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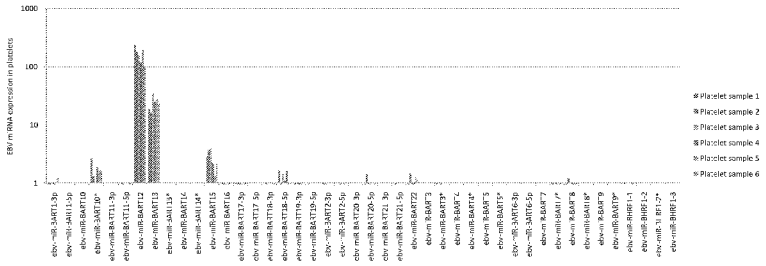
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(54) Title: A METHOD OF ANALYSING A BLOOD SAMPLE OF A SUBJECT FOR THE PRESENCE OF AN INFECTIOUS DISEASE MARKER

Figure 2




(57) Abstract: The present invention relates to a method of analysing a blood sample of a subject for the presence of an infectious disease marker, said method comprising the steps of a) extracting nucleic acid from anucleated blood cells in said blood sample to provide an anucleated blood cell-extracted nucleic acid fraction, and b) analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of an infectious disease marker, wherein said infectious disease marker is a nucleic acid of an infectious disease agent, or wherein said infectious disease marker is an infectious disease- specific expression profile of genes of a nucleated cell of said subject.

Title: A method of analysing a blood sample of a subject for the presence of an infectious disease marker

## FIELD OF THE INVENTION

The invention is in the field of medical diagnostics, in particular in the field of infectious disease diagnostics and monitoring. The invention is directed to methods for detecting infectious disease, and to a method for determining the efficacy of treatment of infectious diseases.

## BACKGROUND OF THE INVENTION

In clinical practice there is a strong need to be able to detect disease in its earliest stages, to predict disease progression, and to implement patient-tailored therapy. Early detection of in particular infectious disease agents is critical to ensure favourable treatment of the disease. In spite of numerous advances in medical research, infectious disease remains a major cause of concern for the medical world due to the increase in resistance to antibiotics. When patients seek treatment, they are generally exhibiting symptoms of general malaise and fever that can be caused by one of many conditions and illnesses, meaning that too often the infectious disease is detected too late.

In order to facilitate appropriate remedial action by appropriate medicaments or other known treatment methods there is a need for rapid and simple methods for the early diagnoses of infectious disease. The availability of good diagnostic methods for infectious disease is also important to assess patient responses to treatment, or to assess efficacy of novel treatment methods.

Improved screening and detection methods are needed in order to detect infectious disease in an early phase and to follow the progression of the disease. In the case of HIV infection we are at a state where the disease progression can be arrested, but where the presence of residual pockets

requires very sensitive methods. Also, in an age where antibiotics are rapidly losing effectiveness, detection and containment of infectious agents is becoming more and more important. People at risk, as well as human patients and other non-human carriers of infectious disease, will have to be monitored regularly.

Detection methods for infectious agents include methods for detecting the infectious agent itself or antibodies raised against them in, for instance, serum, urine or tissue samples. However, the levels of detectable molecules may generally be low in serum, and the use of tissue biopsies, in particular of organ tissue, is so invasive that regular monitoring is impractical.

The present invention provides a novel method for detecting the presence of infectious agents in a subject. Further, the present invention aims to provide methods that do not require biopsies, and allow extensive monitoring of patients.

## SUMMARY OF THE INVENTION

The present invention in a first aspect provides a method of analysing a blood sample of a subject for the presence of an infectious disease marker, said method comprising the steps of a) extracting nucleic acid from anucleated blood cells, preferably thrombocytes, in said blood sample to provide an anucleated blood cell-extracted nucleic acid fraction, and b) analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of an infectious disease marker, wherein said infectious disease marker is a nucleic acid of an infectious disease agent, or wherein said infectious disease marker is an infectious disease-specific expression profile of genes of a nucleated cell of said subject. Preferably, said infectious disease marker is a nucleic acid of an infectious disease agent.

The term “anucleated blood cell” as used herein refers to a cell that lacks a nucleus. The term includes reference to both erythrocyte and thrombocyte. Preferred embodiments of anucleated cells in aspects of this

invention are thrombocytes. The term "anucleated blood cell" preferably does not include reference to cells that lack a nucleus as a result of faulty cell division.

The term "nucleated cell" as used herein refers to a cell having a nucleus. The term includes reference to somatic cells, germ cells and stem cells, and may include cells from colon, pancreas, brain, bladder, breast, prostate, lung, breast, ovary, uterus, liver, kidney, spleen, thymus, thyroid, nerve tissue, connective tissue, blood, epithelial tissue, lymph node, bone, muscle and skin tissues. The nucleated cell is preferably a cell from a diseased tissue.

Thus, the present invention is – in a highly preferred embodiment - generally aimed at analysing nucleic acids that have been transferred from cells that have a nucleus into cells that have no nucleus, wherein the cells that have no nucleus can be easily isolated from the blood stream and contain nucleic acid from the nucleated cells.

The term "nucleus" refers to the membrane-enclosed organelle found in eukaryotic cells that contains most of the cell's genetic material organized in the form of chromosomes. The genes within these chromosomes are the cell's nuclear genome. The interior of the nucleus contains a number of subnuclear bodies including the RNA-comprising nucleolus, which is mainly involved in the assembly of RNA-comprising ribosomes. After being produced in the nucleolus, ribosomes are exported to the cytoplasm where they translate mRNA.

An anucleated blood cell-extracted nucleic acid fraction preferably refers to a fraction comprising chromosomal DNA, ribosomal RNA, nucleolus RNA, and/or messenger RNA.

The term "gene" as used herein, and in particular in the phrasing "mutation in a gene of a nucleated cell" is meant to refer to any nucleic acid sequence, both chromosomal and extra-chromosomal, of a nucleated cell, preferably a nuclear nucleic acid sequence, and may include transcribed and

non-transcribed sequences as well as ribosomal RNA sequences, most preferably chromosomal sequences that are transcribed into RNA.

In a preferred embodiment of a method of the invention said nucleic acid of an infectious disease agent is chromosomal or genomic nucleic acid of said infectious disease agent. In another preferred embodiment, said infectious disease marker is a nucleic acid of an infectious disease agent that has not infected said anucleated blood cell. An infectious disease agent has not infected said anucleated blood cell when the intact agent itself is not present in anucleated blood cells, or when the infectious disease agent-derived nucleic acids are incomplete for it to propagate in said anucleated blood cell. In another preferred embodiment, said infectious disease marker is a nucleic acid of an infectious disease agent that has infected a nucleated cell, including a nucleated blood cell, of said subject.

In another preferred embodiment, said expression profile of genes is not an expression profile of genes from an anucleated blood cell.

In a preferred embodiment of a method of the invention said infectious disease-specific expression profile is the expression profile of chromosomal genes in (a cell of) said subject, the expression of which is altered do to infection of (a cell of) said subject with an infections disease agent. Preferably, the profile is the expression profile of chromosomal genes from a nucleated cell of said subject the mRNA of which is present in an anucleated blood cell.

In another preferred embodiment of a method of the invention said nucleic acid is ribonucleic acid (RNA), more preferably messenger ribonucleic acid (mRNA) or miRNA.

In a preferred embodiment of a method of the invention said nucleic acid is not mitochondrial DNA or mitochondrial RNA. Hence, mitochondrial nucleic acid is preferably not an aspect of the present invention.

In another preferred embodiment of a method of analysing a blood sample according to the invention said step b) of analysing said anucleated

blood cell-extracted nucleic acid fraction for the presence of an infectious disease marker comprises the selective amplification of

i) at least a part of said nucleic acid of an infectious disease agent by (reverse transcriptase) polymerase chain reaction amplification using at least one infectious disease agent-specific amplification primer or probe, or

ii) a plurality of mRNAs (or at least a part of the mRNAs) by reverse transcriptase polymerase chain reaction amplification to determine the expression level of the chromosomal genes encoding said mRNAs to thereby provide an expression profile for said genes and comparing said expression profile to a reference profile.

In a preferred embodiment of aspects of the invention the infectious disease is caused by an agent selected from the group consisting of viruses, bacteria, parasites, and fungi.

In a more preferred embodiment of aspects of the invention said infectious disease is viral or bacterial.

In alternatively preferred embodiments of aspects of the invention said infectious disease is selected from the infectious agents listed in Table 1.

Table 1. Infectious disease agents subject of embodiments of the present invention.

**Bacteria Superkingdom**

- *CFB group*
  - *Bacteroides* (e.g. *B. fragilis*)
  - *Flavobacterium* (e.g. *F. meningosepticum*)
  - *Prevotella* (e.g. *P. intermedia*)
  - *Capnocytophaga* (e.g. C. *canimorsus*)
- *Chlamydiales order*
  - *Chlamydia* (e.g. *C. trachomatis*, C. *pneumoniae and C. psittaci*)
- *Fusobacteria*
  - *Fusobacterium necrophorum*
  - *Streptobacillus moniliformis*
- *Spirochaetales order*
  - *Borrelia (e.g. B. burgdorferi* **and** *B. recurrentis)*
  - *Treponema* (e.g. *T. pallidum)*
  - *Leptospira* (e.g. *L. interrogans)*
- Firmicutes phylum (Gram positive group)
  - *Bifidobacteriales order*
    - *Gardnerella* (e.g. *G. vaginalis*)
  - *Lactobacillales order*
    - *Streptococcus* (e.g. *S. pneumoniae*, α-, β- and γ-haemolytische and viridans and S. *pyogenes*)
    - *Enterococcus* (e.g. *E. faecium and E. faecalis)*
    - *Aerococcus* (e.g. *A. viridans*)
    - *Pediococcus* (e.g. *P. acidilactici*)
    - *Leuconostoc* (e.g. *L. pseudomesenteroides*)
  - *Actinomycetales order*
    - *Mycobacterium* (e.g. *M. tuberculosis, M. lepra, M. africanum, M. bovis and M. avium)*
    - *Nocardia* (e.g. *N. asteroides)*
    - *Corynebacterium* (e.g. *C. diphtheriae)*
    - *Micrococcus* (e.g. *M. luteus*)
    - *Actinomyces (e.g. A. israelii)*
    - *Propionibacterium propionicum*
    - *Brevibacterium* (e.g. *B. linens*)
  - *Mycoplasmatales order*
    - *Mycoplasma* (e.g. *M. pneumoniae)*
  - *Bacillales order*
    - *Staphylococcus* (e.g. *S. aureus and S. pyogenes)*
    - *Alicyclobacillus* (e.g. *A. acidocaldarius*)
    - *Listeria* (e.g. *L. monocytogenes*)
    - *Bacillus* (e.g. *B. anthracis*)

*Gemella* (e.g. *G. morbillorum*)
*Clostridiales order*
*Clostridium* (e.g. *C. botulinum*, *C. diffilie*, *C. perfringens* and *C. tetani)*
*Peptostreptococcus* (e.g. *P. prevotii*)
*Veillonella* (e.g. *V. parvula*)
Proteobacteria phylum
*Alpha subdivision Class*
*Rickettsiales order*
*Rickettsiaceae family*
*Rickettsia* (e.g. *R. prowazekii and R. typhi*)
*Ehrlichia* (e.g. *E canis, E. chaffeensis* and *E. phagocytophila*)
*Cowdria* (e.g. *C. ruminantium*)
*Neorickettsia* (e.g. *N. helminthoeca*)
*Anaplasma* (e.g. *A. marginale and A. ovis)*
*Wolbachia* (e.g. *W. pipientis*)
*Rhizobacteriaceae* group
*Brucellaceae family*
*Brucella* (e.g. *B. melitensis* biovar *abortus and B. m.* biovar *canis)*
*Bartonellaceae family*
*Bartonella* (e.g. *B. bacilliformis, B. henselae and B. quintana)*
*Beta subdivision Class*
*Alcaligenaceae family*
*Alcaligenes* (e.g. *A. faecalis)*
*Bordetella* (e.g. *B. pertussis)*
*Neisseriaceae family*
*Neisseria* (e.g.. *N. meningitidis and N. gonorrhoeae*)
*Kingella* (e.g. *K. denitrificans*)
*Eikenella* (e.g. *E. corrodens*)
*Chromobacterium* (o..a. *C. violaceum*)
*Burkholderia group*
*Burkholderia* (e.g. *B. cepacia)*
*Gamma subdivision Class*
*Aeromonadaceae family*
*Aeromonas*
*Moraxellaceae family*
*Acinetobacter* (e.g. *A. lwoffii and A. baumannii)*
*Moraxella* (e.g. *M. catarrhalis)*
*Enterobacteriaceae family*
*Escherichiae* (e.g. *E. coli)*
*Klebsiellae* (e.g. *K. pneumoniae)*
*Salmonellae* (e.g. *S. typhimurium and S. enteritidis)*
*Shigella* (e.g. *S. dysenteriae*)
*Edwardsiella* (e.g. *E. tarda*)
*Yersinia* (e.g. *Y. pestis*)

*Citrobacter (e.g. C. freundii)*
*Proteus* (e.g. *P. mirabilis*)
*Morganella morganii*
*Providencia* (e.g. *P. alcalifaciens*)
*Serratia* (e.g. *S. marcescens*)
*Plesiomonas (e.g. P. shigelloides)*
*Legionellaceae family /Coxiella group*
*Legionella* (e.g. *L. pneumophila and L. micdadei)*
*Coxiella* (e.g. *C. burnetii*)
*Rickettsiella* (e.g. *R. popilliae*)
*Tatlockia* (e.g. *T. micdadei*)
*Fluoribacter* (e.g. *F. dumoffii*)
*Pasteurellaceae family*
*Haemophilus* (e.g. *H. influenzae and H. ducreyi)*
*Pasteurella* (e.g. *P. multocida)*
*Pseudomonadaceae family*
*Pseudomonas* (e.g. *P. aeruginosa and P. cepacia*)
*Francisella group*
*Francisella* (e.g. *F. tularensis*)
*Vibrionaceae family*
*Vibrio* (e.g. *V. cholerae, V. vulnificus and V. parahaemolyticus*)
*Xanthomonas group*
*Stenotrophomonas* (e.g. *S. maltophila*)
*Epsilon subdivision Class*
*Campylobacter group*
*Campylobacter* (e.g. *C. jejuni*)
*Helicobacter* (e.g. *H. pylori, H. cinaedi and H. fennelliae*)

**Eukaryota Superkingdom**
*Protista Kingdom*
*Trichomonadida order*
*Trichomonas (e.g. T. vaginalis)*
*Microsporida order*
*Enterocytozoon bieneusi*
*Amoebida order*
*Acanthamoeba* (e.g. *A. castellani)*
*Entamoeba (e.g. E. histolytica)*
*Eucoccidiida order*
*Cryptosporidium (e.g. C. parvum)*
*Diplomonadidae order*
*Giardia lamblia*
*Eimeriida order*
*Cryptosporidium (e.g. C. parvum)*
*Toxoplasma gondii*
*Neospora caninum*

*Haemosporida order*
*Plasmodium* (e.g. *P. falciparum)*
*Kinetoplastida order*
*Trypanosoma (e.g. T. brucei)*
*Leishmania donovani*
*Fungi Kingdom*
*Acremonium*
*Aspergillus* (e.g. *A. fumigatus*)
*Beauveria*
*Fusarium*
*Histoplasma* (e.g. *H. duboisii*)
*Paecilomyces*
*Penicillium*
*Scopulariopsis*
*Trichophyton* (e.g. *T. rubrum and T. mentagrophytes*)
*Cryptococcus* (e.g. *C. neoformans*)
*Coccidioides* (e.g. *C. immitis*)
*Candida* (e.g. *C. albicans*)
*Blastomyces*
*Malassezia*
*Pneumocystosis* (e.g. *P. carinii*)

**Viruses**
*DNA viruses*
*Herpesviridae*
*Herpes simplex virus type 1*
*Varicella zoster virus*
*Epstein Barr virus*
*Human cytomegalovirus*
*Human herpesvirus 6* (*Roseolovirus*)
*Kaposi's sarcoma-associated herpesvirus*
*Cercopithecine herpesvirus-1*
*Murine gammaherpesvirus-68*
*Adenoviridae*
*Human adenoviruses*
*Polyomaviridae*
*Human polyomavirus*
*Papillomaviridae*
*Human papillomaviruses*
*Hepadnaviridae*
*Hepatitis B virus*
*Poxviridae*
*Vaccinia virus*
*Smallpox virus (variola)*
*Cowpox virus*

*Monkeypox virus*
*Orf virus*
*Pseudocowpox*
*Bovine papular stomatitis virus*
*Tanapox virus*
*Yaba monkey tumor virus*
*Molluscum contagiosum virus*
*Parvoviridae*
*B19 parvovirus*

*RNA viruses*
*Picornaviridae*
*Polioviruses*
*Echoviruses*
*Coxsackieviruses*
*Hepatitis A virus*
*Human rhinoviruses*
*Caliciviridae*
*Norovirus*
*Paramyxoviridae*
*Parainfluenza viruses*
*Measles virus*
*Respiratory syncytial virus*
*Orthomyxoviridae*
*Influenza virus A, B and C*
*Thogotovirus*
*Rhabdoviridae*
*Rabies virus*
*Filoviridae*
*Ebola and Marburg viruses*
*Retroviridae*
*Human immunodeficiency virus type-1 and –2*
*Togaviridae*
*Rubella virus*
*Sindbis virus*
*Flaviviridae*
*Yellow fever virus*
*Dengue virus*
*Hepatitis C virus*
*West Nile virus*
*Japanese encephalitis virus*
*Tick-borne encephalitis virus*
*Reoviridae*
*Human rotaviruses*
*Bunyaviridae*

*Andes virus*
*Bayou virus*
*Black Creek Canal virus*
*Cano Delgadito virus*
*Choclo virus*
*Dobrava-Belgrade virus*
*Hantaan virus*
*Isla Vista virus*
*Khabarovsk virus*
*Laguna Negra virus*
*Muleshoe virus*
*New York virus*
*Prospect Hill virus*
*Puumala virus*
*Rio Mamore virus*
*Rio Segundo virus*
*Seoul virus*
*Sin Nombre virus*
*Thailand virus*
*Thottapalayam virus*
*Topografov virus*
*Tula virus*
*Pulmonary Syndrome Hantavirus*

*Arenaviridae*

*Lassa virus*
*Lymphocytic choriomeningitis virus*
*Junin virus*
*Machupo virus*
*Guanarito virus*
*Sabiá virus*
*Tacaribe virus*
*Flexal virus*
*Whitewater Arroyo virus*

*Coronaviridae*

*Human coronaviruses*

*Astroviridae*

*Human astroviruses*

In another aspect, the present invention provides a method of diagnosing infectious disease in a subject using the method of analysing a blood sample according to the invention. Hence, in another preferred embodiment of a method of the invention, said method of analysing a blood sample according to the invention is part of a method of diagnosing infectious

disease in a subject, wherein the presence of said infectious disease marker in said anucleated blood cell-extracted nucleic acid fraction is indicative of said subject suffering from said infectious disease.

In another aspect, the present invention provides a method for determining the efficacy of an infectious disease treatment in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of an infectious disease marker using the method of analysing a blood sample according to the invention at a first time point to thereby provide a first value for the level of said infectious disease marker in said subject;
- analysing a blood sample of said subject for the presence of an infectious disease marker using the method of analysing a blood sample according to the invention at a second time point that is earlier or later, preferably later, than said first time point, to thereby provide a second value for the level of said infectious disease marker in said subject, wherein said subject has been subjected to a infectious disease treatment between said first and second time point, and
- comparing said first and second value to determine the efficacy of said infectious disease treatment in said subject.

Infectious disease treatment may comprise treatment with antiviral agents, antibiotic agents, antifungal agents, or antiparasitic agents.

The skilled artisan will understand that treatment prior to the first time point and subsequent measurements at a second, later, time point without any infectious disease treatment having occurred between said time points, is included in aspects of the invention for determining the efficacy of an infectious disease treatment.

In another aspect, the present invention provides a method for determining the stage of infectious disease. In order to determine the stage of infectious disease, it is beneficial to correlate infectious disease marker values as determined by methods of this invention to infectious disease stages. A

single measurement of the infectious disease marker may than be compared to one or more reference values to obtain an indication of the stage of the infectious disease.

In another aspect, the present invention provides a method for determining the stage of infectious disease in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of an infectious disease marker using the method of analysing a blood sample of a subject for the presence of an infectious disease marker according to the present invention as described above to thereby provide a test value for the level of said infectious disease marker in said subject,
- providing a reference value for the level of said infectious disease marker wherein said reference value is correlated to a particular stage of infectious disease, and
- comparing said test and reference value to determine the stage of infectious disease in said subject.

In another aspect, the present invention provides a kit of parts adapted for performing a method of the invention as described herein above, the kit comprising a packaging material which comprises at least one of:

- a container for holding anucleated blood cells, preferably thrombocytes, separated from a blood sample;
- an agent for extracting nucleic acids from said anucleated blood cells;
- an agent for selectively amplifying from said nucleic acids extracted from said anucleated blood cells an infectious disease marker (i.e. an infectious disease agent-specific nucleic acid sequence or an infectious disease-specific gene expression profile of chromosomal genes of said subject as indicated above), by (reverse transcriptase) polymerase chain reaction amplification, and
- a printed or electronic instruction for performing a method of the invention as described herein above,

the kit further comprising:

- a reference for said disease marker, wherein said reference is indicative for the presence or absence of said infectious disease marker in said anucleated blood cells-extracted nucleic acid fraction.

In a preferred embodiment of a kit according to the present invention said reference is a reference value for the level of nucleic acids comprising said infectious disease agent-specific nucleic acid sequence in anucleated blood cells in a healthy control subject or in a control subject suffering from infectious disease, or wherein said reference is a reference expression profile for said plurality of mRNAs in anucleated blood cells from a healthy control subject or from a control subject suffering from infectious disease.

In another preferred embodiment of a kit according to the present invention said agent or instruction is selected from a particle or fluorescent marker-labeled anti-anucleated blood cell antibody (preferably a fluorescent marker-labeled anti-thrombocyte antibody), an instruction for bead-based anucleated blood cells isolation (preferably thrombocyte isolation), an instruction for FACS sorting of anucleated blood cells (preferably of thrombocytes), an instruction for anucleated blood cell (preferably thrombocyte) recovery by centrifugation, or negative selection of non-anucleated blood cell components (preferably non-thrombocyte components).

In yet another aspect, the present invention provides a device for diagnosing infectious disease, the device comprising a support and at least one agent for specifically determining a level and/or activity of at least one nucleic acid mutant in a anucleated blood cells sample of the subject, said agent being attached to said support, and a computer-readable medium having computer-executable instructions for performing a method of the invention as described herein above.

In a preferred embodiment of a device according to the present invention, said at least one agent is an oligonucleotide probe or sequencing primer.

In a preferred embodiment of a device according to the present invention, the device comprises a lateral flow device, a dipstick or a cartridge for performing a nucleic acid hybridization reaction between:

- an anucleated blood cells-extracted nucleic acid and at least one infectious disease agent-specific amplification primer or oligonucleotide probe, wherein said infectious disease agent-specific amplification primer or oligonucleotide probe hybridizes specifically to an infectious disease agent-specific nucleic acid sequence, or
- an anucleated blood cells-extracted nucleic acid and a plurality of gene-specific amplification primers or oligonucleotide probes for providing an disease-specific gene expression profile, wherein said genes are genes of a nucleated cell of the subject in which the infectious disease is to be diagnosed.

## DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of the experiments described in Example 1. Platelet RNA was subjected to PCR in order to detect EBV RNA. EBV-EBER RNA was detected, as indicated by the amplification curves following 30 cycles of amplification.

Fig. 2 shows the results of the experiments described in Example 2. Platelet RNA was subjected to miRNA microarrays in order to detect EBV RNA. EBV-miR-BART12 and EBV-miR-BART13 RNA were detected, as indicated by the bar graphs. The horizontal line below expression level 10 indicates an arbitrary significance threshold.

## DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "infectious disease" refers to a disease or disorder resulting from the residence or proliferation of infectious agents in (cells of) a subject.

As used herein the term “infectious disease marker” refers to in particular to nucleic acid sequence that is specific for an infectious disease or

that can be used to detect the presence of a nucleic acid of an infectious disease in a sample, or to gene expression profile in a subject that is correlated with infection by an infectious disease agent in a subject, and wherein the mRNA of said genes is present in the nucleic acid fraction extracted from anucleated blood cells (preferably thrombocytes).

As used herein, the term “stage of infectious disease” refers to a qualitative or quantitative assessment of the level of advancement of an infectious disease. Criteria used to determine the stage of an infectious disease include, but are not limited to, the size of lesion caused by infection, or the amount of infectious disease agent in the blood or a tissue of a subject.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single-or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e. g., peptide nucleic acids).

The term “RNA” refers to ribonucleic acid, a molecule of RNA encoding for a protein product or non-coding for a protein product (such as miRNAs but not excluding other non-coding RNAs). RNA is transcribed from a DNA template.

By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e. g., Diagnostic Molecular Microbiology. Principles and Applications, D. H. Persing et al., Ed., American Society for Microbiology, Washington, D. C. (1993). The product of amplification is termed an amplicon.

The term "hybrid" refers to a double-stranded nucleic acid molecule, or duplex, formed by hydrogen bonding between complementary nucleotides. The terms "hybridise" or "anneal" refer to the process by which single strands of nucleic acid sequences form double-helical segments through hydrogen bonding between complementary nucleotides.

The term "oligonucleotide" refers to a short sequence of nucleotide monomers (usually 6 to 100 nucleotides) joined by phosphorous linkages (e.g., phosphodiester, alkyl and aryl-phosphate, phosphorothioate), or non-phosphorous linkages (e.g., peptide, sulfamate and others). An oligonucleotide may contain modified nucleotides having modified bases (e.g., 5-methyl cytosine) and modified sugar groups (e.g., 2'-O-methyl ribosyl, 2'-O-methoxyethyl ribosyl, 2'-fluoro ribosyl, 2'-amino ribosyl, and the like). Oligonucleotides may be naturally-occurring or synthetic molecules of double- and single-stranded DNA and double- and single-stranded RNA with circular, branched or linear shapes and optionally including domains capable of forming stable secondary structures (e.g., stem-and-loop and loop-stem-loop structures).

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxy ribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. A "pair of bi-directional primers" as used

herein refers to one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

The term "probe" refers to a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative.

The terms "stringency" or "stringent hybridization conditions" refer to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, formamide concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridise to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point ($T_m$) for the specific sequence at a defined ionic strength and pH. The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M $Na^+$ ion, typically about 0.01 to 1.0 M $Na^+$ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringent conditions or "conditions of reduced stringency" include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 2x SSC at 40°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1x SSC at 60°C.

Hybridization procedures are well known in the art and are described in e.g. Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons Inc., 1994.

"Subject" as used herein includes, but is not limited to, mammals, including, e.g., a human, a non-human primate, a mouse, a pig, a cow, a goat, a cat, a rabbit, a rat, a guinea pig, a hamster, a degu, a horse, a monkey, a sheep, or other non-human mammal; and non-mammal animals, including, e.g., a non-mammalian vertebrate, such as a bird (e.g., a chicken or duck) or a fish, and an invertebrate. The subject may be a healthy animal or human subject undergoing a routine well-being check up. Alternatively, the subject may be at risk of having cancer (e.g., a genetically predisposed subject, a subject with medical and/or family history of cancer, a subject who has been exposed to carcinogens, occupational hazard, environmental hazard] and/or a subject who exhibits suspicious clinical signs of cancer [e.g., blood in the stool or melena, unexplained pain, sweating, unexplained fever, unexplained loss of weight up to anorexia, changes in bowel habits (constipation and/or diarrhoea), tenesmus (sense of incomplete defecation, for rectal cancer specifically), anaemia and/or general weakness). According to another embodiment, the subject may be a diagnosed cancer patient and is performing a routine check-up, in-between treatments.

The term “thrombocyte”, as used herein, refers to blood platelets, i.e. the small, irregularly-shaped cell fragments do not have a nucleus containing DNA, and that circulate in the blood of mammals. Thrombocytes are 2-3 μm in diameter, and are derived from fragmentation of precursor megakaryocytes. The average lifespan of a thrombocyte is 5 to 9 days. Thrombocytes are involved and play an essential role in haemostasis, leading to the formation of blood clots.

The term “blood” as used herein refers to whole blood (including plasma and cells) and includes arterial, capillary and venous blood.

Proper infectious disease therapy is critically depending on disease profiling and the development of diagnostics. However, obtaining easily accessible high-quality nucleic acids for detecting infectious disease remains a significant developmental hurdle. Blood generally contains 150,000-350,000 thrombocytes (platelets) per microliter, providing a highly available biomarker source for research and clinical use. Moreover, thrombocyte isolation is relatively simple and is a standard procedure in blood bank/haematology labs. Since platelets do not contain a nucleus, their RNA transcripts - needed for functional maintenance - are derived from bone marrow megakaryocytes during thrombocyte origination. It has now been found that thrombocytes may take up RNA during circulation via various transfer mechanisms. Infectious agents and infected cells of a subject release an abundant collection of genetic material, some of which originates from infectious disease agents or infectious disease agent-infected cells. During circulation in the blood stream thrombocytes absorb the genetic material secreted by infected cells or infectious agents themselves, serving as an attractive platform for the diagnostics of infectious disease.

In the Examples below it is shown that platelets isolated from human patients suffering from infection with the infectious disease agent Epstein-Barr Virus (EBV) have the ability to take up EBV RNA. Hence, it was determined that circulating platelets isolated from EBV patients contain RNA biomarkers for EBV.

The present invention provides a novel and easy-to-use method to isolate circulating infectious disease markers as used herein for genetic analysis. The present inventors isolated virus-derived RNA from circulating thrombocytes, yielding pure RNA and an easy way to extract high quality RNA from low amounts of blood. Thrombocyte RNA isolation and subsequent analysis presents a marked increase in the diagnostic sensitivity of circulating RNA in blood. The same accounts for erythrocytes.

The present inventors found that in infectious disease patients these circulating thrombocytes contain significant amounts of infectious disease agent-derived RNA. These infectious disease agent-derived RNAs presents unique genetic information about the infectious disease agent, which may be used to determine infectious agent type, extent of the disease and possibly the susceptibility of the infectious agent to therapeutic treatment. The thrombocyte RNA can be analyzed for the presence of specific infectious disease agent-derived RNAs, as demonstrated herein for the EBV RNA derived from latently infected patients.

The present invention describes a method of finding specific transcripts derived from cells of infectious disease origin within anucleated cells extracted from blood. This approach is robust and easy. This is attributed to the rapid and straight forward extraction procedures and the quality of the extracted RNA. Within the clinical setting, extraction or isolation of anucleated cells such as thrombocytes is already implemented in general biological sample collection and therefore it is foreseen that the implementation into the clinic is relatively easy.

The present invention provides a general method for analysing blood of a subject for the presence of an infectious disease nucleic acid marker and a method of diagnosing infectious disease in a subject using said general method. When reference is herein made to a method of the invention, both embodiments are referred to.

A method of the invention can be performed on any suitable body sample comprising anucleated blood cells, such as for instance a tissue sample comprising blood, but preferably said sample is whole blood.

A blood sample of a subject can be obtained by any standard method, for instance by venous extraction.

The amount of blood needed is not particularly limited. Depending on the methods employed, the skilled person will be capable of establishing the amount of sample required to perform the various steps of the method of the

present invention and obtain sufficient nucleic acid for genetic analysis. Generally, such amounts will comprise a volume ranging from 0.01 µl to 100 ml.

The body sample may be analyzed immediately following collection of the sample. Alternatively, analysis according to the method of the present invention can be performed on a stored body sample or on a stored anucleated blood cells fraction thereof. The body sample for testing or the anucleated blood cells fraction thereof can be preserved using methods and apparatuses known in the art. In a collected anucleated blood cell fraction, the thrombocytes are preferably maintenance in inactivated state (ie. in non-activated state). In that way, the cellular integrity and the infectious disease-derived nucleic acids are best preserved.

In case the fraction of anucleated blood cells is a thrombocyte fraction, this platelet isolated fraction does preferably not include platelet poor plasma or platelet rich plasma (PRP). Further isolation of the platelets is preferred for optimal resolution.

The body sample may suitably be processed otherwise, for instance, it may be purified, or digested, or specific compounds may be extracted therefrom. Depending upon the method of characterizing the nucleic acids present in the anucleated blood cells in said sample, which method preferably involves isolation of RNA and RT-PCR, the anucleated blood cells may be extracted from the sample by methods known to the skilled person and be transferred to any suitable medium for extraction of the nucleic acids therefrom should the analysis method so require. The recipient subject's body sample may be treated to remove abundant nucleic acid degrading enzymes (like RNases, DNases) therefrom, in order to prevent early destruction of the thrombocyte nucleic acids.

Anucleated blood cell extraction from the body sample of the subject may involve any available method. In transfusion medicine, thrombocytes are often collected by apheresis, a medical technology in which the blood of a donor

or patient is passed through an apparatus that separates out one particular constituent and returns the remainder to the circulation. The separation of individual blood components is done with a specialized centrifuge. Plateletpheresis (also called thrombopheresis or thrombocytapheresis) is the apheresis process of collecting thrombocytes. Modern automatic plateletpheresis allows blood donors to give a portion of their thrombocytes, while keeping their red blood cells and at least a portion of blood plasma. Although it is possible to provide the body sample comprising anucleated blood cells as envisioned herein by apheresis, it is often easier to collect whole blood and isolate the anucleated blood cells fraction therefrom by centrifugation. Generally, in such a protocol, the thrombocytes are first separated from the other blood cells by a centrifugation step of about 120 x g for about 20 minutes at room temperature to obtain a platelet rich plasma (PRP) fraction. The thrombocytes are then washed (for instance in PBS-EDTA) to remove plasma proteins and enrich for thrombocytes. Wash steps are generally carried out at 850 - 1000 x g for about 10 min at room temperature. Further enrichments can be carried out to yield more pure thrombocyte fractions.

Platelet isolation generally involves blood sample collection in Vacutainer tubes containing anticoagulant citrate dextrose (e.g. 36 ml citric acid, 5 mmol/l KCl, 90 mmol/l NaCl, 5 mmol/l glucose, 10 mmol/l EDTA pH 6.8). A suitable protocol for platelet isolation is described in Ferretti et al. (J Clin Endocrinol Metab 2002; 87:2180–2184). This method involves a preliminary centrifugation step (1,300 rpm per 10 min) to obtain platelet-rich plasma (PRP). Platelets are then washed three times in an antiaggregation buffer (Tris–HCl 10 mmol/l; NaCl 150 mmol/l; EDTA 1 mmol/l; glucose 5 mmol/l; pH 7.4) and centrifuged as above, to avoid any contamination with plasma proteins and to remove any residual erythrocytes. A final centrifugation at 4,000 rpm for 20 min may then be performed to isolate platelets. The platelet pellet may be washed (e.g. in phosphate buffered saline). For quantitative determination of cancer marker levels, the protein

concentration of platelet membranes may be used as internal reference. Such protein concentrations may be determined by the method of Bradford (Anal Biochem 1976; 72:248–254), using serum albumin as standard.

Following the provision of the body sample of the subject, and the extraction therefrom of the anucleated blood cells, the anucleated blood cells of the subject are screened for the presence of infectious disease agent-specific nucleic acids (e.g. in the form of specific sequences or in the form of RNA profiles indicative of infection). If infectious disease-specific nucleic acids are encountered in the anucleated blood cells of the subject, or if infectious disease-specific nucleic acids are encountered in the anucleated blood cells of the subject at a higher level than in the thrombocytes in an unaffected blood sample of a control subject, which infectious disease-specific nucleic acids are considered to originate from a infectious disease agent residing in the subject, said subject is diagnosed with infectious disease as defined herein. Also, if infectious disease-specific nucleic acid (expression) profiles are encountered in the anucleated blood cells of the subject, which nucleic acid (expression) profiles are profiles (expressed) of genes of infectious agent-infected nucleated cells, said subject is diagnosed with infectious disease as defined herein.

A further step in a method of the invention is the provision of an anucleated blood cells-extracted nucleic acid fraction. Such a nucleic acid fraction is subsequently used for the detection of an infectious disease marker therein. An anucleated blood cells-extracted nucleic acid fraction may be obtained by any nucleic acid (NA) extraction method available. Usually NA extraction is performed by using chaotropic reagents. The first step in isolating total NA from cells or tissue is to break open the cells under denaturing conditions. In 1979, Chirgwin et al. (Biochemistry, 18[24]:5294-9, 1979) devised a method for the efficient isolation of total RNA by homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate with 0.1 M 2-mercaptoethanol to break protein disulfide bonds. RNA was then isolated by ethanol extraction or by ultracentrifugation through cesium

chloride. In 1987 Chomczynski and Sacchi (Analytical Biochemistry, 162[1]:156-9, 1987) modified this method to devise a rapid single-step extraction procedure using a mixture of guanidinium thiocyanate and phenol-chloroform, a method especially useful for processing large numbers of samples or for isolation of RNA from small quantities of cells or tissue. Any commercial kit can also be used for the extraction of RNA, non-limiting examples thereof include Ambion's RNAqueous™ system, Bio101's RNaid Plus kit, Bioline Ltd.'s RNAce kits, CLONTECH's NucleoSpin® RNA II and NucleoTrap mRNA kits, Invitrogen Corp.'s S.N.A.P. Total RNA Isolation Kit and QIAGEN's RNeasy kits.

The detection of an infectious disease-derived nucleic acid in the extracted nucleic acid sample may occur by any genetic analysis technique available that is suitable for the detection of infectious disease specific nucleic acid sequences in nucleic acids that are specific for the infectious disease. Usually, such sequences can be easily detected by selective nucleic acid hybridization, involving the formation of a duplex nucleic acid structure formed by selective hybridization with each other of two single-stranded nucleic acid sequences. Selective hybridization includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e. g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i. e., complementary) with each other.

Alternatively, detection of an infectious disease-derived nucleic acid may occur through sequencing technologies such as DNA and RNA sequencing.

When detecting infectious disease specific nucleic acid sequences in the form of RNA, it is preferred that the RNA is transcribed into cDNA prior to the detection of the infectious disease specific nucleic acid sequences therein.

RNA can be reverse transcribed into cDNA using RNA-dependent DNA polymerases such as, for example, reverse transcriptases from viruses, retrotransposons, bacteria, etc. These can have RNase H activity, or reverse transcriptases can be used that are so mutated that the RNase H activity of the reverse transcriptase was restricted or is not present (e.g. MMLV-RT RNase H$^-$). RNA-dependent DNA synthesis (reverse transcription) can also be carried by enzymes that show altered nucleic acid dependency through mutation or modified reaction conditions and thus obtain the function of the RNA-dependent DNA polymerase. Commercial kits are available to reverse transcribe RNA into cDNA.

Once the RNA is reverse transcribed into cDNA, the DNA sequence can be analysed for the presence of infectious disease-specific mutations using for instance selective nucleic acid hybridization as described above. Such techniques are well known in the art and may comprise selective amplification using amplification primers that are specific for the mutation to be detected. Alternatively, general primers can be used to amplify the DNA comprising the suspected mutation and the mutation can than be detected in the amplicon by selective nucleic acid hybridization using probes that are specific for the mutation.

Methods of the invention can in principle be performed by using any nucleic acid amplification method, such as the Polymerase Chain Reaction (PCR; Mullis 1987, U.S. Pat. No. 4,683,195, 4,683,202, en 4,800,159) or by using amplification reactions such as Ligase Chain Reaction (LCR; Barany 1991, Proc. Natl. Acad. Sci. USA 88:189-193; EP Appl. No., 320,308), Self-Sustained Sequence Replication (3SR; Guatelli et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), Strand Displacement Amplification (SDA; U.S. Pat. Nos. 5,270,184, en 5,455,166), Transcriptional Amplification System (TAS;

Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al., 1988, Bio/Technology 6:1197), Rolling Circle Amplification (RCA; U.S. Pat. No. 5,871,921), Nucleic Acid Sequence Based Amplification (NASBA), Cleavase Fragment Length Polymorphism (U.S. Pat. No. 5,719,028), Isothermal and Chimeric Primer-initiated Amplification of Nucleic Acid (ICAN), Ramification-extension Amplification Method (RAM; U.S. Pat. Nos. 5,719,028 and 5,942,391) or other suitable methods for amplification of DNA.

In order to amplify DNA with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency (e.g. a PCR amplification using an annealing temperature of 38°C, or the presence of 3.5 mM $MgCl_2$). The person skilled in the art will be able to select conditions of suitable stringency.

The primers herein are selected to be "substantially" complementary (i.e. at least 65%, more preferably at least 80% perfectly complementary) to their target regions present on the different strands of each specific sequence to be amplified. It is possible to use primer sequences containing e.g. inositol residues or ambiguous bases or even primers that contain one or more mismatches when compared to the target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target DNA oligonucleotide sequences, are considered suitable for use in a method of the present invention. Sequence mismatches are also not critical when using low stringency hybridization conditions.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The detection fragments may be directly stained or labelled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA fragments may be detected by incorporation of labelled dNTP bases into the synthesized DNA fragments. Detection labels

which may be associated with nucleotide bases include e.g. fluorescein, cyanine dye or BrdUrd.

When using a probe-based detection system, a suitable detection procedure for use in the present invention may for example comprise an enzyme immunoassay (EIA) format (Jacobs et al., 1997, J. Clin. Microbiol. 35, 791795). For performing a detection by manner of the EIA procedure, either the forward or the reverse primer used in the amplification reaction may comprise a capturing group, such as a biotin group for immobilization of target DNA PCR amplicons on e.g. a streptavidin coated microtiter plate wells for subsequent EIA detection of target DNA amplicons (see below). The skilled person will understand that other groups for immobilization of target DNA PCR amplicons in an EIA format may be employed.

Probes useful for the detection of the target DNA as disclosed herein preferably bind only to at least a part of the DNA sequence region as amplified by the DNA amplification procedure. Those of skill in the art can prepare suitable probes for detection based on the nucleotide sequence of the target DNA without undue experimentation as set out herein. Also the complementary sequences of the target DNA may suitably be used as detection probes in a method of the invention, provided that such a complementary strand is amplified in the amplification reaction employed.

Suitable detection procedures for use herein may for example comprise immobilization of the amplicons and probing the DNA sequences thereof by e.g. southern blotting. Other formats may comprise an EIA format as described above. To facilitate the detection of binding, the specific amplicon detection probes may comprise a label moiety such as a fluorophore, a chromophore, an enzyme or a radio-label, so as to facilitate monitoring of binding of the probes to the reaction product of the amplification reaction. Such labels are well-known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), ß-galactosidase, horseradish peroxidase,

streptavidin, biotin, digoxigenin, $^{35}S$ or $^{125}I$. Other examples will be apparent to those skilled in the art.

Detection may also be performed by a so called reverse line blot (RLB) assay, such as for instance described by Van den Brule et al. (2002, J. Clin. Microbiol. 40, 779787). For this purpose RLB probes are preferably synthesized with a 5'amino group for subsequent immobilization on e.g. carboxylcoated nylon membranes. The advantage of an RLB format is the ease of the system and its speed, thus allowing for high throughput sample processing.

The use of nucleic acid probes for the detection of DNA fragments is well known in the art. Mostly these procedures comprise the hybridization of the target DNA with the probe followed by post-hybridization washings. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the $T_m$ (the thermal melting point, i.e. the temperature under defined ionic strength and pH at which 50% of a complementary target sequence hybridizes to a perfectly matched probe) can be approximated from the equation of Meinkoth and Wahl (Anal. Biochem., 138: 267-284 (1984)): $T_m$ = 81.5 °C + 16.6 (log M) + 0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The $T_m$ is reduced by about 1 °C for each 1 % of mismatching; thus, the hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with > 90% identity are sought, the $T_m$ can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the $T_m$ for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than $T_m$; moderately stringent conditions can utilize a hybridization and/or wash at 6,7,8,9, or 10 °C

lower than the $T_m$; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than $T_m$. Using the equation, hybridization and wash compositions, and desired $T_m$, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a $T_m$ of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier. New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995).

Detection probes are preferably selected to be "substantially" complementary to one of the strands of the double stranded DNA amplicons generated by an amplification reaction in a method of the invention. Preferably the probes are substantially complementary to the immobilizable (e.g. biotin labelled) antisense strands of the amplicons generated from the target DNA.

It is allowable for detection probes to contain one or more mismatches to their target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target DNA oligonucleotide sequences are considered suitable for use in a method of the present invention.

The step of analysing the thrombocyte-extracted nucleic acid fraction for the presence of an infectious disease-derived nucleic acid can thus be performed by standard nucleic acid analysis techniques. The step of determining whether there is an alteration in the level of said nucleic acid in said nucleic acid fraction with respect to an unaffected blood sample will involve (semi-) quantitative measurements of the amount of the nucleic acid marker or the amount of mRNA in the thrombocytes. A much preferred

protocol for the detection of infectious disease-specific mutations in the nucleic acids isolated from thrombocytes is therefore quantitative reverse-transcription PCR (qRT-PCR) (Freeman et al., BioTechniques 26:112-125 (1999)).

An "unaffected blood sample" as referred to above refers to the level of the infectious disease nucleic acid in thrombocytes of a healthy control subject or from the same subject prior to the onset of the infectious disease. Since anucleated blood cell characteristics and quantities of anucleated blood cell components depend on, amongst other things, species and age, it is preferable that the non-infected control anucleated blood cells come from a subject of the same species, age and from the same sub-population (e.g. smoker/nonsmoker). Alternatively, control data may be taken from databases and literature. It will be appreciated that the control sample may also be taken from the diseased subject at a particular time-point, in order to analyze the progression of the disease.

Infectious disease-specific markers may include a wide variety of markers known to be associated with infectious disease.

The invention further provides a kit for diagnosing infectious disease in a subject, the kit comprising a packaging material which comprises at least one agent for specifically determining a level and/or activity of at least one nucleic acid mutant in an anucleated blood cell sample of the subject. As used herein, the term "diagnosing" refers to determining the presence of an infectious disease, classifying an infectious disease, determining a severity of infectious disease (grade or stage), monitoring infectious disease progression, forecasting an outcome of the infectious disease and/or prospects of recovery.

It will be appreciated that the tools necessary for detecting the infectious disease-derived nucleic acid may be provided as a kit, such as an FDA-approved kit, which may contain one or more unit dosage form containing the active ingredient for detection of the infectious disease-derived nucleic acid in thrombocytes by a method of the present invention.

Alternatively, the kit may comprise means for collecting the sample and specific amplification and/or detection primers packaged separately.

The kit may be accompanied by instructions for performing a method of the present invention.

For example, the kit may be comprised in a device such as a dipstick or a cartridge, (optionally comprised in a housing) to which a blood sample or an isolated and/or amplified thrombocyte nucleic acid sample may be applied and which detects a infectious disease-derived nucleic acid in said sample. The device may comprise any agent capable of specifically detecting the infectious disease-derived nucleic acid. For example, the device may comprise one or a combination of immobilized mutation-specific hybridization probes that bind the infectious disease-derived nucleic acid and an indicator for detecting binding. In an embodiment of this invention, supports are provided in the device to which the hybridization probes are removably or fixedly attached.

According to one embodiment, the device may be a lateral flow device comprising inlet means for flowing a blood sample or an isolated and/or amplified thrombocyte nucleic acid sample into contact with the agents capable of detecting the infectious disease-derived nucleic acid. The test device can also include a flow control means for assuring that the test is properly operating. Such flow control means can include control nucleic acids bound to a support which capture detection probes added to the sample as a means of confirming proper flow of sample fluid through the test device. Alternatively, the flow control means can include capture probes in the control region which capture control nucleic acids naturally present in said sample or added thereto as control, again indicating that proper flow is taking place within the device.

In another aspect, the present invention provides the use of device of the present invention for diagnosing infectious disease in a subject using any one of the methods described herein above. Very suitable devices for use in diagnosing infectious disease in a subject using any one of the methods described herein above include Platelet RNA chips such as for instance

described in Nagalla & Bray (2010) Blood 115 (1): 2-3 and Gnatenko et al. Blood 115 (1): 7-14.

EXAMPLES

Example 1. Detection of Epstein-Barr Virus Small RNA in thrombocytes.

The pathogenic lymphocryptovirus Epstein–Barr virus (EBV), also called human herpesvirus 4 (HHV-4), is one of the most common viruses in humans. It is best known as the cause of infectious mononucleosis. It is also associated with particular forms of cancer, particularly Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, and central nervous system lymphomas associated with HIV. Finally, there is evidence that infection with the virus is associated with a higher risk of certain autoimmune diseases, especially dermatomyositis, systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, and multiple sclerosis.

Most people become infected with EBV and gain adaptive immunity. After maternal antibody protection, many children become infected with EBV, and these infections usually cause no or only mild symptoms.

The virus has a lytic cycle, involving the production of infectious virions, and a latent cycle, that does not result in production of virions, but wherein a limited, distinct set of viral proteins are produced. These include Epstein–Barr nuclear antigens (EBNA), latent membrane proteins (LMP), and the Epstein–Barr encoded RNAs (EBERs). In addition, EBV codes for at least twenty microRNAs which are expressed in latently infected cells

In the present example it was determined whether Epstein-Barr Virus EBERs could be detected in nucleic acids isolated from platelets of patients.

An RT-PCR detection assay was set up for detection Epstein-Barr Virus Small RNA (EBERl RNA), essentially as described by Tierney et al. 1994. J. Virol. 68(11):7374-7385.

Platelet RNA was isolated using a commercial RNA isolation kit (miRVANA RNA isolation kit, Applied Biosystems/Ambion, Austin, TX, USA). The isolated RNA was converted into cDNA using a commercially available system (Omniscript cDNA kit, Qiagen, Hilden, Germany). Subsequently, a SYBR Green PCR amplification process (Qiagen) using 3 µl of the cDNA was performed using the following primers in a final concentration of 200 nM.

Fw: 5'AGGACCTACGCTGCCCTAGA 3'
Rev: 5'AAAACATGCGGACCACC 3'

The PCR amplification program was run on an ABI RT-PCR machine (Applied Biosystems Inc. Foster City, USA): 1 cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 15 sec. The results are displayed in Figure 1.

It was concluded that Epstein-Barr Virus (EBV) EBERl RNA could successfully be detected in nucleic acids isolated from platelets of patients.

Example 2. EBV miRNA microarray analysis

EBV is known to express a large number of distinct microRNAs (miRNAs) in latently infected cells. These are arranged in clusters: many miRNAs are located in the introns of the viral BART gene while another set of miRNAs is located adjacent to BHRF1. The BART miRNAs are expressed at high levels in latently infected epithelial cells and at lower, albeit detectable, levels in B cells (Cai et al. 2006. PLoS Pathog. 2:e23). Hence, EBV miRNAs can be used to detect latent EBV infection.

In the present example it was determined whether Epstein-Barr Virus miRNAs could be detected in nucleic acids isolated from platelets of patients.

Platelet RNA was subjected to miRNA microarrays. In short, Platelet RNA was isolated using a commercial RNA isolation kit (miRVANA RNA isolation kit, Applied Biosystems/Ambion, Austin, TX, USA). The isolated RNA was labelled and hybridized with miRNA-specific probes using the Agilent miRNA microarray analysis according the manufacturer's instructions as described in great detail in the document "miRNA Microarray System with miRNA Complete Labeling and Hyb Kit Protocol" Version 2.3, December 2010, Agilent Technologies, Inc., Santa Clara, USA. The results of the analysis are displayed in Figure 2.

EBV-miR-BART12 RNA and EBV-miR-BART13 RNA were detected, as indicated by the bars in Fig. 2. Hence, it was concluded that Epstein-Barr Virus (EBV) miRNA could successfully be detected in nucleic acids isolated from platelets of patients.

## Claims

1. A method of analysing a blood sample of a subject for the presence of an infectious disease marker, said method comprising the steps of
a) extracting nucleic acid from anucleated blood cells in said blood sample to provide an anucleated blood cell-extracted nucleic acid fraction, and
b) analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of an infectious disease marker,
wherein said infectious disease marker is a nucleic acid of an infectious disease agent, or
wherein said infectious disease marker is an infectious disease-specific expression profile of genes of a nucleated cell of said subject.

2. The method of claim 1, wherein said anucleated blood cells are thrombocytes or erythrocytes, preferably thrombocytes.

3. The method of claim 1 or 2, wherein said infectious disease is selected from the group consisting of viral disease, bacterial infection, parasitic infection and fungal infection.

4. The method of claim 3 wherein said disease is viral disease or bacterial infection.

5. The method of any one of the preceding claims, wherein said infectious disease marker is a nucleic acid of an infectious disease agent.

6. The method of any one of the preceding claims, wherein said expression profile is based on ribonucleic acid (RNA), preferably mRNA in said anucleated blood cell.

7. The method of any one of the preceding claims, wherein said step b) of analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of an infectious disease marker comprises the selective amplification of

i) at least a part of said nucleic acid of an infectious disease agent by (reverse transcriptase) polymerase chain reaction amplification using at least one infectious disease agent-specific amplification primer or probe, or

ii) a plurality of mRNAs (or at least a part of the mRNAs) by reverse transcriptase polymerase chain reaction amplification to determine the expression level of the chromosomal genes encoding said mRNAs to thereby provide an expression profile for said genes and comparing said expression profile to a reference profile.

8. The method of any one of the preceding claims, wherein said method is part of a method of diagnosing said infectious disease in a subject, and wherein the presence of said infectious disease marker in said anucleated blood cell-extracted nucleic acid fraction is indicative of said subject suffering from said infectious disease.

9. A method for determining the stage of infectious disease or the efficacy of an infectious disease treatment in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of an infectious disease marker using the method according to any one of claims 1-8 at a first time point to thereby provide a first value for the level of said infectious disease marker in said subject,
- analysing a blood sample of said subject for the presence of an infectious disease marker using the method according to any one of claims 1-8 at a second time point to thereby provide a second value for the level of said infectious disease marker in said subject, wherein said subject has been

subjected to an infectious disease treatment between said first and second time point, and

- comparing said first and second value to determine the efficacy of said infectious disease treatment in said subject.

10. A method for determining the stage of an infectious disease in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of an infectious disease marker using the method according to any one of claims 1-8 to thereby provide a test value for the level of said infectious disease marker in said subject,
- providing a reference value for the level of said infectious disease marker wherein said reference value is correlated to a particular stage of infectious disease, and
- comparing said test and reference value to determine the stage of infectious disease in said subject.

11. A kit of parts adapted for performing the method recited in any one of claims 1-10, the kit comprising a packaging material which comprises at least one of:

- a container for holding anucleated blood cells separated from a blood sample of a subject;
- an agent for extracting nucleic acids from said anucleated blood cells;
- an agent for selectively amplifying from said nucleic acids extracted from said anucleated blood cells an infectious disease agent-specific nucleic acid sequence or an infectious disease-specific gene expression profile of chromosomal genes of said subject, and
- a printed or electronic instruction for performing the method recited in any one of claims 1-10,

the kit further comprising:

- a reference for said infectious disease marker, wherein said reference is indicative for the presence or absence of said infectious disease marker in said anucleated blood cells-extracted nucleic acid fraction.

12. The kit of claim 11, wherein said reference is a reference value for the level of nucleic acids comprising said infectious disease agent-specific nucleic acid sequence in anucleated blood cells in a healthy control subject or in a control subject suffering from said infectious disease, or
wherein said reference is a reference expression profile for said plurality of mRNAs in anucleated blood cells from a healthy control subject or from a control subject suffering from said infectious disease.

13. A kit of claim 11 or 12, wherein said agent is selected from a particle or fluorescent marker-labeled anti-anucleated blood cell antibody, or wherein said instruction is selected from an instruction for bead-based anucleated blood cells isolation, an instruction for FACS sorting of anucleated blood cells, an instruction for anucleated blood cell recovery by centrifugation, or negative selection of non-anucleated blood cell components.

14. A device for diagnosing infectious disease in a subject, the device comprising a support and at least one agent for specifically determining a level and/or activity of at least one nucleic acid in an anucleated blood cell sample of the subject attached to said support, and
a computer-readable medium having computer-executable instructions for performing the method recited in any one of claims 1-10.

15. The device of claim 14, wherein said at least one agent is an oligonucleotide probe or sequencing primer.

16. The device of claim 14 or 15, comprising a lateral flow device, a dipstick or a cartridge for performing a nucleic acid hybridization reaction between:

- an anucleated blood cells-extracted nucleic acid and at least one infectious disease agent-specific amplification primer or oligonucleotide probe, wherein said infectious disease agent-specific amplification primer or oligonucleotide probe hybridizes specifically to an infectious disease agent-specific nucleic acid sequence, or

- an anucleated blood cells-extracted nucleic acid and a plurality of gene-specific amplification primers or oligonucleotide probes for providing an disease-specific gene expression profile, wherein said genes are genes of a nucleated cell of the subject in which the infectious disease is to be diagnosed.

Figure 1

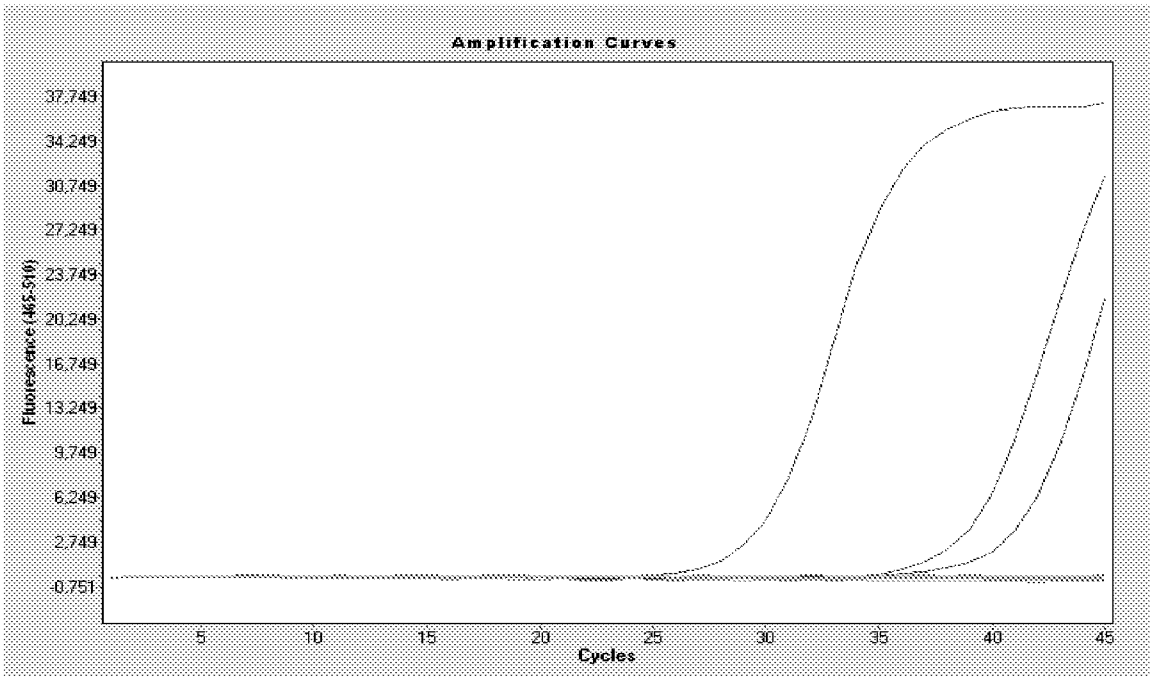
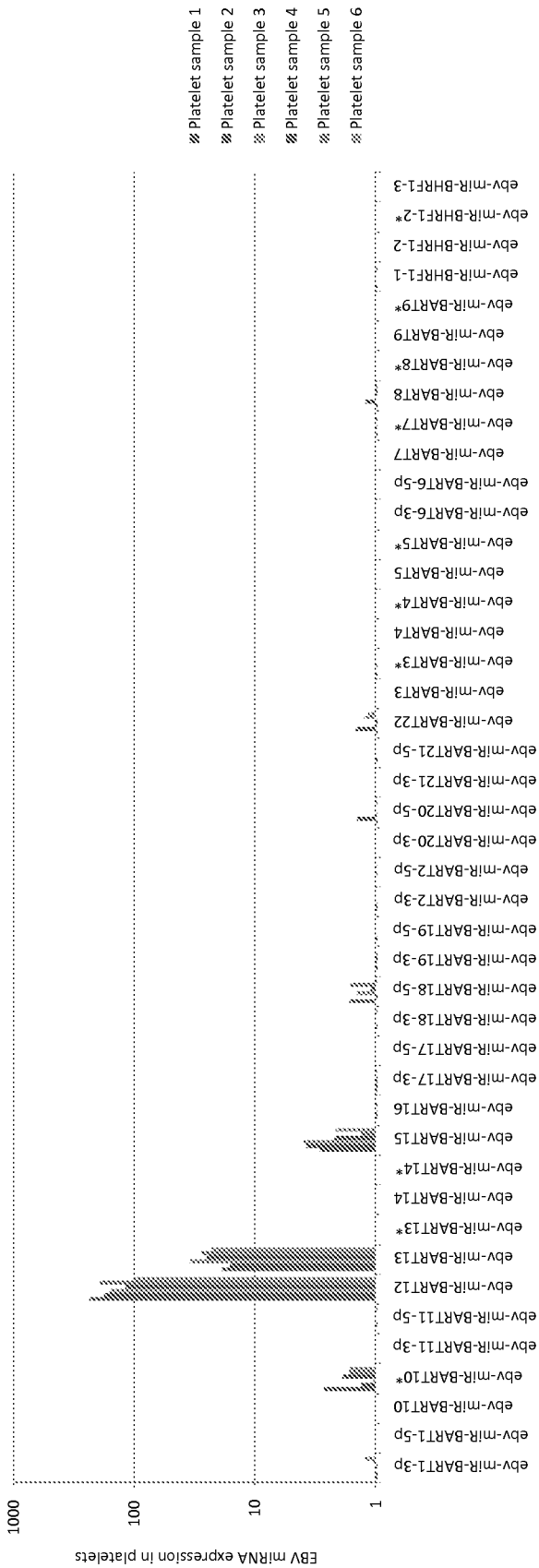

Amplification Curves
37,749
34,249
30,749
27,249
23,749
20,249
16,749
13,249
9,749
6,249
2,749
-0,751
Fluorescence (465-510)
5
10
15
20
25
30
35
40
45
Cycles

Figure 2


EBV miRNA expression in platelets
1000
100
10
1
Platelet sample 1
Platelet sample 2
Platelet sample 3
Platelet sample 4
Platelet sample 5
Platelet sample 6
ebv-miR-BART1-3p
ebv-miR-BART1-5p
ebv-miR-BART10
ebv-miR-BART10*
ebv-miR-BART11-3p
ebv-miR-BART11-5p
ebv-miR-BART12
ebv-miR-BART13
ebv-miR-BART13*
ebv-miR-BART14
ebv-miR-BART14*
ebv-miR-BART15
ebv-miR-BART16
ebv-miR-BART17-3p
ebv-miR-BART17-5p
ebv-miR-BART18-3p
ebv-miR-BART18-5p
ebv-miR-BART19-3p
ebv-miR-BART19-5p
ebv-miR-BART2-3p
ebv-miR-BART2-5p
ebv-miR-BART20-3p
ebv-miR-BART20-5p
ebv-miR-BART21-3p
ebv-miR-BART21-5p
ebv-miR-BART22
ebv-miR-BART3
ebv-miR-BART3*
ebv-miR-BART4
ebv-miR-BART4*
ebv-miR-BART5
ebv-miR-BART5*
ebv-miR-BART6-3p
ebv-miR-BART6-5p
ebv-miR-BART7
ebv-miR-BART7*
ebv-miR-BART8
ebv-miR-BART8*
ebv-miR-BART9
ebv-miR-BART9*
ebv-miR-BHRF1-1
ebv-miR-BHRF1-2
ebv-miR-BHRF1-2*
ebv-miR-BHRF1-3

# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050517

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 C12Q1/70
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SANSANEE NOISAKRAN ET AL: "Detection of dengue virus in platelets isolated from dengue patients.", SOUTHEAST ASIAN JOURNAL OF TROPICAL MEDICINE AND PUBLIC HEALTH, vol. 40, no. 2, 1 March 2009 (2009-03-01), pages 253-262, XP055009527, ISSN: 0125-1562 the whole document<br>----- | 1-5,7-16 |
| X | DE 199 37 234 A1 (UNIV LEIPZIG [DE]) 22 February 2001 (2001-02-22) claims 3-4, 6 page 2, paragraph 1<br>----- | 1-5,7-16 |
| | -/-- | |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 24 January 2012 | 06/02/2012 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Persson, Anna |

# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050517

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JINLIN ZHOU ET AL: "cDNA Cloning of a Rab5 Homologue in Babesia gibsoni", JOURNAL OF VETERINARY MEDICAL SCIENCE, vol. 65, no. 4, 1 January 2003 (2003-01-01), pages 519-521, XP055009586, ISSN: 0916-7250, DOI: 10.1292/jvms.65.519 the whole document | 1-3,5, 7-16 |
| X | US 2007/015172 A1 (ZHANG HONG [US] ET AL) 18 January 2007 (2007-01-18) | 11-13 |
| A | paragraphs [0009], [0010], [0045] | 1-4, 6-10, 14-16 |
| A | T. YOUSSEFIAN: "Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation", BLOOD, vol. 99, no. 11, 30 April 2002 (2002-04-30), pages 4021-4029, XP055009547, ISSN: 0006-4971, DOI: 10.1182/blood-2001-12-0191 the whole document | 1-5,7-16 |
| A | D. M. PEGTEL ET AL: "Functional delivery of viral miRNAs via exosomes", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 107, no. 14, 18 March 2010 (2010-03-18), pages 6328-6333, XP55017264, ISSN: 0027-8424, DOI: 10.1073/pnas.0914843107 the whole document | 1-5,7-16 |
| A | A. J. ANTCZAK ET AL: "Internalization of IgG-Coated Targets Results in Activation and Secretion of Soluble CD40 Ligand and RANTES by Human Platelets", CLINICAL AND VACCINE IMMUNOLOGY, vol. 18, no. 2, 22 December 2010 (2010-12-22), pages 210-216, XP055009550, ISSN: 1556-6811, DOI: 10.1128/CVI.00296-10 the whole document | 1-16 |

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# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050517

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | NALINI RAGHAVACHARI ET AL: "Amplified Expression Profiling of Platelet Transcriptome Reveals Changes in Arginine Metabolic Pathways in Patients With Sickle Cell Disease", CIRCULATION, LIPPINCOTT WILLIAMS & WILKINS, US, vol. 115, 27 March 2007 (2007-03-27), pages 1551-1562, XP002663671, ISSN: 0009-7322, DOI: 10.1161/CIRCULATIONAHA.106.658641 [retrieved on 2007-03-12] the whole document | 1-16 |
| A | L.-M. LI ET AL: "Serum microRNA Profiles Serve as Novel Biomarkers for HBV Infection and Diagnosis of HBV-Positive Hepatocarcinoma", CANCER RESEARCH, vol. 70, no. 23, 1 December 2010 (2010-12-01), pages 9798-9807, XP55017222, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-10-1001 the whole document | 1-4,6-16 |
| A | RAMILO O ET AL: "Gene expression patterns in blood leukocytes discriminate patients with acute infections", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 109, no. 5, 1 March 2007 (2007-03-01) , pages 2066-2077, XP002580520, ISSN: 0006-4971, DOI: 10.1182/BLOOD-2006-02-002477 [retrieved on 2006-11-14] the whole document | 1-4,6-16 |
| T | R. J. A. NILSSON ET AL: "Blood platelets contain tumor-derived RNA biomarkers", BLOOD, vol. 118, no. 13, 29 September 2011 (2011-09-29), pages 3680-3683, XP55017258, ISSN: 0006-4971, DOI: 10.1182/blood-2011-03-344408 the whole document | |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2011/050517

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/** 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5(completely); 1-4, 7-16(partially)

   A method of analysing an anucleated blood cell-extracted nucleic acid for the presence of an infectious disease marker, wherein said marker is a nucleic acid of an infectious disease agent. Related methods of determining the stage of infection or efficacy of treatment. Related kits and devices.

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2. claims: 6(completely); 1-4, 7-16(partially)

   As for Invention 1, but wherein said marker is an infectious disease-specific expression profile of genes of a nucleated cell of the subject.

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2011/050517

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| DE 19937234 A1 | 22-02-2001 | NONE | |
| US 2007015172 A1 | 18-01-2007 | NONE | |