CHAPTER 2

Materials and Methods

2.1 Production of recombinant cell surface and secreted *P. falciparum* and erythrocyte proteins

Secreted proteins, and the full-length ectodomains of membrane embedded proteins were produced in soluble recombinant form, by using an established system based on transient transfection of the HEK293E (Human Embryonic Kidney-293-EBNA1 variant) human cell line (Durocher *et al.*, 2002). The cells, adapted for suspension culture, were maintained in Freestyle media (*Invitrogen*) supplemented with 1% fetal bovine serum, 50 µg/ml of geneticin (*Sigma*), 100 units/ml penicillin (*Invitrogen*) and 100 µg/ml streptomycin (*Invitrogen*), incubated at 37°C, 5% CO2 and 70% humidity with orbital shaking at 120 r. p. m.

2.1.1 Design and construction of protein expression plasmids

Transmembrane protein coding sequences were truncated for removal of the transmembrane and cytoplasmic regions (as predicted by TMHMM Server; http://www.cbs.dtu.dk/services/TMHMM/) enabling recombinant expression in a soluble, secreted form. The sequences encoding for the full-length secreted proteins or for the ectodomains of cell surface proteins (from 3D7 reference genome), were codon optimised for expression in mammalian cells, using the *GeneArt* gene synthesis service (*Invitrogen).* The optimised sequences were provided already sub-cloned into a pTT3-based expression vector (Durocher *et al.*, 2002) containing a region coding for the immunoglobulin-like domains 3 and 4 of rat Cd4 (Brown and Barclay, 1994) together with either

i. A 17-amino acid peptide substrate for the *Escherichia coli* biotin ligase BirA (Brown and Barclay, 1994; Bushell *et al.*, 2008), and a hexa-His tag (BLH; Fig. 2.1), or

ii. The pentamerisation domain of the rat cartilage oligomeric matrix protein (COMP) (Tomschy *et al.*, 1996), an ampicillin resistance protein β-lactamase (Bushell *et al.*, 2008), a Flag tag and a hexa-His tag (BLFH; Fig. 2.1)

Endogenous signal peptides were included for erythrocyte cell surface/secreted proteins (Crosnier *et al.*, 2011), however those of *Plasmodium* proteins (as predicted by using SignalP; http://www.cbs.dtu.dk/services/ SignalP/)were replaced by the leader sequence of the mouse variable κ light chain 7-33 (Crosnier *et al.*, 2010) during gene synthesis.

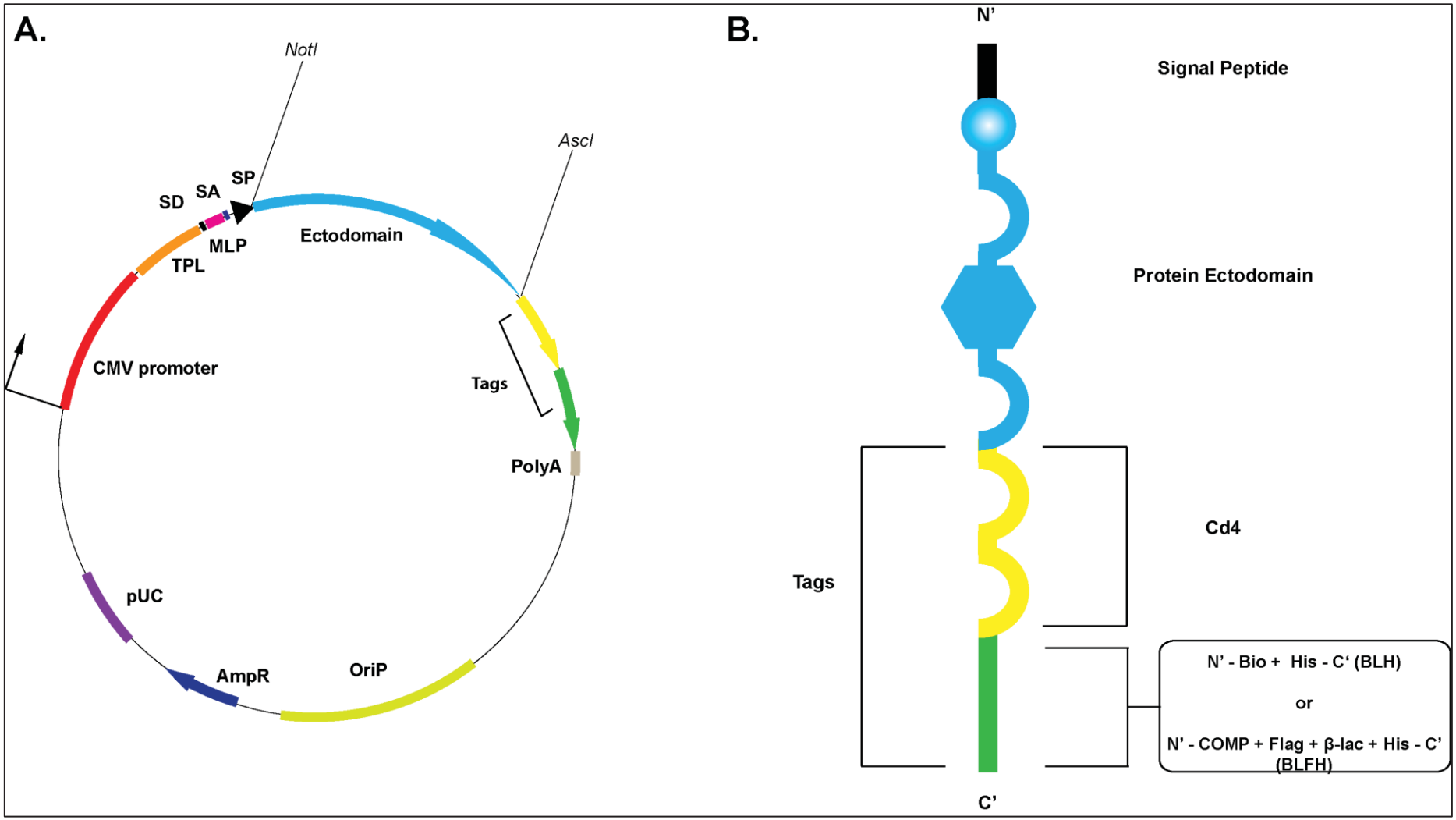
A.
NotI
AscI
SP
SD
SA
MLP
TPL
Ectodomain
CMV promoter
Tags
PolyA
pUC
AmpR
OriP
B.
N'
Signal Peptide
Protein Ectodomain
Tags
Cd4
N' - Bio + His - C' (BLH)
or
N' - COMP + Flag + β-lac + His - C' (BLFH)
C'

Figure 2.1 Design of recombinant protein expression vectors

A. The transfection vector which was used for recombinant protein expression.

B. A simplified cartoon of a typical recombinant protein as expressed from the transfection vector. The protein is tagged either with the Cd4, Bio and His or with the Cd4, COMP, β-lac, Flag and His tags.

Abbreviations: CMV promoter, Cytomegalovirus promoter; TPL, Tripartite Leader Sequence; SD, Splicing Donor; SA, Splicing Acceptor; MLP, Adenovirus Major Late Promoter enhancer; SP, Signal Peptide; NotI, NotI resctriction enzyme recognition site; AscI, AscI restriction enzyme recognition site; polyA, SV40 polyadenylation sequence; OriP, Epstein Barr virus origin of replication; AmpR, β-lactamase gene; pUC origin, bacterial origin of replication; Cd4, domains 3 and 4 of rat Cd4 ; Bio, Biotinylation signal for BirA; Flag, Flag tag ; His, hexa-histidine; COMP, coiled-coil sequence from the rat Cartilage Oligomeric Matrix Protein; β-lac, β-lactamase.

Evidence suggests that *Plasmodium* proteins are not N-linked glycosylated *in vivo* (Dieckmann-Schuppert *et al.*, 1992; Gowda and Davidson, 1999, 2000; Kimura *et al.*, 2000). To prevent spurious addition of glycans in the human cell secretory pathway the potential N-linked glycosylation sites (N-XS/T, with X≠proline) of *Plasmodium* proteins (as predicted by using NetNGlyc; http://www.cbs.dtu.dk/services/NetNGlyc/) were also systematically removed by substituting alanine for serine/threonine at these sites.

During gene assembly, flanking unique *NotI* (5') and *AscI* (3') restriction endonuclease recognition sites were introduced to all sequences encoding for recombinant proteins; these restriction sites were used for the subcloning of protein coding sequences, into expression vectors carrying different tags (Fig. 2.1). Briefly, after digestion with *NotI* (*New England BioLabs*, *NEB*) and *AscI* (*NEB*) for 2h at 37°C, both the vectors and inserts were resolved by agarose gel electrophoresis (section 2.8) and purified using the *QIA*quick Gel Extraction kit (*QIAGEN*) as per manufacturer's instructions. Ligations were performed for 1h at room temperature with T4 DNA Ligase (*NEB*) in ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, pH 7.5). Chemically-competent *E.coli* TOP10 cells (*Invitrogen*) were transformed with the ligation products and positive clones were selected on LB-agar plates containing 100 µg/ml of ampicillin.

Plasmids were then purified using the *QIA*prep Miniprep Spin kit (*QIAGEN*) according to manufacturer's instructions, tested for the correct insert via *NotI* and *AscI* digestion and sequenced with primers 462, 463, 497, 498, 3732, 3381, 3811, 3397, 3851 (Table 2.1 at the end of this chapter). Sequence-verified expression plasmids were transformed into TOP10 competent cells. Single positive clones were then inoculated into 100 ml cultures of LB broth supplemented with 100 µg/ml of ampicillin and grown overnight at 37°C with shaking at 200 r.p.m. Plasmids were purified from pelleted bacterial cultures using the PureLinkTM HiPure Plasmid Maxiprep kit (*Invitrogen*). The purified plasmid DNA was assessed for quantity and quality by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using Nanodrop (*Thermo Scientific)*. In terms of purity, A260/A280 ratios between 1.8-1.95 were considered to be acceptable. All plasmid DNA preparations were diluted to 1 mg/ml in EB buffer (10 mM Tris-Cl, pH 8.5) for use in transfections.

2.1.2 Recombinant protein expression by transient transfection of protein expression plasmids

The recombinant protein expression plasmids (Fig. 2.1) were transiently transfected into HEK293E cells by using the cationic reagent polyethylenimine (PEI; Tom *et al.*, 2008). Cells were split into 50 ml of fresh media at a density of $2.5x10^5$ cells/ml and allowed to recover for 24 hours prior to transfection. For recombinant erythrocyte or *Plasmodium* protein expression each culture was inoculated with the following transfection mixture: 25 µl of the expression plasmid (at 1 mg/ml), 50 µl of linear PEI (at 1 mg/ml) and 2 ml of non-supplemented Freestyle media. The transfection mixture was incubated for 10 min at room temperature before it was added to the cell culture. Six days after transfection cells and cell debris were removed by centrifugation at 3220*g* for 20 min, supernatants were then filtered (0.2-µm filter) and stored at 4°C until use. When producing biotinylated proteins, the culture media was supplemented with 100 µM D-biotin (*Sigma*) and 2.5 µl of a plasmid (at 1 mg/ml) coding for a secreted form of BirA (Bushell *et al.*, 2008) was included in the transfection mix. After harvesting, filtered culture supernatants containing biotinylated proteins were dialysed against 5 L of Hepes Buffered Saline (HBS; 0.14 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES) over 2 days (7 changes of buffer) in Snakeskin dialysis tubing (10 kDa MWCO, *Thermo Scientific*) to remove excess D-biotin.

2.1.3 Purification of His-tagged recombinant proteins by using *Ä*KTAxpress purification system

His-tagged proteins were purified from harvested culture supernatants on nickel-charged Sepharose columns (HisTrap HP 1 ml; *GE Healthcare*) using the ÄKTAxpress purification system (*GE Healthcare*). In each case, the column was pre-equilibrated with binding buffer (20 mM sodium phosphate, 40 mM imidazole, 0.5 M NaCl, pH 7.4) at a flow rate of 1 ml/min. The harvested supernatant (> 200 ml) was supplemented with imidazole (10 mM) and NaCl (100 mM), before passed through the column at 1 ml/min. When loading was complete, the column was washed with 15 column volumes (15 CV) of binding buffer to remove non-specific adherents and with 10 CV of elution buffer (20 mM sodium phosphate, 0.4 M imidazole, 0.5 M NaCl, pH 7.4) to recover specifically-bound protein. The elutant was monitored at 280 nm in real-time and collected in 0.5 ml fractions. The two/three fractions with the highest

concentration of purified protein (as estimated by measuring the absorbance at 280 nm) were analysed by SDS-PAGE and pooled for downstream applications.

His-tagged proteins recovered from nickel column purification were further purified by gel filtration prior to use in surface plasmon resonance experiments (section 2.12). This step was necessary for removing protein aggregates. The gel filtration column (Superdex Tricorn 200 10/600 GL, *GE Healthcare*) connected to *Ä*KTAxpress was pre-equilibrated with ~100ml (~2CV) of the running buffer, HBS-EP (10 mM HEPES, 150 mM NaCl, 30 mM EDTA, 0.05% polyoxyethylenesorbitan 20, pH 7.4) before injecting each protein sample. One millilitre fractions were collected once 22 ml of running buffer (equivalent to the void volume of the column) had passed through the column. Absorbance at 280 nm and *in silico* predicted extinction coefficients were used to estimate the concentrations of peak fractions. The actual sizes of proteins and their conformational states (i.e. monomer, dimer or oligomer) were deduced from the elution volumes of the peak fractions, by comparison against a standard curve generated using well-defined protein standards from the low molecular weight and high molecular weight gel filtration calibration kits (*GE Healthcare*).

2.1.4 Purification of His-tagged recombinant proteins by using the Protein Press

His-tagged merozoite cell surface and secreted proteins were purified from harvested culture supernatants on a 96-well, nickel-charged Sepharose column plate (*His Multitrap HP; GE Healthcare*), using the protein press. Protein press is a device designed and developed in our laboratory (by Dr Yi Sun) which allows high throughput protein purification in a 96-well plate format. The column plate was pre-equilibrated with binding buffer (20 mM sodium phosphate, 40 mM imidazole, 0.5 M NaCl, pH 7.4) at a flow rate of 1 ml/min. The harvested supernatants (~200 ml for each protein) were supplemented with imidazole (10 mM) and NaCl (100 mM), before passed through the column plate (1 well per sample) at 1 ml/min. When loading was completed, each well was washed with 1.6ml (2CV) of binding buffer to remove non-specific adherents and with 0.2ml of elution buffer (20 mM sodium phosphate, 0.4 M imidazole, 0.5 M NaCl, pH 7.4) to recover specifically-bound protein.

2.2 Production of humanised or chimeric recombinant antibodies and DVD-Igs

2.2.1 Immunisation of animals

Six-week-old male BALB/c mice were immunised subcutaneously - three times with four weeks interval in between immunisations - with 10µg purified His-tagged Basigin in Titermax Gold adjuvant (*Sigma)*. Mice selected for hybridoma production were boosted intraperitoneally (without adjuvant) 3 days before dissecting the spleen.

2.2.2 Cell culture and hybridoma generation

The SP2/0 myeloma and SP2/mIL6 cell lines were grown in advanced DMEM medium (*GIBCO*) supplemented with 20% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM). SP2/mIL6 conditioned medium was harvested every 3 days. Hybridomas were generated as described previously (Crosnier *et al.*, 2010). Briefly, following spleen dissection and dissociation, 10^8 splenocytes were fused to 10^7 SP2/0 myeloma cells in 50% PEG (PEG 1500; *Roche*). The resulting hybridomas were plated over ten 96-well plates (*COSTAR*) and initially grown in advanced DMEM medium (*GIBCO*) supplemented with 20% fetal bovine serum, 20% SP2/mIL6 conditioned medium, penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM) before addition of Hypoxanthine Aminopterin Thymidine (HAT) selection medium 24 h after the fusion; after a regular exchange of selection medium for ten days, hybridoma supernatants were harvested for screening (section 2.2.3). Positive clones were re-plated in 48-well plate and grown for a further five days before further cloning by limiting dilution

2.2.3 Antibody screening

Hybridoma supernatants were screened using an ELISA-based assay as previously described (Crosnier *et al.*, 2010). Briefly, the biotinylated ectodomain of Basigin was bound to streptavidin-coated plates (*NUNC*) and incubated for 1 h with 50 µL hybridoma supernatant diluted 1:2 in phosphate buffered saline (PBS; 135 mM NaCl, 1.3 mM KCl, 3.2 mM Na2HPO4, 0.5 mM KH2PO4, pH 7.4) containing 2% w/v bovine serum albumin (BSA). The plates were washed in PBS/0.1% Tween20 (PBST) before incubation with an anti-mouse immunoglobulin (anti-mIgG) antibody coupled to alkaline phosphatase (*Sigma*) for 1 h at room temperature. After washes

in PBST and PBS positive wells were detected with *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well).

2.2.4 RNA preparation and amplification of antibody variable regions

Amplification of antibody variable regions was performed as previously described (Crosnier *et al.*, 2010). Total RNAs were prepared from selected hybridoma using the RNeasy mini kit (*QIAGEN*) and the reverse transcription was performed with a polydT primer, using SuperscriptIII (*Invitrogen*). The functionally rearranged light variable region of a selected hybridoma was amplified without its signal peptide sequence, using a set of 17 degenerate forward primers (primers 3 to 19; Table 2.1) and one unique reverse primer corresponding to the constant region of the κ light chain (primer 20; Table 2.1). Similarly, the functionally rearranged heavy variable region was amplified without its signal peptide sequence, using a set of 18 degenerate forward primers (primers 27 to 44; Table 2.1) and four reverse primers (primers 45 to 48; Table 2.1). In total, the primers can recognise 97 and 98% of functional heavy and kappa (κ) light chain variable regions, respectively (Crosnier *et al.*, 2010). Lambda light chains only constitute 5% of those used in mouse immunoglobulins and primers were, therefore, not designed to this light chain (Crosnier *et al.*, 2010).

Both heavy and light chain variable region sequences were amplified with *Pfu*Ultra II Fusion HS DNA polymerase (*Agilent*) using the PCR conditions: 95°C for 2 min; ten cycles at 95°C for 45 s, 62°C for 30 s, 72°C for 1 min; 20 cycles at 95°C for 45 s, 61°C to 57 °C for 30 s with 1°C decrement every five cycles, 72°C for 1 min; ten cycles at 95°C for 45 s, 56 °C for 30 s, 72°C for 1 min, and one cycle 72°C for 10 min.

2.2.5 Identification of Complemetarity Determining Regions (CDRs) and antibody humanisation by CDR grafting

PCR-amplified antibody variable regions were analysed by gel electrophoresis (section 2.8) and gel extracted by using *QIA*quick Gel Extraction kit (*QIAGEN*) as per manufacturer's instructions. Gel extracted DNA was cloned by blunt end ligation in pCR®-Blunt II-TOPO® vector (*Invitrogen)* according to manufacturer's instructions. Chemically-competent *E.coli* TOP10 cells (*Invitrogen*) were transformed with the ligation products and positive clones were selected on LB-agar plates containing 50

μg/ml of kanamycin. Forty eight clones for each antibody heavy and light chain were sequenced by using T7, SP6, M13F and M13R primers (Table 2.1).

Sequencing results were analysed by using Seqman Pro (*DNAStar Lasergene Core Suite 11)*, and assembled sequences were subjected to variable region identification as well as to CDR and FR assignment by using IMGT/V-Quest (www.imgt.org). The sequences identified to correspond to the aberrantly rearranged κ chain (IGKV3-12) which is transcribed from SP2/0 myeloma genome (Strohal *et al.*, 1987) were excluded from further analysis. For the identification of the closest human variable regions, the verified mouse variable region sequences were blasted against the complete human variable region repertoire by using IMGT/DomainGapAlign (www.imgt.org). The human variable region sequence with the highest similarity in FRs was chosen to serve as CDR acceptor during antibody humanisation. If two or more human sequences were equally similar, the human sequence classified as I (genomic and rearranged evidence) on Vbase2 database (www.vbase2.org; Dr Mike Clark, personal communication) and/or had evidence of existence on protein level (www.imgt.org) was preferred. The latter provided evidence that the chosen CDR acceptor human variable region sequence is naturally expressed, increasing the probabilities of the engineered-humanised sequence to be functional.

For antibody humanisation, the human CDRs of the chosen human variable regions were replaced *in silico* by their mouse counterparts.

2.2.6 Design and construction of antibody and DVD-Ig expression vectors

2.2.6.1 Design and generation of anti-Basigin antibody expression vectors

For the construction of anti-Basigin chimeric and humanised antibody expression vectors the coding sequences of human Immunoglobulin kappa constant *(hIgCκ;* ENST00000390237*)* and a modified version *(*E233P / L234V / L235A / G236Δ / A327G / A330S / P331S / K214T / D356E / L358M; Greenwood and Clark, 1993; Armour *et al.*, 1999, 2003, 2006; Ghevaert *et al.*, 2008) of human Immunoglobulin heavy constant gamma 1 *(hIgG1;* ENST00000390549), each flanked by unique (5') *NotI* and (3') *EcoRI* restriction sites were synthesised by gene synthesis (*GeneArt)*. The synthesised sequences were cloned into the pTT3 vector vector by using the *NotI-EcoRI* restriction sites.

The engineered or mouse antibody variable heavy or light chain sequences, were fused (*in silico*) downstream to the stated signal peptides which carry a *NotI* restriction site to their 5' end. The oligonucleotide GCTAGC was incorporated to the 3' end of each heavy chain variable region sequence. The latter sequence is in fact an *NheI* site and corresponds to the 5' end sequence of hIgG1. Similarly the oligonucleotide CGGACCGTGGCCGCTCCCAGCGTGTTCATCTTCCCACCCAG-CGACGAGCAGCTGAAG*TCCGGA* was incorporated to the 3' end of light chain variable region sequences. The latter sequence corresponds to the 5' end of *hIgCκ* and includes a *BspEI* cutting site (underlined).

The sequences consisting of the signal peptide, antibody (heavy or light) variable region and 3' oligonucleotide were synthesised by gene synthesis (*GeneArt).* The synthesised sequences carrying the heavy variable regions were cloned in pTT3 vector (by using the *NotI-NheI* restriction sites) in which the modified hIgG1 had been previously incorporated. Similarly the sequences encoding for light variable regions were cloned in pTT3 vector carrying the hIgCκ, by using the *NotI-BspEI* restriction sites. Chemically-competent *E.coli* TOP10 cells (*Invitrogen*) were transformed with the ligation products and positive clones were selected on LB-agar plates containing 100μg/ml ampicillin. Correct clones were verified by sequencing, using the primers V022F, V022R and hIgG1Rnew for heavy chain expression vector and V022F, V022R and hIgCκR for light chain expression vector (Table 2.1).

2.2.6.2 Design and construction of anti-RH5 expression vectors

For the construction of anti-RH5 chimeric antibody expression vectors, ch6D9 heavy and light variable regions were deleted from ch6D9 heavy and light chain encoding vectors, respectively, by inverted PCR by using the primer pairs LeaderHR-hIgG1F and LeaderLR-hIgCκF respectively (Table 2.1.). For the PCR reactions the Phusion High Fidelity DNA Polymerase (*Thermo Scientific*) was used under the following conditions: (1) 98^{o}C for 30s; (2) 98^{o}C for 10s; (3) 65^{o}C for 30s; (4) 72^{o}C for 1min and 45s; repeat steps 2-4 for 34 times; 72^{o}C for 10min. PCR products were analysed by agarose gel electrophoresis (section 2.8) and the bands corresponding to linear ch6D9 heavy and light chain expression vectors lacking the variable regions (~7 and ~6kb respectively) were extracted from the gel by using the *QIA*quick Gel Extraction kit (*QIAGEN*) as per manufacturer's instructions.

The variable heavy and light chain sequences of 9AD4 and 2AC7 anti-RH5 antibodies were cloned and verified as described in sections 2.2.4 and 2.2.5. 9AD4 and 2AC7 variable heavy chain sequences were amplified from pCR®-Blunt II-TOPO® vector (*Invitrogen)* by using the primer pairs 7H27F-4H12R and 7H27F-7H27R respectively (Table 2.1). Similarly, TOPO-cloned 9AD4 and 2AC7 variable light chain sequences were amplified by using the primer pairs LL2F-2L1R and 2L1F-2L1R respectively (Table 2.1). For the PCR reactions the *Pfu*Ultra II Fusion HS DNA polymerase (*Agilent*) was used under the following conditions: (1) 95^{o}C for 2min; (2) 95^{o}C for 20s; (3) 60^{o}C for 20s; (4) 72^{o}C for 15s; (5) repeat steps 2-4 for 34 times; (6) 72^{o}C for 3min

PCR products were analysed by agarose gel electrophoresis (section 2.8) and the bands corresponding to variable heavy and light sequences were extracted from the gel by using the *QIA*quick Gel Extraction kit (*QIAGEN*) as per manufacturer's instructions. Gel extracted DNA encoding for antibody variable heavy and light chain, was cloned by blunt end ligation (*T4 ligase, NEB*; room temperature, overnight) to the empty linear ch6D9 heavy and light chain respectively. Chemically-competent *E.coli* TOP10 cells (*Invitrogen*) were transformed with the ligation products and positive clones were selected on LB-agar plates containing 100µg/ml ampicillin. Correct clones were verified by sequencing, using the primers V022F, V022R and hIgG1Rnew for heavy chain expression vectors, and V022F, V022R and hIgCκR for light chain expression vectors (Table 2.1)

2.2.6.3 Design and generation of 2AC7-6D9 DVD-Ig expression vectors

Ch6D9 heavy and light chain encoding vectors were linearised by inverted PCR by using the primer pairs 6D9HF-LeaderHR and 6D9LF-LeaderLR respectively (Table 2.1). The PCR conditions for the linearisation of ch6D9 heavy chain expression vector are as follows: (1) 98^{o}C for 30s; (2) 98^{o}C for 10s; (3) 60^{o}C for 30s; (4) 72^{o}C for 1 min and 50 sec; (5) repeat steps 2-4 for 34 times; (6) 72^{o}C for 10min. The same PCR conditions were used for the amplification and linearisation of ch6D9 light chain encoding vector, with the difference of using 1min and 40s extension time in step 4.

For the construction of 2AC7-6D9 DVD-Ig long, 2AC7 variable heavy and light chain encoding sequences were PCR amplified from ch2AC7 heavy and light chain

expression vectors by using the primer pairs 7H27F-LG1Linker and 2L1F-LCkLinker, respectively (Table 2.1). Likewise, for the generation of the short version of 2AC7-6D9 DVD-Ig, 2AC7 variable heavy and light chains were amplified by using the primer pairs 7H27F-SG1Linker and 2L1F- SCkLinker (Table 2.1). The conditions used for all PCR reactions are as follows: (1) 98^{o}C for 30s; (2) 98^{o}C for 10s; (3) 60^{o}C for 30s; (4) 72^{o}C for 10 sec; (5) repeat steps 2-4 for 34 times; (6) 72^{o}C for 10min.

PCR products were analysed by agarose gel electrophoresis (section 2.8) and the bands corresponding to the 2AC7 variable heavy and light sequences were gel extracted by using the *QIA*quick Gel Extraction kit (*QIAGEN*) as per manufacturer's instructions. Gel extracted DNA, encoding for 2AC7 variable heavy and light chain was cloned by blunt ligation (*T4 ligase, NEB*; room temperature overnight) to the linearised ch6D9 heavy and light chain respectively. Chemically-competent *E.coli* TOP10 cells (*Invitrogen*) were transformed with the ligation products and positive clones were selected on LB-agar plates containing 100µg/ml ampicillin. Correct clones were verified by sequencing, using the V022F, V022R, 7H27F, 7H27R for heavy chain expression vectors, and V022F, V022R, 2L1F and 2L1R for light chain expression vectors (Table 2.1)

For all PCR reactions the Phusion High Fidelity DNA Polymerase (*Thermo Scientific*) was used.

2.2.7 Site directed mutagenesis of hu6D9

Hu6D9 mutants were generated by using the QuikChange® XL Site-Directed Mutagenesis Kit (*Agilent*) according to manufacturer's instructions. For the generation of C58P and R80V the primer pairs CYS58PROF-CYS58PROR and ARG80VALF-ARG80VALR were used respectively. C58P/R80V double mutant was developed by introducing the R80V mutation in hu6D9VHC58P mutant.

2.2.8 Recombinant antibody and DVD-Ig expression by transient transfection of expression plasmids

Recombinant antibodies and DVD-Igs were produced by using the HEK-293F (Human Embryonic Kidney-293-Fast growing variant) protein expression system. The cells, adapted for suspension culture, were maintained in non-supplemented Freestyle media (*Invitrogen*) incubated at 37°C, 5% CO2 and 70% humidity with orbital shaking at 120 r. p. m. Expression plasmids for recombinant antibodies were

transiently transfected into HEK293F by using the cationic reagent polyethylenimine (PEI; Tom *et al.*, 2008). Cells were split into 50 ml of fresh media at a density of $2.5x10^5$ cells/ml and allowed to recover for 24 hours prior to transfection. For recombinant antibody or DVD-Ig expression a transfection mixture consisting of 12.5 µl (at 1 mg/ml) heavy chain expression vector mixed with 12.5 µl (at 1 mg/ml) light chain expression vector, 2 ml of non-supplemented Freestyle medium and 50 µl of linear PEI (at 1 mg/ml) was added to the cell culture. Five to six days after transfection cells and cell debris were removed by centrifugation at 3220*g* for 20 min, supernatants were then filtered (0.2-µm filter) and stored at 4°C until use.

2.2.9 Purification of antibodies by affinity chromatography on protein G loaded column

Antibodies were purified from harvested culture supernatants on protein G Sepharose columns (HiTrap Protein G HP, 1 ml; *GE Healthcare*) using the ÄKTAxpress purification system (*GE Healthcare*). In each case, the column was pre-equilibrated with binding buffer (20 mM sodium phosphate, pH 7) at a flow rate of 1 ml/min, before loading of the harvested supernatant. When loading was complete, the column was washed with 15 column volumes (15 CV) of binding buffer to remove non-specific adherents and with 10 CV of elution buffer (0.1 M glycine HCl, pH 2.7) to recover specifically-bound protein. The elutant was monitored at 280 nm in real-time and collected in 0.5 ml fractions. The two/three fractions with the highest concentration of purified antibody (as estimated by measuring the absorbance at 280 nm) were pooled and dialysed (by using Slide-A-Lyzer Dialysis cassettes; *Thermo Scientific*) against HBS or PBS for the removal of glycine and to restore the pH to neutral. In several cases were antibodies were used in *P. falciparum* erythrocyte invasion assays, antibodies were also dialysed against RPMI 1640 (*GIBCO)* after dialysis against HBS or PBS.

2.2.10 Purification of antibodies with ammonium sulfate

Precipitation by ammonium sulfate is one of the oldest and widely used approaches for partial antibody purification. The principle is simple: at a specific concentration of ammonium sulfate, the number of water molecules that are available to keep a given protein moiety in solution is insufficient, resulting in protein

precipitation. The purity is not high because proteins with similar chemical properties to antibodies are salted out of the solution.

A saturated solution of ammonium sulfate was gradually added to harvested hybridoma supernatant with concomitant stirring, until the mixture became cloudy. Then, the stirring was stopped and the solution was left in 4^{o}C until the cloud completely precipitated. After the supernatant was discarded, the pellet (containing IgG) was dissolved in PBS, followed by dialysis against PBS (by using Slide-A-Lyzer Dialysis cassettes; *Thermo Scientific*) before use.

2.2.11 Antibody isotyping

Antibody isotyping was performed by using IsoQuick Strips (*Sigma*). Fifty microliters of 1µg/ml of m6D9 were loaded to the isotyping strip and incubated in room temperature for 20 minutes, before the results were detected.

2.3 Enzyme-linked-immunosorbent assays (ELISAs)

ELISAs were performed as previously described in (Bushell *et al.*, 2008). For quantitation of recombinant proteins from merozoite cell surface and secreted protein library, serially diluted biotinylated proteins were immobilised on streptavidin-coated 96-well plates (*NUNC*) for 1 h before incubation with 100µl of the mouse anti-rCd4 monoclonal OX68 (1µg/ml in HBS containing 0.1% Tween-20 and 2% BSA; HBST-BSA) for another hour. The plates were then washed in HBST before incubation with an anti-mIgG (*Sigma*) secondary antibody conjugated to alkaline phosphatase, for 1h. Plates were then washed three times in HBST and once in HBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well).

Similarly, for the assessment of mouse, humanised or chimeric antibody binding, biotinylated RH5 or Basigin were immobilised on streptavidin-coated 96-well plates (*NUNC*) for 1h, at concentrations sufficient for complete saturation of the available binding surface, followed by incubation with 100µl of serially diluted purified or tissue culture supernatant contained antibody. The plates were then washed in HBST before incubation with an anti-mIgG (*Sigma*) or anti-hIgG (*Sigma*) secondary antibody conjugated to alkaline phosphatase for 1h. Plates were then washed three times in HBST and once in HBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well).

In all ELISAS, absorbance was measured at 405 nm on a PHERAstar plus *(BMG Labtech)* plate reader after 10-15 mins. All the steps in the procedure were carried out at room temperature.

2.4 Immunoreactivity of recombinant merozoite proteins

In addition to quantifying biotinylated proteins and assessing antibody binding, ELISAs were used for testing the conformational state of merozoite cell surface and secreted proteins by means of specific antibodies present in immune sera, that recognise non-linear, heat-labile epitopes. For the latter analysis, proteins were heat-treated for 10 min at 80°C and then incubated on ice for a further 10 min, prior to use in ELISAs with untreated controls. Heat treated or untreated proteins were immobilised on streptavidin-coated 96-well plates (*NUNC*), at concentrations sufficient for complete saturation of the available binding surface/well (as determined by ELISA). In the cases where the concentration of biotinylated proteins was below saturating levels, 200µl of protein were used for protein immobilisation.

After washing the plates three times in HBST immobilised proteins were incubated for 2h, in the presence of 100µl of purified IgGs (provided by Dr Faith Osier; used at dilution 1:1000 in PBST, 2% BSA) from pooled immune sera obtained from previously malaria infected Malawian adults, or IgG purified from pooled non-immune sera from people who had never been exposed to malaria (provided by Dr Faith Osier; used at dilution 1:1000 in PBST, 2% BSA; negative control). The anti-rCd4 monoclonal OX68 (at concentration 1µg/ml) was used to confirm immobilisation of proteins. The plates were then washed in PBST before incubation with an anti-mIgG (*Sigma*) or anti-hIgG (*Sigma*) secondary antibody conjugated to alkaline phosphatase for 1h. Plates were then washed three times in PBST and once in PBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well). Absorbance was measured at 405 nm on a PHERAstar plus *(BMG Labtech)* plate reader after 10-15 mins. All the steps were carried out at room temperature.

2.5 Normalisation of β-lactamase tagged membrane protein ectodomains

Beta-lactamase tagged pentameric proteins were normalised as described by Bushell *et al.*, 2008, by monitoring their enzymatic activity over time, in a nitrocefin (*Calbiochem*) turnover assay. Twenty microliters of harvested culture supernatants

were incubated with 60 µl of nitrocefin (at 250 µg/ml) for a period of 20 mins at room temperature, during which nitrocefin turnover was quantified by monitoring the absorbance at 485 nm on a PHERAstar plus *(BMG Labtech)* plate reader. Prey activity was considered sufficient for AVEXIS if the available nitrocefin was completely turned over within 5-10 mins (Bushell *et al.*, 2008). Normalisation of proteins was achieved either by concentrating with 20 kDa MWCO spin concentrators (*Vivascience*) or diluting as required in HBST, 2% BSA.

2.6 Avidity-based extracellular interactions screen (AVEXIS)

This protocol was adapted from Bushell *et al.*, 2008. Streptavidin-coated 96-well plates (*NUNC*) were blocked with HBST, 2%BSA (100µl/well) for 1h. Biotinylated proteins (the baits) were diluted in HBST, 2% BSA and immobilised on the pre-blocked streptavidin-coated 96-well plates at concentrations sufficient for complete saturation of the available binding surface/well (as determined by ELISA). In the cases where the concentration of biotinylated proteins was below saturating levels, 200µl of purified protein (unknown concentration) were used for protein immobilisation. The plates were then washed three times in HBST before addition of normalised β-lactamase tagged pentameric proteins (the preys) and incubation for 2h. The plates were then washed three times in HBST and once in HBS. Nitrocefin (250 µg/ml; 60 µl per well) was added and developed for ~3h, before absorbance was measured at 485 nm on a PHERAstar plus *(BMG Labtech)* plate reader. All steps were performed at room temperature.

The binding events in the AVEXIS screen were analysed as follows: the binding of each prey to Cd4 bait was subtracted from the binding to a specific bait (actual binding). The average actual binding of a prey across the whole merozoite bait panel was then subtracted from the actual binding to a specific bait. The latter value was finally divided to the standard deviation of the binding of the prey under investigation to the whole panel of merozoite baits, to obtain a z-score for each interaction. A z-score represents the number of standard deviations, a given interaction is above or below the mean binding of a prey to the whole panel of merozoite baits.

2.7 High-throughput screen for the identification of glycan binding specificities of merozoite membrane and secreted proteins

Mono-biotinylated synthetic carbohydrate probes (*GlycoTech*) were immobilised at 50 µg/ml (100 µl/well) on pre-blocked (HBST, 2%BSA; 100µl/well; 1h) streptavidin-coated 96-well plates (*NUNC*) for 1 h at room temperature. The plates were then washed twice in HBST and blocked with HBS-BSA (100 µl per well) for 30 mins before addition of normalised β-lactamase tagged pentameric proteins and incubation for 2 h at room temperature. The plates were then washed three times in HBST and once in HBS before detection of the prey proteins by using nitrocefin as described before in section 2.6.

2.8 Agarose gel electrophoresis

DNA samples diluted in loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were loaded onto 1% agarose, TAE-buffered (40 mM tris, 20 mM acetic acid, 1 mM EDTA) gels containing 0.1 µg/ml ethidium bromide. After electrophoresis at a constant voltage of 80V, DNA was visualised using a UV transilluminator (*BIORAD*).

2.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE using Novex NuPage 4-12% Bis-Tris pre-cast gels (*Invitrogen*), as per manufacturer's instructions. Electrophoresis was performed at a constant voltage of 200 V for 50 mins and proteins were visualized using Coomassie brilliant blue (G250) staining solution (*Thermo Scientific*) according to the manufacturers' protocols.

2.10 Western blotting

Western blotting was used to confirm the size of recombinant antibodies or biotinylated proteins. Tissue culture supernatant or two micrograms of each protein or antibody were separated by SDS-PAGE (section 2.9), before transferring to PVDF membrane using a XCell II blotting module (*Novex*), at a constant voltage of 30 V, for 1 hour, at room temperature, in NuPage transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 0.05 mM Chlorobutanol) (*Invitrogen*), supplemented with 10% methanol. The blots were incubated for 2h in room temperature, in PBST containing 5% milk to block non-specific binding sites. Biotinylated proteins or recombinant antibodies were then probed with horseradish peroxidase (HRP)-conjugated

streptavidin (1:37 000 dilution in PBST, 2% milk; *Jackson Immunoresearch Laboratories*) or anti-human-HRP (1:20 000 in PBST, 2% milk; *Sigma*) respectively, and detected by SuperSignal® West Pico Chemiluminescent Substrate (*Thermo Scientific*) after exposure to Hyperfilm (*GE Healthcare*).

2.11 *In vitro* biotinylation of antibodies and DVD-Igs

Purified mouse or recombinant antibodies and DVD-Igs were diluted at 2µg/ml in PBS at a final volume of ~150µl. Diluted antibodies and DVD-Igs were biotinylated *in vitro* using a 20-fold molar excess of EZ-link sulfo-NHS-biotin (*Thermo Scientific*) for 1h at room temperature. The biotinylated antibody and DVD-Ig preparations were then dialysed against 5 L HBS overnight (5 changes of buffer) by using Slide-A-Lyzer Dialysis cassettes (*Thermo Scientific*) to remove free, unconjugated biotin.

2.12 Surface plasmon resonance (SPR)

Surface plasmon resonance was used to derive affinity and kinetic parameters for protein-protein (including antibody-antigen) interactions. All SPR experiments were performed on a Biacore T100 instrument (*GE Healthcare*) using HBS-EP as the running buffer at 25°C.

2.12.1 Regeneration scouting by using SA chip

For the regeneration scouting experiment the Series S Sensor Chip SA (*GE Healthcare*) was used. The sensor chip surface was activated with three 1 min injections of 1 M NaCl and 50 mM NaOH at a flow rate of 30 µl/min. Regeneration scouting experiment was performed by using the Regeneration Scouting setup of Biacore T100 Control software (*GE Healthcare*), according to manufacturer's instructions. Briefly biotinylated MEM-M6/4 was captured on the activated chip surface, at about 400 response units (RU). Basigin contained in tissue culture supernatant was injected over immobilised antibody at a flow rate of 20µl/min for 30s, followed by injection of the stated regeneration solution at a flow rate of 30µl/min for 30s. Five consecutive analyte (Basigin) binding and regeneration cycles were tested for each regeneration solution before the results were analysed by using the Biacore T100 Control software (*GE Healthcare*) and Graphpad Prism 5 (*Graphpad Software Inc*.).

2.12.2 Analysis of protein-protein interactions by using CAP chip

SPR analysis of protein-protein (including antibody antigen) interactions were performed by using Biotin CAPture Kit (*GE Healthcare*), according to manufacturer's instructions. Briefly, Sensor CAP Chip was docked on Biacore T100 instrument and left on standby for at least 24h, in order to become rehydrated. Sensor CAP Chip surface was then conditioned by 3 one minute injections of regeneration buffer (3 parts of 8M guanide-HCl mixed with 1 part of 1M NaOH) at a flow rate of 20μl/min. After conditioning, the Biotin CAPture Reagent was immobilised on chip surface at 3800-4300 RU, followed by immobilisation of the stated ligand (biotinylated protein or antibody) and relevant control. The stated concentrations of analytes were then injected over sensor-surface captured ligands (at the indicated flow rates and for the stated times). Results were analysed by using the BIAevaluation software (*GE Healthcare*), and kinetic parameters were obtained by fitting a 1:1 binding model to the reference subtracted sensorgrams.

2.13 Erythrocyte binding assay

The binding of anti-Basigin antibodies to human red blood cells was investigated by a flow cytometric based method. In round-bottom 96-well plates (*COSTAR*) $5x10^5$ human O^+ erythrocytes were incubated with each anti-Basigin antibody at a final concentration of 10μg/ml (at a final volume of 100μl) for 2h at 4^oC. Mouse IgG or hIgG isotype controls (*Abcam*) and the anti-Basigin monoclonal MEM-M6/6 (*Abcam*) were used as negative and positive controls, respectively. After three washes in PBS, red blood cells were incubated for 2h at 4^oC with goat anti-mIgG (at dilution 1:500; *Abcam)* or goat anti-hIgG (at dilution1:200; *Abcam)* FITC conjugated secondary polyclonals. Erythrocytes were washed twice in PBS, and antibody binding was examined by counting the FITC positive antibody-cell complexes by flow cytometry (BD LSRII flow cytometer; *BD Biosciences*). The data collected were then further analyzed with FlowJo (*Tree Star*).

2.14 C1q binding assay

The binding of C1q to ch6D9 was examined by using an ELISA based assay. One hundred microliters of 1.5μg/ml of ch6D9 or hIgG1 isotype (*SouthernBiotech)* were used to coat overnight at 4^oC the binding surface of a 96-well maxisorb plate (*NUNC)*. The plate was washed three times in PBST and blocked with PBST, 2%

BSA for 1h at room temperature, followed by incubation with a 2-fold dilution series of complement component C1q from human serum (*Sigma)* for 2h. The plate was then washed again three times in PBST followed by 1h serial incubations with goat anti-hC1q polyclonal (at dilution 1:1000; *Calbiochem*) and rabbit anti-goat IgG conjugated with alkaline phosphatase. The plate was finally washed three times in PBST and once in PBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well). Absorbance was measured at 405 nm on a PHERAstar plus *(BMG Labtech)* plate reader after 10-15 mins

2.15 FcγRIIA binding assay

The binding of $FcγRIIA_{131His}$ (α-chain) to ch6D9 was assessed by using an ELISA based assay. Recombinant monomeric biotinylated FcγRIIA was expressed as described in section 2.1.2. Biotinylated FcγRIIA was immobilised on a pre-blocked (with HBST, 2%BSA) streptavidin-coated 96-well plate (*NUNC*) at concentrations sufficient for complete saturation of the available binding surface (as determined by ELISA). The plate was washed with HBST and incubated for 2h at room temperature with a dilution series of ch6D9 or hIgG1 isotype (*SouthernBiotech)*. The plate was then washed again three times in PBST followed with 1h incubation with a donkey anti hIgG $F(ab)_2$ polyclonal, conjugated to alkaline phosphatase (*Abcam)*. The plate was finally washed three times in PBST and once in PBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well). Absorbance was measured at 405 nm on a PHERAstar plus *(BMG Labtech)* plate reader after 10-15 mins.

2.16 *P. falciparum* culture, and invasion assays

All *P. falciparum* parasite strains were routinely cultured in human O^+ erythrocytes at 5% haematocrit in complete medium (RPMI-1640 supplemented with 10% human serum), under an atmosphere of 1% O_2, 3% CO_2 and 96% N_2. Invasion assays were carried out in round-bottom 96-well plates (*COSTAR*), with a culture volume of 100 µl per well at 2% haematocrit and 0.75% parasitemia, as previously described (Theron *et al.*, 2010; Bustamante *et al.*, 2013). *P. falciparum* cultures were synchronised on early stages by treatment with 5% w/v D-sorbitol solution (*Sigma)*. Parasites in trophozoite stage were mixed with monoclonal antibodies (or DVD-Igs) and incubated overnight at 37 °C inside a static incubator culture chamber (VWR),

gassed with 1% O_2, 3% CO_2 and 96% N_2. At the end of the incubation period, red blood cells were collected, fixed with a fixative solution (2% paraformaldehyde, 0.2% glutaraldehyde) and permeabilized with 0.3% Triton-X 100 (*Sigma*). Erythrocytes were then treated with 0.5mg/ml RNAse (Ribonuclease A; *MP Biomedicals)* before staining with SYBR Green I (at dilution 1:5000; *Invitrogen)*. Parasitized RBC (pRBC) were counted as SYBR Green I positive cells by using flow cytometry (BD LSRII flow cytometer; *BD Biosciences*). The data collected were then further analyzed with FlowJo (*Tree Star*).

Table 2.1 **The primers that were used in this study.**

Primer Name	Sequence
462	CCACTTTGCCTTTCTCTCCA
463	ATGTCCTTCCGAGTGAGAGA
497	TGAGATCCAGCTGTTGGGGT
498	AGAAGGGGCAGAGATGTCGT
3732	TTTAGGGAGAAGGTGATCCAGG
3381	TAGAGATCTCCGAGGGATCTCGACC
3811	AGAAGGGGCAGAGATGTCGTAG
3397	AGGAAGTGAACCTGGTGGTG
3851	AGAAGGGGCAGAGATGTCGTAG
3	TCAATAGTTGAACATAGCGGCCGCASAAAWTGTKCTCACCCAGTC
4	TCAATAGTTGAACATAGCGGCCGCAGAWATTGTGCTMACTCAGTC
5	TCAATAGTTGAACATAGCGGCCGCAGACATTGTGCTRACACAGTC
6	TCAATAGTTGAACATAGCGGCCGCAGACATTGTGATGACMCAGTC
7	TCAATAGTTGAACATAGCGGCCGCAGAYATCMAGATRAMCCAGTC
8	TCAATAGTTGAACATAGCGGCCGCAGAYATCCAGATGAYTCAGTC
9	TCAATAGTTGAACATAGCGGCCGCAGATATCCAGATGACACAGAC
10	TCAATAGTTGAACATAGCGGCCGCAGACATTGTGCTGACCCAATC
11	TCAATAGTTGAACATAGCGGCCGCAGACATYSTRATGACCCARTC
12	TCAATAGTTGAACATAGCGGCCGCAGATRTTKTGATGACYCARAC
13	TCAATAGTTGAACATAGCGGCCGCAGAYATTGTGATGACBCAGKC
14	TCAATAGTTGAACATAGCGGCCGCAGATATTGTGATAACCCAGGA
15	TCAATAGTTGAACATAGCGGCCGCAGACATCYTGCTGACYCAGTC
16	TCAATAGTTGAACATAGCGGCCGCAGAAAWTGTGYTGACCCAGTC
17	TCAATAGTTGAACATAGCGGCCGCAGAAACAACTGTGACCCAGTC
18	TCAATAGTTGAACATAGCGGCCGCAGACATTRTGATGWCACAGTC
19	TCAATAGTTGAACATAGCGGCCGCAGACATCCAGMTGACMCARTC
20	GGATACAGTTGGTGCAGCATCAGCCC
27	TTCACGAGTCCAGCCTCAAGCAGTGAKRTRCAGCTTMAGGAGTC
28	TTCACGAGTCCAGCCTCAAGCAGTGAGKTYCAGCTBCAGCAGTC
29	TTCACGAGTCCAGCCTCAAGCAGTCAGGTGCAGMTGAAGSAGTC
30	TTCACGAGTCCAGCCTCAAGCAGTGAGRTCCAGCTGCAACARTC
31	TTCACGAGTCCAGCCTCAAGCAGTCAGGTYVAGCTGCAGCAGTC
32	TTCACGAGTCCAGCCTCAAGCAGTCAGGTYCARCTGCAGCAGTC

33	TTCACGAGTCCAGCCTCAAGCAGTGAGGTGMAGCTGGTGGAATC
34	TTCACGAGTCCAGCCTCAAGCAGTGAVGTGMWGCTSGTGGAGTC
35	TTCACGAGTCCAGCCTCAAGCAGTGARGTGCAGCTGKTGGAGWC
36	TTCACGAGTCCAGCCTCAAGCAGTGAGGTGAAGCTGATGGAATC
37	TTCACGAGTCCAGCCTCAAGCAGTGAGGTGCAGCTTGTTGAGTC
38	TTCACGAGTCCAGCCTCAAGCAGTGAGGTGAAGCTTCTCRAGTC
39	TTCACGAGTCCAGCCTCAAGCAGTGAAGTGAARMTTGAGGAGTC
40	TTCACGAGTCCAGCCTCAAGCAGTCAGGTTACTCWGAAAGWGTCTG
41	TTCACGAGTCCAGCCTCAAGCAGTCAGGTCCAACTGCAGCAGCC
42	TTCACGAGTCCAGCCTCAAGCAGTGATGTGAACCTGGAAGTGTC
43	TTCACGAGTCCAGCCTCAAGCAGTCAGATCCAGTTSGTRCAGTC
44	TTCACGAGTCCAGCCTCAAGCAGTGAGGTRCAGCTKGTAGAGAC
45	CTATTCTAGCTAATCTAGGCGCGCCGAGGAGACGGTGACCGTGGTCC
46	CTATTCTAGCTAATCTAGGCGCGCCGAGGAGACTGTGAGAGTGGTGC
47	CTATTCTAGCTAATCTAGGCGCGCCGCAGAGACAGTGACCAGAGTCC
48	CTATTCTAGCTAATCTAGGCGCGCCGAGGAGACGGTGACTGAGGTTC
M13F	TGTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGACC
T7	TAATACGACTCACTATAGGG
SP6	ATTTAGGTGACACTATAG
LeaderHR	GCTGTGCACGCCAGCTGTGCC
hIgG1F	GCTAGCACCAAGGGCCCCAGCG
LeaderLR	GCCTCTGCTGGCGGGGATCCAG
hIgCκF	CGGACCGTGGCCGCTCCCAG
7H27F	GAGGTGAAGCTGGTGGAGTCTGGGGG
7H27R	CGAGGAGACGGTGACCGTGGCCC
4H12R	CGAGGAGACGGTGACCGTGGTCC
2L1F	GACATTGTGCTGACCCAATCTCCAACTTCTTT
2L1R	TTTGATTTCCAGCTTGGTGCCTCCACC
LL2F	GACATTGTTCTCACCCAATCTCCAGCTTCTTTG
V022F	TCTCTCCACAGGTGTCCACT
hIgG1Rnew	GGGGAAGTAGTCCTTGACCA
V022R	TTATTAGCCAGAGGTCGAGG
hIgCκR	TTGTCCACCTTCCACTGCAC
6D9HF	GAAGTGCAGCTGCAGCAGAGCGG

6D9LF	GACATCGTGCTGACCCAGAGCCCC
LG1Linker	GGGGGCCAGAGGGAACACGC
LCkLinker	GGGTGGGAAGATGAACACGCTGGG
CYS58PROF	TGGGCTGGATCAGCCCCTACAACGGCGTGCC
CYS58PROR	GGCACGCCGTTGTAGGGGCTGATCCAGCCCA
ARG80VALF	GAGTGACCATCACCGTGGACACAAGCGCCAG
ARG80VALR	CTGGCGCTTGTGTCCACGGTGATGGTCACTC

Degenerate base code: M = A+C; R = A+G; W = A+T; S = C+G; Y = C+T; K = G+T; V = A+C+G; B = C+G+T