Chapter 5

Development of a biochemical co-purification assay to detect interactions between *Plasmodium* merozoite proteins and human serum proteins

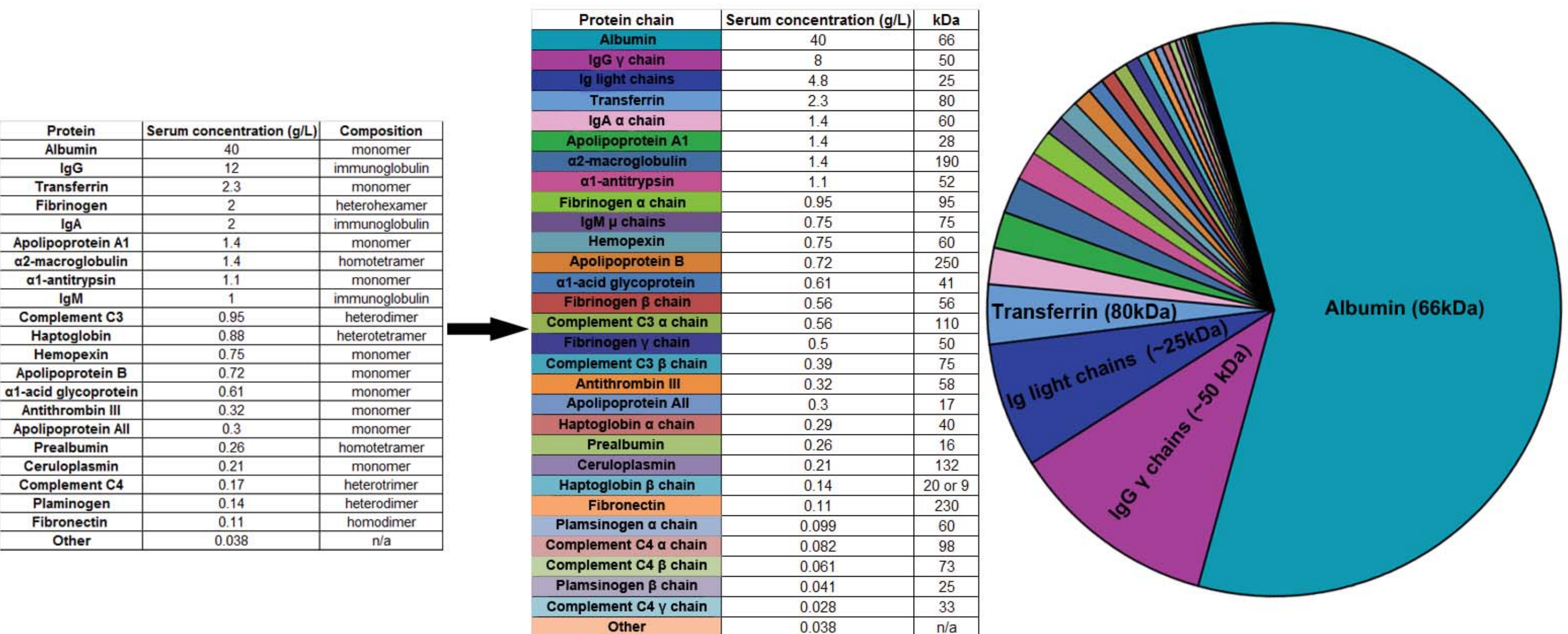
5.1 Introduction

This chapter describes the development and application of a method using recombinant *P. falciparum* protein-coated paramagnetic beads to co-purify interacting proteins from human serum, with the aim of identifying novel, potentially immunomodulatory, interactions involved in the pathogenesis of malaria.

5.1.1 *Plasmodium* merozoites are exposed to human serum

During the blood stage of infection, *Plasmodium* parasites are predominantly intracellular and are thus largely shielded from the body's immune defences. Between cycles of erythrocyte invasion, however, free merozoites are exposed to immune effectors, perhaps more so than at any other point in the parasites' life cycle. *Plasmodium* parasites are highly adept at immune evasion, such that true sterile immunity is probably never achieved, even in highly exposed individuals[185]. With a multitude of mechanisms at work to eliminate the parasite from the blood, it is very likely that a number of *P. falciparum*'s 5,300 genes - the majority of which have as-yet undetermined functions - are involved in manipulating the immune system. One such example whereby a parasite-secreted molecule subverts the functioning of leukocytes has

been identified[320] and in this laboratory we have recently determined that certain proteins belonging to the PfMSP3 family are able to bind IgM, in another possibly immunomodulatory mechanism. We hypothesise that *Plasmodium* parasites possess a large repertoire of these defences and that merozoite proteins in particular play a key role in immune evasion.



Protein	Serum concentration (g/L)	Composition
Albumin	40	monomer
IgG	12	immunoglobulin
Transferrin	2.3	monomer
Fibrinogen	2	heterohexamer
IgA	2	immunoglobulin
Apolipoprotein A1	1.4	monomer
α2-macroglobulin	1.4	homotetramer
α1-antitrypsin	1.1	monomer
IgM	1	immunoglobulin
Complement C3	0.95	heterodimer
Haptoglobin	0.88	heterotetramer
Hemopexin	0.75	monomer
Apolipoprotein B	0.72	monomer
α1-acid glycoprotein	0.61	monomer
Antithrombin III	0.32	monomer
Apolipoprotein AII	0.3	monomer
Prealbumin	0.26	homotetramer
Ceruloplasmin	0.21	monomer
Complement C4	0.17	heterotrimer
Plaminogen	0.14	heterodimer
Fibronectin	0.11	homodimer
Other	0.038	n/a

Protein chain	Serum concentration (g/L)	kDa
Albumin	40	66
IgG γ chain	8	50
Ig light chains	4.8	25
Transferrin	2.3	80
IgA α chain	1.4	60
Apolipoprotein A1	1.4	28
α2-macroglobulin	1.4	190
α1-antitrypsin	1.1	52
Fibrinogen α chain	0.95	95
IgM μ chains	0.75	75
Hemopexin	0.75	60
Apolipoprotein B	0.72	250
α1-acid glycoprotein	0.61	41
Fibrinogen β chain	0.56	56
Complement C3 α chain	0.56	110
Fibrinogen γ chain	0.5	50
Complement C3 β chain	0.39	75
Antithrombin III	0.32	58
Apolipoprotein AII	0.3	17
Haptoglobin α chain	0.29	40
Prealbumin	0.26	16
Ceruloplasmin	0.21	132
Haptoglobin β chain	0.14	20 or 9
Fibronectin	0.11	230
Plamsinogen α chain	0.099	60
Complement C4 α chain	0.082	98
Complement C4 β chain	0.061	73
Plamsinogen β chain	0.041	25
Complement C4 γ chain	0.028	33
Other	0.038	n/a

Figure 5.1: **Protein composition of human serum**
Breakdown by mass of the 21 most abundant proteins in human serum, which account for over 99% of the total protein content. The left hand table ranks the proteins by abundance in serum, presented as an average from a number of means of measurement[131]. The second table and pie chart break down of these proteins by chain, as they would appear in the denaturing SDS-PAGE step of the purification procedure.

5.1.2 A modified biochemical co-purification assay to discover novel merozoite-serum interactions

As a starting point to identify novel immunomodulatory mechanisms, I developed a screening approach for detecting interactions between merozoite proteins and human serum components. Human serum is a complex mixture of over 1000 proteins including antibodies, cytokines and complement proteins, as well as many others involved in homeostasis. It therefore represents an abundant (and readily obtainable) source of potential interacting partners to screen against merozoite proteins. In brief, the modified biochemical co-purification assay is a six-step process (Figure 5.2). The first step in this process involves creating a paramagnetic, multivalent *Plasmodium* protein reagent via the interaction of N-terminally biotinylated 'bait' proteins and streptavidin coated super-paramagnetic beads. Unbound proteins are removed by the isolation of the beads using a magnet. The beads are resuspended in buffer and re-isolated to wash. These reagents are then incubated with human serum and subsequently washed to remove proteins that have bound to the beads non-specifically. Proteins remaining bound to the beads are eluted, denatured and separated by SDS-PAGE for further analysis[1].

5.2 Assay development

5.2.1 Requirements of a sensitive assay

In order to recover sufficient material to identify a protein from an SDS-PAGE gel band using mass spectrometry, I aimed to pull down at least 100ng of protein from a single assay. To achieve this, the assay must fulfill three main criteria. In the first instance, sufficient serum protein must be bound to the merozoite protein-loaded beads following incubation. This means that interaction detection will be dependent on the successful capture of the merozoite protein on the bead and subsequently the strength of the interaction between the protein-coated bead and the serum component. Second, there must be sufficient serum protein left bound to the beads after washing. This will require a washing procedure that is sufficient to remove non-specifically bound proteins, but not so stringent as to remove the bound serum protein-of-interest. Finally, the elution process must release at least 100ng bound serum protein from the beads, which will be dependent on the previous two criteria, as well as the rigour of the elution process itself. In developing this assay I worked through each stage to optimise pro-

[1] For more details of the method see 2.7.

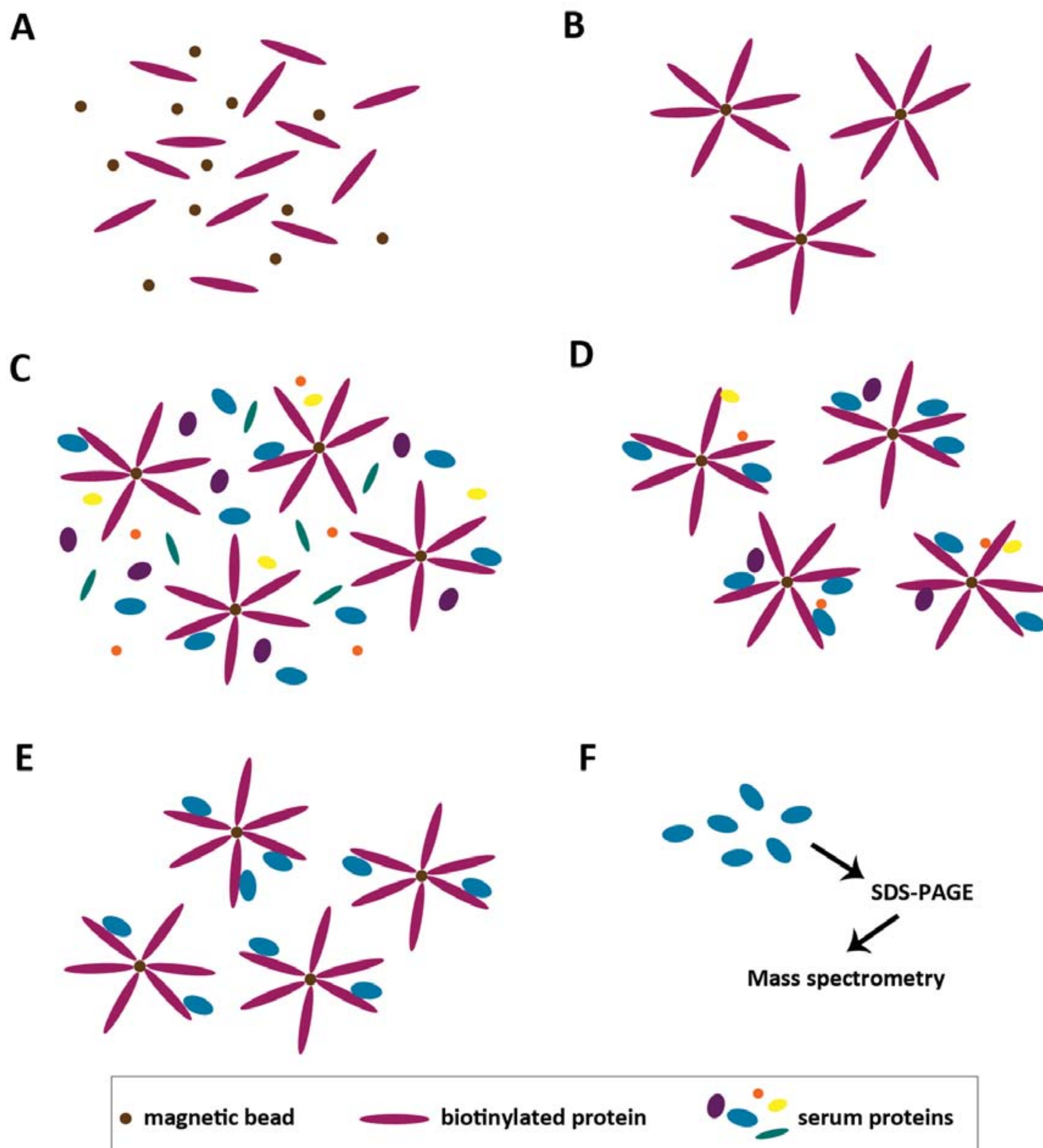


Figure 5.2: **A biochemical co-purification assay for the discovery of merozoite-serum protein interactions**

A. Biotinylated proteins are incubated with streptavidin-coated paramagnetic beads (not to scale).

B. Binding reagents are isolated using a magnet.

C. Binding reagents are incubated with human serum.

D. Binding reagents and their interacting proteins are isolated using a magnet.

E. Reagents are washed to remove loosely-bound proteins.

F. Interacting proteins are eluted from the beads, resolved by SDS-PAGE and identified by mass spectrometry.

cesses where protein losses could occur and to determine the theoretical range of interactions that could be detected.

5.2.2 Ensuring specific capture of biotinylated merozoite proteins

To create merozoite protein-conjugated ’bait’ reagents on which to capture interacting serum proteins, I coupled biotin-tagged merozoite proteins to streptavidin-coated paramagnetic beads. To ensure that the beads were saturated with merozoite proteins, I incubated the beads with purified biotinylated proteins for 30 minutes, isolated the beads and performed ELISAs to detect biotinylated proteins (as described in Section 2.3.1.1) in the supernatant. If biotinylated protein was successfully captured on the beads, I observed a decrease in the amount of protein recovered from the supernatant when compared with an equivalent amount of input protein (Figure 5.3A). To achieve the highest sensitivity for the assay I would ideally capture the maximum amount of merozoite proteins on the beads[2]. Hence, it was important to ensure that sufficient amounts merozoite proteins were incubated with the beads to saturate their biotin-binding capacity. I demonstrated saturation of this binding by recovering biotinylated protein from the supernatant following the isolation of protein-coated beads from 30-minute incubations of beads and biotinylated proteins (Figure 5.3A). Before every biochemical co-purification experiment, these ELISA-based bead saturation assays were performed, so as to determine the amount of protein required to completely coat the bead surface. The interaction between biotin and streptavidin has a very high affinity[69], such that merozoite proteins bound to the beads via this interaction will not be removed by even very stringent wash steps. It was therefore important to confirm that the merozoite proteins were binding to the beads specifically via the biotin-streptavidin interaction, and not via weak, transient interactions that would not withstand the subsequent steps of the assay. To demonstrate this, I pre-incubated the beads with biotin prior to adding the biotinylated proteins. This should block the available sites for the biotinylated merozoite proteins to bind and hence prevent their capture. By performing ELISAs on the supernatant following isolation of the beads after their 30-minute incubation with biotinylated PfMSP3.4, I was able to demonstrate that saturating the beads with biotin could completely block subsequent capture of biotinylated protein, indicating that merozoite proteins are indeed being captured specifically via their biotin tag (Figure5.3B).

[2] In theory 100μL beads can bind 40pmoles of biotinylated protein, which, for example, would be 2μg of a 50kDa protein

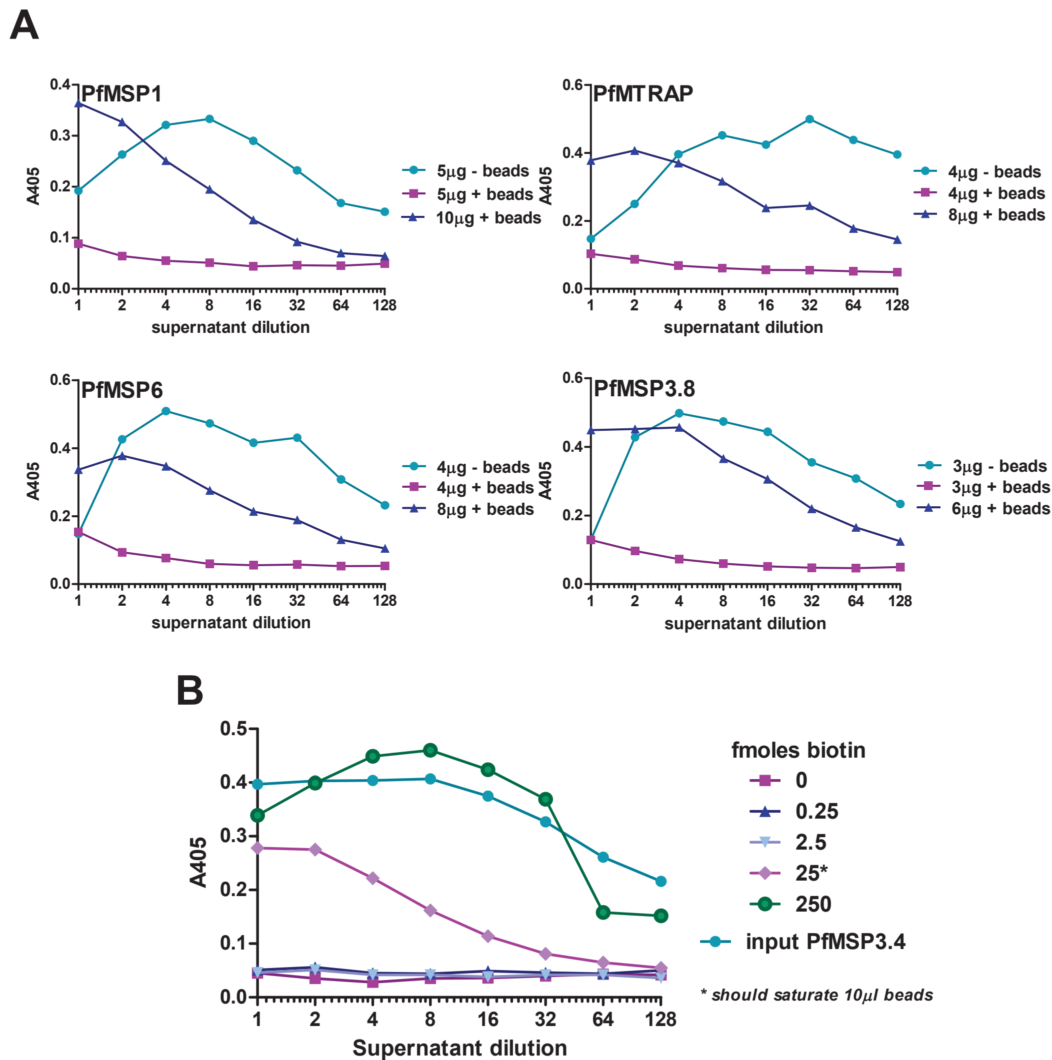


Figure 5.3: **Biotinylated merozoite proteins were specifically captured on paramagnetic beads**

A. ELISA-based analyses on the supernatants from the protein/bead incubations demonstrated saturable capture of biotinylated protein. The absorbance readings reflect the amount of biotinylated protein captured from the supernatant after 33µL beads were incubated with the indicated biotinylated proteins. Where beads had been incubated with insufficient biotinylated protein to saturate the bead surface, very little biotinylated protein remained in the supernatant, reflected in negligable turnover of phosphatase substrate and consequent low-level absorbance at 405nm (A405) in ELISAs (magenta squares). These signals can be compared to the cyan data series, which shows the ELISA A405 readings for the equivalent amount of each protein without incubation with the beads. Where beads had been incubated with an excess of biotinylated proteins, protein could be recovered from the supernatant following bead isolation, and A405 signals were observed by ELISA (blue triangles).

B. Capture of biotinylated PfMSP3.4 can be blocked by preincubation of beads with biotin. 20µL beads were incubated with biotin prior to incubation with 10µg PfMSP3.4. A405 readings reflect the amount of PfMSP3.4 that could be re-isolated from the beads (i.e. that which could not bind to the biotin-blocked beads).

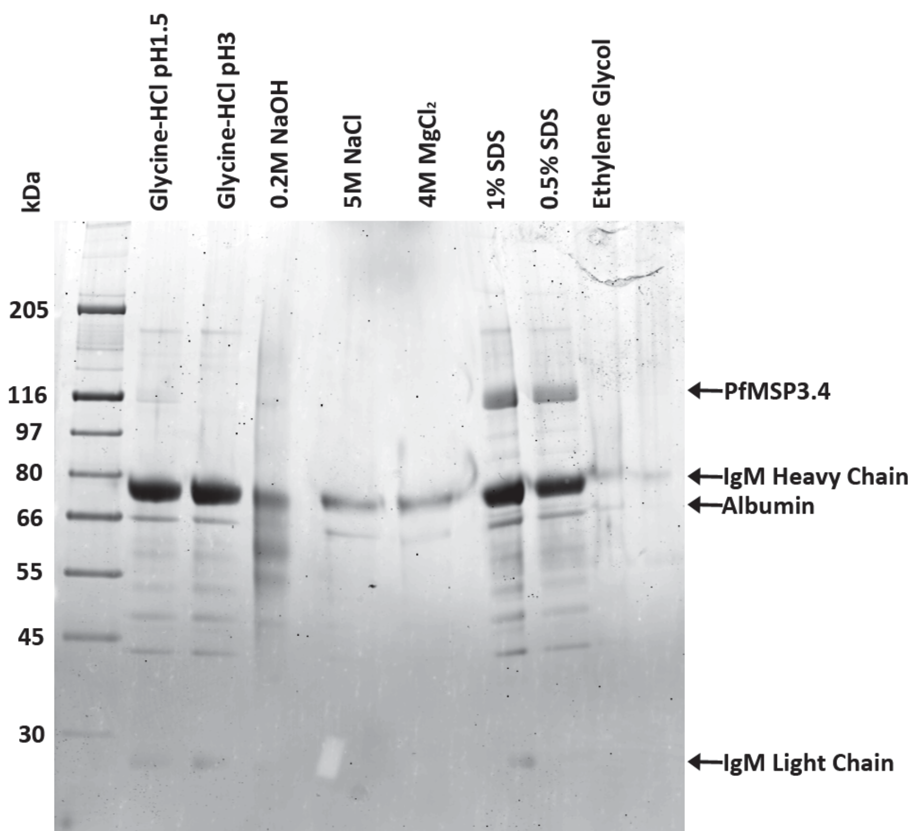


Figure 5.4: **Identification of appropriate elution buffers**
SDS-PAGE analysis of the eluate from biochemical co-purifications using a range of elution reagents indicated that Glycine-HCl or SDS solutions were appropriate elution buffers. Biochemical co-purifications were performed using 100μL PfMSP3.4-coated beads, 1mL serum and three 60-second washes. Proteins were eluted by five minute incubations with the indicated solutions.

5.2.3 Optimising elution efficiency

Using effective elution conditions, it should be possible to release a high proportion of bound protein from the beads. There are various types of solutions that can be used to disrupt protein-protein interactions including those with high salt concentrations, low or high pH and denaturing reagents. To test the compatibility of various elution buffers with the proposed biochemical co-purification method, I performed a series of preliminary experiments using the known interaction between PfMSP3.4 and human serum IgM (see Figure 5.4).

High salt solutions (5M NaCl and 4M $MgCl_2$) were not compatible with the assay, as they did not allow a particularly clear resolution of bands and also distorted the running of the surrounding lanes on the gel. Low pH and SDS solutions successfully brought down proteins

of the correct size to be the IgM heavy chain. SDS also brought down a band of the correct size to be the input PfMSP3.4, indicating that the denaturing conditions may be strong enough to dissociate the streptavidin-biotin interaction. However PfMSP3.4, like other members of the PfMSP3 family, is thought to oligomerise[118], so this band may also be PfMSP3.4 that is covalently associated to the beads via a PfMSP3.4 homotetrameric interaction, as opposed to via its biotin tag. Hence I chose SDS for the assay's elution buffer, as it returned the highest yield of PfMSP3.4-interacting protein.

5.2.4 Optimising washing steps

Serum is a complex matrix from which to isolate proteins, so it is important that the washing of the beads following incubation with serum is sufficiently stringent to remove non-specifically bound serum proteins. However, this needs to be balanced against washing away the interacting proteins and losing the beads themselves between wash steps, which will reduce the yield of any interacting proteins. To advise the number and type of wash steps, I performed a series of theoretical calculations. Assuming first-order dissociation kinetics during wash steps, mass (in g) of interacting protein bound to the beads declines in a time-dependent manner such that $mass(t) = mass(0) \times e^{-\lambda t}$, where $mass(t)$ is the mass of serum protein bound to the beads after t seconds of washing, $mass(0)$ is the mass of serum protein bound prior to washing and λ is the dissociation rate constant, which is inversely related to the interaction half life ($\lambda = \frac{\ln 2}{t_{\frac{1}{2}}}$). The mass of bound serum protein required prior to washing can therefore be found using the following equation:

$$mass(0) = \frac{mass(t)}{e^{-\lambda t}}$$

By applying this equation to a range of bead-serum protein interaction half-lives, I could estimate the $mass(0)$ required to ensure $mass(t) > 10^{-7}$, i.e. the mass of serum protein present prior to washing necessary to leave 100ng for elution, as a function of washing time (Figure 5.5A). These calculations indicate that this method cannot be expected to detect interactions that dissociate rapidly during washing; those with a half-life in the order of seconds are unlikely to be detectable, but the method has promise to detect those with a half-life in the order of minutes. As serum is a complex mixture of proteins, many of which are highly abundant (see Figure 5.1), multiple wash steps will be required to remove proteins that are bound non-specifically to the protein-coated beads. By iterative application of exponential decay calculations, I estimated $mass(0)$ required to isolate 100ng serum protein after a succession of

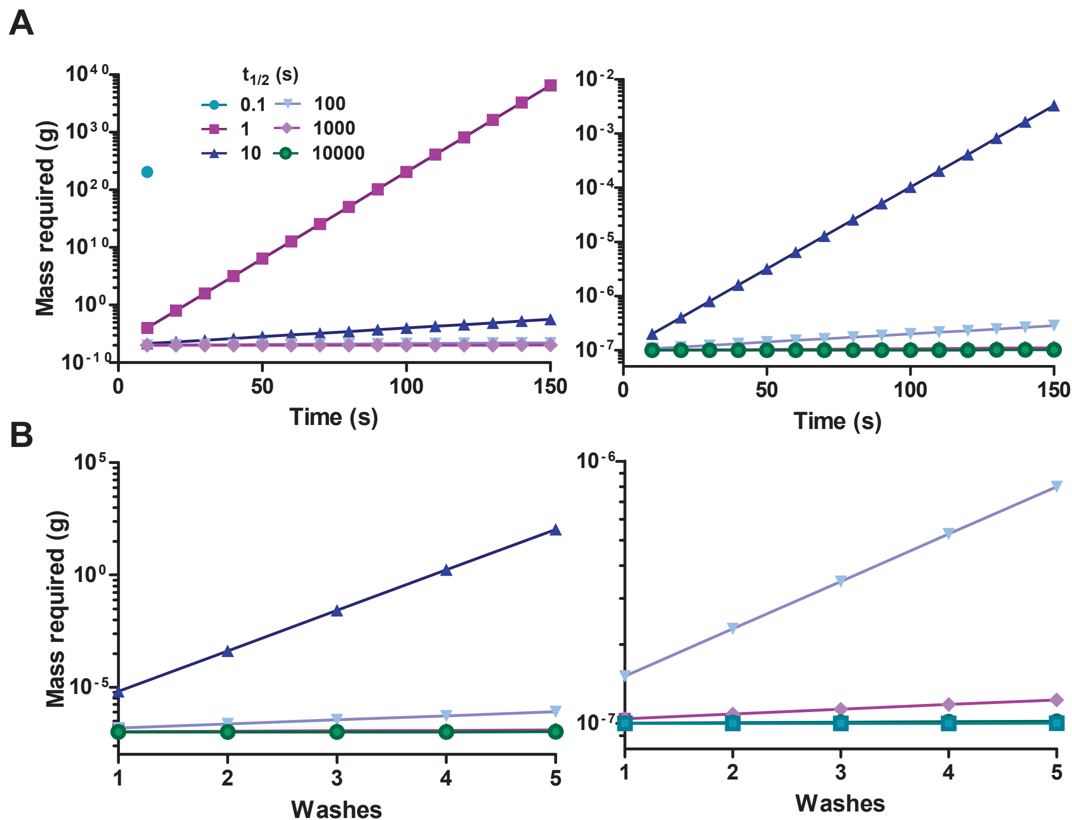


Figure 5.5: **Theoretical assessment of wash-step dependency of interaction detection**
A. The minimum required mass(0) - the amount of a 50kDa serum protein bound to the beads prior to washing to ensure that 100ng can be recovered from co-purifications using 100µL beads and 1mL serum - was plotted against as a function of washing time, assuming first-order dissociation kinetics, for a range of given interaction half-lives.
B. The minimum required mass(0) was plotted as a function of the number of successive 60-second wash steps.

60-second wash steps[3] (Figure 5.5B). These calculations indicate that the requirement for repeated washing will only slightly increase the threshold interaction half-life needed to ensure detection.

To investigate the effectiveness of washing for removing non-specifically bound proteins, it was necessary to perform some additional preliminary biochemical co-purification assays. I observed that the beads had a tendency to adhere to the walls of the polypropylene tubes in which they were being handled, so I tested whether transferring the resuspended beads to a

[3]Note that a washing time of less than 60s is impractical, as at least 45s are required for the magnetic separation of the beads for the supernatant

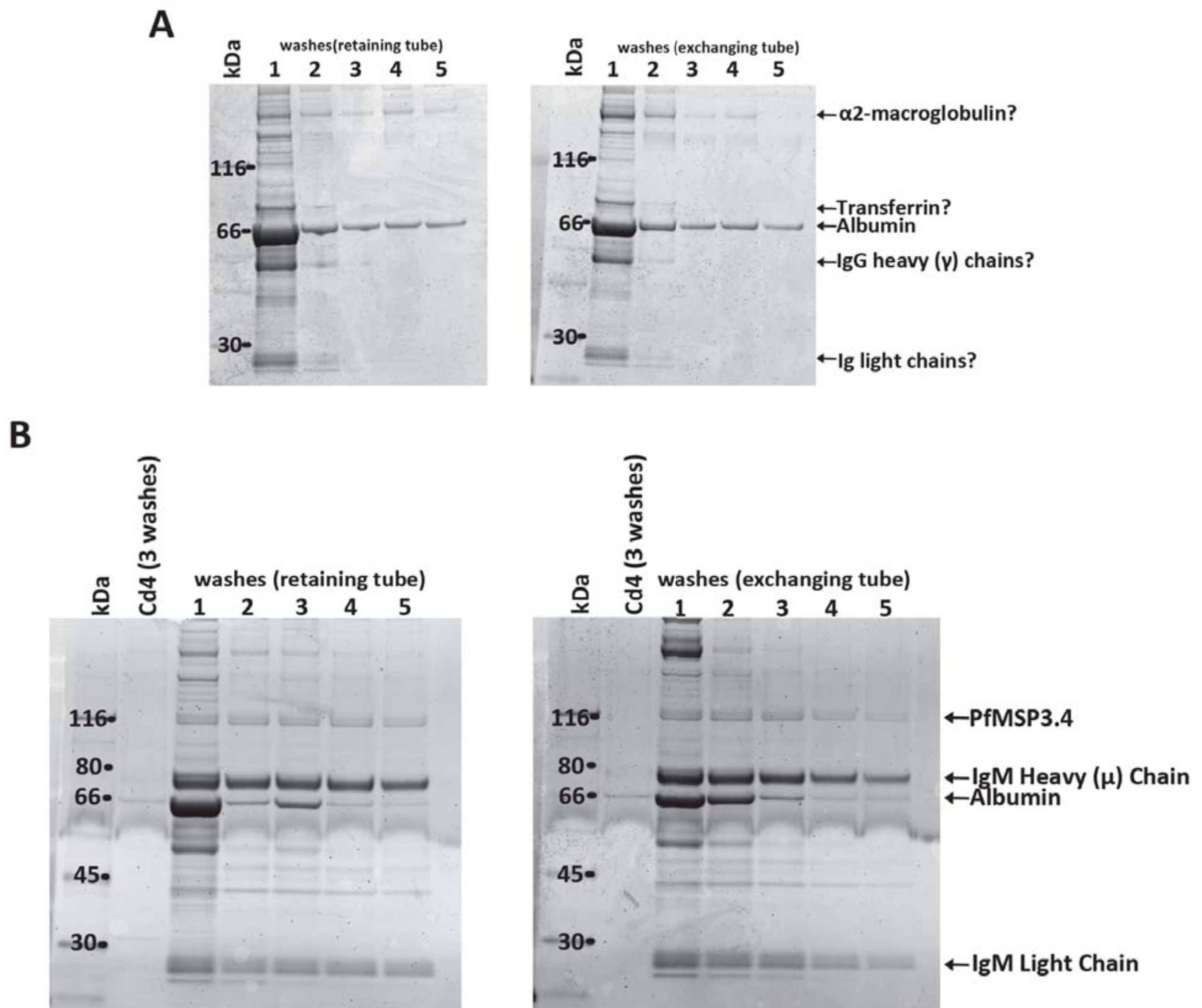


Figure 5.6: **Experimental optimisation of washing steps using negative and positive control bait proteins**

A. 100μL beads coated with the Cd4 tag region were used in preliminary co-purification assays with 1mL serum and the indicated number of 60-second wash steps. On the left-hand panel the same tube was used for all wash steps, whereas in the right-hand picture the beads were transferred to a fresh tube between each wash step. In either case, only albumin was recovered after three washes. The approximate locations of other common contaminating serum proteins, identified in Figure 5.1 are indicated on the right.

B. PfMSP3.4-coated beads were used in similar preliminary co-purification assays. At least two washes were required to remove contaminating serum proteins. IgM remained stably bound to the beads during washing, though IgM yields were slightly decreased after multiple washes when fresh tubes were used for each wash.

fresh tube between 60 second washes stood to increase the purity of eluted protein, without compromising the yield. To systematically determine the optimum number and type of wash steps, I performed a series of co-purifications using a rat Cd4 domains 3&4 bait, which we do not expect to bind serum proteins (Figure 5.6A), and the PfMSP3.4 bait which we know to bind IgM (Figure 5.6B). The results of these preliminary experiments demonstrated that:

- Even after five washes, it is not feasible to remove all non-specifically bound albumin protein, shown by the consistent presence of a 65kDa band in all lanes in Figure 5.6.
- There is no significant increase in the purity of the eluted IgM after four washes, shown in Figure 5.6B.
- Exchanging tubes between wash steps does not significantly impact the purity of the eluted IgM (Figure 5.6B).
- Losses of the interacting IgM are more noticeable when changing tube between washes. This is most easily observed by examining the light chain band in Figure 5.6B.
- Higher levels of non-specifically bound proteins are observed when performing the biochemical co-purification using PfMSP3.4-coated beads than the Cd4-coated beads (as seen by comparing Figure 5.6B with Figure 5.6A). This might mean that serum proteins have a greater propensity to interact with other proteins than with the beads themselves, or the comparatively small Cd4-tag region.
- Recurring contaminant bands likely represent the most abundant serum proteins, which are summarised in Figure 5.1. The hypothesised identities of some of these bands are indicated in Figure 5.6A.

Based on these observations, pull down assays were subsequently performed using four 60-second wash steps, without exchanging the tube between each.

5.2.5 Theoretical assessment of assay sensitivity

For a receptor-ligand interaction $R + L \rightleftharpoons RL$, at equilibrium

$$K_D = \frac{[R][L]}{[RL]}$$

And if a is the concentration of *Plasmodium* protein added into serum, and x the initial concentration of the ligand in serum, then

$$a = [RL] + [R]$$

and

$$x = [RL] + [L]$$

Combining these equations gives

$$K_D = \frac{(x - [RL])(a - [RL])}{[RL]}$$

where $[RL]$ can be determined from $mass(0)$ in the wash-step dependency calculations in Section 5.2.4. By applying this formula to a hypothetical experiment using 100μL beads and 1mL human serum, and involving a medium-sized (50kDa) serum protein, it is possible to approximate the ranges of interaction affinities and target protein abundances into which detectable interactions would fall (Figure 5.7). These rough calculations indicate that this method could detect interactions with a K_D in the micromolar range, provided the concentration of serum protein is in the micromolar, or even the high nanomolar range. Using these calculations we can speculate that an assay using 100μL beads and 1mL human serum would be well suited to the detection of interactions:

- with half-lives in the order of minutes, so as not to be lost during washing (Figure 5.5);
- with K_Ds in the micromolar range, or lower
- provided their serum binding partner is present at high nanomolar concentrations, or higher. At least 30 serum proteins can be expected to be present in this concentration range [131][4].

However, these calculations are highly simplified and make several assumptions, and are thereby likely to underestimate the potential this screening approach has to discover novel interactions. The calculations have been based on biochemical co-purification assays using 1mL of serum and 100μL of beads; by increasing the amount of beads used in the assay, the amount of serum protein that can be captured also increases so number of interacting serum proteins that we can hope to detect will rise[5]. These calculations also assume that proteins are interacting monovalently. In reality a number of serum proteins, including IgM, are multimeric and may thus interact with the merozoite protein-coated beads with a high avidity, increasing the sensitivity of the detection system. Perhaps the biggest flaw with these calculations is the

[4] As proteins are denatured prior to detection, the abundance of the particular interacting chain of multimeric proteins will also be important. See Figure 5.1.

[5] Taking into account the assumptions made in the above calculations, doubling the amount of beads would theoretically double the maximum detectable K_D for a given concentration of serum protein

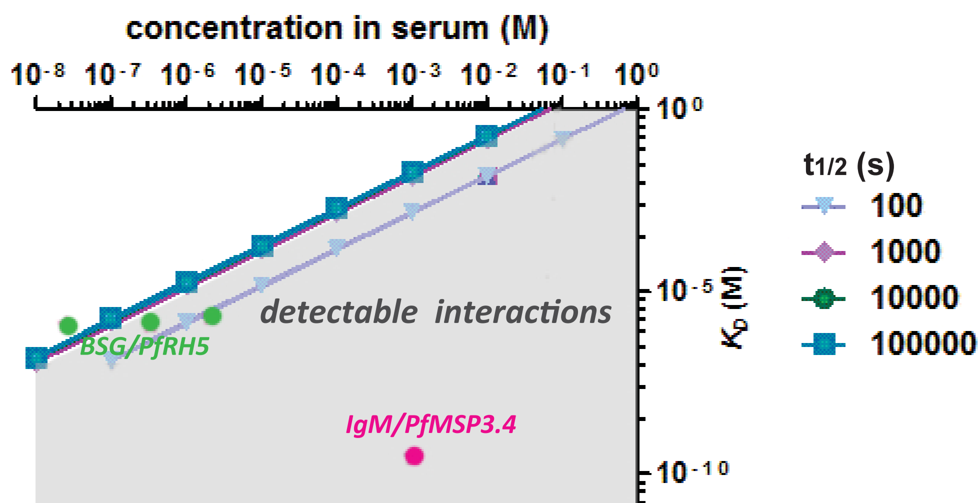


Figure 5.7: **Theoretical determination of the range of detectable interactions**
For given interaction half lives (during washing), the maximal K_D that would allow the recovery sufficient serum protein is shown as a function of the protein's original concentration in the serum. The interaction between serum IgM and PfMSP3.4 falls well within the predicted detectable range. The indicated BSG concentrations correspond to those used in the experiment depicted in Figure 5.10.

simplification that the proteins are interacting in free solution, when in fact they are interacting around the surface of a bead. This may significantly increase the effective half-life of the bead-serum protein interaction, as there is a very high local concentration of *Plasmodium* bait protein. This way, when an interacting serum protein dissociates from an individual *Plasmodium* bait it can readily re-associate with a neighbouring bait protein and remain bound to the bead surface for longer than would be expected for an interaction between free, monomeric proteins. This single simplification may mean that assuming exponential decay during wash steps massively underestimates the range of half-lives of detectable interactions, and also that lower-affinity interactions have a higher chance of detection than the calculations suggest.

5.3 Results

5.3.1 The optimised assay reproducibly detected the interaction between PfMSP3.4 and IgM

In developing the assay, I used the previously-identified interaction between PfMSP3.4 and serum IgM. In a biochemical co-purification assay using PfMSP3.4 bait, we would therefore expect to elute 72kDa and 25kDa proteins corresponding to the heavy and light chains of IgM. We might also expect to recover the 115kDa PfMSP3.4 protein. These three species were all observed by SDS-PAGE (Figure 5.6B). To support the assumption that the identity of bands are as expected, I ran purified IgM, biotinylated PfMSP3.4 and bovine serum albumin (BSA, which is almost identical in size to human serum albumin) alongside the eluate by SDS-PAGE (Figure 5.8); each band of the eluate was identical in size to their purified expected counterpart. To confirm the identity of the IgM heavy chain, I extracted peptides from the appropriate bands from PfMSP3.4 and PfMSP3.8 biochemical co-purification assays and submitted them for in-house tandem mass spectrometry analysis (LC-MS/MS). The most abundant peptides identified from these bands mapped to human IgM, indicating that this protocol can be used to accurately determine the identity of eluted interacting serum proteins (Tables 5.1 & 5.2).

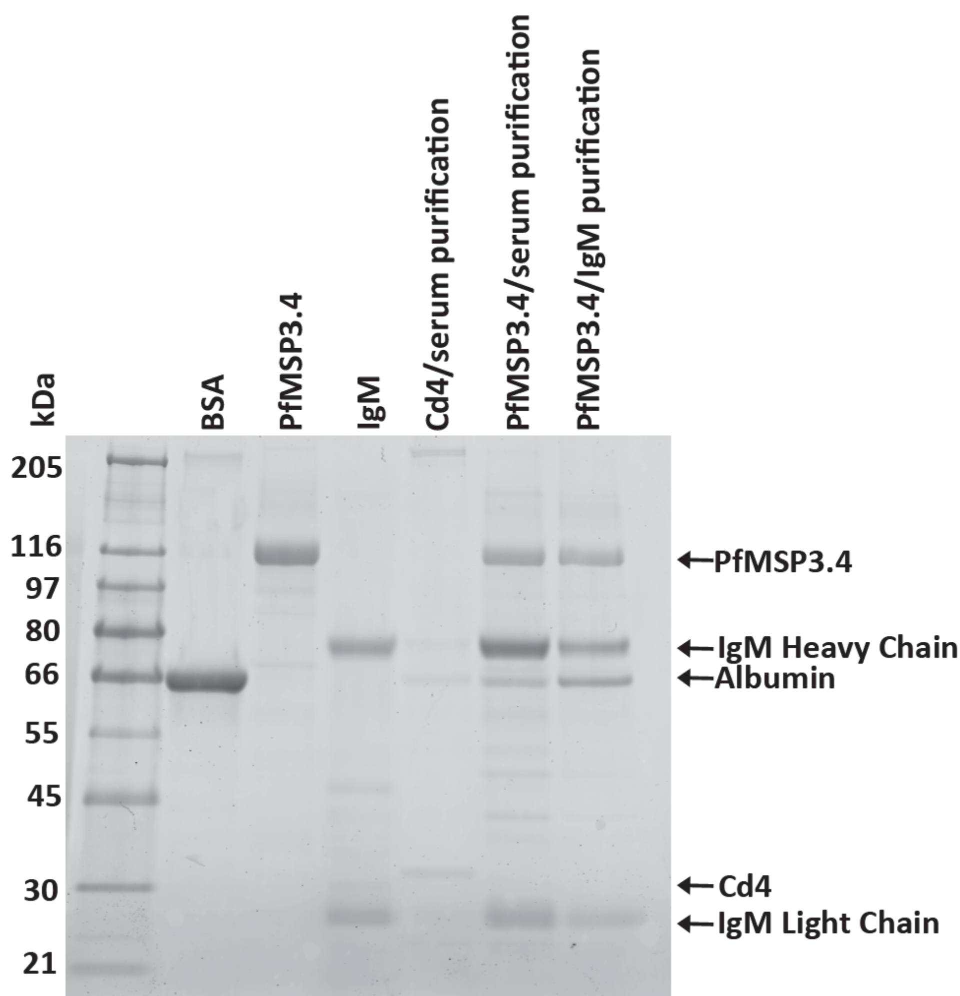


Figure 5.8: **Identification of IgM from PfMSP3.4 biochemical co-purification experiments** Purified BSA, biotinylated PfMSP3.4 and IgM were resolved by SDS-PAGE alongside eluates from serum biochemical co-purification experiments using Cd4- and PfMSP3.4-coated beads. The biochemical co-purification protocol was also performed using PfMSP3.4-coated beads, replacing serum with purified IgM in 5% BSA (far-right lane).

Protein	Example Uniprot ID	Unique peptides
IgM heavy chain constant region	P01871	54
Keratin	P35527	37
Complement C4	B0UZ83	27
Ig heavy chain variable region	P01765	24
Heat shock protein	P07900	6
RNA-binding protein	H3BPE7	6
Desmoplakin	P15924	6
IgG heavy chain constant region	P01857	4
Dermicidin	P81605	4
Serotransferrin	P02787	4
Arginase 1	P05089	3
Desmoglien 1	Q02413	1
Caspase 14	P31944	1
Coagulation factor V	P12259	1

Table 5.1: **Peptides identified by mass spectrometry from hypothesised IgM heavy chain band from PfMSP3.4 biochemical purification** In this experiment, the β chain of Complement C4 was a significant contaminant, which is perhaps unsurprising as it as an abundant component of human serum and is equivalent in mass to the IgM heavy chain (Figure 5.1).

Protein	Example Uniprot ID	Unique peptides
IgM heavy chain constant region	P01871	57
Ig heavy chain variable region	P10766	22
Keratin	P35527	8
IgG heavy chain constant region	P01857	3
Complement C4	B0UZ83	1

Table 5.2: **Peptides identified by mass spectrometry from hypothesised IgM heavy chain band from PfMSP3.8 biochemical purification**

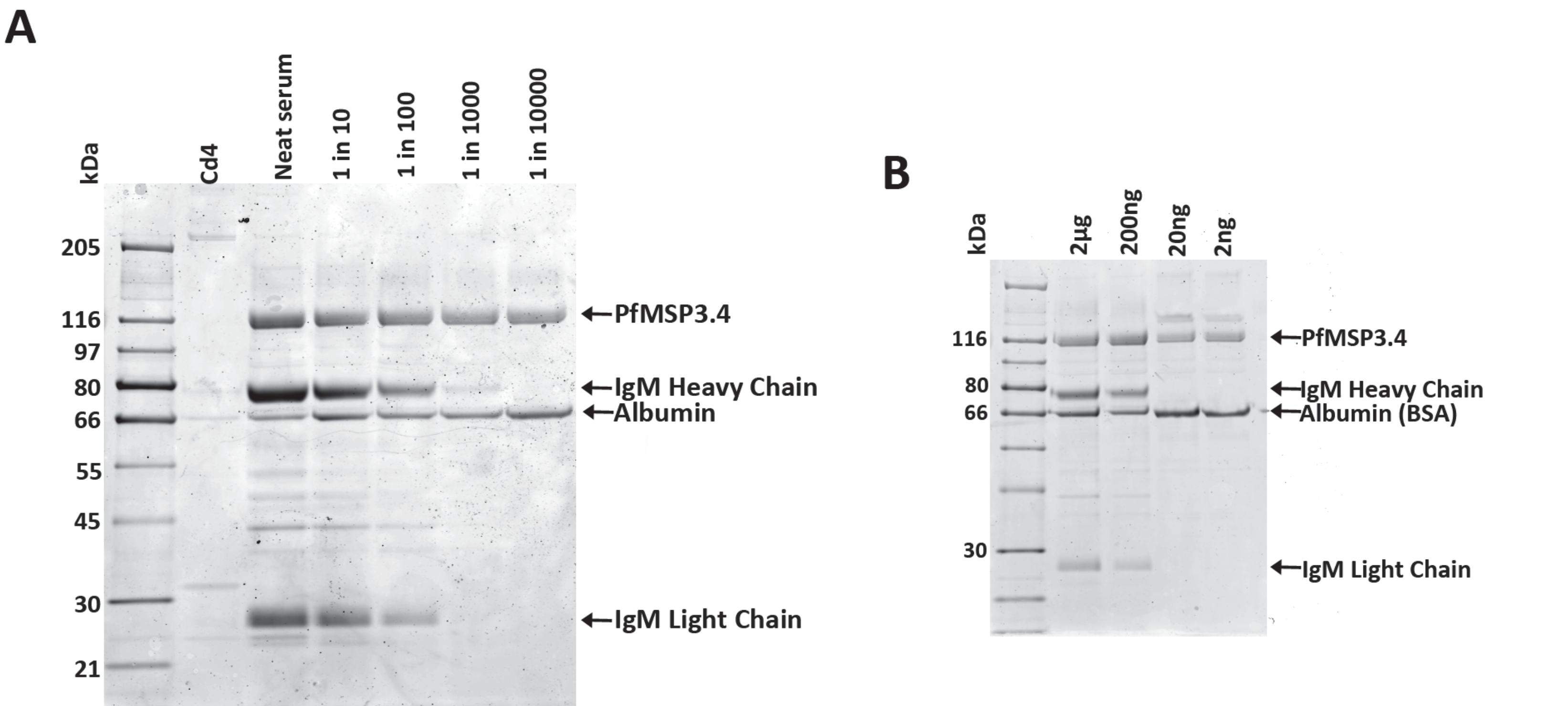


Figure 5.9: **PfMSP3.4 was used to detect IgM with high sensitivity**

A. SDS-PAGE analyses of the eluates from biochemical co-purifications performed using 1mL serial dilutions of serum indicate that 100µL PfMSP3.4-coated beads can purify IgM even when its serum concentration is diminished 1000-fold.

B. Similarly, biochemical co-purifications performed using purified IgM/BSA instead of serum indicated that 100µL PfMSP3.4-coated beads can purify IgM when only 200ng is present in a 1mL volume.

5.3.2 The PfMSP3.4 bait could be used to co-purify IgM with a very high sensitivity

To test the sensitivity of the optimised IgM co-purification assay, I performed it using serial dilutions of serum. I diluted the serum in PBS containing 5% BSA, such that the albumin content of the dilutions remained approximately constant, but the concentration of IgM was reduced (Figure 5.9A). The interaction between recombinant PfMSP3.4 (in its oligometric form) and IgM has an estimated K_D of 0.3nM, determined by SPR. According to the theoretical calculations in 5.2.5, this high-affinity means that the PfMSP3.4 should be able to purify IgM, even if the antibody is present at very low levels (Figure 5.7). IgM can still be detected when serum is diluted 1000-fold, when its expected concentration would be approximately 2μg/mL. I performed the same assay replacing serum with purified IgM diluted in PBS + 5% BSA (Figure 5.9B). Using the purified IgM it was possible to detect the interaction when only 200ng of IgM was added, approximating to a 1 in 10,000 dilution of serum. According to the manufacturers instructions, SYPRO staining can be used to detect as little as 4ng of protein in an SDS-PAGE band, so when 20ng was added to the biochemical co-purification assay, this is probably at the very limit of detection of the assay[6]. We might expect to be able to detect IgM at a 1 in 10,000 dilution of serum, when the IgM concentration is thought to be 200ng/mL, however IgM bands are not present for this dilution in Figure 5.9A. Despite this, IgM bands are seen in Figure 5.9B when 200ng purified IgM was present, indicating that the assay is a highly sensitive method to detect this interaction.

5.3.3 The biochemical co-purification assay was capable of detecting a low affinity interaction

IgM interacts with PfMSP3.4 with a high affinity, but the interactions we wish to identify may not be so strong. To experimentally determine whether the optimised assay is capable of detecting low affinity interactions, I tested it using the interaction between PfRH5 and BSG, which has a micromolar affinity[64]. Even under equilibrium binding conditions, the interaction between monomeric proteins has a half-life of only a few seconds[347], so if the assumption that exponential decay occurs during washing is correct, it may not be possible to detect the interaction (Figure 5.5). BSG is not a known component of human serum, so I added a range of concentrations of purified BSG into the serum before use in the assay using

[6] only 25μL of the 100μL elutant was analysed by SDS-PAGE

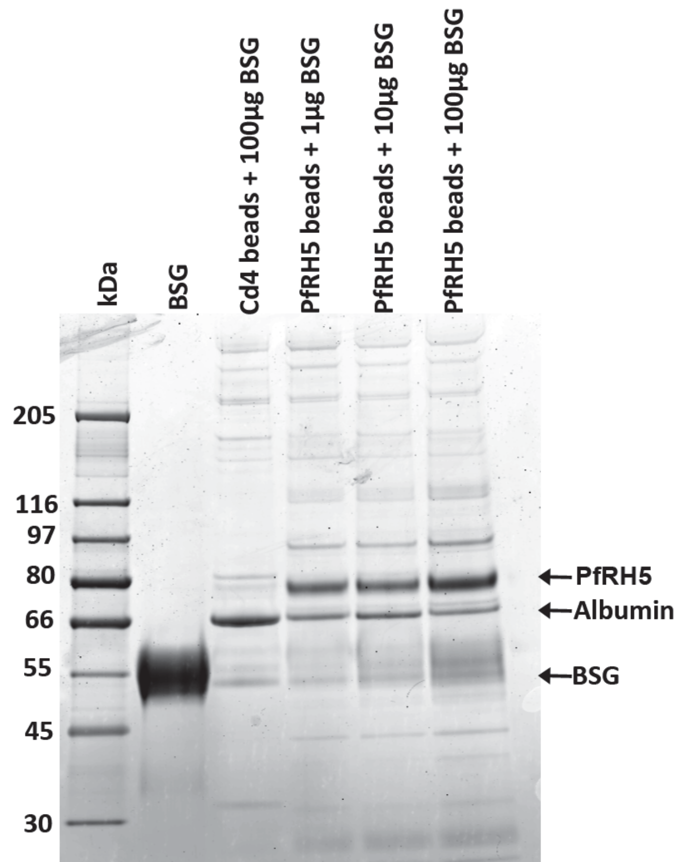


Figure 5.10: **PfRH5-coated beads could be used to co-purify BSG**
The biochemical co-purification assay was performed using 100μL PfRH5-coated beads in 1mL serum spiked with purified human BSG. When sufficient BSG was added, a enhancement of SYPRO staining is seen in the 55kDa region of the SDS-PAGE resolved eluate from co-purifications using PfRH5-coated beads when compared with that using Cd4-coated beads.

PfRH5 as the bait protein (Figure 5.10). Even if BSG remains bound to the PfRH5 beads following washing, these concentrations are at the very limits of the expected detection range for this interaction, given its micromolar K_D (indicated in Figure 5.7). High concentrations of BSG were required to be able to see an appropriately-sized band in the PfRH5 biochemical co-purification. When 100μg BSG was added into 1mL serum, there was a clear differential between the elution profiles when using Cd4- and PfRH5-coated beads, such that a 55kDa band corresponding to BSG was specifically observed in the PfRH5 lane. This implies that the stability of the BSG/bead interaction is higher than anticipated, and that the assay is potentially more sensitive than the above calculations suggest (as discussed in Section 5.2.5).

5.3.4 Serum interaction screens using purified merozoite baits

To screen for novel merozoite-serum interactions, I initially selected a range of merozoite proteins that are known to be exposed to the bloodstream. This shortlist included proteins known to be cleaved from the surface of the merozoite during the invasion process. PfMSP1, and hence the peripherally associated fragments of PfMSP6 and PfMSP7, as well as PfAMA1 are released as the parasite enters the host blood cells via the activity of the PfSUB2 [135]. PfM-TRAP has also been implicated in RBC invasion and is potentially cleaved from the surface by a rhomboid protease upon the parasite's entry into the cell[12, 22]. PfRH5 was also included because it is of particular interest to vaccine research taking place within the laboratory, and elsewhere[41]. The elution profiles obtained appeared to contain a number of bands specific to the *Plasmodium* protein used to coat the beads. However, the majority of the bands were the correct size to be those of the input *Plasmodium* protein (indicated by grey circles in Figure 5.11). The elution profile when using PfAMA1 protein initially indicated that the protein was interacting with a serum component consisting of 75 and 25kDa fragments, but analysis of the protein size and re-sequencing of the expression plasmid revealed that the protein was in fact PfMSP3.8. We can therefore safely assume that these bands correspond to the heavy and light chains of IgM. The elution profile using PfRH5 bait also looked potentially interesting, as three unique bands were observed. SDS-PAGE analysis of the input recombinant PfRH5 showed that each of these bands were in fact fragments of the full-length PfRH5 precursor (Figure 5.11C). Proteolytic processing of recombinant PfRH5 has been previously reported[41, 277], so it is likely that we are observing a similar phenomenon in these preparations.

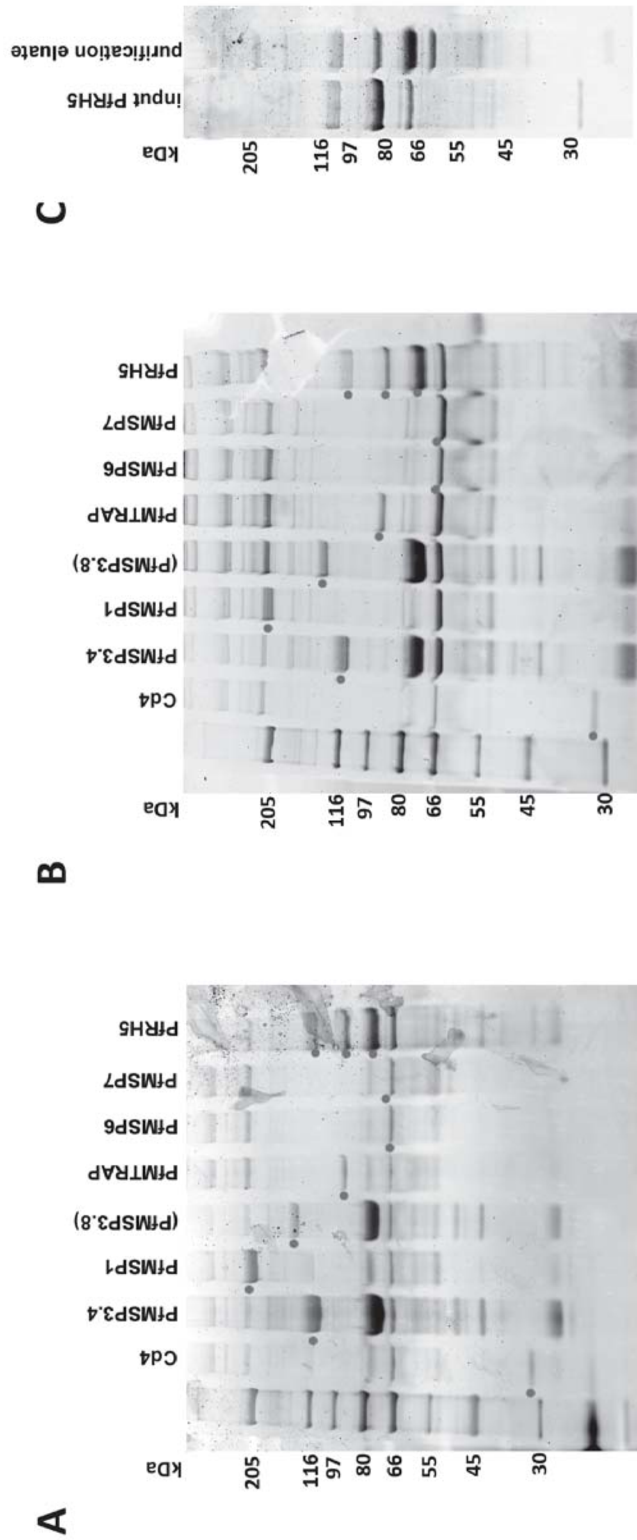


Figure 5.11: **An initial serum protein interaction screen identified no novel interactions**
A,B. SDS-PAGE elution profiles from biochemical co-purification assays using a range of serum-exposed *Plasmodium* baits. Grey circles to the left of bands indicate that the band corresponds to the input *Plasmodium* protein.
C. The PfRH5 input protein was resolved alongside the PfRH5 elution profile. PfRH5 appears to be proteolytically processed such that biotinylated species of 3 major lengths are used in the biochemical co-purification assay.

In two additional screens, all members of the PfMSP3 family and six members of the PfMSP7 family were loaded onto beads and incubated with human serum. These multi-gene families are enigmatic in that multiple members are preserved in parasite genomes, yet the reason for this is unclear. Most of the members of these families have no currently-identified function. We already know that PfMSP3.4 and PfMSP3.8 bind serum IgM in a potential immunomodulatory mechanism; however none of the other PfMSP3 family members appear to demonstrate the same antibody-binding behavior, nor did I reproducibly identify any other serum proteins to which PfMSP3 proteins could additionally bind (Figure 5.12). Recognising that the sensitivity of the assay may be limited by the amount of serum used, I repeated the assay using serum volumes up to 15mL. I also increased the bead volume to 200µL so as to expand the range of interactions that could potentially be discovered. Neither of these enhancements, individually or in combination, resulted in any novel bands in SDS-PAGE analyses of their eluates. Similarly, biochemical co-purification assays did not identify any serum protein binding partners for PfMSP7 proteins (Figure 5.13).

5.3.5 High-throughput screening of the merozoite protein library

In the screens described above, the major limitation on the throughput of the assay was the need to purify the recombinant biotinylated merozoite proteins with which to coat the beads. Although purified protein is preferable (as it can be more easily quantified and quality checked), it should be possible to use transfected HEK293E cell culture supernatant[7] as a direct source this biotinylated protein to coat the beads. To test whether this would be a viable approach, I selected a panel of eight merozoite protein baits for a pilot screen. I incubated 100µL beads with 1.5mL cell culture supernatant for 30 minutes, isolated the beads and performed ELISAs on the resulting supernatant. In most cases 1.5mL cell culture supernatant was insufficient to clearly saturate the binding capacity of 100µL beads, such that that no biotinylated protein could be detected following isolation of the beads. Where this occurred I re-incubated the partially coated beads with a further 1.5mL cell culture supernatant for 30 minutes and repeated the bead saturation ELISA. If I was still unable to observe saturation, I repeated the incubations with 1.5mL culture supernatant until unbound biotinylated protein could be detected by ELISA (Figure 5.14). Having demonstrated that bead saturation was possible without the use of purified proteins, I screened these proteins against human serum

[7] Dialysed as described in 2.1.2.3 to remove excess D-biotin

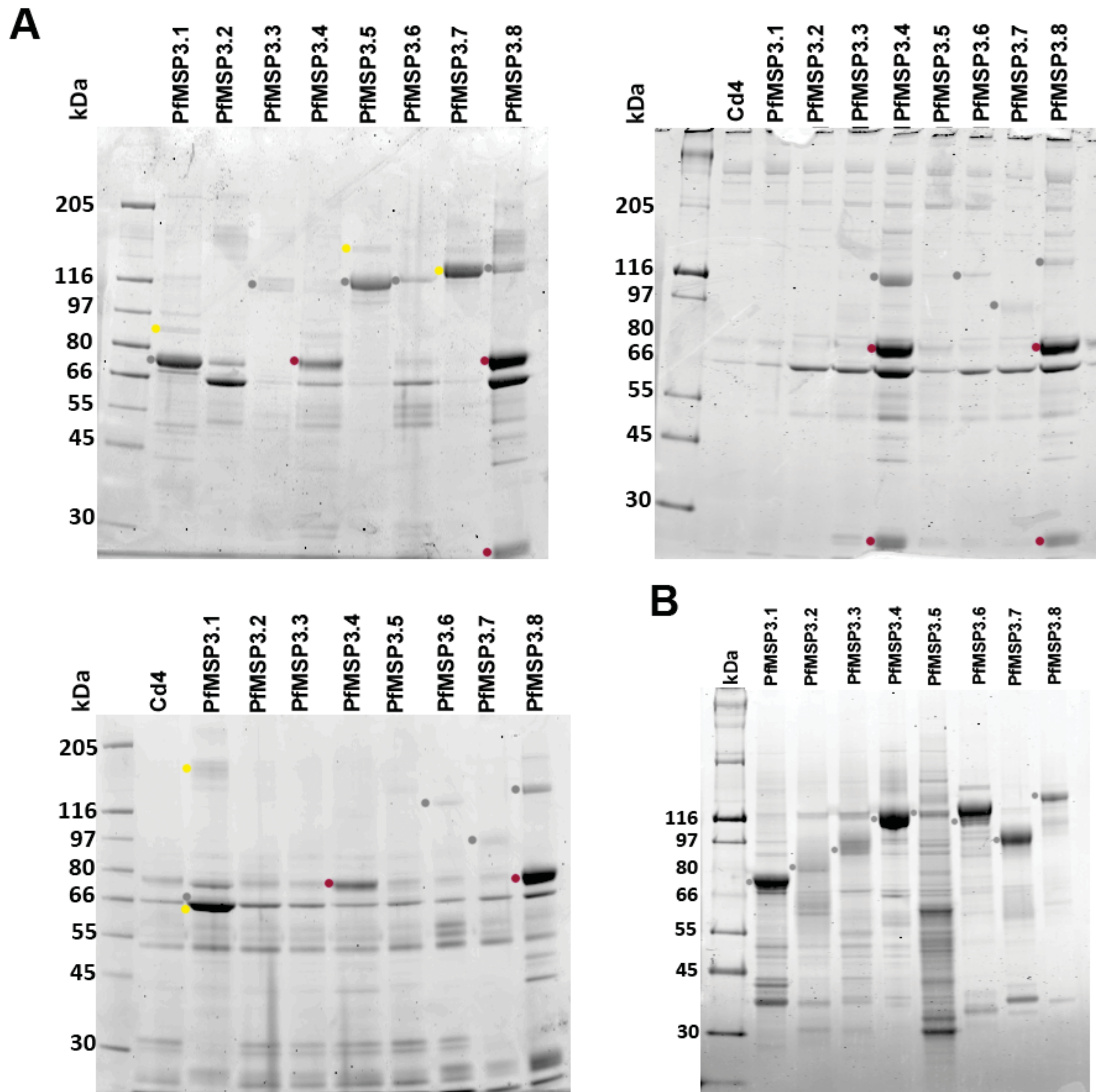


Figure 5.12: **PfMSP3-family serum biochemical co-purification screen revealed no novel interactions**

A SDS-PAGE elution profiles from biochemical co-purification assays using eight PfMSP3 family baits. Grey dots to the left of bands indicate that the band corresponds to the input *Plasmodium* protein. Pink dots indicate bands corresponding to the heavy or light chain of IgM, and yellow dots are placed to the left of bands that are potentially unique and do not correspond to the input bait protein or IgM. The three gels are a selection from six sets of biochemical co-purification experiments, each using a different batch of human serum.

B. SDS-PAGE analysis of the nickel-purified proteins incubated with the beads to produce the binding reagents. Grey dots indicate the expected protein sizes.

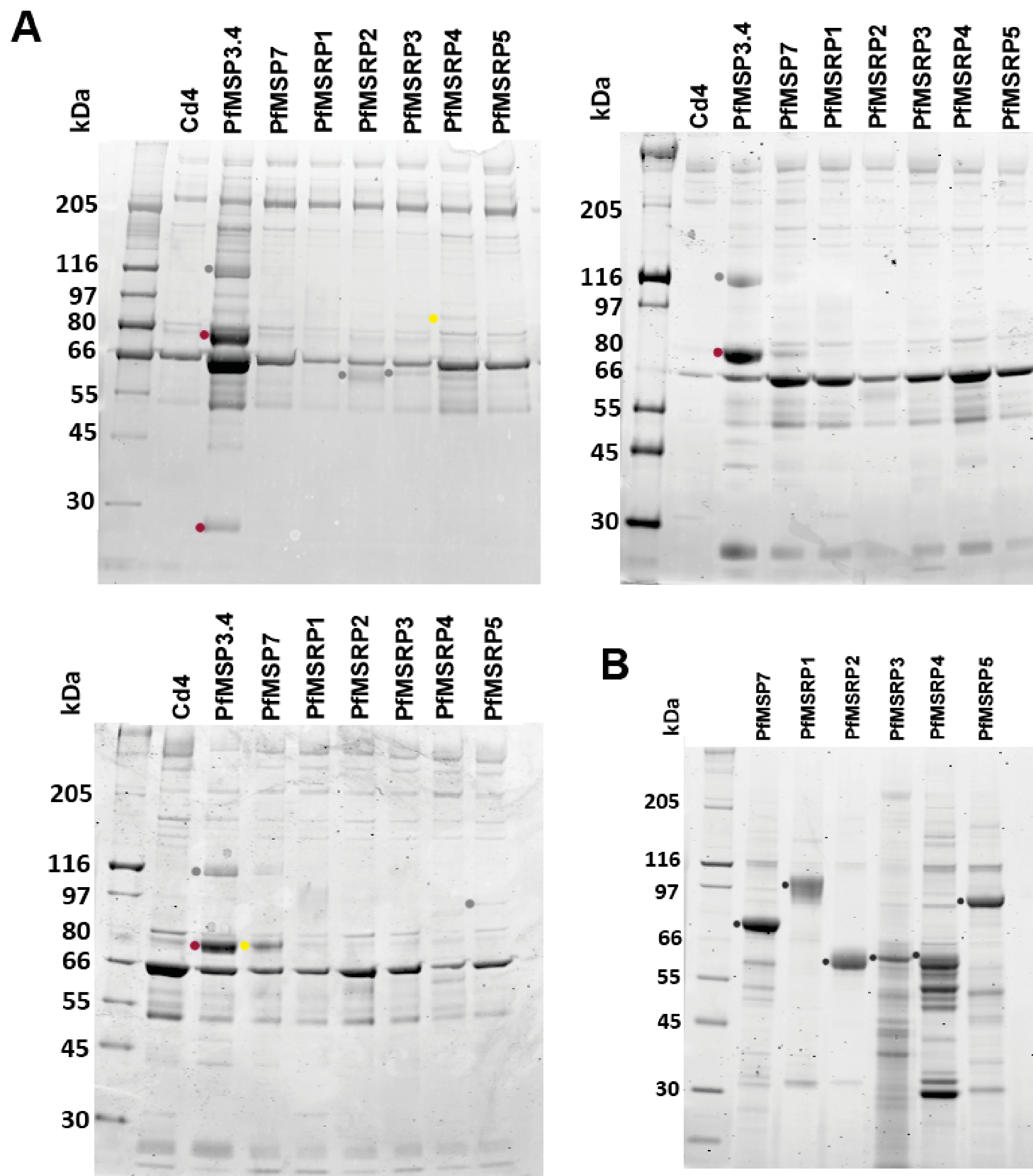


Figure 5.13: **PfMSP7-family serum biochemical co-purification screen revealed no novel interactions**

A SDS-PAGE elution profiles from biochemical co-purification assays using six PfMSP7 family baits. Grey dots to the left of bands indicate that the band corresponds to the input *Plasmodium* protein.

B. SDS-PAGE analysis of the nickel-purified proteins incubated with the beads to produce the binding reagents. Grey dots indicate the expected protein sizes.

as before, but without the bait protein purification step used originally. Encouragingly, the interaction between PfMSP3.4 and IgM was still readily detectable, as I reliably observed a clear band corresponding to the IgM heavy chain. A few bands appeared to be specific to the input bait protein, thus representing potential 'hits' in this screen. A proportion of these were of the correct size to be the bait protein itself, but a number of them were not. Of these, no single specific band was observed reproducibly in three independent screens. Following this pilot screen, I produced 32 additional proteins from the *P. falciparum* merozoite for to screen for interactions with human serum. I coated the beads by 3 successive 30-minute incubations with 1.5mL filtered, dialysed transfected cell culture supernatant, retaining the supernatant from the final incubation to confirm bead saturation by ELISA. I screened panels of up to ten proteins using 100µL beads and 1.5mL serum, and did so at least twice, using a different batch of serum on each occasion. I used these high throughput screens to select candidates for screening using 250µL beads and 5mL serum (Figure 5.17). None of the unique bands observed in Figures 5.15 & 5.16 were repeated or validated using this approach.

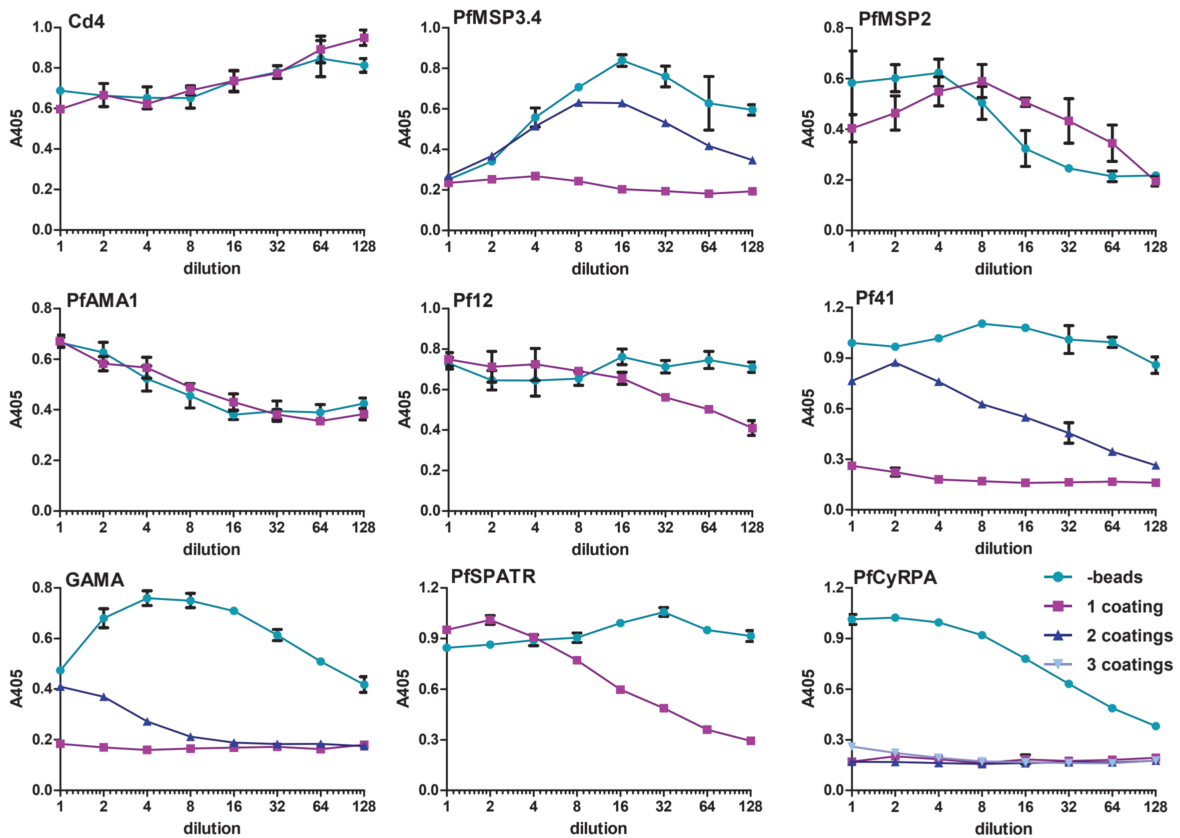


Figure 5.14: **Recombinant protein from HEK cell culture supernatant could saturate beads**
Bead-saturation ELISA measurements were performed on the supernatants following the isolation of 100µL beads after 30 minute incubations with 1.5mL filtered, dialysed HEK293 cell culture supernatants. The cyan data series shows the phosphatase substrate turnover, measured by absorbance at 405nm, resulting from the capture of biotinylated protein from the cell culture supernatant prior to its incubation with the beads. The remaining data series show the detection of biotinylated proteins following the indicated number of successive incubations of 1.5mL supernatants with 100µL beads. Error bars repesent the mean +/- SD, *n*=2.

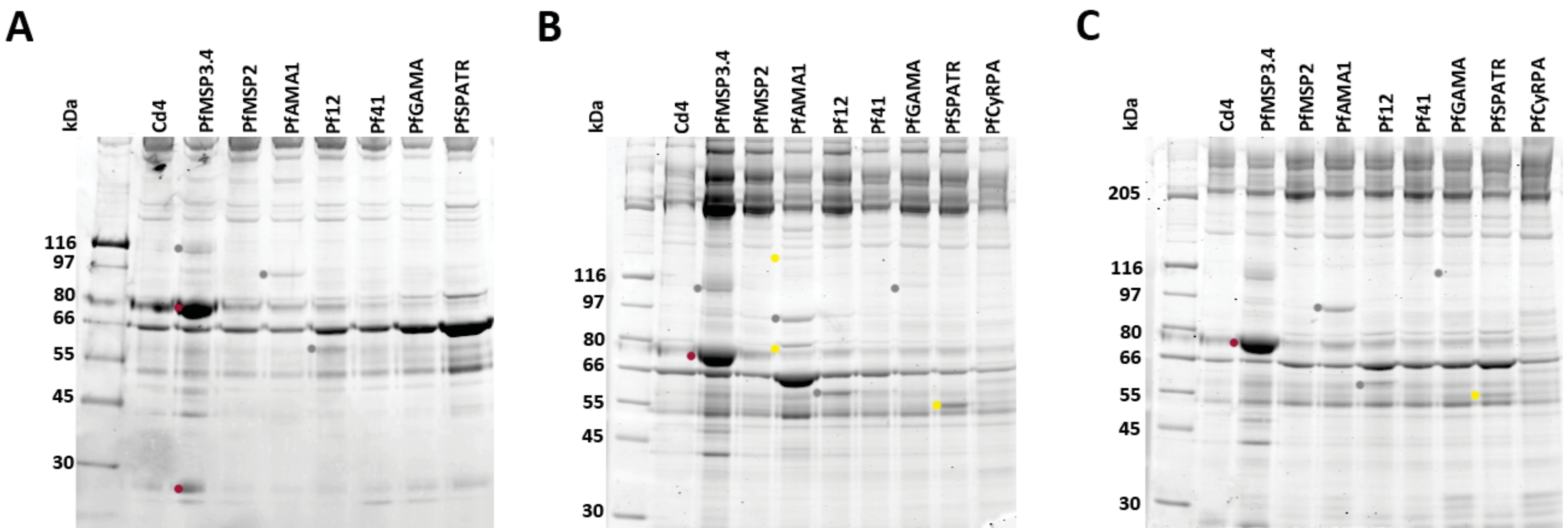


Figure 5.15: **A pilot high-throughput screen identified candidate *P. falciparum* proteins for further investigation**
Eight *P. falciparum* proteins were used to coat 100µL beads, which were used to probe 1.5mL human serum for potential host-pathogen interactions. The pink circles to the left of the indicated bands indicated the expected IgM chains purified using the PfMSP3.4 baits. Yellow circles to the left of bands indicate that they appeared specific to the bait protein used, and could hence represent hits in the screen. A subset of these are liable to be the bait proteins themselves (grey circles), for instance the PfAMA1-specific band around 90kDa, the Pf41-specific band around 60kDa, PfGAMA-specific band around 100kDa and the PfSPATR-specific band around 55kDa. Specific bands around 80 and 150kDa were seen when PfAMA1 bait was used in B, and significantly more albumin appeared to be detected when PfSPATR protein were used as a bait in A; this might indicate that more albumin interacted with PfSPATR-coated beads or that PfSPATR interacted with a serum protein of approximately 65kDa.

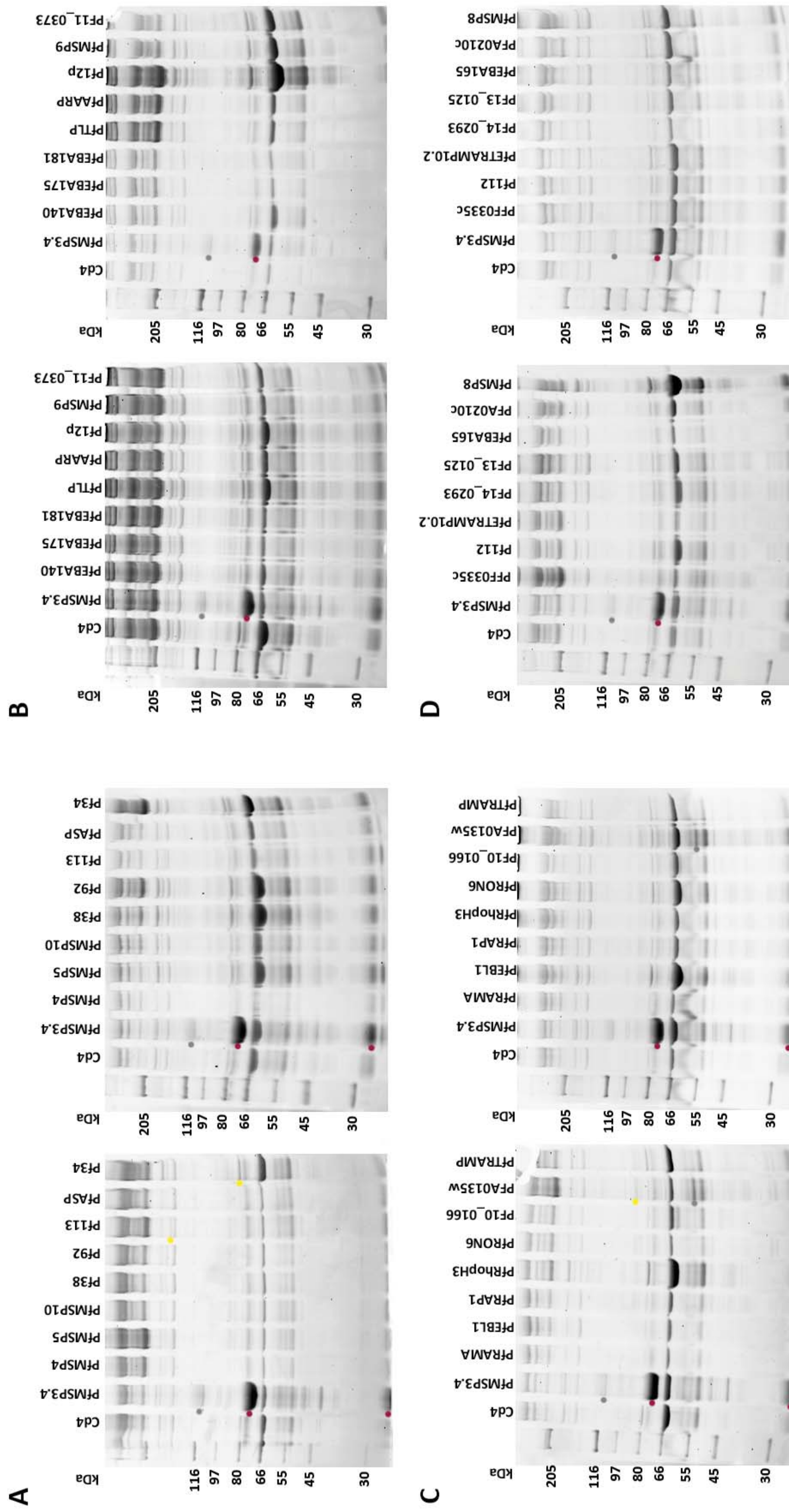


Figure 5.16: **Additional high-throughput interaction screens**
32 additional merozoite proteins were screened for interactions with human serum. Screens were performed in duplicate, using panels of eight proteins.

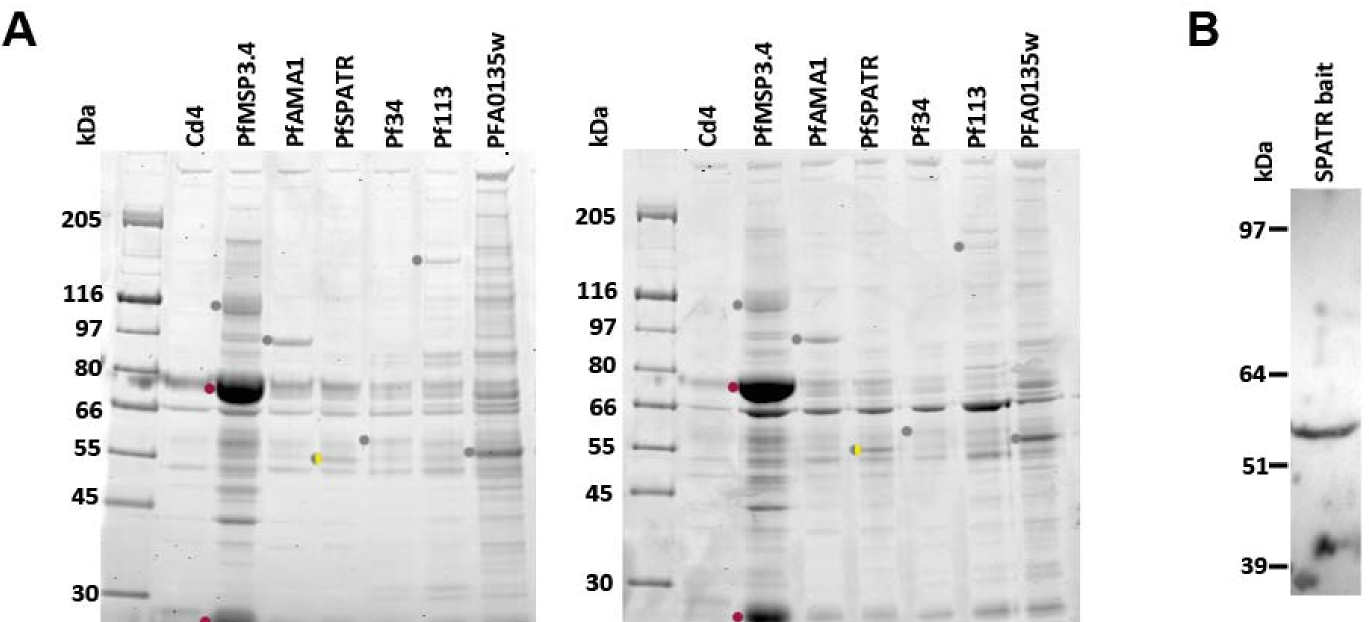


Figure 5.17: **Follow-up screening did not validate potential hits from high-throughput screening**
A Five *P. falciparum* proteins that potentially co-purified specific proteins from serum in earlier screens were screened, in duplicate, using 250μL beads and 5mL serum. Higher background was observed when PFA0335w was used as a bait protein, which could indicate that an interaction is occurring between PFA0335w and a human serum component. However, it is not possible from this information to speculate on an identity for a PFA0335w-interacting serum protein. Aside from those resulting from the PFA0335w co-purification, all unique bands observed were consistent in size with the respective bait proteins, indicated with a grey dot. The yellow-grey dot indicates a band that appeared to be significantly larger than the expected 49kDa size of the SPATR bait.
B Western blotting confirmed that the electrophoretic mobility of this band is consistent with being the input biotinylated SPATR protein.

5.4 Discussion

5.4.1 Strengths of the assay

This assay is capable of detecting interactions with a wide range of affinities, and can be combined with mass spectrometry to determine the identity of binding partners for *Plasmodium* proteins. Serum is an extremely protein-rich medium to sample for interactions (Figure 5.1), yet this assay is able to yield elution profiles with fairly low levels of contaminating proteins (Figures 5.6 & 5.8). Whilst the screens using 56 *P. falciparum* bait proteins did not lead to the identification of any novel interactions (Figures 5.11, 5.12, 5.13, 5.15, 5.16 & 5.17), there are still many more serum-exposed *Plasmodium* proteins that have yet to be screened. Hence this assay could be a useful tool for screening proteins for which there is a rational basis to assume that they might interact with a component of human serum. Serum is not the only matrix that can be screened using this approach; the method can be used to sample any other fluid. For instance I used SELP-coated beads to try to detect PfMSP7 fragments in parasite culture supernatant. In summary the assay has demonstrated potential for interaction detection, and could be used to discover novel interactions between recombinant proteins and partners in serum and beyond.

5.4.2 Potential improvements to the assay

Despite these successes, the assay is limited by the low abundances of certain serum proteins and the potentially transient nature of many host-pathogen interactions. However, there are a number of steps that could be taken to overcome these challenges.

- *Increase volumes:* Interaction detection capacity is certainly limited by the amount of serum protein that can be captured, either due to a low abundance of protein present in serum or a low interaction affinity that means that fewer molecules of interacting protein are bound to beads at equilibrium. As serum is abundantly available, it is very straightforward to increase its volume, although it is more cumbersome experimentally. Increasing the volume of beads has no negative effect on running the experiment, but is associated in a significantly increased cost. I performed the PfMSP3-family biochemical co-purification assay using 200μL beads and 15mL serum to see whether this would reveal any additional bands, but recovered very similar elution profiles to those presented in Figure 5.12.

- *Further increase the avidity and stability of interactions:* Coating *Plasmodium* bait pro-

***P. falciparum* bait**	**Accession number**	**Tags**	**Mass (kDa)**	**Plasmid constructor**
AARP	PF3D7_0423400	Bio	43.1	Dr C. Crosnier
AMA1	PF3D7_1133400	Bio	82.7	Dr C. Crosnier
ASP	PF3D7_0405900	Bio	103	Dr C. Crosnier
CyRPA	PF3D7_0423800	Bio, His	64.5	Dr Z. Zenonos
EBA140	PF3D7_1301600	Bio	152	Dr C. Crosnier
EBA165	PF3D7_0424300	Bio	178	Dr Z. Zenonos
EBA175	PF3D7_0731500	Bio	186	Dr C. Crosnier
EBA181	PF3D7_0102500	Bio	192	Dr C. Crosnier
EBL1	PF3D7_1371600	Bio	318	Dr C. Crosnier
ETRAMP10.2	PF3D7_1033200	Bio	26.5	Dr C. Crosnier
GAMA	PF3D7_0828800	Bio	103	Dr C. Crosnier
MSP1	PF3D7_0930300	Bio, His	216	A. J. Perrin
MSP2	PF3D7_0206800	Bio	45.9	Dr C. Crosnier
MSP3.1	PF3D7_1035400	Bio, His	62.4	A. J. Perrin
MSP3.2	PF3D7_1035500	Bio, His	65.5	A. J. Perrin
MSP3.3	PF3D7_1035600	Bio, His	70.5	A. J. Perrin
MSP3.4	PF3D7_1035700	Bio	100	Dr C. Crosnier
MSP3.4	PF3D7_1035700	Bio, His	102	A. J. Perrin
MSP3.5	PF3D7_1035800	Bio, His	104	A. J. Perrin
MSP3.6	PF3D7_1035900	Bio, His	87.8	A. J. Perrin
MSP3.7	PF3D7_1036000	Bio, His	68.7	A. J. Perrin
MSP3.8	PF3D7_1036300	Bio, His	110	A. J. Perrin
MSP4	PF3D7_0207000	Bio	49.1	Dr C. Crosnier
MSP5	PF3D7_0206900.1	Bio	50.1	Dr C. Crosnier
MSP7	PF3D7_1335100	Bio, His	63.1	A. J. Perrin
MSP8	PF3D7_0502400	Bio, His	89.0	Dr Z. Zenonos
MSP9	PF3D7_1228600	Bio	107	Dr C. Crosnier
MSP10	PF3D7_0620400	Bio	78.5	Dr C. Crosnier
MSRP1	PF3D7_1335000	Bio, His	65.4	A. J. Perrin
MSRP2	PF3D7_1334800	Bio, His	55.2	A. J. Perrin
MSRP3	PF3D7_1334600	Bio, His	57.4	A. J. Perrin
MSRP4	PF3D7_1334400	Bio, His	59.0	Dr Z. Zenonos
MSRP5	PF3D7_1334300	Bio, His	76.8	Dr Z. Zenonos
MTRAP	PF3D7_1028700	Bio	73.1	Dr C. Crosnier
Pf12	PF3D7_0612700	Bio	56.7	Dr C. Crosnier
Pf12p	PF3D7_0612800	Bio	61.4	Dr C. Crosnier
Pf34	PF3D7_0419700	Bio	57.0	Dr C. Crosnier
Pf38	PF3D7_0508000	Bio	58.8	Dr C. Crosnier
Pf41	PF3D7_0404900	Bio	63.6	Dr C. Crosnier
Pf92	PF3D7_1364100	Bio	110	Dr C. Crosnier
Pf112	PF3D7_1436300	Bio	133	Dr C. Crosnier
Pf113	PF3D7_1420700	Bio	130	Dr C. Crosnier

Table 5.3: ***P. falciparum* merozoite proteins screened against human serum**

P. falciparum bait	Accession number	Tags	Mass (kDa)	Plasmid constructor
PF10_0166	PF3D7_1017100	Bio, His	58.8	Dr Z. Zenonos
PF11_0373	PF3D7_1136200	Bio	94.4	Dr C. Crosnier
PF13_0125	PF3D7_1321900	Bio, His	76.9	Dr Z. Zenonos
PF14_0293	PF3D7_1431400	Bio	133	Dr C. Crosnier
PFA0135w	PF3D7_0102700	Bio, His	57.1	Dr Z. Zenonos
PFA0210c	PF3D7_0104200	Bio, His	76.1	Dr Z. Zenonos
PFF0335c	PF3D7_0606800	Bio	55.0	Dr C. Crosnier
RAMA	PF3D7_0707300	Bio	122	Dr C. Crosnier
RAP1	PF3D7_1410400	Bio	111	Dr C. Crosnier
RH5	PF3D7_0424100	Bio, His	85.2	A. J. Perrin
RhopH3	PF3D7_0905400	Bio	125	Dr C. Crosnier
RON6	PF3D7_0214900	Bio	133	Dr C. Crosnier
SPATR	PF3D7_0212600	Bio	49.7	Dr C. Crosnier
TLP	PF3D7_0616500	Bio	173	Dr C. Crosnier
TRAMP	PF3D7_1218000	Bio	56.0	Dr C. Crosnier

Table 5.4: ***P. falciparum* merozoite proteins screened against human serum (continued)**

teins onto paramagnetic beads has the dual purpose of facilitating the isolation of interacting proteins and increasing the stability of interactions with serum proteins. It may be possible to increase the strength of binding interactions further by changing the way that merozoite bait is arrayed on the surface of the bead. For instance, it might be possible to coat the bead surface with pentamerised proteins, so as to increase the number and density of receptors on the surface of the bead. Stabilisation of interactions could also be achieved by chemically cross-linking beads and interacting proteins prior to washing, although this might also compromise the purity of the eluate.

- *Improve quality of serum*: Serum was purchased as frozen, pooled isolates from a number of healthy donors. However, even before performing any assays with the serum, there was observable variation between the batches received. For instance, some batches were seen to contain large, visible protein aggregates upon thawing. I removed these aggregates by centrifugation and subsequent filtration, but in doing so could have removed an important source of serum protein that could potentially interact with one (or even many) of the merozoite baits. I expect that variability in the serum batches might account for differences in backgrounds of the serum screens, for example in Figures 5.11, 5.12 and 5.13. In the future it may be possible to improve the source of serum, such that steps can be taken to reduce protein aggregation and increase the consistency of screens.

- *Alter elution conditions*: The experiment shown in Figure 5.4 indicated that SDS or Glycine-HCl would be appropriate elution buffers for the assay. The main difference between these buffers was that SDS was able to elute PfMSP3.4 as well as IgM from PfMSP3.4/serum biochemical co-purification experiments. Knowing that PfMSP3.4 oligomerises *in vitro* I had hypothesised that the best elution process would bring down IgM and the PfMSP3.4 that was peripherally associated with the beads (i.e. not via the biotin-streptavidin interaction). However, given the appearance of input protein in the elution profiles of each screen (Figures 5.11, 5.12 and 5.13), I expect that the denaturing conditions used are able to dissociate the biotin-streptavidin interaction that binds the merozoite protein to the beads. By using an acidic elution buffer, such as pH 1.5 Glycine-HCl, the biotin-streptavidin interaction may remain intact and only serum proteins may be eluted. This would remove the input protein bands from the gels, making the presence of additional interesting bands more immediately identifiable.

- *Decrease stringency:* These experiments were designed with the intention of observing a single, unique band in a denaturing gel electrophoresis-based analysis of the eluate from washed beads. This way the protein-of interest could be specifically isolated from the gel and identified by tandem mass spectrometry. This is perhaps an unrealistically stringent expectation and, as mass spectrometry techniques advance, it might be possible to compare more of the eluate from a large numbers of biochemical co-purifications and identify human proteins that are specifically enriched when particular merozoite baits are used[261], without an SDS-PAGE step.