# APPENDIXES

## Appendix A: Proof of principle of MARTI-assay on IAsys biosensor

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Research paper

### A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients

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**Abstract**

Tuberculosis has re-emerged as a global health problem due to co-infection with HIV and the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. HIV co-infection introduced a 30% underestimation in TB diagnosis based on sputum analysis, calling for a reliable and fast serodiagnostic assay to assist in the management of TB in HIV-burdened populations. Serodiagnosis with mycobacterial lipid cell wall antigens gave promising results, in particular with LAM and cord factor. Free mycolic acids have also been considered because they are unique in structure to each species of *Mycobacterium* and can be economically extracted and purified. In a standard immunoassay such as ELISA, however, an unacceptable number of false positive and false negative test results were obtained. Here we report a much improved biosensor method to detect antibodies to mycolic acids in patient serum as surrogate markers of active tuberculosis. Mycolic acid (MA) liposomes were immobilized on a non-derivatized twin-celled biosensor cuvette and blocked with saponin. A high dilution of serum was used to calibrate the binding signal of the two cells, followed by contact with patient serum at a lesser dilution, but pre-incubated with either antigen-carrying, or empty liposomes. The serum, or the protein A purified IgG thereof, from sputum-positive tuberculosis patients could be inhibited from binding to the MA in the biosensor by prior incubation with MA-containing liposomes. The accuracy of the inhibition test was 84% if HIV-positive patients for whom a negative TB sputum analyses could not be relied upon to serve as a reference standard were excluded. If biosensor technology could be made suitable for high throughput screening, then it may provide the solution to the serodiagnosis of tuberculosis against a background of HIV.


*Keywords:* Antibodies; Mycolic acids; Biosensor; *Mycobacterium tuberculosis*; Serodiagnosis

#### 1. Introduction

Tuberculosis (TB) is a chronic pulmonary disease caused by infection with *Mycobacterium tuberculosis*. It is a major scourge in developing countries as well as an increasing problem in many developed areas of the world, with about 8 million new cases and 3 million deaths each year (Hendrickson et al., 2000). Although tuberculosis is a curable disease that responds well to antibiotics, it has re-emerged as a growing, global health problem because of the development of drug-resistant strains. The resurgence of TB and the increased risk for TB in HIV-infected persons has magnified the need for rapid, inexpensive and accurate methods for the

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diagnosis of TB (Cheon et al., 2002). People with TB that are co-infected with HIV have a 5–15% risk yearly of developing active tuberculosis (Winkler et al., 2005). World Health Organization (WHO) guidelines suggest starting antiretroviral drugs within 2 months of tuberculosis treatment. Patients who start antiretroviral drugs too early in their TB treatment can be predisposed to immune reconstitution syndrome (IRS). Immune reconstitution syndrome has symptoms overlapping with worsening TB and can be life threatening to the patients (Lawn et al., 2005). A major challenge with immunological diagnosis of tuberculosis is to distinguish between mere physical exposure to TB, latent infection and chronic or severe active disease (Pai et al., 2006). Other factors that affect the performance of immune-based assays include previous BCG vaccination, exposure to non-tuberculous mycobacteria and co-infection with *M. tuberculosis* and HIV.

The tuberculin skin test, based on the cellular response to protein antigen, is currently the most generally used method of identifying TB infection. The specificity is low as purified protein derivative (PPD) contains many antigens widely shared among mycobacteria. Several studies have demonstrated that PPD cannot reliably distinguish between previous *Mycobacterium bovis* BCG vaccination, exposure to environmental mycobacteria, or infection with *M. tuberculosis* (Doherty et al., 2002; Chan et al., 2000). Sputum analysis for the presence of live *M. tuberculosis* is done in a variety of ways and is generally employed for the diagnosis of TB. Acid-fast microscopy is quick and easy, but needs confirmation by other tests, as some acid-fast bacilli are not *M. tuberculosis* (Hamasur et al., 2001). Culture of *M. tuberculosis* from sputum is the gold standard for the diagnosis of tuberculosis. The technique is very sensitive, such that even a few mycobacteria can be detected. However, primarily due to the slow growth of the bacteria, this method usually requires 4–8 weeks for completion (Samanich et al., 2000) and is subject to contamination. This often results in delayed diagnosis, adversely affecting patient care and TB control and allows for the spread of infection. In addition, HIV-infected patients often cannot produce sputum of the desired quality for TB detection (Frieden et al., 2003). PCR DNA detection kits are faster, more sensitive and accurate, but also require a sputum sample and a sophisticated laboratory with matching staff competency. Samples from other sources, in particular blood, showed much less sensitivity with PCR amplification tests (Saltini, 1998).

A simple diagnostic assay that does not require highly trained personnel or a complex technological infrastructure is essential for global control of TB (Foulds and O'Brien, 1998). A serologic test, such as ELISA, is a simple and inexpensive alternative to other TB diagnostic methods (Simonney et al., 1996; Moran et al., 2001). The disadvantage of ELISA is that it detects only the high affinity antibodies to the antigen, due to the need for washing steps after contact between the antigen and serum antibodies. Irrespective of the antigen(s) used, no single ELISA test has hitherto succeeded as a reliable test to confirm tuberculosis. Due to the limitations of conventional TB diagnostic tests, there is a widely felt need for a novel approach to develop a diagnostic test that is accurate and convenient to use, especially in HIV patients (Pavlou et al., 2004; Reid et al., 2006).

Mycolic acids are unique, 60–90 carbons long, branched α-alkyl, β-hydroxy fatty acids, which form an outer waxy lipid layer around the mycobacteria (Barry et al., 1998). Our previous studies (Schleicher et al., 2002) and those of others (e.g. Pan et al., 1999) have shown the prevalence of anti-mycolic acid antibody in TB patients with ELISA. Schleicher et al. (2002) investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *M. tuberculosis* mycolic acids in the serum of HIV-seropositive and HIV-seronegative tuberculosis patients, in a population with a high prevalence of HIV. Although they observed a higher signal of antibody to mycolic acids in TB-positive patients than in TB-negative patients, they also found quite a number of false positive and false negative results. From their studies, they concluded that the ELISA has inadequate sensitivity and specificity to detect anti-mycolic acid antibody and is therefore not suitable as a reliable serodiagnostic assay for the diagnosis of pulmonary TB.

This study aimed to assess the potential of detecting anti-mycolic acid antibodies as a surrogate marker for tuberculosis with an IAsys affinity biosensor in the serum of patients with active pulmonary tuberculosis. The IAsys biosensor can monitor and quantify the binding of interacting analytes in real time, by detecting changes in refractive index in the vicinity of the immobilized ligand. The changes in refractive index values are proportional to the change in the accumulated mass (Cush et al., 1993; Buckle et al., 1993). The benefit of not having to label any of the reagents, combined with low sample consumption has made the optical biosensor a useful instrument in both research and commercial laboratories (Myszka, 1999). Although wave guide and SPR assays are currently still experimentally cumbersome, there is considerable technological development in this field such that one can realistically expect that the prevailing technical challenges can be overcome to

make such tests suitable for a routine diagnostic lab in the not too distant future.

Here, we describe how the technology could be applied in the detection of anti-mycolic acid antibodies as surrogate markers for active TB. To confirm that the active binding agents in the human sera were of antibody nature, the IgG antibodies were isolated from a healthy control and a TB-positive patient using protein A and tested in the biosensor assay. The advantages of protein A-based affinity chromatography (Verdoliva et al., 2002) were exploited to confirm that binding to immobilized mycolic acid on the IAsys cuvette surface was due to IgG from patient serum.

## 2. Materials and methods

### 2.1. Human sera

Serum samples collected for another study (Schleicher et al., 2002) were used; they were obtained from 61 patients (aged between 18 and 65 years), who were admitted to the general medical wards of the Helen Joseph Hospital, Johannesburg, South Africa, including a number with active pulmonary tuberculosis. The study population consisted of 32 tuberculosis-positive ($TB^+$) and 29 control tuberculosis-negative ($TB^-$) patients. The $TB^+$ group consisted of patients with newly diagnosed smear-positive pulmonary tuberculosis of which some were HIV-seropositive (23 patients). The $TB^-$ patients that were used as controls had medical conditions other than TB and were recruited from the general medical wards. None of the $TB^+$ patients were on anti-TB chemotherapy at the time of serum collection.

For the purpose of IgG isolation and testing, a $TB^+$ patient serum was selected from a collection of sera supplied by the Medical Research Council (MRC) (Clinical and Biomedical Research Unit, King George V Hospital, Durban, 1994). Healthy volunteer sera were used as negative controls for IgG isolation.

### 2.2. ELISA of patient sera

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum et al. (2001). Mycolic acids (250 µg) were dissolved in 4 ml of hot phosphate-buffered saline (PBS, pH 7.4) for 20 min at 85 °C and sonicated (Virsonic 600, United Scientific, USA) at 20% duty cycle and optimal output level for 1 min. The solution was kept at 85 °C during pipetting into ELISA plates (Sero-Well®, Bibby sterilin Ltd., UK), after which the plates were placed in plastic bags and incubated overnight at 4 °C. The final antigen load was approximately 3 µg/well. Control wells were coated with hot PBS only. After overnight incubation, the ELISA plates were flicked out and the wells blocked with 0.5% (m/v) carbohydrate-and fatty acid-free casein in PBS for 2 h at room temperature. The solution was flicked out, the wells were filled with 50 µl of serum, serum precipitate in triplicate and incubated for 1 h at room temperature, flicked out and washed three times with PBS/0.5% casein. The wells were aspirated to remove proteinaceous froth. The plates were incubated for 30 min at room temperature with peroxidase-conjugated goat anti-human IgG (whole molecule, Sigma) diluted 1/1000 in PBS/0.5% casein, flicked out, washed three times with PBS/0.5% casein and aspirated. The presence of antibody was revealed using 50 µl/well of hydrogen peroxide (40 mg) and *o*-phenylenediamine (50 mg) in 50 ml of 0.1 M citrate buffer (pH 4.5). Measurement of the yellow colour was done after 30 min at 450 nm using a Multiskan Ascent photometer (Thermo-Labsystems, Finland). To correct for background binding in the serum, the signal generated for the samples in PBS-coated wells was subtracted from that generated in mycolic acid-coated wells.

### 2.3. Preparation of liposomes

Stock solution of phosphatidylcholine (100 mg/ml) (Sigma, St Louis, MO) was prepared by dissolving the weighed amounts in chloroform. Mycolic acids-containing liposomes were prepared by adding 90 µl of phosphatidylcholine stock to 1 mg of dried mycolic acids. Empty liposomes, i.e. with no mycolic acids, were prepared by using 90 µl of phosphatidylcholine stock solution only. During pipetting, everything was kept on ice to avoid evaporation of chloroform. The liposome ingredients were dried with nitrogen gas in a heat block at 85 °C for about 10 min. Liposome formation was induced by addition of 2 ml of saline (0.9% NaCl) and placing in a heat block at 85 °C for 20 min, with vortexing every 5 min. The liposomes were then sonicated for 2 min at 30% duty cycle at an output of 3% with a Model B-30 Branson sonifier (Sonifer Power Company, USA). The sonicator tip was washed with chloroform and rinsed with distilled water before and after use. The liposomes (200 µl) were aliquoted into ten tubes and kept at −20 °C overnight before freeze-drying. After freeze-drying, 2 ml of phosphate-buffered saline (PBS) azide EDTA (Sigma, St Louis, MO) buffer (PBS/AE, pH 7.4) was added to each tube containing liposomes. The tubes were placed in a heat block for 20 min and sonicated as before.

### 2.4. Isolation of IgG using protein A Sepharose

A TB-positive patient serum was selected from a collection of sera supplied by the Medical Research Council (MRC) (Clinical and Biomedical Research Unit, King George V Hospital, Durban, 1994). The only criterion for selecting this serum for isolation was that it had previously given good responses to mycolic acid on ELISA (unpublished results). TB-negative samples, which consisted of pooled sera from healthy students working in the Biochemistry Department of the University of Pretoria, were used as negative controls. A modified method for IgG purification was used compared to that given in the literature (Seppala et al., 1990). Protein A, immobilized on Sepharose (3 ml; Fluka, BioChemika) was transferred to a 50-ml tube. The Sepharose was washed twice by centrifuging at 3000 rpm for 5 min, the supernatant removed by pipette and 10 mM Tris–HCl buffer (pH 8.08) added until the final volume in the tube was 20 ml. After the last washing step, the supernatant was removed, leaving the Sepharose just covered in buffer. Serum aliquots (500 µl each) were thawed at 37 °C for 30 min. Two aliquots (1 ml) were added to the Sepharose beads and the volume was adjusted to 20 ml with 10 mM Tris–HCl to effect a final serum dilution of 1/20. The serum was incubated with the Sepharose at room temperature with rotation for 2 h and then left to stand for the Sepharose to settle out. The supernatant was removed by pipette and transferred to a labelled 50-ml tube. This represented the IgG-depleted serum fraction.

The remaining bound IgG was removed from the protein A beads by washing with 100 mM Tris–HCl, keeping the final volume in each tube at 20 ml and then removing the supernatant after gentle mixing. This was repeated twice, after which the buffer was changed to 10 mM Tris–HCl for another three washes. The supernatant of the last washing step was measured at 280 nm against a suitable blank for the efficiency of washing. The Sepharose in the tube was finally washed once more with 10 mM Tris.

IgG was eluted with 0.1 M glycine (3 ml, pH 2.7) into tubes containing 1 M Tris–HCl (200 µl) to neutralize the acid. This process was repeated seven times using 0.1 M glycine (2 ml) and 2 min incubation before removing the next IgG supernatant. The efficiency of elution of protein from the beads was monitored at 280 nm against a suitable blank. The IgG fractions were pooled and dialyzed against 1.5 l of PBS with two exchanges after 12 h and 18 h. The IgG-depleted serum fraction isolated earlier was also dialyzed in the same manner. After dialysis, the solutions were transferred to 50 ml tubes, and 100 µl were removed for protein concentration determination. To the IgG (12 ml) and depleted serum fractions (12 ml), 99% glycerol (8 ml, 40% final concentration) was added and filtered through a 0.2-µm filter (Ministart Plus, CA-membrane and GF-prefilter) into sterile 50-ml tubes that were subsequently stored at 4 °C. This isolation process was repeated for the TB-negative serum sample. The protein A Sepharose beads were regenerated by washing twice with 0.1 M glycine (5 ml) and four times with 100 mM Tris–HCl (10 ml), until the pH was above 7. The Sepharose was stored in 100 mM Tris–HCl solution (10 ml, containing 1% benzyl alcohol).

### 2.5. Removal of glycerol for biosensor

To prepare the IgG and depleted serum fractions for IAsys biosensor analysis, the glycerol had to be removed. VetaSpin Micro columns (400 µl, Whatman) were pre-washed with PBS (400 µl) by centrifuging at 10 000 × *g* for 15 min. The glycerol-containing samples (400 µl) were then transferred to the spin columns and centrifuged at 10 000 × *g* for 40 min. The filtrate was removed and 200 µl of PBS added to the retentate and again centrifuged. This process was repeated another five times for each glycerol-containing sample. The final volume was reconstituted to the original sample volume (400 µl) with PBS. The concentration of IgG in the purified samples was determined by absorbance measurement at 280 nm using a Shimadzu spectrophotometer.

### 2.6. SDS-PAGE of isolated IgG and depleted serum

The protein concentration of the IgG fractions was determined using the absorbance at 280 nm and an extinction coefficient of 1.4, whereas the concentration of depleted serum and other protein fractions was determined using the Bradford assay. An amount corresponding to 10 µg per sample was aliquoted into Eppendorf tubes and four volumes of acetone added. The samples were then left to precipitate overnight at −70 °C. The next day the samples were centrifuged at 14 000 × *g* for 1 h at 4 °C. The supernatant was discarded and the samples dried under vacuum for 20 min. Deionized water (8 µl) was added to re-suspend the protein pellet and non-reducing sample buffer (8 µl) was added. The samples were boiled at 100 °C for 5 min. SDS-PAGE analysis of purified IgG fractions and depleted sera was performed according to Ey et al. (1978) in a MINIVE complete vertical electrophoresis system (Amersham Pharmacia Biotech AB, USA). Approximately 10 µg of protein was loaded per lane. The gel

consisted of a 10% separating gel and a 4% stacking gel. The low molecular weight markers contained 97, 66, 45, 30, 20.1 and 14.4 kDa standards. The loaded gel was first run at 60 V for 1 h and then at 120 V for 3 h until the indicator was 0.5 cm from the end of the gel.

### *2.7. Detection of anti-mycolic acid antibody (IgG) with the IAsys affinity biosensor*

The PBS/AE buffer (8.0 g NaCl, 0.2 g KCl, 0.2 g $KH_2PO_4$ and 1.05 g $Na_2HPO_4$ per litre, 1 mM EDTA and 0.025% (m/v) sodium azide) was prepared in double-distilled water and adjusted to pH 7.4. Cetyl-pyridinium chloride (CPC, 0.02 mg/ml) and saponin (1 mg/ml) were prepared in PBS/AE. The IAsys resonant mirror biosensor system and twin-cell non-derivatized cuvettes were from Affinity Sensors (Cambridge, UK). The sensor was set to a data-sampling interval of 0.4 s, a temperature of 25 °C and a stirring rate of 75% for all experiments. The cells were rinsed three times prior to use with 96% ethanol (Saarchem, SA), followed by extensive washing with PBS/AE. A 60-µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 µl of CPC for 10 min. This was followed by washing five times with 60 µl of PBS/AE and then substituting with 25 µl of PBS/AE for a new baseline before immobilization of mycolic acids-containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed five times with 60 µl of PBS/AE, substituted with 50 µl of saponin and incubated for 10 min. This latter step was to avoid non-specific binding on the surface of the cuvette during the subsequent binding events. The cells were then washed five times with PBS/AE, the content of each cell substituted with 25 µl of PBS/AE and left for about 5–10 min to achieve a stable baseline. Inhibition studies were performed using patient serum that was first left at room temperature to thaw completely. After obtaining a stable baseline, a 1/1000 dilution of serum antibodies (10 µl) in PBS/AE was added to each cell, to compare the responses of the two cells over 10 min. A pre-incubation of 1/500 dilutions of serum with solutions of liposomes containing mycolic acids and empty liposomes (phosphatidylcholine alone) were allowed for 20 min. These were then added (10 µl) for binding inhibition studies in different cells, one with mycolic acid liposomes and the other with empty liposomes as a control, and allowed to bind for 10 min. Inhibition studies using IgG antibody were also performed, but differently. After obtaining a stable baseline, 10 µl of IgG solution (0.04 mg/ml) in PBS/AE were added to both cells and the response measured over 10 min as above. Subsequently, 10 µl of 0.08 mg/ml IgG diluted with either mycolic acids or phosphatidylcholine liposomes were added and the response measured for 10 min. Finally, dissociation of antibodies was effected by washing three times with PBS/AE and measurement of the response for 5 min.

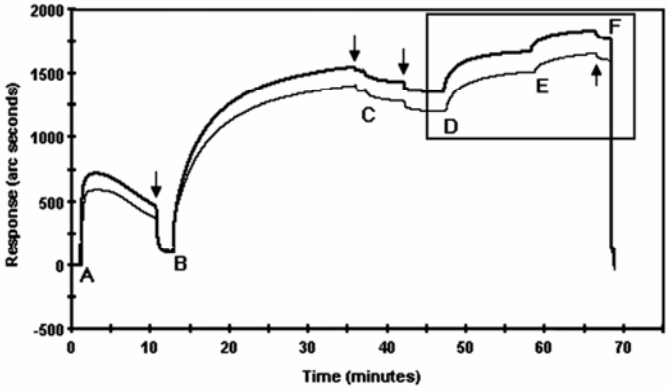


Fig. 1. A typical graph summarizing the process of measuring antibody binding or inhibition of binding by mycolic acid and empty (phosphatidylcholine only) liposomes, in the two cells of an IAsys biosensor cuvette surface coated with mycolic acid liposomes. The surface was activated with cetyl-pyridinium chloride (A), coated with mycolic acid liposomes (B), blocked with saponin (C), calibrated with a high dilution of serum (D), applied to measure the binding and dissociation of inhibited sera at lesser dilution (E), and regenerated with potassium hydroxide (12.5 M) and 96% ethanol (F). The arrows indicate washing with PBS/AE and the response from the two cells are differentiated by thick lines (channel 1, upper curve) and thin lines (channel 2, lower curve).

*2.8. Regeneration of non-derivatized cuvettes*

Regeneration was effected by three initial washes with 96% ethanol for 1 min, followed by washing seven times with 70 µl of PBS/AE for 1 min. The surface was then finally treated with 50 µl of potassium hydroxide (12.5 M) for 2 min followed by seven washes with 70 µl of PBS/AE for 1 min.

## 3. Results

The six main stages involved in measuring the binding of specific antibodies to lipid antigens in liposomes in real time on the biosensor are: (A) activation of the non-derivatized cuvette surface with CPC; (B) immobilization of the liposomes containing mycolic acids to the surface; (C) blocking with saponin to prevent non-specific protein binding; (D) binding (association) of antibodies from a high dilution of serum to calibrate the signal of the two cells of the cuvette; (E) binding and dissociation of inhibited patient serum at lesser dilution; and finally (F) surface regeneration (Fig. 1). The dilutions of serum used were estimated from a dilution range of one positive and one negative serum sample and are not necessarily optimal for all sera. The cuvette cell calibration curves for the high dilution serum in the two cells of one cuvette had to

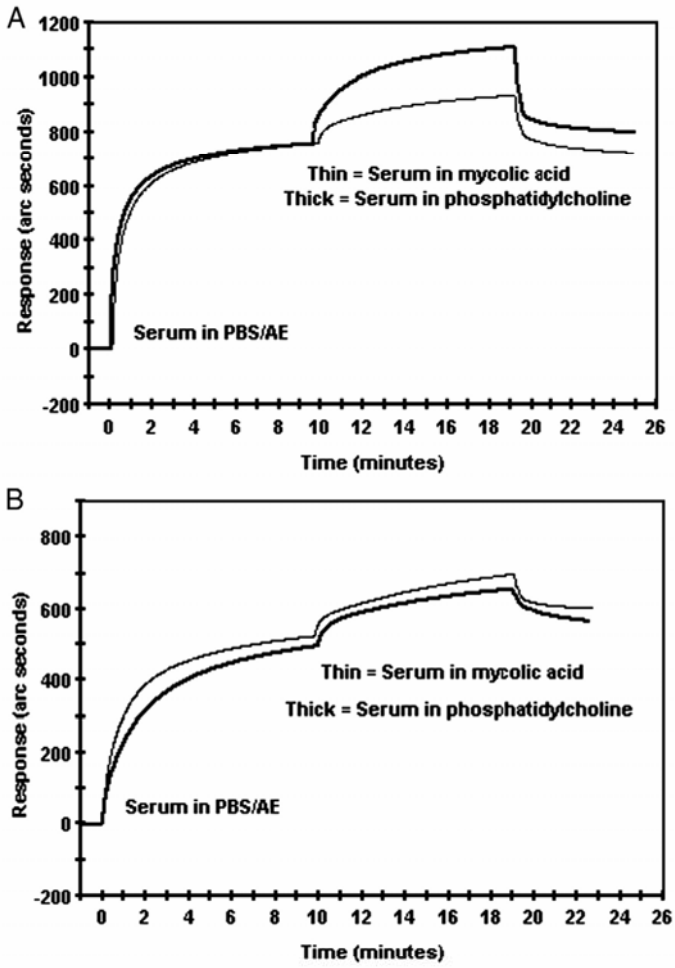


Fig. 2. Inhibition of human $TB^+$ (A) and $TB^-$ (B) patient serum antibody binding with mycolic acid liposomes or empty liposomes on an IAsys cuvette surface coated with immobilized mycolic acid liposomes. For the first 10 min, a 1/1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1/500 was then added with the thin line (A, lower curve; B, upper curve) representing the binding response of serum in mycolic acids and the thick line (A, upper curve; B, lower curve) representing that of serum in empty liposomes as control.

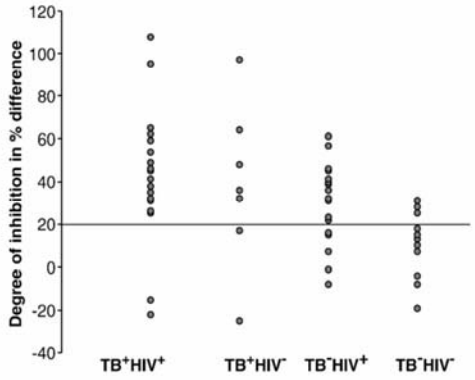


Fig. 3. The percentage of inhibition of binding of the biosensor signal for the 61 patient sera of $TB^+$ and $TB^-$ controls after pre-incubation of sera with mycolic acid liposomes and empty liposomes before testing on mycolic acid-coated cuvettes.

fall within 90–100% identity in terms of the relative response amplitudes in order to be accepted. A limitation of the IAsys system was found to be the difference in quality from one cuvette to another when using liposomes as antigen coat. In rare cases, new cuvettes were not usable at all. Usually, new cuvettes were found to be reliable only after a succession of regenerations, while in other rare cases, new cuvettes could be reliably applied after a single regeneration cycle. The results (those in the rectangle, Fig. 1) were aligned using the Fastplot programme from IAsys.

### *3.1. Detection of anti-mycolic acid antibodies in human serum*

Patient sera selected from the collection of Schleicher et al. (2002) were used to detect antibodies against mycolic acids on the optical IAsys biosensor. The ELISA experiments were performed as described in Schleicher et al. (2002). Of the 61 patient sera that were analyzed on the IAsys biosensor, 17 were re-analyzed on ELISA to confirm that the original antibody activity as reported by Schleicher et al. (2002) was still intact and to compare them with the results found on the IAsys biosensor during the same period of assessment. The inhibition studies on the IAsys were determined by pre-incubating test serum with mycolic acids-containing liposomes and applying these on biosensor cuvettes coated with mycolic acids. In the control experiments, sera were pre-incubated with empty liposomes. The pre-incubation of a sputum-positive TB patient serum with mycolic acid liposomes resulted in inhibition of antibody binding to mycolic acids when compared to the signal generated by the same serum pre-incubated with empty liposomes (Fig. 2A). This confirmed the specificity of binding of antibodies to mycolic acids in sputum-positive TB patient sera.

There was no inhibition of binding observed when a sputum-negative control serum ($TB^-HIV^-$) was pre-incubated with liposomes containing mycolic acids and tested on the biosensor to determine binding of antibodies to mycolic acids (Fig. 2B). This shows that specific anti-mycolic acid antibodies can be demonstrated in $TB^+$ patients, after pre-incubation of serum with mycolic acids. TB-negative sera from patients infected with HIV tested negative on the IAsys biosensor, with inhibition values of less than 20% (Fig. 3).

From 23 $TB^+HIV^+$ patient sera selected, two serum samples tested false negative on the biosensor (Fig. 3). Thirteen $TB^-HIV^+$ patient sera tested false positive, showing an inhibition of greater than 20% on the biosensor (Fig. 3). It is noteworthy that these patients were HIV-positive. Some patient sera that were false negative (e.g. Fig. 4A) and false positive (e.g. Fig. 4B) on the ELISA tested positive and negative respectively on the biosensor. The normalized signals on ELISA that were above two were regarded as positive and below two as

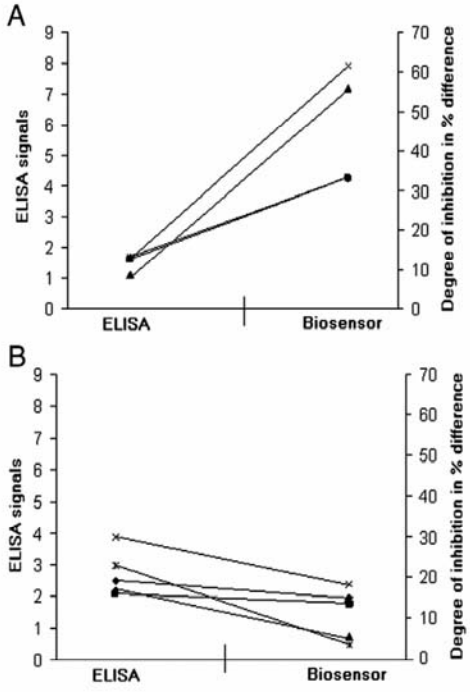


Fig. 4. Normalized ELISA signals and the percentage of inhibition of binding of the biosensor signal of false negative (A) and false positive (B) patients on ELISA who tested correctly on the biosensor.

negative. The $TB^+$ patients who showed truly positive responses of antibodies to mycolic acids on ELISA also tested truly positive on the biosensor. Our previous studies have also addressed the problems of detecting *M. tuberculosis*-specific antibodies to mycolic acid in TB patients co-infected with HIV on ELISA (Schleicher et al., 2002). Three of the patient sera tested from the 13 $HIV^-TB^-$ tested false positive on the biosensor, and only two serum samples tested false negative in the $TB^+HIV^-$ population (Table 1). An apparently lower specificity (27.8%) was observed in the $TB^-HIV^+$ sub-groups. However, all these patients were hospitalized with diseases other than TB based on the prevailing diagnostic methods. The low specificity obtained amongst the $HIV^+$ population could reflect true positive results, since it is known that the sputum culture assay is not sensitive enough to detect TB in HIV-positive patients (Frieden et al., 2003). This may reflect the fact that the serum test is better able to detect TB in $HIV^+$ patients. The IAsys affinity biosensor was found to be more sensitive (91.3%) in detecting TB amongst the TB-positive patients co-infected with HIV. The overall specificity and sensitivity of the assay after analyzing 61 patient sera was 48.4% (15/31) and 86.7% (26/30), respectively.

It is known that the gold standard of sputum growth of mycobacteria does not measure accurately in the $TB^-HIV^+$ cohort (Table 1). As the serum collection was actually made for an earlier study, follow-up data were not available to determine the true TB status of the $TB^-HIV^+$ cohort tested here. When the 18 $TB^-HIV^+$ sera were omitted in the calculation of the performance parameters of the test based on the 61 data points, the accuracy of the assay was found to be 83.7% (36/43). The sensitivity (86.7%, 26/30) remained the same after exclusion of the $TB^-HIV^+$ population, and the specificity was 76.9% (10/13). The assay showed a high sensitivity (91.3%) in sera from patients who were TB-positive and co-infected with HIV. It is known that HIV-positive patients generally have lower levels of *M. tuberculosis*-specific antibodies to protein and certain lipid antigens than HIV-negative patients. This shows that the IAsys biosensor can detect anti-mycolic acid antibodies in an HIV endemic population.

Table 1
Specificity and sensitivity of the IAsys affinity biosensor assay for detecting anti-mycolic antibody in pulmonary TB and negative control patient sera

| Patient group | No. of patients | False positive | False negative | Specificity (%) | Sensitivity |
|---|---|---|---|---|---|
| $TB^+HIV^+$ | 23 | – | 2 | – | 91.3 (21/23) |
| $TB^+HIV^-$ | 7 | – | 2 | – | 71.4 (5/7) |
| $TB^-HIV^+$ | 18 | 13 | – | 27.8 (5/18) | – |
| $TB^-HIV^-$ | 13 | 3 | – | 76.9 (10/13) | – |
| Total | 61 | 16 | 4 | 48.4 (15/31)[a] | 86.7 (26/30)[a] |

[a] Accuracy=81.8% (the data for the specificity of the $TB^-HIV^+$ group is omitted because of the known underestimation of TB positives by standard culture growth assays).

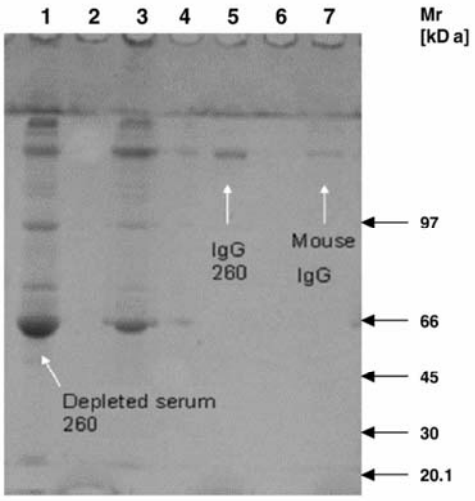


Fig. 5. SDS-PAGE gel indicating the purity of the IgG fraction in relation to a standard mouse IgG sample. The depleted serum fraction in lane 1 still contains IgG along with albumin at 66 kDa and other unidentified proteins. Samples were loaded in non-reducing buffer bovine serum albumin (BSA): (1) depleted serum no. 260, (2) blank, (3) whole serum no. 260, (4) blank, (5) IgG fraction from patient no. 260, (6) blank, (7) mouse IgG.

### 3.2. Isolation of IgG

To assess whether the inhibited signal on the IAsys affinity biosensor was due to the IgG in human sera, isolation of IgG was done using protein A Sepharose beads, which are specific for IgG but not for the other classes of immunoglobulin. The serum was not fully depleted of IgG as is evident from Fig. 5. The IgG fractions were expected to represent a repertoire of antigen binding specificities found in serum. The eluted IgG fractions were tested to bind mycolic acid on the IAsys biosensor.

### 3.3. Analysis of IgG on the IAsys biosensor

The isolated IgG from TB-positive serum no. 260 and TB-negative serum was analysed on the IAsys resonant mirror biosensor to confirm that the binding of

antibody to mycolic acid is due to the IgG. The liposomes containing mycolic acids were immobilized on non-derivatized cuvettes after activation with CPC as described previously. The response of IgG in PBS/AE to mycolic acid liposomes was measured in arc seconds at an IgG concentration of 0.04 mg/ml to detect high affinity binding. When comparing the binding of IgG from the TB-positive serum and IgG from negative serum to mycolic acid liposomes, there was a significant difference (paired Student's *t*-test with alpha=0.05; Table 2).

Inhibition experiments are necessary in the biosensor analyses of sera, as no adequate difference in direct binding in PBS/AE was observed between TB-positive and TB-negative sera to provide improved diagnosis over that obtained by ELISA. This was thought to be due to serum components binding non-specifically to the liposomes. By incubating the serum in mycolic acid liposomes, the highly specific antibodies would be complexed and prevented from binding to the immobilized mycolic acid liposomes on the cuvette surface. We attempted to apply the same principle to the IgG samples. The purified IgG in PBS was mixed with either phosphatidylcholine or mycolic acid liposomes to afford a final concentration of 0.08 mg/ml of IgG in PBS/AE and the response was measured over 10 min as previously done for serum. The degree of binding of IgG to the immobilized mycolic acid liposomes after mycolic acid or phosphatidylcholine incubation was compared to determine whether the IgG could be inhibited.

The inhibition signals of IgG from patient 260 and the IgG negative control were measured on different channels of the IAsys biosensor. A significant difference in the ability of IgG 260 and IgG negative to be inhibited by mycolic acid liposomes was determined with Student's *t*-test using an alpha value of 0.05 (Table 2). These results correlate with previously obtained data using whole serum and confirm that it is the IgG fraction of serum that is inhibited from binding by preincubation with the mycolic acid antigen.

Table 2
Binding of IgG antibody purified from $TB^+$ and $TB^-$ serum to mycolic acid liposomes

| Biosensor signal | Patient (±SEM) | |
|---|---|---|
| | $TB^+$ (no. 260) | $TB^-$ control |
| Serum binding (arc seconds) | 462±35 | 248±24 |
| IgG binding (arc seconds) | 70±10 | 31±1 |
| IgG binding inhibited with mycolic acid (%) | 54±3.4 | 24±6 |

SEM, standard error of the mean; $n>4$.

## 4. Discussion

In Africa, TB is often the first manifestation of HIV infection; it is the leading cause of death among HIV-infected patients. Corbett et al. (2006) stated that every opportunity should be taken to screen HIV-infected patients for active TB in order to prevent rapid death when both diseases manifest themselves in an individual, and to provide safe antiretroviral (ARV) treatment. The shorter the time from sampling to the diagnostic result, the more lives will be saved. Serodiagnosis with mycolic acids as antigen provides such an opportunity (Verschoor and Onyebujoh, 1999).

Pan et al. (1999) indicated that the anti-cord factor antibodies (IgG) in TB patients specifically recognized the mycolic acid structure, especially methoxy mycolic acid methyl esters. Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve major histocompatibility complex (MHC)-class I or MHC-class II molecules (Moody et al., 1999). The anti-mycolic acid immune response could therefore be independent of the participation of $CD8^+$ or $CD4^+$ T cells that respond to antigen presented on MHC I and MHC II surface proteins, respectively. Other than the MHC-presented protein antigens, mycolic acid is presented on CD1, with the ability to induce proliferation of T cell lines, with or without the CD4 or CD8 molecules (Beckman et al., 1994; Goodrum et al., 2001). The production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells. It is known that infection with HIV results in depletion of $CD4^+$ T cells and inhibition of the function of the remaining T cells (Price et al., 2001). Thus, Hendrickson et al. (2000) showed a decreased antibody specificity and sensitivity to a mycobacterial 30-kDa protein antigen with ELISA when screening patients in a population that had a high prevalence of HIV. Ratanasuwan et al. (1997) showed that when a lipoarabinomannan (LAM) was used in serological tests on HIV-negative and TB-positive patients, it showed sensitivities varying from 21% to 89%, but only 7% to 40% in HIV-positive patients. Antunes et al. (2002) described the MycoDot serological assay for tuberculosis, which is based on the detection of specific IgG antibodies against the LAM antigen, fixed onto a solid support consisting of a plastic comb designed to fit into the wells of a microtitre plate. The sensitivity values observed were definitely lower in cases of TB associated with HIV, which refuted the usefulness of the test in regions where HIV is highly endemic. They concluded that LAM as an antigen is only satisfactory in the serodiagnosis of TB as long as HIV is not highly prevalent in the population. Daniel

et al. (1994) performed a field test in Mexico, and showed that an ELISA based on the mycobacterial 30-kDa protein antigen had a sensitivity of 70% in patients with culture-positive or smear-positive pulmonary TB and a specificity of 100% in 125 control donors. The same test was evaluated with HIV-positive and -negative patients in Uganda. Although the sensitivity and specificity in HIV-negative donors were similar to the results of the Mexico test, the ELISA gave a sensitivity of 28% in 128 sera from HIV-positive donors. However, the immune response to mycolic acid could in principle proceed independently of the $CD4^+$/$CD8^+$ T cells. The human CD1 protein is known to mediate T cell responses by presenting at least three classes of mycobacterial lipids, i.e. free mycolates, glycosylated mycolates and diacylglycerol-based glyco-phospholipids. The alkyl chains of the mycolic acid antigen have been proposed to bind directly within the hydrophobic groove of CD1 resulting in presentation of the hydrophilic caps to the T cell's antigen receptor (Porcelli et al., 1996; Moody et al., 1999). The CD1-restricted lipid antigen presentation pathway could provide a possible explanation why the antibody response to mycolic acids is maintained in HIV-seropositive patients, despite a declining CD4 T lymphocyte count (Schleicher et al., 2002). Simonney et al. (2007) also suggested that the CD1-restricted lipid antigen presentation pathway is the likely mechanism accounting for the perseverance of high circulating antibody responses to PGL-Tb1 antigen in HIV-infected patients with TB. Simonney et al. (2007) showed that about half of HIV-positive individuals produce specific anti-glycolipid antibody several months before a diagnosis of TB disease can be made.

Here, a significant increase in sensitivity and specificity was shown for anti-mycolic acid antibody detection in patient serum with the inhibition assay on the biosensor, compared to that reported in our previous study using an ELISA assay (Schleicher et al., 2002). The false positive results observed amongst the $TB^-$ $HIV^+$ population could show that the patients were true positive on the IAsys biosensor, since a sputum culture was used as the gold standard method for confirming their TB status. However, it is known that sputum culture of HIV-infected patients needs more incubation time than that of patients without HIV infection, which is consistent with the lower bacillary load seen in the sputum of HIV-infected patients (Brindle et al., 1993). The culture requires 10–100 viable *M. tuberculosis* per millilitre of sputum to give positive results (Colebunders and Bastian, 2000). It has also been shown that 15–20% of adults with pulmonary TB whose diagnosis was based on clinical, radiographic, and histopathological findings and response to anti-TB treatment have negative sputum cultures (Frieden et al., 2003).

The IAsys affinity biosensor was able to detect low affinity antibody binding to mycolic acids, in addition to high affinity antibody, which the conventional methods cannot generally achieve. In an ELISA, these antibodies would have been washed away before the final step and the patient would have tested false negative. The advantage of the biosensor lies in its real-time detection of antibody binding, without the need for prior washing away of the unbound antibody excess. In addition, the inhibition of binding as an endpoint eliminates much non-specific binding interference, which adds to the increased specificity of the biosensor assay. A disadvantage of the biosensor is that it is blind to the identity of the binding ligand from the serum sample. Here, the binding of IgG to mycolic acids was confirmed by showing that its binding inhibition could be reproduced with purified IgG from the same serum sample.

The few false negative results that still remain with the biosensor analysis are probably due to the inhibition of antibody activity by circulating mycolic acid antigen. Should this be the case, one can envisage that a duplicate test be run that is spiked with a stable source of anti-mycolic acid antibodies, such as monoclonal antibodies. A true negative will then return the spike signal, whereas a false negative will consume the signal. False positive results pose a more daunting technical challenge, but may be due to the cross-reactivity of antibodies to mycolic acids of non-tuberculous pathogenic mycobacteria, e.g. *M. avium*, which do occur at low frequency, especially in HIV-positive patients. More work is required to manage the specificity of the assay by, for instance, screening sera from patients that are TB negative, but test positive for *M. avium* disease. This work is currently underway.

Many serological assays have been developed for detection of specific antibody to lipid cell wall antigens in tuberculosis patients (Lyashchenko et al., 1998; Pan et al., 1999; Pottunarthy et al., 2000; Julian et al., 2002; Schleicher et al., 2002; Lopez-Marin et al., 2003; Fujita et al., 2005), but generally they do not meet the requirements on specificity and sensitivity (Attalah et al., 2005). The biosensor approach may improve that by means of its unique benefits reported here. However, the technique is technically quite difficult to perform in the laboratory and the technology is not yet suitable for large scale screening of patients.

Since only 61 patients were analyzed with the biosensor in this study, more patient and control sera will have to be analyzed to properly validate it as a reliable technique to determine anti-mycolic acid antibodies as surrogate markers for active tuberculosis. However, the

detection of anti-mycolic acid antibodies with the IAsys affinity biosensor appears to be technically feasible, quick and may also be made affordable by further optimization and innovation of the biosensor hardware. Moreover, the biosensor assay may even prove to be more sensitive than the microbiological sputum growth assay, as suggested here with the serum samples from $HIV^+$ patients that tested positive with the biosensor, but negative with the sputum assay.

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# Appendix B: Mycolic acids and cholesterol

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## Cholesteroid nature of free mycolic acids from *M. tuberculosis*

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**Abstract**

Mycolic acids (MAs) are a major component of the cell walls of *Mycobacterium tuberculosis* and related organisms. These α-alkyl β-hydroxy long fatty acids have been the subject of numerous studies for their immunological properties. We previously reported that an interaction between cholesterol and mycolic acids could be responsible for the low accuracy in the serodiagnosis of TB when using free mycolic acid in an ELISA assay. The aim of this work was to investigate if this interaction could be due to a similarity in the structural properties between mycolic acids and cholesterol. The investigation revealed that patient sera cross-reacted with mycolic acids and cholesterol in an ELISA experiment suggesting that both molecules may present related functionality in a similar structural orientation. This relation was further supported by the interaction of mycolic acids with Amphotericin B (AmB), a known binding agent to ergosterol and cholesterol. Using a resonant mirror biosensor, we observed that AmB recognised both cholesterol and mycolic acids. In addition, a specific attraction was observed between mycolic acid and cholesterol by the accumulation of cholesterol from liposomes in suspension onto immobilized mycolic acids containing liposomes, detected with a biosensor technique. Combined, these results suggest that mycolic acids can assume a three-dimensional conformation similar to a sterol. This requires that mycolic acid exposes its hydroxyl group and assumes rigidity in its chain structure to generate a hydrophobic surface topology matching that of cholesterol. A particular folded conformation would be required for this, of which a few different types have already been proven to exist in monolayers of mycolic acids.


*Keywords:* Mycolic acids; Cholesterol; Lipid conformation; Amphotericin B; Resonant mirror biosensor

### 1. Introduction

Tuberculosis is one the most life-threatening infectious diseases. Its re-emergence has brought new interest in finding novel methods for its treatment and diagnosis, especially in developing countries where the population is most burdened by this pandemic and where the connection between TB and HIV/AIDS infection is particularly worrying. In fact, TB is the major cause of death in HIV/AIDS co-infected individuals (Uma Devi et al., 2003).

In the search for new surrogate markers for the diagnosis of tuberculosis, antibodies to cord factor (trehalose-6,6′-dimycolate, TDM) have attracted much attention. Although having a hydrophobic nature, TDM is a very immunogenic and biologically active substance present in the mycobacteria and a few related genera. TDM is a glycolipid consisting of trehalose, which is identical for all cord factors, and two of a set of mycolic acids (MAs) (**1**, Fig. 1), which differ within and among species and genera (Ryll et al., 2001). For example, *Mycobac-*

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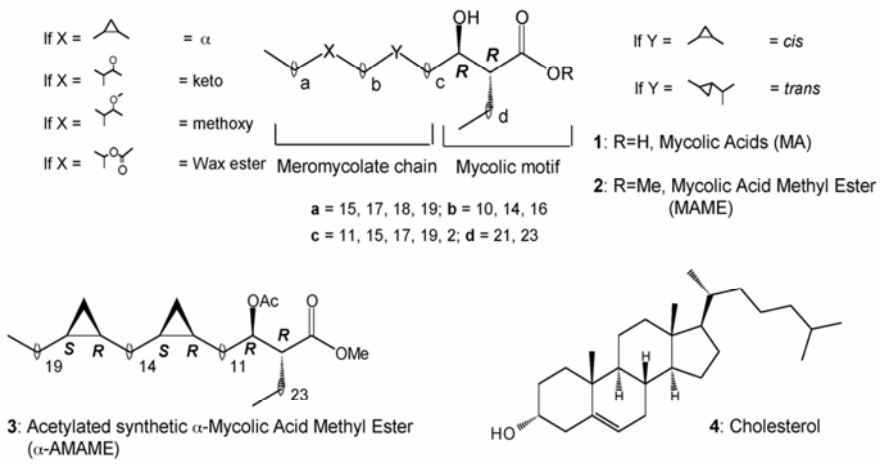



Fig. 1. Molecules under investigation for their similarity in function.

*terium tuberculosis* contains α-, methoxy- and keto-mycolic acids subclasses, whereas *Mycobacterium avium* contains wax ester—instead of methoxymycolic acids (Dobson et al., 1985). Fujiwara et al. (1999) identified the antigenic epitope in cord factor molecules to be the mycolic acids by proving that antibodies against TDM from TB patients could distinguish between mycolic acids subclasses.

We have reported the use of free mycolic acids as antigens for the serodiagnosis of tuberculosis; they have considerable potential because HIV–TB co-infected patients maintain high antibody levels to mycolic acids, despite the severity of the immune deficiency measured by the fall in CD4 T cell count (Schleicher et al., 2002). However, in an ELISA assay, the use of free mycolic acids was not adequate for serodiagnosis of tuberculosis (accuracy = 57%). An association between mycolic acids and cholesterol was hypothesised as a possible reason for this low accuracy (Schleicher et al., 2002). Cholesterol may be non-specifically attracted to mycolic acids by means of hydrophobic Van der Waals type of binding, or by a more specific interaction such as a hydrogen bond, arising from conformational features present in the two molecules.

Recently, free mycolic acids have been demonstrated to be able to adopt a folded conformation to give a hydrophobic surface. In particular, the "W" conformation has the alkyl chains folded to give four parallel arms, while in the "Z" conformation three folded arms provide the three-dimensional, curved hydrophobic surface (Villeneuve et al., 2007; Sekanka et al., 2007). The existence of these conformations has been suggested by analyses of Langmuir monolayers consisting of free mycolic acids over a range of temperatures (Villeneuve et al., 2005, 2007). In an extended form, the shape and structure of a mycolic acid appears very different to that of cholesterol and does not suggest particularly strong interactions between these molecules. However, the folded conformations of mycolic acids could be imagined to assume a shape similar to that of cholesterol.

The current study was designed to investigate the possibility that the low accuracy measured for a serodiagnostic ELISA assay based on the use of mycolic acid as antigen could be the result of the folding of free mycolic acids to resemble a "cholesteroid" shape. In order to investigate this, we have used an approach similar to that employed by Prendergast et al. (1998) to suggest molecular mimicry between microbial and self-structures in autoimmune diseases. The basic assumption of this approach is that the specific molecular recognition of two substances by an established binding agent indicates resemblance in the three-dimensional structure of the two compounds. Therefore we further investigated the interaction of mycolic acids and cholesterol with sera from human TB patients, using the ELISA technique described by Schleicher et al. (2002). Secondly, using a resonant mirror biosensor (Cush et al., 1993; Buckle et al., 1993; Athanassopoulou et al., 1999), we compared the interaction of these two lipids with Amphotericin B (AmB), a known cholesterol binding molecule (Baginski et al., 2002, 2005). Thirdly, the attraction between cholesterol and mycolic acids was studied, also using the resonant mirror biosensor.

## 2. Materials and methods

### 2.1. General procedures

$^{1}H$, $^{13}C$ and COSY nuclear magnetic resonance (NMR) were recorded on a Bruker advance DRX-500 spectrometer or Bruker AC-300 spectrometer with chemical shifts in ppm. Analytical thin layer chromatography (TLC) was performed on Alugram SIL G/UV, layer: 0.2 mm silica gel 60 with fluorescent indicator $UV_{254}$ plates. The plates were viewed under ultraviolet light (254 and 366 nm). Flash column chromatography was performed using Merck silica gel. FT-IR was obtained with a

PerkinElmer BX-1 instrument of 2.00 $cm^{-1}$ resolution fitted with a MIR source, an internal LiT $aO_3$ detector and FT-IR Spectrum Software version 5.1. Optical rotations were measured on a PerkinElmer model 341 polarimeter. For mycolic acids analyses HPLC was performed using a Merck Hitachi Chromatograph fitted with a Phenomenex Luna 5 μm C18 column and a Merck Hitachi L-4500 Diode Array detector. For AmB derivative purification a Waters high performance liquid chromatography (HPLC) system was used with a Waters 610 fluid unit, Waters 600 controller and a Waters 996 Photodiode Array detector. A Waters C18 reverse phase column (150 mm × 4.6 mm) was used for optimization of methods and a Phenomenex Luna C-18 reverse phase column (250 mm × 10 mm, 10 μm) was used to collect the product. Electrospray mass spectrometry performed on a Micromass Micro Triple quadrupole mass spectrometer in full scan mode, range 500–2000 amu, was used to characterise the product. Sample infusion was directly into the electron spray source by means of a Hamilton syringe pump at 10 μl/min.

### 2.2. Preparation of methyl mycolic acids isolated from M. tuberculosis (H37Rv)

A mixture of mycolic acids (**1**) was isolated from the cell wall of the virulent Erdman strain of *M. tuberculosis* as described by Goodrum et al. (2001). HPLC and NMR spectra were in agreement with the data reported in literatures (Steck et al., 1978; Watanabe et al., 2001).

To form the methyl esters, mycolic acids (**1**, 100 mg, ~0.1 mmol) were dissolved in a mixture of toluene:methanol (5:1, 18 ml), trimethylsilyldiazomethane (TDM, 2 M solution, 0.2 ml, 0.4 mmol) added, followed by a further 4 additions of TDM (0.1 ml, 0.2 mmol) every 45 min. The mixture was stirred for 72 h, and then quenched by evaporation. The residue was dissolved in dichloromethane (15 ml) and water (10 ml) was added. The two layers were separated and the water layer extracted with dichloromethane (2× 10 ml). The combined organic layers were dried and the solvent evaporated to give the desired compound (98 mg, ~97%). The NMR spectra of the compounds obtained corresponded to those reported in the literatures for methyl esters of mycolic acids (MAME, **2**) (Watanabe et al., 2001; Al Dulayymi et al., 2003, 2007; Koza and Baird, 2007).

The acetylated synthetic alpha-mycolic acid methyl ester (α-AMAME, **3**) was kindly provided by Dr. J. Al Dulayymi, University of Bangor, UK (Al Dulayymi et al., 2003, 2005). Cholesterol (**4**), isoniazid, AmB, $NaBH_4$, terephthalaldehyde and phosphatidylcholine were purchased from Fluka or Sigma–Aldrich. All organic solvents were purchased from Merck while the inorganic materials were from Sigma.

### 2.3. Synthesis of Amphotericin B derivative

#### 2.3.1. Preparation of N′-(4-formylbenzylidene)isonicotinohydrazide (6)

Isoniazid (**5**, 0.5 g, 3.65 mmol) was added in portions over an hour to a solution of terephthalaldehyde (0.49 g, 3.65 mmol) in ethanol (50 ml) and stirred at room temperature (RT) overnight. The precipitate that formed was removed by filtration and the mother liquor was concentrated to give a crude product (0.15 g) that was purified by flash column chromatography on silica gel in ethyl acetate:methanol:ethanol containing 1% triethylamine (90:9:1, v/v) to give pure *N′*-(4-formylbenzylidene) isonicotinohydrazide (**6**, 0.12 g, 14% yield).

Molecular weight 253.2599 g $mol^{-1}$. $R_f$ 0.57 (ethyl acetate:methanol:ethanol with 1% $Et_3N$ (v/v) (90:9:1); mp 219–220 °C; IR $\nu_{max}$ 3465, 3197 (NH), 1698 (CHO) $cm^{-1}$. $^1H$ NMR (300 MHz, $(CD_3)_2$ SO) $\delta$ 12.25 (1H, s, NH), 10.06 (1H, s, 1a), 8.80 (2H, d, $J$ = 5.4 Hz, 10a), 8.55 (1H, s, 6a), 8.01 (2H, d, $J$ = 9 Hz, 3a), 7.97 (2H, d, $J$ = 9 Hz, 4a), 7.84 (2H, d, $J$ = 5.4 Hz, 9a); $^{13}C$ NMR (300 MHz, $(CD_3)_2$ SO) $\delta$ 193.1, 162.3, 150.7, 148, 140.7, 139.9, 137.4, 130.3, 128.1, 121.9 ppm; HR–MS (EI) calculated for $C_{14}H_{10}O_2N_3$ [$M^+$] 253.08513 g $mol^{-1}$ found 253.08389 g $mol^{-1}$, *m/z* (EI) 122 (74), 106 (100), 79 (10), 78 (47), 51 (22).

#### 2.3.2. Preparation of N-(4-((2-isonicotinoylhydrazono)methyl)benzyl)-AmB (7, AmB derivative)

In the next step, the aldehyde (**6**, 0.01 g, 0.04 mmol) and Amphotericin B (0.043 g, 0.05 mmol) were stirred at RT in 2 ml dimethylsulfoxide in the dark for an hour. The derivative that formed was reduced *in situ* with $NaBH_4$ (0.001 g, 0.04 mmol) overnight and purified by RP-HPLC (methanol:$H_2O$ a gradient from 20% to 100% methanol). The column eluent was monitored at 300 and 407 nm and AmB derivative collected and concentrated.

Molecular weight 1159.35 g $mol^{-1}$ (calculated), melting point not determined due to decomposition of compound at about 40 °C, $[\alpha]_D^{20}$ (*c* 2.0, DMF), IR $\nu_{max}$ 3391, (NH, OH's), 1564 (double bonds) $cm^{-1}$. $^1H$ NMR (500 MHz, $(CD_3)_2$ SO) $\delta$ 12.06 (1H, s, H–NH), 8.79 (2H, d, $J$ = 3.3 Hz, H-10a), 8.46 (1H, s, H-6a), 7.83 (2H, d, $J$ = 4.4 Hz, H-6a), 7.51 (2H, d, $J$ = 7.9 Hz, H-4a), 7.7 (2H, d, $J$ = 7.9 Hz, H-3a), 6.45–6.07 (m, 12H, olefinic), 5.95 (1H, dd, $J$ = 13, 5.3 Hz, H-20), 5.43 (1H, dd, $J$ = 11.3, 12 Hz, H-33), 5.21 (1H, m, H-37), 4.48 (1H, broad-s, H-1′), 4.4 (1H, m, H-19), 4.24 (1H, m, H-11), 4.23 (1H, t, H-17), 4.06 (1H, m, H-3), 3.99 (1H, dt, H-15), 3.89 and 3.67 (2H, s, H-1a), 3.73 (1H, d, H-2′), 3.54–3.46 (HOD plus 3H), 3.2 (1H, m, H-5′), 3.09 (2H, m, H-4′, H-35), 2.82 (1H, m, H-3′), 2.28 (1H, s, H-34), 2.16 (3H, m, H-2, H-18), 1.88–1.05 (14H, m, $CH_2$, CH), 1.87 (1H, t, $J$ = 6.7 Hz, H-16), 1.16 (3H, d, $J$ = 5.5 Hz, $CH_3$), 1.11 (3H, d, $J$ = 5.6 Hz, $CH_3$), 1.04 (3H, d, $J$ = 5.6 Hz, $CH_3$), 0.91 (3H, d, $J$ = 6.5 Hz, $CH_3$), $^{13}C$ NMR (300 MHz, $(CD_3)_2$ SO) $\delta$ 174.4, 170.6, 161.54, 150.3, 149.1, 143.6, 140.51, 133.9, 136.8, 133.7–128.5, 131.9, 127.2, 121.5, 97.2, 77.1, 73.8, 74.5, 74.4, 69.8, 73.6, 69.4, 68.8, 66.2, 65.4, 65.2, 63, 57.1, 44.7, 49.5–44.3, 42.3, 42, 35.1, 29, 18.5, 12.1 ppm.

### 2.4. ELISA

The human sera used in the ELISA experiments were from two sources: the first was from a pulmonary TB positive collection that was made in 1994 by the MRC Clinical and Biomedical TB Research Unit at King George V Hospital, Dur-

ban, KwaZulu-Natal and donated by Dr. P.B. Fourie. The second source was from a collection made in the year 2000 from patients for another study by Schleicher et al. (2002). From the latter, five serum samples were selected from patients who were determined to be negative for both tuberculosis and HIV ($TB^-$). These TB negative patients were hospitalised for various reasons other than TB or AIDS.

Mycolic acids (MA, **1**), acetylated synthetic α-mycolic acid methyl ester (α-AMAME, **3**) and cholesterol were used at final concentrations of 60 μg/ml. To prepare the coating solutions, the antigens were heated in PBS buffer for 20 min at 85 °C. The hot solutions were sonicated and kept at 85 °C during loading into the ELISA plates at 3 μg antigen per well. The assay with sera was done according to Schleicher et al. (2002). The results obtained were analysed for statistical differences using the Student's *t*-test.

### 2.5. Preparation of liposomes

Empty liposomes were prepared consisting of only phosphatidyl choline (PC), whereas the other liposomes consisted of PC in some ratio to the lipid under investigation. Mycolic acids (MA, **1**) liposomes and other chemically prepared mycolic acid liposomes contained MA or MAME (**2**) or α-AMAME (**3**) (7.8 mol%; mycolic acid: PC = 1 mg: 9 mg), whereas the cholesterol (Chol, **4**) liposomes contained Chol (50 mol%, Chol:PC = 3 mg:6 mg). The appropriate lipids were initially dissolved in chloroform in an amber glass vial and vortexed to ensure mixing. The samples were then dried at 80 °C under a stream of $N_2$ and sonicated in 2 ml saline for 5 min at room temperature. Sonication was done using a Branson sonifier (model B30, Branson Sonicpower Co. USA) with 30% duty cycles and output of 5. Subsequently the liposomes were divided into 200 μl aliquots, freeze-dried and stored at −70 °C until required for use. Before use, the liposomes were reconstituted with 2 ml of phosphate buffered saline (PBS/AE) pH 7.4 containing EDTA (1 mM), sodium azide (0.025%, m/v), heated at 80 °C for 15 min and then sonified as above. The final liposome concentration came to 500 μg/ml. The liposome suspensions were analysed for their mycolic acid content by HPLC according to Goodrum et al. (2001). Recovery of mycolic acids ranged between 70% and 110% of the expected values.

### 2.6. Measurements of interaction between mycolic acids, cholesterol and Amphotericin B derivatives on an IAsys affinity biosensor

The binding interactions among Amphotericin B, cholesterol, mycolic acids and acetylated synthetic α-mycolic acid methyl ester were measured by means of an IAsys resonant mirror biosensor (IAsys Affinity Sensors, Bar Hill, Cambridge, UK) according to the activity sequence outlined in Fig. 2. IAsys software was used to set the device at a data-sampling interval of 0.4 s, temperature of 25 °C and stirring rate of 75% for all experiments on the biosensor. The cells of the cuvette were rinsed with ethanol (95%) 3 times prior to use, followed by extensive washing with PBS/AE. A 60-μl volume of PBS/AE was

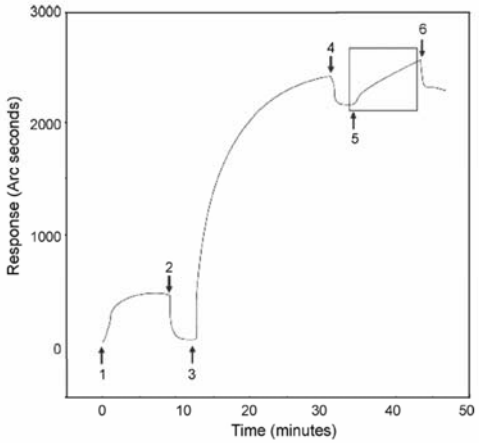
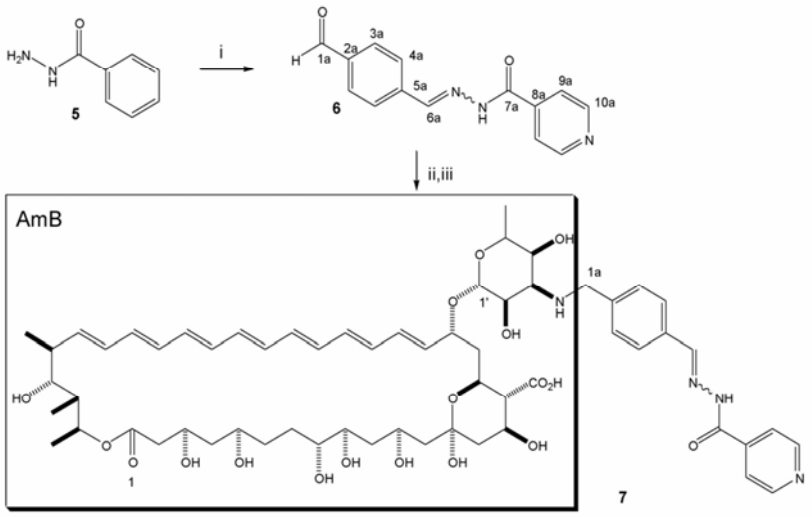



Fig. 2. Biosensor activity sequence to obtain sensorgrams of the binding of cholesterol from cholesterol-containing liposomes onto various immobilized lipid-containing liposomes. 1: activation of surface with CPC, 2: PBS/AE wash step, 3: addition of liposomes for coating, 4: PBS/AE wash step, 5: addition of the test solution, 6: PBS/AE wash step. The cholesterol binding sensorgram appears in the rectangle.

pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 μl of cetyl-pyridinium chloride (CPC, 0.02 mg/ml in PBS/AE) for 10 min. This was followed by washing 5 times with 60 μl PBS/AE and then substituting with 25 μl PBS/AE for a new baseline before immobilization of mycolic acids (or cholesterol, or a synthetic α-AMAME, or PC only) containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed 5 times with 60 μl PBS/AE. The cuvette cell content was substituted with 25 μl of PBS/AE and allowed to equilibrate for 5–10 min to achieve a stable baseline. An addition of either 25 μl liposomes under investigation or 25 μl of a solution of AmB ($1 \times 10^{-4}$ M) or the AmB derivative (**7**, Scheme 1, $1 \times 10^{-4}$ M) in PBS/AE was made. Direct interaction between the immobilized cholesterol/mycolic acids and the dissolved Amphotericin B compounds or lipids contained in the suspended liposomes was monitored for 5–10 min, after which the cuvette was washed 3 times with 60 μl PBS/AE. Finally, regeneration of the cuvette was effected by 5 times washing with 50 μl 95% ethanol for 1 min, followed by 7 times washing with 70 μl PBS/AE for 1 min. The surface was then finally treated for 5 times with 50 μl potassium hydroxide (12.5 M) for 2 min, followed by 7 times washing with 70 μl PBS/AE for 1 min. The results obtained were analysed for statistical differences using the Student's *t*-test.

### 2.7. Measurements of direct interaction between mycolic acids and cholesterol

After immobilization of either the mixture of natural mycolic acids (**1**), their methyl esters (**2**), or protected acetylated synthetic α-mycolic acid methyl ester (**3**) containing liposomes using the same method as described above and illustrated in




Scheme 1. Preparation of an AmB derivative: (i) terephthalaldehyde, (ii) AmB and (iii) $NaBH_4$.

Fig. 2, 25 μl cholesterol-containing liposomes were added. Direct interaction between the immobilized mycolic acids and cholesterol was monitored for 10 min, after which the cuvette was washed 3 times with 60 μl PBS/AE and regenerated as before.

## 3. Results and discussion

### *3.1. Interactions of patient sera to mycolic acids and cholesterol*

It is known that all humans have anti-cholesterol antibodies (ACHA), which have been proven to be very specific for their interaction with the sterol. ACHA recognise selectively 3β-hydroxy-sterols in a stereospecific manner, but they cannot distinguish between enantiomers (Geva et al., 2001; Bíró et al., 2007). Concurrently, tuberculosis patients have been shown to produce antibodies against mycolic acids (Schleicher et al., 2002). Pan et al. (1999) reported that even small changes in the structure of the mycolic acids present in TDM are important for their antigenicity. In particular, among the three subclasses present in *M. tuberculosis*, methoxymycolic acids were shown to be the most antigenic. For this reason, we expected TB negative patient sera to react to cholesterol, but not to mycolic acids.

TB positive and TB negative sera were tested in ELISA for antibody binding to natural mycolic acid and cholesterol. To verify the specificity of the interaction between sera and mycolic acids, a synthetic α-mycolic acid (**3**), with the carboxylic acid protected as methyl ester and the hydroxyl group as acetyl ester, was used as negative control. This compound was chosen because it lacks a hydrogen donor at either the α-carboxylic acid, the β-hydroxyl group or in the meromycolate chain, which diminishes the possibility of hydrogen bonds with antibodies without alleviating the hydrophobic nature of the molecule that can be expected to be the major force of non-specific binding from serum components. Moreover, the protection of the polar groups in the mycolic motif also discourages any folded arrangement with the merochain (Villeneuve et al., 2005, 2007). Therefore, this change in the three-dimensional structure may be hypothesised to preclude specific interactions with antibodies against mycolic acids or cholesterol.

Coating of the wells with the lipid antigens was confirmed under the microscope as visible fatty deposits adsorbed on the polystyrene. Eleven TB positive patient sera were randomly chosen from the 1994 collection and five TB and HIV negative patients were randomly selected from the 2000 collection. For the TB negative controls, it was deemed important to exclude HIV positive individuals, for whom false negative TB diagnosis is known to occur at high frequency with the currently available TB diagnostics (Uma Devi et al., 2003). The sera were diluted 1:20, which was found to be the highest dilution where significant binding to the antigens on the plate could still be observed with most sera.

As expected there was a tendency for TB positive patient antibodies to bind more strongly to mycolic acid than those of the TB negative patients (average absorbance value 0.76 compared to 0.37 respectively, Fig. 3, $P<0.1$). The selectivity of binding with MAs was confirmed by the negligible antibody activity of the same patients to the synthetic alpha-mycolic acid in both groups of sera (Fig. 3, $P<0.0001$). Both TB positive and TB negative sera also recognised cholesterol as an antigen. In either TB positive or TB negative patients, the difference in ELISA signal between mycolic acid and cholesterol was not significant (Fig. 3, $P>0.25$). Although TB negative patients should not present antibodies against *M. tuberculosis* mycolic acids, the results obtained show that their sera recognise in a similar fash-

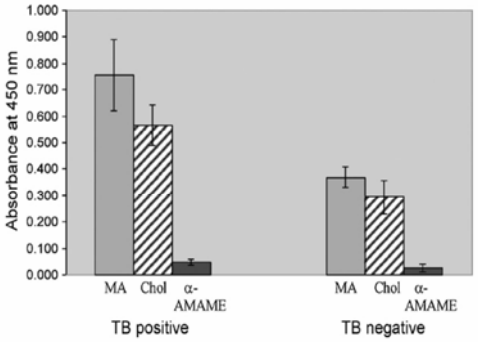



Fig. 3. Normalised ELISA results of TB positive ($n = 11$) and TB negative sera ($n = 5$) against natural mycolic acids (1) (MA, grey), cholesterol (4) (Chol, hatched) and an acetylated, synthetic α-mycolic acid methyl ester (3) (α-AMAME, black). The error bars indicate the standard error of the mean.

ion either cholesterol or mycolic acids. Such congruence of the titres of both antibody specificities could suggest cross-reactivity of recognition of cholesterol and mycolic acid by antibodies.

### 3.2. AmB cross-reactivity to mycolic acids and cholesterol

To further probe the possibility of resemblance in the nature of mycolic acid and cholesterol to bind to a ligand, an alternative experiment was designed using Amphotericin B (AmB) as a cholesterol binding agent. AmB is a macrolide antibiotic that exerts its antifungal activity by binding to ergosterol and cholesterol (Baginski et al., 2002). The major interaction between the macrolide and the sterol is based on the formation of a hydrogen bond between the amine or hydroxyl groups of the mycosamine moiety of AmB and the hydroxyl group of ergosterol (Herve et al., 1989; Matsumori et al., 2005). Other sterols, in particular cholesterol, are also recognised and bound by AmB, albeit with one order of magnitude weaker affinity. This is due to the more rigid hydrophobic sterol surface of ergosterol (which has two extra double bonds and a methyl group compared to that of cholesterol), making the Van der Waals interaction with the hydrophobic part of AmB stronger (Readio and Bittman, 1982; Baginski et al., 2002). The difference in binding affinity of AmB to ergosterol and cholesterol proves the specificity of the interaction between AmB and its sterol ligands. According to the approach of Prendergast et al. (1998), the specific molecular recognition of two substances by an established binding agent of one of them indicates a resemblance in the three-dimensional structure of the two compounds. For this reason, AmB was selected to assess its binding activity to cholesterol and to mycolic acid using resonant mirror biosensor technology. This technique was preferred to ELISA because it does not need a fluorescent tag on the binding agent that may affect the structure and interaction properties of AmB (Cush et al., 1993). Moreover using this technique it is possible to use liposomes which better mimic the biological environment within which these lipids are presented (MacKenzie et al., 1997).

In order to immobilize the liposomes onto the surface of the biosensor, a new procedure was used. This is based on the activation of the cuvette cell surface with cetyl-pyridinium chloride, a

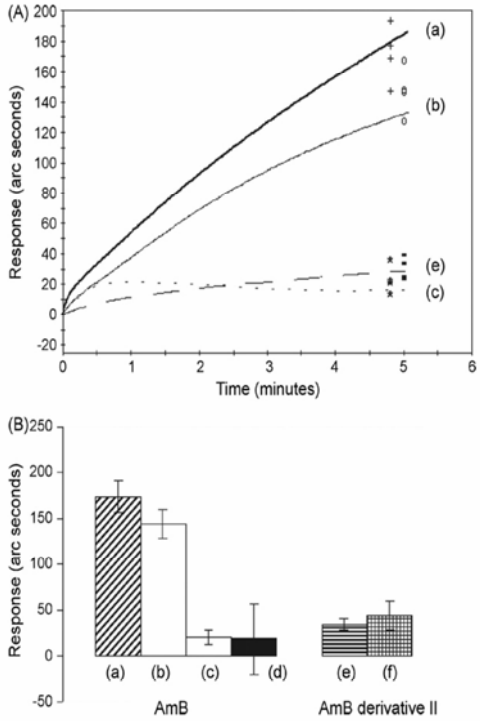



Fig. 4. Resonant mirror biosensor binding curves (A) and binding capacity (B) of AmB or AmB derivative (7) on immobilized lipid antigens. Binding of AmB on (a) mycolic acids (1) (thick line, +, hatched bar); (b) cholesterol (thin line, o, white bar); (c) synthetic protected α-MA (3) (dotted line, *, grey bar) or (d) PC liposomes (black bar). Binding of AmB derivative (7) on immobilized (e) cholesterol liposomes (dashed line, -, horizontal stripes bar) or (f) mycolic acid (1) containing liposomes (grid bar); $n = 5$ for each set. Immobilization of lipid ligate containing liposomes was monitored to achieve binding of at least 2000 arc seconds before contacting with AmB or AmB derivative (7) solutions.

frequently employed cationic, amphipathic compound that gives glazed and metal surfaces a hydrophobic character. Hydrophobic lipid antigen-containing liposomes can then adhere to the surface (see Fig. 2). Following the coating of the cuvette cells with either cholesterol-containing or empty liposomes, consisting only of phosphatidylcholine, the biosensor registered a direct accumulation of AmB on cholesterol liposomes, while it did not show any binding to empty liposomes (Fig. 4). Remarkably, a binding sensorgram comparable to that of immobilized cholesterol was obtained when AmB was allowed to interact with immobilized mycolic acids.

The binding between the AmB macrolide and cholesterol could be abrogated by covalent modification of the single amino group on the AmB macrolide by linking it to isoniazid to give Amphotericin B-derivative (**7**). The mycolic acid binding was similarly abrogated by the covalent modification of the AmB macrolide amino group. This modification to the amino group of the AmB molecule destroyed the latter's ability to bind to both cholesterol and the mycolic acid, showing that the attraction of AmB for both these lipids is determined by the fine structure of the ligand–receptor pair and possibly that this requires a specific hydrogen bond between the binding partners. Such an analysis would be in agreement with the literature, confirming the impor-

tance of the hydrogen bond for a stable interaction (Baginski et al., 2002). Matsumori et al. (2005) demonstrated that AmB's binding to sterols could be hindered when its amino-sugar moiety was forced in a conformation that prevented hydrogen bonding with its ligate. This suggests that the interaction between AmB and mycolic acids could also be based on the formation of a hydrogen bond with the amino-sugar moiety of AmB. It is noteworthy that AmB did not bind to the synthetic protected alpha-mycolic acid (**3**) immobilized on the sensor surface (Fig. 4), thus supporting the specificity of this interaction in terms of the requirement for both Van der Waal's and hydrogen bonding in the recognition event. These results imply that mycolic acid and cholesterol share structural features that are similarly recognised by AmB and that may provide the basis for the cross-reactivity observed with the TB patient antibodies.

### 3.3. Interaction between mycolic acids and cholesterol

In order to determine whether the presumed structural relatedness between mycolic acid and cholesterol would also lead to their interaction directly, the interaction between mycolic acids and cholesterol liposomes was analysed using the biosensor technique.

Following coating of the cuvette surface with either mycolic acid (**1**), mycolic acid methyl ester (**2**) or synthetic acetylated α-mycolic acid methyl ester (**3**), the cuvettes were exposed to cholesterol-containing test liposomes and the sensorgrams for the binding of cholesterol onto the different surfaces were recorded (Fig. 5). A mixture of methyl esters of natural mycolic acids (MAME), still containing hydrogen binding groups (β-hydroxyl and the oxygenated groups in the meromycolate chain), was also used as a negative control for this test. The experiments described earlier compared the interactions of mycolic acids and cholesterol with binding agents known to be able to differentiate between small conformational changes. The present experiment measured the attraction between two lipids and therefore it was important to verify that this is not just due to Van der Waals interactions between two hydrophobic molecules but also to a more specific conformational match between cholesterol and

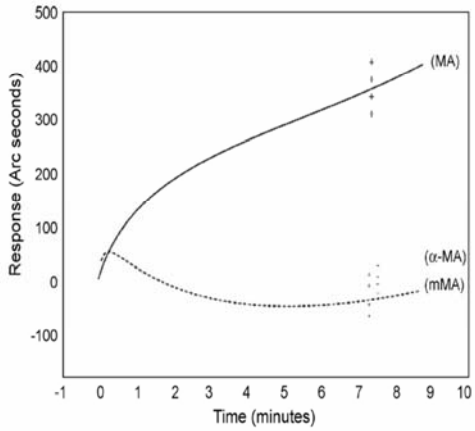



Fig. 5. Resonant mirror biosensor binding curves for the attraction of cholesterol to immobilized mycolic acids (1) (MA, thick line/+), their methyl esters (2) (mMA, dashed line/*) or synthetic protected alpha-mycolic acid (3) (α-MA, thin line/-). Each line represents a typical curve of five repeats with the end points of each indicated after 7.5 min of contact.

mycolic acid. The formation of a hydrogen bond between the carboxylic and the β-hydroxyl of mycolic acid is particularly favoured for the natural erythro configuration of 2*R*,3*R* 2-alkyl-3-hydroxy acids and has been shown to have a stabilising effect on the alignment of the alkyl chains, affecting the physical properties of these acids (Durand et al., 1979a,b). Therefore, the use of methyl esters of mycolic acids as a negative control is particularly important, as this modification will destroy the specific hydrogen bond in the mycolic motif that is expected to destabilise the natural conformations of the alkyl chains to assume a sterol fold, similar to that assumed by the steroid-precursor, the linear 2,3-oxidosqualene, before its conversion into lanosterol in cholesterol biosynthesis (Van Tamelen, 1982).

Fig. 5 shows that the mycolic acid liposomes-coated cuvette surface accumulated cholesterol from the solution while the methyl esters of mycolic acid (**2**) and the synthetic, acetylated alpha-mycolic acid methyl ester (**3**) were unable to do so (no significant difference between binding of cholesterol to (**2**) and (**3**), $P > 0.1$, but highly significant difference of cholesterol bind-

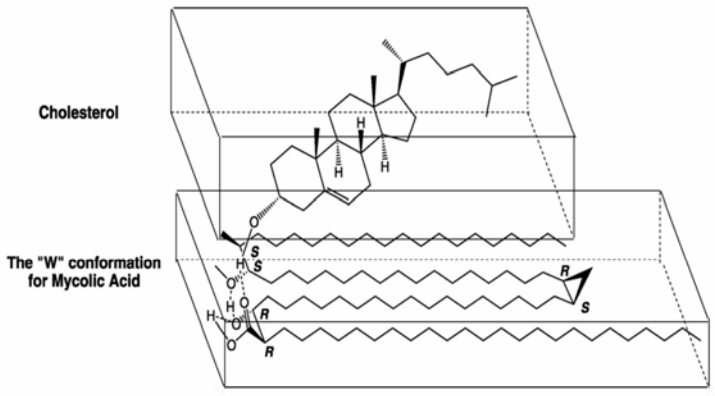



Fig. 6. A pictorial representation of a hypothesised mechanism of interaction between a "W"-folded methoxy MA (Villeneuve et al., 2005, 2007) and cholesterol. The absolute stereochemistry of the cyclopropane and the methoxy group are not completely clarified. Here for simplicity the S,R stereochemistry has been used for the cyclopropyl group and the SS for the methoxy-group. RR is known to be the absolute configuration of the mycolic motif. The "W" fold is drawn here in a flat plane, whereas in practice the fold shown in earlier models had a cylindrical three-dimensional structure (Villeneuve et al., 2005, 2007).

ing to mycolic acid or either the methyl esters of mycolic acid (**2**) and the synthetic, acetylated alpha-mycolic acid methyl ester (**3**), $P < 0.001$). These results demonstrate a pronounced attraction between cholesterol and mycolic acid that is determined by the presence of a free carboxylic acid group and the degree of structural fit by a particular conformation that free mycolic acids assume, which can be eliminated by formation of a simple methyl ester of the acid. In Fig. 6 a graphical representation is attempted of a mycolic acid, folded to assume a cholesteroid nature. A more accurate representation can be expected from virtual models based on Langmuir–Blodgett analysis of synthetic subclasses of mycolic acids interacting with cholesterol. The current advances in the stereo-controlled chemical synthesis of mycolic acids may make this possible in the near future.

## 4. Conclusion

The following observations support the hypothesis of a similarity in nature between the mycolic acids of *M. tuberculosis* and cholesterol that can affect the accuracy of serodiagnostic assays based on antibodies to mycolic acids as surrogate markers of active TB: (i) both mycolic acid and cholesterol are recognised by AmB and human TB sera, (ii) both human TB sera and AmB are able to distinguish between natural occurring mycolic acid (**1**) and a closely related chemically synthetic structure, i.e. acetylated alpha-mycolic acid methyl ester (**3**), (iii) TB negative patient sera containing antibodies to cholesterol (Bíró et al., 2007) bind to mycolic acid comparatively well, but not to the acetylated alpha-mycolic acid methyl ester (**3**) and (iv) the association of AmB to both cholesterol and mycolic acid could be disrupted by covalent modification of the AmB that prevented the formation of hydrogen bonds but not Van der Waals association. Likewise, modification of mycolic acid, aiming to disfavour its arranging into the "W" or any other folded conformation and preclude hydrogen bonding with ligate, abrogated its interaction with patient sera, AmB and cholesterol. The specific attraction between mycolic acid and cholesterol in liposomes probably depends on a particular conformation that free MAs assume in the phospholipid bilayer of liposomes, and this will probably also apply to biological membranes. The existence of a condensed conformation for MAs has already been proposed by several groups, based on Langmuir studies. Here we present the first evidence that these folded conformations could possibly exist in biological conditions to determine the antigenicity of mycolic acids.

The implications of such a discovery and of the relatedness between cholesterol and mycolic acids may be significant for several reasons.

First it could confirm the explanation of the poor results obtained with ELISA testing the usefulness of antibodies to mycolic acids as surrogate markers for tuberculosis (Schleicher et al., 2002).

Second, it impacts on the observation that mycolic acids, intraperitoneally or intratracheally administered as liposomes in mice, resulted in a pathogen-associated type of innate immune response that mainly affects macrophages, converting them into cholesterol-rich foam cells (Korf et al., 2005). Moreover, when administered intratracheally, mycolic acid could also prevent experimentally induced asthma in mice (Korf et al., 2006). The ability of mycolic acid to attract cholesterol to the macrophage may be critical for these phenomena.

Third, cholesterol has been shown to play a role in the entry and survival of *M. tuberculosis* in the host macrophage (Gatfield and Pieters, 2000; de Chastellier and Thilo, 2006). The cholesteroid nature of at least some mycolic acids may imply their active participation in this manifestation of virulence.

The different subclasses of mycolic acids from *M. tuberculosis* are currently under investigation to learn if the cholesteroid nature of mycolic acid is due only to the mycolic motif or to the other functional groups in the meromycolate chain. Finally, an improved understanding of the nature of the mycolate coat of *M. tuberculosis* could lead to the development of improved diagnostics and treatments of tuberculosis.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemphyslip.2008.01.004.

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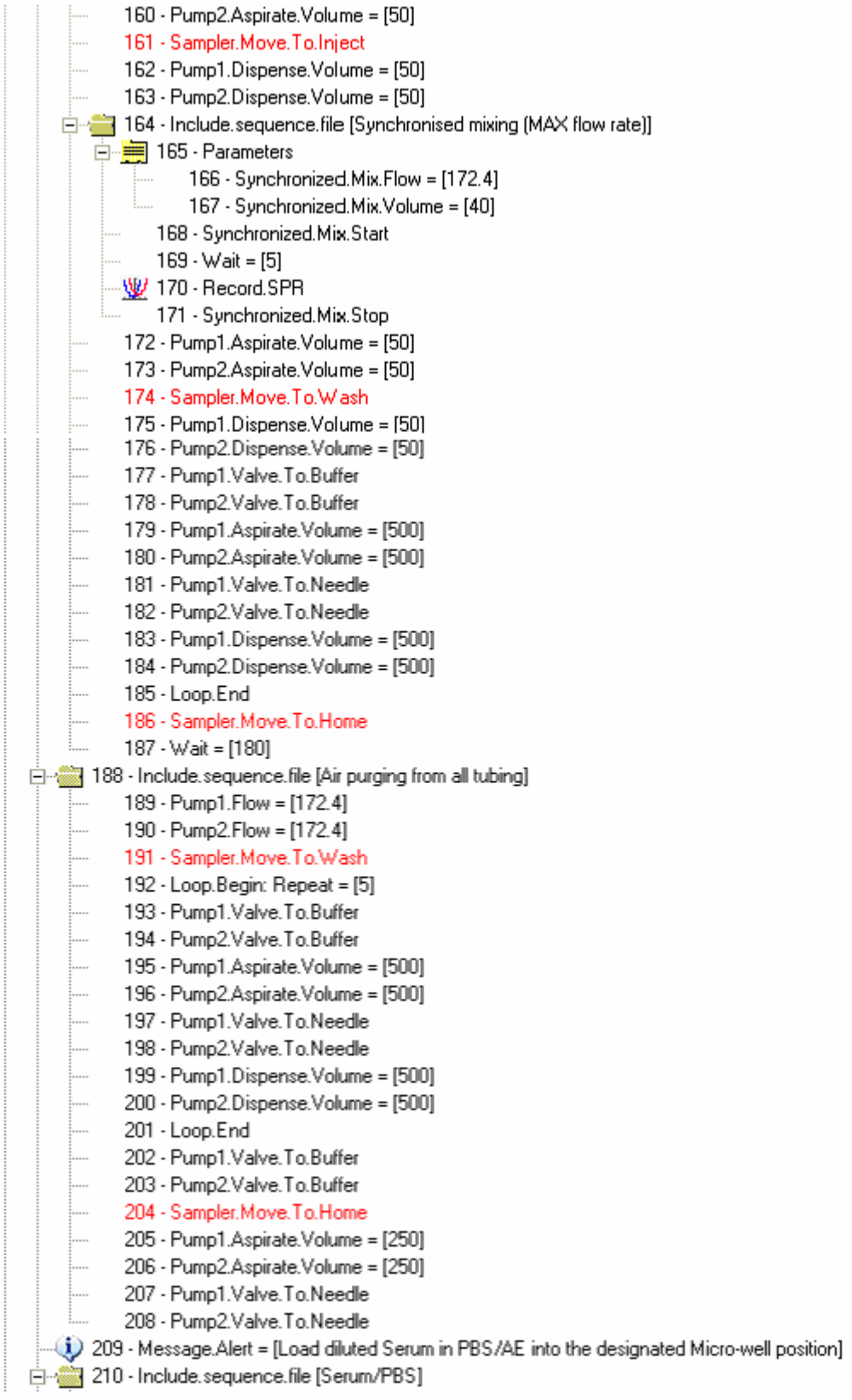
## Appendix C: The optimal MARTI-test sequence

- 1 - Synchronized.Mix.Flow = [172.4]
- 2 - Measurement.Interval = [1]
- 3 - Measurement.Start.Both Channels
- 4 - Include.sequence.file [Full NEW sequence (without 'airlock' sequences)]
  - 5 - Pump1.Flow = [172.4]
  - 6 - Pump2.Flow = [172.4]
  - 7 - Include.sequence.file [BASELINE SETTING (leaving 50 uL in cuvette)]
    - 8 - Update/Add.Event.Item [Baseline setting BEGIN]
    - 9 - Sampler.Move.To.Stock[2]
    - 10 - Pump1.Aspirate.Volume = [50]
    - 11 - Pump2.Aspirate.Volume = [50]
    - 12 - Sampler.Move.To.Inject
    - 13 - Pump1.Dispense.Volume = [50]
    - 14 - Pump2.Dispense.Volume = [50]
    - 15 - Sampler.Move.To.Home
    - 16 - Record.SPR
    - 17 - Wait.Baseline (s)
    - 18 - Wait = [180]
    - 19 - Sampler.Move.To.Inject
    - 20 - Pump1.Aspirate.Volume = [50]
    - 21 - Pump2.Aspirate.Volume = [50]
    - 22 - Sampler.Move.To.Wash
    - 23 - Record.SPR
    - 24 - Update/Add.Event.Item [Baseline setting END]
    - 25 - Pump1.Dispense.Volume = [50]
    - 26 - Pump2.Dispense.Volume = [50]
    - 27 - Sampler.Move.To.Home
  - 28 - Include.sequence.file [Air purging from all tubing]
    - 29 - Pump1.Flow = [172.4]
    - 30 - Pump2.Flow = [172.4]
    - 31 - Sampler.Move.To.Wash
    - 32 - Loop.Begin: Repeat = [5]
    - 33 - Pump1.Valve.To.Buffer
    - 34 - Pump2.Valve.To.Buffer
    - 35 - Pump1.Aspirate.Volume = [500]
    - 36 - Pump2.Aspirate.Volume = [500]
    - 37 - Pump1.Valve.To.Needle
    - 38 - Pump2.Valve.To.Needle
    - 39 - Pump1.Dispense.Volume = [500]
    - 40 - Pump2.Dispense.Volume = [500]
    - 41 - Loop.End
    - 42 - Pump1.Valve.To.Buffer
    - 43 - Pump2.Valve.To.Buffer
    - 44 - Sampler.Move.To.Home
    - 45 - Pump1.Aspirate.Volume = [250]
    - 46 - Pump2.Aspirate.Volume = [250]
    - 47 - Pump1.Valve.To.Needle
    - 48 - Pump2.Valve.To.Needle
  - 49 - Message.Alert = [Load liposomes into the designated Micro-well plate position]
  - 50 - Include.sequence.file [Liposomes immobilisation]
    - 51 - Update/Add.Event.Item [Liposome immobilisation BEGIN]
    - 52 - Sampler.Move.To.Sample[137]

- 53 - Pump1.Aspirate.Volume = [50]
- 54 - Pump2.Aspirate.Volume = [50]
- 55 - Sampler.Move.To.Inject
- 56 - Pump1.Dispense.Volume = [50]
- 57 - Pump2.Dispense.Volume = [50]
- 58 - Include.sequence.file [Synchronised mixing (70% flow rate)]
  - 59 - Parameters
    - 60 - Synchronized.Mix.Flow = [172.4]
    - 61 - Synchronized.Mix.Volume = [20]
  - 62 - Synchronized.Mix.Start
  - 63 - Wait = [1200]
  - 64 - Record.SPR
  - 65 - Synchronized.Mix.Stop
- 66 - Pump1.Aspirate.Volume = [50]
- 67 - Pump2.Aspirate.Volume = [50]
- 68 - Sampler.Move.To.Wash
- 69 - Pump1.Dispense.Volume = [50]
- 70 - Pump2.Dispense.Volume = [50]
- 71 - Record.SPR
- 72 - Message.Alert = [Change to Non-Degassed Buffer]
- 73 - Include.sequence.file [PBS/AE wash sequence]
  - 74 - Update/Add.Event.Item [PBS/AE wash]
  - 75 - Record.SPR
  - 76 - Loop.Begin: Repeat = [5]
  - 77 - Sampler.Move.To.Stock[2]
  - 78 - Pump1.Valve.To.Needle
  - 79 - Pump2.Valve.To.Needle
  - 80 - Pump1.Aspirate.Volume = [50]
  - 81 - Pump2.Aspirate.Volume = [50]
  - 82 - Sampler.Move.To.Inject
  - 83 - Pump1.Dispense.Volume = [50]
  - 84 - Pump2.Dispense.Volume = [50]
  - 85 - Include.sequence.file [Synchronised mixing (70% flow rate)]
    - 86 - Parameters
      - 87 - Synchronized.Mix.Flow = [172.4]
      - 88 - Synchronized.Mix.Volume = [20]
    - 89 - Synchronized.Mix.Start
    - 90 - Wait = [5]
    - 91 - Synchronized.Mix.Stop
  - 92 - Pump1.Aspirate.Volume = [50]
  - 93 - Pump2.Aspirate.Volume = [50]
  - 94 - Sampler.Move.To.Wash
  - 95 - Pump1.Dispense.Volume = [50]
  - 96 - Pump2.Dispense.Volume = [50]
  - 97 - Pump1.Valve.To.Buffer
  - 98 - Pump2.Valve.To.Buffer
  - 99 - Pump1.Aspirate.Volume = [500]
  - 100 - Pump2.Aspirate.Volume = [500]
  - 101 - Pump1.Valve.To.Needle
  - 102 - Pump2.Valve.To.Needle
  - 103 - Pump1.Dispense.Volume = [500]
  - 104 - Pump2.Dispense.Volume = [500]
  - 105 - Loop.End

- 106 - Record.SPR
- 107 - Sampler.Move.To.Home
- 108 - Include.sequence.file [Air purging from all tubing]
  - 109 - Pump1.Flow = [172.4]
  - 110 - Pump2.Flow = [172.4]
  - 111 - Sampler.Move.To.Wash
  - 112 - Loop.Begin: Repeat = [5]
  - 113 - Pump1.Valve.To.Buffer
  - 114 - Pump2.Valve.To.Buffer
  - 115 - Pump1.Aspirate.Volume = [500]
  - 116 - Pump2.Aspirate.Volume = [500]
  - 117 - Pump1.Valve.To.Needle
  - 118 - Pump2.Valve.To.Needle
  - 119 - Pump1.Dispense.Volume = [500]
  - 120 - Pump2.Dispense.Volume = [500]
  - 121 - Loop.End
  - 122 - Pump1.Valve.To.Buffer
  - 123 - Pump2.Valve.To.Buffer
  - 124 - Sampler.Move.To.Home
  - 125 - Pump1.Aspirate.Volume = [250]
  - 126 - Pump2.Aspirate.Volume = [250]
  - 127 - Pump1.Valve.To.Needle
  - 128 - Pump2.Valve.To.Needle
- 129 - Message.Alert = [Load saponin into the designated Micro-well plate position]
- 130 - Include.sequence.file [Saponin blocking (0.5 mg/mL)]
  - 131 - Update/Add.Event.Item [Saponin blocking (0.5 mg/mL) BEGIN]
  - 132 - Sampler.Move.To.Sample[139]
  - 133 - Pump1.Aspirate.Volume = [50]
  - 134 - Pump2.Aspirate.Volume = [50]
  - 135 - Sampler.Move.To.Inject
  - 136 - Pump1.Dispense.Volume = [50]
  - 137 - Pump2.Dispense.Volume = [50]
  - 138 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 139 - Parameters
      - 140 - Synchronized.Mix.Flow = [50.0]
      - 141 - Synchronized.Mix.Volume = [20]
    - 142 - Synchronized.Mix.Start
    - 143 - Wait = [300]
    - 144 - Record.SPR
    - 145 - Synchronized.Mix.Stop
  - 146 - Pump1.Aspirate.Volume = [50]
  - 147 - Pump2.Aspirate.Volume = [50]
  - 148 - Pump1.Valve.To.Needle
  - 149 - Pump2.Valve.To.Needle
- 150 - Sampler.Move.To.Wash
- 151 - Pump1.Dispense.Volume = [50]
- 152 - Pump2.Dispense.Volume = [50]
- 153 - Include.sequence.file [PBS/AE wash sequence]
  - 154 - Update/Add.Event.Item [PBS/AE wash]
  - 155 - Loop.Begin: Repeat = [5]
  - 156 - Sampler.Move.To.Stock[2]
  - 157 - Pump1.Valve.To.Needle
  - 158 - Pump2.Valve.To.Needle
  - 159 - Pump1.Aspirate.Volume = [50]


160 - Pump2.Aspirate.Volume = [50]
161 - Sampler.Move.To.Inject
162 - Pump1.Dispense.Volume = [50]
163 - Pump2.Dispense.Volume = [50]
164 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
165 - Parameters
166 - Synchronized.Mix.Flow = [172.4]
167 - Synchronized.Mix.Volume = [40]
168 - Synchronized.Mix.Start
169 - Wait = [5]
170 - Record.SPR
171 - Synchronized.Mix.Stop
172 - Pump1.Aspirate.Volume = [50]
173 - Pump2.Aspirate.Volume = [50]
174 - Sampler.Move.To.Wash
175 - Pump1.Dispense.Volume = [50]
176 - Pump2.Dispense.Volume = [50]
177 - Pump1.Valve.To.Buffer
178 - Pump2.Valve.To.Buffer
179 - Pump1.Aspirate.Volume = [500]
180 - Pump2.Aspirate.Volume = [500]
181 - Pump1.Valve.To.Needle
182 - Pump2.Valve.To.Needle
183 - Pump1.Dispense.Volume = [500]
184 - Pump2.Dispense.Volume = [500]
185 - Loop.End
186 - Sampler.Move.To.Home
187 - Wait = [180]
188 - Include.sequence.file [Air purging from all tubing]
189 - Pump1.Flow = [172.4]
190 - Pump2.Flow = [172.4]
191 - Sampler.Move.To.Wash
192 - Loop.Begin: Repeat = [5]
193 - Pump1.Valve.To.Buffer
194 - Pump2.Valve.To.Buffer
195 - Pump1.Aspirate.Volume = [500]
196 - Pump2.Aspirate.Volume = [500]
197 - Pump1.Valve.To.Needle
198 - Pump2.Valve.To.Needle
199 - Pump1.Dispense.Volume = [500]
200 - Pump2.Dispense.Volume = [500]
201 - Loop.End
202 - Pump1.Valve.To.Buffer
203 - Pump2.Valve.To.Buffer
204 - Sampler.Move.To.Home
205 - Pump1.Aspirate.Volume = [250]
206 - Pump2.Aspirate.Volume = [250]
207 - Pump1.Valve.To.Needle
208 - Pump2.Valve.To.Needle
209 - Message.Alert = [Load diluted Serum in PBS/AE into the designated Micro-well position]
210 - Include.sequence.file [Serum/PBS]

- 211 - Update/Add.Event.Item [Diluted serum blocking START]
- 212 - Sampler.Move.To.Sample[141]
- 213 - Pump1.Aspirate.Volume = [35]
- 214 - Pump2.Aspirate.Volume = [35]
- 215 - Sampler.Move.To.Inject
- 216 - Pump1.Dispense.Volume = [35]
- 217 - Pump2.Dispense.Volume = [35]
- 218 - Include.sequence.file [Synchronised mixing (70% flow rate)]
  - 219 - Parameters
    - 220 - Synchronized.Mix.Flow = [172.4]
    - 221 - Synchronized.Mix.Volume = [20]
  - 222 - Synchronized.Mix.Start
  - 223 - Wait = [480]
  - 224 - Synchronized.Mix.Stop
- 225 - Record.SPR
- 226 - Message.Alert = [Load Serum diluted in Liposomes into the designated Micro-well position]
- 227 - Include.sequence.file [Serum/liposomes]
  - 228 - Update/Add.Event.Item [Inhibition study START]
  - 229 - Sampler.Move.To.Sample[143]
  - 230 - Pump1.Aspirate.Volume = [35]
  - 231 - Pump2.Aspirate.Volume = [35]
  - 232 - Sampler.Move.To.Inject
  - 233 - Pump1.Dispense.Volume = [35]
  - 234 - Pump2.Dispense.Volume = [35]
  - 235 - Include.sequence.file [Synchronised mixing (70% flow rate)]
    - 236 - Parameters
      - 237 - Sampler.Move.To.Sample[227.3]
      - 238 - Synchronized.Mix.Volume = [20]
    - 239 - Synchronized.Mix.Start
    - 240 - Wait = [480]
    - 241 - Record.SPR
    - 242 - Synchronized.Mix.Stop
    - 243 - Pump1.Aspirate.Volume = [70]
    - 244 - Pump2.Aspirate.Volume = [70]
    - 245 - Sampler.Move.To.Wash
    - 246 - Pump1.Dispense.Volume = [70]
    - 247 - Pump2.Dispense.Volume = [70]
    - 248 - Loop.Begin: Repeat = [3]
    - 249 - Pump1.Valve.To.Buffer
    - 250 - Pump2.Valve.To.Buffer
    - 251 - Pump1.Aspirate.Volume = [500]
    - 252 - Pump2.Aspirate.Volume = [500]
    - 253 - Pump1.Valve.To.Needle
    - 254 - Pump2.Valve.To.Needle
    - 255 - Pump1.Dispense.Volume = [500]
    - 256 - Pump2.Dispense.Volume = [500]
    - 257 - Loop.End
  - 258 - Sampler.Move.To.Home
  - 259 - Update/Add.Event.Item [Inhibition study END]
- 260 - Include.sequence.file [PBS/AE wash sequence]
  - 261 - Update/Add.Event.Item [PBS/AE wash]
  - 262 - Loop.Begin: Repeat = [5]
  - 263 - Sampler.Move.To.Stock[2]

- 264 - Pump1.Valve.To.Needle
- 265 - Pump2.Valve.To.Needle
- 266 - Pump1.Aspirate.Volume = [50]
- 267 - Pump2.Aspirate.Volume = [50]
- 268 - Sampler.Move.To.Inject
- 269 - Pump1.Dispense.Volume = [50]
- 270 - Pump2.Dispense.Volume = [50]
- 271 - Include.sequence.file [Synchronised mixing (70% flow rate)]
  - 272 - Parameters
    - 273 - Synchronized.Mix.Flow = [172.4]
    - 274 - Synchronized.Mix.Volume = [40]
  - 275 - Synchronized.Mix.Start
  - 276 - Wait = [5]
  - 277 - Record.SPR
  - 278 - Synchronized.Mix.Stop
- 279 - Pump1.Aspirate.Volume = [50]
- 280 - Pump2.Aspirate.Volume = [50]
- 281 - Sampler.Move.To.Wash
- 282 - Pump1.Dispense.Volume = [50]
- 283 - Pump2.Dispense.Volume = [50]
- 284 - Pump1.Valve.To.Buffer
- 285 - Pump2.Valve.To.Buffer
- 286 - Pump1.Aspirate.Volume = [500]
- 287 - Pump2.Aspirate.Volume = [500]
- 288 - Pump1.Valve.To.Needle
- 289 - Pump2.Valve.To.Needle
- 290 - Pump1.Dispense.Volume = [500]
- 291 - Pump2.Dispense.Volume = [500]
- 292 - Loop.End
- 293 - Sampler.Move.To.Home
- 294 - Wait = [120]
- 295 - Include.sequence.file [Air purging from all tubing]
  - 296 - Include.sequence.file [Air purging from all tubing]
    - 297 - Pump1.Flow = [172.4]
    - 298 - Pump2.Flow = [172.4]
    - 299 - Sampler.Move.To.Wash
    - 300 - Loop.Begin: Repeat = [5]
    - 301 - Pump1.Valve.To.Buffer
    - 302 - Pump2.Valve.To.Buffer
    - 303 - Pump1.Aspirate.Volume = [500]
    - 304 - Pump2.Aspirate.Volume = [500]
    - 305 - Pump1.Valve.To.Needle
    - 306 - Pump2.Valve.To.Needle
    - 307 - Pump1.Dispense.Volume = [500]
    - 308 - Pump2.Dispense.Volume = [500]
    - 309 - Loop.End
    - 310 - Pump1.Valve.To.Buffer
    - 311 - Pump2.Valve.To.Buffer
    - 312 - Sampler.Move.To.Home
    - 313 - Pump1.Aspirate.Volume = [250]
    - 314 - Pump2.Aspirate.Volume = [250]
    - 315 - Pump1.Valve.To.Needle

- 316 - Pump2.Valve.To.Needle
- 317 - Message.Alert = [Put Isopropanol + 50 mM NaOH (2/3, v:v) in Stock 1]
- 318 - Include.sequence.file [NaOH and Isopropanol wash]
  - 319 - Update/Add.Event.Item [NaOH wash ]
  - 320 - Loop.Begin: Repeat = [5]
  - 321 - Sampler.Move.To.Stock[1]
  - 322 - Pump1.Aspirate.Volume = [100]
  - 323 - Pump2.Aspirate.Volume = [100]
  - 324 - Sampler.Move.To.Inject
  - 325 - Pump1.Dispense.Volume = [100]
  - 326 - Pump2.Dispense.Volume = [100]
  - 327 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 328 - Parameters
      - 329 - Synchronized.Mix.Flow = [172.4]
      - 330 - Synchronized.Mix.Volume = [40]
    - 331 - Synchronized.Mix.Start
    - 332 - Wait = [20]
    - 333 - Synchronized.Mix.Stop
  - 334 - Pump1.Aspirate.Volume = [100]
  - 335 - Pump2.Aspirate.Volume = [100]
  - 336 - Sampler.Move.To.Wash
  - 337 - Pump1.Dispense.Volume = [100]
  - 338 - Pump2.Dispense.Volume = [100]
  - 339 - Loop.End
- 340 - Include.sequence.file [PBSAE wash sequence]
  - 341 - Include.sequence.file [PBS/AE wash sequence]
    - 342 - Update/Add.Event.Item [PBS/AE wash]
    - 343 - Loop.Begin: Repeat = [5]
    - 344 - Sampler.Move.To.Stock[2]
    - 345 - Pump1.Valve.To.Needle
    - 346 - Pump2.Valve.To.Needle
    - 347 - Pump1.Aspirate.Volume = [100]
    - 348 - Pump2.Aspirate.Volume = [100]
    - 349 - Sampler.Move.To.Inject
    - 350 - Pump1.Dispense.Volume = [100]
    - 351 - Pump2.Dispense.Volume = [100]
    - 352 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
      - 353 - Parameters
        - 354 - Synchronized.Mix.Flow = [172.4]
        - 355 - Synchronized.Mix.Volume = [40]
      - 356 - Synchronized.Mix.Start
      - 357 - Wait = [5]
      - 358 - Record.SPR
      - 359 - Synchronized.Mix.Stop
    - 360 - Pump1.Aspirate.Volume = [100]
    - 361 - Pump2.Aspirate.Volume = [100]
    - 362 - Sampler.Move.To.Wash
    - 363 - Pump1.Dispense.Volume = [100]
    - 364 - Pump2.Dispense.Volume = [100]
    - 365 - Pump1.Valve.To.Buffer
    - 366 - Pump2.Valve.To.Buffer
    - 367 - Pump1.Aspirate.Volume = [500]
    - 368 - Pump2.Aspirate.Volume = [500]

- 369 - Pump1.Valve.To.Needle
- 370 - Pump2.Valve.To.Needle
- 371 - Pump1.Dispense.Volume = [500]
- 372 - Pump2.Dispense.Volume = [500]
- 373 - Loop.End
- 374 - Sampler.Move.To.Home
- 375 - Wait = [60]
- 376 - Message.Alert = [Put 96 % Ethanol in Stock 1]
- 377 - Include.sequence.file [EtOH wash sequence]
  - 378 - Include.sequence.file [EtOH wash]
    - 379 - Update/Add.Event.Item [EtOH wash]
    - 380 - Loop.Begin: Repeat = [5]
    - 381 - Sampler.Move.To.Stock[1]
    - 382 - Pump1.Aspirate.Volume = [100]
    - 383 - Pump2.Aspirate.Volume = [100]
    - 384 - Sampler.Move.To.Inject
    - 385 - Pump1.Dispense.Volume = [100]
    - 386 - Pump2.Dispense.Volume = [100]
    - 387 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
      - 388 - Parameters
        - 389 - Synchronized.Mix.Flow = [172.4]
        - 390 - Synchronized.Mix.Volume = [40]
      - 391 - Synchronized.Mix.Start
      - 392 - Wait = [20]
      - 393 - Synchronized.Mix.Stop
    - 394 - Pump1.Aspirate.Volume = [100]
    - 395 - Pump2.Aspirate.Volume = [100]
    - 396 - Sampler.Move.To.Wash
    - 397 - Pump1.Dispense.Volume = [100]
    - 398 - Pump2.Dispense.Volume = [100]
    - 399 - Loop.End
  - 400 - Include.sequence.file [PBSAE wash sequence]
    - 401 - Include.sequence.file [PBS/AE wash sequence]
      - 402 - Update/Add.Event.Item [PBS/AE wash]
      - 403 - Loop.Begin: Repeat = [5]
      - 404 - Sampler.Move.To.Stock[2]
      - 405 - Pump1.Valve.To.Needle
      - 406 - Pump2.Valve.To.Needle
      - 407 - Pump1.Aspirate.Volume = [100]
      - 408 - Pump2.Aspirate.Volume = [100]
      - 409 - Sampler.Move.To.Inject
      - 410 - Pump1.Dispense.Volume = [100]
      - 411 - Pump2.Dispense.Volume = [100]
      - 412 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
        - 413 - Parameters
          - 414 - Synchronized.Mix.Flow = [172.4]
          - 415 - Synchronized.Mix.Volume = [40]
        - 416 - Synchronized.Mix.Start
        - 417 - Wait = [5]
        - 418 - Record.SPR
        - 419 - Synchronized.Mix.Stop
      - 420 - Pump1.Aspirate.Volume = [100]

421 - Pump2.Aspirate.Volume = [100]
422 - Sampler.Move.To.Wash
423 - Pump1.Dispense.Volume = [100]
424 - Pump2.Dispense.Volume = [100]
425 - Pump1.Valve.To.Buffer
426 - Pump2.Valve.To.Buffer
427 - Pump1.Aspirate.Volume = [500]
428 - Pump2.Aspirate.Volume = [500]
429 - Pump1.Valve.To.Needle
430 - Pump2.Valve.To.Needle
431 - Pump1.Dispense.Volume = [500]
432 - Pump2.Dispense.Volume = [500]
433 - Loop.End
434 - Sampler.Move.To.Home
435 - Wait = [180]
436 - Measurement.End

## Appendix D: SPR dips preparation and regeneration sequence

- 1 - Include.sequence.file [SPR dip calibration]
  - 2 - Message.Alert = [SPR dip calibration will now commence]
  - 3 - Include.sequence.file [Air purging from all tubing]
    - 4 - Pump1.Flow = [172.4]
    - 5 - Pump2.Flow = [172.4]
    - 6 - Sampler.Move.To.Wash
    - 7 - Loop.Begin: Repeat = [2]
    - 8 - Pump1.Valve.To.Buffer
    - 9 - Pump2.Valve.To.Buffer
    - 10 - Pump1.Aspirate.Volume = [500]
    - 11 - Pump2.Aspirate.Volume = [500]
    - 12 - Pump1.Valve.To.Needle
    - 13 - Pump2.Valve.To.Needle
    - 14 - Pump1.Dispense.Volume = [500]
    - 15 - Pump2.Dispense.Volume = [500]
    - 16 - Loop.End
    - 17 - Pump1.Valve.To.Buffer
    - 18 - Pump2.Valve.To.Buffer
    - 19 - Sampler.Move.To.Home
    - 20 - Pump1.Aspirate.Volume = [250]
    - 21 - Pump2.Aspirate.Volume = [250]
    - 22 - Pump1.Valve.To.Needle
    - 23 - Pump2.Valve.To.Needle
  - 24 - Update/Add.Event.Item [SPR dip calibration START]
  - 25 - Pump1.Valve.To.Needle
  - 26 - Pump2.Valve.To.Needle
  - 27 - Sampler.Move.To.Wash
  - 28 - Pump1.Dispense.Volume = [250]
  - 29 - Pump2.Dispense.Volume = [250]
  - 30 - Sampler.Move.To.Stock[1]
  - 31 - Pump1.Aspirate.Volume = [100]
  - 32 - Pump2.Aspirate.Volume = [100]
  - 33 - Sampler.Move.To.Inject
  - 34 - Pump1.Dispense.Volume = [100]
  - 35 - Pump2.Dispense.Volume = [100]
  - 36 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 37 - Parameters
      - 38 - Synchronized.Mix.Flow = [172.4]
      - 39 - Synchronized.Mix.Volume = [50]
    - 40 - Synchronized.Mix.Start
    - 41 - Wait = [30]
    - 42 - Record.SPR
    - 43 - Synchronized.Mix.Stop
  - 44 - Pump1.Aspirate.Volume = [50]
  - 45 - Pump2.Aspirate.Volume = [50]
  - 46 - Sampler.Move.To.Wash
  - 47 - Pump1.Dispense.Volume = [50]
  - 48 - Pump2.Dispense.Volume = [50]
  - 49 - Include.sequence.file [Air purging from all tubing]
    - 50 - Pump1.Flow = [172.4]
    - 51 - Pump2.Flow = [172.4]
    - 52 - Sampler.Move.To.Wash
    - 53 - Loop.Begin: Repeat = [3]

- 54 - Pump1.Valve.To.Buffer
- 55 - Pump2.Valve.To.Buffer
- 56 - Pump1.Aspirate.Volume = [500]
- 57 - Pump2.Aspirate.Volume = [500]
- 58 - Pump1.Valve.To.Needle
- 59 - Pump2.Valve.To.Needle
- 60 - Pump1.Dispense.Volume = [500]
- 61 - Pump2.Dispense.Volume = [500]
- 62 - Loop.End
- 63 - Pump1.Valve.To.Buffer
- 64 - Pump2.Valve.To.Buffer
- 65 - Sampler.Move.To.Home
- 66 - Pump1.Aspirate.Volume = [250]
- 67 - Pump2.Aspirate.Volume = [250]
- 68 - Pump1.Valve.To.Needle
- 69 - Pump2.Valve.To.Needle
- 70 - Loop.Begin: Repeat = [4]
- 71 - Sampler.Move.To.Stock[1]
- 72 - Pump1.Aspirate.Volume = [50]
- 73 - Pump2.Aspirate.Volume = [50]
- 74 - Sampler.Move.To.Inject
- 75 - Pump1.Dispense.Volume = [50]
- 76 - Pump2.Dispense.Volume = [50]
- 77 - Include.sequence.file [Synchronised mixing (MAX flow
  - 78 - Parameters
    - 79 - Synchronized.Mix.Flow = [172.4]
    - 80 - Synchronized.Mix.Volume = [50]
  - 81 - Synchronized.Mix.Start
  - 82 - Wait = [30]
  - 83 - Record.SPR
  - 84 - Synchronized.Mix.Stop
- 85 - Pump1.Aspirate.Volume = [50]
- 86 - Pump2.Aspirate.Volume = [50]
- 87 - Sampler.Move.To.Wash
- 88 - Pump1.Dispense.Volume = [50]
- 89 - Pump2.Dispense.Volume = [50]
- 90 - Include.sequence.file [Air purging from all tubing]
  - 91 - Pump1.Flow = [172.4]
  - 92 - Pump2.Flow = [172.4]
  - 93 - Sampler.Move.To.Wash
  - 94 - Loop.Begin: Repeat = [1]
  - 95 - Pump1.Valve.To.Buffer
  - 96 - Pump2.Valve.To.Buffer
  - 97 - Pump1.Aspirate.Volume = [500]
  - 98 - Pump2.Aspirate.Volume = [500]
  - 99 - Pump1.Valve.To.Needle
  - 100 - Pump2.Valve.To.Needle
  - 101 - Pump1.Dispense.Volume = [500]
  - 102 - Pump2.Dispense.Volume = [500]
  - 103 - Loop.End
  - 104 - Pump1.Valve.To.Buffer
  - 105 - Pump2.Valve.To.Buffer

- 106 - Sampler.Move.To.Home
- 107 - Pump1.Aspirate.Volume = [250]
- 108 - Pump2.Aspirate.Volume = [250]
- 109 - Pump1.Valve.To.Needle
- 110 - Pump2.Valve.To.Needle
- 111 - Loop.End
- 112 - Include.sequence.file [PBS/AE wash sequence]
  - 113 - Loop.Begin: Repeat = [6]
  - 114 - Sampler.Move.To.Stock[2]
  - 115 - Pump1.Valve.To.Needle
  - 116 - Pump2.Valve.To.Needle
  - 117 - Pump1.Aspirate.Volume = [50]
  - 118 - Pump2.Aspirate.Volume = [50]
  - 119 - Sampler.Move.To.Inject
  - 120 - Pump1.Dispense.Volume = [50]
  - 121 - Pump2.Dispense.Volume = [50]
  - 122 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 123 - Parameters
      - 124 - Synchronized.Mix.Flow = [172.4]
      - 125 - Synchronized.Mix.Volume = [50]
    - 126 - Synchronized.Mix.Start
    - 127 - Wait = [10]
    - 128 - Record.SPR
    - 129 - Synchronized.Mix.Stop
  - 130 - Pump1.Aspirate.Volume = [50]
  - 131 - Pump2.Aspirate.Volume = [50]
  - 132 - Sampler.Move.To.Wash
  - 133 - Pump1.Dispense.Volume = [50]
  - 134 - Pump2.Dispense.Volume = [50]
  - 135 - Loop.Begin: Repeat = [1]
  - 136 - Pump1.Valve.To.Buffer
  - 137 - Pump2.Valve.To.Buffer
  - 138 - Pump1.Aspirate.Volume = [500]
  - 139 - Pump2.Aspirate.Volume = [500]
  - 140 - Pump1.Valve.To.Needle
  - 141 - Pump2.Valve.To.Needle
  - 142 - Pump1.Dispense.Volume = [500]
  - 143 - Pump2.Dispense.Volume = [500]
  - 144 - Loop.End
  - 145 - Loop.End
- 146 - Pump1.Valve.To.Buffer
- 147 - Pump2.Valve.To.Buffer
- 148 - Pump1.Valve.To.Needle
- 149 - Pump2.Valve.To.Needle
- 150 - Update/Add.Event.Item [SPR dip calibration END]
- 151 - Sampler.Move.To.Home
- 152 - Record.SPR
- 153 - Update/Add.Event.Item [50 uL now in cuvette]
- 154 - Include.sequence.file [Air purging from all tubing]
  - 155 - Pump1.Flow = [227.3]
  - 156 - Pump2.Flow = [227.3]
  - 157 - Sampler.Move.To.Wash
  - 158 - Loop.Begin: Repeat = [2]
  - 159 - Pump1.Valve.To.Buffer

160 - Pump2.Valve.To.Buffer
161 - Pump1.Aspirate.Volume = [500]
162 - Pump2.Aspirate.Volume = [500]
163 - Pump1.Valve.To.Needle
164 - Pump2.Valve.To.Needle
165 - Pump1.Dispense.Volume = [500]
166 - Pump2.Dispense.Volume = [500]
167 - Loop.End
168 - Pump1.Valve.To.Buffer
169 - Pump2.Valve.To.Buffer
170 - Sampler.Move.To.Home
171 - Pump1.Aspirate.Volume = [250]
172 - Pump2.Aspirate.Volume = [250]
173 - Pump1.Valve.To.Needle
174 - Pump2.Valve.To.Needle

## Appendix E: Sequence for cleaning the needles and cuvette

- 1 - Loop.Begin: Repeat = [4]
- 2 - Sampler.Move.To.Wash
- 3 - Include.sequence.file [Safety lines]
  - 4 - Pump1.Flow = [227.3]
  - 5 - Pump2.Flow = [227.3]
  - 6 - Pump1.Valve.To.Buffer
  - 7 - Pump2.Valve.To.Buffer
  - 8 - Synchronized.Mix.Stop
- 9 - Wash.Speed = [150]
- 10 - Drain.Speed = [200]
- 11 - Loop.Begin: Repeat = [2]
- 12 - Wash.Start
- 13 - Drain.Start
- 14 - Pump1.Aspirate.Volume = [250]
- 15 - Pump2.Aspirate.Volume = [250]
- 16 - Pump1.Valve.To.Needle
- 17 - Pump2.Valve.To.Needle
- 18 - Pump1.Dispense.Volume = [500]
- 19 - Pump2.Dispense.Volume = [500]
- 20 - Wait = [2]
- 21 - Wash.Stop
- 22 - Pump1.Valve.To.Buffer
- 23 - Pump2.Valve.To.Buffer
- 24 - Synchronized.Mix.Stop
- 25 - Drain.Stop
- 26 - Loop.End
- 27 - Pump1.Dispense.Volume = [50]
- 28 - Pump2.Dispense.Volume = [50]
- 29 - Sampler.Move.To.Home
- 30 - Pump1.Flow = [16.7]
- 31 - Pump2.Flow = [16.7]
- 32 - Pump1.Valve.To.Needle
- 33 - Pump2.Valve.To.Needle
- 34 - Synchronized.Mix.Stop
- 35 - Loop.End

**Appendix F: ESPRIT Biosensor signal percentage inhibition of patient serum antibody binding to MA using different dilutions of serum and inhibitory liposomes**

| Serum dilutions | | % inhibition of serum antibody binding to MA | | | |
|---|---|---|---|---|---|
| High diluted in PBS/AE | Low diluted in MA or PC Liposomes | P135 ($TB^+HIV^+$) | P129 ($TB^+HIV^-$) | P94 ($TB^-HIV^-$) | P96 ($TB^+HIV^-$) |
| 1/4000 | 1/250 | -10.43* | 17.29 | - | - |
| | 1/500 | -21.51* | 25.09 | -0.23* | 29.53 |
| | 1/1000 | -45.85* | 24.73 | - | - |
| | 1/2000 | -23.32* | 2.00* | - | - |
| 1/2000 | 1/250 | - | 16.58* | - | - |
| | 1/500 | - | 19.22* | 7.61* | 30.41 |
| | 1/1000 | - | 41.67 | - | -5.12* |

MA: mycolic acid, PC: phosphatidylcholine, TB: tuberculosis, HIV: human immunodeficiency virus, +: positive, -: negative. Results represent the average of triplicate values and *: no significant difference.