# IDENTIFICATION OF POTENTIAL RECEPTORS FOR SURFACE PROTEINS OF *TOXOPLASMA GONDII* IN HUMANS

**LAI MENG YEE**

**FACULTY OF MEDICINE**
**UNIVERSITY OF MALAYA**
**KUALA LUMPUR**

**2018**

# IDENTIFICATION OF POTENTIAL RECEPTORS FOR SURFACE PROTEINS OF *TOXOPLASMA GONDII* IN HUMANS

**LAI MENG YEE**

**THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

**FACULTY OF MEDICINE**
**UNIVERSITY OF MALAYA**
**KUALA LUMPUR**

**2018**

**UNIVERSITY OF MALAYA**
**ORIGINAL LITERARY WORK DECLARATION**

Name of Candidate: **LAI MENG YEE**

Matric No: **MHA 120031**

Name of Degree: **DOCTOR OF PHILOSOPHY**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

**IDENTIFICATION OF POTENTIAL RECEPTORS FOR SURFACE PROTEINS OF *TOXOPLASMA GONDII* IN HUMANS**

Field of Study: **PARASITOLOGY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

**IDENTIFICATION OF POTENTIAL RECEPTORS FOR SURFACE PROTEINS OF *TOXOPLASMA GONDII* IN HUMANS**

## ABSTRACT

*Toxoplasma gondii* (*T. gondii*) is an obligate intracellular protozoan parasite that invades any nucleated cells in humans and other warm-blooded animals, with a great infection rate. Approximately 25% to 30% of the world's human population is infected by *T. gondii*. Many proteins involved in *T. gondii* invasion have been characterized, and the contribution for parasite entry has been proposed. The identification of receptors or binding host proteins for surface antigens is an important activity throughout the study. A better understanding of the interplay between *T. gondii* and its hosts may provide a starting point for the discovery of novel therapeutics. The surface antigens (SAGs) of *T. gondii* play a major role during the host cell invasion process. In this study, the yeast two-hybrid system was used to analyze the interaction of *T. gondii* SAG1 and SAG2 with the human host cell. Protein interaction was performed using commercial human cDNA in pGADT7-RecAB. A total of 39 and 25 clones which interacted with the respective SAG1 and SAG2 were detected based on a series of the selection procedures. Twenty-nine and 18 clones for SAG1 and SAG2 were sent for sequencing after colony PCR. Following analysis of sequencing results, Y187 cells transformed with each of these potential prey plasmids (22 and 13 prey clones of each SAG1 and SAG2) was mated with the respective Y2HGold containing pGBKT7-SAG2 or pGBKT7-SAG1 and Y2HGold (pGBKT7). *Homo sapiens* lysine rich coil-coiled (abbreviated as HLY) and *Homo sapiens* zinc finger (abbreviated as HZF) proteins were identified as potential candidate interacting with SAG1 and SAG2, respectively. The interaction were further examined by beta-galactosidase assay and the enzyme activity between SAG1 and SAG2 with their host proteins were 449.4 U and 437.7 U, respectively. In comparison to positive control (424.3 U), both interaction between prey and SAG1 and SAG2 were demonstrated. The

interaction between prey and bait proteins was further determined using co-immunoprecipitation assay. The result indicated the binding between these prey and SAG1 and SAG2 proteins were significant. Finally, with the aid of isothermal titration calorimetry (ITC) method, binding strength for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins were 0.0075 μM and 18.75 μM, respectively. Both thermodynamic curves revealed that there were exothermic and endothermic reaction for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins, respectively. These prey proteins may serve as the potential drug candidates during the vaccination study in future.

**Keywords:** *Toxoplasma gondii*, protein-protein interaction, yeast two-hybrid, co-immunoprecipitation, isothermal titration calorimetry

**IDENTIFICATION OF POTENTIAL RECEPTORS FOR SURFACE PROTEINS OF *TOXOPLASMA GONDII* IN HUMANS**

## ABSTRAK

*Toxoplasma gondii* (*T. gondii*) adalah sejenis parasit protozoa obligat intrasel yang menyerang mana-mana sel bernukleus manusia dan haiwan yang berdarah panas. Lebih kurang 25% hingga 30% daripada populasi manusia di dunia telah dijangkiti *T. gondii*. Banyak protein yang terlibat dalam serangan *T. gondii* telah dicirikan dan sumbangan bagi kemasukan parasit telah diusulkan. Pengenalpastian reseptor atau protein pengikat perumah bagi protein ini dari manusia adalah satu aktiviti penting. Pemahaman yang mendalam terhadap interaksi di antara *T. gondii* dan perumah dapat memberikan titik permulaan dalam penemuan terapeutik novel. Antigen permukaan (SAGs) bagi *T. gondii* memainkan peranan penting semasa proses serangan sel perumah. Dalam kajian ini, sistem dua-hibrik yis telah digunakan untuk menganalisis interaksi di antara *T. gondii* SAG1 dan SAG2 dengan sel perumah manusia. Interaksi protein telah dilakukan dengan menggunakan cDNA manusia komersial di dalam pGADT7-RecAB. Sebanyak 39 dan 25 klon yang berinteraksi masing-masing dengan SAG1 dan SAG2 telah dikesan menerusi siri prosedur pemilihan. Dua puluh sembilan dan 18 klon untuk SAG1 dan SAG2 telah dihantar untuk penjujukan selepas koloni PCR. Berikutan analisis keputusan penjujukan menunjukkan setiap transformasian oleh Y187 dengan plasmid pemangsa yang berpotensi (22 dan 13 klon pemangsa bagi setiap SAG1 dan SAG2) telah digabungkan dengan Y2HGold yang masing-masing mengandungi pGBKT7-SAG2 atau pGBKT7-SAG1 dan Y2HGold (pGBKT7). Protein *Homo sapiens* kaya dengan lysine (singkatan sebagai HLY) dan protein *Homo sapiens* zink jari (singkatan sebagai HZF) telah dikenalpasti sebagai calon yang berpotensi untuk berinteraksi masing-masing dengan SAG1 dan SAG2. Interaksi ini diuji seterusnya dengan esei beta-galactosidase dan aktiviti enzim di antara SAG1 dan SAG2 dengan protein perumah masing-masing adalah 449.4

U dan 437.7 U. Perbandingan di antara aktiviti enzim bagi kawalan positif (424.3 U) menunjukkan kedua-dua interaksi di antara pemangsa dan SAG1 dan SAG2 terbukti wujud. Interaksi antara protein pemangsa dan protein umpan ditentukan selanjutnya dengan esei imunopresipitasi bersama. Keputusan ujian menunjukkan pengikatan antara pemangsa dan protein SAG1 dan SAG2 adalah signifikant. Akhir sekali, dengan bantuan kaedah kalorimetri titrasi isoterma (ITC), kekuatan pengikatan antara rekombinan pRSET-A-SAG1 dengan protein rekombinan pRSET-A-HLY dan rekombinan pRSET-A-SAG2 dengan protein rekombinan pRSET-A-HZF masing-masing adalah 0.075 μM dan 18.75 μM. Kedua-dua lengkung termodinamik mendedahkan bahawa terdapat reaksi eksotermik dan endotermik masing-masing bagi rekombinan pRSET-A-SAG1 dengan protein rekombinan pRSET-A-HLY dan bagi rekombinan pRSET-A-SAG2 dengan protein rekombinan pRSET-A-HZF. Protein pemangsa mungkin berperanan sebagai calon ubat berpotensi semasa kajian vaksinasi di masa akan datang.

**Kata kunci**: *Toxoplasma gondii*, interaksi protein-protein, yis dua-hibrik, imunopresipitasi bersama, kalorimetri titrasi isoterma

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Associate Professor Dr. Lau Yee Ling for their guidance and support throughout the entire course of my project. She spent a lot of time in teaching and correcting my papers during the entire project. Her help will be highly appreciated.

Also, I would like to acknowledge Professor Dr. Fong Mun Yik who gave me a lot of suggestions and guidance when doing the project. Apart from that, I would like to thank Professor Dr. Rohela Mahmud that always encourage me not to give up. Thanks again.

I would like to take this opportunity to thank my seniors especially Dr. Parthasarathy Sonaimuthu. He is the person who showed and taught me all the procedures during the beginning of my study. I am really appreciated.

Also, my sincere thanks to Higher Impact Research and Institute of Research Management & Monitoring Grant Research Board, University of Malaya for providing me research grant.

Last but not least, I would like to express my deepest appreciation to my parents and other members of my family that have supported me throughout the entire course of my work.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| % | percent |
| $^{o}$C | degree Celcius |
| *et al.*, | et alia (and others) |
| x g | gravitational field (centrifuging) |
| µg | microgram |
| ng | nanogram |
| mg | miligram |
| g | gram |
| µl | microlitre |
| ml | millilitre |
| L | litre |
| µM | micromolar |
| mM | milimolar |
| M | molar |
| N | Normality |
| min | minute |
| rpm | revolutions per minute |
| sp. | species |
| v/v | volume per volume |
| w/v | weight per volume |
| V | volt |
| s | second |
| U | Unit |
| bp | base pair |
| UV | ultraviolet |

| | |
|---|---|
| *T. gondii* | *Toxoplasma gondii* |
| SAG | surface antigen |
| MIC | microneme |
| GRA | dense granules |
| ROP | rhoptry |
| RON | rhoptry neck |
| kDa | kilodalton |
| *H. sapiens* | *Homo sapiens* |
| HLY | *H. sapiens* lysine-rich coil-coiled |
| HZF | *H. sapiens* zinc finger |
| Y2H | Yeast two-hybrid |
| Co-IP | Co-immunoprecipitation |
| ITC | Isothermal titration calorimetry |
| PCR | polymerase chain reaction |
| RFLP | restriction fragment length polymorphism |
| PPI | protein-protein interaction |
| BD | binding domain |
| AD | activation domain |
| DNA | deoxyribonucleic acid |
| RNA | ribonucleic acid |
| mRNA | messenger ribonucleic acid |
| N | number of binding sites |
| $K_a$ | association constant |
| $K_d$ | dissociation constant |
| $\Delta H$ | enthalpy changes |
| $\Delta S$ | entrophy changes |

## LIST OF APPENDIX

# CHAPTER 1: INTRODUCTION

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii* (*T. gondii*) that infects all warm-blooded animals (Dubey, 2008). *T. gondii* is an extraordinarily successful parasite, infecting an estimated one third of humans worldwide (Reese *et al*., 2011; Tenter *et al*., 2000). Members of the Felidae family are the definitive hosts for this parasite. Humans may become infected by consuming raw meats containing tissue cysts of *T. gondii*, water or raw vegetables contaminated with *T. gondii* oocysts, through the placenta from mother to foetus if the mother has a primary infection, blood transfusion or organ transplantation from an infected donor. Normally, people with a weak immune response, immunocompromised patients, and newborn infants may develop severe diseases, such as pneumonia, encephalitis, mental retardation, and other life-threatening conditions (Wilking *et al*., 2016). Healthy individuals are usually asymptomatic (70-80%) or sometimes suffer from fever, swollen glands, muscle aches and pains, and tiredness at the beginning of infection (Gharavi *et al*., 2011). However they are usually self-recovering and treatment is not needed (da Silva *et al*., 2015; Striepen *et al*., 1997).

Surface antigens (SAG1 and SAG2) are glycophosphatidylinositol (GPI)-anchored antigens. GPI-anchored antigens (SAG1, SAG2A, SAG2B, SAG3 and SRS2) are distributed all over the surface of *T. gondii* (Nagel & Boothroyd, 1989; Tomavo *et al*., 1989). These molecules play a crucial role in host-cell attachment during the invasion process and provide protection needed by parasites in order to survive in host cell environment (Lekutis *et al*., 2001).

SAG1, 30 kDa, is a tachyzoite-specific antigen and is present in the tachyzoite stage only (Wu *et al*., 2009; Zhang *et al*., 2007). This is different from malaria, considering that *T. gondii* can infect any nucleated cells while *Plasmodium* merozoite specifically marks the red blood cells. SAG1 is a predominant protein (3% to 5%) and a key mediator for attachment to host cells (Grimwood & Smith, 1996; Kazemi *et al*.,

2007). SAG1 is very immunogenic and elicits high titers of antibodies in infected individuals (Nagel & Boothroyd, 1989). On the other hand, SAG2, 22 kDa, is present in both tachyzoite and bradyzoite stages (Lekutis *et al*., 2000). SAG2 is related to the SAG1 family, following a sequence analysis that had been reported by Lekutis *et al*. (2001). The sequence analysis revealed that SAG2 family members shared ~30% similar identity of their cysteine residues with SAG1 family. However, there exists a significant differences in the length of the open reading frame of SAG2 family. SAG2A and SAG2B have a much shorter open reading frame (~200 amino acids) than SAG1 family (~300 amino acids) and other SAG2-related family members (Lekutis *et al*., 2001).

Identifying receptors or binding partners in humans during parasitism of *T. gondii* is of great importance. This is not an easy task as human genomes consist of 20,000-30,000 genes that code for over 500,000 different proteins. More than 80% of the proteins in *T. gondii* do not operate alone but in complex or through protein-protein interaction networks (Beggard *et al*., 2007). These proteins are vital parts of living organisms and they are the main components of the physiological metabolic pathways of cells.

Yeast two-hybrid (Y2H) system is an easy, inexpensive and *in vivo* approach to study protein interaction. Y2H system does not require purified proteins to initiate the screening and is therefore less time-consuming and cheaper compared to the more classical methodologies. The Y2H system is often more sensitive than the *in vitro* techniques, therefore more suitable for detection of weak or transient interactions (Bruckner *et al*., 2009). The advent of the Y2H system has stimulated significant increase in the number of protein interactions reported in the scientific works within the last 30 years because these assays allow rapid detection and discovery of new protein interactions via DNA library screening. In Y2H assay, a given protein is assayed against a mixture of protein fragments expressed from a cDNA library, followed by isolation of the interacting

partner protein. We attempted to identify host cellular proteins that interact with *T. gondii* SAG1 and SAG2 by means of this powerful system.

**Objectives of this study:**

**(a)** To identify the potential host binding proteins of *T. gondii* SAG1 and SAG2 by Y2H system.

**(b)** To analyse interactions between positive clones with *T. gondii* SAG1 and SAG2 by beta-galactosidase assay.

**(c)** To confirm the interaction between *T. gondii* SAG1 and SAG2 with selected host binding protein by chemiluminescent Co-IP assay.

**(d)** To measure binding strength between *T. gondii* SAG1 and SAG2 with selected host binding protein by ITC.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 *Toxoplasma gondii* (*T. gondii*)

*T. gondii* was first described in 1908 by Nicolle and Manceaux in the tissue of North Africa rodent, *Ctenodactylus gondi*. In the same year, Splendore in Brazil also reported the identification of this organism in tissue of a rabbit. The genus was named by Nicolle and Manceaux as *Toxoplasma* according to its crescent shape. Several years later, Wolf *et al*. (1939) identified *T. gondii* as a causative agent to human disease (Wolf *et al*., 2009).

*T. gondii* belongs to the phylum Apicomplexa, and causes substantial morbidity and mortality worldwide. The morbidity and mortality rates vary between groups of patients. For example, patients with acquired immune deficiency syndrome (AIDS), toxoplasmosis encephalitis causes morbidity and mortality of 27% and 12%, respectively (Renold *et al*., 1992). Figure 2.1 reveals a phylogenetic tree for Apicomplexa family members (Pappas *et al*., 2009). In addition to *T. gondii*, other parasites that are considered as Apicomplexa family are *Plasmodium* (causative agent of malaria), *Cryptosporidium* (causative agent of cryptosporidiosis), *Eimeria* (causative agent of chicken coccidiosis), *Neospora* (causative agent of neosporosis in dogs and cattles), *Babesia* (causative agent of Babesiosis), and *Theileria* (causative agent of Theileriosis). Most of Apicomplexa parasites are causative agents of humans (estimating killing over 1 million people every year) and livestocks diseases (estimating causing agricultural losses over US$ 1 billion per year) (Shirley *et al*., 2007). Their importance has been recognized by the European Commission and this research was supported by COST (Cooperation in Science and Technology) Action 857 ‘Apicomplexan Biology in the Post-Genomic Era’ (Beck *et al*., 2009).

Among the family members of Apicomplexan, *T. gondii* is usually employed as a model for studies of parasitism in this family as it can be cultured both *in vitro* and *in vivo*

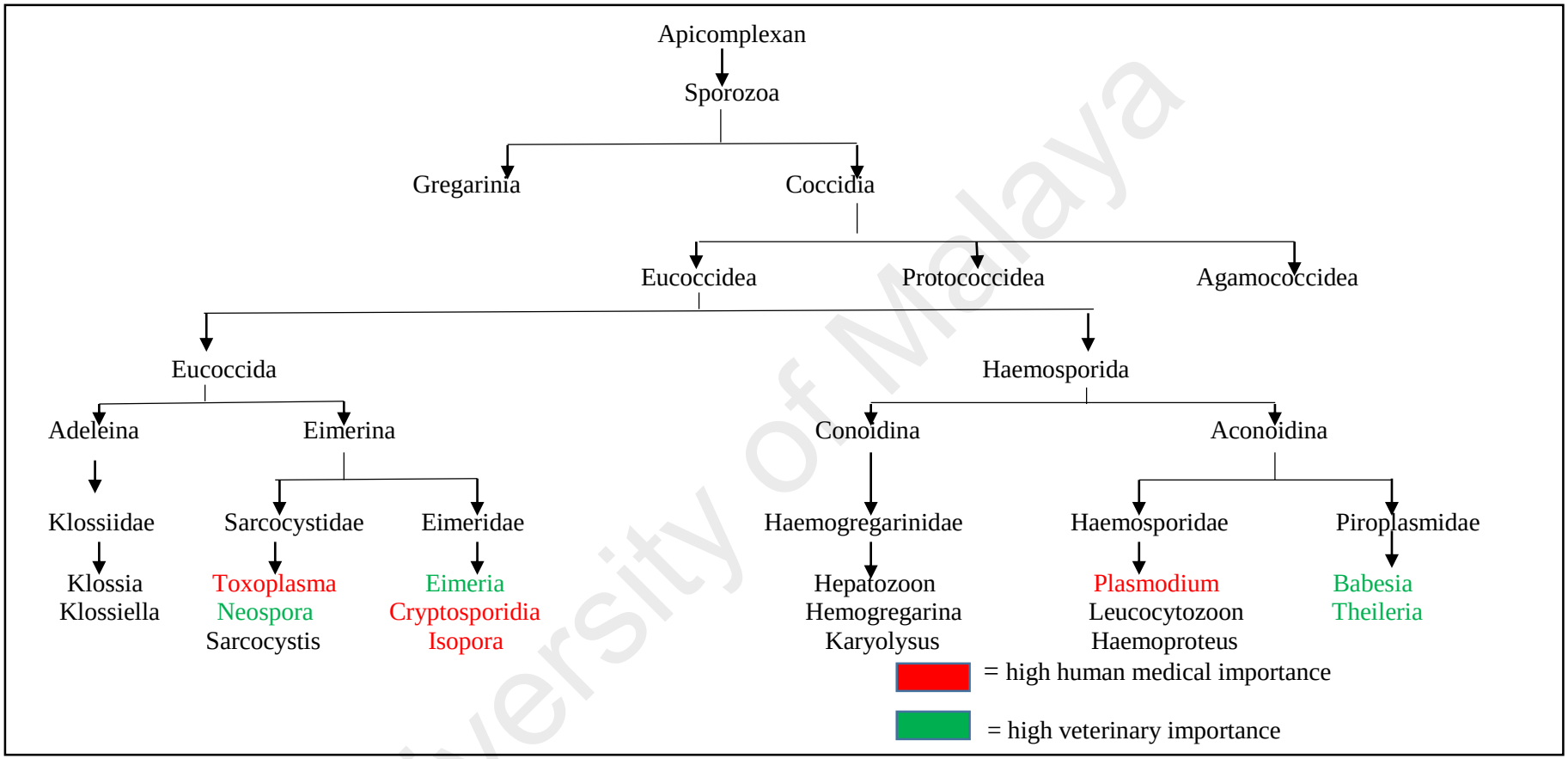



Figure 2.1: Phylogenetic tree of Apicomplexa members. *Toxoplasma* parasite belongs to cysts-forming Coccidia family and they are classified as highly human medical importance (Beck *et al*., 2009).

easily. The tachyzoite form of this parasite can be propagated in a variety of mammalian cell lines and mouse animal models (Kim & Weiss, 2004; Roos *et al*., 1994; Saeij *et al*., 2005).

Moreover, this parasite is able to infect all warm blooded animals. This is significantly different from other more restricted host range Coccidia members such as *Sarcocystis* sp.. On top of that, this parasite can be transmitted between different intermediate hosts via carnivorous or omnivorous feeding owing to its asexual form to invade, penetrate and propagate virtually in all types of animal cells. Also, this parasite is able to adapt in various ecological systems due to its long term survival rate as tissue cysts (Beck *et al*., 2009).

### 2.2 Life cycle of *T. gondii*

*T. gondii* life cycle is categorized into two main stages, namely, sexual and asexual stage. Figure 2.2 indicates the life cycle of *T. gondii*. Sexual stage of *T. gondii* occurs in felines only (wild or domesticated cats). Cats play an important role in the spread of toxoplasmosis as they are the only animals known to excrete resistant oocysts into environment (Silva *et al*., 2001). Sexual stage begins with ingestion of tissue cysts by cats, probably after eating an infected rodent. Inside the body of felines, the cysts make their way to the stomach and intestines. Once toxoplasma cysts reach the intestines, the cysts release bradyzoites. Bradyzoites penetrate the epithelial cells of the small intestinal. Then, the bradyzoites undergo sexual reproduction, the male gametes fertilizes the female gametes to produce zygotes and form the oocysts. These unsporulated oocysts develop a thick, impermeable wall and are shed in the faeces. Sporulation occurs outside the body, and the oocyst becomes infectious one to five days after excretion. Each sporulated oocyst contains two sporocysts and each sporocyst contains four sporozoites. Sexual stage ends

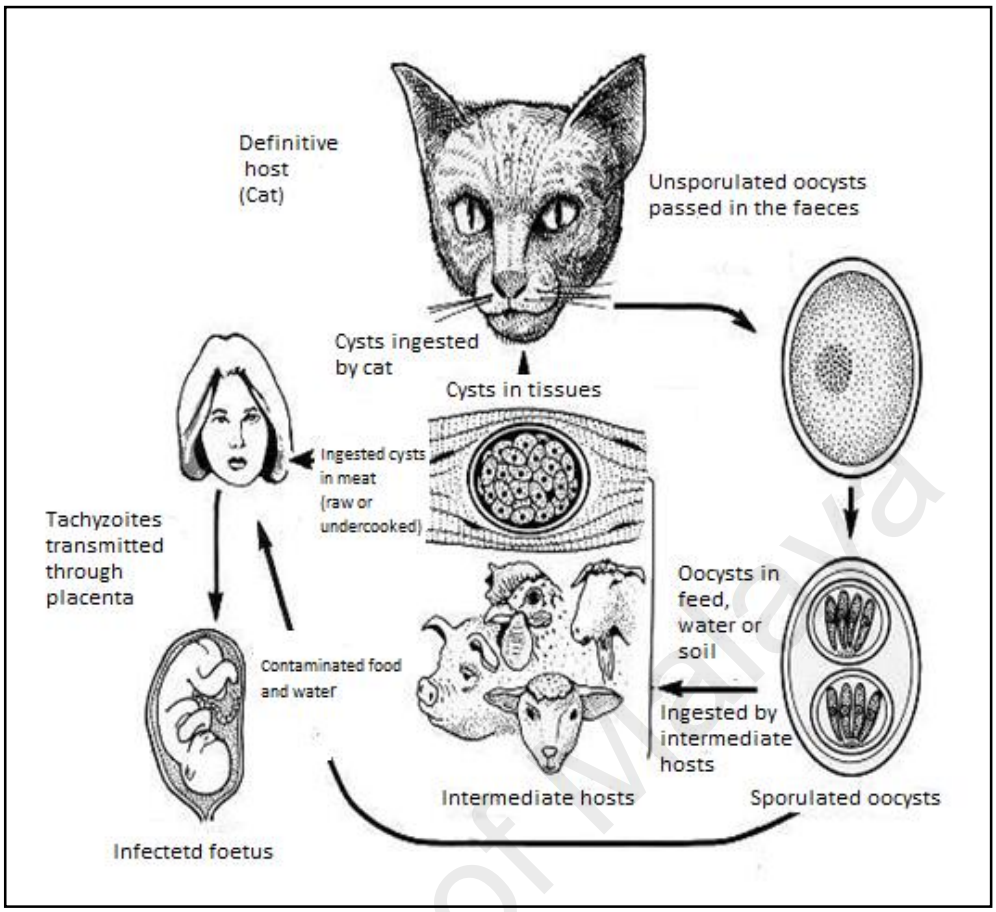



Figure 2.2: Life cycle of *T. gondii*. *T. gondii* life cycle consists of sexual (only occurs in definitive host) and asexual stage (occurs in intermediate hosts) (Dubey *et al*., 1998).

when these oocysts are excreted by cats in their faeces, thereby contaminating water and soil. The oocysts can survive in the environment for several months (Dubey *et al*., 1998).

While asexual stage of *T. gondii* occurs in intermediate hosts such as birds, chickens, cattle, lamb, goat, rabbit, sheep and humans. Humans acquired this parasite after eating raw meat and contaminated foods (that contain oocysts). When sporozoites enter the stomach and small intestinal of the hosts, the sporozoite cyst wall is dissolved by the host's proteolytic enzymes such as pepsin and trypsin. Bradyzoites are released and converted into tachyzoites. Tachyzoites are then distributed to other parts of the body via blood circulation. Tachyzoites can invade all nucleated cells and propagate until the host cell is filled with parasites and dies. The released tachyzoites enter new host cells and propagate. This cycle may cause tissue necrosis in the host. The host usually overcomes this stage of infection. The parasite then enters the "resting" stage in which bradyzoites are isolated in tissue cysts. Tissue cysts are found most commonly in the brain, liver, and muscles. Tissue cysts usually cause no harm to host and may remain for the life of the host (Dubey, 1998).

### 2.3 *T. gondii* clonal lineages

*T. gondii* has been classified into three major genotypes which are types I, II, and III (Fuentes *et al*., 2001). All strains of *T. gondii* are found in both humans and animals with considerable variability of virulence. Type I strains (RH, and GT-1) are highly virulent, whereas type II (ME49) and type III (CEP and VEG) are relatively less virulent. Type I strains are usually found in patients with recurrent ocular toxoplasmosis. While type II strain is commonly found in congenital toxoplasmosis and AIDS patients ((Roos *et al*., 1994; Khan *et al*., 2005). Within each lineage, comparative sequence analysis of individual genes indicates the rate of divergence are definitely low which is only 1% divergence at the DNA level (Howle & Sibley, 1995). Hence, genotyping has been used

to differentiate these clonal lineages. Chen *et al*. (2012), Khan *et al.* (2007) and Su *et al.* (2006) used methods such as PCR-restriction fragment length polymorphism (PCR-RFLP) or multilocus sequence typing (MLST) analysis. In PCR-RFLP, specific fragment of the genome is amplified and then cut with a restriction enzyme. The resulting restriction fragments are separated according to their lengths by gel electrophoresis. Strains with the same pattern of digested fragments are considered identical.

The RH strain was initially isolated from a child with lethal toxoplasmic encephalitis and has been maintained in the laboratory since 1939 (Ware & Kasper, 1987). RH is a commonly used laboratory strain due to its rapid replication rate and high productivity (Roos *et al*., 1994).

### 2.4 Transmission of *T. gondii*

Consumption of contaminated water and undercooked meat are major factors of acquisition of toxoplasmosis. The raw meat and water may be contaminated by tissue cyst (containing bradyzoites) or oocysts (containing sporozoites) from cat faeces, respectively. Humans are infected after consuming contaminated water and meat which are not cooked properly, leading to waterborne and foodborne outbreaks (Choi *et al*., 1997; Dawson, 2005; Mead *et al.*, 1999; Bahia-Oliveira *et al*., 2003; Moura *et al*., 2006).

*T. gondii* can also be transmitted to the foetus through placenta from mother. Freyre *et al*. (2001) reported that the incidence of congenital toxoplasmosis is 1-6/1000 births in the world. Toxoplasmosis can be observed in organ transplant and blood transfusion recipients as well. Most of the recipients are infected from primary infection (from seropositive donors to negative recipients). This is a rare mode of transmission where an infected organ is transplanted to an uninfected recipient (Sukthana *et al.*, 2000).

Workers in research and diagnostic laboratories have a high risk of infection with this parasite, particularly researchers who work with infected experimental animal such

as mice. They might be infected through samples, needles, sharp objects and other contaminated laboratory equipment. Some accidents are directly linked to poor laboratory practices such as working barehanded and recapping a needle after injection of tachyzoites into mice. The laboratory staffs may be infected through needlestick injury, spillage onto skin and splash into an eye (Herwaldt, 2001).

**2.5 Symptoms**

In pregnant women, *T. gondii* infection can cause abortion or still birth during the first trimester of pregnancy. The parasites pass through the placenta to the foetus and causing severe neonatal malformation, neurological damage, blindness, mental retardation or other congenitive defects. In comparison to first trimester of pregnancy, *T. gondii* infection can cause mild damages to the foetus in second and third trimesters of pregnancy. Infant born with asymptomatic infection may show symptoms months to years later. The obvious signs of infection are lymphadenopathy, jaundice, bruises or bleeding under the skin, enlarged liver or spleen and hydrocephalus (Fatoohi *et al*., 2002).

In immunocompromised individuals such as human immunodeficiency virus (HIV) patients and patients receiving immunosuppressive therapy with corticosteroid and cytotoxic drugs, toxoplasmosis can cause damage especially to their brain and eyes. On the onset of toxoplasmosis, the T-lymphocytes and lymphokines produced during the cellular mediated immune response and the antibodies produced during the initial humoral response are responsible for the eradication of the parasite. However, not all parasites are destroyed with the decline in T-cell numbers in patients with immune deficiency syndrome (Renold *et al*., 1992; Denkers & Gazzinelli, 1998). Toxoplasma-seropositive patients that are chronically infected with *T. gondii* can develop toxoplasmic encephalitis (damage in brain), pneumonitis and myocartiditis (Sibley & Boothroyd, 1992).

## 2.6 Diagnosis of *T. gondii* infection

Proper diagnosis of *T. gondii* infection may reduce morbidity and mortality particularly in the foetuses and newborns as prompt treatment could be provided (Singh, 2003). However, diagnostic tools or approaches for detection of *T. gondii* infection are scarce. Generally, diagnostic approaches may include microscopic examination, serological tests and molecular methods.

Microscopic examination is a traditional method used to examine the appearance of *T. gondii* stages in feline faeces, water, environmental and tissue samples from infected animals. The tissue cysts can be stained in order to differentiate parasites from host cells. Such staining solutions are Giemsa, haematoxylin and eosin. Also, amylopectin granules in bradyzoites can be stained by periodic acid Schiff (PAS) (da Silva *et al*., 2010). However, these staining methods are time consuming and adequate skill is needed to obtain a reliable result.

Therefore, serological methods are alternative ways to detect *T. gondii* infection. Laboratory serological tests for toxoplasmosis have been used to detect antibody produced in toxoplasmosis suspected patients (Montoya, 2002). The detection is performed by quantification of IgG and IgM class antibodies against *T. gondii* in human serum (Cong *et al*., 2015). Antibodies IgG and IgM are detectable around eight days after infection. However, IgM antibodies will disappear after a few months while IgG antibodies persist lifelong. Serological methods that have been used for diagnosis of toxoplasmosis are indirect hemagglutination (IHA) test, indirect fluorescent antibody tests (IFAT), avidity test, enzyme-linked immunosorbent assay (ELISA), latex agglutination test (LAT) and immunosorbent agglutination assay (ISAGA) (Cambiaso *et al*., 1992; de Ory *et al*., 1995; Liu *et al*., 2015; Remington *et al*., 2004). IgM antibodies are produced in the case of an exposure to toxoplasma infection. IgG antibodies are the long term response of body to a disease. IgM is an indicator of current infection while

IgG indicates a recent or past exposure to the disease. IgG antibodies are abundant in body but it does not indicate the timing of infection (Liu *et al*., 2015).

Sabin-Feldman dye test is the gold standard for detecting *T. gondii* antibodies in human. This method was first described in 1948 (Thomas, 1979). The test is able to detect specific antibodies to *T. gondii* at low levels. The principle of the test is based on complement-mediated cytolysis of antibody-coated live *T. gondii* parasites. The patient's serum is mixed with live tachyzoites, follow by methylene blue dye. In positive serum samples which contains anti-Toxoplasma antibodies, by the effect of complement factors, the parasites are unable to capture the methylene blue dye, and as a result they are observed without colour under the microscope. If there are no anti-Toxoplasma antibodies, the live parasites are stained and appear blue under the microscope. Since this test uses live tachyzoites thus it is only performed in some reference laboratories (Reiter-Owana *et al*., 1999).

To date, diagnosis of toxoplasmosis has been improved by the emergence of molecular techniques during the past two decades. Molecular approaches are more specific than serological detection methods since these techniques only make use of the parasite's nucleic acid. Polymerase chain reaction (PCR) is an ultimate molecular technique to detect *T. gondii* parasites. To amplify *T. gondii* DNA effectively, the 35-fold-repeated B1 gene is used as the PCR target (Costa *et al*., 2013; Liu *et al*., 2015). Burg *et al*. (1989) showed that the B1 gene is highly specific for *T. gondii* and is well conserved among all strains. This method has been used to detect *T. gondii* infection among pregnant women and immunocompromised people (Hohlfeld *et al*., 1994; Khalifa *et al*., 1994). Jones *et al.* (2000) reported that B1 gene is the most sensitive and successful gene in detection of *T. gondii* DNA in ocular fluids and retinal sections.

PCR has been successfully used to detect *T. gondii* DNA in brain tissue, amniotic fluid, cerebrospinal fluid (CSF), vitreous and aqueous fluids, bronchoalveolar lavage

(BAL) fluid and blood in patients with HIV/AIDS (Montoya, 2002; Remington *et al*., 2004; Tonkal *et al*., 2008). Although PCR is more sensitive, rapid and safer than conventional diagnostic procedures, it cannot distinguish between latent and acute *T. gondii* infection (Remington *et al*., 2004).

Additionally, several other molecular methods such as real-time PCR, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) also have been used for *T. gondii* diagnosis (Kasper *et al*., 2009; Lau *et al*., 2010; Meganathan *et al*., 2010; Wu *et al*., 2017; Yahaya, 1991). Besides, genotyping based molecular technologies also can serve as alternative approaches for diagnosis. Such methods are microsatellite analysis (MS), multilocus sequence typing (MLST), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA-PCR (RAPD-PCR) and high-resolution melting (HRM) (Liu *et al*., 2015).

### 2.7 Treatment of *T. gondii* infection

All drugs for treatment of *T. gondii* infections mainly target tachyzoites. In order to treat patients effectively, drugs such as sulfonamides and pyrimethamine are given to the patients for up to 6 months. Other drugs such as sulfadiazine, trisulfapyrimidine, quinine and anti-malarials can be used to treat *T. gondii* infections as well (Thirumudi *et al*., 2015). However, pyrimethamine is not suitable for pregnant women during the first trimester of pregnancy since this drug causes serious side effects for both mother and unborn child. Instead, alternative drug, spiramycin is given as this drug is non-toxic and does not cross the placenta. However, if early diagnostic test indicates that the foetus is infected by *T. gondii*, treatment with pyrimethamine will be considered (Dubey, 2008; Thomas, 1979).

Besides, some other drugs have been used to treat toxoplasmosis by specifically targeting certain proteins of *T. gondii*. For example, atovaquone was used to treat

toxoplasmosis, targeting cytochrome B protein in *T. gondii*. Cytochrome B is a mitochondrial protein and plays an important role as part of the electron transport chain. Atovaquone interferes with the electron transport pathway at the cytochrome B and subsequently blocks the production of adenosine triphosphate (ATP). As ATP plays a vital role to living cells, the parasites are not able to propagate within the cells (McFadden *et al*., 2000).

Clindamycin targets at apicoplast of *T. gondii* which involves in lipid metabolism. This drug destroys the apicoplast and prevents the synthesis of fatty acids. Fatty acids are the main components of parasitophorous vacuole (PV). Due to inability to form PV, the parasites are not able to invade host cells (Camps *et al.*, 2002).

## 2.8 Epidemiology

Toxoplasmosis is becoming a global health disease as 30% to 50% of world's human population is infected by *T. gondii* (asymptomatic and symptomatic), making it amongst the most successful human parasites (Flegr *et al.*, 2014). Figure 2.3 depicts the global status of *T. gondii* seroprevalence. The distribution of the infection depends on regional socioeconomic parameters and population habits (Pappas *et al*., 2009).

In the United States, toxoplasmosis-associated disease such as chorioretinitis occurs in an estimated 21,000 persons each year. Out of 21,000 persons, 4,800 people suffered from vision loss as the consequence of symptomatic chorioretinitis. Congenital toxoplasmosis is another issue of concern. An estimated 30% of women who acquire infection during their first and third trimesters of pregnancy, lead to 400 to 4,000 infants being born with congenital toxoplasmosis every year (Jones *et al*., 2014). In China, Cong *et al*. (2015) reported that there is high seroprevalence of *T. gondii* infection in pregnant woman in eastern China in contrast to other regions in China or East Asian countries with

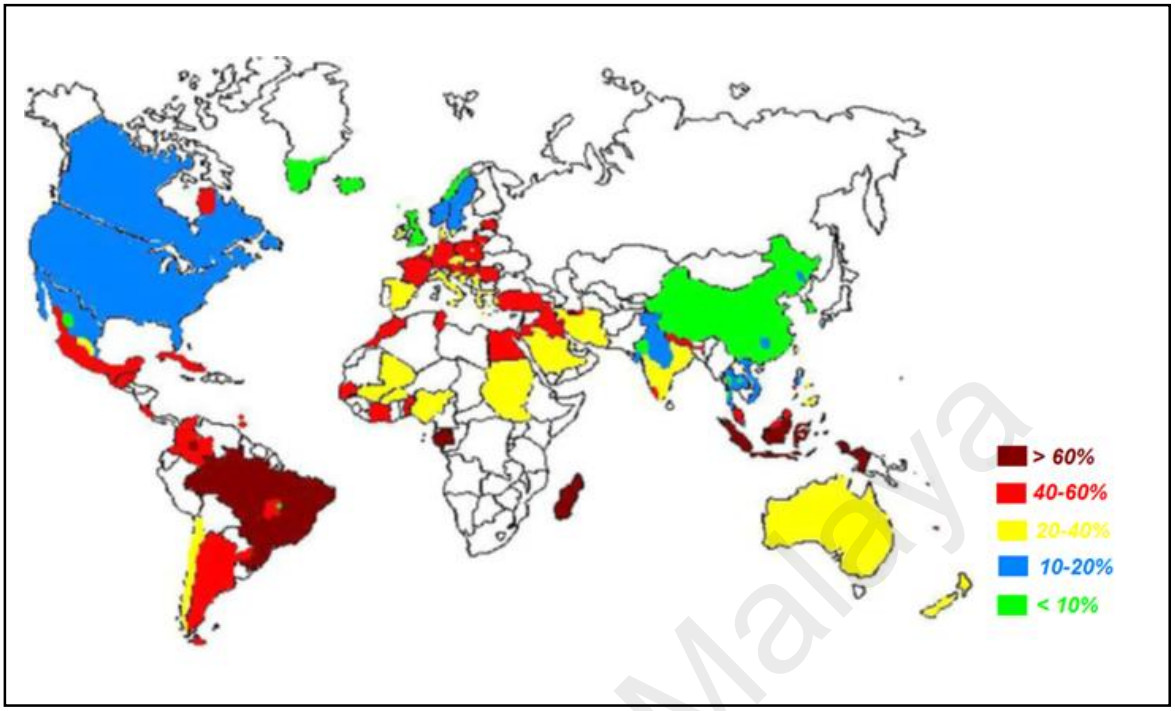



Figure 2.3: Global status of *T. gondii* seroprevalence (Pappas *et al*., 2009).

same climate conditions. In Germany, high prevalence among the populations is due to their improper eating habits such as consumption of freshly prepared raw minced meat (Wilking *et al*., 2016). In Brazil, overall toxoplasmosis in pregnant woman and vertical transmission was elevated among 487 pregnant women involved in the study. Of these, 63.03% of women had chronic toxoplasmosis infection, 5.33% of them had acute infection suspected by IgM antibodies detection in the peripheral blood, and 28% developed vertical transmission (da Silva *et al*., 2015).

In Southeast Asia, several reports on human and animal toxoplasmosis from different countries have been published such as Cambodia (Senya *et al*., 2003), Thailand (Chintana *et al*., 1998; Sukthana *et al*., 2000), Indonesia (Konishi *et al*., 2000; Kusumawati *et al*., 2011), Laos (Nissapatorn *et al*., 1991), Philippines (Eduardo, 1991; Kawashima *et al*., 2000), Singapore (Mohan *et al*., 1991; Singh *et al*., 1968), Malaysia (Chan *et al*., 2008; Ngui *et al*., 2011), Vietnam (Huong & Dubey, 2007) and Myanmar (Khine, 2005; Nyunt, 2005).

In Malaysia, human toxoplasmosis occurred among different races. Nissapatorn *et al*. (2004) reported that Malays have the highest prevalence followed by Indians and Chinese. Nimir *et al*. (2010) reported a hospital based study regarding the latent toxoplasmosis in patients with different malignancy and found highest toxoplasmosis among Malays (32%), followed by Chinese (17%) and Indians (1%). This could be explained by the fact that Malays like to keep cats as pets and thus they are easily exposed to contaminated cat faeces. Recently, Brandon-Mong *et al*., (2015) reported on the seroepidemiology of *Toxoplasma* infection in Malaysia and its risk association among people having close contact with animals. The study was conducted by collecting 312 blood samples from veterinarians, technicians, students and pet owners from veterinary clinics and hospitals in Klang Valley. Out of 312 samples, toxoplasmosis was prevalent in 62 participants, 33.3% (veterinary technician), 31.4% (pet owner), 18.4%

(veterinarian) and 14.9% (veterinary student). Additionally, the researchers found a high prevalence of toxoplasmosis in people who live in villages (33.3%) when compared to those living in the city (19.3%).

### 2.9 Ultrastructure of *T. gondii*

Several structures and secretory organelles such as surface antigens (SAGs), micronemes (MICs), rhoptries (ROPs) and dense granules (GRAs) of *T. gondii* are involved in host cell attachment, invasion, penetration, gliding, motility and propagation (Figure 2.4). SAGs of *T. gondii* play a pivotal role in host cell attachment during the invasion process. Surface antigen glycoprotein related sequences (SRSs) family was recognized as the main member in directing parasite attachment and improve survival rate in host cell. There are 160 members related to this family which are SAG1-like sequence branch and SAG2-like sequence branch (Cong *et al*., 2015). However, SAG1, SAG2A and SAG3 were the first members of SRSs family to be discovered (Burg *et al*., 1998; Cesbron-Delauw *et al*., 1989). Most of them are glycophosphatidylinositol (GPI)-anchored proteins. GPI-anchored proteins are membrane proteins containing a soluble protein attached with a conserved glycolipid at the C-terminus during posttranslational modification (Nagel & Boothroyd, 1989; Striepen *et al*., 1997).

ROPs and MICs are unique structures of apicomplexan parasite and play an important role in host cell invasion. The contents of ROPs and MICs are critical for biogenesis of PV that envelopes the parasite during invasion (Perkins, 1992; Saffer *et al*., 1992; Soldati *et al*., 2001). Apart from ROPs and MICs, GRAs proteins are secreted during and after host cell invasion. The secreted GRAs proteins remain either soluble in the lumen of the PV or they become associated with the parasitophorous vacuole membrane (PVM). According to Nam (2009), GRAs proteins are thought to modify the environment within the PV, so that the parasites survive and replicate continuously.

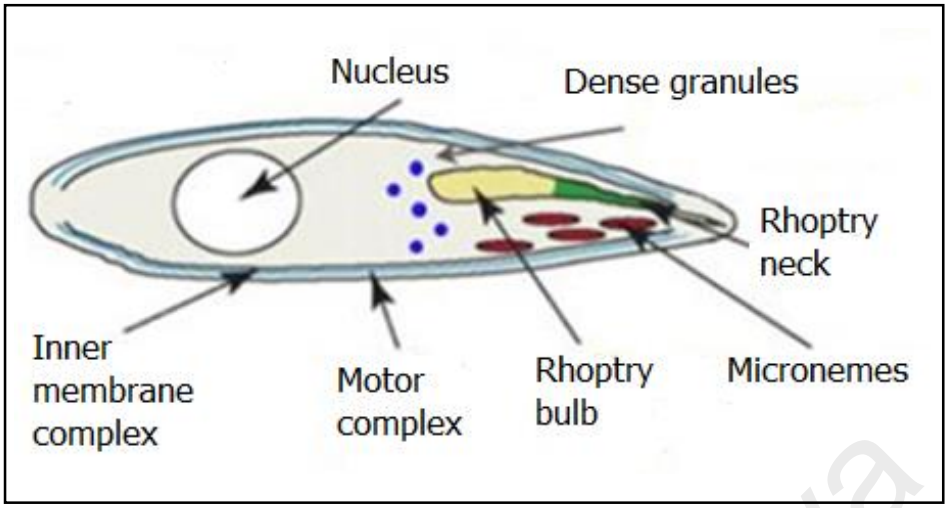



Figure 2.4: Ultrastructure of *T. gondii*. There are several important organelles (surface antigens, rhoptry bulb, rhoptry neck, micronemes and dense granules) involved in invasion, attachment, penetration, gliding and motility of parasite within host cells (Jones *et al.*, 2017).

## 2.10 Host cell invasion by *T. gondii*

For Apicomplexan, a prerequisite for invasion is attachment of the parasite to the host cell, as well as reorientation prior to entering the host cell. Cellular invasion by *T. gondii* is a rapid multiple step event (Hoff & Carruthers, 2002; Soldati-Favre, 2008). Figure 2.5 indicates host cell invasion by *T. gondii*. Approximately 3 mins are needed to accomplish the invasion process (Alexander *et al*., 2005).

As *T. gondii* enters, it moves by gliding motility and attaches to the host cell surface via SAGs. Following the host cell attachment, the parasite undergoes a series of reorientation processes and the conoid is extended to penetrate the host cell. During the apical contact, MICs and rhotry neck (RONs) contents are released. Secreted microneme-derived AMA-1 and RON form a moving junction (MJ) between host cell and parasite. The parasite reorientates and ROPs proteins are discharged from rhoptries into the host cytoplasm through MJ. The parasite is enclosed in the parasitophorous membrane. As parasite grows and multiplies within the cell, it forms a PVM by using part of the host cell membrane. Then, GRA contents which are associated to PVM are released at the final stage (Jones *et al*., 2017; Lecordier *et al*., 1999).

## 2.11 SAG1 and SAG2

Both SAG1 and SAG2 are members of SRSs family. SRSs proteins are expressed in a stage specific manner. SAG1, SAG2A, SAG2B, SAG3, SRS1, SRS2 and SRS3 are expressed on the tachyzoite surface only and play an important role in invasion and attachment. Meanwhile, SAG2C, SAG2D, SAG2X and SAG2Y are expressed at bradyzoite stage and these proteins are important for persistence of cysts in brain and tissues (Cong *et al*., 2013).

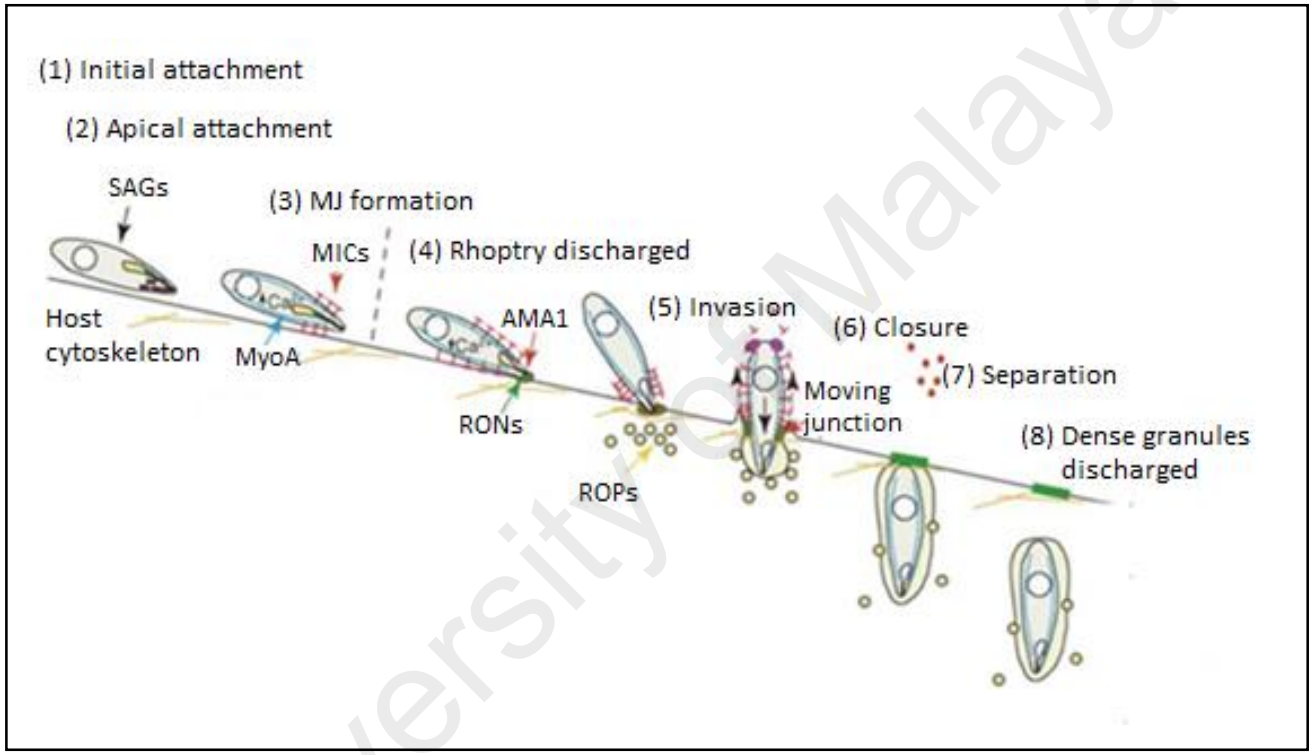



Figure 2.5: Host cell invasion by *T. gondii*. Host cell invasion by *T. gondii* is a multi-step process includes attachment to host cells (by SAGs), discharge by the micronemes (MICs), formation of moving junction (MJ) (by AMA1 and RONs), discharge of rhoptries (ROPs), invasion, internalisation of parasites into host cells, formation and sealing of PV, secretion of dense granules (GRAs) and intracellular parasite replication (Alexander *et al.*, 2005).

SAG1 was first identified by Handman *et al.* (1980) from *T. gondii* surface membrane antigens using a monoclonal antibody technique and subsequently isolated by mAB-affinity chromatography (Handman *et al*., 1980; Kasper *et al*., 1983). SAG1 anchors to the cell membrane through a GPI anchor. *sag1* is a single-copy gene that does not have introns in coding region or a TATA box in the promoter region.

SAG1 transcription is managed by five 27 bp repeat sequences 35-190 bp upstream of the first two transcription start sites. These repeat elements are conserved regions that are related with virulence of different *T. gondii* strains (Soldati & Boothroyd, 1995; Windeck & Gross, 1996). Basically, highly virulent strains have five intact 27 bp repeats located 70 bp upstream of the transcription initiation site, whereas less virulent strains have only four repeats (Wang & Yin, 2014). The precursor of SAG1 mRNA transcript contains hydrophobic regions in both the N- and C-terminal that are not present in most mature SAG1 proteins. The N-terminal hydrophobic region is believed to play a role in surface transport of the protein. However, both signal peptide and C-terminal hydrophobic region are cleaved and form a mature SAG1 protein (Johnson *et al*., 1983; Nam *et al.*, 1996). SAG1 has a main role in promoting invasion of tachyzoites into host cells and allowing tachyzoites binding to host receptors. To facilitate the attachment of SAG1 to host cells, SAG2 and SAG3 play an important role in facilitating rapid parasite invasion (Tomavo, 1996).

SAG2 is a major surface protein known as an attachment ligand to host. SAG2 has good antigenicity and immunogenicity properties. The recombinant SAG2 is effective in detecting the IgG antibody to *T. gondii* in patients with acute toxoplasmosis. Several studies have shown that C-terminal of SAG2 is related to host immune system especially in acute infection (Cong *et al*., 2013; Macedo *et al*., 2013).

*sag2* is selected as this gene requires a small amount of DNA for genetic analysis. The PCR is able to amplify this gene from as low as five parasites in the presence of host

tissues. Thus allowing *sag2* to be amplified directly from clinical samples (Howe *et al*., 1997). Besides, *sag2* is used as the genetic marker for genotyping *T. gondii* isolates as it contains multiple lineage-specific polymorphisms. The *T. gondii* isolates can be characterized as genotype I, II and III based on their digested patterns. Howe *et al*. (1997) reported that genotype II is the most prevalent in humans with toxoplasmosis.

SAG2 is a membrane protein with a signal peptide which is located at the N-terminal region. *sag2* gene used in this study contains no intron within the sequence.

**2.12 Protein-protein interactions (PPIs)**

PPIs are essential in all cellular processes including DNA replication, transcription and translation to signal transduction, cell cycle control and intermediary metabolism (Mukherjee *et al*., 2001). Proteins have to work together with other domains, ligands, receptors or with other complexes in order to function properly (Berggard *et al*., 2007; Rao *et al*., 2014). Mechanisms of interaction may involve numerous types of forces and bonds such as van der Waals forces, electrostatic bonds, hydrogen bonds, non-covalent bonds and hydrophobic interaction (Veselovsky *et al*., 2002).

Apicomplexa parasites cause some of the deadly parasitic diseases in humans and livestock globally, there is an urgent need for development a new drug that targets on apicomplexan invasion pathways. Blocking assay is an alternative method to study the interaction between *T. gondii* and its' binding host. Gaji *et al*. (2015) found that members of the family of calcium dependent protein kinases (CDPK's) are responsible for initiation of motility and parasite egress from host cell. They managed to prove that *T. gondii* calcium dependent protein kinases (TgCDPK3) control the rapid exit of parasite by phosphorylating motor protein *T. gondii* myosin A (TgMyoA) which is important for parasite's motility (Gaji *et al*., 2015). Apart from this, protein interaction study may provide insights into certain *T. gondii* organelles involved in other biological functions in

addition to host cell invasion. For example, Wang *et al.* (2014) reported that *T. gondii* micronemal adhesin (MIC2) plays a role in modulating host signal transduction and other biological processes in addition to binding host cells. They discovered MIC2 interacted with two mouse proteins, late endosomal/lysosomal adaptor, MAPK and mTOR activator 1 (LAMTOR1) and ribonuclease H2 subunit B (RNaseH2B) (Wang *et al*., 2014).

Generally, PPIs between two interacting partners can be detected by a pull down assay and molecular size-based chromatography. The pull down assay method utilize a tagged or labeled bait to form a specific affinity matrix that will allow binding and purification of a prey protein from a lysate or protein mixture. A pull down assay was used by Sloves *et al.* (2012) during their investigation of interaction between MIC and ROP proteins with *T. gondii* sortilin-like receptors. This method is slightly different from the commonly used Western blot in terms of protein detection. The sample is detected by using a labeled bait protein as the antibody probe. Western blot has been used by Tanaka *et al*. (2003) during their detection of bovine lactoferrin binding protein on *T. gondii*.

In addition to that, microarray is another approach that can be used to study PPIs. In 2001, Blader *et al.* (2001) used microarray to analyse the transcriptional profile during *T. gondii* infection on human foreskin fibroblasts (HFFs). The researchers managed to differentiate genes that were secreted by the parasite or host cell and those that were activated only in the presence of parasite invasion.

Alternatively, tandem affinity purification-mass spectroscopy (TAP-MS) is another approach for PPIs detection. TAP-MS is based on double tagging of the protein of interest on its chromosomal locus, followed by a two-step purification process and mass spectroscopic analysis. This method has been used by Hester *et al*. (2012) in identification of *T. gondii* antigens involved in active proliferation of the tachyzoites in mice brain.

In addition to that, imaging of PPIs in living subjects using reporter genes could also be employed as a tool to examine binding activity between interacting proteins. Such approaches include split luciferase complementation assay (Fujikawa *et al*., 2014), split synthetic renilla luciferase complementation assay (Massoud *et al.*, 2004), fluorescence resonance energy transfer (FRET) (Typas & Sourjik, 2015), bioluminescence resonance energy transfer (BRET) (Mo & Fu, 2016) and biomolecular fluorescence complementation (BiFC) (Miler *et al*., 2015; Schutze *et al*., 2009) assays.

Though some of these traditional tools, for example complementation assays are a choice to analyse large scale protein interaction networks, these methods were not used in this study due to their low sensitivity. Some of these methods also involve usage of different fluorescent tags and detection antibodies which are not affordable. Hence, in this project, Y2H assay was used to characterize and identify a large number of protein interactions at the same time.

### 2.13 Yeast two-hybrid (Y2H) system

Y2H is a useful method for detection of interacting partners from a complex and confirmation of interaction between two proteins (Fields & Song, 1989). The principle of Y2H assay was first described by Fields & Songs (1989) and has been subsequently modified and improved by Chien *et al*. (1991) and Gyuris e*t al*. (1993).

Figure 2.6 reveals the components involved in Y2H system. This assay involves two important domains, the DNA-binding domain (BD) and activation domain (AD). DNA-BD mediates binding of transcription factor to the gene promoter and the DNA-AD recruits transcriptional apparatus to the gene for mRNA production. If bait and prey protein interacts, the reporter gene will be expressed. RNA polymerase II will then catalyze the transcription of mRNA However, no gene expression will be seen if the two transcription factor modules are not in close contact (Coates & Hall, 2003).

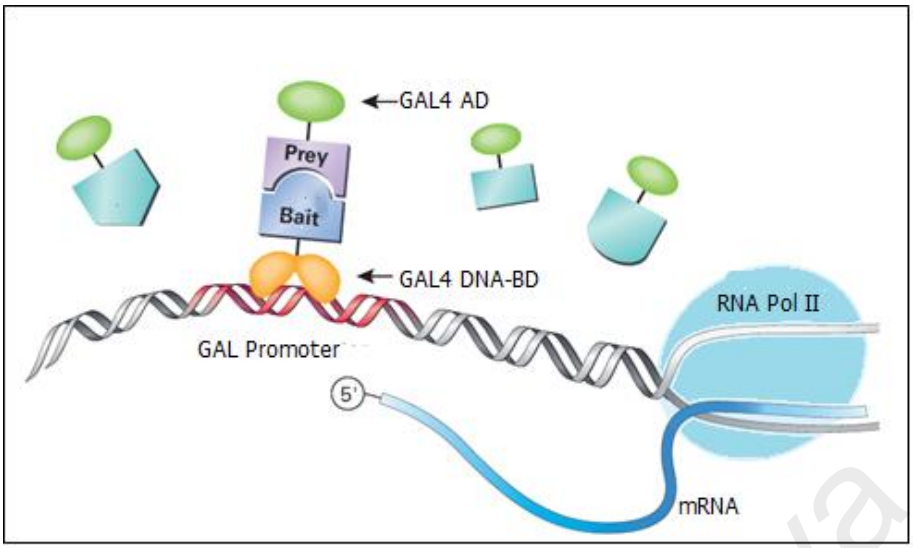



Figure 2.6: Components involved in Y2H system. Two important domains which is DNA-BD and AD are involved in Y2H system. When bait and prey protein interact, the reporter genes will be switched on (Clontech manual, USA).

Fields & Song (1989) tested this system on two yeast proteins, serine-threonine-specific protein kinase (SNF1) and glucose-repressible protein (SNF4) (Figure 2.7) (Celenze *et al*., 1989). SNF1 was fused to DNA-BD (DB) while SNF4 was fused to AD. When these two proteins interacted, DB and AD formed a functional transcriptional factor and subsequently induced reporter gene expression (Fields & Song, 1989).

Y2H system has been used by numerous researchers during the investigation between two interacting proteins. Miyake *et al*. (2017) used Y2H screening to identify relevant host factors (pyruvate kinase M2) that interacted with RNA-dependent RNA polymerase (RdRp) of influenza virus. They found that C-terminal region of pyruvate kinase M2 interacted with C-terminus of the viral protein subunit A (PA) at which the expression level of human host protein increased with influenza virus infection over time. This proves that this enzyme is essential for influenza virus multiplication (Miyake *et al*., 2017). Mignolet *et al.* (2010) reported that fumarase FumC of *Brucella abortus* is a specific partner for N-terminal 'sensing' domain of PdhS (old-pole-localized histidine kinase) using a Y2H screen. Ramalingam *et al*. (2015) identified galactinol-sucrose galactosyltransferase 2, calcineurin B-like (CBL) -interacting serine/threonine-protein kinase 25, and ABA responsive 17-like proteins are co-regulators for drought tolerance mechanism in chickpea (Ramalingam *et al*., 2015).

## 2.14 Normalized Mate and Plate library

A commercial cDNA human library (Normalized Mate & Plate Library) that was pre-transformed into Y187 yeast was used as prey during a Y2H experiment (Clontech, USA). cDNA human library was used since cDNA contains only exons and no introns. The cDNA human library was constructed by using switching mechanism at 5' end of RNA template (SMART) technology. This is a high-complexity cDNA library that is cloned into a GAL4 pGADT7-RecAB vector.

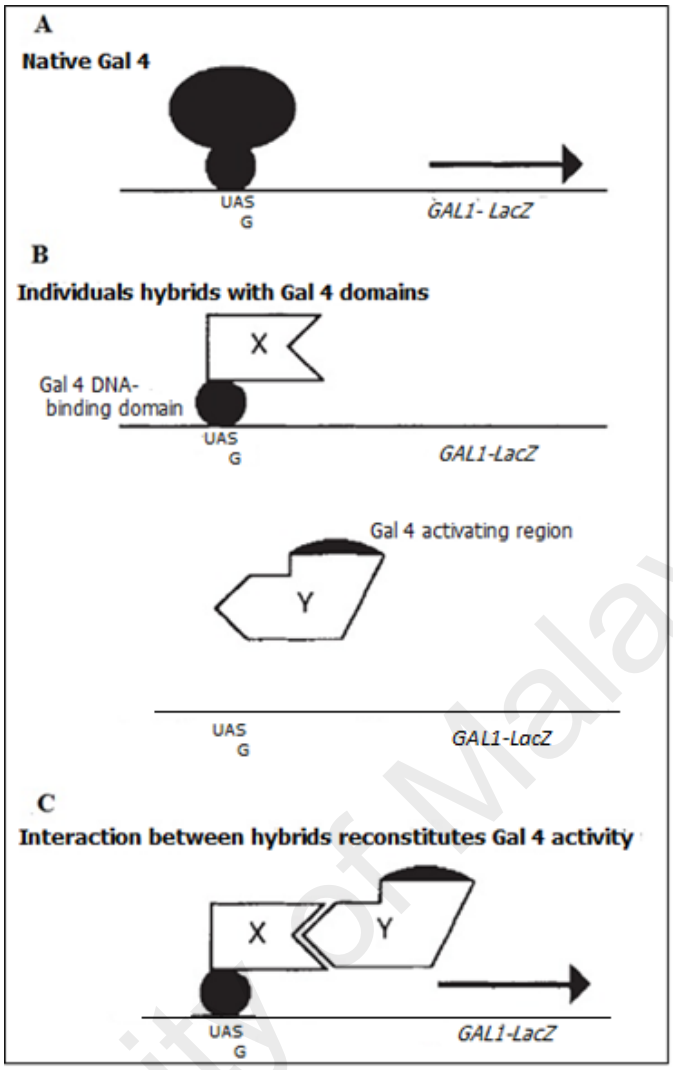



Figure 2.7: Model of transcription activation by reconsitutional of GAL4 activity. A) the native GAL4 protein containing both DNA-binding and activating domains induces GAL1-lacZ transcription. B) individual hybrids containing either the DNA binding domain (upper) or activating region (lower) are incapable of activating transcription. C) interaction between two proteins occurs when protein SNF1 (X) and SNF4 (Y) bring the GAL4 domains into close proximity and turn on transcription activity (Fields & Song, 1989).

SMART technology is a technique that helps incorporation of known sequences at both ends of cDNA during first strand synthesis without DNAse treatment and adaptor ligation. This commercial library significantly reduced the laborious work and time required to perform a Y2H screen since yeast transformation has already been done.

This Y2H library was constructed from human cDNAs that had been previously normalized to remove abundant cDNAs derived from high-copy-number mRNAs. The normalization process involves a duplex-specific nuclease (DSN) treatment and SMART technology and increases the representation of low copy number transcripts in the library. This greatly reduces the possibility of obtaining false positives during screening.

Finally, the efficiency of cDNA normalization was checked by Northern blot analysis (Clontech, USA). Since this universal human cDNA library was cloned into pGADT7-RecAB vector and pre-transformed into Y187 yeast strain, it is ready to mate with Y2HGold yeast.

### 2.15 Co-Immunoprecipitation study (Co-IP)

Once a protein and its binding partner have been identified using Y2H system, it is important to verify the interaction between them. Several methods can be used to verify the interacting partner such as affinity chromatography, protein microarrays, confocal microscopy, Co-IP, surface plasmon resonance and spectroscopic studies (Berggard *et al.*, 2007). All these methods are *in vitro* techniques (Rao *et al*., 2014).

Co-IP experiment is one of the most commonly used method. A bait protein is captured from the cell lysate by using the specific primary antibody. This antibody is then immobilized with protein A, protein G or protein A/G sepharose beads and washed several times before elution. The complex mixtures of bait, beads and antibody is usually eluted by elution buffer provided in the kit. Bound protein or bait protein is then analyzed by Western blot, dot blot or mass spectrometry methods. To avoid significant

background, Co-IP experiments need to be carried out in parallel with negative controls. This system employs a fluorescent AcGFP1 tag and an enzymatic ProLabel reporter for detection of interactions between proteins that are expressed in mammalian cells.

Figures 2.8 and 2.9 indicate the chemiluminescent Co-IP system and ProLabel screening assay which are conducted in this study.

## 2.16 Isothermal titration calorimetric (ITC)

It is imperative to study and measure binding strength between two interacting proteins. Most of the binding assays fail to measure the affinity between the interacted molecules. In fact, many experiments show only qualitaitive answers. This is a loss as binding reactions between molecules are not digital. However, the answer to whether two molecules interact with each other should always be quantitative, with a number that describes the affinity.

The binding strength is calculated by measurement of the affinity binding constant ($K_d$) which is obtained through an instrument. Several methods available are surface plasmon resonance (SPR), protein-fragment complementation assays (PCAs), single-molecule imaging (SMI), dynamics light scattering (DLS), dual polarization interferometry (DPI), microscale thermophoresis (MST) and isothermal titration calorimetry (ITC) (Dixon *et al*., 2016; Drescher *et al*., 2009; Hibino *et al*., 2009; Karim *et al*., 2007; Leavitt & Freire, 2001; Lorber *et al*., 2012; Liang, 2008; Seidel *et al*., 2013; Xing *et al*., 2016). These methods provide complete thermodynamics information ($K_a$, $\Delta H$ and $\Delta S$) for PPIs in order to calculate binding affinity between bait and prey proteins.

In the present study, ITC was selected as it is a robust method and has been used by numerous researchers from various fields of research since last decade. ITC can be used to study protein-DNA interactions, protein-RNA interactions and protein-small molecule interactions. ITC measures the heat change when two molecules interact.

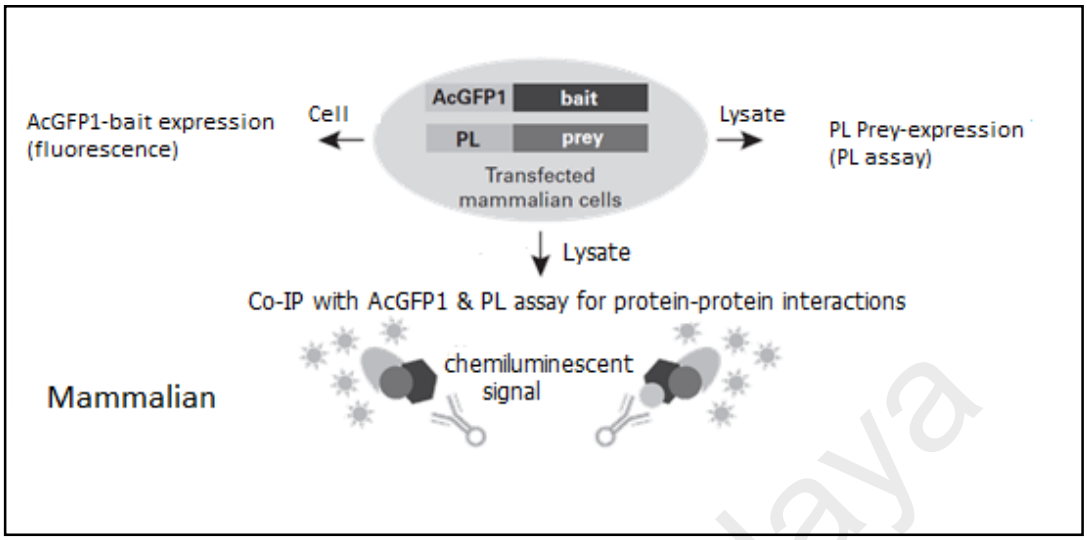



Figure 2.8: The chemiluminescent Co-IP system used in this study. Both bait and prey are co-transfected into mammalian cells. Cells are harvested after 72 h of transfection and cell lysate is prepared. The cell lysate is used in pProLabel enzymatic assay and chemiluminescent signal is measured by a luminometer (Clontech manual, USA).

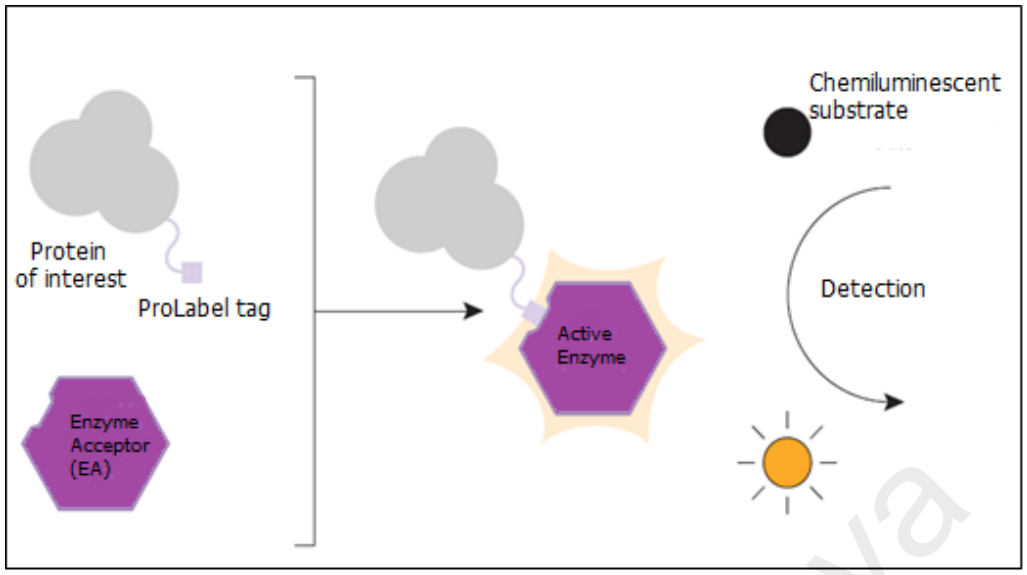



Figure 2.9: ProLabel screening assay in this study. An active enzyme is formed by combination of ProLabel tag together with enzyme acceptor that cleaves the chemiluminescent substrate. The resulting signal is measured by a luminometer (Clontech manual, USA).

Heat change is commonly due to heat released or absorbed during a molecular interaction event. Figure 2.10 indicates the schematic representation of the essential components of an ITC system. ITC regulates the heat changes by maintaining zero temperature difference between reference and sample cells as the binding partners are mixed. From Figure 2.10, 'reference cell' refers to water and 'sample cell' consists of binding partner protein while stirring syringe contains 'ligand'. ITC has been demonstrated to be a very powerful and sensitive method for assessing binding strength between two proteins as this is the only experimental approach to discern all relevant thermodynamic parameters with a single titration, including enthalpy (ΔH), entropy (ΔS), binding stoichiometry (N) and binding constant ($K_d$). For instance, the ligand or macro molecules involved in ITC measurement are label-free, avoiding the requirement of radio-labeled or fluorescence-labeled ligands or proteins (Leavitte & Freire, 2001; Pierce *et al*., 1999).

ITC is a popular technique for determining the affinity of interacting biomolecules. Strauss & Wuttke (2007) employed the ITC approach to examine binding affinity between RNA-dependent RNA polymerase (3D) with the viral proteins (VPg) during their investigation for proteins related with poliovirus replication. They found that VPg mutant R17A does not bind to 3D as no peak was observed from the thermodynamic graph (no heat released upon addition ligand to 3D). However, VPg mutant T15A binds to 3D with a weak binding affinity as a curve showing smaller peak was observed compared to wild-type VPg (Strauss & Wuttke, 2007).

Also, Bonsor *et al.* (2013) reported that protein CagF was required for translocation of *Helicobacter pylori* oncogenic protein CagA into host cells. The interaction between these two proteins was shown by using the ITC approach. The experiment was set up by using CagF as ligand and CagA in the sample cell. Titrations were performed with 15 injections of 2.49-4.49 µl aliquots at 25 ℃. The intervals between

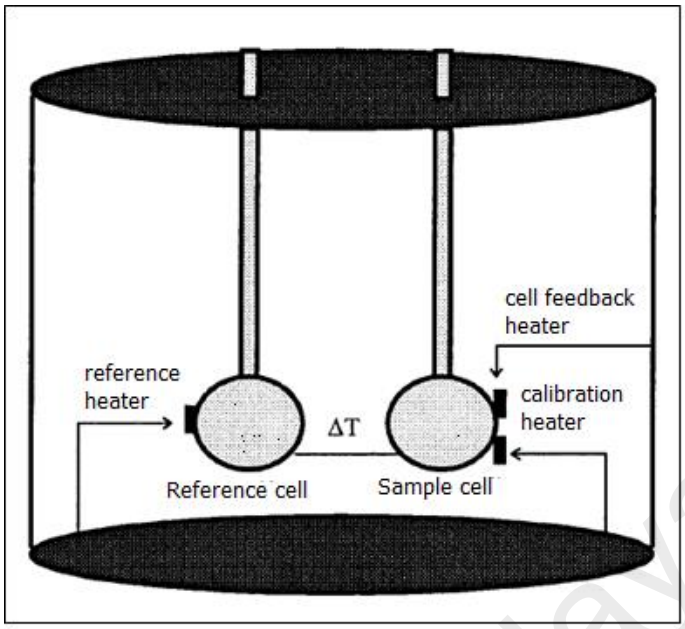



Figure 2.10: Schematic diagram of an ITC equipment. Two main components involved are ligand protein and sample cell. Reference cell refers to sterile distill water and is used to maintain equilibrium of heat transfer between ligand and sample cell (Pierce *et al*., 1999).

each of the injections were at least 210 s. Heats released was measured and a typical thermodynamic curve was obtained (Bonsor *et al.*, 2013).

Unlike SPR, ITC technique involves both interacting proteins in solution without immobilization, thereby potential artifacts arising from surface immobilization can be avoided. Additionally, there is no molecular weight limitations for the binding proteins. Also, full thermodynamic profiles for the interacting proteins can be obtained in a single experiment (Leavitt & Freire, 2001; Rajarathnam & Rosgen, 2014).

However, there are some limitations of this approach. This technique requires a high concentration of protein for both ligand or macromolecules to be measured which is in the range of μM to nM. Thus, a large amount of protein is required. Additionally, both ligand and macromolecules need to be diluted with the same buffer to avoid any other interfering components (Berggard *et al.*, 2007; Rajarathnam & Rosgen, 2014).

To avoid errorous measurements arising from false positive and false negative result, several parameters need to be considered such as both ligand and macromolecules need to be pure and dialyzed to remove the undesired salt. Mostly, source of the salt may arise from guanidinium lysis buffer, binding buffer and washing buffer during protein purification steps. On top of that, both the ligand and macromolecule should be degased in order to remove all bubbles as they will disturb the measurement of binding activity between two proteins (Malvern, UK).

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Overview

*T. gondii* was cultured and maintained in HS27 human foreskin cell lines. Tachyzoites DNA was extracted and purified from Blood and Tissue Extraction kit (Qiagen, Germany). There is no ethical approval required as live tachyzoites was not used in this project. *sag1* and *sag2* genes were used as bait to fish out the potential binding proteins from cDNA human library. PCR amplification of *T. gondii sag1* and *sag2* were performed by specific primers according to standard amplification procedures. Amplified products were then purified by using DNA Extraction kit (Qiagen, Germany) and cloned into pGEM-T vector (Promega, USA). Ligated mixtures were transformed into *E. coli* TOP10F' strain and incubated overnight on ampicillin plates at 37$^{o}$C. Colony PCR was performed on the next day by using M13 primers and positive colonies were inoculated. Plasmids were extracted and sent for sequencing. The recombinant plasmids (pGEM-T-SAG1 and pGEM-T-SAG2) were double digested with *Eco*RI/*Sal*I and *Nco*I/*Pst*I enzymes (New England Biolabs, USA), respectively and ligated into yeast vector, pGBKT7 (Clontech, USA) before transformed into *E. coli* TOP10F' strain. The constructs, pGBKT7-SAG1 and pGBKT7-SAG2 were transformed into Y2HGold yeast competent cells. Positive clones were sent for sequencing following colony PCR amplification. Recombinant constructs, pGBKT7-SAG1 and pGBKT7-SAG2 with 99% and 100% identity were applied as 'bait' and mated with cDNA human library (Clontech, USA) as the 'prey' during Y2H experiment. Mated culture was plated on double dropout medium (DDO) plate and incubated at 30$^{o}$C for 5 d. Positive clones were patched out on DDO with X-alpha-gal and aureobasidin A (DDO/X/A) plates and followed by quadruple dropout medium (QDO) plates and QDO with X-alpha-gal and aureobasidin A (QDO/X/A) plates. Following a series of selection procedures, colonies from the highest stringency plates (QDO/X/A) were subjected to PCR amplification by using vector insert

primers provided in the kit. Recombinant plasmids of successful amplified clones were extracted and sent for sequencing. Additionally, these clones were subjected to beta-galactosidase activity assay using Yeast β-galactosidase Assay kit (Thermo Scientific, USA).

To confirm the binding activity between SAG1 and SAG2 with their respective host proteins, chemiluninescent co-immunoprecipitation assay was performed. Luminescent signal from interacted protein was detected by using ProLabel Detection kit II (Clontech, USA). The relative luminescence units (RLU) of samples was measured by using Multimode Reader & Hydroflex microplate washer (Tecan, Switzerland).

Binding strength of the recombinant pRSET-A-SAG1 or recombinant pRSET-A-SAG2 with their binding partners were performed by isothermal titration calorimetry (ITC) method. Expression of recombinant proteins (pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF) was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Invitrogen Corp., USA). The expressed proteins were purified by ProBond$^{TM}$ purification system (Invitrogen Corp., USA). Purified proteins were dialyzed, concentrated and analysed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assay. The binding strength between pRSETA-SAG1 and pRSET-A-HLY or pRSET-A-SAG2 and pRSET-A-HZF proteins were measured by MicroCal Auto-iTC$_{200}$ machine. Overall procedures that involved throughout the whole study was listed as Figure 3.1.

## 3.2 Parasites

*T. gondii* RH strain used in this study was obtained from Tissue Culture Lab, Department of Parasitology, Faculty of Medicine, University Malaya. This parasite was cultured and maintained in HS27 human foreskin cell lines. The cells were allowed to grow until 90% confluent before harvesting.

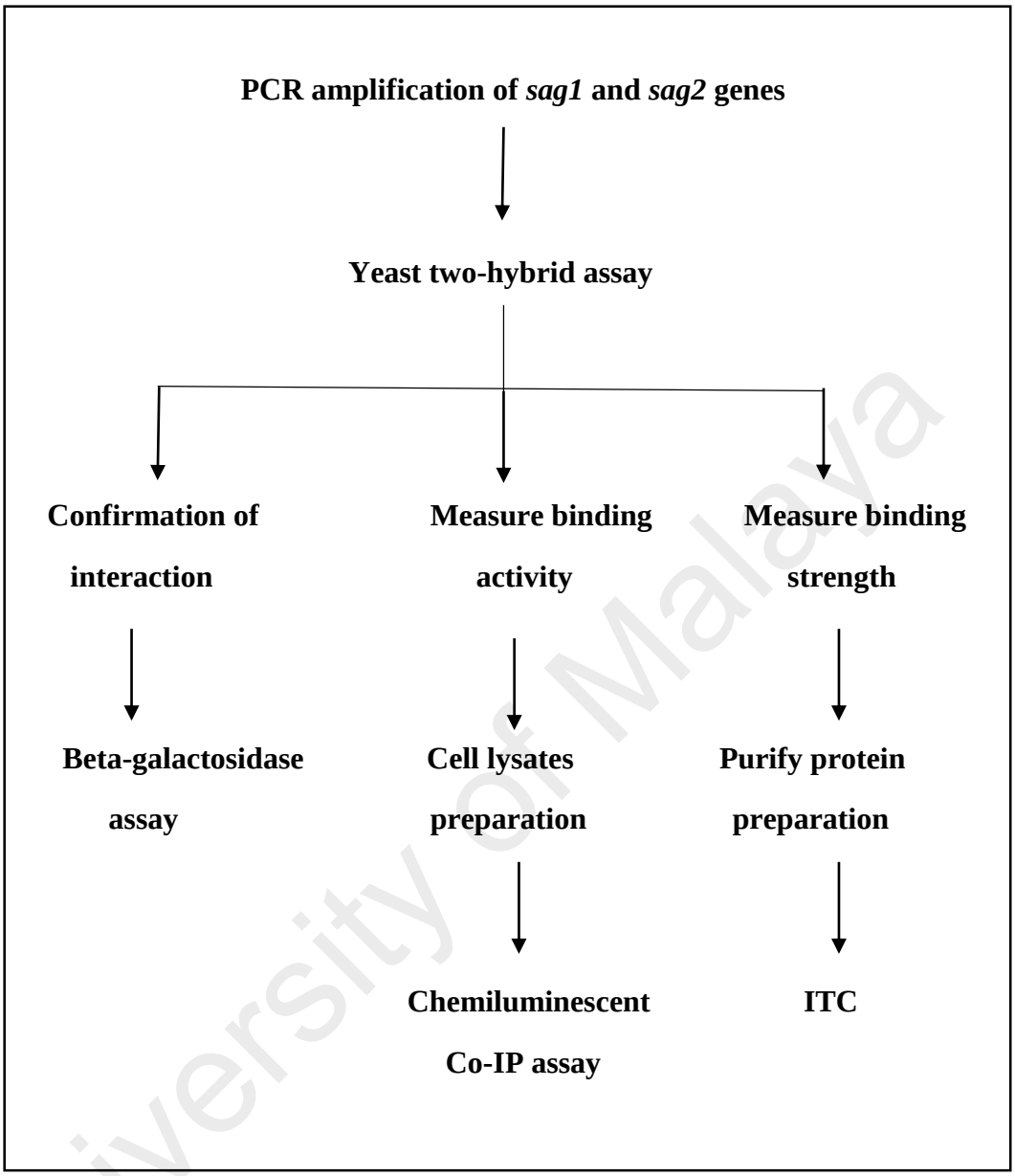



Figure 3.1: Flow chart of overall procedures involved in this study.

## 3.3 Oligonucleotide primers

All primers used in this study were synthesized by MyTACG Bioscience Enterprise, Malaysia and listed in Table 3.1. The primers were designed by Gene Runner software. Stock concentration for all primers was 100 µM and diluted to 10 µM as working solutions.

## 3.4 Genomic DNA extraction of tachyzoites

*T. gondii* genomic DNA was extracted by using DNeasy® Blood and Tissue kit (Qiagen, USA) following manufacturer's standard protocol. The DNA was extratced from dead tachyzoites in Class II Biological Safety Cabinet. *T. gondii* tachyzoites were briefly centrifuged to remove the supernatant. Pellet was re-suspended in 200 µl of PBS buffer followed by additional of 20 µl of proteinase K and 200 µl of Buffer AL. The mixture was incubated at 56$^{o}$C for 10 min. Two hundred µl of ethanol (96-100%) was added, mixed well and transferred to DNeasy Mini spin column placed in a 2-ml collection tube and centrifuged at 8,000 rpm for 1 min. Flow through and collection tube was discarded. Spin column was then put into a new 2-ml collection tube, 500 µl of Buffer AW1 was added and centrifuged at 8,000 rpm for 1 min. The flow through and collection tube was discarded. Five hundred µl of Buffer AW2 was added and centrifuged at 14,000 rpm for 3 min. The spin column was transferred to a new 1.5-ml microcentrifuge tube. DNA was eluted by adding 200 µl of Buffer AE and incubated for 1 min. Spin column was then centrifuged for 1 min at 8,000 rpm to elute the DNA.

Table 3.1: Primers used in this study.

| Name | | Sequence (5'-3') | Size (bp) |
|---|---|---|---|
| SAG1-pRSET-A | F | AAGGATCCTTCACTCTCAAGTGCCC | 735 |
| | R | GAGGATCCTTAGGCAAAAATGGAAACGTG | |
| SAG1-pGBKT7 | F | AAGAATTCTTCACTCTCAAGTGCCC | 735 |
| | R | GAGTCGACTTAGGCAAAAATGGAAACGTG | |
| SAG1-pAcGFP1 | F | AAGTCGACTTCACTCTCAAGTGCCC | 735 |
| | R | GAAAGCTTTTAGGCAAAAATGGAAACGTG | |
| SAG2-pRSET-A | F | AAGGATCCTCCACCACCGAGACGCCA | 483 |
| | R | GCGGATCCTTACACAAACGTGATC | |
| SAG2-pGBKT7 | F | GACCATGGCGTCCACCACCGGACGCCA | 483 |
| | R | GCCTGCAGTTACACAAACGTGATC | |
| SAG2-pAcGFP1 | F | AAGGATCCTCCACCACCGAGACGCCA | 483 |
| | R | GCGGATCCTTACACAAACGTGATC | |
| HLY-pProLabel | F | CCGTCGACATGAAGCATTCAAAGAAGAC | 564 |
| | R | CCGGATCCTCAATTTCCTCGCAGCTTTTTTTTTCTC | |
| HLY-pRSET-A | F | CCGGATCCATGAAGCATTCAAAGAAGAC | 564 |
| | R | CCGGATCCTCAATTTCCTCGCAGCTTTTTTTTTCTC | |
| HZY-pProLabel | F | GCGTCGACATGGCTCAAGAAACTAATCAC | 627 |
| | R | GCGGATCCTCAAATCTTTTGGATCTTTTCACCAACAACTACTGG | |
| HZF-pRSET-A | F | GCGGATCCATGGCTCAAGAAACTAATCAC | 627 |
| | R | GCGGATCCTCAAATCTTTTGGATCTTTTCACCAACAACTACTGG | |
| ADLD | F | CTATTCGATGATGAAGATACCCCACCAAACCC | 221 |
| | R | GTGAACTTGCGGGGTTTTTCAGTATCTACGATT | |
| M13 | F | GTTTTCCCAGTCACGAC | ~200 |
| | R | AGCGGATAACAATTTCACACAGGA | |
| T7 Promoter | | TAATACGACTCACTATAGGG | |

Note: GGATCC (*Bam*HI); GAATTC (*Eco*RI); GTCGAC (*Sal*I); AAGCTT (*Hind*III); CCATGG(*Nco*I); CTGCAG (*Pst*I)

## 3.5 Yeast two-hybrid assay (Y2H)

### 3.5.1 Cloning of SAG1 and SAG2 fragments into pGEM-T vector

#### 3.5.1.1 PCR amplification of *sag1* and *sag2* genes

Extracted DNA was used as DNA template to amplify *sag1* and *sag2* since there is no intron in between the fragment. Primers (SAG1-pGBKT7 and SAG2-pGBKT7) used for amplification of *sag1* and *sag2* were listed as in Table 3.1. Restriction sites were inserted into these primers and indicated by underlined. PCR reaction mixtures (25 µl) contained 2 µl of DNA, 0.4 µM of each primer, and 5 µl of 5X PCR buffer, 200 µM of dNTP mix, 1.5 mM of $MgCl_2$ and 1U of *Taq* DNA polymerase (Promega, USA) PCR premix. PCR was conducted as follows: denaturation at 94 ℃ for 4 min, followed by 30 cycles of 94 ℃ for 45 s, 55 ℃ for 60 s (*sag1*), or 60 ℃ for 45 s (*sag2*), 72 ℃ for 60 s, and a final extension of 72 ℃ for 10 min.

#### 3.5.1.2 Agarose gel electrophoresis

DNA fragments were separated by using 1% agarose gel electrophoresis. Agarose powder (Promega, USA) was dissolved in 1X TAE buffer and stained with SYBR Safe DNA gel stain (Invitrogen Corp., USA). Samples were loaded on the solidified gel and electrophoresed at 100 mA for 30 min using Power Pac Basic Power Supply (BioRad Laboratories, USA). Molecular weight marker, 100 bp or 1 kb DNA ladder (Thermo Scientific, USA) was used to analyse the samples. Gel images were taken by using Gel Doc$^{TM}$ XR+ imaging system (BioRad Laboratories, USA).

#### 3.5.1.3 Purification of PCR products

PCR products of SAG1 and SAG2 were purified by QIAquick PCR Purification kit (Qiagen, Germany) according to manufacturer's protocol. Five volumes of Buffer PB was added to 1 volume of PCR product. The mixture was added to a QIAquick column

that was placed on a 2-ml collection tube and centrifuged at 13,000 rpm for 1 min. Flow through was discarded and 750 µl of Buffer PE was added and centrifuged at the same condition. Flow through was discarded and spun once again at the same condition. The column was placed into a clean 1.5-ml microcentrifuge tube and 30 µl of Buffer EB was applied onto the center of membrane. This column was spun at 13,000 rpm for 1 min for DNA elution. To analyse the purified DNA on gel, 1 volume of 5X loading dye was mixed with 5 volumes of purified DNA before loading onto gel.

### 3.5.1.4 Ligation of SAG1 and SAG2 fragments into pGEM-T vector

Purified SAG1 and SAG2 fragments were ligated into pGEM-T vector according to manufacturer's standard protocol. Ligation mixture consisted of the following ingredients:

| | |
|---|---|
| Purified fragment | 3 ul |
| 2X buffer solution | 5 ul |
| pGEM-T vector | 1 ul |
| T4 DNA ligase | 1 µl |

Cloning reaction was performed in a final volume of 10 µl solution and incubated overnight at 4°C before transformation into *E. coli* TOP10F' competent cells.

### 3.5.1.5 Preparation of *E. coli* TOP10F' competent cells

Competent cells were prepared by using calcium chloride ($CaCl_2$) method. TOP10F' *E. coli* competent cells were revised from stock (Invitrogen Corp., USA) and re-streaked on plate containing 15 µg/ml tetracycline antibiotic and incubated overnight at 37°C. A single colony was selected and inoculated into 5 ml LB broth and incubated overnight with shaking at 37°C. Forty µl of overnight culture was inoculated into 40 ml LB broth and incubated with shaking at 200 rpm at 37°C until $OD_{600}$ reached at 0.4.

Meanwhile, 40 ml of 0.1 M calcium chloride ($CaCl_2$) was prepared and filtered by 0.02 µM pore size filter. Culture was centrifuged at 5,000 rpm at 4°C for 5 min and supernatant was discarded. Pellet was re-suspended with 20 ml 0.1 M ice-cold $CaCl_2$ solution and incubated in ice for 3 h. Then, the culture was centrifuged at 5,000 rpm at 4°C for 5 min. Supernatant was discarded and pellet was re-suspended with a solution containing 3.4 ml 0.1 M ice-cold $CaCl_2$ solution and 0.6 ml glycerol. Cells were then aliquoted out into 40 1.5-ml microcentrifuge tubes and stored at -80°C.

#### 3.5.1.6 Transformation of ligation mix into *E. coli* TOP10F' competent cells

Ligation mixture was mixed gently and incubated overnight at 4°C. Two µl of reaction mixture was added to *E. coli* TOP10F' competent cells after thawing and incubated on ice for 30 min. The cells were then heat-shock at 42°C without shaking for 1 min and incubated on ice for another 2 min. One ml of LB broth was added and incubated at 37°C shaking incubator. The tube was capped tightly and shake horizontally at 200 rpm for 1 h. The reaction mixture was spun at 13,000 rpm for 1 min. Supernatant was discarded and the pellet was re-suspended with 100 µl of LB broth and spread on a pre-warmed LB plate containing 100 mg/ml ampicillin. The plate was incubated overnight at 37°C.

#### 3.5.1.7 Colony PCR for screening transformants

Colonies on the transformation plates were picked by sterile toothpick and dipped into the sample reaction PCR tubes, while no colony was chosen for negative control tube. The PCR was carried out in a final volume of 25 µl containing 5 µl of 1X buffer, 0.4 µM of each primer (specific for *sag1* or *sag2*), 200 µM of dNTPs, 2 mM of $MgCl_2$ and 1U of *Taq* DNA polymerase (Promega, USA). Cycling parameters of each PCR consisted of denaturation at 95℃ for 10 min, followed by 30 cycles at 95℃ for 30 s,

55 ℃ for 1 min, 72 ℃ for 2 min, and a final extension at 72 ℃ for 5 min. PCR product was resolved on a 1% (w/v) agarose gel stained with SYBR Safe DNA gel stain (Invitrogen Corp., USA) and visualized using Gel Doc$^{TM}$ XR+ imaging system (BioRad Laboratories, USA).

#### 3.5.1.8 Extraction of plasmid DNA

A single colony from positive clone was inoculated into a 10 ml LB broth containing 100 mg/ml ampicillin and incubated overnight at 37$^{o}$C in a shaking incubator. Overnight culture was centrifuged at 13,000 rpm for 3 min at room temperature. QIAprep Spin Miniprep kit (Qiagen, Germany) was used to extract plasmid. The pelleted bacterial cells were re-suspended with 250 µl of Buffer P1 and transferred into a 1.5-ml microcentrifuge tube. Two hundred fifty µl of Buffer P2 was added to tube and mixed thoroughly by inverting the tube four to six times until solution became clear. Then, 350 µl of Buffer N3 was added to tube and mixed thoroughly by inverting the tube four to six times until solution became clear. Tube was centrifuged at 13,000 rpm for 10 min. Supernatant was applied to spin column and centrifuged for 1 min at 13,000 rpm. Flow-through was discarded, 500 µl of Buffer PB was added into spin column and centrifuged at 13,000 rpm for 1 min. Flow-through was then discarded, 750 µl of Buffer PE was added into spin column and centrifuged at 13,000 rpm for 1 min. The spin column was centrifuged one more time to remove residual wash buffer. The column was placed in a clean 1.5-ml microcentrifuge tube and 50 µl of Buffer EB was added to the center of column. This spin column was incubated for 1 min and centrifuged for 1 min at 13,000 rpm for plasmid DNA elution.

#### 3.5.1.9 DNA sequencing

Extracted plasmids were sent to MyTACG Bioscience Enterprise, Malaysia for sequencing by using M13 forward and reverse primers. Data was BLAST against the database available from GenBank to confirm their identities.

#### 3.5.1.10 Maintenance of recombinant clones

Recombinant clones with 100% similarity to the identity from GenBank were grown in LB medium with additional of 100 µg/ml ampicillin overnight at 37°C, 200 rpm. Five hundred µl of overnight culture was mixed together with 500 µl of 50% glycerol and kept at -80°C. To maintain the viability of these clones, the cultures were re-streaked regularly on LB agar plates with 100 µg/ml ampicillin.

### 3.5.2 Cloning of SAG1 and SAG2 fragments into pGBKT7 vector

#### 3.5.2.1 Digestion of pGEM-T-SAG1 and pGEM-T-SAG2 fragments

To clone into pGBKT7 vector, recombinant pGEM-T-SAG1 was double digested by *Eco*RI/*Sal*I restriction enzyme. Digestion mixture was carried out in a total volume of 40 µl of mixture, consisted of 25 µl of pGEM-T-SAG1 vector, 1 µl of each of *Eco*RI and *Sal*I restriction enzyme, 4 µl of Buffer 3, and 9 µl of $ddH_2O$. Digestion mixture was incubated at 37°C for 6 h. Empty pGBKT7 vector was digested concurrently in a same constitution with digestion of pGEM-T-SAG1. For *T. gondii* tachyzoite DNA containing SAG2, digestion was carried out in a total volume of 40 µl of mixture, consisted of 25 µl of pGEM-T-SAG2 vector, 1 µl of each *Nco*I and *Pst*I restriction enzyme, 4 µl of Buffer 3 and 9 µl of $ddH_2O$. The digestion mixture was incubated at 37°C for 6 h. Empty pGBKT7 vector was digested concurrently in a same constitution with digestion of pGEM-T-SAG2.

### 3.5.2.2 Gel purification of digested fragments

Digested DNA fragments with insert and pGBKT7 vector were tested on 1% agarose gel to confirm their intensity. DNA fragments were excised from the agarose gel with a clean and sharp scalpel. Gel purification was performed by using QIAquick Gel Extraction kit (Qiagen, Germany). Gel slices were weight in a colourless tube. Three volumes of Buffer QG were added to 1 volume gel (100 mg~100 µl). Tubes were incubated at 50ºC for 10 min with vortexing every 2 to 3 min. After gel slices were dissolved completely, colour of the mixture was yellow. One gel volume of isopropanol was added to sample and mixed. This sample was then applied to the QIAquick column that placed in a 2-ml collection tube and centrifuged at 13,000 rpm for 1 min. Supernatant was discarded, 500 µl of Buffer QG was added to QIAquick column and centrifuged at 13,000 rpm for 1 min. Flow-through was discarded, 750 µl of Buffer PE was added to QIAquick column and centrifuged at 13,000 rpm for 1 min. Flow-through was discarded. The sample was centrifuged once more time at 13,000 rpm for 1 min to remove residual wash buffer. QIAquick column was placed into a clean 1.5-ml microcentrifuge tube. For DNA elution, 30 µl of Buffer EB was added to the centre of membrane and incubated at room temperature for 1 min. The tube was then centrifuged at 13,000 rpm for 1 min. Purified DNA was analyzed on 1% gel.

### 3.5.2.3 Ligation of digested pGEM-T-SAG1 and pGEM-T-SAG2 into pGBKT7 vector

Purified DNA fragments were ligated to purified pGBKT7 vector. For SAG1 and SAG2 fragments of *T. gondii* tachyzoite DNA, the ligation conditions were same. These ligation mixtures were consisted of 1 µl of 10X ligation Buffer, 1 µl of T4 DNA ligase (New England Biolabs, USA), 2 µl of pGBKT7 vector and 6 µl of insert with SAG1 or SAG2 DNA. The ligation mixture was incubated overnight in at 4ºC.

#### 3.5.2.4 Transformation of ligation mixture into *E. coli* TOP10F' competent cells

Overnight ligation mixtures were transformed into *E. coli* TOP10F' competent cells according to the methods described in section 3.5.1.6.

#### 3.5.2.5 Colony PCR for screening of transformants

To screen positive clones with correct orientation for pGBKT7-SAG1 and pGBKT7-SAG2, colony PCR was performed by using T7 promoter primer and insert's reverse primer according to section 3.5.1.7.

#### 3.5.2.6 Extraction of plasmid DNA

Following the colony PCR, positive clones were inoculated and incubated overnight at 37°C. Recombinant plasmids with insert were extracted according to section 3.5.1.8 and sent for sequencing (section 3.5.1.9). Clones of pGBKT7-SAG1 and pGBKT7-SAG2 with correct orientation were maintained and kept as glycerol stocks at -80°C according to section 3.5.1.10.

### 3.5.3 Transformation into Y2HGold yeast competent cells

#### 3.5.3.1 Preparation of yeast competent cells

Y2HGold yeast was re-streaked on YPDA plate and incubated at 30°C for 5 d. A single colony was inoculated into 3 ml YPDA broth in a sterile 15-ml Falcon conical tube and incubated at 30°C with shaking at 250 rpm for 8 to 12 h. Five µl of culture was then transferred to 50 ml of YPDA broth and incubated with shaking at 250 rpm until $OD_{600}$ reached to 0.15-0.30. The cells were centrifuged at 1,500 rpm for 5 min at room temperature. Supernatant was discarded and re-suspended with 100 ml of fresh YPDA broth. Cells were incubated at 30°C until $OD_{600}$ reached to 0.4-0.5. Culture was divided into two 50-ml Falcon conical tubes. Cells were centrifuged at 1,500 rpm for 5 min at

room temperature and supernatant was discarded. The pellet was re-suspended with 30 ml of sterile dd$H_2O$. Again, cells were centrifuged at 1,500 rpm for 5 min at room temperature and supernatant was discarded. The pellet was re-suspended with 1.5 ml of 1.1X Tris-acetate with lithium acetate (TE/LiAc) solution. The cell suspensions were transferred into two 1.5-ml microcentrifuge tubes and centrifuged at high speed (14,000 rpm) for 15 s. Supernatant was discarded and the pellet was re-suspended with 600 µl of 1.1X TE/LiAc solution. The cells were ready to be transformed with plasmid DNA.

### 3.5.3.2 Transformation of pGBKT7-SAG1 and pGBKT7-SAG2 into yeast

Bait plasmids were constructed by cloning SAG1 and SAG2 fragments into pGBKT7 plasmids and transformed into Y2HGold yeast strain. The concentration of recombinant pGBKT7-SAG1 and pGBKT7-SAG2 were measured by NanoDrop (Thermo Scientific, USA) prior to transformation into yeast cells. One hundred ng of plasmid DNA and 5 µl of Yeastmaker Carrier DNA (Clontech, USA) were added into 50 µl of yeast competent cells. Five hundred µl of PEG/LiAC was added and mixed gently. The mixture was incubated at 30°C for 30 min. Cells were gently mixed every 10 min during the incubation period. Twenty µl of DMSO was added into mixture. The tube was incubated in a 42°C water bath for 15 min. While incubating, cells were gently mixed for every 5 min. Cells were centrifuged at high speed for 15 s. Supernatant was then removed and the pellet was re-suspended with 1 ml YPD Plus Medium (Clontech, USA). Cells were centrifuged at high speed (14,000 rpm) for 15 s and supernatant was discarded. The pellet was re-suspended with 1 ml 0.9% (w/v) NaCl solution and spread on SD selection plates. For those DNA transformed into pGBKT7 plasmid, the culture were spread on SD/-Trp plates, while for pGADT7 plasmid, the culture were spread on SD/-Leu plates and incubated at 30°C for 5 d.

### 3.5.3.3 Screening of transformants by colony PCR

To screen positive clones with correct inserts for pGBKT7-SAG1 and pGBKT7-SAG2, colony PCR was performed by using T7 promoter primer and insert's reverse primer. The protocol was described in section 3.5.1.7.

### 3.5.3.4 Extraction of yeast plasmids

Yeast plasmid was isolated by using Easy Yeast Plasmid Isolation kit (Clontech, USA). Before using the kit, 1 ml of Y1 Resuspension Buffer was added to the vial containing RNase A and mixed by vortexing. This solution was ready to use and stored at 4$^{o}$C.

A single yeast colony growing on selective medium was picked by using sterile toothpick and re-suspended in a 1.5-ml microcentrifuge tube containing 500 µl of supplied 10 mM EDTA. Cells were centrifuged at 13,000 rpm for 1 min. Supernatant was discarded and each cell pellet was re-suspended in 200 µl of ZYM Buffer. Meanwhile, 20 µl of zymolyase suspension was added to cell pellet the vial was inverted two to three times to ensure a uniform suspension of zymolyase. Tube was gently mixed by vortexing and incubated at 30$^{o}$C for 1 h. Spheroplasts were then centrifuged at 3,000 rpm for 10 min and supernatant was discarded. The spheroplasts were re-suspended with 250 µl of Y1 Buffer/RNase A solution. Two hundred fifty µl of Y2 Lysis Buffer was added. The tube was mixed gently by inverting six to eight times and incubated at room temperature for 5 min. Then, 300 µl of Y3 Neutralization Buffer was added and mixed gently. The lysate was cleared by centrifuging at 13,000 rpm for 5 min at room temperature and supernatant was transferred to a clean microcentrifuge tube. This centrifugation was repeated once more. Supernatant was loaded into a Yeast Plasmid Spin Column with a 2-ml collection tube. The tube was centrifuged at 13,000 rpm for 1 min and column flow-through was discarded. Spin column was placed back into 2-ml collection tube and 450

µl of Y4 Wash Buffer was added. The tube was centrifuged at 13,000 rpm for 3 min. Column flow-through was discarded and centrifuged once more to completely remove any residual wash buffer. Spin column was placed in a clean 1.5-ml microcentrifuge tube and 50 µl of YE Elution Buffer was added. The tube was incubated at room temperature for 1 min and centrifuged at 13,000 rpm for 1 min to elute plasmid DNA.

The extracted pGBKT7-SAG1 and pGBKT7-SAG2 plasmids in yeast were transformed into *E. coli* TOP10F' cells according to method as described in section 3.5.1.6. Colony PCR was then performed by using insert's specific primer according to method as described in section 3.5.1.7. Recombinant plasmid of pGBKT7-SAG1 and pGBKT7-SAG2 in *E. coli* were extracted and sent for sequencing (as mentioned in section 3.5.1.8 and 3.5.1.9). Only clones with correct orientation were used as bait in the following Y2H assay.

#### 3.5.3.5 Maintenance of yeast recombinant clones

Yeast cells with positive pGBKT7-SAG1 and pGBKT7-SAG2 constructs were grown overnight in YPDA broth at 30$^{o}$C. Five hundred µl of overnight culture were mixed with 500 µl of 50% glycerol. The mixtures were kept as glycerol stocks at -80$^{o}$C.

### 3.5.4 Yeast two-hybrid screening

#### 3.5.4.1 Autoactivation test

An autoactivation test was carried out to confirm the bait (pGBKT7-SAG1) or (pGBKT7-SAG2) did not autonomously activate reporter genes in Y2HGold in the absence of prey protein. One hundred ng of (pGBKT7-SAG1) or (pGBKT7-SAG2) was transformed into Y2HGold according to method as described in section 3.5.3. One hundred µl of transformation mixture were spread on SD/-Trp, SD/-Trp/X and single

dropout medium without tryptophan but with X-alpha-gal and aureaobasidin A (SD/-Trp/X/A) plates. The plates were incubated at 30°C for 5 d.

### 3.5.4.2 Toxicity test

To confirm bait protein was not toxic when expressed in yeast, the bait plasmid need to be tested for toxicity. One hundred ng of pGBKT7 empty vector, (pGBKT7-SAG1) or (pGBKT7-SAG2) was transformed into Y2HGold according to section 3.5.3. One hundred µl of transformation mixture were spread on SD/-Trp plates. The plates were incubated at 30°C for 5 d. Only the colonies containing pGBKT7-SAG1 or pGBKT7-SAG2 with the similar size of the colonies containing empty pGBKT7 vector were selected.

### 3.5.4.3 Mating between bait and human cDNA library

A concentrated overnight culture of bait strain was prepared by inoculation of a single colony of Y2HGold [pGBKT7-SAG1] or Y2HGold [pGBKT7-SAG2] into 50 ml SD/-Trp broth. The culture was incubated by shaking at 250 rpm at 30°C until $OD_{600}$ reached 0.8. Cells were then centrifuged at 2,000 rpm for 5 min. Supernatant was discarded and the pellet was re-suspended with 5 ml SD/-Trp broth.

This bait was ready to mate with Normalized human cDNA library that was pre-transformed into Y187. The library was thawed in water bath at room temperature and combined with 5 ml of bait strain in a 2-L flask. Forty five ml of 2X YPDA broth containing 50 µg/ml kanamycin was added to the flask. Meanwhile, the remaining cells from library vial were rinsed twice with 1 ml of 2X YPDA broth and added to flask. The culture was then incubated by gently shaking (50 rpm) at 30°C for 24 h.

The overnight culture was centrifuged at 2,000 rpm for 10 min while the flask was rinsed twice with 50 ml of 0.5X YPDA broth (50 µg/ml kanamycin). Rinses were

combined and used to re-suspend the pelleted cells. The cells were centrifuged at 2,000 rpm for 10 min. Supernatant was discarded and pelleted cells were re-suspended with 10 ml of 0.5X YPDA/Kan liquid medium. From the mated culture, 100 µl of each 1/10/ 1/100, 1/1,000 and 1/10,000 dilutions were spread on SD/-Trp, SD/-Leu and DDO plates. The plates were incubated at 30ºC for 5 d until colonies grow.

### 3.5.4.5 Confirmation of positive clones by PCR

Colony PCR was performed by using ADLD-Insert Screening Amplimer primer (Clontech USA) according to method as described in section 3.5.1.7.

### 3.5.4.6 Rescuing prey plasmid

To rescue true positive prey plasmids, a single green colony from Y2H experiment was re-streaked on DDO/X plates twice and incubated for 5 d at 30ºC. Yeast plasmids were extracted according to the method described in section 3.5.3.4. The extracted plasmids were transformed into *E. coli* Top10F' cells according to the method as indicated in section 3.5.1.6. The plasmids were sent for sequencing following colony PCR amplification according to section 3.5.1.7, 3.5.1.8 and 3.5.1.9.

### 3.5.4.7 Confirmation of interacted protein by small scale mating

To further confirm genuine positive interaction of SAG1 or SAG2 with their potential prey proteins, a small scale Y2H assay was carried out. Generally, prey plasmids were transformed with Y187 yeast and mated with their respective Y2HGold(pGBKT7-SAG1) or Y2HGold(pGBKT7-SAG2) and Y2HGold(pGBKT7). The mated culture was plated on QDO/X/A plates. The same positive and negative controls as Y2H experiment were included.

### 3.6 Beta-galactosidase (β-galactosidase) assay

Genuine positive clones were further analyzed using Yeast β-galactosidase Assay kit (Thermo Scientific, USA). A portion of single colony from DDO plate was suspended with Y-PER reagent in order to lyse the yeast cells. Wavelength of mixture was measured at OD 660 nm and 250 µl of 2X β-galactosidase Assay Buffer was added. The reaction was incubated at 37°C until a colour change was observed and 200 µl of β-galactosidase Assay Stop Solution was added. The cell debris were then removed by centrifuging and the supernatant was measured at OD 420 nm. Positive control used in this assay was Y2HGold(pGBKT7-53)/ Y187(pGADT7-T). The enzyme activity was measured three times and average of beta-galactosidase activity was recorded. Beta-galactosidase activity was calculated based on this equation, (1000 x OD 420)/(t x V x OD 660) with 't' referred to incubation time (min) and 'V' referred to volume of cells (ml) used in this assay

### 3.7 Chemiluminescent co-immunoprecipitation assay (Co-IP)

#### 3.7.1 Construction of pAcGFP1-SAGs recombinant

PCR amplification of *T. gondii sag1* and *sag2* genes were performed by using extracted tachyzoites DNA as template. PCR conditions and cycling parameters were same as described in section 3.5.1.1. Primers (SAG1-pAcGFP1 and SAG2-pAcGFP1) used were listed in Table 3.1. The amplified PCR products were purified according to the method as mentioned in section 3.5.1.3. Purified products were ligated into pGEM-T vector system according to the procedures as indicated in section 3.5.1.4. Overnight ligation mixture was transformed into *E. coli* TOP10F' competent cells according to the method as described in section 3.5.1.6. By using M13 forward and reverse primers, colony PCRs were performed to select positive clones. The PCR conditions and cycling parameters were same as showed in section 3.5.1.7. Following colony PCR, a single colony from positive clone was inoculated into LB broth containing ampicillin and

incubated overnight at 37ºC. Recombinant plasmids were extracted according to the method as indicated in section 3.5.1.8 and sent for sequencing (section 3.5.1.9).

### 3.7.2 Cloning into pAcGFP1-C1 vector

Recombinant plasmids for positive clone of pGEM-T-SAG1 and pGEM-T-SAG2 were digested by *Sal*I/*Hind*III and *Bam*HI enzymes, respectively following sequencing result. The digestion reaction for insert was carried out in a total volume of 30 µl of mixture, consisted of 25 µl of pGEM-T-SAG1 recombinant plasmid, 1 µl of *Sal*I enzyme, 1 µl of *Hind*III enzyme and 3 µl of Buffer 3. Similarly, the digestion of pAcGFP1-C1 vector was carried out in a total volume of 30 µl of mixture, consisted of 25 µl of pAcGFP1-C1 vector, 1 µl of *Sal*I enzyme, 1 µl of *Hind*III enzyme and 3 µl of Buffer 3. Both digestion reactions were carried out at 37ºC for 6 h. For digestion of pGEM-T-SAG2, the digestion mixture was consisted of 26 µl of pGEM-T-SAG2 recombinant plasmid, 1 µl of *Bam*HI enzyme and 3 µl of Buffer 3. Similarly, the digestion of pAcGFP1-C1 vector was carried out in a total volume of 30 µl of mixture, consisted of 26 µl of pAcGFP1-C1 vector, 1 µl of *Bam*HI enzyme and 3 µl of Buffer 3.

Digested pGEM-T-SAG1, pGEM-T-SAG2 and pAcGFP1-C1 vector were loaded on 1% agarose gel and fragments were excised according to procedure as showed in section 3.5.2.2. The purified DNA fragments were ligated to purified pAcGFP1-C1 vector and transformed into *E. coli* TOP10F' competent cells according to the methods as described in section 3.5.1.4 and 3.5.1.6. Colony PCR was performed in the same conditions as mentioned in section 3.5.1.7. Primes used for screening was insert's specific primer. Following the colony PCR, a single colony from positive clones was inoculated into LB broth containing kanamycin and incubated overnight at 37ºC. Plasmids were extracted and sent for sequencing according to methods as indicated in section 3.5.1.8 and 3.5.1.9.

### 3.7.3 Construction of pProLabel-C-prey recombinant plasmids

Following sequencing analysis, two human proteins, *H. sapiens* lysine rich coil-coiled (abbreviated as HLY) and *H. sapiens* zinc finger (abbreviated as HZF) were obtained for SAG1 and SAG2 respectively. PCR amplification of prey fragments were performed by using human DNA as template. The template was extracted from healthy human DNA according to section 3.4. PCR conditions were performed as described in section 3.5.1.1. Primers used were listed in Table 3.1.

The amplified PCR products of HLY and HZF fragments were purified and ligated into pGEM-T vector according to the methods as indicated in section 3.5.1.3 and 3.5.1.4. The overnight ligation mixture was transformed into *E. coli* TOP10F' competent cells according to the methods as described in section 3.5.1.6. By using M13 forward and reverse primers, colony PCRs were performed to select positive clones. The PCR conditions and cycling parameters were same as mentioned in section 3.5.1.7. A single colony from positive clones was inoculated into LB broth containing ampicillin and incubated overnight at 37°C. Recombinant plasmids were extracted and sent for sequencing according to the methods as mentioned in section 3.5.1.8 and 3.5.1.9.

### 3.7.4 Cloning into pProLabel-C vector

Recombinant plasmid for positive clones were digested using *Sal*I and *Bam*HI enzyme following sequencing analysis result. The digestion reaction for insert was carried out in a total volume of 30 µl of mixture, consisted of 25 µl of pGEM-T-HLY or pGEM-T-HZF recombinant plasmid, 1 µl of *Sal*I enzyme, 1 µl of *Bam*HI enzyme and 3 µl of Buffer 3. Similarly, the digestion of pProLabel-C vector was carried out in a total volume of 30 µl of mixture, consisted of 25 µl of pProLabel-C vector, 1 µl of *Sal*I enzyme, 1 µl of *Bam*HI enzyme and 3 µl of Buffer 3. Both digestion reactions were performed at 37°C for 6 h.

The digested pGEM-T-HLY, pGEM-T-HZF and pProLabel-C vector were loaded on 1% agarose gel and fragments were excised according to the procedure as described in section 3.5.2.2. Digested pGEM-T-HLY and pGEM-T-HZF were ligated into pProLabel-C vector after purification and transformed into *E. coli* TOP10F' competent cells according to methods as showed in section 3.5.2.3 and 3.5.1.6. Five colonies were then selected for colony PCR amplification. The PCR condition and cycling parameters were same as mentioned in section 3.5.1.7. A single colony from positive clones was inoculated into LB broth containing kanamycin and incubated overnight at 37$^{o}$C. Recombinant plasmids were extracted and sent for sequencing according to the methods as indicated in section 3.5.1.8 and 3.5.1.9.

### 3.7.5 Transfection into human embryonic kidney (HEK) 293 mammalian cells

#### 3.7.5.1 Co-transfection into HEK 293 mammalian cells

Transfection of plasmid DNA into mammalian cells was carried out by using Turbofect transfection reagent kit (Invitrogen Corp., USA). HEK 293 mammalian cells were seeded in 24-well plate with one ml groth medium one day before using. The optimal confluency for adherent cells was 70-90% during transfection. To co-transfect pAcGFP1-C1-SAG1/pProLabel-C-HLY and pAcGFP1-C1-SAG2/pProLabel-C-HZF plasmids, one µg of each plasmids was diluted in 100 µl of serum free DMEM medium. Two µl of transfection reagent was added into the diluted plasmids and incubated for 20 min in room temperature. The transfection reagent and DNA plasmid mixture was added drop wise into each well. The plate was then gently rocked to distribute the cells evenly and incubated at 37$^{o}$C in $CO_2$ incubator. Co-transfection of pAcGFP1-C1-SAG1/pProLabel-C vectors and pProLabel-C-HLY/ pAcGFP1-C1 vectors into cells were used as negative experimental control.

After 24 h post-transfection, the cells were examined under fluorescent microscope. Cells in each plate were washed twice with 5 ml of 1X PBS (pre-warmed to 37ºC) after 48 h post-transfection. The PBS was aspirated off and cells in each 60-mm plate were trypsinized with two ml of trypsin and incubated at 37ºC for 10 min. Five ml of DMEM containing 10% FBS (pre-warmed to 37ºC) was added to stop trypsinization process and the cells were transferred to a clean 15-ml conical tube. Any remaining adherent cells were dislodged by either gently pipetting or by knocking the plate sideways against the palm of hand. The cells were pelleted at 1,000 rpm for 5 min and supernatant was discarded. Cell pellets were washed for two more times, each time with 10 ml of ice cold PBS. The PBS was removed as much as possible from each tube without disturbing the pellet. At this stage, the cell pellets could be used immediately to prepare cell lysate for Co-IP assay or stored at -70 ℃ until used. If cell pellets were intended to lyse at this stage, the pellets must be kept on ice at all times.

**3.7.5.2 Preparation of cell lysates**

Tubes containing cell lysates were thawed on ice. Total volume of cell lysis buffer containing of 1X PMSF and 1X protease inhibitor cocktail was prepared. The cell pellet was re-suspended with 500 µl of cell lysis buffer. PMSF was added into the cell lysis buffer just before using as PMSF was labile in aqueous solution. Tube containing mixture of cell pellet and cell lysis buffer cocktail was capped and mixed thoroughly by inversion several times and chilled on ice. Five hundred µl of cold cell lysis buffer (containing 1X PMSF and 1X protease inhibitor cocktail) was added to cell pellet and disbursed by pipetting up and down. The entire volume of each crude lysate sample was transferred to a clean 1.5-ml microcentrifuge tube and placed on ice for 30 min, with brief (5-10 s) vortexing at 10 min intervals. Then, the cellular debris was pelleted by centrifugation at 11,000 rpm for 20 min at 4ºC. To prevent degradation of proteins, the microcentrifuge

was cooled down to 4°C prior to placing lysate samples into it. The pre-cleared lysate (supernatant) was then transferred to another clean 1.5-ml microcentrifuge tube and immediately placed on ice.

Total protein concentration in each sample was determined by Quick Start Bradford Assay kit (BioRad Laboratories, USA). Based on the protein concentration measurement, 250 µg of each lysate was transferred to a clean 1.5-ml microcentrifuge tube and volume of the lysate was brought to a total of 500 µl using remaining cold cell lysis buffer cocktail. Tubes containing diluted lysates were placed on ice and proceeded with Co-IP experiment.

### 3.7.5.3 Washing of protein G plus/A agarose beads

Protein G Plus/Protein A agarose beads was pipetted up and down several times to obtain an even suspension of the beads. Twenty-five µl of agarose beads suspension was transferred to a 1.5-ml microcentrifuge tube that placed on ice. Beads were washed twice, each time with 500 µl of cold lysis buffer by inverting the tube gently three to four times. The beads were pelleted at 12,000 rpm for 30 s at 4°C. The lysis buffer was removed as much as possible without disturbing or removing the agarose beads. Twenty-five µl of cold lysis buffer was added to form a suspension again.

### 3.7.5.4 Co-immunoprecipitation assay

To each 500-ml diluted lysate sample, one µl of anti-AcGFP polyclonal antibody was added. The tubes were capped tightly and placed on a rotator at 4°C for 1 h. Entire volume of each sample was transferred to the tube containing 25 µl of washed agarose beads. The sample was then allowed to rotate gently overnight at 4°C. Beads were gently pelleted at 4°C, 6,000 rpm for 10 s on the next day. Supernatant was discarded without pipetting off the beads as these contained protein complexes. The beads were washed for

five times with 500 µl Wash Buffer 1. The tube was capped and re-suspended with wash buffer by inverting several times. Beads were pelleted at 4ºC, 6,000 rpm for 10 s, and supernatant was discarded. The beads were washed for another four times with 500 µl Wash Buffer 2. The final wash was maintained. Tubes containing beads were placed on ice.

### 3.7.5.5 ProLabel detection of protein-protein interactions

Lysis/complementation buffer and substrate mix were prepared. The lysis/complementation buffer was consisted of 3 volumes of cell lysis buffer and 1 volume of enzyme acceptor (EA). For each ProLabel assay, a substrate mix of 1.2 µl of Galacton, 6 µl of Emerald and 22.8 µl substrate buffer was prepared. The final Wash Buffer 2 was removed and discarded as much as possible from tubes without disturbing the beads. Each sample of beads was re-suspended with 80 µl of lysis/complementation buffer by gently pipetting up and down to prevent bubbles. The entire content (beads and buffer) was transferred to a well in 96-well plate. To each well, thirty µl of substrate mix was added and ProLabel activity was measured by Multimode Reader & Hydroflex Microplate Washer (Tecan, USA).

The luminescent activity for interacted group was compared with a negative experimental control which were consisted of culture from pAcGFP1-Lam/pProLabel-T, pAcGFP1-C1-SAG1/pProLabel-C, pAcGFP1-C1-SAG2/pProLabel-C, pProLabel-C-HLY/pAcGFP1-C1 and pProLabel-C-HZF/pAcGFP1-C1 plasmids. The interaction was measured three times and average RLU was calculated. Statistical significance difference between two groups was analyzed with Prism5 software (GraphPad), using a Mann-Whitney test.

## 3.8 Isothermal titration calorimetry (ITC)

### 3.8.1 Construction of recombinant pRSET-A-SAGs, pRSET-A-HLY and pRSET-A-HZF plasmids

PCR amplification of *T. gondii sag1* and *sag2* were performed by using the extracted tachyzoites DNA as template. The same PCR conditions and cycling parameters as described in section 3.5.1.1 were performed. Primers (SAG1-pRSET-A and SAG2-pRSET-A) used were listed in Table 3.1.

Two prey proteins, HLY and HZF for the respective SAG1 and SAG2 were obtained following a series of selection media and sequencing analysis. PCR amplification of prey fragments were performed by using human DNA as template. DNA template was extracted from healthy human blood according to method as indicated in section 3.4. The same PCR conditions and cycling parameters as described in section 3.5.1.1 were performed. Primers (HLY-pRSET-A and HZF-pRSET-A) used were specifically designed and listed in Table 3.1.

The amplified PCR products of SAG1, SAG2, HLY and HZF fragments were purified and cloned into pGEM-T vector according to the methods as mentioned in section 3.5.1.3 and 3.5.1.4. The overnight ligation mixture was transformed into *E. coli* TOP10F' competent cells according to the methods as described in section 3.5.1.6. By using M13 forward and reverse primers, colony PCRs were performed to select positive clones. PCR conditions and cycling parameters were same as mentioned in section 3.5.1.7. Following the colony PCR, a single colony from a positive clone was inoculated into LB broth containing ampicillin and incubated at overnight at 37$^{o}$C. Recombinant plasmids containing insert were extracted and sent for sequencing according to the methods as shown in sections 3.5.1.8 and 3.5.1.9.

### 3.8.2 Cloning into pRSET-A vector

The recombinant plasmids for positive clones were digested by *Bam*HI enzyme following the sequencing analysis result. The digestion reaction for insert was performed in a total volume of 30 µl of mixture, consisted of 25 µl of pGEM-T-SAGs or pGEM-T-HLY or pGEM-T-HZF recombinant plasmid, 1 µl of $ddH_2O$, 1 µl of *Bam*HI enzyme and 3 µl of Buffer 3. Similarly, the pRSET-A vector was digested in a total volume of 30 µl of mixture, consisted of 25 µl of pRSET-A vector, 1 µl of *Bam*HI enzyme, 1 µl of $ddH_2O$ and 3 µl of Buffer 3. Both digestion reactions were incubated at 37$^{o}$C for 6 h.

The digested pGEM-T-SAG1, pGEM-T-SAG2, pGEM-T-HLY, pGEM-T-HZF and pRSET-A vectors were loaded on 1% agarose gel. The fragments were excised and purified according to section 3.5.2.2. The digested products were ligated into pRSET-A vector and transformed into *E. coli* TOP10F' competent cells according to the methods as mentioned in section 3.5.2.3 and 3.5.1.6. Five colonies were selected for colony PCR amplification. The same PCR condition and cycling parameters as mentioned in section 3.5.1.7 were performed. Primes used for PCR was T7 promoter primer and insert's reverse primer. A single colony from a positive clone was inoculated into LB broth containing ampicillin and incubated overnight at 37$^{o}$C. The recombinant plasmids were extracted and sent for sequencing according to the procedures as mentioned in section 3.5.1.8 and section 3.5.1.9.

### 3.8.3 Transformation into *E. coli* BL21 (DE3) pLysS

#### 3.8.3.1 Preparation of *E. coli* BL21 (DE3) pLysS competent cells

*E. coli* BL21 (DE3) pLysS competent cells were prepared by using calcium chloride ($CaCl_2$) method. The *E. coli* BL21(DE3) pLysS was revised from stock and re-streaked on plate containing 100 µg/µl ampicillin and chloramphenicol antibiotic and

incubated overnight at 37ºC. Competent cells was then prepared according to section 3.5.1.5.

#### 3.8.3.2 Transformation of recombinant pRSET-A-SAGs, pRSET-A-HLY and pRSET-A-HZF plasmids into *E. coli* BL21 (DE3) pLysS

Following sequencing analysis, recombinant pRSET-A with insert in the sense of orientation was transformed into *E. coli* BL21 (DE3) pLysS expression host. The protocol for transformation was same as mentioned in section 3.5.1.6 except the medium used was LB plate containing 100 µg/µl ampicillin and chloramphenicol antibiotic.

#### 3.8.3.3 Screening of positive clones by PCR

Five colonies were selected for colony PCR amplification following the transformation. The same PCR conditions and cycling parameters as mentioned in section 3.5.1.7 were performed. Primes used for PCR was T7 promoter primer and insert's reverse primer. A single colony from a positive clone was inoculated into LB broth containing ampicillin and chloramphenicol and incubated overnight at 37ºC. The recombinant plasmids were extracted and sent for sequencing according to the methods as mentioned in sections 3.5.1.8 and 3.5.1.9.

### 3.8.4 Protein expression

A single colony was picked by using a sterile toothpick and inoculated into a 10 ml LB broth containing ampicillin and chloramphenicol. The culture was incubated at shaking incubator at 250 rpm, 37ºC for overnight. OD reading of overnight culture was measured at 600 nm wavelength. The culture was diluted to 0.1 OD using LB broth without additional of any antibiotics and allowed to grow until 0.6 OD. One ml of culture was collected as 0 h time point. Then, 1mM IPTG was added into the culture to induce

the protein expression. One ml of culture was collected every hour as time point until 4 h. The culture was then spun at 5,000 rpm for 10 min. Supernatant was discarded. The pellet was re-suspended with 20 µl of each 2X sample buffer and PBS. The pellet was boiled for 10 min and 5 µl of pellet was loaded into the SDS-PAGE gel.

To proceed with large scale protein expression, a 30-ml overnight culture of a single colony from *E. coli* BL21 (DE3) cells containing recombinant clones from each of pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF were prepared. After measuring OD at 600 nm on next day, ~22 ml of the overnight culture was added into 500 ml of LB broth. The subsequent protein expression procedures were same as small scale protein expression.

#### 3.8.4.1 SDS-PAGE analysis

Purified fusion proteins were separated by 12% SDS-PAGE gels. The reactions were run for 20 min in 80 V followed by 60 min in 120 V. Gels were stained with coomassie brilliant blue solution and shake on belly dancer for overnight. The gels were de-stained by adding 20 ml de-staining solution and shake for 2 h at room temperature.

#### 3.8.4.2 Western blot

Purified proteins were separated by 12% SDS-PAGE gels and transferred onto a PVDF membrane. PVDF membrane was blocked with 5% skim milk overnight at 4$^{o}$C. The membrane was washed three times with TBS-T buffer and probed with anti-Xpress$^{TM}$ monoclonal antibody (1:5000 dilution) for 1 h. Membrane was washed three times with TBS-T (TBS containing 0.2% Tween-20) and incubated with biotin-labelled goat anti-mouse IgG (1:2500 dilution) (KPL Inc., USA) as secondary antibody for 1 h. Again, the membrane was washed three times with TBS-T followed by streptavidin-AP (1: 2500

dilution) (KPL Inc., USA) for 1 h. Finally, the membranes were developed by chromogenic substrate NBT/BCIP (Sigma, USA) at room temperature in dark.

### 3.8.4.3 Protein purification

Expressed recombinant pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF proteins were purified by using ProBond$^{TM}$ purification system (Invitrogen Corp., USA) under hybrid condition. Cell lysates were prepared from 500 ml culture volume and re-suspended with 6M guanidinium lysis buffer, pH 7.8. The cell lysates were rocked for 10 min at room temperature following by ultrasonication on ice with three 5 s pulses high intensity. The lysates were then centrifuged for 15 min at 5,000 rpm and supernatant was added into column containing nickel-NTA resin. Protein lysates incubated for 30 min at room temperature with gentle agitation. Resin was allowed to settle down and columns were washed twice with denaturing binding buffer, pH 7.8 followed by twice denaturing wash buffer, pH 6.0. Protein lysates were washed four times with native wash buffer, pH 8. The purified recombinant pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF proteins were finally eluted with 6 ml native elution buffer and stored at -80$^{o}$C.

### 3.8.4.4 Dialysis recombinant proteins

Purified proteins were dialyzed against 1X PBS buffer at cold room. Five ml of purified proteins were loaded into dialysis cassettes. The dialysis buffer was changed after incubation for 2, 4 h and overnight.

### 3.8.4.5 Concentrated of recombinant proteins

Purified proteins were concentrated by using viva spin. Five ml of dialyzed protein was loaded into viva spin and centrifuged at 4$^{o}$C for 1 h at 2,000 rpm.

### 3.8.4.6 Measurement of protein concentration

Concentration of purified proteins was determined by using Quick Start Bradford Assay kit. For each sample, 10 µl of each purified and dialyzed protein was diluted in 500 µl of 1X Quick Start Bradford dye reagent. The mixture was incubated for 10 min at room temperature and loaded into each well of 96-well plate. Readings were then taken at 595 nm by using M200 Pro Nanoquant plate reader (Tecan, USA).

### 3.8.4.7 Isothermal titration calorimetry (ITC)

ITC experiments were performed at 25 ℃ in a MicroCal Auto-iTC$_{200}$ calorimeter from MicroCal™ (GE Healthcare, USA). The calorimeter was calibrated according to standard protocol of the instrument before starting of ITC experiment. Dialyzed and concentrated protein samples were briefly centrifuged and degassed for 20 min before each ITC experiments.

To measure binding strength for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins, 30 µM of recombinant pRSET-A-SAG1 and 4 µM of recombinant pRSET-A-HLY protein were used. The ITC experiments were performed by adding aliquots of recombinant pRSET-A-SAG1 protein into sample cell which consisted of recombinant pRSET-A-HLY protein. Total injections for measuring the binding strength between these two proteins was 20. The injection volume was setup at 0.4 µl per injection and duration of injection was 0.8 s, with an interval of 150 s between injections. The reaction mixture was continuously stirred at 1,000 rpm during titration period. ORIGIN version 7.0 software from MicroCal™ was used to analyse the ITC data. Titrations were performed in duplicate using the same set of stock solutions. Titration curve showed all information of thermodynamic stoichiometry such as enthalpy (ΔH), entropy (ΔS), binding stoichiometry (N) and binding constant ($K_d$).

To measure binding strength for recombinant pRSET-A-SAG2/pRSET-A-HZF proteins, 20 μM of recombinant pRSET-A-SAG2 and 3 μM of recombinant pRSET-A-HZF protein were used. ITC experiment conditions were performed in the same way as recombinant pRSET-A-SAG1/pRSET-A-HLY proteins.

The titration curve of the recombinant pRSET-A-SAG1/pRSET-A-HLY and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins were observed. Negative controls used in this assay were recombinant pRSET-A-SAG1/pRSET-A vector proteins and recombinant pRSET-A-SAG2/pRSET-A vector proteins.

# CHAPTER 4: RESULTS

## 4.1 Yeast two-hybrid system

### 4.1.1 Construction of bait plasmid (pGBKT7-SAGs)

#### 4.1.1.1 PCR amplification of *T. gondii sag1* and *sag2*

*sag1* and *sag2* were amplified with their respective primer pairs. Figure 4.1 indicates agarose gel electrophoresis for PCR amplification of *sag1* (~750 bp) and *sag2* (~500 bp). The actual band size generated by SAG1 and SAG2 PCR were 735 and 483 bp, respectively.

#### 4.1.1.2 Cloning of purified fragments into pGEM-T vector

PCR products of SAG1 and SAG2 were purified and ligated into pGEM-T vector system. Overnight ligation mixtures were transformed into TOP10F' cells. Colony PCR amplification was performed by using M13 forward and reverse primer pairs (Figure 4.2). Five colonies from each of recombinant pGEM-T-SAG1 and pGEM-T-SAG2 in TOP10F' cells were selected for PCR. The amplicons generated by SAG1 and SAG2 PCR were ~950 bp (735 bp of SAG1 plus ~200 bp of vector) and ~700 bp (483 bp of SAG2 plus ~200 bp of vector), respectively.

Sequences of these amplicons were analyzed by BLAST. The SAG1 was found to be 99% similar to *T. gondii* major surface antigen P30 gene (Accession number X14080.1) (Appendix P). There was one nucleotide different in between the sequence. But it's still coding for the same amino acid (ACA=ACG). So, the function of this protein would not be affected. While SAG2 was found to be 100% similar to *T. gondii* strain RH surface antigen P22 (SAG2) mRNA, complete cds (Accession number FJ825705.1) (Appendix Q).

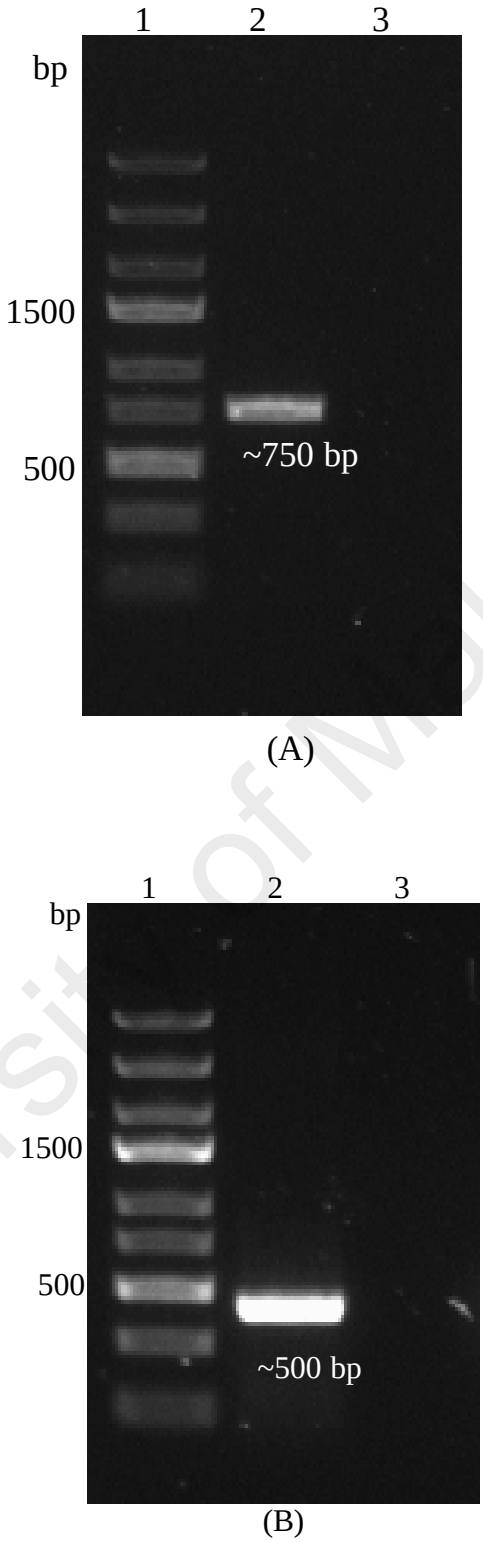
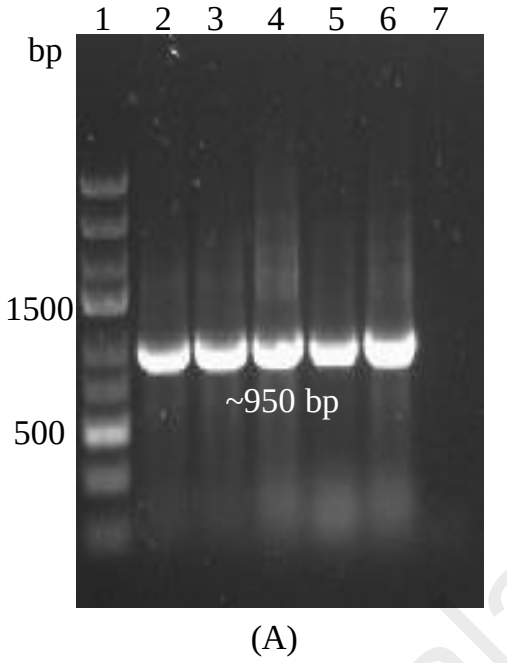
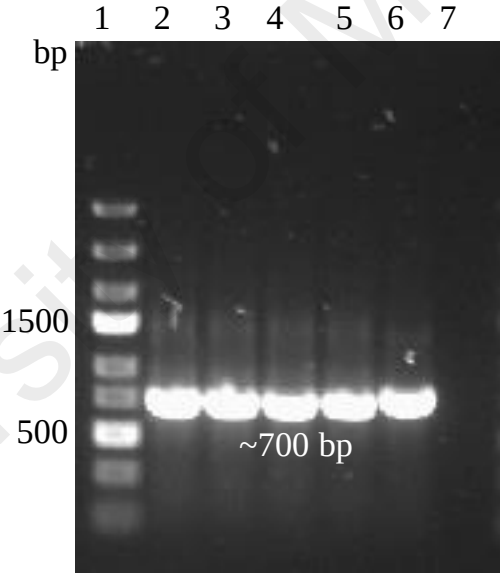



Figure 4.1: PCR amplification of *T. gondii sag1* and *sag2* genes using gene specific primers. The amplified DNA fragments were (A) ~750 bp for *T. gondii sag1* gene (lane 2) and (B) ~500 bp for *sag2* gene (lane 2), respectively. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 3 in both panel (A) and (B) are negative control ($ddH_2O$)




(A)




(B)

Figure 4.2: Colony PCR of recombinant pGEM-T-SAG1 and pGEM-T-SAG2 in TOP10F' cells. The amplified DNA fragments were (A) ~950 bp for positive pGEM-T-SAG1 clones (lanes 2 to 6) and (B) ~700 bp for positive pGEM-T-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

#### 4.1.1.3 Digestion of recombinant pGEM-T-SAGs and pGBKT7 plasmids

Complete digestion of recombinant pGEM-T-SAG1 plasmid generated a ~750 bp of SAG1 insert fragment and a ~3 kb pGEM-T plasmid (Figure 4.3 A), while complete digestion of recombinant pGEM-T-SAG2 generated a ~500 bp SAG2 fragment and a ~3 kb pGEM-T plasmid (Figure 4.3 C). Figure 4.3 B and D show completely digested pGBKT7 plasmid with a size of 7.3 kb.

#### 4.1.1.4 Ligation of digested fragments and transformation into TOP10F' cells

Digested DNA fragments of SAG1 and SAG2 were excised from agarose gel and proceeded to gel purification procedure. Purified SAG1 and SAG2 fragments were ligated into digested pGBKT7 vector and then transformed into TOP10F' cells. Colony PCR was performed on 5 selected recombinant clones from each of pGBKT7-SAG1 and pGBKT7-SAG2 using gene specific primers (Figure 4.4 A and B). PCR amplification of positive recombinant pGBKT7-SAG1 and pGBKT7-SAG2 clones generated an expected band of ~750 bp and ~500 bp, respectively.

#### 4.1.1.5 Transformation of pGBKT7-SAGs plasmids into Y2HGold yeast cells

Recombinant pGBKT7-SAG1 and pGBKT7-SAG2 with insert of 100% similarity to the sequences available in GenBank were extracted and transformed into Y2HGold yeast cells. The culture was plated on single dropout medium without tryptophan (SD/-Trp) plate.

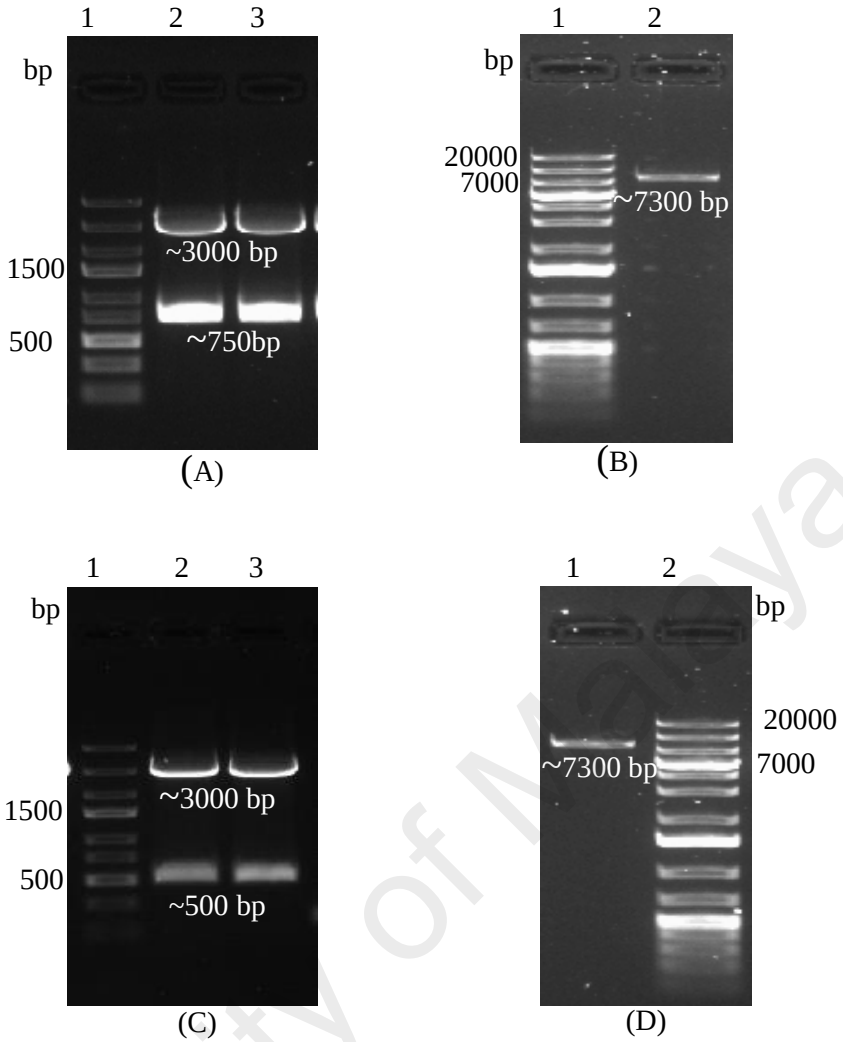
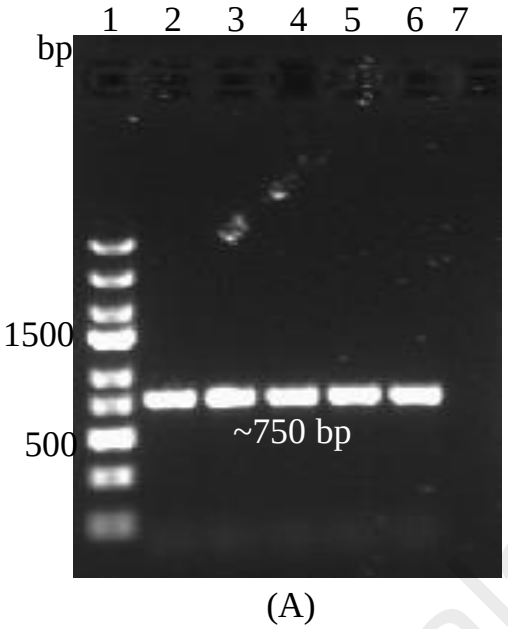
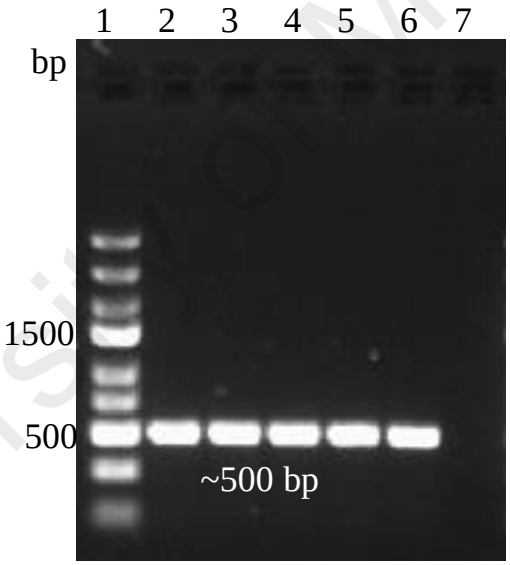



Figure 4.3: Restriction digestion of recombinant pGEM-T-SAG1, pGEM-T-SAG2 and pGBKT7 plasmids. (A) Double digested recombinant pGEM-T-SAG1 plasmid with *Eco*RI and *SaI*I restriction enzymes (lanes 2 and 3). (B) Double digested pGBKT7 plasmid with *Eco*RI and *SaI*I restriction enzymes (lane 2). (C) Double digested recombinant pGEM-T-SAG2 plasmid with *Nco*I and *Pst*I restriction enzymes (lanes 2 and 3). (D) Double digested pGBKT7plasmid with *Nco*I and *Pst*I restriction enzymes (lane 1). Lane 1 in both panel (A) and (C) are 1 kb DNA ladder. Lane 1 in panel (B) and lane 2 in panel (D) are 1 kb plus DNA ladder.




(A)




(B)

Figure 4.4: Colony PCR of recombinant pGBKT7-SAG1 and pGBKT7-SAG2 in TOP10F' cells. The amplified DNA fragments were (A) ~750 bp for positive pGBKT7-SAG1 clones (lanes 2 to 6) and (B) ~500 bp for positive pGBKT7-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

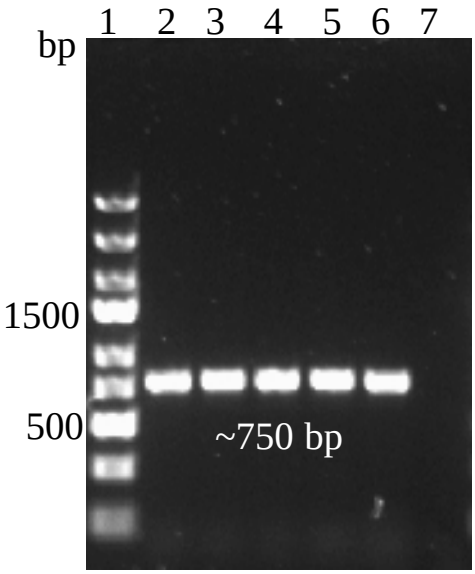
After incubation for 5 d at 30ºC, PCR amplification was performed by using gene specific primers to identify positive clones with insert. Five clones of respective pGBKT7-SAG1 and pGBKT7-SAG2 were selected (Figure 4.5 A and B). The size of amplicon generated for positive pGBKT7-SAG1 and pGBKT7-SAG2 clones were ~750 bp and ~500 bp, respectively.

### 4.1.2 Autoactivation test

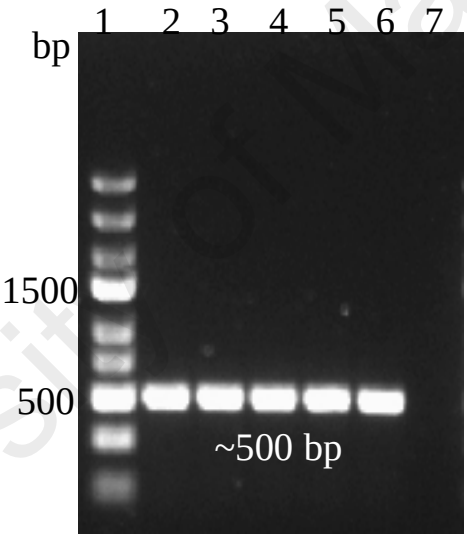
Autoactivation activity of the bait proteins was tested before the Y2H screening. These tests were carried out by plating the pGBKT7-SAG1 or pGBKT7-SAG2 transformed in Y2HGold cells on selective plates, including SD/-Trp and single dropout medium without tryptophan but with X-alpha-gal and aureobasidin A (SD/-Trp/X/A) plates. Autoactivation activities would allow the expression of the reporter genes and result in green colonies on SD/-Trp/X/A plates.

White colonies appeared on SD/-Trp plate plated with Y2HGold transformed with pGBKT7-SAG1 or pGBKT7-SAG2 after 5 d at 30ºC (Figure 4.6 A). However, no colonies were observed for Y2HGold transformed with pGBKT7-SAG1 or pGBKT7-SAG2 plated on SD/-Trp/X/A plate. The results showed that no autoactivation activities was detected from Y2HGold transformed with pGBKT7-SAG1 or pGBKT7-SAG2.

In comparison to positive control [Y2HGold(pGBKT7-53) with Y187(pGADT7-T)] which was provided from the kit, green colonies were observed on SD/-Trp/X/A plate (Figure 4.6 B).

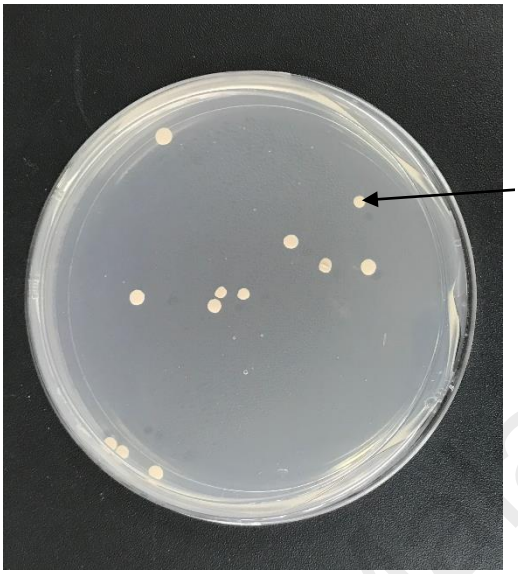



(A)

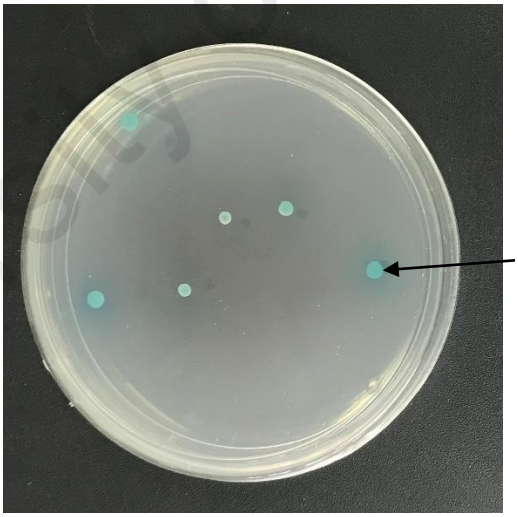



(B)

Figure 4.5: Colony PCR of recombinant pGBKT7-SAG1 and pGBKT7-SAG2 in Y2HGold cells. The amplified DNA fragments were (A) ~750 bp for positive pGBKT7-SAG1 clones (lanes 2 to 6) and (B) ~500 bp for positive pGBKT7-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).




(A)




(B)

Figure 4.6: Representative plates for autoactivation test of pGBKT7-SAG1 or pGBKT7-SAG2 in Y2HGold cells. (A) White colonies were observed on SD/-Trp plate plated with pGBKT7-SAG1 or pGBKT7-SAG2 clones (B) Green colonies were observed on DDO/X/A plate plated with positive control.

### 4.1.3 Toxicity test

Figure 4.7 A shows colonies of Y2HGold transformed with pGBKT7-SAG1 or pGBKT7-SAG2 plated on SD/-Trp plate. Only bait that are not toxic to yeast cells will be selected to proceed with Y2H experiment. Toxicty causes the size of the colonies containing bait is smaller than the colonies containing empty vector. However, Figure 4.7 B shows the size of colony of pGBKT7-SAG1 or pGBKT7-SAG2 was similar with the empty pGBKT7 vector.

### 4.1.4 Control experiment

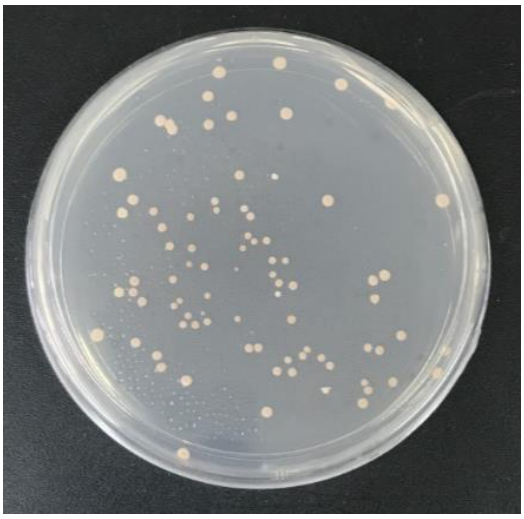
Control experiments were performed using bait and prey controls provided from the kit. These mated cultures were plated on DDO and DDO/X/A plates.

Figure 4.8 A indicates the representative plate for negative control, Y2HGold (pGBKT7-Lam) mated with Y187 (pGADT7-T). White colonies were observed on DDO plate after incubation for 5 d at 30$^{o}$C and no colonies were observed on DDO/X/A plate.

Figure 4.8 B shows the representative plate for positive control, Y2HGold (pGBKT7-53) mated with Y187 (pGADT7-T). Green colonies were observed on DDO/X/A plate after incubation for 5 d at 30$^{o}$C and white colonies were observed on the DDO plate.

### 4.1.5 Mating experiments

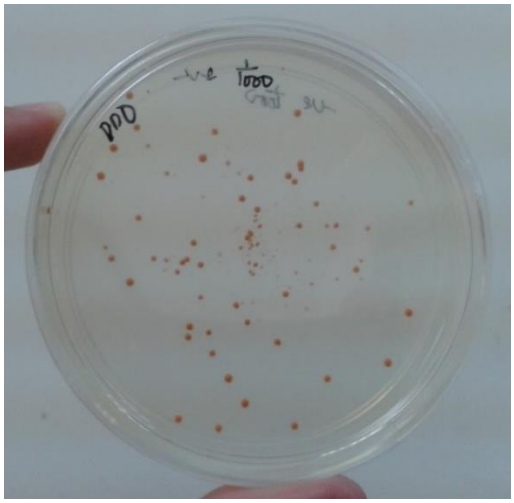
For Y2H screening, both mated cultures were plated on a series of selective plates which is DDO, DDO/X/A, QDO and QDO/X/A plates (Table 4.1). A total amount of 65 and 40 colonies on 1/1,000 dilution DDO plates were found for SAG1 and SAG 2 mated with human cDNA library, respectively.



(A)

(B)

Figure 4.7: Representative plates for toxicity test of pGBKT7-SAG1 and pGBKT7-SAG2 in Y2HGold cells. (A) White colonies of pGBKT7-SAG1 or pGBKT7-SAG2 in Y2HGold yeast growing on SD/-Trp plate which is having almost same size with colonies containing the empty pGBKT7 vector. (B) White colonies of empty pGBKT7 in Y2HGold yeast growing on SD/-Trp plate.




(A)

(B)

Figure 4.8: Colonies growing on selective plates for control experiment. (A) Colonies of negative control plated on DDO plate. (B) Green colonies of positive control plated on DDO/X/A plate.

Table 4.1: Total clones interacting with SAG1 and SAG2 after Y2H assay.

| Selective agar plates | Total colonies | |
|---|---|---|
| | SAG1 | SAG2 |
| DDO | 65 | 40 |
| DDO/X/A | 58 | 36 |
| QDO | 49 | 28 |
| QDO/X/A | 39 | 25 |

Note: Double dropout medium, DDO; Double dropout medium with X-alpha-gal and aureobasidin A, DDO/X/A; Quadruple dropout medium, QDO; Quadruple dropout medium with X-alpha-gal and aureobasidin A, QDO/X/A .

To increase specificity of selection, these colonies were plated on more stringent plates, DDO/X/A, After incubarion for 5 d at 30$^{o}$C, green colonies from DDO/X/A plates (58 and 36 colonies from SAG1 and SAG2, respectively) were restreaked on QDO plates and incubated for another 5 d with same temperature. Then, colonies from QDO plates were plated on the highest stringency plates, QDO/X/A plates and incubated with the same conditions.

Colony PCR was performed by using ADLD insert screening primers. A total of 39 and 25 clones interacting with SAG1 and SAG2, respectively were observed on QDO/X/A. Most of the clones generated a PCR band of more than 1 kb size (Figures 4.9).

### 4.1.6 Rescuing of putative yeast plasmids

Following ADLD colony PCR screening, these clones were re-streaked on DDO with X-alpha-gal (DDO/X) plates and incubated at 30$^{o}$C for 5 d. This process was repeated for 3 times in order to confirm that only green colonies (representing positive interaction) were found. Eventually, 33 and 20 prey interacting with SAG1 and SAG2 were found. These clones (containing plasmid with *leu* gene) were then re-steaked on selective agar plate with single dropout medium without leucine (SD/-Leu). After incubation for 5 d at 30$^{o}$C, colony PCR was performed by using ADLD insert screening primer (Figure 4.10). Plasmids from the recombinant clones were extracted and transformed into TOP10F' cells. After propagation in TOP10F' cells, these plasmids were extracted and sent for sequencing. Table 4.2 and Table 4.3 indicate sequencing results for prey of SAG1 and SAG2 respectively. Results show that 22 and 13 clones were found to be possible prey for SAG1 and SAG2, respectively. Based on the analysis results from UniProt, these proteins were localized at cytoplasm, nucleus, membrane and transmembrane of the cell. The proteins were selected based on their predicted functions from BLAST result and literature.

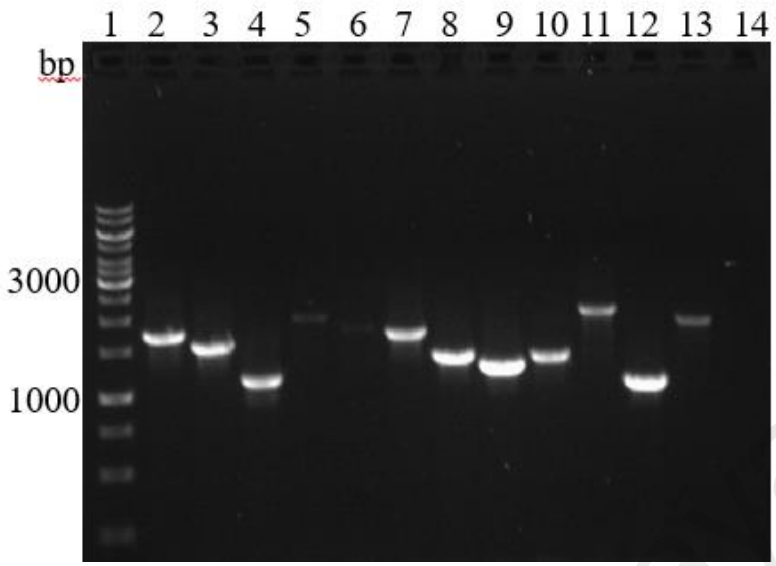



Figure 4.9: Representative agarose gel of colony PCR for selection of colonies after Y2H experiment. Prey colonies interacting with SAG1 or SAG2 were selected. The size of amplicon generated for most of the clones was above 1 kb. Lane 1 is 1 kb DNA ladder. Lanes 2 to 13 are colonies from QDO/X/A plates. Lane 14 is negative control ($ddH_2O$).

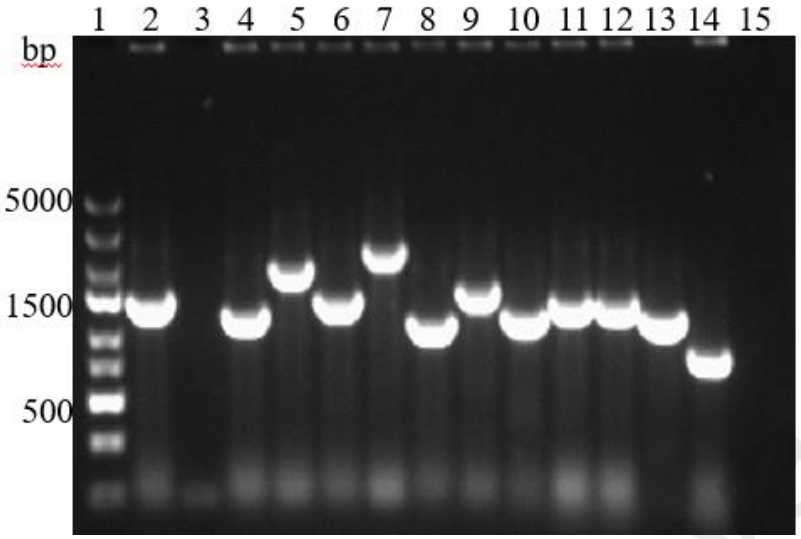



Figure 4.10: Representative agarose gel of colony PCR for positive interaction colonies using ADLD insert screening primers. Prey colonies interacting with SAG1 or SAG2 were selected. Lane 1 is 1 kb DNA ladder. Lanes 2 to 14 are 13 colonies from SD/-Leu plates. Lane 15 is negative control ($ddH_2O$).

**Table 4.2:** BLAST result for interacting proteins between SAG1 and cDNA human library

| Clone | Name | Functions | References | Accession number |
|---|---|---|---|---|
| 1 | *H. sapiens* sortilin 1 (SORT1), transcript variant 2, mRNA | Formation of apical secretory organelles | Sloves *et al*., 2012 | NM_001205228 |
| 2 | *H. sapiens* cytochrome c, somatic, mRNA | Facilitate the invasion and motility | Pomel *et al.*, 2008 | BC021994 |
| 3 | *H. sapiens* tetratricopeptide repeat, ankyrin repeat | Facilitate invasion | Lamarque *et al*., 2012 | NM_025185 |
| 4 | *H. sapiens* cytochrome P450 family 2 | Protection | Pomel *et al*., 2008 | NG_007961 |
| 5 | *H. sapiens* lysine-rich coiled-coil 1, mRNA | Facilitate the motility | Heaslip *et al*., 2011 | BC107580 |
| 6 | *H. sapiens* cytochrome P450 family 2 | Facilitate the invasion and motility | Pomel *et al*., 2008 | NG_007961 |
| 7 | *H. sapiens* zinc finger, MYM-type 6, mRNA | Differentiation of tachyzoite to bradyzoite | Vanchinathan *et al*., 2005 | BC029439 |
| 8 | *H. sapiens* cytochrome P450 family 2 | Facilitate the invasion and motility | Pomel *et al*., 2008 | NG_007961 |
| 9 | *H. sapiens* CORTBP1 cortactin-binding protein, mRNA | Gliding motility | Chen *et al*., 2003 | AB208027 |
| 10 | *H. sapiens* ankyrin 3 (ANK3), transcript variant 2, mRNA | Attachment to the host cell plasma | Lamarque *et al*., 2012 | NM_001149 |
| 11 | *H. sapiens* lectin, mannose binding 1 (LMAN1) | Attachment to the host cell plasma | Carruthers *et al*., 2000 | NG_012097 |
| 12 | *H. sapiens* ERGIC and golgi 2 (ERGIC2), mRNA | Daughter cell division of *T. gondii* | Sinai *et al*., 1997 | NM_016570 |
| 13 | *H. sapiens* mannosyl (alpha-1,6-)-glycoprotein | GPI biosynthesis | Kimmel *et al.*, 2006 | NM_002410 |
| 14 | *H. sapiens* NAD(P) dependent steroid dehydrogenase | Energy production for growth and replication | Possenti *et al*., 2013 | NG_009163 |
| 15 | *H. sapiens* ERGIC and golgi 2 (ERGIC2), mRNA | Daughter cell division of *T. gondii* | Sinai *et al*., 1997 | NM_016570 |
| 16 | *H. sapiens* zinc finger protein 432 (ZNF432), mRNA | Differentiation of tachyzoite to bradyzoite | Vanchinathan *et al*., 2005 | AK292827 |
| 17 | *H. sapiens* ras-related GTP-binding protein, mRNA | Assisting in replication | Nepomuceno-Silva *et a*l., 2003 | AK025136 |
| 18 | *H. sapiens* zinc finger and SCAN domain | Differentiation of tachyzoite to bradyzoite | Vanchinathan *et al*., 2005 | NM_001111039 |
| 19 | *H. sapiens* zinc finger and SCAN domain | Differentiation of tachyzoite to bradyzoite | Vanchinathan *et al*., 2005 | NM_001111039 |
| 20 | *H. sapiens* glutamate receptor interacting protein 1 | Energy production for growth and replication | Possenti *et al*., 2013 | NG_021400 |
| 21 | *H. sapiens* peroxiredoxin 1, mRNA | Assisting in proliferation | Guimaraes *et al*., 2010 | BC021683 |
| 22 | *H. sapiens* ubiquitin-conjugating enzyme E2 | Facilitating in DNA repair and cellular division | Braun *et al*., 2009 | BC007051 |
| 23 | *H. sapiens* acyl-CoA synthetase long-chain | Produce energy for growth and replication | Possenti *et al*., 2013 | NM_001205251 |
| 24 | *H. sapiens* leucine rich repeats | Produce energy for growth and replication | Possenti *et al*., 2013 | NM_020871 |
| 25 | *H. sapiens* transferrin variant, mRNA | Promote the growth | Laliberte & Carruthers, 2008 | AK222755 |
| 26 | *H. sapiens* transferrin variant, mRNA | Promote the growth | Laliberte & Carruthers, 2008 | AK222755 |
| 27 | *H. sapiens* ankyrin 3 (ANK3), transcript variant 2, mRNA | Attachment to the host cell plasma | Lamarque *et al*., 2012 | NM_001149 |
| 28 | *H. sapiens* laminin subunit gamma 1 (LAMC1), mRNA | Attachment of tachyzoite to host cell | Guimaraes *et al*., 2010 | NM_002293 |
| 29 | *H. sapiens* ATP synthase, H+ transporting, mitochondrial Fo | Energy production for growth and replication | Possenti *et al*., 2013 | NM_001003703 |

**Table 4.3.** BLAST result for interacting proteins between SAG2 and cDNA human library

| Clone | Name | Functions | References | Accession number |
|---|---|---|---|---|
| 1 | *H. sapiens* phosphatase 4 | Involved in Krebs Cycle | Possenti *et al*., 2013 | NR0035105 |
| 2 | *H. sapiens* cathepsin B, mRNA | Cell division, PV and PVM formation | Que *et al.*, 2002 | L22569 |
| 3 | *H. sapiens* cyclin C, mRNA | Cell differentiation | Kvaal *et al*., 2002 | BC010135 |
| 4 | *H. sapiens* Na+/Ca2+ exchanger, partial mRNA | Cell egression | McCoy *et al*., 2012 | AJ508602 |
| 5 | *H. sapiens* cytochrome P450 family 2 | Facilitate the invasion and motility | Pomel *et al*., 2008 | NG_007961 |
| 6 | *H. sapiens* kinase, cAMP dependent regulatory, type 1 | Promote the growth of *T. gondii* | Kurokawa *et al*., 2011 | BC002470 |
| 7 | *H. sapiens* cyclin C, mRNA | Cell differentiation | Kvaal *et al*., 2002 | BC010135 |
| 8 | Human HS1 binding protein HAX-1, mRNA | Promote the growth of *T. gondii* | Pomel *et al*., 2008 | U68566 |
| 9 | Human HS1 binding protein HAX-1, mRNA | Promote the growth of *T. gondii* | Pomel *et al*., 2008 | U68566 |
| 10 | Human cAMP-dependent protein kinase | Promote the cell division of *T. gondii* | Possenti *et al*., 2013 | NM_001028 |
| 11 | *H. sapiens* Na+/Ca2+ exchanger isoform 4, partial mRNA | Cell egression | McCoy *et al*., 2012 | AJ508602 |
| 12 | Human HS1 binding protein HAX-1, mRNA | Promote the growth of *T. gondii* | Pomel *et al*., 2008 | U68566 |
| 13 | *H. sapiens* Na+/Ca2+ exchanger isoform 4, partial mRNA | Cell egression | McCoy *et al*., 2012 | AJ508602 |
| 14 | *H. sapiens* cyclin C, mRNA | Cell differentiation | Kvaal *et al*., 2002 | BC010135 |
| 15 | *H. sapiens* cathepsin B, mRNA | Cell division, PV and PVM formation | Que *et al.*, 2002 | L22569 |
| 16 | *H. sapiens* endomucin, mRNA | Promote the growth of *T. gondii* | Zahr *et al*., 2016 | AF205940 |
| 17 | *H. sapiens* HS1 binding protein HAX-1, mRNA | Promote the growth of *T. gondii* | Pomel *et al*., 2008 | U68566 |
| 18 | *H. sapiens* zinc finger AN1-type | Differentiation of tachyzoite to bradyzoite | Vanchinathan *et al*., 2005 | NM_001242914 |

### 4.1.7 Small scale Y2H assay

The selected 22 and 13 prey interacted with SAG1and SAG2, respectively were re-streaked onto SD/-Leu plates which were selectively for prey plasmid (pGADT7-RecAB vector) and incubated at 30$^{o}$C for 5 d. The re-streaked clones were screened by colony PCR to confirm their inserts still existed (Figure 4.11).

Each of prey constructs were mated with Y2HGold(pGBKT7-SAG1) or Y2HGold(pGBKT7-SAG2), respectively. Mated cultures were plated on DDO/X/A plates. These prey constructs were mated with Y2HGold(pGBKT7) as well.

Figure 4.12 shows result for mating between prey with Y2HGold(pGBKT7) and prey with Y2HGold(pGBKT7-SAG1). Since Clone C5 (*Homo sapiens* lysine-rich coiled-coil, abbreviated as HLY) showed no colony growing on plate after mating with Y2HGold(pGBKT7), this clone (designated as pGADT7-RecAB-HLY) was selected to proceed with downstream experiment. No colonies were observed due to no interacting between this clone with Y2HGold(pGBKT7).

Meanwhile, Figure 4.13 shows result for mating between prey with Y2HGold(pGBKT7) and prey with Y2HGold(pGBKT7-SAG2). Since Clone C18 (*Homo sapiens* zinc finger, abbreviated as HZF) showed no colony growing on plate after mating with Y2HGold(pGBKT7), this clone (designated as pGADT7-RecAB-HZF) was selected to proceed with downstream experiment.

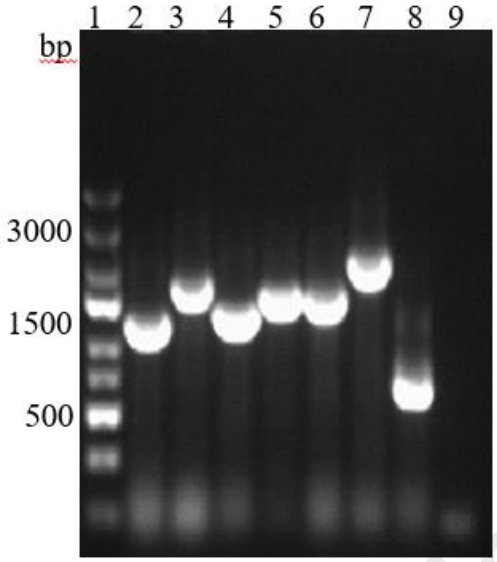



Figure 4.11: Representative agarose gel for colony PCR of prey clones in pGADT7-RecAB plasmid. PCR was performed by using ADLD insert screening primer. Lane 1 is 1 kb DNA ladder. Lanes 2 to 8 are 7 colonies from SD/-Leu palte. Lane 9 indicates negative control ($ddH_2O$).

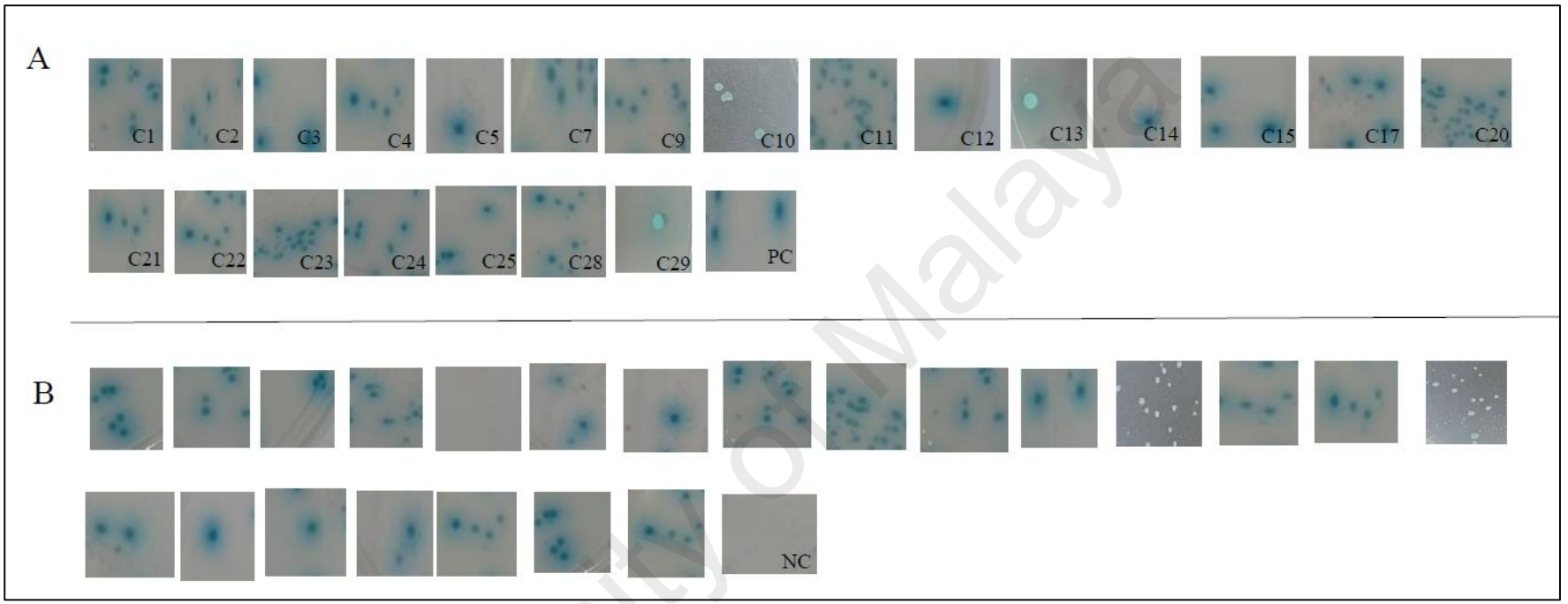



Figure 4.12: Confirmation of true positive clones interacting with Y2HGold(pGBKT7-SAG1) by small scale Y2H assay. Panel A: Zygotes formed following the mating between pGBKT7-SAG1 plasmid and the respective prey 1 to prey 29 plasmids. Panel B: Zygotes formed following the mating between pGBKT7 empty vector and the respective prey 1 to prey 29 plasmids. PC: Positive control which was zygotes formed following the mating between Y2HGold transformed with pGBKT7-53 and Y187 transformed with pGADT7-T vector. NC: Negative control with zygotes formed following the mating between Y2HGold transformed with pGBKT7 empty vector and Y187 transformed with pGADT7-T vector.

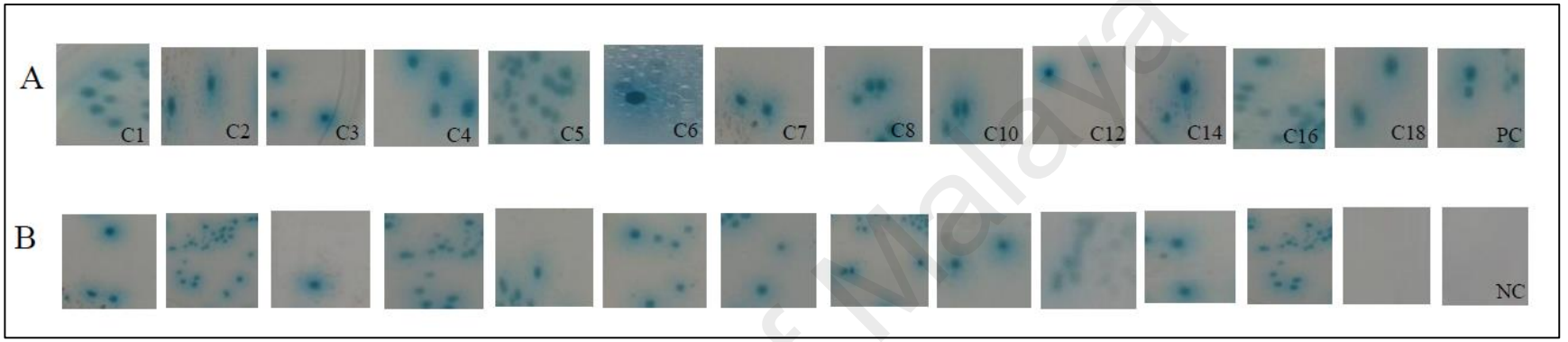



Figure 4.13: Confirmation of true positive clones interacting with Y2HGold(pGBKT7-SAG2) by small scale Y2H assay. Panel A: Zygotes formed following the mating between pGBKT7-SAG2 plasmid and the respective prey 1 to prey 18 plasmids. Panel B: Zygotes formed following the mating between pGBKT7 empty vector and the respective prey 1 to prey 18 plasmids. PC: Positive control which was zygotes formed following the mating between Y2HGold transformed with pGBKT7-53 and Y187 transformed with pGADT7-T vector. NC: Negative control with zygotes formed following the mating between Y2HGold transformed with pGBKT7 empty vector and Y187 transformed with pGADT7-T vector.

## 4.2 Beta-galactosidase assay

To further confirm interaction between SAG1 and SAG2 with their respective prey protein, beta-galactosidase assay was performed. A single colony from DDO plate (containing both bait and prey culture) was inoculated into Y-PER reagent.

Beta-galactosidase activity for Y2HGold(pGBKT7-SAG1)/Y187(pGADT7-RecAB-HLY) and Y2HGold(pGBKT7-SAG2)/Y187(pGADT7-RecAB-HZF) were 431.3 and 437.7 Unit, respectively. Meanwhile, beta-galactosidase activity for Y2HGold(pGBKT7-53)/Y187(pGADT7-T) (positive control) was 401.3 Unit (Table 4.4). The solution turned yellow at 25 and 22 mins for Y2HGold(pGBKT7-SAG1)/Y187(pGADT7-RecAB-HLY) and Y2HGold(pGBKT7-SAG2)/Y187(pGADT7-RecAB-HZF), respectively. However, the solution for positive control turned yellow at 23 mins.

## 4.3 Chemiluminescent Co-IP assay

### 4.3.1 Construction of pAcGFP1-C1-SAGs plasmids

#### 4.3.1.1 PCR amplification of *sag1* and *sag2* genes

*sag1* and *sag2* genes were amplified by using primers as indicated in Table 3.1. Figure 4.14 A and B indicate agarose gel electrophoresis for SAG1 and SAG2 fragments. The amplicons generated by SAG1 and SAG2 were ~750 bp and ~500 bp, respectively.

#### 4.3.1.2 Cloning of SAG1 and SAG2 fragments into pGEM-T vector system

By using M13 forward and reverse primer pairs, colony PCR was performed for 5 selected recombinant clones of pGEM-T-SAG1 and pGEM-T-SAG2 (Figure 4.15 A and B).

Table 4.4: Beta-galactosidase activity for SAG1 and SAG2 with their respective prey protein.

| Sample | OD 420 nm | OD 660 nm | Time (mins) | RLU (U) |
|---|---|---|---|---|
| Y2HGold(pGBKT7-SAG1)/Y187(pGADT7-RecAB-HLY) | 0.62 | 0.23 | 25 | 431.3 |
| Y2HGold(pGBKT7-SAG2)/Y187(pGADT7-RecAB-HZF) | 0.65 | 0.27 | 22 | 437.7 |
| Y2HGold(pGBKT7-53)/Y187(pGADT7-T) | 0.6 | 0.26 | 23 | 401.3 |

Note: Positive control, Y2HGold(pGBKT7-53)/Y187(pGADT7-T); Relative light unit, RLU; *Homo sapiens* lysine-rich coil-coiled protein, HLY; *Homo sapiens* zinc finger protein, HZF.

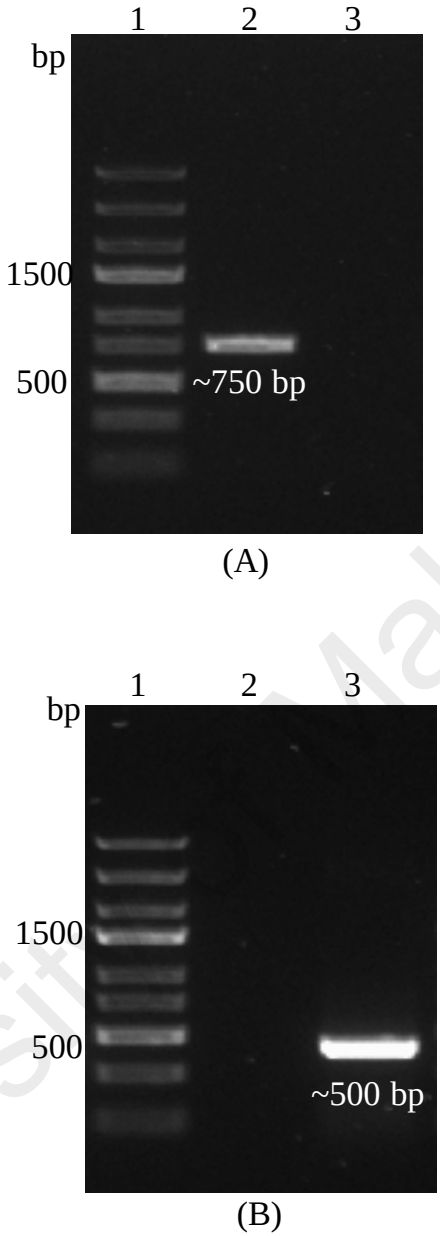



Figure 4.14: PCR amplification of *sag1* and *sag2* using gene specific primer pairs. The amplified DNA fragments were (A) ~750 bp for *T. gondii sag1* gene (lane 2) and (B) ~500 bp for *sag2* gene (lane 3), respectively. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 3 in panel (A) and lane 2 in panel (B) are negative control ($ddH_2O$).

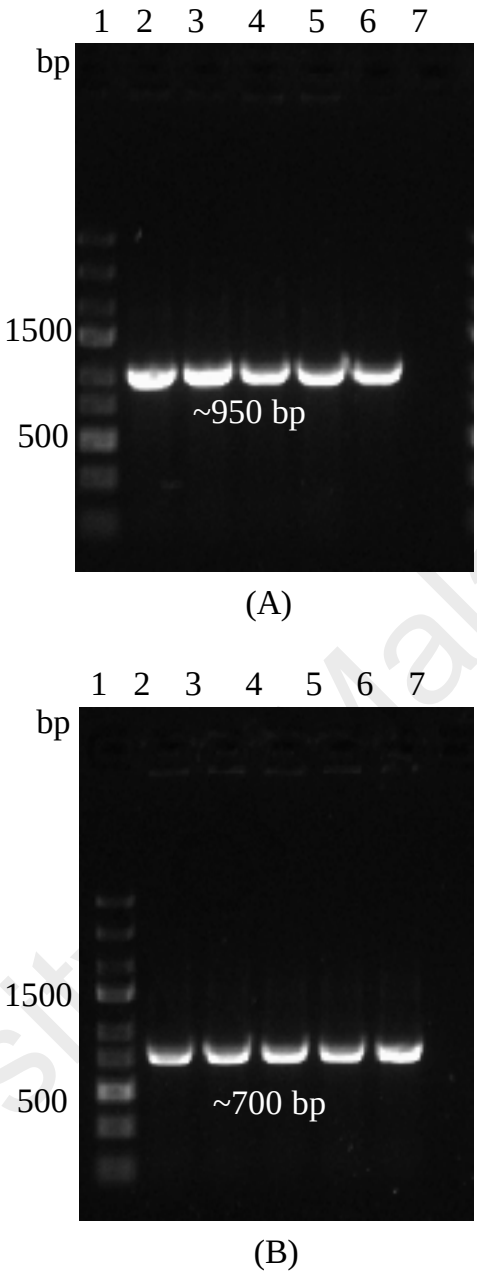



Figure 4.15: Colony PCR of recombinant pGEM-T-SAG1 and pGEM-T-SAG2 in TOP10F' cells. The amplified DNA fragments were (A) ~950 bp for positive pGEM-T-SAG1 clones (lanes 2 to 6) and (B) ~700 bp for positive pGEM-T-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

The amplicons generated by SAG1 and SAG2 PCR were ~950 bp (735 bp of SAG1 plus ~200 bp of vector) and ~700 bp (483 bp of SAG2 plus ~200 bp of vector), respectively. Recombinant pGEM-T-SAG1 and pGEM-T-SAG2 plasmids from positive clones were extracted and sent for sequencing. Nucleotide sequences were analysed by using BLAST analysis. Result showed that sequences of SAG1 and SAG2 were 99 and 100%, respectively similarity to the sequences available in GenBank (Appendix P and Q).

#### 4.3.1.3 Construction of pAcGFP1-C1-SAG1 and pAcGFP1-C1-SAG2 plasmids

Positive recombinant pGEM-T-SAG1 and pGEM-T-SAG2 plasmids were double digested with *SaI*I/*Hind*III and *Bam*HI, respectively. Both digestion mixtures were incubated at 37$^{o}$C for 6 h. Similarly, pAcGFP1-C1 vector was digested with same restriction enzymes and incubated at the same condition.

Complete digestion of recombinant pGEM-T-SAG1 plasmid generated a ~750 bp of SAG1 insert fragment and a ~3 kb pGEM-T plasmid (Figure 4.16 A), while complete digestion of recombinant pGEM-T-SAG2 generated a ~500 bp SAG2 insert fragment and a ~3 kb pGEM-T plasmid (Figure 4.16 B). Figure 4.16 B and D show completely digested pAcGFP1-C1 plasmid with a size of 4.7 kb.

Digested DNA fragments of SAG1 and SAG2 were excised from agarose gel and proceeded to gel purification procedure. Purified SAG1 and SAG2 fragments were ligated into digested pAcGFP1-C1 vector and then transformed into TOP10F' cells. Colony PCR was performed on 5 selected recombinant pAcGFP1-C1-SAG1 and pAcGFP1-C1-SAG2 clones, respectively using gene specific primers (Figure 4.17 A and B). PCR amplification of positive recombinant pAcGFP1-C1-SAG1 and pAcGFP1-C1-SAG2 clones generated expected band of ~750 bp and ~500 bp, respectively.

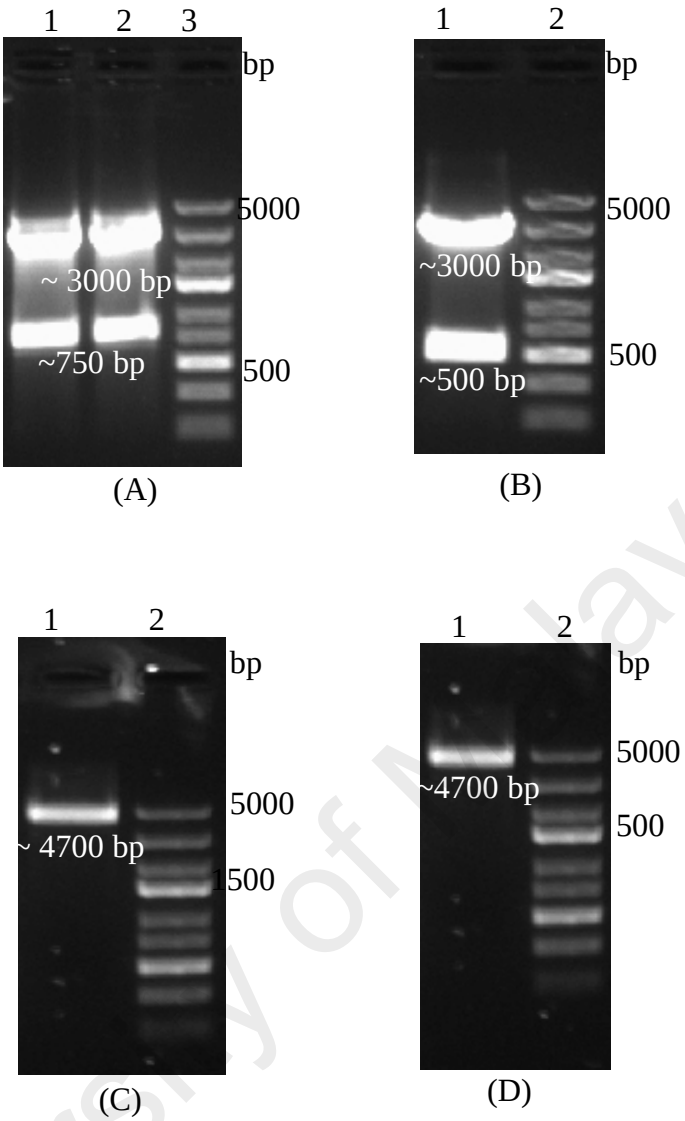



Figure 4.16: Restriction digestion of recombinant pGEM-T-SAG1, pGEM-T-SAG2 and pAcGFP1-C1 plasmids. (A) Double digested recombinant pGEM-T-SAG1 plasmid with *SaI*I and *Hind*III restriction enzymes (lanes 1 and 2). (B) Digested recombinant pGEM-T-SAG2 plasmid with *Bam*HI restriction enzymes (lane 1). (C) Double digested recombinant pAcGFP1-C1 plasmid with *SaI*I and *Hind*III resctritcion enzymes (lane 1). (D) Digested recombinant pAcGFP1-C1 plasmid with *Bam*HI resctritcion enzyme (lane 1). Lane 3 in panel (A), lane 2 in panel (B), (C) and (D) are 1 kb DNA ladder.

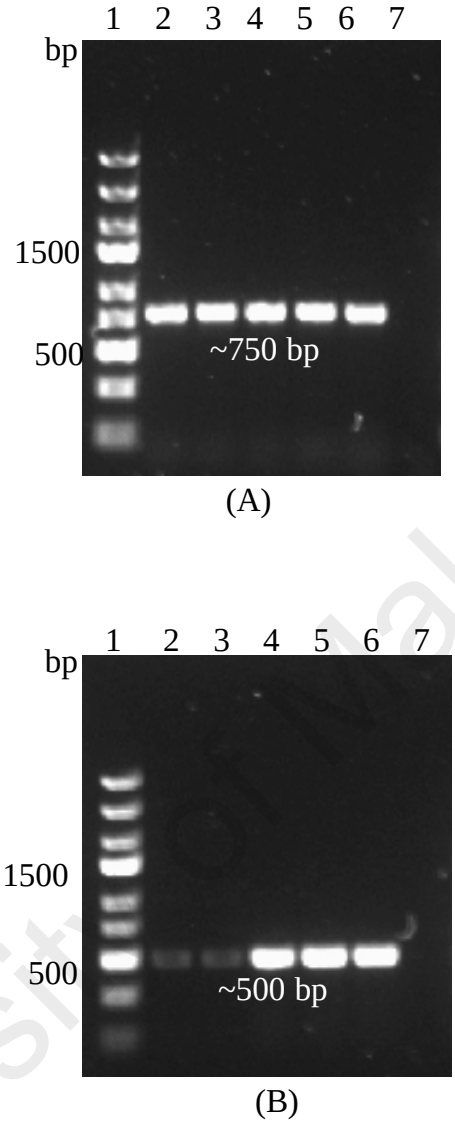



Figure 4.17: Colony PCR of recombinant pAcGFP1-C1-SAG1 and pAcGFP1-C1-SAG2 in TOP10F' cells. The amplified DNA fragments were (A) ~750 bp for positive pAcGFP1-C1-SAG1 clones (lanes 2 to 6) and (B) ~500 bp for positive pAcGFP1-C1-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

Plasmids from positive recombinant clones were extracted and sent for sequencing. Sequences of these amplicons were analyzed by BLAST. The SAG1 was found to be 99% similar to *T. gondii* major surface antigen P30 gene (Accession number X14080.1) (Appendix P). While SAG2 was found to be 100% similar to *T. gondii* strain RH surface antigen P22 (SAG2) mRNA, complete cds (Accession number FJ825705.1) (Appendix Q).

### 4.3.2 Construction of pProLabel-C-HLY and pProLabel-C-HZF plasmids

#### 4.3.2.1 PCR amplification of *hly* and *hzf* genes

*hly* and *hzf* were amplified with their respective primer pairs. Figure 4.18 A and B indicate the agarose gel electrophoresis for PCR amplification of *hly* (~600 bp) and *hzf* (~650 bp). The actual band size generated by HLY and HZF PCR were 564 and 627 bp, respectively.

#### 4.3.2.2 Cloning of HLY and HZF fragments into pGEM-T vector system

PCR products of HLY and HZF were purified and ligated into pGEM-T vector system. Overnight ligation mixtures were transformed into TOP10F' cells. Colony PCR amplification was performed by using M13 forward and reverse primer pairs (Figure 4.19 A and B). Five colonies from each of recombinant pGEM-T-SAG1 and pGEM-T-SAG2 in TOP10F' cells were selected for PCR. The amplicons generated by HLY and HZF were ~800 bp (564 bp of HLY plus ~200 bp of vector) and ~850 bp (627 bp of HZF plus ~200 bp of vector), respectively.

Plasmids from positive recombinant clones were extracted and sent for sequencing. Sequences of these amplicons were analyzed by BLAST. The HLY was found to be 100% similar to *Homo sapiens* lysine-rich coiled-coil 1, mRNA, complete CDS (Accession number BC107580.1) (Appendix R). While HZF was found to be 100%

similar to *Homo sapiens* zinc finger AN1-type containing 6 (ZFAND6), transcript variant 5, mRNA (Accession number NM_001242914.1) (Appendix S).

#### 4.3.2.3 Ligation of HLY and HZF fragments into pProLabel-C plasmids

Positive recombinant pGEM-T-HLY and pGEM-T-HZF plasmids were double digested with *SaI*I and *Bam*HI, respectively. Both digestion mixtures were incubated at 37ºC for 6 h. Similarly, pProLabel-C vector was digested with same restriction enzymes and incubated at the same condition.

Complete digestion of recombinant pGEM-T-HLY plasmid generated a ~600 bp of HLY insert fragment and a ~3 kb pGEM-T plasmid (Figure 4.20 A), while complete digestion of recombinant pGEM-T-HZF generated a ~650 bp HZF fragment and a ~3 kb pGEM-T plasmid (Figure 4.20 B). Figure 4.20 C show completely digested pProLabel-C plasmid with a size of ~4.1 kb.

Digested DNA fragments of HLY and HZF were excised from agarose gel and proceeded to gel purification procedure. Purified HLY and HZF fragments were ligated into digested pProLabel-C vector and then transformed into TOP10F' cells. Colony PCR was performed on 5 selected recombinant clones from each of pProLabel-C-HLY and pProLabel-C-HZF using gene specific primers (Figure 4.21 A and B). PCR amplification of positive recombinant pProLabel-C-HLY and pProLabel-C-HZF clones generated expected band of ~600 bp and ~650 bp, respectively.

Plasmids from positive recombinant clones were extracted and sent for sequencing. Sequences of these amplicons were analyzed by BLAST. The HLY was found to be 100% similar to *Homo sapiens* lysine-rich coiled-coil 1, mRNA, complete CDS (Accession number BC107580.1) (Appendix R). While HZF was found to be 100% similar to *Homo sapiens* zinc finger AN1-type containing 6 (ZFAND6), transcript variant 5, mRNA (Accession number NM_001242914.1) (Appendix S).

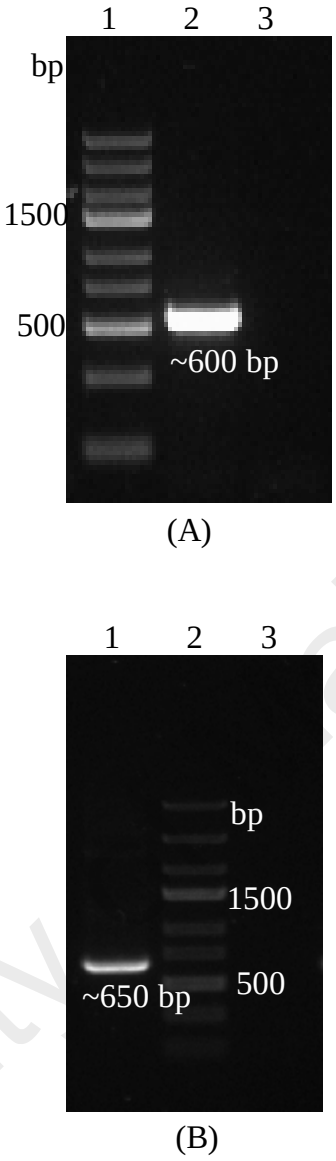
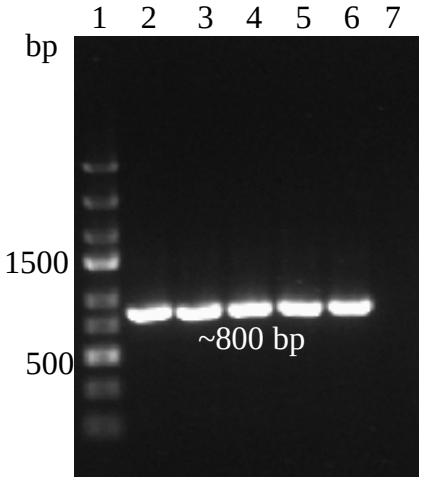
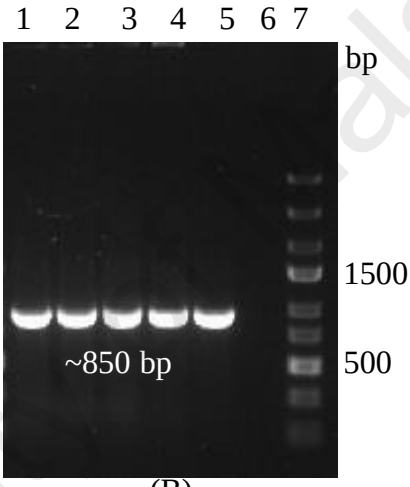



Figure 4.18: PCR amplification of *hly* and *hzf* genes using gene specific primer pairs. The amplified DNA fragments were (A) ~600 bp for *hly* gene (lane 2) and (B) ~650 bp for *hzf* gene (lane 1), respectively. Lane 1 in panel (A) and lane 2 in panel (B) are 1 kb DNA ladder. Lane 3 both in panel (A) and (B) are negative control (dd$H_2O$).




(A)




(B)

Figure 4.19: Colony PCR of recombinant pGEMT-HLY and pGEMT-HZF in TOP10F' cells. The amplified DNA fragments were (A) ~800 bp for positive pGEMT-HLY clones (lanes 2 to 6) and (B) ~850 bp for positive pGEMT-HZF clones (lanes 1 to 5), respectivey. Lane 1 in panel (A) and lane 7 in panel (B) are 1 kb DNA ladder. Lane 7 in panel (A) and lane 6 in panel (B) are negative control ($ddH_2O$).

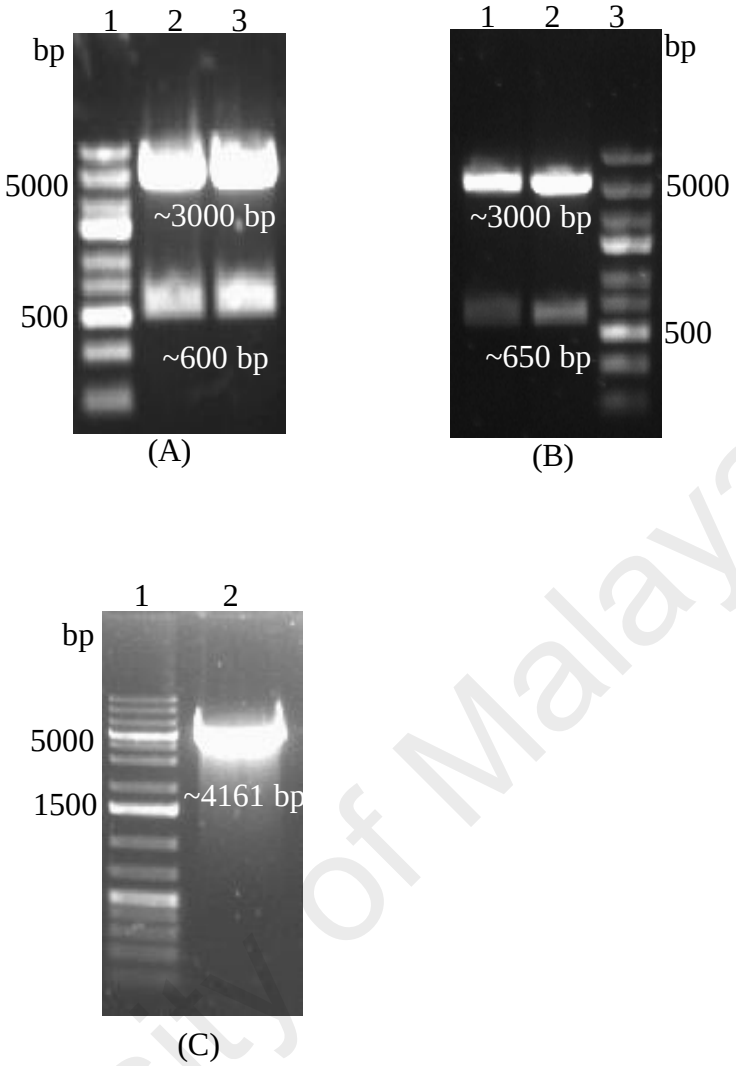
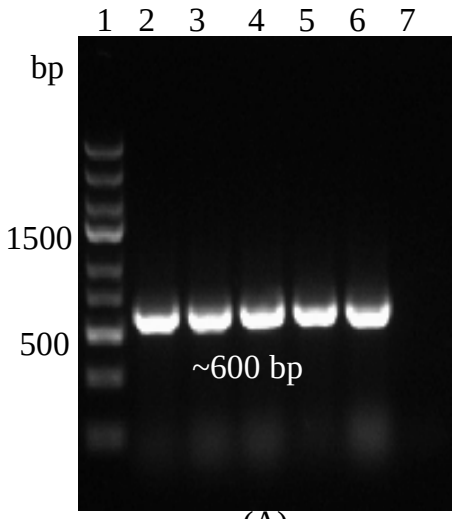
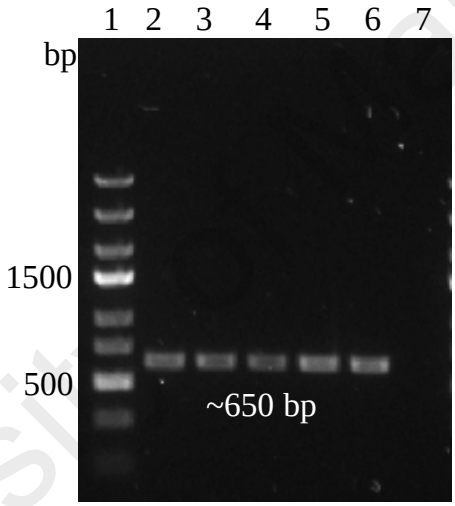



Figure 4.20: Restriction digestion of recombinant pGEM-T-HLY, pGEM-T-HZF and pProLabel-C vector. (A) Double digested recombinant pGEM-T-HLY plasmid with *Sal*I and *Bam*HI restriction enzymes (lanes 2 and 3). (B) Double digested pGEM-T-HZF plasmid with *Sal*I and *Bam*HI restriction enzymes (lanes 1 and 2). (C) Double digested pProLabel-C plasmid with *Sal*I and *Bam*HI restriction enzymes (lanes 2). Lane 1 in both panel (A) and (C), and lane 3 in panel (B) are 1 kb DNA ladder.




(A)




(B)

Figure 4.21: Colony PCR of recombinant pProLabel-C-HLY and pProLabel-C-HZF in TOP10F' cells. The amplified DNA fragments were (A) ~600 bp for positive pProLabel-C-HLY clones (lanes 2 to 6) and (B) ~650 bp for positive pProLabel-C-HZF clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negtaive control ($ddH_2O$).

### 4.3.3 Transfection into HEK 293 mammalian cells

In order to detect interaction, recombinant pAcGFP1-C1-SAG1/pProLabel-C-HLY and pAcGFP1-C1-SAG2/pProLabel-C-HZF plasmids were co-transfected into HEK 293 mammalian cells and incubated at 37$^{o}$C in $CO_2$ incubator. On the next day, successfully transfected cells presented green colour under the light microscope (Figure 4.22). Cells were harvested after 72 h. Cell lysates were further examined by pProLabel enzymatic assay. Figure 4.23 and 4.24 indicate that the binding between these prey and SAG1 and SAG2 proteins were significant (Mann-Whitney U test, pAcGFP1-C1-SAG1/pProLabel-C-HLY, Z= -1.964, P=0.05; pAcGFP1-C1-SAG2/pProLabel-C-HZF, Z=-1.964, P≤0.05).

Negative controls for this experiment include: co-transfection of pAcGFP1-Lam/pProLabel-T, pAcGFP1-C1-SAG1/pProLabel-C, pAcGFP1-C1-SAG2/pProLabel-C, pProLabel-C-HLY/pAcGFP1-C1 and pProLabel-C-HZF/pAcGFP1-C1 vectors into HEK 293 mammalian cells. Also, a cell lysate from non-transfected cells was included. The averaged value obtained for non-transfected cells was subtracted from each experimental result.

## 4.4 Isothermal titration calorimetry experiment (ITC)

### 4.4.1 Construction of pRSET-A-SAGs and pRSET-A-prey plasmids

#### 4.4.1.1 PCR amplification of *sag1*, *sag2*, *hly* and *hzf* genes

*sag1* and *sag2* were amplified with their respective primer pairs. Figure 4.25 A and B indicate agarose gel electrophoresis for PCR amplification of *sag1* (~750 bp) and *sag2* (~500 bp). The actual band size generated by SAG1 and SAG2 PCR were 735 and 483 bp, respectively.

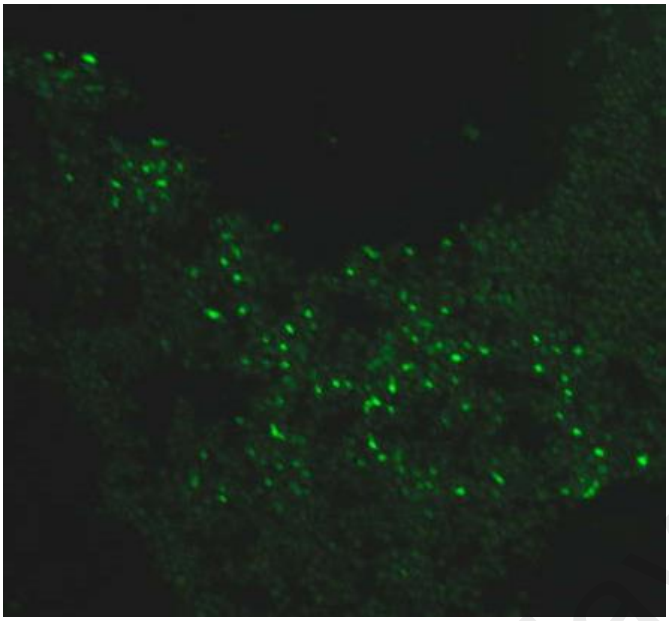


Figure 4.22: Transfected HEK 293 mammalian cells with recombinant plasmid.

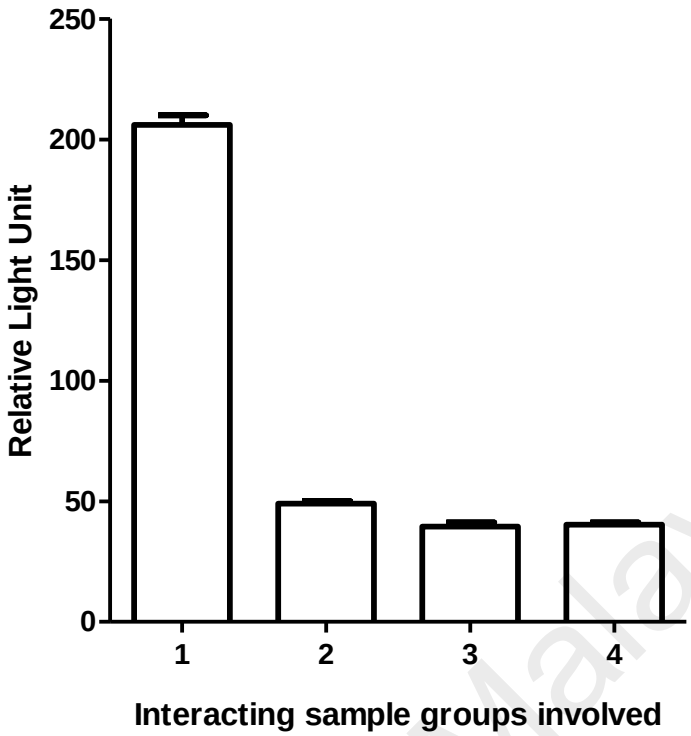



Figure 4.23: Detection of chemiluminescent interaction for recombinant pAcGFP1-C1-SAG1/pProLabel-C-HLY proteins. Based on Mann-Whitney test, the RLU reading of interaction for recombinant pAcGFP1-C1-SAG1/pProLabel-C-HLY proteins was found to be significantly different when compared to recombinant pAcGFP1-SAG1/pProLabel-C proteins (Mann-Whitney U test, Z= -1.964, P=0.05). Group 1: interaction for recombinant pAcGFP1-C1-SAG1/pProLabel-C-HLY proteins; Group 2: interaction for recombinant pAcGFP1-Lam/pProLabel-T proteins; Group 3: interaction for recombinant pAcGFP1-C1-SAG1/ProLabel-C proteins; Group 4: interaction for recombinant pProLabel-C-HLY/pAcGFP1-C1 proteins; HLY: *Homo sapiens* lysine-rich coiled-coil protein.

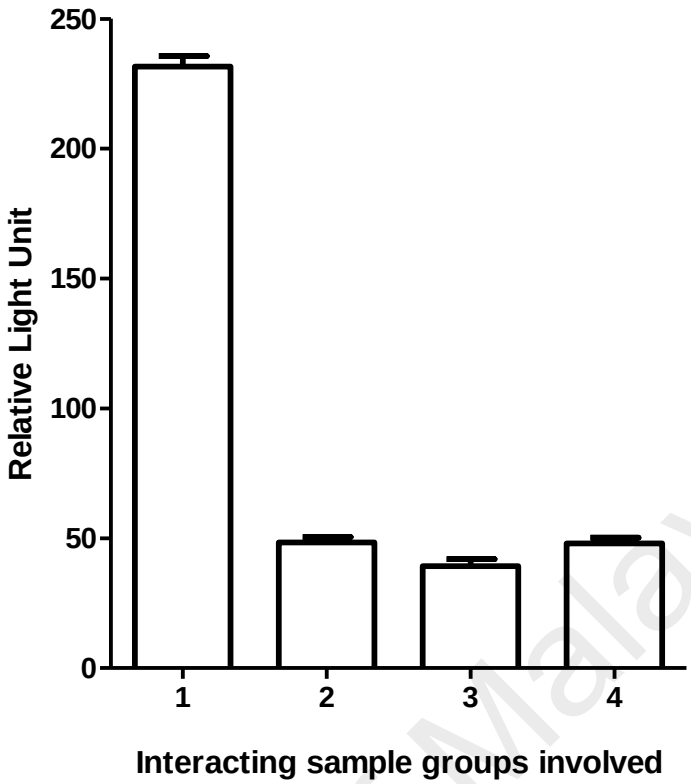



Figure 4.24. Detection of chemiluminescent interaction for recombinant pAcGFP1-C1-SAG2/pProLabel-C-HZF proteins. Based on Mann-Whitney test, the RLU reading for interaction of recombinant pAcGFP1-C1-SAG2/pProLabel-C-HZF proteins was found to be significantly different when compared to recombinant pAcGFP1-C1-SAG2/pProLabel-C proteins (Mann-Whitney U test, Z= -1.964, P≤0.05). Group 1: interaction for recombinant pAcGFP1-C1-SAG2/pProLabel-C-HZF proteins; Group 2: interaction for recombinant pAcGFP1-Lam/pProLabel-T proteins; Group 3: interaction for recombinant pAcGFP1-C1-SAG2/ProLabel-C proteins; Panel 4: interaction for recombinant pProLabel-C-HZF/pAcGFP1-C1 proteins; HZF: *Homo sapiens* zinc finger protein.

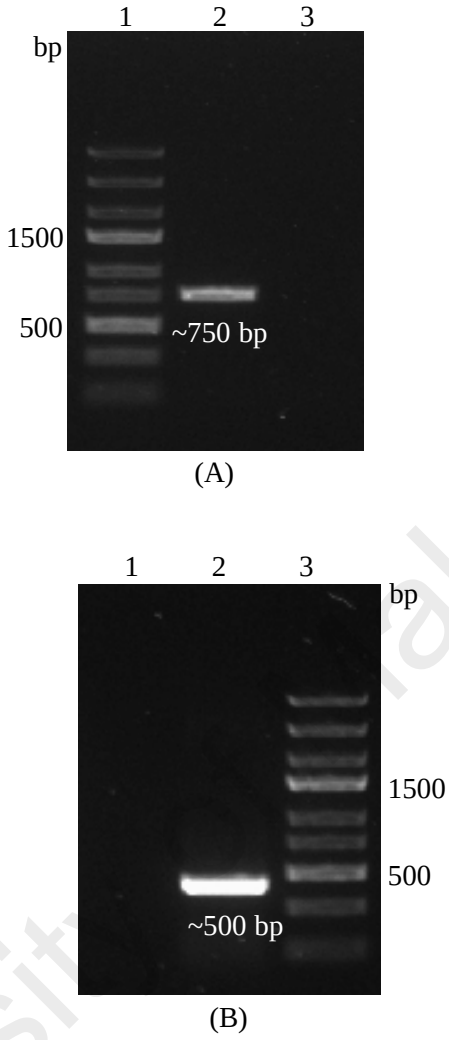



Figure 4.25: PCR amplification of *sag1* and *sag2* genes using newly designed primers. The amplified DNA fragments were (A) ~750 bp for *T. gondii sag1* gene (lane 2) and (B) ~500 bp for *sag2* gene (lane 2), respectively. Lane 1 in panel (A) and lane 3 in panel (B) are 1 kb DNA ladder. Lane 3 in panel (A) and lane 1 in panel (B) are negative control ($ddH_2O$).

Both of *hly* and *hzf* were amplified with their respective primer pairs. Figure 4.26 A and B indicate agarose gel electrophoresis for PCR amplification of *hly* (~600 bp) and *hzf* (~650 bp). The actual band size generated by HLY and HZF PCR were 564 and 627 bp, respectively.

#### 4.4.1.2 Cloning of SAG1, SAG2, HLY, HZF fragments into pGEM-T vector system

PCR products of SAG1, SAG2, HLY and HZF were purified and ligated into pGEM-T vector system. The overnight ligation mixtures were transformed into TOP10F' cells. Colony PCR amplification was performed by using M13 forward and reverse primer pairs (Figure 4.27 A, B and Figure 4.28 A and B). Five colonies from each of recombinant pGEM-T-SAG1, pGEM-T-SAG2, pGEM-T-HLY and pGEM-T-HZF in TOP10F' cells were selected for PCR. The amplicons generated by SAG1, SAG2, HLY and HZF PCR were ~950 bp (735 bp of SAG1 plus ~200 bp of vector), ~700 bp (483 bp of SAG2 plus ~200 bp of vector), ~800 bp (564 bp of HLY plus ~200 bp of vector) and ~850 bp (627 bp of HZF plus ~200 bp vector), respectively.

Plasmids from positive recombinant clones were extracted and sent for sequencing. Sequences of these amplicons were analyzed by BLAST. The SAG1 was found to be 99% similar to *T. gondii* major surface antigen P30 gene (Accession number X14080.1) (Appendix P). While SAG2 was found to be 100% similar to *T. gondii* strain RH surface antigen P22 (SAG2) mRNA, complete cds (Accession number FJ825705.1) (Appendix Q). The HLY was found to be 100% similar to *Homo sapiens* lysine-rich coiled-coil 1, mRNA, complete CDS (Accession number BC107580.1) (Appendix R). While HZF was found to be 100% similar to *Homo sapiens* zinc finger AN1-type containing 6 (ZFAND6), transcript variant 5, mRNA, (Accession number NM_001242914.1) (Appendix S).

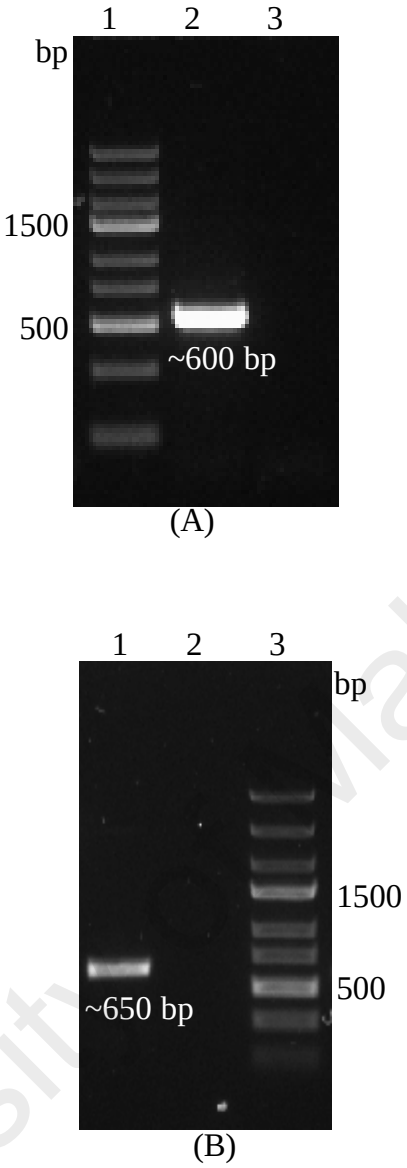



Figure 4.26: PCR amplification of *hly* and *hzf* genes using newly designed primers. The amplified DNA fragments were (A) ~600 bp for *hly* gene (lane 2) and (B) ~650 bp for *hzf* gene (lane 1), respectively. Lane 1 in panel (A) and lane 3 in panel (B) are 1 kb DNA ladder. Lane 3 in panel (A) and lane 2 in panel (B) are negative control ($ddH_2O$).

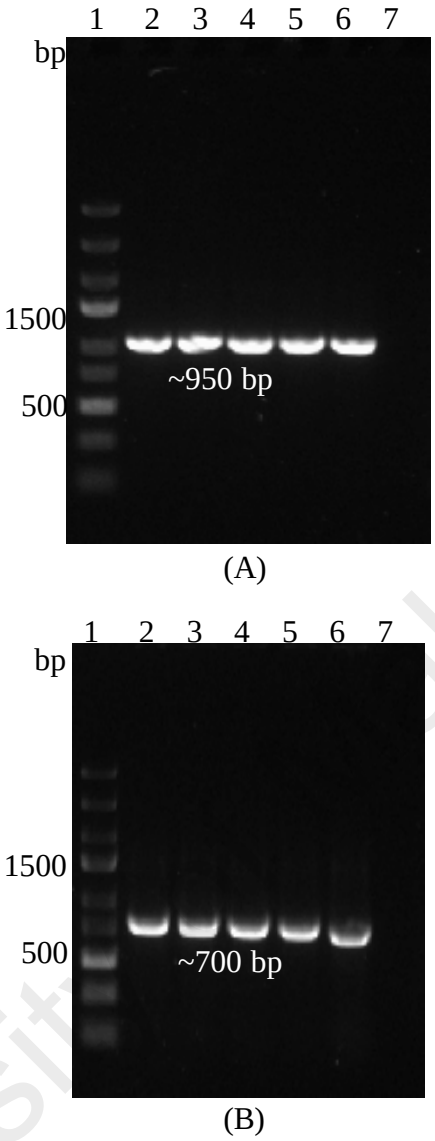



Figure 4.27: Colony PCR of recombinant pGEM-T-SAG1 and pGEM-T-SAG2 in TOP10F' cells. The amplified DNA fragments were (A) ~950 bp for positive pGEM-T-SAG1 clones (lanes 2 to 6) and (B) ~700 bp for positive pGEM-T-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

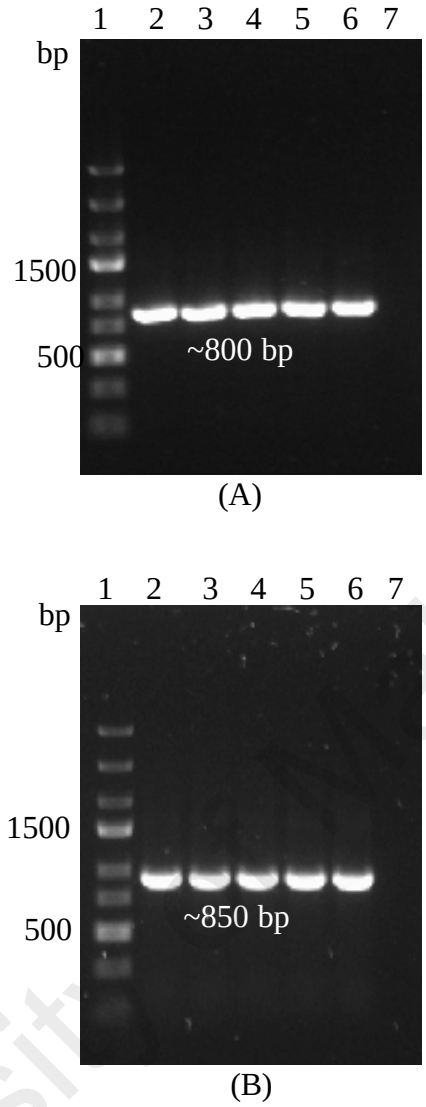



Figure 4.28: Colony PCR of recombinant pGEM-T-HLY and pGEM-T-HZF in TOP10F' cells. The amplified DNA fragments were (A) ~800 bp for positive pGEM-T-HLY clones (lanes 2 to 6) and (B) ~850 bp for positive pGEM-T-HZF clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

#### 4.4.1.3 Digestion of fragments and pRSET-A vector

Complete digestion of recombinant pGEM-T-SAG1 plasmid generated a ~750 bp of SAG1 insert fragment and a ~3 kb pGEM-T plasmid (Figure 4.29 A), while complete digestion of recombinant pGEM-T-SAG2 generated a ~500 bp SAG2 fragment and a ~3 kb pGEM-T plasmid (Figure 4.29 C). Figure 4.29 B show completely digested pRSET-A plasmid with a size of ~2.89 kb.

Complete digestion of recombinant pGEM-T-HLY plasmid generated a ~600 bp of HLY insert fragment and a ~3 kb pGEM-T plasmid (Figure 4.30 A), while complete digestion of recombinant pGEM-T-HZF generated a ~650 bp HZF fragment and a ~3 kb pGEM-T plasmid (Figure 4.30 B). Figure 4.30 C show completely digested pRSET-A plasmid with a size of ~2.89 kb.

#### 4.4.1.4 Ligation of digested fragments into pRSET-A vector

Digested DNA fragments of SAG1, SAG2, HLY and HZF were excised from agarose gel and proceeded to gel purification procedure. Purified SAG1, SAG2, HLY and HZF fragments were ligated into digested pRSET-A vector and then transformed into TOP10F' cells. Colony PCR was performed on 5 selected recombinant clones from each of pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF using gene specific primers (Figure 4.31 A, B and Figure 4.32 A and B). PCR amplification of positive recombinant pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF clones generated expected PCR band of ~900 bp (735 bp of SAG1 fragment plus ~150 bp of vector), ~650 bp (483 bp of SAG2 fragment plus ~150 bp of vector), ~750 bp (564 bp of HLY fragment plus ~150 bp of vector), and ~800 bp (627 bp of HZF fragment plus ~150 bp of vector), respectively.

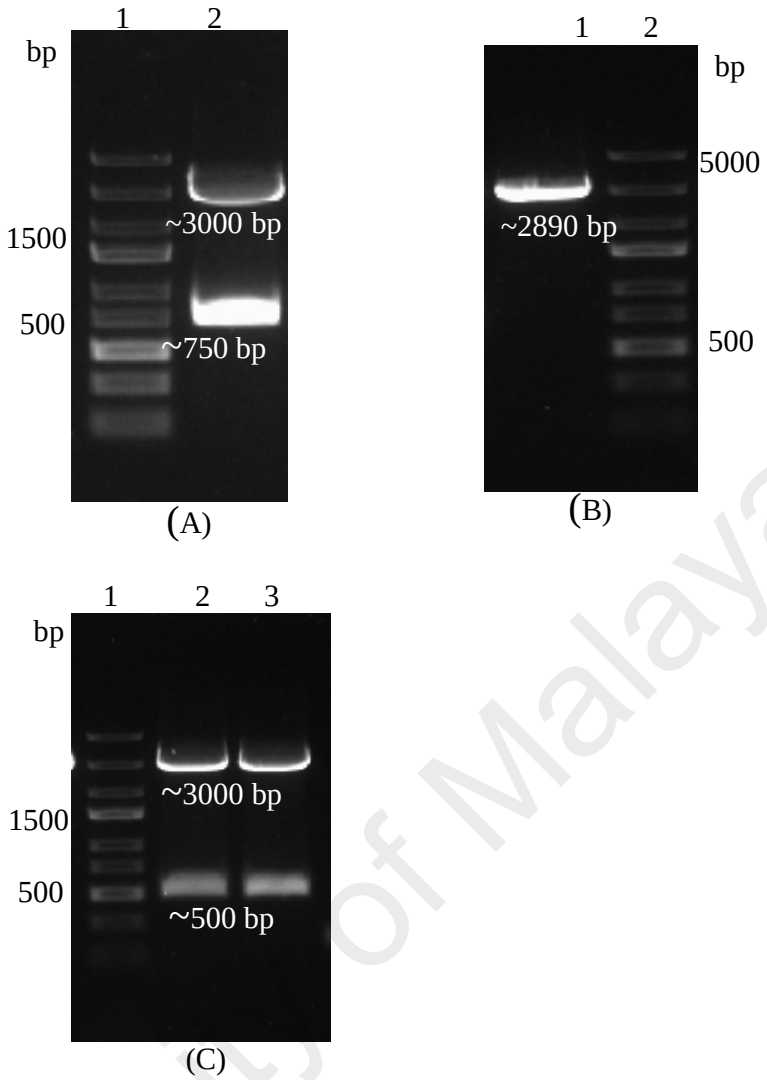



Figure 4.29: Restriction digestion of recombinant pGEM-T-SAG1, pGEM-T-SAG2 and pRSET-A plasmid. (A) Digested recombinant pGEM-T-SAG1 plasmid with *Bam*HI restriction enzymes (lanes 2 and 3). (B) Digested pRSET-A plasmid with *Bam*HI restriction enzyme (lane 1). (C) Digested pGEM-T-SAG2 plasmid with *Bam*HI restriction enzymes (lanes 2 and 3). Lane 1 in both panel (A) and (C) and lane 2 in panel (B) are 1 kb DNA ladder.

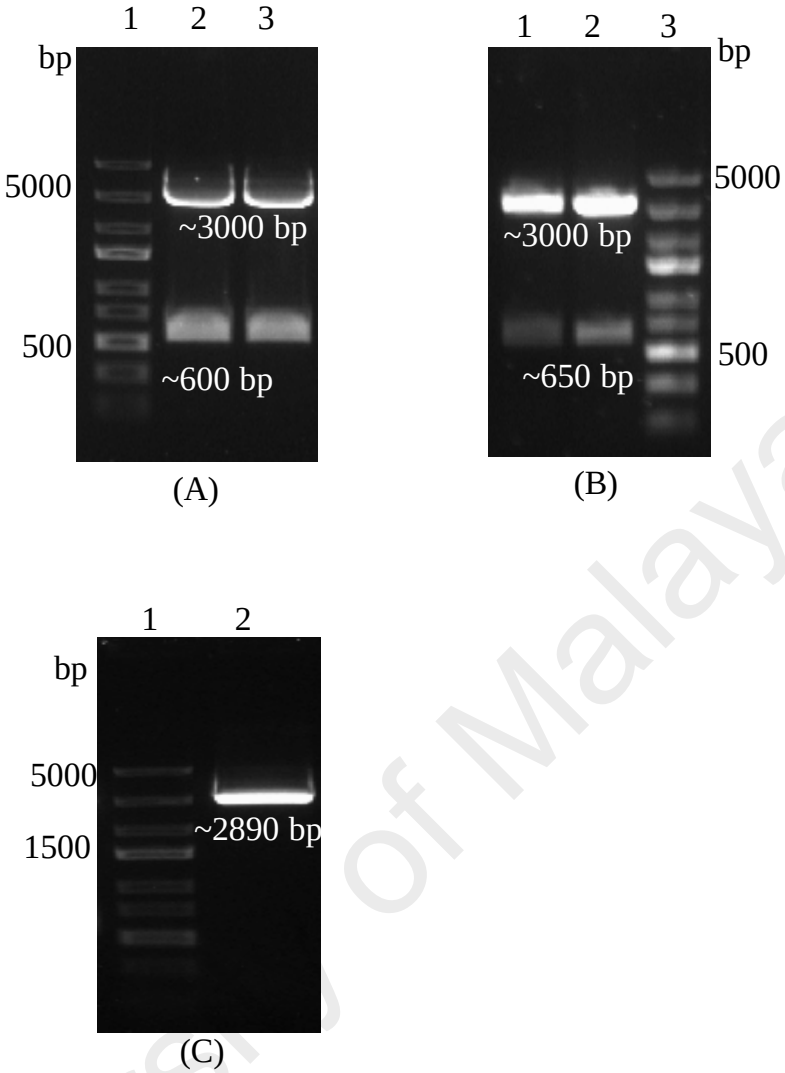
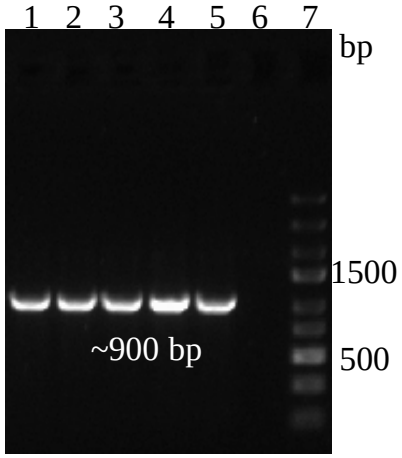
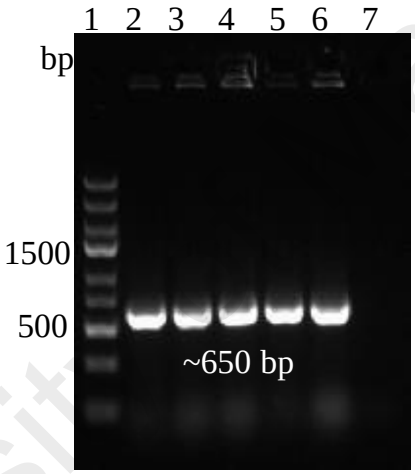



Figure 4.30: Restriction digestion of recombinant pGEM-T-HLY, pGEMT-HZF and pRSET-A vector. (A) Digested recombinant pGEM-T-HLY plasmid with *Bam*HI restriction enzymes (lanes 2 and 3). (B) Digested pGEM-T-HZF plasmid with *Bam*HI restriction enzymes (lanes 1 and 2). (C) Digested pRSET-A plasmid with *Bam*HI restriction enzyme (lane 1). Lane 1 in both panel (A) and (C) and lane 3 in panel (B) are 1 kb DNA ladder.

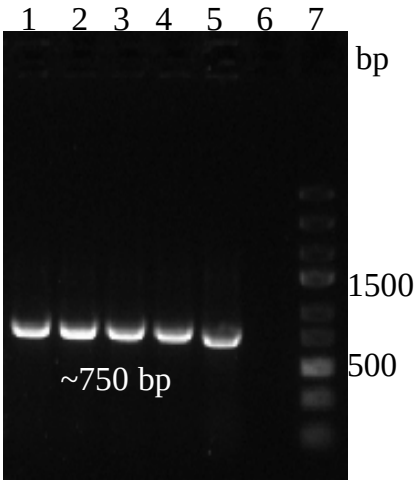



(A)

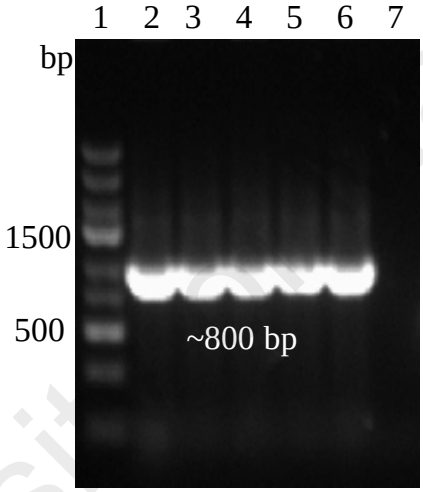



(B)

Figure 4.31: Colony PCR of recombinant pRSET-A-SAG1 and pRSET-A-SAG2 in TOP10F' cells. The amplified DNA fragments were (A) ~900 bp for positive pRSET-A-SAG1 clones (lanes 1 to 5 ) and (B) 650 bp for positive pRSET-A-SAG2 clones (lanes 2 to 6), respectivey. ). Lane 7 in panel (A) and lane 1 in panel (B) are 1 kb DNA ladder. Lane 6 in panel (A) and lane 7 in panel (B) are negative control (dd$H_2O$).




(A)




(B)

Figure 4.32: Colony PCR of recombinant pRSET-A-HLY and pRSET-A-HZF in TOP10F' cells. The amplified DNA fragments were (A) ~750 bp for positive pRSET-A-HLY clones (lanes 1 to 5) and (B) ~800 bp for positive pRSET-A-HZF clones (lanes 2 to 6), respectivey. Lane 7 in panel (A) and lane 1 in panel (B) are 1 kb DNA ladder. Lane 6 in panel (A) and lane 7 in panel (B) are negative control ($ddH_2O$).

Plasmids from positive recombinant clones were extracted and sent for sequencing. Sequences of these amplicons were analyzed by BLAST. The SAG1 was found to be 99% similar to *T. gondii* major surface antigen P30 gene (Accession number X14080.1) (Appendix P). While SAG2 was found to be 100% similar to *T. gondii* strain RH surface antigen P22 (SAG2) mRNA, complete cds (Accession number FJ825705.1) (Appendix Q). The HLY was found to be 100% similar to *Homo sapiens* lysine-rich coiled-coil 1, mRNA, complete CDS (Accession number BC107580.1) (Appendix R). While HZF was found to be 100% similar to *Homo sapiens* zinc finger AN1-type containing 6 (ZFAND6), transcript variant 5, mRNA, (Accession number NM_001242914.1) (Appendix S).

#### 4.4.1.5 Transformation into *E. coli* BL21 (DE3) expression host

Following sequencing result, the constructs of pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF with insert in the sense orientation were extracted and transformed into *E. coli* BL21 (DE3) expression host cells. By using T7 promoter and gene specific reverse primer pair, colony PCR was performed on five selected recombinant clones from each of the constructs. PCR of the clones with insert generated an expected PCR band of ~900 bp (735 bp of SAG1 fragment plus ~150 bp of vector), ~650 bp (483 bp of SAG2 fragment plus ~150 bp of vector), ~750 bp (564 bp of HLY fragment plus ~150 bp of vector) and ~800 bp (627 bp of HZF fragment plus ~150 bp of vector (Figure 4.33 A, B and 4.34 A, B).

Plasmids from positive recombinant clones were extracted and sent for sequencing. Sequences of these amplicons were analyzed by BLAST. The SAG1 was found to be 99% similar to *T. gondii* major surface antigen P30 gene (Accession number X14080.1) (Appendix P). While SAG2 was found to be 100% similar to *T. gondii* strain RH surface antigen P22 (SAG2) mRNA, complete cds (Accession number FJ825705.1)

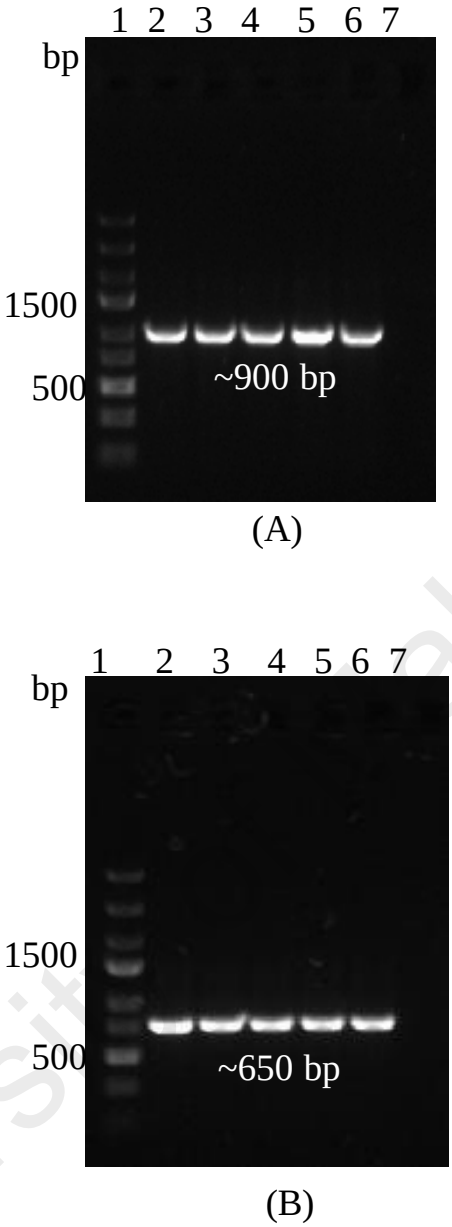



Figure 4.33: Colony PCR of recombinant pRSET-A-SAG1 and pRSET-A-SAG2 in *E. coli* BL21 (DE3) cells. The amplified DNA fragments were (A) ~900 bp for positive pRSET-A-SAG1 clones (lanes 2 to 6) and (B) ~650 bp for positive pRSET-A-SAG2 clones (lanes 2 to 6), respectivey. Lane 1 in both panel (A) and (B) are 1 kb DNA ladder. Lane 7 in both panel (A) and (B) are negative control ($ddH_2O$).

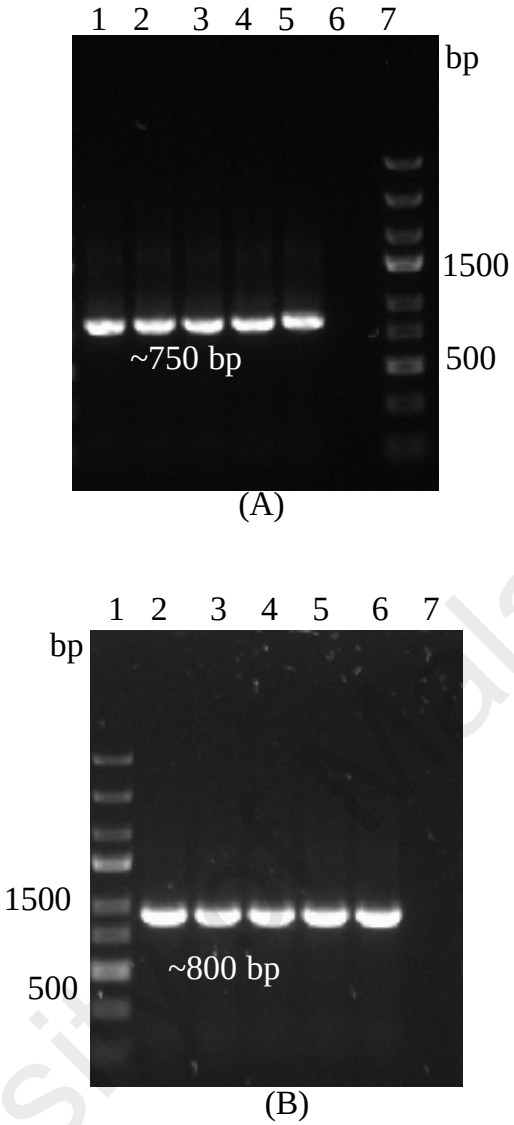



Figure 4.34: Colony PCR of recombinant pRSET-A-HLY and pRSET-A-HZF in *E. coli* BL21 (DE3) cells. The amplified DNA fragments were (A) ~750 bp for positive pRSET-A-HLY clones (lanes 1 to 5) and (B) ~800 bp for positive pRSET-A-HZF clones (lanes 2 to 6), respectivey. Lane 7 in panel (A) and lane 1 in panel (B) are 1 kb DNA ladder. Lane 6 in panel (A) and lane 7 in panel (B) are negative control ($ddH_2O$).

(Appendix Q). The HLY was found to be 100% similar to *Homo sapiens* lysine-rich coiled-coil 1, mRNA, complete CDS (Accession number BC107580.1) (Appendix R). While HZF was found to be 100% similar to *Homo sapiens* zinc finger AN1-type containing 6 (ZFAND6), transcript variant 5, mRNA, (Accession number NM_001242914.1) (Appendix S).

### 4.4.2 Protein expression

A single colony was picked and inoculated into 10 ml LB broth. The cultures were allowed to grow at 37$^{o}$C, 250 rpm and induced with 1 mM IPTG for 4 h. One ml of the harvested culture was collected at each time point (0, 2 and 4 h) and pelleted before loading on the 12% SDS PAGE. Negative control sample included a strain carrying the pRSET-A plasmid only. This negative control provided the background native proteins of *E. coli* BL21 (DE3) and those encoded by the vector. Inclusion of these control samples helped to differentiate the novel recombinant proteins from the native ones.

SDS-PAGE analysis revealed appearance of novel proteins 32, 23 and 28 kDa (expected size plus 6 kDa 6X His tag) for recombinant pRSET-A-SAG1, recombinant pRSET-A-SAG2 and pRSET-A-HLY (Figure 4.35 A, B and 4.36 A). Size of pRSET-A-HZF was larger with the additionally of 7 kDa as the result of posttranslational modification (Figure 4.36 B). These bands were absent in the negative controls. Qualitative estimation based on the intensity of the bands in the gel revealed that the expression peaked at 4 h. This time interval was used as a guide when growth and expression was carried out in a larger volume of culture. Meanwhile, the expressed proteins were further confirmed in Western blot by using anti-His monoclonal antibody (Figure 4.37 A, B and 4.38). Figure 4.37 and 4.38 indicate a gel image for non-purified recombinant protein. Molecular weight of each recombinant protein was correctly detected at their respective size.

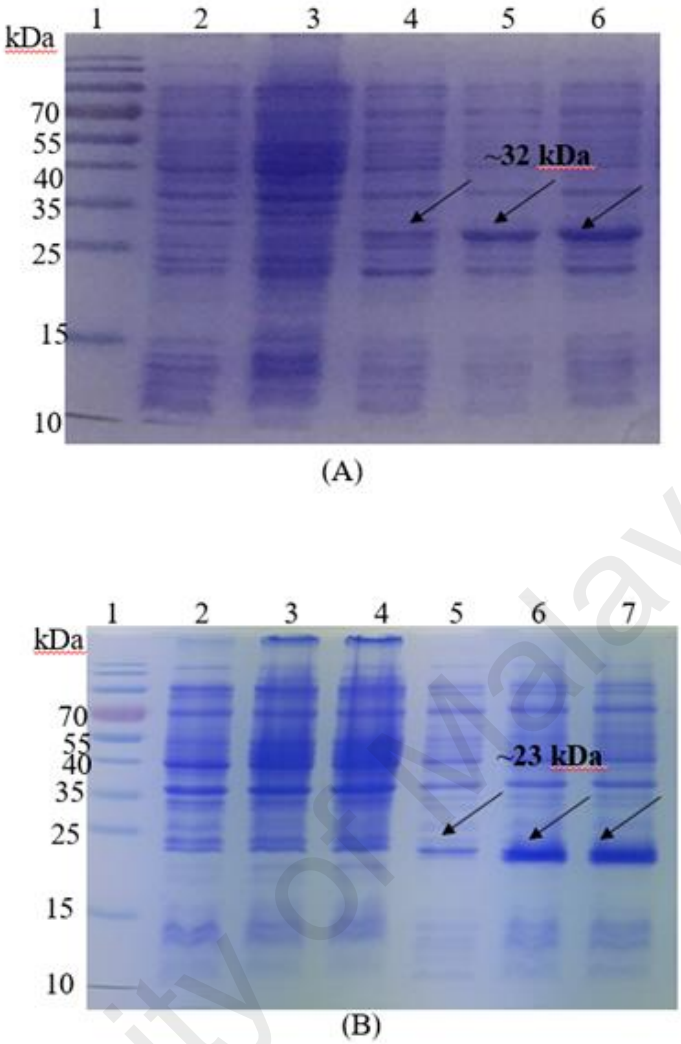
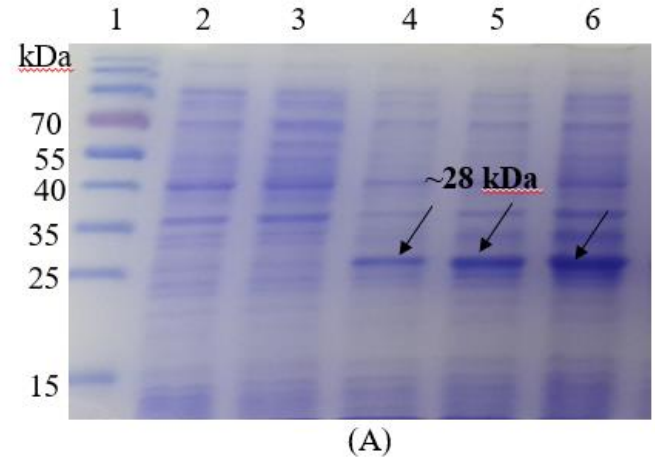
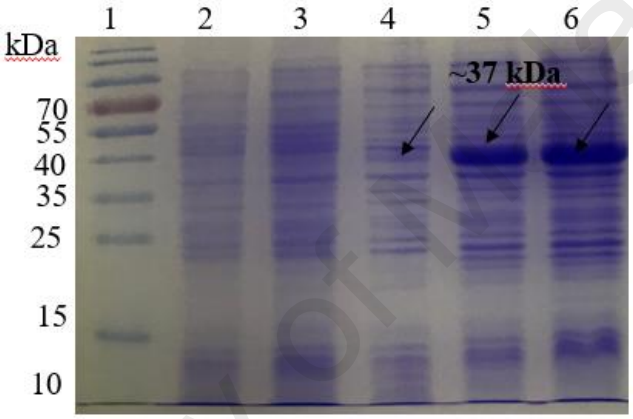



Figure 4.35: SDS-PAGE analysis of the expression of recombinant pRSET-A-SAG1 and pRSET-A-SAG2 proteins in BL21 (DE3) cells. (A) Expression of recombinant pRSET-A-SAG1 protein at 0, 2 and 4 h (lanes 4, 5 and 6). Size of recombinant pRSET-A-SAG1 protein was detected at ~32 kDa. (B) Expression of recombinant pRSET-A-SAG2 protein at 0, 2 and 4 h (lanes 5, 6 and 7). Size of recombinant pRSET-A-SAG2 protein was detected at ~23 kDa. Lanes 2 and 3 in panel (A) indicates protein expression of pRSET-A empty vector at 2 and 4 h. Lanes 2, 3 and 4 in panel (B) indicates protein expression pf pRSET-A empty vector at 0, 2 and 4 h. Lane 1 in both panel (A) and (B) are PageRuler Prestained Protein Ladder.




(A)




(B)

Figure 4.36: SDS-PAGE analysis of the expression of recombinant proteins pRSET-A-HLY and pRSET-A-HZF in BL21 (DE3) cells. (A) Expression of recombinant HLY protein at 0, 2 and 4 h (lanes 4, 5 and 6). ). Size of recombinant pRSET-A-HLY protein was detected at ~28 kDa. (B) Expression of recombinant HZF protein at 0, 2 and 4 h (lanes 4, 5 and 6). Size of recombinant pRSET-A-HZF protein was detected at ~37 kDa. Lanes 2 and 3 in panel (A) indicates protein expression of pRSET-A empty vector at 2 and 4 h. Lanes 2 and 3 in panel (B) indicates protein expression of pRSET-A empty vector at 0 and 4 h. Lane 1 in both panel (A) and (B) are PageRuler Prestained Protein Ladder.

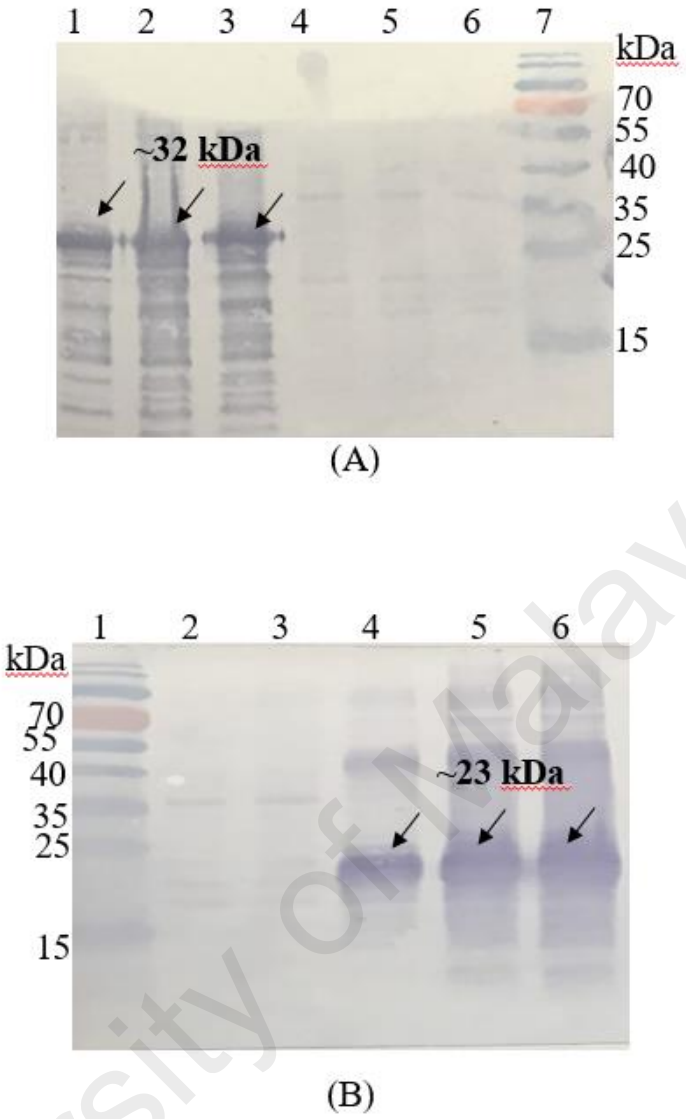



Figure 4.37: Western blot analysis of the expression of recombinant pRSET-A-SAG1 and pRSET-A-SAG2 proteins in BL21 (DE3) cells. (A) Expression of recombinant pRSET-A-SAG1 protein at 0, 2 and 4 h (lanes 1, 2 and 3). Size of recombinant pRSET-A-SAG1 protein was detected at ~32 kDa. (B) Expression of recombinant pRSET-A-SAG2 protein at 0, 2 and 4 h (lanes 4, 5 and 6). Size of recombinant pRSET-A-SAG2 protein was detected at ~23 kDa. Lanes 4, 5 and 6 in panel (A) indicates protein expression of pRSET-A empty vector at 0, 2 and 4 h. Lanes 2 and 3 in panel (B) indicates protein expression of pRSET-A empty vector at 2 and 4 h. Lane 7 in panel (A) and lane 1 in panel (B) are PageRuler Prestained Protein Ladder.

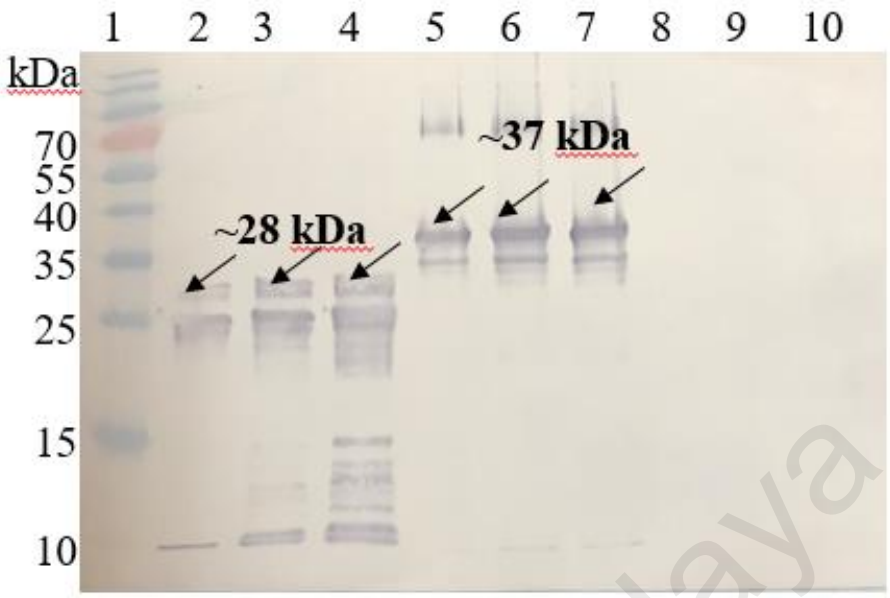



Figure 4.38: Western blot analysis of the expression of recombinant pRSET-A-HLY and pRSET-A-HZF proteins in *E. coli* BL21 (DE3) cells. Expression of recombinant pRSET-A-HLY at 0, 2 and 4 h (lanes 2, 3 and 4) while expression of recombinant pRSET-A-HZF at 0, 2 and 4 h were indicated at lanes 5, 6 and 7. Size of recombinant pRSET-A-HLY and pRSET-A-HZF protein were detected at ~28 and ~37 kDa, respectively. Expression of empty pRSET-A vector at 0, 2 and 4 h (lanes 8, 9 and 10). Lane 1 indicates PageRuler Prestained Protein Ladder.

### 4.4.3 Protein purification

Non-specific bands from expressed proteins were purified by using ProBond$^{TM}$ purification method. The purified recombinant protein of pRSET-A-SAG1 and pRSET-A-SAG2 were successfully detected at ~32 kDa and ~23 kDa, respectively (Figures 4.39 A and B). Meanwhile, Figures 4.40 A and B indicate the purified recombinant protein of pRSET-A-HLY and pRSET-A-HZF were detected at the size of ~28 kDa and ~37 kDa, respectively.

The fragments of purified proteins were excised from SDS-PAGE and sent for MALDI-TOF service at University of Malaya Centre for Proteomics Research (UMCPR). Meanwhile, the expressed proteins were further confirmed in Western blot by using anti-His monoclonal antibody (Figure 4.41 A, B and 4.42 A, B). Molecular weight of each recombinant protein was correctly detected at their respective size.

### 4.4.4 Isothermal titration calorimetry (ITC)

Concentration for each recombinant protein was then measured by Bradford assay kit and calculated based on standard curve of Bradford protein assay (Appendix T). To measure the binding strength for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins, 20, 30 and 40 µM of recombinant pRSET-A-SAG1 protein and 2, 3 and 4 µM of recombinant pRSET-A-HLY protein were used. After optimization, a combination of 30 µM recombinant pRSET-A-SAG1 protein and 4 µM of recombinant pRSET-A-HLY protein showed a better result. Thus, a total volume of 585 µl from recombinant pRSET-A-SAG1 protein (30 µM) and 300 µl of recombinant pRSET-A-HLY protein (4 µM) were loaded into 96 well plates. Figure 4.43 indicates thermodynamic curve of interaction for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG1/pRSET-A vector proteins. An exothermic reaction was observed.

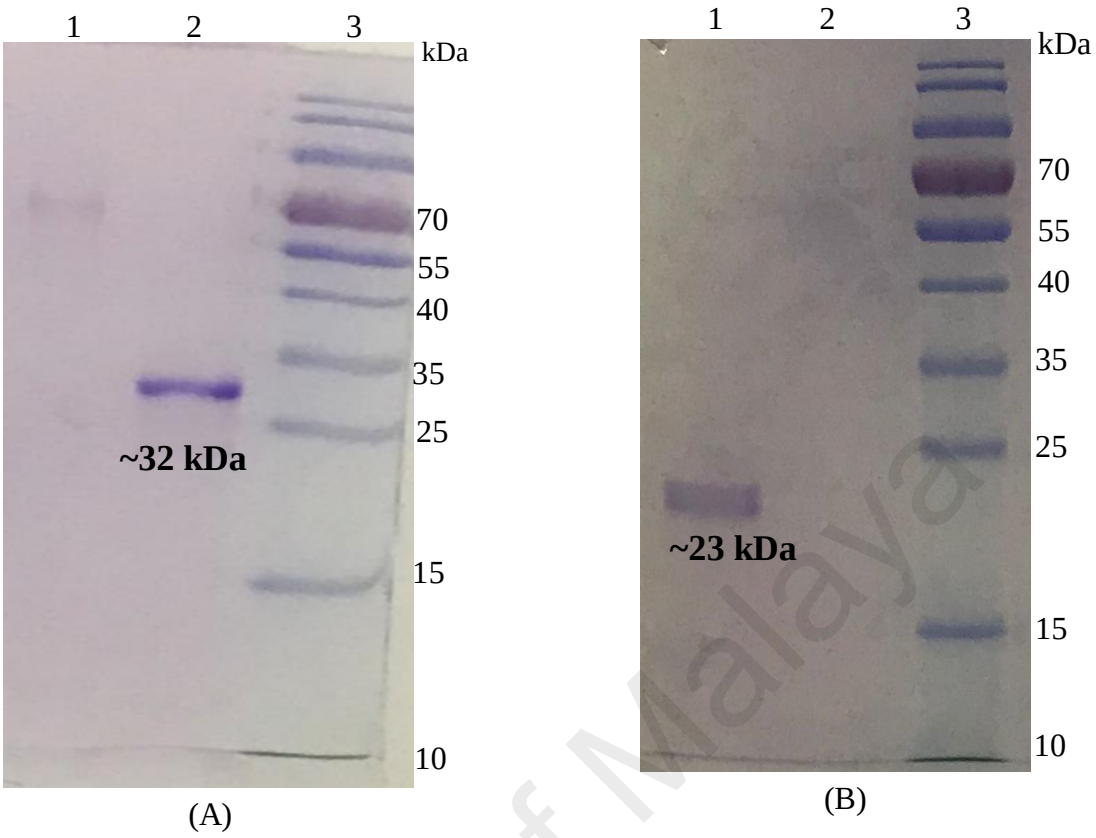



Figure 4.39: SDS-PAGE analysis of the purified recombinant pRSET-A-SAG1 and pRSET-A-SAG2 proteins. (A) A distinct band of ~32 kDa was detected in purified pRSET-A-SAG1 clone (lane 2), but absent in purified pRSET-A clone (lane 1). (B) A thick band with size of ~23 kDa was observed in purified pRSET-A-SAG2 clone (lane 1), which was not detected in purified pRSET-A clone (lane 2). Lane 3 in panel (A) and lane 3 in panel (B) were PageRuler Prestained Protein Ladder.

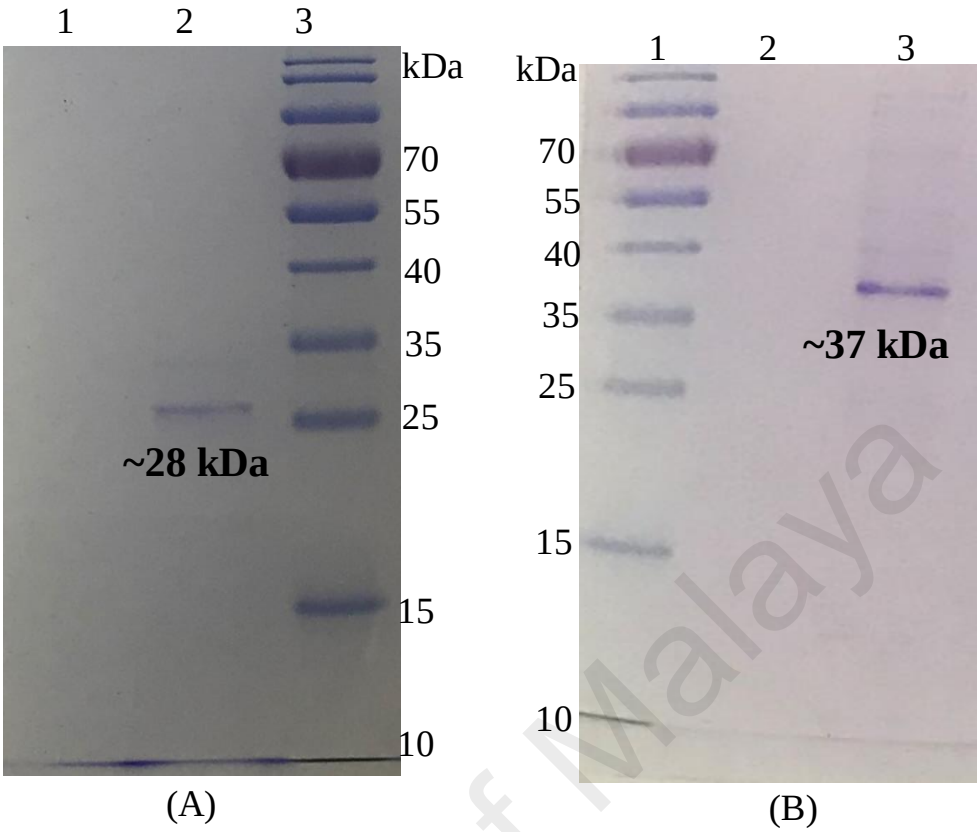



Figure 4.40: SDS-PAGE analysis of the purified recombinant pRSET-A-HLY and pRSET-A-HZF proteins. (A) A distinct band of ~28 kDa was detected in purified pRSET-A-HLY clone (lane 2), but absent in purified pRSET-A clone (lane 1). (B) A band with size of ~37 kDa was observed in purified pRSET-A-HZF clone (lane 3), which was not detected in purified pRSET-A clone (lane 2). Lane 3 in (A) and lane 1 in (B) were PageRuler Prestained Protein Ladder.

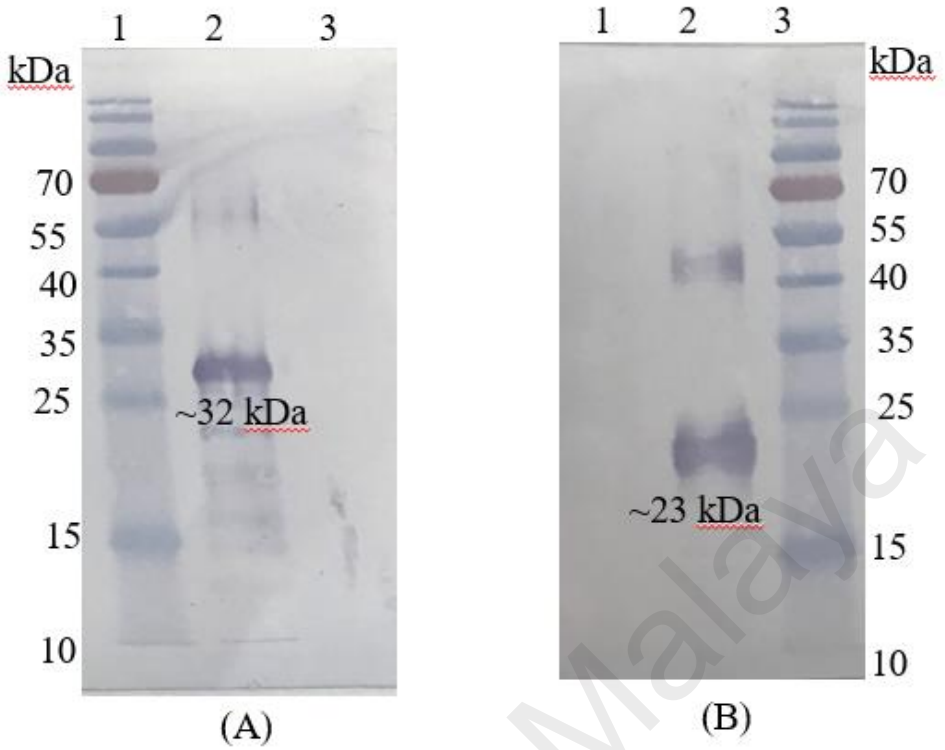



Figure 4.41: Western blot analysis of the purified recombinant pRSET-A-SAG1 and pRSET-A-SAG2 proteins. (A) Detection of purified recombinant pRSET-A-SAG1 protein at the size of ~32 kDa (lane 2) which is not detected in purified pRSET-A clone (lane 3). (B) Detection of purified recombinant pRSET-A-SAG2 protein at the size of ~23 kDa (lane 2) which is not observed in purified pRSET-A clone (lane 1). Lane 1 in panel (A) and lane 3 in panel (B) are PageRuler Prestained Protein Ladder.

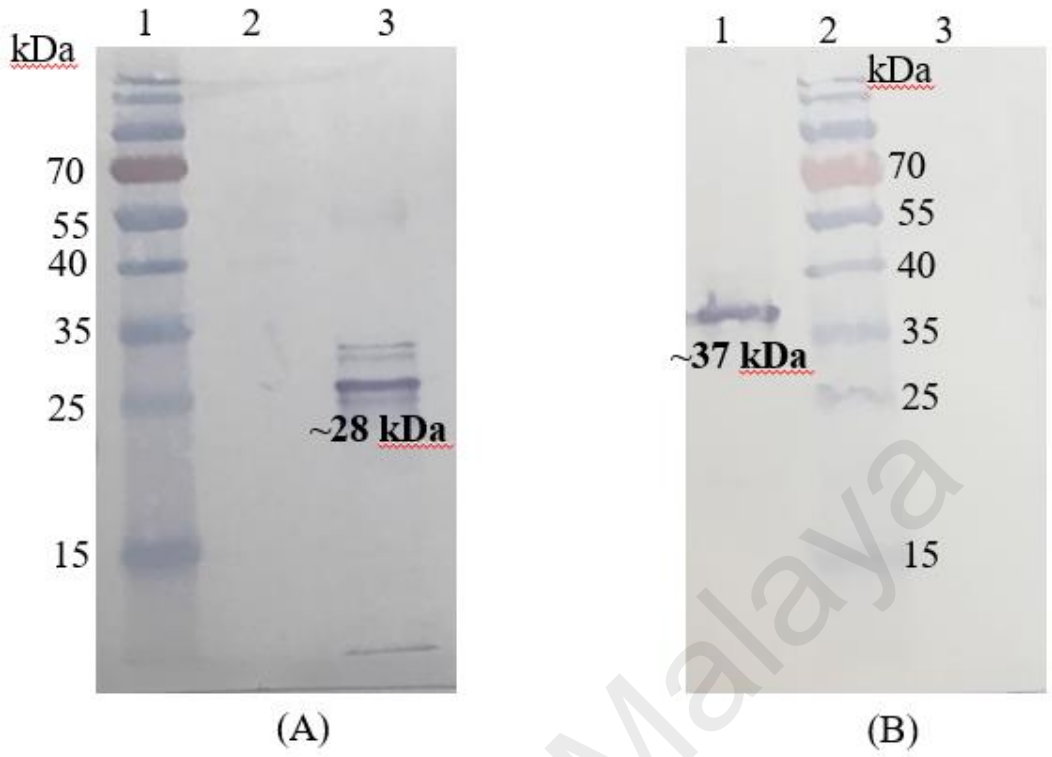
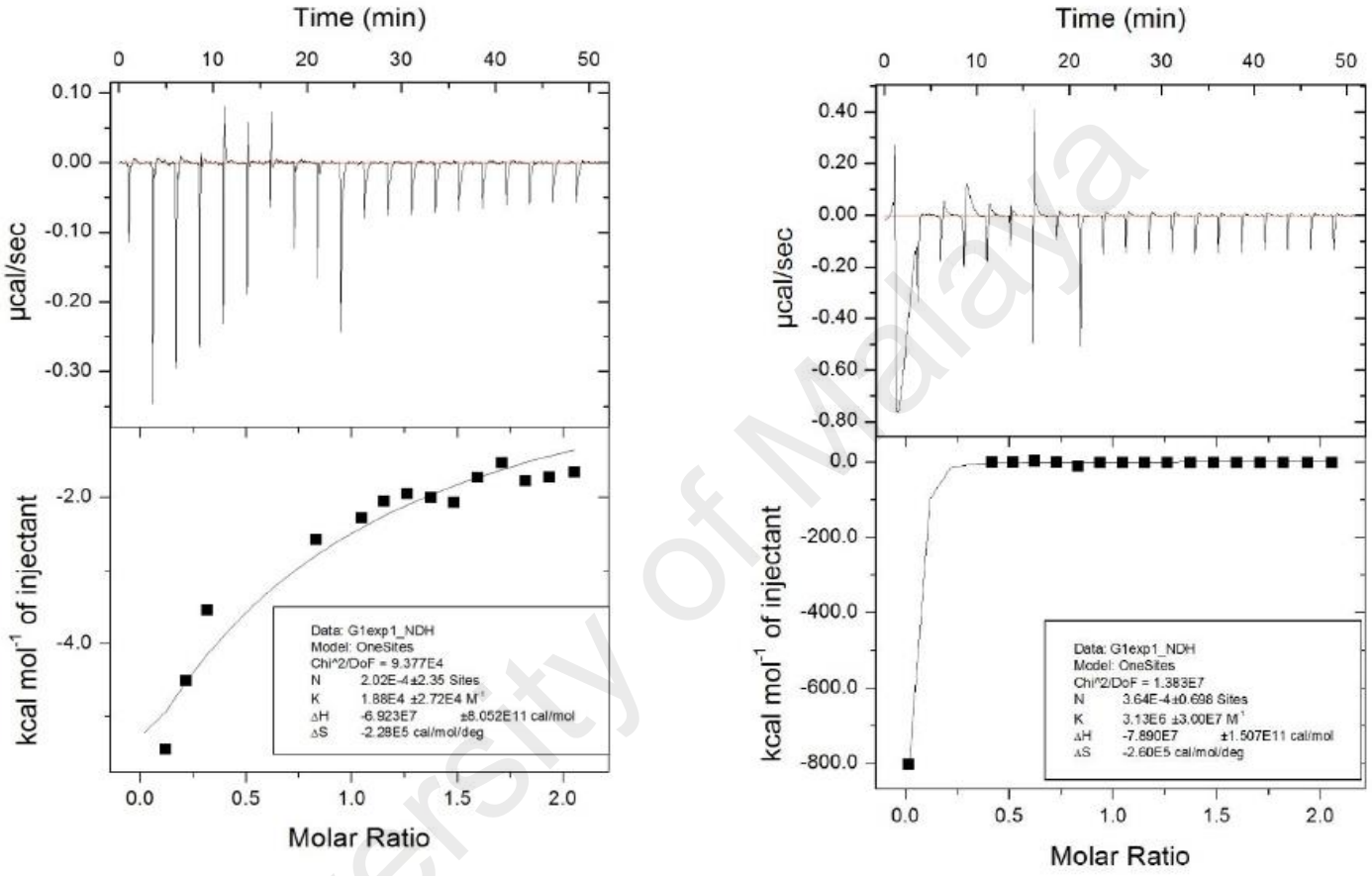



Figure 4.42: Western blot analysis of the purified recombinant pRSET-A-HLY and pRSET-A-HZF proteins. (A) Detection of purified recombinant pRSET-A-HLY protein at the size of ~28 kDa (lane 3) which is not observed in purified pRSET-A clone (lane 2). (B) Detection of purified recombinant pRSET-A-HZF protein at the size of ~37 kDa (lane 1) which is not detected in purified pRSET-A clone (lane 3). Lane 1 in panel (A) and lane 2 in panel (B) are PageRuler Prestained Protein Ladder.

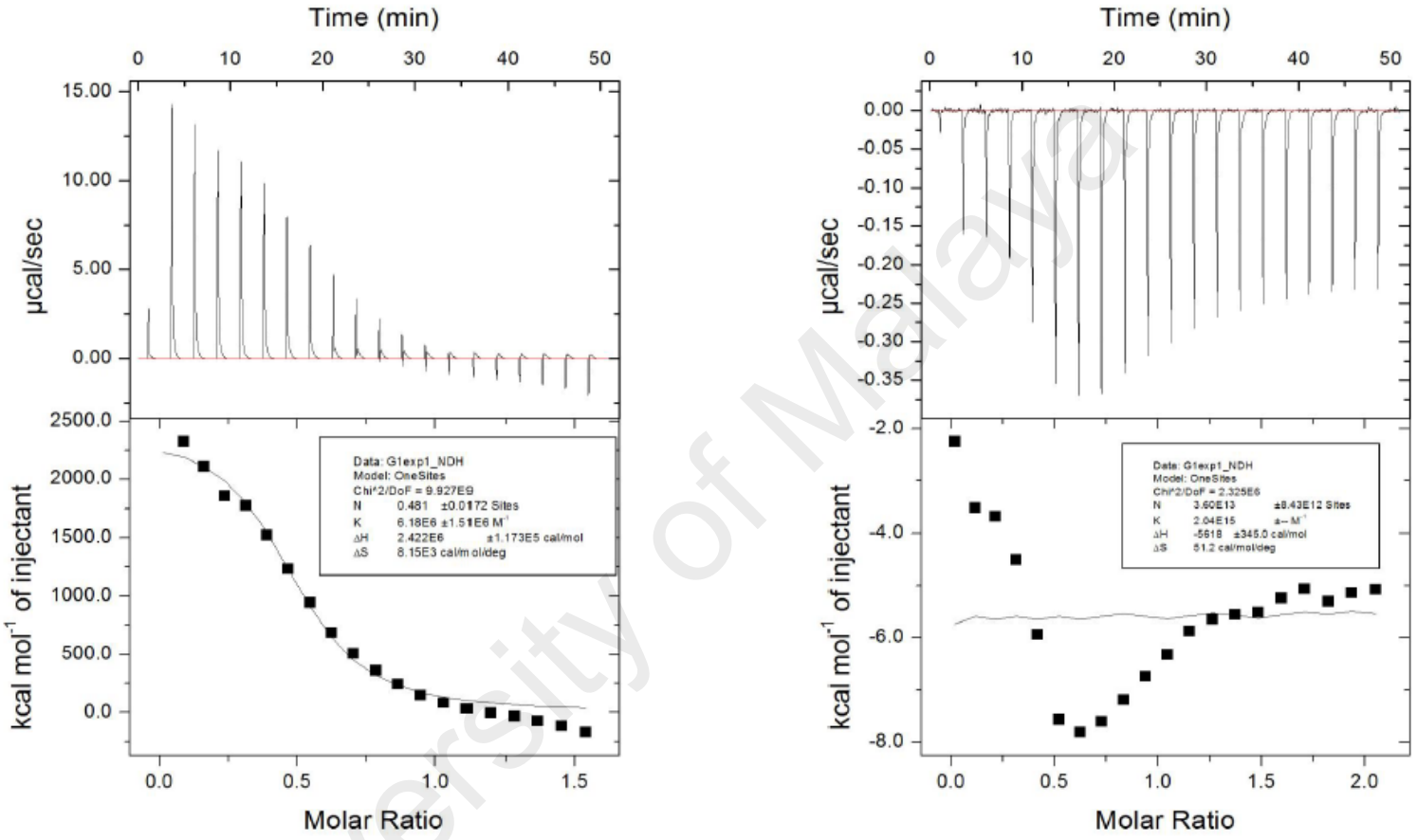
To measure the binding strength for recombinant pRSET-A-SAG2/pRSET-A-HZF proteins, 20, 30 and 40 µM of recombinant pRSET-A-SAG2 protein and 2, 3 and 4 µM of recombinant pRSET-A-HZF protein were used. After optimization, a combination of 20 µM of recombinant pRSET-A-SAG2 protein and 3 µM of recombinant pRSET-A-HZF protein showed a satisfied result. Therefore, a total volume of 240 µl from recombinant pRSET-A-SAG2 protein and 300 µl of recombinant pRSET-A-HZF proteins were loaded into 96 well plates. Figure 4.44 shows thermodynamic curve of interaction for recombinant pRSET-A-SAG2/pRSET-A-HZF proteins and recombinant pRSET-A-SAG2/pRSET-A vector proteins. An endothermic reaction was observed.

Figure 4.43 and 4.44 indicate a typical and atypical curve, respectively. ITC machine functions automatically where ΔH refers to entaphy changes and 'K' refers to binding constant. ΔH is measuring total heat is released when binding is occured. 'K' is needed to calculate binding strength between the interacted proteins. Binding affinity was calculated based on dissociation constant ($K_d$) and formula for it was $K_d = 1/K_a$. It was observed that binding affinity of recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins were 53.2 µM and 0.016 µM, respectively. To determine the strength between two interacting partners, 'c' value was took into consideration and it was calculated based on the formula, concentration of sample protein/$K_d$. Binding affinity between two interacted proteins was considered as good binding if the 'c' value falls between 1 and 1000. Conversely, if 'c' value was less than 1 or more than 1000, the binding affinity was considered as weak binding. Following the calculation, interaction of recombinant pRSET-A-SAG1/pRSET-A-HLY proteins was considered as weak binding as the 'c' value was only 0.075 µM. However, binding affinity between recombinant pRSET-A-SAG2/pRSET-A-HZF proteins was good binding as 'c' value was 18.75 µM.




(A) (B)

Figure 4.43: Thermodynamic curve for measurement of binding strength for (A) recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and (B) recombinant pRSET-A-SAG1/pRSET-A vector proteins (Negative control). N, number of binding sites; K, binding constant; ΔH, enthalpy changes; ΔS, entropy changes; HLY, *H. sapiens* lysine-rich coiled-coil protein.




(A) (B)

Figure 4.44: Thermodynamic curve for measurement of binding strength for (A) recombinant pRSET-A-SAG2/pRSET-A-HZF proteins and (B) recombinant pRSET-A-SAG2/pRSET-A vector proteins (Negative control). N, number of binding sites; K, binding constant; ΔH, enthalpy changes; ΔS, entrophy changes , HZF, *H. sapiens* zinc finger protein.

# CHAPTER 5: DISCUSSION

## 5.1 Overview

Toxoplasmosis has been wide spread all over the world. As an intracellular parasite, *T. gondii* encounters and respons to host cell stress in order to maintain its survival and propagation within the cell. Many proteins involved in *T. gondii* invasion had been characterized, and their contribution for parasite entry had been proposed. Identification of receptors or binding host proteins from humans is an essential activity.

Attachment and penetration of parasite into host cell are mediated by both parasite and host cell surface molecules. SAGs are the first elements to contact human body during the invasion process. *sag1* and *sag2* were employed as target genes in this study to determine the potential binding partners from human cDNA library. Y2H experiment was used to perform this assay. By cloning SAG1 and SAG2 fragments into pGBKT7 vector, the expressed proteins were used as bait protein and mated with commercial cDNA human library in pGADT7-RecAB. Following analysis of sequencing results, Y187 cells transformed with each of these potential prey plasmids (22 and 13 prey clones of each SAG1 and SAG2) were mated with the respective Y2HGold(pGBKT7-SAG1) or Y2HGold(pGBKT7-SAG2) and Y2HGold(pGBKT7). This step was done so that only genuine positive interactive clones being selected. *H. sapiens* lysine rich coil-coiled (abbreviated as HLY) and *H. sapiens* zinc finger (abbreviated as HZF) proteins were identified as potential candidates interacting with SAG1 and SAG2, respectively.

Though both interacted host proteins are cytoplasmic protein, it is believed that cell surface receptors may used 'second messenger' to transmit a signal to the cytoplasm. Such 'second messengers' may include amino acids, nucleotides, steroids and other lipids (Stiegler *et al*., 2014).'

Interaction between prey and SAG1 or SAG2 were further examined by beta-galactosidase assay. Result indicates that enzyme activity between SAG1 and SAG2 with

their prey proteins were 449.4 U and 437.7 U, respectively. In comparison to positive control (424.3 U), the existence of interactions between prey and SAG1 or SAG2 were proven. Additionally, the interaction between prey and bait proteins was further determined by a co-immunoprecipitation assay. After analysis with non-parametric method, interaction between these prey and SAG1 or SAG2 proteins were significant as compared to negative control. Finally, the binding strength for SAG1/ HLY and SAG2/ HZF were measured by ITC method. Thermodynamic curves revealed that there were good binding for recombinant SAG2/HZF proteins and weak binding for recombinant SAG1/HLY proteins.

## 5.2 Structure of SAG1 and SAG2

SAG1 was selected as it is the most abundant surface protein of *T. gondii* tachyzoites even though it only accounts 3-5% of total *T. gondii* protein (Velge-Roussel *et al*., 2001). Since *T. gondii* lacks of specific motility and gliding organelles such as flagella, pseudopodia and cilia, SAG1 plays an important role as a receptor-ligand during invasion of *T. gondii* into host cell (Wang & Yin, 2014). Several studies indicated that SAG1 is crucial in promoting attachment of tachyzoite to host cell during the invasion. For example, *in vitro* antibody neutralization assays indicated that a polyclonal and monoclonal specific to *T. gondii* SAG1 (P30) managed to inhibit the invasion of tachyzoites into host cells (Grimwood & Smith, 1996; Mineo *et al.*, 1993; Mineo & Kasper, 1994). Also, soluble SAG1 was shown to bind to host cells directly as the surface of SAG1 consisted of a groove to accommodate its dimerization. Since the groove faces away from parasite surface and therefore can assist the binding of cognate ligand into host cell surface. Wang & Yin (2014) published a review paper on SAG1 structure. Tachyzoite is a crescent shape. The SAG1 proteins are located on the ‘notch’ and subsequently

helping in promoting the penetration of cognate into the host cells (Robinson *et al*., 2004; Wang & Yin, 2014).

Structure of SAG1 used here were truncated. Hydrophobic regions in both of the N-terminus and C-terminus were trimmed off as it will disturb the solubility of mature protein (Wang & Yin, 2014). Protein expression yield of the truncated form increased dramatically as compared to the full length SAG1. Due to its high yield of protein expression, truncated *sag1* had been widely used for protein expression. Yan *et al*. (2004) successfully cloned truncated SAG1 into pET-32a (+) and expressed in *E. coli* BL21. Their report revealed that high level of truncated SAG1 expression was shown in SDS-PAGE and the size was ~40 kDa. The expressed protein was in soluble form and thus easily purified by Ni-NTA agarose (Yan *et al*., 2004). Besides, truncated SAG1 was expressed by Velmurugan *et al*. (2008) to detect toxoplasmosis via ELISA. This was the first report of serological detection of caprine toxoplasmosis by using a cocktail of two recombinant *Toxoplasma* proteins, SAG1 and GRA7 (Velmurugun *et al*., 2008).

SAG2 was found to be closely related to SAG1 and distributed on the surface of tachyzoites and bradyzoites. Although both of these families were diverged, SAG1 and SAG2 play a crucial role in attachment and reorientation during the *T. gondii* invasion (Grimwood & Smith, 1996; Lekutis *et al.*, 2000). As well, structure of SAG2 used in this study was in a truncated form as the signal peptide was removed, resulting approximately 17 kDa of SAG2. Cleaved form of recombinant SAG2 protein was easier to be expressed than precursor SAG2 protein (Macedo *et al.*, 2013).

### 5.3 Y2H

Y2H assay allows the simultaneous isolation of interacting proteins along with the genes that encode them inside a yeast cell nucleus. In this study, Y2H was used to screen binding proteins for *T. gondii* SAG1 and SAG2 since this method was easy to

perform and not required a specific equipment. Y2H is an *in vivo* assay as live *S. cerevisiae* is involved. Yeast cells are eukaryotic member, which display the reality closer than *in vitro* approaches or bacterial expression techniques. The Y2H assay can be used to identify a novel interacting protein partner. Y2H also provides a functional screens as this system can be used to predict an unknown protein's function. Considering the advantages of Y2H technique, this approach has been employed by other researchers during their investigation of protein-protein interaction studies. Ahn *et al.* (2006) used Y2H assay to detect interactions between secreted GRA proteins and host cell proteins involved in parasitism of *T. gondii*. They found that GRA proteins interacted with a variety of host cell proteins such as enzymes, structural and functional proteins related to PV and PVM formation during the host cell invasion by *T. gondii*. Pan *et al*. (2017) used Y2H assay to screen for host proteins interacting with *T. gondii* rhoptry protein 16 (TgROP16). TgROP16 plays an important role in activation of host STAT (signal transducer and activator of transcription) signaling pathway through phosphorylation of STAT3 and STAT6. By using mouse cDNA library as the prey, two proteins, DnaJ heat shock protein family member A1 (Dnaja1) and gamma-aminobutyric acid A receptor, subunit alpha 4 (Gabra4) were identified. Based on the gene ontology analysis software, Dnaja1 is predicted to involve in stress response while Gabra4 is participated in the system development process. The discovery of new host proteins that interact with TgROP16 will help to further investigate the functions of this effector proteins during *T. gondii* invasion.

Y2H system uses reporter genes and nutritional markers for the detection of interaction of proteins inside a yeast cell nucleus. When a target protein binds to another protein in the cell, their interaction brings two halves of a transcriptional factor together, which is then able to turn on the expression of the reporter genes and nutritional markers, allowing the yeast cells to grow on a selective medium. In the present study, protein-

protein interactions were confirmed when four independent nutritional markers and reporter genes, *aur1-C, his3, ade2*, and *mel1* were transcribed simultaneously in yeast cell. The *aur1-C* gene from Y2HGold yeast encodes for enzyme inositol phosphoryl ceramide synthase and this enzyme confers strong resistance ($AbA^r$) to the highly toxic aureobasidin A antibiotic. Besides, *mel1* gene that was incorporated into both Y2HGold and Y187 yeast strains, was another reporter gene used. This gene encodes for α-galactosidase enzyme which is able to catalyze X-alpha-gal into green precipitates. Therefore, yeast colonies show green colour when *mel1* gene is turned on. Additionally, *his3* and *ade2* genes that were incorporated into Y2HGold yeast were used to synthesize histidine and adenine, respectively when these nutritional markers are turned on. Y2HGold was not able to synthesize these amino acids during the absence of interaction between bait and prey proteins. Hence, a positive protein-protein interaction is indicated when all reporter genes and nutritional markers are turned on simultaneously.

### 5.3.1 *S. cerevisiae*

*S. cerevisiae* is an established model for studying and understanding cellular and biological processes in higher eukaryotic organism as well as in humans. This yeast strain was recruited as the host throughout the Y2H study. Culturing yeasts are simple and economical. Cells divide mitotically by forming a bud, which is then segregated to form daughter cells. Yeast can also be grown on a completely defined medium, allows the identification of several nutritional auxotrophs. Nearly 40% of yeast genes share a similarity of amino acid sequences to human protein which some of them are related with human diseases (Galao *et al*., 2007; Parson *et al*., 2003; Foury, 1997).

Yeast can exist stably in haploid and diploid states. Yeast with either ‘a ‘or ‘α’ type is called haploid while the diplod yeast is formed by the mating of a ‘a’ cell and a ‘α’ cell. Two strains of *S. cerevisiae* that were involved throughout the study were

Y2HGold and Y187 yeasts. Both strains were genetically modified by integration of several type of reporter genes, nutritional markers as well as bacterial *lacZ* gene. The *aurI-C*, *his3*, *ade2* and *mel1* genes were integrated into Y2HGold genome. For Y187 strain, both of *mel1* and *lacZ* reporter genes were integrated into yeast genome (Clontech, USA). The two-hybrid assay was performed between the 'Y2HGold-MATa GAL4BD-SAGs' cells and 'Y187-MATα' human cDNA library' cells. The mated cells were incubated at 30$^{o}$C with gentle shaking at 50 rpm only. This prevents the yeast from settling to the bottom of the flask, moreover rapid shaking reduces mating efficiency. If interaction between proteins were not existed, either reporter genes or nutritional markers were not activated. Thus, no colonies or no changes in the colour of the yeast colonies were observed. During Y2H experiment, only clones that are not toxic will be selected as bait in Y2H experiment. If the bait is toxic to the yeast cells, both solid and liquid cultures will grow more slowly. Also, the colonies containing the bait vector are significantly smaller than colonies containing the empty vector. However, the toxicity test result in this study indicates the size of colonies containing pGBKT7-SAG1 or pGBKT7-SAG2 was similar with the colonies containing empty pGBKT7 vector (Figure 4.7 B). It was observed that some of the pink colonies growing on plates. This is because amount of adenine in the media is insufficient. Then, yeast *ade2* was activated, leading to accumulation of pink pigment and colonies appeared pink colour. Since accumulation of pink pigment slows the growth, thus adenine may need to be added into the media. Adenine is used by yeast cells as the building blocks for adenosine triphosphate (ATP) to synthesis energy (Joseph & Hall, 2004).

### 5.3.2 pGBKT7 vector in Y2H assay

SAG1 and SAG2 bait proteins were expressed as fusion protein in Y2HGold under control of ADH1 promoter which was located at locus 7-1478. The transcription

was terminated by T7 and ADH1 transcription termination signals ($T_{T7\ \&ADH1}$). The pGBKT7 vector also contains T7 promoter, c-myc epitope tag and multiple cloning sites (MCS). C-myc tag enable the expressed proteins to be analyzed by Western blot using anti-C-myc antibodies. Multiple restriction enzyme cutting sites were incorporated into this vector to facilitate the cloning of digested fragments. Moreover, this vector contains 5'DNA-BD and 3'DNA-BD fragments enabling the colony PCR amplification by using ADLD insert screening primers.

The pGBKT7 plasmid also plays an important role as a shuttle vector since it contains both features of *E. coli* and *S. cerevisiae*. Such features are 2 µ ori and pUC origin replication. This vector is able to replicate autonomously in both *E. coli* and *S. cerevisae*. The pGBKT7 vector also contains Trp1 and $Kan^r$ selectable markers. Both SAG1 and SAG2 fragments that were inserted into pGBKT7 vector can be selected easily in *E. coli* and *S. cerevisiae* system after propagation.

### 5.3.3 Mating experiment

Bait and prey fusion proteins are generated by standard recombinant DNA techniques. In most cases, a single bait protein is used to fish out interacting partners from a cDNA library. Following the mating experiment, interaction between bait and prey proteins was confirmed by plating on a series of selective plates. Three replicates of screenings were employed to negate leakiness of positive interactions. SAG1 and SAG2 were tested for autoactivation and toxicity in order to verify that both genes were not autoactivators and nontoxic when expressed in yeast.

SAG1 and SAG2 proteins were found to interact with a number of host cell proteins including functional enzymes and organelle proteins. However, most of these clones were false positives. A list of proteins that were involved in the attachment, invasion, penetration, PV and PVM formation, cell division, cell proliferation and cell

egression were compiled (Table 4.2 and 4.3). Bioinformatics investigation from *in silico* software (UniProtKB) and literature reviews revealed that these proteins were involved in many cellular procedures.

## 5.4 Yeast plasmid extraction and recovery

Isolation of yeast plasmid was important to analyse the molecular function of the clones following Y2H experiment. However, procedures involved were tedious as compared to plasmid extraction from the bacterial cells. Yeast plasmid was initially extracted from yeast cells and re-introduced into *E. coli* cells to recover DNA plasmid. Re-introducing the plasmid into *E. coli* was important due to the low yield and poor quality of DNA after extraction directly from yeasts cell (Singh & Weil, 2002).

Currently, several approaches are available for plasmid extraction from yeast including mechanical (Gunn and & Nickoloff, 1995), chemical (Oshiro *et al*., 1987) and enzymatic method (Holm *et al*., 1986; Brozmanova & Holinova, 1988). In this study, enzymatic method was used to extract the yeast plasmids following Y2H assay. With the aid of zymolase enzyme, yeast cell walls were first disrupted to release the spheroplasts (yeast cells with partial loss of the wall) followed by SDS or alkaline lysis of spheroplasts. This method was reliable and efficient to extract the plasmid from yeast cells, however the yield was low (Suzuki & Iwahashi, 2013). Cell lysate was then purified and the eluted plasmid DNA was recovered by transformation into *E. coli*. From the observation, no significant band was observed on agarose gel for the extracted yeast plasmids without pre-propagation into *E. coli*. It may due to the low copy number of yeast plasmids. The prey plasmids were then extracted from *E. coli* and was successfully to be sequenced.

## 5.5 Confirmation of genuine positive interaction clones

To confirm the selection of genuine potential interacted proteins, segregation of prey plasmids in yeast was important. These clones may contain a plasmid that express a non-interacting protein in addition to having prey vector which express a protein responsible for activating the reporter genes. Segregation is performed by repeated streaking the clones onto DDO/X plates. Thus, plasmid extraction from yeast cells without the first segregation of non-interacting prey may have a possibility to rescue other non-interacting prey plasmids. Subsequently, some of the potential partners are hindered. In fact, mixture of green and white colonies still can be observed after first streaking showing that segregation of positive interactors (green) from non-interactors (white).

Following Y2H experiment, the interacted proteins were further verified by performing a small scale mating experiment between potential prey proteins and their respective Y2HGold(pGBKT7-SAG1) or Y2HGold(pGBKT7-SAG2 and Y2HGold(pGBKT7). It was observed that some colonies growing on the plate that mated with empty pGBKT7 vector. This was because their prey may has self-autoactivated characteristic, where the prey itself is able to turn on the reporter genes without the presence of bait. Thus, only clone that showed no colonies on plate after mating with empty pGBKT7 vector was selected for downstream work. As the result, *H. sapiens* lysine rich coil-coiled and *H. sapiens* zinc finger which interacted with SAG1 and SAG2, respectively were selected.

## 5.6 Functions of interacted host proteins

Proteins involved virtually in most of the cellular process of living cells. They control cell division, metabolism, and the flow of materials and signals into and out of the cell. Understanding how cells work requires the understanding of how proteins function. Based on the protein and nucleic acid sequences that are available in genome

databases, the function of a gene and its encoded protein can be predicted by comparing its sequence with those of previously characterized genes. Since amino acid sequence determines protein structure and structure decides biochemical function, proteins that share a similar amino acid sequence will also perform a similar biochemical function, even when they are found in distantly related organisms (Kosloff & Kolodny, 2008). At present, *H. sapiens* lysine rich coil-coil and *H. sapiens* zinc finger proteins were found to be interacted with *T. gondii* SAG1 and SAG2, respectively. Their functions were presumptively discussed based on the literature search. These two human proteins were selected after a series of selective plates, colony PCR, sequencing anaysis, small scale Y2H and beta galactosidase assay. During the small scale Y2H, only these two proteins shown no interaction when mated with empty vector (Figure 4.12 and Figure 4.13). The results have been published in 2017 and 2018.

### 5.6.1 *H. sapiens* lysine rich coil-coiled rich (HLY) protein

According to *in silico* software (UniProtKB) analysis, HLY protein is involved in modulating of p53 transcription factor pathways in the host cells. Lysine methylalted p53 is able to drive the activation or repression of a various number of promoters. It is a tumor suppressor and related to pathologic processes in neurodegenerative diseases, diabetes and myocardial infarction. Besides, p53 is involved in numerous post-translation modification (PTM) such as phosphorylation, acetylation, methylation and ubiquitination, which alter its activity, localization, stability and thus modulating its response to several forms of genotoxic stress. Four lysines are found in p53 C-terminal region. Lysine methylation may generate different groups of p53 which each p53 is methylated at a specific C-terminal lysine residue. Lysine methylation is believed to modulate the surface structure of host cells in order to promote or subvert the binding pathway by the effector proteins (West & Gozani, 2011).

In 2014, p53 and ROP16 has been shown that they are co-localized at the nucleus of neuroblastoma SH-SY5Y cells via subcellular localization assay. During the invasion of *T. gondii*, ROP16 that is released from the rhoptries can quickly migrate into the host cell nucleus and bind to p53 transcription factor. The interaction between lysine methylated p53 and ROP16 intersect the host signal transducer and activator pathways. Consequently, the host cell immune responses are supressed, leading to cell cycle G1 phase arrest and cell apoptosis (Chang *et al*., 2015).

Apart from p53 binding function, HLY protein may involve in gliding and motility of *T. gondii* into host cells. During the invasion process, *T. gondii* moves along the host cell surface using actomyosin-based gliding motor, attaches, penetrate and establish a PV inside the host cell. Inside the host cell, the parasite become non-motile to avoid premature rupture of the host cells. Thereby permits the multiple rounds of parasite replication within a same cell. When the host cell environment is favourable for propagation, the parasites switch back to motile stage and disrupt the host cell membrane, propagate and invade a new host cell. The successful motility, invasion and egress of *T. gondii* within the host cell is regulated by human lysine methyltransferase (Heaslip *et al*., 2011).

### 5.6.2 *H. sapiens* zinc finger (HZF) protein

Based on the zinc finger protein's structures, they are classified into several groups such as Cys2His2, Cys4, Cys5, Gag knuckle, Treble clef, Zinc ribbon, Zn2/Cys6 and TAZ2 domain-like group. Zinc finger protein's functions are as diverse as their structures. Variation of zinc finger domain is associated with their binding specificity to the particular proteins. Zinc finger proteins are involved in the regulation of several cellular processes including gene expression, transcriptional regulation, ubiquitin-mediated protein degradation, signal transduction, DNA repair, cell migration, and cell

apoptosis. Perturbations in zinc finger protein domains can cause neurodegenerative disease, diabetes, skin disease, and cancer (Laity *et al*., 2001; Cassendri *et al.*, 2017).

Gissot *et al*. (2016) reported that zinc finger protein is involved in *T. gondii* propagation in the host cell via mRNA nuclear export pathway. During the gene expression process, the mRNA transcripts are synthesized inside the nucleus. Gissot *et al*. (2016) confirmed that the parasite is able to bind *in vivo* to these transcripts and facilitates the movement of the mRNA transcripts to the cytoplasm where the proteins can be translated. The synthesized proteins are important for parasite propagation in cell cycle G1 phase (Gissot *et al*., 2016).

Besides, zinc finger proteins play an important role in facilitating the parasite differentiation from tachyzoites to bradyzoites inside the host cells. During the asexual life cycle in humans, interconversion between these stages are crucial for survival and pathogenicity. Vanchinathan *et al*. (2005) demonstrated that a single insertion mutagenesis on the transcription site of a gene encoding zinc finger protein can efficiently disrupt the stage conversion from tachyzoite to bradyzoite. Thus, the parasite propagation process within the host cell is disturbed.

Zinc finger protein is involved in the regulation of cell growth. To maintain the survival rate of the cell, zinc finger protein has to bind to double stranded RNA (dsRNA). Yang *et al*. (1999) has reported that interruption of the binding pathway to human fibroblast cell can cause cell apoptosis. Zinc finger protein is an example to show binding capability of protein to nucleic acid. Thus, during the host cell invasion, it is believed that HZF may bind to *T. gondii* dsRNA in order to maintain its survival rate. This finding may suggest that dsRNA-binding zinc finger proteins also play an essential role in eukaryotic cell growth and survival.

## 5.7 Chemiluminescent Co-immunoprecipitation (Co-IP)

### 5.7.1 HEK 293 mammalian cells in chemiluminescent Co-IP study

HEK 293 mammalian cells was selected as the transfection host in chemiluminescent Co-IP assay. Several advantages of using this cell lines are rapid division rate, high transfection efficiency, ease of propagation and maintenance. It is noted that these cells possess post-translational modification machineries and thus assisting in proper folding of expressed target protein. These cell lines can be used for transient expression (Jager *et al*., 2013; Ooi *et al*., 2016; Song *et al*., 2011) and stable expression in viral based expression vectors (Kim *et al*., 2009). Since the discovery of HEK 293 mammalian cell lines, it has been used by many researchers to express various types of recombinant proteins such as transmembrane proteins, soluble proteins, secreted mammalian and viral proteins and complete membrane proteins (Bandaranayake & Almo, 2014; Bussow, 2015).

### 5.7.2 Selection of pAcGFP1-C1 and pProLabel-C vectors in Co-IP study

The pAcGFP1-C1 vector is a mammalian expression vector. This vector contains AcGFP1 fragment, encoding green fluorescent protein (GFP) which is located at the C-terminus of the vector. SAG1 and SAG2 were inserted in the downstream of AcGFP1 coding sequences and expressed as a fusion protein together with GFP protein under the control of $P_{CMVIE}$ promoter. The transcription was terminated by simian virus 40 (SV40) poly $A^+$ signal. Green fluorescence is observed for successfully transfected cells under microscope due to the presence of GFP protein. Besides, this vector also contains pUC origin and $Kan^r$ to allow the propagation and selection of constructs in *E. coli*. Additionally, pAcGFP1-C1 vector contains MCS sites which is located downstream of AcGFP1 fragment. These fusion bait proteins were used in the following Co-IP experiment.

Similarly, human *hly* and *hzf* were amplified and subcloned into linearized pProLabel-C vector after purification. The pProLabel-C vector is a linearized mammalian expression vector. Both HLY and HZF fragments were ligated into the MCS sites after linearizing by *Sal*I/*Bam*HI restriction enzymes. Both fusion proteins containing ProLabel tag were expressed under control of $P_{CMVIE}$ promoter. The transcription was terminated by SV40 poly $A^+$ signal. pProLabel-C vector also contains pUC origin and $Kan^r$ to allow propagation and selection of the constructs in *E. coli*. The expressed fusion proteins containing pProLabel tag are used as the prey proteins and co-transfected together with SAG1 and SAG2, respectively into HEK 293 mammalian cells.

The ProLabel enzymatic assay system is an enzyme fragment complementation assay involving a functional reporter protein and an enzyme acceptor (EA). The recombinant plasmids, pAcGFP1-C1-SAG1/pProLabel-HLY and pAcGFP1-C1-SAG2/pProLabel-HZF were co-transfected into HEK 293 mammalian cells. The cell culture were harvested and lysed. The cell lysates were then incubated overnight at $4^oC$ with AcGFP1 antibody, which is specific to the GFP tag on pAcGFP1-C1-SAG1 and pAcGFP1-C1-SAG2 proteins. Overnight incubation is needed and it may increase the binding affinity of lysates to antibody. Then, the antibody-protein complex was pelleted using protein A/G sepharose which binds to most antibodies, while the unpelleted proteins were washed away during the washing step. The pelleted proteins contain a 6 kDa ProLabel tag which is located upstream of pProLabel-HLY or pProLabel-HZF proteins, thus enabling the detection of luminescent signal. The combination between ProLabel-tag and EA will form a functional active enzyme. The active enzyme cleaves the chemiluminescent substrate and emits a signal which can be quantified by a luminometer in relative light unit (RLU). In this study, a mixture of galacton, emerald and substrate buffer were served as the chemiluminescent substrate. The results indicate that binding for pAcGFP1-C1-SAG1/pProLabel-C-HLY proteins and pAcGFP1-C1-

SAG2/pProLabel-C-HZF proteins were significant as compared to positive control (Lai & Lau, 2017).

## 5.8 Isothermal titration calorimetry (ITC)

### 5.8.1 Selection of pRSET-A vector as expression vector

The pRSET-A vector was used to express recombinant protein in *E. coli* BL21 cells. This is a pUC derived vector and designed for high-level of protein expression. It has been used by many researchers such as Ghosh *et al*. (2012), Ramkumar *et al*. (2017) and Dumon-Seignovert *et al*. (2004). Protein expression level from the recombinant proteins is controlled by T7 promoter. During the induction of IPTG, IPTG binds to lac repressor and releases it from operator. T7 RNA polymerase is then expressed and bound to T7 promoter sequence which is located upstream of *sag1*, *sag2*, *hly* and *hzf* sequences and thus turn on the transcription.

MCS is located downstream from T7 promoter and used to facilitate the cloning of gene interest into respective cutting sites. Besides, this vector contains 6X histidine tag that functions as a metal chelating resins during protein purification. Xpress epitope tag was used for protein detection in Western blot by Xpress monoclonal antibody. This vector also contains enterokinase cleavage site, allowing removal of 6X histidine tag from purified fusion proteins by using protease if needed. In addition to that, pRSET-A vector also possess bla promoter and ampicillin (*bla*) resistance gene enables the vector propagation in *E. coli* on ampicillin selective plate.

### 5.8.2 *E. coli* BL21 (DE3) pLysS expression host

BL21 (DE3) pLysS was used as the host for recombinant protein expression in this study. This strain is frequently used as expression host in prokaryotic protein expression system (Gopal *et al.*, 2011). BL21 (DE3) pLysS contains DE3 lysogen, a gene

encoding for T7 RNA polymerase. Expression of T7 RNA polymerase was induced by IPTG. BL21 (DE3) pLysS is able to reduce toxic protein production due to the T7 lysozyme expression. T7 lysozyme is able to inhibit the function of T7 RNA polymerase in the absence of IPTG. As a consequence, the basal level expression of pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF were eliminated.

The size of recombinant pRSET-A-SAG1, pRSET-A-SAG2, pRSET-A-HLY and pRSET-A-HZF proteins were detected at ~33, ~23, ~28 and ~37 kDa, respectively on SDS gel. A few concentration of IPTG were tested including 1 mM and 1.5 mM IPTG. Additionally, different temperatures (30$^{o}$C and 37$^{o}$C) of incubation were also tested. It was observed that optimum expression condition for the recombinant proteins were 1 mM IPTG and 37$^{o}$C.

### 5.8.3 Purification of protein

Protein Purification was carried out by using 6X histidine tag located at either N- or C- terminus. This method has been performed successfully in several expression systems such as *E. coli*, baculovirus-infected insect cells, *S. cerevisiae*, and mammalian cells (Bornhorst & Falke, 2000). In ProBond$^{TM}$ purification system, the polyhistidine binds strongly to divalent metal ions such as nickel. The protein can pass through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. Proteins that were bound to the resin were eluted with low pH buffer, which decreases the affinity of the tag for the resin. Hybrid conditions were used to purify all recombinant proteins in this study.

Some non specific bands were observed in Figure 4.40 A, 4.41 and 4.42. The bands may be removed by two steps of purification systems. During the whole study, there is only ProBond$^{TM}$ purification involved. Further purification method such as size

exclusion chromatography may be used to solve this problem. Due to budget constraint, only ProBond$^{TM}$ purification system was involved.

Dialysis was performed by using dialysis cassette with 10K molecular weight cut off membrane. This cassette membrane is made of low-binding regenerated cellulose in order to maintain the protein purity and maximum recovery of sample. During the dialysis process, unwanted salts, buffers and urea were diffused from higher concentration to lower concentration until an equilibrium reached. By changing dialysis buffer occasionally (at 2, 4 and 8 h), the unwanted molecules were removed. Dialyzed proteins were then concentrated by using viva spin columns.

In the present study, it was observed that protein size of HZF was larger than the expected size on SDS-PAGE. Based on the information from a protein software, predicted size of this protein was 23 kDa. But, the size indicated on SDS-PAGE gel was approximately 37 kDa (23 kDa of HZF plus 6 kDa 6X histidine tag and additional of 8 kDa). The size discrepancy can be attributed to the presence of the 6 histidine residues in the recombinant proteins and the intrinsic error (+10%) of molecular mass determination by SDS-PAGE. Besides, it is also possible that these differences arise from unique amino acid composition of HZF, such as the proline composition. In this study, the deduced proline (P) content in recombinant pRSET-A-HZF was 5.8%. The presence of peptidyl-prolyl cis-trans-isomerase in *E. coli* may contribute to the catalysis of proline isomerization during protein-folding activity, which subsequently resulting the mobility shift and discrepancies of size on SDS-PAGE (Lin *et al.*, 1988; Liu & Walsh, 1990). Nonetheless, the identity of this protein has been further confirmed by MALDI-TOF, indicating that this protein was 100% homogenous to HZF (NM_001242914.1) (Lai & Lau, 2018).

### 5.8.4 Measurement by ITC approach

From the ITC results, the binding affinity for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins were 1/1.88E4 (~53.2 μM) and 1/6.18E6 (~0.16 μM), respectively. As compared to negative control, both thermodynamic curves revealed that the binding interaction between SAGs with their respective prey proteins were proven to exist. The negative controls used in this assay was recombinant pRSET-A-SAG1/empty pRSET-A proteins or recombinant pRSET-A-SAG2/empty pRSET-A proteins.

The thermodynamic curve describing the interaction for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins was not of a 'sigmoidal' shape. In other words, the binding affinity (*K*a) between these two proteins was not that strong despite three replicates being carried out at the same temperature, being 25$^{o}$C. Temperature may has been a factor worth for examining. According to the manual from Malvern, UK, the binding temperature need to set below the actual temperature of the protein source. Therefore, HLY protein binds better to SAG1 protein at the low human body and parasite's temperature (which is 37$^{o}$C). To date, there is no published report regarding the binding affinity for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins. Thus, different binding temperatures can be set on the machine during the measurement of binding strength for pRSET-A-SAG1/pRSET-A-HLY proteins in order to obtain a strong binding curve.

Meanwhile, the thermodynamic curve describing interaction for recombinant pRSET-A-SAG2/pRSET-A-HZF proteins was an endothermic reaction and showing an atypical curve in comparison to negative control. According to Rajarathnam and Rosgen (2014), typical binding gives rise to sigmoid curve; whereas, in atypical binding, the curve shifts in the opposite way. For negative control, the binding curve be a flat line or non-smooth fluctuate line since there is no binding between proteins involved in negative

control. Also, atypical binding indicates that there might be a high-affinity binding between the interacted proteins (Rajarathnam & Rosgen, 2014).

The success rate to show the existence of interactions between proteins here was not high. It may be related to some technical issues that should be taken into account. Both ligand and macromolecule should be diluted in the same buffer. Furthermore, air bubbles has to be avoided in the sample cell. Any air bubble left in the syringe after filling it can cause variation in the injected volume and lead to additional heat signals. Both of these have been observed closely in this experiment. However, numerous ratios of (ligand to macromolecule) need to be optimized in order to get a typical sigmoid curve. Additionally, to find the proper binding protein, a more tissue specific human library can be used as prey during the Y2H assay, for example, the human brain tissue and human liver tissue libraries (Lai & Lau, 2018).

ITC is a reliable method and has seen widely used for examining the binding affinity between two interacting proteins. For instance, Parker *et al*. (2015) employed ITC to investigate the interactions between apical membrane antigens (AMAs) on the apicomplexan surface and rhoptry neck 2 (RON2) proteins discharged from parasites during invasion. By using a panel of RON2 peptides as bait, they observed that there is only weak binding between *Eimeria tenella* RON2 (EtRON2) peptides but there is binding with high affinity to the TgAMA1ΔDII loop. As well, Yadav *et al*. (2011) measured the binding affinity between TgADF and rabbit muscle G actin by using ITC technique and found that there is strong binding affinity between them with an equilibrium constant of 23.81 nM (Yadav *et al*., 2011). Sevinc *et al*. (2011) used the ITC approach to study the interaction between four mutants of alpha spectrin (αII) with beta spectrin (βII) after performing the Y2H assay. Among the four studied mutants, αII-V22D, V22M and V22W are bound to beta spectrin (βII) protein with the same binding constant ($K_d$) value compared to the wild type of alpha spectrin (Sevinc *et al*., 2011).

Apart from studying the interaction between proteins, ITC also can be used to study interaction between other biomolecules as well as DNA, RNA, drugs, lipids and any other inhibitors. Additonally, ITC has been widely used to investigate domain-domain interaction, protein recognition, ligand-receptor blocking and antibody-receptor binding activities (Leavitt & Freire, 2001).

## 5.9 Limitation of study

The Y2H technology presented here are a starting point rather than a complete study to the elucidation of interaction networks. After screening of Y2H assay, many false positive clones were found. This is an obvious weakness that need to be highlighted. Since a large amount of colonies growing on the plates, there might be bias during the clones selection step. Subsequently, some of the potential proteins may be hindered. To further confirm the interaction between these proteins, the interaction of *T. gondii* SAG1 and SAG2 with their respective HLY and HZF proteins may need to be further examined by using other approach such as immunofluorescent assay (IFA).

# CHAPTER 6: CONCLUSION

In this study, Y2H was used to analyze the interactions of *T. gondii* SAG1 and SAG2 with human host cell proteins. As the result, HLY and HZF proteins were identified as potential candidates interacting with SAG1 and SAG2, respectively. The interaction were further examined by beta-galactosidase assay and the enzyme activity between SAG1 and SAG2 with their host proteins were 449.4 U and 437.7 U, respectively. In comparison to positive control (424.3 U), both interaction between prey and SAG1 and SAG2 were proven to exist. The interaction between preys and bait proteins was further determined by Co-IP assay. The results indicate that interaction between preys and their respective SAG1 and SAG2 were significant as compared to positive control.

Finally, the binding strength between these interacting proteins was measured via the ITC approach. The binding strength for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins were 0.0075 μM and 18.75 μM, respectively. Both thermodynamic curves revealed that there was an exothermic and endothermic reaction for recombinant pRSET-A-SAG1/pRSET-A-HLY proteins and recombinant pRSET-A-SAG2/pRSET-A-HZF proteins, respectively. These results indicate that the binding for SAG1 and SAG2 with their respective prey proteins were significant as compared to negative control.

Since the present study is a preliminary research for investigating the interactions of host proteins with *T. gondii* SAG1 and SAG2, the obtained data were insufficient to prove these proteins as receptors for SAG1 and SAG2. In future, more confirmation assays such as blocking assay, localization assay and protein modelling may need to be performed in order to fulfill this requirement.

## REFERENCES


Ahn, H., Kim, S., Kim, S., & Nam, H. (2006). Interactions between secreted GRA proteins and host cell proteins across the parasithophorous vacuolar membrane in the parasitism of *Toxoplasma gondii*. *Korean Journal of Parasitology*, *44*(4), 303-312.

Alexander, D. L., Mital, J., Ward, G. E., Bradley, P., & Boothroyd, J. C. (2005). Identification of the moving junction complex of *Toxoplasma gondii*: A collaboration between distinct secretory organelles. *Plos Pathogens, 1*(2), e17. doi: 10.1371/journal.ppat.0010017

Bandaranayake, A. D., & Almo, S. C. (2014). Recent advances in mammalian protein production. *Federation of European Biochemical Societies Letter*, *588*(2), 253-260.

Bahia-oliveira, L. M., Jones, J. L., Azevedo-Silva, J., Alves, C. C., Orefice, F., & Addiss, D. G. (2003). Highly endemic, waterborne toxoplasmosis in North Rio de Janeiro State, Brazil. *Emerging Infectious Diseases*, *9*(1), 55-62.

Beck, H. P., Blake, D., Darde, M. L., Felger, I., Pedraza-Diaz, S., Regidor-Cerrillo, J., Gomez-Bautista, M., Ortega-Mora, L. M., Putignani, L., Shiels, B., Tait, A., & Weir, W. (2009). Molecular approaches to diversity of populations of apicomplexan parasites. *International Journal for Parasitology*, *39*, 175-189.

Berggard, T., Linse, S., & James, P. (2007). Methods for the detection and analysis of protein-protein interactions. *Proteomics*, *7*(16), 2833-2842.

Black, M. W., & Boothroyd, J. C. (2000). Lytic cycle of *Toxoplasma gondii*. *Microbiology and Molecular Reviews*, *64*(3), 607-623.

Blader, I. J., Manger, I. D., & Boothroyd. J. C. (2001). Microarray analysis reveals previously unknown changes in *Toxoplasma gondii*-infected human cells. *The Journal of Biological Chemistry*, *276*, 24223-24231.

Bonsor, D. A., Weiss, E., Iosub-Amir, A., Reingewertz, T. H., Chen, T., W., Haas, R., Friedler, A., Fischer, W., & Sundberg, E. J. (2013). Characterization of the translocation-competent complex between the *Helicobacter pylori* oncogenic protein CagA and the accessory protein CagF. *The Journal of Biological Chemistry*, *288*(64), 32897-32909.

Bornhorst, J. A., & Falke, J. J. (2000). Purification of proteins using polyhistidine affinity tags. *Methods in Enzymology*, *326*, 245-254.

Brandon-Mong, G. J., Seri, N. A. A. C. M., Sharma, R. S. K., Andiappan, H., Tan, T. C., Lim, Y. A. L., & Nissapatorn, V. (2015). Seroepidemiology of toxoplasmosis among people having close contact with animals. *Frontiers in Immunology*, *6*(143), doi: 10.3389/fimmu.2015.00143

Braun, L., Cannella, D., Pinheiro, A. M., Kieffer, S., Belrhali, H., & Garin, J. (2009). The small ubiquitin–like modifier (SUMO)-conjugating system of *Toxoplasma gondii*. *International Journal of Parasitology*, *39*, 81-90.

Brozmanova, J., & Holinova, Z. (1988). A rapid preparation of plasmid DNA from *Saccharomyces cerevisiae*. *Folia Microbiologica*, *33*(1), 34-37.

Bruckner, A., Polge, C., Lentze, N., Auerbach, D., & Schlattner, U. (2009). Yeast two-hybrid, a powerful tool for systems biology. *International Journal of Molecular Sciences*, *10*, 2763-2788.

Burg, J. L., Grover, C. M., Pouletty, P., & Boothroyd, J. C. (1989): Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii* by PCR. *Journal of Clinical Microbiology*, *27*, 1787-1792.

Bussow, K. (2015). Stable mammalian producer cell lines for structural biology. *Current Opinion in Structural Biology*, *32*, 81-90.

Cambiaso, C. L., Galanti, L. M., Leautaud, P., & Masson, P. L. (1992). Latex agglutination assay of human immunoglobulin M antitoxoplasma antibodies which uses enzymatically treated antigen-coated particles. *Journal of Clinical Microbiology*, *30*, 882-888.

Camps, M., Arrizabalaga, G., & Boothroyd, J. (2002). An rRNA mutation identifies the apicoplast as the target for clindamycin in *Toxoplasma gondii*. *Molecular Microbiology*, *43*(5), 309-318.

Carruthers, V. B., Hakansson, S., Giddings, O. K., & Sibley, L. D. (2000). *Toxoplasma gondii* uses sulfated proteoglycans for substrate and host cell attachment. *Infection and Immunity*, *68*, 4005-4011.

Cassandri, M., Smirnov, A., Novelli, F., Pitolli, C., Agostini, M., Malewicz, M., Melino, G., & Raschellà, G. (2018). Zinc-finger proteins in health and disease. *Cell Death Discovery*, *3*(17071), doi: 10.1038/cddiscovery.2017.71

Castello, A., Fischer, B., Frese, C. K., Horos, R., Alleaume, A., Foehr, S., Curk, T., Krijgsveld, J. & Hentze1, M.W. (2016). Comprehensive identification of RNA-binding domains in human cells. *Molecular Cell*, *63*(4), 696-710.

Celenza, J. L., Eng, F. J., & Carlson, M. (1989). Molecular analysis of the SNF4 gene of *Saccharomyces cerevisiae*: evidence for physical association of the SNF4 protein with the SNF1 protein kinase. *Molecular and Cellular Biology*, *9*(11), 5045-5054.

Cesbron-Delauw, M. F., Guy, B., Torpier, G., Pierce, R. J., Lenzen, G., Cesbron, J. Y., Charif, H., Lepage, P., Darcyf, J., Lecocq, P., & Capro, A. (1989). Molecular characterization of a 23-kilodalton major antigen secreted by *Toxoplasma gondii* (cDNA cloning/Ca2+-binding protein). *Proceedings of the National Academy of Science of the United States America*, *86*, 7537-7541.

Chan, B. T. E., Amal, R. N., Hayati, M. I. N., Kino, H., Anisah, N., Norhayati, M., Sulaiman, O., Abdullah, M. M., Fatmah, M. S., Roslida, A. R., & Ismail, G.

(2008). Seroprevalence of Toxoplasmosis among migrant workers from different ASIAN countries working in Malaysia. *The Southeast Asian Journal Tropical and Medicine and Public Health*, *39*(1), 9-13.

Chang, S., Shan, X., Li, X., Fan, W., Zhang, S. Q., Zhang, J., Jiang, N., Ma, D., & Mao, Z. (2015). *Toxoplasma gondii* rhoptry protein ROP16 mediates partially SH-SY5Y cells apoptosis and cell cycle arrest by directing Ser15/37 phosphorylation of p53. *International Journal of Biological Sciences*, *11*(10), 1215-1225.

Chen, J., Li, A. Y., Zhou, D. H., Liu, G. H., & Zhu, X. Q. (2012). Genetic diversity among *Toxoplasma gondii* strains from different hosts and geographical regions revealed by sequence analysis of GRA5 gene. *Parasites & Vectors*, *5*(279), https://doi.org/10.1186/1756-3305-5-279

Chen, X. M., Huang, B. Q., Splinter, P. L., Cao, H., Zhu, G., & Mc Niven M. A. (2003). *Cryptosporidium parvum* invasion of biliary epithelia requires host cell tyrosine phosphorylation of cortactin via c-Src. *Gastroenterology*, *125*, 216-228.

Chien, C., Bartel, P. L., Sternglanz, R., & Fields, S. (1991). The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proceedings of the National Academy of Sciences United States of America*, 88, 9578-9582.

Chintana, T., Sukthana, Y., Bunyakai, B., & Lakkla, A. (1998). *Toxoplasma gondii* antibody in pregnant women with and without HIV infection. *Southeast Asean Journal Tropical Medicine and Public Health*, *29*(2), 383-386.

Choi, W., Nam, H., Kwak, N., Huh, W., Kim, Y., Kang, M., Cho, S., & Dubey, J. P. (1997). Foodborne outbreaks of human toxoplasmosis. *Journal of Infectious Diseases*, *175*(5), 1280-1282.

Coates, P. J., & Hall, P. A. (2003). The yeast two-hybrid system for identifying protein-protein interactions. *The Journal of Pathology*, *199*(1), 4-7.

Cong, H., Zhang, M., Zhang, A., Gong, J., Cong, H., Xin, Q., & He, S. (2013). Analysis of Structures and epitopes of surface antigen glycoproteins expressed in bradyzoites of *Toxoplasma gondii*. *BioMed Research International*, *2013*(2013), http://dx.doi.org/10.1155/2013/165342

Cong, W., Dong, X. Y., Meng, Q. F., Zhou, N., Wang, X. Y., Huang, S. Y., Zhu, X. Q., & Qian, A. D. (2015). *Toxoplasma gondii* infection in pregnant women: A seroprevalence and case-control study in Eastern China. *BioMed Research International*, *2015*(2015), http://dx.doi.org/10.1155/2015/170278

Costa, J. M., Alanio, A., Moukoury, S., Clairet, V., Debruyne, M., Poveda, J. D., & Bretagne, S. (2013). Direct genotyping of *Toxoplasma gondii* from amniotic fluids based on B1 gene polymorphism using minisequencing analysis. *BMC Infectious Diseases*, *13*(552), doi: 10.1186/1471-2334-13-552

da Silva, M. G., Vinaud, M. C., & de Castro, A. M. (2015). Prevalence of toxoplasmosis in pregnant women and vertical transmission of *Toxoplasma gondii* in patients

from basic units of health from Gurupi, Tocantins, Brazil, from 2012 to 2014. *PLoS One*, *10*(11), e0141700. doi: 10.1371/journal.pone.0141700

da Silva, P. C., Shiraishi, C. S., Silva, A. V., Goncalves, G. F., Sant'Ana Dde, M. & Araujo, E. J. (2010). *Toxoplasma gondii*: a morphometric analysis of the wall and epithelial cells of pigs intestine. *Experimental Parasitology*, *125*, 380-383.

Dawson, D. (2005). Foodborne protozoan parasites. *International Journal of Food Microbiology*, *84*, 621-622.

de Ory, F., Casas, I., Domingo, C. J., & Echevarria, J. (1995). Application of fluoroimmunoassay to the identification of low avidity specific IgG against pathogenic human viruses and *Toxoplasma gondii*. *Clinical and Diagnostic Virology*, *3*, 323-332.

Denkers, E. Y., & Gazzinelli, R. T. (1998). Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clinical Microbiology Reviews*, *11*(4), 569-588.

Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., Butler, B. L., Binkowski, B. F., Machleidt, T., Kirkland, T. A., Wood, M. G., Eggers, C. T., Encell, L. P., & Wood, K. V. (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *American Chemical Society Chemistry Biology*, *11*, 400-408.

Drescher, D. G., Ramakrishnan, N. A., & Drescher, M. J. (2009). Surface plasmon resonance (SPR) analysis of binding interactions of proteins in inner-ear sensory epithelia. *Methods in Molecular Biology*, *493*, 323-343.

Dubey, J. P. (2008). The history of *Toxoplasma gondii*-The first 100 years. *Journal of Eukaryotic Microbiology*, *55*(6), 467-475.

Dubey, J. P., Lindsay, D. S., & Speer, C. A. (1998). Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clinical Microbiology Reviews*, *11*(2), 267-299.

Dumon-Seignovert, L., Cariot, G., & Vuillard, L. (2004). The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). *Protein Expression and Purification*, *37*(1), 203-206.

Eduardo, S. L. (1991). Food-borne parasitic zoonoses in the Philippines. *The Southeast Asian Journal of. Tropical Medicine and Public Health*, *22*, 16-22.

Fatoohi, A. F., Cozon, G. J., Greenland, T., Ferrandiz, J., Bienvenu, J., Picot, S., & Peyron, F. (2002). Cellular immune responses to recombinant antigens in pregnant women chronically infected with *Toxoplasma gondii*. *Clinical and Diagnostic Laboratory Immunology*, *9*(3), 704-707.

Fields, S., & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature*, *340*, 245-246.

Flegr, J., Prandota, J., Sovičková, M., & Israili, Z. H. (2014). Toxoplasmosis - A Global Threat. Correlation of latent Toxoplasmosis with specific disease burden in a set of 88 countries. *PLoS One*, *9*(3), e90203. doi: 10.1371/journal.pone.0090203

Foury, F. (1997). Human genetic diseases: a cross-talk between man and yeast. *Gene*, *195*(1), 1-10.

Freyre, A., Falcon, J., Mendez, J., Rodriguez, A., Correa, L., & Gonzalez, M. (2001). Refinement of the mouse model of congenital toxoplasmosis. *Experimental Parasitology*, *113*(3), 154-160.

Fuentes, I., Rubio, J. M., Ramirez, C., & Alvar, J. (2001). Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. *Journal of Clinical Microbiology*, *39*(4), 1566-1570.

Fujikawa, Y., Nakanishi, T., Kawakami, H., Yamasaki, K., Sato, M. H., Tsuji, H., Matsuoka, M., & Kato, N. (2014). Split luciferase complementation assay to detect regulated protein-protein interactions in rice protoplasts in a large-scale format. *Rice*, *7*(11), doi: 10.1186/s12284-014-0011-8

Garcia-Fuentes, L., Ramiro, Tellez-Sanz, Quesada-Soriano, I., & Baron, C. (2011). *Thermodynamics of molecular recognition by calorimetry: thermodynamics-physical chemistry of aqueous systems*. Europe: InTech.

Gaji, R. Y., Johnson, D. E., Treeck, M., Wang, M., Hudmon, A., & Arrizabalaga, G. (2015). Phosphorylation of a myosin motor by TgCDPK3 facilitates rapid initiation of motility during *Toxoplasma gondii* egress. *PLoS Pathogens*, *11*(11), e1005268. doi: 10.1371/journal. ppat.1005268

Galao, R., Scheller, N., Alves-Rodrigues, I., Breinig, T., Meyerhans, A., & Diez, J. (2007). *Saccharomyces cerevisiae*: a versatile eukaryotic system in virology. *Microbial Cell Factories*, *6*(32), doi: 10.1186/1475-2859-6-32

Gharavi, M. J., Jalali, S., Khademvatan, S., & Heydari, S. (2011). Detection of IgM and IgG anti-Toxoplasma antibodies in renal transplant recipients using ELFA, ELISA and ISAGA methods: comparison of pre- and post- transplantation status. *Annals of Tropical Medicine & Parasitology*, *105*(5), 367-371.

Ghosh, C., Gupta, R., & Mukherjee, K. J. (2012). An inverse metabolic engineering approach for the design of an improved host platform for over-expression of recombinant proteins in *Escherichia coli*. *Microbial Cell Factories*, *11*(93), doi: 10.1186/1475-2859-11-93

Gissot, M., Hovasse, A., Chaloin, L., Schaeffer-Reiss, C., Van Dorsselaer, A. & Tomavo, S. (2016). An evolutionary conserved zinc finger protein is involved in *Toxoplasma gondii* mRNA nuclear export. *Cellular Microbiology*, *19*(2), e12644. https://doi.org/10.1111/cmi.12644

Grimwood, J., & Smith, J. E. (1996). *Toxoplasma gondii*: The role of parasite surface and secreted proteins in host cell invasion. *International Journal for Parasitology*, *26*(2), 169-173.

Guimaraes, E. V., Acquarone, M., de Carvalho, L., & Barbosa, H. S. (2010). Detection of fibronectin and laminin in *Toxoplasma gondii* tissue cysts: immunocytochemical assays. *Scientia Medica (Porto Alegre)*, *20*, 83-87.

Gunn, L., & Nickoloff, J. A. (1995). Rapid transfer of low copy-number episomal plasmids from *Saccharomyces cerevisiae* to *Escherichia coli* by electroporation, *Molecular Biotechnology*, *3*, 79-84.

Gyuris, J., Golemis, E., Chertov, H., & Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*, *75*, 791-803.

Handman, E., Goding, J. W., & Remington, J. S. (1980). Detection and characterization of membrane antigen of *Toxoplasma gondii*. *Immunology*, *124*, 2578-2583.

Heaslip, A. T., Nishi, M., Stein, B., & Hu, K. (2011). The motility of a human parasite, *Toxoplasma gondii*, is regulated by a novel lysine methyltransferase. *PLoS Pathogen*, *7*(9), e1002201. doi: 10.1371/journal.ppat.1002201

Herwaldt, B. L. (2001). Laboratory-acquired parasitic infections from accidental exposures. *Clinical Microbiology Reviews*, *14*(4), 659-688.

Hester, J., Mullins, J., Sa, Q., Payne, L., Mercier, C., Cesbron-Delauw, M., & Suzuki, Y. (2012). *Toxoplasma gondii* antigens recognized by IgG antibodies differ between mice with and without active proliferation of tachyzoites in the brain during the chronic stage of infection. *Infection Immunity*, *80*(10), 3611-3620.

Hibino, K., Hiroshima, M., Takahashi, M., & Sako, Y. (2009). Single-molecule imaging of fluorescent proteins expressed in living cells. *Methods in Molecular Biology*, *48*, 451-460

Hoff, F. H., & Carruthers, V. B. (2002). Is Toxoplasma egress the first step in invasion? *Trends in Parasitology*, *18*(6), 251-255.

Hohlfeld, P., Daffos, F., Costa, J. M., Thulliez, P., Forestier, F., & Vidaud, M. (1994). Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. *The New England Journal of Medicine*, *331*, 695-699.

Holm, C., Meeks-Wagner, D. W., Fangman, W. L., & Botstein, D. (1986). A rapid, efficient method for isolating DNA from yeast. *Gene*, *42*(2), 169-173.

Howe, D. K., Honore, S., Deroin F., & Sibley, L. D. (1997). Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *Journal of Clinical Microbiology*, *35*(6), 1411-1414.

Howle, D. K., & Sibley, L. D. (1995). *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *Journal of Infectious Diseases*, *172*(6), 1561-1566.

Huong, L. T. T., & Dubey, J. P. (2007). Seroprevalence of *Toxoplasma gondii* in pigs from Vietnam. *Journal of Parasitology*, *93*(4), 951-952.

Jager, V., Bussow, K., Wagner, A., Weber, S., Hust, M., Frenzel, A., & Schirrmann, T. (2013). High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. *BMC Biotechnology*, *13*(52), doi: 10.1186/1472-6750-13-52

Johnson, A. M., McDonald, P. J., & Neoh, S. H. (1983). Molecular weight analysis of soluble antigens from *Toxoplasma gondii*. *Journal of Parasitology*, *69*, 459-464.

Jones, C. D., Okhravi, N., Adamson, P., Tasker, S., & Lightman, S. (2000). Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor. *Investigative Ophthalmology & Visual Science*, *41*(3), 634-644.

Jones, J. L., Parise, M. E., & Fiore, A. E. (2014). Neglected Parasitic Infections in the United States: Toxoplasmosis. *The American Journal of Tropical Medicine and Hygiene*, *90*(5), 794-799.

Jones, E. J., Korcsmaros, T., & Carding, S. R. (2017). Mechanisms and pathways of *Toxoplasma gondii* transepithelial migration. *Tissue Barriers*, *5*(1), e1273865. http://dx.doi.org/10.1080/21688370.2016.1273865

Joseph, S. B., & Hall, D. W. (2004). Spontaneous mutations in diploid *Saccharomyces cerevisiae*: more beneficial than expected. *Genetics*, *168*, 1817-1825.

Karim, J., Taylor, D., & Cullen, D. C. (2007). Measurement of conformational changes in the structure of transglutaminase on binding calcium ions using optical evanescent dual polarisation interferometry. *Analytical Chemistry*, *79*, 3023-3031.

Kasper, L. H., Crabb, J. H., & Pfefferkorn, E. R. (1983). Purification of a major membrane protein of *Toxoplasma gondii* by immunoabsorption with a monoclonal antibody. *The Journal of Immunology*, *130*, 2407-2412.

Kasper, D. C., Sadeghi, K., Prusa, A. R., Reischer, G. H., Kratochwill, K., Förster-Waldl, E., Gerstl, N., Hayde, M., Pollak, A., & Herkner, K. R. (2009). Quantitative real-time polymerase chain reaction for the accurate detection of *Toxoplasma gondii*in amniotic fluid. *Diagnostic Microbiology and Infectious Disease*, *63*(1), doi: 10.1016/j.diagmicrobio.2008.09.009

Kawashima, T., Khin-Sane-Win, Kawabata, M., Barzaga, N., Matsuda, H., & Konishi, E. (2000). Prevalence of antibodies to *Toxoplasma gondii* among urban and rural residents in Philippines. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *31*(4), 742-746.

Kazemi, B., Bandehpour, M., Maghen, L., & Solgi, G. H. (2007). Gene cloning of 30 kDa *Toxoplasma gondii* tachzyzoite surface antigen (SAG1). *Iranian Journal of Parasitology*, *2*(2), 1-8.

Khalifa, K. S., Roth, A., Roth, B., Arasteh, K. N., & Janitschke, K. (1994). Value of PCR for evaluating occurrence of parasitemia in immunocompromised patients with cerebral and extracerebral toxoplasmosis. *Journal of Clinical Microbiology*, *32*, 2813-2819.

Khan, A., Fux, B., Su, C., Dubey, J. P., Darde, M. L., Aijoka, J. W., Rosenthal, B. M., & Sibley, L. D. (2007). Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. *Proceedings of National Academy Sciences of the United States of America, 104*, 14872-14877.

Khan, A., Su, C., German, M., Storch, G. A., Clifford, D. B., & Sibley, L. D. (2005). Genotyping of strains from immunocompromised patients reveals high prevalence of type I strains. *Journal of Clinical Microbiology*, *43*(12), 5881-5887.

Khine, D. A. A. (2005). Case report 3: Congenital Toxoplamosis. *Myanmar Journal of Current Medical Practice*, *9*, 157-158.

Kim, K. S., Kim, M. S., Moon, J. H., Jeong, M. S., Kim, J., Lee, G. M., Myung, P. K., & Hong, H. J. (2009). Enhancement of recombinant antibody production in HEK 293E cells by WPRE. *Biotechnology and Bioprocess Engineering*, *14*, 633-638.

Kim, K., & Weiss, L. M. (2004). *Toxoplasma gondii*: the model apicomplexan. *International Journal of Parasitology*, *34*(3), 423-432.

Konishi, E., Houki, Y., Harano, K., Mibawani, R. S., Marsudi, D., Alibasah, S., & Dachlan Y. P. (2000). High prevalence of antibody to *Toxoplasma gondii* among humans in Surabaya, Indonesia. *Japanese Journal of Infectious Diseases*, *53*, 238-241.

Kosloff, M., & Kolodny, R. (2008). Sequence-similar, structure-dissimilar protein pairs in the PDB. *Proteins*, *71*(2), 891-902.

Kurokawa, H., Kato, K., Iwanaga, T., Sugi, T., Sudo, A., & Kobayashi, K. (2011). Identification of *Toxoplasma gondii* cAMP dependent protein kinase and its role in the tachyzoite growth. *PLOS One*, 6(e22492), doi: 10.1371/journal.pone.0022492

Kusumawati, A., Septiana, N., & Hartati, S. (2011). Digoxigenin (DIG) labeled probe candidate of surface antigen 1 (SAG1) for *Toxoplasma gondii* detection. *Indonesian Journal of Biotechnology*, *16*(1), 17-23.

Kvaal, C. A., Radke, J. R., Guerini, M. N., & White, M. W. (2002). Isolation of a *Toxoplasma gondii* cyclin by yeast two-hybrid interactive screen. *Molecular and Biochemical Parasitology, 120*, 187-194.

Lai, M. Y., & Lau, Y. L. (2017). Screening and identification of host proteins interacting with *Toxoplasma gondii* SAG2 by yeast two-hybrid assay. *Parasites & Vectors*, *10*(1), 456. doi: 10.1186/s13071-017-2387-y

Lai, M. Y., & Lau, Y. L. (2018). Measurement of binding strength between prey proteins interacting with *Toxoplasma gondii* SAG1 and SAG2 using isothermal titration calorimetry (ITC). *Acta Parasitologica*, *63*(1), doi: 10.1515/ap-2018-00

Laity, J. H., Lee, B. M., & Wright, P. E. (2001). Zinc finger proteins: new insights into structural and functional diversity. *Current Opinion in Structural Biology*, *11*, 39-46.

Laliberte, J., & Carruthers, V. B. (2008). Host cell manipulation by the human pathogen *Toxoplasma gondii. Cellular and Molecular Life Science*, *65*(12), 1900-1915.

Lamarque, M. H., Papoin, J., Finizio, A., Lentini, G., Pfaff, A. W., Candolfi E, Dubremetz, J. F., & Lebrun, M. (2012). Identification of a new rhoptry neck complex RON9/RON10 in the apicomplexa parasite *Toxoplasma gondii*. *PLOS One*, *7*(e32457), doi: 10.1371/journal.pone.0032457.

Lau, Y. L., Meganathan, P., Sonaimuthu, P., Thiruvengadam, G., Nissapatorn, V., & Chen, Y. (2010). Specific, sensitive and rapid diagnosis of active toxoplasmosis by a loop-mediated isothermal amplification (LAMP) method using blood samples from patients. *Journal of Clinical Microbiology*, *48*(10), 3698-3702.

Leavitt, S., & Freire, E. (2001). Direct measurement of protein binding energetics by isothermal titration calorimetry. *Current Opinion in Structural Biology*, *11*, 560-566.

Lecordier, L., Mercier, C., Sibley, L. D., & Cesbron-Delauw, M. (1999). Transmembrane insertion of the *Toxoplasma gondii* GRA5 protein occurs after soluble secretion into the host cell. *Molecular Biology of the Cell*, *10*(4), 1277-1287.

Lekutis, C., Ferguson, D. J., Grigg, M. E., Camps, M., & Boothroyd, J. C. (2001). Surface antigens of *Toxoplasma gondii*: variations on a theme. *International Journal of Parasitology*, *31*, 1285-1292.

Lekutis, C., Ferguson, D. J. P., & Boothroyd, J. C. (2000). *Toxoplasma gondii*: identification of a developmentally regulated family of genes related to SAG2. *Experimental Parasitology*, *96*, 89-96.

Liang, Y. (2008). Applications of isothermal titration calorimetry in protein science. *Acta Biochimica Et Biophysica Sinica*, *40*(7), 565-576.

Lin, L. N., Hasumi, H., & Brandts, J. F. (1988). Catalysis of proline isomerization during protein-folding reactions. *Biochimica et Biophysica Acta*, *956*, 256-266.

Liu, J., & Walsh, C. T. (1990). Peptidyl-prolyl cis-trans-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proceedings of the National Academy Sciences United States America*, *87*, 4028-4032.

Liu, Q., Wang, Z. D., Huang, X. Y., & Zhu, X. Q. (2015). Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. *Parasites & Vectors*, *8*(292), doi: 10.1186/s13071-015-0902-6

Lorber, B., Fischer, F., Bailly, M., Roy, M., & Kern, D. (2012). Protein analysis by dynamic light scattering: Methods and techniques for students. *Biochemistry and Molecular Biology Education*, *40*(6), 377-382.

Macedo, J. A. G., Junior, J. P. C., Cardoso, T. H. S., Silva, M. V., Santiago, F. M., Silva, J. S., Pirovani, C. P., Silva, D. A. O., Mineo, J. R., & Mineo, T. W. P. (2013). SAG2A protein from *Toxoplasma gondii* interacts with both innate and adaptive immune compartments of infected hosts. *Parasites & Vectors*, *6*(163), doi: 10.1186/1756-3305-6-163

Massoud, T. F., Paulmurugan, R., & Gambhir, S. S. (2004). Molecular imaging of homodimeric protein-protein interactions in living subjects. *Federation of European Biochemical Societies Journal*, *18*, 1105-1107.

McCoy, J. M., Whitehead, L., van Dooren, G. G., & Tonkin, C. J. (2012). TgCDPK3 regulates calcium-dependent egress of *Toxoplasma gondii* from host cells. *PLOS Pathogen, 8*(e1003066), doi: 10.1371/journal.ppat.1003066

McFadden, D. C., Tomavo, S., Berry, E. A., & Boothroyd, J. C. (2000). Characterization of cytochrome b from *Toxoplasma gondii* and Q(o) domain mutations as a mechanism of atovaquone-resistance. *Molecular and Biochemical Parasitology*, *108*(1), 1-12.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging of Infectious Disease*, *5*, 607-625.

Meganathan, P., Singh, S., Lau, Y. L., Singh, J., Subrayan, V., & Nissapatorn, V. (2010). Detection of *Toxoplasma gondii* DNA by PCR following microwave treatment of serum and whole blood. The *Southeast Asian Journal of Tropical Medicine and Public Health*, *41*(2), 265-273.

Mignolet, J., Van der Henst, C., Nicolas, C., Deghelt, M., Dotreppe, D., Letesson, J. J., & De Bolle, X. (2010). PdhS, an old-pole-localized histidine kinase, recruits the fumarase FumC in *Brucella abortus*. *Journal of Bacteriology*, *192*(12), 3235-3239.

Miller, K. E., Kim, Y., Huh, W. K., & Park, H. O. (2015). Bimolecular fluorescence complementation (BiFC) analysis: advances and recent applications for genome-wide interaction studies. *Journal of Molecular Biology*, *427*(11), 2039-2055.

Mineo, J. R., McLeod, R., Mack, D., Smith, J., Khan, I. A., Ely, K. H., & Kasper, L. H. (1993). Antibodies to *Toxoplasma gondii* major surface protein (SAG1-P30) inhibit infection of host cells and are produced in murine intestine after peroral infection. *The Journal of Immunology*, *150*, 3951-3964.

Mineo, J. R., & Kasper, L. H. (1994). Attachment of *Toxoplasma gondii* to host cell involves major surface protein, SAG-1(P30). *Experimental Parasitology*, *79*, 11-20.

Miyake, Y., Ishii, K., & Honda, A. (2017). Influenza virus infection induces host pyruvate kinase M which interacts with viral RNA-dependent RNA polymerase. *Frontiers in Microbiology*, *8*(162), doi: 10.3389/fmicb.2017.00162

Mo, X. L., & Fu, H. (2016). BRET: NanoLuc-based bioluminescence resonance energy transfer platform to monitor protein-protein interactions in live cells. *Methods in Molecular Biology*, *1439*, 263-271.

Mohan, T. C., Jalil, H. A., Nadarajah, M., & Sng, E. H. (1991). Anti-Toxoplasma antibodies in healthy adults and in different patient categories. *Singapore Medical Journal*, *32*, 344-347.

Montoya, J. G. (2002). Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. *The Journal of Infectious Diseases*, *85*, S73-82.

Moura, L., Bahia-Oliveira, L. M. G., Wada, M. Y., Jones, J. L., Tuboi, S. H., Carmo,E. H., Ramalho, W. M., Camargo, N. J., Trevisan, R., Graça, R. M. T., da Silva, A. J., Moura, I., Dubey, J. P., & Garrett, D. O. (2006). Waterborne toxoplasmosis, Brazil, from field to gene. *Emerging of Infectious Diseases*, *12*, 326-329.

Mukherjee, S., Bal, S., & Saha, P. (2001). Protein interaction maps using yeast two-hybrid assay. *Current Science*, *81*, 458-464.

Nagel, S. D., & Boothroyd, J. C. (1989). The major surface antigen, P30, of *Toxoplasma gondii* is anchored by a glycolipid. *The Journal of Biochemistry*, *264*(10), 569-5574.

Nam, H. (2009). GRA Proteins of *Toxoplasma gondii*: Maintenance of host-parasite interactions across the parasitophorous vacuolar membrane. *Korean Journal of Parasitology*, *47*, S29-S37.

Nam, H. W., Im, K. S., Baek, E. J., Choi, W. Y., & Cho, S. Y. (1996). Analysis of antigenic domain of GST fused major surface protein (p30) fragments of *Toxoplasma gondii*. *Korean Journal of Parasitology*, *34*, 135-141.

Ngui, R., Lim, Y. A. L., Amir, N. F. H., Nissapatorn, V., & Mahmud, R. (2011). Seroprevalence and sources of Toxoplasmosis among orang asli (Indigenous) communities in Peninsular Malaysia. *American Journal of Tropical Medicine and Hygiene*, *85*(4), 660-664.

Nepomucena-Silva, J. L., de Melo, L. D. B., Mendonca, S. M., Paixao, J. C., & Lopes, U. G. (2003). RJLs: a new family of Ras-related GTP-binding proteins. *Gene, 327*, 221-232.

Nimir, A., Othman, A., Ee, S., Musa, Z., Majid, I. A., Kamarudin, Z., Xian, C., & Isa, N. H. (2010). Latent toxoplasmosis in patients with different malignancy: A hospital based study. *Journal of Clinical Medical Research*, *2*(3), 117-120.

Nissapatorn, V., Kuppusamy, I., Rohela, M., Anuar, A. K. & Fong, M. Y. (2004). Extrapulmonary tuberculosis in Peninsular Malaysia: retrospective study of 195

cases. *Southeast Asian Journal of Tropical Medicine and Public Health*, *35*(2), 39-45.

Nissapatorn, V., Teoh, H. L., Lee, R., Ithoi, I., Ibrahim, J., & Tan, S. Y. (1991). Seroepidemiological study of toxoplasmosis in Laos. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *42*(3), 111-114.

Nyunt, U. T. (2005). Report on laboratory diagnosis of toxoplasmosis in Myanmar at the National Health Laboratory. *Myanmar Journal of Current Medical Practice*, *9*, 120-124.

Oshiro, S., Katsura, N., Kitada, K., & Gunge, N. (1987). A simple method for extraction of DNA from fungi and yeasts with anhydrous hydrogen fluoride. *Federation of European Biochemical Societies Letters*, *220*, 383-386.

Ooi, A., Wong, A., Esau, L., Lemtiri-Chlieh, F., & Gehring, C. (2016). A guide to transient expression of membrane proteins in HEK-293 cells for functional characterization. *Frontiers in Physiology*, *7*(300), doi: 10.3389/fphys.2016.00300

Pan, M., Zhou, Y., Wang, Y., Li, L., Song, Y., Hou, L., & Zhao, J. (2017). Screening and identification of the host proteins interacting with *Toxoplasma gondii* rhoptry protein ROP16. *Frontiers in Microbiology*, *8*(2408), doi: 10.3389/fmicb.2017.02408

Pappas, G., Roussos, N., & Falagas, M. E. (2009). Toxoplasmosis snapshots: Global status *of Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *International Journal for Parasitology*, *39*, 1385-1394.

Parker, M. L., Penarete-Vargas, D. M., Hamilton, P. T., Guérin, A., Dubey, J. P., Perlman, S. J., Spano, F., Lebrun, M., & Boulanger, M. J. (2015). Dissecting the interface between apicomplexan parasite and host cell: Insights from a divergent AMA-RON2 pair. *Proceedings of the National Academy of Science of the United States America*, *113*(2), 398-403.

Parsons, A. B., Geyer, R., Hughes, T. R., & Boone, C. (2003). Yeast genomics and proteomics in drug discovery and target validation. *Progress in Cell Cycle Research*, *5*, 159-166.

Perkins, E. M. (1992). Rhoptry organelles of apicomplexan parasites. *Parasitology Today*, *8*(1), 28-32.

Pierce, M. M., Raman, C. S., & Nall, B. T. (1999). Isothermal titration calorimetry of protein-protein interactions. *Methods*, *19*, 213-221.

Pomel, S., Luk, F. C. Y., & Beckers, C. J. M. (2008). Host cell egress and invasion induce marked relocations of glycolytic enzymes in *Toxoplasma gondii* tachyzoites. *PLOS Pathogen*, *10*(e1000188), doi: 10.1371/journal.ppat.1000188

Possenti, A., Fratini, F., Fantozzi, L., Pozio, E., Dubey, J. P., & Ponzi, M. (2013). Global proteomic analysis of the oocyst/sporozoite of *Toxoplasma gondii* reveals commitment to a host-independent lifestyle. *BioMed Central Genomics*, *14*(183), doi: 10.1186/1471-2164-14-183

Que, X., Ngo, H., Lawton, J., Gray, M., Liu, Q., & Engel, J. (2002). The cathepsin B of *Toxoplasma gondii*, toxopain-1, is critical for parasite invasion and rhoptry protein processing. *The Journal of Biological Chemistry*, *277*, 25791-25797.

Rahman, W. A., Manimegalai, V., Chandrawathani, P., Nurulaini, R., Zaini, C. M., & Premaalatha, B. (2011). Seroprevalence of *Toxoplasma gondii* in Malaysian cattle. *Malaysian Journal of Veterinary Research*, *2*(2), 51-56.

Rajarathnam, K., & Rosgen, J. (2014). Isothermal titration calorimetry of membrane proteins-Progress and challenges. *Biochimica et Bipphysica Acta*, *1838*, 69-77.

Ramalingam, A., Kudapa, H., Pazhamala, L. T., Garg, V., & Varshney, R. K. (2015). Gene expression and yeast two-hybrid studies of 1R-MYB transcription factor mediating drought stress response in chickpea (*Cicer arietinum L.*). *Frontiers in Plant Science*, *6*(1117), doi: 10.3389/fpls.2015.01117

Ramkumar, S., Pai, V. R., Thangadurai, R., & Murugan, V. P. (2017). Chemical complexity of protein determines optimal *E. coli* expression host; A comparative study using erythropoietin, streptokinase and tumor necrosis factor receptor. *Journal of Genetic Engineering and Biotechnology*, *15*(1), 179-185.

Rao, V. S., Srinivas, K., Sujini, G. N., & Kumar, G. N. S. (2014). Protein-protein interaction detection: Methods and analysis. *International Journal of Proteomics*, *2014*, 1-12. doi: 10.1155/2014/147648

Reese, M. L., Zeiner, G. M., Saeij, J. P. J., Boothroyd J. C., & Boyle, J. P. (2011). Polymorphic family of injected pseudokinases is paramount in *Toxoplasma* virulence. *Proceedings of the National Academy of Sciences*, *108*(23), 9625-9630.

Reiter-Owana, I., Peterson, E., Joynson, D, Aspock, H., Darde, M. L., Disko, R., Dreazen, O., Dumon, H., Grilo, R., Gross, U., Holliman, R., Ho-yen, D. O., Janitschke, K., Jenum, P. A., Naser, K., Olszewski, M., Thulliez, P., & Seitz, H. M. (1999). The past and present role of the Sabin-Feldman dye test in the serodiagnosis of toxoplasmosis. *Bulletin of the World Health Organization*, *77*(11), 929-935.

Remington, J. S., Thulliez, P., & Montoya, J. G. (2004). Recent developments for diagnosis of toxoplasmosis. *Journal of Clinical Microbiology*, *42*(3), 941-945.

Renold, C., Sugar, A., Chave, J. P., Perrin, L., delavelle, J., Pizzolato, G., & Hirschel, B. (1992). *Toxoplasma* encephalitis in patients with the acquired immunodefiency syndrome. *Medicine (Baltimore)*, *71*(4), 224-239.

Robinson, S. A., Smith, J. E., & Millner, P. A. (2004). *Toxoplasma gondii* major surface antigen (SAG1): *in vitro* analysis of host cell binding. *Parasitology*, *128*, 391-396.

Roos, D. S., Donald, R. G. K., Morrissette, N. S., & Moulton A. L. C. (1994). Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods in cell Biology*, *45*, 27-63.

Saeij, J. P. J., Boyle, J. P., & Boothroyd, J. C. (2005). Among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. *Trends in Parasitology*, *21*(10), 476-481.

Saffer, L. D., Mercereau-puijalon, O., Dubremetz, J. F., & Schwartzman, J. D. (1992). Localization of a *Toxoplasma gondii* rhoptry protein by immunoelectron microscopy during and after host cell penetration. *The Journal of Protozoology*, *39*, 529-530.

Schutze, K., Harter, K., & Chaban, C. (2009). Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in living plant cells. *Methods in Molecular Biology*, *479*, 189-202.

Senya, C., Mehta, A., Harwell, J. I., Pugatch, D., Flanigan, T., & Mayer, K. H. (2003). Spectrum of opportunistic infections in hospitalized HIV-infected patients in Phnom Penh, Cambodia. *International Journal of STD & AIDS*, *14*(6), 411-416.

Seidel, S. A. I., Dijkman, P. M., Lea, W. A., Bogaart, G. V. D., Jerabek-Willemsen, M., Lazic, A., Joseph, J. S., Srinivasan, P., Baaske, P., Simeonov, A., Katritch, I., Melo, F. A., Ladbury, J. E., Schreiber, G., Watts, A., Braun, A., & Duhr, S. (2013). Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. *Methods*, *59*(3), 301-315.

Sevinc, A., Witek, M. A., & Fung, L. W. M. (2011). Yeast two-hybrid and ITC studies of alpha and beta spectrin interaction at the tetramerization site. *Cellular & Molecular Biology Letters*, *16*(3), 452-461.

Shirley, M.W., Smith, A.L., & Blake, D.P. (2007). Challenges in the successful control of the avian coccidia. *Vaccine*, *25*, 5540-5547.

Sinai, A. P., Webster, P., & Joiner, K. A. (1997). Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii* parasitophorous vacuole membrane: high affinity interaction. *Journal of Cell Science*, *110*, 2117-2128.

Sibley, L. D., & Boothroyd, J. C. (1992). Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature*, *359*(6390), 82-85.

Silva. J. C., Ogassawara, S., Adania, C. H., Ferreira, F., Genari S. M., Dubey, J. P., & Ferreira-Neto, J. S. (2001). Seroprevalence of *Toxoplasma gondii* in captive neotropical felids from Brazil. *Veterinary Parasitology*, *102*(3), 217-224.

Singh, M. V., & Weil, P. A. (2002). A method for plasmid purification directly from yeast. *Analytical Biochemistry*, *307*, 13-17.

Singh, M., Zaman, V., & Goh, T. K. (1968). A report on the prevalence of toxoplasmic antibodies in Singapore. *Singapore Medical Journal*, *9*, 108-110.

Singh, S. (2003). Mother-to-child transmission and diagnosis of *Toxoplasma gondii* infection during pregnancy. *Indian Journal of Medical Microbiology*, *21*(2), 69-76.

Sloves, P. J., Delhaye, S., Mouveaux, T., Werkmeister, E., Slomianny, C., Hovasse, A., Alayi, T. D., Callebaut, I., Gaji, R. Y., Schaeffer-Reiss, C., Dorsselear, A. V., Carruthers, V. B., & Tomavo. S. (2012). *Toxoplasma* sortilin-like receptor regulates protein transport and is essential for apical secretory organelle biogenesis and host infection. *Cell Host & Microbe, 11*, 515-527.

Soldati, D., & Boothroyd, J. C. (1995). A selector of transcription initiation in the protozoan the protozoan parasite *Toxoplasma gondii*. *Molecular and Cellular Biology, 15*, 87-93.

Soldati, D., Dubremetz, J. F., & Lebrun, M. (2001). Microneme proteins: Structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii. International Journal for Parasitology, 31*, 1293-1302.

Soldati-Favre, D. (2008). Molecular dissection of host cell invasion by the apicomplexans: the glideosome. *Parasite, 15*, 197-205.

Song, K., Raphaelli, M., Jones, L., Aliabadi-Zadeh, K., Leung, K., Crowley, D., Hughes, B., Mahler, S., Gray, P. P., Huang, E. P., & China.D. Y. (2011). Clonal selection of high producing, stably transfected HEK293 cell lines utilizing modified, high-throughput FACS screening. *Journal of Chemical Technology and Biotechnology, 86*, 935-941.

Stiegler, A. L., Zhang, R., Liu, W., & Boggon, T. J. (2014). Structural determinants for binding of sorting nexin 17 (SNX17) to the cytoplasmic adaptor protein krev interaction trapped 1 (KRIT1). *The Journal of Biological Chemistry, 289*(36), 25362-25373.

Strauss, D. M., & Wuttke D. S. (2007). Characterization of protein-protein interactions critical for poliovirus replication: analysis of 3AB and VPg binding to the RNA-dependent RNA polymerase. *Journal of Virology, 81*(12), 6369-6378.

Striepen, B., Zinecker, C. F., Damm, J. B., Melgers, P. A., Gerwig, G. J., Koolen, M., Vliegenthart, J. F., Dubremetz, J. F., & Schwarz, R. T. (1997). Molecular structure of the "low molecular weight antigen" of *Toxoplasma gondii*: a glucose alpha 1-4 N-acetylgalactosamine makes free glycosyl-phosphatidylinositols highly immunogenic. *Journal of Molecular Biology, 266*(4), 797-813.

Su, C., Zhang, X., & Dubey, J. P. (2006). Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *International Journal for Parasitology, 36*, 841-848.

Sukthana, Y., Chintana, T., Supathanapong, W., Siripanth, C., Lekkla, A., & Chiabchalard, R. (2000). Prevalence of Toxoplasmosis in selected populations in Thailand. *Journal of Tropical Medicine and Parasitology, 23*, 53-58.

Suzuki, T., & Iwahashi, Y. (2013). RNA Preparation of *Saccharomyces cerevisiae* using the digestion method may give misleading results. *Applied Biochemistry and Biotechnology, 169*(5), 1620-1632.

Tanaka, T., Abe, Y., Kim, W. S., Xuan, X., Nagasawa, H., Igarashi, I., Kumura, H., & Shimazaki, K. (2003). The detection of bovine lactoferrin binding protein on *Toxoplasma gondii. The Journal of Veterinary Medical Science*, *65*(12), 1377-1380.

Tenter, A. M., Heckeroth, A. R., & Weiss, L. M. (2000). *Toxoplasma gondii*: From animals to humans. *International Journal for Parasitology*, *30*, 1217-1258.

Thirumudi, I., Vetrivel, U., Mahalakshmi, B., Therese, K, L., & Madhavan, H. N. (2015). Insights on drug targeting of *Toxoplasma gondii* host invasion proteins: A review. *Asian Journal of Pharmaceutical and Clinical* Research, *8*(3), 52-57.

Thomas, V. (1979). Toxoplasmosis with special reference to peninsular Malaysia and Singapore. *The Malaysian Journal of Pathology*, *2*, 23-31.

Tomavo, S., Schwarz, R. T., & Dubremetz, J. F. (1989) Evidence for glycosyl-phosphatidylinositol anchoring of *Toxoplasma gondii* major surface antigens. *Molecular and Cellular Biology*, *9*, 4576-4580.

Tomavo, S. (1996). The major surface proteins of *Toxoplasma gondii* structures and functions. *Current Topics in Microbiology and Immunology, 219*, 45-54.

Tonkal, A. M. D. (2008). PCR versus ELISA in diagnosis of human toxoplasmosis in Jeddah, Saudi Arabia. *Journal of the Egyptian Society of Parasitology, 38*(3), 707-714.

Typas, A., & Sourjik, V., (2015). Box 2: Fluorescence-based measurements of protein-protein interactions. *Nature Reviews Microbiology*, *13*, 559-572.

Vanchinathan, P., Brewer, J. L., Harb, O. S., Boothroyd, J. C., & Singh, U. (2005). Disruption of a locus encoding a nucleolar zinc finger protein decreases tachyzoite to bradyzoite differentiation in *Toxoplasma gondii*. *Infection and Immunity, 73*, 6680-6688.

Velge-Roussel, F., Dimier-Poisson, I., Buzoni-Gatel, D., & Bout, D. (2001). Anti-SAG1 peptide antibodies inhibit the penetration of *Toxoplasma gondii* tachyzoites into enterocyte cell lines. *Parasitology, 123*, 571-578.

Velmurugan, G. V., Tewari, A. K., Rao, J. R., Baidya, S., Kumar, M. U., & Mishra, A. K. (2008). High-level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. *Veterinary Parasitology*, *154*, 185-192.

Veselovsky, A. V., Ivanov, Y. D., Ivanov, A. S., Archakov, A. I., Lewi, P., & Janssen, P. (2002). Protein-protein interactions: mechanisms and modification by drugs. *Journal of Molecular Recognition*, *15*(6), 405-422.

Wang, Y. F., Fang, R., Yuan, Y., Hu, M., Zhou, Y. Q., & Zhao J. L. (2014). Identification of host proteins interacting with the integrin-like A domain of *Toxoplasma gondii* micronemal protein MIC2 by yeast-two-hybrid screening. *Parasites & Vectors*, *7*(543), doi: 10.1186/s13071-014-0543-1

Wang, Y., & Yin, H. (2014). Research progress on surface antigen 1 (SAG1) of *Toxoplasma gondii*. *Parasites & Vectors*, *7*(180), doi: 10.1186/1756-3305-7-180

Ware, P. L., & Kasper, L. H. (1987). Strain specific antigens of *Toxoplasma gondii*. *Infection and Immunity*, *55*(3), 778-783.

Weiss, L. M. & Dubey, J. P. (2009). Toxoplasmosis: A history of clinical observations. *International Journal of Parasitology*, *39*(8), 895-901.

West, L. E., & Gozani, O. (2011). Regulation of p53 function by lysine methylation. *Epigenomics*, *3*(3), 361-369.

Wilking, H., Thamm, M., Stark, K., Aebischer, T., & Seeber, F., (2016). Prevalence, incidence estimations, and risk factors of *Toxoplasma gondii* infection in Germany: a representative, cross-sectional, serological study. *Scientific Reports*, *6*(22551), doi: 10.1038/srep22551

Windeck, T., & Gross, U. (1996). *Toxoplasma gondii* strain specific transcript level of SAG1 and their association with virulence. *Parasitology Research*, *82*(8), 715-719.

Wolf, A., Cowen, D., & Paige, B. (1939). Human toxoplasmosis: occurrence in infants as an encephalomyelitis verification by transmission to animals. *Science*, *89*, 226-227.

Wu, K., Chen, X. G., Li, H., Yan, H., Yang, P. L., Lun, Z. R., & Zhu, X. Q. (2009). Diagnosis of human toxoplasmosis by using the recombinant truncated surface antigen 1 of *Toxoplasma gondii*. *Diagnostic Microbiology and Infectious Disease*, *64*(3), 61-66.

Wu, Y. D., Xu, M. J., Wang, Q. Q., Zhou, C. X., Wang, M., Zhua, X. Q., & Zhou, D. H. (2017). Recombinase polymerase amplification (RPA) combined with lateral flow (LF) strip for detection of *Toxoplasma gondii* in the environment. *Veterinary Parasitology*, *243*, 199-203.

Xing, S., Wallmeroth, N., Berendzen, K. W., & Grefen, C. (2016). Techniques for the analysis of protein-protein interactions *in vivo*. *Plant Physiology*, *171*(2), 727-758.

Yadav, R., Pathak, P. P., Shukla, V., Jain, A., Srivastava, S., Tripathi, S., Pulavarti, S. V. S. R., Mehta, S., Sibley, L. D., & Arora, A. (2011). Solution structure and dynamics of ADF from *Toxoplasma gondii*. *Journal of Structural Biology*, *176*(1), 97-111.

Yan, H., Li, H., Zhou, X. H., Wu, K., & Chen, X. G. (2004). Efficient soluble expression, purification and identification of the truncated SAG1 gene of *Toxoplasma Gondii* in *Escherichia coli*. *Di Yi Jun Yi Da Xue Xue Bao*, *24*(4), 412-414.

Yang, M., May, W. S., & Ito, T. (1999). JAZ requires the double-stranded RNA-binding zinc finger motifs for nuclear localization. *The Journal of Biological Chemistry*, *274*(39), 27399-27406.

Yahaya, N. (1991). Review of toxoplasmosis in Malaysia. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *22*, 102-106.

Zahr, A., Alcaide, P., Yang, J., Jones, A., Gregory, M., dela Paz, N. G., Patel-Hett, S., Nevers, T., Koirala, A., Luscinskas, F. W., Saint-Geniez, M., Ksander, B., D'Amore, P. A., & Argu ëso, P. (2016). Endomucin prevents leukocyte–endothelial cell adhesion and has a critical role under resting and inflammatory conditions. *Nature Communications*, *7*(10363), doi: 10.1038/ncomms10363

Zhang, J., He, S., Jiang, H., Yang, T., Cong, H., Zhou, H., Zhang, J., Gu, Q., Li, Y., & Zhao, Q. (2007). Evaluation of the immune response induced by multiantigenic DNA vaccine encoding *SAG1* and *ROP2* of *Toxoplasma gondii* and the adjuvant properties of murine interleukin-12 plasmid in BALB/c mice. *Parasitology Research*, *101*, 331-338.

## LIST OF PUBLICATIONS

Publications from this research project

1. Lai, M. Y., & Lau, Y. L. (2017). Screening and identification of host proteins interacting with *Toxoplasma gondii* SAG2 by yeast two-hybrid assay. *Parasites & Vectors*, *10*(456), doi: 10.1186/s13071-017-2387-y

2. Lai, M. Y., & Lau, Y. L. (2018). Measurement of binding strength between prey proteins interacting with *Toxoplasma gondii* SAG1 and SAG2 using isothermal titration calorimetry (ITC). *Acta parasitologica*, *63*(1), doi: 10.1515/ap-2018-00

3. Lai, M. Y., Nazia A. M., & Lau, Y. L. (2018). Identification of potential host proteins interacting with *Toxoplasma gondii* SAG1 by yeast two-hybrid assay. Submitted to *Acta Parasitologica*.