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Malaria parasites: Oral and Nasal inoculation of mice with iRBC's & development of P. falciparum mutants with the CRISPR/cas9 system

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Award date:
2017

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Malaria parasites:

Oral and Nasal inoculation of mice with iRBC's & development of *P.*

falciparum mutants with the CRISPR/cas9 system

Ph.D. Thesis

By

Cátia S. R. Antunes

School of Biological Sciences

19th October 2017

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Summary

Malaria is a parasitic disease caused by parasites of the *Plasmodium* genus and in 2015 around 214,000,000 new cases of malaria infection occurred resulting in 438,000 deaths worldwide. Currently, there is no available vaccine against malaria and despite efforts, mainly channelled into subunit vaccines, immunogenicity is often low. In this work, whole live blood stage *Plasmodium* was used in oral and nasal inoculation of mice to test for infectivity and immunogenicity. Oral inoculation resulted in infection of some mice while none of those inoculated nasally became infected. Both oral and nasal inoculation, despite not providing protection, allowed for extended survival of inoculated individuals. Given that survival was increased by inoculating animals solely with live parasites, the results detailed in this work show that this approach holds great applicational potential, as coupling oral and nasal inoculation with live parasites and a powerful adjuvant may be the key to unlock full protection.

In connection with inoculation of live parasites, this work also aimed at producing a *Plasmodium falciparum* mutant expressing *Salmonella*'s FimH⁺ glycoprotein as to allow its detection by M cells in the gut. This was to be achieved by a modified version of the CRISPR/Cas9 system which would bear a double guide RNA instead of a single. However, this was not possible as the resulting CRISPR plasmid contained an extremely high AT-content becoming toxic to *E. coli*.

In addition to these two main goals, a few molecular techniques crucial for malaria research were improved and a detection method of avian malaria was developed.

Acknowledgments

I would like to extend thanks to the several people who so generously, in one way or another, contributed to this work.

Special mention goes to my enthusiastic supervisor, Dr. Henk Braig for his tremendous academic support.

I am also hugely grateful to Dr. Henrique Silveira, Dr. Dinora Lopes and in particular to Joana Gomes without whom the experimental work with rodents would have not been possible. I would like to thank Joana Gomes in particular, whose patience and dedication went far beyond duty.

And finally a special mention to my family and Boyin for the support that kept me going through the roughest times.

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1.

Introduction

1.1. Malaria

Malaria is a parasitic disease caused by parasites of the *Plasmodium* genus (**Figure 1**). In humans, infection occurs when an infected female mosquito from the genus *Anopheles* takes a blood meal resulting in the injection of the parasites with its bite. The World Health Organization estimates that in 2015 around 214.000.000 new cases of malaria infection occurred resulting in 438.000 deaths worldwide (W.H.O. 2015). Malaria's pathology can be classified as uncomplicated or severe (Satoskar, Simon et al. 2009). Uncomplicated malaria is characterized by periodic fever, chills, anaemia and splenomegaly. It is seldom fatal unless left untreated (Grobusch and Kremsner 2005, Satoskar, Simon et al. 2009). Severe malaria, with an extremely high mortality rate of 80%, is mainly caused by *Plasmodium falciparum* and it can lead to life threatening conditions such as severe anaemia, cerebral malaria and acidosis (Satoskar, Simon et al. 2009, Sanklecha, Mehta et al. 2012).

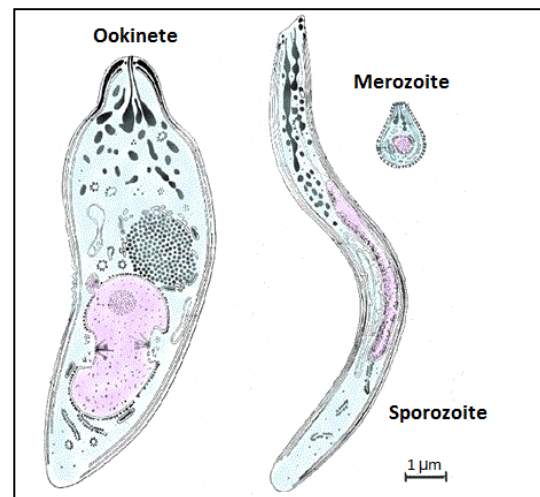


Figure 1 - Invasive stages of *Plasmodium* (Kneil 1991)

Plasmodium falciparum life cycle

There are five species of *Plasmodium* known to infect humans. The majority of morbidity and mortality are associated with *Plasmodium falciparum* and *Plasmodium vivax*. The species *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlsei* comprise a much lower distribution and prevalence (Lamb 2012).

The life cycle of *P. falciparum* (**Figure 2**) starts when an infected *Anopheles* female mosquito bites a vertebrate host, in this case a human, and injects sporozoite forms of the parasite through the skin. These move on to the liver and invade the hepatocytes, where they develop to produce exoerythrocytic merozoite forms that are then released into the blood stream. Merozoites invade the erythrocytes and grow into trophozoites that mature into schizonts

which contain many merozoites. Merozoites are released and reinvade new erythrocytes. When parasitemia is high, some invaded erythrocytes stop cycling and develop to the transmissible stage known as gametocyte so that they can be taken up by a feeding mosquito into the gut where they mature to form male and female gametes. The fertilized zygote develops into an ookinete followed by an oocyst and finally sporozoites that migrate to the salivary gland thus completing the cycle (Cowman, Berry et al. 2012, Lamb 2012).

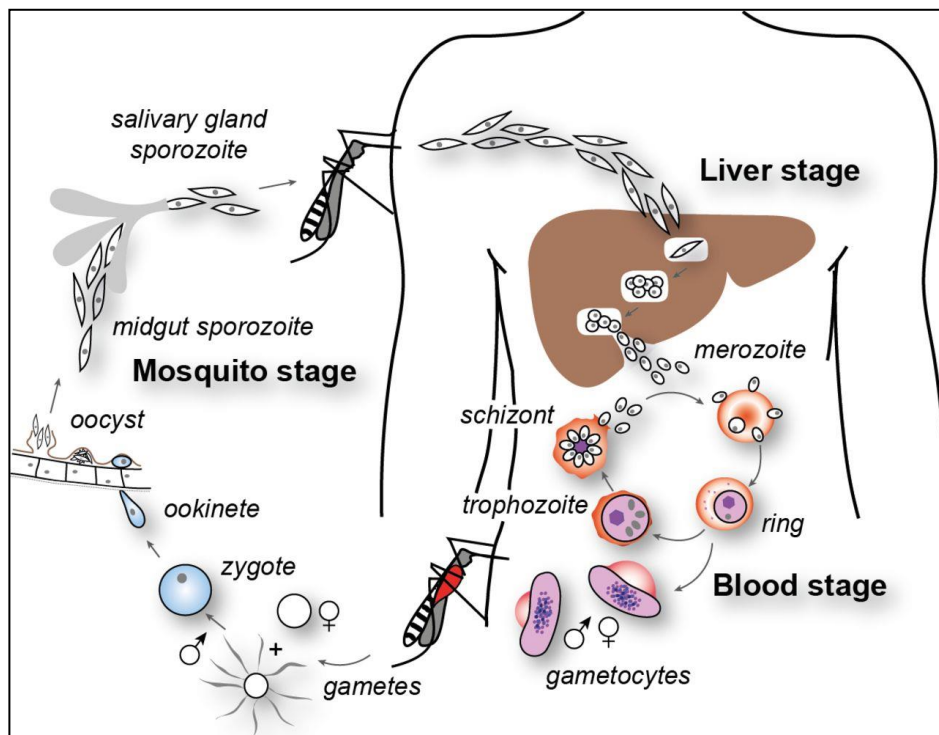


Figure 2 - *Plasmodium* lifecycle (Cowman, Berry et al. 2012) (CC BY-NC-ND 4.0).

1.2. Immunity to Malaria

Like modern humans, *Plasmodium* is thought to be as old as 100,000 years, which strongly points to co-evolution between parasite and the human host (Mu, Duan et al. 2002, Prugnolle, Durand et al. 2011). High mortality rates of *P. falciparum* infections induced significant selective pressure in human populations resulting in shaping of the host's immune system (Evans and Wellems 2002).

It is now well known that the type of immune responses mounted against *P. falciparum* tend to dictate the course and outcome of the disease (Rogerson, Wijesinghe et al. 2010). Acquisition of immunity is complex, developing initially resistance to severe disease and evolving into resistance to uncomplicated infections, but never leading to sterile resistance (Crompton, Moebius et al. 2014). Immunity to malaria develops only after long exposure in endemic areas with high transmission rates and where the host is exposed to hundreds of infectious mosquito bites per year (Guilbride, Guilbride et al. 2012). In children, immunity in the first year of life can be divided into two crucial stages: from birth to approximately 6 months of age, where infants are less vulnerable to infection due to maternal antibodies; and from 6 months to 1 year old, where infants become highly susceptible to infection due to metabolizing of maternal antibodies. By the age of 5 children gain resistance to the most severe form of malaria but remain susceptible to uncomplicated infections showing occasional episodes of febrile malaria. Only by early adolescence do they gain resistance and rarely develop clinical malaria (Dobbs and Dent 2016).

Despite acquiring immunity to clinical malaria, resistance to liver infections is never achieved explaining why adults residing in areas where transmission rates are high still present asymptomatic infections (Marsh and Kinyanjui 2006).

Malaria parasites induce specific immune responses that stimulate the release of cytokines from peripheral blood mononuclear cells which play an important role in activating the host's neutrophils, monocytes, T cells and natural killer (NK) cells to react to liver and blood stage parasites (Malaguarnera and Musumeci 2002). The antigens present in the invading sporozoites are thought to be rapidly processed by the host cells and presented on the surface of infected hepatocytes complexed to MHC-I molecules (Weiss, Mellouk et al. 1990). This

presentation results in recognition by cytotoxic T lymphocytes and killing of infected cells. Also, it can lead to the stimulation of natural killer (NK) and CD4+ T cells to produce interferon γ (INF- γ) triggering a cascade of immune reactions leading ultimately to the death of the parasite (Weiss, Mellouk et al. 1990, Wang, Charoenvit et al. 1996, Malaguarnera and Musumeci 2002). After leaving the liver, the surviving merozoites enter the red blood cells by receptor mediated endocytosis and continue their development. When erythrocytes rupture they not only release parasites but also parasite antigens that end up stimulating the production of tumour necrosis factor α (TNF α) and other factors causing the cyclical fevers associated with malaria infection (Kern, Hemmer et al. 1989, Kwiatkowski, Hill et al. 1990, Malaguarnera and Musumeci 2002). However, many merozoites escape these immune reactions and carry to infect fresh red blood cells thus continuing the cycle of infection and immune response stimulation. During a malaria crisis, the pathogenic manifestations result from proinflammatory cytokines produced by T cells and macrophages in response to malaria parasites and their products (Malaguarnera and Musumeci 2002).

1.3. Malaria vaccines

The beginning

The name malaria originated from *mal'aria*, an Italian term for “bad air”, when it was thought the disease was transmitted via miasmas or contaminated air (Snowden 2006). The first evidence of malaria parasites was observed in mosquitos preserved in 30 million years old amber from the Palaeogene period (Poinar 2005). However, the causative agent of malaria was first identified in 1880 by Charles Laveran who associated the presence of a foreign organism in patients suffering from malaria with the disease (Snowden 2006, Sherman 2009). This discovery was followed by many other crucial studies such as blood smear staining, parasite life cycle and infectivity, and culturing of the different life stages (Sherman 2009). The modern malaria vaccine development, however, started in the 1960's, with immunisation studies of mice with irradiated sporozoites (Nussenzweig, Vanderberg et al. 1967). This was followed by challenge studies in humans that showed that a high level of protection could be achieved (Clyde 1975). Great advances in the field kept being made when in the beginning of the 1980's the circumsporozoite protein was identified, cloned and sequenced (Hill 2011). This sparked the beginning of the identification and expression of multiple blood-stage antigens raising the expectations for peptide based blood-stage vaccines (Ballou, Hoffman et al. 1987).

Malaria vaccination – Where do we stand?

Currently, there is no available vaccine against malaria. The efforts on developing a Malaria vaccine have been mainly channelled to subunit vaccines. Many targets have been pinpointed but in general immunogenicity is often low (Schwartz, Brown et al. 2012). Presently, the vaccine with furthest clinical development is RTS,S/AS01 and targets the pre-erythrocytic circumsporozoite protein (CSP) of *P. falciparum*, which is the major surface protein present in sporozoites. Trials in children naturally exposed to malaria revealed an efficacy of 30 to 56% against clinical disease and up to 66% against infection (Alonso, Sacarlal et al. 2004, Bejon, Lusingu et al. 2008, Thera and Plowe 2012). Despite being well below the expected level of

protection of common vaccines these rates brought optimism showing that at least some protection was being achieved. This optimism however was short-lived. A recent follow-up spanning a period of 7 years, found that RTS,S/AS01's efficacy was only protective through the first year after vaccination. In fact, in the cohort with high exposure to malaria parasites, unvaccinated individuals suffered less episodes of clinical malaria (Olotu, Fegan et al. 2016).

Compared with viruses and bacteria to which vaccines have been successfully developed *Plasmodium* presents a much higher level of complexity that needs to be considered. It comprises a genome size of 23 Mbp organised into 14 chromosomes and 5000 genes (Gardner, Hall et al. 2002). Many of these genes are expressed differently during the several stages of the parasite's life cycle. Adding to this, mutations during the mitotic reproduction in the haploid liver and blood stages result in broad genetic diversity driven by selection pressure by the immune system and drugs (Takala and Plowe 2009).

Although several subunit vaccines are at the trial stage (Schwartz, Brown et al. 2012), the ideal vaccine would have to achieve a much higher degree of efficacy than the ones being presently tested (Thera and Plowe 2012). So far, only one vaccine has accomplished such prominent results: a whole parasite vaccine comprising irradiated sporozoites.

Why whole Parasite?

Live attenuated vaccines were the earliest of vaccine development and some still remain the best vaccines against some pathogens. Such cases include polio (Hird and Grassly 2012), varicella, measles and rubella (Watson, Laufer et al. 1996). Whole organism vaccines can be divided into two categories: Live attenuated and inactivated vaccines (Kindt, Goldsby et al. 2006). Attenuation of live vaccines can be achieved by genetic modification or irradiation whilst inactivated vaccines are produced by inactivation of whole organisms by high temperature, chemical treatment or physical treatment (irradiation) (Khan, Janse et al. 2012, Todd, Tibi et al. 2013, Fernandes, Frank et al. 2014). Live vaccines cause a subclinical infection that induces a protective immune response in the recipient, and because live attenuated vaccines are the closest to a natural infection, they elicit strong cellular and antibody responses and often confer lifelong immunity with only one or two doses (Detmer and Glenting 2006, Vignuzzi, Wendt et al. 2008, Todd, Tibi et al. 2013).

For malaria vaccines, three parasite stages can be used in a vaccine: Liver, blood and vector stages (Waheed, Sameeullah et al. 2016). Almost 40 years ago, protection against *P. falciparum* was in fact achieved in human volunteers using an attenuated live vaccine with whole sporozoites (mosquito stage) (Clyde, McCarthy et al. 1975). These results were seen as a possibility to develop a subunit vaccine instead of pursuing a whole organism vaccine. Yet, the basis of protective immunity against malaria is still not fully understood and a correlation of clinical protection with a specific immune response is yet to be established (Thera and Plowe 2012). Now, almost after 30 years of failed attempts to produce a subunit vaccine that would elicit lifelong protective immune response, immunologists are once again turning to the whole parasite approach (Epstein, Tewari et al. 2011, Butler, Vaughan et al. 2012, Seder, Chang et al. 2013). Example of that is the work of Hoffman and colleagues who have successfully administered irradiated sporozoites in a new way that enabled to protect 12 of 15 volunteers from malaria infection (Seder, Chang et al. 2013). Using mosquitoes raised in sterile conditions and fed on blood infected with malaria they then proceeded to irradiate them and weaken the parasite so when intravenously administered it would still infect but not cause disease. However many obstacles still remain. Aiding to the fact that this vaccine must be stored in liquid nitrogen unlike conventional vaccines, it also needs to be administered intravenously (IV). 70% of deaths caused by malaria are of children under 5 years old and the most adequate time to immunize a child would be between 5-17 months (Agnandji, Lell et al. 2011, W.H.O. 2012). Administration of an IV vaccine is impossible in children at such young age since not only the parasite load is too high but also because an infant's vein is too small and frail to cope with such procedures. On top of this is also the lack of trained personnel and the fact that HIV still remains a big problem in malaria endemic countries (Idemyor 2007, Reithinger, Kamya et al. 2009). These reasons make oral vaccines the best delivery candidates as they are not only less expensive but also less invasive.

Oral Vaccines

Creating an effective malaria vaccine is far from being the only obstacle for malariologists. An equally challenging objective is to deliver these hypothetical vaccines to the populations residing in countries where malaria is endemic (Wang, Webster et al. 2004). The success of

immunisation is also intrinsically associated to vaccine production, distribution and delivery, and because these populations are almost exclusively located in economically disadvantageous countries the cost of a vaccine is a significant factor to a widespread deployment (Gething, Patil et al. 2011, W.H.O. 2012). Oral vaccines are not only less expensive but also less invasive, which is a key factor for vaccine administration in countries with high prevalence of HIV. As previously mentioned, most countries where malaria is endemic show elevated rates of HIV infection. Eliminating needles from the vaccination process will reduce the need of skilled personnel but also put to rest any concerns regarding transmission of blood-borne pathogens associated with needle re-use and disposal (Wang, Webster et al. 2004, W.H.O. 2012).

Oral immunisation: Achievements

Despite all the attractive features mentioned, studies on oral vaccination have been limited almost exclusively to protection against mucosally transmitted pathogens (Wang and Coppel 2008). Oral vaccination can induce local responses in gut as well as systemic humoral and cellular immune responses (Ruedl, Rieser et al. 1996). These vaccines characteristically generate large amounts of secretory immunoglobulin A (sIgA) that plays a key role in mucosal defence (Macpherson, Hunziker et al. 2001). Thus, studies have been mainly focused on pathogens entering the body through mucosal surfaces leaving the field of oral vaccines against non-mucosally transmitted pathogens largely unexplored.

Early studies with recombinant *Salmonella typhimurium* expressing *P. berghei* CSP showed induction of antigen specific cell-mediated immunity resulting in protection of mice against sporozoite challenge. This immunity was mediated by the induction of specific CD8⁺T cells, which were directed against the same peptide targeted by cytotoxic T lymphocytes (CTL's) induced by sporozoite immunization (Aggarwal, Kumar et al. 1990, Flynn, Weiss et al. 1990). Among some of the recent work in oral immunisation against malaria is the work of Wang and colleagues that were able to protect mice against lethal malaria infections using *E. coli* to express the *P. yoelii* merozoite surface protein 4/5 (PyMSP4/5) (Wang, Goschnick et al. 2004). They proved that contrary to the general perception that soluble proteins are poorly immunogenic by oral immunization, PyMSP4/5 and its homologue in *P. falciparum* (PfMSP4)

are in fact able to induce antigen-specific serum antibodies when delivered orally (Wang, Kedzierski et al. 2003, Wang, Goschnick et al. 2004).

Malaria vaccines delivered orally have been mainly subunitary focused on the circumsporozoite protein (CSP) and to date there has been no published work on immunologic responses to orally administered whole organism malaria vaccines (Sadoff, Ballou et al. 1988, Aggarwal, Kumar et al. 1990, Zhang, Jiang et al. 2005, Ramasamy, Yasawardena et al. 2006, Moorthy and Ramasamy 2007). As referred above, impressive work has been done with whole parasites so it's only a matter of time until it can be extended to the field of oral immunisation. Meanwhile, a better understanding of the gastrointestinal and gut non-mucosal vaccine immunology is essential.

1.4. The GALT and the NALT

The Gut Associated Lymphoid Tissue (GALT)

The gut mucosal immune system is exposed to a broad variety of antigens derived from food, resident microflora and invading pathogens. These need to be limited by a barrier that allows absorption of nutrients but provides immune defences against harmful antigens (Wittig and Zeitz 2003). The Gut Associated Lymphoid Tissue (GALT) (**Figure 3**) is often seen as the immune system of the digestive tract and it is in fact the major collection of lymphoid tissues in the body. It comprises organized lymphoid tissues such as mesenteric lymph nodes (MLN) and Peyer's Patches (PP's), and more diffusely spread lymphocytes in the intestinal lamina propria (LP) (Forchielli and Walker 2005). The GALT can be roughly divided into inductive and effector sites (Wittig and Zeitz 2003). The inductive sites comprise the Peyer's patches located in the small intestinal wall. These enclose naïve B Cells, dendritic cells (DC) and T cells. The effector site is the lamina propria comprising mature T and B cells migrated from the Peyer's patches after induction.

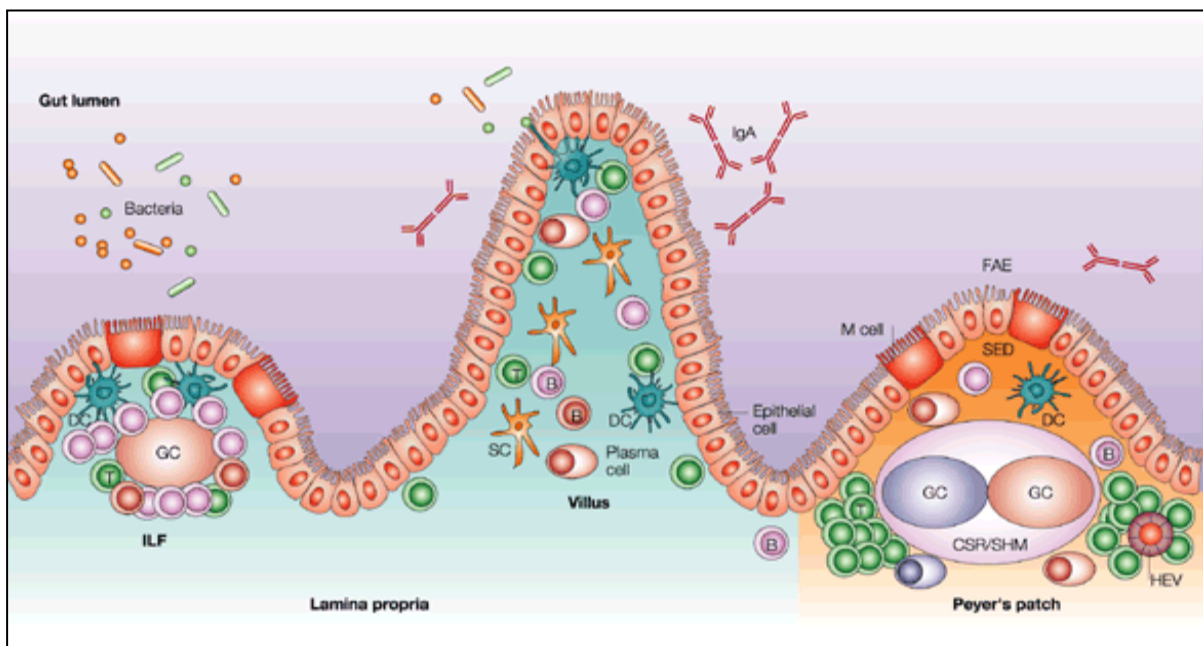


Figure 3 – Schematic representation of the GALT. Peyer's patches and Lymphoid follicles (ILFs) containing the specialised follicle-associated epithelium (FAE) with M cells, a subepithelial dome (SED) rich in dendritic cells (DCs), and B-cell follicle(s) that contain germinal centres (GCs), where follicular B cells efficiently undergo class-switch recombination (CSR) and somatic hypermutation (SHM). Migration of B cells into the mucosa takes place through high endothelial venules (HEVs), located in the interfollicular regions of Peyer's patches, which contain mostly T cells. The diffuse tissues of the lamina propria contain a large number of immunoglobulin A (IgA)⁺ plasma cells, T and B cells, macrophages, dendritic cells (DCs) and stromal cells (SCs) (Fagarasan and Honjo 2003).

M Cells – Gateway

Peyer's patches are covered by follicle associated epithelium (FAE) containing specialised cells termed Microfold cells (M cells) (**Figure 4**) (Spahn and Kucharzik 2004). These are unique epithelial cells dedicated to transepithelial transport of particles, macromolecules and microorganisms, and can be identifiable by their flattened apical surfaces, lack of glycocalyx, low cytoplasmic lysosomes, and high number of mitochondria (Neutra, Frey et al. 1996, Azizi, Kumar et al. 2010). M cells take up microorganisms and antigens from the intestinal lumen and deliver them to the underlying immune cells of the mucosae that activate or inhibit the immune response leading to either tolerance or systemic immune cell response (Azizi, Kumar et al. 2010, Jung, Hugot et al. 2010). For this, M cells have a deeply invaginated basolateral membrane

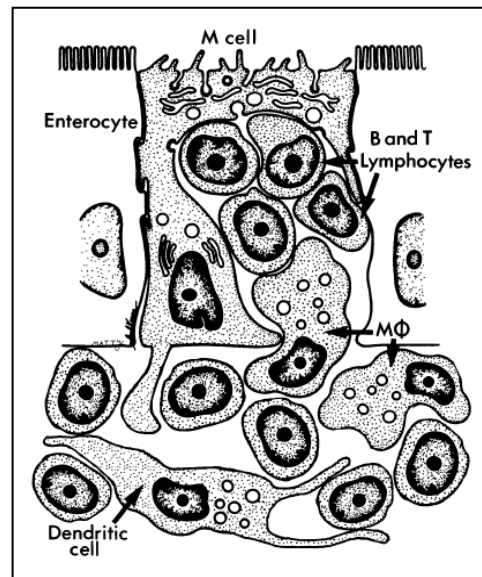


Figure 4 - Diagram of an M cell in the intestinal follicle-associated epithelium depicting the basolateral membrane with B and T lymphocytes, macrophages (MΦ), and dendritic cells (DC's) (Neutra, Frey et al. 1996).

that forms an intraepithelial pocket that contains B and T lymphocytes and macrophages (Neutra, Frey et al. 1996, Ermak and Giannasca 1998). Phagocytic or endocytic uptake of foreign particles is followed by transcytosis directly into the intraepithelial pocket. After being transported, the particles are processed and presented by macrophages, B cells and DC's within and below the epithelium. This results in the generation of antigen-specific B lymphoblasts that proliferate locally and migrate via bloodstream to distant mucosal and glandular tissues where they end up differentiating into plasma cells (Neutra, Frey et al. 1996, Neutra and Kraehenbuhl 1996). This ability of M cells to take up and transcytose diverse numbers of microorganisms to antigen-presenting cells (APCs) turned M cells into the ideal target for vaccine delivery to the mucosal immune system (Azizi, Kumar et al. 2010). Oral vaccination can induce immune responses locally in the gut and at distant mucosal sites, as well as systemic humoral and cellular immune responses. This naturally generates a large amount of secretory IgA (sIgA), which plays a major role in mucosal defence (Wang and Coppel 2008). The next step will be to prove that oral immunization can in fact be applied in the

prevention of infections transmitted through non-mucosal routes, such as malaria (Wang and Coppel 2008).

The Nasopharynx Associated Lymphoid Tissue (NALT)

The Nasopharynx Associated Lymphoid Tissue (NALT) is a constitutive structure of the nasal immune system and, much like the GALT, is a main inductive site for immune responses in both natural infection and vaccination (Debertin, Tschernig et al. 2003, Kang, Yan et al. 2013). The NALT has been identified in rat, mouse, hamster and primates with most studies being done on rodents (Kang, Yan et al. 2013).

The NALT is considered a key site for immune induction in the upper airways. Here, antigens can be directly sampled by M cells for processing and presentation by antigen presenting cells such as DC to naïve lymphocytes (Mestecky 2015). These M cells have been recently identified in rodent airways, and function similarly to the M cells within the mucosal associated lymphoid tissue (MALT). CD11C⁺ dendritic cells roam under these cells to receive antigen during a nasal infection (Kim, Sato et al. 2011). Like the GALT, this makes the NALT another great target for vaccine delivery.

Targetting M cells

Unlike a typical enterocyte, M cells, as antigen-sampling cells, have the capability to transcytose a wide range of pathogen materials and because of this are an effective gateway for drug and vaccine delivery (Clark, Hirst et al. 2000, Kraehenbuhl and Neutra 2000). Although, the molecular mechanisms behind antigen uptake are largely unknown, glycoprotein 2 (GP2), which is exclusively expressed in the apical plasma membrane of M cell, has been proven to serve as transcytotic receptor for mucosal antigens (Hase, Kawano et al. 2009). Hase *et al.* (2009) have shown that this protein selectively binds to *Salmonella enterica* by recognising FimH⁺, a component of type I pili on the bacterial outer membrane. Because previous studies have demonstrated the importance of *Salmonella* translocation into Peyer's patches for initiation of antigen specific responses (Martinoli, Chiavelli et al. 2007, Hashizume, Togawa et al. 2008), FimH⁺ could be the key to unlock targeted vaccine delivery via M cells.

An interesting approach could entail expression of FimH⁺ in pathogens such as *Plasmodium*. This would obviously require editing *Plasmodium*'s genome.

1.5. Genome editing with CRISPR/Cas9

Many prokaryote genomes contain clustered regularly interspaced short palindromic repeats (CRISPRs) which are structures composed of 21-48 bp repeats separated by unique sequence spacers of 26-72 bp (Rath, Amlinger et al. 2015). These structures, flanked by CRISPR associated (Cas) genes, make-up a highly diverse system of adaptive immunity in approximately 50% of bacteria and 90% of archaea (Wright, Nunez et al. 2016). Because the spacers, also known as proto-spacers, are usually derived from phage or plasmid nucleic acids, the idea arose that these sequences are used for recognition and destruction of invading pathogens. The more spacers are added to the host's genome, the more invading viruses can be recognised, making this an effective adaptive immune system that can learn to recognize specific sequences (Rath, Amlinger et al. 2015, Waddington, Privolizzi et al. 2016).

It all starts when an invading pathogen's DNA is inserted into the host and is then integrated into the CRISPR array (**Figure 5a**). This first stage is also known as "Adaptation" or "Spacer acquisition" and can be seen as host immunization. Later on, when immunity needs to be executed, a second stage known as "CRISPR RNA (crRNA) biogenesis" is initiated where a guide RNA with the relevant spacer is transcribed (**Figure 5b**) and coupled with a Cas endonuclease (**Figure 5c**). As the name suggests, the RNA serves as guide to direct the cleavage of foreign nucleic acid with the aid of the Cas complex resulting in the last stage of this system: "Interference" (Marraffini 2015, Wright, Nunez et al. 2016).

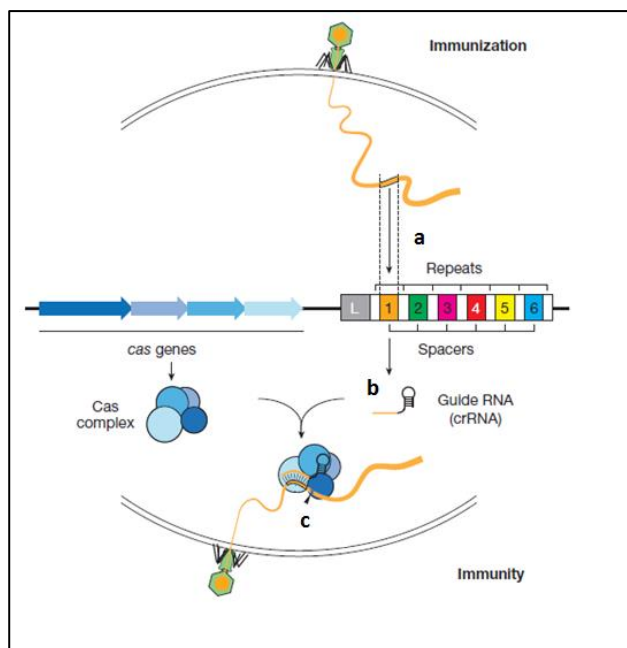


Figure 5 – Stages of CRISPR-Cas immunity (Marraffini 2015).

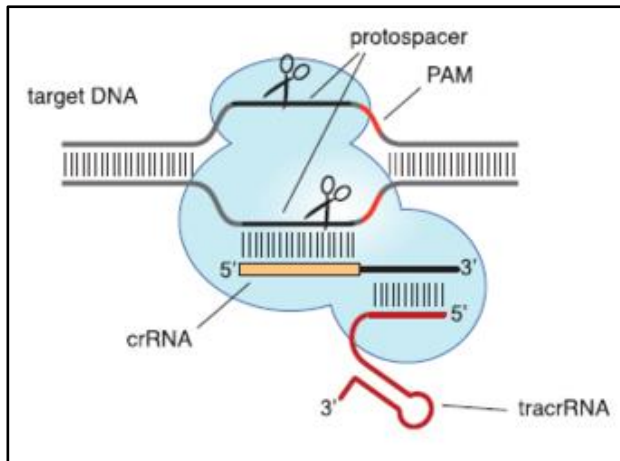


Figure 6 - Wild-type *S. pyogenes* CRISPR system with crRNA:tracrRNA:Cas9 assembly (Jinek, Chylinski et al. 2012).

Fundamental to the interference stage of all CRISPR-Cas systems are the Cas genes that, depending on its accessory contents, can be used to differentiate the systems into three (Chylinski, Makarova et al. 2014). Of the three systems, type II has been the most studied and is the best candidate for genome engineering. To execute immunity, Type II CRISPR-Cas system (**Figure 6**) requires the cas9 gene and two small RNAs: the crRNA and the transcribed RNA

(tracrRNA). When all three are assembled they become activated and specific to the DNA sequence complimentary to the crRNA. In addition to this assembled structure, the type II system also requires a protospacer adjacent motif (PAM), which is found immediately downstream of the protospacer (target sequence) of the non-target DNA strand (Chylinski, Makarova et al. 2014, Marraffini 2015, Waddington, Privolizzi et al. 2016).

The CRISPR-Cas9 system allows for a very specific and precise cleavage of DNA which is why it became a great tool for targeted genome editing. Due to its simplicity, the type II system has been adapted for this task by replacement of the dual tracrRNA:crRNA with a single guide RNA (Jinek, Chylinski et al. 2012). The genomic target may be any DNA sequence of approximately 20bp and only two conditions need to be met: 1. The sequence is unique and not present in any other part of the genome; 2. The target sequence is immediately upstream of the PAM sequence. With this, a variety of guide RNAs (gRNAs) can be designed and, when associated with the Cas9 endonuclease, create a multitude of site specific molecular “scissors” allowing not only for gene editing but also deletions and insertions.

1.6. Malaria in other vertebrates

Rodent Malaria

Plasmodium parasites can infect an extensive range of hosts, from mammals to birds and even reptiles (Dronamraju and Arese 2006). Rodent malaria parasites have been expansively used as models of human malaria (Craig, Grau et al. 2012). From African rodents, three species have been adapted and vastly used in laboratory research: *Plasmodium yoelii*, *Plasmodium berghei* and *Plasmodium chabaudi*.

These three species behave slightly different from each other and, when in different laboratory mice strains, can be used to study different aspects of human malaria (Otto, Böhme et al. 2014).

Avian Malaria

Avian malaria played a key role in the initiation of human malaria research, being the first model to provide insights into parasite life cycle, development of therapies, *in vitro* cultivation amongst many other crucial findings (Valkiūnas 2005). Much of the initial research on avian parasites started in 1884 with Vassily Danilewsky who accomplished a very detailed report on intracellular blood parasites of birds (Valkiūnas 2005, Cox 2010). Research on bird *Plasmodium* thrived for almost 90 years, however, most efforts were directed at using avian malaria as a model for human malaria research. In addition to this, most avian parasites were not known to cause disease and so, had no veterinary interest (Mehlhorn and Bunnag 1988). This idea is now beginning to be challenged with some reports that show that some species of *Plasmodium* can indeed induce pathology in some bird species (Williams 2005). It is clear that much investigation is still needed but this is made even more problematic when it is taken into account that much research involves invasive sampling techniques. In addition to bird capturing, many of species of interest are also protected by law (Braga, Silveira et al. 2011). In order to advance knowledge about avian *Plasmodium* species it is critical that non-invasive techniques be implemented.

1.7. Objective

The main objective of this project was to look at the possibility of developing a needle-free vaccine by exploiting both nasal and oral inoculation routes for immunisation with live *Plasmodium* blood stage parasites. This approach was expected to prime specific immune cells in the GALT and NALT leading to *Plasmodium*-specific immune responses. Because this involved the use of live parasites, the possibility of infection via oral and nasal inoculation was considered and also tested.

A secondary objective focused on developing a mutant strain of *Plasmodium falciparum* capable of

As a side project, the possibility of using bird faecal samples in a non-invasive approach of malaria detection in bird was also explored.

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2.

Synthetic peptides

2.1. Introduction

In order to perform direct and indirect ELISA's, one must first acquire the antigen that will be used for plate coating. This antigen is usually a protein of interest that in many cases needs to be produced in-house. Production of these proteins requires cloning, expression, purification and purity testing (Hnasko 2015). This is a laborious procedure that on top of being expensive might not even be achievable in some laboratories as equipment and material needed isn't always available.

A way to circumvent this is to outsource peptides from biotech companies which now offer affordable solutions for custom peptide synthesis. However, when using synthetic peptides, one must regard its two greatest drawbacks: peptide length, with a quality threshold between 30 and 50 amino acids, and three dimensional structure, since mimicry of nonlinear motives with linear peptide constructs is still under development (Cretich and Chiari 2016).

In Chapter 3, oral and nasal inoculation of mice with live parasites is explored as an approach to elicit protective immune responses. In order to validate the experiment, it was necessary to produce *Plasmodium* proteins for ELISA assays.

Because in-house production of proteins was not viable, several peptides were outsourced and tested for reactivity with serum from immunised mice. The objective of this experiment was to confirm if small peptides could be used for antibody titer determination of serum samples from immunised mice.

2.2. Methods

2.2.1. Serum

Serum for this test was sourced from the animals used in experiments described in Chapter 3. The serum was collected from animals that were still alive 15 days after Challenge (and therefore containing higher titers of antibodies).

2.2.2. Peptides

P. yoelii and *P. falciparum* peptides used for ELISA were sourced from published. *P. berghei* peptides were derived from unique regions of the following aligned sequences: MSP1 *P. berghei* ANKA (GenBank accession number CDS4641.1), MSP1 *P. berghei* (Genbank accession number AAC2887.1) and MSP1 *P. yoelii* (GenBank accession number CDU17703.1). After alignment, 4 peptide sequences were extrapolated ensuring the whole extracellular domain was covered (**Table 1**). All peptides spanned 30 a.a. (except the last one) with a 10 a.a. overlap (5 on each end) (**Figure 7**). All were synthesised by GenScript® and prepared as manufacturer's specifications.

Table 1 - List of synthesized *P. berghei* peptides and corresponding sequences

Name	Protein	Species	Sequence	Reference
MSPE24	MSP1	<i>P. yoelii</i>	EPTPNAYYEGVFCSSSS	(Wipasa, Hirunpetcharat et al. 2002)
PEP45B1	AMA1	<i>P. yoelii</i>	CSNSDKPKCGGGS	(Narum, Ogun et al. 2006)
PEPAMAP1	AMA1	<i>P. yoelii</i> / <i>P. berghei</i>	CSASDQPKCGGGS	(Narum, Ogun et al. 2006)
AMAPB1	MSP1	<i>P. berghei</i>	MDLLGIDPKHVCINTRDIPANAGCFRYDNG	Current work
AMAPB2	MSP1	<i>P. berghei</i>	CFRYDNGNEEWRCLLGYKKNNTCIEDSNP	Current work
AMAPB3	MSP1	<i>P. berghei</i>	CIEDSNPTCGNNGGCDPTAGCQTAENREN	Current work
AMAPB4	MSP1	<i>P. berghei</i>	TAENRENSKKIICTCKEPTPNAYYDGVFCS	Current work
MSPPF	MSP1	<i>P. falciparum</i>	NSGCFRHLDEREECKLL	(Biswas, Seth et al. 2008)
AMAPF	AMA1	<i>P. falciparum</i>	DGNCEDIPHVNEFSAIDL	(Biswas, Seth et al. 2008)

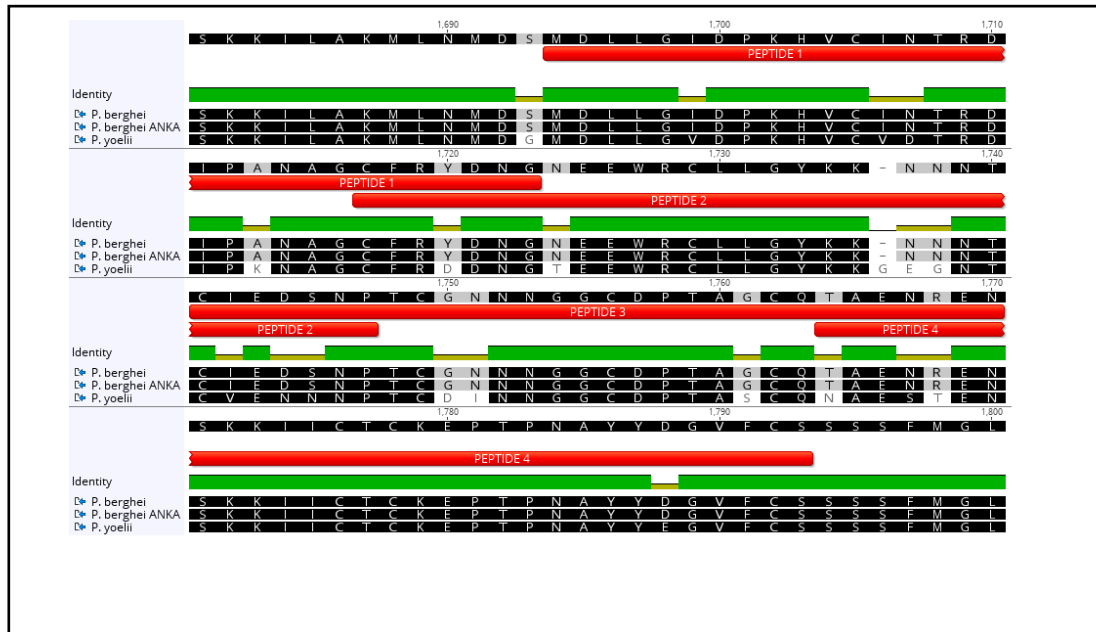


Figure 7 - Alignment of *P. berghei* ANKA, *P. berghei* and *P. yoelii* MSP1 amino acid sequence, and peptides selected for synthesis, (Geneious, Version 9.1.3.).

2.2.3. ELISA

ELISA plates (VWR, 735-0079) were coated over night at 4°C with 50µL of 0.01µg/mL of synthetic peptide diluted in sterile PBS. Plates were washed 3 times with 200µL of PBST (1X PBS with 0.05% Tween-20) prior to blocking for two hours with blocking buffer (4% milk powder in PBST). Serum was then diluted in PBS at 1:2500 and 50µL were added per well. Plates were incubated for three hours at 37 °C before washing three times as previously described. Next, HRP-conjugated anti-mouse IgG1 (Abcam) was diluted separately in blocking buffer at 1:2000 and 50µL was added per well. After a two-hour incubation at 37°C plates were washed followed by the addition of 50µL of 3,3',5,5'-Tetramethylbenzidine (TMB) (Abcam ab171523). The substrate was incubated at room temperature for 15 minutes and reaction stopped with 1N Sulphuric acid prior to measuring the absorbance at 450nm with a SpectraMax® 384 microplate reader.

2.3. Results

2.3.1. *Plasmodium berghei* peptides

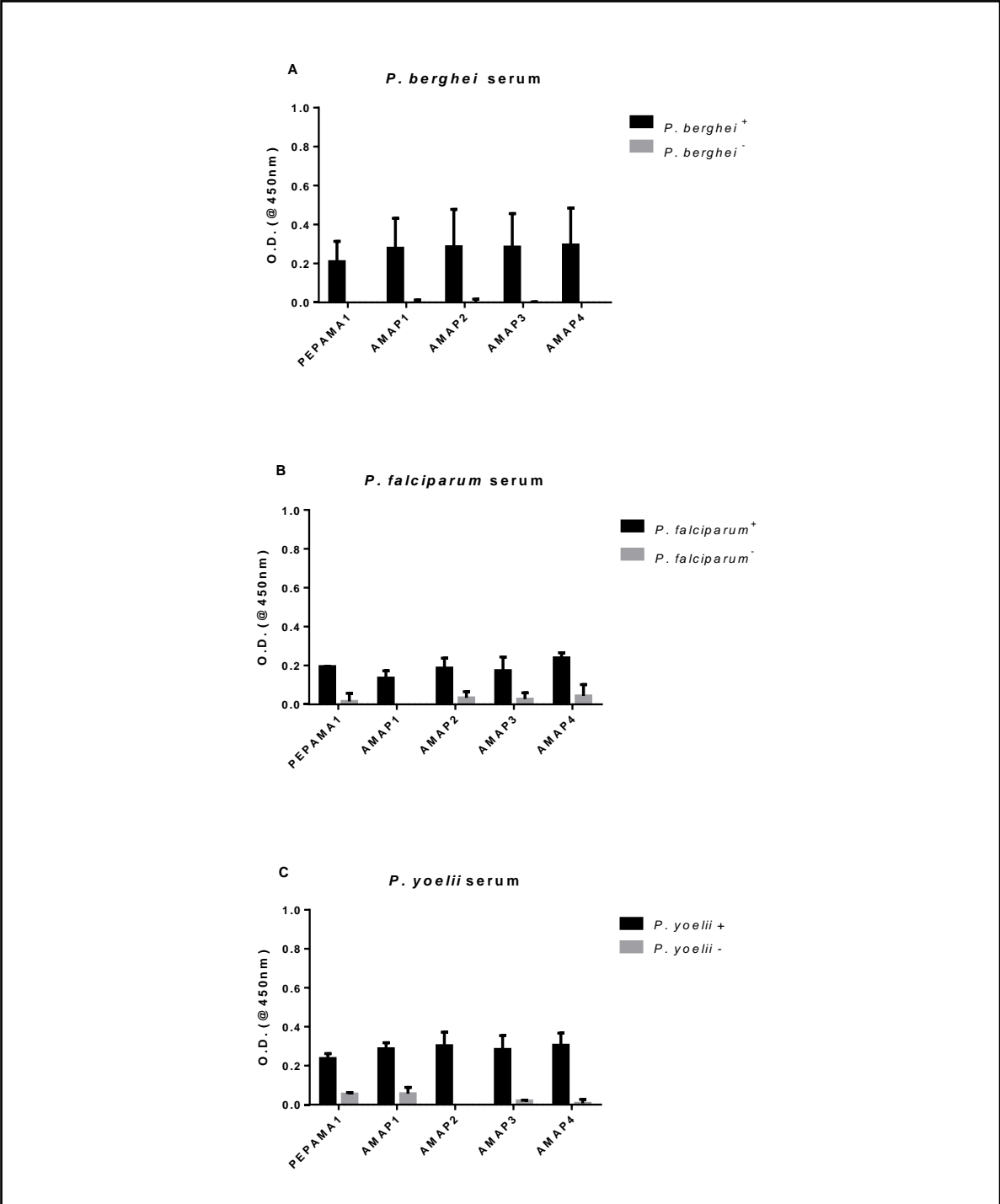


Figure 8 – Reactivity of serum from infected mice to *P. berghei* synthetic peptides measured with anti-IgG1 as secondary antibody. Bars represent standard deviation. **A.** Reactivity of serum from mice immunised with *P. berghei*; **B.** Reactivity of serum from mice immunised with *P. falciparum*; **C.** Reactivity of serum from mice immunised with *P. yoelii*.

2.3.2. *Plasmodium falciparum* peptides

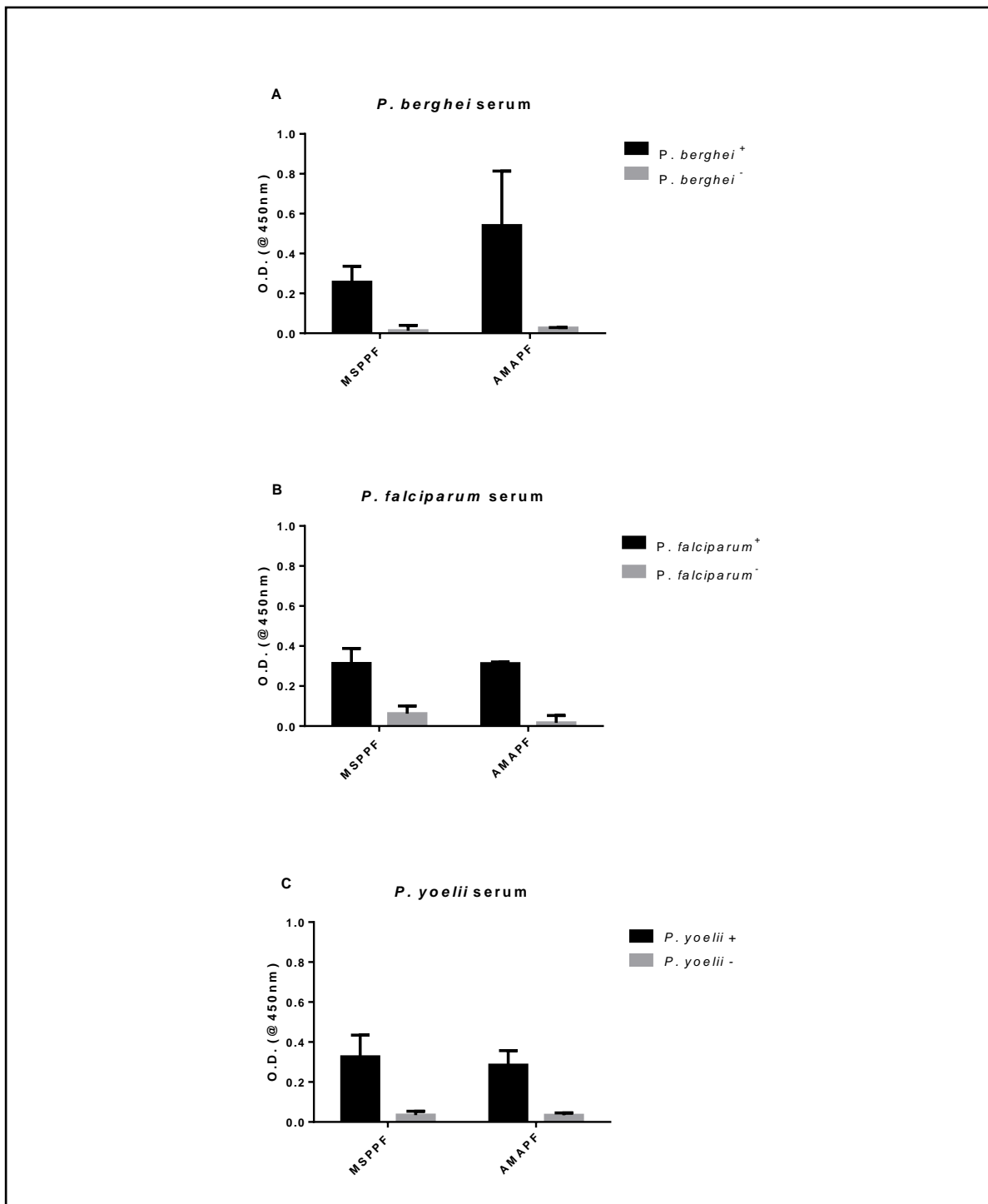


Figure 9 - Reactivity of serum from infected mice to *P. falciparum* synthetic peptides measured with anti-IgG1 as secondary antibody. Bars represent standard deviation. **A.** Reactivity of serum from mice immunised with *P. berghei*; **B.** Reactivity of serum from mice immunised with *P. falciparum*; **C.** Reactivity of serum from mice immunised with *P. yoelii*.

2.3.3. *Plasmodium yoelii* peptides

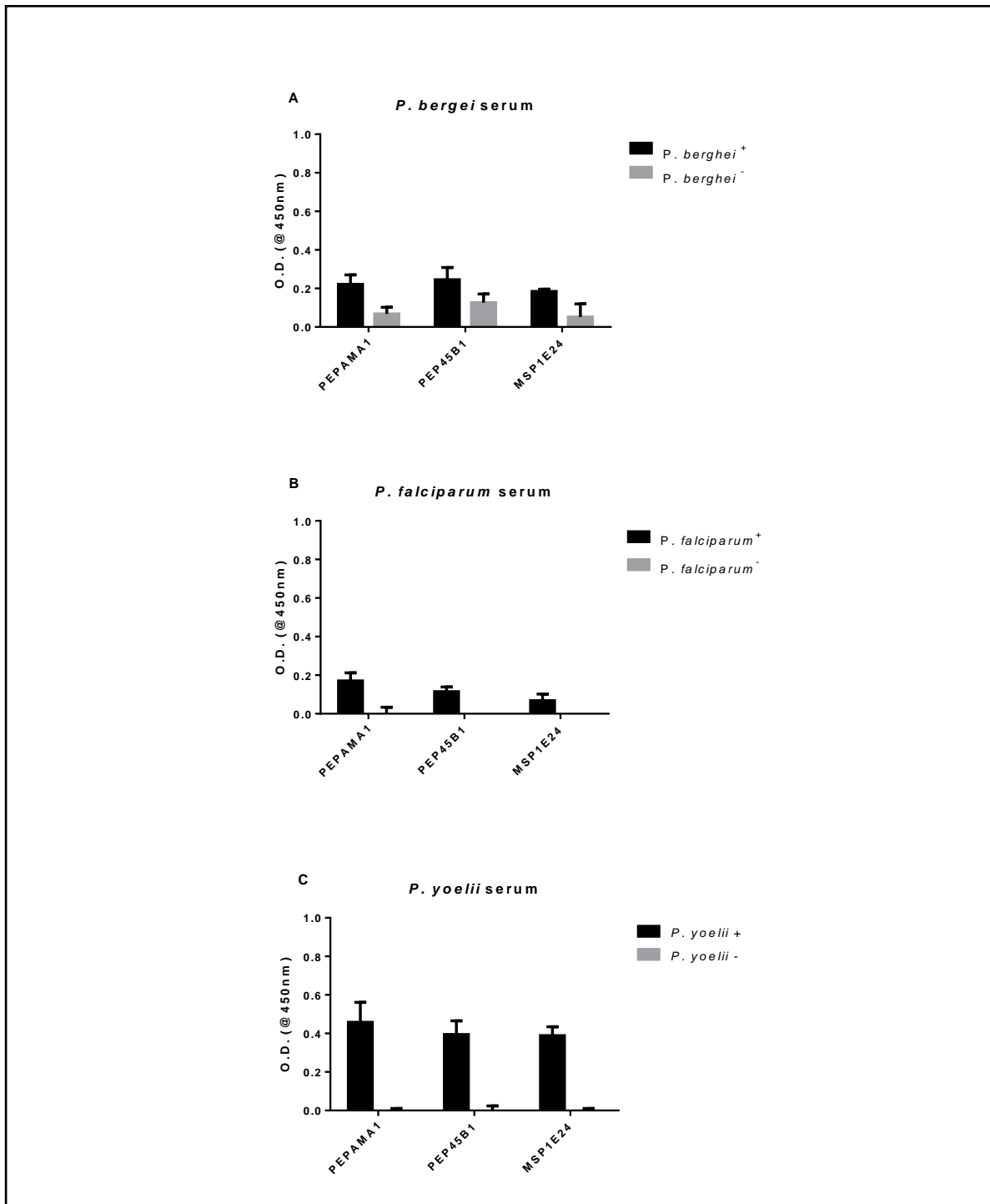


Figure 10 - Reactivity of serum from infected mice to *P. yoelii* synthetic peptides measured with anti-IgG1 as secondary antibody. Bars represent standard deviation. **A.** Reactivity of serum from mice immunised with *P. berghei*; **B.** Reactivity of serum from mice immunised with *P. falciparum*; **C.** Reactivity of serum from mice immunised with *P. yoelii*.

2.4. Discussion

Aside from *P. yoelii* peptides, that were visibly more reactive to serum from mice immunised with *P. yoelii*, all other peptides showed no visible specificity towards a particular parasite species. Despite unexpected, this shows that synthesised peptides not only can be used for ELISA assays but also, the ones described in this work, can be used in general detection assays.

Synthesis of peptides is without doubt a much quicker, cheaper and less laborious approach for applications such as epitope mapping, immune profiling and antibody titer determination.

2.5. References

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3.

Inoculation of mice with
live *Plasmodium* parasites

3.1. Introduction

The first attempt with a blood stage whole parasite malaria vaccine was in the 1940's, when Thomson and colleagues successfully inoculated both ducks and monkeys with killed *P. lophurae* and *P. knowlesi*, respectively (Freund, Thomson et al. 1946, Thomson, Freund et al. 1947, Freund, Thomson et al. 1948, Xu, Wipasa et al. 2002). Similar studies were carried out using *P. falciparum* in Aotus monkeys where protection was also achieved (Siddiqui 1977). However, in that study, the immunization approach included the use of Freund's adjuvant, which is banned from human application. Killed parasites were tested with other adjuvants but protection was never achieved (Freund, Thomson et al. 1948). Because of this, and the lack of massive parasite production techniques at the time, whole parasite vaccine development was hampered. Instead, the efforts were channelled into sub-unit vaccines, which became more accessible with the development of *Plasmodium* culturing methods and cloning (Trager and Jensen 1976, Ellis, Ozaki et al. 1983, Kemp, Coppel et al. 1983). The efficacy of sub-unit vaccines has been disappointing with even the most advanced candidate vaccine, RTS,S, showing extremely low efficacy rates (Olotu, Fegan et al. 2013). This disillusionment with sub-unit vaccines renewed the interest in whole parasite approaches, particularly after the promising results were achieved with whole attenuated sporozoites (Seder, Chang et al. 2013). Whole parasite vaccines have the major advantage of presenting a broad range of antigens to the immune system and currently offer the most successful means to achieve sterile protection (Butler, Vaughan et al. 2012, Staniscic and Good 2015). With this in mind, the objective of this work was to explore the possibility of using whole live blood stages of *Plasmodium* to induce a protective immune response, at the same time as exploring two different routes of inoculation: oral and nasal. This was tested with three species of *Plasmodium* (*P. yoelii*, *P. berghei* and *P. falciparum*) in two strains of mice (BALB/c and C57BL/6) by measuring serum reactivity to parasite's proteins and comparing host survival rates.

3.2. Methods

3.2.1. Animals and Parasites

Female BALB/c and C57BL/6 mice aged between 6 to 8 weeks were used. Animals were housed at the Instituto de Higiene e Medicina Tropical (IHMT), Lisbon, Portugal, in strict accordance with the recommendations of the European Directive 86/609/ EEC and Portuguese law (Decreto-Lei No. 129/92). Animal experiments were conducted with the approval of the Divisão Geral de Alimentação e Veterinária (DGAV), Portugal, under Art° 8, Portaria n°1005/92 from 23rd October (permit number n° 023357).

To grow rodent *Plasmodium* species, female BALB/c aged 5 – 8 weeks were infected with *Plasmodium yoelii nigeriensis* and *Plasmodium bergheii* ANKA. *Plasmodium falciparum* was grown in complete RPMI medium (11875093).

Animals were divided into two main groups according to administration route: Oral or Nasal. In each of these groups four groups were defined according to the species of parasite inoculated: *P. falciparum* 3D7, *P. berghei* ANKA, *P. yoelii nigeriensis* and Control (K) (**Table 2**). In order to facilitate group identification **Table 3** is provided with the acronyms used in this work.

All work associated with animal housing and handling was carried by staff of the IHMT and all remaining tasks were carried by the author.

Table 2 - Animal grouping and respective numbers according to inoculation approach and species

Species	ORAL				NASAL			
	<i>P.b</i>	<i>P.y</i>	<i>P.f</i>	K	<i>P.b</i>	<i>P.y</i>	<i>P.f</i>	K
BALB/c	5	5	5	5	5	5	5	5
C57BL/6	5	5	5	5	5	5	5	5

Table 3 - Group terminology according to mice strain, parasite species and inoculation route

Species	ORAL				NASAL			
	<i>P.b</i>	<i>P.y</i>	<i>P.f</i>	K	<i>P.b</i>	<i>P.y</i>	<i>P.f</i>	K
BALB/c	BPBO	BPYO	BPFO	BKO	BPBN	BPYN	BPFN	BKN
C57BL/6	CPBO	CPYO	CPFO	CKO	CPBN	CPYN	CPFN	CKN

3.2.2. Immunization, Booster and Challenge

3.2.2.1. Preparation of Oral and Nasal Inoculum

Inoculum, booster and challenge were prepared from infected whole blood collected from infected mice. For preparation of both inoculum and booster, the blood was collected when parasitemia reached 20 to 30% and the total volume obtained was separated in two tubes: Oral and Nasal. Blood smears were prepared in order to estimate the number of infected red blood cells (iRBC's) present in each dose of inoculum. The volumes of blood and PBS needed to create a concentration of approximately 1×10^8 iRBC's per inoculum were calculated considering the following method of iRBC/ μ L estimation:

$$iRBC's/\mu L = \frac{\text{Avg. RBC's}/\mu L \times \text{Parasitemia}}{100}$$

Avg. RBC's/ μ L was assumed to be $1.E+07$ (Roscoe and Green 1975)

3.2.2.1.1. Oral inoculum

Blood in the Oral tube was washed three times by centrifuging at 6,000 rpm, discarding the supernatant and adding PBS. At the last washing step, RBC's were gently resuspended with a specific volume of PBS as to produce an approximate concentration of 1×10^8 iRBC's per inoculum.

3.2.2.1.2. Nasal inoculum

Instead of iRBC's, the nasal inoculum was constituted of extracted parasites via RBC lysis. For this, the infected blood and PBS volumes were calculated as to produce an approximate concentration of what would be of 1×10^8 iRBC's per inoculum. The resulting iRBC volume was then lysed by centrifuging the nasal tube at 6,000 rpm for 3 minutes, the serum discarded and the RBC's resuspended in 1mL of 0.15% Saponin. The tube was incubated on ice for 5 minutes, vortexed every minute during incubation and washed 3 times. Each washing step included centrifugation at 4,000 rpm for 3 minutes at 4 °C, aspiration of supernatant and addition of

1mL of cold PBS. During the last wash, the pellet was resuspended with the specific volume of PBS.

3.2.2.1.3. Challenge

The challenge was prepared from blood with a parasitemia of 20-30% to which a specific volume of PBS was added in order to create a concentration of 1×10^6 iRBC's per dose.

3.2.2.2. Administration of Oral and Nasal Inoculum

Inoculation, booster and challenge were carried out 30 days apart (**Figure 11**) and the combination of parasite species administered to each experimental group can be found in **Table 4**.

Table 4 - Combination of parasite species administered during Inoculation, Booster and Challenge. *P.b.*, *P.y.* and *P.f.* groups were inoculated with *P.b.*, *P.y.* and *P.f.* respectively. During challenge, groups *P.b.*, *P.y.* and *P.f.* were infected with *P.b.*, *P.y.* and *P.b.*. Control groups (K), were inoculated with PBS and challenged with either *P.b.* or *P.y.*

	Groups	ORAL				NASAL							
		<i>P.b.</i>	<i>P.y.</i>	<i>P.f.</i>	K	<i>P.b.</i>	<i>P.y.</i>	<i>P.f.</i>	K				
<i>Inoculation</i>	BALB/c	<i>P.b.</i>	<i>P.y.</i>	<i>P.f.</i>	PBS	<i>P.b.</i>	<i>P.y.</i>	<i>P.f.</i>	PBS				
	C57BL/6												
<i>Booster</i>	BALB/c												
	C57BL/6												
<i>Challenge</i>	BALB/c			<i>P.b.</i>	<i>P.y.</i>			<i>P.b.</i>	<i>P.b./P.y.</i>	<i>P.b.</i>	<i>P.y.</i>	<i>P.b.</i>	<i>P.b./P.y.</i>
	C57BL/6												

3.2.2.2.1. Oral route

Oral inoculations and boosters were conducted with the aid of a micropipette by gently and slowly depositing 50 μ L (approximately 1×10^8 iRBC's) of inoculum into the oral cavity and allowing the animal to swallow the volume. This method was preferred in order to avoid any potential damage to the oral epithelium that oral gavage could cause. Controls were

inoculated with sterile PBS. For all species, an aliquot of 50 μL of inoculum and booster was saved in RNAlater and stored at $-20\text{ }^{\circ}\text{C}$ for posterior DNA quantification.

3.2.2.2. Nasal route

Nasal inoculations and booster were carried by depositing 5 μL (approximately 1×10^8 lysed iRBC's) of inoculum in each nostril and allowing for the animal to inhale the total volume. Again, forced administration was avoided to prevent epithelial damage. Controls were given sterile PBS. For all species, an aliquot of 10 μL was saved in RNAlater and stored at $-20\text{ }^{\circ}\text{C}$ for posterior DNA quantification.

3.2.2.3. Challenge

Inoculated and control mice were challenged with 100 μL (approximately 1×10^6 iRBC's) of respective parasite species (**Table 4**), by intraperitoneal injection (i.p.). For all species, an aliquot of 100 μL was saved in RNAlater and stored at $-20\text{ }^{\circ}\text{C}$ for posterior DNA amplification as to verify parasite content in the challenge.

3.2.3. Sample collection: Blood, smears and livers

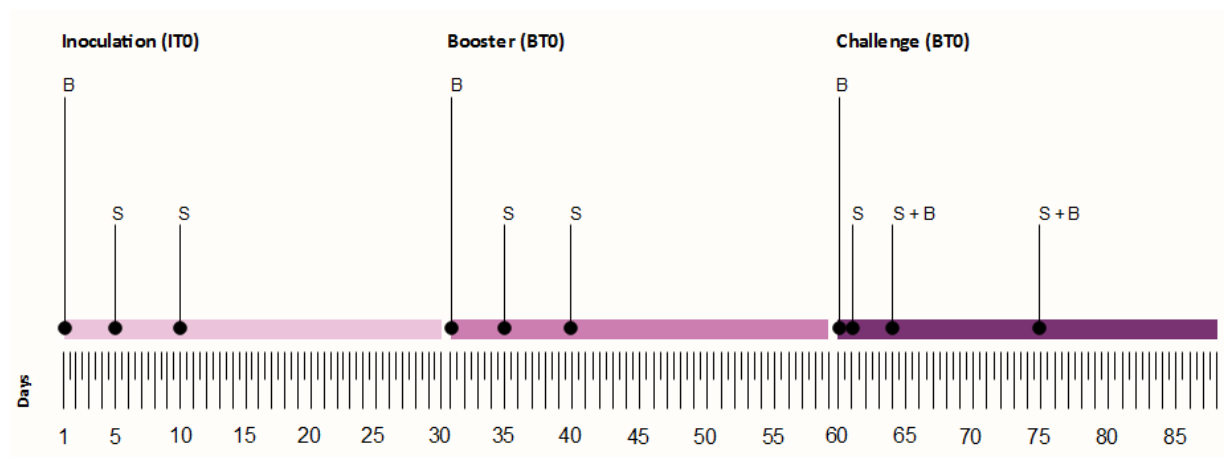


Figure 11 - Experimental timeline. Inoculation (IT0) of animals at day one where blood (B) was also collected right before the procedure was initiated. Booster (BT0) was carried at day 30 and Challenge (CT0) at day 60, with blood collections in the same days. Blood smears (S) were collected 5 and 10 days after Inoculation and Booster, and 2, 4 and 15 days after challenge.

Blood samples were collected at specific times points (**Figure 11**) from individual mice into heparin-coated microcentrifuge tubes by serial cheek puncture. Collected volumes varied from 80 to 150 μ L. Blood smears were collected at specific time points (**Figure 11**) using standard thin film smear procedure and staining. Livers were collected *post-mortem* from all animals, kept in a microcentrifuge tube with RNAlater (25 mM Sodium citrate, 10mM EDTA, 70 g Ammonium sulfate / 100 mL solution, pH5.2) and stored at – 20 °C.

3.2.4. ELISA

3.2.4.1. Antigens: Crude extracts and peptides

3.2.4.1.1. Crude extracts

Blood was collected from infected mice by heart puncture when parasitemia reached 40% of schizonts. Collected blood was then centrifuged at 6000 rpm and the supernatant discarded. Three volumes of cold ddH₂O were added and the pellet resuspended. After leaving the tube to incubate on ice for approximately 5 minutes, centrifugation was carried once again and supernatant discarded. The resulting pellet was washed three times with two volumes of cold PBS. During the last washing step, the pellet was resuspended in 200 μ L of PBS and stored at - 80°C.

3.2.4.1.2. Peptides

Peptides used for antibody titer determination were all synthesised and derived from the apical membrane antigen 1 (AMA1). Sequences of *P. yoelii* and *P. falciparum* peptides were sourced from published works (**Table 5**). For *P. berghei* peptides were derived from unique regions of the following aligned sequences: MSP1 *P. berghei* ANKA (GenBank accession number CDS4641.1), MSP1 *P. berghei* (GenBank accession number AAC2887.1) and MSP1 *P. yoelii* (GenBank accession number CDU17703.1). The process by which these peptides were selected and tested is further detailed in chapter 2. All peptides were synthesised by GenScript® and prepared as manufacturer's specifications.

Table 5 - List of peptides and corresponding sequences used for ELISA

Name	Protein	Species	Sequence	Reference
PEP45B1	AMA1	<i>P. yoelii</i>	CSNSDKPKCGGGS	(Narum, Ogun et al. 2006)
AMAPB4	AMA1	<i>P. berghei</i>	TAENRENSKKIICTCKEPTPNAYYDGVFCS	Current work
AMAPF	AMA1	<i>P. falciparum</i>	DGNCEDIPHVNEFSAIDL	(Biswas, Seth et al. 2008)

3.2.4.2. ELISA protocol

ELISA plates (VWR, 735-0079) were coated over night at 4°C with 50µL of either 0.1µg/mL of synthetic peptide or 5µg/mL crude extract diluted in sterile PBS. Plates were washed 3 times with 200µL of PBST (1X PBS with 0.05% Tween-20) prior to blocking for two hours with blocking buffer (4% milk powder in PBST). Serum was then diluted in PBS at 1:2500 and 50µL were added per well. Plates were incubated for three hours at 37 °C before washing three times as previously described. Next, HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgM (Abcam) were diluted separately in blocking buffer at 1:1000 and 50µL was added per well. After a two-hour incubation at 37°C plates were washed followed by the addition of 50µL of 3,3',5,5'-Tetramethylbenzidine (TMB) (Abcam ab171523). The substrate was incubated at room temperature for 15 minutes and reaction stopped with 1N Sulphuric acid prior to measuring the absorbance at 450nm with a SpectraMax® 384 microplate reader.

3.2.5. Blood clot and liver DNA extractions

3.2.5.1. Blood clots DNA extractions

Blood clot extractions were carried with DNeasy Blood & Tissue kit (Qiagen, 69504) with modifications to manufacture's protocol. Tubes containing the blood clots were centrifuged at 8000 rpm for 1 min and the volume of each clot annotated followed by addition of 150µL of PBS. The full content of each tube was transferred to a bead tube (BeadBug™ 0.5 mm Silica glass beads, Z763748-50EA) with 200µL of AL buffer followed by shaking at 400rpm for 150s in a BeadBug microtube homogenizer (Benchmark Scientific). After shaking, tubes were centrifuged at 4000 rpm for 1 min to dissipate foam and 15µL of proteinase K was added. Tubes were incubated at 56°C for 10min followed by addition of 200µL of ethanol and

vortexing. The mixture was transferred to a Spin column and centrifuged at 8000 rpm for 1 min. Flow-through was discarded and column placed in a new collection tube after which 500µL of AW1 buffer were added and tubes centrifuged at 8000 rpm for 1 min. Again, flow-through was discarded and column placed in new collection tube followed by addition of 500µL of AW2 buffer and centrifugation at 14000 for 3 min. The flow-through was discarded and the column was now placed in a clean 1.5mL Eppendorf tube followed by addition of 70µL of AW buffer. After a 10min incubation at room temperature, tubes were centrifuged at 8000rpm for 1 min. DNA extractions were then stored at -20°C until used.

3.2.5.2. Liver DNA extractions

Liver extractions were carried with DNeasy Blood & Tissue kit (69504) with modifications to manufacture's protocol. A Small piece of liver was cut and placed in a bead tube (BeadBug™ 0.5 mm Silica glass beads, Z763748-50EA) with 200µL of AL buffer followed by shaking at 400rpm for 150s in a BeadBug microtube homogenizer (Benchmark Scientific). After shaking, tubes were centrifuged at 4000 rpm for 1 min to dissipate foam and 15µL of proteinase K was added. Tubes were incubated at 56°C for 35min followed by addition of 200µL of ethanol and vortexing. The mixture was transferred to a Spin column and centrifuged at 8000 rpm for 1 min. Flow-through was discarded and column placed in a new collection tube after which 500µL of AW1 buffer were added and tubes centrifuged at 8000 rpm for 1 min. Again, flow-through was discarded and column placed in new collection tube followed by addition of 500µL of AW2 buffer and centrifugation at 14000 for 3 min. The flow-through was discarded and the column was now placed in a clean 1.5mL Eppendorf tube followed by addition of 70µL of AW buffer. After a 10min incubation at room temperature, tubes were centrifuged at 8000rpm for 1 min. DNA extractions were then stored at -20°C until used.

3.2.6. qPCRs

Amplification of a 18S rRNA highly conserved region found only in the genus *Plasmodium* and qPCR measurements were performed using a Bio Rad CFX96 qPCR system. The thermal program used for the qPCR comprised of an initial denaturation at 95°C for 2min, 30 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s and extension at 60°C for 30s. Each

25µL reaction mixture contained 10µL of 1x PrecisionPLUS™ mastermix, 1µL of DNA template and 6pmol of forward (5'-GCTCTTTCTTGATTTCTTGGATG-3') and reverse (5'-AGCAGGTTAAGATCTCGTTCG-3') primers described by Kamau *et al.* (Kamau, Alemayehu et al. 2013). All qPCR assays were run with duplicates and appropriate controls including negative (blood from non-infected mice) and positive controls (blood from infected mice). Data was analyzed with Bio-Rad CFX Manager 2.0 software using a regression Cq determination mode and negative samples were denoted as N/A (no amplification).

3.2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Version 6.01). Mantel-Cox tests were used to compare survival distribution between groups, while Mann-Whitney and unpaired t-tests were used to evaluate statistical significance of optical densities (O.D.) of immunoglobulins between groups. A p value < 0.05 was considered significant and the following representation was used: * = P<0.05; **=P<0.01; ***=P<0.001, ****=P<0.0001.

3.3. Results

3.3.1. Disregarding subjects from control group

In this work 20 animals were used as controls and these were challenged according to **Table 2**. In the beginning of the project it was expected to use the adjuvant GPI-0100 (Hawaii Biotech, USA) in all inoculations and so it was important to have a control group for both Oral and Nasal route, hence the grouping depicted in **Table 6**.

Table 6 - Mice strain and respective parasite species used for challenge

	Oral		Nasal	
	<i>P. yoelii</i>	<i>P. berghei</i>	<i>P. yoelii</i>	<i>P. berghei</i>
BALB/c	2	3	3	2
C57BL/6	3	2	2	3

However, the selected adjuvant was not available and authorised by the company at the time the experiment was due to start. Because of that, it was decided that experiments would still be carried out but without adjuvant. In terms of animal grouping this meant that, in practice, despite the original experimental segregation of control groups according to route, the animals challenged with *P. yoelii* and *P. berghei* were 5 for each of the mice strains (**Table 7**). Because all controls were inoculated solely with PBS, the only distinction between them was the species of parasite used for challenge.

Table 7 - Mice strain and respective parasite species used for challenge

	Controls	
	<i>P. yoelii</i>	<i>P. berghei</i>
BALB/c	5	5
C57BL/6	5	5

At the end of the experiment animals from the control group survived on average 8 days. However, from the 20 animals, 3 survived much longer than the rest. These 3 animals belonged to the same challenge group: Oral BALB/c challenged with *P. berghei* (Table 4). Because all three survived remarkably longer than the rest of the control group, and all were challenged at the same time, it is believed that something influenced this extended survival. Before this experiment was carried out, a similar pilot study, where mice were inoculated orally and nasally, was initiated in the same facilities. It is possible, that some of the control animals used in the current experiment have been mistakenly sourced from the previous pilot study. Unfortunately, and because this part of the project was not carried by the author, it was not possible to verify the origin of those 3 animals. Because of this, it was decided to disregard them from the current study.

3.3.2. Survival curves

3.3.2.1. Before challenge: Orally inoculated mice can become lethally infected

Oral inoculation resulted in some lethal infections, three cases after initial inoculation (**Figure 12 B; Figure 14A and B**) and one case after booster administration (**Figure 12A**).

In all groups inoculation orally, with the exception of those inoculated with *P. falciparum*, 1 out of 5 animals developed a lethal infection. This shows that infection was not dependant on the *Plasmodium* species nor on the mice strain. Both *P. yoelii* and *P. berghei* are, therefore, capable of infecting the host through the oral route.

Plasmodium yoelii

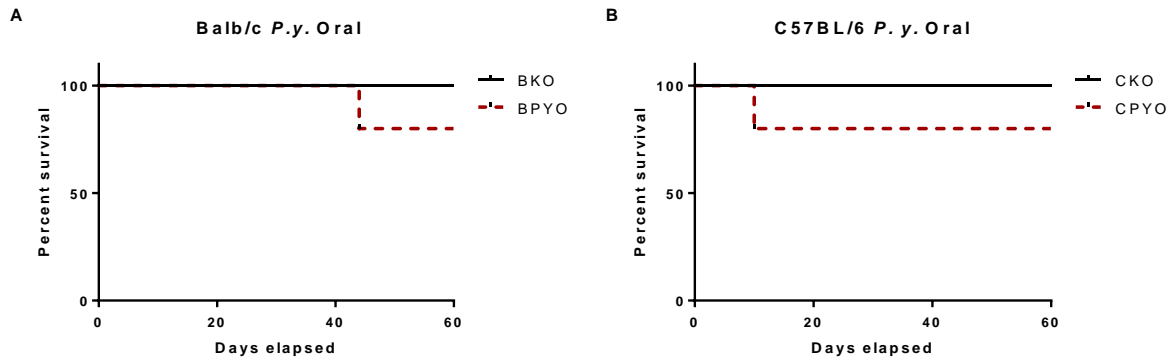


Figure 12 - Survival curve of mice orally inoculated with *P. yoelii*, from inoculation day (day 0) to challenge day (day 60). **(A)** BKO group: BALB/c control orally inoculated with PBS; BPYO group: BALB/c orally inoculated with *P. yoelii*. **(B)** CKO group: C57BL/6 control orally inoculated with PBS; CPYO group: C57BL/6 orally inoculated with *P. yoelii*.

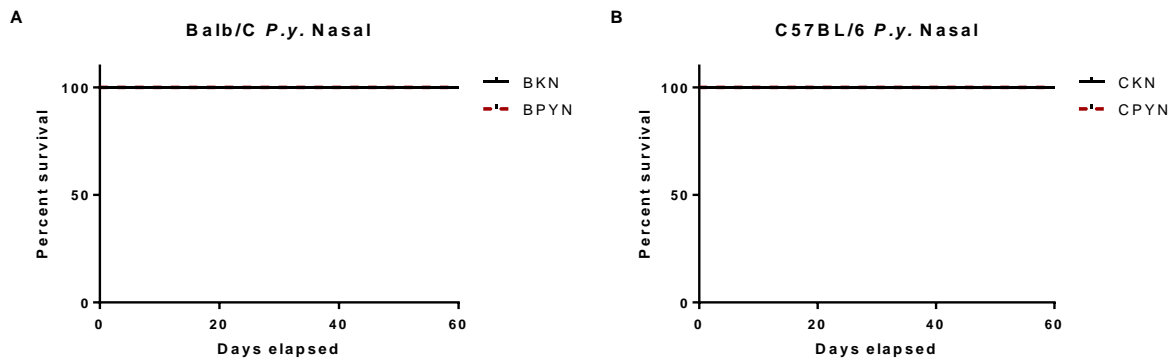


Figure 13 - Survival curve of mice nasally inoculated with *P. yoelii*, from inoculation day (day 0) to challenge day (day 60). **(A)** BKN group: BALB/c control nasally inoculated with PBS; BPYN group: BALB/c nasally inoculated with *P. yoelii*. **(B)** CKN group: C57BL/6 control nasally inoculated with PBS; CPYN group: C57BL/6 orally inoculated with *P. yoelii*.

Plasmodium berghei

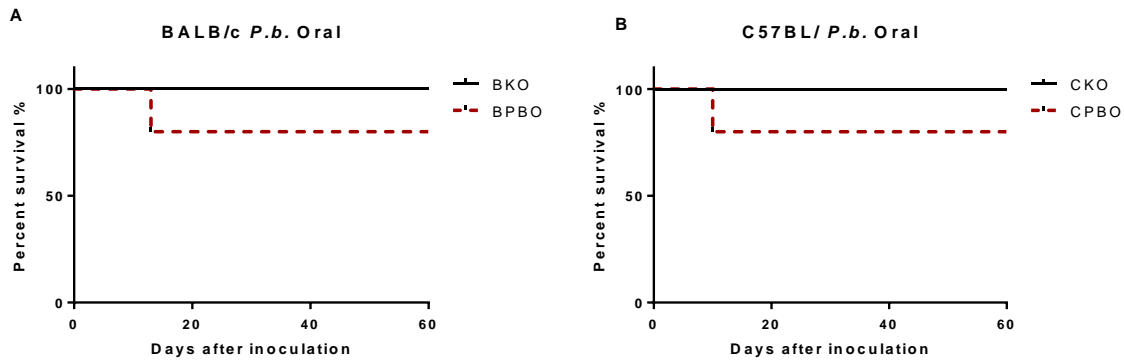


Figure 14 - Survival curve of mice orally inoculated with *P. berghei*, from inoculation day (day 0) to challenge day (day 60). **(A)** BKO group: BALB/c control orally inoculated with PBS; BPBO group: BALB/c orally inoculated with *P. berghei*. **(B)** CKO group: C57BL/6 control orally inoculated with PBS; CPBO group: C57BL/6 orally inoculated with *P. berghei*.

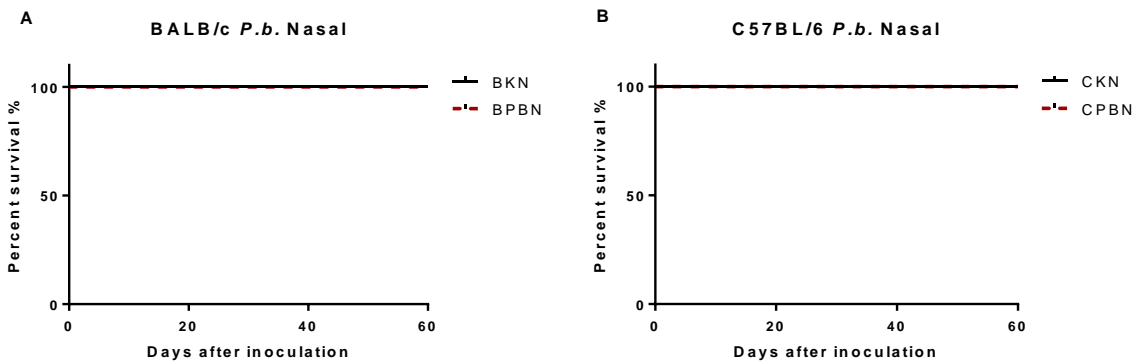


Figure 15 - Survival curve of mice nasally inoculated with *P. berghei*, from inoculation day (day 0) to challenge day (day 60). **(A)** BKN group: BALB/c control nasally inoculated with PBS; BPBN group: BALB/c nasally inoculated with *P. berghei*. **(B)** CKN group: C57BL/6 control nasally inoculated with PBS; CPBN group: C57BL/6 orally inoculated with *P. berghei*.

Plasmodium falciparum

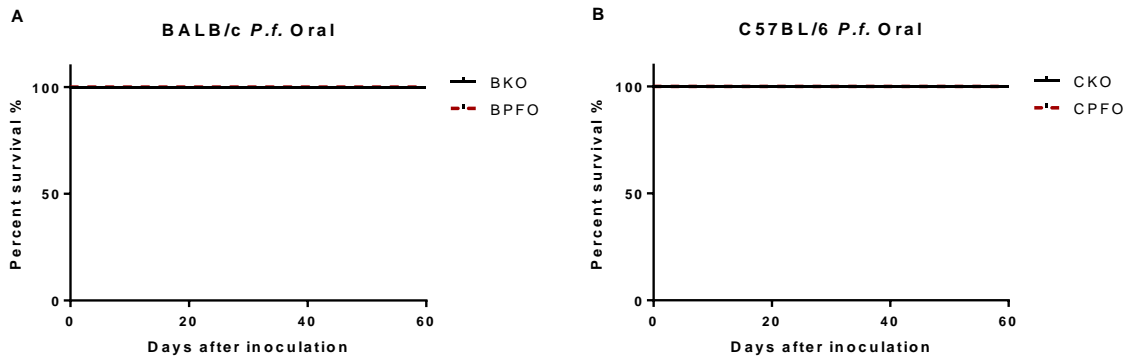


Figure 16 - Survival curve of mice orally inoculated with *P. falciparum*, from inoculation day (day 0) to challenge day (day 60). **(A)** BKO group: BALB/c control orally inoculated with PBS; BPFO group: BALB/c orally inoculated with *P. falciparum* **(B)** CKO group: C57BL/6 control orally inoculated with PBS; CPFO group: C57BL/6 orally inoculated with *P. falciparum*.

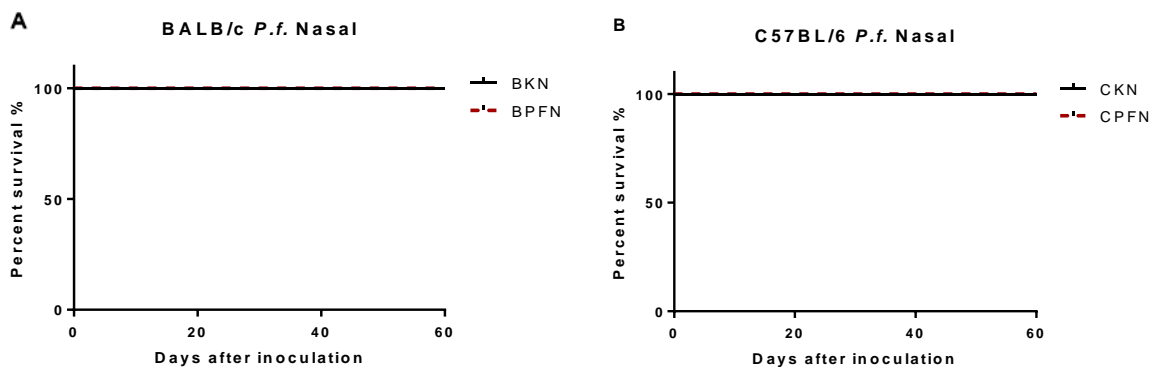


Figure 17 - Survival curve of mice nasally inoculated with *P. falciparum*, from inoculation day (day 0) to challenge day (day 60). **(A)** BKN group: BALB/c control nasally inoculated with PBS; BPFN group: BALB/c nasally inoculated with *P. falciparum*. **(B)** CKN group: C57BL/6 control nasally inoculated with PBS; CPFN group: C57BL/6 orally inoculated with *P. falciparum*.

3.3.2.2. After challenge: All inoculated groups survived longer than the control groups

From all groups, mice who were inoculated tended to survive longer than control mice. This was particularly evident in groups inoculated nasally where the majority presented a significant survival rate.

3.3.2.2.1. *Plasmodium yoelii*

The strain of *P. yoelii* used for inoculation and challenge chosen was *P. yoelii nigeriensis*, which is known to be lethal in BALB/c mice (Sharma, Sharma et al. 1994). Although protection was not achieved, nasally inoculated groups survived significantly longer than control groups (**Figure 19, A and B**).

Groups orally inoculated with *P. yoelii* did not show a significant survival rate when compared with respective control groups, however, an interesting proportion of inoculated subjects outlived the individuals from the control group (**Figure 18 - A and B**). In addition, there was also no statistical difference between mice strain. For the groups inoculated nasally, both mice strains showed a significant survival rate over the respective control groups (p value = 0.0023 in BPYN and 0.0339 in CPYN) (**Figure 19 - A and B**). There was also no statistical difference between inoculated mice strains.

Plasmodium yoelii

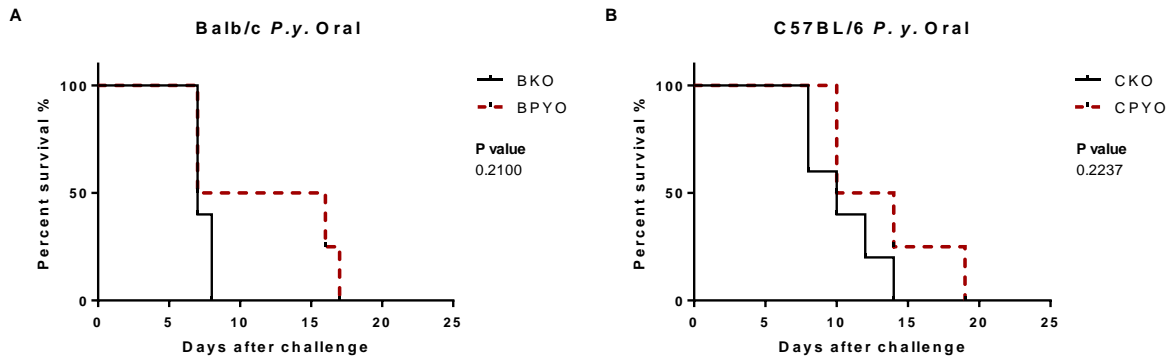


Figure 18 - Survival curve of mice orally inoculated and i.p. challenged with *P. yoelii*. **(A)** BKO group: BALB/c control orally inoculated with PBS and challenged with *P. yoelii*; BPYO group: BALB/c orally inoculated and i.p. challenged with *P. yoelii*. **(B)** CKO group: C57BL/6 control orally inoculated with PBS and challenged with *P. yoelii*; CPYO group: C57BL/6 orally inoculated and i.p. challenged with *P. yoelii*.

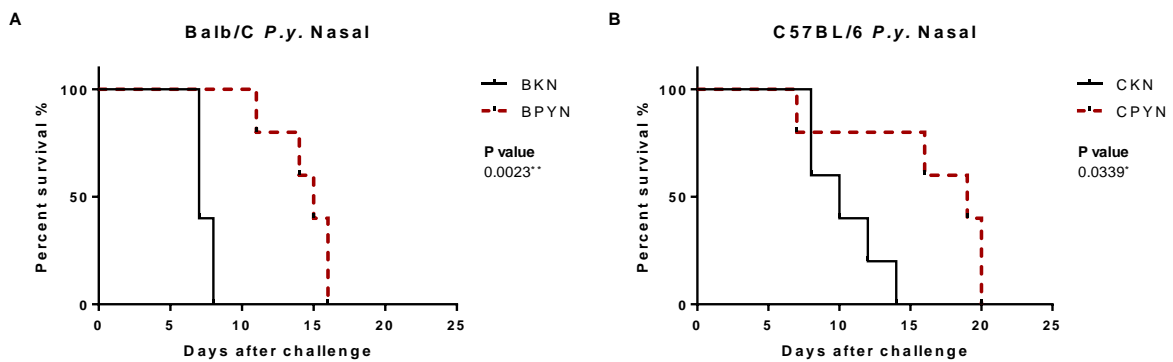


Figure 19 - Survival curve of mice nasally inoculated and i.p. challenged with *P. yoelii*. **(A)** BKN group: BALB/c control nasally inoculated orally with PBS and challenged with *P. yoelii*; BPYN group: BALB/c nasally inoculated and i.p. challenged with *P. yoelii*. **(B)** CKN group: C57BL/6 control nasally inoculated orally with PBS and challenged with *P. yoelii*; CPYN group: C57BL/6 orally inoculated and i.p. challenged with *P. yoelii*.

3.3.2.2.2. *Plasmodium berghei*

The strain of *P. berghei* used for immunization and challenge chosen was *P. berghei* ANKA known to be lethal in BALB/c mice. Once again protection was not achieved but C57BL/6 mice from inoculated groups did survive longer than control groups.

In group BPBO, despite a large proportion of mice having outlived individuals from BKO, the survival rate did not seem to be statistically significant (**Figure 20A**). In CPBO, however, all mice outlived subjects from CKO, which was reflected on a significant survival rate (p value = 0.0220) (**Figure 20B**).

In nasally inoculated groups, all inoculated mice outlived the respective control group. However, only CPBN had a statistically significant survival rate (p value = 0.0090) (**Figure 21B**).

Plasmodium berghei

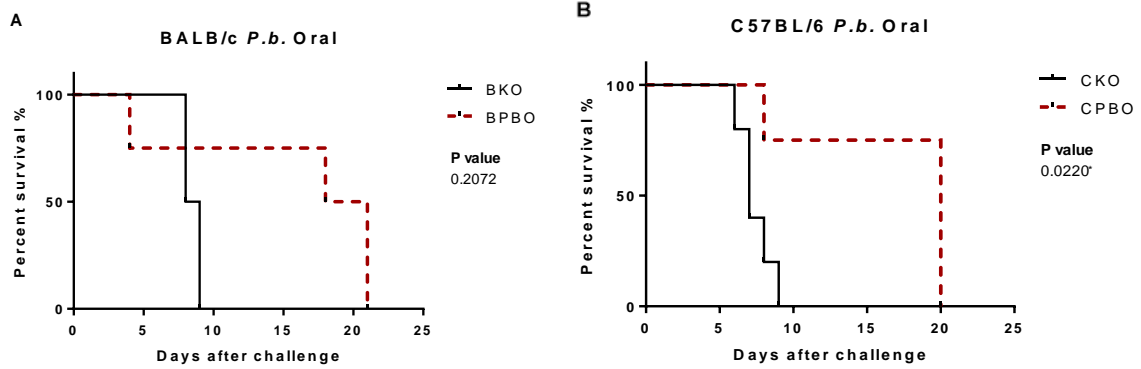


Figure 20 - Survival curve of mice orally inoculated and i.p. challenged with *P. berghei*. **(A)** BKO group: BALB/c control orally inoculated with PBS and challenged with *P. berghei*; BPBO group: BALB/c orally inoculated and i.p. challenged with *P. berghei*. **(B)** CKO group: C57BL/6 control orally inoculated with PBS and challenged with *P. berghei*; CPBO group: C57BL/6 orally inoculated and i.p. challenged with *P. berghei*.

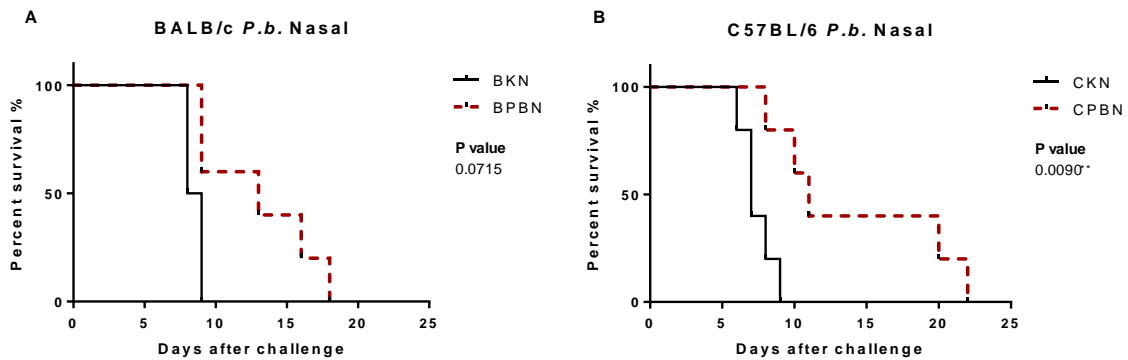


Figure 21 - Survival curves of mice nasally inoculated and i.p. challenged with *P. berghei*. **(A)** BKN group: BALB/c control nasally inoculated with PBS and challenged with *P. berghei*; BPBN group: BALB/c nasally inoculated and i.p. challenged with *P. berghei*. **(B)** CKN group: C57BL/6 control nasally inoculated with PBS and challenged with *P. berghei*; CPBN group: C57BL/6 nasally inoculated and i.p. challenged with *P. berghei*.

3.3.2.2.3. *Plasmodium falciparum*

Plasmodium falciparum is the deadliest of the human malaria species to exclusively infect humans and therefore, mice inoculated with *P. falciparum* had to be challenged with *P. berghei* ANKA.

From the groups inoculated with *P. falciparum*, only BALB/c mice presented a statistical significant survival rate (p value = 0.0082 for both BPFO and BPFN) when compared to respective controls, regardless of the inoculation route (**Figure 22A** and **Figure 23A**). Unlike previous groups, there was a statistical difference between mice strains when comparing BPFO with CPFO (p value = 0.0071) and BPFN with CPFN (p value = 0.0018).

Plasmodium falciparum

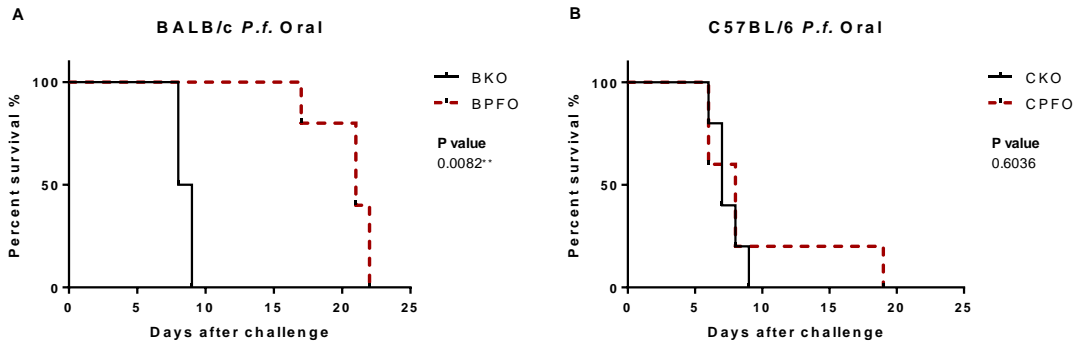


Figure 22 - Survival curve of mice orally inoculated and i.p. challenged with *P. falciparum*. **(A)** BKO group: BALB/c control orally inoculated with PBS and challenged with *P. berghei*; BPFO group: BALB/c orally inoculated and i.p. challenged with *P. falciparum*. **(B)** CKO group: C57BL/6 control orally inoculated with PBS and challenged with *P. berghei*; CPFO group: C57BL/6 orally inoculated and i.p. challenged with *P. falciparum*.

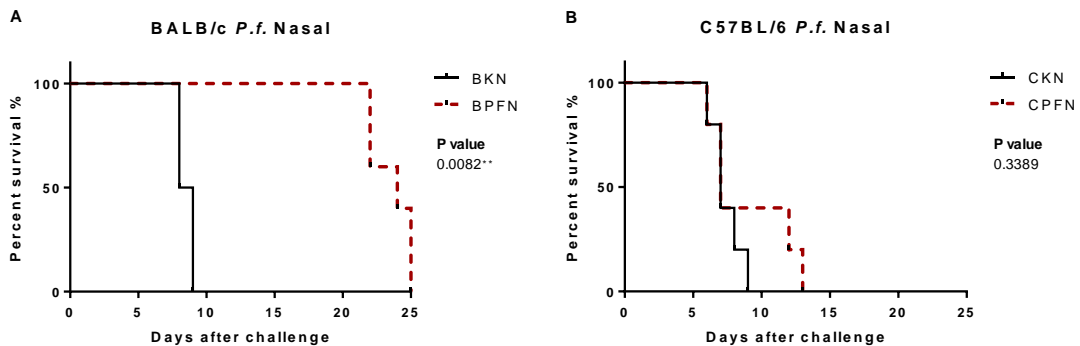


Figure 23 - Survival curves of mice nasally inoculated and i.p. challenged with *P. falciparum*. **(A)** BKN group: BALB/c control nasally inoculated with PBS and challenged with *P. berghei*; BPFN group: BALB/c nasally inoculated and i.p. challenged with *P. falciparum*. **(B)** CKN group: C57BL/6 control nasally inoculated with PBS and challenged with *P. berghei*; CPFN group: C57BL/6 nasally inoculated and i.p. challenged with *P. falciparum*.

Overall, groups inoculated nasally presented a more significant survival rate when compared with correspondent control groups (**Table 8**). This was particularly evident in groups inoculated with *P. yoelii*. A pattern was also noted regarding mice strains. In the groups inoculated with *P. berghei*, significance was observed only in C57BL/6 mice contrary to the groups inoculated with *P. falciparum* where significance was seen only in BALB/c mice.

Table 8 – Summary of statistical significance with p values for the survival rates of inoculated groups against respective control groups. Statistical significance is assumed when p value < 0.05 (in bold).

Route	ORAL			NASAL		
Species	<i>P.y.</i>	<i>P.b.</i>	<i>P.f.</i>	<i>P.y.</i>	<i>P.b.</i>	<i>P.f.</i>
BALB/c	0.2100	0.2072	0.0082**	0.0023**	0.0715	0.0082**
C57BL/6	0.2237	0.0220*	0.7807	0.0339*	0.0090**	0.3389

3.3.3. ELISA

3.3.3.1. *Plasmodium yoelii*

ELISAs in which mice serum was tested for reactivity to *P. yoelii* CE (crude extract), the overall O.D. (optical density) was very low. This was observed in both BALB/c and C57BL/6 mice strains and in both oral and nasal routes. Despite that, in many cases, the difference in O.D. between treated and control groups was statistically significant, the overall intensity was considered low for a positive antibody reactivity expected from an positive immune response, as seen in results from chapter 2. The same was observed when serum was tested against *P. yoelii*'s peptide PEP45B1.

Regardless of this, it was noticeable that IgG2a was elevated at time point CT15 in all BALB/c mice. This was observed with both crude extract and peptide essays eliminating the possibility of assay variation. In addition, IgM was elevated in all groups

Interestingly, it was noticeable that despite an overall low reactivity of most immunoglobulins, almost all groups showed an elevated O.D. for IgM.

3.3.3.1.1. Reactivity to *P. yoelii* Crude Extract

Oral route (BALB/c)

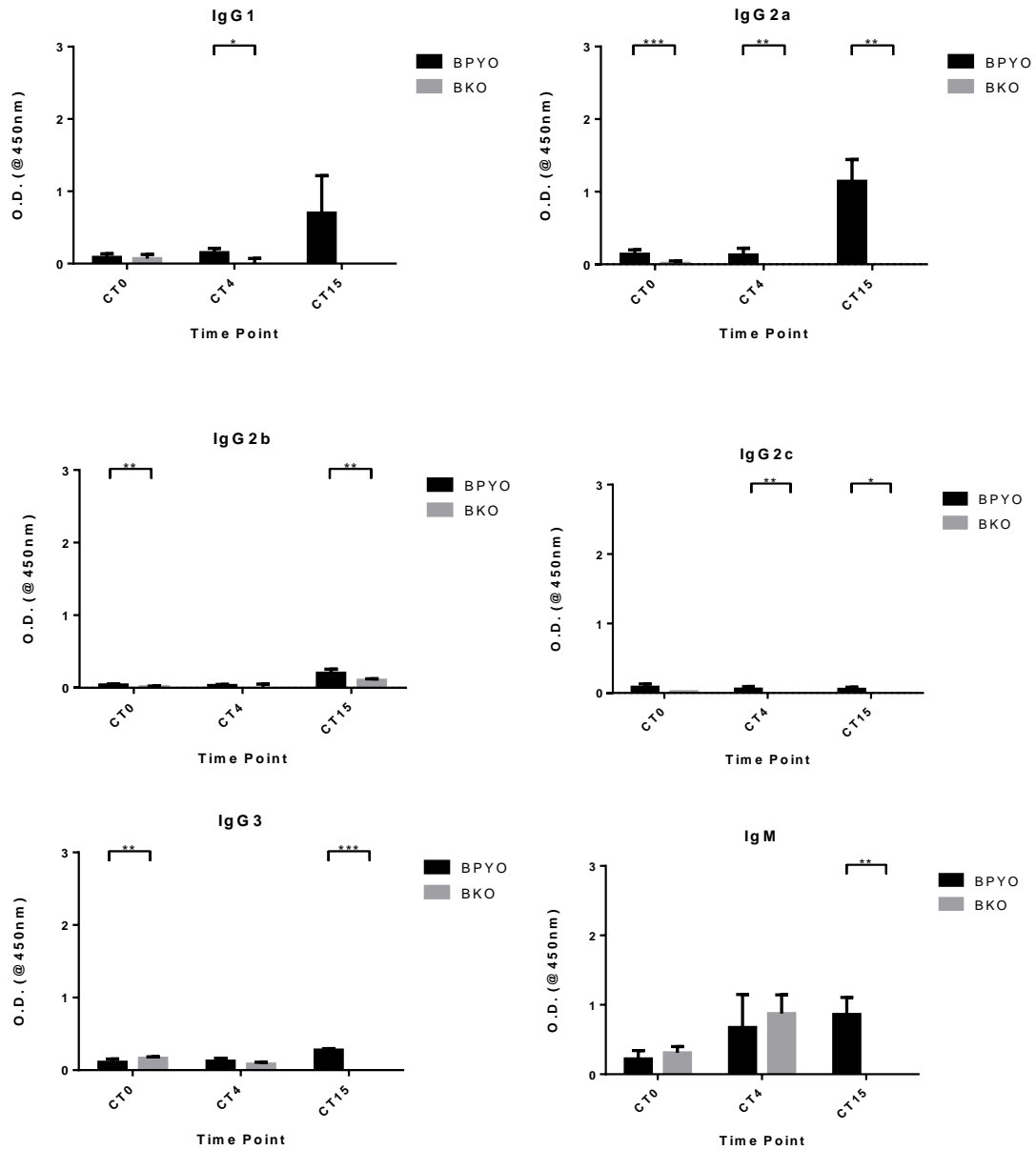


Figure 24 - Variation of immunoglobulin reactivity to *P. yoelii* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated orally with *P. yoelii* (BPYO) and PBS (BKO). At CT15, only animals from group BPYO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; ** = P<0.01; *** = P<0.001.

Nasal route (BALB/c)

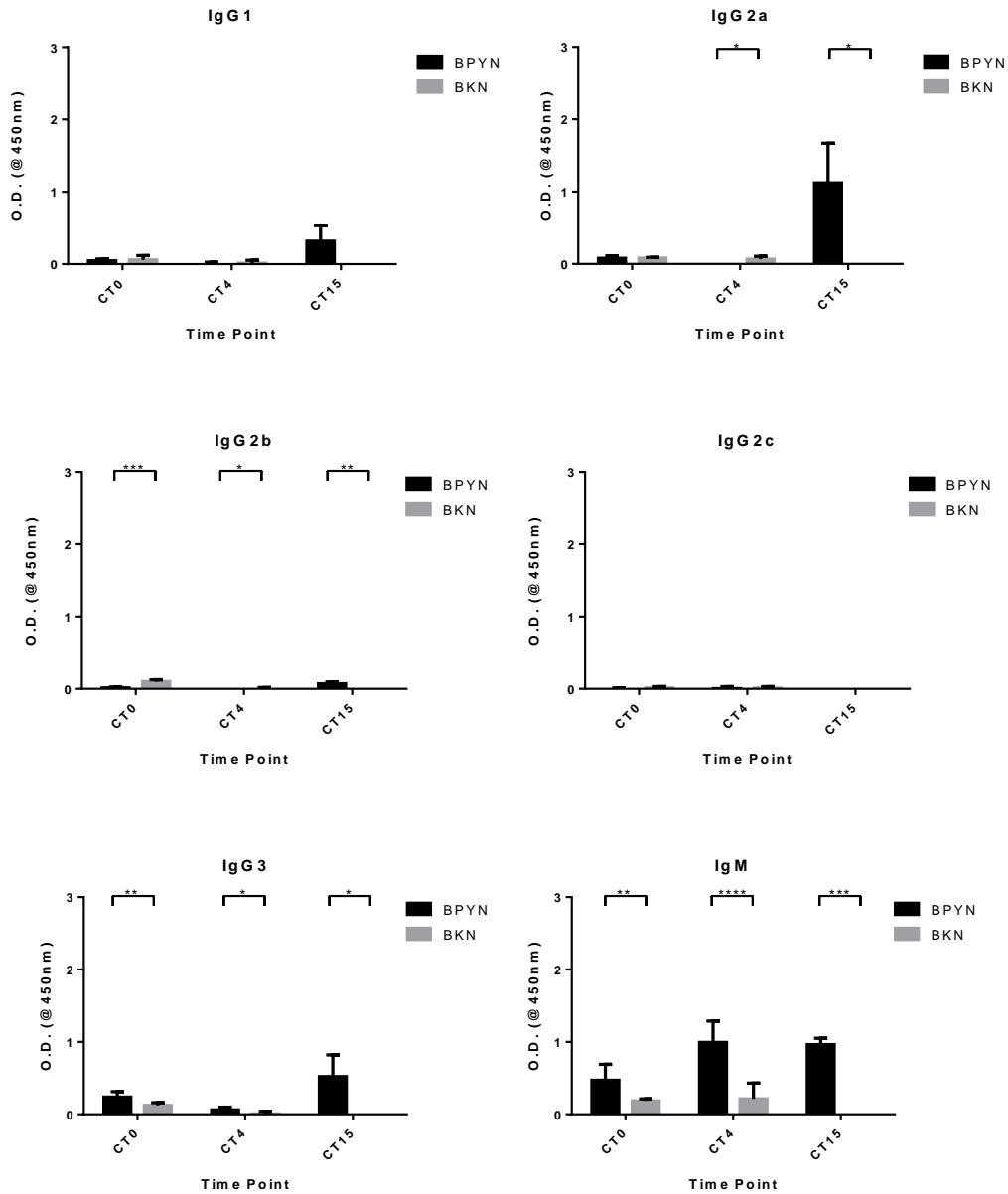


Figure 25 - Variation of immunoglobulin reactivity to *P. yoelii* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated nasally with *P. yoelii* (BPYN) and PBS (BKN). At CT15, only animals from group BPYN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; ** = P<0.01; *** = P<0.001.

Oral route (C57BL/6)

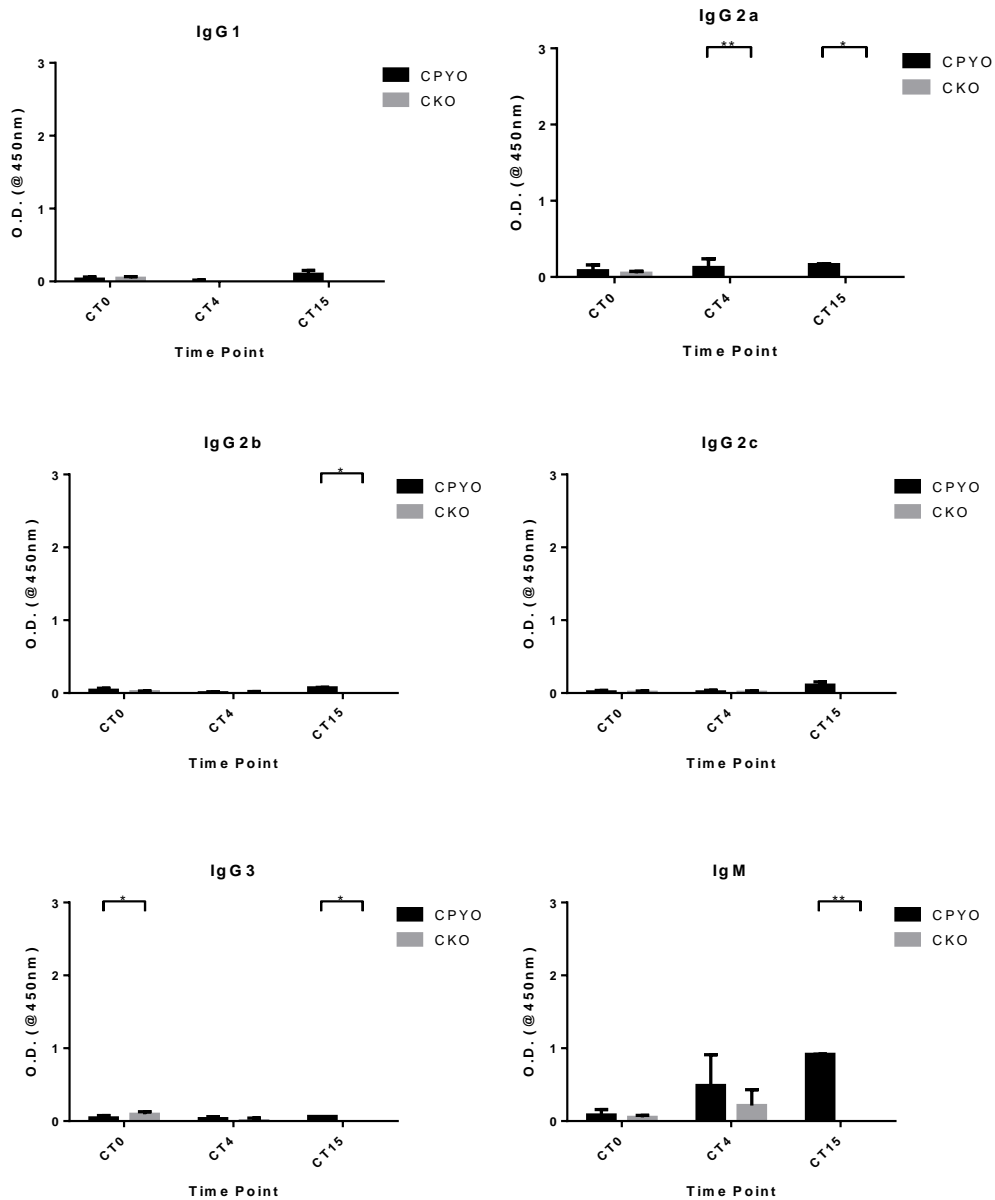


Figure 26 - Variation of immunoglobulin reactivity to *P. yoelii* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated orally with *P. yoelii* (CPYO) and PBS (CKO). At CT15, only animals from group CPYO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Nasal route (C57BL/6)

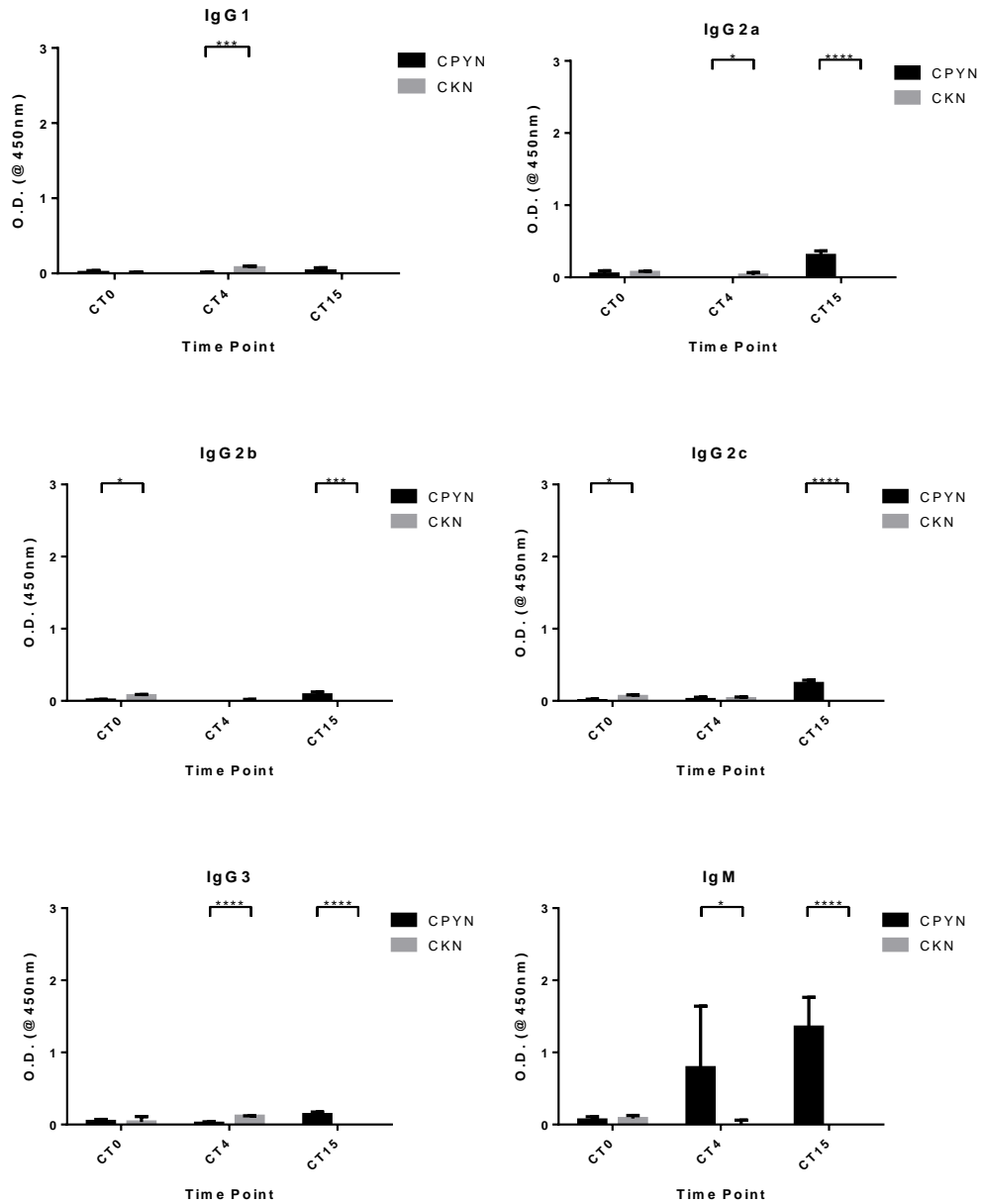


Figure 27 - Variation of immunoglobulin reactivity to *P. yoelii* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated nasally with *P. yoelii* (CPYN) and PBS (CKO). At CT15, only animals from group CPYN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

3.3.3.1.2. Reactivity to *P. yoelii* PEP45B1

Oral route (BALB/c)

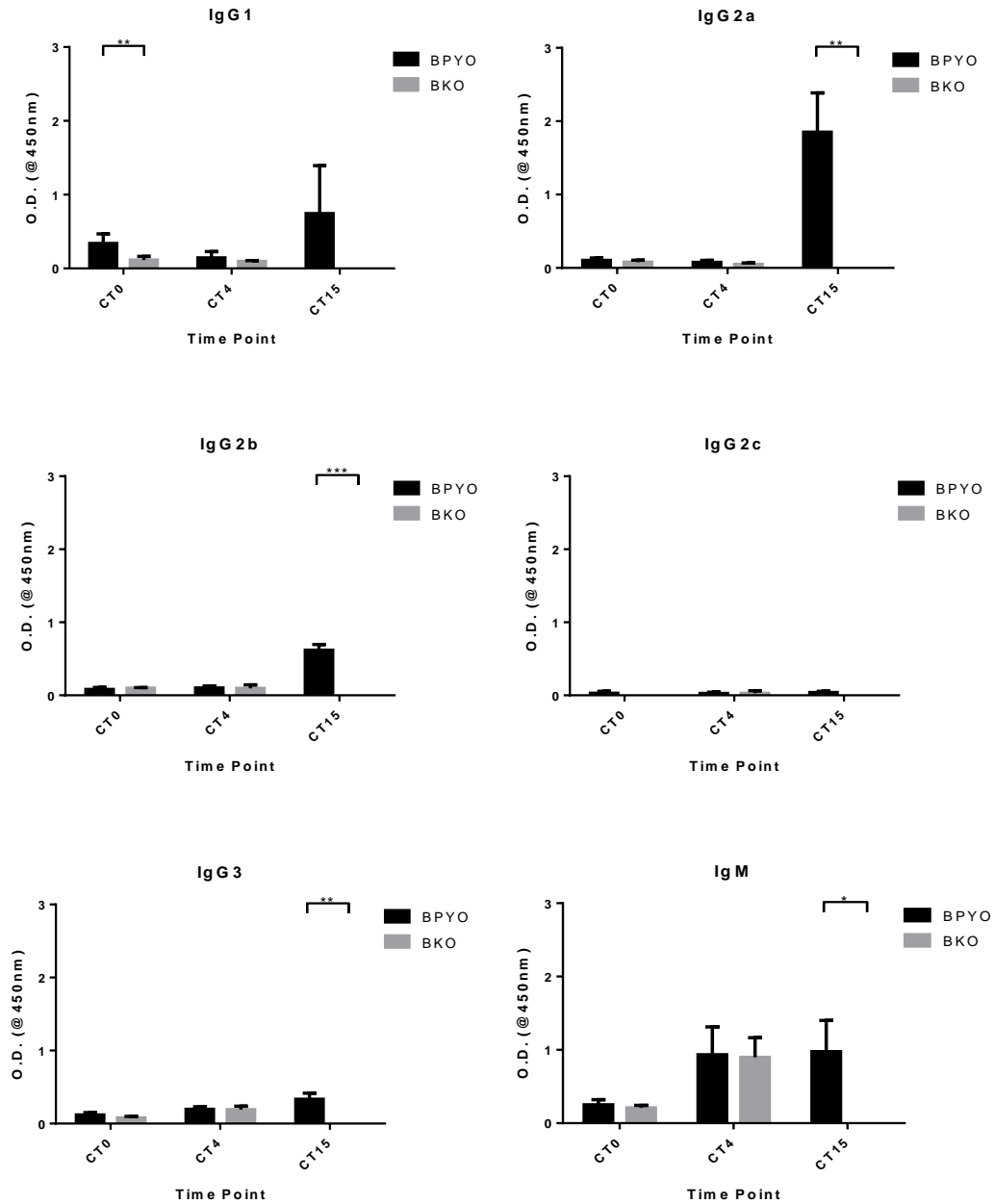


Figure 28 – Variation of immunoglobulins against *P. yoelii* peptide PEP45B1 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated orally with *P. yoelii* (BPYO) and PBS (BKO). At CT15, only animals from group BPYO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Nasal route (BALB/c)

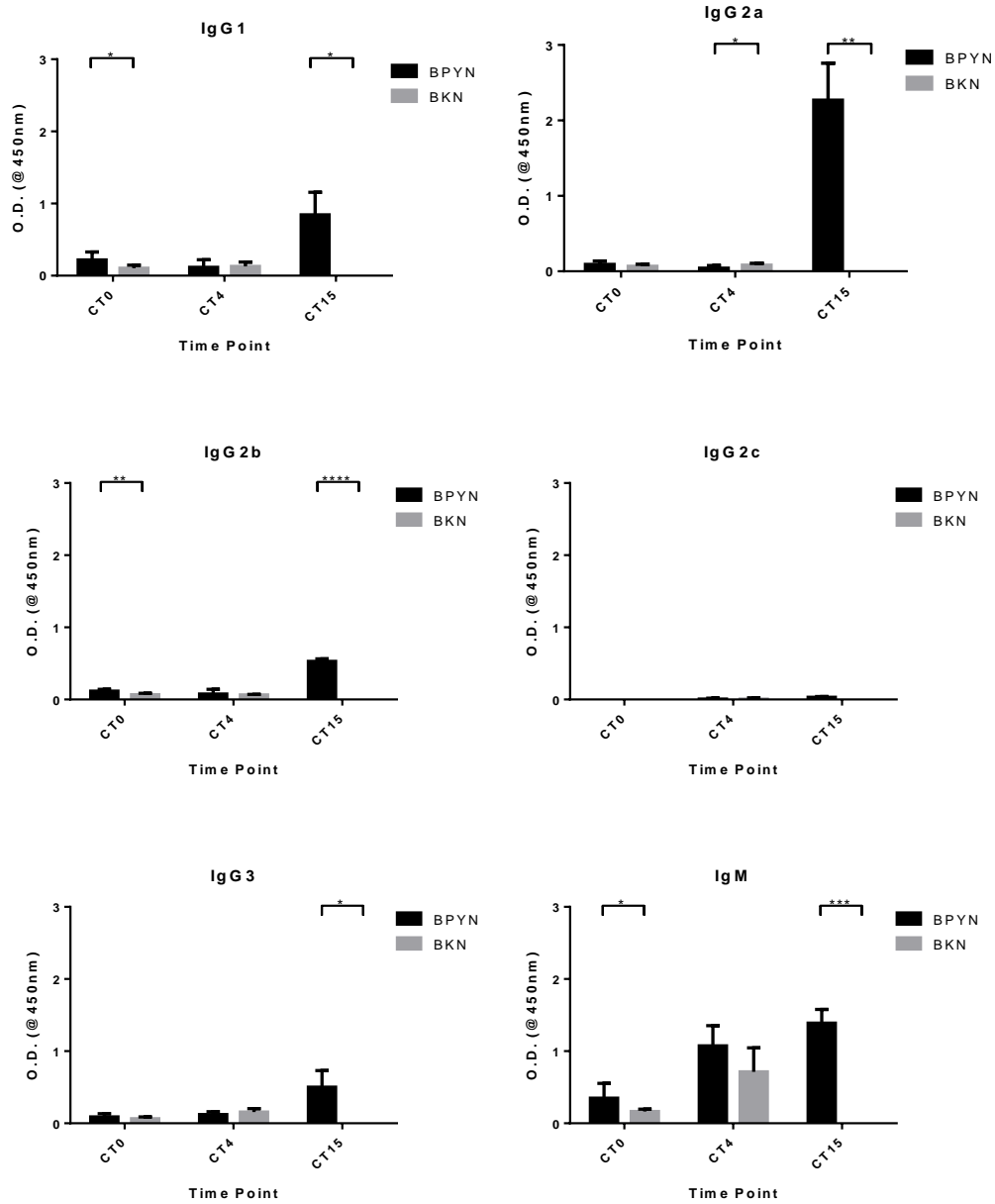


Figure 29 - Variation of immunoglobulins against *P. yoelii* peptide PEP45B1 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated nasally with *P. yoelii* (BPYN) and PBS (BKN). At CT15, only animals from group BPYN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Oral route (C57BL/6)

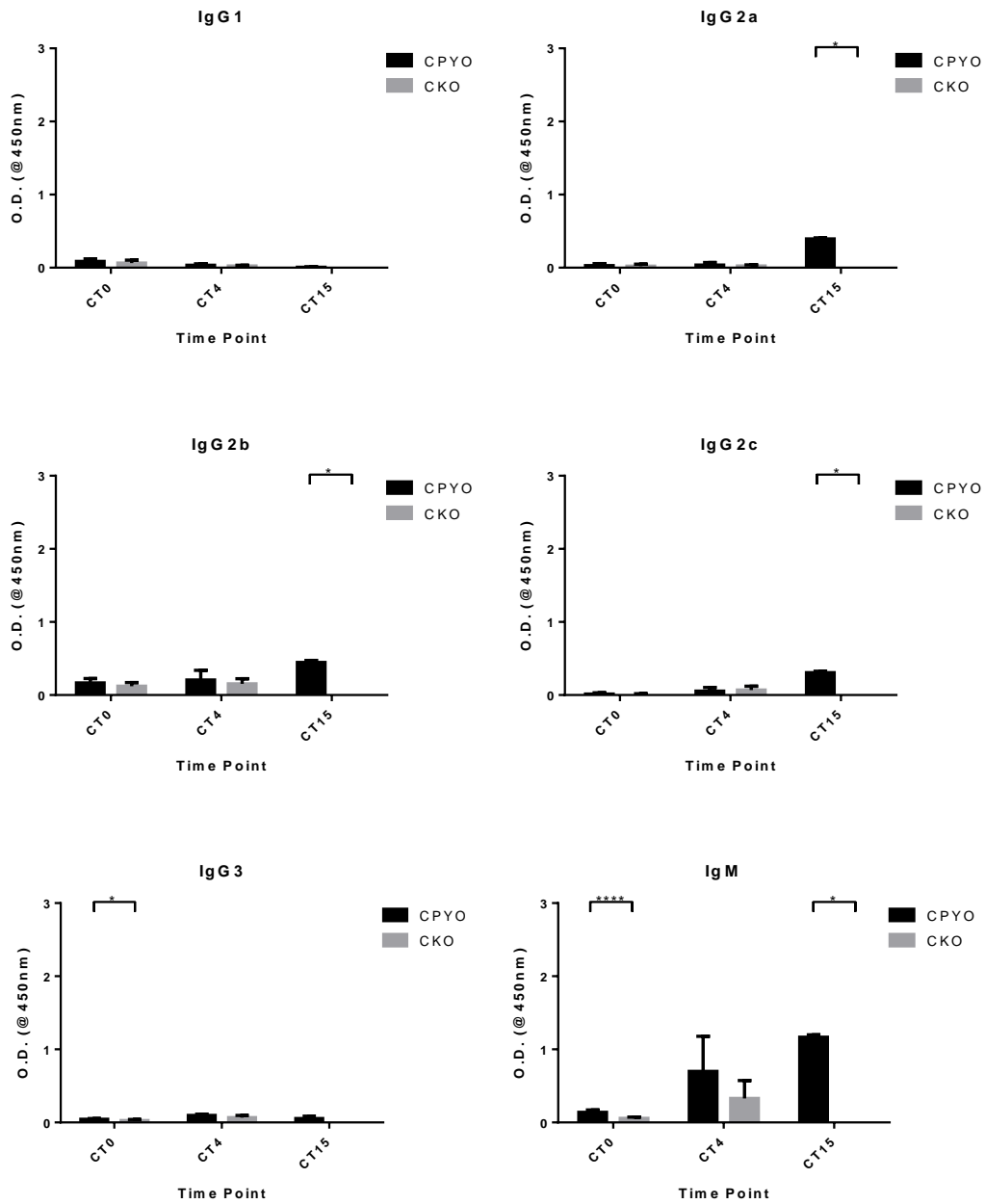


Figure 30 - Variation of immunoglobulins against *P. yoelii* peptide PEP45B1 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated orally with *P. yoelii* (CPYO) and PBS (CKO). At CT15, only animals from group CPYO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Nasal route (C57BL/6)

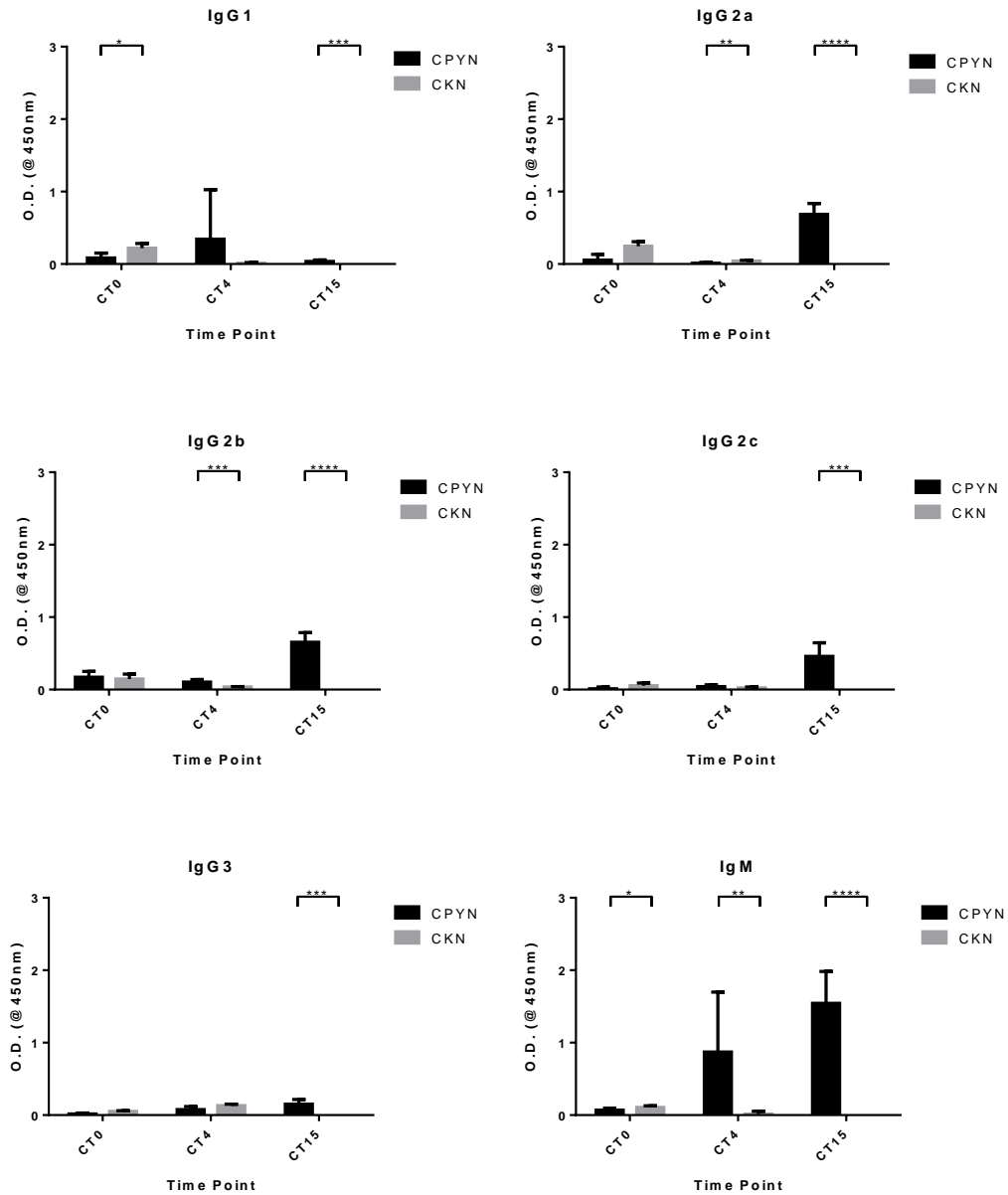


Figure 31 - Variation of immunoglobulins against *P. yoelii* peptide PEP45B1 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated nasally with *P. yoelii* (CPYN) and PBS (CKN). At CT15, only animals from group CPYN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

3.3.3.2. *Plasmodium berghei*

Again, ELISAs in which mice serum was tested for reactivity to *P. berghei* CE (crude extract), the overall O.D. (optical density) was very low. This was observed in both BALB/c and C57BL/6 mice strains and in both oral and nasal routes. The same was observed when serum was tested against *P. berghei*'s peptide PEP4AMA1.

As with *P. yoelii*, it was observed that IgM was slightly higher than all other immunoglobulins.

3.3.3.2.1. Reactivity to *P. berghei* crude extract

Oral route (BALB/c)

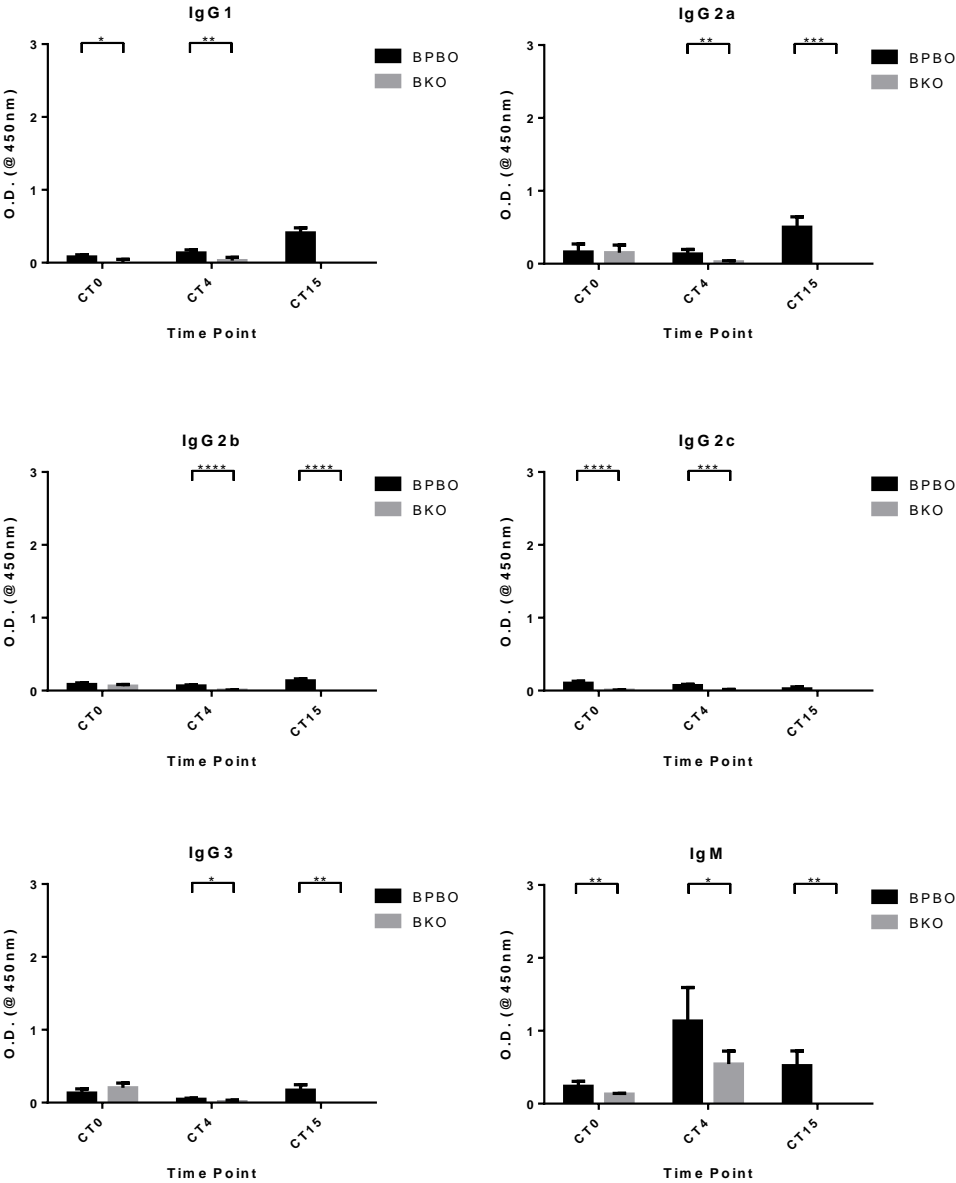


Figure 32 - Variation of immunoglobulins against *P. berghei* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated orally with *P. berghei* (BPBO) and PBS (BKO). Bars represent standard deviation and significance was determined using unpaired t test with Welch’s correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Nasal route (BALB/c)

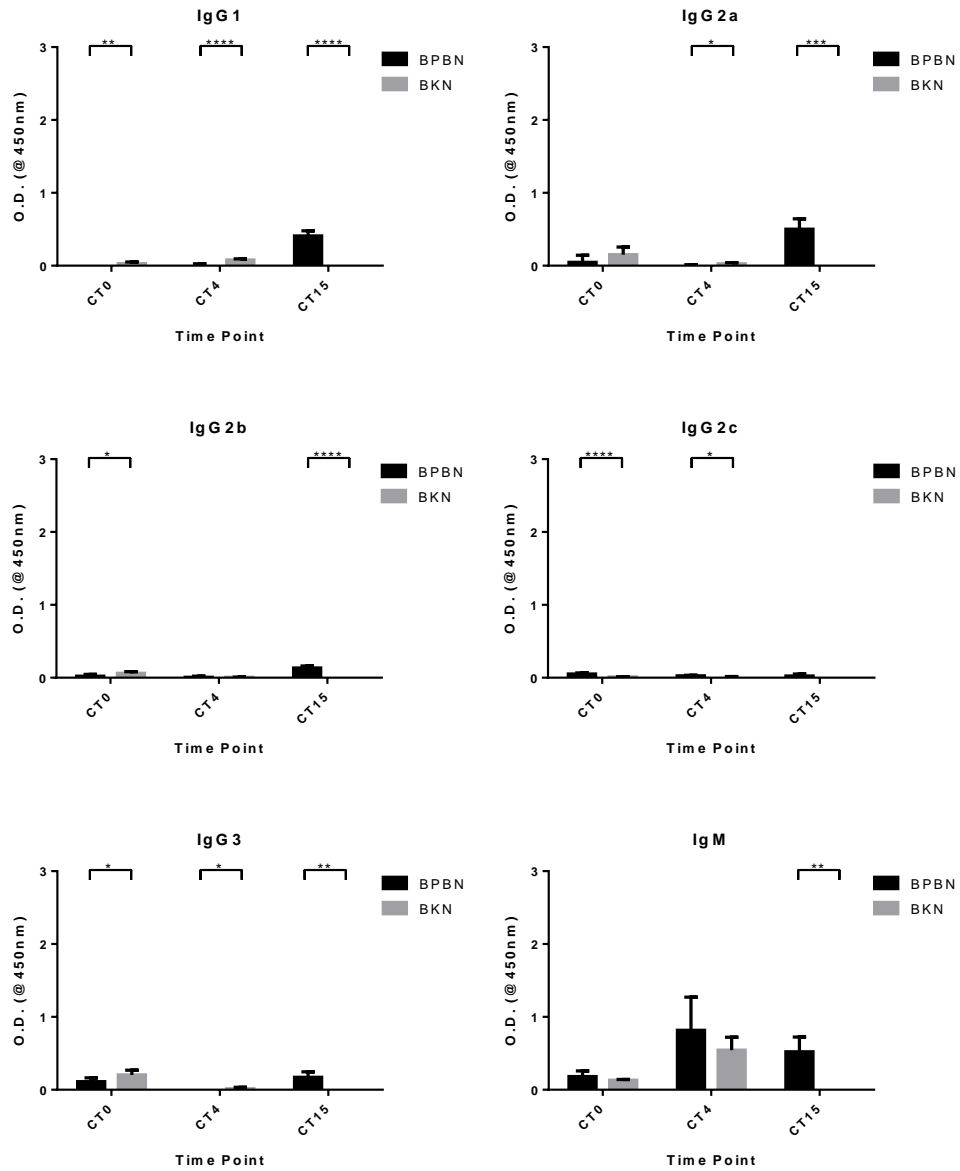


Figure 33 - Variation of immunoglobulins against *P. berghei* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated nasally with *P. berghei* (BPBN) and PBS (BKN). At CT15, only animals from group BPBN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001

Oral route (C57BL/6)

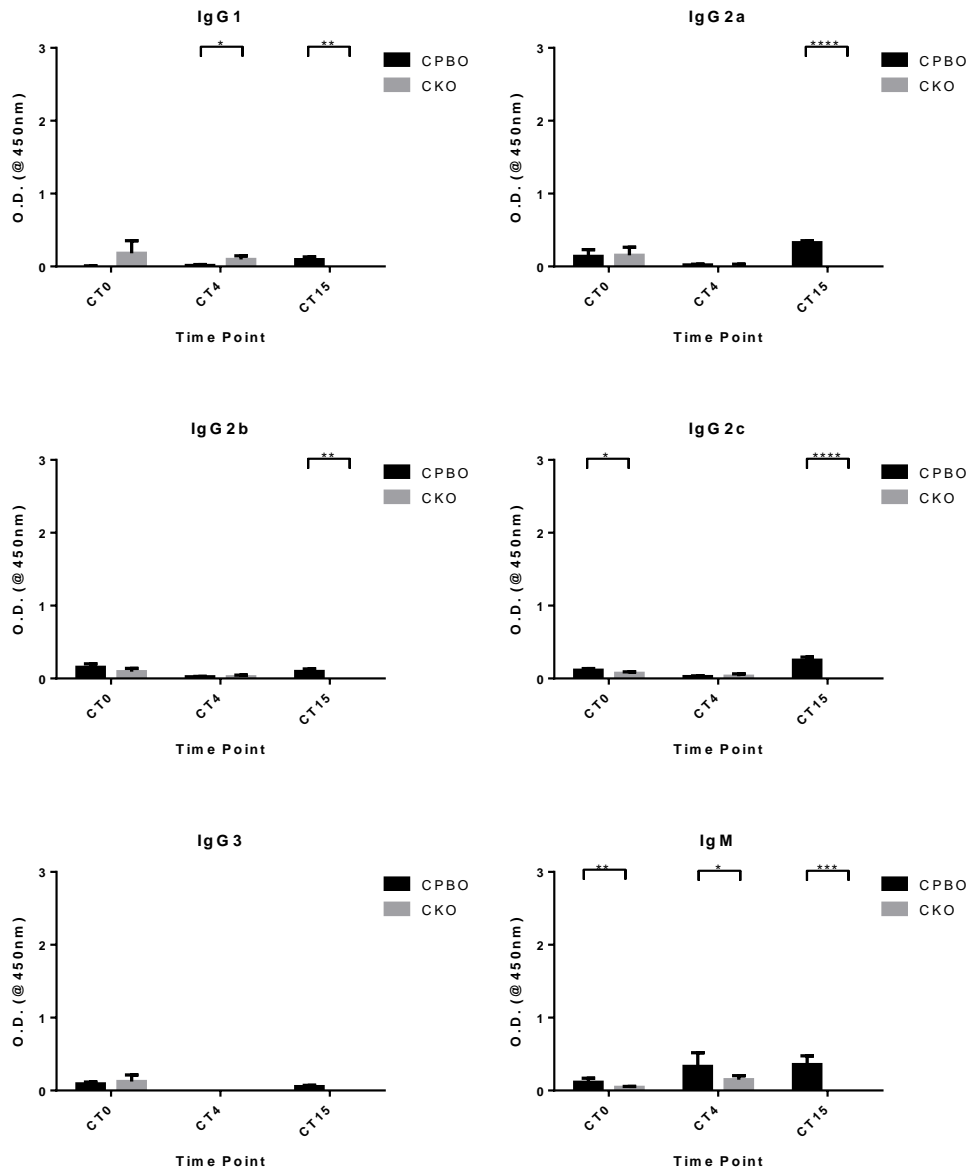


Figure 34 - Variation of immunoglobulins against *P. berghei* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated orally with *P. berghei* (CPBO) and PBS (CKO). At CT15, only animals from group CPBO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Nasal route (C57BL/6)

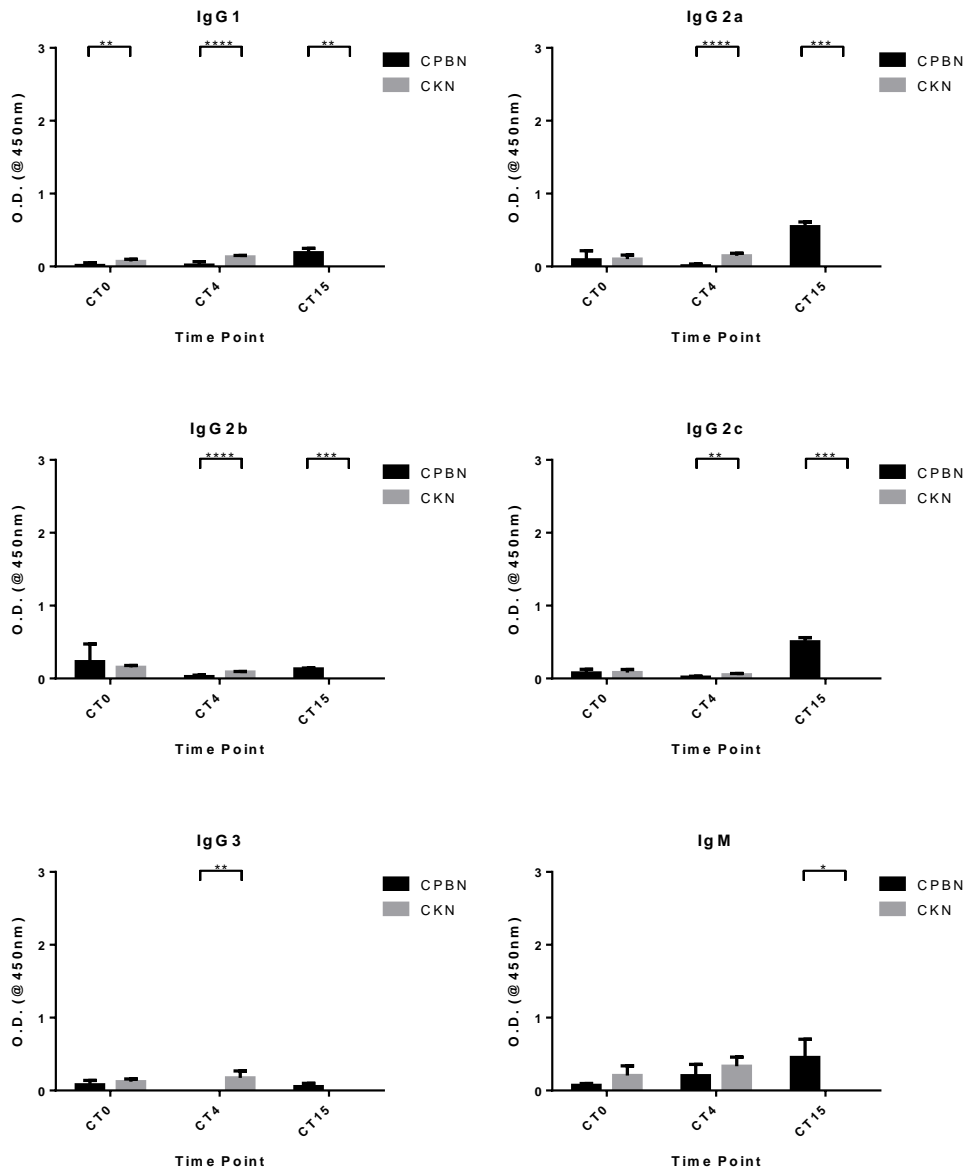


Figure 35 - Variation of immunoglobulins against *P. berghei* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated nasally with *P. berghei* (CPBN) and PBS (CKN). At CT15, only animals from group CPBN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

3.3.3.2.2. Reactivity to *P. berghei* PEPAMA4

Oral route (BALB/c)

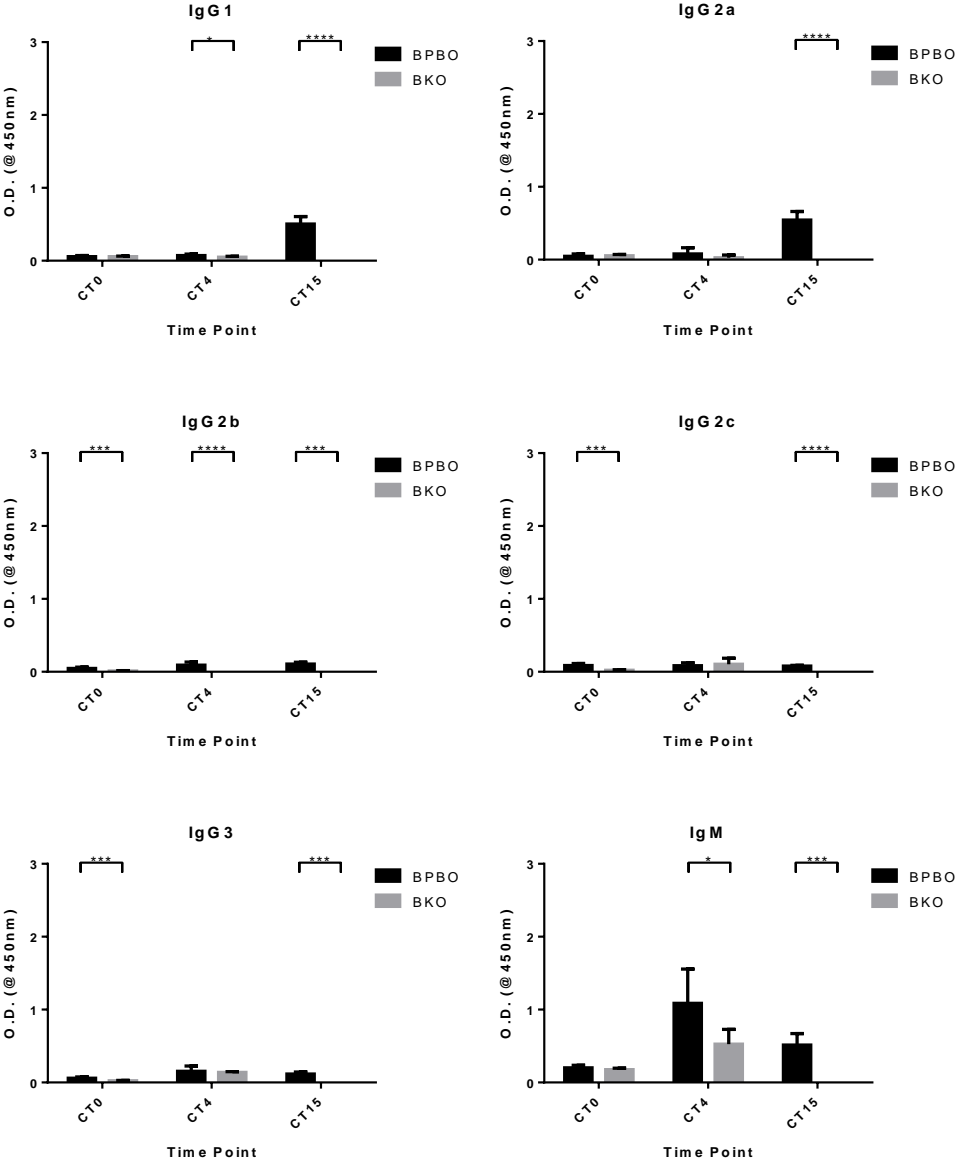


Figure 36 - Variation of immunoglobulins against *P. berghei* peptide PEPAMA4 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated orally with *P. berghei* (BPBO) and PBS (BKO). Bars represent standard deviation and significance was determined using unpaired t test with Welch’s correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Nasal route (BALB/c)

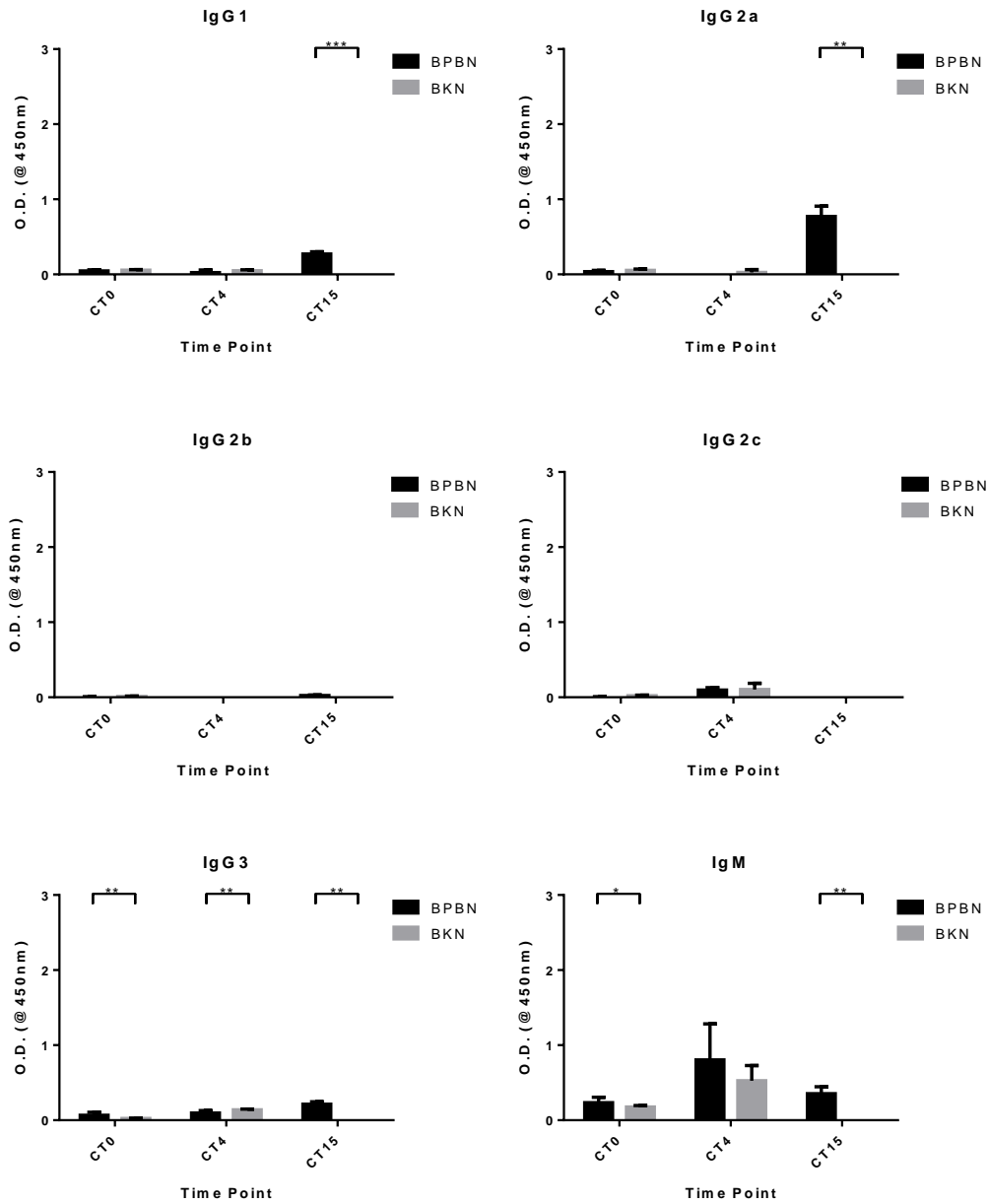


Figure 37 - Variation of immunoglobulins against *P. berghei* peptide PEPAMA4 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated nasally with *P. berghei* (BPBN) and PBS (BKN). At CT15, only animals from group BPBN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Oral route (C57BL/6)

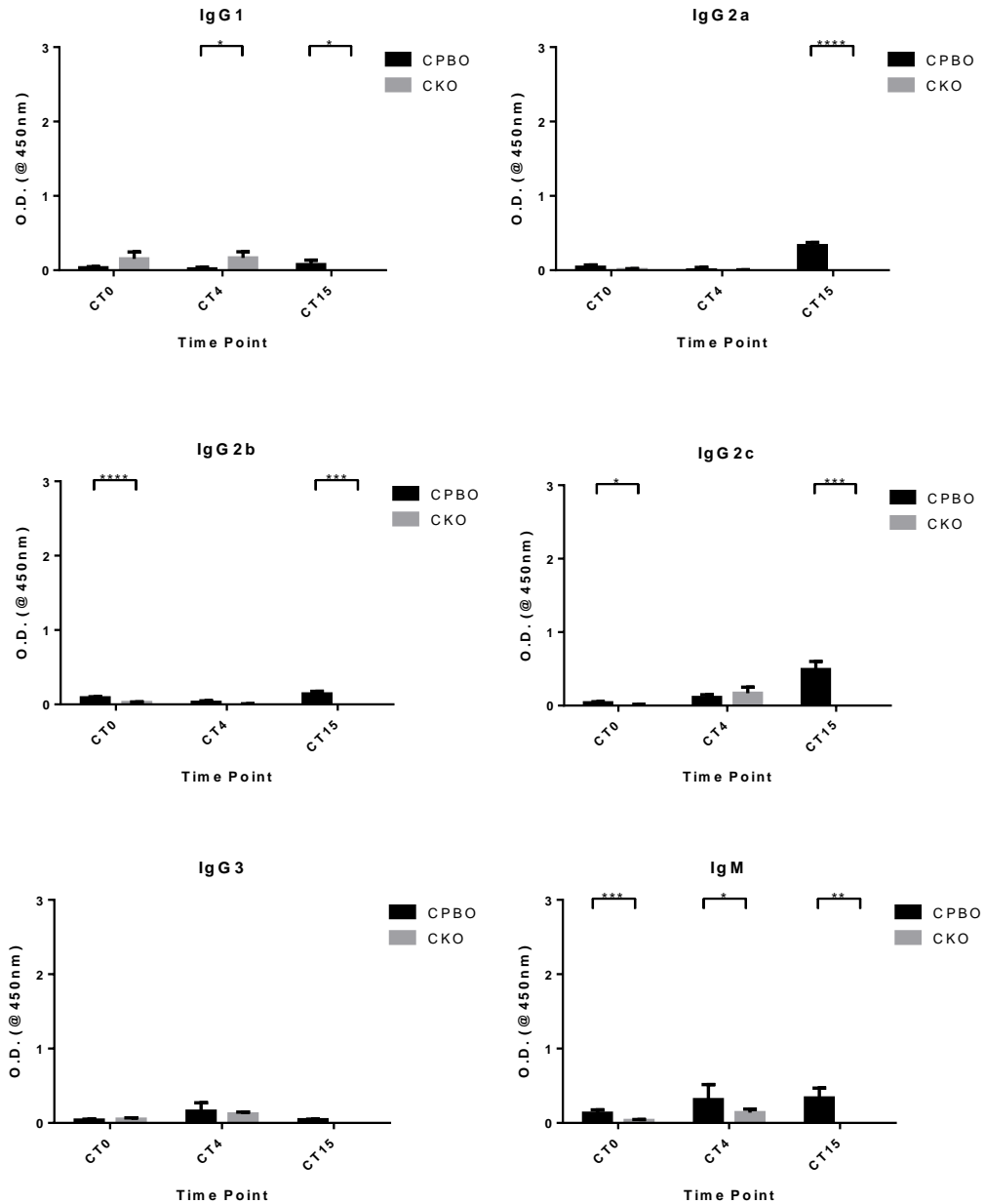


Figure 38 - Variation of immunoglobulins against *P. berghei* peptide PEPAMA4 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated orally with *P. berghei* (CPBO) and PBS (CKO). At CT15, only animals from group CPBO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; ** = P<0.01; *** = P<0.001.

Nasal route (C57BL/6)

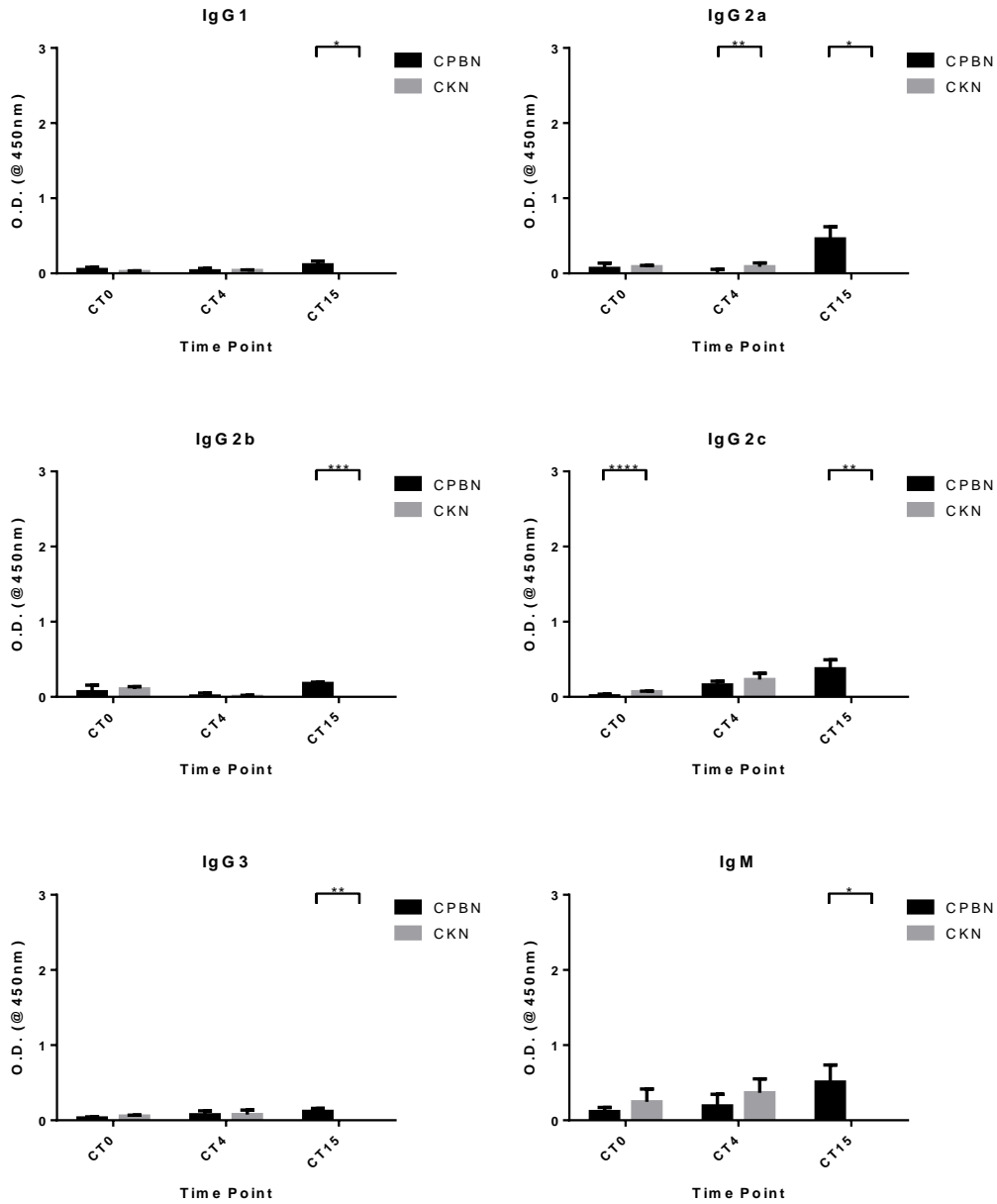


Figure 39 - Variation of immunoglobulins against *P. berghei* peptide PEPAMA4 across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated nasally with *P. berghei* (CPBN) and PBS (CKN). At CT15, only animals from group CPBN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

3.3.3.3. *Plasmodium falciparum*

Unlike *P. yoelii* and *P. berghei*, ELISA essays in which mice serum was tested for reactivity to *P. falciparum*, the O.D. was substantially high. However, this was observed in both control and treated groups, with the same proportion, which could indicate that this increase of O.D. in *P. falciparum* groups is associated to assay issues. This is further emphasised by comparing results of assays with crude extract against assays with peptide seen as immunoglobulin variation is very different across both assays. This was not seen in *P. yoelii* and *P. berghei*, where results were very similar between the two assays.

3.3.3.3.1. Reactivity to *P. falciparum* crude extract

Oral route (BALB/c)

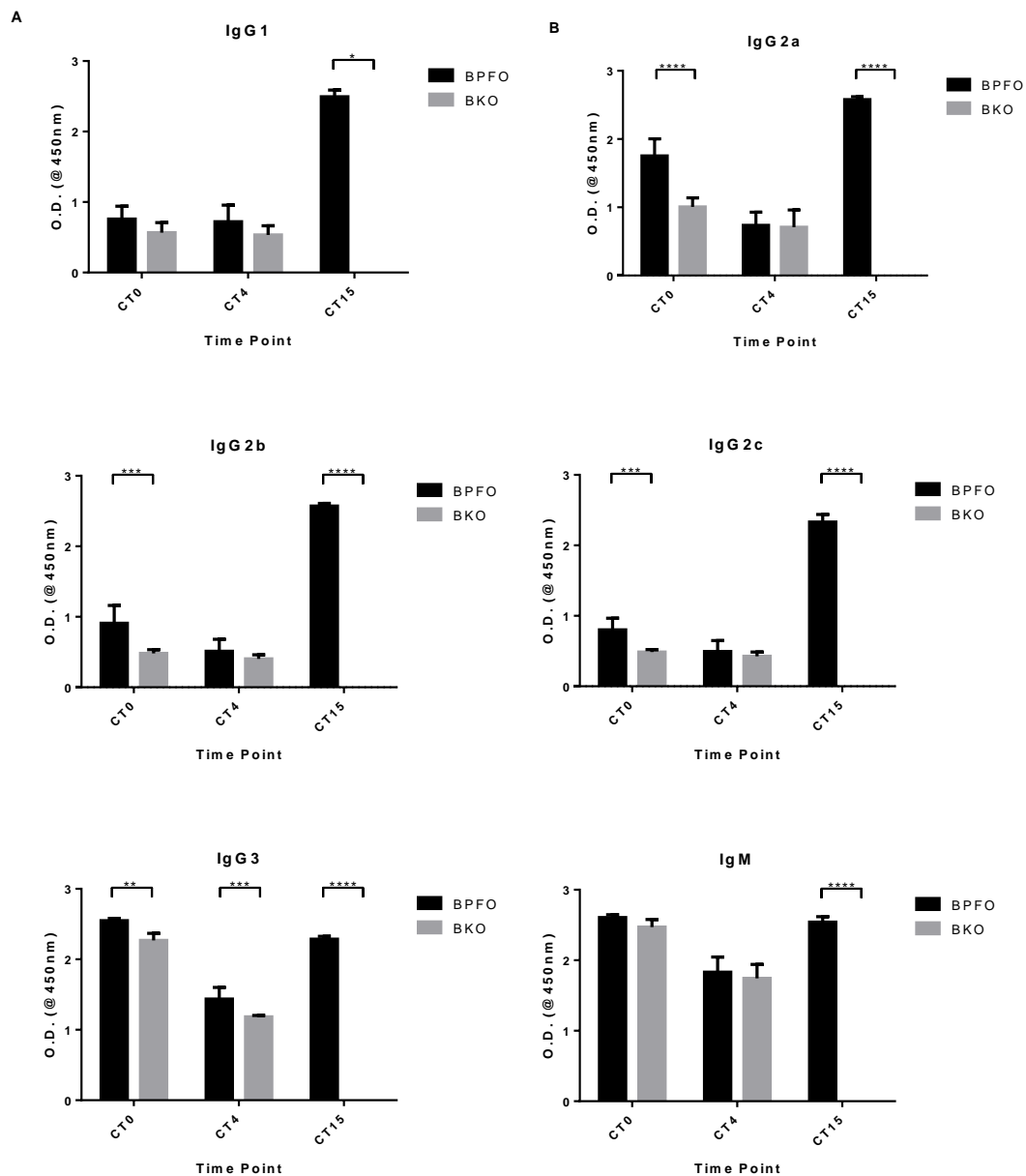


Figure 40 - Variation of immunoglobulins against *P. falciparum* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated orally with *P. falciparum* (BPFO) and PBS (BKO). Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; ** = P<0.01; *** = P<0.001

Nasal route (BALB/c)

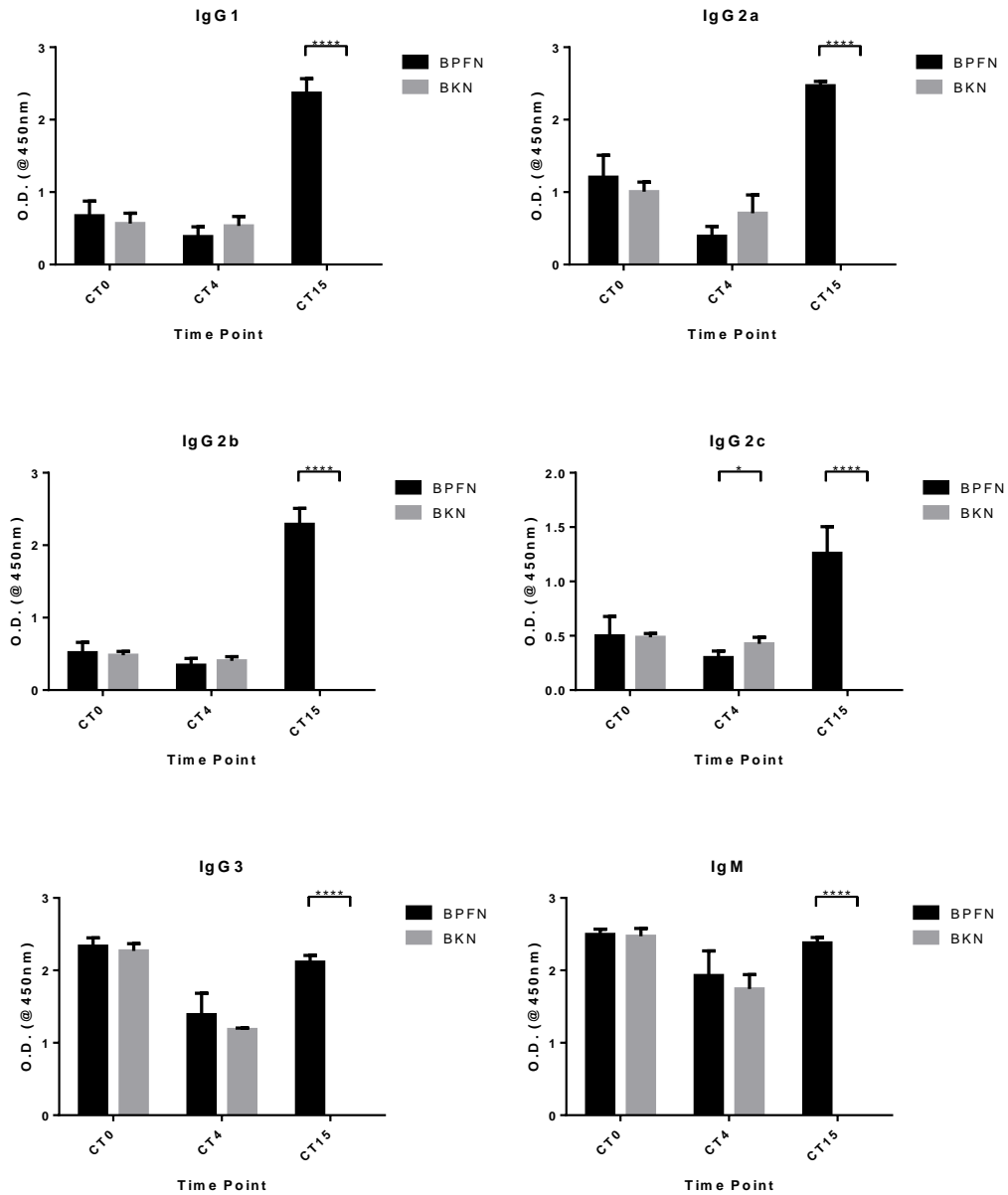


Figure 41 - Variation of immunoglobulins against *P. falciparum* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated nasally with *P. falciparum* (BPFN) and PBS (BKN). At CT15, only animals from group BPFN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Oral route (C57BL/6)

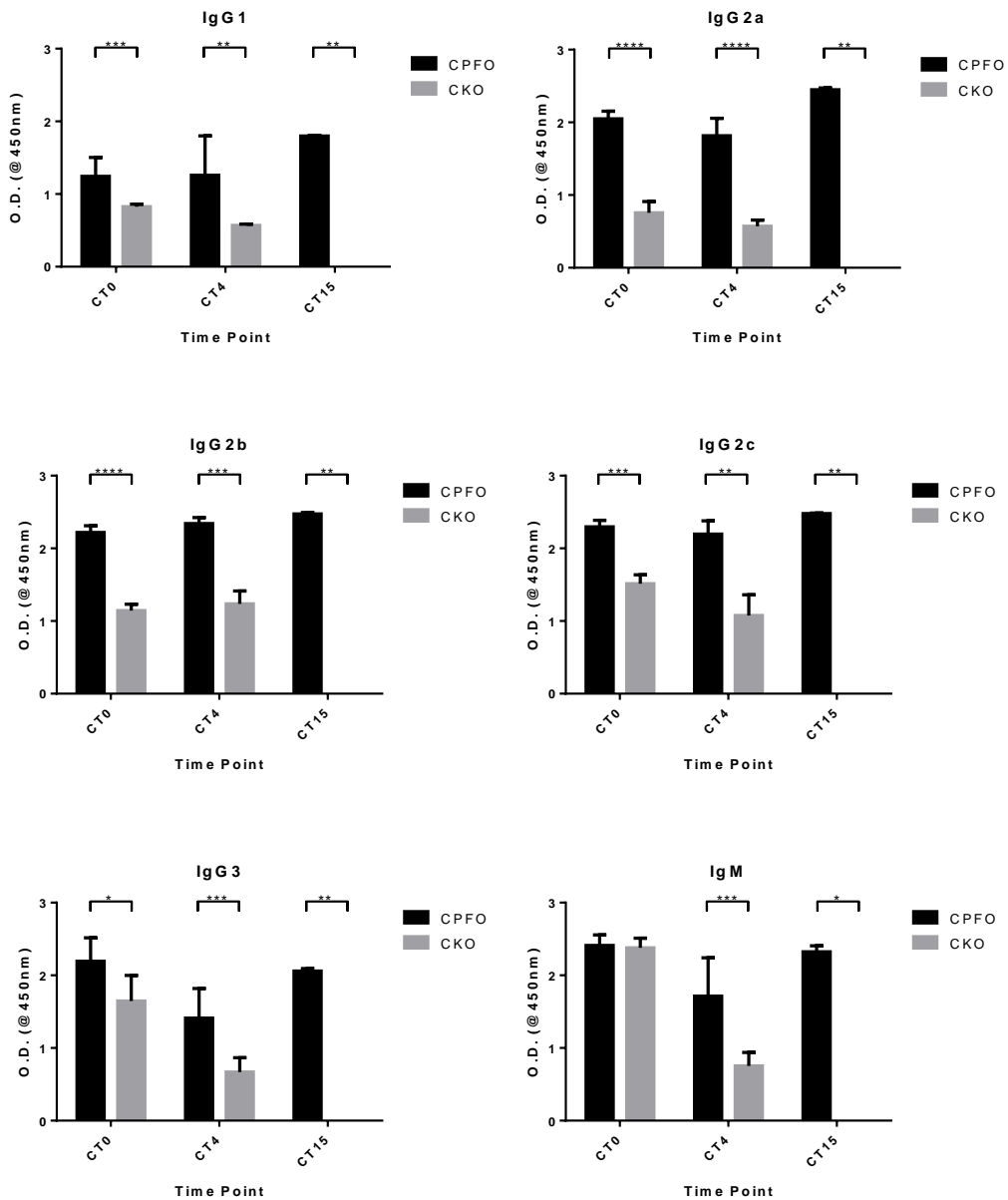


Figure 42 - Variation of immunoglobulins against *P. falciparum* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated orally with *P. falciparum* (CPFO) and PBS (CKO). At CT15, only animals from group CPFO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; ** = P<0.01; *** = P<0.001.

Nasal route (C57BL/6)

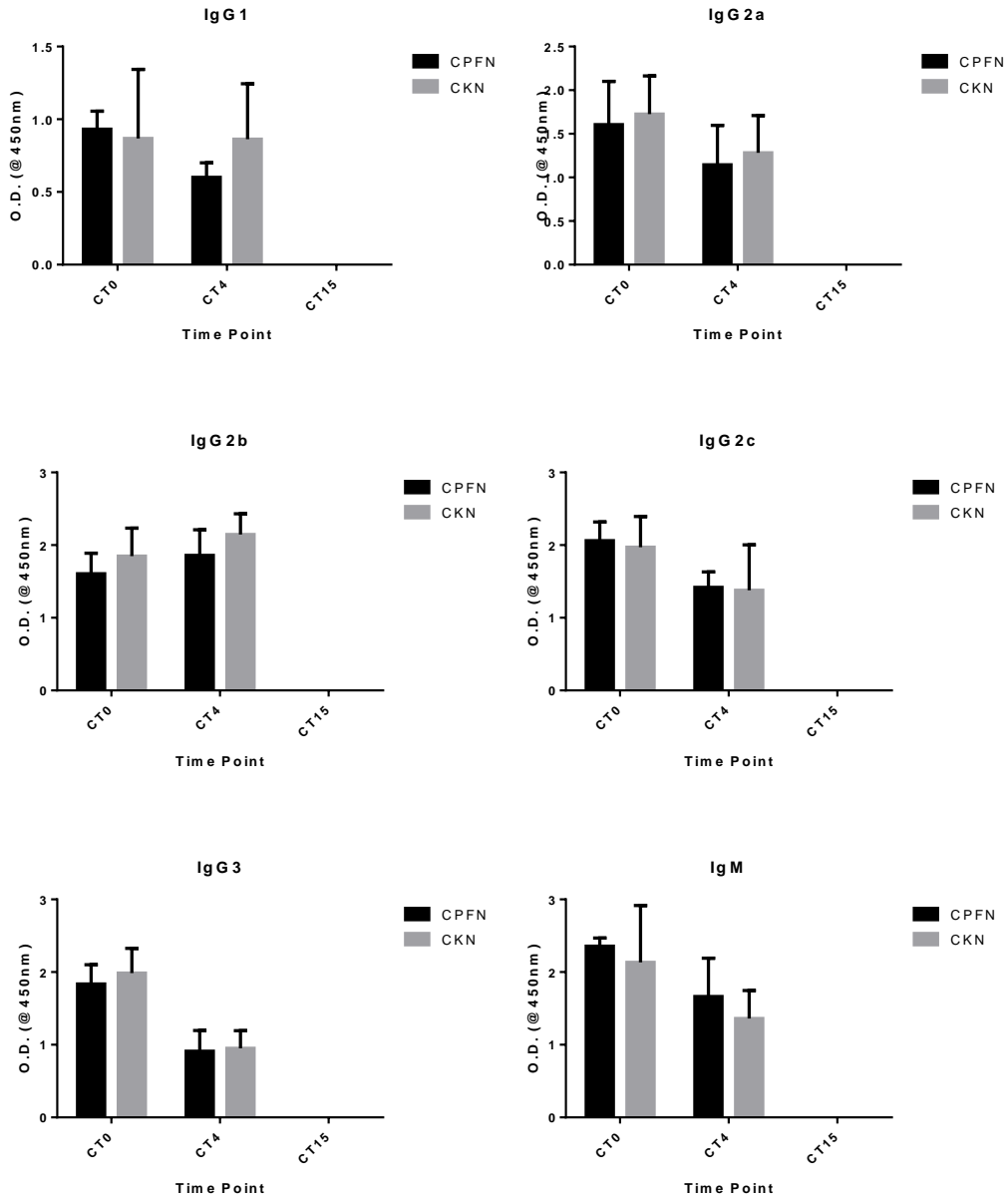


Figure 43 - Variation of immunoglobulins against *P. falciparum* crude extract across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated nasally with *P. falciparum* (CPFN) and PBS (CKN). At CT15 no animals remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

3.3.3.3.2. Reactivity to *P. falciparum* AMAPF

Oral route (BALB/c)

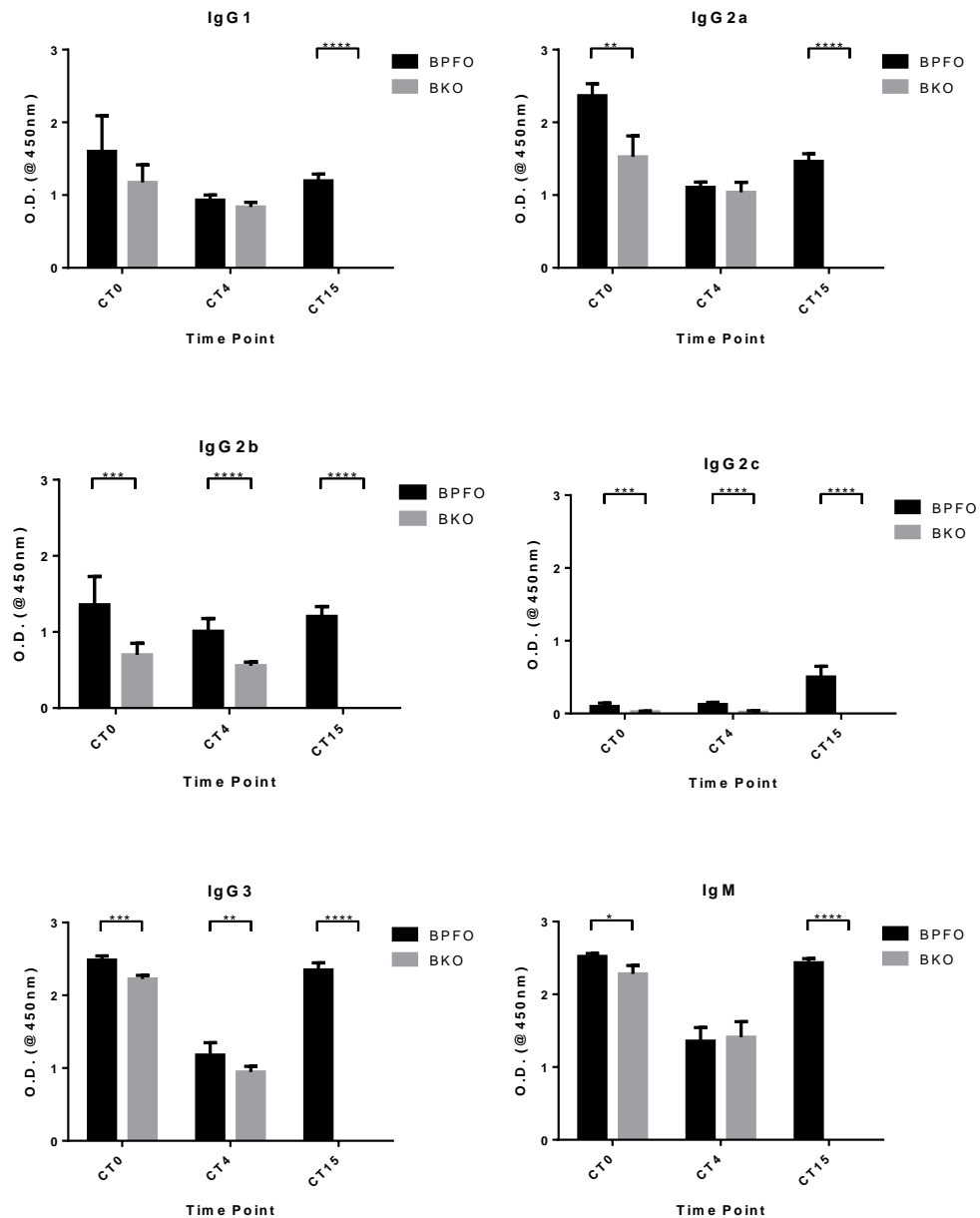


Figure 44 - Variation of immunoglobulins against *P. falciparum* peptide AMAPF across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated orally with *P. falciparum* (BPFO) and PBS (BKO). At CT15, only animals from group BPFO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; ** = P<0.01; *** = P<0.001.

Nasal route (BALB/c)

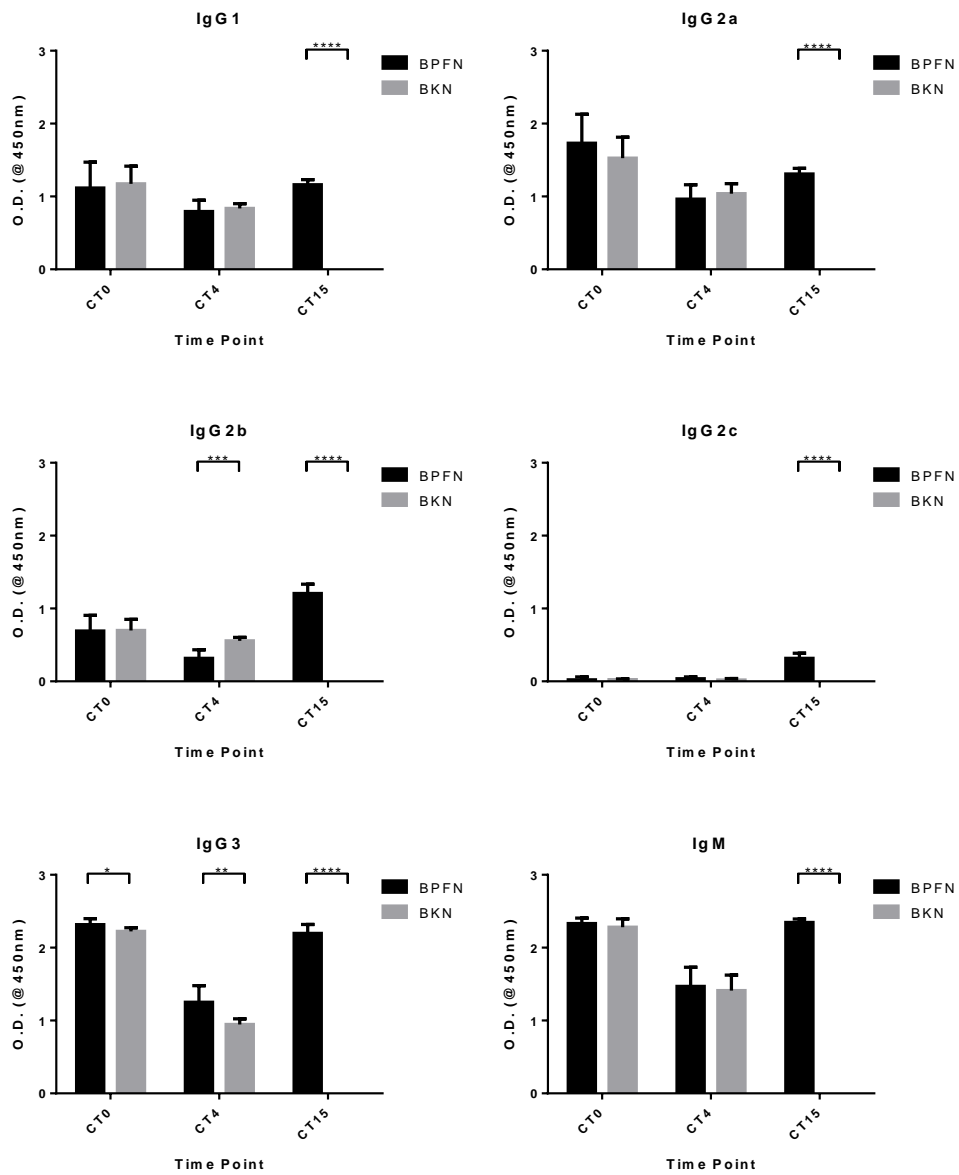


Figure 45 - Variation of immunoglobulins against *P. falciparum* peptide AMAPF across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of BALB/c mice inoculated nasally with *P. falciparum* (BPFN) and PBS (BKN). At CT15, only animals from group BPFN remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Oral route (C57BL/6)

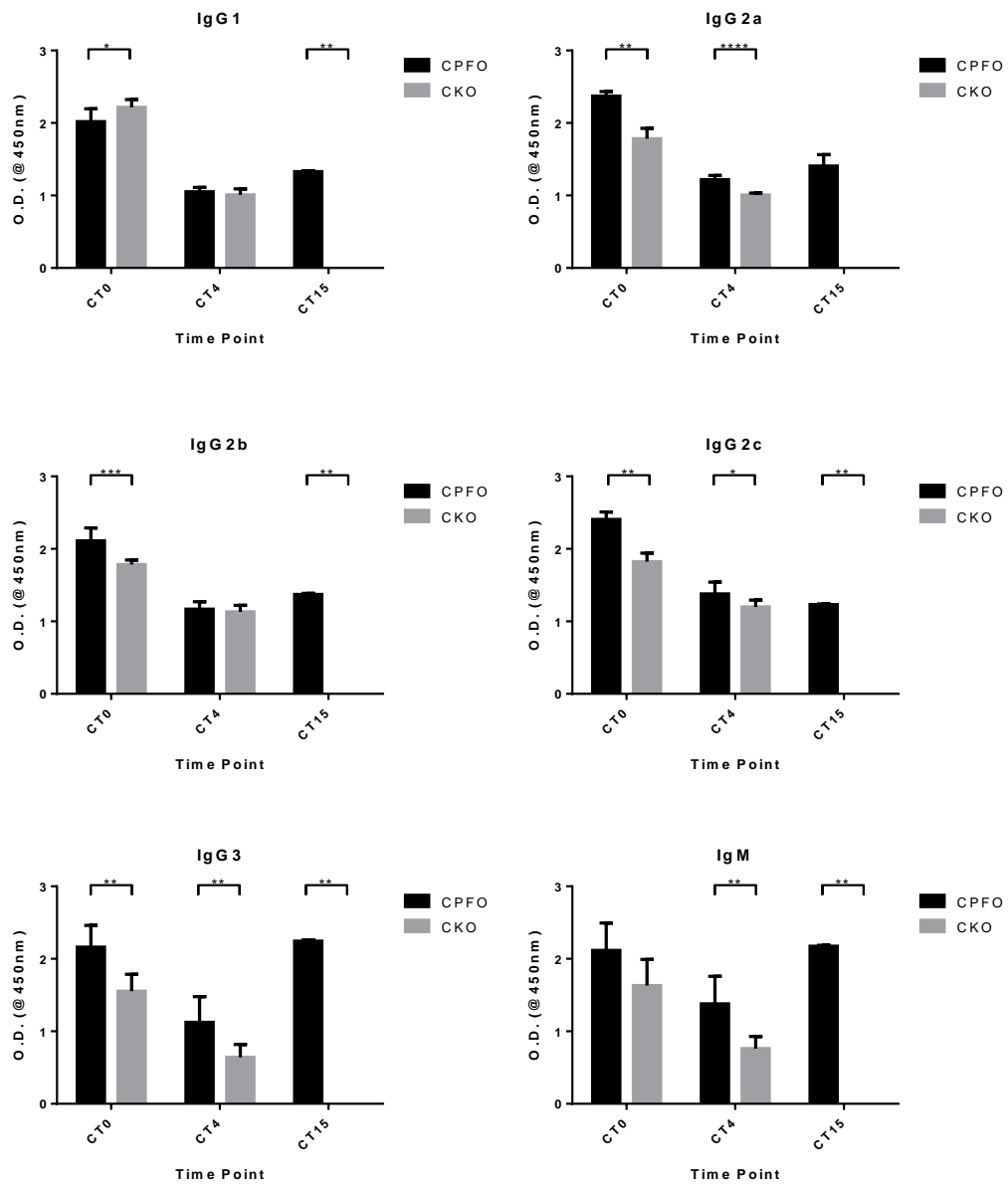


Figure 46 - Variation of immunoglobulins against *P. falciparum* peptide AMAPF across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated orally with *P. falciparum* (CPFO) and PBS (CKO). At CT15, only animals from group CPFO remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = P<0.05; **=P<0.01; ***=P<0.001.

Nasal route (C57BL/6)

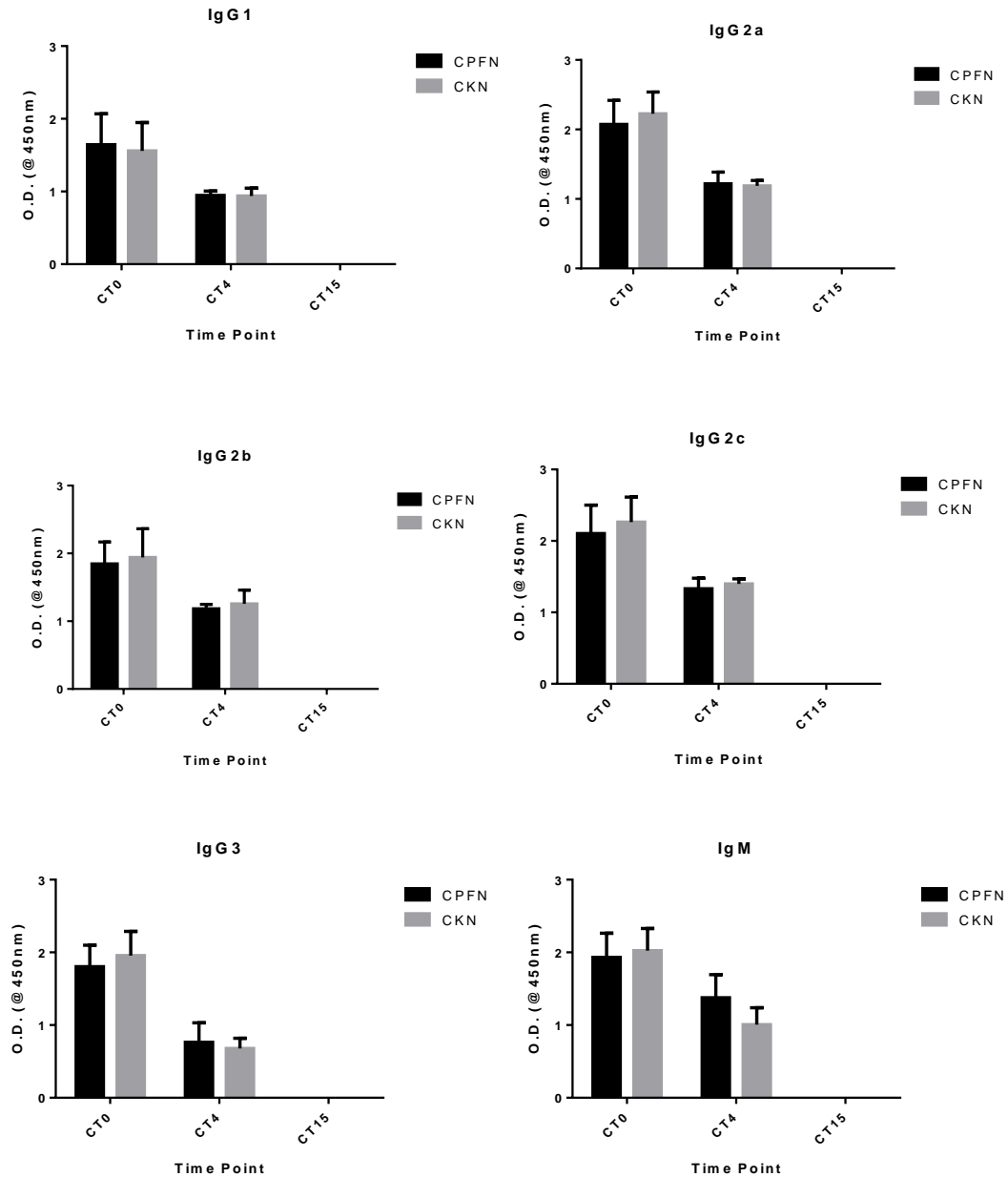


Figure 47 - Variation of immunoglobulins against *P. falciparum* peptide AMAPF across challenge days 0 (CT0), 4 (CT4) and 15 (CT15), in groups of C57BL/6 mice inoculated nasally with *P. falciparum* (CPFN) and PBS (CKN). At CT15 no animals remained. Bars represent standard deviation and significance was determined using unpaired t test with Welch's correction: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

3.3.4. qPCRs

In order to accurately track infections, qPCR assays were used for each of the following experimental time points: Inoculation (IT0), booster (BT0), challenge (CT0) and 4 days after challenge (CT4) (Table 9). The qPCR assays targeted the 18S rRNA conserved region found only in the genus *Plasmodium*. This meant that only infected animals would render positive results.

As expected all samples were negative for *Plasmodium spp.* at time points IT0, BT0 and CT0.

After challenge, on day 4 (CT4), it was noticeable and statistically significant that parasitemia was higher in the Oral group than in the Nasal group ($p < 0.0013$).

Table 9 – Averaged Cq values of qPCR on blood clots obtained from all subjects at inoculation (IT0), booster (BT0), challenge (CT0) and 4 days after challenge (CT4).

Route	Plasmodium	Mice Strain	IT0	BT0	CT0	CT4	
Oral	PY	BALB/c	N/A	N/A	N/A	17.57	
		C57BL/6	N/A	N/A	N/A	18.86	
	PB	BALB/c	N/A	N/A	N/A	19.31	
		C57BL/6	N/A	N/A	N/A	20.56	
	PF	BALB/c	N/A	N/A	N/A	20.15	
		C57BL/6	N/A	N/A	N/A	19.80	
	K	BALB/c	N/A	N/A	N/A	19.35	
		C57BL/6	N/A	N/A	N/A	19.34	
	Nasal	PY	BALB/c	N/A	N/A	N/A	21.60
			C57BL/6	N/A	N/A	N/A	20.68
PB		BALB/c	N/A	N/A	N/A	22.66	
		C57BL/6	N/A	N/A	N/A	21.03	
PF		BALB/c	N/A	N/A	N/A	20.80	
		C57BL/6	N/A	N/A	N/A	23.88	
K		BALB/c	N/A	N/A	N/A	19.95	
		C57BL/6	N/A	N/A	N/A	21.35	
Positive Controls		PY	-	19.41	19.66	19.79	24.15
		PB	-	21.32	21.81	22.18	24.61
	PF	-	16.20	15.59	16.54	17.72	

3.4. Discussion

The present study was aimed at exploring the capability of live blood stage *Plasmodium* parasites to infect and elicit immune responses via oral and nasal route.

3.4.1. Mice inoculated orally with live parasites can become infected

There have been several reports of oral transmission with different species of *Plasmodium* (Young 1941, Robinson and Comer 1968, Yoeli and Most 1971, Malagon, Castillo et al. 1993). A particular study by Malágon *et al.* (1993) reported that 30% of mice orally inoculated with *P. yoelii* infected blood developed and died of malaria. In experiments carried in the present work it was also observed that animals could become infected after oral inoculation with 10% of the oral group becoming lethally infected. These results are of extreme importance if oral, or even whole-live organism, applications are to be developed. Although the oral route is not *Plasmodium's* natural infection route, it seems it is still capable of causing infection when administered orally. Like other live vaccines, attenuation would be crucial if this approach was to be deployed as amongst other challenges that stand in the way of production of whole parasite blood stage malaria vaccines, underattenuation/reversion of attenuated parasites constitutes one of the most significant factors (Stanisic and Good 2015).

Nevertheless, in the current work it was also observed that mice inoculated nasally with “extracted” parasites not only did not become infected from the inoculum, but also presented a higher survival rate. To the date of writing, and to the authors knowledge, this is the first description of whole live parasite nasal inoculation of *Plasmodium* species and this new approach could potentially bypass the need for attenuation.

3.4.2. Inoculation Route

In most of the present experiments, and in particular the ones carried with *P. yoelii*, the factor that seemed to influence survival the most was the route of inoculation as survival was significantly increased in groups inoculated nasally. This difference could be associated with two factors: The first one being the fact that the nasal inoculum was composed of crude

parasites “extracted” from red blood cells (RBCs). The nasal inoculum was therefore mostly composed of “exposed” parasites, thus providing a much more direct and wider spectrum of antigens to be recognised by the immune system. The oral approach was meant to have the same effect as, although the oral inoculum contained infected RBCs (iRBCs), the red cell membrane was intended to serve as a natural delivery system to the gut, by protecting the parasites from the harsh conditions of the digestive system. The first argument that comes to mind for the lack of significant immune response to some of the oral inoculations would be the effect of gastric acid on parasites, since despite being protected with RBC’s could still have been affected. However, one of the first experiments on oral transmission of *Plasmodium* showed that gastric acid presented no detectable effect on the outcome of infection on oral transmission (Robinson and Comer 1968). Instead, and this takes us to factor two, it is likely that this could be related to the distance between inoculation and induction sites. During nasal inoculation, the inoculum was virtually deposited at the induction site, while oral inoculation required a lengthier route allowing for a higher rate of parasites to be degraded, resulting in a smaller percentage of inoculum reaching the gut-associated lymphoid tissue (GALT). Some effect was still observed in the survival rates of some of the orally inoculated groups, which could mean that in some cases, a sufficient amount of parasites did reach the GALT to elicit immunity.

Because this is the first report of live parasite inoculation via nasal route, it is vital that more research be undertaken, particularly with the regarding the use of adjuvants. Many studies have underlined the importance of adjuvant requirement in intranasal applications (Arakawa, Komesu et al. 2005, Bargieri, Rosa et al. 2007, Yoshida, Araki et al. 2010). It is very likely that a nasal live blood stage vaccine coupled with adjuvant will allow for the possibility to achieve full protective immunity.

3.4.3. Differences between mice strains and parasite species

Immunization with *P. falciparum* sporozoites had previously been proven to confer cross protection against *P. berghei* (Sina, do Rosario et al. 1993). In this work it was shown that the same can potentially also be achieved with blood stages via nasal route as BALB/c mice nasally inoculated with *P. falciparum* survived much longer than the control group after challenge

with *P. berghei*. The same was not observed in C57BL/6 mice, which showed no significant survival rate over the control group. BALB/c and C57BL/6 strains are known to exhibit specific Th2 and Th1 type immune responses, respectively (Watanabe, Numata et al. 2004). T cells from BALB/c mice tend to produce Th2 cytokine with low interferon γ (IFN- γ) and high interleukin 4 (IL-4), whilst those from C57BL/6 favour Th1 cytokine with high IFN- γ and low IL-4 (Watanabe, Numata et al. 2004). This outcome seems to be connected with natural killer T (NKT) cells that in C57BL/6 mice, during a natural infection of *P. berghei* ANKA, produce IFN- γ and promote pathology, while in BALB/c mice promote Th2 polarization and resistance to infection (Hansen, Siomos et al. 2003). High IFN- γ has been shown to mediate depletion of parasite-specific CD4⁺ T cells during infection, which ultimately affected the development of long-term immunity to malaria (Xu, Wipasa et al. 2002). Interestingly enough, the opposite trend was observed when using *P. berghei* for inoculation, where statistical significance was only observed in C57BL/6 groups. Following the same principal, this could mean that *P. berghei* inoculation elicits a Th1 immune response that seems to be less effective.

In groups inoculated with *P. yoelii*, mice strain appeared to not present any relevance towards survival. Instead, as previously mentioned, the inoculation route seemed to be the relevant factor. This points to different immunity pathways and could be the reason why there is no cross protection between *P.y* and *P.b*. (Sina, do Rosario et al. 1993). If *P. berghei* tends to produce a Th1 immune response, cross-protection with *P. yoelii* will not be likely unless a similar immune response is elicited by *P. yoelii*.

3.4.4. Low antibody production

In 1950, MacGregor and Cohen established that antibodies were crucial for antimalarial immunity (Cohen, Mc et al. 1961). In the 20 years that followed, the first whole parasite vaccines conducted with irradiated sporozoites lead to the identification of essential antigens such as the circumsporozoites protein (CSP) (Clyde 1975). The identification of CSP and many other antigens steered most efforts in the direction of subunit vaccines that resulted, however, in mixed results (Schwartz, Brown et al. 2012). While the importance of antibody responses in achieving protection is widely recognised, the role and importance of cellular mechanisms involved in acquisition of immunity is only now starting to be explored. In this

work, inoculation with whole live parasites did not result in significant antibody levels that could be correlated with extended subject survival. Nonetheless, inoculated groups did survive longer than the correspondent control groups, which could indicate a key role of cellular immunity in achieving protective immunity.

Work by Good et al. (2013) has shown that i.v. administration of attenuated parasites leads to parasite-specific CD4⁺ and CD8⁺ T cell responses, with protection mediated by CD4 with no parasite-specific antibodies being detected. These CD4⁺ T cells seem to be reactivated by different stages of *Plasmodium* promoting cell-mediated immune responses that prevent further parasite growth (Good, Reiman et al. 2013). These cell mediated mechanisms include macrophage activation, phagocytosis and release of inflammatory molecules (Stevenson and Zavala 2006). Although full protection was not achieved in the current work, inoculated groups did survive longer which could indicate that these mechanisms were at work controlling parasitemia levels.

3.4.5. Elevated levels of IgM

Across all inoculated groups, immunoglobulin M (IgM) rates were particularly elevated. The serum antibody IgM mediates the clearance of altered and apoptotic cells through both complement-dependent and independent mechanisms. Because IgM is particularly good at recognizing conserved structures and possesses a multimeric conformation, it is particularly equipped to agglutinate invading pathogens (Pleass, Moore et al. 2016). In addition, its poly-reactivity allows for binding of different structures of the same pathogen or multiple pathogens simultaneously, thus enhancing neutralisation (Ehrenstein and Notley 2010). IgM is also known to mediate protection against infection and recent research is starting to uncover its importance in controlling *Plasmodium spp.* (Pleass, Moore et al. 2016). Despite not much being currently known about the role of parasite-specific IgM in the immune response to malaria, data from the few studies available point towards a protective role of IgM by limiting parasite replication during infection (Couper, Phillips et al. 2005, Arama, Skinner et al. 2015, Pleass, Moore et al. 2016).

The elevated levels of IgM observed in the current work could explain why survival was seen but protection was not achieved. It is likely that IgM, along with mechanisms described above,

were limiting replication of parasites allowing for an extended survival of inoculated groups. In addition, since high levels were detected solely in inoculated subjects, it is possible that inoculation did elicit parasite-specific IgM memory B cells.

3.4.6. Nasal route: the backdoor to Tregs

Regulatory T cells are a subpopulation of T cells that mediate immunological tolerance by suppression or downregulation of induction and proliferation of other effector cells (Corthay 2009). When activated, produce high levels of tolerogenic cytokines (TGF- β and IL-10) and interact with other lymphocyte subsets such as lymph node (LN) B cells, Dendritic cells (DCs) and natural killer (NK) T cells triggering TGF- β and IL-10 secretion and further promoting immunosuppressive cascades (Guilbride, Guilbride et al. 2012). In the skin matrix there are multiple powerful immunosuppressive mechanisms that are generally meant to prevent allergic overreactions and autoimmunity (Tomura, Honda et al. 2010). These mechanisms however, have been suggested to affect the efficacy of malaria vaccines (Guilbride, Gawlinski et al. 2010, Guilbride, Guilbride et al. 2012). Human trials with malaria vaccines that showed fully protective results involved either avoidance or blockage of potential Treg induction in the skin triggered by the parasite during inoculation (Guilbride, Gawlinski et al. 2010). Therefore, Tregs can negatively affect immunity to malaria limiting the generation of immune memory and controlling immunopathology and disease severity (Walther, Tongren et al. 2005, Todryk, Bejon et al. 2008, Clemente, Caporale et al. 2011). So, what if it was possible to bypass Treg activation? Would this promote a long lasting immunity to malaria?

A particular experiment by Costalonga *et al.* (2008) with commensal intranasal bacterial demonstrated that after intranasal inoculation of mice, antigens are locally processed by CD11c⁺ DCs known play a key role in regulating immune responses. The processed antigens are then presented to specific T cell that after being primed divide locally and in the lymph nodes followed by migration to the spleen to act as central memory T cells. Unexpectedly, these T cells are not regulatory T cells (Tregs) seen as when restimulation occurs, they release proinflammatory rather than immunosuppressive cytokines. These findings are particularly surprising seen as commensal organisms are hypothesised to induce immunosuppressive and regulatory cytokines (Costalonga, Cleary et al. 2008). This new evidence is vital for malarial

vaccine applications as activation of Tregs seems to be correlated with failure of whole parasite inoculation through the skin (Guilbride, Guilbride et al. 2012).

Interestingly, work by Nacer *et al.* (2013) has shown that nasal inoculation with flagellin modified circumsporozoite (CS) increases the number of CD11c⁺ DCs in the NALT leading to protective immunity against sporozoites (Nacer, Carapau et al. 2014). This proves that the intranasal route could indeed be the gateway for an effective and protective malaria vaccine.

3.5. Conclusion

In this study it was shown that oral inoculation of live parasites can lead to lethal infections, unlike with nasal inoculation where none of the subjects became infected in addition to showing longer overall survival. It was also hypothesised that different parasite species seem to trigger different immune responses, which was particularly evident with *P. berghei* and *P. falciparum*, each being more biased towards Th1 and Th2 immune responses, respectively. Despite different cellular responses, all groups showed elevated IgM concentrations. Because IgM has recently been associated with successful parasite clearance, these results show that inoculation can potentially elicit parasite-specific IgM memory B cells leading to parasite control.

This work provides evidence that nasal inoculation with live parasites has the potential to become an effective needle free vaccine. However, further research on adjuvant options is crucial, as including an effective adjuvant to this approach could present a robust method of eliciting protective immunity.

3.6. References

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4.

Plasmodium Mutants:
Creating Immunogenic parasites with
CRISPR/Cas9

4.1. Introduction

4.1.1. Creating an immunogenic parasite for Oral administration

Much about the urgency of creating an effective malaria vaccine has been covered in Chapter 1 and 3 so in this chapter the main focus will be the approach selected and the reasoning behind it.

Despite the considerable efforts made to develop oral vaccines against mucosal pathogens, little progress has been made in oral vaccines against non-mucosal infectious agents such as malaria (Wang, Webster et al. 2004). A few reports on subunit vaccines against malaria can be found (Wang, Kedzierski et al. 2003, Wang, Goschnick et al. 2004) but the recurrent drawback continues to be their inability to elicit a strong immune response (Halbroth and Draper 2015). The favourite approach for oral vaccination is the use of live attenuated or killed microorganisms that can resist intestinal degradation by either replicating in the gut or by having digestion-resistant structures (Silin, Lyubomska et al. 2007). In addition, vectors such as salmonella have the added capability of invading M cells in the intestinal induction sites, making them excellent vaccine delivery systems (Jepson and Clark 2001).

Although, the molecular mechanisms behind antigen uptake by M cells are largely unknown, glycoprotein 2 (GP2), which is exclusively expressed in the apical plasma membrane of M cells, has been proven to serve as transcytotic receptor for mucosal antigens (Hase, Kawano et al. 2009). This protein has been shown to selectively binds to *Salmonella enterica* by recognising FimH⁺, a component of type I pili on the bacterial outer membrane. Because previous studies have demonstrated the importance of *Salmonella spp.* translocation into Peyer's patches for initiation of antigen specific responses (Martinoli, Chiavelli et al. 2007, Hashizume, Togawa et al. 2008), FimH⁺ could be the key to unlock targeted vaccine delivery via M cells.

With this in mind, it was proposed that, using the CRISPR/Cas9 system, a mutant *Plasmodium falciparum* strain would be created expressing *Salmonella's* extracellular domain of FimH⁺. Once in the intestinal tract, this would allow for the parasite to be necessarily recognized and transcytosed into the GALT. In order to achieve this, the extracellular domain of *Plasmodium's*

AMA1 (Apical Membrane Antigen 1) would be replaced by *Salmonella's* FimH⁺ with the aid of the much acclaimed CRISPR/Cas9 system. AMA1 was selected since, in addition to being one of *Plasmodium's* protein to have been most extensively studied, it has also been reported to be dispensable in during parasite invasion (Bargieri, Andenmatten et al. 2013). This last factor allows for the protein to be modified or replaced without affecting the parasite's fitness.

4.1.2. A double guide CRISPR/Cas9 system

The current CRISPR/Cas9 method (**Figure 48**) allows for a single cleavage of the DNA, sequence which is then either: repaired by non-homologous end joining creating deletions or insertions (**Figure 48a**), or by homologous recombination provided that a donor sequence is present (**Figure 48b**).

With this work, the development of a two cleavage system by addition of a second gRNA was proposed (**Figure 49**). This system aimed to create a double cleavage system (**Figure 49a**), which by providing a donor plasmid with a sequence of interest (SOI) (**Figure 49b**) allows for deletion and/or insertions of significantly bigger sequences (**Figure 49c**).

As for its application in the current work (**Figure 50**), two specific guide sequences present in AMA1 were selected as to allow cleavage of the AMA1 gene (**Figure 50a**). A plasmid containing the FimH⁺ sequence flanked by AMA1 homology regions (**Figure 50b**) provided the donor DNA needed for homologous recombination and generation of the mutant gene (**Figure 50c**).

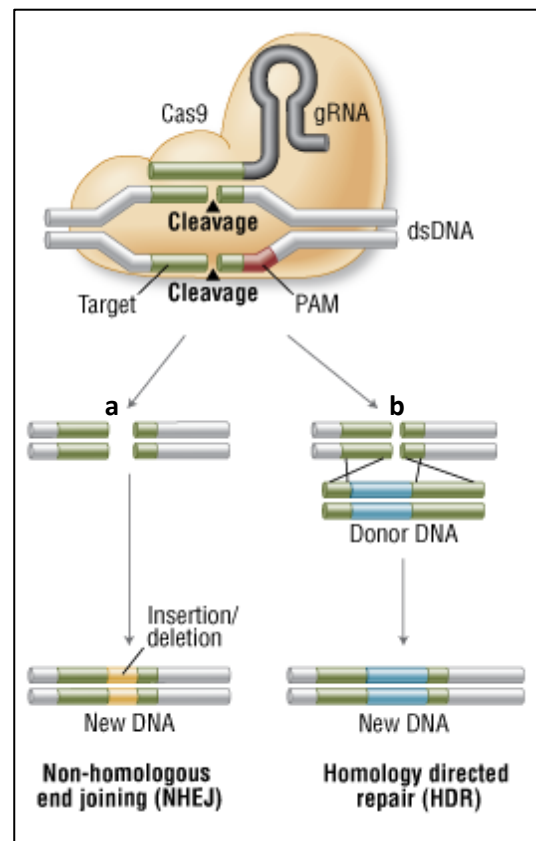


Figure 48 – Genome engineering with CRISPR/Cas 9 nuclease

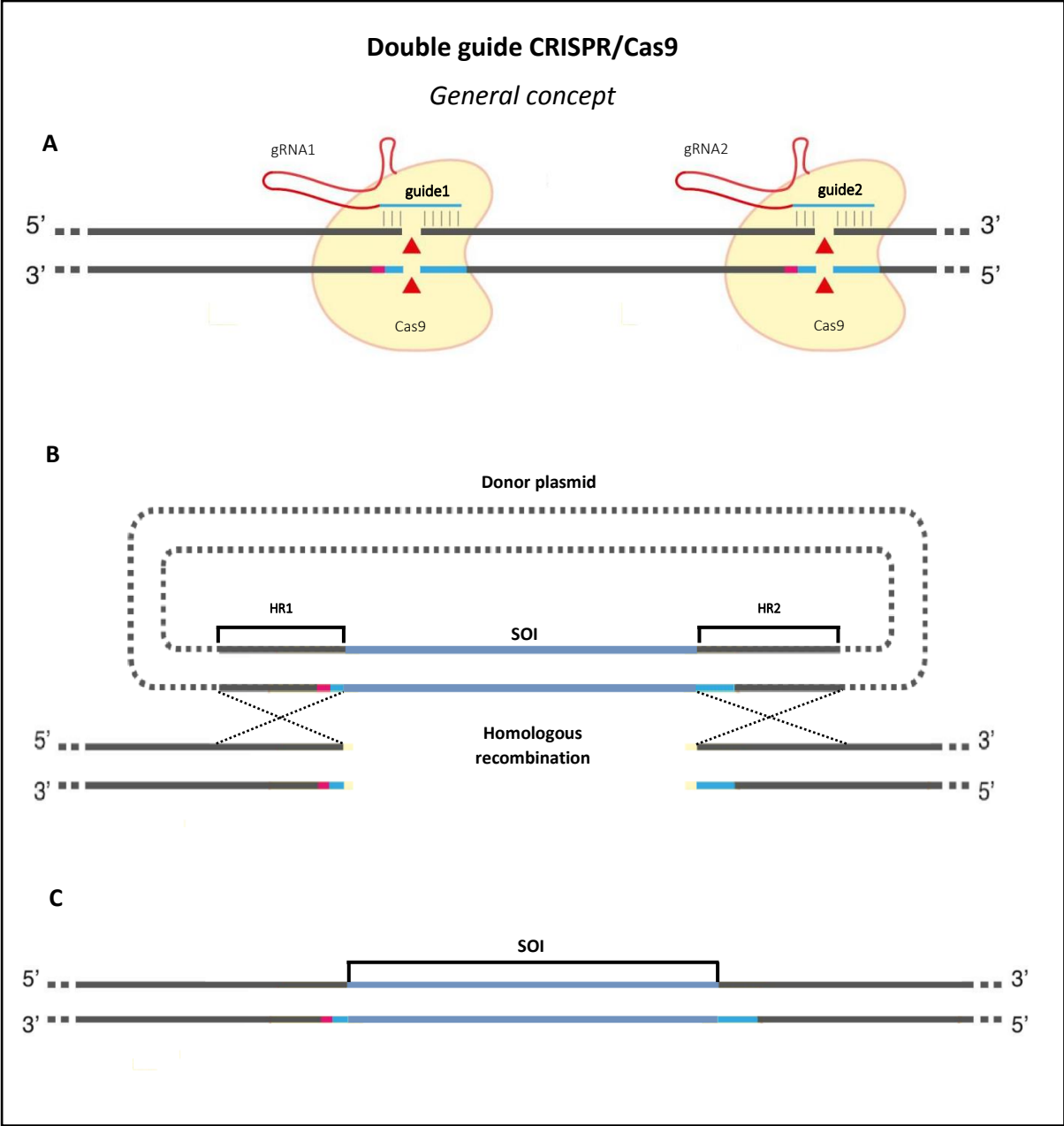


Figure 49 - Double gRNA CRISPR/Cas9 system

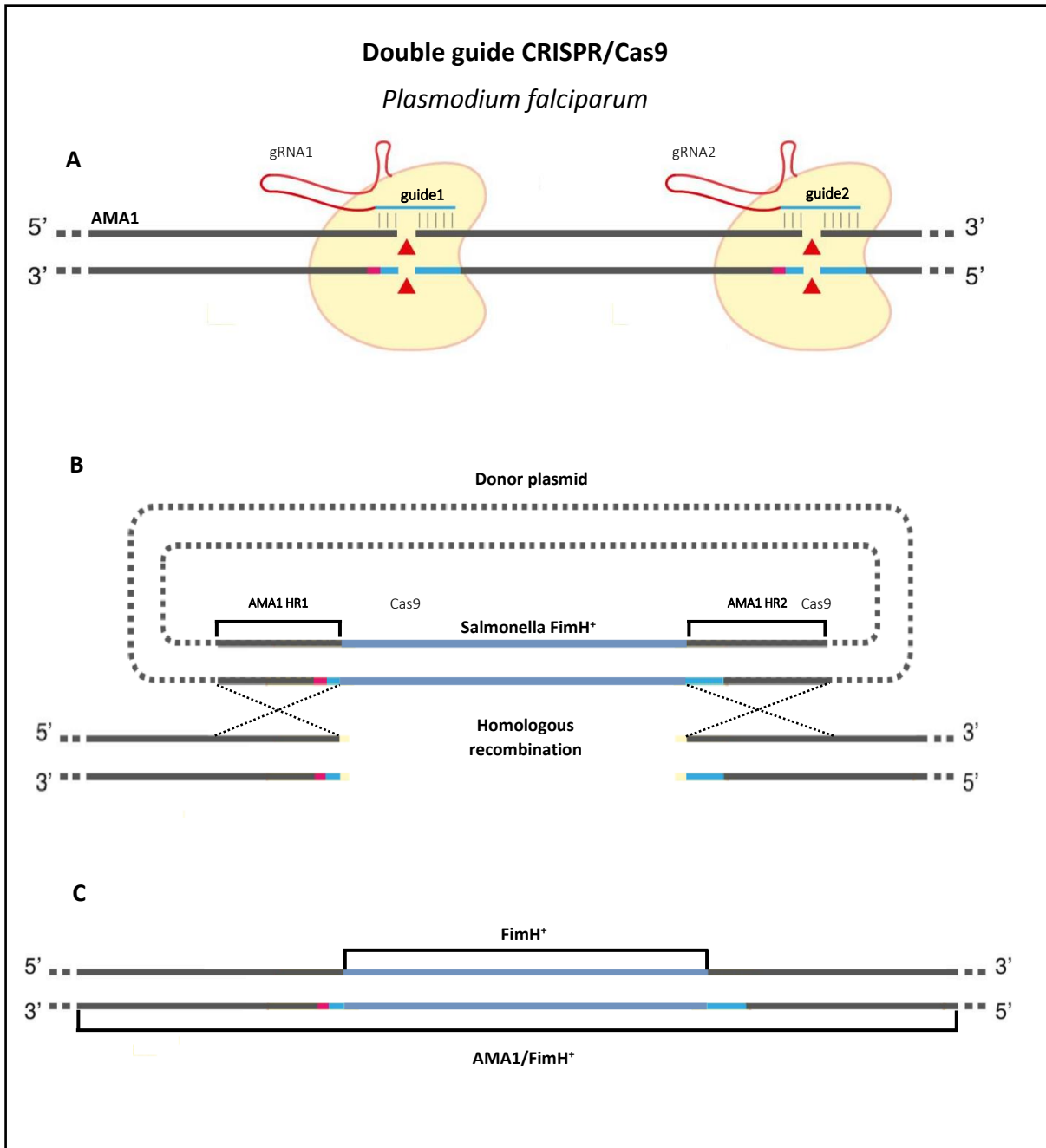


Figure 50 - Double guide CRISPR/Cas9 system for insertion of *Salmonella*'s FimH⁺ into *Plasmodium*'s AMA1

4.2. Methods

4.2.1. Outline of molecular approaches

Recent work by Ghorbal *et al.* (2014), which described the use of the CRISPR/cas9 system for genome editing in *P. falciparum*, employed the use of two plasmids: pL6-GFP and pUF1-Cas9. The pL6-GFP plasmid contains the guide RNA and the donor DNA while pUF1-Cas9 contains the Cas9 coding sequence.

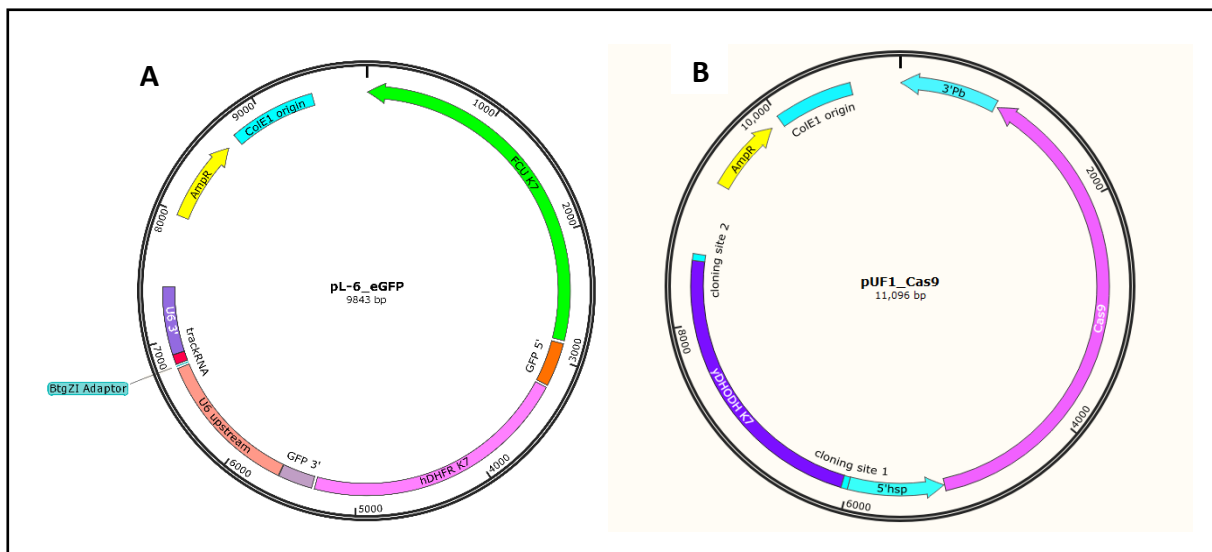


Figure 51 – Plasmids used for genome editing of *P. falciparum*. **A** – pL6-GFP plasmid with gRNA and donor DNA. **B** – pUF1-Cas9 plasmid with Cas9 endonuclease sequence.

Plasmid pL6-GFP (**Figure 51A**) contained: the suicide gene yFCU (yeast cytosine deaminase and uridyl phosphoribosyl transferase) allowing 5-fluorocytosine selection to be used to kill parasites carrying the plasmid; a donor DNA Template, the drug-selectable marker-cassette hDHFR (human dihy-drofolate reductase), which confers resistance to the antifolate drug WR99210; A GFP (green fluorescent protein) marker flanking the donor DNA template; the gRNA expression cassette containing *P. falciparum* U6 small nuclear RNA (snRNA) polymerase III promoter, trackRNA and *BtgZ1* adaptor; an ampicillin resistance gene; and ColE1 origin of replication. Plasmid pUF1-Cas9 (**Figure 51B**) contained: *S. pyrogenes* endonuclease Cas9 flanked by plasmidial regulatory elements; the drug selectable marker yeast dihydroorotate (yDHODH) which gives resistance to DSM1, a *P. falciparum* dihydroorotate dehydrogenase (PFDHODH) inhibitor; an ampicillin resistance gene; and ColE1 origin of replication.

One of the objectives of this project was to develop a CRISPR/cas9 plasmid with a double guide RNA (gRNA) system and different approaches were used in an effort to achieve the plasmid depicted in **Figure 52**.

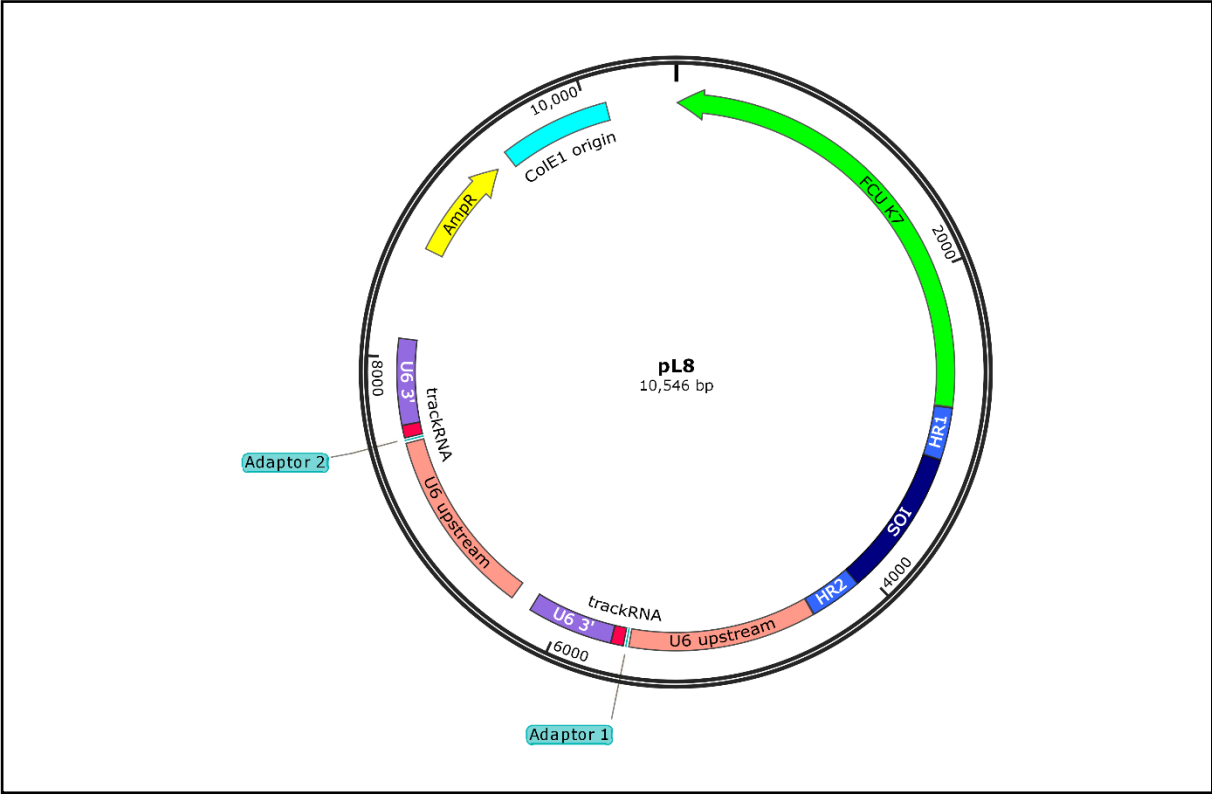


Figure 52 - pL8 plasmid with double gRNA system. Plasmid includes a suicide gene (FCUK7); the sequence of interest (SOI) flanked by homologous regions 1 and 2 (homologous to the sequence where SOI is to be inserted); two gRNA cassettes each containing a promoter, a trackRNA and a specific adaptor;

4.2.1.1. Approach 1

When approach 1 was initiated, the main goal was only to produce plasmid pL8 (**Figure 53**), which was to be used only for this experiment in particular. The first step was to replace the *BtgZI* adaptor with the respective desired guide sequence (Figure 54a). This would generate pL6.1 (Figure 54b) and pL6.2 (Figure 54c), each with a different guide sequence. Next, a restriction site (*BclI*) was added to pL6.1 (Figure 54d) in order to allow for posterior ligation of gRNA amplified from pL6.2 (Figure 54e). The amplification of the gRNA DNA sequence from pL6.2 would be carried with primers containing a restriction site (*BglII*) so the product could be digested and ligated to pL6.1 (Figure 54f). Finally the sequence of interest would be ligated along with the homologous regions in a single reaction using the In-Fusion kit (**Figure 54g**). In this case, the sequence of interest was the partial sequence of *Salmonella*'s FimH⁺ and the homologous regions partial sequences of *P. falciparum*'s AMA1. All of these steps would create pL8 (**Figure 53**).

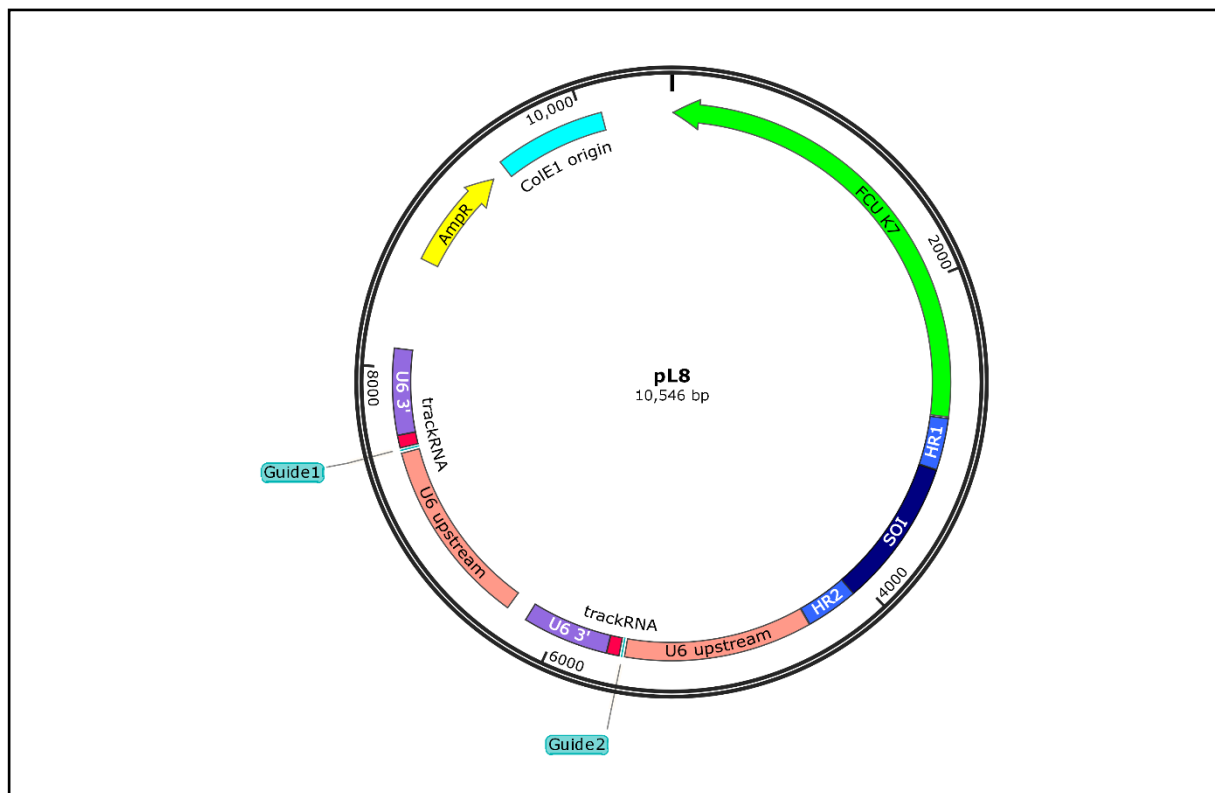


Figure 53 – Map of pL8 plasmid, a double guide CRISPR/Cas9 system for *P. falciparum*, specifically designed to replace part of *P. falciparum*'s AMA1 gene with *Salmonella*'s FimH⁺.

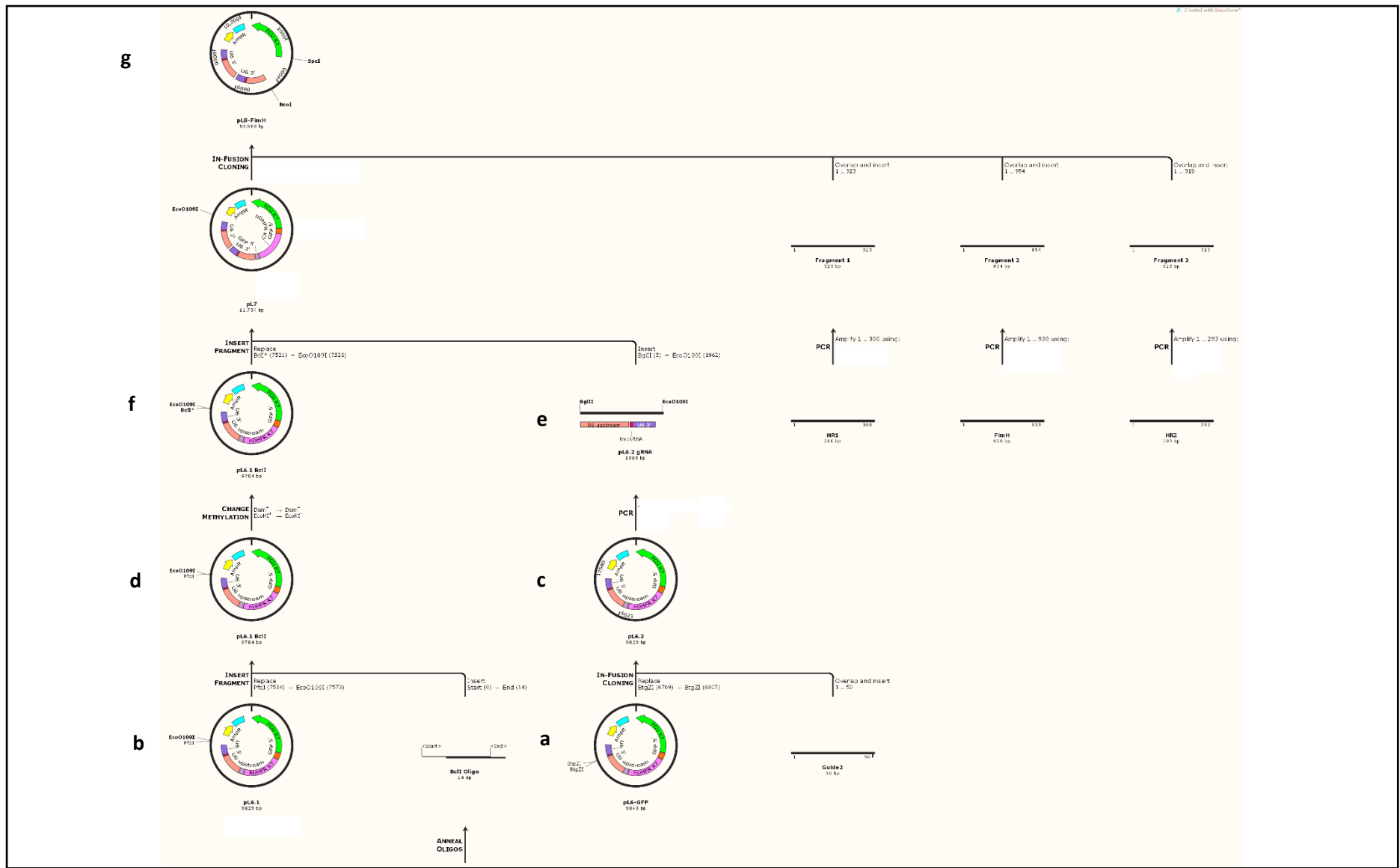


Figure 54 – Map history of steps required to generate pL8-FimH

4.2.1.2. Approach 2

In approach 2, the objective was to create a double gRNA plasmid that could be easily modified and used for genome editing of *P. falciparum*. For this, instead of replacing the *BtgZI* adaptor with a guide sequence, one of the adaptors was replaced with a *BpII* adaptor, as to have each gRNA with it's own adaptor (**Figure 55**). In order to achieve this, the *BtgZI* adaptor of pL6-GFP was replaced with a *BpII* adaptor in the same way a guide sequence would be added (**Figure 56a**). This generated pL6-BpII from which the gRNA sequence was amplified (**Figure 56b**) so that it could be ligated to a pL6-GFP (**Figure 56c**). This ligation was attempted with both standard (**Figure 56**) and In-Fusion cloning (**Figure 57**, **Figure 58** & **Figure 59**) employing three different methods.

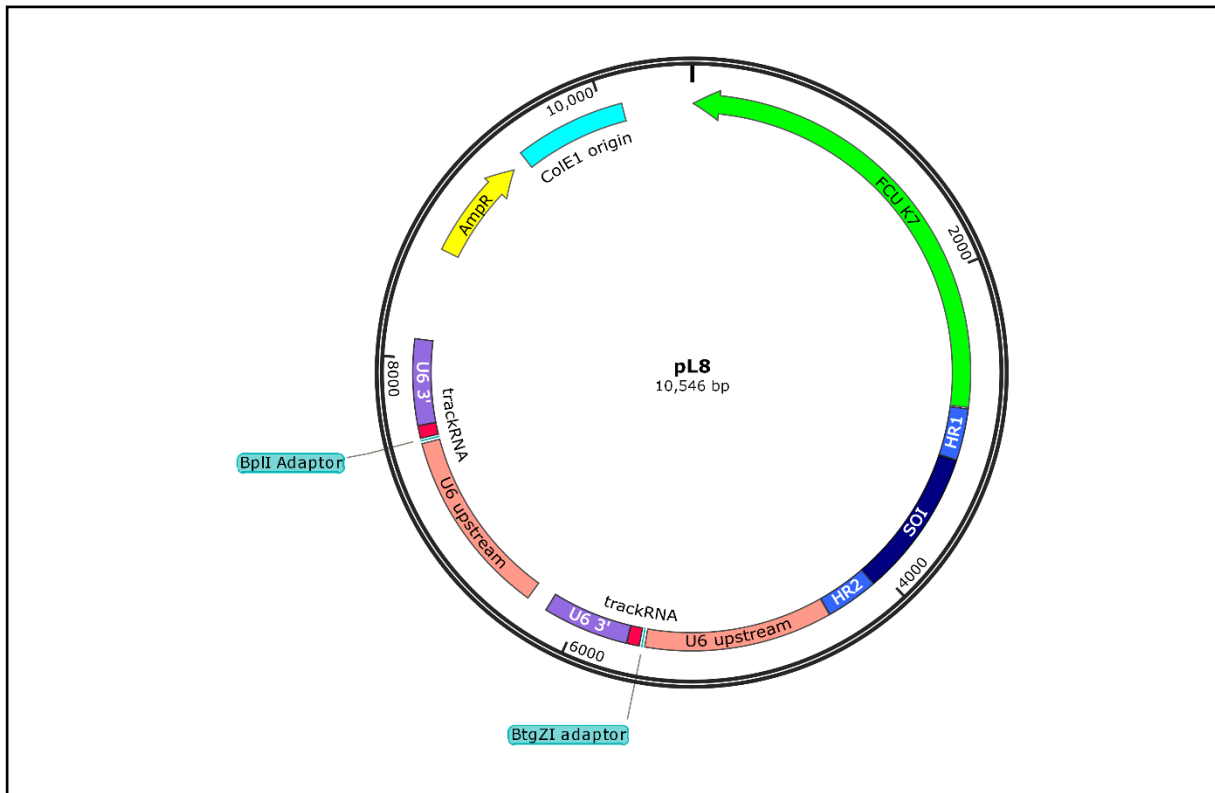


Figure 55 – Map of pL8 plasmid, a double guide CRISPR/Cas9 system for *P. falciparum*.

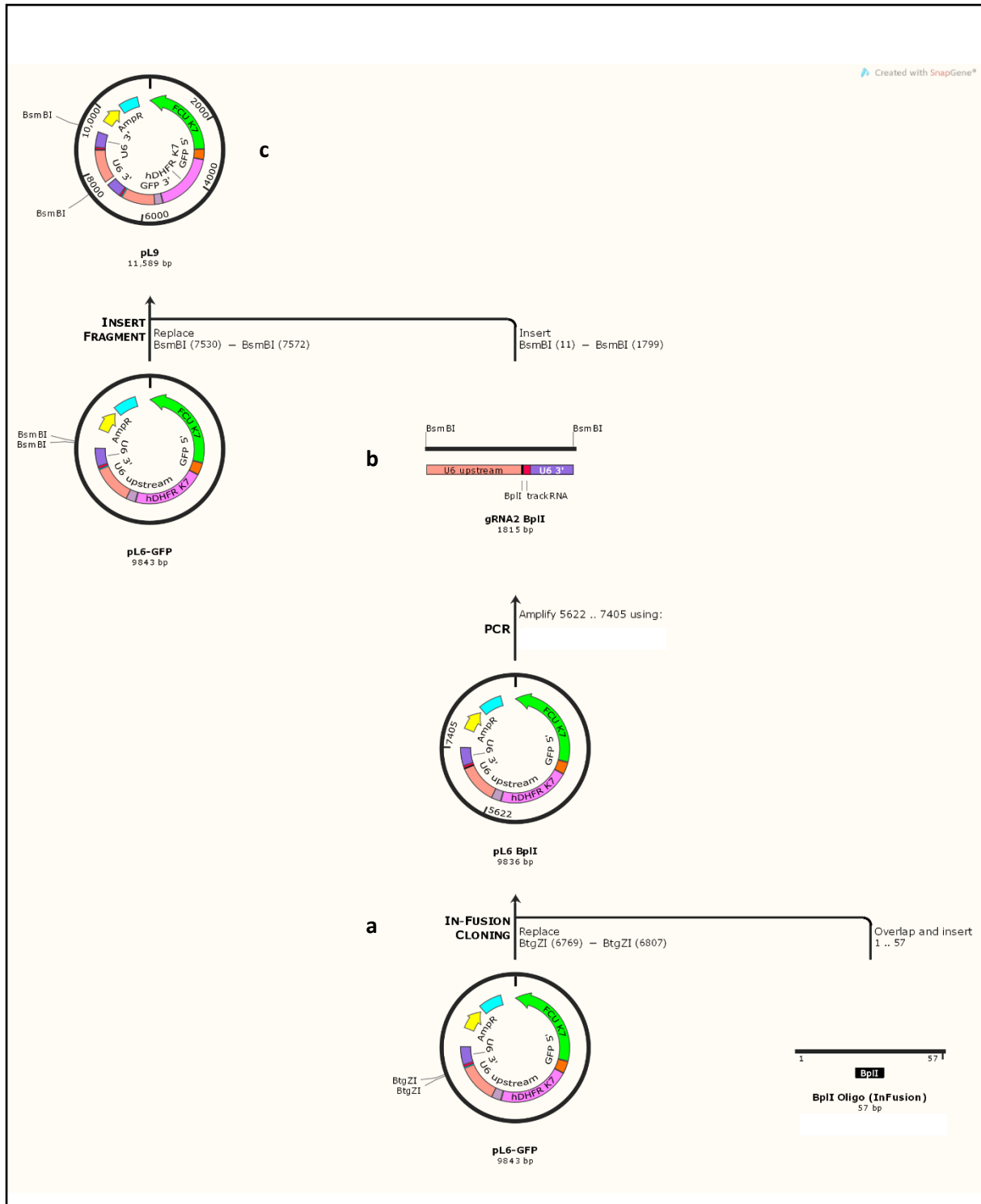


Figure 56 – Map of steps required for generation of pL9 through standard cloning

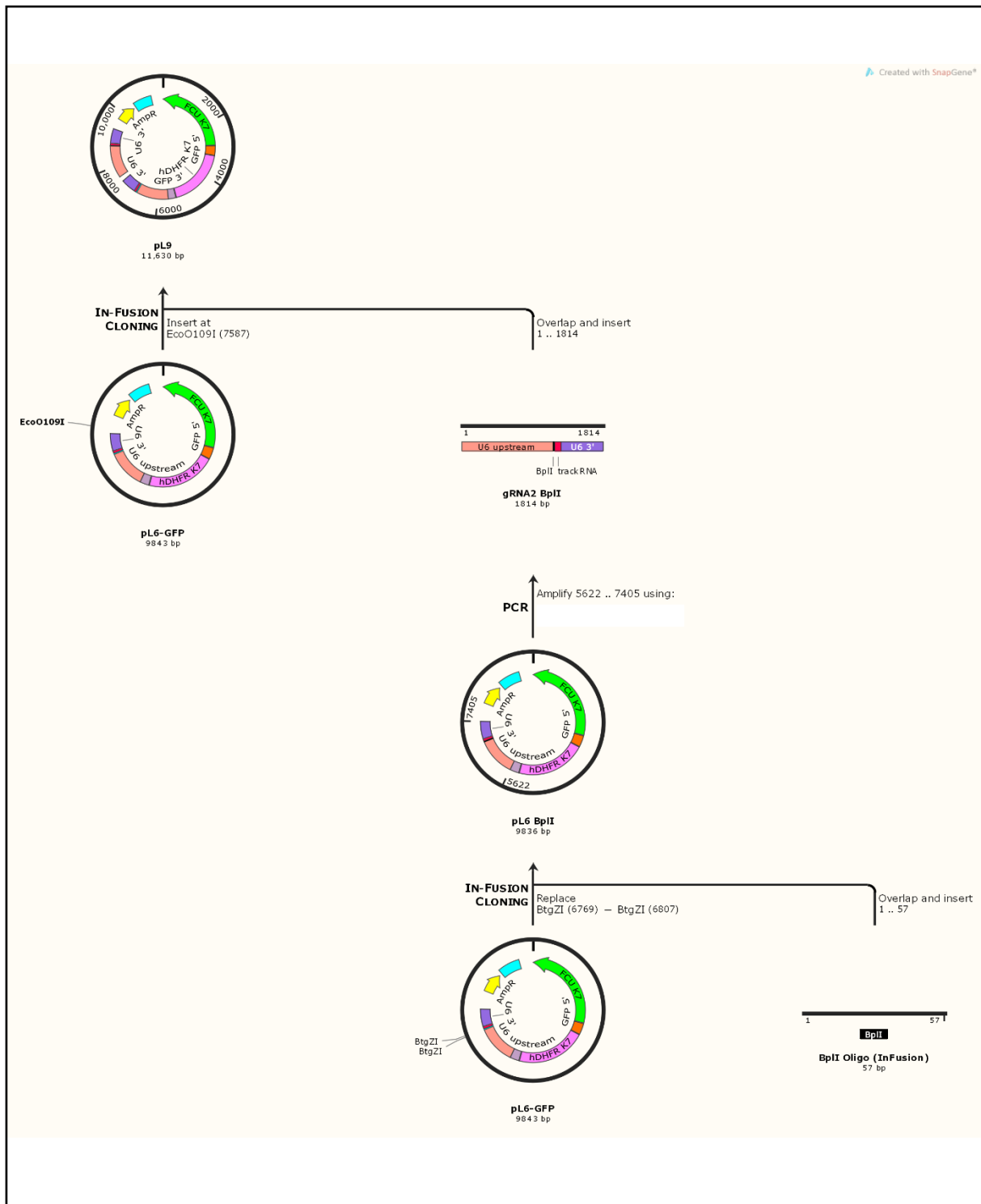


Figure 57 - Map of steps required for generation of pL9 through In-Fusion cloning

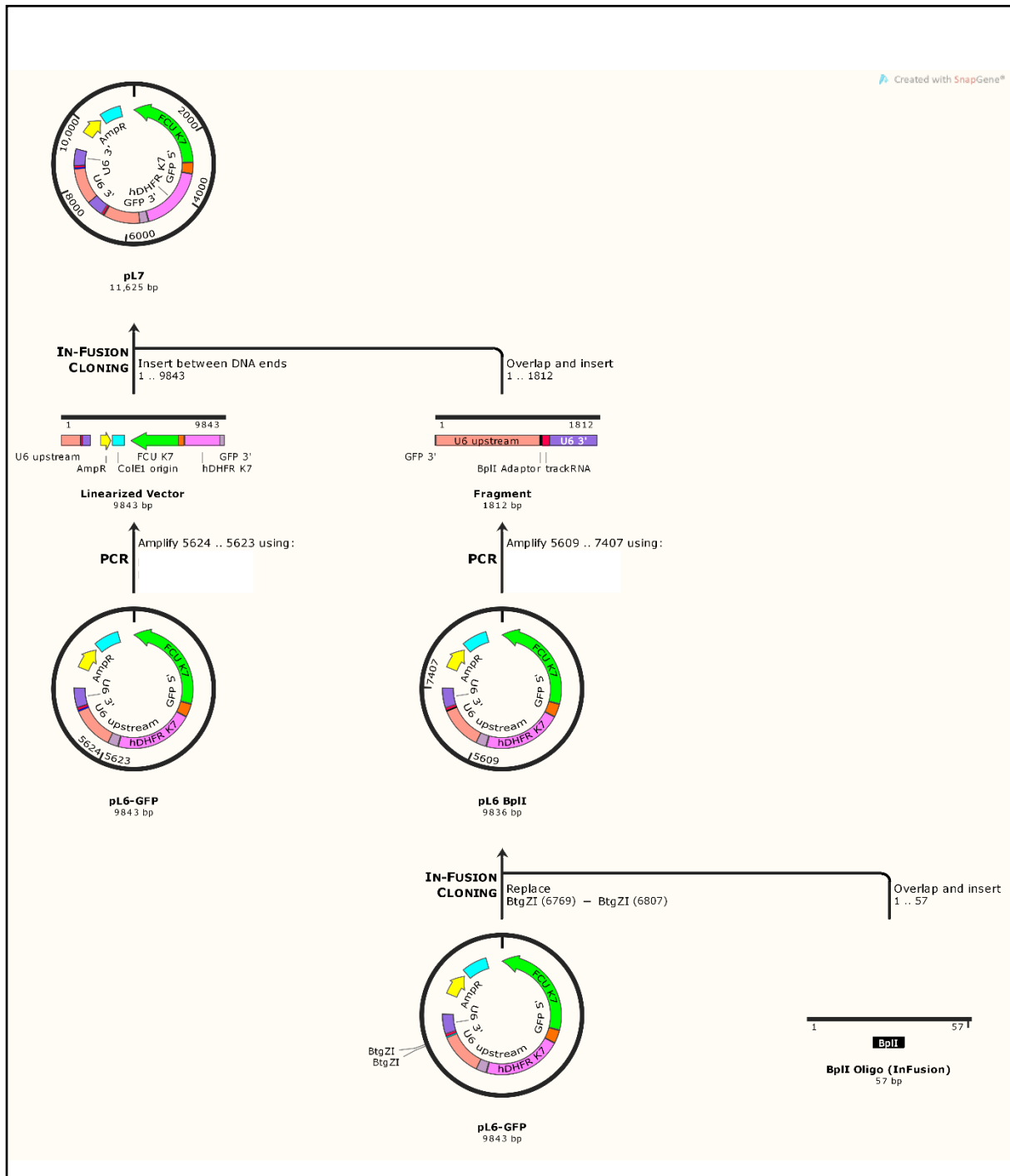


Figure 58 - Map of steps required for generation of pL9 through In-Fusion PCR cloning (anterior placement of gRNA2)

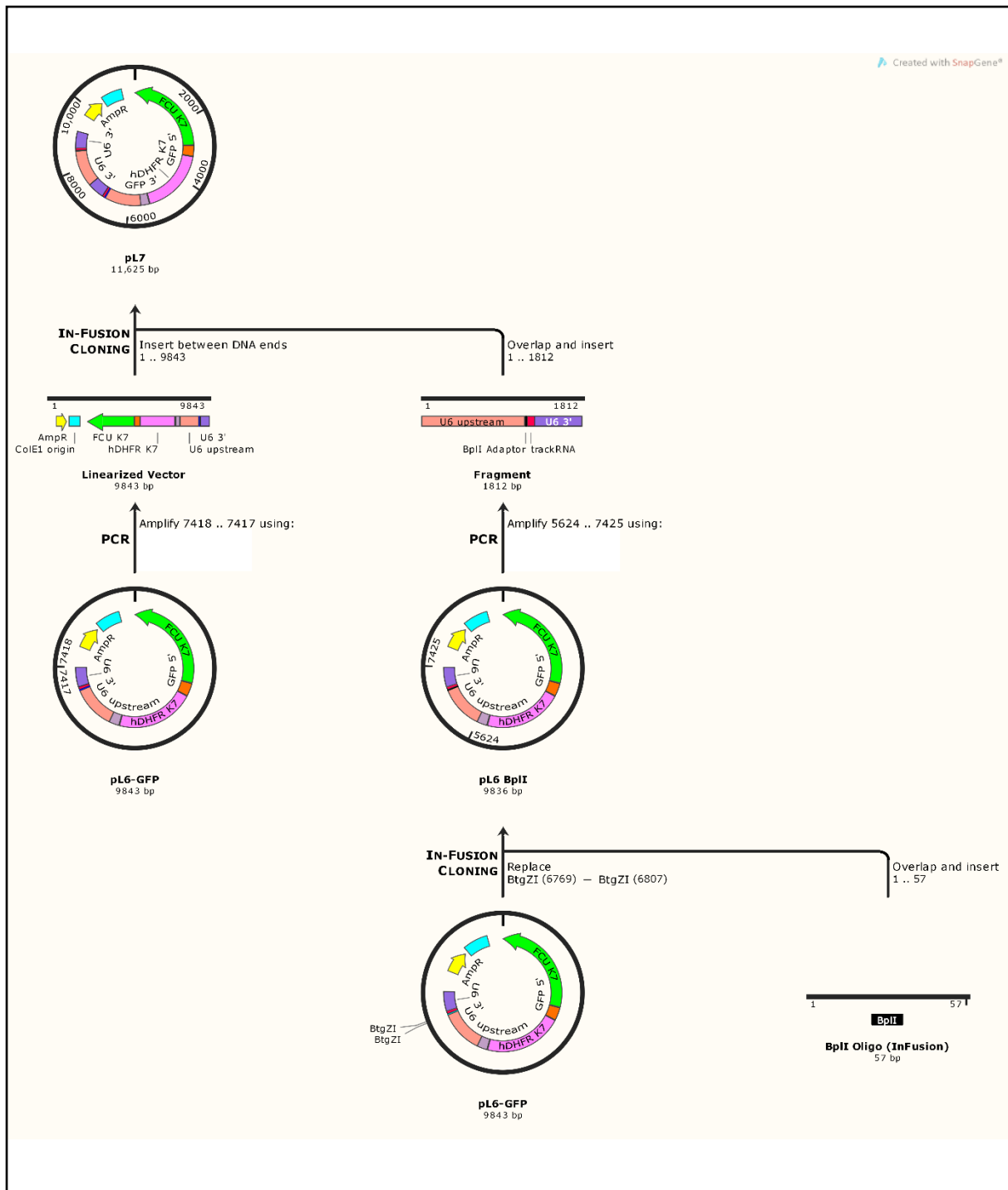
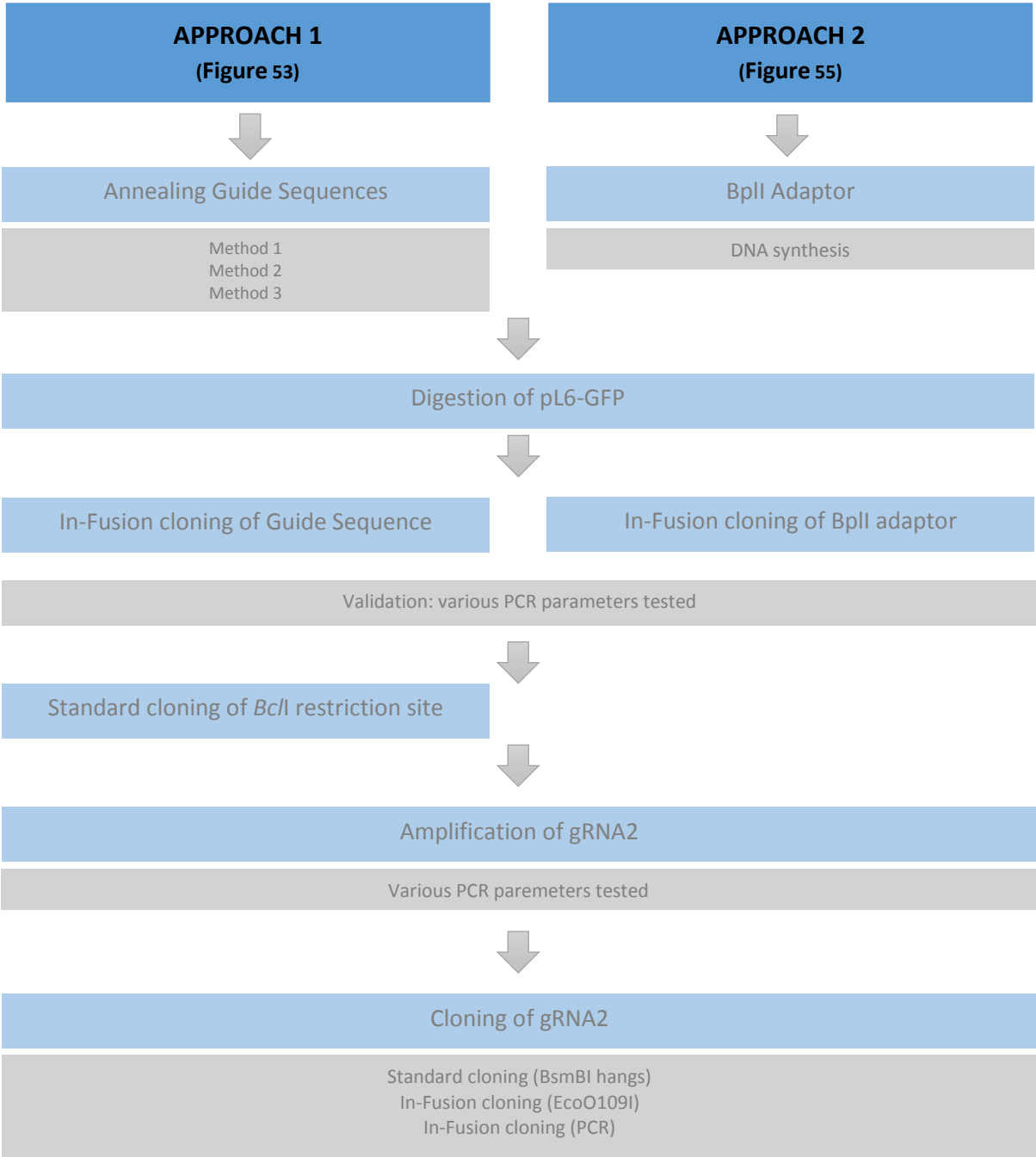


Figure 59 - Map of steps required for generation of pL9 through In-Fusion PCR cloning (posterior placement of gRNA2)

4.2.2. Outline of molecular methods



4.2.2.1. Approach 1

4.2.2.1.1. Annealing the guide oligonucleotides

The guide DNA sequences were ordered as two oligonucleotides each from Eurofins Genomics for subsequent annealing. Three annealing methods were tested.

Table 10 – Sequences of guide oligonucleotides. The 20bp guide RNA is shown in uppercase surrounded by the lowercase 15bp homology sequence needed for In-Fusion cloning.

Oligonucleotide	Sequence
Guide 1 F	taagtataatatt AATAATTTATTTGATAATTT gttttagagctagaa
Guide 1 R	ttctagctctaaaac AAATTATCAAATAAATTATTA aatattatatactta
Guide 2 F	taagtataatatt ATGTGACCCTGAAATGGTAA gttttagagctagaa
Guide 2 R	ttctagctctaaaac TTACCATTTCAAGGGTCACAT aatattatatactta

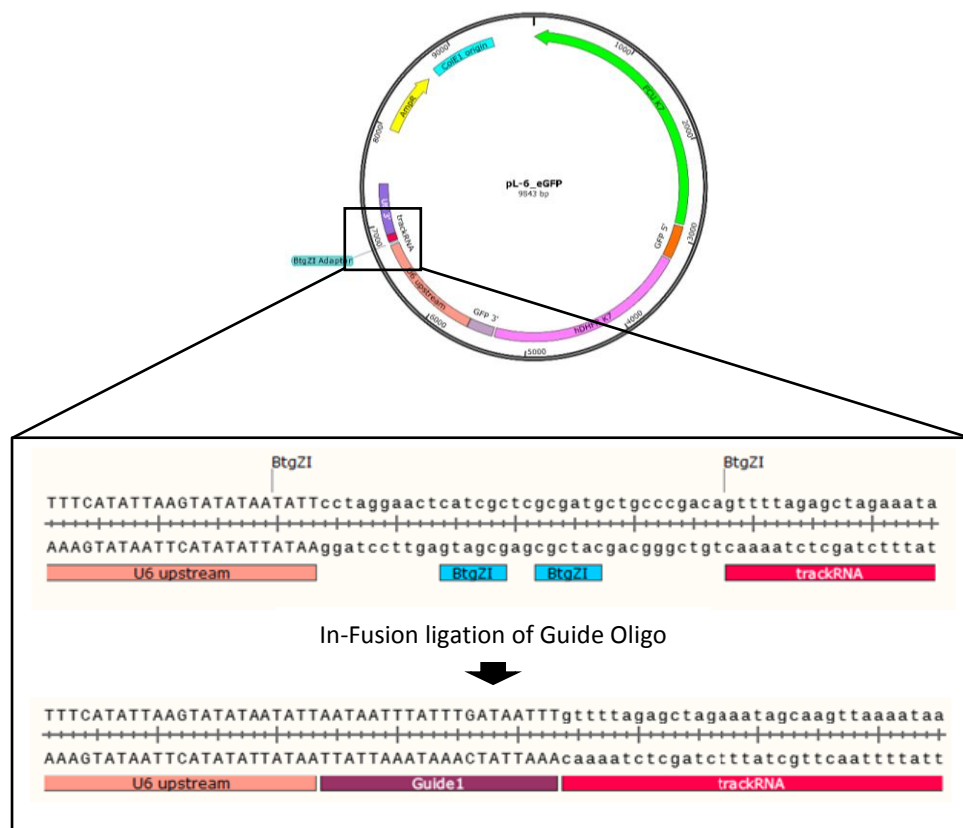


Figure 60 – Replacement of *BtgZI* adaptor with guide sequence

4.2.2.1.1.1. Method 1

Annealing was carried by adding 10 μL of each of the two corresponding oligonucleotide (100pM/ μL) to 80 μL of annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA). The tube was placed on a heating block that was pre-heated to 95 °C and then turned off. After 15 minute, the block was removed from the heating unit and allowed to slowly cool on the bench for 45 minute. The annealed oligonucleotides were immediately store in the freezer at -20 °C.

4.2.2.1.1.2. Method 2

Annealing was carried by adding 10 μL of each of the two corresponding oligonucleotide (100pM/ μL) to 80 μL of annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA). Tubes were placed on a thermal cycler running on the following program: 95 °C for 10 minutes; Ramp to 85 °C at 0.6 °C/second for 16 seconds; 85 °C for 1 minutes; Ramp to 75 °C at 0.6 °C/second for 16 seconds; 75 °C for 1 minutes; Ramp to 65 °C at 0.6 °C/second for 16 seconds; 65 °C for 1 minutes; Ramp to 55 °C at 0.6 °C/second for 16 seconds; 55 °C for 1 minutes; Ramp to 45 °C at 0.6 °C/second for 16 seconds; 45 °C for 1 minutes; Ramp to 35 °C at 0.6 °C/second for 16 seconds; 35 °C for 1 minute; Ramp to 25 °C at 0.6 °C/second for 16 seconds; 25 °C for 1 minute; Store at 4 °C infinitely. Samples were immediately stored in the freezer at -20 °C.

4.2.2.1.1.3. Method 3

Annealing was carried by adding 10 μL of each of the two corresponding oligonucleotide (100pM/ μL) to 25 μL of nuclease free water and 5 μL of restriction digest buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 $\mu\text{g}/\text{ml}$ BSA, pH 7.9). Using a floating tube rack, the tube was then placed in a beaker filled with water pre-heated at 95 °C and left to incubate for 5 minutes. The beaker was then transferred into an ice box filled with water and ice and allowed to cool for 10 minutes. Finally, 50 μL of nuclease free water were added and the tube was stored in the freezer at -20 °C.

4.2.2.1.2. Digestion of pL6-GFP with *Btg*ZI

To digest pL6-GFP the following components were added to a 1.5 mL tube: 2 μ L of *Btg*ZI (NEB, R0703S), 2 μ g of vector, 5 μ L of CutSmart[®] Buffer 10 x (NEB, B7204S) and ddH₂O to 50 μ L. The tube was then incubated at 60 °C for 2 hours followed by 20 min at 80 °C for enzyme inactivation. After allowing the tube to cool, 2 μ L of rSAP (NEB, M0371S) were added and the tube was incubated at 37 °C for 1h followed by inactivation at 50 °C for 20 min. Results were visualised by running a 0.7% gel electrophoresis and specific bands isolated. Purification of isolated DNA was purified with Macherey-Nagel NucleoSpin[®] Gel and PCR Clean-up kit (740609) according to manufacturer's instructions.

4.2.2.1.3. In-Fusion Cloning of guide DNA sequence into pL6-GFP

To ligate the guide DNA to pL6-GFP the In-Fusion[®] HD cloning kit (Clontech) was used. In a 0.5 mL tube the following components were added: 100ng of linearized/purified pL6-GFP, 50 ng of annealed guide RNA, 2 μ L of 5x In-Fusion HD Enzyme Premix and ddH₂O to 10 μ L. The tube was then incubated at 50 °C for 15 minutes followed by transformation with XL10-Ultracompetent Cells (Stratagene) according to manufacturer's instructions.

4.2.2.1.4. Checking for guide DNA sequence insert in pL6-GFP

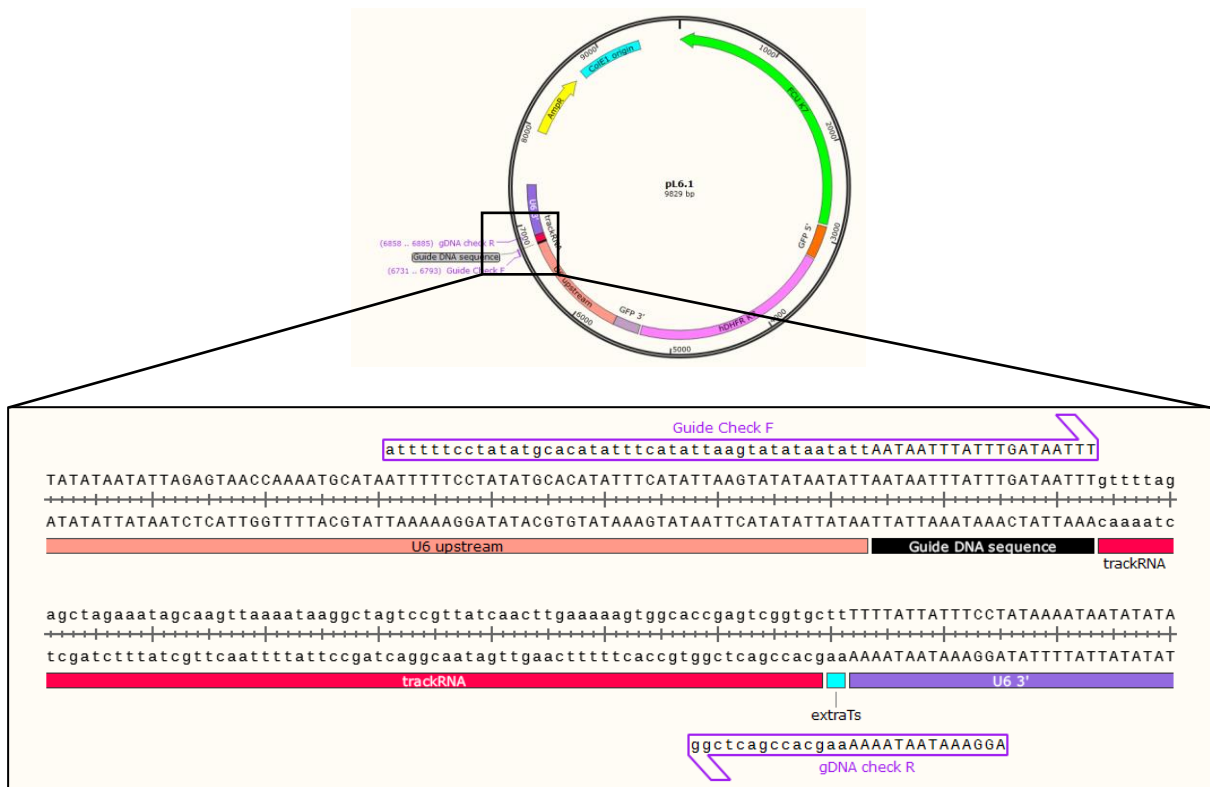


Figure 61 – Map of pL6.1 where *BtgZI* adaptor has been replaced with guide sequence 1.

In order to check for successful insertion of the guide DNA sequence, several PCR's were carried at different conditions. For all reactions GoTaq® G2 Flexi Polymerase (Promega, M7801) was used and for each component the following volumes were added: 5 µL (1x) of 5x Green GoTaq® Flexi Buffer, 1 µL (1 mM) of MgCl₂ solution, 0.25 µL (0.2 mM each dNTP) of Nucleotide mix, 0.25 µL (0.2 mM) of forward primer, 0.25 µL (0.2 mM) of reverse primer, 0.15 µL (0.75u) of GoTaq® Flexi DNA Polymerase and ddH₂O to 25 µL. (add template)

Table 11 - List of PCR programs tested

	Program	Temperature °C								Δt
		A	B	C	D	E	F	G	H	-
30x	Initial Denaturation	95	95	95	95	95	95	95	95	2 m
	Denaturation	95	95	95	95	95	95	95	95	30 s
	Annealing	49	50	60.5	61.5	60.5	60.5	59	58	30 s
	Extension	72	72	72	72	68	60	72	72	45 s
	Final Extension	72	72	72	72	68	60	72	72	5 m

Table 12 - List with combinations of PCR programs and primer sets tested

Primer	Sequence	Program
Guide1Check F* (6)	CCTAATAATTTATTTGATAATTTTTCAAAT	A, B
gDNA check R (10)	TAGGAAATAATAAAAAAGCACC	
Guide2check F (9)	ATGTGACCCTGAAATGGTAA	A, B,
gDNA check R (10)	TAGGAAATAATAAAAAAGCACC	
Guide1Check F* (6)	CCTAATAATTTATTTGATAATTTTTCAAAT	C, D, E, F, G
gDNA check R New (23)	AGGAAATAATAAAAAAGCACCGACTCGG	
Guide2check F (9)	ATGTGACCCTGAAATGGTAA	C, D, E, F, G
gDNA check R New (23)	AGGAAATAATAAAAAAGCACCGACTCGG	
Guide 1 F check (27)	ATTTTCCTATATGCACATATTTTCATATTAAGTATATAATATTAATAATTTATTTGATAATTT	C, D, E, F, G
gDNA check R New (23)	AGGAAATAATAAAAAAGCACCGACTCGG	
Guide 2 F (28)	GCACATATTTTCATATTATGTGACCCTGAAATGGTAA	C, D, E, F, G
gDNA check R New (23)	AGGAAATAATAAAAAAGCACCGACTCGG	
Guide seq F (31)	CATGACATTTGGATTCTACACATCTTGAGG	H
Guide seq R (32)	GAAATAATAAAAAAGCACCGACTCGG	

4.2.2.1.5. Standard cloning of *Bcl*I restriction site into pL6.1

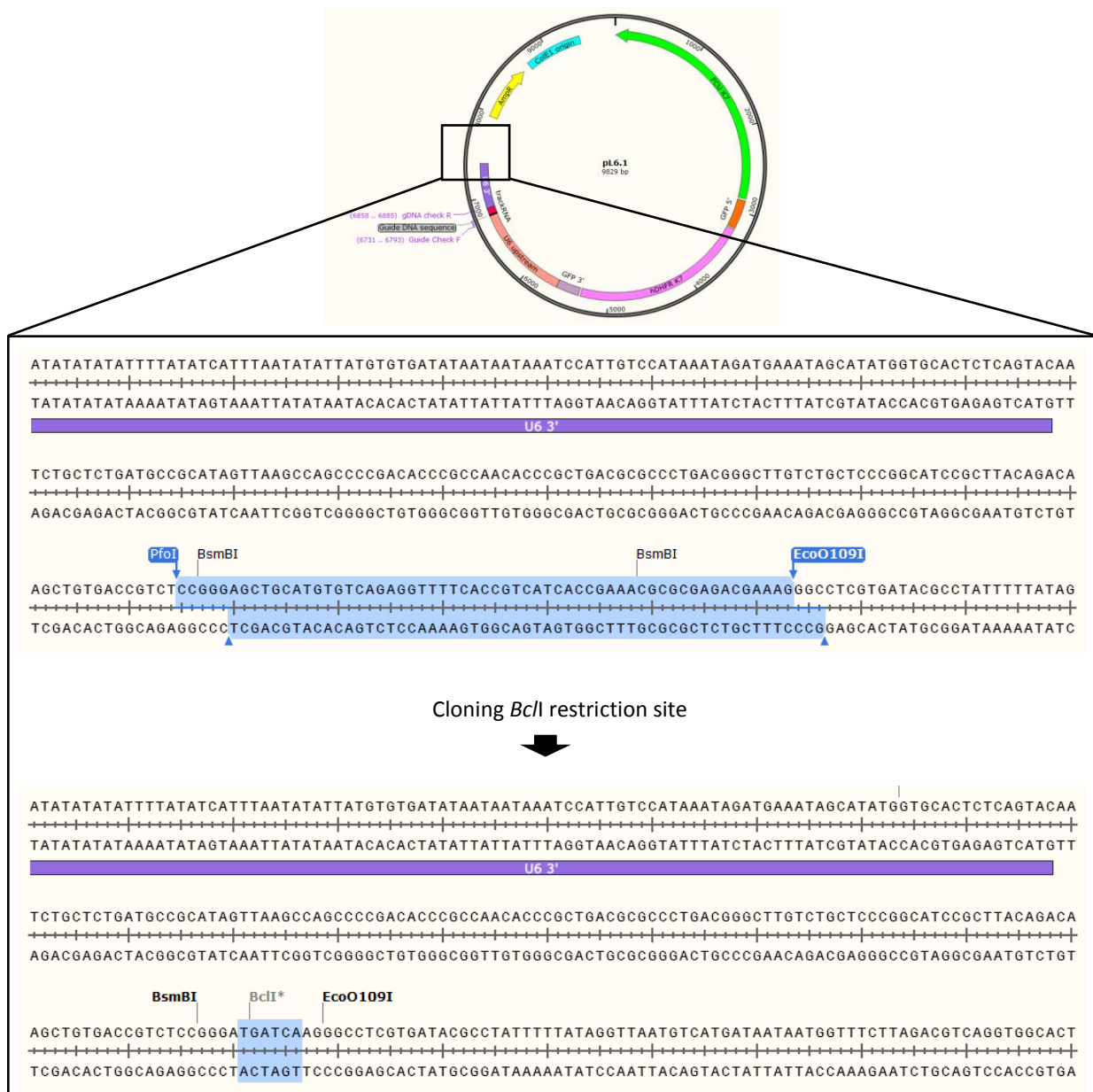


Figure 62 - Map of pL6.1 and location of *Bcl*I restriction site insertion

4.2.2.1.5.1. Annealing *Bcl*I restriction oligonucleotide

To anneal the *Bcl*I restriction site oligonucleotides method 1 described section 4.2.2.1.1.1. was followed.

4.2.2.1.5.2. Double digestion of pL6-GFP with PfoI and Eco109I

To digest pL6-GFP the following components were added to a 1.5 mL tube: 2 μ L of PfoI (ThermoFisher, ER1751), 2 μ L of EcoO109I (NEB, R0503S), 2 μ g of vector, 5 μ L of CutSmart[®] Buffer 10 x (NEB, B7204S) and ddH₂O to 50 μ L. The tube was then incubated at 37 °C for overnight followed by 20 min at 65 °C for enzyme inactivation. After allowing the tube to cool, 2 μ L of rSAP (NEB, M0371S) were added and the tube was incubated at 37 °C for 1h followed by inactivation at 50 °C for 20 min. Results were visualised by running a 0.7 % gel electrophoresis and specific bands isolated. Purification of isolated DNA was purified with Macherey-Nagel NucleoSpin[®] Gel and PCR Clean-up kit (740609) according to manufacturer's instructions.

4.2.2.1.5.3. Ligation of *Bcl*I restriction oligonucleotide into pL6-GFP

In order to add a *Bcl*I restriction site to pL6-GFP, the annealed oligonucleotides obtained in section .2.2.2.1. were ligated to vector pL6-GFP using a standard ligation reaction. In a 0.5 mL tube, the following components were added: 50 ng of linearized pL6-GFP (from section 4.2.2.1.2.), 1 pmol of annealed oligonucleotides (from section 4.2.2.2.1.), 1 μ L of T4 DNA ligase (NEB, M0202S) and ddH₂O to 20 μ L. The tube was then incubated at room temperature for 30 minutes followed by inactivation of enzyme at 65 °C for 10 minutes and finally transformation in Dam⁻ /Dcm⁻ competent cells (NEB, C2925I) according to manufacturer's instructions.

4.2.2.1.5.4. Checking for *Bcl*I insert in pL6-GFP

In order to check for successful insertion of the *Bcl*I restriction site, a PCR was carried using GoTaq[®] G2 Flexi Polymerase (Promega, M7801) and for each component the following volumes were added: 5 μ L (1x) of 5x Green GoTaq[®] Flexi Buffer, 1 μ L (1 mM) of MgCl₂ solution, 0.25 μ L (0.2 mM each dNTP) of Nucleotide mix, 0.25 μ L (0.2 mM) of forward primer (**Table 13**, P25), 0.25 μ L (0.2 mM) of reverse primer (**Table 13**, P26), 0.15 μ L (0.75u) of GoTaq[®] Flexi DNA Polymerase and ddH₂O to 25 μ L. The PCR protocol used comprised of an initial denaturation step at 95 °C for 2 minutes; a 30x cycle of denaturation at 95 °C for 1 minute, annealing at 64 °C for 1 minute, extension at 72 °C for 1.5 minutes; and lastly a final extension at 72 °C for 5 minutes. After PCR amplification the products were ran in a 0.7% agarose gel.

Table 13 – Primer set used to verify insertion of *Bcl*I restriction oligo

Primer	Sequence
<i>Bcl</i> I check F (25)	CCGTCTCCGGGATGATCAAGGGC
<i>Bcl</i> I check R (26)	CGATCAAGGCGAGTTACATGATCCCCCA

4.2.2.2. Approach 2

4.2.2.2.5. Cloning BpII adaptor

4.2.2.2.1.1. BpII adaptor synthesis

Unlike the guide sequences described earlier, the BpII adaptor was not obtained as two oligonucleotides. As an alternative, the gene synthesis service by Eurofins Genomics was used as to avoid the process of oligonucleotide annealing. The desired sequence was submitted to the company and delivered in a plasmid, which was later digested with *BtgZI* producing a BpII adaptor fragment that was used directly for standard cloning producing pL6-BpII.

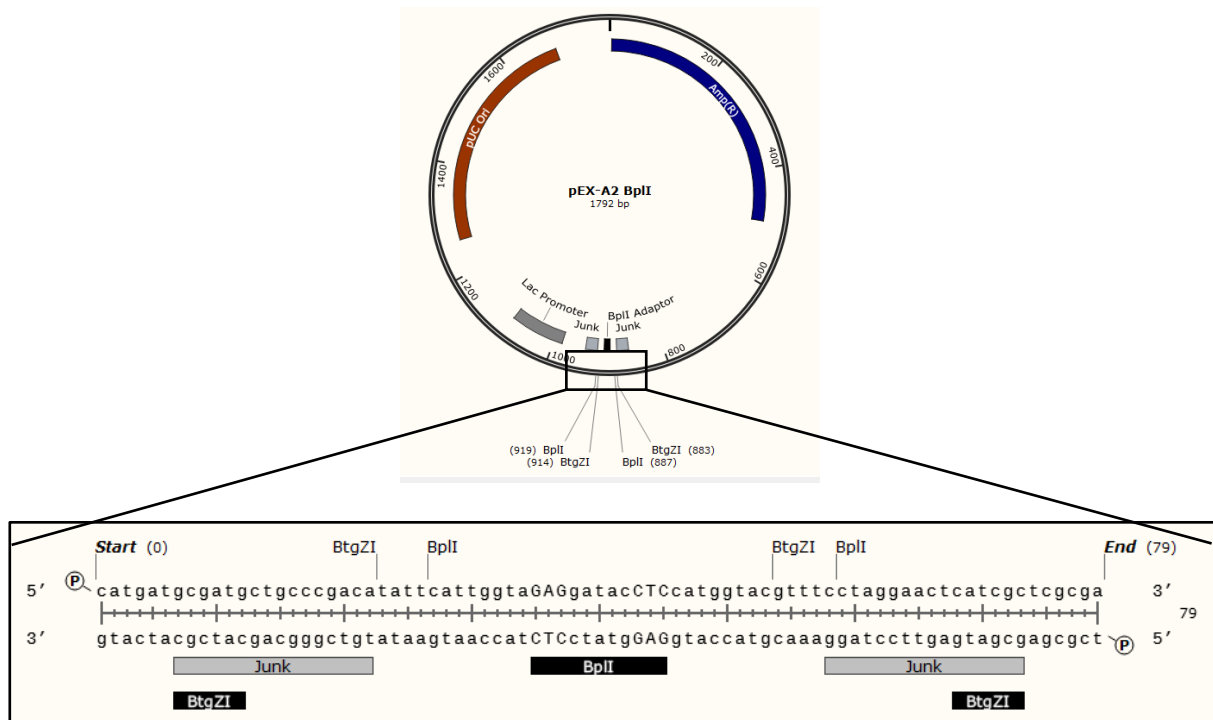


Figure 63 – BpII adaptor sequence submitted for DNA synthesis. The adaptor is flanked by *BtgZI* restriction sites which in turn are fringed by “junk” sequences in order to improve digestion.

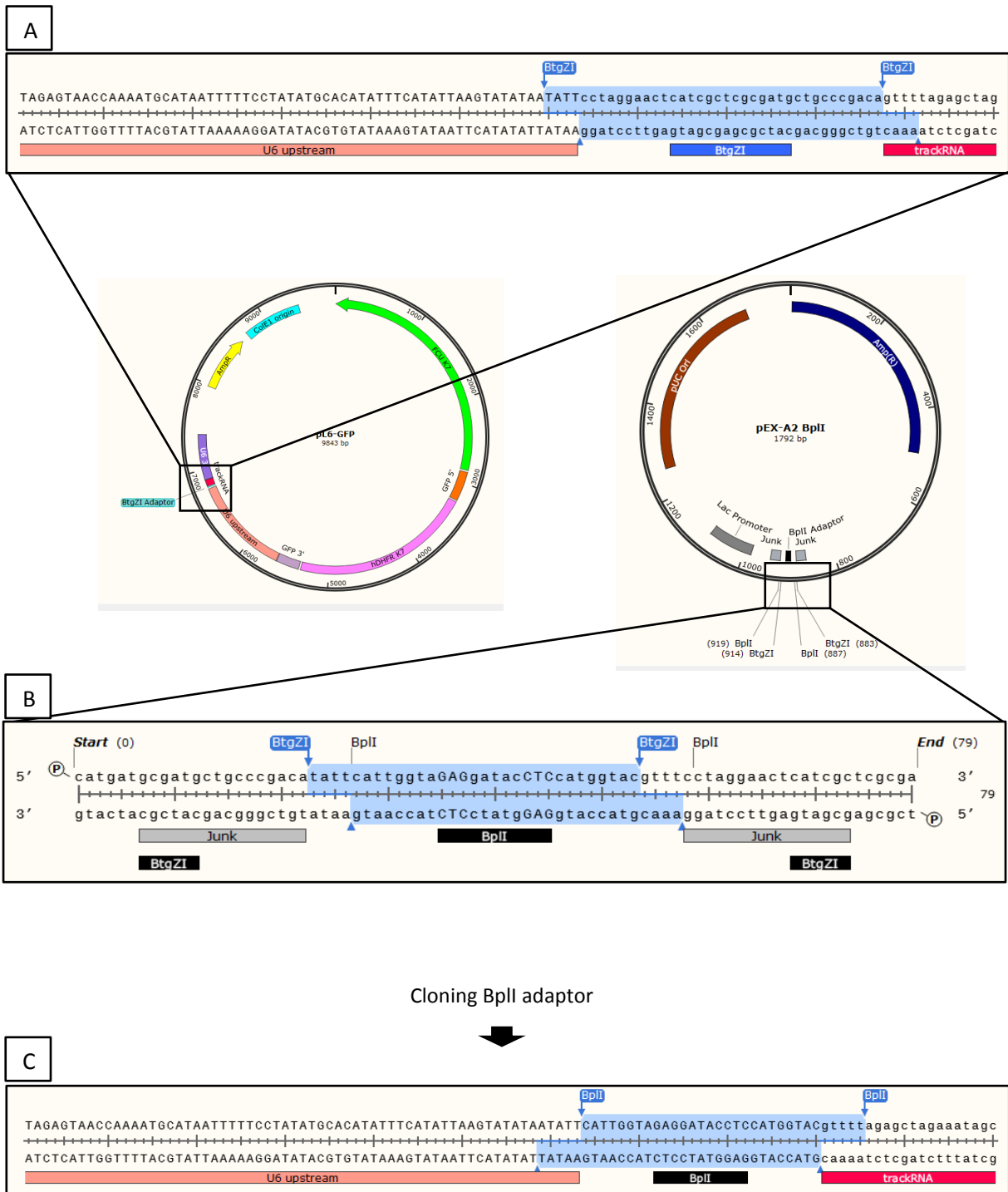


Figure 64 - Replacement of the *BtgZI* adaptor with the *BpII* adaptor. **A** – Digestion of pL6-GFP with *BtgZI*; **B** – Digestion of *BpII* adaptor in pEX-A2 *BpII* with *BtgZI*; **C** – *BpII* adaptor after cloning into pL6-GFP creating pL6-*BpII*

4.2.2.2.1.2. Digestion of pEX-A2 BpII

The BpII adaptor was contained in a pEX-A2 plasmid which was initially propagated in Stellar™ competent cells (Clontech, Cat. 636763) and extracted with NucleoSpin® Plasmid (Macherey-Nahel, REF 740588), according to manufacturer's instructions. The plasmid was then digested with *BtgZI* as described in section 4.2.2.1.2.

4.2.2.2.1.3. Ligation of BpII adaptor

Ligation was accomplished with standard cloning according to the procedure described in section 4.2.2.1.3., but transformed with Stellar™ competent cells (Clontech, Cat. 636763).

4.2.2.2.1.4. Checking for BpII adaptor insert

In order to check for successful insertion of the BpII adaptor, a PCR was carried using the same PCR parameters in section 4.2.2.1.4. with primers P31 and P32 (**Table 12**). PCR products were then sent for sequencing.

4.2.2.2.2. Amplification and cloning of gRNA2 sequence

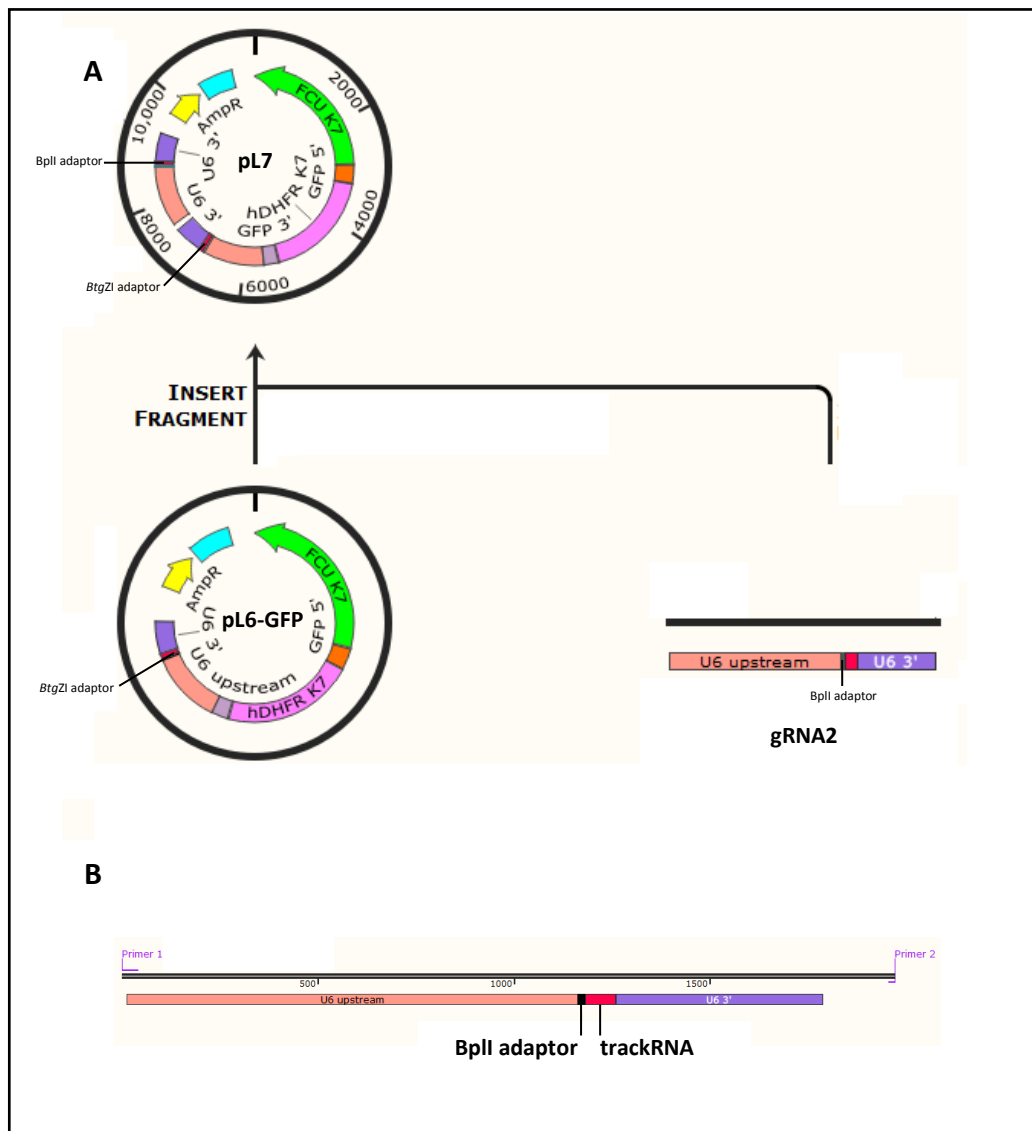


Figure 65 – Creating a double gRNA plasmid. **A** - Schematic representation of gRNA2 ligation into pL6-GFP creating pL7. **B** - Full DNA sequence of the gRNA2 amplified with flanking primers (primers depend on method used).

In order to create a double cutting system, a second gRNA (gRNA2) DNA sequence was amplified from the pL6-BtgZI plasmid (containing the BtgZI adaptor) and subsequently ligated into pL6-GFP (containing the BtgZI adaptor). The result was pL7, a plasmid with double gRNA's but each with its own restriction adaptor. Several PCR's were carried with various sets of primers and at different conditions in order to find a protocol that would result in successful amplification and ligation of the desired fragment.

4.2.2.2.1. Amplification of gRNA2 with *Bg*III 5' and a *Eco*O109I 3' hangs

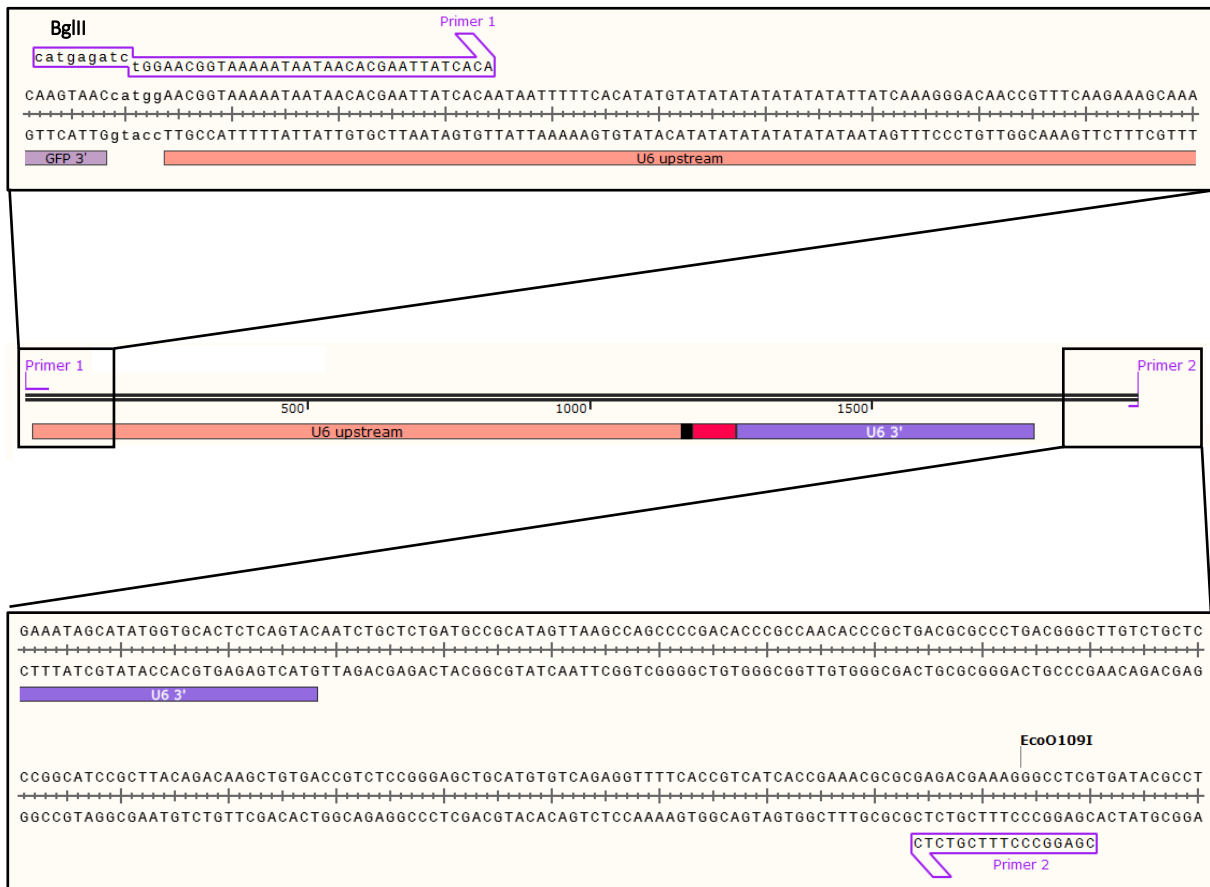


Figure 66 – Schematic view of primers used for amplification of the gRNA DNA sequence from pL6-GFP. Primer 1 contained an overhang with the restriction site for *Bg*III while Primer 2 included the sequence for *Eco*O109I restriction site.

Several PCR's were carried at different conditions, and with two sets of primers, in order to find a protocol that would result in successful amplification of the desired fragment. Amplification was attempted with both Phusion® High Fidelity Polymerase (NEB, M0530S) and GoTaq® G2 Flexi Polymerase (Promega, M7801) with different components and concentrations. For assays in which Phusion® High Fidelity Polymerase was used, the reagent combinations tested can be found in **Table 14**. For samples amplified with GoTaq® G2 Flexi Polymerase (Promega, M7801), all tubes contained the following volumes and concentrations: 4 µL (1x) of 5x Green GoTaq® Flexi Buffer, 2 µL (2.5 mM) of MgCl₂ solution, 0.5 µL (200µM) of dNTP's, 0.5 µL (0.5 mM) of forward primer, 0.5 µL (0.5 mM) of reverse primer, 0.15 µL (0.75u) of GoTaq® Flexi DNA Polymerase and ddH₂O to 20 µL. In addition to

the various concentrations and volumes (Table 14), different cycling programs (Table 15) and primers (Table 16) were also tested.

Table 14 - Phusion PCR mix combinations tested for amplification of gRNA2 sequence

Component	1	2	3	4	Concentration
5x Phusion® HF Buffer	5	5	-	-	1x
5x Phusion® CG Buffer	-	-	5	5	1x
DMSO	0.75	1.5	-	1.5	3 - 7.5%
dNTP's	0.5	0.5	0.5	0.5	0.2mM each
Primer F / R	1.25	1.25	1.25	1.25	1.25mM
DNA template	0.5	0.5	0.5	0.5	~125ng
Phusion® DNA Polymerase	0.25	0.25	0.25	0.25	0.1u
ddH ₂ O	10.5	9.75	10.5	9.75	To 20µL

Table 15 - PCR programs tested

Program	Temperature °C						Δt
	I	J	K	L	M	N	-
Initial Denaturation	98	98	95	95	95	95	2 m
Denaturation	98	98	95	95	95	95	30 s
Annealing	56	57	65	64	65.5	58	30 s
Extension	72	72	72	72	72	60	1.5 m
Final Extension	72	72	72	72	72	60	5 m

Table 16 - List of the combinations of primers with the respective PCR programs used

Primer	Sequence	Program
<i>Bgl</i> II site to U6 5' F (11)	CATGAGATCTGGAACGGTAAAAATAATAACACGAATTATCACA	I, J and K
EcoO109I U6 3' R (12)	CGAGGCCCTTCGTCTC	
<i>Bgl</i> II site to U6 5' F (11)	CATGAGATCTGGAACGGTAAAAATAATAACACGAATTATCACA	L, M and N
EcoO109I R (24)	CGAGGCCCTTCGTCTCGCGCG	

4.2.2.2.2.2. Amplification of gRNA2 with BsmBI hangs

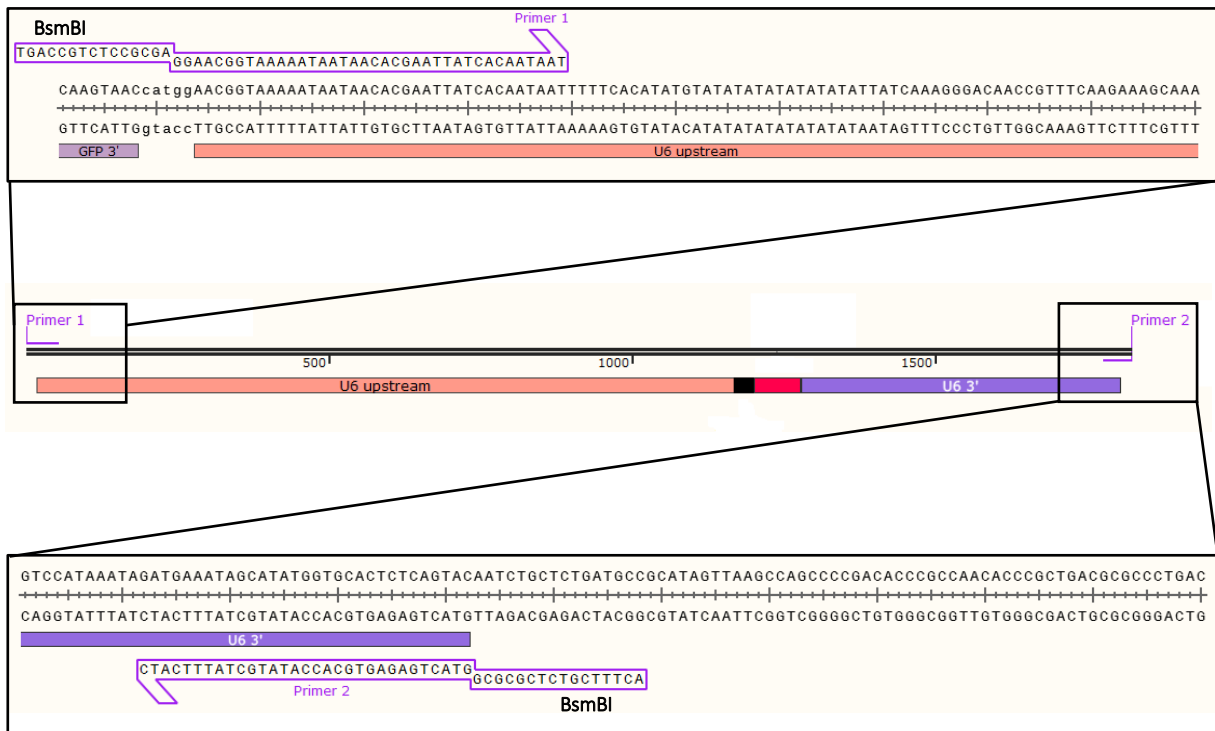


Figure 67 - Schematic view of primers used for amplification of the gRNA2 (BpII adaptor) DNA sequence from pL6-BpII. Primer 1 and 2 contained an overhang with the restriction site for BsmBI.

Amplification of the gRNA2 DNA sequence with BsmBI hangs was attempted with Phusion® High Fidelity Polymerase (NEB, M0530S) and GoTaq® G2 Flexi Polymerase (Promega, M7801) with the concentrations described in the previous section (4.2.2.2.2.1.). In addition to the aforementioned polymerases tested, Q5® High Fidelity Polymerase (NEB, M0491S) and CloneAmp™ HiFi PCR Premix (Clontech, 639298) were also used. The Q5® High Fidelity Polymerase (NEB, M0491S) was tested at the following concentrations: 5µL (1x) of 5x Q5® Reaction buffer, 0.5µL (200µM) of dNTP's, 1 µL of (0.5µM) of forward primer, 1 µL of (0.5µM) of reverse primer, 1µL (~800ng) of DNA template, 0.15 µL of (0.01u) Q5 High-fidelity DNA polymerase and ddH₂O to 25 µL. Where CloneAmp™ HiFi PCR Premix (Clontech, 639298) was used, each assay tube contained the following volumes and concentrations: 10 µL (2x) of CloneAmp HiFi PCR Premix, 0.8 µL (0.5 mM) of forward primer, 0.8 µL (0.5 mM) of reverse primer, 1 µL of DNA template and ddH₂O to 20 µL. Program N from section 4.2.2.2.2.1. was used with various annealing temperatures as two sets of primers were also tested.

Table 17 - Primers used for amplification of gRNA2 (BplI adaptor) with BsmBI hangs and corresponding annealing temperatures (Ta) tested

Primer	Sequence	Ta tested (°C)
gRNA2 BsmBI hg F (47)	TGACCGTCTCCGCGAGGAACGGTAAAAATAATA	43, 58, 60, 62
gRNA2 BsmBI hg R (48)	ACTTTCGTCTCGCGGGTACTGAGAGTGCACCAT	
gRNA2 BsmBI hg2 F (49)	TGACCGTCTCCGCGAGGAACGGTAAAAATAATAACACGAATTATCACAATAAT	58
gRNA2 BsmBI hg2 R (50)	ACTTTCGTCTCGCGGGTACTGAGAGTGCACCATATGCTATTCATC	

4.2.2.2.3. Amplification of gRNA2 with In-Fusion hangs

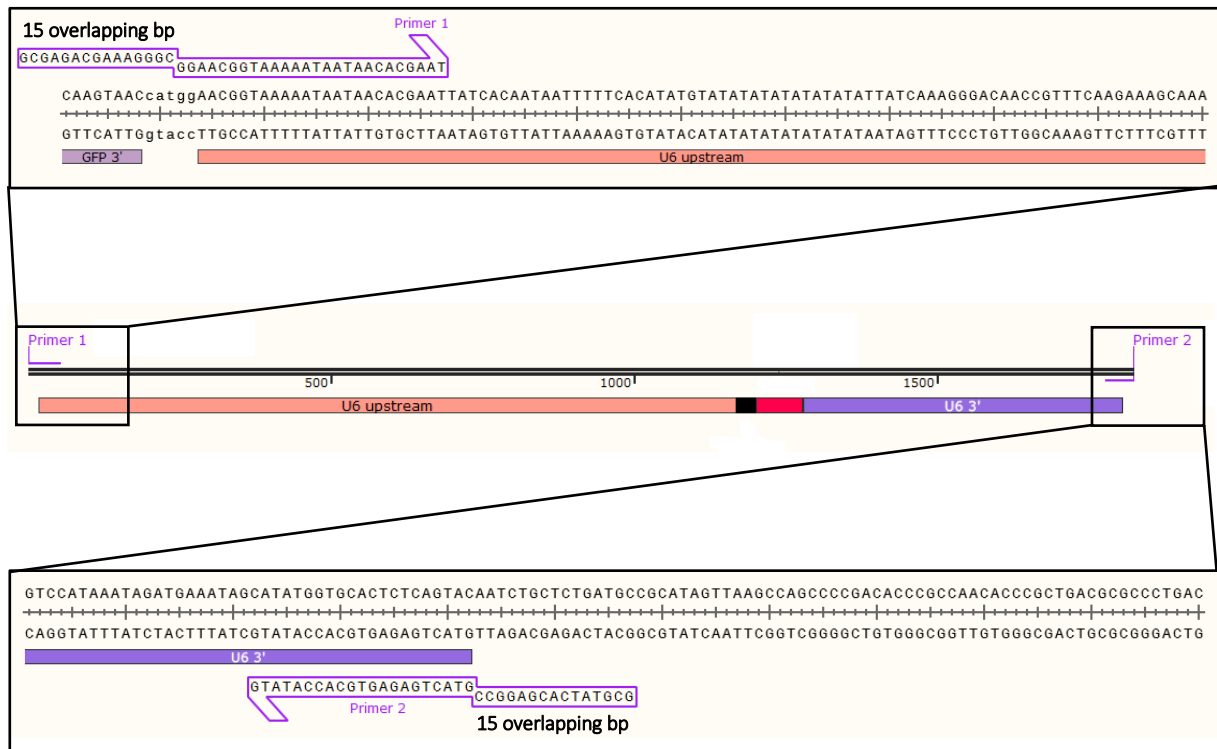


Figure 68 - Schematic view of primers used for amplification of the gRNA DNA sequence from pL6-BpII for In-Fusion cloning. Primer 1 and 2 contained an overhang with the 15 overlapping base pairs necessary for In-Fusion cloning.

Amplification of gRNA2 (BpII adaptor) with In-fusion hangs was tested with program N (4.2.2.2.1) using both CloneAmp™ HiFi PCR Premix (Clontech, 639298) and Q5® High Fidelity Polymerase (NEB, M0491S) with the same concentrations described in the section 4.2.2.2.2..

Table 18 - Primers used for amplification of gRNA2 (BpII adaptor) DNA sequence with In-Fusion hangs and corresponding annealing temperature (Ta) tested

Primer	Sequence
gRNA2 In-Fusion F (41)	TGACCGTCTCCGCGAGGAACGGTAAAAATAATA
gRNA2 In-Fusion R (42)	ACTTTCGTCTCGCGGTTACTGAGAGTGCACCAT

58

4.2.2.2.4. Amplification of gRNA2 and pL6-GFP for In-Fusion PCR cloning

PCR cloning requires linearization of vector by PCR amplification and because the vector in question contained AT-rich sequences, several programs were tested in order to produce an adequate quantity of amplicon. PCR amplification, of both vector and insert, was attempted with Q5® High Fidelity Polymerase (NEB, M0491S), Phusion® High Fidelity Polymerase (NEB, M0530S) and CloneAmp™ HiFi PCR Premix (Clontech, 639298) with the concentrations described in **Table 19**. Two tactics were attempted, insertion of gRNA2 (BpII adaptor) in the anterior region of gRNA1 (*BtgZI* adaptor) – Method 1 (**Figure 69**); and insertion of gRNA2 (BpII adaptor) in the posterior region of gRNA 1 (*BtgZI* adaptor) – Method 2 (**Figure 72**).

Table 19 – Combinations of polymerases and master mixes used in amplification of both vector (pL6-GFP) and insert (gRNA2)

Component	5	6	7	8	9	10	11	Concentration
5x Phusion® HF Buffer	5	5	5	5	-	-	-	1x
5x Phusion® CG Buffer	-	-	-	-	5	-	-	1x
5x Q5® Reaction Buffer	-	-	-	-	-	5	-	1x
HiFi Buffer	-	-	-	-	-	-	12.5	1x
dNTP's	0.5	0.5	0.5	0.5	0.5	0.5	-	0.2mM each
Primer F / R	1	1	1	1	1	-	1	1.25mM
DNA template	0.5	0.5	0.5	0.5	0.5	1	1	~125ng
Phusion® DNA Polymerase	0.25	0.25	0.25	0.25	0.25	-	-	0.1u
Q5® Polymerase	-	-	-	-	-	0.15	-	0.1u
DMSO 3%	-	0.75	-	-	-	-	-	3%
DMSO 5%	-	-	1.25	-	-	-	-	5%
DMSO 7%	-	-	-	1.75	-	-	-	7%
ddH ₂ O	To 25µL							

4.2.2.2.4.1. Method1 (Anterior insertion)

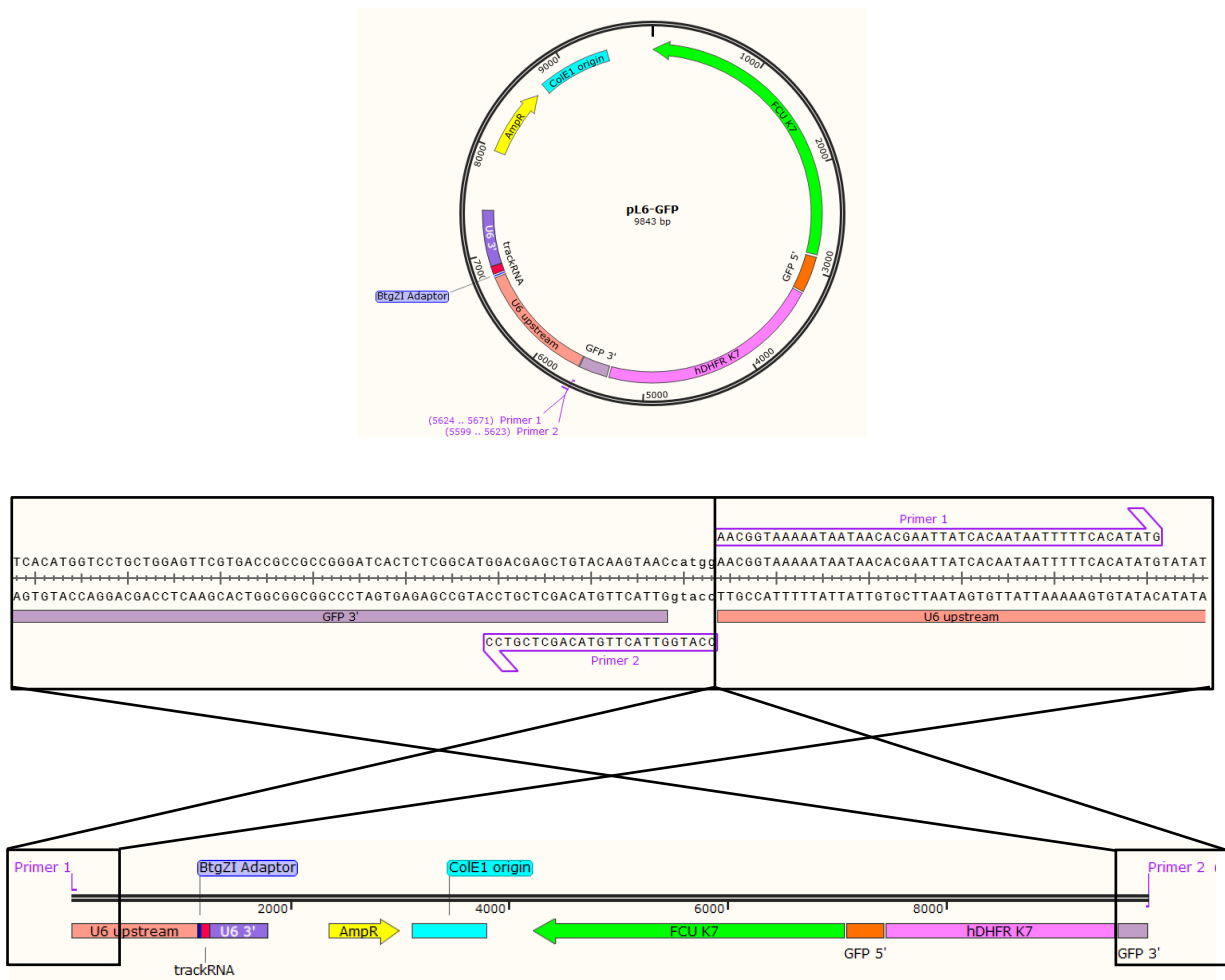


Figure 69 – Linearization of pL6-GFP for In-Fusion PCR cloning. Primers are located at the anterior region of the gRNA (*BtgZ1* adaptor).

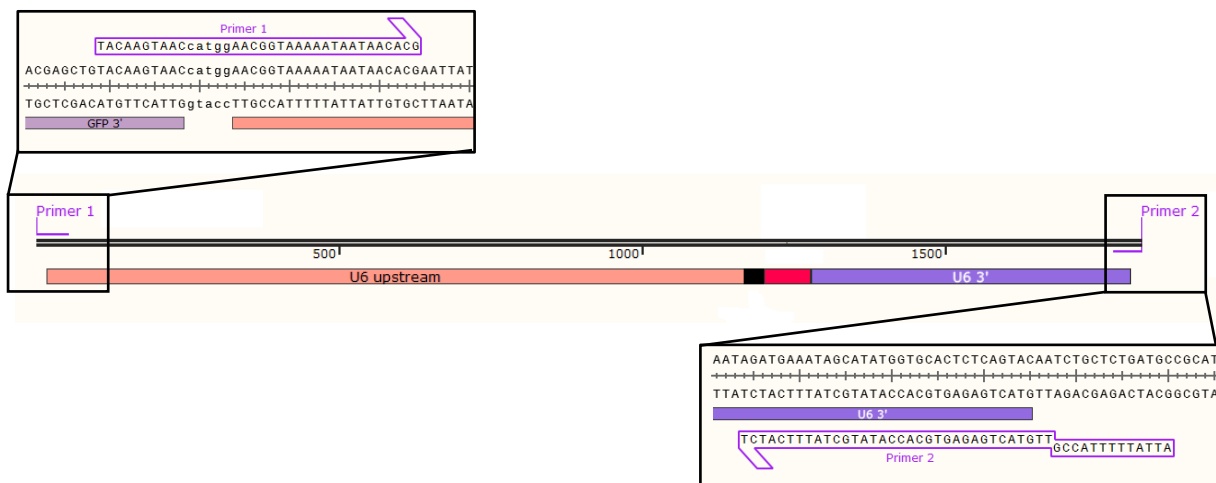


Figure 70 – Amplification of gRNA2 (BplI Adaptor) for In-Fusion PCR cloning. Primers contain the necessary overlapping base pairs necessary for ligation of sequence into the anterior region of gRNA in pL6-GFP.

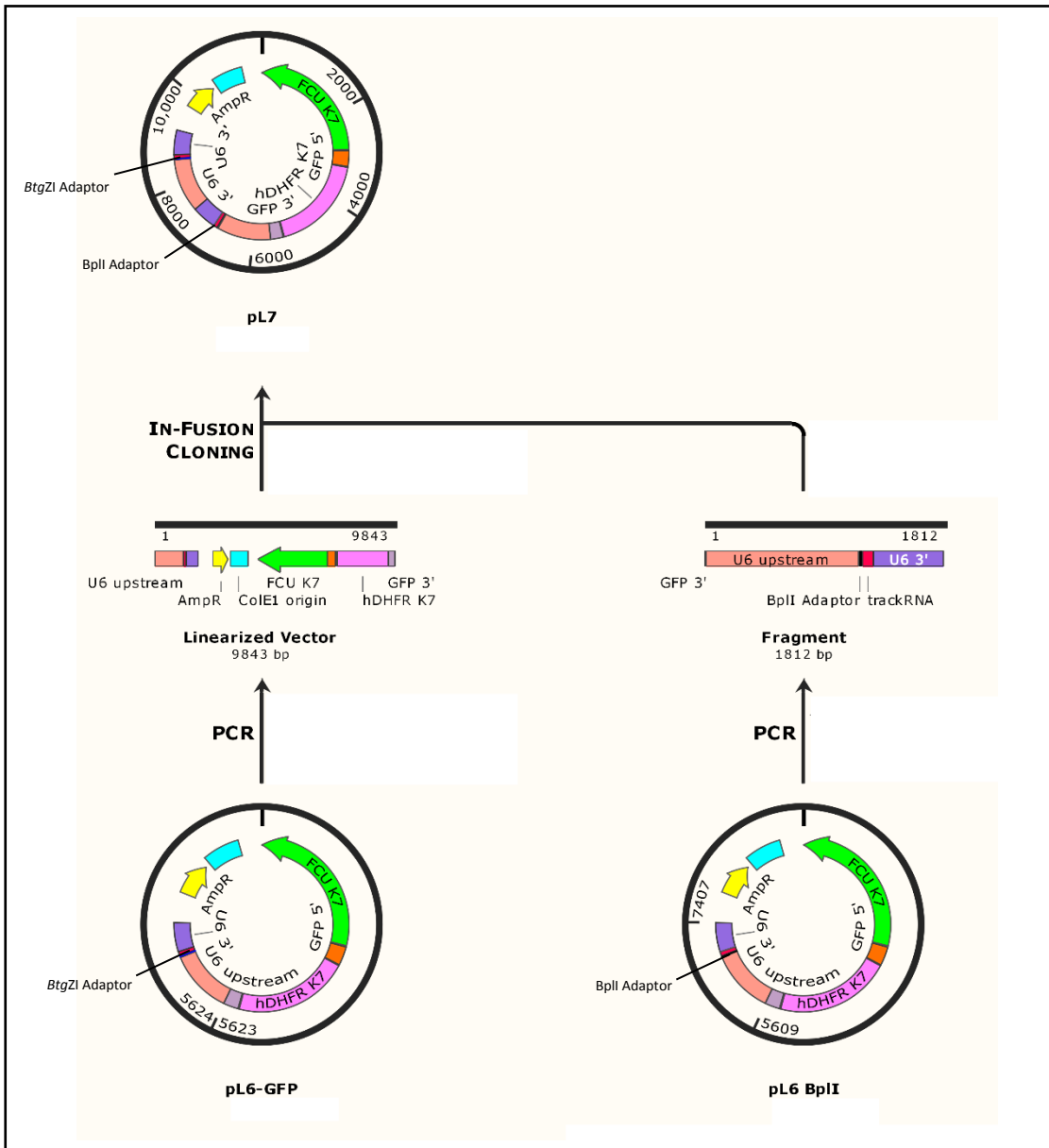


Figure 71 - Schematic view of In-Fusion PCR cloning of gRNA2 (BpII Adaptor) into the anterior region of gRNA (*BtgZI* adaptor) of pL6-GFP creating the double gRNA plasmid pL7.

For method 1, amplification of pL6-GFP (plasmid) and gRNA2 (insert) was carried with the following cycling programs (Table 20) and primers (Table 21).

Table 20 – PCR programs used for amplification of pL6-GFP and gRNA2 into the anterior region of gRNA1

	Plasmid		Insert		
	°C	Δt	°C	Δt	
Initial Denaturation	98	1 m	98	1 m	
35x	Denaturation	98	40 s	98	40 s
	Annealing	58	50 s	58	50 s
	Extension	60	9 m	60	1.3 m
Final Extension	60	20 m	60	72	

Table 21 – Primer sets used for amplification of pL6-GFP and gRNA2 and subsequent cloning of gRNA2 into the anterior region of gRNA1

Primer	Sequence
pL6 Linear BEG F (57)	AACGGTAAAAATAATAACACGAATTATCACAATAATTTTTCACATATG
pL6 Linear BEG R (58)	CCATGGTTACTTGACAGCTCGTCC
gRNA2 In-Fusion BEG F (61)	TACAAGTAACcatggAACGGTAAAAATAATAACACG
gRNA2 In-Fusion BEG R (62)	ATTATTTTACCGTTGACTGAGAGTGACCATATGCTATTTTCATCT

4.2.2.2.4.2. Method2 (Posterior insertion)

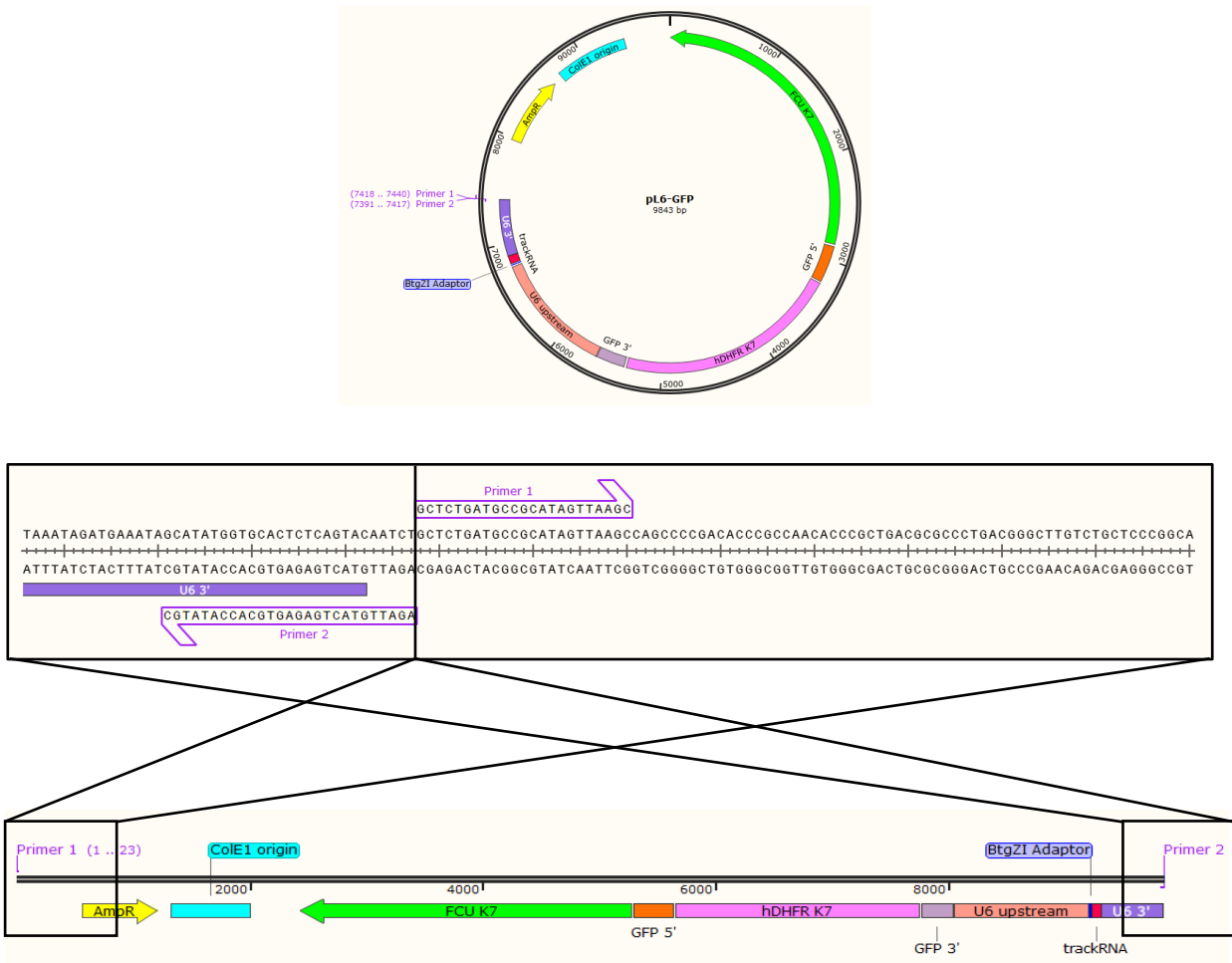


Figure 72 - Linearization of pL6-GFP for In-Fusion PCR cloning. Primers are located at the posterior region of the gRNA (*BtgZI* adaptor).

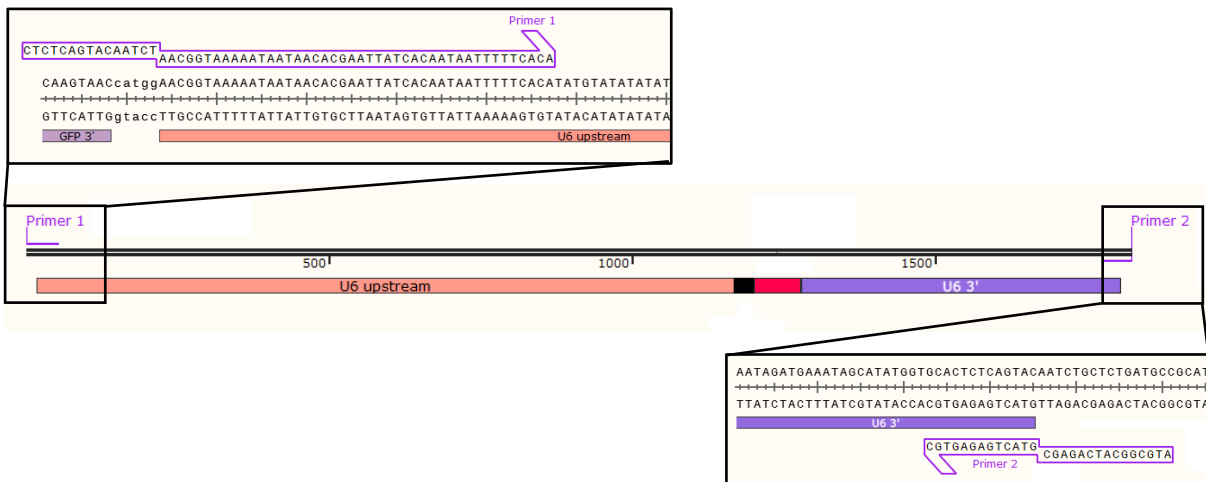


Figure 73 - Amplification of gRNA2 (BplI Adaptor) for In-Fusion PCR cloning. Primers contain the necessary overlapping bp necessary for ligation of sequence into the posterior region of gRNA in pL6-GFP.

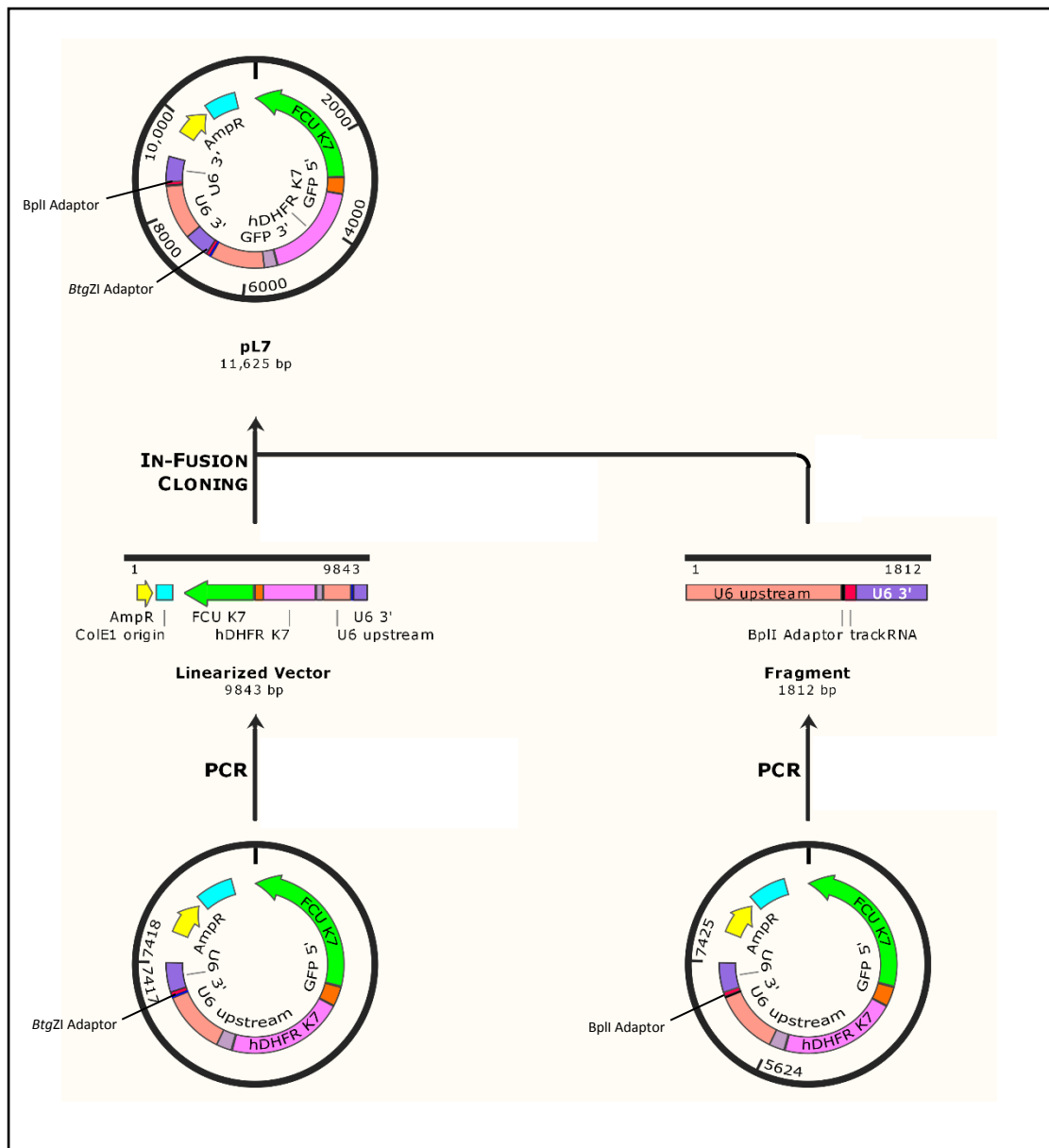


Figure 74 - Schematic view of In-Fusion PCR cloning of gRNA2 (BplI Adaptor) into the posterior region of gRNA (*BtgZI* adaptor) of pL6-GFP creating the double gRNA plasmid pL7.

For method 2, amplification of pL6-GFP (vector) and gRNA2 (insert) was carried with the programs described previously (Table 20) and primers from Table 22.

Table 22 - Primer sets used for amplification of pL6-GFP and gRNA2 and subsequent cloning of gRNA2 into the posterior region of gRNA1

Primer	Sequence
pL6 Linear END F (55)	GCTCTGATGCCGCATAGTTAAGC
pL6 Linear END R (56)	AGATTGTA CTGAGAGTGACCCATATGC
gRNA2 In-Fusion End F (59)	CTCTCAGTACAATCTAACGGTAAAAATAATAACACGAATTATCACAATAATTTTTCACA
gRNA2 In-Fusion End R (60)	ATGCGGCATCAGAGCGTACTGAGAGTGC

For both methods, the results were visualised by running a 0.7 % gel electrophoresis and desired bands isolated. Purification of DNA was carried with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, 740609) according to manufacturer’s instructions.

4.2.2.2.2.5. Synthesis of gRNA2 with In-Fusion hangs

Because amplification of gRNA2 DNA was problematic, synthesis of the fragment was outsourced from GeneArt® Services at Thermo Fisher Scientific Inc. by submission of gRNA2 with In-Fusion hangs full sequence.

4.2.2.2.2.6. In-Fusion ligation of vector and insert

To ligate the gRNA2 to pL6-GFP the following components were added to a 0.5 mL tube: 50ng of linearized/purified pL6-GFP, 30 ng of gRNA, 2 µL of 5x In-Fusion HD Enzyme Premix and ddH₂O to 10 µL. The tube was then incubated at 50 °C for 15 minutes followed by transformation with XL10-Ultracompetent Cells (Stratagene) according to manufacturer’s instructions. Positive colonies were picked and grown overnight at 37 °C with shaking at 220 rpm followed by plasmid extraction with NucleoSpin® Plasmid (Macherey-Nagel, 740588.250) according to manufacturer’s instructions.

To test for insertion, the resulting plasmid was digested with BpII (ThermoFisher, ER1311) by combining, in a 0.5mL tube, 6 µL of 10x buffer Tango, 0.4 µL of 50x SAM, 1µg of DNA, 1µL of

BpII and ddH₂O to 20µL. The tube was then incubated at 37 °C for 4h followed by inactivation at 65 °C for 20 minutes. Results were observed by running a 0.7% agarose gel.

4.2.2.2.7. Standard ligation

To ligate the gRNA2 into pL6-GFP, using a standard ligation with T4 DNA ligase (NEB, M0202), the following components were added to a 0.5 mL tube: 50ng of linearized/purified pL6-GFP, 30 ng of gRNA, 2 µL of 10x T4 DNA ligase buffer and ddH₂O to 10 µL. The tube was then incubated at room temperature for 15 minutes followed inactivation at 65 °C for 10 minutes. Transformation was carried with XL10-Ultracompetent Cells (Stratagene) according to manufacturer's instructions. Positive colonies were picked and grown overnight at 37 °C with shaking at 220 rpm followed by plasmid extraction with NucleoSpin® Plasmid (Macherey-Nagel, 740588.250) according to manufacturer's instructions. Insertion was tested as described previously (section 4.2.2.2.6.).

4.2.2.2.8. Cloning outsourcing

Because cloning of the gRNA2 sequence was attempted in a variety of ways with no success, one last effort was made by outsourcing cloning of the gRNA2 with BsmBI hangs amplified from pL6-BII into pL6-GFP (Section). The following products were sent to Dundee Cell Products for cloning: uncut pL6-GFP (150ng/µL); purified gRNA2 with BsmBI hangs (70 ng/µL); Primers P49 and P50 (10pmol/µL).

4.3. Results

4.3.1. Approach 1

4.3.1.1. Annealing the guide oligonucleotides

Although there was no direct way to test for successful oligonucleotide annealing, it was assumed that all three methods resulted in very poor annealing. This was assumed based on cloning results, where all attempts to clone the guide oligonucleotide failed. Digestion of pL6-GFP with *BtgZI* was successful (**Figure 75A**), cloning reactions produced positive colonies, and PCR's assays employed to check for insertion produced the desired amplicon (**Figure 75B**). However, when these fragments were sent for sequencing the obtained sequences showed the *BtgZI* adaptor was still in place and had not been replaced with the guide oligo.

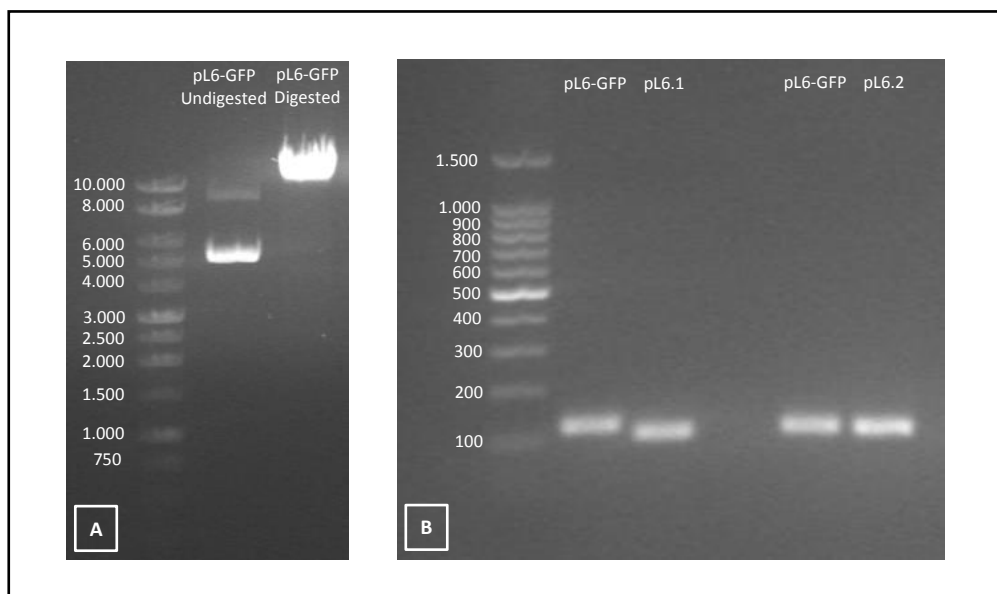


Figure 75 – Agarose gels of pL6-GP and pL6.1/6.2. **A.** Digestion of pL6-GFP with *BtgZI* (0.7% agarose gel), where first band shows undigested plasmid and second band the linearized plasmid. **B.** PCR amplification of pL6.1 guide sequences for posterior sequencing (2% agarose gel).

4.3.1.2. Standard cloning of *Bcl*I restriction site into pL6.1

Insertion of the *Bcl*I restriction site was attempted several times but was always unsuccessful. Cloning with $\text{Dam}^-/\text{Dcm}^-$ competent cells yielded a few positive colonies that were tested by PCR (**Figure 76A**) and enzymatic digestion (**Figure 76B**). Both methods showed that the restriction oligonucleotide failed to be inserted.

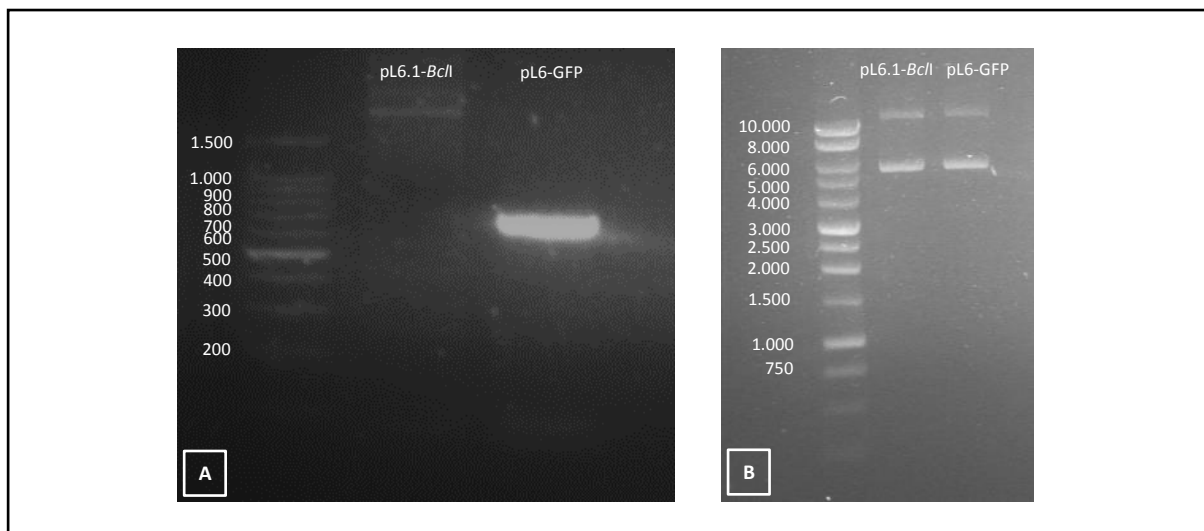


Figure 76 - Agarose gels of pL6-*Bcl*I. **A.** PCR amplification of pL6-*Bcl*I and pL6-GFP with primers designed to check for *Bcl*I insertion. PCR amplification of pL6.1-*Bcl*I plasmid did not produce any detectable amplicon, contrary to pL6--GFP which yielded a defined band of 650bp. **B.** Digestion of pL6-*Bcl*I and pL6-GFP with *Bcl*I where neither plasmids were digested.

4.3.2. Approach 2

4.3.2.1. Cloning *Bp*II adaptor

Because cloning of guide sequences was unsuccessful, it was decided that the *Bp*II adaptor would be synthesised instead of annealed. After digestion of DNA fragment from donor plasmid and In-Fusion cloning into pL6-GFP many colonies were obtained. After PCR amplification, insertion was validated by sequencing, which showed that the *Btg*ZI adaptor had been successfully replaced with the *Bp*II adaptor, thus creating pL6-*Bp*II.

4.3.2.2. Amplification and cloning of gRNA2 sequence

The next step was to amplify the second gRNA (gRNA2) from pL6-BpII so it could be ligated into pL6-GFP creating a double gRNA system. For this, several methods and attempts were made as this stage proved to be unexpectedly challenging.

4.3.2.3. Amplification of gRNA2 with *Bg*III 5' and a *Eco*O109I 3' hangs

Amplification of gRNA2 with *Bg*III 5' and *Eco*O109I 3' hangs was attempted with both Phusion and Taq polymerases with varied concentrations and with different PCR programs. In general, all amplifications failed with exception of those in which the extension temperature was 60 °C - Program N (Table 23).

Table 23 – Results of PCR amplification of pL6.1 with primers P11/P12 and P11/P24 using different polymerase kits, concentrations and PCR programs.

Primer	Polymerase	Mix Comb.	Program	Result
P11 / P12	Phusion	1	I	☒
	Taq	1	J	☒
		2	J	☒
		3	J	☒
		4	J	☒
		1	K	☒
P11 / P24	Phusion	1	N	☒
	Taq	1	M	☒
		1	L	☒
		1	N	☑

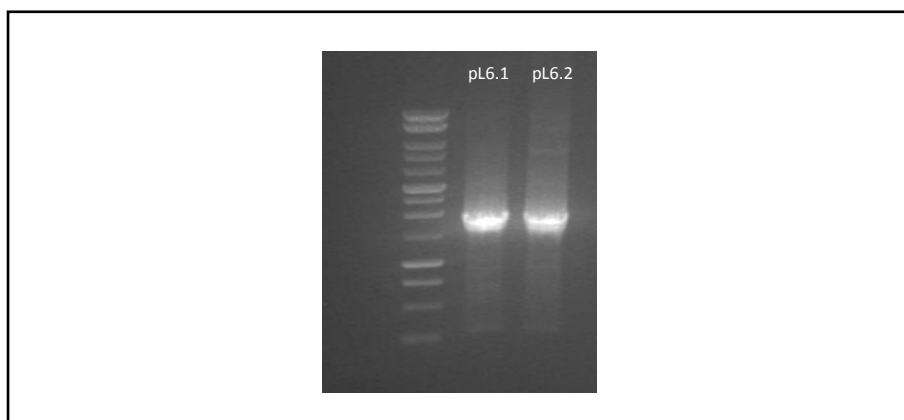


Figure 77 – PCR amplification of pL6.1 and pL6.2 with primers P11/24 and Taq polymerase with cycling program N, producing a band of approximately 1800bp.

4.3.2.4. Amplification of gRNA2 with BsmBI hangs

Amplification of gRNA2 with BsmBI hangs from pL6-BpII was attempted with cycling program N and a wide range of polymerases. Amplification with Q5 at 58 °C produced very faint bands (**Figure 78A**). However, when the same cycling program was used but with the HiFi CloneAmp polymerase, amplification was improved producing high amounts of specific amplicon (**Figure 78B**).

Table 24 - Results of PCR amplification of pL6-BpII with primers P47/P48 and P49/50 using different polymerase kits, concentrations and PCR programs.

Primer	Polymerase	Ta tested (°C)
P47 / P48	Q5	43 <input type="checkbox"/> ; 58 <input checked="" type="checkbox"/> ; 60 <input type="checkbox"/> ; 62 <input type="checkbox"/>
	Phusion	58 <input type="checkbox"/> ; 60 <input type="checkbox"/> ; 62 <input type="checkbox"/>
	Taq	58 <input type="checkbox"/> ; 60 <input type="checkbox"/> ; 62 <input type="checkbox"/>
	HiFi	58 <input checked="" type="checkbox"/>
P49/ P50	Q5	58 <input type="checkbox"/>
	Phusion	58 <input type="checkbox"/>
	HiFi	58 <input checked="" type="checkbox"/>

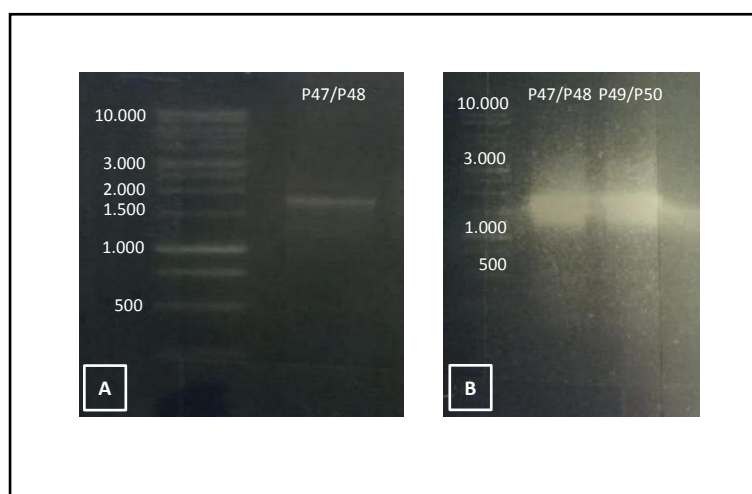


Figure 78 - PCR amplification of gRNA2 from pL6-BpII. **A.** Amplification with primers P47/48 with Q5 polymerase at 58 °C producing a band of approximately 1800bp. **B.** Amplification with P47/45 and P49/P50 with HiFi at 58 °C producing a band of approximately 1800bp.

4.3.2.5. Amplification of gRNA2 with In-Fusion hangs

Amplification of pL6-BpII with In-Fusion hangs with cycling program N and Q5 polymerase produced a few unspecific bands along with a bright band of approximately 1800bp (**Figure 79**).

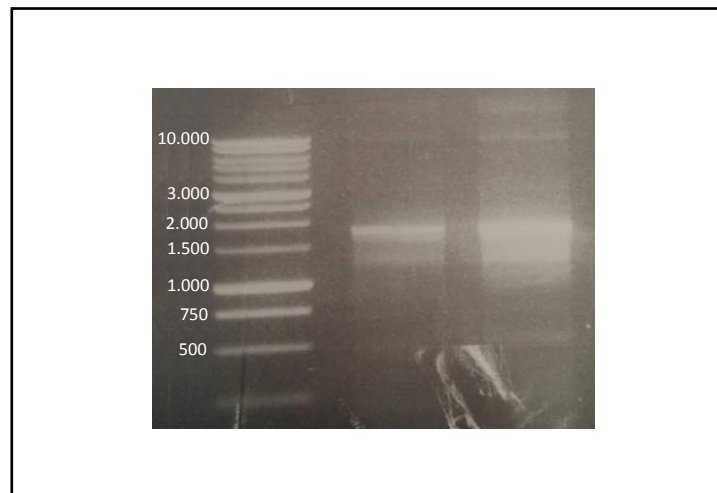


Figure 79 - PCR amplification of gRNA2 from pL6-BpII primers P41/42 with Q5 polymerase at 58 °C producing a main band of approximately 1800bp and other unspecific faint bands.

4.3.2.6. Amplification of gRNA2 and pL6-GFP for In-Fusion PCR cloning

4.3.2.6.1. Method 1 (Anterior insertion)

For PCR In-Fusion with method 1, where the gRNA2 was to be inserted in the anterior region of pL6-GFP, three types of polymerases were tested, each with a variety of reagent mix combinations (**Table 25**). Amplification of the whole plasmid (pL6-GFP) with primer P57/P58 was expected to produce a fragment of approximately 9900bp. With Phusion polymerase this was only possible with mix combination 8, which included 7% of DMSO (**Figure 80A**). However, this led to simultaneous amplification of many unspecific bands. With CloneAmp HiFi, however, not only amplification was successful, but also produced a single, specific, band (**Figure 80B**). The same was observed during amplification of the insert, gRNA2 sequence from

pLBpII, where the desired DNA fragment was obtained only with CloneAmp HiFi polymerase (Figure 80C).

Table 25 – Amplification results of PCR assays carried in method 1. For each primer set different polymerases were tested with varied reagent mix combinations.

Primer	Polymerase	Mix Comb.	Result
P57 / P58 (plasmid)	Phusion	5	✗
		6	✗
		7	✗
		8	✓
		9	✗
	Q5	10	✗
	HiFi	11	✓
P61 / P62 (insert)	Phusion	5	✗
		6	✗
		7	✗
		8	✗
		9	✗
	Q5	10	✗
	HiFi	11	✓

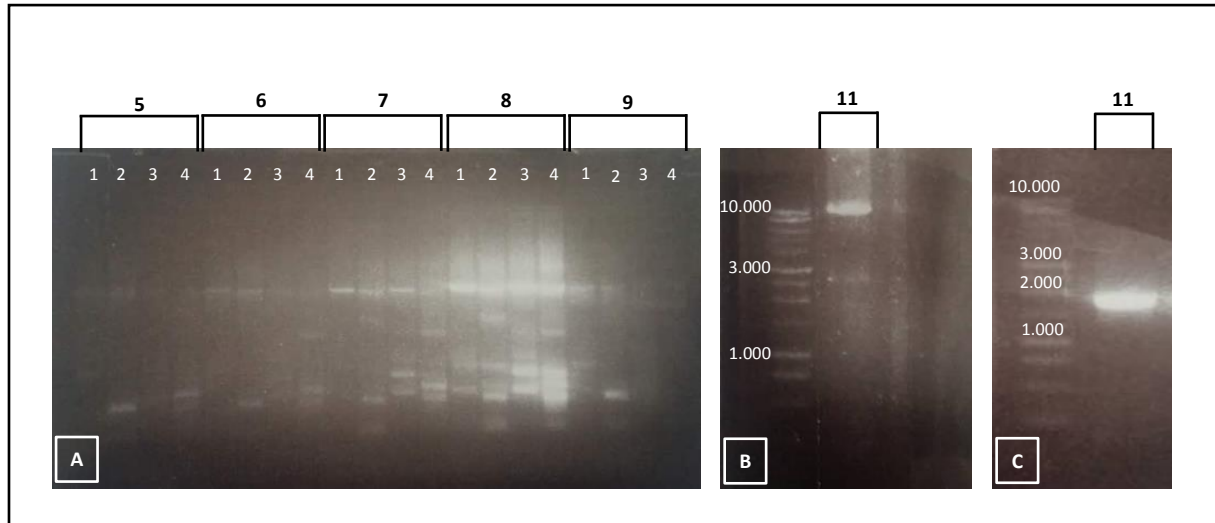


Figure 80 - PCR amplification of pL6-BpII (Plasmid) and gRNA2 (Insert). **A.** Amplification of pL6-GFP with primers P57/P58 (method 1) and P55/56 (method 2). Assay was carried with Phusion polymerase with mix combinations 5 to 9. Samples 1 and 2: pL6-GFP amplified with primers P57/58 (method 1); Samples 3 and 4: pL6-GFP amplified with primers P55/P56 (method 2). Despite DNA Ladder not being visible, the bands of interest (top bands) were of approximately 10.000bp. **B.** Amplification of pL6-GFP with primers P57/P58 (method 1) and CloneAmp HiFi polymerase producing a band of approximately 10.000 bp. **C.** Amplification of gRNA2 from pL6- BpII with primers P61/P62 and CloneAmp HiFi polymerase.

4.3.2.6.2. Method 2 (Posterior insertion)

For PCR In-Fusion with method 2, where the gRNA2 was to be inserted in the posterior region of pL6-GFP, the same combinations used in method 1 were applied and the results were fairly similar (**Table 26**). However, amplification of the whole plasmid (pL6-GFP) was only successful with the Phusion polymerase with mix combination 8 (7% DMSO) (**Figure 81**). Unlike with the previous method, CloneAmp HiFi failed to produce any detectable amplification of the whole pL6-GFP plasmid. The amplification results of gRNA2 were the same as described for method 1. Amplification was only possible with CloneAmp HiFi polymerase.

Table 26 - Amplification results of PCR assays carried in method 2. For each primer set different polymerases were tested with varied reagent mix combinations.

Primer	Polymerase	Mix Comb.	Result
P55 / P56 (plasmid)	Phusion	5	✗
		6	✗
		7	✗
		8	✓
		9	✗
	Q5	10	✗
	HiFi	11	✗
P59 / P60 (insert)	Phusion	5	✗
		6	✗
		7	✗
		8	✗
		9	✗
	Q5	10	✗
	HiFi	11	✓

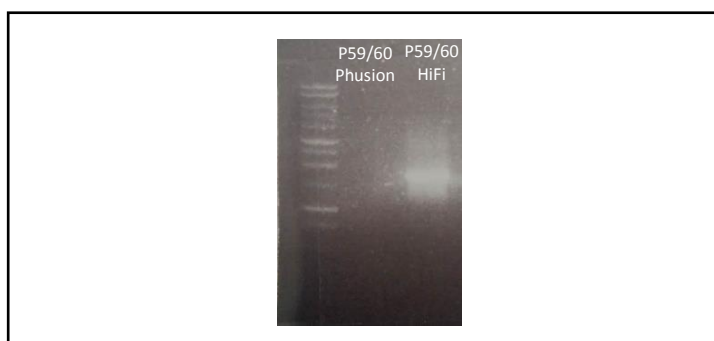


Figure 81 - Amplification of gRNA2 with primers P59/P60 for anterior insertion into pL6-GFP. Amplification was only successful with HiFi polymerase, producing a band of approximately 1800bp.

4.3.2.7. Synthesis of gRNA2 with In-Fusion hangs

Synthesis of the gRNA2 DNA fragment by GeneArt® Thermo Fisher was unsuccessful as AT content was too elevated for synthesis.

4.3.2.8. Ligation of gRNA2 into pL6-GFP

All attempts to ligate the gRNA2 fragment into pL6-GFP were unsuccessful. Both In-Fusion and standard T4 ligation failed to produce a ligated version of the plasmid. The same was described by the Dundee Cell Products, the company

4.4. Discussion

4.4.1. Approach 1

4.4.1.1. Annealing the guide oligonucleotides vs DNA fragment synthesis

The annealing of guide and restriction oligonucleotides was proven to be difficult and in all cases futile. Several attempts were made with different methods but all ultimately failed to be cloned. This led to the idea of ordering the oligonucleotides as synthesised DNA fragments.

The desired fragments were designed with flanking restriction sites compatible with the preferred cloning method. Because the synthesised DNA is cloned and delivered into a donor plasmid, this means that after propagation of plasmid all that is needed is to digest the DNA fragment from the donor plasmid. In this way not only many copies of the fragment can be obtained, but also the resulting fragments are more stable. In addition, and despite not being described in this work, copies of the fragment can easily be attained in high quantities by PCR amplification of the plasmid. Another factor to consider is that oligonucleotide annealing is impossible to validate, and so one must go through the whole cloning process in order to determine if annealing was indeed successful. With synthesised fragments this is not an issue, as not only fragments are undoubtedly annealed but also can be amplified and visualised.

In this work, DNA synthesis was noticeably much more efficient, with positive colonies obtained at first try. Despite being costly, DNA synthesis provides an extremely reliable method of DNA fragment production.

4.4.1.2. Standard cloning of *Bcl*I restriction site into pL6.1

Failure to insert the *Bcl*I restriction oligonucleotide could be associated with several difficulties. Considering that double restriction was necessary, it is possible that one or both enzymes used did not digest the fragment efficiently. However, considering the results from section 4.3.1.1. with guide oligonucleotides, the more likely justification is that the oligonucleotide was not successfully annealed, thus leading to failure of the whole process.

4.4.2. Approach 2

4.4.2.1. Cloning BpII adaptor

As mentioned in section 4.3.2.1., cloning of BpII adaptor was easy and straight forward as many positive clones were obtained at first try. This was a refreshing surprise as much time and effort had been spent with attempts to clone annealed oligonucleotides. The convenience that DNA synthesis provides over the alternative of annealing oligonucleotides has already been discussed in section 4.4.1.1.

4.4.2.2. Amplification of gRNA2 sequences

Overall, amplification of gRNA2 sequences was somewhat challenging. Many polymerases had to be tested, and with a variety of different protocols. This was mainly associated with the AT content of the gRNA2 DNA sequence, which was extremely high at 84%. *Plasmodium falciparum* has a genome with tremendously high AT content (>80%) making molecular studies of the genus a particularly difficult endeavour (Gardner, Hall et al. 2002). In this work, from all PCR assays tested, the only ones providing reliable amplification of *Plasmodium* DNA were the ones where the extension step was carried at 60 °C (Su, Wu et al. 1996). For amplification of regular *Plasmodium* sequences (not shown in this chapter), this setup accompanied with standard polymerases was sufficient for successful amplification of desired fragments. However, the same was not observed with the gRNA2 fragment demonstrating that this was a particularly challenging sequence. For that reason, outsourcing of DNA synthesis of the fragment was also attempted but equally with no success. After combining many polymerases with different cycling programs, the only reliable way to achieve amplification of the difficult gRNA2 DNA sequence was to use the CloneAmp™ HiFi PCR premix by Clone Tech together with cycling program N (extension at 60°C). This cycling program, already described by Su *et al.* (1996) was proven to be very efficient in amplification of *Plasmodium* DNA, however, when coupled with the CloneAmp HiFi polymerase, it was shown to be an exceptionally accurate and efficient method of DNA amplification with high sensitivity, specificity, and extension efficiency.

4.4.2.3. Cloning of gRNA2 sequence

Cloning the gRNA2 sequence into pL6-GFP was unsuccessful despite the many attempts with several different approaches (Restriction, In-Fusion, PCR In-Fusion, Outsourcing). A plausible explanation as to why this occurred could be, once again, related to the AT-richness of the gRNA2 DNA fragment. It has been reported that many *E. coli* strains with GC-rich genes seem to have better fitness, demonstrating that there might be an underlying selective bias toward higher CG contents (Raghavan, Kelkar et al. 2012). Consequently, bacteria have evolved to maintain this ratio and minimise the harmful effects of foreign genes with high AT content. This can be achieved by xenogeneic silencing proteins that bind to incoming AT-rich DNA and silence transcription (Singh, Milstein et al. 2016). When cells lack these proteins, AT-rich genes end up causing cell toxicity by sequestration of RNA polymerase. Because AT-rich sequences contain a high number of promotor like regions, toxicity arises from constitutive transcription initiation of the coding regions. If left unhindered, the amount of available RNA polymerase decreases leading to a downshift of host's gene expression and therefore low fitness (Lamberte, Baniulyte et al. 2017). A double gRNA plasmid would have an AT content of approximately 84% thus constituting an extremely AT-rich plasmid. It is plausible to assume that this is the reason why cloning of this plasmid in particular was unsuccessful.

4.4.2.4. Failure to develop a double guide system

Due to failure of integrating the second gRNA sequence, a double CRISPR/Cas9 system was not achieved, and genome editing was not carried. The alternative to this issue could have been the use of two CRISPR/Cas9 plasmids, each containing a different gRNA sequence necessary for double cleavage. However, in the author's opinion, this would be much less likely to succeed for several reasons. In order to implement this approach, a transfection of three plasmids (gRNA1, gRNA2 and Cas9) would have been necessary, making it less likely to get a population of parasites with all three plasmids. In addition, each plasmid would require a different drug-selectable marker in order to select parasites carrying all plasmids. In the unlikely event that all plasmids are transfected, there is still no guarantee that all will be expressed simultaneously, rendering this approach unlikely to succeed.

4.5. Conclusion

Despite unsuccessful development of a double guide CRISPR/Cas9 system for *P. falciparum*, this work allowed the significant improvement of two standard techniques required for routine molecular work on *Plasmodium*. Amplification of *Plasmodium*'s AT-rich genome can be challenging. Nonetheless, with this work it was shown that by simply combining the CloneAmp™ HiFi polymerase with a cycling program in which the extension step is carried at 60 °C, even fragments with 85% AT content can be successfully amplified. In addition, and because many experiments require cloning of small DNA fragments, such as restriction sites or guide sequences (CRISPR/Cas9 system), many labs rely on oligonucleotide synthesis and posterior annealing. However, it was shown here that synthesis of these fragments ordered as dsDNA fragments incorporated into a plasmid, provide a much easier and reliable alternative.

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5.

PLASMODIUM DETECTION IN FAECAL SAMPLES

5.1. Introduction

Plasmodium parasites affect a wide range of vertebrate species including birds (Valkiūnas 2005). Avian *Plasmodium* species have a cosmopolitan distribution and can impact avian populations in a variety of ways (Delhaye, Jenkins et al. 2016, Paxton, Camp et al. 2016). Avian malaria infections include two stages: acute and chronic. The acute stage begins right after infection and is characterised by an elevated parasitemia with hosts presenting symptoms such as lethargy, weight loss and sometimes even death (Williams 2005). Surviving hosts experience a chronic stage characterised by a low level of parasitemia and some seasonal relapses (Lapointe, Atkinson et al. 2012). Both stages affect the individual's survival. During the acute stage the host may have difficulty foraging and escaping predators, while during the chronic stage, despite symptoms being less pronounced there is still a significant impact on the individual's fitness (Knowles, Palinauskas et al. 2010).

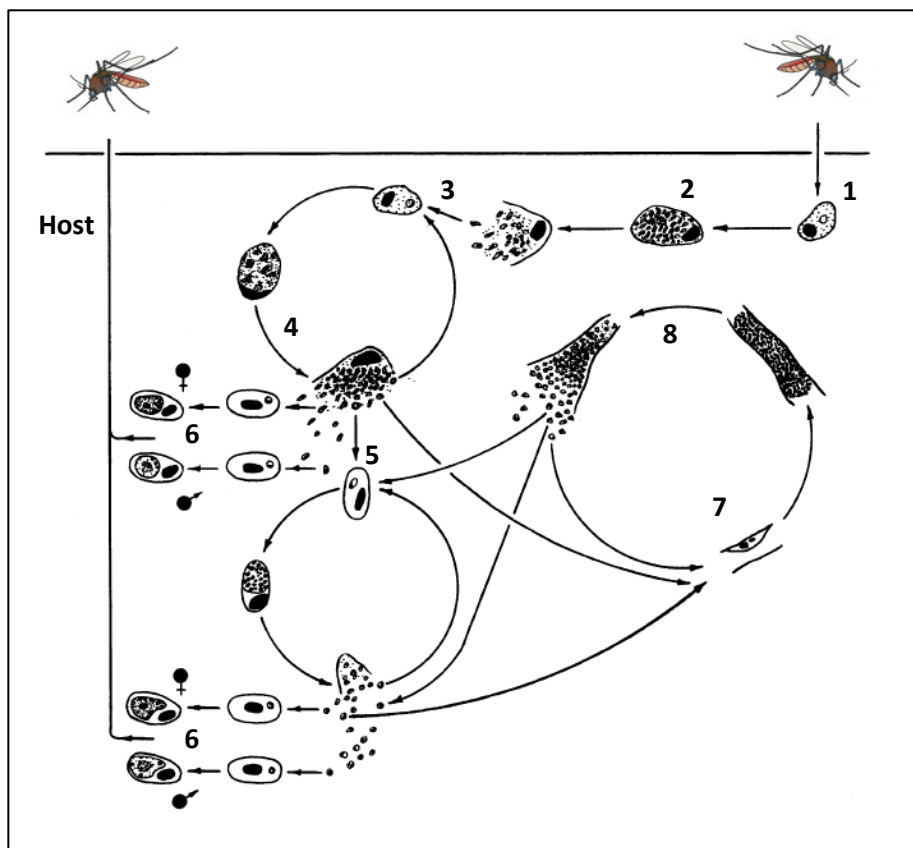


Figure 82 – Life cycle of *Plasmodium relictum*, one of the causative species of avian malaria (Valkiūnas 2005). 1. Sporozoite; 2. Cryptozoites; 3. First generation merozoites; 4. Metacryptozoite; 5. Merozoites; 6. Gametocytes; 7. Infected endothelial capillary cell; 8. Phanerozoites.

The life cycle of avian malaria is slightly different from its mammalian counter parts. Using *Plasmodium relictum* as an example (**Figure 82**), infection starts when an infected mosquito feeds from a bird depositing sporozoites (**Figure 82.1**) that instead of migrating to the liver, give rise to the first generation of exoerythrocytic meronts known as cryptozoites (**Figure 82.2**). This form is capable of developing in reticular cells of many organs including the skin. Cryptozoites produce small amounts of a first generation of merozoites (**Figure 82.3**), which at this stage are not capable of infecting blood cells. The first generation of merozoites induce the development of a second generation called metacryptozoites (**Figure 82.4**) that can only develop inside macrophages. These will produce higher amounts of merozoites (**Figure 82.5**) which are now capable of infecting blood cells and turning into gametocytes (**Figure 82.6**) (Valkiūnas 2005). The merozoites are also capable of infecting endothelial cells of capillaries (**Figure 82.7**) in many organs and then mature into phanerozoites (**Figure 82.8**).

Plasmodium belongs to the order *Haemosporida* characterised by heteroxenous protists that infect amphibians, reptiles, birds, and mammals and use blood-sucking dipteran insects as vectors. Haemosporidians include many diseases that infect domestic birds affecting their productivity and even lead to high mortality rates, which in turn can have a potential devastating economic impact. Amongst these pathogens, the most common are *Leucocytozoon* spp., *Haemoproteus* spp. and *Plasmodium* spp. and all can produce malaria-like symptoms (Sehgal 2015).

Bird haemosporidians were the first models in the study of human malaria and it was with these that the first investigations on life cycles, in vitro cultivation and vaccination were advanced (Valkiūnas 2005). However, with attention focused mainly on human *Plasmodium*, the practical importance of bird haemosporidians became underrated. This is also correlated with the fact that birds are especially difficult to sample in the wild and in some cases it is even forbidden due to protection laws. As a result there is a lack of molecular studies in prevalence and diversity of avian malaria haemoparasites in avian populations (Braga, Silveira et al. 2011). This chapter will be focused on detection of *Plasmodium* spp. in faecal samples of rodents and birds with the ultimate goal of developing a method for detection avian *Plasmodium* species from bird faecal samples.

5.2. Methods

5.2.1. Biological material

5.2.1.1. Rodent faeces from non-infected animals

Samples were acquired from the Bangor University's rodent facility where non-infected healthy BALB/c mice were reared for the purpose of snake feeding. Samples were collected in the same day as cages were cleaned in order to ensure collected samples were not older than 24h.

5.2.1.2. Rodent faeces from infected animals

Faecal samples from BALB/c mice infected with *P. berghei* were provided by the Sinden Lab, Imperial College London, UK.

5.2.1.3. Faeces and blood from wild birds

Samples from wild birds were collected from animals admitted to the Centre for Wild Animal Recovery of the Monsanto Forest Park (Lisbon, Portugal). A total of 11 birds (**Table 27**) were surveyed and from each, one blood sample and three faecal samples were collected. Faecal and blood collections were carried by staff at the centre.

For blood sampling, a few drops were collected into an Eppendorf tube pre-filled with RNAlater stabilization solution (25 mM sodium citrate, 10 mM EDTA, 70 g ammonium sulphate/100 ml) and tubes were kept in the freezer until shipping.

For faecal sampling, three samples were collected with a 24-hour interval between collections and stored in RNAlater stabilization solution in the freezer until shipping.

Table 27 - List of birds surveyed from which blood and faecal samples were collected

#Subject	Species	Reason for admittance
1	<i>Buteo buteo</i>	Debilitation
2	<i>Falco tinnunculus</i>	Trauma (collision)
3	<i>Larus ridibundus</i>	Trauma (unkown)
4	<i>Larus michahellis</i>	Trauma (unkown)
5	<i>Corvus corone</i>	Debilitation
6	<i>Buteo buteo</i>	Gunshot
7	<i>Larus sp.</i>	Trauma (unkown)
8	<i>Accipiter gentilis</i>	Not available
9	<i>Buteo buteo</i>	Not available
10	<i>Larus sp.</i>	Intoxication
11	<i>Strix aluco</i>	Trauma (Collision)

5.2.1.4. *Plasmodium falciparum* DNA

The WHO international standard for *P. falciparum* DNA nucleic acid amplification technology (NAT) essays, obtained from the National Institute for Biological Standards and Control (NIBSC, UK) was used as positive control and calibration reference.

5.2.2. DNA and RNA extractions

5.2.2.1. DNA extraction from Faecal samples with Qiagen DNeasy Blood and Tissue kit

Extraction of DNA from faecal samples with the aid of the Qiagen DNeasy® blood and tissue kit followed the manufacturer's protocol with some modifications.

Approximately 25mg of faecal material was incubated for 5 minutes in 50 µL of PBS and 100 µL of ATL buffer. With the aid of a pestle, the material was macerated as much as possible and 20 µL of proteinase K were added. The sample was then incubated in a heating block at 56 °C for 1 hour. After incubation, 200 µL of 100% ethanol was added to the sample and vortexed thoroughly. The tube containing the sample was then centrifuged for 20 seconds at 400 rpm in order to precipitate the larger fragments of faecal matter. The supernatant was transferred into a spin column with respective collection tube and centrifuged at 8000 rpm for 1 minute. The resulting flow-through was discarded and 500 µL of AW1 buffer was added to the column, followed by centrifugation at 6000 rpm for 1 min. Once again, the flow-through

was discarded and 500 µL of AW2 buffer was added to the column followed by centrifugation at 14000 rpm for 3 min. The flow-through was discarded and the column transferred to a clean Eppendorf tube. Finally, 70 µL of AE buffer was added to the column followed by centrifugation at 8000 rpm for 1 min. The eluted DNA was then stored at – 20 °C until needed.

5.2.2.2. DNA extraction from Faecal samples with Zymo Research Fecal DNA kit

DNA extraction with the Zymo Fecal DNA kit was carried exactly as per manufacturer's instructions.

5.2.2.3. RNA extraction from Faecal samples with Promega SV Total RNA Isolation system

RNA extraction with the SV Total RNA Isolation kit was carried exactly as per manufacturer's instructions.

5.2.2.4. DNA extraction from *P. falciparum* blood standard with Qiagen DNAeasy® blood and tissue kit

DNA extraction of the *P. falciparum* NAT was extracted with Qiagen DNAeasy® blood and tissue kit as per manufacturer's instructions.

5.2.3. Template amplification from faecal material: finding a reliable technique

Four amplification methods were initially tested in order to find the most reliable and consistent detection method. The DNA/RNA used in these assays was extracted from faecal samples of BALB/c mice infected with *P. berghei* from which parasitemia was confirmed with blood smears.

5.2.3.1. PET-PCR (Photo-Induced Electron Transfer PCR)

The PET-PCR was carried using QuantiTect™ Probe PCR kit (Qiagen, 204343) by adding the following volumes: 12.5 µL of 2x QuantiTect™ Probe Master Mix, 0.5 µL (0.4 mM) of both

forward primer and reverse primers (**Table 28**), 2 μL of template and ddH₂O to 25 μL . The cycling parameters comprised an initial denaturation step at 95 °C for 15 minutes, 45 cycles of denaturation at 95 °C for 15 seconds and annealing at 56 °C for 1 minute. Reactions were performed in a Bio-Rad CFX96™ Real Time PCR detection system with the fluorophore channel for FAM and the cycle threshold (Ct) values recorded at the end of the annealing step. Data was analysed with Bio-Rad CFX Manager 2.0 software using the regression Cq determination mode and negative samples were denoted as N/A (no amplification).

Table 28 – List of primers used for PET-PCR amplification of rodent faecal samples

Primer	Sequence	Fluorophore	Source
Plasm 18S PET F	GGCCTAACATGGCTATGACG	-	(Lucchi, Narayanan et al. 2013)
Plasm 18S PET R	agg cgcatagcgctggCTGCCTCCTTAGATGTGGTAGCT	FAM	

5.2.3.2. qPCR (quantitative PCR)

The qPCR was carried using QuantiTect™ Probe PCR kit (Qiagen, 204343) by adding the following volumes: 12.5 μL of 2x QuantiTect™ Probe Master Mix, 0.5 μL (0.4 mM) of both forward primer and reverse primer (**Table 29**), 0.25 μL (0.2 mM) of probe, 2 μL of template and ddH₂O to 25 μL . The cycling parameters comprised an initial denaturation step at 95 °C for 15 minutes, 45 cycles of denaturation at 95 °C for 15 seconds and annealing at 56 °C for 1 minute. Amplifications were performed in a Bio-Rad CFX96™ Real Time PCR detection system with the fluorophore channel for FAM and the cycle threshold (Ct) values recorded at the end of the annealing step. Data was analyzed with Bio-Rad CFX Manager 2.0 software using the regression Cq determination mode and negative samples were denoted as N/A (no amplification).

Table 29 - List of primers used for qPCR amplification of rodent faecal samples

Primer	Sequence	Fluorophore	Source
Plas 18S Kamau F	GCTCTTTCTTGATTTCTGGATG	-	(Kamau, Alemayehu et al. 2013)
Plas 18S Kamau R	AGCAGGTTAAGATCTCGTTTCG	-	
Plas 18S Kamau P	ATGGCCGTTTTAGTTCGTG	FAM	

5.2.3.3. RT-qPCR (Quantitative Reverse Transcription Real Time PCR)

The RT-qPCR was carried using QuantiTect™ Probe RT-PCR kit (Qiagen, 204443) by adding the following volumes: 12.5 µL of 2x QuantiTect™ Probe Master Mix, 0.25 µL of QuantiTect™ RT Mix 0.5 µL (0.4 mM) of forward primer and reverse primer (**Table 30**), 0.25 µL (0.2 mM) of probe, 2 µL of template and ddH₂O to 25 µL. The cycling parameters comprised a reverse transcription at 50 °C for 30 minutes, initial denaturation step at 95 °C for 15 minutes, 45 cycles of denaturation at 95 °C for 15 seconds and annealing at 56 °C for 1 minute. Amplifications were performed in a Bio-Rad CFX96™ Real Time PCR detection system with the fluorophore channel for FAM and the cycle threshold (Ct) values recorded at the end of the annealing step. Data was analyzed with Bio-Rad CFX Manager 2.0 software using the regression Cq determination mode and negative samples were denoted as N/A (no amplification).

Table 30 - List of primers used for RT-qPCR amplification of rodent faecal samples

Primer	Sequence	Source
Plas 18S Kamau F	GCTCTTCTTGATTTCTGGATG	(Kamau, Alemayehu et al. 2013)
Plas 18S Kamau R	AGCAGGTTAAGATCTCGTTCG	
Plas 18S Kamau P	ATGCCCGTTTTAGTTCGTG	

5.2.3.4. Nested PCR

For the Nested PCR the GoTaq® G2 Flexi Polymerase (Promega, M7801) was used with the following volumes for both the outer and inner reactions: 4 µL (1x) of 5x Green GoTaq® Flexi Buffer, 2.4 µL (1 mM) of MgCl₂ solution, 0.4 µL (0.2 mM each dNTP) of Nucleotide mix, 0.4 µL (0.2 mM) of forward primer and reverse primer (**Table 31**), 2 µL of template/outer reaction, 0.15 µL (0.75u) of GoTaq® Flexi DNA Polymerase and ddH₂O to 25 µL. The outer reaction was carried under 35 cycles of denaturation at 94 °C for 20 seconds, annealing at 60 °C for 20 seconds and extension at 72 °C for 1 minute. The inner reaction was carried under 40 cycles of denaturation at 94 °C for 20 seconds, annealing at 50 °C for 20 seconds, extension at 72 °C for 1 minute and lastly, the final extension at 72 °C for 7 minutes. After PCR amplification the products were ran in a 0.7% agarose gel.

Table 31 - List of primers used for Nested PCR amplification of rodent faecal samples

	Primer	Sequence	Source
Outer	DW2	TAATGCCTAGACGTATTCCTGATTATCCAG	(Perkins and Schall 2002)
	DW4	TGTTTGCTTGGGAGCTGTAATCATAATGTG	
Inner	DW1	TCAACAATGACTTTATTTGG	
	DW6	GGGAGCTGTAATCATAATGTG	

5.2.4. Effects of incubation on DNA degradation and detection

5.2.4.1. Incubation of samples

For incubation, 14 Eppendorf tubes were prepared, each containing approximately 25mg of faecal material and 10 μ L of *P. falciparum* NAT blood. Tubes were incubated at room temperature in duplicates for 1, 2, 8, 16 and 32 days. After the respective incubation period, the samples were extracted with Zymo Research Fecal DNA kit according to manufacturer's instructions.

5.2.4.2. Detection by qPCR

Degradation of DNA was investigated by amplification of both rodent and *Plasmodium* DNA with the SensiMix™ SYBR® & Fluorescein Kit (Bioline, QT615-05) in a reaction mixture of 20 μ L containing 10 μ L of 2x SensiMix™ SYBR® & Fluorescein mastermix, 1 μ L of DNA template and 0.2 μ L of each primer (**Table 32**) and ddH₂O to 20 μ L. All qPCR assays included duplicates and appropriate controls such as negative and positive controls. The cycling conditions used for amplification with mammalian primers comprised an initial denaturation at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 56 °C for 15 seconds, extension at 72 °C for 15 seconds. For *Plasmodium* primers the cycling conditions included an initial denaturation at 95 °C for 15 minutes, 40 cycles of denaturation at 95 °C for 1 minutes an annealing at 53 °C for 1.3 minutes. Amplifications were performed in a Bio-Rad CFX96™ Real Time PCR detection system with the fluorophore channel for SYBR green and the cycle threshold (Ct) values recorded at the end of the extension step. Data was analyzed with Bio-

Rad CFX Manager 2.0 software using the regression Cq determination mode and negative samples were denoted as N/A (no amplification).

Table 32 - List of primers used for qPCR amplification of mammalian and plasmodium DNA from rodent faecal samples

Name	Sequence	Target	Source
16Smam1	CGGTTGGGGTGACCTCGGA	Mammalian	(Ficetola, Coissac et al. 2010)
16Smam2	GCTGTTATCCCTAGGGTAACT		
Plas 18S Kamau F	GCTCTTTCTTGATTCTTGGATG	Plasmodium	(Kamau, Alemayehu et al. 2013)
Plas 18S Kamau R	AGCAGGTTAAGATCTCGTTCG		

5.2.5. Detection of *Plasmodium* in bird samples

The first step was to test for *Plasmodium* DNA amplification in all blood samples with positives being sent for sequencing (Eurofins, Germany). The second step involved attempting DNA amplification in faecal samples of individuals whose blood produced a positive result in the previous step.

5.2.5.1. Standard PCR amplification of blood samples

A series of primers were designed with Geneious software version 9.1.3. (Kearse, Moir et al. 2012) based on the following mitochondrion sequences: *Plasmodium relictum* mitochondrion (NC_012426.1), *Plasmodium gallinaceum* mitochondrion (NC_008288.1), *Plasmodium floridense* mitochondrion (NC_009961.2), *Parahaemoproteus vireonis* mitochondrion (NC_012447.1), *Leucocytozoon sabraezesi* mitochondrion (NC_009336.1), *Leucocytozoon majoris* mitochondrion (NC_012450.1), *Leucocytozoon fringillinarum* mitochondrion (NC_012451.1), *Leucocytozoon caulleryi* mitochondrion (NC_015304.1) and *Haemoproteus columbae* mitochondrion (NC_012448.1) (Table 33). Primers were tested on DNA isolated from blood samples using the GoTaq® G2 Flexi Polymerase (Promega, M7801) with the following volumes: 4 µL (1x) of 5x Green GoTaq® Flexi Buffer, 0.8 µL (1 mM) of MgCl₂ solution, 0.2 µL (0.2 mM each dNTP) of Nucleotide mix, 0.2 µL (0.2 mM) of forward primer and reverse primer (Table 33), 1 µL of DNA template, 0.1 µL (0.75u) of GoTaq® Flexi DNA Polymerase and ddH₂O

to 20 µL. The cycling conditions used for amplification comprised an initial denaturation at 95 °C for 2 minutes, 32 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute, extension at 60 °C for 1.5 minutes and a final extension at 60 °C for 5 minutes. Resulting products were analysed in a 2% agarose gel.

Table 33 – List of primers used for standard amplification of avian blood and faecal samples

Set	Name	Sequence	Source	
Set 1	P. relictum CyBF1	CACACCGCTCGTCACGCAAGA	Present work	
	P. relictum CyBR1	CAGTCCCAGCGACAGCGGTT		
Set 2	P. relictum CyBF2	GGGTCTTACCGTCGGGCCGT		
	P. relictum CyBR2	CGGGAAACCGGCGCTACCAT		
Set 3	P. relictum CyBF2	GGGTCTTACCGTCGGGCCGT		
	P. relictum CyBR3	CCGGGAAACCGGCGCTACC		
Set 4	P. berghei CyBF1	ATGGTAGCGCCGGTTTCCCG		
	P. berghei CyBR1	AGCGATGCGTGAGCTGGGTT		
Set 5	Plasm 18S PET F	GGCCTAACATGGCTATGACG		(Lucchi, Narayanan et al. 2013) <i>modified</i>
	Plasm 18s PET NoP R	CTGCCTTCCTTAGATGTGGTAGCT		

5.2.5.2. qPCR amplification of faecal samples

From the sequenced fragments obtained from the blood samples, new sets of primers and a probe were designed in order to ensure specificity (**Table 34**).

Table 34 - List of primers used for qPCR amplification of avian blood and faecal samples

Primer Set	Name	Sequence	Source
Set 6	Plasm CyB6 F	AGGAACTCGACTGGCATAAC	Present work
	Plasm CyB5 R	GCGACAGCGTTATACTTTTG	
Probe	Plasm CyB Probe	GAACGAACGCTTTTAACGCCTGACAT	

A qPCR amplification was attempted with faecal samples belonging to the subjects whose blood produced a positive result with the previous assay. Primer set 6 (**Table 34**) was used with the QuantiNova™ Probe PCR kit (Qiagen, 208252) in a reaction mixture of 20 µL containing 10 µL of 1x QuantiNova Probe PCR Master Mix, 1µL of DNA template, 0.5 µL of each primer, 0.25 µL of probe and ddH₂O to 20 µL. All qPCR assays included duplicates and appropriate controls such as negative and positive controls. The cycling conditions were as follows: Initial

denaturation at 95 °C for 2 minutes, 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 60 °C for 30 seconds. Amplifications were performed in a Bio-Rad CFX96™ Real Time PCR detection system, with the fluorophore channel for FAM and the cycle threshold (Ct) values recorded at the end of the extension step. Data was analyzed with Bio-Rad CFX Manager 2.0 software using a regression Cq determination mode and negative samples were denoted as N/A (no amplification).

5.3. RESULTS

5.3.1. DNA and RNA extractions

DNA extractions from faecal samples with Qiagen DNeasy blood and tissue kit were made difficult because of the nature of the samples. Most elution columns were obstructed and attempts to clear them resulted in occasional membrane damage. This made the extraction procedure difficult and long. The same problem was observed with SV RNA isolation kit, which was not particularly manufactured for extraction from faecal samples. The Zymo Research Fecal MiniPrep™ being specifically designed for faecal extraction was the easiest to use with no issues to report.

5.3.2. Template amplification from faecal material: finding a reliable technique

Because amplification of faecal samples is in many cases difficult, four sensitive PCR assays were tested in order to determine which would be the most adequate.

5.3.2.1. PET-PCR (Photo-Induced Electron Transfer PCR)

With the PET-PCR, faecal samples from infected rodents extracted with the DNeasy and Zymo kits, showed no detectable amplification of *Plasmodium* DNA (**Table 35**). All faecal samples were negative despite both positive controls being detectable.

5.3.2.2. qPCR (Kamau)

With qPCR a total of 3 samples were positive for *Plasmodium* DNA (**Table 36**), one extracted with the DNeasy kit and two with the Zymo kit.

5.3.2.3. RT-qPCR (Quantitative Reverse Transcriptase Real Time PCR)

With RT-qPCR only one sample was positive for *Plasmodium* DNA (**Table 37**), interestingly showing a lower C_q than the positive control.

5.3.2.4. NESTED PCR

With NESTED PCR none of the faecal samples produced any detectable band.

Table 35 - Results of PET-PCR amplification of rodent faeces. Positive *P.f.* consisted of extracted *P. falciparum* NAT blood; Positive rodent comprised rodent faecal sample mixed with *P. falciparum* NAT blood. Samples categorised as Zymo consisted of faecal material extracted with the Zymo kit while samples labelled DNeasy were extracted with the DNeasy kit. Samples 1 to 3 comprised faecal material from mice infected with *P. berghei* while Negatives were from non-infected mice.

Sample	Cq
Blank	N/A
Positive <i>P.f.</i>	20.19
Positive rodent	29.71
Zymo 1	N/A
Zymo 2	N/A
Zymo 3	N/A
Zymo Negative	N/A
Dneasy 1	N/A
DNeasy 2	N/A
DNeasy 3	N/A
Dneasy Negative	N/A

Table 36 - Results of qPCR amplification of rodent faeces. Positive *P.f.* consisted of extracted *P. falciparum* NAT blood. Samples categorised as Zymo consisted of faecal material extracted with the Zymo kit while samples labelled DNeasy were extracted with the DNeasy kit. Samples 1 to 3 comprised faecal material from mice infected with *P. berghei* while Negatives were from non-infected mice.

Sample	Ct
Blank	N/A
Positive <i>P.f.</i>	18.02
Zymo1	33.66
Zymo2	N/A
Zymo3	N/A
Zymo Negative Control	N/A
Dneasy1	36.02
DNeasy2	37.34
DNeasy3	N/A
Dneasy Negative Control	N/A

Table 37 - Results of RT-qPCR amplification of rodent faeces extracted with the SV Total RNA Isolation System. Positive *P.f.* consisted of extracted *P. falciparum* NAT blood; Samples 1 to 3 comprised faecal material from mice infected with *P. berghei* while Negatives were from non-infected mice.

Sample	Cq
Blank	N/A
Positive <i>P.f.</i>	33.52
SV RNA 1	N/A
SV RNA 2	26.07
SV RNA 3	N/A
SV RNA Negative	N/A

5.3.3. Effects of incubation on DNA degradation and detection

Incubation of faecal samples at room temperature did not seem to affect detection of DNA as in all samples it was possible to amplify both mammalian and *Plasmodium* DNA with no loss in sensitivity (**Table 38**).

Table 38 - qPCR amplification from faecal samples mixed with *P. falciparum* NAT blood and incubated at room temperature over a period of 32 days. Mammal: values for mammal DNA; *Plasmodium*: values for *Plasmodium* DNA.

Sample	Cq	
	Mammal	Plasmodium
Blank	N/A	N/A
Negative	18.07	N/A
<i>P.f.</i> NAT	22.61	22.55
12h	15.60	21.16
24h	17.30	26.54
2d	16.09	25.07
4d	17.05	26.22
8d	17.61	26.28
16d	18.32	26.00
32d	18.71	26.24

5.3.4. Detection of *Plasmodium* in bird samples

5.3.4.1. Standard PCR amplification of blood samples

Standard PCR amplification of bird blood samples with primer Set 1 resulted in amplicon production for samples B1, B3, B5 and B9 (**Figure 83**). These products were sent for sequencing and later confirmed to be *Plasmodium relictum* sequences. Primer Set 2 produced faint bands for samples B3 and B8 which when sent for sequencing failed to be sequenced (**Figure 84**). Primer Set 3 produced many unspecific bands in particular for samples B4, B7, B10 and B11 (**Figure 85**). The few bands to be successfully sequenced did not match any sequences from the National Center for Biotechnology Information (NCBI) database (Coordinators 2016). With Primer Set 4, in addition to unspecific amplification in sample B5, target amplification was only present in sample B3 (**Figure 86**) and obtained bands failed to be sequenced. Finally, Primer Set 5 completely failed to amplify the target sequence (**Figure 87**).

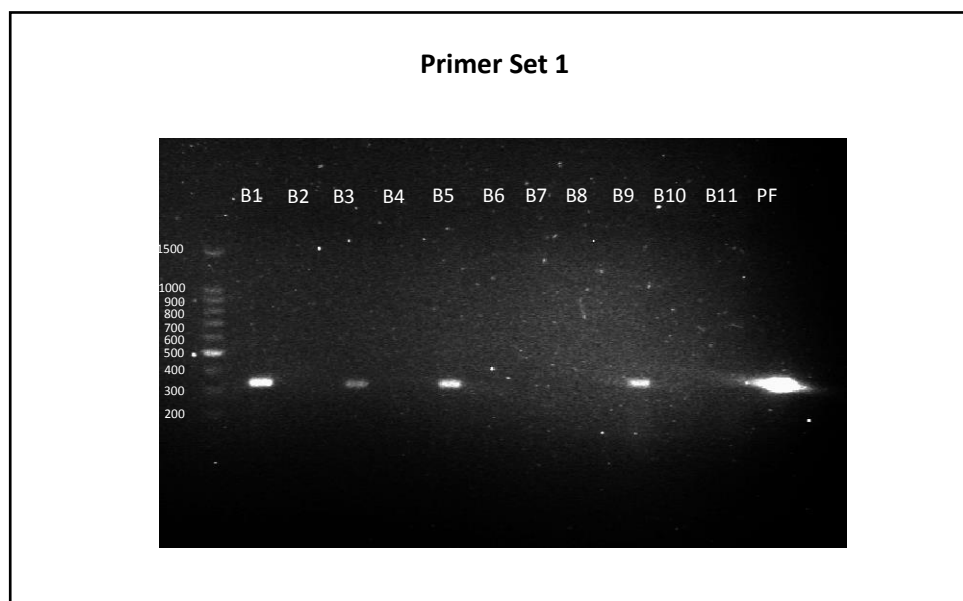


Figure 83 - Ethidium bromide stained agarose gel (2%) with PCR products amplified with GoTaq buffer and primer set 1. Samples B1 to B12 (blood samples).

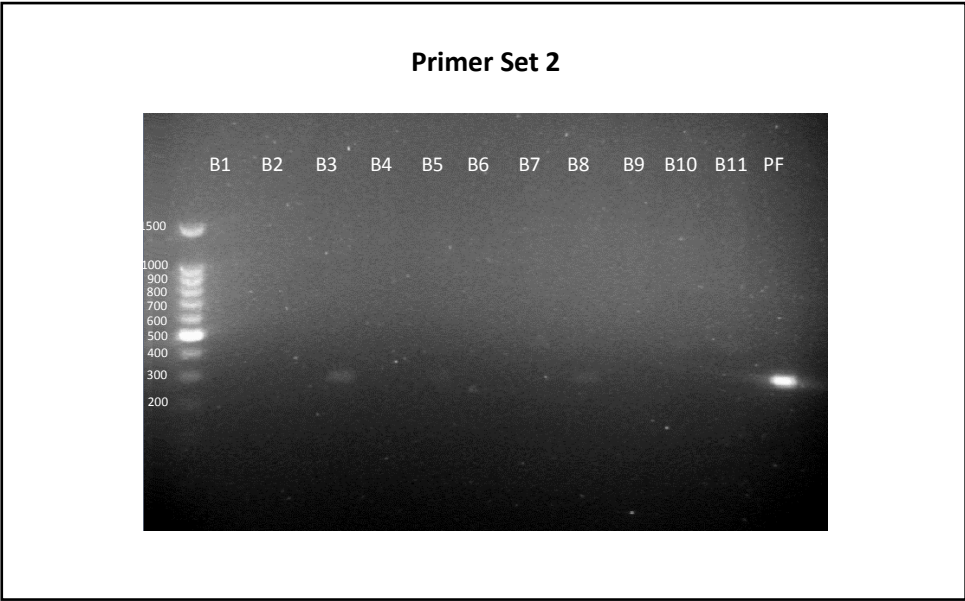


Figure 84 - Ethidium bromide stained agarose gel (2%) with PCR products amplified with GoTaq buffer and primer set 2. Samples B1 to B12 (blood samples).

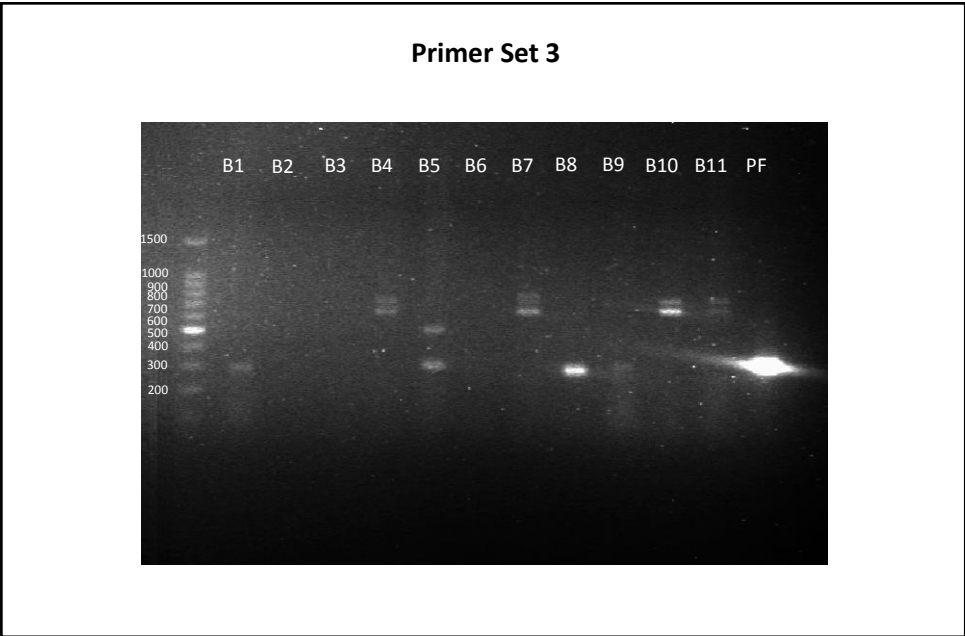


Figure 85 - Ethidium bromide stained agarose gel (2%) with PCR products amplified with GoTaq buffer and primer set 3. Samples B1 to B12 (blood samples).

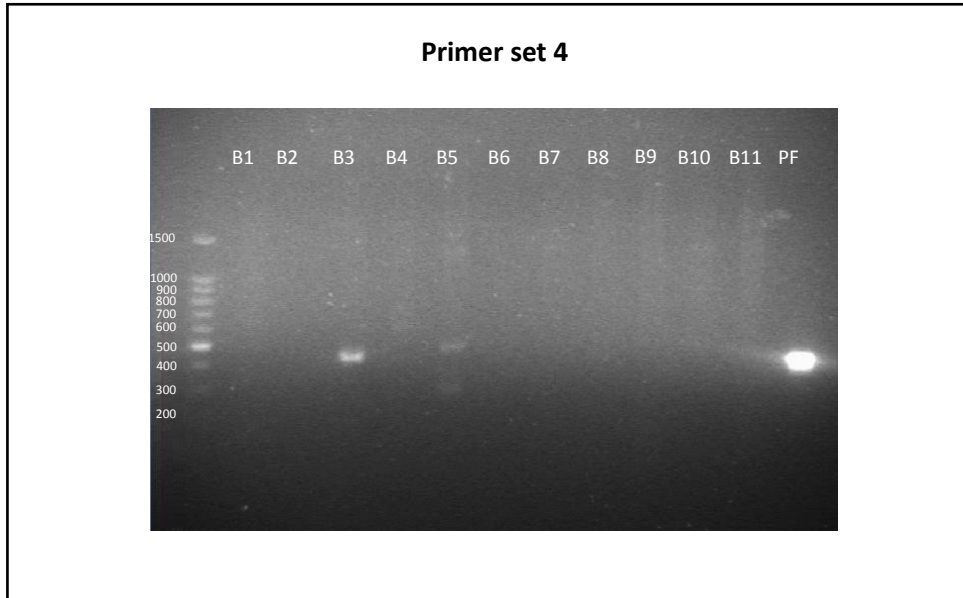


Figure 86 - Ethidium bromide stained agarose gel (2%) with PCR products amplified with GoTaq buffer and primer set 4. Samples B1 to B12 (blood samples) and *P.f.* NAT as positive control.

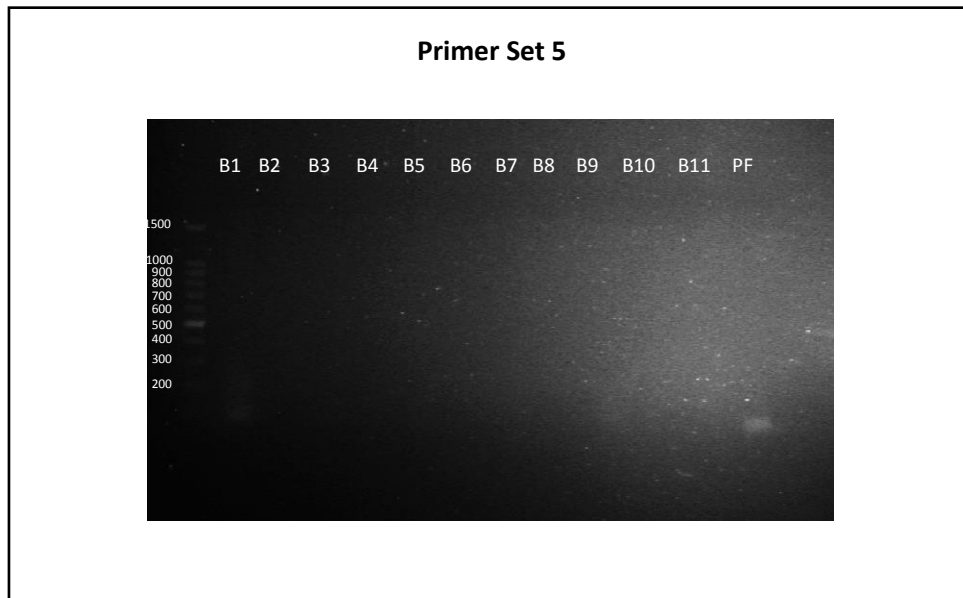


Figure 87 - Ethidium bromide stained agarose gel (2%) with PCR products amplified with GoTaq buffer and primer set 5. Samples B1 to B12 (blood samples) and *P.f.* NAT as positive control.

5.3.4.2. qPCR amplification of faecal samples

In order to explore the possibility of detection of *Plasmodium* DNA in bird faecal samples, a qPCR assay was used using both blood and faecal samples (**Table 39**). The blood samples, which produced a positive result with the standard PCR, were once again positive. The blood sample of subject 2, which had shown no detectable amplification previously, was once more negative when tested with qPCR. This sample was added to this essay in order to test if a blood negative sample could still produce a positive faecal result, which indeed occurred. The faecal samples of the remaining positive subjects all produced a positive result in at least one of the three time points collected.

Table 39 – qPCR Cq values of bird blood and faecal samples. B1 to 9: Subjects previously positive for *Plasmodium*. Blood: Cq values for respective subjects. Faecal: Cq values of samples collected at days 1, 2 and 3 for respective subjects. Positive *P.f.*: *Plasmodium falciparum* NAT.

Subject	Blood	Faecal		
		Day 1	Day 2	Day 3
B1	26.23	33.58	32.72	N/A
B2	N/A	31.54	N/A	N/A
B3	29.30	N/A	N/A	35.28
B5	27.91	30.69	N/A	N/A
B9	29.13	N/A	N/A	35.69
Positive (<i>P.f.</i>)	14.05	-	-	-
Negative	N/A	-	-	-

5.4. Discussion

The first step of this project was to explore several molecular techniques and find which would be more suitable and sensitive for detection of *Plasmodium* DNA in rodent faecal samples. An additional experiment was also carried out to evaluate the possibility of faecal DNA degradation over a period of 30 days. After establishing appropriate DNA extraction and amplification protocols, faecal samples from malaria infected birds were tested for the presence of avian *Plasmodium*.

5.4.1. qPCR assay was the best method for DNA amplification from faecal samples

From the four assays tested (PET-PCR, qPCR, RT-qPCR and Nested PCR), qPCR was the only to produce reliable amplification from faecal samples. However, it was noticeable that even with this technique not all faecal samples produced a positive result. This could be related to an inconstant detectable amount of *Plasmodium* DNA in faeces rather than the technique itself and this issue will be discussed in more detail further into this section.

Regarding extraction methods, the Zymo Research Fecal DNA MiniPrep™ ended up being selected as primary faecal extraction kit instead of Qiagen's DNeasy Blood and Tissue kit. The main factor for this was that the Zymo kit was specifically designed for extraction from faecal samples making the process easier. With the DNeasy kit, there was a big issue with filter obstruction resulting in longer extraction times and sometimes even column membrane damage.

5.4.2. Room temperature incubation does not impact DNA amplification from faecal samples

Endurance of faecal DNA can be affected by exposure to the weather, preservation and storage method and most importantly, sample age (DeMay, Becker et al. 2013).

Here it was shown that rodent faecal samples up to at least 30 days old still contain significant amplifiable amounts of DNA. Although incubations were carried at room temperature in the

laboratory, this result correlates with a similar study on the effects of sample age and season collection where samples were exposed to the elements (Piggott 2004). In the aforementioned study, faecal samples of red fox (*Vulpes vulpes*) and rock-wallaby (*Petrogale penicillata*) were collected during summer and winter months and tested for DNA amplification after up to 180 days of exposure. Decline in amplifiable DNA was observed only from day 21, on average. This further proves that even when exposed to the elements, faecal samples should still be viable for DNA amplification making them an excellent non-invasive source for genetic sampling and monitoring of rare and/or protected populations.

5.4.3. *Plasmodium* DNA can be detected in bird faecal samples

A recent study reported that PCR amplification from bird faecal samples was not suitable for detection of *Plasmodium* infections (Martinsen, Brightman et al. 2015). However, here it was shown that *Plasmodium* DNA can in fact be detected if sensitive assays, such as qPCR, are used. The abovementioned study employed standard and nested PCR assays, which were shown here not to produce any amplicons in faecal samples from both rodents and birds infected with *Plasmodium*. In most qPCR assays, the amount of amplicon is measured at each cycle with fluorescent markers that are incorporated into the product. This means that amplification is measured by fluorescence and the increase in signal is directly proportional to the number of amplicons (Muniesa, Ferreira et al. 2014). This makes qPCR an extremely sensitive tool for detection of difficult samples. In addition, and as it was used in the present study, the fluorescent markers can be probes that bind specifically to the desired amplicon adding to the assay's specificity.

Interestingly, not all faecal samples from infected individuals produced positive results. Samples were collected in triplicates with a 24h interval between each collection. This suggests that *Plasmodium* DNA is not continuously being passed in the faeces. The reason for this is unknown but it is possible that the amount of DNA in faeces is connected to the rate of plasma clearance, the parasite cycle and infection stage

Subjects 1 and 5 were both admitted to the centre as a result of debilitation and although it is unclear if their physical condition was due to high *Plasmodium* parasitemia, it is still interesting to note that, especially for subject one, two of the three faecal samples collected

were positive for *Plasmodium*. In addition, both standard PCR and qPCR assays of blood from subject 1 produced the highest amount of amplicon, which could indicate high parasitemia. This further points to a connection between the infection stage and detectable DNA in faeces, seen as it is likely that subject 1 was experiencing an acute stage of infection.

For subject 2 (B2), the blood sample was negative with both standard PCR and qPCR assays. However, it was possible to detect *Plasmodium* DNA in one of the subject's faecal samples. It is known that in birds the chronic stage is usually followed by the latent stage where parasites completely disappear from the peripheral blood and persist in the internal organs (Valkiūnas 2005). It is plausible to assume that this was the case with subject B2.

Inconsistent rates of *Plasmodium* DNA in faeces could pose as a complication for accurate incidence surveys of *Plasmodium* species in bird populations. However, this could be easily mended if the sampling pool is increased and so collected information should still provide valuable information on species present in different populations.

5.5. References

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6.

CONCLUSION

6.1. Conclusion

The two main goals of this work were to test the viability of blood stage wild type parasites in oral and nasal inoculations, and develop a mutant strain with increased immunogenicity for future similar application.

Inoculation of mice with blood stage *Plasmodium* showed that despite not providing protection, this approach allowed for extended survival of inoculated individuals. It is important to note that due to circumstances beyond the author's control, it was not possible to conduct the inoculations with an adjuvant. Seeing that survival was increased by inoculating animals solely with live parasites, the results detailed in this work show that this approach holds great applicational potential. The next step clearly needs to be a more comprehensive look into this approach by coupling oral and nasal inoculation with live parasites and a powerful adjuvant as this may be the key to unlock full protection. More research into this topic is urgently needed as recent studies suggest that the efficacy of leading malaria vaccine candidate, RTS,S/AS01 seems to wane over time. In fact, after a seven year follow up of patients immunised with this vaccine, it was observed that in areas with high malaria exposure, vaccinated subjects suffered more malaria episodes than non-vaccinated (Clemens and Moorthy 2016, Olotu, Fegan et al. 2016).

The second goal of this work was to produce a *Plasmodium falciparum* mutant expressing *Salmonella's* FimH⁺ glycoprotein as to allow its detection by M cells in the gut. This was to be achieved with a modified version of the CRISPR/Cas9 system which would bear a double guide RNA instead of a single. This however, was not accomplished as the resulting CRISPR plasmid contained an extremely high AT-content becoming toxic to *E. coli*. This issue is a recurrent road block in malaria research and, despite the unsuccessful achievement of a double guide CRISPR/Cas9 system for *Plasmodium*, with this work it was possible to improve crucial malaria molecular techniques previously hampered by AT-rich sequences. In order to achieve long genomic deletions on *Plasmodium*, a different approach is clearly needed. Up to four gRNA's have been reported to be used in large genomic deletions (Zhou, Wang et al. 2014, Song, Yuan et al. 2016, Song, Lai et al. 2017). Despite the author's belief that multiple plasmids would

hinder successful transfection and expression in *Plasmodium*, it is still important to look at this approach as a potential alternative.

In addition to the two main goals mentioned, other unrelated work was carried regarding avian malaria. Because blood sampling of wild birds for detection of malaria is complicated and in some cases impossible, the possibility of using faecal samples was considered. With this work it was found that faecal samples can in fact be used for PCR detection of *Plasmodium* parasites. This is a great advance for future field applications which comprise the next step of this project.

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