Chapter 2

Materials and Methods

2.1 Protein production

2.1.1 Plasmid preparation

All plasmids used for protein expression were based on pTT3 which contains an ampicillin resistance marker for selection, an EBV origin of replication to allow amplification in HEK293E cells, and a series of promoter and leader elements to enhance expression[79] (Figure 2.1A). For the expression of mammalian proteins, the endogenous signal peptide was cloned into the vector, whereas the coding sequences of *Plasmodium* proteins were cloned downstream of the leader sequence of the mouse variable κ light chain (Figure 2.1B)[1]. *Plasmodium* protein sequences were codon-optimised for expression in mammalian cells using Life Technologies' GeneArt Service[240]. Since N-linked glycosylation is uncommon in *Plasmodium* (and prevalent in HEK cells), N-X-S/T glycosylation site-encoding motifs in *Plasmodium* constructs were mutated to N-X-A-encoding DNA, prior to gene synthesis.

[1] N-terminally truncated mammalian proteins were also cloned downstream of this leader sequence, without their endogenous signal peptide

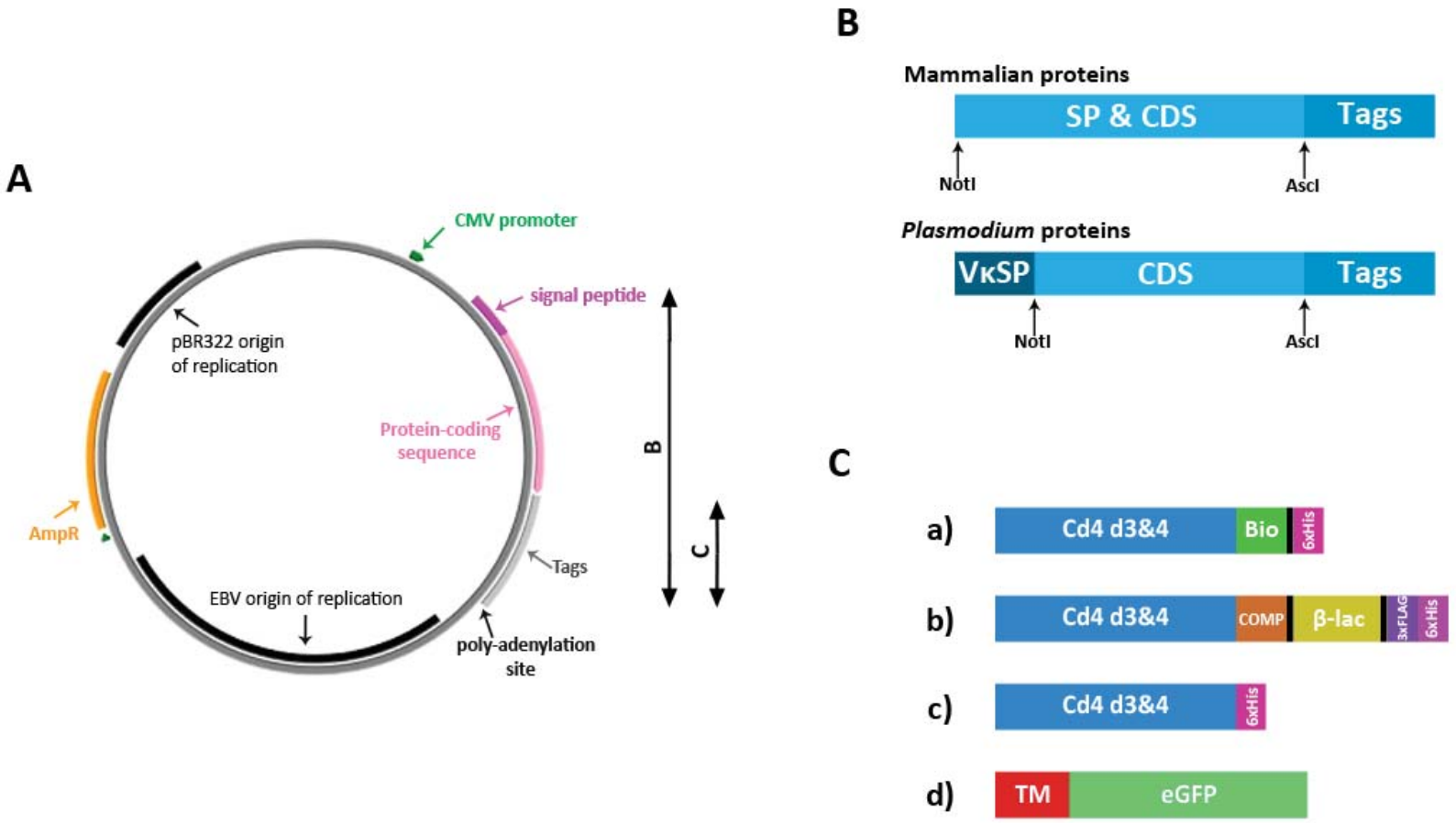

Figure 2.1: **Construction of plasmids for protein expression**
A. Generalised map of pTT3-based expression plasmids used in this work
B. Signal peptide (SP), protein ectodomain-coding sequence (CDS) and restriction-ligation cloning site arrangements for different expression plasmids
C. N-terminal tags on expression constructs included Cd4 expression tags, biotinylation sites (Bio), pentamerisation sequences (COMP), β-lactamase (β-lac) or eGFP reporters, and FLAG- or His-tags. Proteins destined for the cell surface were cloned upstream of the rat Cd200 transmembrane (TM) domain.

Depending on the intended use of the recombinant protein, a selection of different C-terminal tags were incorporated (Figure 2.1C). All secreted proteins were produced as fusions with rat Cd4 domains 3 & 4 (Cd4), which is likely to increase the proteins' solubility and hence expression, and also means that they could be detected via the use of a mouse anti-rat Cd4 OX68 monoclonal antibody[35]. For proteins that were to be biotinylated, an *Escherichia coli* BirA biotin ligase substrate motif was included[36, 40]. A six-histidine tag[141] and/or 3xFLAG® (Sigma) were also included for protein purification and detection. Pentamerisation of proteins was achieved via the inclusion of a pentamerisation domain from cartilage oligometric matrix protein (COMP)[333]. Pentameric proteins were also tagged with a β-lactamase reporter that could be used as a proxy to normalise the amount used in assays (as described in 2.3.1.2). To produce GFP-tagged proteins on the surface of cells, a transmembrane (TM) domain from rat Cd200 was included, along with an eGFP reporter[60].
All plasmid DNA sequences were validated by in-house capillary sequencing. Details of the expression plasmids used as part of this work can be found in Tables 2.4 & 2.5.

2.1.1.1 PCR

Several mammalian protein-coding sequence inserts were cloned from cDNA. PCR primers were designed so as to introduce NotI and AscI sites 5' of the signal peptide and 3' of transmembrane domain respectively. 1µg cDNA for human *SELE*, *SELL* and *SELPLG* (encoding PSGL1), as well as mouse *Selp* (Origene), were used as templates. The reaction was performed using 1U KOD Hot Start DNA polymerase (Novagen) in the manufacturer's buffer supplemented with 1.5mM $MgSO_4$, 1µM primers and 0.2 µM dNTPs. The thermocycler was programmed to carry out an initial five-minute denaturation step at 95°C followed by 25 cycles of denaturation for 15 seconds at 95°C, primer annealing at between 60 and 65°C for 30 seconds and elongation at 72°C for one minute. A final elongation step at 72°C was then carried out for five minutes before purifying the fragments using a QIAquick PCR Purification Kit (QIAGEN). In order to make truncated protein fragments of SELP and PfMSP7, similar PCR methods were applied using existing plasmids as templates. A list of primers can be found in Table 2.1 and details of the boundaries of the SELP and PfMSP7 fragments are provided in Tables 2.2 and 2.3.

2.1.1.2 Restriction-ligation cloning

New plasmids for these studies were constructed using restriction-ligation cloning methods. Vectors and inserts were digested for two hours at 37°C (most commonly using NotI and

AscI, NEB) and the resulting fragments were resolved by agarose gel electrophoresis. Bands were visualised under ultraviolet light and extracted using a QIAquick Gel Extraction Kit (QIAGEN). 20ng of vector and 60ng of insert were used in ligation reactions with T4 ligase (Roche) which were performed at 16°C for 3-12 hours. A 45-second 42°C heat shock was used to transform chemically-competent *E. coli* cells (Agilent) with the ligation products. Cells were plated out on LB-agar containing 100µg/mL ampicillin for selection. Positive clones were cultured overnight in liquid LB and plasmids were prepared for use at 1mg/mL using a PureLink HiPure Plasmid Maxiprep kit (Life Technologies). In-house capillary sequencing was used to verify the success of cloning new plasmids.

2.1.2 Protein expression using HEK293 cells

All recombinant proteins were produced in a mammalian expression system based on the transient transfection of cells from the Human Embryonic Kidney (HEK293) line. The majority of proteins were expressed using HEK293E cells which are stably transformed with Epstein Barr Virus Nuclear Antigen (EBNA1), so as to increase the number of plasmids maintained in transfected cells during protein production. HEK293F cells, which do not possess this EBNA1-based amplification system, were used for the expression of GFP-tagged recombinant proteins targeted to the cell surface.

2.1.2.1 Cell culture

HEK293 cells were maintained in suspension in 50mL Freestyle medium (Life Technologies) at 37°C, 70% humidity, 5% CO_2 and 120rpm orbital shaking. When HEK293E cells were grown, the medium was supplemented with 1% FCS (Life Technologies) and 50µg/mL G418 antibiotic (Sigma).

2.1.2.2 Transfection

24 hours prior to transfection, 50mL fresh medium was seeded with cells to give a final density of $2.5x10^5$ cells/mL. For each transfection, 2mL of Freestyle medium, 25µg of expression plasmid and 50µg polyethylenimine (PEI) transfection regent were mixed and incubated together at room temperature for 10 minutes. The mixture was added to the 50mL culture which was then returned to the incubator. In order to produce biotinylated proteins, cells were seeded into Freestyle medium containing the same FCS and G418 supplements along with 100µM D-biotin (Sigma). During the transfection procedure, 2.5µg of a plasmid encoding a secreted

form of *E. coli* biotin ligase (BirA) was added alongside the expression plasmid. The BirA enzyme catalyses the addition of D-biotin to recombinant protein produced with a biotinylation site at their C-terminus (Figure 2.1C).

2.1.2.3 Collection

After four to six days of incubation post-transfection, secreted proteins were collected from the culture supernatant. Cellular material was removed following centrifugation at 3220g for 10 minutes and the resulting supernatant was filtered using 0.2µm filters. The filtered supernatants were stored at 4°C with 50µg/mL polymixin B antibiotic (Sigma). To remove free D-biotin, supernatants containing biotinylated proteins were transferred to 10kDa MWCO Snakeskin dialysis tubing (Thermo Scientific) then dialysed against 4.5L of HBS (0.14M NaCl, 10mM HEPES, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$) at 4°C. To ensure sufficient D-biotin removal, the HBS was replaced seven times over two days.

2.2 Protein purification and quantification

2.2.1 Immobilised metal ion affinity chromatography

6xHis tagged proteins were enriched from cell culture supernatants using 1mL HisTrap nickel columns and ÄKTAxpress purification apparatus (GE Healthcare). To decrease non-specific protein retention in the columns, 10mM imidazole and 200mM NaCl were included in the input protein sample. The HisTrap column was equilibrated with binding buffer (0.5M NaCl, 40mM imidazole, 20mM sodium phosphate buffer, pH7.4), loaded with the input protein, then washed with binding buffer. Elution buffer (0.5M NaCl, 0.4M imidazole, 20mM sodium phosphate buffer, pH7.4) was then flowed through the column and 0.5mL eluate fractions were collected by the apparatus. The ÄKTAxpress readout estimates the absorbance of the output samples at 280nm and was used to identify fractions with a high protein yield, which were then visualised and quality-checked using SDS-PAGE (see 2.2.4). The elution buffer was replaced with HBS during subsequent size-exclusion chromatography (see 2.2.2) or by dialysis (see 2.1.2.3) using 3.5kDa MWCO D-tube Dialysers (Novagen).

2.2.2 Size-exclusion chromatography

To remove protein aggregates from IMAC-purified proteins, fractions were further purified by size-exclusion chromatography (SEC), using a Superdex Tricorn 200 10/600 GL or Superdex

200 Increase 10/300 GL gel-filtration column (GE healthcare). Columns were connected to an ÄKTAxpress system which was used to pre-equilibrate the column with HBS buffer prior to the injection of the sample. After the protein was loaded onto the column, HBS buffer was pumped through the column at a flow rate of 1mL/min and 0.5mL fractions were collected once the void volume of the column was reached. Protein content of the eluting solution was inferred by the absorbance at 280nm, measured in real time by the ÄKTAxpress instrument.

2.2.3 Determination of protein concentration

The approximate total protein content of recombinant protein solutions was measured using their absorbance at 280nm, determined using a benchtop spectrophotometer or Nanodrop (Thermo). The extinction coefficients of proteins of interest were calculated *in silico* allowing the estimation of protein concentration using the Beer-Lambert law.

2.2.4 SDS-PAGE

NuPAGE SDS-PAGE reagents, including 4-12% pre-cast gradient gels (Life Technologies) were used for protein gels. All proteins were denatured proir to electrophoresis. Gels were stained with colloidal Coomassie blue G250 (Fisher) or SYPRO Orange (Sigma), according to the manufacturer's instructions. SYPRO-stained gels were visualised using a Typhoon phosphoimager.

2.2.5 Western blotting

Following SDS-PAGE, proteins were transferred from the gel to a PVDF (GE Healthcare) membrane. The transfer was carried out using NuPage transfer buffer (Life Technologies) supplemented with 10% methanol, an XCell II blot module (Novex) and 40V voltage for two hours at room temperature. To reduce non-specific streptavidin or antibody binding, the membrane was then blocked by incubation with HBS containing 2% BSA overnight at 4°C. To detect biotinylated proteins, the membrane was incubated in 25mL HBS containing 0.2% BSA and 25ng/mL streptavidin-HRP conjugate for one hour at room temperature. When rabbit polyclonal antibodies were used to detect proteins, 25mL HBS containing 2% BSA and 1µg/mL antibody were incubated together overnight at 4°C then incubated with the membrane for one hour at room temperature. The antibody-stained membranes were then transferred to 25mL HBS containing 200ng/mL HRP-conjugated anti-rabbit secondary antibody for one hour. All membranes were washed with HBST for at least one hour at room temperature. 1mL

SuperSignal West Pico enhanced chemiluminescent HRP substrate (Thermo) was applied to the membrane, which was then used to expose Hyperfilm (GE Healthcare).

2.2.6 Polyclonal antibody production and purification

His-tagged Cd4-tagged PfMSP7 was expressed and purified as described in 2.2.1. Approximately 1mg of the protein was sent to Cambridge Research Biochemicals and used to immunise one rabbit. 50mL of the harvest bleed was filtered through a 0.2µm filter then purified using a 1mL HiTrap Protein G HP column (GE healthcare) and the ÄKTAxpress apparatus. The Protein G column was equilibrated with 20mM sodium phosphate buffer (pH7.0), loaded with the input protein, then washed with sodium phosphate buffer. Bound antibody was eluted using 0.1M glycine HCl (pH2.7) buffer. 0.5mL fractions were collected and neutralised by the addition on 60µL 2M Tris-HCl (pH 9.0). The purified antibodies were dialysed against PBS and tested for reactivity against recombinant PfMSP7 and Cd4 domains 3 & 4 bait proteins by ELISA (see 2.3.1.1).

2.3 AVEXIS

Avidity-based extracellular interaction screening (AVEXIS) was performed as directed in Bushell *et al.* (2008), with some alterations[40]. In brief, 100µL bait proteins, normalised as described in 2.3.1.1, were captured on a 96-well streptavidin-coated plate, which was incubated at room temperature for one hour. The plate was then washed three times with HBST and once again with HBS. 100µL normalised prey protein was then added to the wells and incubated at room temperature for 90 minutes. Washes were performed as before and 60µL nitrocefin substrate was added to the wells. Absorbance at 485nm was used to quantify the colour changes after one hour, unless otherwise stated.

2.3.1 Standardisation

To standardise the amounts of cell culture supernatants to be used in AVEXIS, ELISAs were carried out on serial dilutions of bait proteins and the nitrocefin hydrolysis rates of serial dilutions of prey protein were also assessed[40]. If necessary, proteins were concentrated using Vivaspin 20 spin concentrators (Sartorius-stedim) or diluted with HBS containing 1% BSA.

2.3.1.1 ELISA

100µL two-fold serial dilutions of biotinylated proteins were captured on a streptavidin-coated plate (Nunc). After one hour the plate was washed three times with HBS containing 0.02% Tween (HBST), then once with HBS alone. OX68 is a mouse monoclonal antibody with a high affinity for the CD4 tag, and 100µL of a 1.4µg/mL solution was used as the primary antibody. After a further hour's incubation, the plate was washed again and 100µL of anti-mouse IgG coupled to alkaline phosphatase (Sigma A4656, 1:5000) was added to the wells. Following another hour's incubation and washing, 100µL of 1µg/µL phosphatase substrate (Sigma) was added. Substrate hydrolysis was assessed after 30 minutes by measuring absorbance at 405nm on a PHERAstar plus instrument (BMG Labtech).

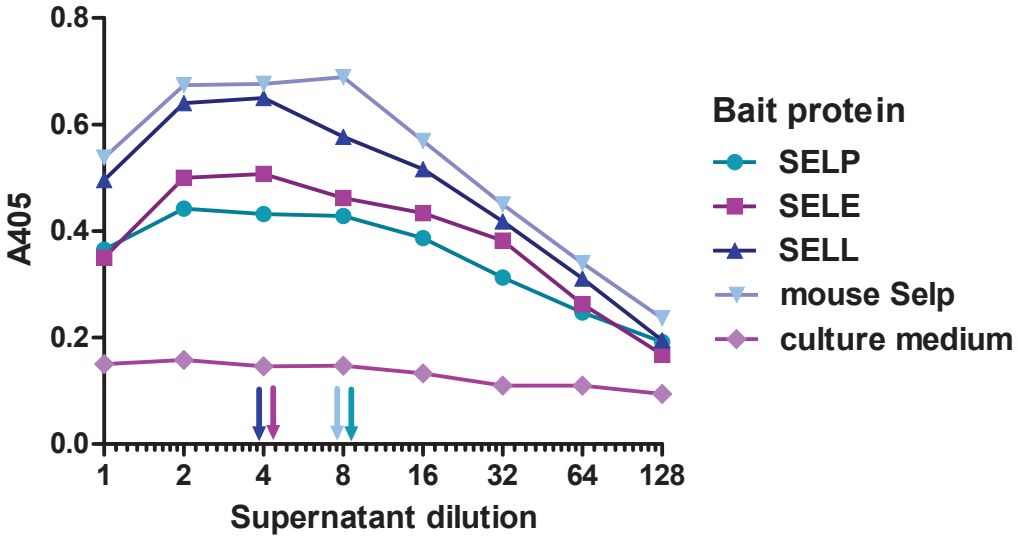

Figure 2.2: **Standardisation of bait proteins by ELISA**
Typical ELISA profiles for biotinylated bait protein supernatants. The arrows indicate the dilution of each protein that was used in subsequent AVEXIS-based assays. Serial dilutions of cell culture medium were used as a negative control, to demonstrate the signals observed were due to the presence of the transfected protein only.

The shapes of the ELISA curves were used to assess protein abundance. If the plate is saturated with biotinylated protein a consistent, high A405 at low dilution factors is expected, with this signal decreasing at higher dilutions of the protein (as gradually less bait is bound to the plate). In reality 'humped' ELISA profiles were often observed (Figure 2.2), for which there could be a number of explanations. Perhaps the most likely explanation when supernatants are used is that the dialysis process has not removed all the available biotin, such that free biotin competes

with biotinylated proteins for the strepatavidin, an effect which is not observed upon dilution as competition for biotin-binding sites decreases. For particularly highly-expressed or purified proteins the curves' shapes may reflect the 'over-crowding' of the plate at low dilutions, such that so much protein is captured on the plate that this impedes access of the OX68 antibody to the Cd4 tag (a manifestation of the prozone effect[42]). It might also be that other abundant proteins in the cell culture supernatant interfere with the specific capture of the biotinylated bait. For AVEXIS, bait proteins were used at the lowest dilution at which absorbance was maximised, so as to balance high levels of protein capture against over-crowding of the bait on the plate surface (indicated in Figure 2.2). Bait proteins that did not show a saturable ELISA signal were spin-concentrated then tested again by ELISA.

2.3.1.2 Nitrocefin hydrolysis assay

To assess their β-lactamase activity, 20µL serial dilutions of prey proteins were prepared in 96-well plates. The absorbance at 485nm was measured every minute for 15 minutes after the addition of 60µL 125µg/mL nitrocefin (Calbiochem) to each well. Prey proteins were used at the concentration where nitrocefin hydrolysis was saturated after ten minutes (Figure 2.3).

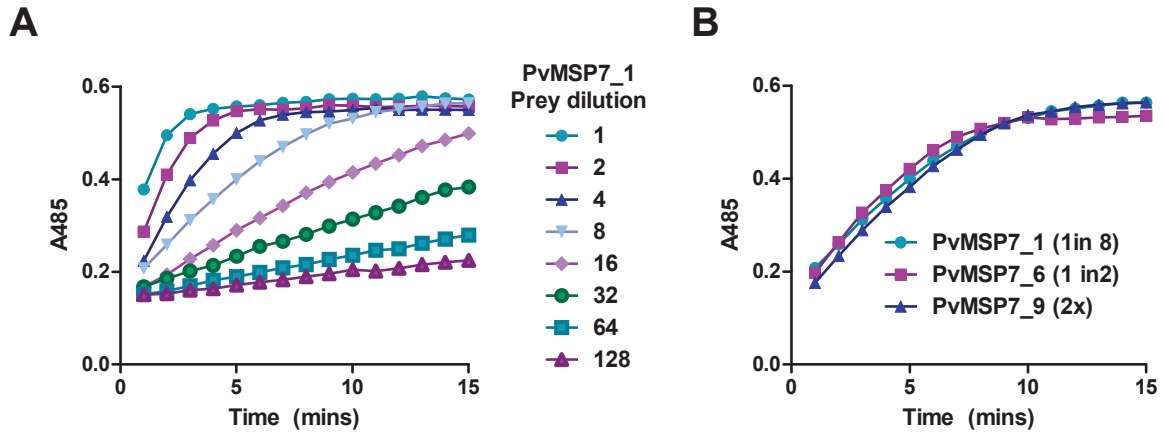

Figure 2.3: **Standardisation of prey protein by measuring nitrocefin hydrolysis**
A. A typical nitrocefin-hydrolysis assay for a single prey protein supernatant.
B. Nitrocefin hydrolysis activity of three *P. vivax* MSP7 protein supernatants at their chosen concentration for use in AVEXIS. The absorbance at 485nm saturates after approximately ten minutes.

2.3.1.3 Data presentation

AVEXIS data in this thesis is predominantly displayed as 485nm absorbance readings (A485). The nitrocefin substrate itself has a measurable A485, so to more clearly delineate binding signals from A485 measurements where no colour change occurs, a reference value was subtracted from each reading. This reference value, usually around between 0.05-0.2 was the mean A485 of the Cd4 bait controls for each prey protein.

2.4 SELP-ligand interaction blocking

2.4.1 sLe^X-SELP interaction blocking

To create a sLe^X-based binding reagent whose presence could be detected by measuring enzymatic activity, biotinylated sLe^X (Glycotech) was incubated with a streptavidin-alkaline phosphatase conjugate (strep-AP, Sigma). To determine the optimum ratio of the two components, a series of binding reagents were created by incubating a range of concentrations of sLe^X with a fixed amount of strep-AP for one hour[2]. The reagents were then used in an AVEXIS-like assay. SELP bait was immobilised on the surface of a 96-well strepatividin-coated plate and incubated with serial dilutions of the reagent. After one hour the plate was washed three times with HBST and once with HBS before 100µL 1mg/ml phosphatase substrate (Sigma) was added. To produce the most avid binding reagent from the sLe^X and strep-AP, it is important to maximise the amount of sLe^X bound to the AP-conjugate. However, if too much 'free' unconjugated sLe^X is present this could bind SELP independently of sLe^X-AP, decreasing the 405nm absorbance (A405) signal observed as a result of SELP binding. Therefore the ratio at highest sLe^X:strep-AP ratio (approximately 5:1) where A405 was still high was used in subsequent assays.

To determine whether PfMSP7 could block the interaction between SELP and sLe^X, SELP bait was immobilised on a 96-well strepatividin-coated plate and incubated with serial dilutions of purified pentameric PfMSP7 for one hour. The plate was washed three times with HBST and once with HBS. The wells were then incubated with sLe^X-AP binding reagent for one hour, washed and incubated with phosphatase substrate. The absorbance at 405nm was measured after 30 minutes and was used to assess the levels of sLe^X binding to SELP.

[2] each strep-AP molecule is expected to bind approximately two sLe^X-bio molecules

2.4.2 THP1 binding assay

THP1 cells were maintained in continuous culture at a density of 10^5-10^6 cells/mL in 50mL RPMI 1640 supplemented with 10% FBS and 2mM L-glutamine at 37°C and 5% CO_2. Saturating quantities of biotinylated proteins were used to coat the wells of a streptavidin-coated microtitre plate as described in 2.3. An anti-PSGL1 (LS-B2507 clone, LSBio) antibody was used as a positive control, to which cells should bind provided they have maintained their expression of PSGL1 receptors on their surface. These immobilised bait proteins were incubated with 10^4 THP1 cells diluted in HBS+1%BSA for one hour. Plates were washed gently by removing the liquid from the wells using a pipette and adding 150µL HBS. Four such washes were performed before counting the cells that remained adhered to the surface using a light microscope. To block interactions between SELP bait and THP1 cells, immobilised baits were incubated with CLB-thromb/6 anti-SELP antibodies (Santa Cruz biotechnology) or purified PfMSP7 pentameric prey each diluted in HBS+1% BSA for 90 minutes, after which plates were washed four times with HBS prior to the addition of cells.

2.5 Surface plasmon resonance

All SPR experiments were performed using a Biacore T100 instrument in combination with SA or CAP chips (GE Healthcare). Both chips use the biotin-streptavidin interaction to capture bait proteins onto their surface. When coating the SA chip, the surface was first 'activated' by three one-minute 30µL/min injection of a 1M NaCl/50mM NaOH solution. CAP chips couple biotinylated proteins onto their surface by means of a DNA-based intermediate, or CAPture reagent. To prepare the surface of the CAP chip for the immobilisation of biotinylated bait proteins, the manufacturer's regeneration solution was injected over the chip surface three times for 60 seconds at a flow rate of 20µL/min followed by a 60-second injection of the HBS running buffer and a five-minute 2µL/min injection of CAPture reagent. In each case, approximately 150 response units (RU), as measured using the Biacore T100 Control software, rat Cd4 domains 3&4 negative control bait was loaded into the first flow cell at a flow rate of 10µL/min. Molar equivalents of each bait protein were loaded into subsequent flow cells. After loading the flow cells, any remaining biotin-binding sites were saturated by injecting a 1nM biotin solution over the chip surface until no further binding responses were observed. All analyte proteins were purified by IMAC and subsequent SEC, and injected over the surface of the chip at a flow rate of 20µL/min for one minute. Binding responses were recorded and subsequently analysed using the Biacore T100 Evaluation software.

2.6 Flow cytometry

2.6.1 Labelling RBC surface proteins

To detect receptors present on the RBC surface, cells were washed, stained with monoclonal antibodies and a fluorescent secondary then analysed by flow cytometry. O-negative RBCs were prepared by centrifugation of 4mL whole blood and 10mL RPMI 1640 at 1800g for 5 minutes. The supernatant, and white blood cells from the top of the pellet, were removed and the remaining RBC diluted to 50% hematocrit with RPMI 1640. These RBCs were diluted 25-fold to 2% hematocrit in PBS containing 2% heat-inactivated FCS. 10µL, approximating to 10^6 cells, were stained on ice for 30 minutes using 1µg primary antibody in a total volume of 100µL PBS/FCS buffer. To remove unbound primary antibody, RBCs were twice pelleted by centrifugation at 450g for 3 minutes then resuspended in 100µL PBS/FCS. A 100µL 1:1000 dilution of Alexa 488-conjugated anti-mouse IgG1 secondary antibody (Abcam) was incubated with the cells on ice for 30 minutes. The washing steps were repeated and the cell pellet was resuspended in 250µL PBS prior to acquisition by flow cytometry using a BD FACSCalibur instrument. The Alexa488 was excited using the 488nm blue laser and its emission was detected using 530/30 filter. 20000 events were counted and the output was analysed using BD FACS Diva and FlowJo (TreeStar) analysis software.

2.6.2 Detecting protein-protein interactions on the surface of HEK cells

HEK293F cells were transfected (as described in 2.1.2.2) to express GFP-tagged receptors on their surface. Successful transfection was verified by fluorescence microscopy. 24 hours post-transfection, 1mL aliquots of 10^6 cells were incubated with 5µg pentameric FLAG-tagged reporter proteins for 1 hour at 4°C, with gentle orbital shaking. Cells were pelleted by centrifugation for five minutes at 200g and resuspended in an HBS buffer supplemented with 1% BSA and 1mM $CaCl_2$. This wash step was repeated before 5µg Cy3-conjugated anti-FLAG antibody (Sigma) was added and incubated at 4°C for one hour. To remove any unbound antibody, the cells were washed three times prior to flow cytometry. A BD LSR Fortessa instrument and FACS Diva software were used to record 10,000 events. The 488nm blue laser was used to excite eGFP, whose fluorescence was detected using a the 530/30 filter. Cy3 was excited via a 561nm yellow laser and fluorescence detected using a 582/15 band pass filter. FlowJo v10 (Tree Star) was used for further analyses. Where appropriate, cells were pre-incubated with 10µg mouse monoclonal IgG1 antibodies for one hour at 4°C, then washed as before, prior addition of the FLAG-tagged prey. These antibodies included the CLB-thromb/6

anti-P-selectin clone and the OX102 anti-rat Cd200R clone (BioLegend).

2.6.3 Platelet staining

Whole blood was isolated from healthy donors on the morning of the experiment. Platelet-rich plasma (PRP) was prepared by isolation of the supernatant following centrifugation of 2mL whole blood for six minutes at 200*g*. When optimising the platelet-staining protocol, 10µL blood or PRP was incubated with 20µL anti-SELP FITC-conjugated antibody or without 10µM ADP agonist in HBS buffer (total volume 50µL). Following a 30-minute incubation the mixture was fixed using 0.5mL formyl saline for ten minutes. A subset of samples were washed twice; the washing process involved centrifugation of fixed blood products for ten minutes at 1000*g*, after which they were resusupended in 0.5mL HBS. When staining platelets with FLAG-tagged reporter protein, 10µL PRP was incubated with 10µM ADP, 0.1-20µM reporter proteins in a total volume of 50µL for one hour, then fixed using 0.5mL formyl saline for ten minutes. Platelets were then washed and incubated with FITC-conjugated anti-FLAG antibody (Sigma) for 30 minutes prior to an additional wash step and analysis by flow cytometry. Stained platelets were examined using a FC500 flow cytometer (Beckman Coulter). FITC was excited using the 488nm blue laser and detected using the 525/40nm filter. 5000 platelets were counted and the data analysed using FlowJo.

2.7 Biochemical co-purifications

Avid merozoite protein reagents were created by conjugating biotinylated IMAC-purified proteins, or biotinylated proteins derived directly from dialysed transfected HEK293E cell-culture supernatant (prepared as described in 2.1.2.3), to streptavidin-coated superparamagnetic beads (Sigma). For each experiment, 100µL beads were washed three times with 1mL PBS using a magnet to isolate beads. Beads were resuspended in 1mL PBS and incubated with merozoite proteins for 30 minutes with rotation. To demonstrate that sufficient protein was provided to saturate the beads, ELISAs were performed on the supernatant as described in 2.3.1.1. If biotinylated protein could be detected then beads were deemed to be saturated. To remove unbound biotinylated protein, the beads were washed three times with 1mL PBS then resuspended in 100µL. Human serum (Sigma) was filtered through a 0.2µm filter before use to remove any aggregated protein before incubation with protein-coated beads for one hour at 4°C. Beads and their bound proteins were isolated using a magnet and washed five times with ice-cold PBS. Proteins remaining associated with the beads were eluted in 100µL 1% SDS for

five minutes. 25µL of the eluate was used in SDS-PAGE (see 2.2.4) and the gel was stained with SYPRO Orange (Sigma). See Figure 5.2 for a schematic representation of the method.

2.7.1 Mass spectrometry

SDS-PAGE-resolved elutants from co-purification assays were fixed with 40% methanol and 2% acetic acid for one hour and stained with a colloidal Coomassie (Sigma) overnight at 4°C. The background was cleared using 25% methanol for two hours then the gel was washed in water. Bands for analysis were isolated, then de-stained by incubation with an equal mixture of 50mM Ammonium Bicarbonate pH8.5 and acetonitrile (AmBic/CH_3CN) for 30 minutes at 37°C and 600rpm shaking. The AmBic/CH_3CN was replaced and incubation repeated until the blue colour of the stain was removed. De-staining was completed by incubating the gel pieces with 1mL CH_3CN for 30 minutes at 37°C, removing the liquid and allowing any remaining CH_3CN to evaporate. To digest any proteins in the bands, gel pieces were covered with 500µL AmBic containing 1µg/mL trypsin (Roche) and incubated for two hours at 37°C, then overnight at 25°C whilst shaking at 600rpm. The resulting peptides were then extracted from the surrounding liquid. Peptides were eluted from the gel by successive incubations of the gel pieces with a 50%CH_3CN/ 0.25% formic acid (Sigma) mixture. Pooled supernatants from each elution were dried completely to leave peptides, which were later resuspended in 40µL 0.5% formic acid prior to mass spectrometry, which was performed by the in-house mass spectrometry team. Peptides were analysed by LC-MS/MS on an Ultimate 3000 RSLCnano System (Dionex) coupled to a LTQ FT Ultra (Thermo Fisher) hybrid mass spectrometer. The raw mass spectrometry data was processed in Proteome Discoverer (V1.4) (Thermo Fisher) using Mascot v2.4 (Matrix Science) to assign protein sources for the detected peptides. The protein databases were a database of human proteins downloaded from Uniprot (as of February 2013) and a database of common contaminants. The reported protein/peptide list used a Mascot ion score cut-off of 30 with 0.05 as significance threshold.

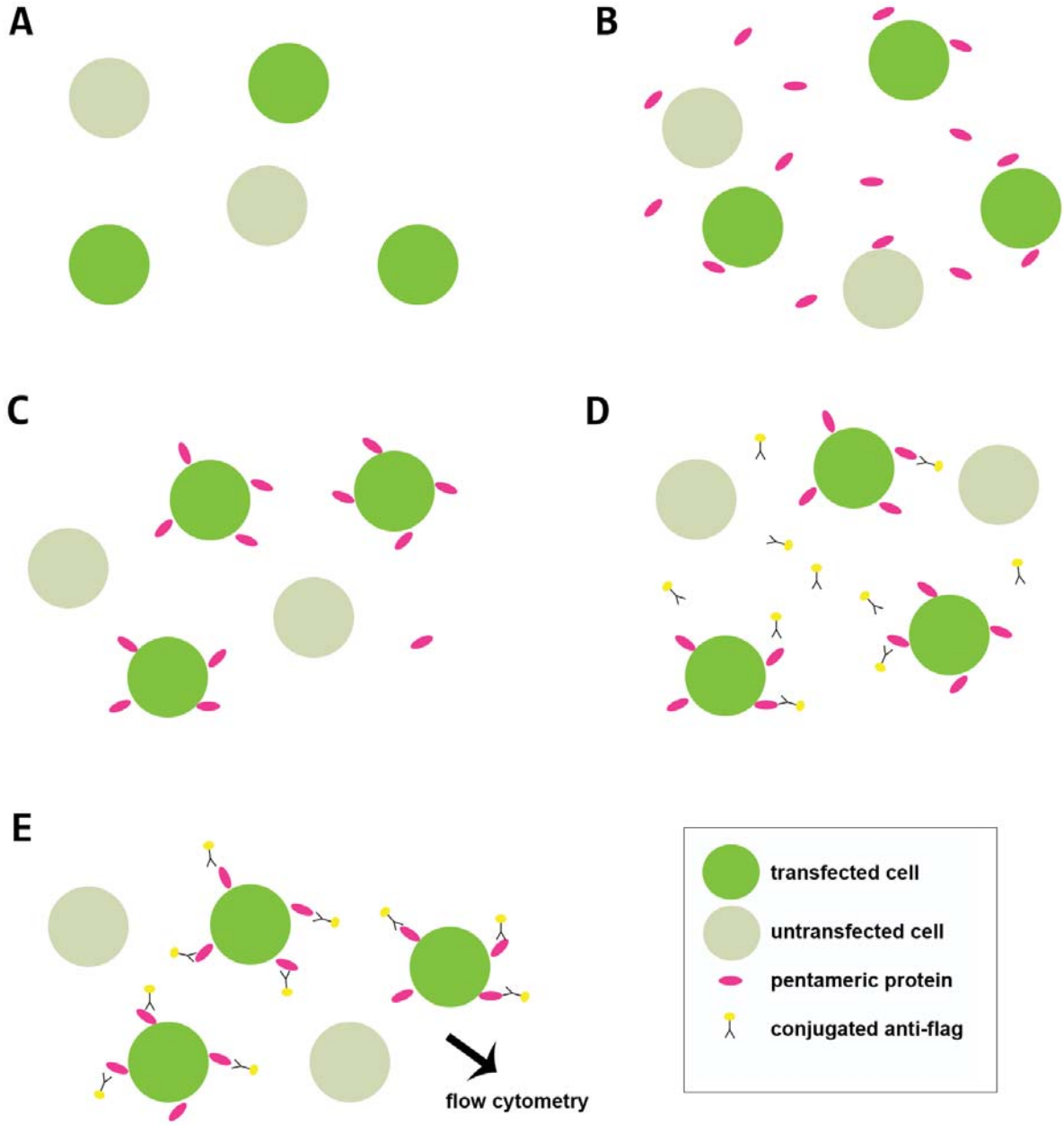

Figure 2.4: **HEK293F cell-surface binding assay**
A. Cells are transfected with recombinant receptor proteins with a cytoplasmic eGFP tag
B. Cells are incubated with FLAG-tagged pentameric *Plasmodium* proteins
C. Cells are washed to remove unbound pentamers
D. Cells are incubated with Cy3-conjugated anti-FLAG antibody.
E. Cells are washed to remove unbound antibody and are analysed by flow cytometry.

Primer name	Template	Sequence
SELP_F	SELP_BioLHis plasmid	TTTAAACTGCGGCCGCCACCATGGCCAACTGCCAGATCGCCATCC
SELP_EGF_F	SELP_BioLHis plasmid	TTTTTGCGGCCGCCACCTACACCGCCAGCTGCCAGGACATGA
SELP_CTL_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCGCTGGCGGTGTAGCACAGGGCGTGC
SELP_EGF_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCCCGCACGTACTCGCACTCAGGGCCG
SELP_S3_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCGCTGATGGCTTCACAGGTGGGCAGA
SELP_S6_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCGATGGCCTCGCACATGGCGGGGCTG
Mouse_SELP_F	cDNA	TTTAAACTGCGGCCGCCACCATGGCTGGCTGCCCAAAAGGTTCCT
Mouse_SELP_R	cDNA	TAGTAGGGCGCGCCACCCAAGTACGTCAGAGCTTCCTGG
SELE_F	cDNA	TTTAAACTGCGGCCGCCACCATGGTTGCTTCACAGTTTCTCTCAG
SELE_R	cDNA	TAGTAGGGCGCGCCGGGAATGTTGGACTCAGTGGGAGCT
SELL_F	cDNA	TTTAAACTGCGGCCGCCACCATGGTATTTCCATGGAAATGTCAGAG
SELL_R	cDNA	TAGTAGGGCGCGCCGGGGTTATAATCACCCTCCTTAATC
SELPLG_F	cDNA	TTTAAACTGCGGCCGCCACCATGGCTCTGCAACTCCTCCTGTTGC
SELPLG_R	cDNA	TAGTAGGGCGCGCCCTGCTTCACAGAGATGTGGTCTGGG
MSP7_Nterm_F	MSP7_Bio plasmid	TGTCTGGTGCGGCCGCCACCCCCGTGAACAACGAAGAGGAC
MSP7_Nterm_R	MSP7_Bio plasmid	TAGTAGGGCGCGCCCTGGGCCTTCACTTTGGACAGCACG
$MSP7_{22}$_F	MSP7_Bio plasmid	TGTCTGGTGCGGCCGCGAGCGAGACAGACACCAGAGCAAAGA
$MSP7_{19}$_F	MSP7_Bio plasmid	TGTCTGGTGCGGCCGCGGAAGTGCAGAAACCAGCCCAGGGCG
MSP7-Cterm_R	MSP7_Bio plasmid	TAGTAGGGCGCGCCCATGGTGTTCAGCAGATTGAAGATG

Table 2.1: **PCR primers**
Restriction sites are higlighted in red, sequences complimentary to pTT3-based plasmids in blue, and those annealing to cDNA in green.

SELP region	Amino acids	Sequence
'Full length'	1-771	MANC.....IQEA
SELP->CTL	1-162	MANC.....YTAS
SELP->EGF	1-208	MANC.....EYVR
SELP->S3	1-385	MANC.....EAIS
SELP->S6	1-570	MANC.....CEAI
SELP_EGF domain	159-208	YTAS.....EYVR

Table 2.2: **SELP protein fragment boundaries**
Truncated SELP proteins were produced comprising the regions indicated in Figure 4.2A using expression plasmids constructed using the primers detailed in Table 2.1. The amino acids from the endogenous SELP protein sequence (P16109 in Uniprot), alongside the four corresponding N-terminal and C-terminal amino acids for each construct, are shown here. The EGF domain was expressed downstream of the mouse variable κ light chain signal peptide. All proteins were expressed with a C-terminal rat Cd4 domains 3&4 tag.

PfMSP7 region	Amino acids	Sequence
'Full length'	28-351	TPVN.....LNTM
MSP7_N	28-176	TPVN.....VKAQ
$MSP7_{22}$	177-351	SETD.....LNTM
$MSP7_{19}$	195-351	EVQK.....LNTM

Table 2.3: **PfMSP7 protein fragment boundaries**
Expression plasmids for recombinant PfMSP7 protein fragments, as depicted in Figure 4.5A, were produced using the primers detailed in 2.1. The amino acids from the PfMSP7 protein sequence (PF3D7_1335100) and the four N-terminal and C-terminal PfMSP7 amino acids included in each construct are indicated here. All PfMSP7 constructs were expressed downstream of the mouse variable κ light chain signal peptide and included the C-terminal rat Cd4 domains 3&4 tag.

Plasmid name	Ectodomain	Accession number	Tags	Use	Constructor
SELP_BioLHis	HsSELP	P16109	Bio, His	AVEXIS, SPR	Dr Y. Sun
BSG_Bio	HsBSG	P35613-1	Bio	AVEXIS	Dr C. Crosnier
Cd4_Bio	RnCd4 domains 3&4	P05540 (partial)	Bio	AVEXIS, SPR, BCP	Dr S. J. Bartholdson
Platelet_BioLHis	HsPECAM1/HsAPLP2/HsCD59...	see Table 3.1	Bio, His	AVEXIS	Dr Y. Sun
MSP7_βlac	PfMSP7	PF3D7_1335100	COMP,β-lac	AVEXIS	Dr C. Crosnier
Merozoite_βlac	PfMSP1/PfMSP2/PfMSP4...	see Table 3.2	COMP,β-lac	AVEXIS	Dr C. Crosnier
MSP7_Bio	PfMSP7	PF3D7_1335100	Bio	AVEXIS	Dr C. Crosnier
MSP1_Bio	PfMSP1	PF3D7_0930300	Bio	AVEXIS	Dr C. Crosnier
SELP_βLFH	HsSELP	P16109	COMP,β-lac, FLAG, His	AVEXIS, FC	Dr Y. Sun
MSP7_His	PfMSP7	PF3D7_1335100	His	SEC, SPR	B. McDade
SELP_His	HsSELP	P16109	His	SEC, SPR	A. J. Perrin
Cd200_βLFH	RnCd200	A0A5D0	COMP,β-lac, FLAG, His	FC	Dr Y. Sun
Cd200R_TMGFP	RnCd200R	Q9ES58	TM, eGFP	FC	Dr G. J. Wright
MSP7_βLFH	PfMSP7	PF3D7_1335100	COMP,β-lac, FLAG, His	AVEXIS, FC	A. J. Perrin
SELP_TMGFP	HsSELP	P16109	TM, eGFP	FC	A. J. Perrin
MSP7_TMGFP	PfMSP7	PF3D7_1335100	TM, eGFP	FC	A. J. Perrin
SELP->CTL_BioLHis	HsSELP CTL domain	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP->EGF_BioLHis	HsSELP CTL & EGF domains	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP->S3_BioLHis	HsSELP CTL, EGF & 3xSCR domains	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP->S6_BioLHis	HsSELP CTL, EGF & 6xSCR domains	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP_EGF_BioLHis	HsSELP EGF domain	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
Cd200R_βlac	RnCd200R	Q9ES58	COMP,β-lac	AVEXIS	Dr K. M. Bushell
Cd200_BioLHis	RnCd200	A0A5D0	Bio, His	AVEXIS	M. Gallagher

Table 2.4: **Summary of expression plasmids used**
Proteins were produced with appropriate C-terminal tags, as described in Figure 2.1C. These proteins were then used in AVEXIS, surface plasmon resonance (SPR), Flow cytometry (FC), size-exclusion chromatography(SEC) and biochemical copurification (BCP) experiments. Plasmids are sorted according to the order in which they are first used in subsequent chapters. Accession numbers correspond to identifiers in Uniprot or PlasmoDB.

Plasmid name	Ectodomain	Accession number	Tags	Use	Constructor
MSP7_N_βLFH	PfMSP7 excluding PfMSP7$_{22}$	PF3D7_1335100(partial)	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
MSP7$_{22}$_βLFH	PfMSP7$_{22}$	PF3D7_1335100(partial)	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
MSP7$_{19}$_βLFH	PfMSP7$_{19}$	PF3D7_1335100(partial)	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
MSP7_N_His	PfMSP7 excluding PfMSP7$_{22}$	PF3D7_1335100(partial)	His	SEC	A. J. Perrin
MSP7$_{22}$_His	PfMSP7$_{22}$	PF3D7_1335100(partial)	His	SEC	A. J. Perrin
EFNB2_βLFH	HsEFNB2	Q2PDH7	COMP,β-lac, FLAG, His	FC	Dr Y. Sun
SELL_BioLHis	HsSELL	P14151	Bio, His	AVEXIS, SPR	A. J. Perrin
SELE_BioLHis	HsSELE	P16581	Bio, His	AVEXIS, SPR	A. J. Perrin
MSRPs_βLFH	PfMSRP1/2/3/4/5	PF3D7_1335000/4800/4600/4400/4300	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
Selp_BioLHis	MmSELP	Q01102	Bio, His	AVEXIS	A. J. Perrin
PbMSP7s_βLFH	PbMSP7/MSRP1/MSRP2	PBANKA_134910/920/900	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
PvMSP7s_βLFH	PvMSP7_1/6/9	PVX_082700/675/655	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
SELP R57H_BioLHis	HsSELP R57->H	rs7529463	Bio, His	AVEXIS	A. J. Perrin
SELP A156T_BioLHis	HsSELP A156->T	rs72712022	Bio, His	AVEXIS	A. J. Perrin
SELP A161P_BioLHis	HsSELP A161->P	rs142790885	Bio, His	AVEXIS	A. J. Perrin
SELP G179R_BioLHis	HsSELP G179->R	rs3917718	Bio, His	AVEXIS	A. J. Perrin
MSP7_COMPHis	PfMSP7	PF3D7_1335100	COMP, His	sLe^X	A. J. Perrin
MSP2_COMPHis	PfMSP2	PF3D7_0206800	COMP, His	sLe^X	A. J. Perrin
Cd4_BioLHis	RnCd4 domains 3&4	P05540 (partial)	Bio, His	BCP	Dr C. Crosnier
Merozoite_bait	PfAARP/PfAMA1/PfASP...	see Table 5.3&5.4	Bio +/- His	BCP	various
EBA175_His	PfEBA175	PF3D7_0731500	His	BSI	Dr M. Wanaguru
Rh5_His	PfRh5	PF3D7_0424100	His	BSI	Dr S. J. Bartholdson

Table 2.5: **Summary of expression plasmids used (continued)**
These proteins were primarily used in AVEXIS, but a some were also analysed by SPR, SEC, in a plate-based assay to demonstrate that PfMSP7 can block the interaction between sLe^X and SELP (sLe^X), in biochemical purifications (BCP) or back-scattering interferometry (BSI) experiments.