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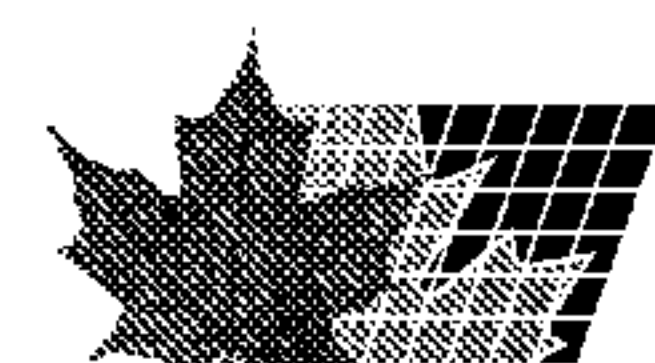
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(54) Title: PLASMODIUM FALCIPARUM AMA-1 PROTEIN AND USES THEREOF

(57) **Abrégé/Abstract:**

In this application is described the expression and purification of a recombinant Plasmodium falciparum (3D7) AMA-1 ectodomain. The method of the present invention produces a highly purified protein which retains folding and disulfide bridging of the native molecule. The recombinant AMA-1 is useful as a diagnostic reagent, for use in antibody production, and as a vaccine.



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(54) Title: *PLASMODIUM FALCIPARUM* AMA-1 PROTEIN AND USES THEREOF

(57) Abstract: In this application is described the expression and purification of a recombinant *Plasmodium falciparum* (3D7) AMA-1 ectodomain. The method of the present invention produces a highly purified protein which retains folding and disulfide bridging of the native molecule. The recombinant AMA-1 is useful as a diagnostic reagent, for use in antibody production, and as a vaccine.



WO 02/077195 A2

TITLE OF THE INVENTION

Plasmodium falciparum AMA-1 Protein and Uses Thereof

5

FIELD OF THE INVENTION

10 The present invention relates to a purified *Plasmodium falciparum* protein. More particularly, this invention is directed to a composition comprising a portion of the *Plasmodium falciparum* AMA-1 protein for use as a vaccine.

INTRODUCTION

15 *Plasmodium falciparum* causes more than three million deaths each year, mostly among children below the age of five (World Health Organization Tropical Disease Research, 1997, TDR Twelfth Program Report, p. 57-76). The spread of multi-drug resistant strains of
20 the parasite has underlined an urgent need for a malaria vaccine. Evidence exists both from animal models and human studies that antibodies to erythrocytic and exo-erythrocytic parasite antigens can induce protection. Apical membrane antigen-1
25 (AMA1) is one of the most promising erythrocytic stage vaccine targets under investigation. Present on the extra-cellular merozoite stage of the parasite, AMA1, is amenable to host immune intervention during the process of invasion. Indeed, immunization in animal
30 models with affinity purified or recombinant forms of AMA1 along with adjuvants permissible for human-use, can induce a protective response against homologous parasite challenge *in vivo* (Deans, et al., 1988, Parasite Immunol. 10, 535-552; Collins et al., 1994,

Am. J. Trop. Med. Hyg. 51, 711-719; Narum et al.,
2000, Infect. Immun. 68, 2899-2906; Anders et al.,
1998, Vaccine 16, 240-247). Homologs of AMA1 gene have
been identified in all of the commonly studied species
5 of *Plasmodium* (Peterson et al., 1989, Mol. Cell. Biol.
9, 3151-3154; Cheng and Saul, 1994, Mol. Biochem.
Parasitol. 65, 183-187; Dutta et al., 1995, Mol.
Biochem. Parasitol. 73, 267-270; Waters et al., 1990,
J. Biol. Chem. 265, 17974-17979; Kocken et al., 2000,
10 Mol. Biochem. Parasitol. 109, 147-156; Peterson et
al., 1990, Mol. Biochem. Parasitol. 39, 279-284;
Marshall et al., 1989, Mol. Biochem. Parasitol. 37,
281-284) and knockout studies have revealed that
expression of AMA1 protein is vital for the parasite
15 survival (Triglia et al., 2000, Mol. Microbiol. 38,
706-718).

P. falciparum AMA1 is an integral membrane
protein, synthesized as a 72 kDa (apparent molecular
weight: 83 kDa) polypeptide (Peterson et al., 1989,
20 supra), it is localized in the apical rhoptries of the
merozoites present within late stage schizont (Narum
and Thomas, 1994, Mol. Biochem. Parasitol. 67, 59-68).
Around the time of schizont rupture and erythrocyte
invasion, AMA1 of *P. falciparum* has been shown to be
25 processed to a smaller 66 kDa protein, which is
further proteolytically cleaved to 44 and 48 kDa
soluble fragments (Kocken et al., 1998, J. Biol. Chem.
273, 15119-24; Howell et al., 2001, J. Biol. Chem.
276, 31311-31320). Compared to several other blood
30 stage antigens, AMA1 of *P. falciparum*, shows limited
inter-strain polymorphism (Escalante et al., 2001,
Mol. Biochem. Parasitol. 113, 279-287). During natural
infection, AMA1, induces both B and T-cell responses
(Thomas et al., 1994, Am. J. Trop. Med. Hyg. 51, 730-
35 740; Lal et al., 1996, Infect. Immun. 64, 1054-1059)

and antibodies to both recombinant *P. falciparum* AMA1 and affinity purified naturally induced anti-AMA1 inhibit growth or invasion of *P. falciparum* parasite *in vitro* (Hodder et al., 2001, Infect. Immun. 69, 3286-3294). The ectodomain of AMA1 comprises a region constituting 16 inter-species conserved cysteine residues. These cysteine residues are cross-linked to form 8 disulphide bridges, which in turn, divide the ectodomain into 3 subdomains (Hodder et al., 1996, J. Biol. Chem. 271, 29446-29452). Correct folding, vis-à-vis the presence of these disulphide bonds, in the case of recombinant *P. chabaudi* and *P. falciparum* AMA1 proteins, has been shown to be critical for the induction of inhibitory anti-AMA1 antibodies (Anders et al., 1998, supra; Hodder et al., 2001, supra).

Although its function remains unclear, there is a growing need to focus resources on a human trial to evaluate the protective potential of AMA1 of *P. falciparum* in human volunteers.

Full length *P. falciparum* AMA1 was first expressed in the eukaryotic insect cell system (Narum et al., 1993, J. Chromatogr. A. 657, 357-363), although the baculovirus product was soluble, the purification strategy was not designed for scale-up production. Prokaryotic expression of AMA1 from various species has been problematic, primarily due to the formation of insoluble aggregates presumably due to incorrect folding of the protein. Previous work on *P. chabaudi* AMA1 expression in *E. coli* showed that it was necessary to include an *in vitro* refolding step in the process in order to obtain correctly folded protein (Anders et al., 1998, supra). A similar approach was successful for obtaining correctly folded AMA1 from *P. falciparum* and the antibodies made against it inhibited parasite growth *in vitro* (Hodder

et al., 2001, supra). A scalable process for the production of recombinant AMA1 has not yet been described.

In this application we describe the expression of a synthetic gene encoding 449 amino acids encompassing the 3 subdomains of the AMA1 ectodomain from *P. falciparum* in *E. coli*. The protein designated as r-AMA1/E ('r' stands for recombinant and 'E' represents the *E. coli* codon bias of the synthetic gene) was refolded and purified and the final protein product was designated as AMA1/E. Biochemical characterization and evidence of correct folding of AMA1/E are presented. In addition, the *in vitro* parasite invasion data with antibodies raised against AMA1/E reaffirms the potential of AMA1 to be an important component of a future malaria vaccine.

SUMMARY OF THE INVENTION

Therefore, the present invention provides a method for proper expression and purification of an AMA-1 3D7 allele. The method of the present invention results in elimination of contaminating proteins and conservation of the native folding and disulfide bridging of the protein. Therefore, the essentially purified protein of the present invention retains proper conformation for optimal reactivity for vaccine and screening purposes.

The protein of the present invention is encoded by a unique DNA fragment of the ectodomain of the AMA-1 protein. Among different DNA fragments isolated from the ectodomain using polymerase chain reaction, only one nucleotide fragment encoding amino acids 83-531 of AMA-1 was found to be stable. This DNA fragment retained all 16 cysteines required for the formation of the 8 intra-molecular disulphide linkages of AMA-1 but did not have the leader sequence. We

were able to successfully, for the first time, express this fragment in a bacterial host and have developed a four-step purification scheme that produces >99% pure protein product, AMA1/E, which is properly folded,
5 i.e. retains its disulfide bridges, and is stable. Furthermore, the AMA1/E protein of the present invention was found to induce a protective immune response in rabbits. This was surprising since the DNA fragment encoding the protein of the present
10 invention did not contain a leader sequence thought to be important for immunogenicity of the protein.

Therefore, according to an aspect of the present invention, there is provided a recombinant *P. falciparum* AMA-1 protein, AMA1/E, for use as a vaccine, in diagnostic
15 assays, and for production of antibodies.

According to another aspect of the present invention, there is provided compositions comprising purified recombinant *P. falciparum* AMA1/E.

According to another aspect of the present invention,
20 there is provided novel vector constructs for recombinantly expressing *P. falciparum* AMA1/E, as well as host cells transformed with said vector.

According to another aspect of the present invention, there is provided a method for producing and purifying
25 recombinant *P. falciparum* AMA1/E protein comprising:

growing a host cell containing a vector expressing *P. falciparum* AMA1/E proteins in a suitable culture medium,

causing expression of said vector sequence as defined above under suitable conditions for production of soluble
30 protein, and

lysing said transformed host cells and recovering said AMA1/E protein such that it retains its native folding and is essentially free of host toxins.

According to another aspect of the present invention, there is provided diagnostic and immunogenic uses of the recombinant *P. falciparum* AMA1/E protein of the present invention, as well as to provide kits for diagnostic use for example in malaria screening and confirmatory antibody tests.

According to another aspect of the present invention, there is provided monoclonal or polyclonal antibodies, more particularly human monoclonal antibodies or mouse monoclonal antibodies which are humanized, which react specifically with AMA1/E epitopes, either comprised in peptides or conformational epitopes comprised in recombinant proteins.

According to another aspect of the present invention, there is provided possible uses of anti-AMA1/E monoclonal antibodies for malaria antigen detection or for therapy of chronic malaria infection.

According to another aspect of the present invention, there is provided a malaria vaccine comprising AMA1/E of the present invention, in an amount effective to elicit an immune response in an animal against *P. falciparum*; and a pharmaceutically acceptable diluent, carrier, or excipient.

According to another aspect of the present invention, there is provided a method for eliciting in a subject an immune response against malaria, the method comprising administering to a subject a composition comprising AMA1/E of the present invention. In one aspect of the invention, the DNA vaccine is delivered along with an adjuvant.

According to another aspect of the present invention, there is provided a method for preventing malaria infection in an animal comprising administering to the animal the AMA1/E of the present invention.

The vaccine according to the present invention is inherently safe, is not painful to administer, and should not result in adverse side effects to the vaccinated individual.

5 The present invention also provides a method for fermenting and inducing the host cells, and a method for isolating and purifying the recombinant protein. Also provided is a method for bulk fermentation and expression of AMA1/E.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram of the plasmid pWRMAL containing the DNA sequence encoding *Plasmodium falciparum* AMA1/E (Pf AMA1/E).

Fig. 2A and 2B. **2A.** SDS-PAGE analysis of AMA1/E during purification: Results from a lab-grade purification starting with 20 g of cell paste. Protein analysis was done on a 4-12% gradient gel under reduced conditions and the gel was stained with Coomassie blue. The elutions off DEAE and SP Sepharose columns were concentrated on a 3.5 K cut-off Centricon concentrator before loading. Lane 1, *E. coli* lysate loaded on the Ni⁺² column; lane 2, Ni⁺² column elution; lane 3, DEAE Sepharose column elution (5 µg protein); lane 4, SP Sepharose column elution (5 µg protein; lab-grade AMA1/E product). **2B.** Western blot analysis for *E. coli* protein detection (Cygnus HCP detection kit). Lanes 1-5, OrigamiTM (DE3) bacterial lysate with 4000, 2000, 1000, 500 and 250 ng protein respectively; lanes 6-11, AMA-1/E product at 1, 10, 100, 500, 1000 and 2000 ng protein per well, respectively.

Fig. 3A and 3B. Analytical HPLC profile of the AMA1/E product: Detector output at 215 nm with

35

absorbance units (AU) plotted against time (min). (A) Gel-permeation Shodex Protein KW-803 column elution profile with 10 µg AMA1/E injected (broken line); equal volume of final formulation buffer with no protein injected (solid line). (B) Reversed-phase C8 Aquapore RP-300 Å, 7 µ, 30x2.1 mm column elution profile, with 4 µg AMA1/E injection (solid line); shift in retention time observed under the same chromatographic conditions with 12 µg AMA1/E reduced in the presence of 6M guanidine HCl and 25 mM DTT and injected (broken line). See Materials and Methods for solvent and gradient information.

Fig. 4A, 4B, and 4C. Relative SDS-PAGE mobility, alkylation analysis and immune reactivity of AMA1/E: Lane 1, AMA1/E protein (~200 ng) in 4 M urea; lane 2, AMA1/E in 4 M urea treated with iodoacetamide; lane 3, AMA1/E in 4 M urea, reduced with DTT followed by iodoacetamide treatment (see Materials and Methods for reaction details). (A) Proteins separated on a non-reducing 4-12% gradient gel and stained with Coomassie blue. (B) Western blot of the gel shown in (A), immuno-stained with mAb 4G2dc1 and developed with HRP-POD substrate. (C) Western blot of the gel shown in (A), immuno-stained with an immune serum pool collected from Western Kenya and developed with HRP-POD substrate.

Fig. 5A and 5B. Recognition of the parasite AMA1 with anti-AMA1/E antibodies produced in rabbit: (A) Anti-AMA1/E antibodies (R-1) used for IFA on *P. falciparum* (3D7) parasites fixed with methanol. Merozoites contained within the late-stage schizonts are shown with bright fluorescence (1000X). (B) Western blot of *P. falciparum* 3D7 parasite, late schizont proteins, extracted with SDS-PAGE loading

buffer, separated on a non-reducing gel, western blotted and immuno-stained with anti-AMA1/E antibodies and developed with a chemiluminescent substrate. The top and bottom arrows represent ~76 and 62 kDa
5 respectively. Lane 1, post-immune rabbit sera (R-1); lane 2, pre-immune serum control.

Fig. 6. Purified anti-AMA1/E IgG inhibited growth by *P. falciparum* 3D7 parasite in a GIA and the inhibition was reversed by addition of the antigen.
10 One-cycle GIA with 0.18, 0.35, or 0.7 mg ml⁻¹ purified rabbit IgG in suspension culture GIA in 48-well plates. solid circle = anti-AMA1/E IgG; open circle = anti-RA-AMA1/E IgG. Antigen (5.3 µg ml⁻¹) added to 0.18 mg ml⁻¹ anti-AMA1/E IgG; solid triangle = RA-AMA1/E
15 antigen added; solid square = AMA1/E antigen added (symbols are offset for clarity). Mean ± SD shown along with number of experiments for each data point are represented within brackets.

Fig. 7. Competitive ELISA using individual sera.
20 Sera samples at 1:1000 dilution were pre-incubated with either RA-AMA1/E (RA), AMA1/E (RF) or with bovine serum albumin protein (AL). ELISA was done to assay for anti-AMA1/E specific antibodies. Shown here is the average OD₄₀₅ at 1:16000 dilution for AMA1/E immunized
25 rabbits (R-1, 2 and 3), represented by clear bars. RA-AMA1/E immunized rabbits (R-7 and 10), represented by black bars. Mean + SD is represented by a line on top of each bar.

DETAILED DESCRIPTION

30 In the description that follows, a number of terms used in recombinant DNA, parasitology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be

given such terms, the following definitions are provided.

In general, an 'epitope' is defined as a linear array of 3-10 amino acids aligned along the surface of a protein. In a linear epitope, the amino acids are joined sequentially and follow the primary structure of the protein. In a conformational epitope, residues are not joined sequentially, but lie linearly along the surface due to the conformation (folding) of the protein. With respect to conformational epitopes, the length of the epitope-defining sequence can be subject to wide variations. The portions of the primer structure of the antigen between the residues defining the epitope may not be critical to the structure of the conformational epitope. For example, deletion or substitution of these intervening sequences may not affect the conformational epitope provided sequences critical to epitope conformation are maintained (e.g. cysteines involved in disulfide bonding, glycosylation sites, etc.). A conformational epitope may also be formed by 2 or more essential regions of subunits of a homo-oligomer or hetero-oligomer. As used herein, 'epitope' or 'antigenic determinant' means an amino acid sequence that is immunoreactive. As used herein, an epitope of a designated polypeptide denotes epitopes with the same amino acid sequence as the epitope in the designated polypeptide, and immunologic equivalents thereof. Such equivalents also include strain, subtype (=genotype), or type(group)-specific variants, e.g. of the currently known sequences or strains belonging to *Plasmodium* such as 3D7, FVO and CAMP, or any other known or newly defined *Plasmodium* strain.

The term 'solid phase' intends a solid body to which the individual *P. falciparum* antigen is bound

covalently or by noncovalent means such as hydrophobic, ionic, or van der Waals association.

The term 'biological sample' intends a fluid or tissue of a mammalian individual (e.g. an anthropoid, a human), reptilian, avian, or any other zoo or farm animal that commonly contains antibodies produced by the individual, more particularly antibodies against malaria. The fluid or tissue may also contain *P. falciparum* antigen. Such components are known in the art and include, without limitation, blood, plasma, serum, urine, spinal fluid, lymph fluid, secretions of the respiratory, intestinal or genitourinary tracts, tears, saliva, milk, white blood cells and myelomas. Body components include biological liquids. The term 'biological fluid' refers to a fluid obtained from an organism. Some biological fluids are used as a source of other products, such as clotting factors (e.g. Factor VIII;C), serum albumin, growth hormone and the like.

The term 'immunologically reactive' means that the antigen in question will react specifically with anti-AMA-1 antibodies present in a body component from a malaria infected individual.

The term 'immune complex' intends the combination formed when an antibody binds to an epitope on an antigen.

The term 'AMA1/E' (also referred to as PfAMA/e, AMA-1/E, PfAMA-1/E, FMP2.0, FMP2.1) as used herein refers to the protein fragment or polypeptide resulting from expression of a DNA fragment encoding amino acids 83 to 531 of *P. falciparum* 3D7 AMA-1. The full length sequence of *P. falciparum* 3D7 AMA-1 has been deposited in GenBank under accession number U65407.1.

The term 'AMA1/E' as used herein also includes analogs and truncated forms that are immunologically identifiable with the native AMA-1. By 'AMA1/E' is intended AMA1/E from other strains of *Plasmodium*
5. *falciparum* such as 3D7, Camp, FVO, and others, or any other newly identified strain of *Plasmodium*.

The term 'purified' as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35% of the total protein
10 component in the composition. The desired protein preferably comprises at least 40%, more preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at
15 least about 90%, and most preferably at least about 95% of the total protein component. The composition may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, without affecting the determination of the percentage purity
20 as used herein. An 'isolated' AMA-1 protein intends a *Plasmodium* protein composition that is at least 35% pure.

The term 'essentially purified proteins' refers to proteins purified such that they can be used for *in*
25 *vitro* diagnostic methods and as a prophylactic compound. These proteins are substantially free from cellular proteins, vector-derived proteins or other *Plasmodium* components. The proteins of the present invention are purified to homogeneity, at least 80%
30 pure, preferably, 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%.

The term 'recombinantly expressed' used within the context of the present invention refers to the
35 fact that the proteins of the present invention are

produced by recombinant expression methods be it in prokaryotes, or lower or higher eukaryotes as discussed in detail below.

The term 'lower eukaryote' refers to host cells
5 such as yeast, fungi and the like. Lower eukaryotes are generally (but not necessarily) unicellular. Preferred lower eukaryotes are yeasts, particularly species within *Saccharomyces*. *Schizosaccharomyces*,
10 *Kluveromyces*, *Pichia* (e.g. *Pichia pastoris*), *Hansenula* (e.g. *Hansenula polymorpha*, *Yarrowia*, *Schwaniomyces*, *Schizosaccharomyces*, *Zygosaccharomyces* and the like. *Saccharomyces cerevisiae*, *S. carlsbergensis* and *K. lactis* are the most commonly used yeast hosts, and are convenient fungal hosts.

15 The term 'prokaryotes' refers to hosts such as *E.coli*, *Lactobacillus*, *Lactococcus*, *Salmonella*, *Streptococcus*, *Bacillus subtilis* or *Streptomyces*. Also these hosts are contemplated within the present invention.

20 The term 'higher eukaryote' refers to host cells derived from higher animals, such as mammals, reptiles, insects, and the like. Presently preferred higher eukaryote host cells are derived from Chinese hamster (e.g. CHO), monkey (e.g. COS and Vero cells),
25 baby hamster kidney (BHK), pig kidney (PK15), rabbit kidney 13 cells (RK13), the human osteosarcoma cell line 143 B, the human cell line HeLa and human hepatoma cell lines like Hep G2, and insect cell lines (e.g. *Spodoptera frugiperda*). The host cells may be
30 provided in suspension or flask cultures, tissue cultures, organ cultures and the like. Alternatively the host cells may also be transgenic animals.

The term 'polypeptide' refers to a polymer of amino acids and does not refer to a specific length of
35 the product; thus, peptides, oligopeptides, and

proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, PNA, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term `recombinant polynucleotide or nucleic acid` intends a polynucleotide or nucleic acid of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation : (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

The term `recombinant host cells`, `host cells`, `cells`, `cell lines`, `cell cultures`, and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer polynucleotide, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

The term `replicon` is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that

behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

The term `vector` is a replicon further
5 comprising sequences providing replication and/or expression of a desired open reading frame.

The term `control sequence` refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are
10 ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include
15 promoters, terminators and, in some instances, enhancers. The term `control sequences` is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous,
20 for example, leader sequences which govern secretion.

The term `promoter` is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the
25 normal transcription initiation site for the adjacent structural gene.

The expression `operably linked` refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their
30 intended manner. A control sequence `operably linked` to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

An `open reading frame` (ORF) is a region of a
35 polynucleotide sequence which encodes a polypeptide

and does not contain stop codons; this region may represent a portion of a coding sequence or a total coding sequence.

A `coding sequence` is a polynucleotide sequence
5 which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop
10 codon at the 3'-terminus. A coding sequence can include but is not limited to mRNA, DNA (including cDNA), and recombinant polynucleotide sequences.

The term `immunogenic` refers to the ability of a substance to cause a humoral and/or cellular response,
15 whether alone or when linked to a carrier, in the presence or absence of an adjuvant. `Neutralization` refers to an immune response that blocks the infectivity, either partially or fully, of an infectious agent. A `vaccine` is an immunogenic
20 composition capable of eliciting protection against malaria, whether partial or complete. A vaccine may also be useful for treatment of an infected individual, in which case it is called a therapeutic vaccine.

25 The term `therapeutic` refers to a composition capable of treating malaria infection.

The term `effective amount` for a therapeutic or prophylactic treatment refers to an amount of epitope-bearing polypeptide sufficient to induce an
30 immunogenic response in the individual to which it is administered, or to otherwise detectably immunoreact in its intended system (e.g., immunoassay). Preferably, the effective amount is sufficient to effect treatment, as defined above. The exact amount
35 necessary will vary according to the application. For

vaccine applications or for the generation of polyclonal antiserum/antibodies, for example, the effective amount may vary depending on the species, age, and general condition of the individual, the severity of the condition being treated, the particular polypeptide selected and its mode of administration, etc. It is also believed that effective amounts will be found within a relatively large, non-critical range. An appropriate effective amount can be readily determined using only routine experimentation. Preferred ranges of AMA1/E for prophylaxis of malaria disease are about 0.01 to 1000 ug/dose, more preferably about 0.1 to 100 ug/dose, most preferably about 10-50 ug/dose. Several doses may be needed per individual in order to achieve a sufficient immune response and subsequent protection against malaria.

More particularly, the present invention contemplates essentially purified AMA1/E and a method for isolating or purifying recombinant AMA1/E protein, characterized in that the recombinantly expressed protein retains the disulfide bonds necessary for proper folding of the protein.

The AMA1/E protein of the present invention spans from amino acid 83 to 531 of AMA-1 3D7 allele (GenBankTM Accession No U65407.1). Upon expression in the parasite system (non-glycosylated), AMA1/E is believed to have an approximate molecular weight of 54 kDa as determined by reduced SDS-PAGE. The term 'AMA1/E' refers to a polypeptide or an analogue thereof (e.g. mimotopes) comprising an amino acid sequence (and/or amino acid analogues) defining at least one AMA1/E epitope. Typically, the sequences defining the epitope correspond to the amino acid sequence of AMA1/E region of *P. falciparum* (either

identically or via substitution of analogues of the native amino acid residue that do not destroy the epitope). Other epitopes of AMA1/E include domain I defined by: amino acids 83-319 of AMA-1, domain II defined by amino acids 303-442 of AMA-1, domain III defined by amino acids 419-531 of AMA-1, and combinations of these domains such as domains I and II encompassing amino acids 83-442 of AMA-1, domains II and III encompassing amino acids 303-531 of AMA-1 and domains I and III encompassing amino acids 83-308 combined with 419-531 of AMA-1 (Hodder, et al., 1996, J. Biol. Chem. 271, 29446-52).

The AMA1/E antigen used in the present invention is preferably a full-length protein, or a substantially full-length version, i.e. containing functional fragments thereof (e.g. fragments which are not missing sequence essential to the formation or retention of an epitope). Furthermore, the *P. falciparum* antigen of the present invention can also include other sequences that do not block or prevent the formation of the conformational epitope of interest. The presence or absence of a conformational epitope can be readily determined through screening the antigen of interest with an antibody as described in the Examples below (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains only linear epitopes (if any).

The *P. falciparum* antigen of the present invention can be made by any recombinant method that provides the epitope of interest. For example, recombinant expression in *E. coli* is a preferred method to provide non-glycosylated antigens in 'native' conformation. This is most desirable because natural *P. falciparum* antigens are not glycosylated.

Proteins secreted from mammalian cells may contain modifications including galactose or sialic acids which may be undesirable for certain diagnostic or vaccine applications. However, it may also be possible
5 and sufficient for certain applications, as it is known for proteins, to express the antigen in other recombinant hosts such as baculovirus and yeast or higher eukaryotes, as long as glycosylation is inhibited.

10 The proteins according to the present invention may be secreted or expressed within compartments of the cell. Preferably, however, the proteins of the present invention are expressed within the cell and are released upon lysing the cells.

15 It is also understood that the isolates used in the examples section of the present invention were not intended to limit the scope of the invention and that an equivalent sequence from a *P. falciparum* isolate from another allele, e.g. FVO, or CAMP, can be used to
20 produce a recombinant AMA1/E protein using the methods described in the present application. Other new strains of *Plasmodium* may be a suitable source of AMA-1 sequence for the practice of the present invention.

The AMA1/E protein of the present invention is
25 expressed as part of a recombinant vector. The present invention relates more particularly to a nucleotide construct (SEQ ID NO:1) encoding 449 amino acids of AMA-1 of *P. falciparum* 3D7 clone, residues 83-531. The present AMA1/E sequence was commercially
30 synthesized with an *E. coli* codon bias (Retrogen, San Diego, CA). It would be evident to those of skill in the art that other codon bias could be used without deviating from the concept of the invention. The final protein construct (SEQ ID NO:2) contained two
35 histidine tags, 18 amino acids fused to the N-terminus

(MAHHHHHHHPGGSGSGTMH (SEQ ID NO:3)) and 11 amino acids fused to the C-terminus (AAALEHHHHHHH (SEQ ID NO:4)). The AMA1/E sequence was cloned into a modified pET32 plasmid pWRMAL. The modifications in the plasmid
5 include the replacement of the thioredoxin and other N-terminal tags with sequences that resulted in minimal non-AMA-1 amino acids fused to the final recombinant protein and a *tet^r* gene for tetracycline resistance added. This plasmid comprises, in sequence,
10 a T7 promoter, optionally a lac operator, a ribosome binding site, restriction sites to allow insertion of the structural gene and a T7 terminator sequence. Examples of other plasmids which contain the T7 inducible promoter include the expression plasmids
15 pET-17b, pET-11a, pET-24a-d(+), pET32a, and pEt-9a, all from Novagen (Madison, Wisconsin); see the Novagen catalogue.

The present invention also contemplates host cells transformed with a recombinant vector as defined
20 above. In a preferred embodiment, *E. coli* strain Origami (DE3) is employed. The above plasmids may be transformed into this strain or other strains of *E. coli* having a DE3 background and a protease deficiency such as *lon⁻*. Other host cells such as insect cells
25 can be used taking into account that other cells may result in lower levels of expression.

Eukaryotic hosts include lower and higher eukaryotic hosts as described in the definitions section. Lower eukaryotic hosts include yeast cells
30 well known in the art. Higher eukaryotic hosts mainly include mammalian cell lines known in the art and include many immortalized cell lines available from the ATCC, including HeLa cells, Chinese hamster ovary (CHO) cells, Baby hamster kidney (BHK) cells, PK15,
35 RK13 and a number of other cell lines. AMA1/E

expressed in these cells will be glycosylated unless the cells have been altered such that glycosylation of the recombinant protein is not possible. It is expected that when producing AMA1/E in a eukaryotic expression system, extensive investigation into methods for expressing, isolating, purifying, and characterizing the protein would be required as eukaryotic cells post-translationally modify this protein and this would alter protein structure and immunogenicity.

Methods for introducing vectors into cells are known in the art. Please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. Host cells provided by this invention include *E. coli* containing pWRMAL-AMA1/E.

A preferred method for isolating or purifying AMA1/E as defined above is further characterized as comprising:

- (i) growing a host cell as defined above transformed with a recombinant vector encoding AMA1/E protein in a suitable culture medium,
- (ii) causing expression of said vector sequence as defined above under suitable conditions for production of a soluble protein,
- (iii) lysing said transformed host cells and recovering said AMA1/E protein such that it retains its native conformation and is essentially pure.

Once the host has been transformed with the vector, the transformed cells are grown in culture in the presence of the desired antibiotic, if necessary. For FDA regulatory purposes, it is preferable to use tetracycline or kanamycin. When cells reach optimal

biomass density, in this case about 0.5-2.0 O.D. 600, preferably about 0.6-1.0 O.D. 600 in small culture flasks or about 5.0-15.0 O.D. 600, preferably about 6.0-10.0 O.D. 600 in bulk fermentors, the cells are
5 induced to produce the recombinant protein. The inventors have found after trial and error that for expression of a soluble AMA1/E, it was necessary to cool the culture to a range of about 25-30°C, more preferably about 28°C, prior to induction. The
10 concentration of inducer added, i.e. IPTG, affects maximal protein synthesis. It was found that a concentration of about 0.2 mM IPTG was best, however, a range of about 0.1 to 1.0 mM would be sufficient to produce 80-100% of maximal.

15 The cells were then collected and lysed to release the recombinant protein. Preferably, lysis should occur at a paste to buffer ratio of about 1 gram paste to about 5 to 10 ml of buffer w/v to reduce viscosity and volume of sample loaded on Ni-NTA
20 column. The recombinant protein of the present invention was fused to 6-His tags at the both the N-terminal and the C-terminal since one 6-His tag at the N-terminal did not result in proper purification on the Ni column. Two 6-His tags are not advised in the
25 field since purification of such a protein on a column usually results in a collection of breakdown products and incomplete synthesis products. However, we found that, with this protein, it is preferable to have two His-tags, one at the N-terminal and one at the C-
30 terminal of the protein, or possibly only one His-tag at the C-terminal.

Preferably, lysis is in the presence of N-lauryl sarcosinate (SLS), or other mild detergent which solubilizes and stabilizes the protein. Even though
35 instructions from the column manufacturer do not

recommend the use of SLS, we found that such a compound facilitates the proper extraction of the recombinant protein. The mild detergent, at about 5%, also prevents aggregation during the refolding step, hence eliminating the need for a cosolvent such as urea or guanidine. Preferably, the detergent is at an initial concentration of about 4-8 M.

Lysis is preferably at a temperature of about 0°C to 24°C, more preferably about 5 to 15°C in order to retain native folding of the AMA1/E protein and to reduce proteolysis. A high salt concentration of about 0.4-1.0 M is preferable though not necessary.

The lysate is applied over a Ni^{+2} -NTA affinity column in a solution containing about 1.25% mild detergent or SLS. The solution is applied at a flow rate of about 113-170 ml/min, at a cell resin to paste ratio of about 0.3-0.6 ml resin / g of paste, preferably about 0.5 ml resin / g of paste. The column is washed with a buffer containing imidazole of about 5-30 mM, preferably about 25 mM at pH of about 8.0 and reduced amount of SLS of about 0.1-0.2%, preferably about 0.125% at a pH of about 7.5-8.5, preferably about 8.0. The recombinant protein can be eluted by addition of high pH buffer of about 7.5 to about 8.5, preferably about pH 8.0, with about 400-600 mM imidazole, preferably about 500 mM of imidazole, and about 0.1-0.2%, preferably about 0.125% of SLS.

At this point the recombinant protein is about 5-10% of total protein. For refolding the protein, the Ni^{+2} elution was diluted about 40 fold (v/v), rapidly, in degassed buffer containing about 0.5-3 mM, preferably about 1 mM reduced glutathione (GSH), about 0.1-0.3 mM oxidized glutathione (GSSG), preferably about 0.25, pH 8.0. The refolding buffer was prepared fresh and refolding was carried out at room

temperature (~22°C) for a minimum of 15 h under nitrogen. The final protein product, resulting from this refolding protocol, was referred to as AMA1/E. Several other variations to the above refolding
5 protocol were also tested. One such variation included reduction of the Ni⁺² eluted proteins with 5 mM DTT for 1 h at 37°C before refolding.

If further purity is required, ion-exchange chromatography can be utilized. The recombinant
10 protein solution can be concentrated by passing through an anion exchange column in a phosphate buffer, in the presence of EDTA (about 1mM) and eluted in a phosphate buffer with EDTA and NaCl (about 100 mM) at about pH 8.0. The flow through protein is then
15 subjected to a pH adjustment step to alter the pH of the solution such that it can be passed through a cation exchange column. The eluted AMA1/E is pH adjusted to about pH 5.7 by the addition of 1 M NaH₂PO₄.H₂O. Other buffers, such as HCl, can be added
20 as is known in the art. The sample is then loaded onto a cation column preequilibrated with a sodium phosphate buffer of about 30-60 mM, preferably about 50 mM sodium phosphate, about 0.05-2.0 mM, preferably about 0.1 mM EDTA, about 50-150 mM, preferably about 100
25 mM NaCl at a pH of about 5.7. After washing the loaded column with a pH exchange buffer of about 3-6 mM, preferably about 5 mM sodium phosphate, about 0.05-2.0 mM, preferably about 0.1 mM EDTA at about pH 7.1, the AMA1/E is eluted from the column in formulation
30 buffer: about 20-25 mM, preferably about 23.5 mM NaH₂PO₄.H₂O, about 30-40 mM, preferably about 37.5 mM NaCl, about 0.05-2.0 mM, preferably about 0.1 mM EDTA at about pH 7.1. NaCl concentration in this SP elution buffer can be as high 150 mM NaCl, which
35 yields approximately 20% more AMA1/E but then the

final product may have to be dialysed against formulation buffer to lower the salt concentration if that is required.

The pH adjustment step is unique and discouraged
5 in the art: changing the charge of a protein from above to below its isoelectric point (5.98) could have caused the protein to separate into a non-soluble form. However, the proteins remains soluble under these conditions. Others usually use size exclusion
10 chromatography to achieve the next degree of purity, however, this presents serious limitation when designing a process for bulk production of the protein.

The bulk process for the isolation of purified
15 AMA1/E differs little from the process described above.

The present invention also relates to a composition comprising peptides or polypeptides as described above, for *in vitro* detection of malaria
20 antibodies present in a biological sample.

The present invention also relates to a composition comprising at least one of the following AMA-1 conformational epitopes and peptides retaining these epitopes:

25 epitope recognized by monoclonal antibodies 4G2 (from Alan Thomas, Primate Research Institute, The Netherlands), domain I defined by: amino acids 83-319 of AMA-1, domain II defined by amino acids 303-442 of AMA-1, domain III defined by amino acids 419-531 of
30 AMA-1, and combinations of these domains such as domains I and II encompassing amino acids 83-442 of AMA-1, domains II and III encompassing amino acids 303-531 of AMA-1 and domains I and III encompassing amino acids 83-308 combined with 419-531 of AMA-1
35 (Hodder, et al., 1996, J. Biol. Chem. 271, 29446-5).

The present invention also relates to a method for *in vitro* diagnosis of malaria antibodies present in a biological sample, comprising at least the following steps

- 5 (i) contacting said biological sample with a composition comprising any of the AMA1/E peptides as defined above, preferably in an immobilized form under appropriate conditions which allow the formation of an immune complex, wherein said peptide or protein can be
10 a biotinylated peptide or protein which is covalently bound to a solid substrate by means of streptavidin or avidin complexes,
- (ii) removing unbound components,
- (iii) incubating the immune complexes formed with
15 heterologous antibodies, with said heterologous antibodies having conjugated to a detectable label under appropriate conditions,
- (iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of
20 densitometry, fluorimetry, colorimetry).

The present invention also relates to a kit for determining the presence of malaria antibodies, in a biological sample, comprising:

- at least one peptide or protein composition as
25 defined above, possibly in combination with other polypeptides or peptides from *Plasmodium* or other types of malaria parasite, with said peptides or proteins being preferentially immobilized on a solid support, more preferably on different microwells of
30 the same ELISA plate, and even more preferentially on one and the same membrane strip,

- a buffer or components necessary for producing the buffer enabling binding reaction between these polypeptides or peptides and the antibodies against
35 malaria present in the biological sample,

means for detecting the immune complexes formed in the preceding binding reaction,

possibly also including an automated scanning and interpretation device for inferring the malaria
5 parasite present in the sample from the observed binding pattern.

The immunoassay methods according to the present invention utilize AMA1/E domains that maintain linear (in case of peptides) and conformational epitopes
10 (proteins) recognized by antibodies in the sera from individuals infected with a malaria parasite. The AMA1/E antigens of the present invention may be employed in virtually any assay format that employs a known antigen to detect antibodies. A common feature
15 of all of these assays is that the antigen is contacted with the body component suspected of containing malaria antibodies under conditions that permit the antigen to bind to any such antibody present in the component. Such conditions will
20 typically be physiologic temperature, pH and ionic strength using an excess of antigen. The incubation of the antigen with the specimen is followed by detection of immune complexes comprised of the antigen.

25 Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. Protocols may, for example, use solid supports, or immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be,
30 for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the immune complex are also known; examples of which are assays which utilize biotin and avidin or streptavidin, and enzyme-labeled and
35 mediated immunoassays, such as ELISA assays.

The immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format, and of a standard or competitive type. In a heterogeneous format, the polypeptide is typically bound to a solid matrix or support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidene fluoride (known as Immunolon.TM.), diazotized paper, nylon membranes, activated beads, and Protein A beads. For example, Dynatech Immunolon.TM.1 or Immunolon.TM. 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptides is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are known in the art.

In a homogeneous format, the test sample is incubated with the combination of antigens in solution. For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

In a standard format, the amount of malaria antibodies in the antibody-antigen complexes is directly monitored. This may be accomplished by determining whether labeled anti-xenogeneic (e.g. anti-human) antibodies which recognize an epitope on anti-malaria antibodies will bind due to complex formation. In a competitive format, the amount of malaria antibodies in the sample is deduced by

monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing ligand) in the complex.

Complexes formed comprising anti-malaria antibody
5 (or in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For example, unlabeled malaria antibodies in the complex may be detected using a conjugate of anti-xenogeneic
10 Ig complexed with a label (e.g. an enzyme label).

In an immunoprecipitation or agglutination assay format the reaction between the malaria antigens and the antibody forms a network that precipitates from
15 the solution or suspension and forms a visible layer or film of precipitate. If no anti-malaria antibody is present in the test specimen, no visible precipitate is formed.

There currently exist three specific types of
20 particle agglutination (PA) assays. These assays are used for the detection of antibodies to various antigens when coated to a support. One type of this assay is the hemagglutination assay using red blood cells (RBCs) that are sensitized by passively
25 adsorbing antigen (or antibody) to the RBC. The addition of specific antigen antibodies present in the body component, if any, causes the RBCs coated with the purified antigen to agglutinate.

To eliminate potential non-specific reactions in
30 the hemagglutination assay, two artificial carriers may be used instead of RBC in the PA. The most common of these are latex particles. However, gelatin particles may also be used. The assays utilizing either of these carriers are based on passive

agglutination of the particles coated with purified antigens.

The AMA1/E proteins, peptides, or antigens of the present invention will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain in separate containers the AMA1/E antigen, control antibody formulations (positive and/or negative), labeled antibody when the assay format requires the same and signal generating reagents (e.g. enzyme substrate) if the label does not generate a signal directly. The AMA1/E antigen may be already bound to a solid matrix or separate with reagents for binding it to the matrix. Instructions (e.g. written, tape, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

Immunoassays that utilize the AMA1/E antigen are useful in screening blood for the preparation of a supply from which potentially infective malaria parasite is lacking. The method for the preparation of the blood supply comprises: reacting a body component, preferably blood or a blood component, from the individual donating blood with AMA1/E proteins of the present invention to allow an immunological reaction between malaria antibodies, if any, and the AMA1/E antigen, and detecting whether anti-malaria antibody--AMA1/E antigen complexes are formed as a result of the reacting. Blood contributed to the blood supply is from donors that do not exhibit antibodies to the AMA1/E antigens.

The present invention further contemplates the use of AMA1/E proteins, or parts thereof as defined above, for *in vitro* monitoring malaria infection or prognosing the response to treatment (for instance with chloroquine, mefloquine, Malarome) of patients suffering from malaria infection comprising:

incubating a biological sample from a patient with malaria infection with an AMA1/E protein or a suitable part thereof under conditions allowing the formation of an immunological complex,

5 removing unbound components,
calculating the anti-AMA1/E titers present in said sample (for example at the start of and/or during the course of therapy),

monitoring the natural course of malaria
10 infection, or prognosing the response to treatment of said patient on the basis of the amount anti-AMA1/E titers found in said sample at the start of treatment and/or during the course of treatment.

Patients who show a decrease of 2, 3, 4, 5, 7,
15 10, 15, or preferably more than 20 times of the initial anti-AMA1/E titers could be concluded to be long-term, sustained responders to malaria therapy.

It is to be understood that smaller fragments of the above-mentioned peptides also fall within the
20 scope of the present invention. Said smaller fragments can be easily prepared by chemical synthesis and can be tested for their ability to be used in an assay as detailed above.

The present invention also relates to a kit for
25 monitoring malaria infection or prognosing the response to treatment (for instance to medication) of patients suffering from malaria infection comprising:

at least one AMA1/E peptide as defined above,
30 a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-AMA1/E antibodies present in a biological sample,

means for detecting the immune complexes formed
35 in the preceding binding reaction,

possibly also an automated scanning and interpretation device for inferring a decrease of anti-AMA1/E titers during the progression of treatment.

5 The present invention also relates to a serotyping assay for detecting one or more serological types or alleles of malaria parasite present in a biological sample, more particularly for detecting antibodies of the different types or alleles of
10 malaria parasites to be detected combined in one assay format, comprising at least the following steps:

- (i) contacting the biological sample to be analyzed for the presence of malaria antibodies of one or more serological types, with at least one of the
15 AMA1/E compositions as defined above, preferentially in an immobilized form under appropriate conditions which allow the formation of an immune complex,
- (ii) removing unbound components,
- (iii) incubating the immune complexes formed with
20 heterologous antibodies, with said heterologous antibodies being conjugated to a detectable label under appropriate conditions,
- (iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of
25 densitometry, fluorimetry, calorimetry) and inferring the presence of one or more malaria serological types present from the observed binding pattern.

It is to be understood that the compositions of proteins or peptides used in this method are
30 recombinantly expressed type-specific or allele-specific proteins or type-specific peptides.

The present invention further relates to a kit for serotyping one or more serological types or alleles of malaria parasite present in a biological
35 sample, more particularly for detecting the antibodies

to these serological types of malaria parasites comprising:

at least one AMA1/E protein or AMA1/E peptide, as defined above,

5 a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-AMA1/E antibodies present in a biological sample,

means for detecting the immune complexes formed
10 in the preceding binding reaction,

possibly also an automated scanning and interpretation device for detecting the presence of one or more serological types present from the observed binding pattern.

15 The present invention also relates to the use of a peptide or protein composition as defined above, for immobilization on a solid support and incorporation into a reversed phase hybridization assay, preferably for immobilization as parallel lines onto a solid
20 support such as a membrane strip, for determining the presence or the genotype of malaria parasite according to a method as defined above. Combination with other type-specific or allele-specific antigens from other malaria parasites also lies within the scope of the
25 present invention.

The present invention further relates to an AMA-1 specific antibody raised upon immunizing an animal with a peptide or protein composition of the present invention, with said antibody being specifically
30 reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a monoclonal antibody.

The present invention also relates to an AMA1/E or AMA-1 specific antibody screened from a variable
35 chain library in plasmids or phages or from a

population of human B-cells by means of a process known in the art, with said antibody being reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a
5 monoclonal antibody.

The AMA1/E specific monoclonal antibodies of the invention can be produced by any hybridoma liable to be formed according to classical methods from splenic or lymph node cells of an animal, particularly from a
10 mouse or rat, immunized against the *Plasmodium* polypeptides or peptides according to the invention, as defined above on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by the ability of the hybridoma to produce
15 the monoclonal antibodies recognizing the polypeptides which has been initially used for the immunization of the animals.

The antibodies involved in the invention can be labelled by an appropriate label of the enzymatic,
20 fluorescent, or radioactive type.

The monoclonal antibodies according to this preferred embodiment of the invention may be humanized versions of mouse monoclonal antibodies made by means of recombinant DNA technology, departing from parts of
25 mouse and/or human genomic DNA sequences coding for H and L chains from cDNA or genomic clones coding for H and L chains.

Alternatively the monoclonal antibodies according to this preferred embodiment of the invention may be
30 human monoclonal antibodies. These antibodies according to the present embodiment of the invention can also be derived from human peripheral blood lymphocytes of patients infected with malaria, or vaccinated against malaria. Such human monoclonal
35 antibodies are prepared, for instance, by means of

human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice, or by means of transgenic mice in which human immunoglobulin genes have been used to replace the mouse genes.

The invention also relates to the use of the proteins or peptides of the invention, for the selection of recombinant antibodies by the process of repertoire cloning.

Antibodies directed to peptides or single or specific proteins derived from a certain strain may be used as a medicament, more particularly for incorporation into an immunoassay for the detection of *Plasmodium* strains for detecting the presence of AMA-1 antigens, or antigens containing AMA-1, or AMA1/E epitopes, for prognosing/monitoring of malaria disease, or as therapeutic agents.

Alternatively, the present invention also relates to the use of any of the above-specified AMA1/E monoclonal antibodies for the preparation of an immunoassay kit for detecting the presence of AMA-1 antigen or antigens containing AMA1/E epitopes in a biological sample, for the preparation of a kit for prognosing/monitoring of malaria disease or for the preparation of a malaria medicament.

The present invention also relates to a method for *in vitro* diagnosis or detection of malaria antigen present in a biological sample, comprising at least the following steps:

(i) contacting said biological sample with any of the AMA1/E specific monoclonal antibodies as defined above, preferably in an immobilized form under appropriate conditions which allow the formation of an immune complex,

(ii) removing unbound components,

(iii) incubating the immune complexes formed with heterologous antibodies, which specifically bind to the antibodies present in the sample to be analyzed, with said heterologous antibodies conjugated to a detectable label under appropriate conditions,

(iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, colorimetry).

10 The present invention also relates to a kit for *in vitro* diagnosis of a malaria antigen present in a biological sample, comprising:

at least one monoclonal antibody as defined above, with said antibody being preferentially immobilized on a solid substrate,

a buffer or components necessary for producing the buffer enabling binding reaction between these antibodies and the malaria antigens present in the biological sample, and

20 a means for detecting the immune complexes formed in the preceding binding reaction.

The kit can possibly also include an automated scanning and interpretation device for inferring the malaria antigens present in the sample from the observed binding pattern.

Monoclonal antibodies according to the present invention are suitable both as therapeutic and prophylactic agents for treating or preventing malaria infection in susceptible malaria-infected subjects. Subjects include rodents such as mice or guinea pigs, monkeys, and other mammals, including humans.

In general, this will comprise administering a therapeutically or prophylactically effective amount of one or more monoclonal antibodies of the present invention to a susceptible subject or one exhibiting

malaria infection. Any active form of the antibody can be administered, including Fab and F(ab')₂ fragments. Antibodies of the present invention can be produced in any system, including insect cells, baculovirus expression systems, chickens, rabbits, goats, cows, or plants such as tomato, potato, banana or strawberry. Methods for the production of antibodies in these systems are known to a person with ordinary skill in the art. Preferably, the antibodies used are compatible with the recipient species such that the immune response to the MAbs does not result in clearance of the MAbs before parasite can be controlled, and the induced immune response to the MAbs in the subject does not induce "serum sickness" in the subject. Preferably, the MAbs administered exhibit some secondary functions such as binding to Fc receptors of the subject.

Treatment of individuals having malaria infection may comprise the administration of a therapeutically effective amount of AMA1/E antibodies of the present invention. The antibodies can be provided in a kit as described below. The antibodies can be used or administered as a mixture, for example in equal amounts, or individually, provided in sequence, or administered all at once. In providing a patient with antibodies, or fragments thereof, capable of binding to AMA1/E, or an antibody capable of protecting against malaria in a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc.

In general, it is desirable to provide the recipient with a dosage of antibody which is in the range of from about 1 pg/kg-100 pg/kg, 100 pg/kg-500

pg/kg, 500 pg/kg-1 ng/kg, 1 ng/kg-100 ng/kg, 100
ng/kg-500 ng/kg, 500 ng/kg- 1 ug/kg, 1 ug/kg- 100
ug/kg, 100 ug/kg-500 ug/kg, 500 ug/kg- 1 mg/kg, 1
mg/kg-50 mg/kg, 50 mg/kg-100 mg/kg, 100 mg/kg-500
5 mg/kg, 500 mg/kg-1 g/kg, 1 g/kg-5 g/kg, 5 g/kg-10 g/kg
(body weight of recipient), although a lower or higher
dosage may be administered.

In a similar approach, another prophylactic use
of the monoclonal antibodies of the present invention
10 is the active immunization of a patient using an anti-
idiotypic antibody raised against one of the present
monoclonal antibodies. Immunization with an anti-
idiotype which mimics the structure of the epitope
could elicit an active anti-AMA1/E response
15 (Linthicum, D.S. and Farid, N.R., Anti-Idiotypes,
Receptors, and Molecular Mimicry (1988), pp 1-5 and
285-300).

Likewise, active immunization can be induced by
administering one or more antigenic and/or immunogenic
20 epitopes as a component of a subunit vaccine.
Vaccination could be performed orally or parenterally
in amounts sufficient to enable the recipient to
generate protective antibodies against this
biologically functional region, prophylactically or
25 therapeutically. The host can be actively immunized
with the antigenic/immunogenic peptide in pure form, a
fragment of the peptide, or a modified form of the
peptide. One or more amino acids, not corresponding
to the original protein sequence can be added to the
30 amino or carboxyl terminus of the original peptide, or
truncated form of peptide. Such extra amino acids are
useful for coupling the peptide to another peptide, to
a large carrier protein, or to a support. Amino acids
that are useful for these purposes include: tyrosine,
35 lysine, glutamic acid, aspartic acid, cyteine and

derivatives thereof. Alternative protein modification techniques may be used e.g., NH₂-acetylation or COOH-terminal amidation, to provide additional means for coupling or fusing the peptide to another protein or peptide molecule or to a support.

The antibodies capable of protecting against malaria are intended to be provided to recipient subjects in an amount sufficient to effect a reduction in the malaria infection symptoms. An amount is said to be sufficient to "effect" the reduction of infection symptoms if the dosage, route of administration, etc. of the agent are sufficient to influence such a response. Responses to antibody administration can be measured by analysis of subject's vital signs.

The present invention more particularly relates to a composition comprising at least one of the above-specified peptides or a recombinant AMA1/E protein composition as defined above, for use as a vaccine for immunizing a mammal, preferably humans, against malaria, comprising administering a sufficient amount of the composition possibly accompanied by pharmaceutically acceptable adjuvant(s), to produce an immune response.

Immunogenic compositions can be prepared according to methods known in the art. The present compositions comprise an immunogenic amount of a recombinant AMA1/E proteins or peptides as defined above, usually combined with a pharmaceutically acceptable carrier, preferably further comprising an adjuvant.

The proteins of the present invention, preferably purified AMA1/E derived from (3D7), are expected to provide a particularly useful vaccine antigen, since the antigen is able to induce invasion inhibitory

antibodies as well as high titer antibodies that react with schizont-infected erythrocytes.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers; and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to : montanide, aluminum hydroxide (alum), N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP) as found in U.S. Pat. No. 4,606,918, N-acetyl-normuramyl-L-alanyl-D-isoglutamine(nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE) and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/TweenTM 80 emulsion. Any of the 3 components MPL, TDM or CWS may also be used alone or combined by 2. Additionally, adjuvants such as Stimulon (Cambridge Bioscience, Worcester, Mass.) or SAF-1 (Syntex) may be used. Further, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) may be used for non-human applications and research purposes. Other adjuvants for human research include AS01, AS02A, AS02B, and AS03, AS04, AS05 (GlaxoSmith Kline, PA), among others known or newly discovered.

The immunogenic compositions typically will contain pharmaceutically acceptable vehicles, such as water, saline, glycerol, ethanol, etc. Additionally,

auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, preservatives, and the like, may be included in such vehicles.

Typically, the immunogenic compositions are
5 prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced
10 adjuvant effect. The AMA1/E proteins of the invention may also be incorporated into Immune Stimulating Complexes together with saponins, for example QuilA (ISCOMS).

Immunogenic compositions used as vaccines
15 comprise a `sufficient amount` or `an immunologically effective amount` of the proteins of the present invention, as well as any other of the above mentioned components, as needed. `Immunologically effective amount`, means that the administration of that amount
20 to an individual, either in a single dose or as part of a series, is effective for treatment, as defined above. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be
25 treated (e.g. nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, the
30 strain of malaria infection, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Usually, the amount will vary from 0.01 to 1000 ug/dose, more particularly from about 1.0

to 100 ug/dose most preferably from about 10 to 50 ug/dose.

The proteins may also serve as vaccine carriers to present homologous (e.g. other malaria antigens, such as MSP-1₄₂, CSP, TRAP, LSA1, LSA3, Pfs25) or heterologous (non-malaria) antigens. In this use, the proteins of the invention provide an immunogenic carrier capable of stimulating an immune response to other antigens. The antigen may be conjugated either by conventional chemical methods, or may be cloned into the gene encoding AMA1/E fused to the 5' end or the 3' end of the AMA1/E gene. The vaccine may be administered in conjunction with other immunoregulatory agents.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A. ed., Mack Easton Pa. (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters,

polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method
5 of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino
10 acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial
15 polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)-microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and
20 nanocapsules or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* (1980).

Administration of the compounds, whether antibodies or vaccines, disclosed herein may be
25 carried out by any suitable means, including parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), *in ovo* injection of birds, orally, or by topical application of the antibodies (typically carried in
30 a pharmaceutical formulation) to an airway surface. Topical application of the antibodies to an airway surface can be carried out by intranasal administration (e.g., by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation
35 intranasally). Topical application of the

antibodies to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and
5 liquid particles) containing the antibodies as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any
10 conventional technique can be employed. Oral administration may be in the form of an ingestible liquid or solid formulation.

The treatment may be given in a single dose schedule, or preferably a multiple dose schedule in
15 which a primary course of treatment may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the response, for example, at 1-4 months for a second dose, and if needed, a
20 subsequent dose(s) after several months. Examples of suitable treatment schedules include: (i) 0, 1 month and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired
25 responses expected to reduce disease symptoms, or reduce severity of disease.

The present invention also provides kits which are useful for carrying out the present invention. The present kits comprise a first container means
30 containing the vaccine or antibodies of the invention. The kit also comprises other container means containing solutions necessary or convenient for carrying out the invention. The container means can be made of glass, plastic or foil and can be a vial,
35 bottle, pouch, tube, bag, etc. The kit may also

contain written information, such as procedures for carrying out the present invention or analytical information, such as the amount of reagent contained in the first container means. The container means may
5 be in another container means, e.g. a box or a bag, along with the written information.

The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent
10 applications) cited throughout this application are hereby expressly incorporated by reference.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for
15 illustration of the invention and are not intended to be limiting thereof.

The following MATERIALS AND METHODS were used in the examples that follow.

Cloning and expression: A nucleotide construct
20 encoding 449 amino acids of AMA1 of *P. falciparum* 3D7 clone, residue # 83_{Gly}-531_{Glu} was commercially synthesized with an *E. coli* codon bias (Retrogen, San Diego, CA). The synthetic gene insert was ligated to the *Nco* I and *Not* I sites of a modified pET32 plasmid,
25 called pWRMAL. The modifications in the plasmid include the replacement of the thioredoxin and other N-terminal tags with sequences that resulted in minimal non-AMA1 amino acids fused to the final recombinant protein and a *tet*^r gene for tetracycline
30 resistance added. (Angov, E. et al.; unpublished). To correct a reading frame error the recombinant vector was cut with *Nco* I, end-filled with Klenow fragment and religated. The final construct contained 18 amino acids fused to the N-terminus and 11 amino acids fused
35 to the C-terminus. The resulting protein construct

was designated as r-AMA1/E (MAHHHHHHHPGGSGSGTMH (SEQ ID NO:3))-(AMA1 amino acids # 83-531)-AAALEHHHHHHH (SEQ ID NO:4)). The recombinant plasmid, pWRMAL-AMA1/E (Figure 1) was transformed into *E. coli* Sure II cells and the
5 insert was sequenced on both strands. For protein expression the recombinant plasmid was transformed into a 'redox modified' host *E. coli* strain (Origami (DE3); Novagen, Madison, WI). Origami (DE3) cells are tetracycline and kanamycin resistant. Expression of
10 r-AMA1/E protein was confirmed by IPTG induction in shake flask cultures and glycerol stocks were prepared.

Fermentation (GMP production): Expression of r-AMA1/E protein was carried out in a 10 L bioreactor
15 (New Brunswick Scientific, Edison, NJ) at the lab scale and in a 300 L bioreactor (New Brunswick Scientific, Edison, NJ) at the Walter Reed Army Institute of Research Pilot Bioproduction Facility. Medium consisting of Super Broth containing 0.8%
20 glycerol and $12.5 \mu\text{g ml}^{-1}$ tetracycline was inoculated with a 3 L overnight culture started from a Production Seed Lot #0788. The bioreactor temperature was maintained at 27°C ; pH 7.2 and agitation at 400 rpm. At a cell density $\text{OD}_{600} = 7.0$, IPTG was added, to a
25 final concentration of 0.1 mM. One hour later cells were harvested by centrifugation and frozen at -80°C . Aliquots (10 to 150 g) from this production were used to develop a purification and refolding process of r-AMA1/E protein at the lab prior to scale-up (1500 g)
30 in a GMP environment.

Plasmid Stability: The presence of recombinant plasmid in *E. coli* Origami (DE3) cells after fermentation was determined by plating an appropriate dilution of cells on LB agar plates containing either
35 tetracycline ($12.5 \mu\text{g ml}^{-1}$) or ampicillin ($100 \mu\text{g ml}^{-1}$)

(selective plates) and on LB agar plates containing no antibiotic (non-selective plates). The percent plasmid retention [No. colonies on selective plates/ No. colonies on non-selective plates] was calculated using colony counts on appropriate dilution plates containing between 30 and 300 colonies.

Metal affinity purification (Lab-scale): All buffers were endotoxin free and maintained chilled; all chemicals used during purification were ACS certified or the next best available grade. Purification was carried out at room temperature on a Waters-600 liquid chromatography system configured to run Pharmacia-Amersham HR columns. Cell paste was thawed and suspended in 5 times w/v of buffer-A (15 mM Na_2HPO_4 , 5.1 mM KH_2PO_4 , 450 mM NaCl; pH 7.4) and mixed until homogenous. A solution of 20% sodium N-lauroyl sarcosine (sarkosyl) was added to a final concentration of 5% detergent. This suspension was mixed and the *E. coli* cells were disrupted by high-pressure microfluidization (Model 1109, Microfluidic Corp., Newton, MA). The cell lysate was cleared by centrifugation at 22,000xg and the supernatant was diluted 4 fold in buffer-A before loading onto a Ni^{+2} -NTA Superflow column (Qiagen, Valencia, CA; 0.5 ml packed resin per gram paste). The Ni^{+2} column was pre-equilibrated with buffer-B (buffer-A containing 1.25 % sarkosyl; pH 7.4). After loading the lysate, the Ni^{+2} column was washed with 20 column volumes (CV) of the buffer-C (buffer-A with 10 mM imidazole, 0.125% sarkosyl; pH 7.4) followed by 20 CV of buffer-D (20 mM sodium phosphate, 25 mM imidazole, 0.125% sarkosyl; pH 8.0). Bound proteins were eluted from the column in buffer-D containing 500 mM imidazole (pH 8.0).

Refolding: The Ni^{+2} elution was diluted 40 fold (v/v), rapidly, in degassed buffer-E (20 mM sodium

phosphate, 1mM EDTA, 1 mM reduced glutathione (GSH),
0.25 mM oxidized glutathione (GSSG); pH 8.0). The
refolding buffer was prepared fresh and refolding was
carried out at room temperature (~22°C) for a minimum
5 of 15 h under nitrogen. The final protein product,
resulting from this refolding protocol, was referred
to as AMA1/E. Several other variations to the above
refolding protocol were also tested. One such
variation included reduction of the Ni^{+2} eluted
10 proteins with 5 mM DTT for 1 h at 37°C before
refolding. The protein after reduction and refolding
followed by ion-exchange purification was referred to
as RR-AMA1/E.

Ion-exchange purification: Ion-exchange
15 column resins were sanitized with 0.2 N NaOH before
use and then equilibrated to initial binding
conditions. After the refolding step, AMA1/E protein
was concentrated on a DEAE SepharoseTM anion-exchange
column (Amersham Pharmacia Biotech, Piscataway, NJ);
20 0.25 ml packed resin per gram paste), the column was
pre-equilibrated with buffer-E without the GSH/GSSG.
After loading the protein, the column was washed with
a minimum of 30 CV of the same equilibration buffer
followed by 10 CV of buffer-F (5 mM sodium phosphate,
25 50 mM NaCl, 1 mM EDTA; pH 8.0). AMA1/E was eluted in
buffer-F containing a final concentration of 100 mM
NaCl (pH 8.0). AMA1/E eluted from the DEAE column was
pH adjusted to 5.7 by the addition of 1M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
and loaded on a SP Sepharose cation-exchange column
30 (Amersham Pharmacia Biotech; 0.15 ml packed resin per
gram paste), pre-equilibrated with buffer-G (50 mM
sodium phosphate, 0.1 mM EDTA, 100 mM NaCl; pH 5.7).
The column was washed with 20 CV of buffer-G
containing a final 275 mM NaCl (pH 5.7), followed by
35 10 CV of a pH exchange buffer (5 mM sodium phosphate,

0.1 mM EDTA; pH 7.1). AMA1/E was eluted from the column in formulation buffer (23.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 37.5 mM NaCl, 0.1 mM EDTA; pH 7.1).

Formulation, lyophilization and storage:

5 Purified AMA1/E protein eluted from the SP column was quantified by Bio-Rad DC protein assay (BioRad, Richmond, CA). AMA1/E was vialled at $100 \mu\text{g ml}^{-1}$, 65 μg protein per vial, in the final formulation buffer (23.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 30 mM NaCl, 0.1 mM EDTA, 3.15% sucrose; pH 7.1) and lyophilized.

Residual sarkosyl and endotoxin content

determination: The residual sarkosyl in purified AMA1/E protein preparations was measured by a reversed-phase HPLC method (Burgess, R.R., 1996, Meth. 15 Enzymol. 273, 145-149). Endotoxin content was estimated using the chromogenic *Limulus* Amebocyte Lysate (LAL) endpoint assay (Associates of Cape Cod, Falmouth, MA). Dilutions of all protein samples and LAL standard were prepared in pyrogen-free vials.

20 Positive control solutions prepared for the standard curves ranged from 1 endotoxin unit (EU) ml^{-1} to 0.06 EU ml^{-1} , in two-fold serial dilutions. A 96-well plate heater was used for incubation at 37°C for 20 min and the assay was carried out as per the manufacturer's

25 instruction. The plates were read at 405 nm on V_{max} kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Purity and stability analysis:

AMA1/E was evaluated for purity on precast polyacrylamide gels (4-12% gradient Bis-Tris, Invitrogen, Carlsbad, CA), 30 run under reduced and non-reduced conditions, with 5-10 μg protein loaded per well. Gels were stained with Coomassie blue, destained, scanned on a Laser densitometer and acquired data was analyzed by

35 ImageQuant 5.1 software (Molecular Dynamics,

Sunnyvale, CA). Residual host cell protein (HCP) content, was assessed by ELISA and Western blotting, using commercially available kits (Cygnus Technologies, Plainville, MA). The HCP standard
5 recommended by the manufacturer was used. In addition to this control, a lysate of the host *E. coli* Origami (DE3) (expressing a *P. vivax* MSP1 protein construct) was also tested as a standard between 1000 and 15 ng ml⁻¹ protein concentration, to determine if the kit was
10 capable of detecting proteins from this specific host *E. coli*. The total protein in the Origami (DE3) lysate was estimated by BCA protein assay (Pierce). HCP ELISA was performed twice, using concentrations of AMA-1/E, between 10,000 and 80 ng ml⁻¹, as per the 'standard
15 procedure' recommended by the manufacturer. Immunoblotting for HCP determination (Cygnus Technologies kit) was carried out using the HCP standard provided by the manufacturer and also using the Origami (DE3) *E. coli* lysate, between 4000 and 250
20 ng protein per well run on a reducing gel. The proteins were electrophoretically transferred to a nitrocellulose membrane and the western blot assay was performed as per the manufacturer's instructions. Stability of AMA1/E was determined by SDS-PAGE and
25 western blotting of protein samples drawn monthly from aliquots stored at -80°C, -30°C, 4°C, 22°C (RT) and 37°C.

Primary structure analysis: Purified AMA1/E protein was sequenced by automated Edman's degradation
30 method on an Applied Biosystems model 477A protein sequencer, in-line with a HPLC (Applied Biosystems model 120A), for detection of phenylthiohydantoin-derived amino acids. Protein samples were analyzed by, Matrix Assisted Laser Desorption Ionization-Time

of flight mass spectrometer (MALDI-TOF; Voyager Biospectrometry RP system, Applied Biosystems), using Sinapinic acid matrix. Lysozyme and Cytochrome C were used as mass standards.

5 **Reduction, alkylation and free thiol**

analysis: AMA1/E protein was reduced with a 100-fold molar excess of DTT over cysteines in presence of either 4 M urea (for SDS-PAGE) or 4 M guanidine-HCl (for RP-HPLC and Ellman's test) at 50°C for 1 h.

10 Alkylation was carried out in presence of either 4 M urea or 4 M guanidine-HCl, along with 1000-fold molar excess of iodoacetamide over cysteines, for 1 h at room temperature in the dark. Free sulfhydryl groups were estimated in the presence and absence of 4 M
15 guanidine-HCl by Ellman's reagent (5,5'-dithio-bis-3-nitrobenzoic acid) (Ellman, G. L., 1959, Arch. Biochem. Biophys. 82, 50-77). L-cysteine was used to plot the standard curve.

Gel-permeation (GPC) and reversed-phase

20 **(RPC) chromatography:** HPLC analysis of purified protein was carried out using a Waters-510 HPLC pump, connected to Waters-712 WISP autosampler and controlled by Millennium Release 3.2 chromatographic software (Waters Corp., Milford, MA). Waters-996 PDA
25 detector was used to monitor the elution profiles. For GPC analysis a Shodex Protein KW-803 column (Waters Corp., Milford, MA) was used with 10 µg protein injection. Buffer system consisted of 20 mM sodium phosphate, 100 mM K₂SO₄ (pH 7.15) at 0.5 ml min⁻¹ flow
30 rate. The column was calibrated with molecular weight standards (BioRad). RPC analysis was done with a C8 Aquapore RP-300 Å column, 7 µ, 30_2.1 mm (PE Brownlee, Norwalk, CT) at 0.5 ml min⁻¹ flow-rate and 4-12 µg protein per load. Solvent A: 0.05% trifluoroacetic acid

(TFA) in H₂O; solvent B: 0.05% TFA in acetonitrile. The solvent gradient consisted of 100% solvent A for 5 min, 100% to 30% solvent A over 15 min, 30% to 0% solvent A over 5 min and back to 100% solvent A over 5 min.

Immune reagents: Rat monoclonal antibody, 4G2dc1 (used at 1.5 µg ml⁻¹ on immunoblots and ELISA), recognizes a disulphide bond dependent conformational epitope on *P. falciparum* AMA1 (Kocken, et al., 1998, supra), was kindly provided by Dr. Alan W. Thomas, Biomedical Primate Research Center, Rijswijk, The Netherlands. A pool of immune human sera (used at 1:1000 dilution on immunoblots) was collected from an endemic area in Western Kenya; the same dilution of a pool of commercially obtained normal human serum (The Binding Site Limited, Birmingham, UK) was used as a negative control.

Immunoblotting: Proteins were separated on SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979, Biotechnology 24, 145-149). The blot was blocked with 0.5% casein and 0.3% Tween-20 containing phosphate buffered saline (PBS). Appropriate dilution of primary antibody in PBST (PBS with 0.05% Tween-20) was incubated for 2 h. The blot was washed with PBST and then incubated with 1:5000 dilution of HRP conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL) for 1 h. After washing with PBST, the blot was developed either with Super Signal Chemiluminescent substrate (Pierce, Rockford, IL) or with BM Blue POD substrate (Roche, Indianapolis, IN) according to the manufacturer's recommendation.

Indirect-immunofluorescence assay (IFA): Recognition of *P. falciparum* 3D7 schizonts, by anti-AMA1 antibodies was tested by IFA. Thin blood smears

were fixed with chilled methanol and serial dilutions of sera in PBST were incubated for 2 h. Slides were washed three times with PBST and incubated with a 1:100 dilution of goat anti-rabbit IgG FITC-labeled antibodies (Southern Biotechnologies Associates) for 1 h. Slides were washed, anti-fade solution (Molecular Probes, Inc, Eugene, OR) was applied and read on a UV fluorescence microscope. IFA titers were determined as the last serum dilution with a positive recognition of the parasite compared to the negative adjuvant control rabbit serum diluted 1:20. The assay was done twice on separate days.

Rabbit Immunization and total IgG

purification: Groups of three NZW rabbits were immunized, three times with 100 µg of lab-grade refolded AMA1/E (animal codes R-1, 2, 3); reduced and refolded protein RR-AMA1/E (R-4, 5, 6) or its reduced and alkylated form RA-AMA1/E (R-7, 8, 10). A group of 3 rabbits received 50 µg (V-2, 3, 4) or 100 µg (V-9, 10, 11) of AMA1/E protein produced under GMP environment. A control group of 3 rabbits (R-9, V45, 45) were given PBS along with the adjuvant. Formulation was prepared by adding 70% (v/v) Montanide® ISA-720 (Seppic Inc. Paris, France) to 30% antigen to make a total 1 ml emulsion per dose. The immunization was given subcutaneous at multiple sites, with a three wk interval between consecutive immunizations. Serum samples were collected 2 wk after each immunization. Rabbits were bled out 2 wk after the last immunization. Total IgG was purified from 9 ml pooled rabbit sera (lab-grade AMA1/E and RA-AMA1/E group). The adjuvant control IgG was purified from a single animal (R9). IgG purification was done on a 5 ml protein-G Sepharose column (Amersham Pharmacia Biotech. Piscataway, NJ) using IgG binding and elution

buffers (Pierce, Rockford, IL), according to the manufacturer's recommendation.

ELISA: Antibody response was evaluated by enzyme-linked immunosorbent assay (ELISA). Ninety six
5 well microtiter plates (Dynax, Chantilly, VA) were coated with 100 ng per well of either RA-AMA1/E or AMA1/E, incubated overnight at 4°C, plates were blocked for 1 h with PBST containing 5% casein (Sigma, St. Louis, MO) and washed with PBST. Consecutive
10 dilutions of individual rabbit sera were incubated for 2 h at room temperature. Plates were washed and 1:4000 diluted HRP-conjugated secondary antibody was incubated for 1 h. Plates were washed and developed for 25 min with ABTS-peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). OD₄₀₅ was
15 recorded and comparative ELISA titers were calculated using regression analysis on the titration curve. The ELISA was repeated 3 times for each individual serum, in triplicate wells, on separate days. Competitive
20 ELISA was carried out using sera from 3 rabbits immunized with lab-grade AMA1/E and two rabbits in the RA-AMA1/E group. The sera were diluted 1:1000 and pre-incubated in solution with 15 µg ml⁻¹ of either AMA1/E or RA-AMA1/E or with BSA, overnight at 4°C on a
25 shaker. The tubes were centrifuged at 15,000 rpm for 15 min and the supernatants were analyzed by ELISA (as described above), with AMA1/E coated on plates. The competition assay was done 3 times.

Parasite culture and Growth Inhibition

30 **Assay (GIA):** *P. falciparum* clone 3D7 cultures were prepared as described previously (Haynes et al., 2002, Erythrocytic Malaria Growth or Invasion Inhibition Assays (GIA) with Emphasis on Suspension Culture GIA, Chapter 51, in Malaria Methods and Protocols, ed. Denise L. Doolan, Methods in Molecular Medicine, The

Humana Press Inc., NJ), in 48-well plates, kept in suspension cultures angled on a rotator platform or under static conditions. All cultures contained a final 10% heat inactivated normal human serum in
5 bicarbonate-containing RPMI 1640. Plates were gassed with 5% O₂, 5% CO₂ and heat-sealed in plastic bags. Synchronized cultures adjusted to 0.2% late ring stages were mixed with test or control serum or IgG (dialyzed into RPMI-NaOH) to a final hematocrit of 4%.
10 In order to assess the antigen specificity of the antibody mediated inhibitions, antigens (AMA1/E or RA-AMA1/E) were added to the IgG preparations before testing in the GIA. The final concentration of antigens in the GIA was 5.3 µg ml⁻¹ (limited by low
15 solubility of RA-AMA1/E protein). Merozoites were released after approximately 34 h and developing ring stages were harvested 14 h post invasion, stained with Hoechst dye-33342 and analyzed by flow-cytometry (Haynes et al., 2002, supra). The fluorescence signal
20 was determined for a minimum of 40,000 erythrocytes gated on forward scatter. The fluorescent signal of ring-infected erythrocytes was about 20 times that of uninfected erythrocytes and schizont-infected erythrocytes, if present, had another 20-fold increase
25 in signal. Almost all (>99%) of the parasites harvested from the assays were ring forms or early trophozoite stages, as confirmed by spot checks of Giemsa-stained thin smears. Percentage inhibition was calculated from the mean parasitaemia of triplicate
30 test and control wells as 100% - (test/control). Sera from rabbits immunized with the adjuvant and PBS were used as controls in the GIA. Prebleeds from individual rabbits was also tested.

Statistical analysis: Microsoft Excel was used to calculate the p values for 2-tailed t tests and the correlation coefficients (R^2).

Example 1

5 **Fermentation of *E. coli* Origami (DE3) expressing the r-AMA1/E protein at 10L and 300L scale:** The synthetic gene cloned in the vector pWRMAL was sequenced and the translation of this gene
10 published 3D7 clone sequence (GenBankTM Accession No U65407.1). Fermentation conditions were developed in a 10 L bioreactor and later scaled-up to a 300 L GMP fermentation. The 10 L fermentation routinely resulted in 150 gm cell paste while the 300L fermentation
15 resulted in 4.5 kg cell paste. The final plasmid stability for the GMP fermentation was 36%. Although the use of Origami (DE3) increased the proportion of r-AMA1/E in the soluble fraction (compared to the conventional BL21 (DE3) strain), protein fractionation
20 experiments showed that a majority of r-AMA1/E was still localized in the insoluble fraction (data not shown).

Example 2

25 **Extraction of r-AMA1/E in sarkosyl and its enrichment by Ni^{+2} affinity chromatography:** Aliquots were taken from the GMP cell paste lot and a scalable refolding and purification process was developed. During cell lysis soluble and insoluble forms of r-AMA1/E were extracted with buffer
30 containing 5% sarkosyl. The r-AMA1/E constituted ~1-2% of total cell protein estimated by laser densitometry of a SDS-PAGE run under reduced conditions (Fig. 2A, lane 1). Following the first step of purification over Ni^{+2} column, r-AMA1/E was enriched to ~ 40 % of total

protein (Fig. 2A, lane 2). A large fraction of r-AMA/E present in the Ni^{+2} elution, was aggregated as seen on a non-reduced SDS-PAGE (data not shown).

Example 3

5 **Optimization of the refolding conditions:**

In order to find the optimal refolding conditions, the Ni^{+2} elution was subjected to rapid dilution in refolding buffers of varying GSH/GSSG ratios, at pH 8.0, in phosphate buffer. Serial dilutions of these
10 test refolding mixtures were coated on a microtiter plate and ELISA reactivity against the conformation specific, inhibitory, monoclonal antibody 4G2dc1, was used as a measure of folding efficiency; while the reactivity to a monoclonal anti-hexa-histidine
15 antibody was used to confirm equivalent coating efficiency. Ratios of GSH/GSSG tested for refolding included 1/0.1 mM, 1/0.25 mM, 1/1 mM, and 0.1/1 mM respectively, while phosphate buffer containing EDTA (pH 8.0) alone was used as a control. The GSH/GSSG
20 ratios of 1/0.1 mM and 1/0.25 mM were found to be equally efficient, both of which gave 4G2dc1 reactivity about 5 times higher than the phosphate buffer control. As the GSH/GSSG ratio of 1/0.25 mM had been previously reported for efficient refolding of *P.*
25 *chabaudi* AMA1 and more recently, the same was used to refold *P. falciparum* AMA1 expressed in *E. coli* (Crewther et al., 1996, Infect. Immun. 64, 3310-3317; Hodder et al., 2001, supra), we chose this ratio to refold r-AMA1/E. After refolding r-AMA1/E was
30 designated as AMA1/E. A minimum 40-fold dilution of the Ni^{+2} elution to about 20 $\mu\text{g ml}^{-1}$ protein during refolding, was found necessary to minimize aggregation. No significant increase in monomer yield of AMA1/E was found if the Ni^{+2} elution was first
35 reduced with DTT prior to refolding, and therefore,

the GMP purification process was carried out using the Ni⁺² elution without reduction. The presence of low concentrations of sarkosyl (0.003%) in the refolding mix eliminated the need for a cosolvent during
5 refolding.

Example 4

Ion-exchange chromatography was used to purify AMA1/E to homogeneity: After 15 h incubation in the refolding buffer, AMA1/E was
10 concentrated on a DEAE anion-exchange column and its monomeric form was eluted with 100 mM NaCl, while the impurities and AMA1/E aggregates remained bound to the column. The percent purity of AMA1/E after this step was ~90 % of the total protein eluted (Fig. 2A, lane
15 3). The pH adjustment step from pH 8.0 to 5.7 was needed to bind the majority of AMA1/E to the SP cation-exchange column. This pH change had no effect on the solubility of AMA1/E or its reactivity to immune reagents. AMA1/E bound to the cation-exchanger
20 was eluted with the final formulation buffer, eliminating the need for an additional buffer exchange step before formulation. The final yield of AMA1/E was about 0.75 to 1 mg L⁻¹ culture with > 99% purity estimated by laser densitometry of Coomassie blue
25 stained gels (Fig. 2A, lane 4). RR-AMA1/E also gave similar yield and purity (data not shown).

Example 5

Lyophilized formulation of AMA1/E along with sucrose and EDTA was stable: A final 3.15% sucrose excipient was added for stabilization and cake
30 formation during lyophilization. AMA1/E, vialled at 100 µg ml⁻¹ in 0.65 ml aliquots, was found to be stable in its lyophilized form at 37°C, 22°C and 4°C over a 24 wk period, with no signs of breakdown or aggregation.

Solution or lyophilized forms of AMA1/E stored at -30°C or -70°C showed equivalent stability (data not shown).

Example 6

5 **Formulated AMA1/E product has very low residual endotoxin, host cell proteins or sarkosyl content:** The endotoxin content of purified AMA1/E under lab conditions was between 3 to 5 EU's per 50 µg protein but dropped to below 0.06 EU (lowest
10 value detectable by LAL assay) per 50 µg protein in GMP purification. No residual sarkosyl was detected with an RP-HPLC based assay (minimum detection limit 0.0005%). The HCP content was determined by ELISA, using an anti-*E. coli* antibody kit, capable of
15 quantitatively detecting 15 ng ml⁻¹ HCP using the Origami (DE3) *E. coli* lysate (the lowest concentration of HCP tested). AMA1/E sample at 10,000 ng ml⁻¹ showed 54 and 44 ng ml⁻¹ HCP (in two tests), giving the final purity of 99.4%. Purity of AMA1/E was also tested by
20 western blot HCP determination kit (Cygnus). The Origami (DE3) lysate was used as positive control at 4000 to 250 ng protein per well (Fig 2B). All the positive bands at 4000 ng per well (Fig. 2B, lane 1) were also observed at 1000 ng per well (Fig. 2B, lane
25 3). Below 1000 ng per well, many *E. coli* protein bands were not detectable. No *E. coli* specific bands were seen in AMA1/E lanes with up to 2 µg AMA1/E loaded per well (Fig 2B, lane 11).

Example 7

30 **AMA1/E had the predicted primary and tertiary structure with no free cysteines:** The primary sequence analysis of AMA1/E identified the first 24 N-terminal amino acids to be
ahhhhhhhpggsgsgtmhGAEPAP (SEQ ID NO:5) (*P. falciparum*

AMA1 specific residues in capital). The methionine at the N-terminal could not be identified. The MALDI-TOF mass spectrometer analysis showed an average mass at 54,656 Da, while the predicted mass of AMA1/E was 54,633 Da. The final product was evaluated for homogeneity and the presence of multimers by analytical RPC and GPC. A single peak was seen on both GPC and RPC elution profiles, giving evidence of a homogenous product (Fig. 3A, B). The RPC elution profile of AMA1/E, shifted towards higher hydrophobicity under reducing condition (Fig. 3B, broken line). This indicates exposure of the protein hydrophobic core, upon DTT reduction, which otherwise, remained buried due to compact folded state, stabilized by disulphide bond formation.

The primary structure of AMA1/E is expected to contain 16 cysteine residues, the presence of any free cysteines in the final product, which would have indicated incorrect folding. The free cysteine content was determined by Ellman's test. Ellman's analysis was also carried out in the presence of 4M GuHCl to unmask any sulfhydryl groups buried in the hydrophobic core of the protein. Ellman's test detected no free sulfhydryl groups in up to 5 μ M AMA1/E, both in the presence and absence of 4M GuHCl (minimum detection limit 0.1 μ M free sulfhydryl). The absence of free cysteines was further confirmed by treating AMA1/E with an alkylating agent before and after reduction. Mobility of AMA1/E on non-reduced SDS-PAGE showed no observable change after treatment with iodoacetamide (Fig. 4A, lanes 1 and 2), while its reductive-alkylation caused significant decrease in mobility (Fig. 4A, lane 3). A recombinant *P. vivax* MSP-1 p42 fragment (Dutta et al., 2001, Infect. Immun. 69, 5464-5470), which was predicted to contain a single free

cysteine, was used as a positive control in both the Ellman's and alkylation analysis and this free cysteine was identified in both tests (data not shown). The above tertiary structure analysis also suggests that, as in the case of *P. chabaudi* AMA1 (Hodder et al., 1996, supra), the majority of AMA1/E molecules also had all 16 cysteines cross-linked by disulphide bonds.

Example 8

10 **AMA1/E reacts with conformation dependent immune reagents:** AMA1/E reacted with the monoclonal antibody 4G2dc1 on immunoblot (Fig. 4B, lane 1). This monoclonal antibody recognizes a reduction sensitive epitope on AMA1 of *P. falciparum* (Kocken et al., 1998 supra). Reactivity on immunoblot was also observed with a hyper-immune malaria endemic serum pool from Kenya (Fig. 4C, lane 1). Alkylation of AMA1/E caused no change in its reactivity to the above two immune reagents (Fig. 4B, lane 2 and 3C, lane 2). However, significant loss of reactivity to both immune reagents was observed upon reductive-alkylation (Fig. 4B, lane 3 and 3C, lane 3), further confirming the presence of critical reduction sensitive epitopes on AMA1/E.

Example 9

25 **AMA1/E was found to be immunogenic in rabbits:** Immunization of rabbits, with lab-grade AMA1/E and RR-AMA1/E at 100 µg per dose was done to determine if one form was immunologically superior to the other. A group of 3 rabbits was immunized with 100 µg per dose RA-AMA1/E to determine if disulphide bond independent epitopes also contributed towards the induction of inhibitory anti-AMA1 antibodies. The AMA1/E protein produced under GMP conditions was

immunized at 50 and 100 μ g per dose to determine the immunogenicity at the two doses (50 μ g is the expected human dose). No apparent signs of toxicity of the antigen-adjuvant combination was observed in the immunized animals. Table 1 shows the mean log ELISA titer of immunized groups with either AMA1/E or RA-AMA1/E coated on plates. Rabbits in the lab-grade AMA1/E group (R-1, 2, 3) showed high titer antibodies against AMA1/E protein. No significant difference in the titer was observed between 50 and 100 μ g GMP protein immunized groups (data not shown), hence all six rabbits are represented by a single group in Table 1. The AMA1/E specific titers observed in the 50 and 100 μ g GMP produced AMA1/E group (V-2, 3, 4, 9, 10, 11) were higher than the 100 μ g lab-grade AMA1/E group (2 tailed t test, $p = 2.5E-04$). The RR-AMA1/E group (R-4, 5, 6) also had high ELISA titer against AMA1/E coated wells. The mean titer in the RR-AMA1/E group was slightly lower than the lab-grade AMA1/E protein immunized group although the difference was not statistically significant ($p = 8.2E-01$). The ELISA titers against the RA-AMA1/E protein coated wells for lab-grade, GMP produced AMA1/E and RR-AMA1/E groups were lower as compared to the refolded AMA1/E coated wells ($p = 2.5E-02$, $1.2E-04$ and $1.8E-02$ respectively). One of the 3 rabbits (R-8) immunized with RA-AMA1/E died while handling. Although, the two remaining RA-AMA1/E immunized rabbits (R-7, 10) had high titer of antibody against RA-AMA1/E coated wells, the titer was lower against the refolded AMA1/E coated wells. This difference was not statistically significant ($p = 3.8E-01$).

Table 1:

Immunization	Number of animals per group	Mean Log ELISA titer		Mean Log IFA titer		Mean GIA data	
		AMA1/E coated	RA-AMA1/E coated	Positive Schizonts	N	percent inhibition	N
Lab-grade AMA1/E, 100 ug	3	5.60 (0.04)	4.94 (0.20)	4.41 (0.35)	2	57 (17) 68 (11)*	3 3*
GMP AMA1/E, 50 & 100 ug	6	6.01 (0.13)	5.29 (0.22)	4.71 (0.25)	1	84 (4)	2
RR-AMA1/E 100 ug	3	5.57 (0.19)	5.01 (0.17)	4.06 (0.35)	2	29 (22) 41(25)*	2 2*
RA-AMA1/E 100 ug	2	4.83 (0.15)	5.01 (0.17)	2.43 (0.11)	2	5 (1)	1
Adjuvant	3	0.00	0.00	0.00	1	-2 (2)	2

Antibody responses and percent inhibition in GIA *in vitro* with rabbit sera: Log of mean ELISA titers of lab-grade, GMP produced AMA1/E, RR-AMA1/E or RA-AMA1/E groups tested against either AMA1/E or RA-AMA1/E coated on wells. Antibody titers were calculated for an OD = 0.5 using regression analysis on titration curves. Log IFA titer of groups tested on methanol fixed *P. falciparum* (3D7) schizonts. The percent-inhibition of parasite growth, in a 1-cycle assay, under suspension conditions, at 1:5 serum dilution are shown. Percentage inhibition was calculated from the mean parasitaemia of triplicate test and control wells as 100% - ((test/control) x 100). Inhibitions in GIA were relative to adjuvant control sera or culture media alone. Initial parasitemias were 0.2% and the final control parasitemias ranged from 2.2% to 3.6%. Compared with growth in media alone (100%), there were no significant differences in the final parasitemia of 12 pre-immune sera (mean $\pm$ SD = 97%, $\pm$ 4% suspension, 102% $\pm$ 1% static) or 3 adjuvant only sera (99% $\pm$ 3% suspension, 106% $\pm$ 2% static). * values under static GIA conditions. N stands for number of times the experiment was repeated. Number in the brackets represents the SD.

5

10

Sera from all AMA1/E immunized rabbits tested positive by IFA with late stage schizonts of 3D7 parasites (Fig. 5A). Table 1 shows the mean log IFA titer of the groups. The IFA titer in the GMP produced AMA1/E group was higher than the lab-grade AMA1/E group, although the difference was not significant ($p = 2.7E-01$). The lab-grade AMA1/E group had slightly higher IFA titers than the RR-AMA1/E group, this difference was also not significant ($p = 2.8E-01$). The IFA titer of lab-grade, GMP produced AMA1/E and the RR-AMA1/E groups, were significantly higher than the RA-AMA1/E group titers ($p = 5.4E-03$, $1.5E-05$ and $8.6E-03$ respectively).

Rabbit antibodies reacted with two bands one ~76 kDa and another at ~62 kDa on western blot of an SDS extract of schizont rich preparation of *P. falciparum* 3D7 parasites (Fig. 5B, lane1). These bands most likely correspond to the previously reported 83 and 66 kDa full length and processed forms of AMA1 in *P. falciparum* (Narum and Thomas, 1994, supra; Howell et al., 2001, supra); difference in apparent molecular weights observed here may be a result of difference in PAGE conditions.

Example 10

Anti-AMA1/E antibodies inhibit *in vitro* growth of the parasite: The growth inhibition assay (GIA) of homologous 3D7 *P. falciparum* parasites was carried out with sera obtained from the immunized rabbits. Table 1 shows the mean percent inhibition, under suspension conditions, at 1:5 dilution obtained for each of the immunized groups. The lab-grade and GMP produced AMA1/E group sera showed significant inhibition of parasite growth, compared to the adjuvant controls ($p = 2.4E-02$, $1.1E-09$ respectively). The lab-grade AMA1/E group sera analyzed under static GIA

conditions, gave even higher inhibition compared to the suspension culture, although the difference between static and suspension culture values was not significant ($p=8.4E-02$) (Table 1). Rabbits immunized with 50 and 100 μ g doses showed no significant difference in the percent inhibition (data not shown). The RR-AMA1/E immunized group sera showed lower level of inhibition when compared lab-grade AMA1/E group, both under suspension (p value = $1.5E-01$) and static conditions, ($p=1.9E-02$); the difference was not statistically significant under suspension conditions. There was a positive correlation between log ELISA and log IFA titers ($R^2 = 0.84$). There was also a positive correlation between log ELISA against the AMA1/E protein coated wells and the percent GIA ($R^2 = 0.81$). A positive correlation was also observed between the log IFA titer and percent inhibition ($R^2 = 0.77$). The above R^2 values were calculated using data from all the immunized rabbits in all the groups. No inhibition was seen in RA-AMA1/E group compared to the lab-grade AMA1/E group ($p=3.1E-02$). In comparison to the growth in media alone (100%) there were no significant differences in the final parasitaemia of 12 pre-immune sera (mean $\pm$ SD = $97 \pm 4\%$) or the 3 adjuvant alone sera ($99 \pm 3\%$). Whole serum from one of the rabbits (R-3), which showed 44% inhibition in the one cycle suspension GIA, was used in a two-cycle suspension GIA at the same dilution. Inhibition of 87% was seen, indicative of cumulative inhibition over two cycles.

Inhibition of parasite growth was also observed with IgG purified from pooled sera of the rabbits within the lab-grade AMA1/E, RA-AMA1/E and the adjuvant control (Fig. 6). The percent inhibition in the AMA1/E group was significantly higher than the

adjuvant control at 0.18, 0.35 and 0.7 mg ml⁻¹ IgG concentrations tested ($p = 7E-04$, $8E-03$ and $6E-03$ respectively). No inhibition was observed with the RA-AMA1/E group IgG was compared to the equivalent IgG concentration from the adjuvant control. In order to determine if the inhibition caused by the IgG could be reversed, identical concentration of AMA1/E or RA-AMA1/E proteins were added to the culture during the GIA. The addition of 5.3 µg ml⁻¹ AMA1/E protein to the 0.18 mg ml⁻¹ anti-AMA1/E IgG significantly reverses inhibition compared to the addition of the same amount of RA-AMA1/E ($p=6E-03$) (Fig 6). This data indicates the critical role of disulphide bonds in the formation of epitopes that can induce inhibitory anti-AMA-1 antibodies.

ELISA, IFA and GIA data with antibodies to recombinant AMA1/E, suggested that the conformational epitopes present on the refolded protein (in addition to the linear epitopes) were indeed highly immunogenic. A competition ELISA using sera from three rabbits in the lab-scale AMA1/E group (R-1, 2, 3) and two rabbits in the RA-AMA1/E group (R-7, 10) was done by pre-incubation with the 15 µg ml⁻¹ of AMA1/E, RA-AMA1/E or with BSA as control. Figure 6, shows the mean OD₄₀₅ at 1:16,000 serum dilution for the two groups along with the SD for 3 experiments. Pre-incubation with the refolded AMA1/E protein resulted on average 86% and 81% reduction in OD₄₀₅ in AMA1/E and RA-AMA1/E groups respectively, in comparison to the BSA control values in the same group ($p = 1.99E-13$ and $1.55E-10$ respectively). Although, RA-AMA1/E protein pre-incubation resulted in an average 89% drop in the OD₄₀₅ in the RA-AMA1/E immunized group ($p = 5.63E-09$), it resulted in an insignificant, 16% drop in the

refolded AMA1/E group ($p = 0.071$). This data further suggests that a large proportion of the antibodies against refolded AMA1/E were against disulphide bond dependent epitopes.

5

DISCUSSION

The availability of a significant quantity of a stable recombinant protein having pharmaceutical levels of purity is an essential step on the path of testing adjuvant combinations capable of inducing a long-lasting and high titer responses in humans. We describe here a process that was successfully scaled-up to produce AMA1/E, a recombinant protein based on the ectodomain of *P. falciparum* AMA-1. A 300 L fermentation generated 4.5 kg of cell paste. Starting from 1.5 kg of cell paste 70 mg AMA1/E, enough material for over 800 doses, was purified, vialled and lyophilized under a GMP environment. While the final yield of AMA1/E was relatively low, the AMA1/E product passed all the major criteria set to proceed into a Phase I clinical trial. These include, purity (>99% done by SDS-PAGE and GPC), endotoxin content (0.06 EU per 50 μ g protein by LAL test), free thiol content (< 0.1 μ M free -SH groups per μ M protein measured by Ellman's test), positive reactivity to immune reagents (monoclonal antibody 4G2dc1 and malaria immune sera done by western blotting), mass analysis (54,648 Da by MALDI-TOF), correct N-terminal sequence (first 21 residues by Edman's method), host cell protein content (<0.5% by ELISA), western blot (no *E. coli* specific band with up to 2 μ g AMA1/E loaded per well), residual sarkosyl content (below detectable limits by RP-HPLC) and product stability (stable at 37°C for more than 6 months) in its lyophilized form (data not shown). The GMP produced AMA1/E product, was immunogenic in rabbits and raised high titer inhibitory antibodies.

The correct folding of AMA1, as in case of several other *Plasmodium* antigens, has been shown to be critical for its immunological activity (Anders et al., 1998, supra; Hodder et al., 2001, supra; Crewther et al., 1996, supra). Full length *P. falciparum* AMA1 was first expressed in the eukaryotic insect cell system (Narum et al., 1993, J. Chromatogr. A. 657, 357-363), although the baculovirus product was soluble, the purification strategy was not designed for scale-up production. Prokaryotic expression of AMA1 from various species has been problematic, primarily due to the formation of insoluble aggregates presumably due to incorrect folding of the protein. Previous work on *P. chabaudi* AMA1 expression in *E. coli* showed that it was necessary to include an *in vitro* refolding step in the process in order to obtain correctly folded protein (Anders et al., 1998, supra). A similar approach was successful for obtaining correctly folded AMA1 from *P. falciparum* and the antibodies made against it inhibited parasite growth *in vitro* (Hodder et al., 2001, supra). A scalable process for the production of recombinant AMA1 has not yet been described. Following the success with another malarial antigen (Dutta et al., 2001, supra), we attempted to express r-AMA1/E as a soluble protein in *E. coli*. A 'redox modified' strain of *E. coli*, Origami (DE3), with mutations in the glutathione and thioredoxin reductase pathways (Bessette et al., 1999, Proc. Natl. Acad. Sci. USA 96, 13703-13708) was used for expression, with induction carried out at low temperature and using a minimal IPTG concentration. Despite attempts to optimize the fermentation conditions to obtain soluble r-AMA1/E, a large fraction was still located in the insoluble pellet. Hence, a downstream purification process was developed

to extract r-AMA1/E from both soluble and insoluble fractions and to refold it *in vitro*.

The use of Origami (DE3) cells was, in fact, a factor that attributed to low protein yield due to
5 plasmid loss during scale-up fermentation. Although the vector pWRMAL contained both ampicillin and tetracycline resistance genes for plasmid maintenance, penicillin derivatives like ampicillin cannot be used during the production of a human-use vaccine. The *E.*
10 *coli* Origami (DE3) strain used to enhance soluble protein expression carried a *tet^r* gene, resulting in low plasmid maintenance in the presence of tetracycline over the long growth period required for scale-up fermentation. Future studies are being
15 directed to the selection of other bacterial hosts that allow the use of a selectable marker to increase plasmid maintenance.

The increase in reactivity to immune reagents observed after the refolding step and the homogeneity
20 of the final product, justified the need for the inclusion of this refolding step in the process, although, this was a limiting factor during scale up production. A minimum of 40-fold dilution was necessary to gain optimal immune reactivity. An anion-
25 exchange step was used after refolding to separate the monomeric AMA1/E from its aggregated forms, which eluted at a higher NaCl concentration. This monomer selection step resulted in some loss of product during purification. After anion-exchange, a doublet at ~ 10
30 kDa, was found to co-elute with AMA1/E and, although GPC was an option, we avoided it due to problems associated with scale-up. Instead, a cation-exchange step using SP-Sepharose was used to purify AMA1/E to homogeneity. Assays based on immuno-detection of
35 HCP's, in combination with laser densitometry of

stained polyacrylamide gels and analytical GPC were used to determine that the final product was >99% pure.

N-terminal sequencing and mass spectrometric
5 analysis confirmed the correct primary structure of AMA1/E. Ellman's test and alkylation analysis confirmed the absence of any free cysteines in the final product. Shift in the RPC elution profile under reduced conditions and immunoblot reactivity to a
10 conformation dependent inhibitory rat monoclonal antibody, 4G2dc1, under non-reduced conditions further confirms the disulphide-bonded nature of the antigen.

AMA1/E was found to be highly immunogenic in rabbits in combination with Montanide ISA720 adjuvant.
15 Antibodies raised against the recombinant protein recognized the native parasite AMA1 protein both on IFA and western blot. Whole serum from the immunized rabbits showed growth inhibition of the homologous *P. falciparum* (3D7) parasites *in vitro* both under
20 suspension and static GIA conditions. During this process development, we refolded the r-AMA1/E protein either directly (AMA1/E) or after DTT reduction of the Ni⁺² column elution (RR-AMA1/E). The RR-AMA1/E product based on its overall lower immunogenicity, lower
25 percent GIA values of its anti-sera, in addition to the observation that DTT reduction gave no significant gain in the monomeric protein yield was not pursued further in the scale-up GMP process.

It has previously been shown that AMA1 based
30 protective immunity can be passively transferred by IgG transfusion into naïve animals (Narum et al., 2000, supra; Anders et al., 1998, supra). Antibodies to recombinant AMA1 from *P. falciparum* have recently been reported to inhibit parasite invasion *in vitro*
35 (Hodder et al., 1996, supra). These data suggest that

AMA1 based protection is probably antibody mediated. *In vitro* growth inhibition observed with whole sera and with purified IgG, in addition to the positive correlations observed between the ELISA titer (against
5 AMA1/E coated wells), and IFA titer, suggests that these measures of antibody response might serve as good correlates of AMA1 based protection *in vivo*. When comparing the same sera in parallel experiments we have observed higher percent GIA values in static
10 culture compared to the suspension GIA (paired *t* test, $p = 5E-06$). Fluid movement in suspension culture may better mimic the blood flow conditions encountered *in vivo* by the parasite, than does static culture GIA. Some antibodies are known to show better inhibition in
15 either static or suspension culture (Haynes et al., 2002, supra) and it remains to be seen whether suspension or static culture GIA better predicts protection following vaccination. However, it is encouraging to report that anti-AMA1/E antibodies are
20 inhibitory under both conditions.

Previous data on mice vaccination with recombinant *P. chabaudi* AMA1 suggested that, the presence of intact disulphide bonds in the vaccinating AMA1 antigen are necessary to induce protection
25 (Crewther et al., 1996, supra). The data presented here also suggests that disulphide-bond dependent motifs play a critical role in the induction of inhibitory anti-AMA1 antibodies. Higher ELISA titer obtained with refolded AMA1/E coated wells compared to
30 the RA-AMA1/E coated wells in the AMA1/E immunized group, lower IFA titers in the RA-AMA1/E group, inability of the anti-RA-AMA1/E antibodies to block parasite invasion, the ability of AMA1/E and not RA-AMA1/E protein to significantly reverse the *in vitro*
35 growth inhibition and the ability of AMA1/E and not of

RA-AMA1/E, to out-compete binding of most of the anti-AMA1/E antibody to AMA1/E protein on ELISA, indicates that a majority of the immunologically significant epitopes of AMA-1 are sensitive to reduction.

- 5 In conclusion this application details the process development for the production of a disulfide cross-linked AMA1 ectodomain recombinant protein that could serve as a malaria vaccine candidate. Safety, stability and potency tests in animals are underway.

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72a

SEQUENCE LISTING

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72b

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Ala Glu Val Ala Gly Thr Gln Tyr Arg Leu
                    75              80
Pro Ser Gly Lys Cys Pro Val Phe Gly Lys
                    85              90
Gly Ile Ile Ile Glu Asn Ser Asn Thr Thr
                    95             100
Phe Leu Thr Pro Val Ala Thr Gly Asn Gln
                   105             110
Tyr Leu Lys Asp Gly Gly Phe Ala Phe Pro
                   115             120
Pro Thr Glu Pro Leu Met Ser Pro Met Thr
                   125             130
Leu Asp Glu Met Arg His Phe Tyr Lys Asp
                   135             140
Asn Lys Tyr Val Lys Asn Leu Asp Glu Leu
                   145             150
Thr Leu Cys Ser Arg His Ala Gly Asn Met
                   155             160
Ile Pro Asp Asn Asp Lys Asn Ser Asn Tyr
                   165             170
Lys Tyr Pro Ala Val Tyr Asp Asp Lys Asp
                   175             180
Lys Lys Cys His Ile Leu Tyr Ile Ala Ala
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Gln Glu Asn Asn Gly Pro Arg Tyr Cys Asn
                   195             200
Lys Asp Glu Ser Lys Arg Asn Ser Met Phe
                   205             210
Cys Phe Arg Pro Ala Lys Asp Ile Ser Phe
                   215             220
Gln Asn Tyr Thr Tyr Leu Ser Lys Asn Val
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```

72c

Val	Asp	Asn	Trp	Glu	Lys	Val	Cys	Pro	Arg	235	240
Lys	Asn	Leu	Gln	Asn	Ala	Lys	Phe	Gly	Leu	245	250
Trp	Val	Asp	Gly	Asn	Cys	Glu	Asp	Ile	Pro	255	260
His	Val	Asn	Glu	Phe	Pro	Ala	Ile	Asp	Leu	265	270
Phe	Glu	Cys	Asn	Lys	Leu	Val	Phe	Glu	Leu	275	280
Ser	Ala	Ser	Asp	Gln	Pro	Lys	Gln	Tyr	Glu	285	290
Gln	His	Leu	Thr	Asp	Tyr	Glu	Lys	Ile	Lys	295	300
Glu	Gly	Phe	Lys	Asn	Lys	Asn	Ala	Ser	Met	305	310
Ile	Lys	Ser	Ala	Phe	Lys	Pro	Thr	Gly	Ala	315	320
Phe	Lys	Ala	Asp	Arg	Tyr	Lys	Ser	His	Gly	325	330
Lys	Gly	Tyr	Asn	Trp	Gly	Asn	Tyr	Asn	Thr	335	340
Glu	Thr	Gln	Lys	Cys	Glu	Ile	Phe	Asn	Val	345	350
Lys	Pro	Thr	Cys	Leu	Ile	Asn	Asn	Ser	Ser	355	360
Tyr	Ile	Ala	Thr	Thr	Ala	Leu	Ser	His	Pro	365	370
Ile	Glu	Val	Glu	Asn	Asn	Phe	Pro	Cys	Ser	375	380
Leu	Tyr	Lys	Asp	Glu	Ile	Met	Lys	Glu	Ile	385	390
Glu	Arg	Glu	Ser	Lys	Arg	Ile	Lys	Leu	Asn	395	400
Asp	Asn	Asp	Asp	Glu	Gly	Asn	Lys	Lys	Ile	405	410
Ile	Ala	Pro	Arg	Ile	Phe	Ile	Ser	Asp	Asp	415	420
Lys	Asp	Ser	Leu	Lys	Cys	Pro	Cys	Asp	Pro	425	430
Glu	Met	Val	Ser	Asn	Ser	Thr	Cys	Arg	Phe	435	440
Phe	Val	Cys	Lys	Cys	Val	Glu	Arg	Arg	Ala	445	450
Glu	Val	Thr	Ser	Asn	Asn	Glu	Val	Val	Val	455	460
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72d

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 His

<210> 5
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 Ser Gly Ser Gly Thr Met His Gly Ala Glu
 15 20
 Pro Ala Pro

CLAIMS:

1. An isolated polynucleotide encoding the *P. falciparum* strain 3D7 apical membrane antigen-1 (AMA-1) ectodomain protein consisting of amino acids 83-531 of AMA-1.
2. The polynucleotide of claim 1, further comprising a polynucleotide encoding a 6-histidine tag on the carboxy terminal end of the encoded protein.
3. The polynucleotide of claim 2, further comprising a polynucleotide encoding a 6-histidine tag on the amino terminal end of the encoded protein.
4. The polynucleotide of claim 3, said polynucleotide defined in SEQ ID NO:1.
5. A recombinant vector comprising the polynucleotide of claim 4.
6. The vector of claim 5, wherein said vector is pWRMAL-AMA1/E.
7. A recombinant vector comprising the polynucleotide of claim 1.
8. An isolated and purified, recombinant *P. falciparum* 3D7 apical membrane antigen 1 ectodomain protein (AMA-1/E) consisting of the amino acid sequence defined in SEQ ID NO:2.
9. An isolated, recombinant AMA-1/E protein consisting of the amino acid sequence encoded by the nucleic acid sequence defined in SEQ ID NO: 1.

10. A host cell transformed with the vector according to claim 6.

11. The host cell of claim 10, wherein said host cell is a redox modified host.

12. The host cell of claim 11, wherein said host cell is Origami™ DE3.

13. A method for isolating and purifying recombinant *P. falciparum* AMA-1 protein comprising:

growing a host cell containing a recombinant vector expressing *P. falciparum* AMA-1 protein according to claim 8 in a suitable culture medium,

causing expression of said vector under suitable conditions for production of soluble AMA-1 protein,

lysing said host cells and recovering said AMA-1 protein, and

refolding said AMA-1 protein such that it reacquires its native folding.

14. The method of claim 13, wherein said expression of said vector is by induction with IPTG at a temperature range of 25°C to 30°C.

15. The method of claim 14, wherein said induction is at 28°C.

16. The method of claim 13, wherein lysing of cells is in the presence of a mild detergent.

17. The method of claim 16, wherein said mild detergent is sarkosyl.

18. The method of claim 13, further comprising removal of *E. coli* proteins, if present.

19. The method of claim 18, wherein said removal of *E. coli* proteins is by application to a Ni-NTA column, followed by anion exchange chromatography, followed by cation exchange chromatography.

20. The method of claim 13, wherein said refolding is in the presence of about 1 mM reduced glutathione and about 0.25 mM oxidized glutathione.

21. The recombinant protein according to claim 8, wherein said protein retains its native folding.

22. The protein of claim 21, wherein said purified protein is at least 95% pure.

23. The protein of claim 21, wherein said purified protein is at least 96% pure.

24. The protein of claim 21, wherein said purified protein is at least 97% pure.

25. The protein of claim 21, wherein said purified protein is at least 98% pure.

26. The protein of claim 21, wherein said purified protein is at least 99% pure.

27. A method for *in vitro* diagnosis of *P. falciparum* malaria antibodies in a biological sample, comprising:

(i) contacting said biological sample with a composition comprising the AMA-1 protein according to claim 21 under

appropriate conditions which allow the formation of an immune complex, wherein said protein is labeled with a detectable label, and

(ii) detecting the presence of said immune complexes visually or mechanically.

28. A kit for determining the presence of *P. falciparum* antibodies in a biological sample, comprising:

a composition comprising the protein according to claim 21,

a buffer or components necessary for producing said buffer, and a

means for detecting immune complexes formed between the protein and antibodies present in the sample.

29. A method for *in vitro* monitoring *P. falciparum* malaria infection or prognosing the response to treatment of patients suffering from malaria infection comprising:

incubating a biological sample from a patient with malaria infection with the AMA-1 protein according to claim 21 under conditions allowing the formation of an immunological complex,

removing unbound components,

calculating the anti-AMA-1 titers present in said sample, and

monitoring the natural course of malaria infection, or prognosing the response to treatment of said patient on the basis of the amount anti-AMA-1 titers found in said sample at the start of treatment, or during the course of treatment, or at the start of treatment and during the course of treatments.

30. A kit for monitoring *P. falciparum* malaria infection or prognosing the response to treatment of patients suffering from malaria infection comprising:

a composition comprising an AMA-1 protein according to claim 21,
a buffer or buffer components,
means for detecting the immune complexes formed between the protein and antibodies present in the sample, and
optionally, a means for determining the amount of immune complex formed.

31. An antibody produced against the AMA-1 protein according to claim 21.

32. A method for *in vitro* diagnosis or detection of *P. falciparum* AMA-1 malaria antigen present in a biological sample, comprising:

- (i) contacting said biological sample with an antibody specific for the protein of claim 21,
- (ii) removing unbound components,
- (iii) incubating the immune complexes formed with heterologous antibodies which specifically bind to the antigens present in the sample to be analyzed, with said heterologous antibodies conjugated to a detectable label under appropriate conditions, and
- (iv) detecting the presence of said immune complexes visually or mechanically.

33. The method of claim 32, wherein step (i) is effected in an immobilized form under appropriate conditions which allow the formation of an immune complex.

34. A kit for *in vitro* detection of *P. falciparum* AMA-1 malaria antigen present in a biological sample, comprising:
at least one antibody which reacts with the protein of claim 21, wherein said antibody is immobilized on a solid substrate,

a buffer, or components necessary for producing the buffer, enabling a binding reaction between these antibodies and the malaria antigens present in the biological sample, and a means for detecting the immune complexes formed in the preceding binding reaction.

35. An immunogenic composition comprising the *P. falciparum* AMA-1/E of claim 21 and a carrier.

36. The composition of claim 35, further comprising an adjuvant.

37. A vaccine against *P. falciparum* malaria comprising the *P. falciparum* AMA-1/E according to claim 21.

38. The vaccine of claim 37, further comprising an adjuvant.

39. The vaccine of claim 38, wherein said adjuvant is montanide.

40. A use for inducing in a subject an immune response against *P. falciparum* malaria infection of a composition comprising an immunologically effective amount of *P. falciparum* AMA-1 of claim 21 in an acceptable diluent.

41. The use of claim 40, wherein said composition further comprises an adjuvant.

42. The use of claim 41, wherein said adjuvant is montanide.

43. A use for inducing a protective immune response to *P. falciparum* malaria in a mammal of a composition comprising the protein according to claim 21 in an amount effective to induce an immune response in said mammal.

44. The use according to claim 43, wherein the composition further comprises an adjuvant.

45. The use according to claim 44, wherein said adjuvant is montanide.

46. A multivalent vaccine for protection against infection with more than one strain of *P. falciparum* comprising the *P. falciparum* protein according to claim 21 from more than one strain of *P. falciparum*, said *P. falciparum* consisting of FVO or CAMP.

47. The multivalent vaccine of claim 46, further comprising an adjuvant.

48. A protein produced by the method of claim 14.

49. An AMA-1/E protein expressed by the vector of claim 6.

50. A method for isolating and purifying recombinant *P. falciparum* AMA-1 protein comprising:

expressing a host cell containing a recombinant vector expressing *P. falciparum* AMA-1 protein according to claim 6 in a suitable culture medium,

causing expression of said vector under suitable conditions for production of soluble AMA-1 protein,

lysing said host cells and recovering said AMA-1 protein, and

refolding said AMA-1 protein such that it reacquires its native folding.

51. A protein produced by the method of claim 50.

FIG. 1

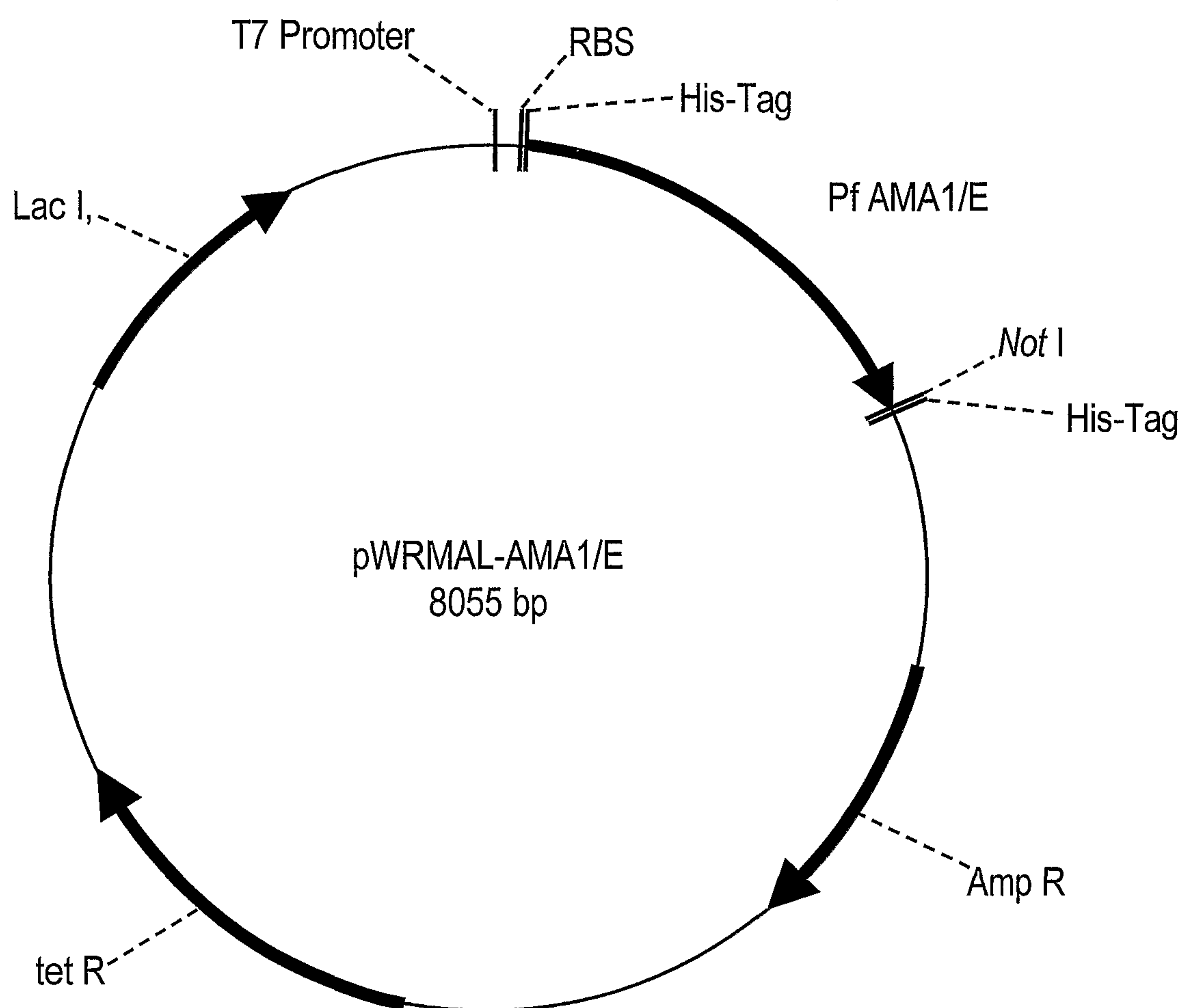


FIG. 2A

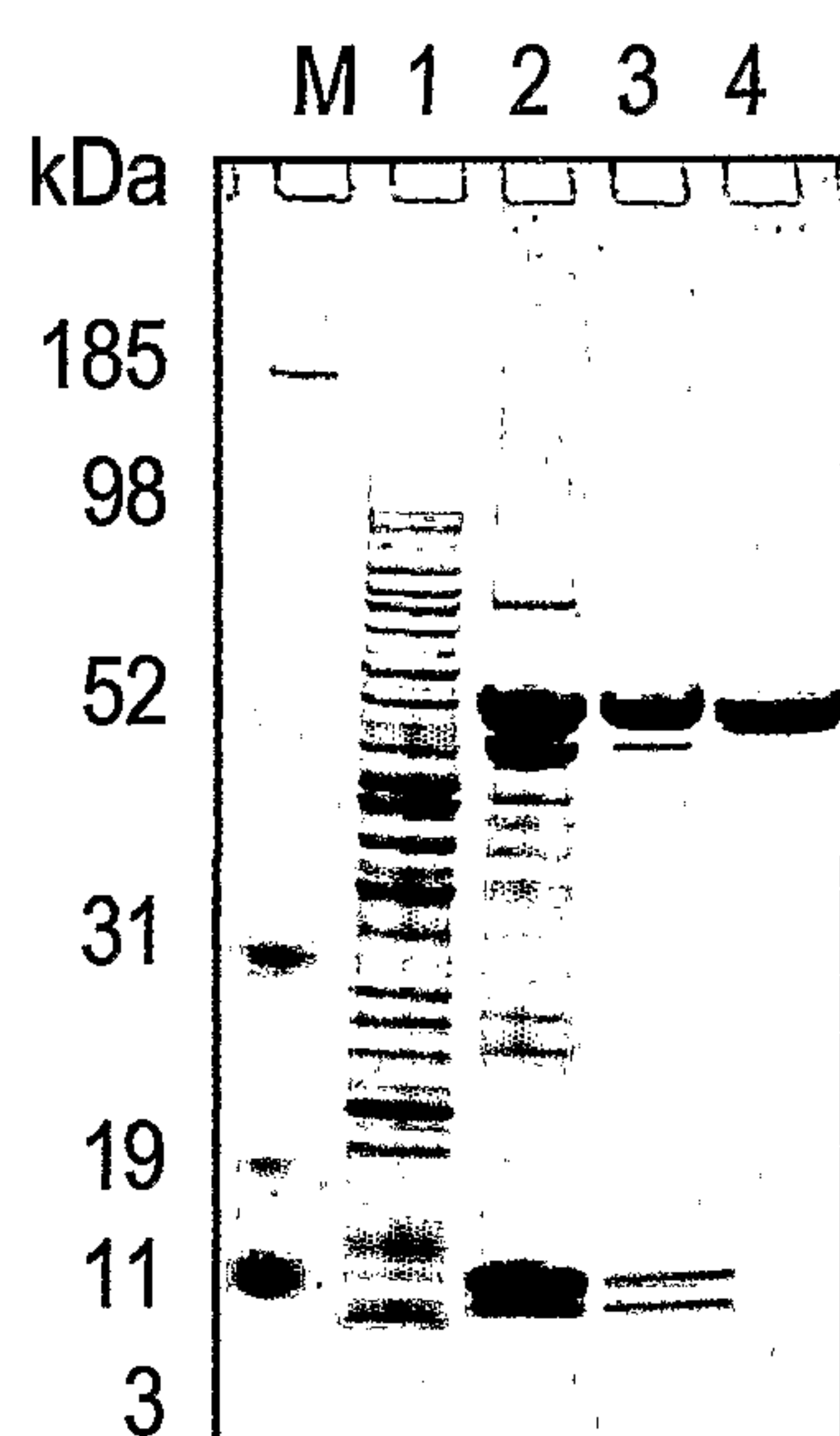


FIG. 2B

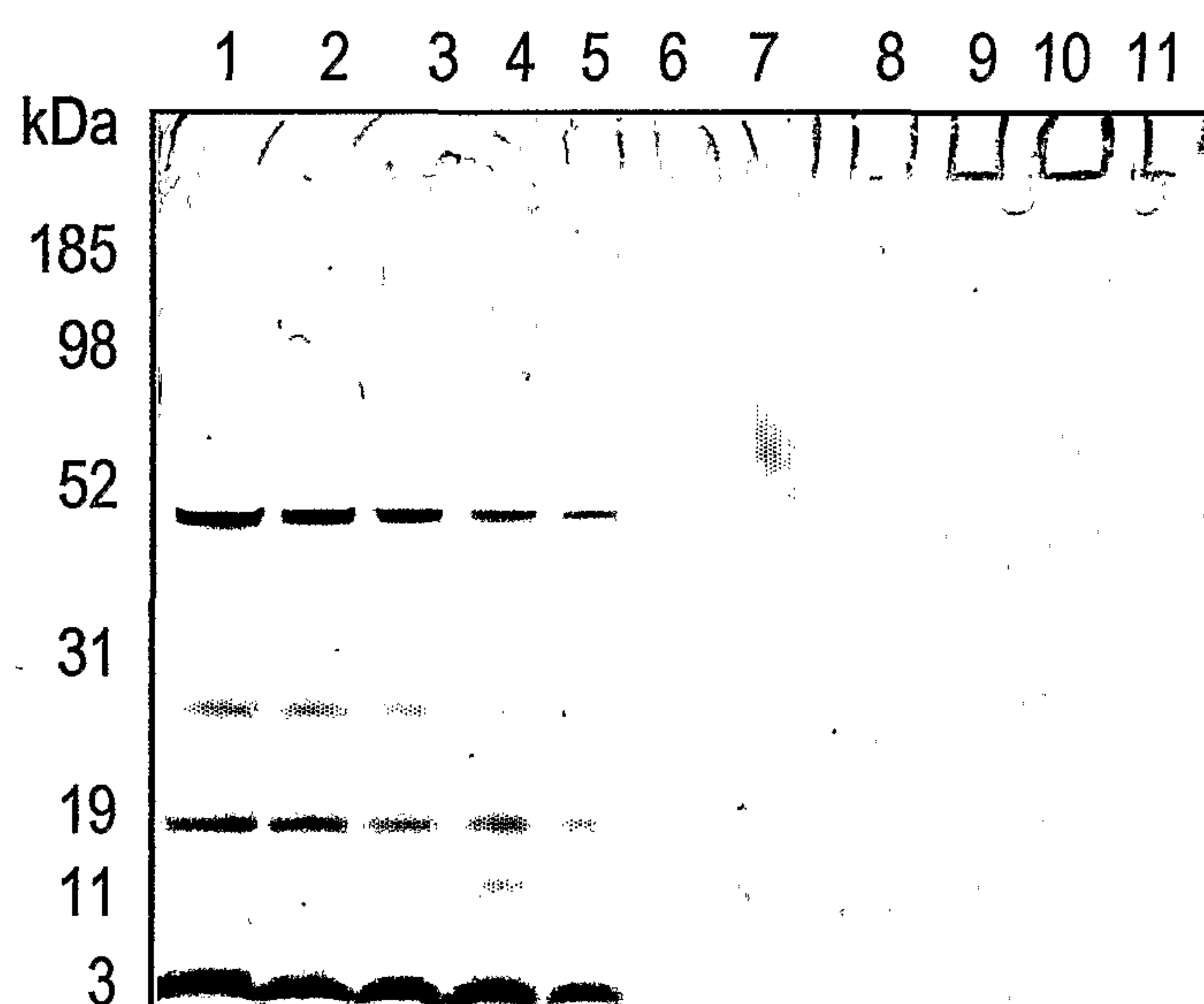


FIG. 3A

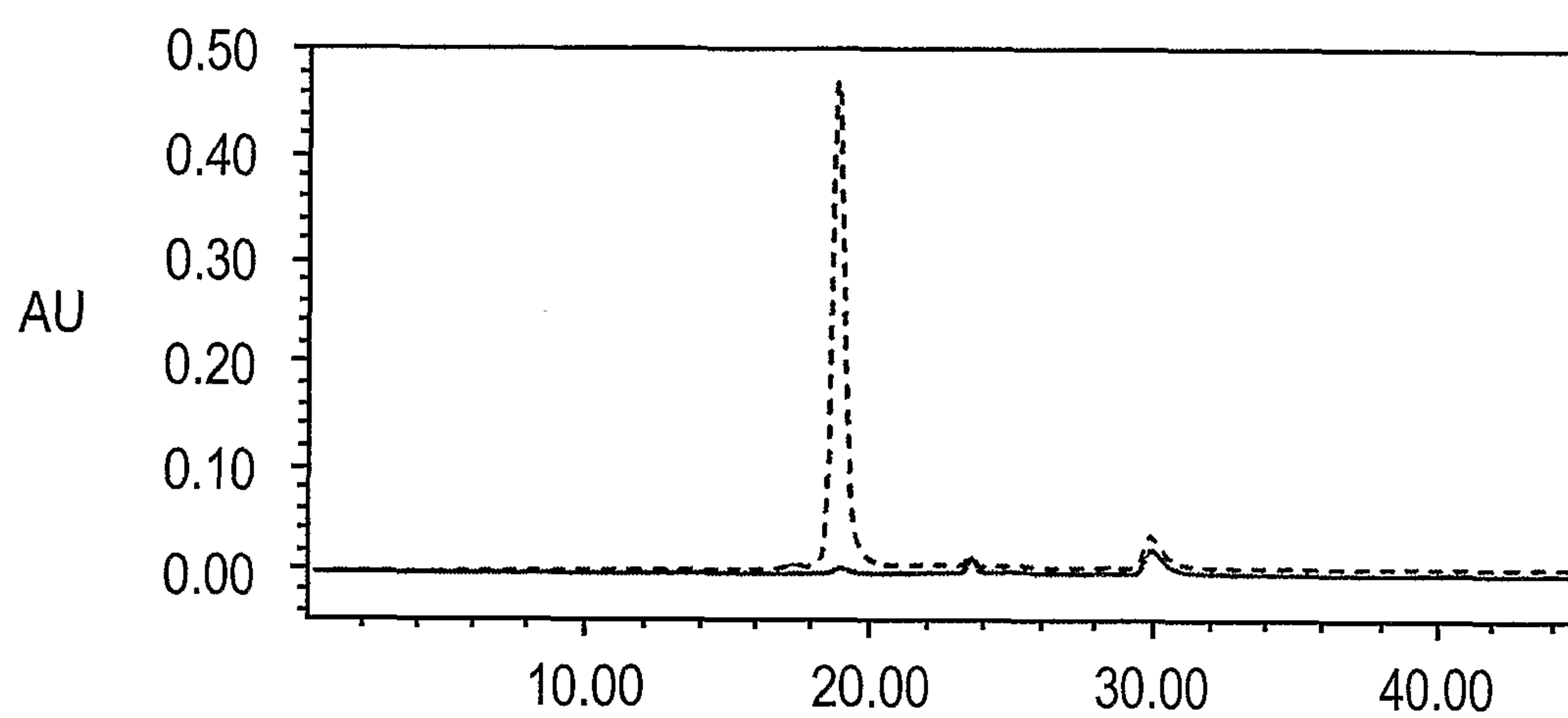


FIG. 3B

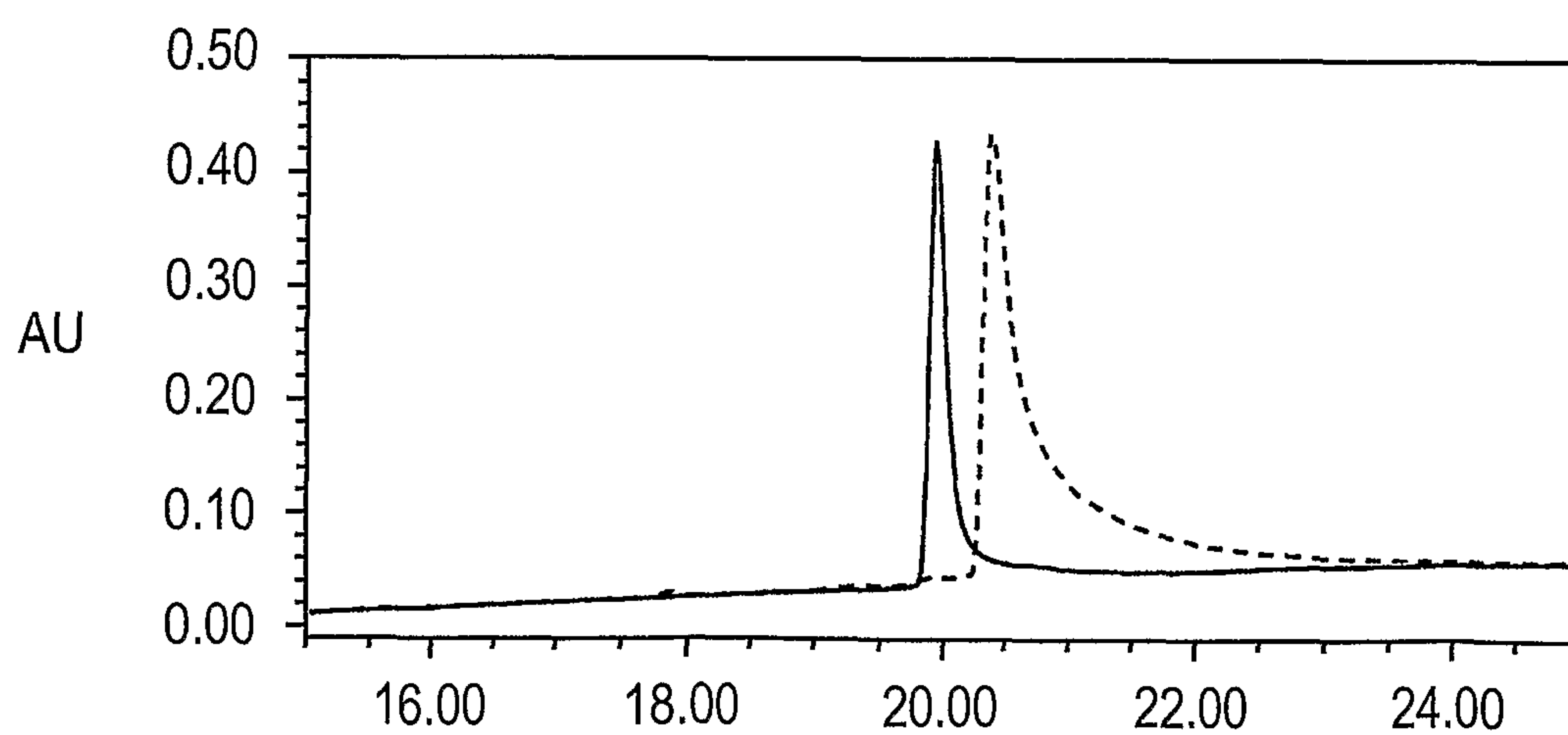


FIG. 4A

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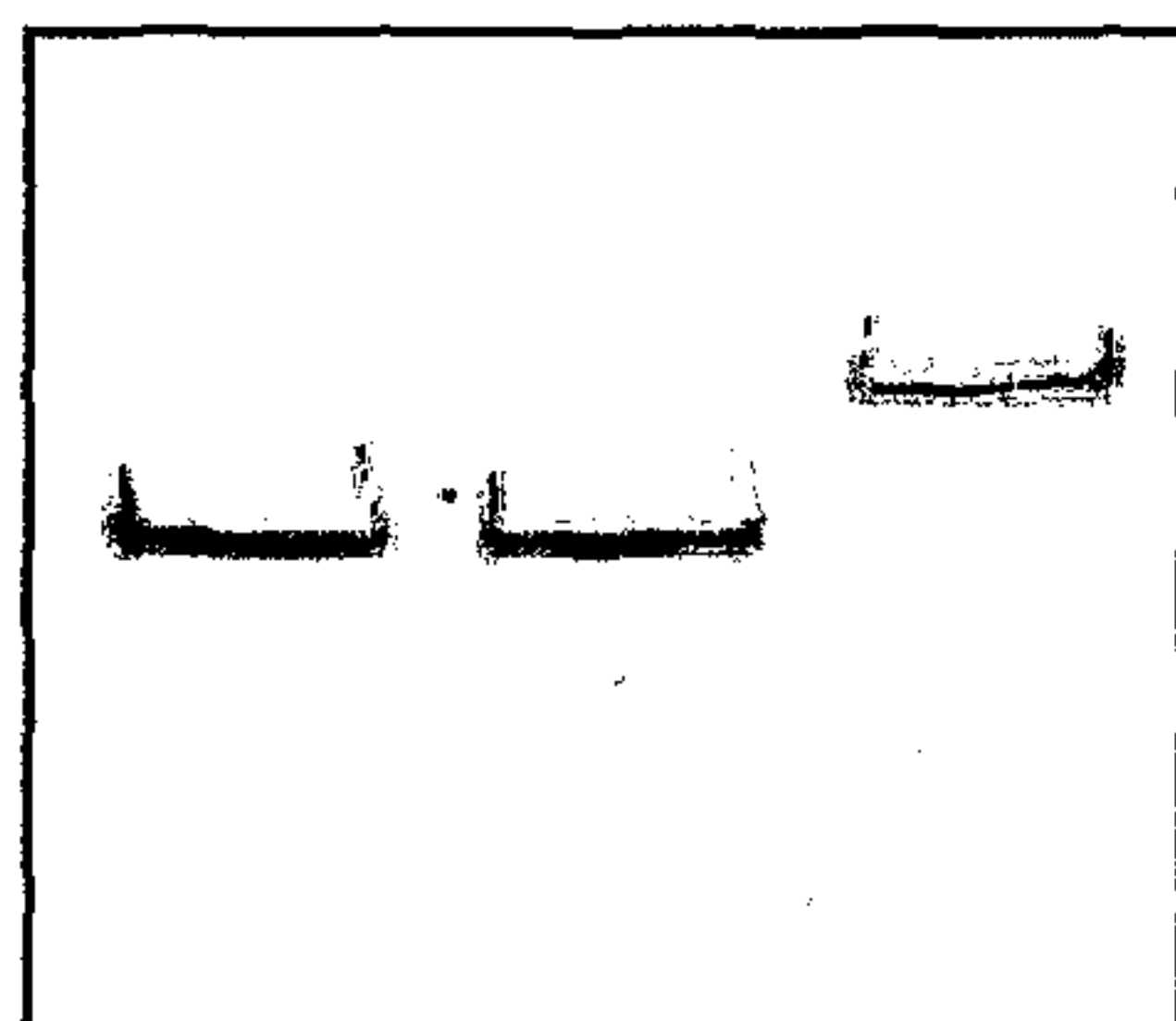


FIG. 4B

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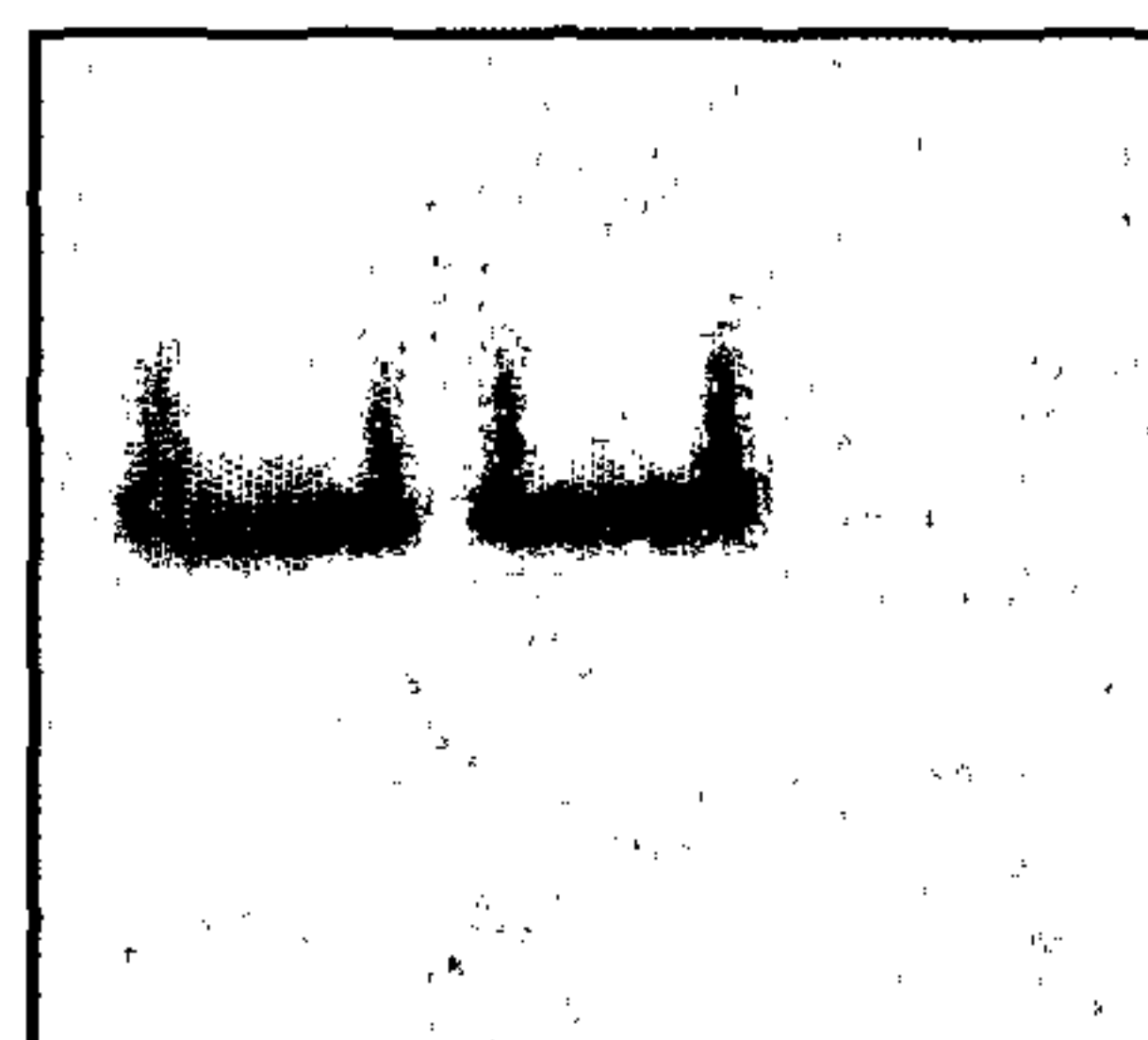


FIG. 4C

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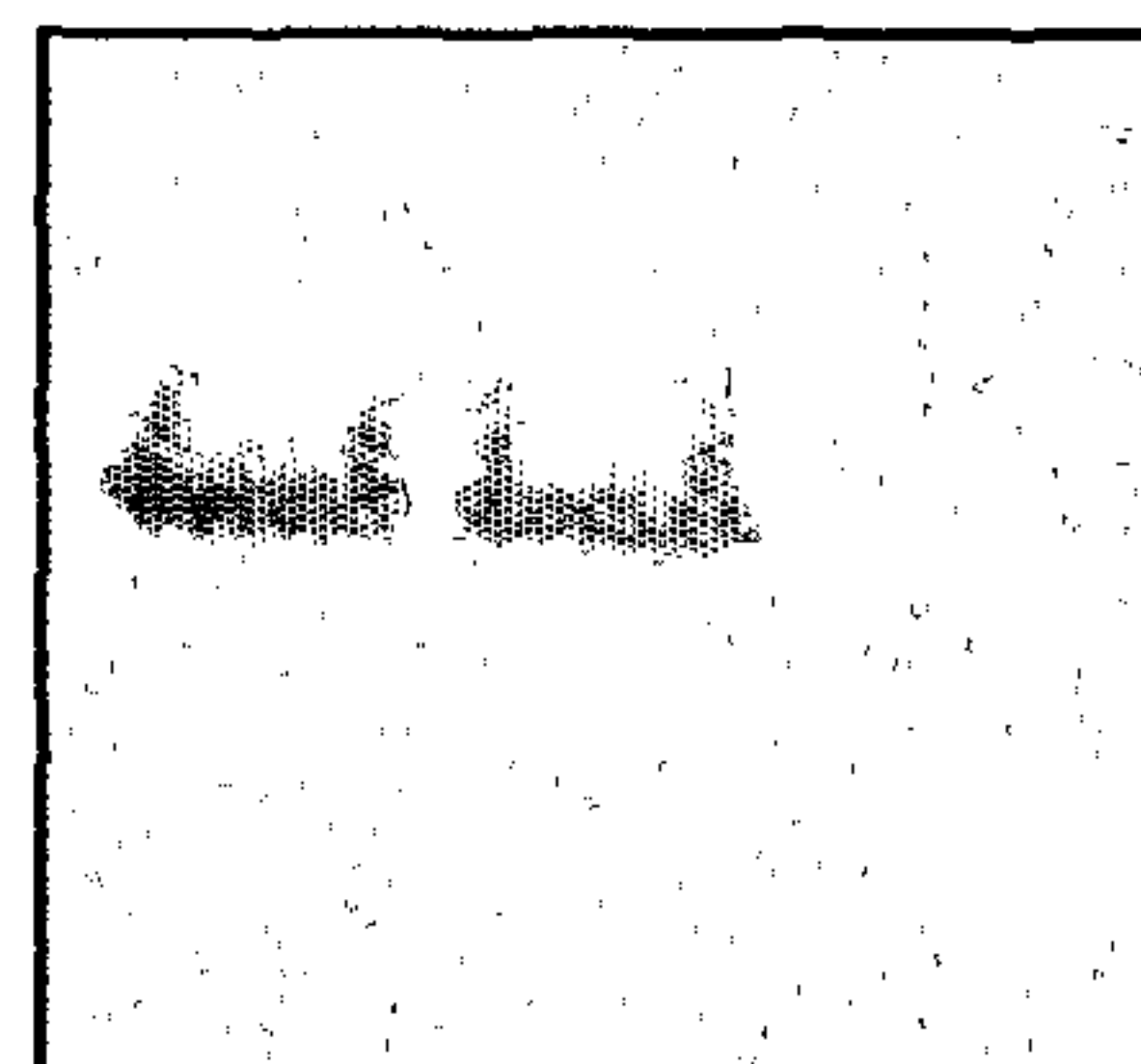


FIG. 5A

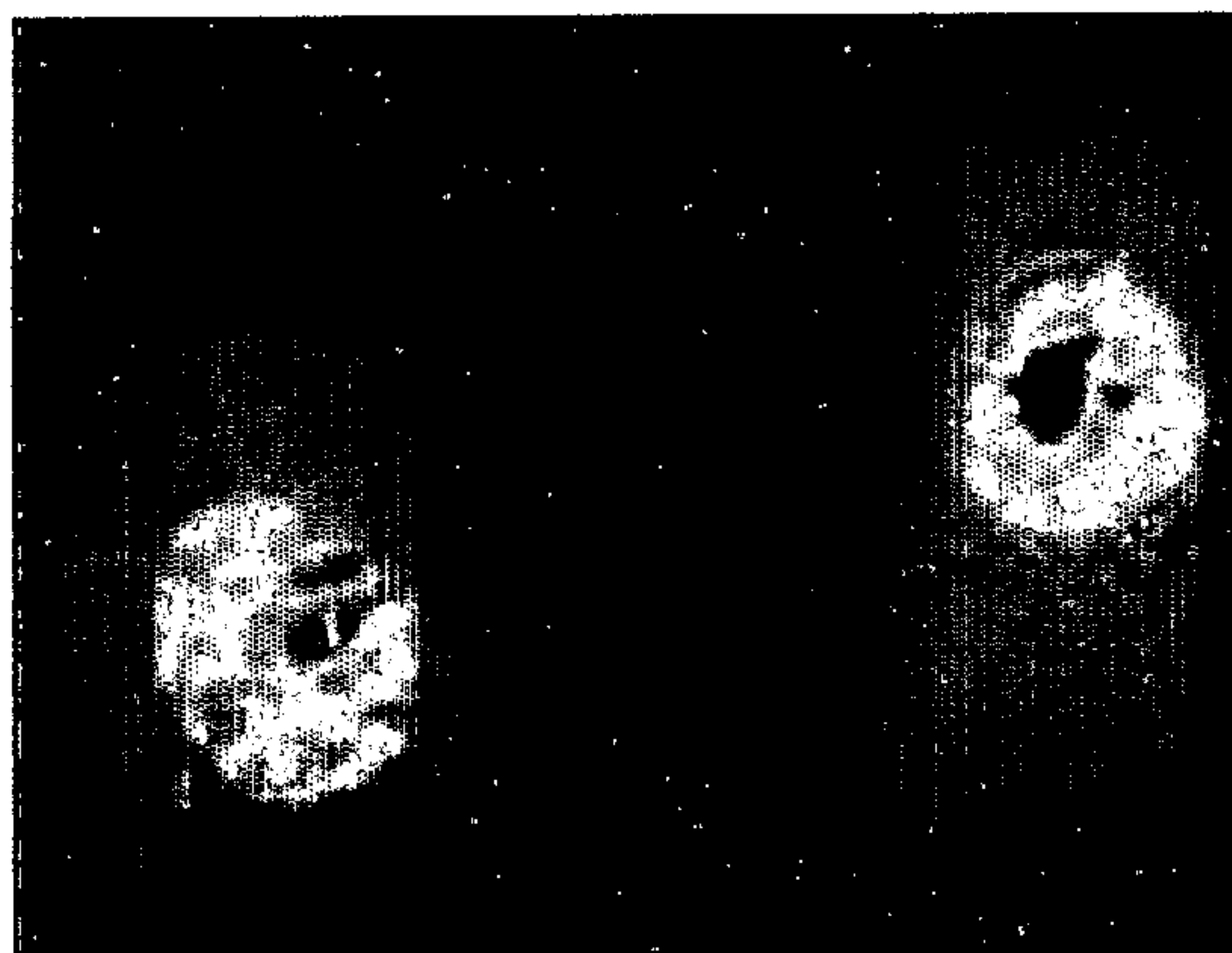


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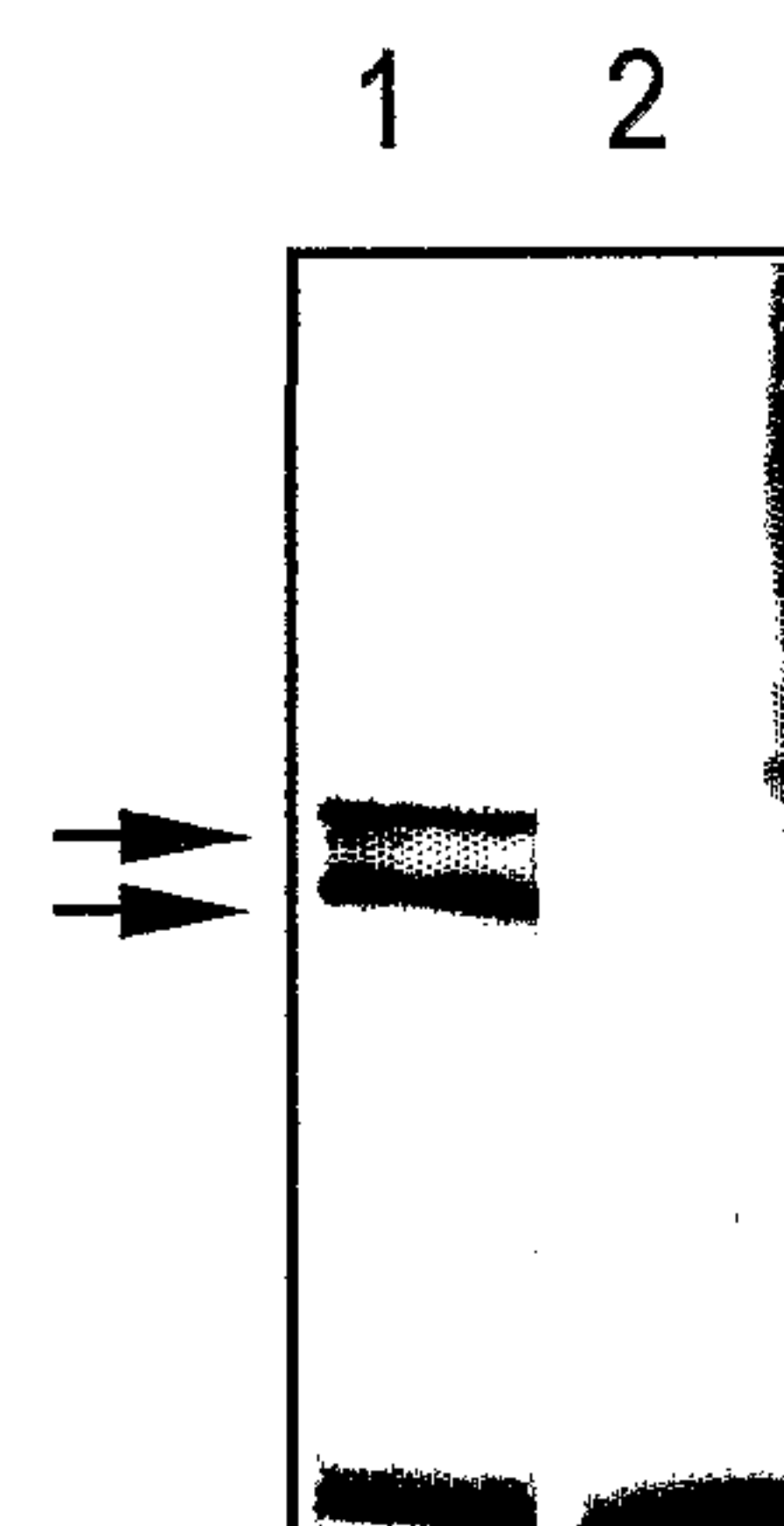


FIG. 6

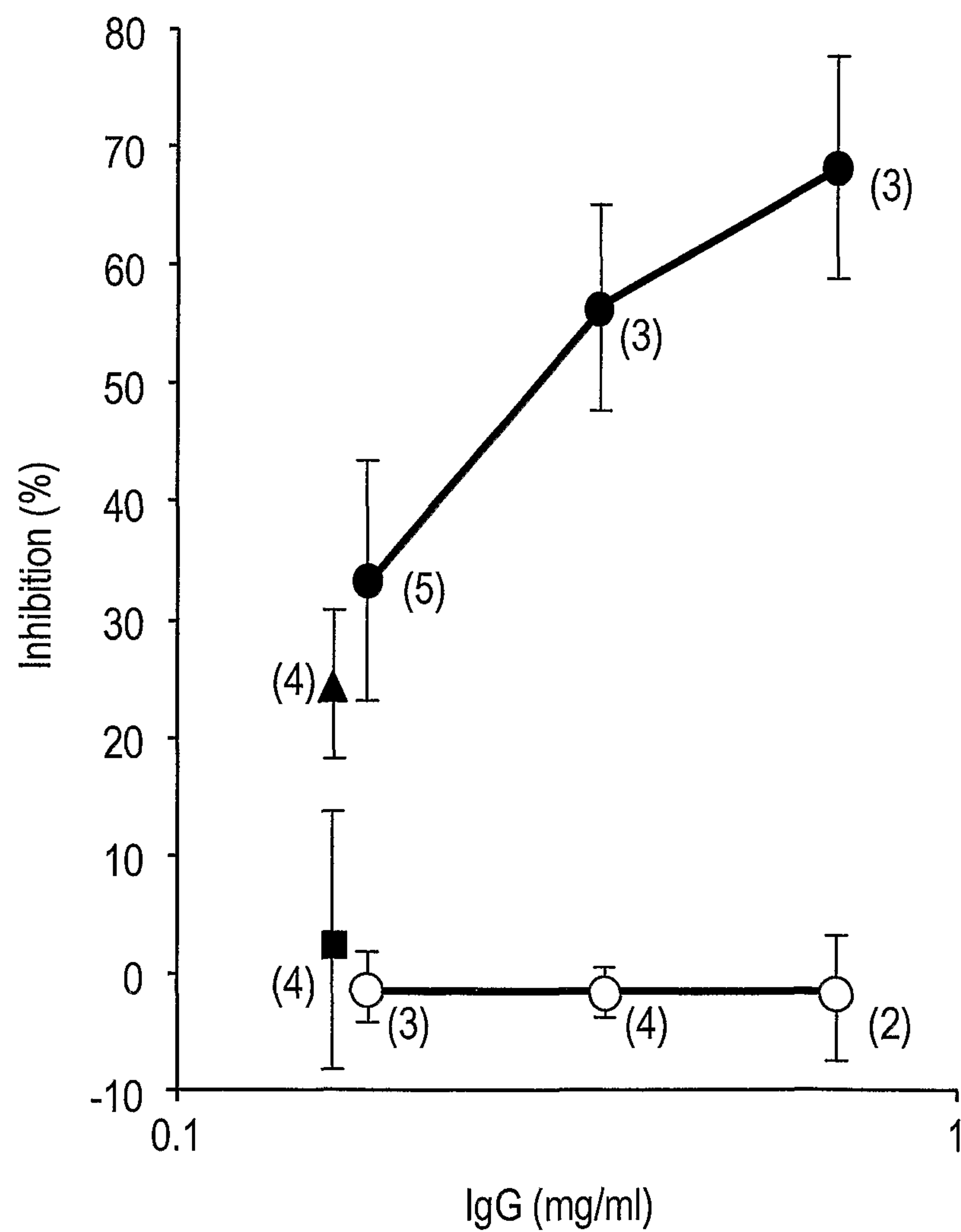


FIG. 7

