(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 July 2017 (13.07.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/118718 A1

(51) International Patent Classification:
C12N 15/10 (2006.01) *C12Q 1/68* (2006.01)

(21) International Application Number:
PCT/EP2017/050250

(22) International Filing Date:
6 January 2017 (06.01.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16150594.6 8 January 2016 (08.01.2016) EP
62/276,650 8 January 2016 (08.01.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

WO 2017/118718 A1

(54) **Title**: MODULATION OF ACCESSIBILITY OF HOST NUCLEIC ACIDS TO NUCLEIC ACID DIGESTING ENZYMES IN ACELLULAR BIOLOGICAL FLUIDS

(57) **Abstract**: The present invention relates to a method for enriching an infectious agent in an acellular fraction of a biological fluid using detergents and nucleic acids-digesting enzymes. The present invention also relates to a method for modulating the accessibility of host nucleic acids (HNA) in an acellular fraction of a biological fluid using detergents and nucleic acids-digesting enzymes, and to a method for detecting infectious agents in an acellular fraction from a biological fluid.

MODULATION OF ACCESSIBILITY OF HOST NUCLEIC ACIDS TO NUCLEIC ACID DIGESTING ENZYMES IN ACELLULAR BIOLOGICAL FLUIDS

FIELD OF INVENTION

The present invention relates to a method for modulating the accessibility of host nucleic acids (HNA) in an acellular fraction of a biological fluid. The present invention further relates to a method for detecting infectious agents in an acellular fraction of a biological fluid.

BACKGROUND OF INVENTION

The detection of pathogenic infectious agents in biological fluids should be performed in the shortest possible time, in particular in the case of septicemia, for which the mortality remains high in spite of the broad range of antibiotics which are available to doctors.

The level of infectious agents in a patient's body fluid is generally very low compared to host components from the subject. Typical biological samples like plasma, serum, cerebrospinal fluid devoid of living or dead cells discarded by centrifugation still contains high amounts of cell free host nucleic acids (HNA). In a particular case, circulating free cells (not associated with other cells), such as host cells, or fetal cells in a case of pregnancy, can release HNA.

Methods to decrease the amount of host nucleic acids are described in WO2010/062354 and WO2014/114896 and consist in treating biological fluids containing cells with detergent for disrupting the cellular membranes and then treating the resulting sample with nucleases (enzymes having DNase and/or RNase activity). However, these approaches have been of partial efficacy as it remains HNA that is resistant to nucleases in the treated sample.

The Applicant surprisingly demonstrates here that incubation of an acellular fraction of a biological fluid with detergent and nucleases improves the detection of infectious agents

in a sample. As a result, the Applicant provides a new method for strongly decreasing the concentration of HNA in an acellular fraction of a biological sample, which results in a better detection of infectious agents in said sample.

SUMMARY

One object of the invention is an *in vitro* method for enriching an infectious agent in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one nucleic acids-digesting enzyme.

In one embodiment, said *in vitro* method further comprises the detection of said infectious agent.

In another embodiment, the detection of said infectious agent is carried out by Next Generation Sequencing.

Another object of the invention is an *in vitro* method for modulating the accessibility of host nucleic acids to nucleic acids-digesting enzymes in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one enzyme digesting nucleic acids.

In one embodiment, the ratio between infectious agents and host nucleic acids is increased.

In another embodiment, the infectious agent is selected from the group comprising bacteria, fungi, viruses, protozoans, and parasites.

In another embodiment, the at least one detergent is selected from the group comprising: non-ionic detergent and/or zwitterionic detergent and/or anionic detergent, preferably the at least one detergent is a non-ionic detergent.

In another embodiment, the non-ionic detergent is selected from the group comprising: saponin, Triton, NP-40, Tween or a derivative detergent thereof, preferably the non-ionic detergent is saponin or a derivative detergent thereof.

In another embodiment, the at least one detergent is at a concentration from about 0.01% to about 10% w/v.

In another embodiment, the at least one nucleic acids-digesting enzyme is a nuclease having DNase and/or RNase activity.

In another embodiment, the at least one nucleic acids-digesting enzyme is at a concentration from about 0.1 units/µg to about 10 units/µg of protein.

In another embodiment, the presence of residual cells within the fraction does not affect the accessibility or the enrichment of the infectious agent in the fraction.

DEFINITIONS

In the present invention, the following terms have the following meanings:

- "Acellular fraction" refers to a biological fluid depleted in about 90%, 95%, preferably 99% of its cellular content. The acellular fraction of a biological fluid may be obtained by any well-known technic in the art. For example, in one embodiment where the biological fluid is blood, said acellular fraction may be obtained from a centrifugation step from about 100 to about 500 g for about 5 minutes to about 20 minutes, preferably at 300 g for 10 minutes.
- "Host nucleic acids" or "HNA" refer to nucleic acids issued from a subject.
- "Modulating" refers to an improvement or increase in the accessibility of HNA.
- "Subject" refers to any species such as for example domestic animals (*i.e.*, farm fish, pets, ruminants, pigs, horses), wide-life animals and human. In one embodiment, a subject may be a "patient", *i.e.*, a mammal or more preferably a human, who/which is awaiting the receipt of, or is receiving medical care or was/is/will be the object of a medical procedure, or is monitored for the development of a disease.

- "Internal control" refers to one or more materials used in an assay as a point of reference and/or comparison in order to make judgements as to the presence, absence or level of one or more factors being analyzed. Some internal control may comprise negative or positive control samples. Negative controls are samples known to lack one or more factors being analyzed. Positive controls are samples known to comprise one or more factors being analyzed. In one embodiment, the internal control can be an internal control nucleic acid sequence that is added in the sample at a given time point or at given time points, at a known concentration. In one embodiment, the internal control can be a virus such as a bacteriophage that is added in the sample at a given time point or at given time points, at a known concentration.
- "About" preceding a figure means plus or less 10% of the value of said figure.

DETAILED DESCRIPTION

One object of the invention is an *in vitro* method for enriching an infectious agent in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one nucleic acids-digesting enzyme.

Another object of the invention is a method for modulating the accessibility of host nucleic acids (HNA) to nucleic acids-digesting enzymes in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one nucleic acids-digesting enzyme.

Another object of the invention is an *in vitro* method for increasing the ratio of the nucleic acids of infectious agents to the total nucleic acids in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one nucleic acids-digesting enzyme.

In one embodiment of the invention, the structure or the entirety of the infectious agent is not affected by the methods of the invention.

The acellular fraction of a biological fluid in the meaning of the present invention refers to a fraction of a biological fluid comprising less than about 10%, 5%, 1% of the cellular material present originally in the biological fluid.

In one embodiment, said acellular fraction can be obtained from a biological fluid having already undergone steps to discard cells from a biological fluid.

In one embodiment, said acellular fraction can be obtained from a biological fluid having already undergone steps to lyse cells from a biological fluid.

In another embodiment, said acellular fraction comprises residual cells that do not affect the enrichment or the accessibility of the infectious agent.

According to the invention, examples of biological fluids include but are not limited to, blood, amniotic fluid, aqueous humor, bile, bladder lavage, breast exudate, bronchio-alveolar lavage, chyle, chyme, cytosol, feces (in semi-fluid or fluid form), interstitial fluid, lymph, menses, mucus, pleural fluid, pus, saliva, sebum, semen, serum, sputum, sweat, synovial fluid, tears, urine and vitreous humor.

In one embodiment, the biological fluid is blood. In another embodiment, the acellular fraction of blood is plasma.

As used herein, the term “infectious agent” refers to microorganisms that are generally unicellular, which can be multiplied and comprises: bacteria, fungi (yeasts or molds), viruses, and protozoans. The term “infectious agent” also refers to microorganisms that are pluricellular, which can be multiplied and comprises: worms and parasites.

Non-limiting examples of bacteria include Gram-positive or Gram-negative bacteria, mycobacteria or mollicutes.

Non-limiting examples of genus of Gram-negative bacteria of this invention include bacteria of the following genera: *Acetobacter*, *Morganella*, *Pseudomonas*, *Escherichia*, *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, *Serratia*, *Proteus*, *Campylobacter*, *Haemophilus*, *Vibrio*, *Yersinia*, *Stenotrophomonas*, *Brevundimonas*, *Ralstonia*, *Achromobacter*, *Fusobacterium*, *Prevotella*, *Branhamella*, *Neisseria*, *Burkholderia*,

Citrobacter, *Hafnia*, *Edwardsiella*, *Aeromonas*, *Moraxella*, *Brucella*, *Pasteurella*, *Providencia*, and *Legionella*.

Non-limiting examples of genus of Gram-positive bacteria of this invention include bacteria of the following genera: *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Listeria*, *Peptostreptococcus*, *Propionibacterium*, *Clostridium*, *Bacteroides*, *Gardnerella*, *Kocuria*, *Lactococcus*, *Leuconostoc*, *Micrococcus* and *Corynebacteria*.

Non-limiting examples of mycobacteria include but are not limited to, *Mycobacterium*.

Non-limiting examples of mollicutes include but are not limited to, *Mycoplasma* and *Ureaplasma*.

List of bacteria comprised within the list of infectious agents can be found in the LPSN website (list of prokaryotic names with standing nomenclatures: http://www.bacterio.net/index.html).

Non-limiting examples of viruses include but are not limited to members of the following families: *Herpesviridae* (e.g., varicella zoster virus, Epstein-Barr virus, herpes simplex virus), *Adenoviridae*, *Poxviridae*, *Astroviridae*, *Bunyaviridae*, *Coronaviridae*, *Arteriviridae*, *Reoviridae* (e.g., rotavirus), *Parvoviridae* (e.g., parvovirus B19), *Picornaviridae* (rhinovirus, coxsackievirus, enterovirus, hepatitis A virus), *Bornaviridae*, *Filoviridae* (e.g., Ebola virus, Marburg virus), *Hepadnaviridae* (e.g., hepatitis B), *Hepeviridae* (hepatitis E virus), *Retroviridae* (e.g., HIV, human T-lymphotropic virus), *Orthomyxoviridae* (e.g., influenza virus A, B, C), *Togaviridae* (e.g., rubella virus), *Paramyxoviridae* (e.g., respiratory syncytial virus, measles virus, mumps virus), *Rhabdoviridae* (e.g., rabies virus, bovine ephemeral fever virus, infectious hematopoietic necrosis virus, spring viraemia of carp virus, vesicular stomatitis indiana virus), *Flavivirus* (e.g., West Nile virus, dengue fever virus), *Papillomaviridae*, bacteriophage (e.g., *Myoviridae*, *Podoviridae*, *Siphoviridae*, *Lipothrixviridae*, *Rudiviridae*) *Poxviridae* (e.g., variola virus, cowpox virus).

Lists of viruses that can be considered as an infectious agent in the meaning of the present application can be found on the International Committee on the Taxonomy of Viruses (ICTV - http://www.ictvonline.org/virustaxonomy.asp).

Non-limiting examples of yeasts or molds include without limitation, *Candida*, *Cryptococcus*, *Nocardia*, *Penicillium*, *Alternaria*, *Rhodotorula*, *Aspergillus*, *Fusarium*, *Saccharomyces*, *Trichosporon*, *Pneumocystis*.

Non-limiting examples of protozoans include without limitation, *Trypanosoma* sp, *Babesia* sp, *Leishmania* sp, *Plasmodium* sp, *Wucheria* sp, *Toxoplasma* sp, *Cryptosporidium* sp.

Non-limiting examples of protozoans include without limitation flagellates (e.g., *Giardia, Trochomonas, Chilomastix, Enteromonas, Retortamoinas, Dientamoeba, Leishmania, Trypanosoma*), amoeboids (e.g., *Entamoeba*, *Acanthamoeba, Massisteria, Naegleria, Arcella, Amoeba, Chaos, Peloxyma, Syringammina*), sporozoans (e.g., *Plasmodium, Babesia, Cryptosporidium, Cyclospora, Isospora, Toxoplasma*), and ciliates (e.g., *Balantidium, Didinium, Colpoda, Stentor, Coleps, Paramecium, Vorticella, Tetrahymena, Cariocothrix*).

In one embodiment, the helminths are excluded from the methods of the invention.

In one embodiment, prion agents are excluded from the methods of the invention.

In one embodiment, the at least one detergent used in the methods of the invention comprises: non-ionic detergent and/or zwitterionic detergent and/or anionic detergent.

Said detergent acts without damaging the structure or the entirety of the infectious agent in order to preserve the nucleic acids of the infectious agent. The term “structure of the infectious agent” refers to complete viral particles, bacteria extracellular or intracellular bodies and the like.

In one embodiment, the detergent can be a non-ionic detergent, including without limitation, saponin, digitonin, Triton® X-100, Triton® X-100-R, Triton® X-114, NP-40, Tween-20, Tween-40, Tween-80, Brij® 98, Brij® 58, Brij® 35, Genapol® C-100,

Genapol® X-100, Igepal®, Brij® 96/97, octyl β-D-glucopyranoside, and nonaethylene glycol monododecyl ether ($C_{12}E_9$) or a derivative thereof. In a preferred embodiment, the non-ionic detergent is saponin, Triton, NP-40, Tween or a derivative thereof.

The term "saponin" as used herein is a compound, usually a secondary metabolite, found in natural sources, in particular in various plants but also in marine species. Specifically, saponins are amphipathic glycosides grouped phenomenologically by the soap-like foaming they produce when shaken in aqueous solutions, and structurally by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. The aglycone (glycoside-free portion) of a saponin is termed sapogenin. The number of saccharide-chains attached to the sapogenin/aglycone core can vary, as can the length of each chain. A typical chain length is from 1 to 11, with the numbers 2 to 5 being the most frequent, and with both linear and branched saccharide-chains being represented.

Monosaccharides such as D-glucose and D-galactose are among the most common components of the attached chains. The lipophilic aglycone can be any one of a wide variety of polycyclic organic structures originating from the serial addition of ten-carbon (C10) terpene units to compose a C30 triterpene skeleton, often with subsequent alteration to produce a C27 steroidal skeleton.

Derivative of saponin can include without limitation, triterpenoid saponin, saponin from *Saponaria (Quillaja saponaria)*, from the family of *Caryophyllaceae*, from the family of *Sapindaceae*, from the families of *Aceraceae* (maples) and *Hippocastanaceae*, gypenosides saponin from *Gynostemma pentaphyllum* (*Gynostemma*, *Cucurbitaceae*), ginsenosides saponin (in ginseng or red ginseng (*Panax*, *Araliaceae*)), steroidal saponin also termed saraponin such as the diosgenin, tigogenin, sarsapogenin, cholestanol, furostanol and spirostanol saponins. Known furostanol steroid saponins are the saponins contained in fresh garlic, such as proto-isoeruboside-B and isoeruboside-B, while aged garlic extract contains also spirostanol steroid saponins. Other examples of spirostanol steroid saponins are the saponin from the underground parts of *Ruscus aculeatus*, from the rhizomes of *Tacca chantrieri*, from *Solanum hispidum*, from the tubers of *Dioscorea polygonoides*, from the harvested *Tribulus terrestris*, from *Lilium candidum*.

Steroid saponins are also the saponins contained in the root of the *Saponaria officinalis*, which were used as a soap before the advent of commercially manufactured soap, the saponins of *Yucca schidigera*, which grows in the arid Mexican desert country of Baja California, used by native American to make soap, the saponins contained in the soap lily, *Chlorogalum pomeridianum*, which were used as soap by native Americans, the saponins from the soapberry tree.

In one embodiment, the at least one detergent used in the methods of the invention is saponin from saponaria (*Quillaja saponaria*, also known as Quillaja bark, soap bark tree or soapbark).

In one embodiment, the at least one detergent used in the methods of the invention is a saponin with a sapogenin content ranging from about 5% to about 50%, preferably from about 10% to about 40%, more preferably from about 20% to about 35%.

In one embodiment, the at least one detergent used in the methods of the invention is a saponin with a sapogenin content of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50%.

In one embodiment, the at least one detergent used in the methods of the invention is saponin from saponaria (*Quillaja saponaria*) with a sapogenin content of about 20-35%.

In another embodiment, the detergent can be a zwitterionic detergent that include without limitation, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), amidosulfobetaine-14 (ASB-14), amidosulfobetaine-16 (ASB-16), sulfobetaine 3-10 (SB3-10), sulfobetaine 3-12 (SB3-12), and sulfobetaine 3-14 (SB3-14).

In another embodiment, the detergent can be an anionic detergent that include without limitation, sodium dodecyl sulfate, N-(alkyl C_{10}-C_{16})-N,N-dimethylglycine betaine (EMPIGEN BB), lithium lauryl sulfate (LLS), sodium deoxycholate, sodium lauryl sulfate, cholesteryl hydrogen succinate, sodium deoxycholate, and sodium taurocholate.

In one embodiment, the final concentration of the at least one detergent used in the methods of the invention ranges from about 0.01% w/v to about 10% w/v, from about 0.1% w/v to about 5% w/v, from about 0.1% w/v to about 1.5% w/v, from about 0.1% w/v to about 1% w/v, from about 0.1% w/v to about 0.5% w/v.

In one embodiment, the final concentration of the at least one detergent used in the methods of the invention ranges from about 0.1% w/v to about 4% w/v, from about 1% w/v to about 4% w/v.

In one embodiment, the detergent is preferably a non-ionic detergent, preferably saponin or a derivative thereof. In another embodiment, the at least one detergent used in the methods of the invention does not affect the structure of the infectious agent.

In another embodiment, the at least one nucleic acids-digesting enzyme used in the methods of the invention refers to enzymes that exhibit DNase and/or RNase activity.

Nucleic acids-digesting enzymes include, without limitation, nucleases such as for example deoxyribonuclease, ribonuclease, exonuclease, exodeoxyribonuclease, exoribonuclease, endonuclease, endodeoxyribonuclease, and endoribonuclease.

Examples of nucleases include, but are not limited to, baseline-zero™ DNase, benzonase®, DNase I, DNase II, endonuclease III, endonuclease IV, endonuclease V, endonuclease VIII, exonuclease I, exonuclease III, exonuclease V, exonuclease VII, exonuclease VIII, exonuclease T, lambda exonuclease, micrococcal nuclease, mung bean nuclease, nuclease BAL-31, nuclease P1, nuclease S1, T4 endonuclease V, T5 exonuclease, T7 endonuclease I, and T7 exonuclease.

In one embodiment, the at least one nucleic acids-digesting enzyme used in the methods of the invention is selected from the group comprising, but not limited to, baseline-zero™ DNase and benzonase®. In one embodiment, the nucleic acids-digesting enzymes used in the methods of the invention comprise or consist of baseline-zero™ DNase and benzonase®.

In one embodiment, the concentration of the at least one nucleic acids-digesting enzyme ranges from about 0.1 units/µg to about 10 units/µg, preferably from about 0.5 units/µg to about 5 units/µg, more preferably about 1 unit/µg of protein.

In one embodiment, the acellular fraction of a biological fluid is contacted with at least one detergent and at least one nucleic acids-digesting enzyme for a period of time ranging from about 5 minutes to about 5 hours, preferably from about 30 minutes to about 2 hours, more preferably for about 1 hour.

In another embodiment, the acellular fraction of a biological fluid is contacted with at least one detergent and at least one nucleic acids-digesting enzyme for a period of time of about 5, 10, 15, 20, 30, 40, 45, 50 minutes, 1, 2, 3, 4 hours.

In another embodiment, the temperature of incubation ranges from about 30°C to about 40°C, preferably 37°C. In another embodiment, the temperature of incubation is about 31, 32, 33, 34, 35, 36, 37, 38, 39°C.

In another embodiment, the acellular fraction of a biological fluid is contacted with at least one detergent for less than 30 minutes, 20 minutes, 10 minutes, then is contacted with the at least one nucleic acids-digesting enzyme for a period of time of about 5, 10, 15, 20, 30, 40, 45, 50 minutes, 1, 2, 3, 4 hours.

In one embodiment, the methods of the invention further comprise a step of inhibition of the nucleic acids-digesting enzyme activity.

Inhibiting nucleic acids-digesting enzymes is well known to the skilled artisan and can be performed using chelating agents or by heating the sample at a temperature ranging from about 60°C to about 80°C, more preferably from about 65°C to about 80°C. In one embodiment, inhibition for nucleic acids-digesting enzymes can be performed by heating the sample at about 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, 80°C.

Inhibitors of nucleic acids-digesting enzymes are well known in the state of the art and include without limitation, ethylenediaminetetraacetic acid (EDTA), ethylene glycol

tetraacetic acid (EGTA), dithiothreitol (DTT), β-mercaptoethanol, diethylpyrocarbonate (DEPC), and guanidine.

In one embodiment, the inhibition step is carried for at least about 5 minutes to about 20 minutes. In one embodiment, the inhibition step is carried out for at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 minutes.

In one embodiment, the concentration of inhibitors of nucleic acids-digesting enzymes, such as for example EDTA, ranges from about 1 to about 10 mM. In one embodiment, the concentration of inhibitors of nucleic acids-digesting enzymes, such as for example EDTA, is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mM.

Optionally, the methods of the invention further comprise an enrichment step for concentrating the infectious agent, such as for example centrifugation, ultra-centrifugation, salt precipitation or filtration steps. The skilled artisan will recognize which technic is the most efficient for such enrichment.

In one embodiment, the methods of the invention do not comprise a step of precipitation of the infectious agent using a precipitating agent such as polyethylene glycol (PEG), glycogen or nucleic acids.

In one embodiment, the methods of the invention may further comprise a step of preparation of homogenate of the infectious agent. Technics to prepare homogenate are well known in the state of the art and include without limitation, mechanical homogenization (e.g., homogenizers, sonication), chemical homogenization (e.g., protease digestion).

In one embodiment, infectious agents are lysed under highly denaturing conditions at room temperature (15-25°C). In one embodiment, infectious agents are lysed in the presence of proteinase K. In one embodiment, infectious agents are lysed in the presence of guanidinium chloride.

Treatment with chemicals and proteinase K is sufficient for complete lysis in the case of many $Gram^-$ bacteria, but the cell walls of $Gram^+$ bacteria and some $Gram^-$ bacteria must be disrupted by additional methods. For maximal lysis efficiency when working with

difficult-to-lyse bacteria, mechanical and/or enzymatic homogenization can be additionally or alternatively carried out.

In one embodiment, the methods of the invention may further comprise a step for the infectious agents' nucleic acid extraction and the detection of said infectious agent.

In one embodiment, the methods of the invention detect a quantity of infectious agent inferior to about 10000 genome copies/mL, preferably inferior to about 1000 genome copies/mL, most preferably inferior to about 100 genome copies/mL.

The extraction/purification of nucleic acids is carried out by technics well known to the skilled artisan. Such technics include without limitation, phenol-chloroform extraction, alkaline extraction, guanidinium thiocyanate-phenol-chloroform extraction, binding on anion exchange resin, silica matrices, glass particle, diatomaceous earth, magnetic particles made from different synthetic polymers, biopolymers, porous glass or based on inorganic magnetic.

The detection of the infectious agent may be carried out by technics for amplifying and sequencing the nucleic acids of the infectious agent, for determining the cellular, physiological, phenotypic activity of the infectious agent. Preferably, the detection method used is a method for amplifying and sequencing the nucleic acids.

Techniques to amplify and sequence nucleic acids are well known to the skilled artisan. Techniques to amplify nucleic acid can include specific amplification methods as well as random amplification methods. Specific amplification techniques include but are not limited to, methods requiring temperature cycling (such as PCR, ligase chain reaction, transcription based amplification) and/or isothermal amplification systems (such as self-sustaining sequence replication, replicase system, helicase system, strand displacement amplification, rolling circle-based amplification and NASBA). In one embodiment, amplification of infectious agents' nucleic acids may be performed by polymerase chain reaction and/or any variations thereof, including, without limitation, allele-specific PCR, asymmetric PCR, hot-start PCR, intersequence-specific PCR, methylation-specific PCR, miniprimer PCR, multiplex ligation-dependent probe amplification, multiplex-PCR, nested PCR1 quantitative PCR, reverse transcription PCR

and/or touchdown PCR. Amplification may be performed using primers and/or a collection of primers that may be selected from those capable of specific binding to nucleic acids of at least one infectious agent.

Random amplification techniques include without limitation, multiple displacement amplification (MDA), random PCR, random amplification of polymorphic DNA (RAPD) or multiple annealing and looping based amplification cycles (MALBAC).

In one embodiment, amplified infectious agents' nucleic acids may be detected by hybridizing a probe and/or a collection of probes capable of specific binding to amplified nucleic acids of at least one infectious agent.

In one embodiment, the detection of the nucleic acids may be carried out by random sequencing by high throughput sequencing (HTS) or next generation sequencing (NGS).

In one embodiment, internal controls may be used throughout the methods of the invention to assess the reliability of said methods.

In one embodiment, an internal control is added in the cellular fraction of a biological fluid. In one embodiment, an internal control is added in the acellular fraction of a biological fluid. In one embodiment, an internal control is added before, after or concomitantly with the at least one detergent. In one embodiment, an internal control is added before, after or concomitantly with the at least one nucleic acids-digesting enzyme. In one embodiment, an internal control is added before, after or during the step of inhibition of the nucleic acids-digesting enzyme activity. In one embodiment, an internal control is added before, after or during the enrichment step for concentrating the infectious agent. In one embodiment, an internal control is added before, after or during the step of preparation of homogenate of the infectious agent. In one embodiment, an internal control is added before, after or during the step of DNA or RNA extraction. In one embodiment, an internal control is added before, after or during the step of amplification and sequencing of the nucleic acids of the infectious agent.

In one embodiment, the internal control is a nucleic acid sequence. In one embodiment, the internal control is a DNA sequence. In one embodiment, the internal control is an RNA sequence.

DNA sequences suitable as an internal control in the methods of the present invention comprise, but are not limited to, the genomic DNA and/or fragments thereof, of bacteriophages, such as bacteriophages from the *Podoviridae*, *Myoviridae*, *Siphoviridae*, *Lipothrixviridae*, *Rudiviridae*, *Ampullaviridae*, *Bicaudaviridae*, *Clavaviridae*, *Corticoviridae*, *Fuselloviridae*, *Globuloviridae*, *Guttaviridae*, *Inoviridae*, *Microviridae*, *Plasmaviridae*, or *Tectiviridae* family.

DNA sequences suitable as an internal control in the methods of the present invention comprise, but are not limited to, the genomic DNA and/or fragments thereof, of bacteriophages, such as T1, T2, T3, T4, T5, T6, T7, M11, λ (Lambda), Φ (Phi), Φ29, P22, P37, μ (Mu), PBSX, P1Puna-like, P2, I3, Bcep 1, Bcep 43, Bcep 78, C2, L5, HK97, N15, Acidianus filamentous virus 1, *Sulfolobus islandicus* rod-shaped virus 1.

RNA sequences suitable as an internal control in the methods of the present invention comprise, but are not limited to, the genomic RNA and/or fragment thereof, of bacteriophages, such as bacteriophages from the *Cystoviridae* or *Leviviridae* family.

RNA sequences suitable as an internal control in the methods of the present invention comprise, but are not limited to, the genomic RNA and/or fragment thereof, of bacteriophages, such as MS2, Qβ.

In one embodiment, the internal control is a virus stock. In one embodiment, the internal control is a bacteriophage stock. In one embodiment, the internal control is a viral nanoparticle-encapsidated nucleic acid sequence.

Bacteriophages suitable as an internal control in the methods of the present invention comprise, but are not limited to, bacteriophages from the *Podoviridae*, *Myoviridae*, *Siphoviridae*, *Lipothrixviridae*, *Rudiviridae*, *Ampullaviridae*, *Bicaudaviridae*, *Clavaviridae*, *Corticoviridae*, *Fuselloviridae*, *Globuloviridae*, *Guttaviridae*, *Inoviridae*, *Microviridae*, *Plasmaviridae*, or *Tectiviridae* family.

Bacteriophages suitable as an internal control in the methods of the present invention comprise, but are not limited to, bacteriophage T3, T1, T2, T4.

In one embodiment, the internal control is a stock solution of bacteriophage T3. In one embodiment, the internal control is an extraction of genomic DNA derived from a stock solution of bacteriophage T3.

In one embodiment, the internal control is a stock solution of bacteriophage MS2. In one embodiment, the internal control is an extraction of genomic RNA derived from a stock solution of bacteriophage MS2.

Another object of the present invention is a kit of parts comprising at least one detergent and at least one nucleic acids-digesting enzyme.

In one embodiment, said kit comprises:

a. at least one detergent,
b. at least one nucleic acids-digesting enzyme,
c. optionally, instructions for use in a method according to the present invention.

By "kit" is intended any manufacture (e.g., a package or at least one container) comprising at least one detergent and at least one nucleic acids-digesting enzyme together (such as, for example, saponin and nucleases) or a container of at least one detergent and a container at least one nucleic acids-digesting enzyme. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention. Furthermore, any or all of the kit reagents may be provided within containers that protect them from the external environment, such as in sealed and sterile containers. The kits may also contain a package insert describing the kit and methods for its use.

In one embodiment, the kit of the invention comprises:

a. at least one detergent, selected from the group comprising, but not limited to, saponin, digitonin, Triton® X-100, Triton® X-100-R, Triton® X-114, NP-40, Tween-20, Tween-40, Tween-80, Brij® 98, Brij® 58, Brij® 35, Genapol® C-100, Genapol® X-100, Igepal®, Brij® 96/97, octyl

β-D-glucopyranoside, and nonaethylene glycol monododecyl ether ($C_{12}E_9$) or a derivative thereof,

b. at least one nucleic acids-digesting enzyme, selected from the group comprising, but not limited to, baseline-zero™ DNase, benzonase®, DNase I, DNase II, endonuclease III, endonuclease IV, endonuclease V, endonuclease VIII, exonuclease I, exonuclease III, exonuclease V, exonuclease VII, exonuclease VIII, exonuclease T, lambda exonuclease, micrococcal nuclease, mung bean nuclease, nuclease BAL-31, nuclease P1, nuclease S1, T4 endonuclease V, T5 exonuclease, T7 endonuclease I, T7 exonuclease,

c. optionally, instructions for use in a method according to the present invention.

In one embodiment, the kit of the invention comprises:

a. at least one detergent, selected from the group comprising, but not limited to, saponin, Triton, NP-40, Tween or a derivative thereof,

b. at least one nucleic acids-digesting enzyme, selected from the group comprising, but not limited to, baseline-zero™ DNase, benzonase®,

c. optionally, instructions for use in a method according to the present invention.

In one embodiment, the kit of the invention comprises:

a. saponin, preferably saponin 20-35% sapogenin,

b. baseline-zero™ DNase and/or benzonase® nuclease,

c. optionally, instructions for use in a method according to the present invention.

EXAMPLES

The present invention is further illustrated by, but is by no means limited to, the following examples. Several aspects are described below with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and

methods are set forth to provide a full understanding of the features described herein. One having ordinary skill in the relevant art, however, will readily recognize that the features described herein can be practiced without one or more of the specific details or with other methods. The features described herein are not limited by the illustrated ordering of acts or events, as some acts can occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with the features described herein.

Example 1: Decrease of HNA concentration in acellular fractions issued from plasma samples

An acellular fraction obtained from the supernatant of blood centrifuged at 1600 rpm (300 g) for 10 minutes, then further centrifuged at 13000 rpm (15000 g) for 10 minutes (to ensure full elimination of vesicles or aggregates), was treated with nucleases (conditions: 1 hour at 37°C) in the absence or in presence of 0.01% to 1% w/v saponin (**Table 1**).

Resulting HNA concentrations was tested by a highly sensitive Alu repetitive sequence qPCR. Incubation of the acellular fraction in presence of saponin was efficient in the range 0.01-1%. An optimal concentration of 0.3 to 1% saponin plus nucleases divided the amount of HNA compared to only nucleases (*i.e.*, the nuclease-resistant fraction) by a factor of at least 18 in this experiment. In fact, much higher HNA load reduction (up to 25 ct, *i.e.*, more than 10^6) can also be demonstrated for acellular fraction richer in HNA, as the treatment by saponin plus nucleases routinely decreases the HNA concentration down to background levels represented by the mix of qPCR.

Table 1

Material EAH-040	Condition	Crossing Point (CP)	Ratio treated/non-treated*	Ratio treated with (nuclease + saponin)/(nuclease only)*
Plasma clarified by high speed centrifugation	**Non-treated**	16.17 16.28		
	Nucleases only	22.74 22.74	91.4	
	Nucleases + Saponin 20-35 0.01%	23.27 23.44	140	1.5
	Nucleases + Saponin 20-35 0.03%	24.65 24.76	357	3.9
	Nucleases + Saponin 20-35 0.1%	24.81 24.92	398	4.3
	Nucleases + Saponin 20-35 0.3%	26.85 26.95	1634	17.9
	Nucleases + Saponin 20-35 0.1%	27.04 26.83	1675	18.31
Mix only	**Non-treated**	27.41 27.24		

* all the calculations were done assuming a PCR efficacy = 2.

In conclusion, we show that saponin increases the accessibility to nucleic acids-digesting enzymes like nucleases of free (non-cell associated) HNA in acellular fractions like plasma samples.

Example 2: Whole-genome next generation sequencing (NGS) of infectious agents in biological samples (in this case nHNA = microbe NA (MNA))

New tools for infectious agents (bacteria, viruses, fungi, protozoans) detection directly from biological samples are emerging: they include random sequencing of biological samples by high throughput sequencing (HTS). The main obstacle to the identification of infectious agents in biological samples is the extreme small quantity of MNAs present, in combination with a relatively high level of HNA. In the initial developments of the techniques, all nucleic acids from the samples were extracted and random-amplified by random polymerase chain reaction (PCR), RCA (rolling circle amplification), MDA (multiple displacement amplification). The drawback of this methodology is the simultaneous amplification of both MNA and HNA. This limits the sensitivity of

downstream MNAs detection: for example, detection with microarrays loses sensitivity, and HTS needs deeper sequencing to reach adequate levels of sensitivity. Even if single molecule sequencing is to be used, increasing the ratio MNA to HNA is pivotal to increase the sensitivity for a given number of reads. To bypass this drawback, hydrolysis of host DNA by nucleases has been proposed.

We show here a relative increase of infectious agents (virus and bacteria) reads from infectious agents spiked in plasma. The readout was the proportion of reads targeted to the infectious agents to the number of reads to HNA. Treatment by nucleases is very strongly improved in the presence of saponin in the range 0.1 to 1%. Saponin at these concentrations preserves MNA within viral and/or bacterial structures while giving access to HNA previously not accessible to nucleases.

Efficacy for the detection of viruses in plasma samples by NGS: experiment 1

In this experiment, blood samples were spiked with three viruses: two DNA viruses (VZV and B19) and one RNA virus (rotavirus). Plasma samples were then obtained following routine procedures in biomedical laboratories, by pelleting blood cells at 1600 rpm (300 g) for 10 minutes. The supernatants, named "*acellular fractions*", were further centrifuged at 13000 rpm (15000 g) to totally ensure lack of blood cells in the resulting supernatant, named "*viral fraction*". This viral fraction was treated with saponin 0.3% w/v plus nucleases, random-amplified and sequenced on the Proton sequencer (Life Technology).

The results show a strong (4.1 to 6.4) enhancement of the number of reads for the viral targets. In parallel, the proportion of human reads was reduced (x 0.68) (**Table 2**). This leads to a ratio virus/human reads increased by 7.1-fold in the presence of saponin, meaning that a lower depth of sequencing would be necessary to get the same number of reads against the viral targets.

Table 2

		# reads normalized for 2.5 M reads (coverage (%))			
Material	**Condition**	**Varicella Zoster virus**	**Rotavirus A**	**Parvovirus B19**	**Human**
S15-0026-01-3	**Nucleases**	1209 (31.3)	246 (36.8)	114 (53.52)	2312500
S15-0026-02-3	**Nucleases + Saponin 20-35 0.3%**	6539 (22.4)	1568 (53.6)	472 (29.61)	1585000
	Ratio saponin/no saponin	5.4	6.4	4.1	0.68
	Ratio total virus/human Nucleases: 0.07% Nucleases + Saponin: 0.5% Relative ratios w/wo saponin: 7.1				

Efficacy for the detection of viruses in plasma samples by NGS: experiment 2

In this experiment, a plasma sample obtained by routine procedures in biomedical laboratories obtained from a donor (id: 076) was spiked with three DNA viruses (VZV, feline herpesvirus, canine parvovirus) and two RNA viruses (rotavirus, feline calicivirus). The plasma was further centrifuged at 13000 rpm (15000 g) and the supernatant (or "*viral fraction*") was treated with saponin 1% w/v plus nucleases, random-amplified and sequenced on the Proton sequencer (Life Technology).

The results show a strong (1.3 to 19.2) enhancement of the number of reads for the viral targets. In parallel, the proportion of human reads was reduced (x 0.50) (**Table 3**). This leads to a ratio virus/human reads increased by 13.6-fold in the presence of saponin, meaning that a lower depth of sequencing would be necessary to get the same number of reads against the viral targets.

Table 3

		# reads normalized for 10 M reads					
Material STP 19	**Condition**	**Feline herpesvirus**	**Varicella zoster virus**	**Feline calicivirus**	**Canine parvovirus 2**	**Rotavirus A**	**Human**
S-14-0118-03	**Nucleases**	75607	18317	148	306	189180	4644353
S-14-0118-04	**Nucleases + Saponin 20-35 1%**	1450498	237195	1505	982	250329	2338926
	Ratio saponin/no saponin	19.18	12.95	10.17	3.21	1.32	0.50
	Ratio total virus/human Nucleases: 6.1% Nucleases + Saponin: 82.9% Relative ratios w/wo saponin: 13.6						

Efficacy for the detection of bacteria in plasma samples

In this experiment, blood samples were spiked with Gram$^-$ bacteria (*Acinetobacter baumannii*, *Morganella morganii*) and Gram$^+$ bacteria (*Enterococcus faecalis*, *Streptococcus agalactiae*). Plasma samples were then obtained following procedures used routinely in biomedical laboratories, by pelleting blood cells at 1600 rpm (300 g). The supernatants (or "*acellular fractions*") were further centrifuged at 13000 rpm (15000 g) for 10 minutes to pellet bacteria from plasma. This pellet, also named herein as "*bacterial fraction*", was treated with or without saponin 1% w/v plus nucleases, random-amplified and sequenced on the Proton sequencer (Life Technology).

The results show a strong (21.5 to 43.5) enhancement of the number of reads from the bacterial targets. In parallel, the proportion of human reads was strongly reduced (x 0.80) (**Table 4**). This leads to a ratio bacteria/human reads increased by 112-fold in the presence of saponin. Genome coverage (*i.e.*, the fraction of the bacterial genome sequenced) was 1.3-7.8% in absence of saponin and 32.2-82.3% in presence of saponin.

Table 4

		# reads normalized for 2.5 M reads (coverage (%))				
Material	**Condition**	***A. baumannii***	***M. morganii***	***E. faecalis***	***S. agalactiae***	**Human**
S-15-0024-04-2	**Nucleases**	430 (1.3)	510 (1.5)	384 (1.6)	1728 (7.8)	2422500
S-15-0024-03-2	**Nucleases + Saponin 20-35 1%**	25029 (35.2)	19447 (32.2)	50623 (67.5)	165816 (82.3)	1930000
	Ratio saponin/no saponin	58 (27)	38.5 (21.5)	131.8 (42.2)	95 (10.6)	0.80
	Ratio total bacteria/human Nucleases: 0.12% Nucleases + Saponin: 13.5% Relative ratios w/wo saponin: 112					

Thus, a privileged sequence of steps is:

1) treatment of an acellular fraction with saponin and nucleases to digest HNAs,
2) inactivation of the nuclease,
3) extraction of NAs from the acellular fraction,
4) readout that might be PCR, NGS following random amplification or NGS on single molecules, hybridization on DNA arrays.

Example 3: Analytical performance characteristics

In this experiment, blood samples were spiked with five infectious agents (**Table 5**).

Table 5

Infectious agent	**Type**	**Concentration (gc/mL)**
Varicella Zoster virus (VZV)	Double strand DNA virus	10^4
BK polyomavirus	Circular double strand DNA virus	10^4
Parainfluenza-3 virus	Single strand RNA virus	10^4
Rotavirus A	Segmented double strand RNA virus	10^3
Parvovirus B19	Single strand DNA virus	2.5×10^3

gc/mL: genome copie/mL

Limits of detection

The limits of detection (LoD) for the five infectious agents were determined from 8 independent blood samples, of which 5 were repeated (starting from the nucleic acid extracts), meaning 13 samples all together.

Amongst the five viruses, three were detected in all situations (13/13): VZV, parvovirus B19 and rotavirus A. The BK polyomavirus was detected in all but one duplicate of the 5 samples processed twice (12/13 positives).

Spiked at the concentration of 10^4 gc/mL, the Parainfluenza-3 virus was detected with only few tens to hundreds sequencing reads in several samples, while undetected in other samples. This data means that the spiking concentration for the Parainfluenza-3 virus corresponds to the extreme limit of the LoD.

The LoD calculated for the viruses are thus:

- VZV 10^4 gc/mL,
- Rotavirus A 10^3 gc/mL,
- Parvovirus B19 $2.5x10^3$ gc/mL,
- BK polyomavirus 10^4 gc/mL,
- Parainfluenza-3 virus 10^4 gc/mL.

Repeatability

Repeatability was assessed at two different levels: by testing identical NGS libraries loaded onto different Illumina MiSeq flowcells, and analyzed independently. This approach allows to assess variability introduced by the NGS sequencing stochastic step on the data output.

Two different NGS libraries were loaded onto two NGS runs. Amongst the 10 conditions (fiveinfectious agents and two different libraries), only 1 condition gave a different result. For two replicates of the same library, the rotavirus A was undetected or detected with a moderate confidence. More importantly, when an infectious agent was detected in a given

point of a sample, it was also detected in the duplicate, indicating a consistent repeatability for detected agents.

Then, experiments starting from the same viral extracts but processed independently up to the NGS sequencing were performed. This was used to monitor the variability introduced by the nucleic acid amplification, the NGS library construction and sequencing steps.

Such type of experiment was performed on five different blood samples, each giving a viral extract. For each viral extract, a duplicate of nucleic acid amplification, NGS library construction and sequencing onto two different MiSeq flowcells was performed. The analysis was performed on ten NGS libraries issued from five blood samples.

Out of the 25 duplicates (five infectious agents spiked into five different blood samples), only 3 were discordant. Regarding the detection, the 7 cases can be classified as follows:

- From positive to moderate confidence level:
  2 cases (2x Parainfluenza-3 virus),
- From positive to negative detection:
  1 case (BK polyomavirus).

Amongst the 3 discordant cases, the parainfluenza-3 virus, the agent showing the less reproducible data, represent 2 cases. More importantly, the extreme variation represented by a positive detection and a non-detection within a single duplicate is only found in a single case out of the 25 studied.

Altogether, this data indicates a very good repeatability for the detection of infectious agents starting from a single nucleic acid extract.

Example 4: Internal controls

Internal control 1

During the preparation of the viral fractions and bacterial fractions, a first internal control, herein termed "*internal control 1*", is used to assess the reliability of the methods of the invention. Internal control 1 is an ultrapure stock solution of bacteriophage T3.

In the viral fraction, internal control 1 is spiked in the plasma, prior to saponin and nuclease treatment, and serves to monitor all the subsequent steps of the procedure.

In the bacterial fraction, internal control 1 is added after the saponin and nuclease treatment, but prior to the extraction of bacterial DNA. It is used to monitor the bacterial DNA purification and the subsequent steps of the procedure.

The differences between the viral and bacterial fractions preparation have an implication on the monitoring of these procedures by the addition of internal control 1. Indeed, extraction of viral nucleic acids from viral particles is achieved by a "soft" treatment (typically, using chaotropic salts), as compared to the strong mechanical lysis required to break-down bacterial cell walls, especially for $Gram^+$ bacteria. Such a hard treatment would inevitably degrade the nucleic acid of internal control 1 and prevent further monitoring of the procedure. Consequently, the addition of internal control 1 in the bacterial fraction is performed right after the mechanical lysis and prior the purification of bacterial nucleic acids.

Internal control 2

During the preparation of the viral fractions, a second internal control, herein termed "*internal control 2*", may be used to assess the reliability of the methods of the invention. Internal control 2 is an extraction of genomic RNA derived from an ultrapure stock solution of bacteriophage MS2.

In the viral fraction, internal control 2 may be added in the viral nucleic acids extract, right after the purification of viral nucleic acids. In this case, it is used to monitor a reverse transcription step, required for the detection and sequencing of RNA viruses, as well as the subsequent steps of the procedure together with internal control 1.

The methods of the invention are deemed to be reliable if enough sequencing reads for internal controls 1 and 2 are recovered, *i.e.*, more than about 10% of the genome covered.

CLAIMS

1. An *in vitro* method for enriching an infectious agent in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one nucleic acids-digesting enzyme.

2. The *in vitro* method according to claim **1**, further comprising the detection of said infectious agent.

3. The *in vitro* method according to claim **2**, wherein the detection of said infectious agent is carried out by Next Generation Sequencing.

4. An *in vitro* method for modulating the accessibility of host nucleic acids to nucleic acids-digesting enzymes in an acellular fraction of a biological fluid comprising contacting the acellular fraction of a biological fluid with at least one detergent and at least one enzyme digesting nucleic acids.

5. The *in vitro* method according to any one of claims **1** to **4**, wherein the ratio between infectious agents and host nucleic acids is increased.

6. The *in vitro* method according to any one of claims **1** to **5**, wherein the infectious agent selected from the group comprising bacteria, fungi, viruses, protozoans, and parasites.

7. The *in vitro* method according to any one of claims **1** to **6**, wherein the at least one detergent is selected from the group comprising: non-ionic detergent and/or zwitterionic detergent and/or anionic detergent, preferably the at least one detergent is a non-ionic detergent.

8. The *in vitro* method according to any one of claims **1** to **7**, wherein the non-ionic detergent is selected from the group comprising: saponin, Triton, NP-40, Tween or a derivative detergent thereof, preferably the non-ionic detergent is saponin or a derivative detergent thereof.

9. The *in vitro* method according to any one of claims **1** to **8**, wherein the at least one detergent is at a concentration from about 0.01% to about 10% w/v.

10. The *in vitro* method according to any one of claims **1** to **9**, wherein the at least one nucleic acids-digesting enzyme is a nuclease having DNase and/or RNase activity.

11. The *in vitro* method according to any one of claims **1** to **10**, wherein the at least one nucleic acids-digesting enzyme is at a concentration from about 0.1 units/µg to about 10 units/µg of protein.

12. The *in vitro* method according to any one of claims **1** to **11**, wherein the presence of residual cells within the fraction does not affect the accessibility or the enrichment of the infectious agent in the fraction.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/050250

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10 C12Q1/68
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)
C12N 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 practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/062354 A1 (BIO MERIEUX INC [US]; HYMAN JONES; CLAY BRADFORD; WALSH JOHN; THORPE T) 3 June 2010 (2010-06-03) cited in the application paragraph [0037] paragraph [0043] - paragraph [0045] -----	1-12
X	US 2015/337362 A1 (TARENDEAU FRANCK [FR]) 26 November 2015 (2015-11-26) paragraph [0143] paragraph [0014] paragraph [0018] - paragraph [0028] paragraph [0072] - paragraph [0084] ----- -/--	1-12

[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

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2017	07/03/2017

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/050250

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/050250

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