Chapter Two

Materials and Methods

The experimental work was conducted between October 2009 and August 2010 and all the protocols are described in this chapter. All work with live *P. falciparum* was carried out *in vitro* in containment level 3 laboratories at The Wellcome Trust Sanger Institute. Except where noted, I performed all experiments described herein.

Cell Culture Reagents

- Incomplete medium
    - 10.43 g RPMI 1640 (Invitrogen)
    - 7.15 g HEPES (Sigma Aldrich)
    - 1 ml 1 mg/mL hypoxanthine (Sigma Aldrich)
    - 0.5 ml 1 mg/mL gentamicin (Sigma Aldrich)
    - 2 g Glucose (Sigma Aldrich)
    - 1 L Milli-Q water (Millipore)
- Complete medium containing 10% sera
    - 450 mL incomplete medium supplemented with:
    - 50ml human sera (NHS Blood Service)
    - 16 mL 7.5% sodium bicarbonate (Sigma Aldrich)
- Glycerolyte 57
    - 57 g glycerol (Sigma Aldrich)
    - 1.6 g sodium lactate (Sigma Aldrich)
    - 30 mg potassium chloride (Sigma Aldrich)
    - 51.7 mg monobasic sodium phosphate (monohydrate) (Sigma Aldrich)
    - 124.2mg dibasic sodium phosphate (anhydrous) (Sigma Aldrich)
    - 100 mL Milli-Q water

Solutions above were filtered through a 0.22 µm membrane to sterilise before use using Stericup® filter units (Millipore). Other solutions used were supplied as a sterile solution from the manufacturer.

In vitro **culture of** ***Plasmodium falciparum*** **parasites**

Samples of *Plasmodium falciparum* infected erythrocytes from patients in Peru were stored at −80°C and thawed in a water bath at +37°C. The volume of the sample was measured and 10 µL aliquots of 12% sodium chloride solution were added to a total of 0.2 times the sample volume. After 5 minutes incubation at room temperature 8 mL of 1.6% sodium chloride solution was added 100 µL at a time. The sample was centrifuged at 800 ***g*** for 5 minutes with no braking, the supernatant solution was removed and the pellet was resuspended in 8 mL of 0.2% D–glucose and 0.9% sodium chloride added in 100 µL drops. The sample was centrifuged as above and the pellet resuspended in complete medium containing human AB sera, with 400 µL of 50% haematocrit O^+ erythrocytes (NHS Blood & Transplant Service) in RPMI 1640 (Invitrogen). Prior to use, all erythrocytes were filtered using a Lymphoprep™ (Axis-Shield) density gradient to remove leucocytes, and washed with RPMI 1640. Samples were cultured in complete medium containing human AB sera for the first 48 hours before this was replaced with pooled sera. As AB sera lacks anti-A and anti-B antibodies, this 48 hour period prevents complement-mediated lysis before O+ erythrocytes have been successfully invaded by merozoites, as the blood group of the patient was unknown (Bakács et al. 1993).

Cultures were maintained at approximately 5% haematocrit, a parasitaemia of less than 10% and in an atmosphere of malaria gas (1% O_2, 3% CO_2 and 96% N_2). Complete medium was changed at least every 48 hours. Routinely, complete medium contained 10% human sera but this was increased to 15% if parasites failed to multiply.

Synchronisation of Parasites

Prior to most experiments, cultures were synchronised at the ring stage by adding a 5% sorbitol (Sigma Aldrich) solution (Lambros & Vanderberg 1979). Cultures were transferred to a Falcon tube and centrifuged at 800 ***g*** for 5 minutes with no braking and the supernatant solution was removed. The volume of the remaining erythrocyte pellet was estimated, and the pellet was resuspended in 5 times the pellet volume of 5% sorbitol solution. The suspension was mixed thoroughly by repeated pipetting and left at room temperature for 5 minutes, before centrifuging as above, washing in RPMI 1640 and finally

resuspending in complete medium and returning to culture conditions. Parasites were routinely sorbitol-treated twice at least 6 hours apart to ensure tight synchronisation.

Phenotyping Invasion Assay

Erythrocyte labelling

50% haematocrit O+ erythrocytes were diluted to 2% haematocrit in RPMI 1640 and centrifuged at 450 ***g*** for 3 minutes. The pellet was resuspended to 2% haematocrit with 10 µM 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE) (Invitrogen) in RPMI 1640 and incubated for 2 hours at 37°C on a MACSmix™ tube rotator (Miltenyi Biotec). Following the incubation, erythrocytes were pelleted at 450 ***g*** for 3 minutes, washed with pre-warmed (37°C) complete medium, then resuspended to 2% haematocrit in complete medium and incubated for 30 minutes at 37°C on a MACSmix™ tube rotator. Erythrocytes were then pelleted at 450 ***g*** for three minutes, washed twice in complete medium, and resuspended at 2% haematocrit in incomplete medium (medium lacking human sera). The suspension was either used immediately or stored at 4°C for a period of no more than 24 hours.

Enzymatic treatment of DDAO-SE labelled O+ human erythrocytes

O+ human erythrocytes labelled with DDAO-SE (as described above) were centrifuged at 450 ***g*** for three minutes and the pellet was resuspended at 2% haematocrit with incomplete medium and aliquoted into individual 1.5 mL microfuge tubes. Neuraminidase from *Vibrio cholerae* (Sigma-Aldrich) was diluted in incomplete medium and added to the appropriate tubes to obtain a final concentration of 20 mU/mL. All tubes were then incubated at 37°C for 1 hour on a MACSmix™ tube rotator.

Following incubation, the tubes were centrifuged and the pellet was washed with incomplete medium. The pellets were resuspended to 2% haematocrit with incomplete medium. Trypsin (Sigma-Aldrich) or chymotrypsin (Sigma-Aldrich) was added to the appropriate tubes. The final concentration of trypsin was 50 µg/mL (low trypsin treatment) or 1 mg/mL (high trypsin treatment). The final concentration of chymotrypsin was 1 mg/mL. All tubes were incubated at 37°C for 1 hour while on a MACSmix™ tube rotator. Following

incubation, the tubes were centrifuged and the pellet was washed twice with incomplete medium before the pellet was resuspended at 2% haematocrit in complete medium.

Invasion Assays

For an invasion assay an aliquot of the culture was diluted with complete medium to 2% haematocrit and a parasitaemia of 1.5% before being transferred to a round-bottom 96-well plate in aliquots of 50 µL per well. Parasitaemia was determined prior to dilution by count of a thin smear stained with Field's stain. 50 µL of DDAO-SE labelled erythrocytes that had undergone differential enzyme treatment were then added to the appropriate wells resulting in a total culture volume of 100 µL in each well with a 1:1 ratio of parasitised erythrocytes (pRBC) to DDAO-SE labelled erythrocytes (sRBC). All invasion assays were carried out in triplicate, although if a post-invasion trypsin treatment was performed (described below) six replicate wells were started. Each well suspension was mixed thoroughly by repeated pipetting to try to ensure an equal probability of parasite reinvasion occurring into stained or unstained erythrocytes. Wells not in use were filled with PBS to reduce evaporation from the plate. The plate was placed in a gas chamber and malaria gas was run through the chamber for three minutes at high flow rate prior to sealing the chamber and incubation at 37°C for 48 hours.

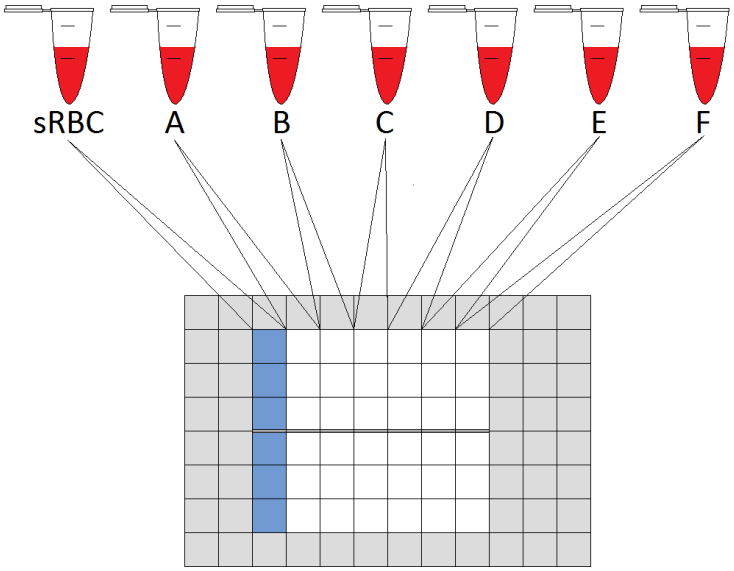


Fig. 2.1. Enzyme treatments in tubes and the 96-well plate set up.
Tubes: sRBC: stained untreated RBC; A: untreated (identical to Tube sRBC); B: neuraminidase treated; C: low trypsin treated; D: high trypsin treated; E: neuraminidase and high trypsin treated; F: chymotrypsin treated.
96-well plate: grey wells contain PBS; blue wells contain no parasites, only sRBC and unstained RBC (a control for flow analysis); white wells contain a 1:1 mix of sRBC and pRBC. Only the top three rows are trypsin treated post-invasion.

Post-Invasion Trypsin Treatment, Fixation and Parasite Labelling

After 48 hours, the plate was removed from incubation and centrifuged at 450 ***g*** for 3 minutes. 50 µL of supernatant solution was removed from each well and the pellets washed in 200 µL of PBS (Sigma Aldrich). A post invasion trypsin treatment was then performed for most assays, necessary because of a specific phenotype observed in some strains, as discussed in Chapter 3 "Results". For the trypsin treatment 100 µL of 1 mg/mL trypsin was added to the appropriate wells, and the wells were mixed thoroughly by repeated pipetting. The plate was incubated for 1 hour at 37°C before being centrifuged at 450 ***g*** for 3 minutes and each well washed once with 200 µL PBS. Fixation was performed by adding 200 µL of a 2% paraformaldehyde (PFA), 0.2% glutaraldehyde (GA) solution in PBS to each well and the plate incubated at 4°C for 1 hour. Each well was then washed once with 200 µL PBS.

Parasite labelling was achieved by using one of two DNA dyes. The first method required the cells to be permeabilised with 200 µL 0.3% Triton®X-100 (Sigma Aldrich, Dorset, UK) in PBS per well. The wells were mixed thoroughly by repeated pipetting and left at room temperature for 10 minutes. The plate was centrifuged at 450 ***g*** for 3 minutes and each well was then washed in 200 µL PBS. The pellet was resuspended with 200 µL 0.5 mg/mL ribonuclease A (MP Biomedicals) in PBS. Each well was mixed thoroughly and the plate was incubated at 37°C for 1 hour. Following incubation the plate was centrifuged at 450 ***g*** for 3 minutes and each well was then washed in 200 µL PBS. 200 µL of 1:5000 SYBR Green I (Invitrogen) in PBS was then added to each well and mixed thoroughly by repeated pipetting before the plate was incubated at 37°C for 1 hour. After incubation, the plate was centrifuged at 450 ***g*** for 3 minutes and each well was washed with 200 µL PBS. The PBS wash was repeated twice before each pellet was resuspended with 200 µL PBS and the plate was stored at 4°C in the dark for up to 72 hours.

The second method of parasite labelling was preferred as it did not require permeabilisation of cells or the RNase treatment. Following fixation and the subsequent wash with PBS, each pellet was resuspended in 200 µL of 2 µM Hoechst 33342 (Invitrogen) in RPMI 1640 and the plate was incubated at 37°C for 1 hour. After incubation, the plate was centrifuged at 450 ***g*** for 3 minutes before each well was washed three times with 200

µL PBS and the cells resuspended in 200 µL PBS and stored at 4°C in the dark for up to 72 hours.

Data Acquisition by Flow Cytometry and Data Analysis

25 µL aliquots were transferred from each stained well into a new flat-bottomed 96-well plate containing 250 µL RPMI 1640 per well. The samples were examined with a 355 nm 20 mW UV laser, a 488nm 20 mW blue laser and a 633 nm 17 mW red laser on a BD LSRII flow cytometer (BD Biosciences). Hoechst 33342 was excited by a UV laser and detected by a 450/50 filter. DDAO-SE was excited by a red laser and detected by a 660/20 filter. BD FACS Diva (BD Biosciences) software was used to detect 100,000 events per well. The data collected were exported to FlowJo (Tree Star) for further analysis. Statistical analysis and phenotype profiles of parasitaemia were generated using GraphPad Prism (GraphPad Software). For the purpose of phenotyping, all experiments were carried out with six replicates (half underwent post-invasion trypsin treatment) and were performed twice in two separate life cycles, with data presented as the mean ± standard error of the mean of both experiments combined unless otherwise stated.

Parasite DNA Extraction and Sequencing

Preparation of Cultures for DNA Extraction

Cultures were synchronised and the volume was expanded to at least 50 mL at 5% haematocrit, with a minimum parasitaemia of 2%. When parasites were in the late trophozoite or schizont stage the culture was transferred to a Falcon tube and centrifuged at 800 ***g*** for 5 minutes with no braking. The pellet was washed in 20 mL RPMI 1640, centrifuged again, and then as much supernatant solution was removed as possible. The tube containing the pellet was then placed in a -20°C freezer prior to DNA extraction.

DNA Extraction

Parasite DNA was extracted and purified from erythrocyte pellets using the QIAamp Blood Maxi Kit (Qiagen®). Sequencing was then undertaken in three steps: library preparation, bridge amplification and sequencing by synthesis.

DNA extraction from isolates was carried out by Professor Dominic Kwiatkowski's team.

Library Preparation

Parasitic DNA was randomly fragmented by sonication and library preparation was carried out using an Illumina Library Prep Kit (Illumina). DNA fragments are purified and the overhangs resulting from sonication are repaired to blunt ends using T4 DNA polymerase and *E. coli* DNA polymerase I Klenow fragment. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs. The 3' ends are adenylated using the polymerase activity of the Klenow fragment. The fragments can then be ligated to adapters that have a single thymine base overhang at their 3' ends. Solid Phase Reversible Immobilisation (SPRI) paramagnetic beads (Beckman Coulter) are then used to purify and size select fragments of 200 – 300 bp.

Bridge Amplification

Oligos complementary to the adapters are irreversibly bound to the surface of the flow cell. When the library fragments are added, the flow cell is heated to denature the DNA molecules into single strands which hybridise to the complementary oligos. Polynucleotide synthesis uses the oligo as a primer and the ssDNA as a template. The result is a double stranded DNA molecule with each strand anchored to the flow cell at one end by the attached oligo. Denaturation leaves two ssDNA templates for the next round of clonal amplification. In this fashion, several million unique ssDNA clusters are generated in each of the eight flow cell channels. The ends of each strand are then blocked and a sequencing primer is hybridised to each strand.

Sequencing by Synthesis

Clusters are sequenced simultaneously, base by base using an Illumina IIx Genome Analyser. Four fluorescently labelled, reversibly terminated bases are added to the flow cell and compete to hybridise to the template, with only the base complementary to the template able to hybridise. The cluster is then excited by a laser, with the colour emitted being different for each of the four bases allowing the sequence to be determined. The

fluorescent label and blocking group that prevents the addition of more than one base at a time are then removed and the cycle is repeated. From the 200 – 300 bp fragments generated in library preparation of Peruvian field isolates, 76 bp were sequenced from each end.

Genotyping Analysis

Whole genome sequencing analysis was carried out using tools developed by Professor Kwiatkowski's team at the Wellcome Trust Sanger Institute.

SNP-o-matic is a tool that aligns paired Illumina reads to the 3D7 reference sequence. Putative variable positions are specified at the outset. Reads are aligned only if they are perfect matches to the reference sequence, unless the variation occurs in one of the specified variable positions. The requirement for perfect matches means that false SNP calls are not made from misaligned reads (Manske & Kwiatkowski 2009).

MapSeq (http://www.sanger.ac.uk/MapSeq) is a browser-based tool for analysing *Plasmodium falciparum* genomes. MapSeq generates a library of all single nucleotide polymorphisms (SNPs) for a given strain, and lists them in order of location in the genome. It allows SNPs to be browsed and analysed across the whole genome, or by specific genome region or gene. Many parameters are user-defined, such as the type of SNP to be analysed (in coding or non-coding regions, synonymous or non-synonymous), or the stringency of evidence required to call SNPs (minimum number of reads required, minimum percentage of samples that a SNP has to be seen in). For analysis of Peruvian strains the default settings were used: a minimum of 10 reads were required for a SNP call, and a minimum of 75% of samples had to be called at that position.

MapSeq also allows the user to link to LookSeq, a browser-based tool to view read alignments from a whole chromosome down to a single base (Manske & Kwiatkowski 2009). Multiple samples can also be viewed and compared simultaneously. SNPs in erythrocyte invasion ligands were all verified by direct inspection of the individual reads via LookSeq.

Mutabo! is a browser-based program that generates a list of all amino acid changes for non-synonymous SNPs. It does not allow the minimum number of reads required for a

call to be pre-determined so all amino acid changes generated in Mutabo! had to be checked with MapSeq to verify sequence quality.

Parasite RNA Extraction and Sequencing

Schizont Enrichment in Preparation for RNA Extraction

Each culture was expanded to 50 mL at 5% haematocrit and synchronised at the ring stage using 5% sorbitol treatments, as detailed previously. A minimum parasitaemia of 2% was required. The development of the parasites was followed by regular blood smear microscopy and Field's staining, as above. When the majority of parasites were found to be schizonts, these were purified using a 60% Percoll™ gradient (GE Healthcare) (Saul et al. 1982). For Percoll™ purification, the erythrocytes were pelleted at 800 ***g*** for 5 minutes with no braking, to produce a 2.5 mL erythrocyte pellet. All subsequent centrifugations were carried out at 4°C and all solutions were kept on ice. The pellet was resuspended with 20 mL complete medium containing 10% heat-inactivated fetal bovine serum (FBS) and centrifuged at 800 ***g*** for 5 minutes with no braking. The supernatant solution was removed and the pellet washed again in FBS-containing complete medium. The 2.5 mL erythrocyte pellet was then resuspended in 22.5 mL of FBS-complete medium and loaded on top of 31.25 mL of a 60% Percoll solution before centrifugation for 20 minutes at 1500 ***g*** with no braking. The top layer containing the enriched schizonts was then collected into a new tube containing 20 mL PBS, the schizonts were pelleted as above and washed with PBS. The purity of the final pellet was evaluated by light microscopy with Field's staining, before the pellet was resuspended in 1 mL RNALater (Ambion®) and stored at -20°C.

RNA Extraction from Schizonts

Purified schizonts stored in RNALater were thawed and pelleted by centrifugation at 8000 ***g*** for 1 minute. The supernatant solution was removed and RNA was extracted from the schizonts using a Qiagen QIAamp® RNA Blood Mini Kit (Qiagen®). The protocol used was "Purification of Total Cellular RNA from Human Whole Blood", and included the "Optional On-Column DNase Digestion with the RNase-Free DNase Set". The final elution with RNase-free water was performed with a final volume of 60 µL. The quantity and purity

of RNA was then assessed by placing 3 – 4 µL on an Implen NanoPhotometer™ (Implen). The samples were stored at -80°C.

Freezing of Parasites

Aliquots were frozen from cultures with a parasitaemia of at least 2% rings. The culture was centrifuged at 800 ***g*** for 5 minutes with no braking and the pellet was washed once with 10 mL RPMI 1640. After centrifugation, the pellet volume was estimated and half the pellet volume of glycerolyte 57 solution was added one 25 µL drop at a time. The suspension was incubated at room temperature for 5 minutes before a further one and a half times the pellet volume of glycerolyte 57 solution was added one 50 µL drop at a time. The suspension was then aliquoted to tubes with approximately 200 µL of pellet per tube. These tubes were placed in a -80°C freezer for at least 24 hours before being transferred to storage in liquid nitrogen vapour at -130°C.

Fluorescence Microscopy DDAO-SE or Hoechst 33342 Stained Samples

Thin blood smears were made on a slide and air dried for a minimum of 4 hours. The samples were fixed by exposure to a 1:1 methanol & acetone solution. After drying, a drop of Fluoro-Gel with Anti-Fading Mounting Medium (Electron Microscopy Sciences) was placed on the slide and a coverslip was put on top. The slide was dried at 4°C for 24 hours. Slides were observed using either a Leica DM2500 fluorescence microscope and a Leica LAS AF camera (Leica Microsystems) or a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss) and a Leica DFC420C camera (Leica Microsystems). Leyla Bustamante lent invaluable assistance in performing confocal microscopy.

Additional Techniques Used

When two Peruvian field isolates (3135 & 3769) were phenotyped by invasion assay, extra populations were observed by flow cytometry. The following techniques were used to investigate the cause of these unforeseen populations.

Erythrocyte Rosetting Assay

An aliquot from Peru strain 3769 was diluted to 2% haematocrit with complete media. 1 mL was centrifuged before the pellet was resuspended with 2 µM Hoechst 33342 in complete media, maintaining a 2% haematocrit. The suspension was incubated at 37°C for 1 hour before being centrifuged and washed with complete media three times. The pellet was then resuspended and diluted to 0.5% haematocrit with complete media and 4 µL was placed on a slide with a coverslip sealed on top with nail varnish. Using a Leica DM2500 fluorescence microscope, 200 parasitised red blood cells were counted. Rosettes were defined as one parasitised erythrocyte binding two or more uninfected erythrocytes (Doumbo et al. 2009).

Electron Microscopy of Parasites

A 10 mL culture at 5% haematocrit was cultured to a high parasitaemia before being fixed in 2% PFA / 0.2% GA in PBS. David Goulding was responsible for preparing the samples further and imaging.

Merozoite Detection Using anti-MSP-1 Antibody

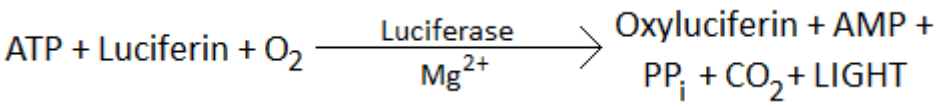
Aliquots from two Peruvian strains, 3769 and 3541, were both adjusted separately to 1% haematocrit, synchronised cultures of 6% rings and 2% schizonts. 100 µL aliquots were transferred to a round-bottom 96 well plate. The plate was centrifuged at 450 ***g*** for 3 minutes, and the supernatant solution was removed. Wells containing schizonts were resuspended in 100 µL 2% PFA / 0.2% GA in PBS, while wells containing rings were resuspended in PBS. The plate was incubated at 4°C for 1 hour before being centrifuged as above, and all wells were washed with PBS. Wells containing schizonts were then permeabilised by resuspending the pellet with 100 µL 0.3% Triton®X-100 in PBS and incubating the plate for 10 minutes at room temperature. Wells containing rings were

resuspended in 100 µL PBS. Following the permeabilisation step, the plate was centrifuged as above, and all pellets were washed three times with 100 µL PBS. 100 µL 1% bovine serum albumin (Sigma Aldrich) in PBS was added to each pellet and the plate was incubated for 30 minutes at room temperature. The plate was centrifuged (as above) and the supernatant solution removed. Mouse monoclonal anti-MSP-1 19 kDa IgG antibody (abcam) was diluted 1:500 in 1% bovine serum albumin (BSA) in PBS and 100 µL was added to each of the appropriate wells. Control samples only being exposed to the secondary antibody were resuspended with 100 µL 1% BSA in PBS. The plate was incubated at room temperature for 1 hour. Subsequently, the plate was centrifuged as above and all wells were washed with 100 µL 1% BSA in PBS three times. Goat polyclonal IgG secondary antibody conjugated with FITC (abcam) was then diluted 1:2000 with 1% BSA in PBS and 100 µL was added to all wells. The plate was incubated at room temperature for one hour before all wells were washed three times with 100 µL 1% BSA in PBS. Wells containing rings were fixed with 2% PFA / 0.2% GA in PBS using the same protocol as above. All pellets were then washed three times with 100 µL PBS, before being resuspended in 100 µL of PBS. Wells were acquired using a BD Calibur flow cytometer (BD Biosciences) and 100,000 events were counted.

Peruvian isolate 3541 at a parasitaemia of 6% rings was also probed with an antibody to CD147. The method was the same as used above for wells containing rings except for the steps involving antibodies. Prior to adding the primary antibody, an aliquot of 6 µL of the 1% haematocrit suspension was added to a new well containing 94 µL 1% BSA in PBS (i.e. the haematocrit of the new well was 0.06%). 0.5 µL of primary monoclonal mouse anti-CD147 IgG antibody (Exbio Antibodies) was added to the appropriate wells and the plate was incubated at room temperature for 1 hour. After centrifugation and three washes with 1% BSA in PBS, the wells were resuspended in 1% BSA in PBS. 0.5 µL of goat polyclonal IgG secondary antibody (abcam) conjugated with FITC was added to the appropriate wells and the plate was incubated at room temperature for 1 hour. After washing with PBS, as above, 10,000 events were acquired using a BD Calibur flow cytometer.

Mycoplasma Detection Assay

Three cultures, Peru 788, 3769 and laboratory strain 7C126 were cultured from 0.6% rings for 72 hours without replacing the media. Each culture was then tested using a MycoAlert® Mycoplasma Detection Kit (Lonza). 100 µL of the culture supernatant solution was transferred to a luminometer cuvette. 100 µL of MycoAlert® Reagent was added and each sample was incubated for 5 minutes at room temperature. The purpose of the MycoAlert® Reagent was to lyse any mycoplasma present. A reading (A) of luminescence was taken using an Orion II Microplate Luminometer (Berthold Detection Systems). 100 µL of MycoAlert® substrate was added and each sample was incubated for 10 minutes at room temperature. A second reading (B) of luminescence was taken. Enzymes released by the lysing of the mycoplasma react with MycoAlert® substrate, catalysing the phosphorylation of ADP to ATP. If mycoplasma are present the rise in ATP is detected by bioluminescence according to the following reaction:



A ratio of luminescence readings B:A >1 is indicative of mycoplasma contamination.