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[Continued on next page]

(54) Title: DETECTION OF DNA SEQUENCES AS RISK FACTORS FOR HIV INFECTION

Figure 1(A)

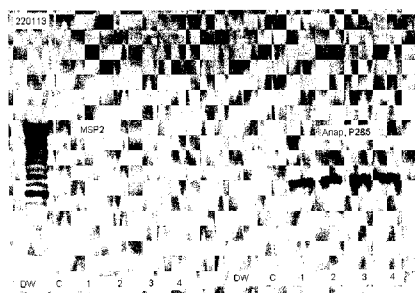


Figure 1(B)



(57) Abstract: A method for identifying a risk factor for diseases, disorders or conditions, such as those caused by human immunodeficiency virus, using the polymerase chain reaction and specific primers. Methods for treating patients having these diseases, disorders or conditions by antimicrobial treatment of the risk factor by combined antiviral and antibacterial treatment or by sustaining or stimulating the subject's immune system. Methods for screening biological products including red blood cell preparations. Primers and methods for detecting nucleic acids or microbial agents associated with red blood cells, such as those associated with red blood cells in subjects infected with HIV and undergoing antiretroviral therapy.



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TITLE

DETECTION OF DNA SEQUENCES AS RISK FACTORS FOR HIV INFECTION

5

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application 61/716,123, filed November 9, 2012 and to U.S. Provisional Application 61/591,111, filed
10 January 26, 2012, which are hereby incorporated by reference. The subject matter disclosed in U.S. Application Nos. 61/186,610; 61/358,282; 61/476,110; 61/476,545; 12/797,286; 13/168,367; 61/591,111; and PCT/US2010/038160 is hereby incorporated by reference.

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BACKGROUND OF THE INVENTION

Field of the Invention

DNA can be amplified from human red blood cell samples by primers designed from
20 DNA sequences encoding a bacterial major surface protein and 16 s ribosomal RNA (16s rRNA). Primer pairs based on DNA sequences for the major surface protein 2 (MSP2) of *Erhlichia/Anaplasma* can amplify DNA homologous to DNA from human chromosomes 1 and 7 from red blood cell samples. Primers based on DNA sequences encoding 16s rRNA from *Anaplasma* species can amplify DNA from human red blood cells, but not from nucleated white
25 blood cells. The amplified DNA is contained in samples of red blood cells from HIV infected individuals as well as from some healthy individuals of Caucasian or African origin and represents a risk factor for HIV infection. These primers can be employed in methods for assessing risk of HIV infection by amplifying DNA from red blood cell samples.

Description of the Related Art

Chronic HIV infection causes strong immune depression (AIDS) in most patients leading to lethal opportunistic infections or cancers. Specific inhibitors of HIV multiplication are currently used for treating HIV infected patients before they reach the full-blown stage of AIDS. Such inhibitors act mostly on the reverse transcriptase and protease of HIV to efficiently
35 suppress virus multiplication and reduce virus load to a low level of less than 40 viral RNA

lessened severity of opportunistic infections. However, this treatment has to be given without interruption in order to prevent rebound of virus multiplication and a subsequent reduction in the numbers of CD4 lymphocytes. Rebound of viral infection is evidence of a reservoir of HIV in infected patients that is not accessible to antiviral treatment and the existence of this
5 reservoir is generally acknowledged. In addition to a reservoir of HIV in infected patients, such patients often carry other microorganisms that are associated with HIV infection or that cause opportunistic infections.

Microorganisms associated with HIV infection that are detectable in human red blood cells, but not in human leukocytes or other kinds of nucleated human cells have not been
10 previously characterized. The identification and characterization of microorganisms associated with HIV infection is of interest for purposes of assessing risk of HIV infection or determining the status of an HIV infected patient, for assessing risk or status of opportunistic infections, and to evaluate modes of treatment for HIV infected subjects.

BRIEF SUMMARY OF THE INVENTION

The primers designed and discovered by the inventor provide ways to pursue these objectives. Three kinds of primers have been developed and studied by the inventor.

The first kind of primer was designed based on the gene encoding the outer surface
20 protein 2 of *Ehrlichia/Anaplasma* a genus of rickettsiales, which are known endosymbionts of other cells. These primers amplified DNA homologous to segments of DNA from human chromosomes 1 and 7. These primers are described in Appendix 2.

This first kind of primers were initially designed to detect DNA encoding the major surface protein 2 (MSP2) of *Erhlichia/Anaplasma* species. However, neither of the two pairs
25 of primers described by Appendix 2 (Primer Pairs 1 and 2) detected at various annealing temperatures any related microorganism in the biological samples investigated.

Surprisingly, it was discovered that this first kind of primer amplified DNA from human red blood cell samples that was highly homologous to DNA sequences on segments of human chromosomes 1 and 7. Primer Pairs 1 and 2 amplified DNA by the polymerase chain
30 reaction ("PCR") that was 100 % homologous with human sequences when the primer sequences themselves were excluded. The amplified DNA was sequenced and the sequences aligned to sequences described for human chromosome 1 (clone RP11-332J14 GI:22024579, clone RP11-410C4 GI:17985906, and Build GRCh37.p5 Primary Assembly-) and in human chromosome 7 (PAC clone RP4-728H9 GI:3980548; human Build GRCh37.p5, and alternate

assembly HuRef SCAF_1103279188381:28934993-35424761). This was not expected since the primer pairs had been designed to detect genes encoding a bacterial MSP2 gene, not human chromosomal sequences. Furthermore, the amplification of such sequences from samples of red blood cells was in itself surprising since red blood cells lack a nucleus
5 containing chromosomes. The ability to amplify DNA homologous to human DNA from red blood cells is evidence that the target DNA amplified by these primers is present as an extranuclear or cytoplasmic element, such as a plasmid, or is contained in or bound by a microorganism that invades or is otherwise associated with red blood cells. This DNA component may be present on a plasmid or otherwise contained in or bound to a microbe
10 associated with red blood cells. Its presence represents a risk factor for HIV infection or progression and/or opportunistic infections.

A second kind of primer was designed based on the sequences homologous to human chromosomes 1 and 7 that were amplified by the first kind of primers (MSP2 primers). This kind of primer is useful for identifying the target DNA homologous to human chromosomes 1
15 and 7 in a sample, such as a red blood cell sample. Such primers, including the first type of MSP2 primers, are used to detect risks of HIV infection, HIV progression, risks of opportunistic infections, disease prognosis and response to drug treatment in subjects where the presence of DNA homologous to segments human chromosomes 1 and 7 is a risk factor. This kind of primer is exemplified in Appendix 3.

A third type of primer was developed based on the genes from *Anaplasma* species encoding 16s rRNA. *Anaplasma* is a genus of rickettsiales. This type of primer was found to amplify a sequence of 700 bp of ribosomal DNA that was about 85% identical to the corresponding genetic regions of *Rickettsia* and about 99% identical to the corresponding genetic regions of *Acinetobacter* genus. *Acinetobacter* is a genus of gram negative bacteria
25 within the class of gammaproteobacteria. The homology of amplified 16s rDNA with *Acinetobacter* DNA may be coincidental because DNA can be amplified from biological samples that pass through a 450 nM filter unlike classical *Acinetobacteria*.

These primers identify a bacterial agent associated with red blood cells that is related to but not identical to known *Rickettsia* species. This bacterial agent has been identified in
30 red blood cells of not only HIV infected patients but also in some healthy individuals of Caucasian or African origin. This third type of primer is used to detect risks of HIV infection, HIV progression, risks of opportunistic infections, disease prognosis and response to drug treatment in subjects where the presence of a target containing the amplifiable 16s rRNA or 16s rDNA is a risk factor. These primers are described in Appendix 4.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1(A) and 1(B) were produced on different dates. Lane numbers 1 and 2 are
5 duplicates (from HIV-negative source) and lanes 3 and 4 are duplicates (from HIV-positive
source). All DNA samples were extracted from a third passage HL60 cells exposed to an
agent originating from red blood cells of HIV-negative or HIV-positive patients. These
panels represent gel electrophoresis pictures of amplicons obtained by 70 cycles of PCR
using primers derived from the *Ehrlichia* MSP2 gene (213 bp) and primers derived from the
10 16s ribosomal gene of *Anaplasma* (690 bp). The bands on the left sides of Figs. 1(A) and
1(B) show the 213 bp fragment mapped to human chromosome 7 amplified by the *Ehrlichia*
MSP2 primers. The bands on the right sides of Figs. 1(A) and 1(B) show the 690 bp fragment
amplified by the *Anaplasma* primers (SEQ ID NOS: 24 and 25).

DETAILED DESCRIPTION OF THE INVENTION

Amplification of DNA from biological samples, including blood, plasma, and serum
samples or samples obtained from cell culture can be performed using PCR or other nucleic
acid amplification methods known in the art. These methods can be used to amplify or detect
target DNA qualitatively or quantitatively to provide a "yes or no" determination of the
20 presence of the target sequence or to quantitatively detect an amount of DNA amplified under
controlled conditions.

The DNA amplified by the first and second kinds of primers is higher frequency in
red blood cells obtained from patients infected with human immunodeficiency virus
compared to healthy individuals. This is especially the case for patients who have undergone
25 or are undergoing antiretroviral therapy. The quantity of DNA amplified by these primers is
reduced after long-term treatment of a patient with antibiotics and the primers were found not
to amplify DNA from white blood cells or from other human cell lines. DNA has also been
amplified from the red blood cells of healthy African subjects not infected with HIV using
these primers. These results suggest the amplified DNA is derived from an antibiotic
30 sensitive microorganism associated with red blood cells.

Despite the apparent human origin of this DNA, the data herein show that the
amplified sequences are associated with a transmissible agent and that the transmissible agent
is closely associated with human immunodeficiency virus as explained below.

These sequences were easily detected in the DNA of anucleated red blood cells

(RBCs) in 100% (35 out of 35 subjects) HIV-infected African and Caucasian patients and they could not be detected in the DNA of nucleated cells including in the white blood cell fraction of the same patients, nor in human DNA from cultured human cells.

These sequences were rarely detected in the red blood cell fraction of healthy African subjects and none were detected in the red blood cells of the healthy Caucasians tested.

Long term antibiotic treatment (*e.g.*, with doxycycline or azithromycin) of HIV-positive patients for more than three months was found to decrease the intensity of the bands amplified using these primers suggesting that these bands are induced, generated or otherwise originate from an antibiotic sensitive microorganism.

Amplification of DNA from a supernatant of a short term culture of human cell line HL60 with an extract of RBC from an HIV-positive patient prepared by freeze-thawing RBC and then by removing heavy components by a low speed (10 mins at 1,500 g) centrifugation produced strong DNA bands. The intensity of these bands suggests growth and multiplication of a microorganism that contains DNA amplified by Primer Pairs 1 and 2.

To further explore this effect, new primers were designed that allow amplification of regions of human genomic DNA adjacent to or including those amplified by Primer Pairs 1 and 2. These new primers include:

primers "hChr1/14179308 S" upstream of, and "hChr1/14179853 AS" encompassing one end of the 237 bp amplicon related to chromosome 1 (546-bp long amplicon);

primers "hChr7/4292976 S" upstream of, and "hChr7/4294619 AS" downstream of the 213 bp amplicon related to chromosome 7 (1,643-bp long amplicon); and the primers described by Appendix 3.

These primers amplify DNA not only from the components of RBCs of HIV infected Caucasian or African patients, but also from the components of RBCs of healthy African subjects. However, these DNAs are lacking in all or most of HIV-negative Caucasian subjects. These sequences are amplified after antibiotic treatment of their carrier subjects indicating that the agent generating them is insensitive to antibiotic treatment. As in the case of the MSP2 primers-amplified sequences, these kinds of primer pairs amplify DNA present in or associated with anucleated RBC and not in white blood cells or human cell lines. It is possible that such a microorganism identified with these primers differs from the carrier of the initial short DNA sequences and will amplify or cause amplification of human genomic DNA sequences in an integrated or unintegrated manner. The primers disclosed herein permit the design of diagnostic tests and treatments aimed at reducing the risk of HIV infection in important segments of the human population in which this agent appears and can

be detected by amplification of these DNA sequences.

The third kind of primers that identify a previously unknown bacterial agent that is associated with human red blood cells and related to, but not identical to, known *Rickettsia* species are provided. These primers are derived from the 16S ribosomal DNA sequences of an *Anaplasma* species and amplify a sequence of 700 bp of ribosomal DNA that is about 89% identical to the corresponding regions of the genome of *Rickettsia*. This sequence is about 99% identical to the corresponding regions of *Acinetobacter* genus DNA. Besides the primers exemplified herein, other primers that amplify the same 700 bp of ribosomal DNA or detectable fragments of this sequences may be designed based on this nucleotide sequence. These primers may amplify 20, 30, 50, 100, 200, 300, 400, 500, 600 or 700 nucleotides of this sequence. They may comprise short portions (e.g., 18-30 bp) of the 700 bp sequence and can be designed based on methods well known in the molecular biological arts. The table below depicts the various kinds of primers.

Appendix 1	Primers designed based on <i>rickettsiales</i> 16S ribosomal DNA gene	SEQ ID NO:	Primers were designed to include a conserved 16S region of DNA which recognizes <i>Rickettsiales</i> and also <i>Propionobacter</i> .
	5'-GCAAC GCGAA AAACC TTACC	1	Rick16S 929S (20 mer) Tm = 45.0°C
	5'-GACGG GCAGT GTGTA CAA	2	Rick16S 1373AS (18 mer) Tm = 45.0°C
Appendix 2	MSP2 Primers		
MSP2 primer pair 1	5' GCCTA CAGAT TAAAG GCT	3	18 mer
	5' ATCAT ARTCA CCATC ACCTA	4	20-mer
MSP2 primer pair 2	5' CYTAC AGAGT GAAGG CT	5	17-mer
	5' ATCAT ARTCA CCATC ACCTA	6	20-mer
Appendix 3	Human chromosome 1 and 7 primers		
Chromosome 1 primers		SEQ ID NO:	
*primer #1	5' - CCT TAC ACT CAG CCA GAC AT	7	hChr1/14179308 S

*primer #2	5' - CCA GGT GTG TGG CTT ATA CA	8	hChr1/14179853 AS
primer #3	5' - CAT AGC TTC CTA GTA AGT AGA CCA G	9	hChr1/14180006 S
primer #4	5' - AGG GGA GTC TGA GAT GGT	10	hChr1/14180401 AS
primer #5	5' - ACT GGA GAG GTG GAG GTT	11	hChr1/14181093 AS
primer #6	5' - GGT AAT TCC TAT GTG CGA GGT	12	hChr1/14181390 AS
primer #7	5' - TCA AAA GAC AGT GGT GAC TCT	13	hChr1/14177990 S
primer #8	5' - ATG TCT GGC TGA GTG TAA GG	14	hChr1/14179327 AS
Chromosome 7 primers		SEQ ID NO:	
primer #1	5' - ATG TAG TTG AGC AGT TTT GAA TGA	15	hChr7/4292976 S
primer #2	5' - TCC TGC CTT AGT GAG GAT CT	16	hChr7/4293490 S
primer #3	5' - ATG AAT AGG TGA TGG TGA TGA CT	17	hChr7/4293941 AS
primer #4	5' - CCG TCA TTT AAG CCT TTA ATC TCA	18	hChr7/4294102 S
primer #5	5' - GTA GTC TTT TGG CAT CTC TTT GTA	19	hChr7/4294619 AS
primer #6	5' - CAG CCT GGA GAA CAG AGT G	20	hChr7/4294983 AS
primer #7	5' - GAT CTA GCA GTT CAT AGG AAG GAA	21	hChr7/4295251 AS
primer #8	5' - GGC TGA AAC AAT GGG GTT TT	22	hChr7/4291579 S
primer #9	5' - TTT AGT	23	hChr7/4293175 AS

	AGA CCC CTC GAC CA		
Appendix 4: Anaplasma 16s rDNA based primers			
		SEQ ID NO:	
Primer	5'-CTG ACG ACA GCC ATG CA	24	sense
Primer	5'-GCA GTG GGG AAT ATT GGA CA	25	antisense

Specific embodiments of the invention include, but are not limited to those described below.

An agent that is associated with red blood cells, especially mature anucleated red blood cells, that passes through a 0.45 micron filter. This agent may be sensitive or insensitive to a particular antibiotic. Agents sensitive to azithromycin or to a cyclin antibiotic have been identified. This agent contains, induces, excises, or otherwise provides DNA that is amplified by (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), (iii), the pair of primers described in Appendix 4 (SEQ ID NOS: 24 and 25); or a pair primers that amplify at least fifteen, twenty, twenty five, thirty, forty, fifty or more consecutive nucleotides of the same DNA as is amplified by the specific primers described herein.

The amplified DNA may be 80%, 85%, 90%, 95%, 99%, up to and including 100% identical or similar to human DNA, wherein sequence identity is determined by BLASTn using the default setting. Preferred parameters for determining the "nucleotide identity" when using the BLASTN program (Altschul, S. et al., Journal of Molecular Biology 215 (1990), pages 403-410) are: Expect Threshold: 10; Word size: 28; Match Score: 1; Mismatch Score: -2; Gap costs: Linear.

An agent that is associated with red blood cells, passes through a 0.45 micron filter, may be sensitive or insensitive to a particular antibiotic, can be detectable in red blood cells of an HIV patient, but not detectable in the white blood cells of said patient. Such an agent may become detectable in the red blood cells of an HIV-infected patient within the first year after HIV infection or after initiation of anti-retroviral treatment. Such an agent may appear or be associated with the red blood cells of an African subject or European subject who is HIV-negative.

The agent may be a microorganism, such as a bacterium, or a specific kind of bacterium such as *Rickettsia* or *Rickettsia*-like bacteria, *Ehrlichia* or *Anaplasma* or a component thereof. Such an agent may contain a plasmid, episome, or extra chromosomal element comprising human chromosomal DNA that is amplified by MPS2 gene primers; or
5 that is contained in or associated with a red blood cell that contains a plasmid or extra chromosomal element comprising human chromosomal DNA that is amplified by MPS2 gene primers.

Isolated red blood cells may contain the agent as described herein as well as disrupted or lysed red blood cells, such as a supernatant produced by freezing and thawing red blood
10 cells after removing white blood cells and then removing material that pellets by a low speed centrifugation, *e.g.*, for 10 mins at 1,500 g. The red blood cells associated with the agent are detected by amplifying DNA from them using (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), (iii), the pair of primers described in Appendix 4 (SEQ ID NOS: 24
15 and 25); or a pair primers that amplify at least fifteen, twenty, twenty five, thirty, forty, fifty or more consecutive nucleotides of the same DNA as is amplified by the specific primers described herein..

Another aspect of the invention is the DNA amplified from the agent or from red blood cells associated with the agent. This DNA is can be produced using the primer pairs
20 described herein. The DNA that is present in a red blood cell may be from an infectious or replicating agent *per se*, from a component of an infectious organism present in the anucleated red blood cell, or from DNA that results from exposure of the red blood cell or its precursor cells to an infectious or replicating agent.

The amplified DNA from a red blood cell may comprise portions of human
25 chromosome 1 or 7 including the sequences described in Appendix 5 or Appendix 6 or fragments of these sequences comprising 10, 20, 30, 40, 50, 100, 200 or more consecutive nucleotides of these sequences.

The DNA according to the invention may be contained or inserted into a vector, such as a plasmid or phage vector containing the isolated or purified amplified DNA. A host cell
30 can be transformed with the isolated or purified amplified DNA from the agent or from red blood cells associated with the agent.

The invention is also directed to a method for detecting an agent as described herein comprising contacting material from anucleated red blood cells of a subject with primer Pair 1, primer Pair 2, or a pair of primers selected from the group consisting of those described in

Appendix 3 under conditions suitable for amplification of DNA by said primers, and detecting said agent when amplified DNA is detected.

The primers used in this method may be selected from the group consisting of (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), or (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23) or a pair primers that amplify at least fifteen, twenty, twenty five, thirty, forty, fifty or more consecutive nucleotides of the same DNA as is amplified by the specific primers described herein.

Alternatively, a set of primers that amplify the same DNA fragment amplified by the two primers described above may be employed. These primers may be designed by methods known in the art and each may comprise 18-30 or more base pairs of the sequence amplified by the primers above.

A method for detecting an agent as described herein comprising: contacting under conditions suitable for amplification of target DNA material from red blood cells of a subject with a primer and detecting or recovering the amplified DNA, where the primers are described by Appendix 4:

Primer (sense)

5'-CTG ACG ACA GCC ATG CA (SEQ ID NO: 24)

Primer (antisense)

5'-GCA GTG GGG AAT ATT GGA CA (SEQ ID NO: 25).

Alternatively, a set of primers that amplify the same DNA fragment amplified by the two primers described above may be employed. These primers may be designed by methods known in the art and each may comprise 18-30 or more base pairs of the sequence amplified by the two primers above.

The biological sample used in the method described above or other methods described herein may be whole blood or a cellular component of whole blood, isolated anucleated red blood cells, isolated red blood cell precursors, such as erythroblasts, bone marrow or spleen cells, or subcellular fractions thereof, such as cellular lysates, supernatants or solid materials. Blood plasma or serum or other bodily fluids or tissues may also be used as a biological sample for the methods described herein. Those of skill in the art can select an appropriate biological sample for performance of PCR or select the appropriate conditions for producing an EMS signalized sample based on the disclosures of the patent applications incorporated by reference above. Representative biological samples include whole blood, isolated RBCs, subcellular components, extracts, or lysates of RBCs or their precursor cells, blood plasma or blood serum, spinal fluid, mucosal secretions, urine, saliva, bone marrow, or tissues.

A method for treating or for reducing the severity of a disease, disorder, or condition associated with the agent comprising treating a patient with an agent that reduces the titer of said agent or that reduces the amount of DNA amplified from a cell associated with it. This method may also comprise treating the patient with one or more antibiotics, such as azithromycin or a cyclin antibiotic; with one or more synthetic or natural immunostimulants, active vaccines, passive vaccines, antioxidants or antibiotics. A patient may also undergo treatment sequentially or simultaneously for viruses or other microorganisms or agents capable of causing an immunodeficient disease, disorder or condition. Treatment may be therapeutic or prophylactic and can include the administration of one or more anti-retroviral drugs or other antiretroviral treatments. The patient may be currently undergoing antiretroviral therapy or therapy to eradicate human immunodeficiency virus infection and treatment for the coinfecting bacterium initiated. Other modes of or supplemental treatments include treating the patient with one or more natural immunostimulants, antioxidants or antibiotics.

The methods described herein may employ samples from subjects or patients of different geographic origins or racial or genetic backgrounds. A subject or patient may be HIV-negative, recently (*e.g.*, less than one year) HIV-positive, a patient who has been HIV-positive for more than one or two years, an HIV-positive patient who has undergone or is undergoing anti-retroviral treatments or other kinds of patients who are HIV-positive such as those with AIDS or subjects at risk of becoming HIV-positive, developing AIDS or opportunistic infections. Patients may be of African origin or may have lived in Africa and exposed to biological and environmental agents there. Similarly, a patient may be of European or Caucasian origin or may have lived in Europe or America and exposed to biological and environmental agents there.

The invention is also directed to a method for treating a disease, disorder or condition associated with an agent described herein comprising contacting red blood cells with a substance that reduces the amount of DNA amplified from a red blood cell using (i) Primer Pairs 1 or 2, (ii) primers described by Appendix 3, (iii) or the primers described in Appendix 4 or primer pairs that amplify at least 20 consecutive nucleotides of the amplicons amplified by the primer pairs described above. Such a method for treating a disease, disorder or condition associated with an agent described herein may comprise contacting red blood cells of a subject with a substance that reduces the transmission of said agent to the red blood cell; may comprise replacing the red blood cells in a subject with red blood cells that are not associated with said agent or by stimulating the development of new red blood cells in said

subject; or may comprise treating blood or red blood cells with an agent that that degrades, crosslinks or otherwise interferes or inactivates nucleic acids inside of or associated with a red blood cell.

Another aspect of the invention is a method for screening blood for red blood cells from which DNA can be amplified using (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), (iii), the pair of primers described in Appendix 4 (SEQ ID NOS: 24 and 25); or a pair primers that amplify at least fifteen, twenty, twenty five, thirty, forty, fifty or more consecutive nucleotides of the same DNA as is amplified by the specific primers described herein. This method comprises contacting a sample of blood or red blood cells with these pairs of primers and detecting amplified DNA and selecting a blood sample from which DNA was amplified or alternatively selecting a blood sample from which no DNA was amplified. For example, a blood sample from which amplified DNA is detected may be further evaluated or cultured to determine the sensitivity of the red blood cells or the agent associated with them to antibiotic or other therapeutic treatments. Alternatively, a blood sample in from which no DNA is amplified may be assessed as being free of the agent associated with the DNA amplified by these primers.

Example 1. Detection of Amplified DNA in Red Blood Cells

Separation of Red Blood Cells

Standard procedures for separating RBCs from buffy coat and other peripheral blood components are known. Peripheral blood was processed on a Ficoll gradient to separate the buffy coat from red blood cells. After such separation it was found that DNA extracted from buffy coat cells was completely negative as determined by PCR using the primers described above while the same primers amplified DNA in the fraction containing the separated red blood cells. While it cannot ruled out that the agent detected is externally associated with the red blood cell membranes, it was found that amplified DNA was only detected in a supernatant prepared by a low speed (1,500 g x 10 mins) centrifugation to remove the heavy components of a red blood cell lysate. This lysate was prepared by repeated freeze-thawing of red blood cells isolated from the buffy coat, strong shaking by vortex, and a low speed centrifugation (1,500 g x 10 mins). A pellet and supernatant fraction were obtained and tested. The primers described above only amplified DNA in the supernatant fraction, but not in the pellet.

Growth on HL-60 cells

HL-60 cells are an ATCC cell line of promyelocytic origin. Samples of HL-60 cells at a density of 5×10^5 cells per ml in RPMI medium supplemented with 10% fetal calf serum were inoculated with the supernatant of the red blood cell lysate described above. This lysate was obtained from the red blood cells of HIV-positive patients after freezing, thawing and vortexing as previously described. After culturing for 3 days at 37°C the low speed (1,500 g x 10 mins) supernatants of the cultures were tested by PCR for DNA amplified using Primer Pairs 1 and 2. DNA was amplified from all of these cultures up to a dilution of 10^{-8} . The same results were obtained from culture supernatant that was passaged through a 0.45 micron filter.

Effects of long-term antibiotic treatment

Five HIV-positive patients were maintained on their antiretroviral therapy, but received for at least three months a daily antibiotic treatment (azithromycin 250 mg/day or doxycycline 100 mg/day). Blood samples were fractionated to recover a red blood cell fraction on day 0 and after 3 months of antibiotic. Results indicated that the amount of DNA amplified after 3 months of antibiotic treatment was significantly less than that amplified under the same conditions from the samples obtained on day 0.

Detection of Amplified DNA in Red Blood Cells of African and Caucasian Patients

Blood samples were obtained from African and European Patients who were HIV-negative or HIV-positive. Red blood cells were isolated from buffy coat and other blood components by separation on a Ficoll gradient as described above. Table 1 shows the results of amplification of red blood cell samples from these patients using Primer Pairs 1 and 2.

Similar results were obtained using the primers described in Appendix 3. No DNA was amplified using Primer Pairs 3 and 4 for Chromosome 1 and 7 from the red blood cells of one European patient who was HIV-positive for a year or less. This suggests that in some Caucasians that the accumulation of this human DNA in the red blood cell fraction occurs late after infection and possibly under the selective pressure of antiretroviral treatment.

However, amplified DNA was detected in this patient using the Primer Pairs 1 and 2 shown in Appendix 2, but the amplified DNA bands were weaker than those for chronically-infected HIV-positive patients.

Table 1

	African				Caucasian				Cultured cells (HL 60)	
	RBC : HIV neg	RBC : HIV +	WBC : HIV +	RBC from HIV + treated with antibiotics for 3 mos	RBC : HIV neg	RBC : HIV +	WBC : HIV +	RBC from HIV + treated with antibiotics for 3 mos (0 vs 3 mos)	No extract	HL 60 + RBC extract from HIV + subject (0 vs 3 days)
<i>App. 2</i>										
Pair 1	rare	100%	0%	↓	0%	100%	0%	↓	-	↑
Pair 2	rare	100%	0%	↓	0%	100%	0%	↓	-	↑
<i>Chr 1</i>										
Pair 1	+	+	-	+	-	+ or - *	-	+	-	
<i>Chr 7</i>										
Pair 1	+	+	-	+	-	+ or - *	-	+	-	

5 + in chronically infected and treated patients

- in recently infected patients

DNA Amplified Using Primer Pairs 1 and 2

MSP2 Primer Pairs 1 and 2 were used to perform PCR on red blood cells of HIV-positive subjects after removal of white blood cells and other blood components by Ficoll gradient separation. Primer Pairs 1 and 2 are shown below.

Primer Pair 1

5' GCCTA CAGAT TAAAG GCT 18 mer

5' ATCAT ARTCA CCATC ACCTA 20 mer

Primer Pair 2:

5' CYTAC AGAGT GAAGG CT 17 mer

5' ATCAT ARTCA CCATC ACCTA 20 mer.

The DNA bands amplified by PCR using Primer Pairs 1 and 2 were 100 % homologous with human sequences (primer sequences excluded) present in data-banks for human genomic sequences, respectively in human chromosome 1 (clone RP11-332J14 GI:22024579, clone RP11-410C4 GI:17985906, and Build GRCh37.p5 Primary Assembly-) and in human chromosome 7 (PAC clone RP4-728H9 GI:3980548; human Build GRCh37.p5, and alternate assembly HuRef SCAF_1103279188381:28934993-35424761).

Human chromosome 1 and 7 DNA Sequences Described in Appendix 5

Appendix 5 shows the identities of human chromosome 1 and Chromosome 7 sequences that are amplified by primers described in Appendix 3. Primer sequences are underlined.

Primer Pair 3: Primer "hChr1/14179308 S" upstream of, and "hChr1/14179853 AS" encompassing one end of the 237 bp amplicon related to chromosome 1 (546-bp long amplicon) were used to perform PCR on material from red blood cells isolated from other blood components by Ficoll gradient.

Primer Pair 4: Primers "hChr7/4292976 S" upstream of, and "hChr7/4294619 AS" downstream of the 213 bp amplicon related to chromosome 7 (1,643-bp long amplicon); and the primers described by Appendix 3.

Example 2. Method for detecting risk of acquiring HIV infection or opportunistic infection associated with HIV infection or risk of the progression of an HIV infection or

opportunistic infection.

Blood is collected from a subject in the presence of EDTA as an anticoagulant. The red blood cells in the sample are separated from buffy coat and plasma components of blood using a Ficoll-Hypaque gradient according to the manufacturer's current protocol. DNA in the red blood cell sample is prepared using a QIAGEN® Blood DNA Extraction Kit (as described in the current QIAGEN® product catalog) and amplified by PCR using MSP2 primer pair 1: 5' GCCTA CAGAT TAAAG GCT (SEQ ID NO: 3) and 5' ATCAT ARTCA CCATC ACCTA (SEQ ID NO: 4) or MSP2 primer pair 2: 5' CYTAC AGAGT GAAGG CT (SEQ ID NO: 5) + 5' ATCAT ARTCA CCATC ACCTA (SEQ ID NO: 6).

Amplified DNA is resolved by gel electrophoresis and detected by staining with ethidium bromide.

A subject is classified as being at a higher risk for acquiring HIV or HIV-associated opportunistic infection or for when amplified DNA is detected.

Example 3. Method for detecting microorganism associated with risk or progression of HIV infection or opportunistic infection associated with infection with HIV.

Blood is collected from a subject in the presence of EDTA as an anticoagulant. The red blood cells in the sample are separated from buffy coat and plasma components of blood using a Ficoll-Hypaque gradient according to the manufacturer's current protocol. DNA in the red blood cell sample is prepared using a QIAGEN® Blood DNA Extraction Kit (as described in the current QIAGEN® product catalog) and amplified by PCR using the primer pair 5'-CTG ACG ACA GCC ATG CA (SEQ ID NO: 24) + 5'-GCA GTG GGG AAT ATT GGA CA (SEQ ID NO: 25).

Amplified DNA is resolved by gel electrophoresis and detected by staining with ethidium bromide.

A subject is identified as being infected with a microorganism when amplified DNA is detected.

APPENDIX 5

The human chromosome 1 sequence amplified by the MSP2 primers shown below:

primer identifier	sequence
MSP2 primer #3)	AC/mMSP2-10198 5'- CTTACAGAGCTGAGCGCT where Y = T or C
MSP2 primer #5)	AC/mMSP2-1128AS 5'- ATCATATTCACCATTCACCTA where R = G or A

Sequence of the 237 bp amplicon generated with these two primers by PCR :

5' - ATGATGTCAGCATCACCTGCCAGCTGTATTAAGCCACACACCTGGAGTCTCTCTAGCCTTTTCTCTCTCTCTCAGCTCCATATCCCATTCGCTCAGGGCCTACTGAGTCTCA
CAGTCCCAATTTTCTTTTAAATCTATGCCACTGGCACTGTGCTAGCTAAAGGCATATCCAGTCTGGTCAACCAAGTATCTTCCATAGCTTCTCTAGTAACTAGACACGCTTACATCCCTGTAAG - 3'

underlined nucleotides : primer sequences
bold nucleotides : divergent nucleotides between MSP2 primers and the corresponding homologous human Chr 1 sequence.

The human chromosome 7 sequence amplified by the MSP2 primers shown below:

primer identifiers	sequences
MSP2 primer #2)	5'- GCCTACAGATTAAAGGCT
MSP2 primer #5)	5'- ATCATARTCACCATCACTTA where R = G or A (same as above)

Sequence of the 213 bp amplicon generated with these two primers by PCR :

5' - GCGTCGACATTAAGGGCTTAAATTGACGGGTGAATAACTTAGTGTTCTTTTTGGGTGGCAATAAGTGAATTTGCACATTTGCCACAAGATGACATGTACAAAAGAGTCAGAAGACC
sequence 8 **3'**

underlined nucleotides : primer sequences

underlined nucleotides : primer sequences
bold nucleotides : divergent nucleotides between MSP2 primers and the corresponding homologous human Chr 7 sequence.

Identities of the human sequences amplified by the human chromosome 1 primers or human chromosome 7 primers described in sections A) and B) respectively below.

The sequences described below, except for the primers and MspI-derived amplicons, are corresponding to human genomic sequences available in NCBI genome databanks (www.ncbi.nlm.nih.gov/projects/genome).

A) Genomic human chromosome 1 primers #1 (hChr1/14179308 S) and #2 (hChr1/14179853 AS) PCR-amplified 546 bp amplicon :

Identifier : AmpliCon hChr1/14179308-14179853

[illegible]

underlined nucleotides : hChr1 genomic primer sequences

underlined nucleotides : human genomic primer sequence
bold nucleotides : homologous extremity of the 237 bp amplicon obtained with the primers MSP2#3-5.

B) Genomic human Chromosome 7 primers #1 (hChr7/4292976 S) and #5 (hChr7/4294619 AS) PCR-amplified 1,644 bp amplicon*

identifier : Amplicon hchr7/4292976-4294619

[illegible]

underlined nucleotides : hChr7 genomic primer sequences

underlined nucleotides : Achr/ genomic primer sequences
bold nucleotides : homologous sequence of the 213 bp amplicon obtained with the primers MSP2#2-5.

bold nucleotides : homologous sequence of the 213 bp amplicon obtained with the primers NS522F and NS522R; **the internal underlined nucleotides** correspond to the sequence of primer hChr7/4293490 8 (hChr7#2).

* please note that this amplicon lenght has been reported as 1,643bp long in previous documents. The correct lenght is 1,644bp.

Other amplicons obtained from HIV positive or HIV-negative patients or from either using the human chromosome 1 or human chromosome 7 primers described in sections C) and D) below.

C) Genomic human Chromosome 1 primers #3 (hChr1/14180006 S) and #4 (hChr1/14180401 AS) PCR-amplified 396 bp amplicon :

Identifier : Amplicon hChr1/14180006-14180401
 5'- CATAGCTCTCTAGTAAATGAGACCGCCCTTCAGCTGAGCCCTCCGCTCTCCGCCAGTCTCTGAGTAATCCTTTACACACACAAATGAAGACAGGTCACTGCGCTCAAAATGATGTCAAGGGCTTT
ATCATCCATGTTGCTGGCTTTTCACAGGATGCTTGCAGTGCAGCCCTTAATACTCTCTCCACACAACTCTGTATCCCTCATCTTTCAAGCTTTTATATGTCAGCCATGTGACACTCCCTTAATATACCATGT
TTCTCTTTTCCCTCCCTCTCTCATTTGAGACTTCCCACTATCTTCCCTAAGACTACTCTTTTGAATGTTTATTCAGGGTTCCTTATCTTTTAAACCATCTCAGACTCCCT -3'

underlined nucleotides : primer sequences
 bold nucleotides : homologous extremity of the 237 bp amplicon obtained with the primers MSP2#3-5.

D) Genomic human Chromosome 1 primers #3 (hChr1/14180006 S) and #5 (hChr1/14181093 AS) PCR-amplified 1,088 bp amplicon :

Identifier : Amplicon hChr1/14180006-14181093

5'- CATAGCTCTCTAGTAAATGAGACCGCCCTTCAGCTGAGCCCTCCGCTCTCCGCCAGTCTCTGAGTAATCCTTTACACACACAAATGAAGACAGGTCACTGCGCTCAAAATGATGTCAAGGGCTTT
ATCATCCATGTTGCTGGCTTTTCACAGGATGCTTGCAGTGCAGCCCTTAATACTCTCTCCACACAACTCTGTATCCCTCATCTTTTGAATGTTTATTCAGGGTTCCTTATCTTTTAAACCATCTCAGACTCCCTTGGGATTAACCCCTT
TTCTCTTTTCCCTCCCTCTCTCATTTGAGACTTCCCACTATCTTCCCTAAGACTACTCTTTTGAATGTTTATTCAGGGTTCCTTATCTTTTAAACCATCTCAGACTCCCTTGGGATTAACCCCTT
TTCTCTTTTCCCTCCCTCTCTCATTTGAGACTTCCCACTATCTTCCCTAAGACTACTCTTTTGAATGTTTATTCAGGGTTCCTTATCTTTTAAACCATCTCAGACTCCCTTGGGATTAACCCCTT
GTTCGCCAATACTTAGTATGCTTAGCATATGCTAGTGTCTCAGTAATATTTGATNTGTGTGACACGAAATGCAATCAATCAATACAAATGACAGACAACTCCACCCCAACCTTAACCAAAAAATC
CAAACTTTCCCTTGCCTTATGAGTACATCTGCTCATCAACATAGGCAAAATTTCTTCCCTCTCAATACAGAGGCGGAGAACTCACAGAAATCAGACAACTAGACCACTGGCTTTGGCATGAGACACCTG
AGTTAAATCTGGCTTCTGCTATTATAGCCACATGACAGTGAATCTCCTTGAAGCTTCTGTTTGTGATCTTATTCCTTTGGTGCATCTGTACACACCCCACTGCTTATTCATA
TGACACTGCTAAACATGCTTGCCTTCTCCGCCACTTTTCTTTTGGACACAGAAATCTCCCTTTCTCACCAGGCTGGAAATTCAGTGGCGTGTATCTCGGCTCACTGCACCTCCCTCCAGT -3'

underlined nucleotides : primer sequences

bold nucleotides : homologous extremity of the 237 bp amplicon obtained with the primers MSP2#3-5.

The sequence of the 396bp amplicon hChr1/14180006-14180401 (C) is included in the larger size amplicon 1,088bp Amplicon hChr1/14180006-14181093 (D). The internal underlined nucleotides correspond to the reverse-complement sequence of primer hChr1/14180401 AS (hChr1#4).

Identifier : Amplicon hchr17:4293490-4294963

unmodified nucleotides : nucleic acid primer sequence
bold nucleotides : homologous sequence of the 213 bp amplicon obtained with the primers MSP2#2-5.

bold nucleotides
: part of the sequence
; homologous sequence of the 213 bp amplicon obtained with the primer pair
1604bp amplicon bChr7/4292876-4294619 (B)
1604bp amplicon bChr7/4292876-4294619 (B) is included in the amplicon 1-494bp Amplicon bChr7/4293490-4294983 (E).

bold nucleotides
: part of the sequence
; homologous sequence of the 213 bp amplicon obtained with the primer pair
: 6040bp amplicon bChr7/4292876-4294619 (B)
: 6040bp amplicon bChr7/4292876-4294619 (B) is included in the amplicon bChr7/4293490-4294983 (E).
: 6040bp amplicon bChr7/4293490-4294983 (E).

The internal underlined nucleotides correspond to the reverse-complement sequence of primer hChr7/4294619 AS (hChr7#5).

For legibility, enlarged versions spanning two side-by-side pages of the sequences shown in Appendix 5 above are provided below.

The human chromosome 1 sequence amplified by the MSP2 primers shown below:

	<u>primer identifiers</u>	<u>sequences</u>
MSP2 primer #3)	AC/mMSP2-1019S	5'- CTTACAGAGTGAGGCT
MSP2 primer #5)	AC/mMSP2-1128AS	5'- ATCATATTCACCATCACCTA

Sequence of the 237 bp amplicon generated with these two primers by :

5'- ATCATATTCACCATCACCTACCAGCTGTATAGCCACACACCTGGGAGTGCTCCTAGCCCTTTTTC
CACTCCAAATTTTCTTTTAAATCTATCCCACTGCCACTGTCTAGTCTAGGCCAATACCATCTGGTCA

underlined nucleotides : primer sequences
bold nucleotides : divergent nucleotides between MSP2 primers

The human chromosome 7 sequence amplified by the MSP2 primers shown below:

<u>primer identifiers</u>	<u>sequences</u>
MSP2 primer #2)	5'- GCCTACAGATTAAAGGCT
MSP2 primer #5)	3'- ATCATATCACCATCACCTA

Sequence of the 213 bp amplicon generated with these two primers by P

5'- GCCTACAGATTAAAGGCTTAATGACGGTGAAAACTTAGTATTCTTTGGTGGACAATAGTGAAN
TTTAAATCTATTTAAGGACTCAAGTAATTTCGTGAAGGCCATAGCGTAABTAACCTTCAGTGGATGG

underlined nucleotides : primer sequences
bold nucleotides : divergent nucleotides between MSP2 primers a

where R = G or A (same as above)

CR :

TTTGCACTTTGGACAGATGACATGTACAAAGAGTCTCAGGAAC
AATGGGATGATGAATAGGATGATGGAGCTATGAT -3'

and the corresponding homologous human Chr 7 sequence.

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Identities of the human sequences amplified by the human chromosome 1 primers described in section A) below.

A) Genomic human Chromosome 1 primers #1 (hChr1/14179308 S) and #2 (hChr

Identifier : Amplicon hChr1/14179308-14179853

5'- **CCTTACACTCAGCCAGACATATATTGTGTTTGTATCCATGTGCACAGAGACTTTGGCATTTCTGGG**
GGCATGGGGAAATGCTCTGCTGGGTCACATTGAAATGGTTCYGAACCACTGTGGGAAATAAAGGAGTTAGAAAGA
GTAGCCTATGGGTAAAGAGAGACAGAGACTGAGGGGAGGACTTTTCCCTGAGTCCACCCCAACCGCCCAT
ATGTCCTACAGGCACCTTCATATTATTAGCAGATCCAAACTTAACCTCAGATACTCCTCTTGCCATATCTGTTC

1/14179853 AS) PCR-amplified 546 bp amplicon :

TGAGGGAAGNAAGAGAGATATACATGGNAACCCAGGGGTAGAGAAAGGACACAGAGATGT
ATCAGATGCCGAGGAGCCAAATTTTCACATATACTCCGAGACTCAGGGCCAAAGCCGCTTGTTC
GGAGCTGAGGCCAGCCCTGAAAGTCTCAGGGGCCATGGGAGGATTTGCCCTTTTGGATTTCTCCAAATGGG
TCCTTGCTGTGTTCCCTGACCATGATATATCACCATACCTACCCCTTTATTAAGCCACACACTGG -3'

Identities of DNA sequences amplified by Human chromosome 7 primers described in section B) below:

B) Genomic human chromosome 7 primers #1 (hChr7/4292976 S) and #5 (hChr7/429

identifier : Amplicon hChr7/4292976-4294619

5'- ATGTAGTGGAGCAGTTTGGATGAGTTTCTTANTCCTGAGTCTAGTTTAAAGAAATATTAATAATAAATAA
GTGAATGCAGTAACCTTGGTTTAAAGCAATATCCAAACCCAGAAATTTGGTCGAGGGTCTACTAAATTCGGTTTTC
TAGAGCTGACAGTTGTGTGGCCATATGCCATGATTCGTGTACAAATGAACAGTATTCNGATTCAAAATCAGTGATAA
AATATTAAACCGCCTTTTGAGTATTAGGGGAAATAACAGAACTAAAGCGAATATATTTTGTTTCTCTGANTTCCTCCCA
GATAAATAACCAATATATAATTTACTCTCTCTCTACCTCACCCCAATATTCACAAATTCCTCTATTTTATTTTGA
CCATAAATACTTCCCTCCAGGGCTTCAGCAGTGTATATTGCATTCCTCATGTGATGTCCTTATAGCTGTACNGG
CTGGTTTATCCATCCCCCAACAAATCCTTTAATCCTCCACTAGAGTTTATTTCTTTTAAAGTGAAATAATCTGGATTCTT
TCATCATCCCATTCCTATCCACTGAAAGTTATTTTACCGCTATGGCCCTTCACAAATTTACTTTTAAATCTTAAATCAGAT
TCCACCCCAAGAACTAAGTTTTCACCGCTATTTAAGCCTTTAATCTCAGGNTCTCACAAATTAATACAAATCACATC
TATATATATTTTAAATTCAGCCACTTCTCTCACTAGAAAGTGAGTTTGTGTGAATATCAGGGTAAGTATATTTTATGT
ATTAAATTGTACATATATAAATACTTTATATATATAAATGTTTCTCTGTAGGGGAGATTTTCTTTGTAAATACTATCCCT
ATGCAAATGAACTTACACTGTGCATATACCAAAAGTGATTAATTTCTTGCTCCTGAACTCACTCTCCCTACCTGCCTA

underlined nucleotides : hChr7 genomic primer sequences

bold nucleotides : homologous sequence of the 213 bp amplicon obtained

The internal underlined nucleotides correspond to the sequence of primer hCh

Other amplicons obtained from HIV positive or HIV-negative patients or from both using the human chromosome 1 or human chromosome 7 primers described in sections C) and D) below.

C) Genomic human Chromosome 1 primers #3 (hChr1/14180006 S) and #4 (hChr1/14180401 Identifier : Amplicon hChr1/14180006-14180401
5' - CTTCTCCTTTCCTCCCTCCCTCTCTCATTTGCAGCTTCCCATACTTATCTTCCTAATACACTACTCTTTTGGAAATGTTTA
ATCATCATGTAGCTGGCTTTTTCACAGGCATGTCTTTCAGTGCAGCCTTATACCTCTCTCAACACAACTCTGTATCCCTCT
TTTCTCTTTTTCCTCCCTCCCTCTCTCATTTGCAGCTTCCCATACTTATCTTCCTAATACACTACTCTTTTGGAAATGTTTA

underlined nucleotides : primer sequences
bold nucleotides : homologous extremity of the 237 bp amplicon obtained with

AS) PCR-amplified 396 bp amplicon :

CTTCTAACAACAATGAAAGCAGGTCACTGCGGCTCAATGATGTCAAGGGCTTT
CATCTTCAGCTTTTATATATGTCAAGCCATGTGACACTCCCTAATATACCATGT
TTTCAAGGGTTCCTTATCTTTTAAACCACTCTCAGACTCCCT -3'

D) Genomic human chromosome 1 primers #3 (hChr1/14180006 S) and #5

Identifier : AMO11CON bchr1/14180006-14181093

5' - CATACCTTCCTGAGTATGATGAGGCTTCAGTCTGAGGCTCTCTGGATGCTTCTCTCCCGAGTCTT
ATCATGCCATGATGAGCTGGCTTTTTCACAGGCATGTCTTGCAGTGCAGCCCTTATTAATCTCTCTCACAACACAACTT
TTACTCTTTTTCCTGCTGCTGCTCTCTCATTTGCGAGCTTCCCATACTTATCTCTCTAAACACTACTCTCTT
TTACCTATGTTTTTATTGTGAGCATCTCTCACAATAATCAGCTTTAGTTCTCTTCCGCTTCTGGTGTGGCTATATA
GTTCCCGCATACTTAGTATTGTGCTTAGCATATGCTAGGTGCTCAGTAAATATATTTGATATATGTTGGTGAAC
CAAACTTTTCCCTTGGCTCTTAGTGTAGATAGTCTCTCATCAACATNAGGCATAATTTCTCTGCGGCTCTCA
AGTTAAANATCTGGCTTCTGCTATTTATTAGCCACATGACAGTGAATCTCCTTGAAGCTTCTGTTTTGTACA
TGACACGTGCTAAACATGGCTTGGCTTCTCCCGCACTTTTTTTTTTTGGAGACAGAACTCCCTTTTGTGAC

underlined nucleotides : primer sequences
 bold nucleotides : homologous extremity of the 237 bp amplicon
 The sequence of the 396bp amplicon hChr1/14180006-14180401 (C) is incl
 The internal underlined nucleotides correspond to the reverse-complement

(hChr1/14181093 AS) PCR-amplified 1,088 bp amplicon :

GCTGGAGTAATCCTTCTAACACACAAATGAAGCAGGTGCACAGCGGCTCAATGATGTTCAGCGGCTTT
CTGTATCCTCCTCATTCCTTCATCCTTTTATATGTCAGCCATGTGACACTCCCTAATATACCATGT
TTGAAATGTTTATTCAGGGTTTCTTATCTTTTANAGCATCTCAGACTCCCTGGGGATTACCCCTT
TTAGTGGGACTATGTCCCTTANCTGTTAGATCTCTTGAGAAAGGGACATGTCTTTTCATCTTGA
GAATGANTCATCATCAATACAAATGACAGACAACTCCACCCCAACCTAATAAANAATC
ATACAGAGGAGCGGAGACTCACAGATCATCAGATTTAGAGCACTGGCTTTGGCATGAGAACACCCCTG
AACTTAACTTTGGCTTTGTGATCTTATCTCTTTGGTGCATCTGTACACCCCACTGCTTATTCATA
CCAGGCTGGAATTCAGTGGGCGTATCTCGGCTCACTGCACCTCCACCTCCCGT -3'

obtained with the primers MSP2#3-5.

included in the larger size amplicon 1,088bp Amplicon hChr1/14180006-14181093
ent sequence of primer hChr1/14180401 AS (hChr1#4).

E) Genomic human chromosome 7 primers #2 (hChr7/4293490 8) and #6 Identifier : Amplicon hChr7/4293490-4294983

[illegible]

underlined nucleotides : bChr7 genomic primer sequences
bold nucleotides : homologous sequence of the 213 bp amplicon
 A part of the sequence 1,644bp Applicon bChr7/4292976-4294619 (2)
 the internal underlined nucleotides correspond to the reverse-com

(hchr7/4294983 AS) PCR-amplified 1,494 bp amplicon :

CCTCTCTACCTCACCCCCCAATATTCACACAAATCCCTATTTTATTTTGTGATTTTACTTCTCTATTTGTCTCTCAGT
 CAGTGTATATTGCAATCCATGAGTGTGATGCCCCCTATATAGCTGTACAGGCACAAACCCAGGCCAAACATACACAA
 TTAAATCCTCCTACTAGAGTTTATCTTTTAAAGTGAATAATCTGGATTTCTATCTCCCTAGTATGTATCTCTTA
 TATTTACCTTAGGCCCTTCACAAATATCTTTGAGTCTTTAATAGATTAATAAGTTTCTTCACTCTTTT
 CATTATTAAGCCCTTAACTCTCAGGATCTCCACAAATAAATACAAATCACTCTTTCTATATGCCCTAATCTTCTGC
 TCTCCTAGAAAGTGAGTTTGTGTAATTCAGGGTAAGTATATTTTATGTGTGGATGAATTCCTCCCTACACAAATA
 ATATAAATGTCTCTTGTGAGGAGATTTTCTTTGTAAATCTATCTCTGAGCTTTGTAGATGTGATGATG
 AAAAGTGTGATAATTTCTTGTCTCCTGAGACTCACCTCTCCCTACCTGCCCTATTAATAATCAGAAATACACAGATCTG
 CTGGATATTTATAGTATAGTATCCAGTATTTTCAATCTAGCTGTACTGAGGCCCGAAGCTAAAGGAATAATTA
 AAAATATATCAATTTTGTGGACCCCTTAATTTTGTTCGGAGTTGAATGCCCTCACTGGGACTTGGGTGA
 TTTTATTTTATTTTATTTTGTGACAGAGCCCTCACTCTGTCTCTCAGGCTG

ion obtained with the primers MSP2#2-5.

is included in the amplicon 1,494bp Amplicon hchr7/4293490-4294983 (E).
 implement sequence of primer hchr7/4294619 AS (hchr7#5).

APPENDIX 6

An amplicon of the 16s rDNA primers (SEQ ID NOS: 24 and 25) is shown below. The amplified DNA originated from the red blood cells of an HIV-negative subject passaged in HL60 cells. Similar DNA is amplified from samples originating from red blood cells of HIV-positive subjects.

5

Amplicon from HIV-negative subject obtained using primers (SEQ ID NOS: 24 and 25).

```
>T56-EMK-4-HIV-_Anae#2-5 wo/ primers seq. = 681bp
GCACCTGTATGTGAATTCCCCGAAGGCACTCCCGCATCTCTGCAGGATTCTCACTATGTCAAGACCAGGTAAGGTTCTTCGCGT
10 TGCATCGAATTAAACCAACATGCTCCACCGCTTGTGCGGCCCGTCAATTCAATTGAGTTTAAACCTTGCGGCCGTAATCCC
CAGGCGGTCTACTTATCGCGTTAACTGCGCCACTAAAGTCTCAAGGACCCCAACGGCTAGTAGACATCGTTTACGGCGTGGAC
TACCAGGGTATCTAATCCTGTTTGTGTACCCACGCTTTCGAAATCTCAGTGTCAATATATGCCAGGAAGCTGCCCTTCGCCATCG
GCATTCCCTCCAGATCTCTACGATTTTACCGCTACACCTGGAAATTCTACTTCCCTCTCACATATTCTAGCACCCACAGTATCA
CATGCAGTTCACAGGTTAAGCCCGGGATTTCACATGTGACTTAATGAGCCACCTACACTCGCTTACGCCCAGTAATTCCGA
15 TTAAAGCTCGCACCCCTCTGTATTACCGGGCTGTGGCACAGAGTTAGCCGTGCTTATTCTGCAGGTAACTTAATCTAAT
GGGTATTAAACCATTAGCCTCTCCTCCCTGCTTAAAGTGTCTTACAACCAAAAGGCCTTCTTCAACACACGCGGCATGGCTGGAT
CAGGGTTGCCCCCCCATT
```

CLAIMS

1. An agent that is associated with red blood cells and passes through a 0.45 micron filter.
2. The agent of claim 1 that is sensitive to antibiotics.
3. The agent of claim 1 that is sensitive to azithromycin or to a cyclin antibiotic.
4. The agent of claim 1 that contains nucleic acid that is amplified by (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), (iii), the pair primers described in Appendix 4 (SEQ ID NOS: 24 and 25); or a pair of primers that amplify at least twenty consecutive nucleotides of the same DNA as said primers.
5. The agent of claim 1 that contains DNA that is amplified by primers that amplify at least twenty consecutive nucleotides of a polynucleotide from *Ehlichia* or *Anaplasma* that encodes the major surface protein 2.
6. The agent of claim 1 that contains DNA that is amplified by MSP2 primers pair 1 (SEQ ID NOS: 3 and 4) or pair 2 (SEQ ID NOS: 5 and 6).
7. The agent of claim 1 that contains DNA that is amplified by a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23).
8. The agent of claim 1 that contains DNA that is amplified by a pair of primers described by Appendix 4 (SEQ ID NOS: 24 and 25).
9. The agent of claim 1 that contains DNA that is amplified by primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6) and that is at least 95% identical to human DNA.

10. The agent of claim 1 that contains DNA that is amplified by primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6) and that is 99% identical to human DNA.

11. The agent of claim 1 that is found in the red blood cells of an HIV-infected patient, but not detectable in the white blood cells of said patient.

12. The agent of claim 1 that becomes detectable in the red blood cells of an HIV-infected patient within the first year after HIV infection or after initiation of anti-retroviral treatment.

13. The agent of claim 1 that is detectable in the red blood cells of an African subject who is HIV-negative.

14. The agent of claim 1 that is found in the red blood cells of an African subject who is HIV-negative and that is insensitive to antibiotic treatment.

15. The agent of claim 1 that is a microorganism.

16. The agent of claim 1 that is a bacterium.

17. An isolated red blood cell that comprises the agent of claim 1.

18. A supernatant that comprises the agent of claim 1, which supernatant is produced by freezing and thawing red blood cells after removing white blood cells and then removing material that pellets by centrifugation for 10 mins at 1,500 g.

19. An isolated red blood cell that contains DNA that is amplified by (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), or (iii), the pair of primers described in Appendix 4 (SEQ ID NOS: 24 and 25).

20. Isolated or purified DNA that is produced by amplifying DNA in a red blood cell using (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), or (iii), the primers described in Appendix 4 (SEQ ID NOS: 24 and 25).

21. The isolated or purified DNA of claim 20 that comprises a DNA sequence or fragment thereof described by Appendix 5.

22. A vector containing the isolated or purified DNA of claim 20.

23. A host cell transformed with the isolated or purified DNA of claim 20.

24. A method for detecting the agent of claim 1 comprising:
contacting material from red blood cells of a subject with (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), (iii), the primers described in Appendix 4 (SEQ ID NOS: 24 and 25) under conditions suitable for amplification of DNA by said primers, and detecting said agent when DNA is amplified.

25. The method of claim 24, wherein said primers are selected from the group consisting of:

pair 1:

5' GCCTA CAGAT TAAAG GCT (SEQ ID NO: 3)

5' ATCAT ARTCA CCATC ACCTA (SEQ ID NO: 4);

pair 2:

5' CYTAC AGAGT GAAGG CT (SEQ ID NO: 5)

5' ATCAT ARTCA CCATC ACCTA (SEQ ID NO: 6)

or from a pair of primers that amplify at least twenty consecutive nucleotides of a DNA molecule amplified by primer pair 1 or 2.

26. The method of claim 24, wherein said primers are selected from the group consisting of primers 1-8 for human chromosome 1 (SEQ ID NOS: 7-14) described in Appendix 3 or from a pair of primers that amplify at least twenty consecutive nucleotides of the DNA amplified by said primers 1-8.

27. The method of claim 24, wherein said primers are selected from the group consisting of primers 1-9 for human chromosome 7 (SEQ ID NOS: 15-23) described in Appendix 3 or from a pair of primers that amplify at least twenty consecutive nucleotides of the DNA amplified by said primers 1-9.

28. The method of claim 24, wherein said primers are selected from the group consisting of the primers described in Appendix 4 (SEQ ID NOS: 24 and 25) or from a pair of primers that amplify at least twenty consecutive nucleotides of the DNA amplified by said primers.

29. The method of claim 24, wherein said biological sample is whole blood or a cellular component of whole blood.

30. The method of claim 24, wherein said biological sample is isolated anucleated red blood cells.

31. The method of claim 24, wherein said biological sample comprises an isolated erythroblast or other precursor cell of an anucleated red blood cell.

32. The method of claim 24, wherein said biological sample comprises isolated bone marrow cells.

33. The method of claim 24, wherein said biological sample is a red blood cell lysate.

34. The method of claim 24, wherein said biological sample is blood plasma or serum.

35. A method for treating or for reducing the severity of a disease, disorder, or condition

associated with the agent of claim 1 comprising treating a patient with an agent that reduces the titer of said agent or that reduces the amount of DNA amplified from a cell associated with said agent.

36. The method of claim 35 comprising treating the patient with one or more antibiotics.

37. The method of claim 35 comprising treating the patient with an azithromycin or a cyclin antibiotic.

38. The method of claim 35 comprising treating the patient with one or more natural immunostimulants, active vaccines, passive vaccines, antioxidants or antibiotics.

39. The method of claim 35, wherein the patient is HIV-negative.

40. The method of claim 35, wherein the subject is African or has lived in Africa.

41. The method of claim 35, wherein the subject is Caucasian or has lived in Europe.

42. The method of claim 35, wherein the patient is HIV-positive.

43. The method of claim 35, wherein said patient is HIV-positive and has or is undergoing antiretroviral therapy or therapy to eradicate human immunodeficiency virus infection.

44. A method for treating a disease, disorder or condition associated with the agent of claim 1 comprising contacting red blood cells with a substance that reduces the amount of DNA amplified from a red blood cell using

Primer Pair 1 or 2, primers described by Appendix 3, primers described by Appendix 4 or primers that amplify a portion of the same DNA as said primers.

45. A method for treating a disease, disorder or condition associated with the agent of claim 1 comprising contacting red blood cells of a subject with a substance that reduces the transmission of said agent to the red blood cell.

46. A method for treating a disease, disorder or condition associated with the agent of claim 1 comprising replacing the red blood cells in a subject with red blood cells that are not associated with said agent or by stimulating the development of new red blood cells in said subject.

47. A method for treating a disease, disorder or condition associated with the agent of claim 1 comprising treating red blood cells with a nucleus or other agent that degrades nucleic acids inside of or associated with a red blood cell.

48. A method for screening blood for red blood cells from which DNA can be amplified using (i) primer pairs 1 (SEQ ID NOS: 3 and 4) or 2 (SEQ ID NOS: 5 and 6), (ii) a pair of primers described by Appendix 3 (SEQ ID NOS: 7-14 or SEQ ID NOS: 15-23), (iii), the pair of primers described in Appendix 4 (SEQ ID NOS: 24 and 25); or a pair primers that amplify at least twenty consecutive nucleotides of the same DNA as said primers, comprising contacting a sample of red blood cells with said primers and detecting amplified DNA and selecting a blood sample from which DNA was amplified or alternatively selecting a blood sample from which no DNA was amplified.

49. An isolated or purified DNA molecule comprising the about 700 bp polynucleotide amplified by the primer pair described by SEQ ID NOS: 24 and 25.

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Figure 1(A)

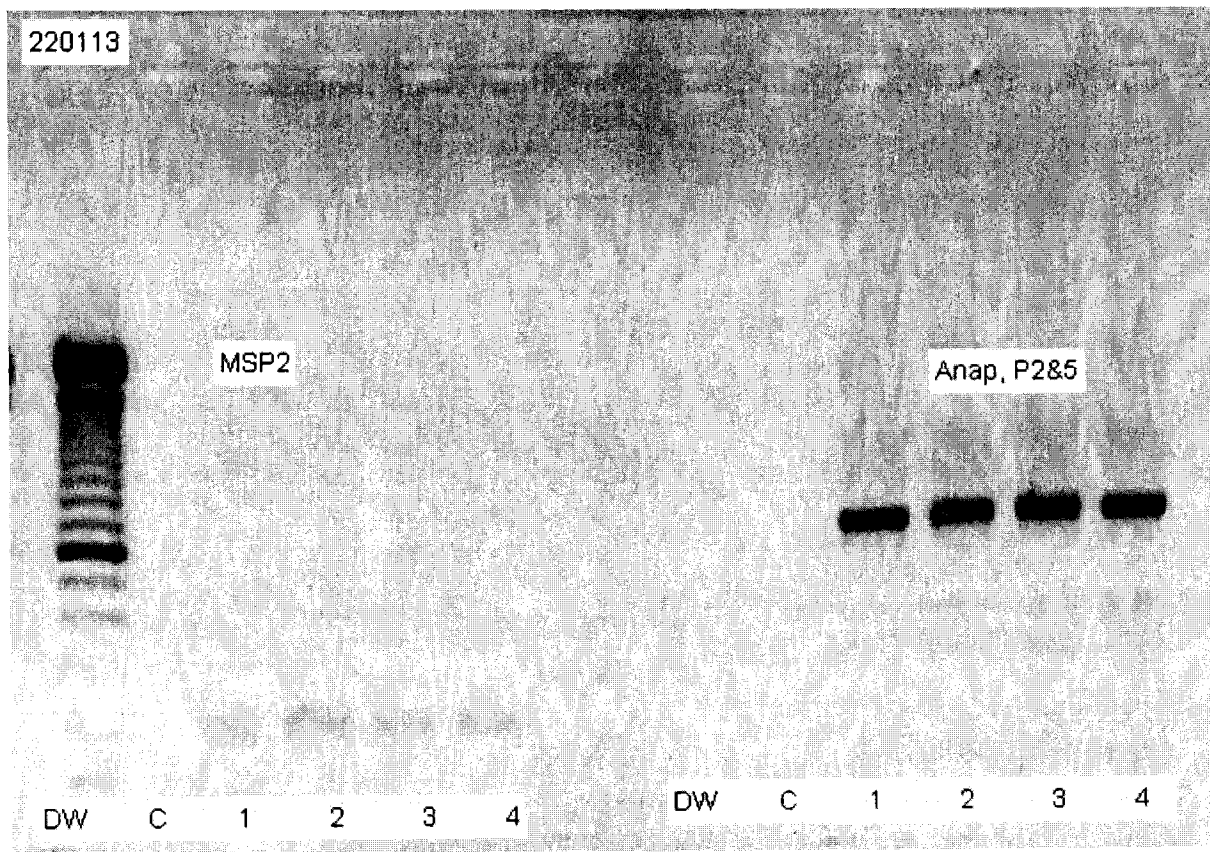


Figure 1(B)

