2, 3 and 4: supernatant obtained after the first round of freeze and thaw followed by sonication, lane 5, 6 and 7: the supernatant obtained after the second round of freeze and thaw followed by

sonication, lane 8, 9 and 10: pellet that remained after removal of supernatants. The experiment was repeated three times, a sample experiment is shown. The arrow indicates the 36 kDa band of *Pf*LDH.

The results are shown in Figure 3.7 and shows that proteins were present in the soluble fraction after the second round of freeze and thaw followed by sonication. There was less protein in the supernatant of the second round of freeze and thaw followed by sonication compared to the first round. This data suggests that there is still more protein present in the pellet.

3.2.4 Comparison of phase of induction

The effect of inducing protein expression at mid-log phase and stationary phase in LB media was evaluated to see which phase of induction produces more soluble proteins. There was an overall higher expression of the *Pf*LDH protein in cultures induced at stationary phase than at mid-log phase, as seen by the intensity of the 36 kDa bands (Figure 3.8) in the supernatant and the pellet on the SDS page gel. More of the *Pf*LDH was seen in the supernatant of cell cultures induced at the stationary phase in all the samples tested. This result is similar to those found by Flick *et al*, (2004). *E. coli* host cells with the same plasmid insert did not always yield the same amount of *Pf*LDH as seen by the intensity of the bands in Figure 3.8 for each of the three individual cultures induced at mid-log or stationary phases.

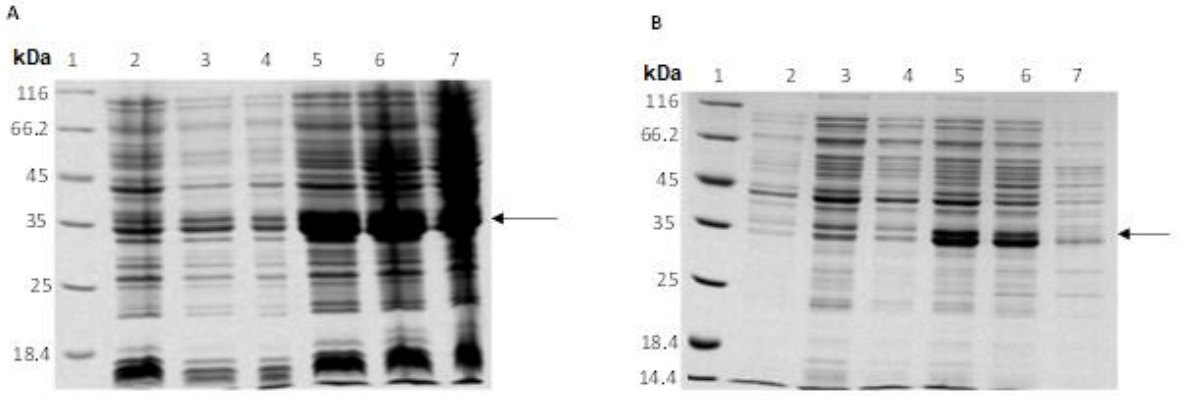


Figure 3.8 Expression of *Pf*LDH in LB broth after induction at mid-log and stationary phases. Recombinant *Pf*LDH was expressed at 37°C in LB media and induced with 0.3 mM IPTG at mid-log (OD_{600} of 0.5-0.6) and stationary phases (OD_{600} of 0.65-0.68). The expression was evaluated using reducing 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Lane 1: molecular weight marker, lane 2, 3 and 4: *Pf*LDH samples induced at mid-log phase, lane 5, 6 and 7: *Pf*LDH samples induced in the stationary phase. **(A)** is the pellet and

(B) is the supernatant from the pellet in A. The experiment was repeated three times, an example experiment is shown. The arrows indicate the 36 kDa *Pf*LDH band.

3.2.5 Comparison of 25°C and 37°C growth temperatures on the expression of *Pf*LDH induced at stationary phase.

One of methods to improve the expression of soluble proteins is to change the growth temperature of cultures. At lower temperatures, bacteria grow slowly and, typically exhibit a low rate of protein synthesis. When bacteria grow slowly there is a low probability of misfolding and aggregation of proteins to form inclusion bodies, favouring a relatively high yield of expressed protein (Vasina and Baneyx, 1997). Results in Figure 3.9 shows higher amounts of *Pf*LDH at 37°C and 25°C in the pellet sample. There were higher amounts of *Pf*LDH in the soluble fraction (supernatant) at 25°C than at 37°C. The amounts of *Pf*LDH in the supernatant at 25°C are high compared to other protein bands in each lane, whereas at 37°C all the protein bands are more intense. This suggests that relatively the expression of *Pf*LDH compared to other proteins is higher at 25°C than at 37°C. This result is similar to what Tsai *et al.*, 2016 found.

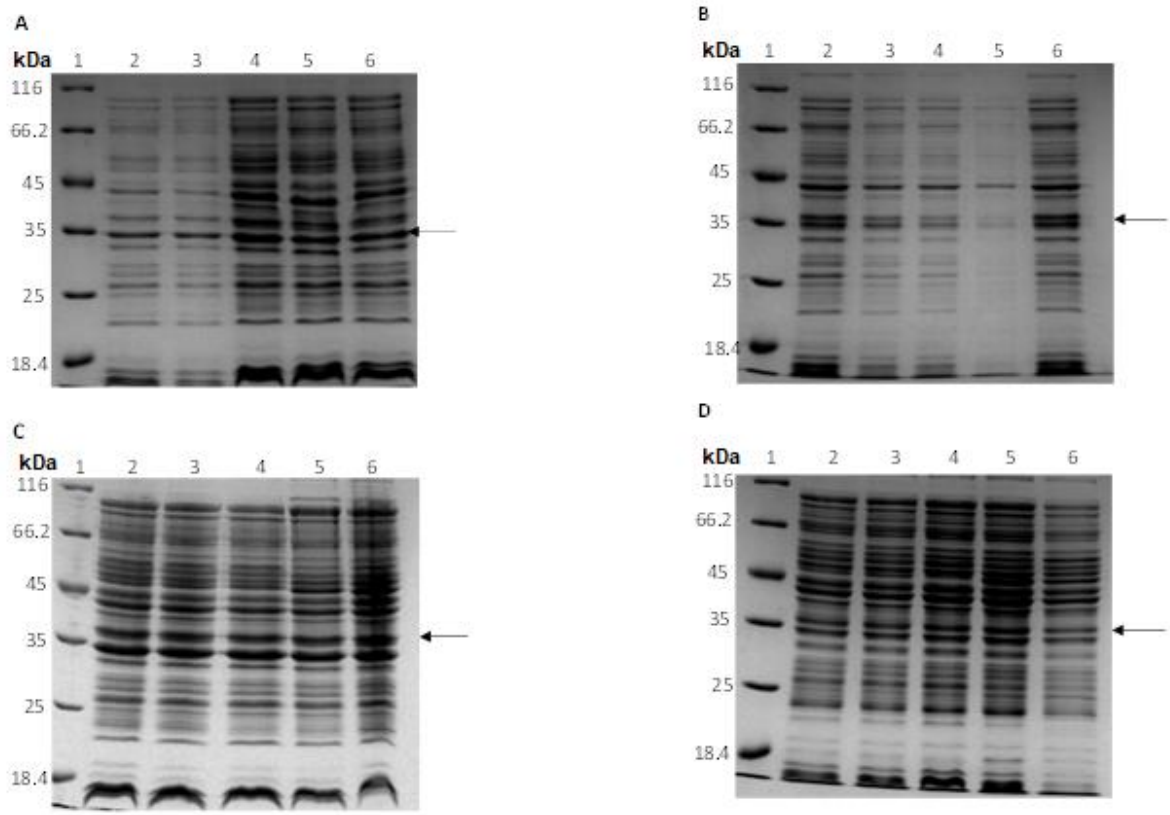


Figure 3.9 Comparing the expression of *Pf*LDH in LB media induced at stationary phase grown at 25°C or 37°C. Bacteria were grown at 25°C and 37°C.The expression was evaluated using 12.5% reducing DSD-PAGE

gel stained with Coomassie Brilliant Blue R-250. Panels **A** and **B** *E. coli* host cells incubated at 25°C, panels **C** and **D** *E. coli* host cells incubated at 37°C. Lane 1: molecular weight marker, lane 2, 3, 4, 5 and 6: *Pf*LDH pellet samples induced in the stationary phase in panel **A** and **C.** Panels **B** and **D** are supernatants from the same pellets. The experiment was repeated three times, a sample experiment is shown. The arrow indicates the 36 kDa *Pf*LDH.

3.2.6 Affinity purification of *Pf*LDH on Talon® (Co^{2+}) resin

After cell lysis, as described above, the clarified soluble protein fraction was passed over a Talon® (Co^{2+}) resin and incubated overnight at either 4°C, or room temperature on a platform shaker. When purifying at 4°C, more recombinant *Pf*LDH compared to room temperature was obtained (Figure 3.10). A doublet was observed with one band at about 36 kDa and a second band at 33 kDa (Figure 3.10 A. lanes 6, 7, 8 and figure 3.10 B. lanes 11, 12, 13).

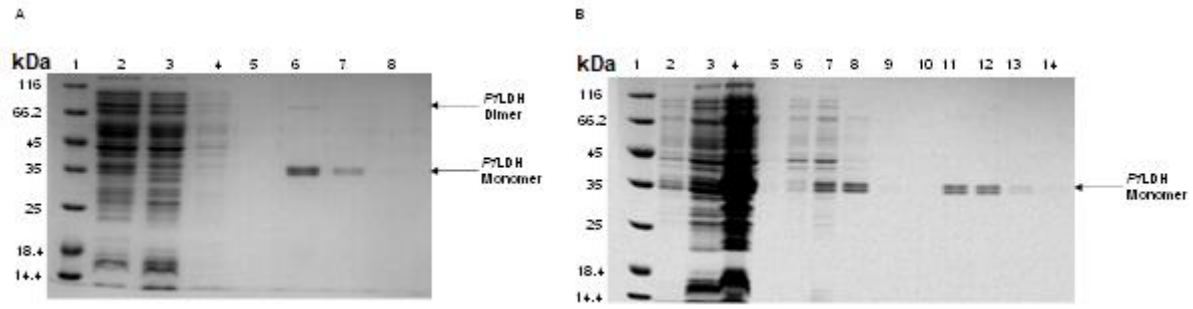


Figure 3.10 Comparison of affinity purification of *Pf*LDH at room temperature and at 4°C. Expression and purification of *Pf*LDH sample passed over a Talon® (Co^{2+}) resin at RT (**A**) and 4°C (**B**) monitored on a reducing 12.5% SDS-PAGE stained with Coomassie Brilliant Blue R-250. Panel **A:** Lane 1: Molecular weight marker, lane 2: supernatant whole cell lysate, lane 3: unbound protein to the column, lane 4: wash samples from the column, lane 5-8: elutions of *Pf*LDH. Panel **B:** Lane 1: Molecular weight marker, lane 2: supernatant from the mid-log phase, lane 3: supernatant from the stationary phase, lane 4: supernatant from the stationary phase induced with IPTG, lane 5: pellet after removal of supernatant at mid-log phase, lane 6: pellet after removal of supernatant at stationary phase, lane 7: pellet after removal of supernatant at stationary phase induced with IPTG, lane 8: wash samples from of the column, lane 9: wash one, lane 10-14: elutions of *Pf*LDH. The experiment was repeated three time, a sample experiment is shown.

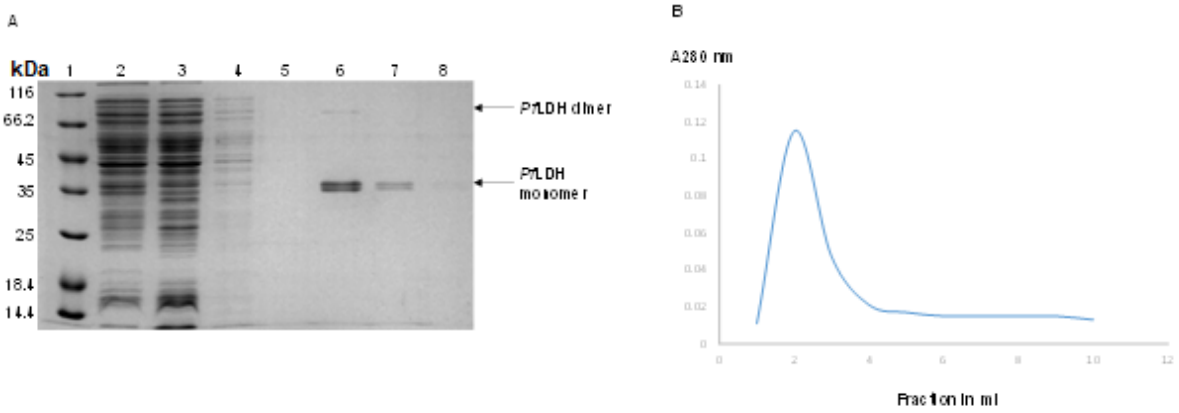


Figure 3.11 Affinity purification of *Pf*LDH at 4°C. Panel **A** is a 12.5% reducing SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker, lane 2: supernatant of cell lysate, lane 3: unbound protein to the column, lane 4: wash sample from the column, lane 5-8: elutions of *Pf*LDH. Panel **B** is an elution profile of *Pf*LDH taken by measuring absorbance at 280 nm of elution 1 to elution 10. Arrows shows the monomer (36 kDa) and dimer (75 kDa) of *Pf*LDH. The experiment was repeated three times, a sample experiment is shown.

The bands in Figure 3.10 B lanes 11, 12 and 13 are sharper compared to Figure 3.10 A lanes 6, 7, and 8. A faint band was seen in Figure 3.10 A at about 75 kDa and was thought to be a dimer of *Pf*LDH similar to the observation by Zocher *et al*., (2012) with *P. falciparum* glutamate dehydrogenase. The 33 kDa band was suggested to be a truncated form of *Pf*LDH (Turgut-*Balik et al*., 2004; Berwal *et al*., 2008; Hurdayal *et al*., 2010) due to an internal Shine Dalgarno sequence (GGAGGA) between base 45 and 51 in the coding sequence of recombinant *Pf*LDH (Cicek *et al*., 2012).

A 50 ml bacterial culture was grown and used for the purification *Pf*LDH in Figure 3.11. An elution profile of the purification shows a high yield of recombinant protein was obtained.

3.2.7 Western blot of recombinant *Pf*LDH

Western blot of each of the steps in the purification protocol of *Pf*LDH showed high yields of recombinant protein as shown by the intensity of bands in Figure 3.12. The total protein obtained as determined by the Bradford method section 2.3.1 from a 200 ml culture yielded 144.77 mg of recombinant *Pf*LDH. The proteins in different fractions were pooled together and concentrated using PEG_{20000} (section 2.2.5). The yield obtained was higher than obtained by others (Berwal *et al*., 2008; Hurdayal *et al*., 2010).

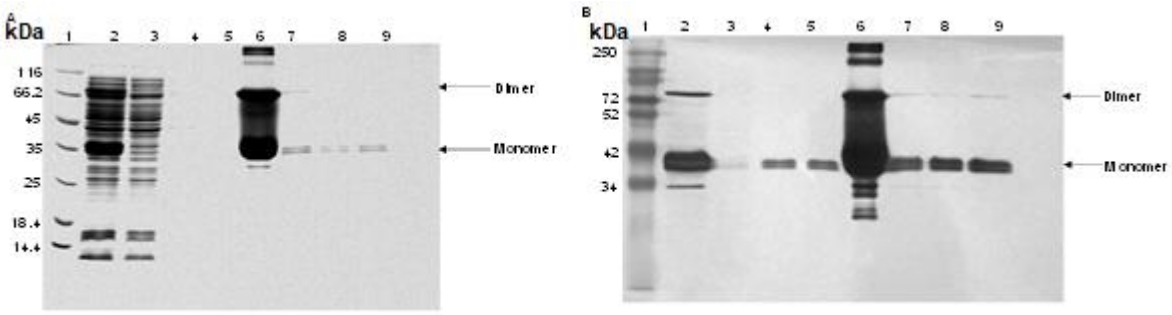


Figure 3.12 Expression, purification and detection of *Pf*LDH using anti-His-tag primary antibodies. Panel **A** is a 12.5% SDS-PAGE reducing gel of recombinant *Pf*LDH stained with Coomassie Brilliant Blue R-250 and panel **B** is a western blot of an identical gel as in A of recombinant *Pf*LDH probed with anti- His-tag monoclonal antibodies at dilution 1 : 1000 and goat anti-mouse HRPO conjugated secondary antibodies at 1: 6000 dilution.

Lane 1: Molecular weight marker, lane 2: supernatant whole cell lysate, lane 3: unbound protein to the column, lane 4: wash sample from the column, lane 5-9: elution of *Pf*LDH. Arrows show the monomer (36kDa) and dimer (75 kDa) of *Pf*LDH. The experiment was repeated three times. A sample experiment is shown.

3.2.8 Enzyme activity of recombinant *Pf*LDH

Preliminary studies of the activity of the recombinant protein were done to see if an active enzyme was expressed. An assay in which the amount of cofactor NAD^+ and substrate L-lactate were kept constant. An increase in the concentration of *Pf*LDH resulted in an increase in activity as indicated by an increase in the absorbance at 345 nm (Figure 3.13). The noise seen at 50 s was when the spectrophotometer was opened to add the substrate to the reaction.

$$\text{Lactate} + NAD^+ \xrightarrow[\text{LDH}]{} \text{Pyruvate} + NADH + H^+$$

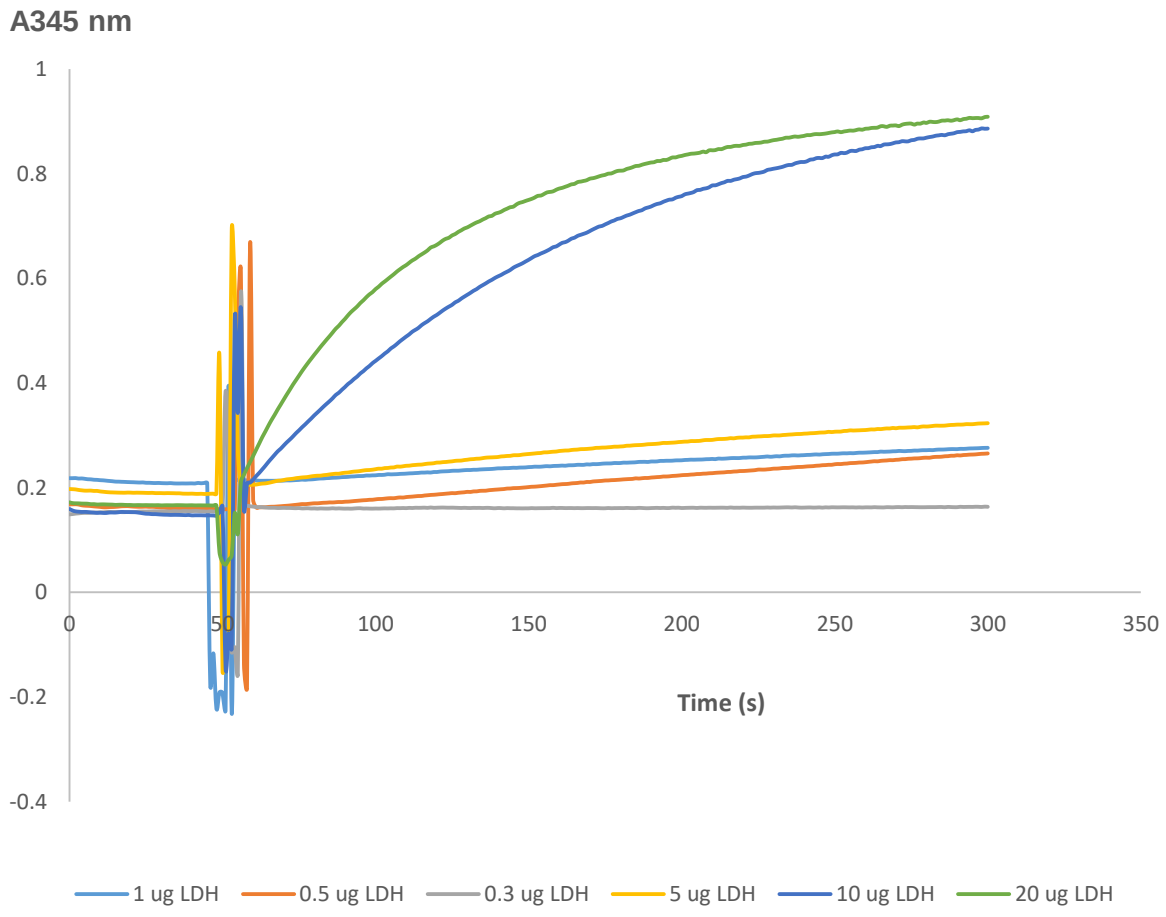


Figure 3.13 Enzyme activity at various amounts of *Pf*LDH are shown. The substrate, lactic acid, and cofactor NAD^+ was kept constant and the enzyme concentration was increased. The experiment was repeated three times. A sample experiment is shown.

3.2.9 Comparison of induced and uninduced cultures in the expression of recombinant *Pf*HSP70-1

Induced *E. coli* cell cultures produced high amounts of recombinant *Pf*HSP70-1 and basal expression of *Pf*HSP70-1 was observed in uninduced *E. coli* cells. Even in the absence of IPTG, basal expression levels of target protein known as 'leaky' expression can occur, as shown in Figure 3.14 lanes 2, 3 and 4. Expression of *Pf*HSP70-1 protein increased after induction with 1 mM IPTG as seen in lanes 5, 6 and 7 (Figure 3.14). The experiment shows that there can be different amounts of protein expressed by each sample of three cultures diluted from a single initial culture.

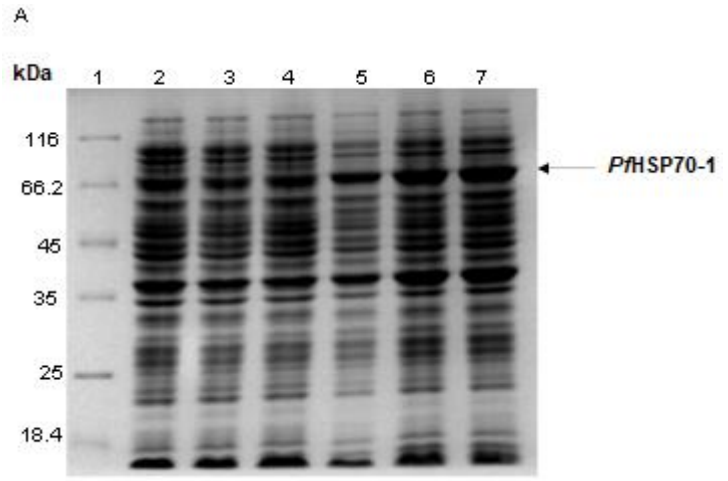


Figure 3.14 Expression of recombinant *Pf*HSP70-1 in LB media at 37°C. Expression of recombinant *Pf*HSP70-1 *E. coli* cell culture induced at mid-log phase (OD_{600} of 0.5-0.6) with 1 mM IPTG in LB media monitored on a 12.5% reducing SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker, lane 2, 3 and 4: uninduced *E. coli* culture, lane 5, 6 and 7 induced *E. coli* culture. Arrow shows the 72 kDa band of *Pf*HSP70-1.

Induction of *E. coli* cultures in LB media at 37°C, at OD_{600} of 0.5 (mid-log phase) showed a minor difference compared to induction at OD_{600} of 0.2 as shown in Figure 3.15 panel A and B, respectively. There was overall poor growth of *E. coli* cells.

The growth of *E. coli* host cells showed earlier increase in cell number after 1 hr and subsequently reached stationary phase earlier when induced at OD_{600} of 0.5 compared at 0.2 (at 2 hr) as seen in Figure 3.16. The difference is not very significant, suggesting poor growth of *E. coli* host cells as observed in Figure 3.15. The *E. coli* host cells reached

stationary phase after approximately 5 hrs and the optical density at stationary phase was about 1.8.

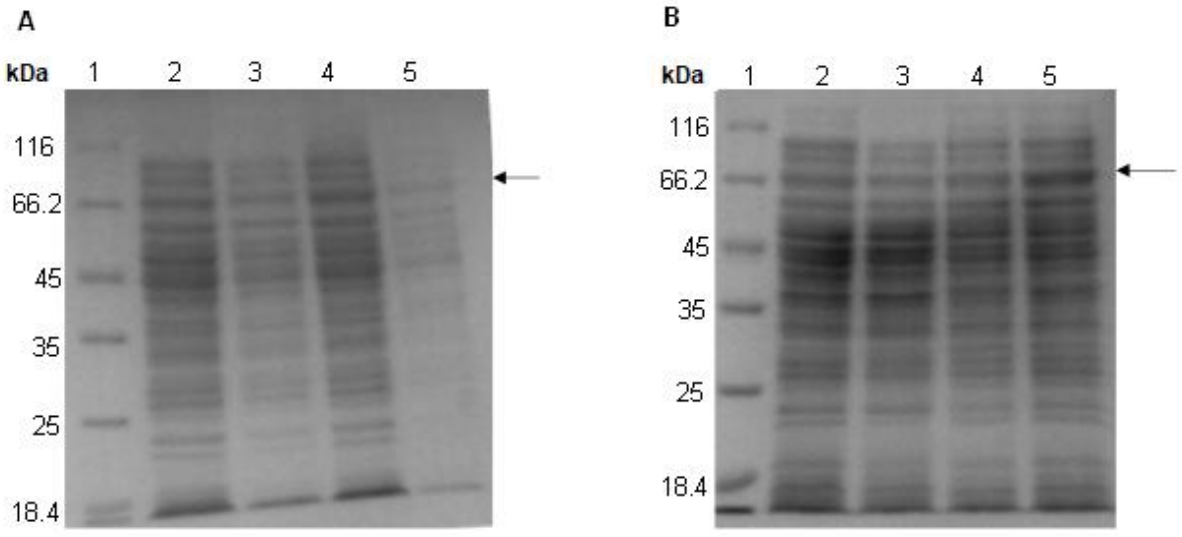


Figure 3.15 Comparison of *Pf*HSP70-1 expression after induction at OD_{600} of 0.2 with that of OD_{600} of 0.5 in LB media at 37°C. The induction of the recombinant protein with 1 mM IPTG at OD_{600} of 0.2 in panel **A** and 0.5 in panel **B** was monitored using a 12.5% SDS-PAGE reducing gel stained with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker, lane 2 and 3: uninduced *E. coli* culture 1, lane 4 and 5: induced *E. coli* cultures. The arrow shows the 72 kDa *Pf*HSP70-1 band.

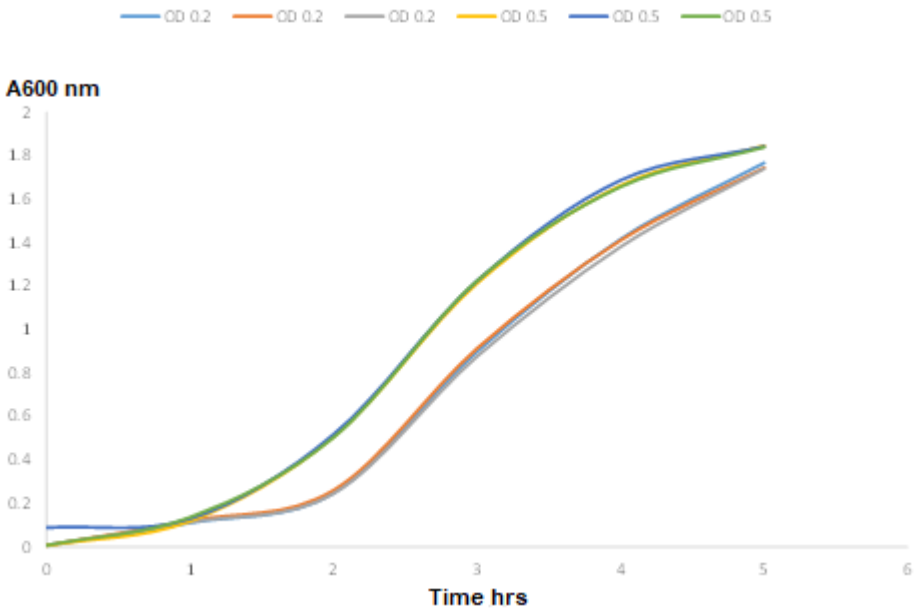


Figure 3.16 *E. coli* host cell growth in cultures induced at OD_{600} 0.2 and 0.5. The *E. coli* host cell were grown in LB media and monitored at OD 600 nm over a 6 hrs period. The experiment was done in triplicate.

3.2.10 Comparison of growth media on the expression of recombinant *Pf*HSP70-1

The effect of TB and LB media on the expression of *Pf*HSP70-1 was evaluated. TB is a self-inducing medium and, therefore, the addition of IPTG is not required. The cultures grown in LB media were induced with 1 mM IPTG at mid-log phase at an OD_{600} of 0.5-0.6. The results in Figure 3.17 shows a difference in the expression of recombinant *Pf*HSP70-1 in TB compared to LB media. Expression of recombinant *Pf*HSP70-1 was higher in TB than LB media. The expression of *Pf*HSP70-1 protein in the *E. coli* lysate was higher relative to other proteins in the TB media.

The effect of inoculating fresh LB broth with overnight cultures at different dilutions was evaluated. A dilution of 1:10 was compared with that of 1:100. The cultures were induced at OD_{600} of 0.5-0.6 with 1 mM IPTG. The results in Figure 3.18 suggest that a dilution of 1:100 is marginally better than that of 1:10.

The expression of *Pf*HSP70-1 using different individual starting colonies of host *E. coli* cells was evaluated and Figure 3.19 shows that the protein profiles in all lanes were different. Lanes 3, 6, 7 and 9 had similar amounts of recombinant *Pf*HSP70-1, lane 11 had the highest amount of recombinant *Pf*HSP70-1 and lane 2 had the least amount of recombinant *Pf*HSP70-1. Inoculating fresh broth with a single colony to express recombinant protein has an advantage over overnight culture dilutions. It involves less steps and can save time.

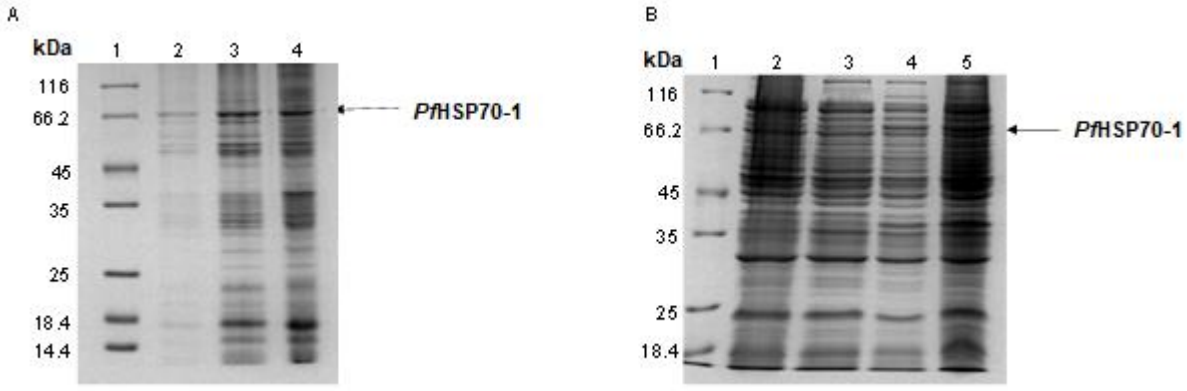


Figure 3.17 Expression of recombinant *Pf*HSP70-1 *E. coli* cell culture in TB and LB media. The expression of *Pf*HSP70-1 *E. coli* cell culture in TB and LB was monitored using a reducing 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Panel **A** is recombinant *Pf*HSP70-1 *E. coli* cell culture grown in TB media. Lane 1: molecular weight marker, lane 2, 3 and 4: lysate of *E. coli* cells. Panel **B** is recombinant *Pf*HSP70-1 *E.*

coli cells grown in LB media. Lane 1: Molecular weight marker, lane 2 and 3: un-induced *Pf*HSP70-1 *E. coli* cells, lane 4 and 5: induced *Pf*HSP70-1 *E. coli* cells. The arrow show the 72 kDa *Pf*HSP70-1 bands.

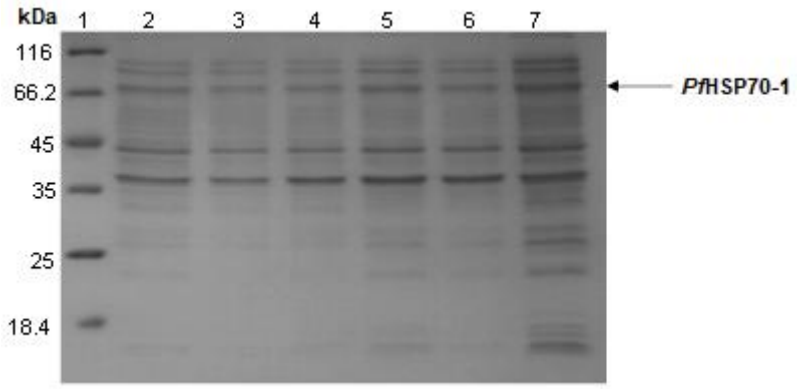


Figure 3.18 Comparing different dilutions of overnight cultures on the growth of *E. coli* host cells. The effect of diluting an overnight culture into fresh LB broth induced at OD_{600} of 0.5-0.6 was tested and monitored using a reducing 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. Lane 1: Molecular weight marker, lane 2, 3 and 4: induced *E. coli* culture diluted at 1:10 from an overnight culture, lane 5, 6 and 7: induced *E. coli* culture diluted at 1:100 from an overnight culture. The arrow shows the 72 kDa band of *Pf*HS70-1 band.

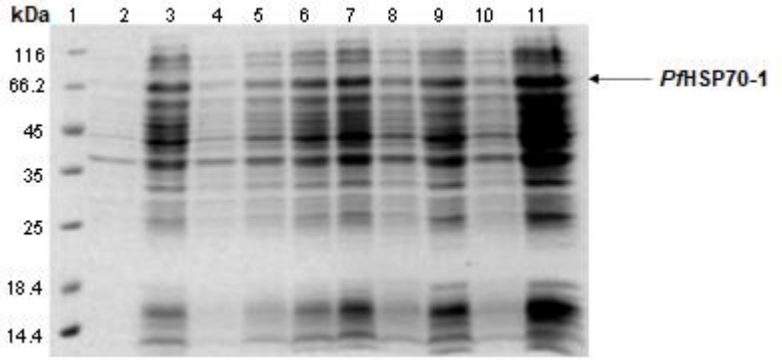


Figure 3.19 Expression of recombinant *Pf*HSP70-1 from different starting colonies in TB media. The expression profile of 10 different *E. coli* colonies containing a 6XHis *Pf*HSP70-1 was analysed on a 12.5% SDS-PAGE reducing gel stained with Coomassie Brilliant Blue R-250. Lane 1: molecular weight marker, lanes 2-11: contain lysate prepared from different individual colonies on the same petri dish. The arrow shows the 72 kDa *Pf*HSP70-1 band.

The data above suggests that TB is a better choice of media to use for expressing recombinant *Pf*HSP70-1 as TB does not require use of IPTG for induction recombinant protein and frequent checking of the optical density during expression. The use of a single colony can be easier. The data indicated that triplicate samples are necessary when looking to optimise bacterial growth and recombinant protein expression.

3.2.11 Comparison of methods for lysing *Pf*HSP70-1 *E. coli* cells

Methods of lysing bacterial cells includes lysozyme digestion, freezing and thawing, sonication, and a combination of all three methods. Combining all three methods yielded the most proteins as seen by intensity of the bands in Figure 3.20. The sample obtained after lysing of *E. coli* host cells containing *Pf*HSP70-1 were always viscous. The samples were prepared and lysed as in section 2.2.4 above were run on 1% agarose gel to determine if these samples contained high amounts of nucleic acids. The agarose gels showed high amount of nucleic acid (Figure 3.21).

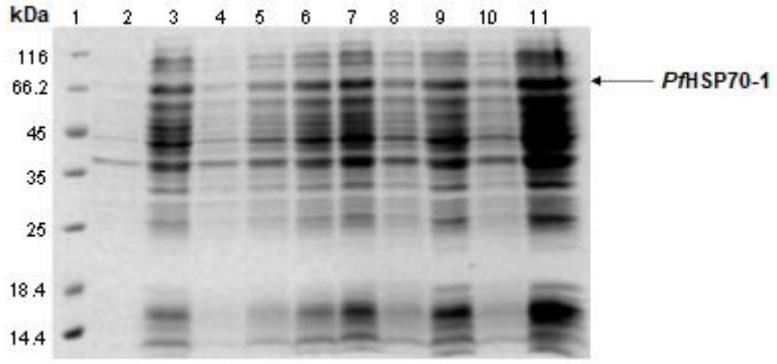


Figure 3.20 Methods used to lyse bacterial cells. The bacterial cells were lysed using lysozyme, freezing and thawing, sonication and a combination of all the methods. The amount of protein released by each method was evaluated on a reducing 12.5% SDS-PAGE stained with Coomassie Brilliant Blue R-250. Lane 1: molecular weight marker, lane 2 and 3: supernatant of lysozyme digested samples, lane 4 and 5: supernatant of freeze and thaw samples, lane 6 and 7: supernatant of sonication samples, lane 8 and 9: supernatant of freeze and thaw then sonication samples, lane 10 and 11: supernatant of all three methods combined. The experiment was repeated three times, a sample experiment is shown. The arrow shows the 72 kDa *Pf*HSP70-1 band.

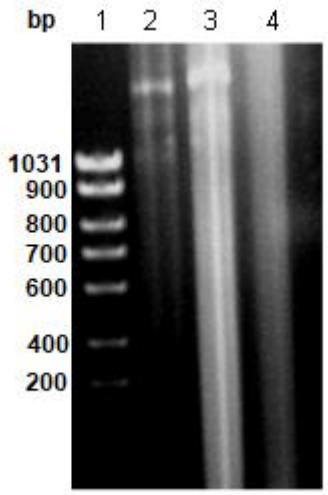


Figure 3.21 A 1% (w/v) agarose gel of *E. coli* host cells containing a *Pf*HSP70-1 insert. The *E. coli* cells were frozen and thawed, then sonicated. A 1% (w/v) agarose gel of expressed *Pf*HSP70-1. Panel **A**: Lane 1:

DNA ladder, lane 2: supernatant of un-induced culture, lane 3 and 4: supernatant of induced culture. The experiment was repeated three times, a sample experiment is shown.

The use of freeze and thaw, followed by sonication was chosen as it was a less viscous solution and contained less contaminants.

3.2.12 Affinity purification of recombinant *Pf*HSP70-1 on Talon® (Co^{2+}) resin and detection of the protein on a western blot

After cell lysis, the clarified soluble protein fraction was poured over a Talon® (Co^{2+}) resin. The expressed and affinity purified recombinant *Pf*HSP70-1 protein were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane and probed with anti-His-tag monoclonal antibodies.

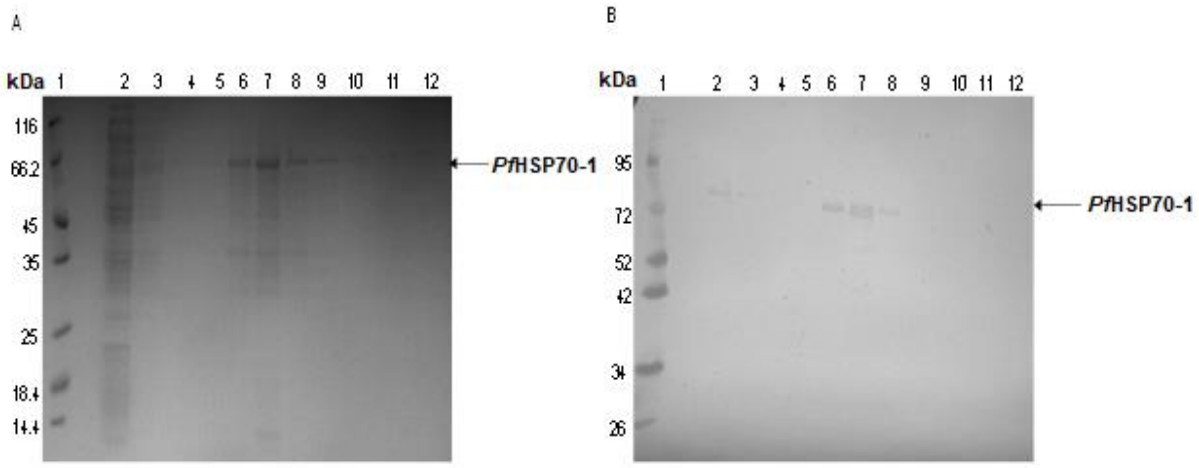


Figure 3.22 Affinity purification of recombinant *Pf*HSP70-1 on Talon® (Co^{2+}) resin. The expression and affinity purification of *Pf*HSP70-1 on Talon® (Co^{2+}) resin was evaluated using a reducing 12.5% SDS-PAGE gel stained with Comassie Brilliant Blue R-250 in panel **A** and western blot probed with anti- His-tag primary antibodies at a dilution of 1:2000 as shown in panel **B**. Lane 1: molecular weight marker, lane 2. proteins that did not bind to resin, lane 3 and 4: 2 washes of the resin, lane 5-12: elution of *Pf*HSP70-1 from resin. The arrows show the 72 kDa *Pf*HSP70-1 bands.

There was a low yield of purified protein as shown by low intensity of the bands in panel A and the western blot in panel B of Figure 3.22. The expression and purification steps were repeated to establish the cause of the low yields of the purified protein. Bacteria from different stocks were compared to see if source of material may make a difference. Low yields were obtained from all sample (Figure 3.23). Each expression round of *Pf*HSP70-1 yielded less and less protein until no protein was detected on western blots. The lower protein band of 34 kDa may be a truncated form of *Pf*HSP70-1 as shown in Figure 3.24 D.

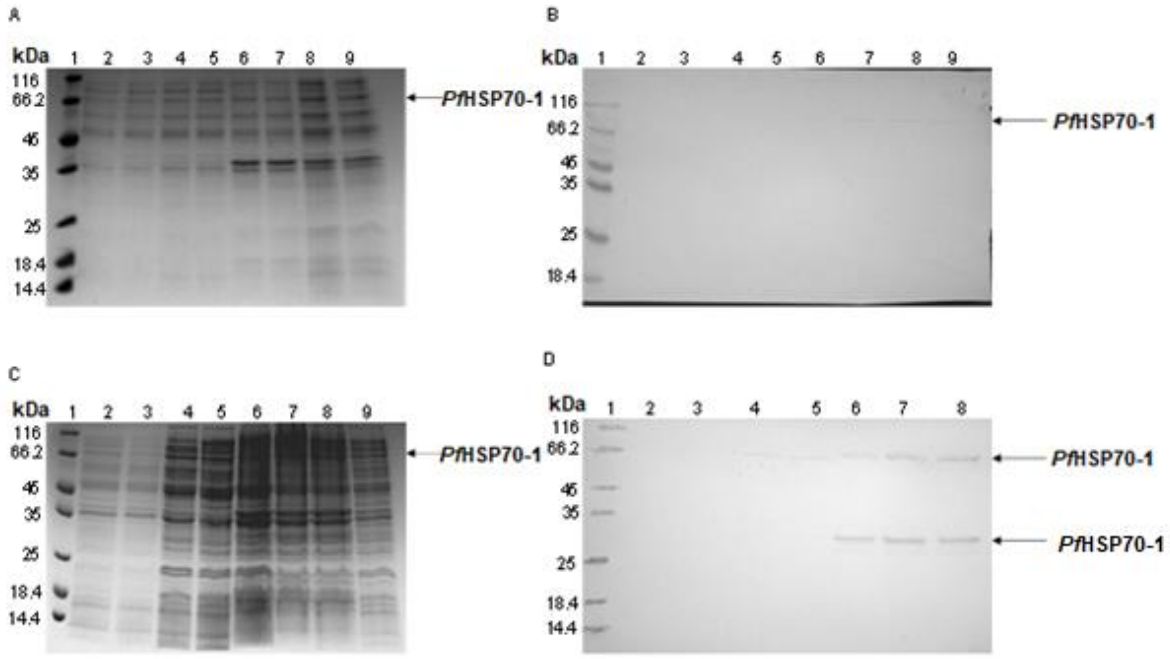


Figure 3.23 Samples of *Pf*HSP70-1 *E. coli* host cells from two different glycerol stocks. 12.5% SDS-PAGE (**A** and **C**) and western blots probed with anti-His-tag (**B** and **D**) of recombinant *Pf*HSP70-1 from bacterial colonies streaked from different glycerol stocks. Lane 1: molecular weight marker, lane 2 and 3 un-induced culture of colonies taken from stock 1 petri dish, lane 4 and 5: un-induced culture of colonies taken from stock 2 petri dish, lane 6 and 7: induced culture of colonies from stock 1 petri dish, lane 8 and 9: induced culture of colonies from stock 2 petri dish. The 30 KDa is the truncated form of *Pf*HSP70-1. The experiments were repeated three times. A sample experiments are shown. The arrows show the 72 kDa *Pf*HSP70-1 bands.

3.2.13 Isolation of pQE30/His$_6$*Pf*HSP70-1 plasmid and restriction digestion

Due to the poor expression of the *Pf*HSP70-1, the original plasmid was obtained as described in section 2.2.1.4 and digested with BamHI.

As a positive control, *E. coli* containing the human HSP70 whose insert is known to be present was used. The *Pf*HSP70-1 insert appeared to have been lost, as there was no restriction digestion with BamHI in lane 4. This explains why very low concentrations of recombinant protein in the western blots (Figures 3.22 panel B, Figure 3.23 panel B and D) and poor expression of the protein (Figures 3.17 – 3.21) were observed.

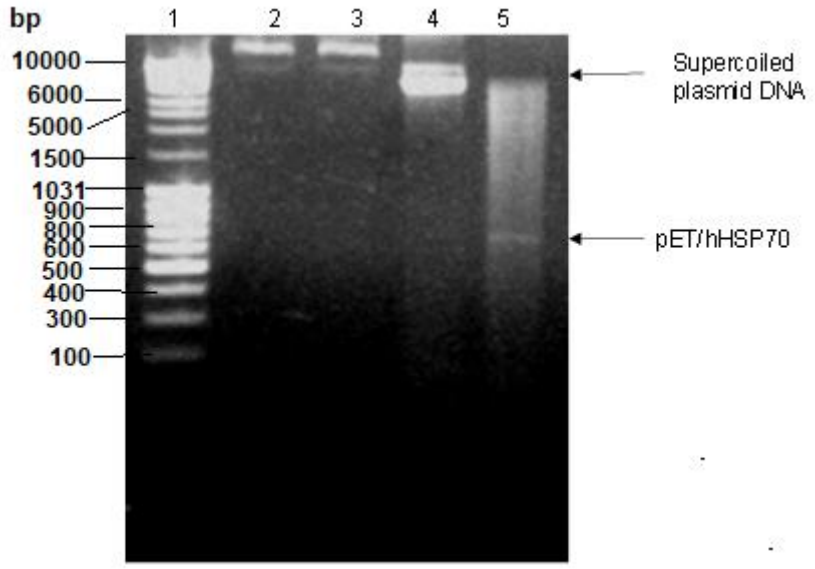


Figure 3.24 Restriction digestion of pQE30/His$_6$*Pf*HSP70-1 and pET/*h*HSP70 by BamHI. A 1% (w/v) agarose gel showing plasmid DNA of *Pf*HSP70-1 and human HSP70-1 (*h*HSP70-1). Lane 1: DNA ladder, lane 2: pQE30/*Pf*HSP70-1 plasmid, lane 3: a 1:10 dilution of pQE30/*Pf*HSP70-1 plasmid, lane 4: pQE30/*Pf*HSP70-1 plasmid digested with BamHI, lane 5: pET/*h*HSP70 plasmid digested with BamHI.

3. 3 Discussion

The conditions that were evaluated for the expression of two recombinant proteins included comparisons of the type of media used, the growth of *E. coli* host cells in TB and LB media, starting bacterial growth from a single colony or an overnight culture, the time during growth when protein expression is induced, two temperatures on the expression of recombinant protein induced at stationary or mid-log phase of growth, methods for lysing *E. coli* cells and conditions for affinity purification of the recombinant proteins.

3.3.1 Media used to express recombinant *Pf*LDH and *Pf*HSP70-1

*Pf*LDH showed better expression in TB than LB media. TB media contains tryptone, yeast extract, glycerol and dibasic phosphates. Tryptone, a pancreatic digest of casein provides source of amino acids. Yeast extract provides a source of amino acids and trace elements to the medium. The extra addition of tryptone and yeast extract in TB medium allows bacteria to produce higher plasmid yield per volume. Glycerol is both a source of carbon and carbohydrates in the medium and unlike glucose, glycerol is not fermented to acetic acid, as such, it prevents the drop of pH during the growth of *E. coli* cell culture. Dibasic potassium phosphates are added to provide potassium for cellular systems and to maintain the pH during growth of the *E. coli* cell culture. TB media is self-induced and does

not require measuring ODs, and as such does not require to be monitored for the correct time of induction (Li *et al.*, 2011). In Figure 3.3.2 the OD_{600} was measured to compare growth in TB and LB medium. Expression of *Pf*HSP70-1 in TB was better than in LB medium. Previously, the amount of IPTG in the expression of *Pf*LDH was determined to 0.3 mM and 1 mM for *Pf*HSP70-1 when expressed in LB media.

3.3.2 Growth of *E. coli* host cells in TB and LB media

The *E.coli* host cells grow faster in TB media than LB media. This may be due to the presence of extra tryptone, yeast extract and glycerol in TB media. The use of a single colony to inoculate media also showed faster growth of *E. coli* host cells in TB media than in LB media. The time it takes to reach stationary phase for *Pf*LDH and *Pf*HSP70-1 was approximately 4 hrs at an optical density measured at 600 nm of about 0.68 and 5 hrs at optical density measured at 600 nm of about 1.8 respectively to monitor the growth of *E. coli* host cells.

3.3.3 Initiating bacterial growth from a single colony or an overnight bacterial culture

Starting with an overnight culture to evaluate recombinant protein expression was compared to starting with a single starting colony. There appeared to be more intense bands of recombinant proteins seen for both *Pf*LDH and *Pf*HSP70-1 from *E. coli* cell cultures grown using a single colony than a dilution of 1:100 as shown in Figure 3.4 (section 3.2.2) and Figure 3.18-19 (section 3.2.10), respectively. Use of a single colony to inoculate fresh media would save a lot of time as there is no requirement to grow an overnight culture for 16 hrs prior to protein expression. Each individual colony and dilution of an overnight culture did not produce the same amount of protein in the lanes examined.

3.3.4 Time of induction of protein expression

The induction of the expression of protein in the culture after the stationary phase OD_{600} of 0.68 for *Pf*LDH yielded higher amounts of *Pf*LDH compared to induction at mid-log phase OD600 of 0.5-0.6 with 0.3 mM ITPG, which was similar to results obtained by Flick *et al*, (2004). Among the reasons suggested for this observation are that proteins expressed at post-log phase tend to be less truncated (Flick *et al.*, 2004). Induction of *E. coli* host cells containing *Pf*HSP70-1 at mid-log phase and early log phase did not show a significant difference as expression was poor.

3.3.5 Two temperatures (25°C and 37°C) on the expression of *Pf*LDH induced at stationary phase

Expression of *Pf*LDH at 25°C produced more *Pf*LDH protein than at 37°C. The result is similar to what Flick *et al*, (2004) and Tsai *et al,* (2016) found when expressing *Plasmodium falciparum* erythrocyte membrane protein I (*Pf*EMP I) at temperatures as low as 16°C. Schein and Noteborn (1988) also found that expression at 30°C produced more recombinant interferon α2 (IFN-α2) and interferon ɣ (IFN-ɣ) proteins than at 37°C. Berwal *et al.*, (2008) also noted the same observation with recombinant *Plasmodium falciparum* LDH at 15°C compared to 37°C.

Expression in *E. coli* BL21 (DE3) has some advantages. The strain is deficient in the outer membrane protease (OmpT) and Lon protease which can digest recombinant proteins as they are expressed (Lee, 2009). BL21 DE3 *E. coli* cells require the T7 RNA polymerase gene to be lysogenised into the host *E. coli* BL21 genome (Sorensen and Mortensen, 2005). The T7 RNA polymerase, which is under control of the lacUV5 promoter, remains suppressed by LacI until IPTG is added to media to induce transcription of the target protein (Divan and Royds, 2013).

3.3.6 Optimising the method of lysis of *E. coli* cells

Bacterial cells are often lysed using three methods: lysozyme digestion, freezing and thawing and sonication. Lysozyme digestion released most proteins in the supernatant after lysing of *E. coli* cells as an individual method compared to sonication and, freezing and thawing. The only drawback in using lysozyme digestion was that the resulting supernatant was viscous and was contaminated with nucleic acids (Figure 3.7 & Figure 3.20). Freezing and thawing released more protein in the supernatant than sonication as an individual method after lysing of *E. coli* cells and contained less contaminants (Figure 3.7 & Figure 3.20). Sonication released the least amount of proteins in the supernatant after lysing *E. coli* cells as an individual method and contained the least contaminants (Figure 3.7 & Figure 3.20).The combination of freezing and thawing followed by sonication and then the two methods repeated yielded high amounts of recombinant *Pf*LDH and *Pf*HSP70-1 compared to lysozyme digestion alone, freeze and thaw alone and sonication alone. The problems of using all three methods is that it produces high contamination of nucleic acids in the samples and the samples were viscous (Figure 3.21). The combination of freeze and thaw followed by sonication repeated was, therefore, considered optimal for the lysing of *E.coli* cells in this study.

3.3.7 Affinity purification of recombinant *Pf*LDH and *Pf*HSP70-1

His-tagged proteins contain a histidine whose imidazole side chain has a pKa of 6.04 (Voet and Voet, 2004). The solubility of a protein is dependent on the overall charge which is negative if the pH is above the protein's pI and positive if the pH is below the protein's pI (Bondansky and Latner, 1975, Voet and Voet, 2004). The pI of *Pf*LDH is around 8.5 (Nirmalan *et al.*, 2004). The buffers used for the purification of *Pf*LDH was 8.0 suggesting that *Pf*LDH will have an overall positive charge and is likely to be soluble. Imidazole binds to cobalt ion (Co^{2+}) in the Talon® (Co^{2+}) resin. At pH 8.0, the recombinant protein will bind with high affinity to the Co^{2+} ions on the Talon® (Co^{2+}) resin. Nguyen *et al.*, (2014) showed that His-tag provides good yields of tagged proteins from inexpensive, high capacity resins. Purification of *Pf*LDH at room temperature was compared to purification at 4°C. Improved purification was observed at 4°C compared to RT (Tsai *et al.*, 2016 and Goto *et al.*, 2016). A doublet was clearly observed at around 33 kDa and 36 kDa and a single band was observed at around 75 kDa when purified at 4°C and a blurred doublet and 75 kDa were observed when purified at 37°C. Hurdayal *et al.*, (2010) expressed a 2X His_6-*Pf*LDH with His tags at both the N- and C- terminal ends of *Pf*LDH and found the size to be approximately 39 kDa and Berwal *et al.*, (2008) expressed a His_6-*Pf*LDH and found the size to be around 36.3 kDa. Purification of *Pf*HSP 70-1 yielded low amounts compared to what was observed by Matambo *et al.*, (2004). The size observed was about 72 kDa.

3.3.8 Western blots detecting *Pf*LDH and *Pf*HSP70-1

Anti-His-tagged antibodies were used to detect *Pf*LDH band on a western blot. A 36kDa band and another band at about 75 kDa were detected. The 75 kDa may be due to dimer formation a result similar to Zocher *et al.,* (2012) with recombinant *Plasmodium falciparum* glutamate dehydrogenase 1 on reducing SDS-PAGE gel which was detected by western blots, and was determined to be a dimer of *Pf*LDH. This could be confirmed by molecular exclusion chromatography. Another small band just below the 36 kDa of about 33 kDa was observed in the western blot which is similar to results found by others (Berwal *et al.*, 2008; Hurdayal *et al.,* 2010 and Turgurt-Balik *et al.*, 2004). This was due to the presence of an internal Shine-Dalgarno like sequence (GGAGGA) between bases 46 to 61 in the coding sequence for recombinant *Pf*LDH (Cicek *et al*., 2013). Berwal *et al.*, (2008) with His6-*rPf*LDH and Goto *et al.,* (2016) with swine LDH found both the recombinant proteins form tetramers and had enzyme activity. Probing western blots of *Pf*HSP70-1 with anti-His-tag antibodies showed low concentration of *Pf*HSP70-1. The size of the recombinant protein was approximately 72 kDa, similar to what was observed by Matambo *et al.*, (2004) who found a monomer of 73.5 kDa and a dimer of 199.5 kDa using SE-HPLC analysis. The 199.5 kDa

was too large to be a dimer and too small to be a trimer. Therefore the dimer may have been formed as an intermediated complex between a dimer and trimer. It was also noted that *Pf*HSP70-1 exists mainly in the monomer form.

3.3.9 Determination of the cause of poor expression of *Pf*HSP70-1

To determine the cause of poor expression of *Pf*HSP70-1, the plasmid was isolated according to manufacturer's instructions in section 2.2.1.4. The plasmid was then subjected to restriction digestion by BamHI to determine if the insert was still present. As a positive control, the plasmid containing human HSP70 whose insert was known to be present in plasmid was isolated and digested with BamHI and electrophoresed on the same 1% agarose gel. The DNA samples were resolved on 1% agarose gel and viewed under UV radiation. The results showed *Pf*HSP70-1 the insert appeared to have been lost. More sensitive methods such as PCR may be used to confirm the loss of the plasmid containing His_6-*Pf*HSP70-1 insert.

Summary

These results suggest that for the recombinant expression of *Pf*LDH in TB media at low temperature and purifying the protein at 4°C ensures production of more soluble recombinant proteins. The use of a single colony as starting material yields a larger amount of recombinant protein compared to dilution of an overnight culture. Therefore, use of a single colony was preferred as it was time saving. Freezing and thawing followed by sonication twice appeared to release more protein in the supernatant and was sufficient for lysing *E. coli* cells. The evidence as shown by poor expression and purification of *Pf*HSP70-1 coupled with results of plasmid digestion of pQE30/*Pf*HSP70-1 as shown on agarose gel electrophoresis (Figure 3.24) suggests loss of plasmid containing His_6-*Pf*HSP70-1 insert.

Chapter 4

Bionformatics

4.1 Introduction

Most of the antibodies used in current malarial RDTs are monoclonal antibodies. These monoclonal antibodies are selected against one of three peptides as opposed to identifying them from a unique epitope. Epitope mapping can be done to determine an epitope target (Saravanan and Kumar, 2009). Identifying a target epitope is important because antigenic variations sometimes affects the detection of certain antigens as in the case of a commonly used RDT target HRPII in malaria (Lee *et al*., 2006; Baker *et al*., 2010). HRPII undergoes antigenic variation leading to false negative results with antibodies in particular test kits (Gamboa *et al*., 2010). In this study, polyclonal and anti-peptide specific antibodies against the target epitopes are intended to be raised as an alternative to monoclonal antibodies. The peptides used to raise anti-peptide antibodies are usually 12-15 amino acid residues in length and cannot elicit an immune response by themselves. They are, therefore, coupled to a carrier protein usually rabbit albumin.

The use of polyclonal antibodies raised against the peptides of target proteins for malaria diagnosis has been validated (Tomar *et al*., 2006). The use of anti-peptide antibodies raised against specific epitopes for diagnosis of malaria and differentiation between the malaria lactate dehydrogenase from different species has been demonstrated by Hurdayal *et al*., 2010.

Many human proteins undergo post translational modifications which may reduce the interactions between antibodies raised against a recombinant protein or a peptide and its detection in the native protein (Glozak *et al*., 2005; Huang *et al.,* 2007; Gupta *et a*l., 1999; Nielsen *et al.,* 2005). This chapter describes the *'in silico*' work done to identify potential immunogenic peptides to be used in the production of antibodies in chicken using a prediction program Predict7™. The chapter describes the prediction of potential post translational modifications such as lysine acetylation, phosphorylation, glycosylation and protease digestion to see if these modifications have the potential to reduce antibody recognition. There is evidence that certain post translational modifications of each protein can affect the activity of native proteins (Zhao *et al*., 2013; Fan *et al*., 2011).

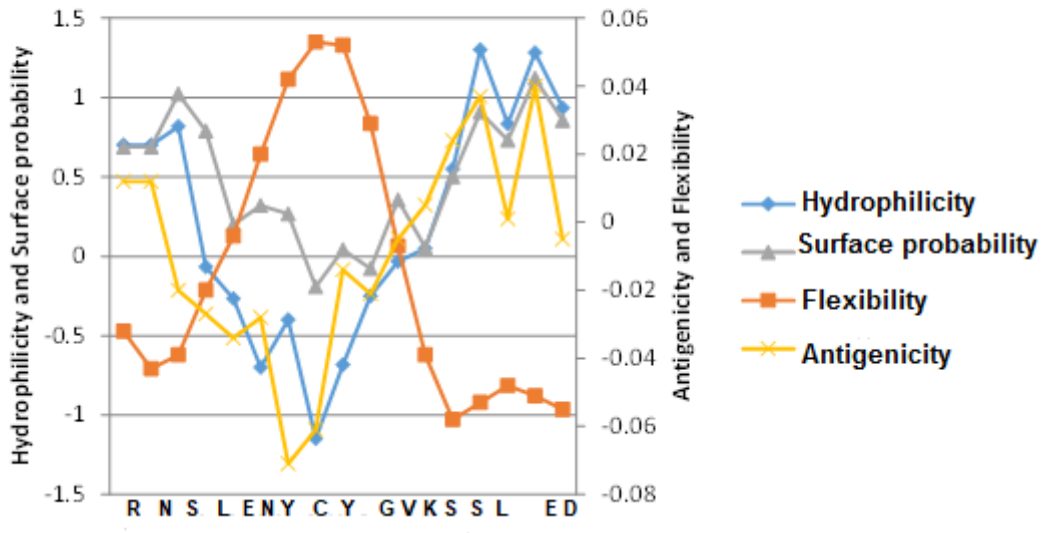
4.2 Selection of immunogenic peptides

The entire amino acid sequence of *Pf*HSP70-1 was analysed using Predict7™ to identify which regions in the sequence had potential as immunogenic peptides as described by Carmenes *et al.*, 1989. The structural features analysed were antigenicity (Welling *et a*l., 1985), flexibility (Karplus and Schulz, 1985), hydrophilicity (Hoop and Woods, 1981) and surface probability (Janin *et al.*, 1978; Emini, 1985). Other features of the program include hydropathy

(Kyte and Doolittle, 1982) and secondary structure (Garnier *et al.*, 1978). The selected peptides need to be on the surface of the protein to increase the probability of recognition and binding by antibodies in solution (Saravanan and Kumar, 2009). Three peptides with high surface probability and hydrophilicity values were selected from the amino acid sequence of *Pf*HSP70-1 and are shown in Figure 4.1. All the three amino acid sequences chosen are located in the α-helical-subdomain (lid and hinge region) of *Pf*HSP70-1, which closes in the ADP state and is open in the ATP state to allow substrate binding. Analysis of *Pf*LDH sequences with Predict7™ to identify immunogenic epitopes was previously done in our laboratory. Within the sequence of *Pf*HSP70-1 three peptides chosen had hydrophilicity and surface probability values above zero, and were, therefore, considered to be potential immunogenic peptides.

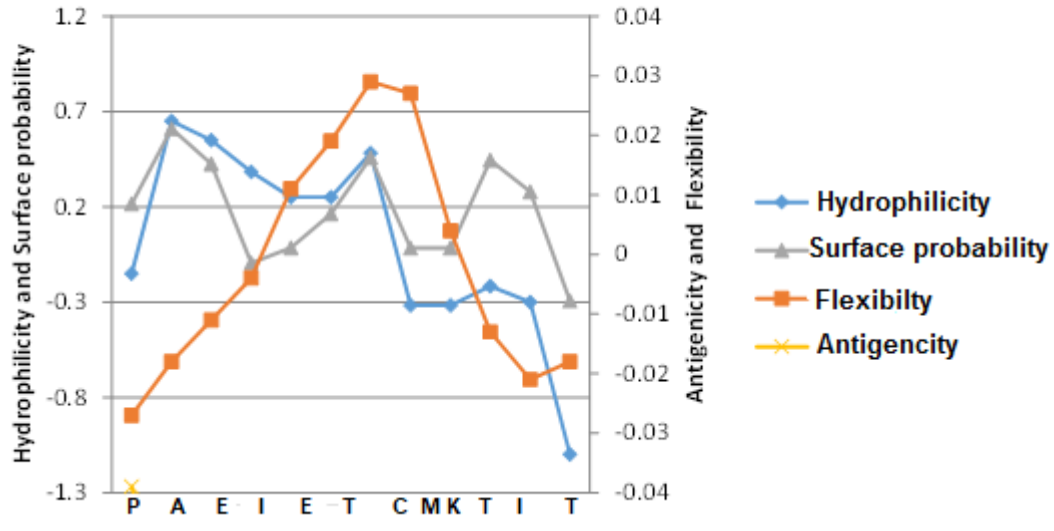
RNSLENYCYGVKSSLED

A



PAEIETCMKTIT

B



AGKDEYEAKQKEAESVC

C

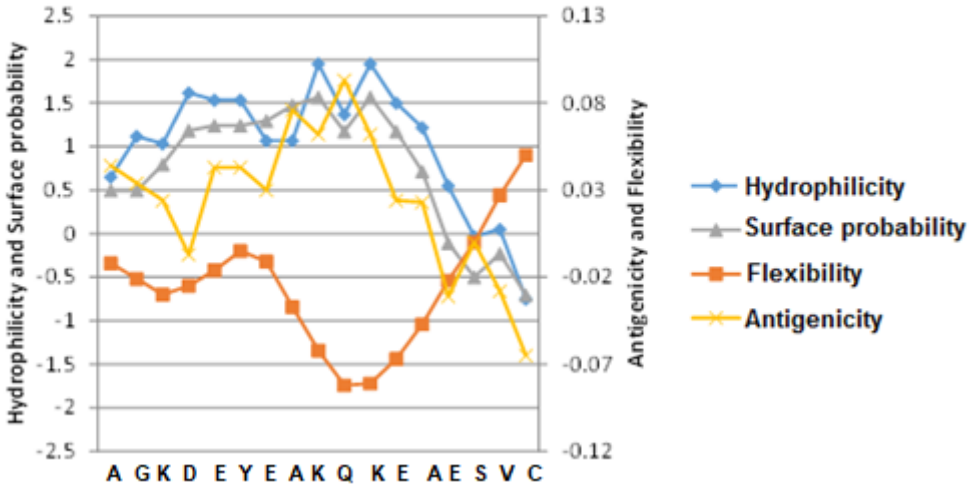
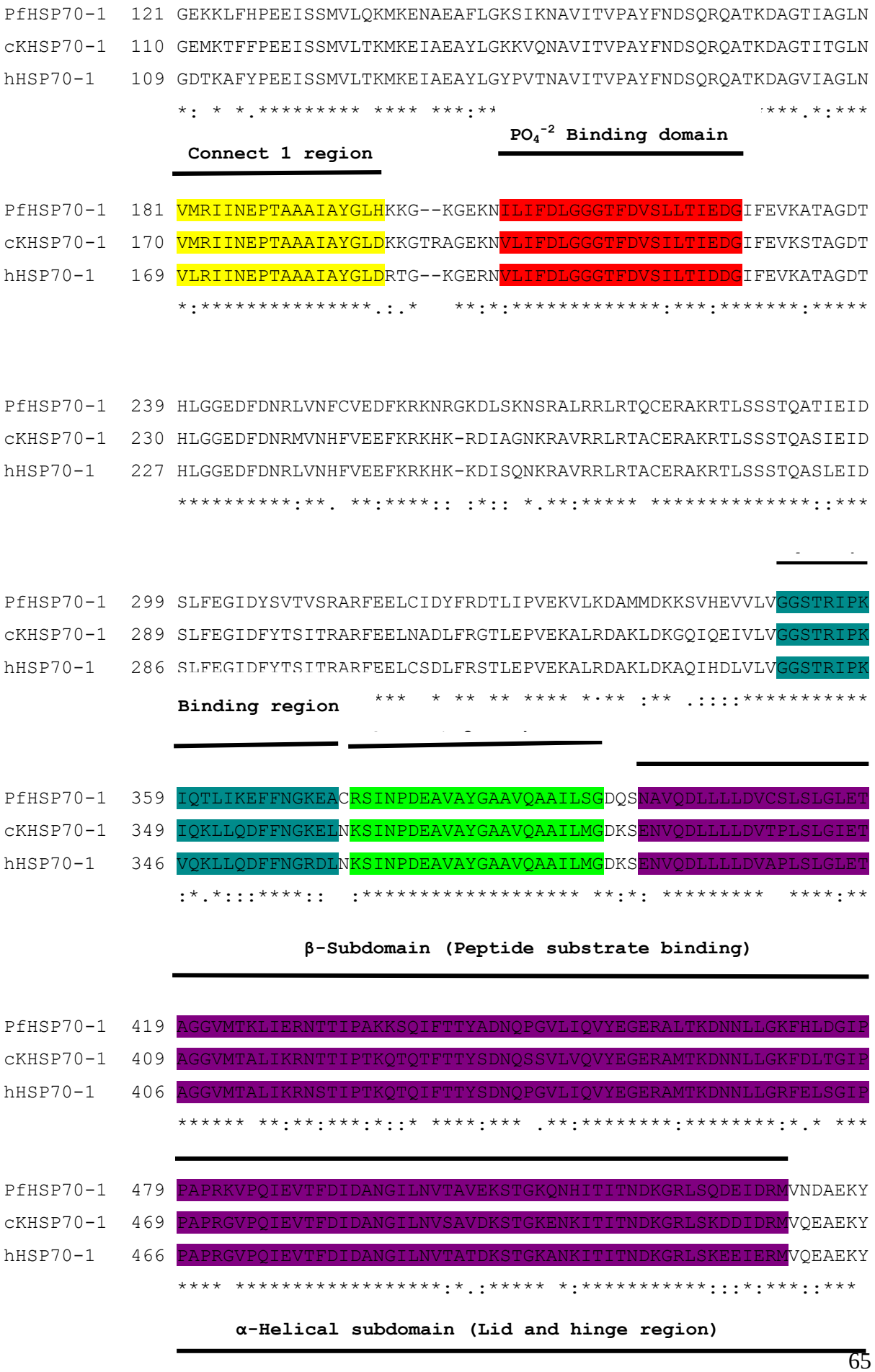


Figure 4.1 Predict7™ analysis of three *Pf*HSP70-1 peptides. Data from the Predict7™ program analysing the three chosen *Pf*HSP70-1 epitopes based on antigenicity, hydrophilicity, surface probability and flexibility. Panel **A; RNSLENYCYGVKSSLED**, panel **B; PAEIETCMKTIT** and panel **C; AGKDEYYAKQLEAESVC** were the epitopes chosen. Surface probability and hydrophilicity were plotted on the primary axis while flexibility and antigenicity were plotted on the secondary axis.

```
                                    PO4-1 Binding
PfHSP70-1      1 MASAKGSKPNLPESNI[AIGIDLGTTYSCVGVWKNENVE]IIANDQGNRTTPSYVAFTDTER
cKHSP70-1      1 ----------MSGKGP[AIGIDLGTTYSCVGVFQHGKVE]IIANDQGNRTTPSYVAFTDTER
hHSP70-1       1 -----------MAKAA[AIGIDLGTTYSCVGVFQHGKVE]IIANDQGNRTTPSYVAFTDTER
                               .  ***************::: :*:**********************

PfHSP70-1     61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQ
cKHSP70-1     51 LIGDAAKNQVAMNPTNTIFDAKRLIGRKYDDPTVQSDMKHWPFRVVNE-GGKPKVQVEYK
hHSP70-1      50 LIGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVIND-GDKPKVQVSYK
                 *********** ** **:**********: :  ********** * .  . ** ::* *:
```


PfHSP70-1 121 GEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPAYFNDSQRQATKDAGTIAGLN
cKHSP70-1 110 GEMKTFFPEEISSMVLTKMKEIAEAYLGKKVQNAVITVPAYFNDSQRQATKDAGTITGLN
hHSP70-1 109 GDTKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIAGLN
PO4-2 Binding domain
Connect 1 region
PfHSP70-1 181 VMRIINEPTAAAIAYGLHKKG--KGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDT
cKHSP70-1 170 VMRIINEPTAAAIAYGLDKKGTRAGEKNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDT
hHSP70-1 169 VLRIINEPTAAAIAYGLDRTG--KGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDT
PfHSP70-1 239 HLGGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEID
cKHSP70-1 230 HLGGEDFDNRMVNHFVEEFKRKHK-RDIAGNKRAVRRLRTACERAKRTLSSSTQASIEID
hHSP70-1 227 HLGGEDFDNRLVNHFVEEFKRKHK-KDISQNKRAVRRLRTACERAKRTLSSSTQASLEID
PfHSP70-1 299 SLFEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPK
cKHSP70-1 289 SLFEGIDFYTSITRARFEELNADLFRGTLEPVEKALRDAKLDKGQIQEIVLVGGSTRIPK
hHSP70-1 286 SLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPK
Binding region
PfHSP70-1 359 IQTLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGLET
cKHSP70-1 349 IQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVTPLSLGIET
hHSP70-1 346 VQKLLQDFFNGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLET
β-Subdomain (Peptide substrate binding)
PfHSP70-1 419 AGGVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIP
cKHSP70-1 409 AGGVMTALIKRNTTIPTKQTQTFTTYSDNQSSVLVQVYEGERAMTKDNNLLGKFDLTGIP
hHSP70-1 406 AGGVMTALIKRNSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFELSGIP
PfHSP70-1 479 PAPRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSQDEIDRMVNDAEKY
cKHSP70-1 469 PAPRGVPQIEVTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKDDIDRMVQEAEKY
hHSP70-1 466 PAPRGVPQIEVTFDIDANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEAEKY
α-Helical subdomain (Lid and hinge region)

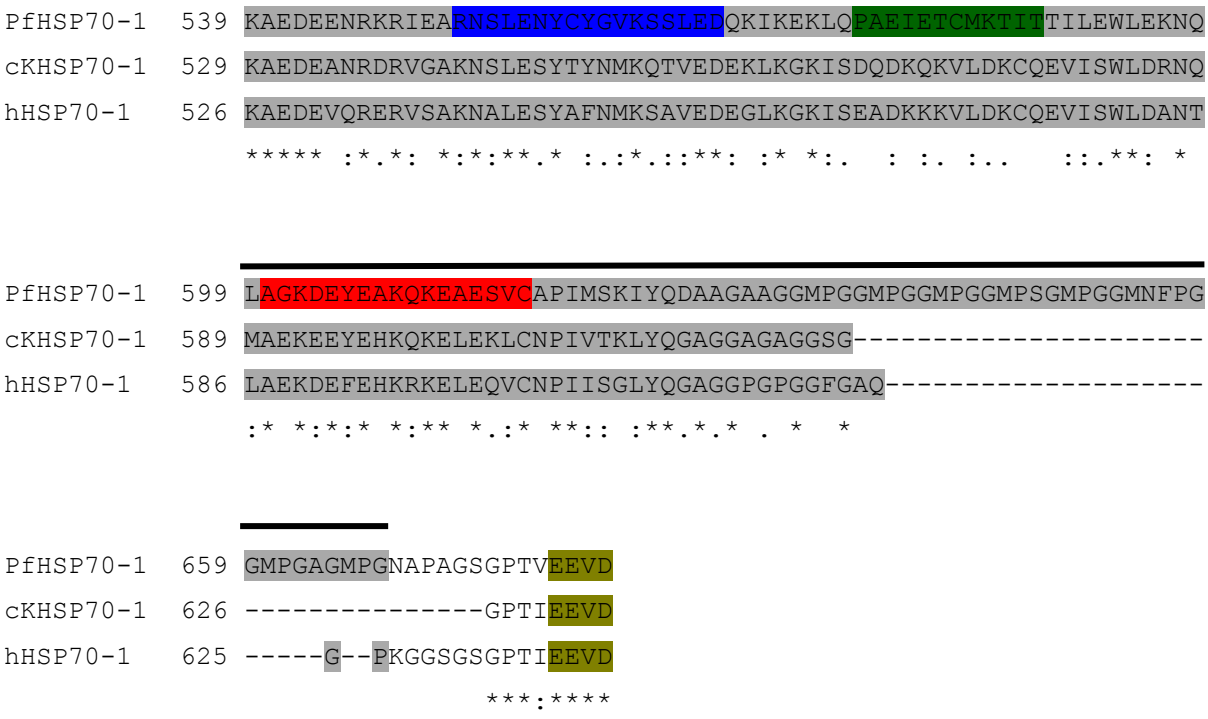


Figure 4.2 Alignment of amino acid sequences from *P. falciparum*, chicken and human HSP70-1 showing the positions of the immunogenic peptides identified by the prediction program. A multiple sequence alignment of *P. falciparum* HSP70-1 (PlasmoDB ID: PF3D7_0818900), *Gallus gallus* HSP70 (NCBI accession ID: NP_001006686 XP-421406) and *Homosapien* HSP70 (NCBI accession ID: PODMV8) amino acids. The annotation '*' refers to identical amino acid residues, ":" refers to conserved amino acid residues, "." refers to semi-conserved amino acid residues and a blank space represented no identity in amino acid residues in the sequences. The blue, green and pink colour show the positions of chosen peptides in the sequence of *Pf*HSP70-1. The blue, green and red colour in the α-helical subdomain (lid and hinge region) in grey colour show RNSLENYCYGVKSSLED, PAEIETCMKTIT and AGKDEYEAKQKEAESVC immunogenic peptide sequences respectively chosen using Predict7™. The underlined regions are: phosphate 1 binding region (dark green), phosphate 2 binding region (red), connect 1 region (yellow), connect 2 region (light green), Adenosine binding region (light blue), β-subdomain for peptide substrate binding (purple), α-helical subdomain for hinge and lid region (grey) and the EEVD motif (greenish).

Multiple alignment (Figure 4.2) of the amino-acid sequence of *Pf*HSP70-1 (PlasmoDB ID: PF3D7_0818900), chicken HSP70 (*Gallus gallus*) (NCBI accession ID: NP_001006686 XP-421406) and human (Homosapien) HSP70 (PODMV8) using clustal W (www.ebi.ac.uk/Tools/msa/clustalo/) was done to ensure that the peptides chosen were unique to *P. falciparum* malaria, so that the antibodies produced in chicken will not cross react with the chicken and human HSP70-1 proteins. The positions of important domains in the sequence of the *Pf*HSP70-1, *ck*HSP70-1 and *h*HSP70-1 proteins are also shown. The peptides selected by Predict7™ analysis are shown on the sequence as

RNSLENYCYGVKSSLED (blue), PAEIETCMKTIT (green) and AGKDEYYAKQLEAESVC (red). The alignment shows that RNSLENYCYGVKSSLED has six identical, six conserved and four semi-conserved amino acid residues. PAEIETCMKTIT has three conserved and three semi-conserved amino acid residues. AGKDEYYAKQLEAESVC has nine identical, four conserved and one semi-conserved amino acid residues. The three peptide sequences were aligned to predict if the anti-peptide raised against these peptides would cross react with the chicken and human HSP70-1 protein sequences. The data suggests that there would be no cross reactivity with chicken and human HSP70-1.

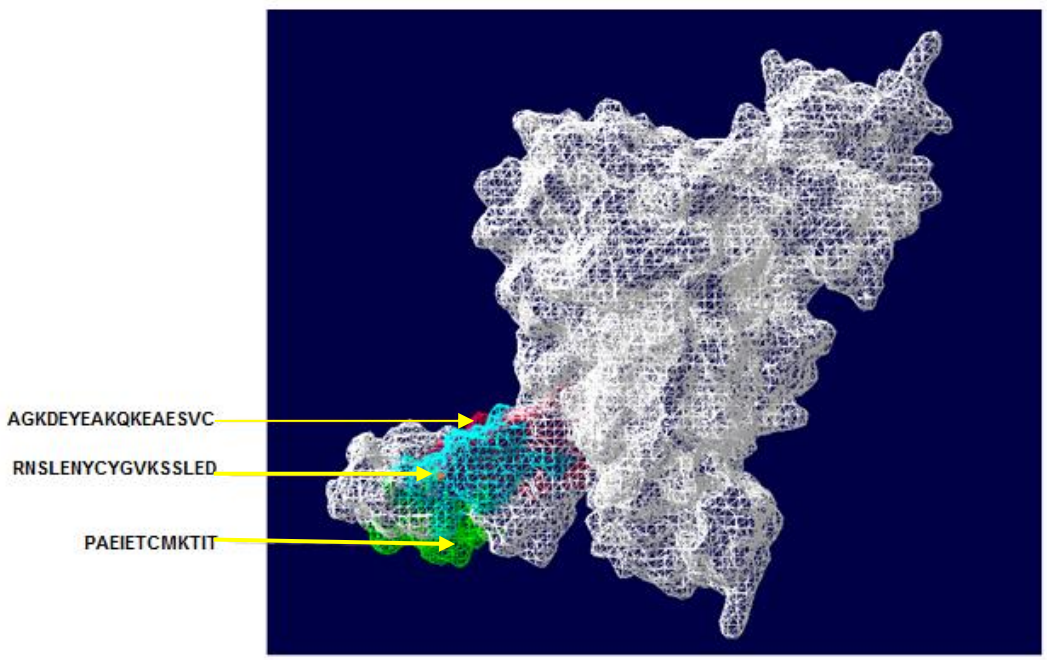


Figure 4.3 An image of the crystal structure of *Pf*HSP70-1 showing the location of the three selected peptides. All the selected peptides (blue, green and pink) were located on the surface of *Pf*HSP70-1. The structure was developed using SwissPDB 4.1.0 (http://www.expasy.org.spdv/). The sequence was obtained from the SWISS-MODEL repository (www.spdv.vital-it.ch/disclaim.html#).

A blast search (data not shown) in http://blast.ncbi.nlm.nih.gov/Blast.cgi showed that RNSLENYCYGVKSSLED and PAEIETCMKTIT are unique to the *Plasmodium falciparum* genome and AGKDEYEAKQKEAESVC was 100% identical in *P. ovale* and *P. vivax* genome and was, therefore, a common peptide. There was 63.58% (433 amino acid residues) identical, 13.8% (94 amino acid residues) conserved, 4.26% (29 amino acid residues) semi-conserved and 18.36% (125 amino acid residues) non-identical amino acid residues in the sequence of *Pf*HSP70-1 that was compared with *ck*HSP70-1 and *h*HSP70-1. The phosphate, adenosine and β-subdomain are highly conserved. The α-helical subdomain is semi conserved. All the three

immunogenic peptides for *Pf*HSP70-1 identified above were located on the surface of the 3D crystal structure model of *Pf*HSP70-1 (spdbv.vital-it.ch/disclaim.html#) as shown in Figure 4.3. The chosen peptides ranged from 15 – 17 amino acid residues in length. The peptide AGKDEYEAKQKEAESVC has cysteine at the C-terminal end and the other two peptides required the addition of a cysteine at the C-terminus during synthesis of the peptides for coupling to carrier proteins. A common carrier is rabbit albumin, and coupling is done with maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). MBS links with SH-group of a cysteine side chain to the ε-amino group of lysine on the carrier protein (Peeters *et al*., 1989). All selected peptides had predicted surface probability and hydrophilicity values. This prediction suggests the surface location of the peptides which ensures the interaction with and binding to antibodies raised against the epitopes on the native protein in solution (Saravanan *et al.*, 2009). All the three immunogenic peptides for *Pf*HSP70-1 identified above were located on the surface of the 3D crystal structure model of *Pf*HSP70-1. This confirmed the data obtained using Predict7™ analysis of the amino-acid sequence of the *Pf*HSP70-1. The peptides will be synthesized and used to produce antibodies in chickens. Peptides selected in the same manner for *Pf*LDH were found to be on the surface of the 3D structure based on the published crystal model by Hurdayal *et al*., 2010 (Dunn *et al.*, 1996). The antibodies will be tested in ELISA and dip-stick format to detect both recombinant and native *Pf*HSP70-1.

4.3 Prediction of post-translational modification

Post translational modification of proteins in cells play an important role in biological functions such as cell signalling (Kiemer *et al.,* 2005), transcription regulation (Faiola *et al.*, 2005), apoptosis (Subramanian *et al*, 2005), cytokine signalling (Yuan *et al.*, 2005) and many other functions. The prediction of possible post translational modification was done to access if the potential post translational sites are close to or on the site of important catalytic amino acids. There is evidence that certain post translational modifications affect the activity of enzymes (Zhao *et al*., 2013; Fan *et al*., 2011). Antibodies produced against a recombinant protein may not interact with the native protein if the native protein is acetylated, phosphorylated, glycosylated or cleaved on its surface.

4.3.1 Acetylation

Acetylation is a widespread post translational modification of eukaryote proteins, in which an acetyl group from acetyl coenzyme A is transferred to either the α-amino group of the amino-terminal residue or to the ε-amino group of internal lysines at specific sites (Glozak *et al.*, 2005; Kouzarids, 2000; Polevoda *et al.*, 2000; Yang, 2004). Acetylation alters the electrostatic properties of proteins as the positive charge of the lysine residue is neutralised. Also formation of hydrogen bonds on lysine side-chains are disrupted (Yang *et al.*, 2004). This leads to loss of

function of the protein. In addition, lysine acetylation also creates a new interface for protein binding, this may results in a gain of function, such as in protein-protein interaction, enzymatic activity, stability and subcellular localization (Glozak *et al*., 2005; Polevoda *et al*., 2002; Yang 2004; Faiola *et al*., 2005).

The recombinant proteins used in this study are produced in *E. coli* cells and, therefore, they are not acetylated. This may result in recombinant proteins having less activity as a result of non-acetylation. Native *Pf*LDH and *Pf*HSP70-1 can be acetylated (Miao *et al.*, 2013; Zhao *et al.*, 2010). The antibodies produced from recombinant proteins may not recognise the epitopes in the native protein if the epitopes contain lysine residues that are acetylated. Two prediction algorithms were used to screen the two protein sequences for potential acetylation sites.

A

>PfLDH

Peptide	Position	Score	Threshold
***MAPKAKIVLV	4	11.04	0.20
*MAPKAKIVLVGS	6	3.01	0.20
ATLIVQKNLGDVV	26	0.23	0.20
KNMPHGKALDTSH	44	0.57	0.20
MAYSNCKVSGSNT	60	1.03	0.20
VTAGFTKAPGKSD	84	1.27	0.20
FTKAPGKSDKEWN	88	1.92	0.20
APGKSDKEWNRDD	91	1.84	0.20
EIGGHIKKNCPNA	114	0.96	0.20
QHSGVPKNKIIGL	144	0.22	0.20
SGVPKNKIIGLGG	146	0.51	0.20
VGAHGNKMVLLKR	185	1.30	0.20
MAESYLKDLKKVL	252	1.03	0.20
SYLKDLKKVLICS	255	1.10	0.20
QLNSEEKAKFDEA	299	0.48	0.20
EAIAETKRMKALA	310	1.69	0.20
AETKRMKALA***	313	3.06	0.20

B

PfHsp70-1

Peptide	Position	Score	Threshold
**MASAKGSKPNL	5	8.42	0.20
ASAKGSKPNLPES	8	2.77	0.20
LIGDAAKNQVARN	67	0.73	0.20
KRLIGRKFTESSV	88	1.50	0.20
VTYQGEKKLFHPE	123	0.91	0.20
TYQGEKKLFHPEE	124	0.78	0.20

MVLQKMKENAEAF	140	0.33	0.20
AEAFLGKSIKNAV	149	0.46	0.20
IAYGLHKKGKGEK	199	0.58	0.20
AYGLHKKGKGEKN	200	1.03	0.20
GLHKKGKGEKNIL	202	1.63	0.20
KKGKGEKNILIFD	205	1.30	0.20
FCVEDFKRKNRGK	258	0.73	0.20
VEDFKRKNRGKDL	260	0.88	0.20
KRKNRGKDLSKNS	264	3.13	0.20
RGKDLSKNSRALR	268	1.96	0.20
TQCERAKRTLSSS	284	1.81	0.20
PVEKVLKDAMMDK	335	0.76	0.20
KIQTLIKEFFNGK	364	0.57	0.20
AGGVMTKLIERNT	425	0.42	0.20
TTIPAKKSQIFTT	437	1.12	0.20
GERALTKDNNLLG	464	0.98	0.20
DNNLLGKFHLDGI	471	0.41	0.20
IPPAPRKVPQIEV	483	0.62	0.20
NVTAVEKSTGKQN	506	1.19	0.20
VEKSTGKQNHITI	510	1.93	0.20
ITITNDKGRLSQD	520	0.59	0.20
MVNDAEKYKAEDE	537	0.51	0.20
NYCYGVKSSLEDQ	563	0.27	0.20
DQKIKEKLQPAEI	574	0.40	0.20
KNQLAGKDEYEAK	602	1.94	0.20
CAPIMSKIYQDAA	622	1.07	0.20

C

```
  NetAcet 1.0 prediction results, 1 sequence
#
#
# Sequence              #     Context   Score      Acetylation
# ------------------------------------------------------------------
PfLDH                    2 A   --MAPKA   0.474           .
```

D

```
# NetAcet 1.0 prediction results, 1 sequence
#
#
```

```
# Sequence              #     Context    Score      Acetylation
# ------------------------------------------------------------
PfHSP70-1                2 A    --MASAK    0.471           .
PfHSP70-1                3 S    -MASAKG    0.506         YES
```

E

Protein	Predicted lysine acetylation sites
*Pf*LDH	17
*Pf*HSP70-1	32

F

Protein	Predicted lysine acetylation sites
*Pf*LDH	0
*Pf*HSP70-1	1

Figure 4.4 The predicted lysine acetylation sites on *Pf*LDH and *Pf*HSP70-1 using two programs. **A** and **B** show the lysine acetylation site using a program PAIL and **C** and **D** using a program NetAcet that uses N-acetyltransferase A as a substrate. **E** and **F** shows the tables the numbers of sites for PAIL and NetAcet, respectively.

***Pf*LDH alignments showing position of acetylation acetylation**

```
PfLDH     1 -----------------MAPKAKIVLVGSGMIGGVMATLIVQKNLGD-VVLFDIVKNMPH
mLDH      1 -ATLKDQLIYNLLK-EEQTPQNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLK
ckLDH     1 MATLKEKLITPVAA-GSTVPSNKITVVGVGQVGMACAISILGKGLCDELALVDVLEDKLK
htLDH     1 -ATLKEKLIAPVAEEEATVPNNKITVVGVGQVGMACAISILGKSLADELALVDVLEDKLK
                              .*. **.:** * :* . *  *: *.* * :.*.*::::  :

PfLDH    43 GKALDTSHTNVMAYSNCKVSGSNTYDDLAGADVVIVTAGFTKAPGKSDKEWNRDDLLPLN
mLDH     59 GEMMDLQHGSLFLR-TPKIVSGKDYNVTANSKLVIITAGARQQEGESRLN-----LVQRN
ckLDH    60 GEMMDLQHGSLFLQ-THKIVADKDYAVTANSKIVVVTAGVRQQEGESRLN-----LVQRN
htLDH    60 GEMMDLQHGSLFLQ-TPKIVADKDYSVTANSKIVVVTAGVRQQEGESRLN-----LVQRN
```

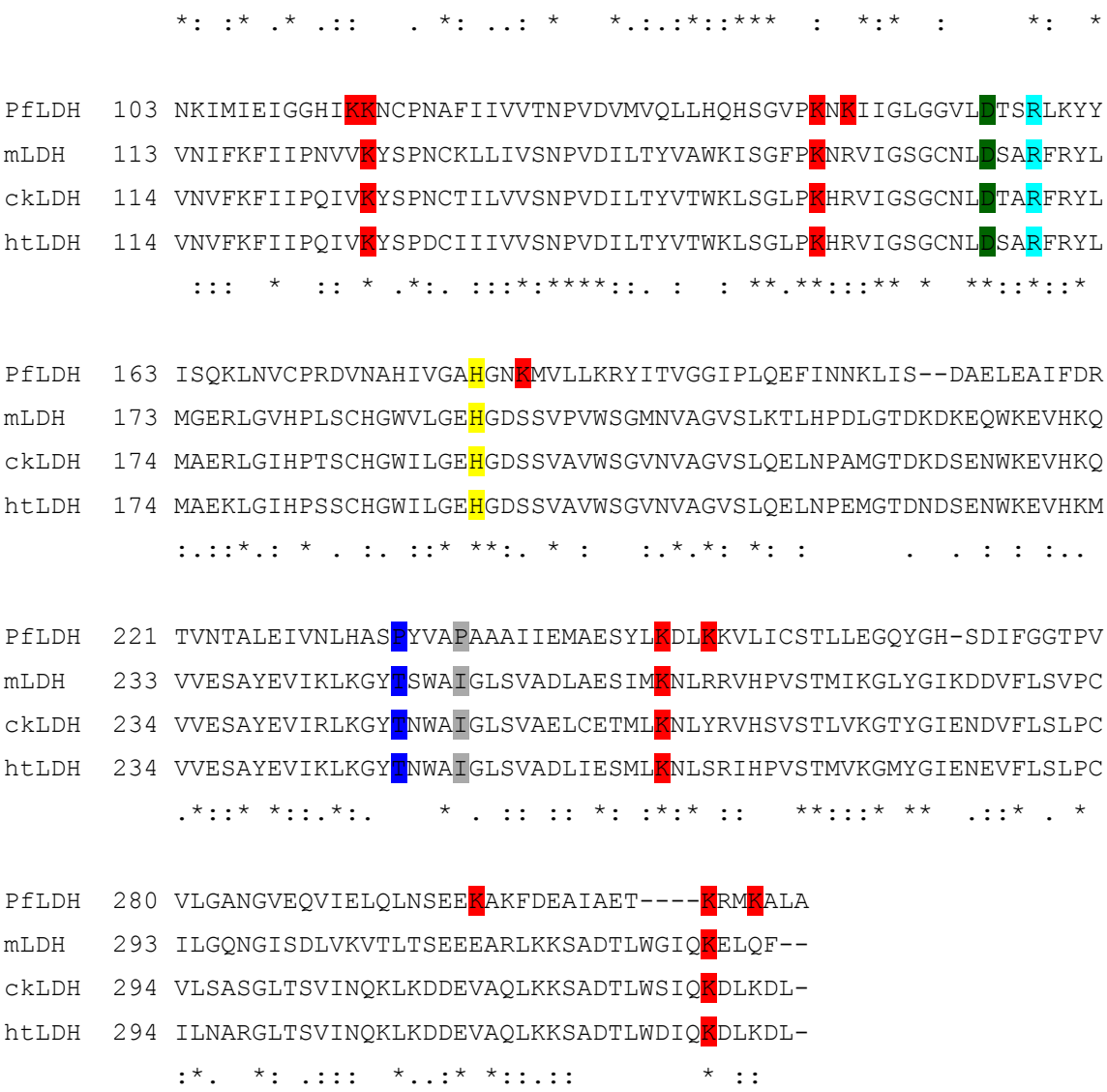
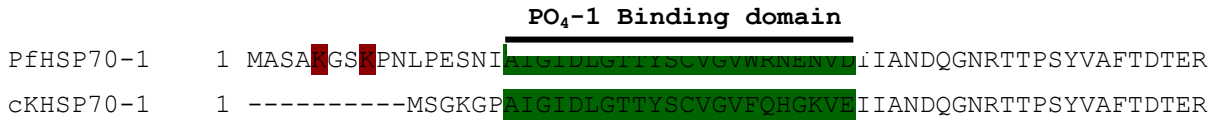
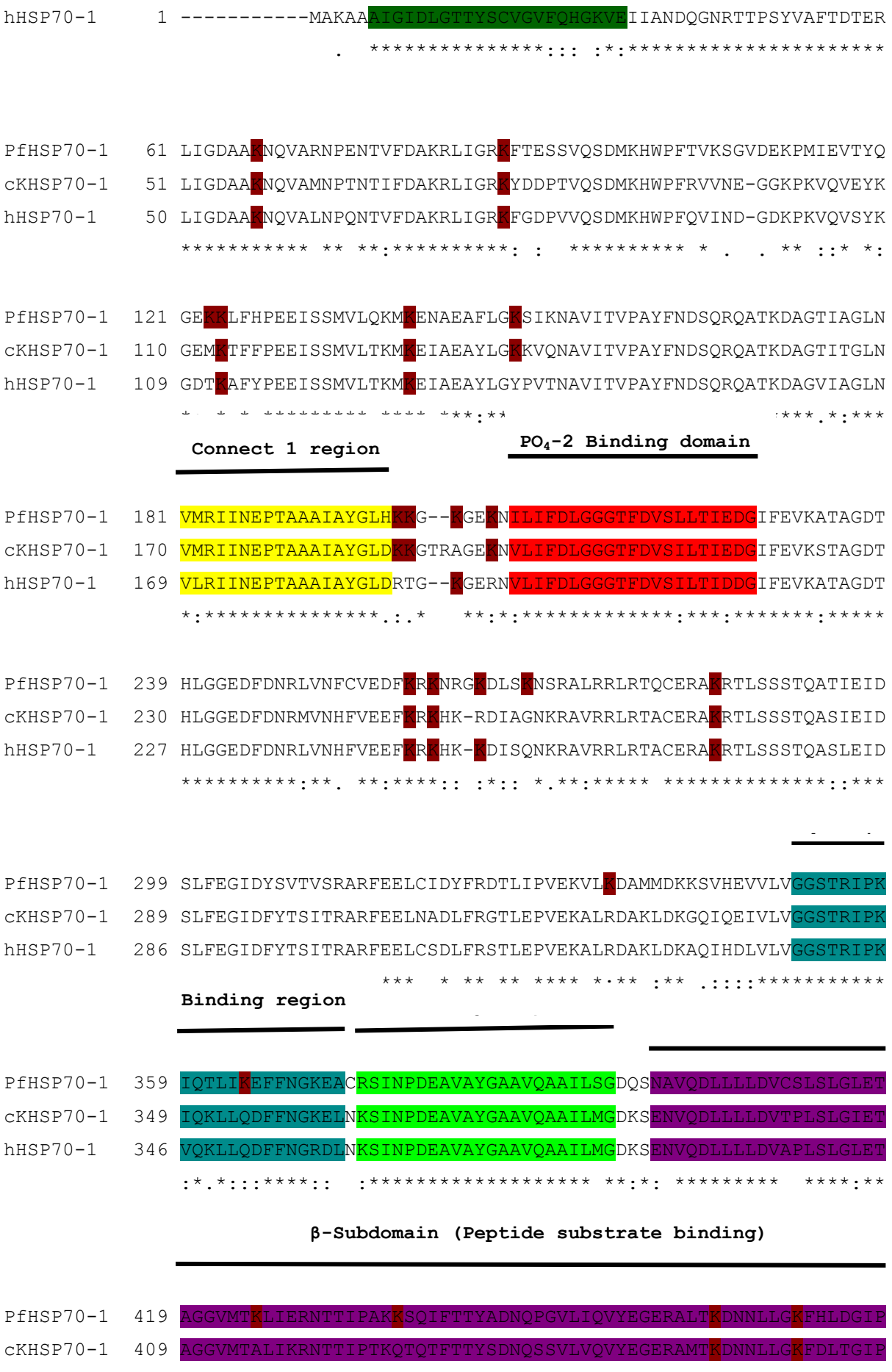


Figure 4.5 Predication of acetylation sites in the sequence of *Pf*LDH aligned with chicken (*Gallus gallus*) cKLDH and human (*Homo sapien*) h**LDH using PAIL.** The dark red colour (with letter K) show the positions of predicted potential lysine acetylation sites by PAIL in the *Pf*LDH sequence. Light blue conserved arginine 109 and 171, Dark green conserved aspartic acid 168, Yellow conserved histidine 195 all conserved in the catalytic site of LDH sequence. The numbering of these conserved amino acids is based on the standard N numbering system (Evenlhoff *et al*., 1977). Dark blue threonine 246 and grey isoleucine 250 are residues in the cofactor binding site of LDH substituted by proline residues in *Pf*LDH.

*Pf*HSP70-1 acetylation




hHSP70-1 1 ----------MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTER
PfHSP70-1 61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQ
cKHSP70-1 51 LIGDAAKNQVAMNPTNTIFDAKRLIGRKYDDPTVQSDMKHWPFRVVNE-GGKPKVQVEYK
hHSP70-1 50 LIGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVIND-GDKPKVQVSYK
PfHSP70-1 121 GEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPAYFNDSQRQATKDAGTIAGLN
cKHSP70-1 110 GEMKTFFPEEISSMVLTKMKEIAEAYLGKKVQNAVITVPAYFNDSQRQATKDAGTITGLN
hHSP70-1 109 GDTKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIAGLN
Connect 1 region
PO4-2 Binding domain
PfHSP70-1 181 VMRIINEPTAAAIAYGLHKKG--KGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDT
cKHSP70-1 170 VMRIINEPTAAAIAYGLDKKGTRAGEKNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDT
hHSP70-1 169 VLRIINEPTAAAIAYGLDRTG--KGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDT
PfHSP70-1 239 HLGGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEID
cKHSP70-1 230 HLGGEDFDNRMVNHFVEEFKRKHK-RDIAGNKRAVRRLRTACERAKRTLSSSTQASIEID
hHSP70-1 227 HLGGEDFDNRLVNHFVEEFKRKHK-KDISQNKRAVRRLRTACERAKRTLSSSTQASLEID
PfHSP70-1 299 SLFEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPK
cKHSP70-1 289 SLFEGIDFYTSITRARFEELNADLFRGTLEPVEKALRDAKLDKGQIQEIVLVGGSTRIPK
hHSP70-1 286 SLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPK
Binding region
PfHSP70-1 359 IQTLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGLET
cKHSP70-1 349 IQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVTPLSLGIET
hHSP70-1 346 VQKLLQDFFNGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLET
β-Subdomain (Peptide substrate binding)
PfHSP70-1 419 AGGVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIP
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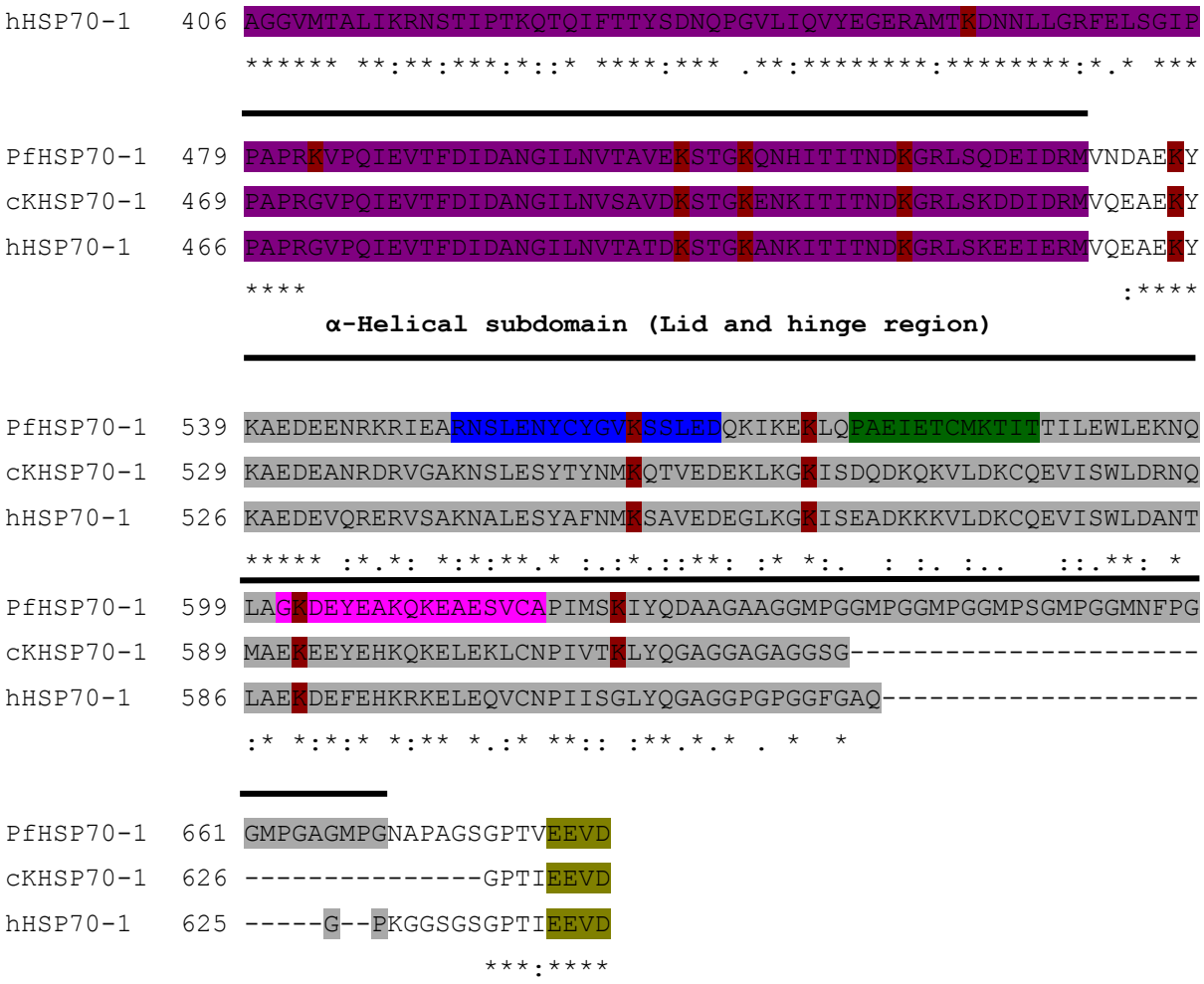


Figure 4.6 Predication of acetylation sites in the sequence of *Pf*HSP70-1 aligned with chicken (*Gallus gallus*) cKHSP70-1 and human (*Homo sapien*) hHSP70-1 using PAIL. Dark red colour (with letter K) potential lysine acetylation sites by PAIL in the *Pf*HSP70-1 sequence. Dark green phosphate 1 binding region, red phosphate 2 binding region, yellow connect 1 region, light green connect 2 region, light blue the adenosine binding region, purple β-subdomain for peptide substrate binding, grey α-helical subdomain for hinge and lid region, greenish is the EEVD mortif. Within the α-subdomain the blue, green and pink colours shows the position of the 3 immunogenic peptide sequences predicted by Predict7™.

The program, "prediction of acetylation on internal lysine" (PAIL) (bdmpail.biocuckoo.org/predictions.php/) which was used to generate the data in Figure 4.4 (A, B and E), predicted 17 possible lysine acetylation sites for native *Pf*LDH and 32 for native *Pf*HSP70-1. The position of the predicted acetylation sites are shown in Figure 4.5 and Figure 4.6 on the aligned sequences of *Pf*LDH and *Pf*HSP70-1, respectively with chicken and human sequences for each protein. The position of the predicted potential lysine acetylation sites relative to the important amino acid residues in the active sites of each protein can be seen on Figure 4.5 and 4.6. Should lysine acetylation occur near or close to essential catalytic residues in the active site and this lysine acetylation is conserved in the species, this may alter activity of the native protein. Another program NetAcet (www.cbs.dtu.dk/services/NetAcet/) predicts substrates of N-acetyltransferase A (NatA) in acetylation (Kiemer *et al.*, 2004). NetAcet predicted no

acetylation site for *Pf*LDH and one acetylation site for *Pf*HSP70-1 as shown in Figure 4.4 (C, D and F). Another important factor is the surface probability of the acetylated lysine residue, because only the acetylated sites on the surface would interfere with the activity of the protein. Only acetylated lysine residues on the surface of the native protein can interact with antibodies. Crystal structure analysis is required to determine which lysines are on the surface. Miao *et al.*, 2013 and Zhao *et al.*, 2010 showed that *Plasmodium* proteins like many parasitic proteins are acetylated.

Zhao *et al.*, (2013) demonstrated that human LDH-A in tumour cells is acetylated at lysine 5 (K5) and that acetylation inhibits LDH-A activity. Furthermore, the K5 acetylated LDH-A is recognised by the HSC70 chaperone and delivered to lysosomes for degradation (Zhao *et al*., 2013). Neumann *et al.*, (2008) showed that a genetically modified Nε-acetyllysine recombinant protein produced in *E. coli* that produces 100% acetylated LDH-A at K5 had less enzyme activity compared to un-acetylated LDH-A activity. In addition, K5 acetylation lead to a time-dependent reduction of LDH-A protein levels. The K5 of *Pf*LDH is a conserved lysine in chicken, muscle and heart LDH, and may, therefore, be acetylated. If that is the case, then the activity of native *Pf*LDH has the potential to alter enzyme activity. Because human LDH and *Pf*LDH are conserved proteins, it would, therefore, be interesting to observe if K5 on *Pf*LDH is acetylated *in vivo* and does this influence *Pf*LDH activity in the parasite. Then it may be responsible for reduced sensitivity in the detection of parasitemia and drug sensitivity assay using *Pf*LDH (Makler and Hinrichs, 1993; Makler *et al*., 1993). The numbering of amino acid residues in LDH is based on the standard N numbering system (Evenhoff *et al*., 1977; Gomez *et al*., 1997).

Yang *et al.*, 2013 demonstrated that cellular stresses could influence increase in intracellular levels of acetylated HSP70s (HSP72, HSP70-1, HSPAIA, GRP78), which bind to Beclin-I-Vps 34 complex and promote KAPI-dependent SUMOylation and activity of Vps34. These findings add to accumulating evidence that supports the role of HSP70 and HSP90 in stabilising and promoting the activity of the molecular effectors upstream in the AV formation, such as ULKI and downstream in the AV expansion and fusion with lysosome (Yang *et al*., 2013). Most of the predicted potential lysine acetylation sites were far from key catalytic domain of *Pf*HSP70-1.The interesting acetylation sites were K563, K574 and K602 which are found in the α-helical subdomain of *Pf*HSP70-1 and conserved in chicken and humans HSP70 proteins. The K563, K574 and K602 acetylations may affect the activity of the native protein if they are acetylated as they have a higher possibility of being on the surface in *P. falciparum*, Chicken and Human HSP70-1. If poly-clonal antibodies against of the whole *Pf*HSP70-1 were produced in chicken and tested on the native protein, there may be a possibility of reduced recognition due to acetylation of K563, K574 and K602. HSP70 proteins are highly conserved proteins and investigation will show if *Pf*HSP70-1 acetylation has an effect on the interaction with antibodies raised against the recombinant proteins.

The results showed potential acetylation sites in both *Pf*LDH and *Pf*HSP70-1 and that similar proteins in humans are acetylated. Human LDH and HSP70 acetylation alter the activities of these proteins. It would be interesting to study the effect of acetylation of *Plasmodial* LDH and *Pf*HSP70-1 in human blood with malaria.

4.3.2 Phosphorylation

Phosphorylation is a post-translational modification of proteins in which amino acid residues threonine, serine or tyrosine undergo the addition of a phosphate group covalently bonded by a protein kinase. Protein phosphorylation is the most fundamental molecular mechanism through which protein function is regulated in response to extracellular stimuli. Virtually all types of extracellular signals, including neurotransmitters, light, hormones, cytokines, neurotropic factors, energy management and cell regulation, produce their diverse physiological effects by regulating the phosphorylation of specific phosphoproteins in their target cells. Potential phosphorylation sites on *Pf*LDH and *Pf*HSP70-1 were predicted using a phosphorylation predicting algorithm Kinasephos (Huang *et al.*, 2005a; Huang *et al.*, 2005b; Hong *et al.*, 2007) at www.kinasephos.mbc.nctu.edu.tw/predict.php/. Phosphorylation plays an important role in protein function modification by either activating or deactivating many enzyme, thereby regulating their function (Nestler *et al.*, 1984). Peace *et al.*, (2013) identified 2767 proteins, 1337 phosphoproteins, and 6293 phosphrylation sites in *P. falciparum*. Post translational regulatory mechanisms have dominant roles in regulation of intraerythrocytic gene expression. Lasonder *et al.,* (2015) identified merozoite proteasome revealing 1765 unique phosphorylation sites, including 785 phosphorylation sites previously identified in schizonts. These merozoite phosphorylation interaction network, a subnet of 119 proteins with potential roles in cellular movement and invasion. Wu *et al.*, 2009 also showed that *P. falciparum* infections greatly increased phosphorylation of a set of proteins in *parasitized* RBC.

A

Protein Name	Predicted phosphorylated site		
	Serine (S)	Threonine (T)	Tyrosine (Y)
PfLDH	3	0	1
PfHSP70-1	7	6	2

B

*Pf*LDH

```
MAPKAKIVLV     GSGMIGGVMA     TLIVQKNLGD     VVLFDIVKNM     PHGKALDTSH     TNVMAYSNCK     60

VSGSNTYDDL     AGADVVIVTA     GFTKAPGKSD     KEWNRDDLLP     LNNKIMIEIG     GHIKKNCPNA     120
```

```
------Y---     ----------     ----------     ----------     ----------     ---------- INSR
------Y---     ----------     ----------     ----------     ----------     ---------- Syk

FIIVVTNPVD     VMVQLLHQHS     GVPKNKIIGL     GGVLDTSRLK     YYISQKLNVC     PRDVNAHIVG     180
----------     ----------     ----------     ----------     ---S------     ---------- ATM

AHGNKMVLLK     RYITVGGIPL     QEFINNKLIS DAELEAIFDR     TVNTALEIVN     LHASPYVAPA     240
----------     ----------     ---------S     ----------     ----------     ---------- CKII

AAIIEMAESY     LKDLKKVLIC     STLLEGQYGH     SDIFGGTPVV     LGANGVEQVI     ELQLNSEEKA     300
--------S-     ----------     ----------     ----------     ----------     ---------- PKG

KFDEAIAETK RMKALA 316
```

C

***Pf*HSP70-1**

```
MASAKGSKPN     LPESNIAIGI     DLGTTYSCVG     VWRNENVDII     ANDQGNRTTP     SYVAFTDTER     60
------S---     ----------     ----------     ----------     ----------     ---------- PKG
----------     ----------     ----------     ----------     --------T-     ---------- MAPK

LIGDAAKNQV     ARNPENTVFD     AKRLIGRKFT ESSVQSDMKH     WPFTVKSGVD     EKPMIEVTYQ     120
----------     ----------     ---------T     ----------     ----------     ---------- PKC
----------     ----------     ---------T     ----------     ----------     ---------- PKA
----------     ----------     ----------     --S-------     ----------     ---------- CKII

GEKKLFHPEE     ISSMVLQKMK     ENAEAFLGKS     IKNAVITVPA     YFNDSQRQAT KDAGTIAGLN     180
----------     ----------     ----------     ----------     ---------T     ---------- PKC
----------     ----------     ----------     ----------     ---------T     ---------- PKA

VMRIINEPTA     AAIAYGLHKK     GKGEKNILIF     DLGGGTFDVS     LLTIEDGIFE     VKATAGDTHL     240

GGEDFDNRLV     NFCVEDFKRK     NRGKDLSKNS     RALRRLRTQC     ERAKRTLSSS     TQATIEIDSL     300
----------     ----------     ----------     ----------     -----T----     ---------- PKC
----------     ----------     ----------     ----------     -----T----     ---------- PKA
----------     ----------     ----------     ----------     -------S--     ---------- PKG
----------     ----------     ----------     ----------     -------S--     ---------- CKI
----------     ----------     ----------     ----------     -------S--     ---------- IKK

FEGIDYSVTV     SRARFEELCI     DYFRDTLIPV     EKVLKDAMMD     KKSVHEVVLV     GGSTRIPKIQ     360
--------T-     ----------     ----------     ----------     ----------     ---------- PKC

TLIKEFFNGK     EACRSINPDE     AVAYGAAVQA     AILSGDQSNA     VQDLLLLDVC     SLSLGLETAG     420

GVMTKLIERN     TTIPAKKSQI     FTTYADNQPG     VLIQVYEGER     ALTKDNNLLG     KFHLDGIPPA     480

PRKVPQIEVT     FDIDANGILN     VTAVEKSTGK     QNHITITNDK     GRLSQDEIDR     MVNDAEKYKA     540
----------     ----------     ----------     ----------     ---S------     ---------- CKII
----------     ----------     ----------     ----------     ---S------     ---------- PKG
----------     ----------     ----------     ----------     ---S------     ---------- ATM
----------     ----------     ----------     ----------     ----------     -------Y-- Syk
```

```
EDEENRKRIE     ARNSLENYCY     GVKSSLEDQK     IKEKLQPAEI     ETCMKTITTI     LEWLEKNQLA     600
----------     ---S------     ----------     ----------     ----------     ---------- PKG
----------     ----------     ----S-----     ----------     ----------     ---------- PKG

GKDEYEAKQK     EAESVCAPIM     SKIYQDAAGA     AGGMPGGMPG     GMPGGMPGGM     NFPGGMPGAG     660
----Y-----     ----------     ----------     ----------     ----------     ---------- EGFR
----Y-----     ----------     ----------     ----------     ----------     ---------- INSR
----Y-----     ----------     ----------     ----------     ----------     ---------- Syk
----------     ---S------     ----------     ----------     ----------     ---------- PKC
----------     ---S------     ----------     ----------     ----------     ---------- CKII
----------     ---S------     ----------     ----------     ----------     ---------- IKK

MPGNAPAGSG                                  PTVEEVD                                   677
---------- -T----- PKC
```

Figure 4.7 Predicted phosphorylation sites on the *Pf*LDH and *Pf*HSP70-1 amino acid sequences using Kinasephos. A is the table showing the number of predicted sites, and B and C the position of the predicted residues in sequence.

*Pf*LDH phosphorylation

```
PfLDH      1 ------------------MAPKAKIVLVGSGMIGGVMATLIVQKNLGD-VVLFDIVKNMPH
mLDH       1 -ATLKDQLIYNLLK-EEQTPQNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLK
ckLDH      1 MATLKEKLITPVAA-GSTVPSNKITVVGVGQVGMACAISILGKGLCDELALVDVLEDKLK
htLDH      1 -ATLKEKLIAPVAEEEATVPNNKITVVGVGQVGMACAISILGKSLADELALVDVLEDKLK
                            .*. **.:** * :* . *  *: *.* * :.*.*::::  :

PfLDH     43 GKALDTSHTNVMAYSNCKVSGSNTYDDLAGADVVIVTAGFTKAPGKSDKEWNRDDLLPLN
mLDH      59 GEMMDLQHGSLFLR-TPKIVSGKDYNVTANSKLVIITAGARQQEGESRLN-----LVQRN
ckLDH     60 GEMMDLQHGSLFLQ-THKIVADKDYAVTANSKIVVVTAGVRQQEGESRLN-----LVQRN
htLDH     60 GEMMDLQHGSLFLQ-TPKIVADKDYSVTANSKIVVVTAGVRQQEGESRLN-----LVQRN
             *: :* .* .::   . *: ..: *   *.:.:*::***  :  *:*  :     *:  *

PfLDH    103 NKIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLHQHSGVPKNKIIGLGGVLDTSRLKYY
mLDH     113 VNIFKFIIPNVVKYSPNCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYL
ckLDH    114 VNVFKFIIPQIVKYSPNCTILVVSNPVDILTYVTWKLSGLPKHRVIGSGCNLDTARFRYL
htLDH    114 VNVFKFIIPQIVKYSPDCIIIVVSNPVDILTYVTWKLSGLPKHRVIGSGCNLDSARFRYL
              :::  *  :: * .*:. :::*:****::. :  : **.**:::** *  **::*::*

PfLDH    163 ISQKLNVCPRDVNAHIVGAHGNKMVLLKRYITVGGIPLQEFINNKLIS--DAELEAIFDR
mLDH     173 MGERLGVHPLSCHGWVLGEHGDSSVPVWSGMNVAGVSLKTLHPDLGTDKDKEQWKEVHKQ
ckLDH    174 MAERLGIHPTSCHGWILGEHGDSSVAVWSGVNVAGVSLQELNPAMGTDKDSENWKEVHKQ
htLDH    174 MAEKLGIHPSSCHGWILGEHGDSSVAVWSGVNVAGVSLQELNPEMGTDNDSENWKEVHKM
             :.::*.: * . :. ::* **:. * :   :.*.*: *: :      .  . : : :..
```

```
PfLDH   221 TVNTALEIVNLHASTYVAPAAAIIEMAESYLKDLKKVLICSTLLEGQYGH-SDIFGGTPV
mLDH    233 VVESAYEVIKLKGYTSWAIGLSVADLAESIMKNLRRVHPVSTMIKGLYGIKDDVFLSVPC
ckLDH   234 VVESAYEVIRLKGYTNWAIGLSVAELCETMLKNLYRVHSVSTLVKGTYGIENDVFLSLPC
htLDH   234 VVESAYEVIKLKGYTNWAIGLSVADLIESMLKNLSRIHPVSTMVKGMYGIENEVFLSLPC
            .*::* *::.*:.    * . :: :: *: :*:* ::   **:::* **  .::* . *

PfLDH   280 VLGANGVEQVIELQLNSEEKAKFDEAIAET----KRMKALA
mLDH    293 ILGQNGISDLVKVTLTSEEEARLKKSADTLWGIQKELQF--
ckLDH   294 VLSASGLTSVINQKLKDDEVAQLKKSADTLWSIQKDLKDL-
htLDH   294 ILNARGLTSVINQKLKDDEVAQLKKSADTLWDIQKDLKDL-
            :*.  *: .:::  *..:* *::.::        * ::
```

Figure 4.8 Predicted phosphorylation sites on the *Pf*LDH sequence with the key catalytic amino acids in the active site of *Pf*LDH aligned with chicken (*Gallus Gallus*) cKLDH and human (*Homo sapien*) m**LDH using Kinasephos.** The light green colour (with letter Y) show the positions of predicted potential tyrosine phosphorylation sites by Kinasephos in the *Pf*LDH sequence. Light blue (with letter S) potential serine phosphorylation sites. Light red conserved arginine 109 and 171, purple conserved aspartic acid 168, yellow conserved histidine 195 are conserved amino-acid residues in the active site of LDH sequence. Dark blue threonine 246 and the greenish isoleucine 250 are residues in the cofactor binding site of LDH are substituted by proline residues in *Pf*LDH. The numbering of these conserved essential catalytic amino acids is based on the standard N numbering system (Evenlhoff *et al*., 1977).

*Pf*HSP70-1 phosphorylation

```
                                  PO4-1 Binding domain

PfHSP70-1     1 MASAKGSKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTER
cKHSP70-1     1 ----------MSGKGPAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTER
hHSP70-1      1 -----------MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTER
                          .  ***************::: :*:**********************

PfHSP70-1    61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQ
cKHSP70-1    51 LIGDAAKNQVAMNPTNTIFDAKRLIGRKYDDPTVQSDMKHWPFRVVNE-GGKPKVQVEYK
hHSP70-1     50 LIGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVIND-GDKPKVQVSYK
                ********** ** **:**********: :  ********** * .  . ** ::* *:

PfHSP70-1   121 GEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPAYFNDSQRQATKDAGTIAGLN
cKHSP70-1   110 GEMKTFFPEEISSMVLTKMKEIAEAYLGKKVQNAVITVPAYFNDSQRQATKDAGTITGLN
hHSP70-1    109 GDTKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIAGLN
                *: * *.********* **** ***:**                  :***.*:***

                Connect 1 region                PO4-2 Binding domain
```

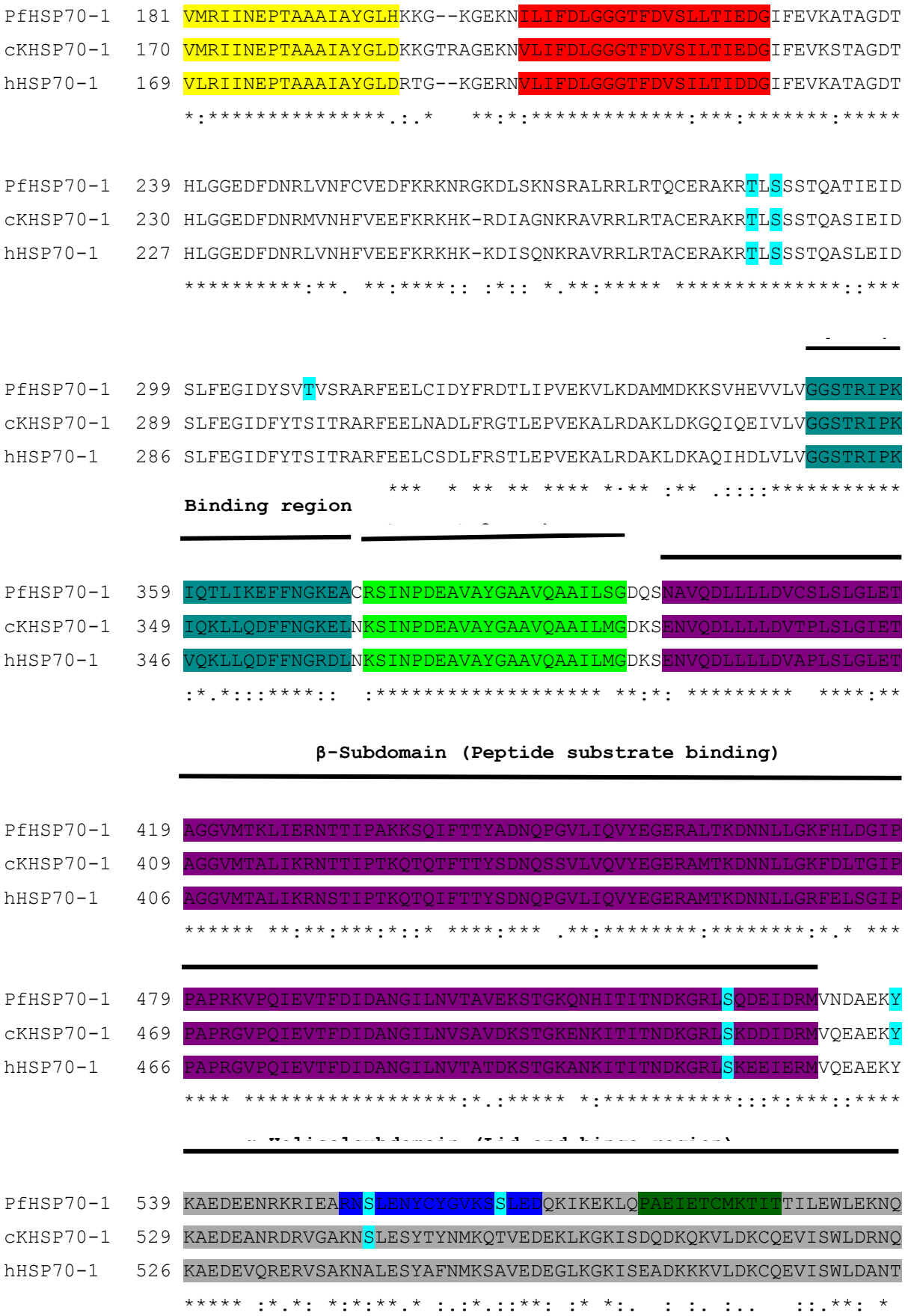

PfHSP70-1 181 VMRIINEPTAAAIAYGLHKKG--KGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDT
cKHSP70-1 170 VMRIINEPTAAAIAYGLDKKGTRAGEKNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDT
hHSP70-1 169 VLRIINEPTAAAIAYGLDRTG--KGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDT
PfHSP70-1 239 HLGGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEID
cKHSP70-1 230 HLGGEDFDNRMVNHFVEEFKRKHK-RDIAGNKRAVRRLRTACERAKRTLSSSTQASIEID
hHSP70-1 227 HLGGEDFDNRLVNHFVEEFKRKHK-KDISQNKRAVRRLRTACERAKRTLSSSTQASLEID
PfHSP70-1 299 SLFEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPK
cKHSP70-1 289 SLFEGIDFYTSITRARFEELNADLFRGTLEPVEKALRDAKLDKGQIQEIVLVGGSTRIPK
hHSP70-1 286 SLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPK
Binding region
PfHSP70-1 359 IQTLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGLET
cKHSP70-1 349 IQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVTPLSLGIET
hHSP70-1 346 VQKLLQDFFNGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLET
β-Subdomain (Peptide substrate binding)
PfHSP70-1 419 AGGVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIP
cKHSP70-1 409 AGGVMTALIKRNTTIPTKQTQTFTTYSDNQSSVLVQVYEGERAMTKDNNLLGKFDLTGIP
hHSP70-1 406 AGGVMTALIKRNSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFELSGIP
PfHSP70-1 479 PAPRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSQDEIDRMVNDAEKY
cKHSP70-1 469 PAPRGVPQIEVTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKDDIDRMVQEAEKY
hHSP70-1 466 PAPRGVPQIEVTFDIDANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEAEKY
PfHSP70-1 539 KAEDEENRKRIEARNSLENYCYGVKSSLEDQKIKEKLQPAEIETCMKTITTILEWLEKNQ
cKHSP70-1 529 KAEDEANRDRVGAKNSLESYTYNMKQTVEDEKLKGKISDQDKQKVLDKCQEVISWLDRNQ
hHSP70-1 526 KAEDEVQRERVSAKNALESYAFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLDANT

Figure 4.9 The positions of the predicted phosphorylation sites on the *Pf*HSP70-1 sequence aligned with chicken (*Gallus Gallus*) cKHSP70-1 and human (*Homosapien*) hHSP70-1 using Kinasephos. The light blue colour (with letter S, Y, T) show the positions of predicted potential serine, tyrosine and threonine phosphorylation sites by Kinasephos in the *Pf*HSP70-1 sequence. Dark green phosphate 1 binding region, red phosphate 2 binding region, yellow connect 1 region, light green connect 2 region, light blue the adenosine binding region, purple β-subdomain for peptide substrate binding, grey α-helical subdomain for hinge and lid region, greenish is the EEVD mortif. Within the α-subdomain the blue, green and pink show the position of the three immunogenic peptide sequences predicted by Predict7™.

Figure 4.7 shows that *Pf*LDH has three possible phosphorylation sites on serine residues and one on a tyrosine residue and *Pf*HSP70-1 has seven possible phosphorylation sites on serine residues, six on threonine and two on tyrosine residues. The position of the predicted phosphorylation sites are shown in Figure 4.8 and Figure 4.9 on the aligned sequences of *Pf*LDH and *Pf*HSP 70-1 respectively. The position of the predicted potential serine, tyrosine and threonine phosphorylation sites relative to the important amino acid residues in the active sites of each protein can be seen on Figure 4.8 and 4.9.

Human LDH-A was found to be directly phosphorylated by the oncogenic receptor tyrosine kinase FGFR1. Phosphorylation at tyrosine 10 (Y10) and tyrosine 83 (Y83) enhances LDH-A activity by enhancing the formation of active, tetrameric LDH-A and the binding of LDH-A substrate NADH, respectively (Fan *et al*., 2011). The Y10 in *Pf*LDH is not present, but the Y83 amino acid is present. If Y83 in *Pf*LDH is phosphorylated, it may increase the activity of *Pf*LDH like in human LDH-A. Most of the predicted potential phosphorylation sites are located far away from the essential catalytic amino acid residues. The numbering of amino acid residues in LDH is based on the standard N numbering system (Evenhoff *et al*., 1977; Gomez *et al*., 1997). The Y10 and Y 83 are conserved in the LDH sequence analysed except *Pf*LDH.

Muller *et al.*, 2011 identified a C-terminal phosphorylation of human HSP70 and HSP90 as a switch for regulating co-chaperone binding and indicated that cancer cells possess an elevated protein folding environment by the concerted action of co-chaperone expression and chaperone modification. S574 in the β-subdomain (peptide substrate binding domain) is a predicted phosphorylation site that is conserve in *P. falciparum*, chicken and human LDH, but

may not be phosphorylated because of its location. The remaining phosphorylation sites are located far from the essential catalytic amino acids.

Both LDH and HSP70 are conserved proteins in humans and malaria parasites, and it would be important to see if native *Pf*LDH and *Pf*HSP70-1 phosphorylation has an effect on the activity of these proteins in malaria parasites. It would be interesting also to find out if native *Pf*LDH and *Pf*HSP70-1 phosphorylation has an effect on the interaction with antibodies raised against the recombinant proteins.

4.3.3 Glycosylation

Glycosylation is post-translational modification by the conjugation of a sugar moiety to proteins. Glycosylation is important in a wide range of biological processes, including cell attachment to the extracellular matrix, protein-ligand interactions in the cell, monitoring the status of protein folding, quality control mechanisms to ensure that only properly folded proteins are trafficked to the Golgi, used in export of proteins to proper destination and can alter the solubility of a protein because sugars are soluble. N-Glycosylation in *P. falciparum* may have a role in protein targeting and both N- and O- glycosylation occur in *P. falciparum* (De Macedo *et al*., 2010). Parasite proteins were found to bind concanavalin A (Con A) which suggested the presence of mannosylated glycans (Kilejian and Olson, 1979). Later, Kilejian and Olson, (1980) demonstrated the presence of glycoproteins in the late trophozoite stage by radioactive labelling with glucosamine. Gowda and Davidson, (1999) also discussed, in their review, that N- and O-glycosylation were present in *P. falciparum* and glycosylation may influence activation of a signal pathway in host cells, and thereby induce expression of cytokines, adhesion molecules and induced nitric oxide synthase (Gowda *et al*., 1997). Cova *et al.*, (2015) suggests that *P. falciparum* protein glycosylation is important for parasite survival. Types of glycosylation include N-, O-, C-, glypiation and phosphoglycoslation.

O-Glycosylation of *Pf*LDH and *Pf*HSP70-1 predictions were done according to Gupta *et al.*, 1999 using a predicting program known as DictyOGlyc (www.cbs.dtu.dk/services/DictyOGlyc/).

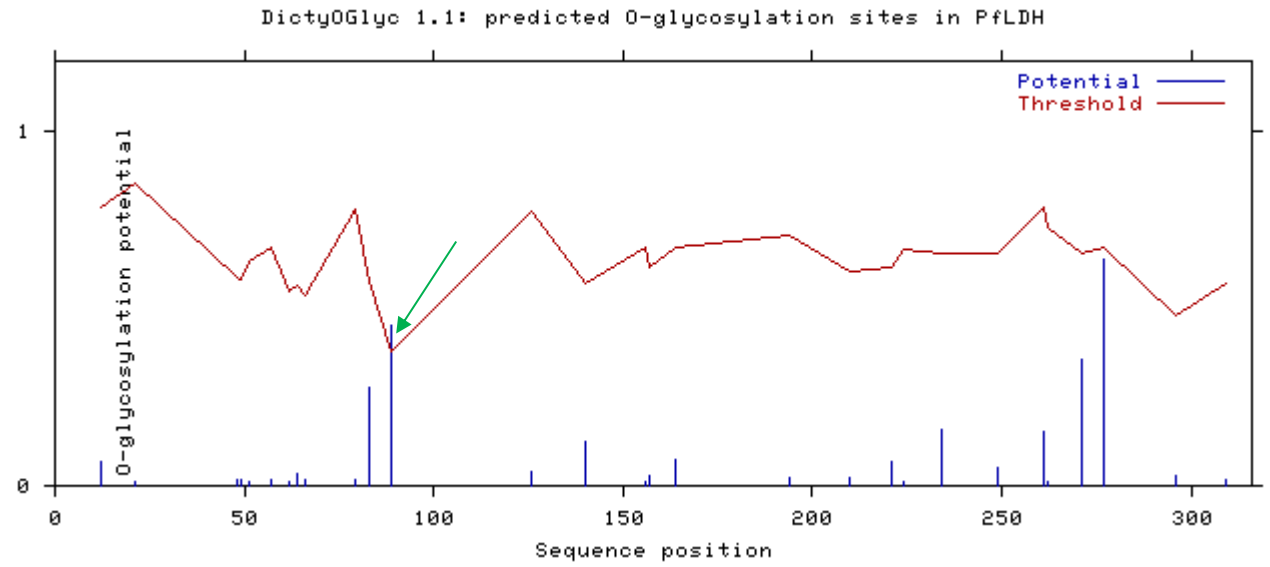
A

```
Name:   PfLDH    Length:  316
MAPKAKIVLVGSGMIGGVMATLIVQKNLGDVVLFDIVKNMPHGKALDTSHTNVMAYSNCKVSGSNTYDDLAGADVVIVTA      80
GFTKAPGKSDKEWNRDDLLPLNNKIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLHQHSGVPKNKIIGLGGVLDTSRLK     160
YYISQKLNVCPRDVNAHIVGAHGNKMVLLKRYITVGGIPLQEFINNKLISDAELEAIFDRTVNTALEIVNLHASPYVAPA     240
AAIIEMAESYLKDLKKVLICSTLLEGQYGHSDIFGGTPVVLGANGVEQVIELQLNSEEKAKFDEAIAETKRMKALA
.........|.....................................................................      80
.........G.....................................................................     160
.....................................................................................     240
.....................................................................
```

B

Name	Residue	Number	Potential	Threshold	Assignment
PfLDH	Ser	0012	0.0683	0.7863	.
PfLDH	Thr	0021	0.0140	0.8543	.
PfLDH	Thr	0048	0.0191	0.5915	.
PfLDH	Ser	0049	0.0193	0.5845	.
PfLDH	Thr	0051	0.0139	0.6325	.
PfLDH	Ser	0057	0.0160	0.6764	.
PfLDH	Ser	0062	0.0133	0.5496	.
PfLDH	Ser	0064	0.0314	0.5636	.
PfLDH	Thr	0066	0.0173	0.5366	.
PfLDH	Thr	0079	0.0152	0.7823	.
PfLDH	Thr	0083	0.2749	0.5795	.
PfLDH	Ser	0089	0.4551	0.3807	G
PfLDH	Thr	0126	0.0419	0.7733	.
PfLDH	Ser	0140	0.1257	0.5745	.
PfLDH	Thr	0156	0.0109	0.6744	.
PfLDH	Ser	0157	0.0309	0.6175	.
PfLDH	Ser	0164	0.0749	0.6714	.
PfLDH	Thr	0194	0.0214	0.7084	.
PfLDH	Ser	0210	0.0211	0.6055	.
PfLDH	Thr	0221	0.0660	0.6185	.
PfLDH	Thr	0224	0.0118	0.6684	.
PfLDH	Ser	0234	0.1610	0.6585	.
PfLDH	Ser	0249	0.0515	0.6555	.
PfLDH	Ser	0261	0.1525	0.7843	.
PfLDH	Thr	0262	0.0130	0.7314	.
PfLDH	Ser	0271	0.3547	0.6575	.
PfLDH	Thr	0277	0.6417	0.6754	.
PfLDH	Ser	0296	0.0256	0.4796	.
PfLDH	Thr	0309	0.0166	0.5705	.

C



D

Name: PfHsp70-1 Length: 677

```
MASAKGSKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVARNPENTVFD      80
AKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQGEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPA     160
YFNDSQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLHKKGKGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDTHL     240
GGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEIDSLFEGIDYSVTVSRARFEELCI     320
DYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPKIQTLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNA     400
VQDLLLLDVCSLSLGLETAGGVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIPPA     480
PRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSQDEIDRMVNDAEKYKAEDEENRKRIEARNSLENYCY     560
GVKSSLEDQKIKEKLQPAEIETCMKTITTILEWLEKNQLAGKDEYEAKQKEAESVCAPIMSKIYQDAAGAAGGMPGGMPG     640
GMPGGMPGGMNFPGGMPGAGMPGNAPAGSGPTVEEVD
.....      80
.....     160
.....     240
.....     320
.....     400
.....     480
.....     560
.....     640
.....G.....
```

E

Name	Residue	Number	Potential	Threshold	Assignment
PfHsp70-1	Ser	0003	0.0427	0.5406	.
PfHsp70-1	Ser	0007	0.0516	0.4966	.
PfHsp70-1	Ser	0014	0.0212	0.6984	.
PfHsp70-1	Thr	0024	0.0120	0.7424	.
PfHsp70-1	Thr	0025	0.0604	0.7833	.
PfHsp70-1	Ser	0027	0.1140	0.7823	.
PfHsp70-1	Thr	0048	0.0214	0.5785	.

PfHsp70-1	Thr	0049	0.0273	0.6155	.
PfHsp70-1	Ser	0051	0.4669	0.7024	.
PfHsp70-1	Thr	0056	0.0174	0.7474	.
PfHsp70-1	Thr	0058	0.0293	0.7114	.
PfHsp70-1	Thr	0077	0.0265	0.6645	.
PfHsp70-1	Thr	0090	0.0146	0.6525	.
PfHsp70-1	Ser	0092	0.1980	0.6964	.
PfHsp70-1	Ser	0093	0.0347	0.6635	.
PfHsp70-1	Ser	0096	0.0297	0.6595	.
PfHsp70-1	Thr	0104	0.0156	0.7164	.
PfHsp70-1	Ser	0107	0.0425	0.6575	.
PfHsp70-1	Thr	0118	0.0356	0.7394	.
PfHsp70-1	Ser	0132	0.0407	0.7713	.
PfHsp70-1	Ser	0133	0.4580	0.8183	.
PfHsp70-1	Ser	0150	0.1532	0.6535	.
PfHsp70-1	Thr	0157	0.0809	0.8233	.
PfHsp70-1	Ser	0165	0.0175	0.6085	.
PfHsp70-1	Thr	0170	0.0101	0.5426	.
PfHsp70-1	Thr	0175	0.0154	0.6984	.
PfHsp70-1	Thr	0189	0.0210	0.6894	.
PfHsp70-1	Thr	0216	0.0748	0.7304	.
PfHsp70-1	Ser	0220	0.0149	0.8283	.
PfHsp70-1	Thr	0223	0.0155	0.7853	.
PfHsp70-1	Thr	0234	0.0105	0.6784	.
PfHsp70-1	Thr	0238	0.0235	0.6625	.
PfHsp70-1	Ser	0267	0.0161	0.5646	.
PfHsp70-1	Ser	0270	0.0179	0.6305	.
PfHsp70-1	Thr	0278	0.0344	0.6575	.
PfHsp70-1	Thr	0286	0.0151	0.6005	.
PfHsp70-1	Ser	0288	0.0434	0.6335	.
PfHsp70-1	Ser	0289	0.0485	0.6445	.
PfHsp70-1	Ser	0290	0.0223	0.6645	.
PfHsp70-1	Thr	0291	0.0119	0.6555	.
PfHsp70-1	Thr	0294	0.0109	0.7264	.
PfHsp70-1	Ser	0299	0.1401	0.7484	.
PfHsp70-1	Ser	0307	0.2203	0.7853	.
PfHsp70-1	Thr	0309	0.0449	0.7683	.
PfHsp70-1	Ser	0311	0.1890	0.7444	.
PfHsp70-1	Thr	0326	0.0876	0.7384	.
PfHsp70-1	Ser	0343	0.0423	0.6355	.
PfHsp70-1	Ser	0353	0.0897	0.7264	.

PfHsp70-1	Thr	0354	0.2519	0.6974	.
PfHsp70-1	Thr	0361	0.0344	0.7244	.
PfHsp70-1	Ser	0375	0.2277	0.6575	.
PfHsp70-1	Ser	0394	0.1559	0.7064	.
PfHsp70-1	Ser	0398	0.0143	0.6425	.
PfHsp70-1	Ser	0411	0.0179	0.8013	.
PfHsp70-1	Ser	0413	0.0117	0.7923	.
PfHsp70-1	Thr	0418	0.0116	0.7044	.
PfHsp70-1	Thr	0424	0.0160	0.7813	.
PfHsp70-1	Thr	0431	0.1538	0.6305	.
PfHsp70-1	Thr	0432	0.0155	0.6395	.
PfHsp70-1	Ser	0438	0.0332	0.6435	.
PfHsp70-1	Thr	0442	0.0245	0.7494	.
PfHsp70-1	Thr	0443	0.0304	0.7454	.
PfHsp70-1	Thr	0463	0.0128	0.6085	.
PfHsp70-1	Thr	0490	0.0570	0.7923	.
PfHsp70-1	Thr	0502	0.0139	0.7713	.
PfHsp70-1	Ser	0507	0.0123	0.5755	.
PfHsp70-1	Thr	0508	0.0179	0.5396	.
PfHsp70-1	Thr	0515	0.0162	0.7284	.
PfHsp70-1	Thr	0517	0.0219	0.6804	.
PfHsp70-1	Ser	0524	0.0137	0.6265	.
PfHsp70-1	Ser	0554	0.0130	0.6704	.
PfHsp70-1	Ser	0564	0.0246	0.6665	.
PfHsp70-1	Ser	0565	0.0187	0.6505	.
PfHsp70-1	Thr	0582	0.1213	0.7104	.
PfHsp70-1	Thr	0586	0.0291	0.7364	.
PfHsp70-1	Thr	0588	0.0558	0.7574	.
PfHsp70-1	Thr	0589	0.1031	0.7753	.
PfHsp70-1	Ser	0614	0.0259	0.7014	.
PfHsp70-1	Ser	0621	0.2921	0.7494	.
PfHsp70-1	Ser	0669	0.1299	0.5636	.
PfHsp70-1	Thr	0672	0.5753	0.5656	G

F

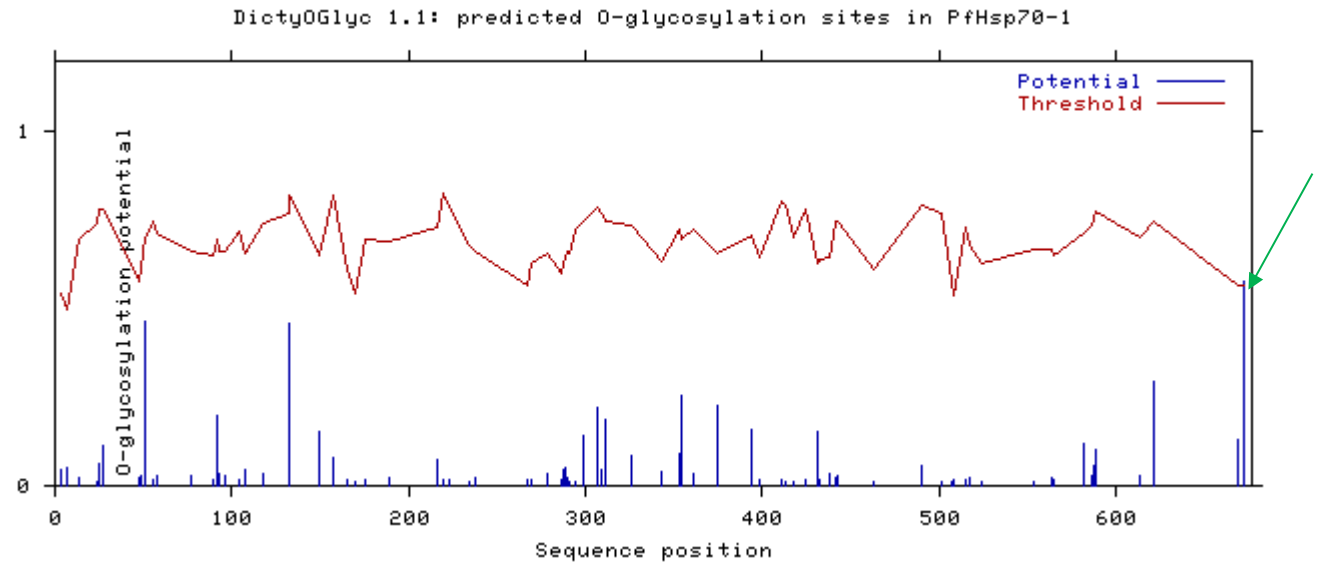


Figure 4.10 The predicted O-glycoslation sites on the *Pf*LDH and *Pf*HSP70-1 amino acid sequences using DictyOGlyc. A and **D** are sequences of *P*fLDH and *Pf*HSP70-1, respectively with the possible gycosylation sites. **B** and **E** shows the threshold and the predicted values. **C** and **F** is a graphical representation of **B** and **E**.

The program predicted 1 glycosylation site for *Pf*LDH and 1 for *Pf*HSP70-1 as shown in figure 4.10 A where a G (where the green arrow is pointing), in B where a G is written on serine 0089 and in C (where the green arrow is pointing) and figure 4.10 D where a G (where the green arrow is pointing), threonine 0672 in E and F (where the green arrow is pointing).

N-Glycosylation of *Pf*LDH and *Pf*HSP70-1 was done according to Gupta *et al.*, 2002 using a predicting program known as NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/).

A

Name: *Pf*LDH Length: 316

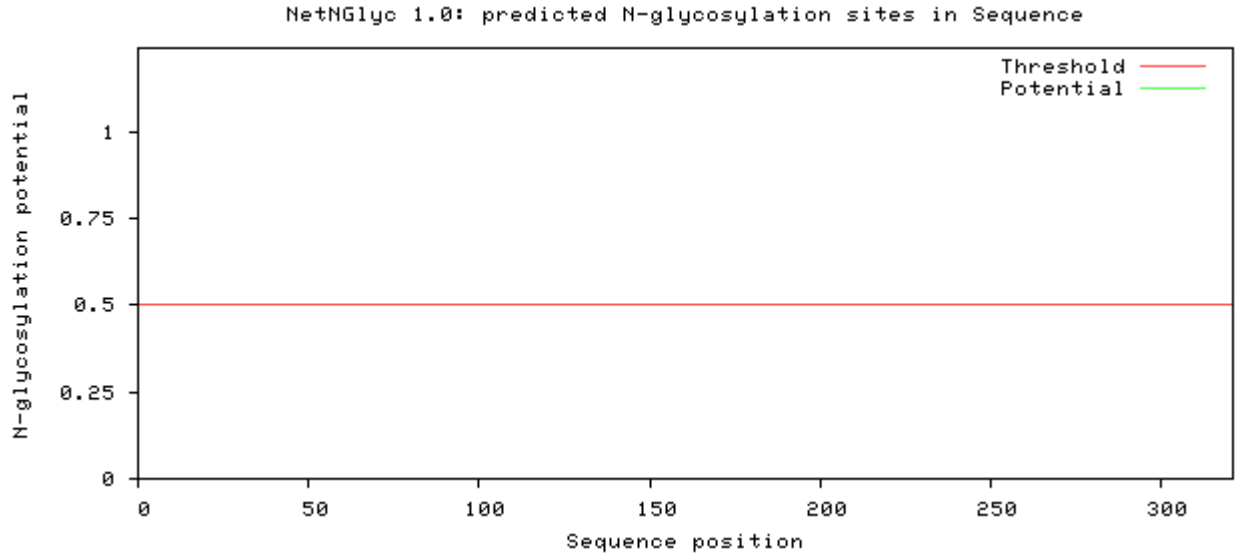
```
MAPKAKIVLVGSGMIGGVMATLIVQKNLGDVVLFDIVKNMPHGKALDTSHTNVMAYSNCKVSGSNTYDDLAGADV      80
VIVTAGFTKAPGKSDKEWNRDDLLPLNNKIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLHQHSGVPKNKIIGLGGVLD    160
TSRLKYYISQKLNVCPRDVNAHIVGAHGNKMVLLKRYITVGGIPLQEFINNKLISDAELEAIFDRTVNTALEIVNLHASP    240
YVAPAAAIIEMAESYLKDLKKVLICSTLLEGQYGHSDIFGGTPVVLGANGVEQVIELQLNSEEKAKFDEAIAETKRMKAL    320
A
.                                                                              80
.                                                                             160
.                                                                             240
.                                                                             320
.                                                                             400
```

(Threshold=0.5)

B

No sites predicted in this sequence.

C



D

Name: *Pf*HSP70-1 Length: 677

```
MASAKGSKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVARNPENT      80
VFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQGEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVIT     160
VPAYFNDSQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLHKKGKGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGD     240
THLGGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEIDSLFEGIDYSVTVSRARFEE     320
LCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPKIQTLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQ     400
SNVQDLLLLDVCSLSLGLETAGGVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIP     480
PAPRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSQDEIDRMVNDAEKYKAEDEENRKRIEARNSLENY     560
CYGVKSSLEDQKIKEKLQPAEIETCMKTITTILEWLEKNQLAGKDEYEAKQKEAESVCAPIMSKIYQDAAGAAGGMPGGM     640
PGGMPGGMPGGMNFPGGMPGAGMPGNAPAGSGPTVEEVD

.................................................N.............................      80
................................................................................     160
.....N..........................................................................     240
................................................................................     320
................................................................................     400
.............................N..................................................     480
....................N........................................................     560
................................................................................     640
.....................................                                           720
```

(Threshold=0.5)

SeqName	Position	Potential	Jury agreement	N-Glyc result
Sequence	49 NRTT	0.6357	(8/9)	+
Sequence	166 NDSQ	0.5906	(8/9)	+
Sequence	432 NTTI	0.5696	(7/9)	+
Sequence	502 NVTA	0.7803	(9/9)	+++

E

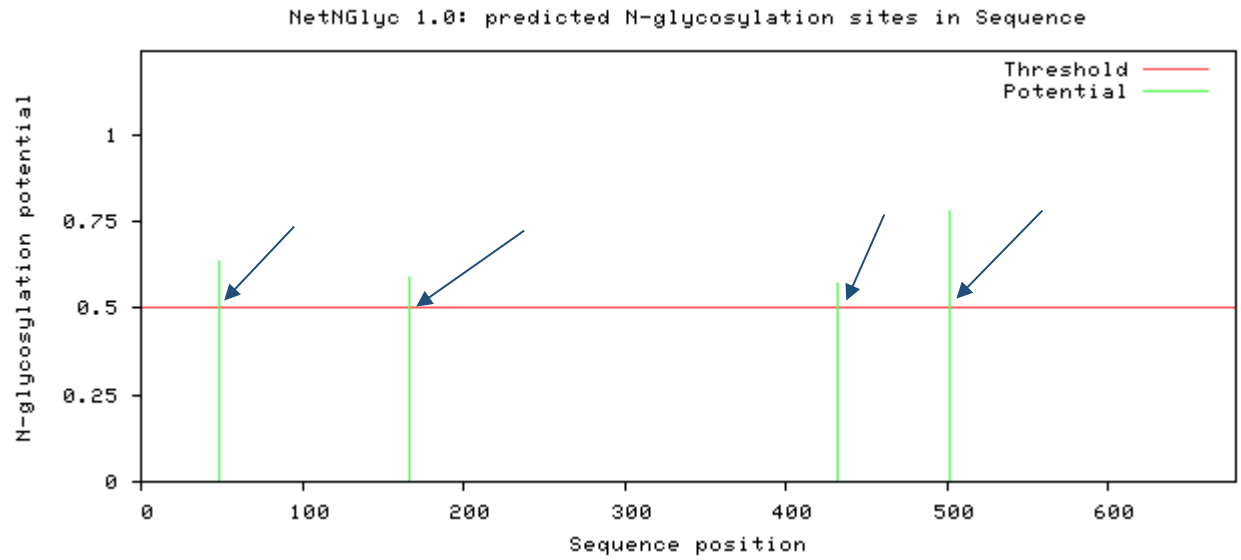


Figure 4.11 Predicted N-glycosylation sites on *Pf*LDH and *Pf*HSP70-1 amino acid sequences using NetNGlyc. A and **D** are sequences of *P*fLDH and *Pf*HSP70-1, respectively with the possible gycosylation sites. **B** and **E** shows the threshold and the predicted values. **C** and **F** is a graphical representation of **B** and **E**.

PO_4^{-1} **Binding domain**

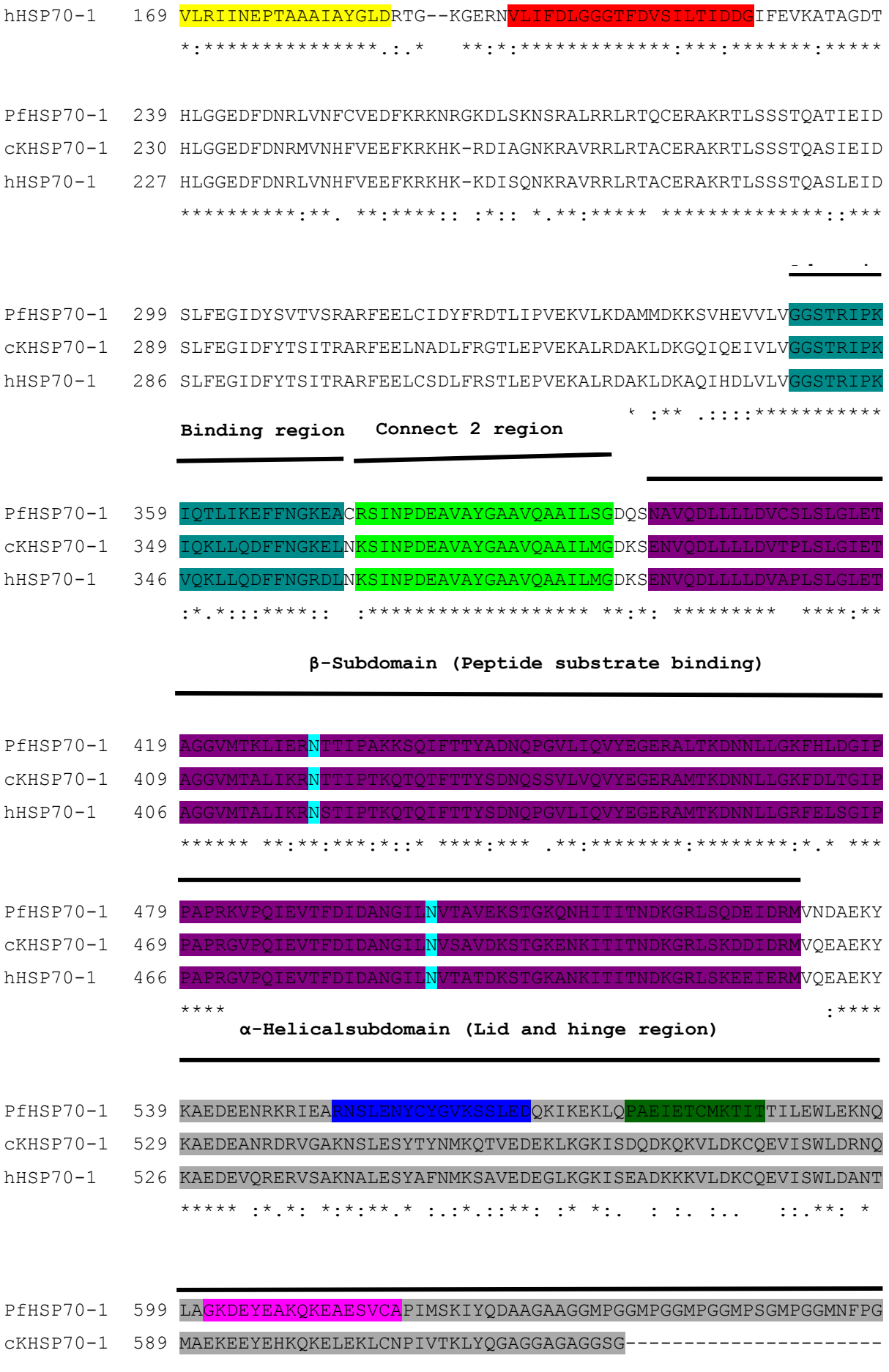
```
PfHSP70-1     1 MASAKGSKPNLPESNIAIGIDLGTTYSCVGVWKNENVDIIANDQGNRTTPSYVAFTDTER
cKHSP70-1     1 ----------MSGKGPAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTER
hHSP70-1      1 -----------MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTER
                          .  ***************::: :*:**********************

PfHSP70-1    61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQ
cKHSP70-1    51 LIGDAAKNQVAMNPTNTIFDAKRLIGRKYDDPTVQSDMKHWPFRVVNE-GGKPKVQVEYK
hHSP70-1     50 LIGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVIND-GDKPKVQVSYK
                ********** ** **:**********: :  ********** * .  . ** ::* *:

PfHSP70-1   121 GEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPAYFNDSQRQATKDAGTIAGLN
cKHSP70-1   110 GEMKTFFPEEISSMVLTKMKEIAEAYLGKKVQNAVITVPAYFNDSQRQATKDAGTITGLN
hHSP70-1    109 GDTKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIAGLN
                            .**:**                          .***.*:***
```

Connect 1 region — PO_4^{-2} **Binding domain**

```
PfHSP70-1   181 VMRIINEPTAAAIAYGLHKKG--KGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDT
cKHSP70-1   170 VMRIINEPTAAAIAYGLDKKGTRAGEKNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDT
```


hHSP70-1 169 VLRIINEPTAAAIAYGLDRTG--KGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDT
PfHSP70-1 239 HLGGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEID
cKHSP70-1 230 HLGGEDFDNRMVNHFVEEFKRKHK-RDIAGNKRAVRRLRTACERAKRTLSSSTQASIEID
hHSP70-1 227 HLGGEDFDNRLVNHFVEEFKRKHK-KDISQNKRAVRRLRTACERAKRTLSSSTQASLEID
PfHSP70-1 299 SLFEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPK
cKHSP70-1 289 SLFEGIDFYTSITRARFEELNADLFRGTLEPVEKALRDAKLDKGQIQEIVLVGGSTRIPK
hHSP70-1 286 SLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPK
Binding region
Connect 2 region
PfHSP70-1 359 IQTLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGLET
cKHSP70-1 349 IQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVTPLSLGIET
hHSP70-1 346 VQKLLQDFFNGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLET
β-Subdomain (Peptide substrate binding)
PfHSP70-1 419 AGGVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIP
cKHSP70-1 409 AGGVMTALIKRNTTIPTKQTQTFTTYSDNQSSVLVQVYEGERAMTKDNNLLGKFDLTGIP
hHSP70-1 406 AGGVMTALIKRNSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFELSGIP
PfHSP70-1 479 PAPRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSQDEIDRMVNDAEKY
cKHSP70-1 469 PAPRGVPQIEVTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKDDIDRMVQEAEKY
hHSP70-1 466 PAPRGVPQIEVTFDIDANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEAEKY
α-Helicalsubdomain (Lid and hinge region)
PfHSP70-1 539 KAEDEENRKRIEARNSLENYCYGVKSSLEDQKIKEKLQPAEIETCMKTITTILEWLEKNQ
cKHSP70-1 529 KAEDEANRDRVGAKNSLESYTYNMKQTVEDEKLKGKISDQDKQKVLDKCQEVISWLDRNQ
hHSP70-1 526 KAEDEVQRERVSAKNALESYAFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLDANT
PfHSP70-1 599 LAGKDEYEAKQKEAESVCAPIMSKIYQDAAGAAGGMPGGMPGGMPGGMPSGMPGGMNFPG
cKHSP70-1 589 MAEKEEYEHKQKELEKLCNPIVTKLYQGAGGAGAGGSG----------

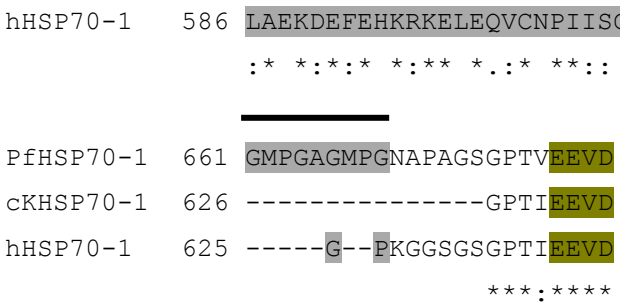


Figure 4.12 Predicted N-glycosylation sites on the *Pf*HSP70-1 sequence aligned with chicken (*Gallus Gallus*) cKHSP70-1) and human (*Homo sapien*) hHSP70-1) using NetNGlyc. The light blue colour (with letter N) show the positions of predicted potential asparagine glycosylation sites by NetNGlyc in the *Pf*HSP70-1 sequence. Dark colour phosphate 1 binding region, red phosphate 2 binding region, yellow connect 1 region, light green connect 2 region, light blue the adenosine binding region, purple β-subdomain for peptide substrate binding, grey α-helical subdomain for hinge and lid region, greenish is the EEVD mortif. Within the α-subdomain the blue, green and pink show the position of the 3 immunogenic peptide sequences predicted by Predict7™.

The program predicted no glycosylation sites for *Pf*LDH and four potential glycosylation sites for *Pf*HSP70-1 (Figure 4.11 A-D and E where the blue arrows point). The position of the predicted N-glycosylation sites in the sequence are shown in Figure 4.12 on the aligned sequences of *Pf*HSP 70-1 with chicken and human *Pf*HSP70-1 sequences. The position of the predicted potential N-glycosylation sites relative to the important amino acid residues in the active site of *Pf*HSP70-1 can be seen in Figure 4.12. The predicted potential glycosylation sites N432 and N502 are located in the β-subdomain (the peptide substrate domain) and may not be phosphorylated. The other two, N46 and N166 are located far away from the essential catalytic residues. The recombinant *Pf*LDH and *Pf*HSP70-1 produced in *E. coli* are non-glycosylated, but the native proteins may be glycosylated leading to differences in their activity compared to the recombinant proteins. Glycosylation can be examined in the native protein by digesting with neuraminidase, then running an SDS-PAGE gel of the digested and undigested native protein and compare the size of the bands. Alternatively, a zymogram of these proteins can be done using neuraminidase to cleave off the glucose moiety (Mensah-Brown *et al*., 2014).

4.3.4 Proteolytic cleavage

Although recombinant proteins produced in *E.coli* are not subject to proteolytic digest because the host cells lack proteases, native proteins may be cleaved by various proteases. The recombinant protein can be subject to protease digestion and probed with antibodies against the protein to determine which portion of the protein the antibody binds to. Netcchop www.cbs.dtu.dk/services/NetChop/ (Kesmir *et al.*, 2002; Nielsen *et al.*, 2003; Nielsen *et al.*, 2005) was used to predict the cleavage sites of *Pf*LDH and *Pf*HSP70-1. The program predicted 92 cleavage sites for *Pf*LDH and 204 for *Pf*HSP70-1. There are so many predicted sites that the data is not shown. Recombinant protein lysate can be immunoprecipitated and an in-gel protease digestion can be done ((Dinglasan *et al*., 2007; Ghosh *et al*., 2009; Chaerkady *et*

al., 2011), after which immunoprecipitated digested peptides can be analysed on a high resolution mass spectrometer (Cha *et al*., 2016).

Three peptides were identified in the structure of *Pf*HSP70-1 as potential immunogenic peptides by Predict7™ and were found to be located on the α-helical subdomain close to the C-terminus of the protein. All the 3 peptides were found to be located on the surface of the 3D crystal structure model of *Pf*HSP70-1.

Potential lysine acetylation sites were identified on both *Pf*LDH and *Pf*HSP70-1. K5 lysine acetylation on *Pf*LDH is implicated in enzyme activity. The amino acid has been shown to be important in human LDH-A as it reduced the activity of human LDH-A. K563, K574 and K602 lysine acetylation of *Pf*HSP70-1 may affect activity of the native protein as it was found to affect activity of human HSP70-1. Only amino acid residues on the surface of the proteins can affect activity of the native proteins because they can interact with antibodies.

Potential phosphorylation sites were identified on both *Pf*LDH and *Pf*HSP70-1. Y83 of *Pf*LDH may enhance activity of the enzyme as Y83 of human LDH-A was found to enhance activity of human LDH-A. The predicted S574 of *Pf*HSP70-1 is found on the β-subdomain which is less likely to be located on the surface and is far from essential catalytic amino acids, and may not affect the activity of *Pf*HSP70-1.

Potential O-glycosylation sites for both *Pf*LDH and *Pf*HSP70-1 were found. No potential N-glycosylation site was found for *Pf*LDH. Four N-glycosylation sites were found on *Pf*HSP70-1. N432 and N502 are located in the β-subdomain which is not likely to be on the surface and may not be phosphorylated. N46 and N166 are located far away from essential catalytic residues, therefore will not affect the activity of the native protein. 92 potential cleavage sites for *Pf*LDH and 204 for *Pf*HSP70-1 were identified. Recombinant proteins can be digested by proteases to see which fragment of the protein antibodies bind to.

Chapter 5

5.1 Brief overview

Malaria is a global heath burden and an estimated 3.3 billion people are at risk of being infected with malaria and 1.2 billion are at high risk (WHO 2014). An estimated 198 million cases of malaria occurred globally in 2013 and 584 000 deaths were recorded (WHO 2014). Although malaria control programs in many countries include vector control, treatment with artemisinin combination therapy, diagnosis in most remote settings is still problematic (Moody, 2002; Kappe *et al*., 2010; Shiff, 2002). RTS, S/AS01 Vaccine development is in the advanced stages of clinical trials (Schwartz *et al*., 2012). Malaria affects mostly developing countries in the tropics, especially sub-Saharan Africa, where there is a lack of infrastructure and funding to control the disease (Hay *et al*., 2009).

Microscopy is the gold standard for the diagnosis of malaria, but most field clinics lack infrastructure, electricity and the experienced manpower needed to use a microscope (Moody, 2002; Suh *et al*., 2004). Field clinics, therefore, require diagnostic methods that are fast, easy to perform and interpret and do not need electricity (Perkins & Bell., 2008; Moody, 2002; Makler *et al*., 1998). Various methods have been developed in an attempt to improve diagnosis including PCR, flow cytometry, LAMP, DNA aptamers (Gupta *et al*., 2015; Echeverry *et al*., 2016; Godonoga *et al*., 2016; Woodrow *et al*., 2015). Most of the methods mentioned require expensive equipment, reagents and skills (Mouatcho & Goldring, 2013).

Malaria immunochromatographic or rapid diagnostic tests (RDTs) were developed to simplify malaria diagnosis as they are fast, easy to use, easy to interpret and do not require the use of electricity. RDTs in current use detect *Plasmodium falciparum* histidine rich protein II (*Pf*HRPII), *Plasmodium* lactate dehydrogenase (LDH) and *Plasmodium* aldolase (Murray *et al*., 2008; Ashley *et al*., 2009; Khairnar *et al*., 2009; Wongrichanalai *et al*., 2007; Leow *et al*., 2014; Verma *et al*., 2014; Ho *et al*., 2014; Shin *et al*., 2013; Otsuki *et al*., 2013; Miao *et al*., 2013). The limit of detection is about 200 parasites/µl and takes an average of 5-20 min to perform (Makler *et al*., 1998; Murray *et al*., 2008; Perkins & Bell, 2008; Ashley *et al*., 2009). To detect asymptomatic malaria RDTs require to have a limit of detection of less than 100 parasites/µl (Suh *et al*., 2011). Other short comings among RDTs in current use include cross reaction with rheumatoid factor, loss of reactivity due to exposure to high temperatures when stored, single nucleotide polymorphism in DNA of parasite genes leading to changes in the proteins and failure of the test to differentiate co-infections with different species of malaria (Mishra *et al*., 1999; Baker *et al*., 2010; Gwer *et al*., 2007).

5.2 Aims and objectives of the current study

The main aim of the study was to optimise the expression and purification of recombinant *Pf*LDH and *Pf*HSP70-1 to produce recombinant protein for use in raising polyclonal antibodies in chickens, antibody-antigen interaction studies and screening of ScFvs in *Nkuku*® library for scFvs. The second aim was to identify immunogenic peptides using an epitope prediction algorithm Predict7™. The peptide could be used to raise anti-peptide antibodies in chickens for comparison with polyclonal antibodies in the detection of each protein. The last aim was to identify possible post-translational modifications that may interfere with detection of *Pf*LDH and *Pf*HSP70-1 by antibodies.

5.3 Main findings

*Pf*LDH showed better expression in TB media (Li *et al*., 2011) (Figure 3.1 and Figure 3.2) and the expression of *Pf*HSP70-1 was better in TB than LB media (Figure 3.17). TB media was found to be a good choice of media by Li *et al.*, (2011) using GFP, gluatathione-S-transferase tagged GFP and human basic fibroblast growth factor recombinant proteins, as cells grow faster in this media and do not require induction. Growing cells in TB media were chosen. Induction in LB media was done using IPTG (a very expensive reagent) although IPTG can be substituted by cheaper substances such as lactose and glucose. The growth, following bacterial cells, showed that the *E. coli* host cells grew faster in TB media reaching the stationary phase at about 4 hrs and 5 hrs for *Pf*LDH and *Pf*HSP70-1, respectively (Figure 3.3 and Figure 3.16). The use of a single colony as starting material for expression of recombinant proteins yielded better results than dilution of an overnight culture 1:100 in fresh growth medium. Induction of cultures at stationary phase was compared with induction at mid-log phase or exponential phase. The results showed that inducing the culture at stationary phase yields higher amounts of recombinant protein than inducing at mid-log phase. This result was similar to that obtained by Flick *et al*., (2004) with recombinant GST-*Pf*EMP1. Expression speed and folding processes appear to influence protein folding, and these appear to be slowed when induction is done at stationary phase. At stationary phase, there is a low bacterial growth rate, which implicates a slow biosynthesis process that results in a low speed of synthesising recombinant proteins. This slow process allows the protein processing machinery to efficiently assemble the freshly synthesised proteins into correctly folded structures. Correctly folded proteins are more likely to remain in the soluble form provided the molecules do not have large numbers of hydrophobic amino acid residues (Flick *et al*., 2004). Expression of recombinant *Pf*LDH protein at 25°C was compared to expression at 37°C. Expression at 25°C produced more

soluble proteins than at 37°C (Figure 3.6) which has been observed by Tsai *et al*., 2016 for recombinant Mani 1 and Flick *et al*., for GST-*Pf*EMP1. Low temperature, before and after induction, improves the amount of soluble protein obtained and activity but reduces the overall final yield (Figure 3.9) (Flick *et al*., 2004).

The optimal condition for lysing of *E. coli* cells was investigated using lysozyme digestion, freezing and thawing, and sonication methods. Lysozymes (muramidase) digest the peptidoglycan layer of the bacterial cell wall. Gram-negative bacteria such as *E. coli* have an outer membrane external to the cell wall and, therefore, require to be permeabilised to expose the peptidoglycan layer to lysozyme digestion. This is achieved by Tris and EDTA added in the buffer. During lysozyme mediated cell lysis, a lot of genomic DNA is released and makes the solution viscous, and addition of DNase can reduce the viscosity. This method is expensive. Freezing and thawing results in the damage of the inner membrane, which leaks the T7 lysozyme in *E. coli* BL21 (DE3) and cytoplasm contents. The resulting solution is a bit viscous and this can be sonicated to break down the genomic DNA. Sonication lyses cells by using shear force and cavitation. The genomic DNA released in the process is sheared. Freezing and thawing followed by sonication done twice was found to be optimal for lysing of *E. coli* cells. Although the combination of all the methods produced more proteins in solution, it contained a lot of contaminants, most likely nucleic acids which were not completely removed by sonication (Figure 3.5 and Figure 3.20). This perhaps was due to the high level of *E. coli* expression in TB media resulting in a lot of genomic DNA released in the solution.

Purifying at 4°C appeared to be better than at room temperature (25°C) (Figure 3.10), a result similar to what was observed by Tsai *et al*., (2016) with recombinant Mani 1.Goto *et al*., (2016) also observed the same trend with swine LDH. Higher yields of purified *Pf*LDH were observed than *Pf*HSP70-1 throughout the study.

Western blots of the purified recombinant *Pf*LDH and *Pf*HSP70-1 showed high yields of recombinant *Pf*LDH (figure 3.12) and low yields of *Pf*HSP70-1 (Figure 3.22). Preliminary enzyme kinetic studies showed that *Pf*LDH was active. It would be interesting to know if the activity of the recombinant *Pf*LDH was more or less compared to the native protein. The recombinant *Pf*LDH produced could be used in the production of antibodies in chickens, study protein activity, antibody-antigen interaction *in vitro*, screen the Nkuku® scFv library (van Wyngaardt *et al*., 2004) for scFvs. The recombinant proteins could be used to test for competition between polyclonal or anti-peptide antibodies produced, with the substrate to see if the antibodies inhibits, increases or does nothing to the activity of the recombinant protein. Poor expression of *Pf*HSP70-1 appeared to be caused by either low copy number or loss of the *Pf*HSP70-1 insert in the plasmid. Because the method used in section 3.2.14 is

less sensitive, primers of the *Pf*HSP70-1 gene can be designed and the gene amplified by PCR to conclusively know if low copy number or loss of insert is the cause of poor expression. The PCR product can be cloned and transformed into *E. coli* for the expression of *Pf*HSP70-1. Alternatively, using specific primers, *Pf*HSP70-1 gene can amplified by PCR from genomic DNA, then transcribed into mRNA of *Pf*HSP70-1. The mRNA can be reverse transcribed into cDNA and then cloned and transformed as mentioned earlier.

Three peptides with high surface probability and hydrophilicity values were identified from the amino acid sequence of *Pf*HSP70-1 using the Predict 7™ program. These peptides were found to be on the surface of *Pf*LDH crystal structure. Production of polyclonal and anti-peptide antibodies against *Pf*HSP70-1 in chicken and isolating lgY from the egg yolk. Testing of these antibodies with the recombinant and native protein would be required. This information would be used to assess the effectiveness in predicting immunogenic epitopes by the algorithm Predict7™ used in the current study to predict the epitopes for *Pf*HSP70-1. There would be need to study the activity of ATPase and substrate binding domains of recombinant *Pf*HSP70-1 and compare this to previous data as proof that the expressed recombinant protein in *Pf*HSP70-1. This study may include studying the ATPase activity of *Pf*HSP70-1 and determine the ATP hydrolysis of *Pf*HSP70-1 in the presence or absence of co-chaperones such as *Plasmodium falciparum* Dnaj 1-4 and *Plasmodium falciparum* HSP90. Potential acetylation, phosphorylation, glycosylation and proteolytic cleavage sites were found in both *Pf*LDH and *Pf*HSP70-1. Polyclonal and anti-peptide antibodies raised from these recombinant proteins would be characterized by comparing their affinity for acetylated, phosphorylated or glycosylated and non-acetylated, non-phosphorylated and non-glycosylated peptides using ELISA formats.

5.4 Conclusion

Recombinant expression and purification of *Pf*LDH and *Pf*HSP70-1 was achieved. *Pf*LDH was expressed and purified in high amounts of 144.77 mg of recombinant protein from 200 ml culture volume compared to 18 mg of recombinant *Pf*LDH per liter of culture volume obtained by Berwal *et al*., 2008. Preliminary studies showed activity of *Pf*LDH. *Pf*HSP70-1 was poorly expressed and purified. The data observed suggests possible low plasmid copy number or loss of *Pf*HSP70-1 insert in the plasmid, and hence poor yields. Purification of recombinant proteins at 4°C is better than at room temperature (25°C). The purified proteins were identified by anti-His-tag antibodies. Potential acetylation, phosphorylation, glycosylation and proteolytic cleavage sites were identified.

The *Pf*LDH plasmid was sequenced, and the sequence was confirmed to be of *Pf*LDH. Probing the recombinant *Pf*LDH protein with anti-peptide antibodies against epitopes

of *Pf*LDH or anti-*Pf*LDH antibodies would further confirm the identity of recombinant *Pf*LDH. The recombinant *Pf*LDH protein amino acid residues can also be sequenced.

A basal ATPase and steady state ATPase activity of recombinant *Pf*HSP70-1 can be done to confirm the identity of recombinant *Pf*HSP70-1. Probing the recombinant *Pf*HSP70-1 with anti-*Pf*HSP70-1 antibodies can confirm the identity of *P*fHSP70-1. Furthermore, recombinant *Pf*HSP70-1 plasmid can be isolated and sequenced or amino acid residues of recombinant *Pf*HSP70-1 can be sequenced to confirm the identity of *Pf*HSP70-1.

Future work will involve cloning of the *Pf*HSP70-1 gene with a His_6-tag at the N-terminal into a pQE30 or another appropriate plasmid vector. Express the recombinant *Pf*HSP70-1 protein, purify and use the purified recombinant protein to raise anti-peptide and whole protein poly clonal antibodies against *Pf*HSP70-1 in chickens. Screening of scFv against *Pf*LDH and *Pf*HSP70-1 recombinant proteins in the *Nkuku*® library will be done. The antibodies will be tested in ELISA, western blots, enhanced chemiluminescence and dip stick format.

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