MULTIPLEX IMMUNO SCREENING ASSAY

Background of the invention

Infectious diseases caused by pathogenic agents, such as bacteria, viruses (e.g., viral hemorrhagic fevers (VHFs)), and parasites pose a significant public health problem, due to the severity of the diseases, high lethality, inter-human contagiousness of certain agents, and lack of effective treatment for most of them.

Control of epidemics crucially depends on the rapid detection and accurate identification of the agent, in order to define and implement timely and appropriate action. In this context, it is essential to produce and validate tools for early detection of outbreaks, precise identification of the etiologic agent, and improved disease surveillance.

In this respect, detection of antibodies in body fluids constitutes a major part of the diagnosis of virally induced diseases, other diseases caused by infectious organisms, autoimmune diseases and the detection of cancer. As a matter of fact, certain antibodies can serve as markers in diagnosis and can lead to prognosis and treatment, as their presence are known to correlate with the outbreak of a pathogen. This is particularly the case for the antibodies targeting viral antigens exclusively.

Current methods for detecting the presence of antibodies include diverse techniques such as immunofluorescence microscopy, chemiluminescence assay, Western blotting, Radio Immuno-Precipitation assay (RIP) and ELISA. The parallel detection of several antibodies simultaneously may be particularly useful by minimizing the matrix effects that exist between individual assays, such as in ELISAs, because the calibrators and the antibodies are analyzed under the same conditions; it therefore will generate comparable results for the measurement of multiple antibodies present within the same sample. One such assay is the Luminex multiplex assay utilizing antigens bound to beads. Pickering et al., Clinical and Diagnostic Laboratory Immunology 9:872-876 (2002).

Complicating the straightforward identification of pathogenically relevant antibodies, however, is that normal sera contain large amounts of natural antibodies which manifest

themselves in complex staining patterns (Avrameas S. *Immunol. Today* 1991). The presence of these natural antibodies can complicate the differentiation of disease-associated antibodies from the complex background of "auto-immune noise", i.e. naturally occurring autoantibodies. As noted in Anderson et al., Methods Mol. Biol. 723:227-238 (2011), cross-talk and interference remain a concern with multiplex assays.

Binding of human antibodies directly to the beads in a multiplex assay has also been reported as a problem. Tainsky et al., Biomarker Insights 2:261-267 (2007); Waterboer et al., J. Immunol Methods 309:200-204 (2006).

Moreover, a low difference between positive and control samples can limit the utility of multiplex assays. Burbelo et al., Exp. Rev Vaccines, 9:567-578 (2010).

Another complication is that ELISA and multiplex assays do not necessary give the same results. For example, Pickering et al., 2002, noted numerous discrepancies between the two assays when measuring protective antibodies against various viral and bacterial antigens. This may reflect alterations in antigenicity relating to how the antigen is attached to the substrate. Ambrosino et al., Malaria Journal 9:317 (2010). Similarly, Cham et al., Malaria Journal 7:108 (2008), reported that some samples showed a marked difference between the ELISA and bead-based assay readings, some of which had higher ELISA readings. This could be due to the fact that different parts of the recombinant proteins are accessible by antibodies when the proteins are bound to a surface or a sphere. *Id.*

The preparation of sufficient quantities of the antigen for commercial applications (i.e., kits) is also required. Burbelo et al., Exp. Rev Vaccines, 9:567-578 (2010).

In view of the foregoing, there exists a need for addressable systems and methods, which can provide additional improvements in high throughput, cost-effectiveness, and accuracy for molecular diagnosis of antibody-generating diseases. The present invention satisfies these and other needs.

Summary

In one aspect, there is provided an *in vitro* assay method for detecting at least two different target antibodies present in a biological sample from a subject, said method comprising the steps of:

(a) contacting a mixture of at least 2 solid supports with a biological sample; wherein at least one of the solid supports comprises a first AGT-Antigen fusion protein covalently coupled to an AGT substrate, and wherein at least one of the solid supports comprises a second AGT-Antigen fusion protein covalently coupled to an AGT substrate; (b) detecting the presence or absence of binding of antibodies to the first AGT-Antigen; and (c) detecting the presence or absence of binding of antibodies to the second AGT-Antigen wherein the AGT of said first and second fusion proteins is the SNAP mutant of SEQ ID NO: 2.

Another aspect relates to a kit for detecting at least different two target antibodies present in a biological sample from a subject comprising: (a) a first solid support comprising a first AGT-Antigen fusion protein covalently coupled to an AGT substrate, and (b) a second solid support comprising a second AGT-Antigen fusion protein covalently coupled to an AGT substrate, wherein said solid supports are mixed together in at least one single compartment, and wherein the AGT of said first and second fusion proteins is the SNAP mutant of SEQ ID NO: 2.

Yet another aspect relates to the use of the kit as defined herein for detecting at least two target antibodies in a biological sample from a subject.

Still another aspect relates to the use of the kit as defined herein for diagnosing at least two target diseases in a subject, wherein said target disease is a viral infection caused by a Papillomavirus or a Dengue, Yellow fever, West Nile, Japanese encephalitis, Tick-Borne Encephalitis, Hepatitis C, Chikungunya, Ross River, Mayaro, Western Equine encephalitis, Eastern Equine Encephalitis, Venezuela Equine Encephalitis, Crimean-Congo hemorrhagic fever, Rift Valley Fever, Schmallenberg, Hepatitis E virus, Lassa, Ebola, Marburg virus; a bacterial infection caused by *Leptospirosa Interrogans*; or an infection caused by *Plasmodium falciparum*.

Another aspect relates to an *in vitro* method for diagnosing at least one target disease in a subject, said target disease being known to induce the synthesis of at least one target antibody in the subject, the method comprising performing the assay method as defined herein, wherein the subject is diagnosed to be suffering from said at least one target disease if the amount of said at least one target antibody is higher than a control value.

A further aspect relates to a method for manufacturing a kit, said method comprising the steps of: (a) providing a first AGT-Antigen fusion protein that is recognized by a first target antibody, (b) contacting said first AGT-Antigen fusion protein with a first solid support, the support being covalently coupled with a substrate of the AGT-Antigen fusion protein, (c) obtaining a first solid support covalently coupled with the first AGT-Antigen fusion protein, d) providing at a second AGT-Antigen fusion protein that is recognized by a second target antibody, but not by said first target antibody, and (e) contacting said second AGT-Antigen fusion protein with a second solid support, the support being covalently coupled with a substrate of said AGT-Antigen fusion protein, and (f) obtaining a second solid support covalently coupled with the second AGT-Antigen fusion protein, wherein the first and second solid supports can be specifically identified from each other, wherein said solid supports are mixed together in at least one single compartment, and wherein the AGT of said first and second fusion proteins is the SNAP mutant of SEQ ID NO: 2.

Figure legends

Figure 1 represents the oriented coupling of chimeric AGT-antigen proteins to substrate-coated microspheres. First step of coupling consists of coupling the AGT substrate BG-PEG-NH2 to the activated microspheres by amine coupling. The second

step consists of contacting the substrate-coated microspheres with fusion proteins containing AGT (for example the SNAP mutant), said enzyme being intended to covalently attach to its BG-PEG-NH2 substrate, that is, to the microspheres.

Figure 2 shows the coupling efficiency of chimeric SNAP-viral antigens proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV.EDIII, SNAP-YF.EDIII, SNAP-JE.EDIII, SNAP-ZIKA.EDIII), as followed by anti-SNAP antibody.

Figure 3 compares the sensitivity of the immunoassay experiment for the detection of purified monoclonal anti-DV2 antibody on chimeric SNAP-DV2.EDIII protein conjugated to microspheres via the substrate of the hAGT protein (coupling of the invention) or coupled through a standard amine coupling procedure, *e.g.* Bio-Plex Amine Coupling Kit, BIORAD.

Figure 4 compares the sensitivity of the immunoassay experiment for the detection of purified monoclonal anti-DV1 antibody on chimeric SNAP-DV1.EDIII protein conjugated to microspheres, either in a singleplex or in a multiplex fomat with other chimeric SNAP-viral Ags proteins (SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV.EDIII, SNAP-YF.EDIII, SNAP-JE.EDIII, SNAP-TBE.EDIII) coupled to microspheres.

Figure 5 shows the reactivity and specificity of the multiplex immunoassay experiment for the detection of dilutions of purified monoclonal anti-WNV antibody on chimeric SNAP-viral Ags proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV.EDIII, SNAP-YF.EDIII, SNAP-JE.EDIII, SNAP-TBE.EDIII) coupled to microspheres.

Figure 6 shows the reactivity and specificity of anti-DV3 IgG detection in mouse polyclonal serum against DV3 (A) and anti-YF IgG detection in mouse polyclonal serum against YF (B) in multiplex immunoassays on chimeric SNAP-viral Ags proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV.EDIII, SNAP-YF.EDIII, SNAP-JE.EDIII, SNAP-WSL.EDIII, SNAP-ROCIO.EDIII, SNAP-MVE.EDIII, SNAP-SLE.EDIII, SNAP-ZIKA.EDIII) coupled to microspheres

Figure 7 shows the reactivity and specificity of anti-DV1 IgM detection (A) and anti-DV1 IgG detection (B) in DV1-infected serum of a human patient in multiplex immunoassays on chimeric SNAP-viral Ags proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV.EDIII, SNAP-YF.EDIII, SNAP-JE.EDIII, SNAP-WSL.EDIII, SNAP-ROCIO.EDIII, SNAP-MVE.EDIII, SNAP-SLE.EDIII, SNAP-ZIKA.EDIII, SNAP-TBE.EDIII) coupled to microspheres.

Figure 8 discloses the structure of the pDeSNAPuniv cassette.

Figure 9 discloses the structure of the pDeSNAPuniv/SBV.N cassette.

Figures 10 A and B show (A) an immunoblot assay performed on the supernatants of S2/SNAP-SBV.N cells induced for 10 days with Cd^{2+} (+) or non induced (-). The secreted chimeric protein SNAP-SBV.N (theorical MW 50 kDa) was detected using an anti-His_{tag} antibody, in comparison to define amounts of highly purified chimeric protein SNAP-TOS.N (theorical MW 49 kDa). (B) Direct visualization of proteins on each fraction of size-exclusion chromatography column (Coomassie blue staining of PAGE-SDS) corresponding to the final purification step of secreted SNAP+SBV.N protein from induced S2/SNAP+SBV.N cells for 10 days.

Figure 11 shows an example of a device containing the antigen-coated microspheres of the invention.

Figures 12 A-F show coding nucleotide (SEQ ID NO:154) and amino acid sequence (SEQ ID NO:155) of a SNAP construct containing the SARS virus N gene.

Figures 13 A-D show coding nucleotide (SEQ ID NO:156) and amino acid sequence (SEQ ID NO:157) of a SNAP construct containing the SARS virus S gene receptor binding domain.

Figures 14 A-E show coding nucleotide (SEQ ID NO:158) and amino acid sequence (SEQ ID NO:159). of a SNAP construct containing the human coronavirus N gene.

Figures 15 A-G show coding nucleotide (SEQ ID NO:160) and amino acid sequence (SEQ ID NO:161). of a SNAP construct containing the human coronavirus S1 gene.

Figures 16 A-D show coding nucleotide (SEQ ID NO:162) and amino acid sequence (SEQ ID NO:163) of a SNAP construct containing the human coronavirus S1 gene receptor binding domain (RBD).

Figures 17 A-D show coding nucleotide (SEQ ID NO:164) and amino acid sequence (SEQ ID NO:165) of a SNAP construct containing the LruA gene of Leptospirosis.

Figures 18 A-D show coding nucleotide (SEQ ID NO:166) and amino acid sequence (SEQ ID NO:167) of a SNAP construct containing the LruB gene of Leptospirosis.

Figures 19 A-D show coding nucleotide (SEQ ID NO:168) and amino acid sequence (SEQ ID NO:169) of a SNAP construct containing the LipL32 gene of Leptospirosis.

Figures 20 A-F show coding nucleotide (SEQ ID NO:170) and amino acid sequence (SEQ ID NO:171) of a SNAP construct containing the C gene of Hepatitis E.

Figures 21 A-E show coding nucleotide (SEQ ID NO:172) and amino acid sequence (SEQ ID NO:173) of a SNAP construct containing the core sequence of the C gene of Hepatitis E.

Figures 22 A-C show coding nucleotide (SEQ ID NO:174) and amino acid sequence (SEQ ID NO:175) of a SNAP construct containing the C gene of Hepatitis C.

Figures 23 A-D show coding nucleotide (SEQ ID NO:176) and amino acid sequence (SEQ ID NO:177) of a SNAP construct containing the core sequence of the C gene of Hepatitis C.

Figures 24 A-C show coding nucleotide (SEQ ID NO:178) and amino acid sequence (SEQ ID NO:179) of a SNAP construct containing sequences encoding the NSs protein from Schmallenberg virus.

Figures 25 A-B show advantageous combinations of antigen-coupled microspheres to be included in the kit of the invention.

Detailed description of the invention

The present invention allows for the simultaneous screening for multiple antigens in a biological sample with higher sensitivity and specificity using an oriented antigen coupling procedure for the antigens. The present inventors have developed and validated immunoassays leading to rapid and simultaneous detection of several antibodies generated by a wide range of diseases, in particular arboviral diseases and VHFs, in biological fluids.

To achieve optimal sensitivity and specificity for the detection of low amount of antibodies using minimal antigen, an oriented antigen coupling procedure has been developed. This oriented antigen coupling procedure is based on the covalent interaction between the AGT enzymes and their substrates, the O6-benzylguanine derivatives, which irreversibly react with AGT enzymes by transferring their benzyl group to the active site cysteine of the enzyme. Accordingly, a number of target antigens can be fused to an AGT enzyme moiety, resulting in different chimeric fusion proteins (hereafter referred to as [AGT- Antigen] fusion proteins), that can be used as capture reagents for the antibodies present in a biological sample. The present inventors have shown that this antibody capture is unexpectedly enhanced when these fusion proteins are bound to solid supports thanks to the specific AGT-substrate interaction.

As shown in the examples, the inventors have generated a number of different antigen-coated fluorescent microspheres with the oriented antigen coupling procedure. Sixteen distinct sets of microspheres coupled with 16 purified chimeric [AGT-Antigen] fusion proteins were used in a titration of 16 serum antibodies specific to different proteins of the dengue serotypes 1 to 4, West Nile, yellow fever, Japanese encephalitis, tick-borne encephalitis, Saint-Louis encephalitis, Murray Valley encephalitis, Wesselsbron, Zika, Rocio, Usutu, Rift Valley fever, and chikungunya virus. Unexpectedly, these 16 distinct sets of microspheres have been mixed in a single sample without affecting the sensitivity and specificity of the detection (see figure 5). The production of this system is highly time- and cost-effective, as only a very small amount of recombinant antigen (< 50 µg) is required to produce one set of antigen-coupled microspheres (~1.25 x 10^6 microspheres), such set being sufficient to perform 1000 individual assays.

Consequently, the invention encompasses methods and kits for performing multiplex assays based on the oriented antigen coupling of a solid support, preferably a labeled microparticle, to an AGT substrate, which is coupled to chimeric fusion proteins, i.e., AGT-Antigen. This oriented coupling allows for increased interaction of the antigen with antibodies in a biological sample. A solid support coated with AGT-substrate can be coupled with the AGT-Antigen through the enzymatic activity of the AGT enzyme in these immunoassays.

The 6-alkylguanine-DNA-alkyltransferase enzyme (AGT, also known as ATase or MGMT, and hereafter referred to as "AGT") is numbered EC 2.1.1.63 in the IUBMB enzyme nomenclature. It is a 6-alkylguanine-DNA-alkyltransferase DNA repair enzyme of 207 amino acid residues whose function in the cells is to repair alkylated DNA. More precisely, AGT acts on O^6-methylated guanine in DNA by irreversibly transferring the methyl group in an S_N2 reaction to a reactive cysteine residue (Cys 145). Recently, a number of O6-benzylguanine derivatives have been shown to irreversibly react with said enzyme by transferring their benzyl group to the active site cysteine of the AGT enzyme (cf. Damoiseaux et al., *ChemBiochem.*, 2001, WO 2004/031404 and WO 2005/085470). Thus, the AGT-Antigen can be coupled to an AGT substrate through this enzymatic reaction.

The solid supports coupled to the AGT-Antigen can be incubated with a biological sample containing immunoglobulins. After immunoglobulins have been bound to the solid supports, detection of the solid supports which are effectively bound to immunoglobulins can be acomplished. The identification of immunoglobulin-coated solid support(s) enables to diagnose which pathogen was infecting the patient (as each solid support matches with a defined pathogenic antigen). This detection step is performed by any usual means, for example by using labeled detection antibodies and by identifying the nature of the solid support.

Advantageously, the method of the invention involves only the detection of the presence of antibodies in diseased patients, but knowledge about the identity of those antibodies is not required.

Methods for detecting multiple target antibodies

The present invention relates to a method for detecting at least 2 target antibodies in a biological sample. The invention encompasses detecting the presence or absence of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. different target antibodies. Thus, the invention comprises a method comprising at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. solid supports and a method comprising at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. different epitopes. The method can comprise at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. solid supports mixed together prior to addition of the biological sample. The epitopes can be recognized by target antibodies.

In one embodiment, the method comprises:

(a) contacting a mixture of at least 2 solid supports with a biological sample;

wherein at least one of the solid supports comprises a first AGT-Antigen fusion protein covalently coupled to an AGT substrate, and

wherein at least one of the solid supports comprises a second AGT-Antigen fusion protein covalently coupled to an AGT substrate;

(b) detecting the presence or absence of binding of antibodies to the first AGT-Antigen; and

(c) detecting the presence or absence of binding of antibodies to the second AGT-Antigen.

In one embodiment, the method comprises:

(a) contacting a mixture of at least 10 solid supports with a biological sample;

wherein a first solid support comprises a first AGT-Antigen fusion protein covalently coupled to an AGT substrate,

wherein a second solid support comprises a second AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a third solid support comprises a third AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a fourth solid support comprises a fourth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a fifth solid support comprises a fifth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a sixth solid support comprises a sixth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a seventh solid support comprises a seventh AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein an eighth solid support comprises an eighth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a ninth solid support comprises a ninth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

wherein a tenth solid support comprises a tenth AGT-Antigen fusion protein covalently coupled to an AGT substrate; and

(b) detecting the presence or absence of binding of antibodies to the each of the AGT-Antigens.

In one embodiment, the method comprises:

(a) contacting a first solid support comprising an AGT substrate covalently coupled to a first fusion protein comprising an AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity and a first epitope that is recognized by a first target antibody with the biological sample;

(b) contacting a second solid support comprising an AGT substrate covalently coupled to a second fusion protein comprising an AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity and a second epitope that is recognized by a second target antibody, but not by said first target antibody with the biological sample; and

(c) detecting the presence or absence of the two target antibodies.

Preferably, the first and second, etc., solid supports are microspheres. Most preferably, the microspheres are labeled. Preferably, the first and second solid supports are labeled with different labels.

The invention encompasses an *in vitro* assay method for detecting at least two different target antibodies present in a biological sample from a subject, said method comprising the steps of:

(a) providing a first fusion protein comprising :

- a polypeptide comprising a first epitope that is recognized by a first target antibody and
- a AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity,

(b) contacting said first fusion protein with a first solid support, said support being covalently coupled with a substrate of said AGT polypeptide,

(c) obtaining a first solid support covalently coupled with a first epitope that is recognized by the first target antibody,

(d) providing a second fusion protein comprising :

- a polypeptide comprising a second epitope, said second epitope being recognized by a second target antibody but not by said first target antibody, and
- a AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity,

(e) contacting said second fusion protein with a second solid support, said support being covalently coupled with a substrate of said AGT polypeptide,

(f) obtaining a second solid support covalently coupled with a second epitope that is recognized by the second target antibody, but not by said first target antibody, wherein said first and second solid supports can be specifically identified from each other,

(g) contacting said biological sample with the first and second solid supports obtained in steps (c) and (f),

(h) detecting the presence of said at least two target antibodies.

As used hereafter, the terms "an antibody", "a fusion protein", "an epitope", "an antigen", "an AGT polypeptide", "a solid support" and the like have obviously to be understood as usual in the art, that is, in a broad manner. In particular, they encompass not only particular single molecules but a number of said molecules. For example, the term "solid support" encompasses a subset of numerous identical solid supports, the term "microparticle" encompasses a subset of numerous identical microparticles, and the term "fusion protein" encompasses a number of identical single protein molecules. In the context of the present invention, it is noteworthy that a solid support carries a number of identical fusion proteins, said fusion proteins containing, apart from the AGT polypeptide, identical antigen, and therefore identical epitopes, so that the antibodies which will be detected on the solid support can be unambiguously identified.

As used herein, the term "fusion protein" means a polypeptide containing a protein or a polypeptide created through the artificial joining of two or more polypeptides(see http://en.wikipedia.org/wiki/peptide). In the immunoassays of the invention, said fusion proteins contain a AGT polypeptide and an antigen, containing at least one epitope. Fusion proteins can be obtained through genetic engineering (see http://en.wikipedia.org/wiki/Genetic_engineering) of a fusion gene. This typically involves removing the stop codon (see http://en.wikipedia.org/wiki/Codon) from a cDNA sequence (see http://en.wikipedia.org/wiki/CDNA) coding for the first protein, then appending the cDNA sequence of the second protein in frame (see http://en.wikipedia.org/wiki/Reading_frame) through ligation (see http://en.wikipedia.org/wiki/Ligase) or overlap extension PCR (see http://en.wikipedia.org/wiki/PCR). That DNA sequence will then be expressed (see http://en.wikipedia.org/wiki/Protein_expression) by a cell (see http://en.wikipedia.org/wiki/Cell_(biology)) as a single protein. The protein can be engineered to include the full sequence of both original proteins, or only a portion of either. If the two entities are proteins, a linker (or "spacer") peptides can be added, which makes it more likely that the proteins fold independently and behave as expected. In particular, the fusion proteins of the invention can be obtained by providing vectors comprising AGT encoding sequences in frame with an epitope or antigen encoding sequences, either attached to the N-terminal or to the C-terminal side of the AGT DNA sequence. These vectors may be introduced in prokaryotic hosts, including eubacteria such as *E.coli* bacteria, or eukaryotic hosts, e.g., yeast, insect cells or mammalian cells and the recombinant fusion proteins may be produced under appropriate conditions. Typical constructions are presented in the experimental part of this application.

The term "antibody" as used herein is intended to include monoclonal antibodies, polyclonal antibodies, and chimeric antibodies. Preferably, the antibodies which are to be detected by the immunoassays of the invention are polyclonal antibodies, which are present in biological samples of diseased patients, and have therefore been generated from different B cell sources. As such, they recognize different epitopes exhibited by a pathogenic antigen (on the other hand, monoclonal antibodies are derived from a single cell line and recognize the same epitope).

An antibody (or "immunoglobulin") consists of a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (or domain) (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" (CDR) or "hypervariable regions", which are primarily responsible for binding an epitope of an antigen, and which are interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g. effector cells) and the first component (Clq) of the classical complement system.

Antibody can be of different isotypes (namely IgA, IgD, IgE, IgG or IgM). Both IgG and IgM type antibodies can be detected by the present method. Of note, these isotypes are composed of two identical heavy chains and two identical light chains that are joined by disulfide bonds. Importantly, IgM antibodies form polymers where multiple immunoglobulins are covalently linked together with disulfide bonds, mostly as a pentamer but also as a hexamer, so that they have a molecular mass of approximately 900 kDa (in their pentamer form). Because each monomer has two antigen binding sites, a pentameric IgM has 10 binding sites. Typically, however, IgM antibodies cannot bind 10 antigens at the same time because the large size of most antigens hinders binding to nearby sites. Due to its polymeric nature, IgM possesses high avidity. Different isotypes of antibodies (e.g., IgG or IgM) can be detected with the same antigen, allowing the temporal discrimination of the antibody response, such as whether the antibody response is an early or later response against an antigen/infection. This can discrimination can provide information as to the timing of infection or exposure to the antigen.

Antibody fragments can also be detected thanks to the present method. This term is intended to include Fab, Fab', F(ab')2, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, and multimers thereof and bispecific antibody fragments.

Monoclonal antibodies can be used in the present immunoassays; for example for detecting the immunoglobulins that are bound to the solid supports. As used herein, "monoclonal antibody" defines an antibody arising from a homogeneous antibody population. More particularly, the individual antibodies of a population are identical except for a few possible naturally-occurring mutations which can be found in minimal proportions. In other words, a monoclonal antibody consists of a homogeneous antibody arising from the growth of a single cell clone (for example a hybridoma, a eukaryotic host cell transfected with a DNA molecule coding for the homogeneous antibody, a prokaryotic host cell transfected with a DNA molecule coding for the homogeneous antibody, etc.) and is generally characterized by heavy chains of one and only one class and subclass, and light chains of only one type. Monoclonal antibodies are highly specific and are directed against a single antigen. In addition, in contrast with preparations of polyclonal antibodies which typically include various antibodies directed against various determinants, or epitopes, each monoclonal antibody is directed against a single epitope of the antigen.

The term "antigen" herein means any substance that causes the immune system to produce antibodies against the said substance. An "immunogenic" antigen is a specific type of antigen which is able to stimulate an adaptive immune response if injected on its own. At the molecular level, an antigen is thus characterized by its ability to be "bound" to the antigen-binding site of an antibody.

In the context of the present invention, an antibody is said to "bind" a define antigen (or epitope) or to "recognize" said antigen (or epitope) if said antibody has an affinity constant K_a (which is the inverted dissociation constant, i.e. $1/K_d$) higher than 10^5 M^{-1}, preferably higher than 10^6 M^{-1}, more preferably higher than 10^7 M^{-1} for said antigen (or epitope). This affinity can be measured for example by equilibrium dialysis or by fluorescence quenching, both technologies being routinely used in the art.

The methods of the invention can be coupled with other techniques for the detection of proteins, nucleic acids, antibodies, immune responses, polysaccharides, etc., for example, in the detection of allergens.

Antigens for use in multiplex immunoassays

In the context of the invention, antigens or epitopes include proteins, lipoproteins, polysaccharides, and glycoproteins. Said proteins include viral, bacterial, parasitic, animal, and fungal proteins such as albumins, tetanus toxoid, diphtheria toxoid, pertussis toxoid, bacterial outer membrane proteins (including meningococcal outer membrane protein), RSV-F protein, malarial derived peptide, B-lactoglobulin B, aprotinin, ovalbumin, lysozyme, linear peptides, oligopeptides etc. The antigens and epitopes include cancer, cytokine, and allergen antigens and epitopes. The antigens can be antigens involved in autoimmune diseases.

The antigens can be tumor associated antigens such as Alphafetoprotein (AFP, http://en.wikipedia.org/wiki/Alphafetoprotein), carcinoembryonic antigen (CEA), CA 15-3, CA27-29, CA 125, CA 19-9, Calrefinin, prostate specific antigen (PSA), MUC-1 (http://en.wikipedia.org/wiki/MUC1), Epithelial membrane protein (EMA), Epithelial tumor antigen (ETA), Tyrosinase (http://en.wikipedia.org/wiki/Tyrosinase), Melanoma-associated antigen (MAGE), TAA complexes, SSX2 or NERCMSL, ras(http://en.wikipedia.org/wiki/Ras_subfamily), P53, CD34, CD99, CD117, Chromogranin, Cytokeratin, Desmin, Factor VIII (http://en.wikipedia.org/wiki/Factor_VIII), CD31 (http://en.wikipedia.org/wiki/CD31), FL1 (http://en.wikipedia.org/wiki/FL1), GFAP, GCDFP-15, HMB-45, hCG, inhibin, keratin, PTPRC (CD45), MART-1, MyoD1, MSA, NSE, PLAP, S100 protein, SMA, synaptophysin, Thyroglobulin, thyroid transcription factor-1, Tumor M2-PK, and vimentin.

The antigens can be cancer biomarkers such as Angiopoietin-2, sCD40L, EGF, Endoglin, sFASL, HB-EGF, IGFBP-1, IL-6, IL-8, IL-18, PAI-1, PLGF, TGF-α, TNF-α, uPA, VEGF-A, VEGF-C, VEGF-D, sEGFR, FGF-basic, Follistatin, G-CSF, HGF, sHER-2/neu, sIL-6Rα, Leptin, Osteopontin, PECAM-1, PDGF-AB/BB, Prolactin, SCF, sTIE-2, sVEGFR-1, and sVEGFR-2.

The antigens can be Kidney toxicity markers such as Calbindin, Clusterin, GST-π, IL-18, KIM-1, MCP-1, Albumin, B2M, Cystatin C, NGAL, Osteopontin, and TFF3.

The antigens can be cytokine, chemokines, and growth factors such as TGF-β1, TGF-β2, TGF-β3, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF, IL-1α, IL-2Rα, IL-3, IL-12 (p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, and TRAIL.

The antigens can be haptens, and other moieties comprising low molecular weight molecules, such as saccharides, oligosaccharides, polysaccharides, peptides, mucins, toxins, and allergens (pollen, egg white).

The antigens can be allergens, such as food, grass, tree, weed, insect, mold, epidermal, or dust mite allergens. The antigens can be animal, shellfish, milk (see http://en.wikipedia.org/wiki/Milk), soy (see http://en.wikipedia.org/wiki/Soy), eggs (see http://en.wikipedia.org/wiki/Egg_(food)), wheat (see http://en.wikipedia.org/wiki/Wheat), peanuts (see http://en.wikipedia.org/wiki/Peanuts), tree nuts (see http://en.wikipedia.org/wiki/Tree_nuts), fish (see http://en.wikipedia.org/wiki/Fish), or seed allergens.

The antigens can be involved in autoimmune diseases, and are preferably human antigens. Preferred antigens are Thyroglobulin, thyroid peroxidase, Rheumatoid Factor (IgA, IgG, IgM), Citrullinated Peptide, histtidyl-tRNA synthetase (Jo-1), PM-Scl, complement, complement components C4c and C3c; total complement (CH50), Sm/RNP, SS-A and –B (Sjogren a and b), Scl-70, topoisomerase, Ribosomal P protein, Actin, Tissue transglutaminase, Myeloperoxidase, Proteinase-3, M-type phospholipase A, liver kidney microsomal antigen type-1 (anti-LKM1), anti-liver cytosol type 1 (anti-LC1), SMA, pyruvate dehydrogenase complex E2 subunit, nuclear pore gp210, nuclear body sp100, High mobility group box 1, Collagen, Collagen type II, Collagen type IV, Collagen VII, hLAMP-2, voltage-gated potassium channel (VGKC), N-methyl-D-aspartic acid receptor (NMDAR), Hu, Yo, Ri, Ma 1/2, CRMP-5, Ampiphysin, heat shock proteins (HSPs), HSP60, clotting factor VIII, proliferating cell nuclear antigen (PCNA), DNA polymerase,

recoverin, α-enolase, transducin-α, myelin basic protein (MBP), factor VIII (FVIII), factor IX (FIX), Amyloid β peptide (Aβ), myelin oligodendrocyte glycoprotein (MOG; see www.discoverymedicine.com/category/species-and-cell-types/human/brain/oligodendrocyte/), myelin basic protein (MBP), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin-(IL)-6, IL-17A, IL-17F, IL-22, histone H1,cytochrome P450, myelin, aquaporin-4 (AQP-4), Ro, La, muscle-specific kinase (MuSK), actinin-α4, α-enolase, elongation factor 2, glutamate receptor (NR2), laminin, myosin, smD1, PM-Scl, fibrillarin, RNA polymerase I, NOR), Scl-70, cyclin I and II, liver-specific protein, formiminotransferase-cyclodeaminase (LC-1), soluble liver antigen/liver-pancreas antigen (SLA/LP), desmoglein 1 or 3, envoplakin, BP180 protein, BP230 protein, myelin-associated glycoprotein, glutamic acid decarboxylase (GAD), tyrosine phosphatase (IA2), insulin, H+/K+-ATPase, elastase, kathepsin G, kactoferrin, BPI, rPAg1 (CUZD1) and rPAg2 (GP2), Phospholipase A2 receptor, Gliadin-analogue fusion peptide, glutamic acid decarboxylase (GAD65), and thyrotropin receptor antigens.

The antigens can be toxins, for example, the botulinum neurotoxins, the Clostridium perfringens epsilon toxin, ricin, saxitoxin, shigatoxin, tetrodotoxin, staphylococcal enterotoxins, etc. Mucins are also well known in the art. MUC5AC, MUC5B and MUC2 are examples thereof. In particular, they can be naturally-occurring polysaccharides such as Group B steptococcal and pneumococcal capsular polysaccharides (including type III), *Pseudomonas aeruginosa* mucoexopolysaccharide, and capsular polysaccharides (including fisher type I), and *Haemophilus influenzae* polysaccharides.

In a preferred embodiment, said antigen or epitope is expressed by a virus which is selected from the group consisting of: the influenza virus, the hepatitis A virus, the Hepatitis B virus, the Hepatitis C virus, the Hepatitis E virus, the Hepatitis G virus, the HIV virus, the yellow fever virus, the dengue virus, the Japanese encephalitis virus, the tick-borne encephalitis virus, the Usutu or West Nile viruses, the Rift Valley fever or Toscana viruses, the chikungunya virus, the Omsk hemorrhagic fever virus, the Alkhurma hemorrhagic fever virus, the Kyasanur forest disease virus, the Venezuelan Equine Encephalitis virus, the Eastern Equine Encephalitis virus, the Western Equine Encephalitis virus, the Ross River virus, the Mayaro virus, the respiratory synticial virus, the Rocio virus, the

morbillivirus, the Murray Valley encephalitis virus, the Wesselbron virus, the Zika virus, the lymphocytic choreomeningitis virus, the Ebola virus, the Marburg virus, the Crimean-Congo hemorrhagic fever virus, the Lassa virus, the Junin virus, the Machupo virus (http://en.wikipedia.org/wiki/Machupo_virus), the Sabia virus, the Guanarito virus, the mumps virus, the rabies virus, the rubella virus, the varicella zoster virus, the herpes simplex virus types 1 and 2, more generally an alphavirus, an adenovirus, a hantavirus, an echovirus, a rotavirus, a flavivirus, a rhinovirus, an orthobunyavirus, a poliovirus, a poxvirus (e.g., smallpox (http://en.wikipedia.org/wiki/Smallpox), monkeypox, blackpox, or canarypox), an orthomyxovirus (e.g., Influenzavirus A, Influenzavirus B, Influenzavirus C, http://en.wikipedia.org/wiki/Influenza_A_virus; http://en.wikipedia.org/wiki/Influenzavirus_B; http://en.wikipedia.org/wiki/Influenzavirus_C), a picornavirus, (e.g., foot and mouth disease virus, FMDV, http://en.wikipedia.org/wiki/Picornavirus), a human parvovirus, an enterovirus, a coronavirus (e.g., SARS or human betacoronavirus), a human papillomavirus, a herpes virus, the human cytomegalovirus, the Epstein-Barr virus, a paramyxovirus (http://en.wikipedia.org/wiki/Paramyxovirus), such as the parainfluenzae viruses from types 1, 2 and 3, or any identified virus.

In a preferred embodiment, said antigen or epitope is expressed by a virus belonging to a family which is selected from the group consisting of: the *Flaviviridae* (Dengue, Yellow fever, West Nile, Japanese encephalitis, Tick-Borne Encephalitis, Hepatitis C viruses), the *Togaviridae* (Chikungunya, Ross River, Mayaro, Western Equine encephalitis, Eastern Equine Encephalitis, Venezuela Equine Encephalitis viruses), the *Bunyaviridae* (Crimean-Congo hemorrhagic fever, Rift Valley Fever, Schmallenberg viruses), the *Caliciviridae* (Hepatitis E virus), the *Arenaviridae* (Lassa) and the *Filoviridae* (Ebola, Marburg).

In another preferred embodiment, said antigen or epitope is expressed by a parasitic protozoa (such as those from the Leishmania genus, or *Toxoplasma Gondii*, *Entamoeba histolytica*, *Plasmodium falciparum* (e.g., MSP1(19)+ AMA-1(III) antigens, separately or co-expressed), *Pneumocystis carinii*, *Cryptosporidium*, *Naegleria fowleri* (http://en.wikipedia.org/wiki/Naegleria_fowleri), *or Giardia lamblia*), worms (such as *Trypanosoma cruzi*, nematodes, cestodes, or trematodes), or arthropods (such as crustaceans, insects, arachnids).

In another preferred embodiment, said antigen or epitope is expressed by an infectious bacterium, for example of the genera *Salmonella*, *Shigella*, *Streptococcus*, *Staphylococcus*, *Mycoplasma*, *Diphteriae*, *Leptospirosa*, *Rickettsia* or *Escherichia*. In a further preferred embodiment, the said bacterium belongs to one of the species selected from *Treponema pallidum (http://en.wikipedia.org/wiki/Treponema_pallidum)*, *Helicobacter pylori*, *Campylobacter jejuni*, *Borrelia burgdorferi*, *Legionella pneumophila*, *Yersinia pestis*, *Yersinia enterocolitica*, *Brucella abortus*, *Burkholderia mallei (http://en.wikipedia.org/wiki/Burkholderia_mallei)*, *Burkholderia pseudomallei (http://en.wikipedia.org/wiki/Burkholderia_pseudomallei)*, *Chlamydia pneumonia*, *Francisella tularensis*, *Mycoplasma pneumonia*, *H. influenzae*, *S. pneumoniae*, *Klebsiella pneumoniae*, *S. aureus*, *Bacillus anthracis*, *Clostridium botulinum (http://en.wikipedia.org/wiki/Clostridium_botulinum)*, *Clostridium perfringens (http://en.wikipedia.org/wiki/Clostridium_perfringens)*, *Listeria monocytogenes*, *Bordetella pertussis*, *Clostridium tetani*, *Neisseria meningitidis (http://en.wikipedia.org/wiki/Neisseria_meningitidis)*, *S. epidermidis*, *N. meningiditis*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, *Coxiella burnetii*, *Rickettsia prowazekii (http://en.wikipedia.org/wiki/Rickettsia_prowazekii)*, *Chlamydia psittaci http://en.wikipedia.org/wiki/Chlamydia_psittaci)*, *Leptospirosa interrogans* and *E.coli*, *(e.g. E coli O157:H7(http://en.wikipedia.org/wiki/Escherichia_coli_O157:H7))*.

In another preferred embodiment, said antigen or epitope is expressed by a fungus or yeast (e.g. from the species *Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Pneumocystis*, or *Stachybotrys*).

The antigen or epitope can be from Prion protein (PrPc).

The epitope can be from a specific immunoglobulin, such as IgD, IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgE, or IgG total.

Antigens usually present several surface features that can act as points of interaction for specific antibodies. Any such distinct molecular feature constitutes an epitope. As used herein, the term "epitope" therefore designates a particular molecular surface feature of an antigen, for example a fragment of an antigen, which is capable of being bound by at least one antibody. On a molecular level, an epitope therefore corresponds to a particular molecular surface feature of an antigen (for example a fragment of an antigen) which is

recognized and bound by a specific antibody. In the context of the present invention, the "fusion proteins" contain at least one epitope that is recognized by a target antibody. Preferably, said fusion proteins contain whole antigens, comprising several epitopes (http://en.wikipedia.org/wiki/Epitope). These epitopes can be linear or conformational epitopes. As used herein, a linear (or sequential) epitope is an epitope that is recognized by antibodies (http://en.wikipedia.org/wiki/Antibody) by its linear sequence of amino acids (http://en.wikipedia.org/wiki/Amino_acid), or primary structure (http://en.wikipedia.org/wiki/Primary_structure). In contrast, a conformational epitope (http://en.wikipedia.org/wiki/Conformational_epitope) is recognized by its specific three-dimensional shape. Preferably, the fusion proteins of the invention contain conformational epitopes, as most polyclonal antibodies recognize same.

It is important however that such antigens do not present cross-reactive epitopes, i.e. epitopes that are recognized by non-specific antibodies that will bind thereto. If it was the case, the specificity of the method of the invention would be decreased.

In a preferred embodiment, said epitope is present on a viral protein, preferably an EDIII protein, which is selected from the group consisting of dengue virus 1, dengue virus 2 , dengue virus 3, dengue virus 4, West Nile virus, yellow fever virus, Japanese encephalitis virus, Zika virus, Wesselbron virus, Rocio virus, Murray encephalitis virus, Saint-Louis encephalitis virus, Japanese encephalitis virus of genotype 1, Japanese encephalitis virus of genotype 2, Japanese encephalitis virus of genotype 4, Japanese encephalitis virus of genotype 5, and Rabensburg virus proteins.

In a more preferred embodiment, said epitope is present on a viral protein which is selected from the group consisting of: the EDIII protein of the dengue virus 1 encoded by SEQ ID NO:3, the EDIII protein of the dengue virus 2 encoded by SEQ ID NO:4, the EDIII protein of the dengue virus 3 encoded by SEQ ID NO:5, the EDIII protein of the dengue virus 4 encoded by SEQ ID NO:6, the EDIII protein of the West Nile virus encoded by SEQ ID NO:7, the EDIII protein of the yellow fever virus encoded by SEQ ID NO:8, the EDIII protein of the Japanese encephalitis virus encoded by SEQ ID NO:9, the EDIII protein of the Zika virus encoded by SEQ ID NO:10, the EDIII protein of the Wesselbron virus encoded by SEQ ID NO:11, the EDIII protein of the Rocio virus encoded by SEQ ID NO:12, the EDIII protein of the Murray encephalitis virus encoded

by SEQ ID NO:13, the EDIII protein of the Saint-Louis encephalitis virus encoded by SEQ ID NO:14, the EDIII protein of the Japanese encephalitis virus of genotype 1 encoded by SEQ ID NO:54, the EDIII protein of the Japanese encephalitis virus of genotype 2 encoded by SEQ ID NO:55, the EDIII protein of the Japanese encephalitis virus of genotype 4 encoded by SEQ ID NO:56, the EDIII protein of the Japanese encephalitis virus of genotype 5 encoded by SEQ ID NO:57, and the EDIII protein of the Rabensburg virus encoded by SEQ ID NO:58 and the viral protein of HIV1, of HIV2, of the Hepatitis B virus, of the Hepatitis C virus, of the Hepatitis E virus, of the West-Nile virus and of oncogenic HPV strains such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

In a preferred embodiment, the first and second epitopes (or antigens) that are fused with the hAGT enzyme in the fusion proteins used in the method of the invention belong to the same taxonomic level, i.e. they belong to the same family (e.g. the *Flaviviridae* family, the *Bunyaviridae* family, the *Arenaviridae* family or the *Filoviridae* family) or genus or species, but which have different serotypes. In other words, the said first and second epitopes can be expressed by closely related viruses, *e.g.* belong to the same family, genus or species but having different serotypes such as the dengue virus 1, 2, 3, or 4.

Alternatively, in another preferred embodiment, said first and second epitopes (or antigens) belong to unrelated biological families or genus or species.

The epitopes can be viral epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from viral epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from Dengue virus (serotypes 1, 2, 3, or 4), West Nile virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, Saint-Louis encephalitis virus, Murray Valley encephalitis virus, Wesselsbron virus, Zika virus, Rocio virus, Usutu virus, Rift Valley fever virus, and chikungunya virus epitopes. More preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from Dengue virus (serotypes 1, 2, 3, or 4), West Nile virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, Saint-Louis encephalitis virus, Murray Valley encephalitis virus, Wesselsbron virus, Zika virus, Rocio virus, Usutu virus, Rift Valley fever virus, chikungunya virus, SARS

coronavirus, human coronavirus, Hepatitic C virus (HCV), Hepatitis E virus (HEV), or Schmallenberg virus epitopes.

Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from Dengue virus (serotypes 1, 2, 3, or 4), West Nile virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, Saint-Louis encephalitis virus, Murray Valley encephalitis virus, Wesselsbron virus, Zika virus, Rocio virus, Usutu virus, Rift Valley fever virus, chikungunya virus, SARS coronavirus, human coronavirus, Hepatitic C virus (HCV), Hepatitis E virus (HEV), Schmallenberg virus, HIV1, HIV2, Hepatitis B virus, and HPV epitopes. The at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes can alternately be chosen from any group of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the above-mentioned viruses.

Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Flaviviridae* epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Togaviridae* eitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Bunyaviridae* epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Caliciviridae* epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Arenaviridae* epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Filoviridae* epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Poxviridae* epitopes.

The epitopes can be bacterial epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from bacterial epitopes. Preferably, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes are selected from *Treponema pallidum (http://en.wikipedia.org/wiki/Treponema_pallidum), Helicobacter pylori, Campylobacter jejuni, Borrelia burgdorferi, Legionella pneumophila, Yersinia pestis (http://en.wikipedia.org/wiki/Yersinia_pestis), Yersinia enterocolitica, Brucella abortus, Burkholderia mallei, Burkholderia pseudomallei, Chlamydia pneumonia, Francisella tularensis, Mycoplasma pneumonia, H. influenzae, S. pneumoniae, Klebsiella pneumoniae, S. aureus, Bacillus anthracis, Clostridium botulinum, Clostridium perfringens, Listeria monocytogenes, Bordetella pertussis, Clostridium tetani, Neisseria meningitidis, S. epidermidis, N. meningiditis, Pseudomonas aeruginosa, Chlamydia*

trachomatis, *Mycobacterium tuberculosis*, *Coxiella burnetii*, *Rickettsia prowazekii*, *Chlamydia psittaci*, *Leptospirosa interrogans* and *E.coli* (e.g., *E.coli*, O157:H7) epitopes. The at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the epitopes can alternately be chosen from any group of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of the above-mentioned bacteria.

Preferably, at least 1, 2, 3, or 4 epitopes are selected from Leptospira epitopes, most preferably LruA, LruB, HbpA, or LipL32 epitopes.

In some embodiments, the first or second fusion protein comprises at least one amino acid sequence encoded by any of SEQ ID NOs 3-14 and 54-58, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof.

In some embodiments, the first or second fusion protein comprises at least one amino acid sequence selected from SEQ ID NOs: 155, 157, 159, 161, 163, 165, 167, 171, 173, 175, 177, and 179, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof.

In some embodiments, the first or second fusion protein comprises at least one amino acid sequence encoded by any of SEQ ID NOs 3-14 and 54-58 and/or comprises at least one amino acid sequence selected from SEQ ID NOs: 155, 157, 159, 161, 163, 165, 167, 171, 173, 175, 177, and 179 or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof.

In some embodiments, the first or second fusion protein comprises at least one amino acid sequence selected from a mutated N protein from SARS coronavirus, an RBD of S protein from SARS coronavirus, a N protein from human betacoronavirus, an S1 protein from human betacoronavirus, an RBD of S protein from human betacoronavirus, a mutated C protein from Hepatitis C (HCV) virus, an HEV core antigen, an HCV core antigen, a short form of the HCV core antigen, the NSs protein from Schmallenberg virus.

In some embodiments, the first or second fusion protein comprises at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids of the viral amino acid sequences shown in Figures 12-16 and 21-24.

In some embodiments, the first or second fusion protein comprises at least one amino acid sequence selected from a LruA, LruB, or LipL32 protein. Preferably, the first or second fusion protein comprises at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids of the LruA, LruB, or LipL32 amino acid sequences shown in Figures 17, 18, or 19.

In some embodiments, the epitope comprises at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive LruA, LruB, or LipL32 amino acids of the sequences shown in Figures 17, 18, or 19.

The immunoassays of the invention can detect a large number of antibodies at the same time, which are known or unknown. By "large number", it is herein understood at least 5, more preferably at least 15, more preferably at least 50 and even more preferably at least 100 antibodies. Therefore, in a preferred embodiment, the assay method of the invention is used to detect at least 5, more preferably at least 15, and preferably at least 50 and even more preferably at least 100 target antibodies in a biological sample from a subject. It is of no relevance for the method of the invention whether the particular antibodies are properly characterized, since the procedure relies only on the detection of the presence of said antibodies, and not on their nature.

In a preferred embodiment of the invention, the said first and second fusion proteins that are coupled with the said first and second solid supports are selected from the group consisting of:

- SEQ ID NO:21 (corresponding to the fusion protein [SNAP-DEN1.EDIII])
- SEQ ID NO:42 (corresponding to the fusion protein [SNAP-SBV.N])
- SEQ ID NO:49 (corresponding to the fusion protein [SNAP-EV71.VP1])
- SEQ ID NO:51 (corresponding to the fusion protein [JE.sE-SNAP])
- SEQ ID NO:53 (corresponding to the fusion protein [SNAP-JE-1.EDIII])
- SEQ ID NO:60 (corresponding to the fusion protein [SNAP- JE-2.EDIII])
- SEQ ID NO:62 (corresponding to the fusion protein [SNAP- JE-4.EDIII])
- SEQ ID NO:64 (corresponding to the fusion protein [SNAP- JE-5.EDIII])
- SEQ ID NO:66 (corresponding to the fusion protein [SNAP- RabV.EDIII])
- SEQ ID NO:68 (corresponding to the fusion protein [SNAP-flavivirus.EDIII])

- SEQ ID NO:70 (corresponding to the fusion protein [RR.sE2 –SNAP])
- SEQ ID NO:72 (corresponding to the fusion protein [MAY.sE2–SNAP])
- SEQ ID NO:74 (corresponding to the fusion protein [WEE.sE2–SNAP])
- SEQ ID NO:76 (corresponding to the fusion protein [EEE.sE2–SNAP])
- SEQ ID NO:78 (corresponding to the fusion protein [VEE.sE2–SNAP])
- SEQ ID NO:80 (corresponding to the fusion protein [SNAP- AKA.N])
- SEQ ID NO:82 (corresponding to the fusion protein [SNAP- AIN.N])
- SEQ ID NO:84 (corresponding to the fusion protein [SNAP- SHA.N])
- SEQ ID NO:86 (corresponding to the fusion protein [SNAP- huCOV.N])
- SEQ ID NO:88 (corresponding to the fusion protein [SNAP- huCOV.S])
- SEQ ID NO:90 (corresponding to the fusion protein [SNAP- HCV.C])
- SEQ ID NO:92 (corresponding to the fusion protein [SNAP- MSP+AMA])
- SEQ ID NO:94 (corresponding to the fusion protein [SNAP- HbpA1])
- SEQ ID NO:96 (corresponding to the fusion protein [SNAP- MUB40])
- SEQ ID NO:98 (corresponding to the fusion protein [SNAP- moCLEC5A])
- SEQ ID NO:100 (corresponding to the fusion protein [SNAP- huCLEC5A])
- SEQ ID NO:102 (corresponding to the fusion protein [SNAP- cxVAGO])
- SEQ ID NO:104 (corresponding to the fusion protein [SNAP- aaVAGO])
- SEQ ID NO:109 (corresponding to the fusion protein [SNAP- CCHF.N])
- SEQ ID NO:111 (corresponding to the fusion protein [SNAP- EBO.N])
- SEQ ID NO:113 (corresponding to the fusion protein [SNAP- MAR.N])
- SEQ ID NO:115 (corresponding to the fusion protein [SNAP- LAS.N])
- SEQ ID NO:117 (corresponding to the fusion protein [SNAP- JUN.N])
- SEQ ID NO:119 (corresponding to the fusion protein [SNAP- MAC.N])
- SEQ ID NO:121 (corresponding to the fusion protein [SNAP- GUA.N])
- SEQ ID NO:123 (corresponding to the fusion protein [SNAP- SAB.N])
- SEQ ID NO:125 (corresponding to the fusion protein [SNAP- OMSK.EDIII])
- SEQ ID NO:127 (corresponding to the fusion protein [SNAP- KYA.EDIII])
- SEQ ID NO:129 (corresponding to the fusion protein [SNAP- ALK.EDIII])
- SEQ ID NO:131 (corresponding to the fusion protein [LAS.ectoGP1–SNAP])
- SEQ ID NO:133 (corresponding to the fusion protein [JUN.ectoGP1–SNAP])
- SEQ ID NO:135 (corresponding to the fusion protein [MAC.ectoGP1–SNAP])
- SEQ ID NO:137 (corresponding to the fusion protein [GUA.ectoGP1–SNAP])

- SEQ ID NO:139 (corresponding to the fusion protein [SAB.ectoGP1–SNAP])
- SEQ ID NO:141 (corresponding to the fusion protein [LAS.ectoGP2–SNAP])
- SEQ ID NO:143 (corresponding to the fusion protein [JUN.ectoGP2–SNAP])
- SEQ ID NO:145 (corresponding to the fusion protein [MAC.ectoGP2–SNAP])
- SEQ ID NO:147 (corresponding to the fusion protein [GUA.ectoGP2–SNAP])
- SEQ ID NO:149 (corresponding to the fusion protein [SAB.ectoGP2–SNAP]), and
- SEQ ID NO:151 (corresponding to the fusion protein [SNAP- HEV.C]).

Consequently, the *in vitro* method of the invention enables to detect target disease(s) that is (are) viral, bacterial, yeast or fungi-mediated infection. Preferably said viral infection is caused by a Papillomavirus or RNA viruses from the families of the *Flaviviridae* (Dengue, Yellow fever, West Nile, Japanese encephalitis, Tick-Borne Encephalitis, Hepatitis C viruses), the *Togaviridae* (Chikungunya, Ross River, Mayaro, Western Equine encephalitis, Eastern Equine Encephalitis, Venezuela Equine Encephalitis viruses), the *Bunyaviridae* (Crimean-Congo hemorrhagic fever, Rift Valley Fever, Schmallenberg viruses), the *Caliciviridae* (Hepatitis E virus), the *Arenaviridae* (Lassa http://en.wikipedia.org/wiki/Lassa_virus) and the *Filoviridae* (Ebola http://en.wikipedia.org/wiki/Ebola_virus, Marburg http://en.wikipedia.org/wiki/Marburg_virus). Preferably, said bacterial infection is caused by *Leptospirosa Interrogans*. Preferably, said infection is caused by *Plasmodium falciparum*.

As used herein, the term "biological sample" refers to any samples which have been obtained from a patient and which might contain antibodies. Preferably, said biological sample is a biological fluid, for example an unfiltered biological fluid such as urine, cerebrospinal fluid, pleural fluid, synovial fluid, peritoneal fluid, amniotic fluid, gastric fluid, blood, serum, plasma, lymph fluid, interstitial fluid, saliva, physiological secretions, tears, mucus, sweat, milk, semen, seminal fluid, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses. It also refers to an extract of tissues including biopsies of normal, malignant, and suspect tissues or any other constituents of the body which may contain antibodies. The said biological sample can be pre-treated prior to use, such as preparing plasma from blood, diluting viscous fluids, or the like; methods of treatment can involve filtration, distillation, concentration, inactivation of interfering compounds, and the addition of reagents. In a preferred embodiment, said biological

sample is chosen from whole blood, serum, plasma, urine, seminal fluid, cerebrospinal fluid and saliva.

AGT polypeptides

Any polypeptide having O^6-alkylguanine-DNA alkyltransferase activity can be used in the method of the present invention. For the purpose of the invention, these polypeptides will be referred to as "AGT polypeptides".

AGT irreversibly transfers the alkyl group from its substrate, O^6-alkylguanine-DNA, to one of its cysteine residues. A substrate analogue that rapidly reacts with AGT is O^6-benzyl-guanine, the second order rate constant being approximately 10^3 sec^{-1} M^{-1}.

In the context of the invention, a polypeptide is said to have "O^6-alkylguanine-DNA alkyltransferase activity" (or "AGT activity") if it is capable of irreversibly transferring an alkyl group from a O^6-alkylguanine-containing molecule to one of its own cysteine residues. The "O6-alkylguanine-DNA alkyltransferase activity" of the said polypeptide can be demonstrated by, for example, contacting known labeled O^6-benzyl-guanine derivatives and monitoring the transfer of said label on to the tested polypeptide. If the assay is performed *in cellulo* or in cell extracts, the reaction of the endogenous AGT of the host cells should be controlled, so that endogenous AGT does not interfere with the said polypeptide. Therefore, known AGT-deficient cell lines are preferably used. Assays for identifying AGT activity are now well described. Several O^6-benzyl-guanine derivatives are commercially available (O^6-benzyl-guanine is distributed for example by Santa Cruz biotechnology, and fluorescently-labeled O^6-benzyl-guanine derivatives can be obtained from New England Biolabs NEB). Some of these assays are disclosed in WO 2005/085470 and in WO 2004/031405.

In the context of the invention, the "catalytic domain" of the AGT polypeptide corresponds to the active site of said enzyme, or, in other words, to the part of the enzyme at which the transfer of the alkyl group from its substrate, O^6-alkylguanine-DNA, to a reactive cysteine residue, occurs. In the structure of hAGT bound with O^6-benzylguanine in its active site, four amino acids are in proximity of either the benzyl ring (Pro140, Ser159, Gly160), or could make contact with the N9 of the nucleobase (Asn157). Mutations at position Pro140 and Gly160 have previously been shown to affect the

reaction of hAGT with O^6-benzylguanine (Xu-Welliver et al., Biochemical Pharmacology 1999): a proline at position 140 is believed to be essential for its interaction with the benzyl ring, and the mutation Gly160Trp has been shown to increase the reactivity of hAGT towards O^6-benzylguanine.

In a preferred embodiment, the AGT polypeptide having O^6-alkylguanine-DNA alkyltransferase activity is the human AGT polypeptide (referenced as NP_002403.2) of sequence SEQ ID NO: 1, the mouse AGT identified as NP_032624.1 (SEQ ID NO: 18), the rat MGMT identified as NP_036993.1 (SEQ ID NO: 19) or a homologous sequence thereof, said homologous sequence having O^6-alkylguanine-DNA alkyltransferase activity.

As used herein, the term "homologous" refers to sequences that have sequence similarity. The term "sequence similarity", in all its grammatical forms, refers to the degree of identity or correspondence between nucleic acid or amino acid sequences. In the context of the invention, two amino acid sequences are "homologous" when at least about 80 %, alternatively at least about 81 %, alternatively at least about 82 %, alternatively at least about 83 %, alternatively at least about 84 %, alternatively at least about 85 %, alternatively at least about 86 %, alternatively at least about 87 %, alternatively at least about 88 %, alternatively at least about 89 %, alternatively at least about 90 %, alternatively at least about 91 %, alternatively at least about 92 %, alternatively at least about 93 %, alternatively at least about 94 %, alternatively at least about 95 %, alternatively at least about 96 %, alternatively at least about 97 %, alternatively at least about 98 %, alternatively at least about 99 % of the amino acids are similar. Preferably the similar or homologous polypeptide sequences are identified by using the algorithm of Needleman and Wunsch.

Preferably, the homologous sequence to the AGT enzyme shares at least 64 % amino acid sequence identity, preferably at least about 65 % amino acid sequence identity, alternatively at least about 66 % amino acid sequence identity, alternatively at least about 67 % amino acid sequence identity, alternatively at least about 68 % amino acid sequence identity, alternatively at least about 69 % amino acid sequence identity, alternatively at least about 70 % amino acid sequence identity, alternatively at least about 71 % amino acid sequence identity, alternatively at least about 72 % amino acid sequence identity, alternatively at least about 73 % amino acid sequence identity, alternatively at least about 74 % amino acid sequence identity, alternatively at least about 75 % amino acid sequence identity,

alternatively at least about 76 % amino acid sequence identity, alternatively at least about 77 % amino acid sequence identity, alternatively at least about 78 % amino acid sequence identity, alternatively at least about 79 % amino acid sequence identity, alternatively at least 80 % amino acid identity, alternatively at least about 81 % amino acid sequence identity, alternatively at least about 82 % amino acid sequence identity, alternatively at least about 83 % amino acid sequence identity, alternatively at least about 84 % amino acid sequence identity, alternatively at least about 85% amino acid sequence identity, alternatively at least about 86 % amino acid sequence identity, alternatively at least about 87 % amino acid sequence identity, alternatively at least about 88 % amino acid sequence identity, alternatively at least about 89 % amino acid sequence identity, alternatively at least about 90 % amino acid sequence identity, alternatively at least about 91 % amino acid sequence identity, alternatively at least about 92 % amino acid sequence identity, alternatively at least about 93 % amino acid sequence identity, alternatively at least about 94 % amino acid sequence identity, alternatively at least about 95 % amino acid sequence identity, alternatively at least about 96 % amino acid sequence identity, alternatively at least about 97 % amino acid sequence identity, alternatively at least about 98 % amino acid sequence identity and alternatively at least about 99 % amino acid sequence identity with SEQ ID NO: 1. In a preferred embodiment, an homologous sequence of SEQ ID NO: 1 is at least 64 %, preferably 70 %, and more preferably 80 % identical to SEQ ID NO: 1.

In a preferred embodiment, the said homologous polypeptide is a fragment or a mutant of the hAGT polypeptide of SEQ ID NO: 1, said fragment or mutant having a O^6-alkylguanine-DNA alkyltransferase activity.

Said fragments can have a size of at least 50, preferably 100, and more preferably 150 amino acids, and contain at least the "catalytic domain" of the AGT polypeptide as defined above, which is responsible of the O^6-alkylguanine-DNA alkyltransferase activity of the AGT enzyme. These fragments can be obtained using common techniques which are known by the skilled person.

Different mutant enzymes derived from native AGT have been described so far (Lim A. et al, 1996; Daniels D.S. et al, 2000; Juillerat A. et al, 2003, WO 2005/085470, WO 2004/031405). In particular, a mutant protein of 20 kDa containing the mutations Cys62Ala, Lys125Ala, Ala127Thr, Arg128Ala, Gly131Lys, Gly132Thr, Met134Leu,

Arg135Ser, Cys150Ser, Asn157Gly, Ser159Glu truncated at amino acid 182 has been obtained (the so-called "AGT26" mutant in WO 2005/085470, also called "SNAP 26" in WO 2006/114409). This particular mutant "SNAP26" has been shown to have enhanced labelling activity.

In the context of the present invention, the sequence of a more preferred AGT polypeptide contains the mutations described in WO 2005/085470, which positions can be easily transposed in view of SEQ ID NO: 1, the starting methionine residue of SNAP26 corresponding to the methionine residue in position 32 of SEQ ID NO: 1 (31 amino acids should therefore be added to the positions disclosed in WO 2005/085470 so as to obtain the corresponding ones in SEQ ID NO: 1).

In a preferred embodiment, the AGT homologous sequence useful in the invention corresponds to the native AGT sequence of SEQ ID NO: 1, in which between 1 and 30, preferably between 6 and 25, and in particular 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 amino acids are substituted by other amino acids, and/or 1 to 40, preferably 1 to 20, in particular 10 to 20 amino acids, more preferably 15 amino acids at the C-terminus are deleted.

In a more preferred embodiment, the AGT homologous sequence contains the following mutations as compared with SEQ ID NO: 1:

(A) Lys31 replaced by Arg, or Met32 replaced by Ser, or Cys93 replaced by Ala, or Lys156 replaced by Ala, or Ala158 replaced by Thr, or Arg159 replaced by Ala, or Gly162 replaced by Lys, or Gly163 replaced by Thr, or Met165 replaced by Leu, or Arg166 replaced by Ser, or Cys181 replaced by Ser, or Asn188 replaced by Gly, or Ser190 replaced by Glu, or Gly214 replaced by Pro, or Ser215 replaced by Ala, or Ser216 replaced by Gly, or Gly217 replaced by Ile, or Leu218 replaced by Gly, or Gly220 replaced by Pro, or Ala221 replaced by Gly, or Trp222 replaced by Ser, or

(B) Lys31-Met32 replaced by Arg-Ser, or Ala158-Arg159 replaced by Thr-Ala, or Gly162-Gly163 replaced by Lys-Thr, or Met165-Arg166 replaced by Leu-Ser, or Gly162-Gly163/Met165-Arg166 replaced by Lys-Thr/Leu-Ser, or Asn188/Ser190 replaced by Gly/Glu, or Gly214-Ser215-Ser216-Gly217-Leu218 replaced by Pro-Ala-Gly-Ile-Gly, or Gly220-Ala221-Trp222 replaced by Pro-Gly-Ser, preferably in combination with any other amino acid replacements cited in (A), or

(C) Truncation after Leu223 (amino acids 224-238 are deleted), preferably in combination with any other amino acid replacement cited in (A) or (B).

Preferred AGT homologous sequences are those being truncated after Leu223.

Preferred AGT homologous sequences are those wherein two out of the modifications (B) are present, and optionally truncation after Leu223.

Preferred AGT homologous sequences are those wherein three out of the modifications (B) are present, and optionally truncation after Leu223.

Preferred AGT homologous sequences are those wherein four out of the modifications (B) are present, and optionally truncation after Leu223.

Preferred AGT homologous sequences are those wherein five out of the modifications (B) are present, and optionally truncation after Leu223.

Preferred AGT homologous sequences are those wherein six out of the modifications (B) are present, and optionally truncation after Leu223.

Other preferred AGT homologous sequences are those containing a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 mutations chosen among the modifications disclosed in (A), and optionally truncated after Leu223.

In a far more preferred embodiment, the AGT polypeptide of the invention is the SNAP mutant of SEQ ID NO: 2, which is homologous to the hAGT enzyme and contains the mutations Lys31Arg, Met32Ser, Cys93Ala, Lys156Ala, Ala158Thr, Arg159Ala, Gly162Lys, Gly163Thr, Met165Leu, Arg166Ser, Cys181Ser, Asn188Gly, Ser190Glu, Gly214Pro, Ser215Ala, Ser216Gly, Gly217Ile, Leu218Gly, Gly220Pro, Ala221Gly, Trp222Ser and truncation after Leu223 as compared with SEQ ID NO: 1. The SNAP mutant of SEQ ID NO: 2 shares 77% homology with the amino acid sequence of the human 6-methylguanine-DNA-methyltransferase (NP_002403.2, SEQ ID NO: 1), and 70 % homology with the amino acid sequence of the mouse 6-methylguanine-DNA-methyltransferase (NP_032624.1, SEQ ID NO: 18).

In an even more preferred embodiment, the AGT enzyme is the SNAP mutant protein of SEQ ID NO: 2 or a homologous thereof, having O^6-alkylguanine-DNA alkyltransferase activity. Preferably, said homologous sequence to the SNAP mutant protein is at least identical at more than 80 %, preferably 81 %, more preferably 82 %, more preferably 83 %, more preferably 84 %, more preferably 85 %, preferably 86 %, more preferably 87 %, more preferably 88 %, more preferably 89 %, more preferably 90 %, more preferably 91 %, more preferably 92 %, more preferably 93 %, more preferably 94 %, more preferably 95 %, more preferably 96 % to the and even more preferably 97 % to the SNAP mutant protein of sequence SEQ ID NO: 2, and has O^6-alkylguanine-DNA alkyltransferase activity as defined above.

Said homologous polypeptides having O^6-alkylguanine-DNA alkyltransferase activity can be produced using protein engineering techniques known to the skilled person and/or using molecular evolution to generate and select new O^6-alkylguanine-DNA alkyltransferases. Such techniques are e.g. targeted mutagenesis, phage display methods, saturation mutagenesis, error prone PCR to introduce variations anywhere in the sequence, DNA shuffling used after saturation mutagenesis and/or error prone PCR, or family shuffling using genes from several species.

In the most preferred embodiment, the AGT polypeptide used in the method of the invention is the SNAP mutant of SEQ ID NO: 2.

AGT Substrates

The AGT enzyme irreversibly transfers the alkyl group from its substrate, O^6-alkylguanine-DNA, to one of its cysteine residues. However, substitutions of O^6-benzylguanine at the C4 of the benzyl ring do not significantly affect the reactivity of AGT against O^6-benzylguanine derivatives. This property has been used to transfer a label attached to the C4 of the benzyl ring to AGT (see WO 2004/031404 and WO 2005/085470).

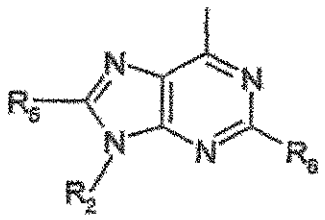
A number of O^6-benzylguanine derivatives have been shown to react with the AGT enzyme by transferring their benzyl group to the active site cysteine of the AGT enzyme (cf. Damoiseaux et al., *ChemBiochem.*, 2001, WO 2004/031404 and WO 2005/085470).

In a preferred embodiment, the AGT substrates used in the method of the invention are O^6 benzyl guanine derivatives having the formula I:

R1-X-CH_2-R3-R4-Y

wherein:

- R1 is a group recognized by said AGT polypeptide as a substrate, such as a heteroaromatic group containing 1 to 5 nitrogen atoms, and preferably a purine radical of the formula:



wherein R5 is hydrogen, halogen, e. g. chloro or bromo, trifluoromethyl, or hydroxy; R6 is hydrogen, hydroxy or unsubstituted or substituted amino; and R2 is hydrogen, an alkyl of 1 to 10 carbon atoms, or a saccharide moiety;

- X is an oxygen or sulfur atom; preferably an oxygen atom;

- R3 is an aromatic or a heteroaromatic group, or an optionally substituted unsaturated alkyl, cycloalkyl or heterocyclyl group with the double bond connected to CH_2; preferably a phenyl, e.g. a phenyl substituted by R4 in para or meta position,

- R4 is a linker moiety,

- Y is a reactive group, preferably an amino group.

In a preferred embodiment, said linker moiety R_4 is a flexible linker. Linker units are chosen in the context of the envisioned application, i.e. in the transfer of the substrate to a fusion protein comprising AGT. The linker does not interfere with the reaction with AGT nor with the target antibody.

For example, it can be a straight or branched chain alkylene group with 1 to 20 carbon atoms, preferably 5 to 15 carbon atoms, wherein :

(a) one or more carbon atoms are replaced by oxygen, in particular wherein every third carbon atom is replaced by oxygen, e.g. a poylethyleneoxy group with 1 to 5 ethyleneoxy units;

(b) one or more carbon atoms are replaced by nitrogen carrying a hydrogen atom, and the adjacent carbon atoms are substituted by oxo, representing an amide function -NH-CO-;

(c) one or more carbon atoms are replaced by oxygen, and the adjacent carbon atoms are substituted by oxo, representing an ester function -O-CO-;

(d) the bond between two adjacent carbon atoms is a double or a triple bond, representing a function -CH=CH- or -C≡C-;

(e) one or more carbon atoms are replaced by a phenylene, a saturated or unsaturated cycloalkylene, a saturated or unsaturated bicycloalkylene, a bridging heteroaromatic or a bridging saturated or unsaturated heterocyclyl group;

(f) two adjacent carbon atoms are replaced by a disulfide linkage -S-S-; or a combination of two or more, especially two or three, alkylene and/or modified alkylene groups as defined under (a) to (f) hereinbefore, optionally containing substituents.

Substituents considered are e.g. lower alkyl, e.g. methyl, lower alkoxy, e.g. methoxy, lower acyloxy, e.g. acetoxy, or halogenyl, e.g. chloro.

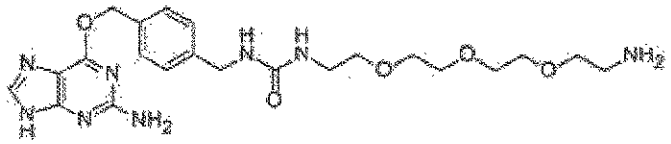
In a preferred embodiment, R4 is a polyethyleneoxy group with 1 to 8 ethyleneoxy units, further comprising one to four nitrogen atoms carrying a hydrogen atom, which adjacent carbon atoms are substituted by oxo, representing an amide function -NH-CO-.

In a more preferred embodiment, R4 is $-CH_2-NH-CO-NH-[C_2H_4-O]_n-$, wherein n is comprised between 1 to 8, preferably 2 to 6, and is most preferably 3.

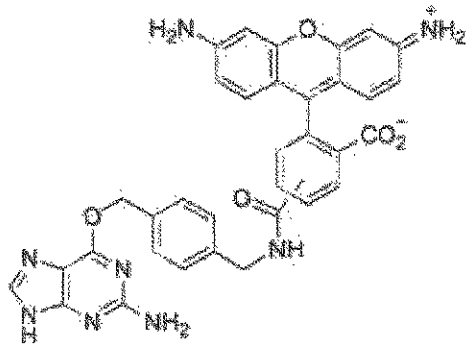
In a preferred embodiment, said reactive group is a functional group that facilitates the attachment and bonding of the substrate on the solid support. Such functional groups are well-known in the art. They include amine, activated esters, acrylamides, acyl azides, acyl halides, acyl nitriles, aldehydes, ketones, alkyl halides, anhydrides, aryl halides, aziridines, boronates, activated carnoxylic acids, carbodiimides, diazoalkanes, epoxides, haloacetamides, haloplatinate, halotriazines, imido esters, isocyanates, isothiocyanates, maleimides, phosphoramidites, solyl halides, sulfonate esters and sulfonyl halides. It is preferably the amine group $-NH_2$.

On the opposite side, the solid support should be functionalized by complementary groups corresponding to such reactive groups. The complementary groups corresponding to each of these reactive groups are well-known in the art. They are given for example on the table I of WO 2010/107433.

In a preferred embodiment, the AGT substrate used in the method of the invention is:



In another preferred embodiment, the AGT substrate used in the method of the invention is the fluorescent linker designated "SNAP-cell® 505", having the following formula:



This benzylguanine derivative possesses one benzyl purine group (guanine) for the specific interaction with the SNAP domain, as well as one free amine group for the covalent

coupling to the microsphere surface. It is commercialized by New England BioLabs and has been successfully coupled to the surface of the microparticles of the invention.

Substrates of the invention are generally prepared by standard methods known in the art. Particular methods are explained e.g. in patent application WO 2005/085470.

Coupling of AGT Substrates to Solid Supports

The invention encompasses AGT substrates covalently coupled to solid supports. In the context of the present invention, an AGT substrate is "covalently coupled" to a solid support if it is permanently attached to the said solid support, and will not desorb or leach over time. According to the invention, an AGT substrate is permanently attached to the said solid support if it stays attached for a long period of storage, e.g., typically, at least 6 months of storage. A number of coupling proceedings have been described so far. Any of these coupling proceedings can be used in the immunoassay of the invention, provided that the AGT substrate becomes permanently attached to the solid support.

Coupling of AGT-Antigen Fusion Proteins to Solid Supports

The invention further encompasses methods for coupling antigens to solid supports comprises the two following steps: i) the coating of solid surfaces with an AGT substrate (e.g. BG-PEG-amino), and ii) the covalent immobilization of chimeric [AGT- Antigen] fusion proteins using the AGT substrate as an anchor (see Figure 1). Before being coated with said AGT substrate, the solid surfaces are advantageously functionalized, preferably by using an optimized two-step carbodiimide process (Kufer SK, *Eur. Biophys.J.* 2005), so that the AGT substrate is covalently attached to the solid surfaces. Once these steps have been performed, the solid surfaces carry AGT substrates that are irreversibly linked to the chimeric [AGT-antigen] fusion proteins. Due to the high specificity of this reaction, the fusion protein is exclusively coupled via the cysteine-containing domain of the AGT enzyme, thus leaving the antigen accessible for its interactions with antibodies.

This coupling procedure is very advantageous as it allows the binding of the antigen in an oriented manner on the solid supports. Also, this antigen coupling procedure advantageously enables to obtain a multimeric antigen organization on a solid surface, so as to enhance immunoglobulin G, and potentially immunoglobulin M, capture efficiency.

Consequently, the antigen-coupled microspheres developed in the experimental part of the application have shown enhanced capture of specific antibodies as compared to antigen-coupled microspheres produced by standard non-oriented amine coupling procedures (see the experimental part below and figure 3). Finally, this antigen coupling procedure enables to obtain a high coupling efficiency and a long-term stability of the antigen-conjugated microspheres (>6 months at 4°C).

In the immunoassay of the invention, the covalent coupling is preferably performed by contacting the AGT substrates (which contain a reactive group Y, as mentioned above) with solid supports which have been previously functionalized with a complementary group such as those disclosed in table I of WO 2010/107433, the disclosure of which is incorporated herein by reference.

Thus, in a preferred embodiment, the methods of the invention use solid supports that have been functionalized with a group which is complementary to the reactive group of the AGT substrate, before being contacted with the AGT substrate.

A preferred and conventional procedure for covalently coupling an AGT substrate to the surface of solid supports is based on the carbodiimide reaction and uses water-soluble carbodiimide. According to this procedure, solid supports have surface carboxyl groups available for attachment of the reactive amine- or sulfhydryl-containing AGT substrate. Thus, in this preferred embodiment, the methods of the invention use solid supports that have been functionalized with surface carboxyl groups prior to be contacted with the AGT substrate.

In this case, the first step of the method of the invention is to activate the carboxyl groups coating the solid supports. This activation is usually performed by adding a so-called "activation buffer", for example a 50 mg/mL EDAC solution or a 50 mg/mL S-NHS solution. These solutions are commercially available. Activation of the solid supports is typically performed by incubating said supports with the activation buffer at room temperature for a few minutes (e.g. 5 minutes to 30 minutes), according to the manufacturer's instructions.

Importantly, covalent coupling of the AGT substrate to the solid support has to be performed under particular conditions, so as to preserve the AGT substrate solubility and

the integrity of the bead (internal fluorochrome). The inventors have observed that the AGT substrates should be suspended in a "covalent coupling" buffer containing between 0 and 20 % of dimethylsulfoxide (DMSO). In particular, the inventors have observed that concentrations of DMSO above 20 % may affect the detection step of the methods of the invention. Preferably, said buffer is a PBS buffer containing between 0 and 20 % of DMSO, more preferably between 10 % and 20 % of DMSO.

Advantageously, the unspecific sites on the solid supports that have not been covalently attached to the AGT substrate can be further blocked by any conventional means, for example, by using a blocking buffer containing 1 % of bovine serum albumin (BSA) or any saturating protein (*e.g.* casein).

Once the solid supports of the invention have been covalently coupled with the AGT substrate (preferably through a carbodiimide covalent linkage), the solid supports are then contacted by the fusion proteins of the invention, so as to couple the epitopes that are specifically recognized by the target antibodies to said supports.

Again, this coupling step has to be performed under particular conditions. As a matter of fact, the catalytic site of the AGT enzyme and the conformational structure of the antigens / epitopes which are carried by the fusion proteins have to be conserved during the coupling proceedings. The inventors identified that the fusion protein should be suspended in a dithiothreitol (DTT)-containing buffer, preferably a PBS/DTT buffer, for the coupling to be efficient. Advantageously, the said coupling buffer contains tween 20; indeed, it has been observed by the present inventors that addition of tween 20 to the coupling medium helps avoiding bead aggregation. Preferably, the coupling buffer contains 0,02 % tween 20. More preferably, the covalent coupling buffer of the invention is a PBS buffer of pH 7,4, containing 0,02 % tween 20, and 1 mM DTT.

Other coupling conditions are usual ones. Preferably, the covalent coupling of the AGT substrate and the coupling of the fusion protein to the solid supports are performed at room temperature. If the solid supports are fluorescently labeled, said proceedings are more preferably performed in darkness.

The invention encompasses methods for covalently coupling a AGT polypeptide having O^6-alkylguanine-DNA alkyltransferase activity, on a functionalized solid support, comprising the following steps:

a) activating the said functionalized solid support,

b) adding a substrate of said AGT polypeptide, said substrate being suspended in a buffer containing between 0 and 20 % of DMSO, in appropriate conditions so that the substrate is covalently attached to said support,

c) contacting the said AGT polypeptide with the substrate-coated support of step b) in a PBS/DTT buffer,

wherein unbound molecules are washed out after steps b) and c).

Washings can be performed by using any kind of appropriate washing buffers. Such buffers are routinely used by the person of skills in the art and need not be further detailed here. Preferably, a PBS buffer is used.

As used herein, "appropriate conditions" are usual ones. Preferably, the covalent coupling of the AGT substrate is performed at room temperature and, if the solid supports are fluorescently labeled, in darkness.

The functionalization of the solid support can be performed by any conventional means (as those reminded above). The activation of said functionalized solid support is performed accordingly. In a preferred embodiment, the said solid supports are functionalized with surface carboxyl groups and further activated with a classical activation buffer, for example a 50 mg/mL EDAC solution or a 50 mg/mL S-NHS solution.

In a preferred embodiment, DTT is at a concentration of 1 mM in the PBS/DTT buffer.

The present invention is also drawn to a solid support which has been obtained by the said method, and to the use of said solid support in the immunoassay of the invention.

Said solid supports can then be stored in conventional storage buffers, for example containing 0.5 g/L sodium azide, 0.1 % BSA, 0.02 % tween 20, and/or 1 mM DTT.

All these coupling steps are preferably performed *in vitro*, in buffers which are devoid of living cells, so that there is no need to take into account the reaction with endogenous

AGT enzymes, and the reaction of the (exogenous) AGT fusion protein is therefore highly specific.

Solid Supports

The solid supports that can be used in the methods of the invention can be of any kind, e.g. test tubes, microtiter wells, sheets, beads, chips, and/or microparticles, provided that they can be specifically identified from each other. Such identification is possible for example when they are separately located in space (e.g. the wells in a microtiter plate, or different locations on a chip) or when they are differently labeled. A "solid support" has therefore to be understood in a broad meaning, that is, by designating either discrete small parts of a whole solid supports (in case of a plate or a biochip) or a large number of identical microparticles that share common detectable characteristics (hereafter referred to as microparticles "subset").

In preferred embodiments, the solid supports used in the immunoassays of the invention should be intrinsically identifiable, so that it is possible to determine precisely which antigen is carried by which solid support. The antigen-coupled and identifiable solid supports are then used as capture reagents for specific human immunoglobulins and are therefore contacted with the biological sample of the patient.

In a preferred embodiment, the solid supports used in this invention can be specifically identified by their specific location, size, diameter, weight, granulometry, and/or labeling. Such labeling is for example a fluorochrome, a fluorophore, a chromophore, a radioisotope, a mass tag, or any kind of detectable tag which is known in the art.

The solid supports used in the invention can be made of any material, for example in polystyrene, cellulose, nitrocellulose, glass, ceramic, resin, rubber, plastic, silica, silicone, metal, and/or polymer. Polymeric materials include brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyamide, polyacrylamide, polyacrolein, polybutadiene, polycaprolactone, polycarbonate, polyester, polyethylene, polyethylene terephthalate, polydimethylsiloxane, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polymethylmethacrylate, polylactide, polyglycolide, poly(lactide-co-glycolide), polyanhydride, polyorthoester, polyphosphazene, polyphosophaze, polysulfone,

or combinations thereof, that are acceptable as well. Most of these supports are commercially available. For example, beads from synthetic polymers such as polystyrene, polyacrylamide, polyacrylate, or latex are commercially available from numerous sources such as Bio-Rad Laboratories (Richmond, Calif.) and LKB Produkter (Stockholm, Sweden). Beads formed from natural macromolecules and particles such as agarose, cross-linked agarose, globulin, deoxyribose nucleic acid, and liposomes are commercially available from sources such as Bio-Rad Laboratories, Pharmacia (Piscataway, NJ), and IBF (France). Beads formed from copolymers of polyacrylamide and agarose are commercially available from sources such as IBF and Pharmacia.

When polymeric supports were used, carboxyl groups can be added to the surface of the solid support by incorporating monomers containing such groups into the polymers (for example, acrylic acid, methacrylic acid, itaconic acid, and the like). Alternatively, they can be added to the support by further chemical reaction of a polymer having other precursor reactive groups which can be converted to carboxyl groups (for example, by hydrolysis of anhydrides, such as maleic anhydride, or by oxidation of surface methylol or aldehyde end groups), as already described.

In a preferred embodiment, the solid supports used in the invention are microparticles. Said microparticles have preferably a diameter of less than one millimeter, preferably a diameter ranging from about 0.1 to about 1,000 micrometers (μm). Even though the microparticles can be of any size, the preferred size is 1-100 μm, more preferably 2-50 μm, more preferably 3-25 μm, and even more preferably about 6-12 μm. Microparticles are made of any regularly shaped material. The preferred shape is spherical; however, particles of any other shape can be employed since this parameter is immaterial to the nature of the invention. The shape of the particle can serve as an additional distinction parameter, which is discriminated by flow cytometry, e.g., by a high-resolution slit-scanning method.

As used hereinafter the terms "microparticles", "microspheres", or "microbeads" are used interchangeably and bear equivalent meanings as they refer to small particles with overall diameter that falls essentially in the micrometer range. The terms "nanospheres", "nanoparticles", or "nanobeads" refer to smaller particles with overall size that falls essentially in the nanometer range. As used hereinafter the general term particles, spheres,

or "beads" refers both to microparticles and nanoparticles, which can effectively serve as solid supports in the methods of the invention.

In the context of the present invention, a "subset" of microparticles corresponds to numerous identical microparticles having the same characteristics and that have been coated with the same epitope. Importantly, each subset of microparticles should be distinguishable from other subsets of the population by at least one characteristic (e.g. location, size, diameter, weight, granulometry, and/or labeling).

In a preferred embodiment, the different subsets of microparticles can be distinguished as they are differently labeled (e.g. with a fluorochrome, a fluorophore, a chromophore, a radioisotope, a mass tag, or any kind of detectable tag which is known in the art).

In a more preferred embodiment, the different subsets of microparticles can be distinguished as they are differently fluorescently labeled, as proposed in US 5,736,330, US 5,981,180, US 6,057,107, US 6,268,222, US 6,449,562, US 6,514,295 , US 6,524,793 and US 6,528,165. More precisely, these different subsets can be dyed with different fluorescent dyes, and/or different concentrations of one or more fluorescent dyes. As such, the different subsets can have different fluorescent signatures (e.g. different fluorescent wavelength(s), different fluorescent intensities, etc.) that can be measured and used by a measurement system to determine the subset that individual microparticles belong to (i.e., to classify the microparticles according to the subset).

In a preferred embodiment, the microparticles used in the invention are internally labeled with fluorescent dyes, as described in EP 1 204 869, which is hereby incorporated by reference.

These microparticles may also incorporate magnet or magnetically responsive metal oxides selected from the group consisting of superparamagnetic, paramagnetic, and ferromagnetic metal oxide. Magnetic beads are for example commercially available from sources such as Dynal Inc. (Great Neck, NY) or can be prepared using known in the art methods as disclosed for example in U.S. 4,358,388; US 4,654,267; US 4,774,265; US 5,320,944; and US 5,356,713. In a preferred embodiment, the solid supports used in the invention are therefore magnetic.

In a more preferred embodiment, the solid supports used in the invention are microparticles internally labeled with fluorescent dyes with magnetite encapsulated in a functional polymer outer coat containing surface carboxyl groups for covalent coupling of ligands, such as those marketed by Luminex Corp under the trade name MagPlex.

It is also possible to use MicroPlex microspheres (sold by Luminex) that are carboxylated polystyrene micro-particles that have been color coded into spectrally distinct regions. These regions can be quickly distinguished by an xMAP Instrument allowing for the interrogation of up to 100 different analytes simultaneously from one single sample volume.

It is also possible to use SeroMAP microspheres (sold by Luminex) which are a special formulation of MicroPlex microspheres which have been optimized to reduce non-specific binding in serology assays.

Detection of Bound Antibodies

The invention encompasses detecting the presence of antibodies that are bound to the epitopes and therefore to the detectable solid support. By analyzing to which subset of microparticles antibodies are bound, it can be easily inferred which antibodies were present in the biological sample, and therefore by which pathogen the tested subject was infected.

Any known technology can be used to detect the presence of the antibodies that are bound to the solid supports. For example, labeled secondary antibodies recognizing specifically the constant part of the subject immunoglobulins can be used, as shown in the experimental part below. It is important to note that the labeling of the detecting-antibodies should be different from the one of the solid support, so as to distinguish between the solid supports that are coupled to antibodies, and those that are not.

Alternatively, immunoglobulins present in sera from infected animals or humans can be directly conjugated to R-phycoerythrin (R-PE), using a one-step antibody labeling protocol (Lightning-Link™ R-Phycoerythrin Conjugation Kit – Innova Biosciences). The hands-on time for the entire procedure is usually 20-30 seconds, and allows the labeling of small quantities of immunoglobulins with 100% recovery. This procedure eliminates the need for

secondary reagents, such as conjugated anti-species antibodies and streptavidin-R-phycoerythrin, in multiplex-immunoassay experiments.

When microparticles internally labeled with fluorescent dyes are used, the fluorescent detection instrument should be equipped with a first laser for detecting the type of microsphere, and a second laser to ensure the quantification of captured IgM or IgG by exciting the fluorophore which is conjugated to the specific detection antibody.

With its extensive multiplexing capabilities and lower limit of detection, this approach offers substantial cost and sample savings over traditional ELISA measurements. Moreover, the selected sets of microspheres are adaptable to an affordable, compact, and robust fluorescent detection system such as the MagPix (Luminex Corporation).

In this embodiment, the method of the invention makes it possible to simultaneously analyze up to 100 types of coupled microspheres per well by using a flow analysis tool, and affords greatly enhanced sensitivity that is expected to be on the order of several orders of magnitude larger than that of currently used systems and methods.

Interestingly, the method of the invention enables to perform high throughput serological screening to diagnose multiple infections in an individual, either a human or an animal.

Multiplex immunoassay kits

The invention encompasses kits suitable for use in the detection of antibodies against multiple antigens. Any of the antigens or epitopes disclosed herein can be incorporated into the kits.

The invention encompasses kits for the detection of antibodies against viral, bacterial, parasitic, animal, prion, yeast, and fungal proteins.

The kit comprises at least two solid supports. The invention encompasses a kit comprising at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. solid supports and a kit comprising at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. different epitopes. The kit can comprise at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. solid supports mixed together. The epitopes can be recognized by target antibodies.

In one embodiment, the kit comprises a first solid support comprising a first AGT-Antigen fusion protein covalently coupled to an AGT substrate, and a second solid support comprising a second AGT-Antigen fusion protein covalently coupled to an AGT substrate.

In one embodiment, the kit comprises:

a first solid support comprising a first AGT-Antigen fusion protein covalently coupled to an AGT substrate,

a second solid support comprising a second AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a third solid support comprising a third AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a fourth solid support comprising a fourth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a fifth solid support comprising a fifth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a sixth solid support comprising a sixth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a seventh solid support comprising a seventh AGT-Antigen fusion protein covalently coupled to an AGT substrate;

an eighth solid support comprising an eighth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a ninth solid support comprising a ninth AGT-Antigen fusion protein covalently coupled to an AGT substrate;

a tenth solid support comprising a tenth AGT-Antigen fusion protein covalently coupled to an AGT substrate.

In other embodiments, the kit can comprise 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 solid supports comprising 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 AGT-Antigen fusion proteins covalently coupled to an AGT substrate.

In one embodiment, the kit comprises a first solid support covalently coupled with a first epitope that is recognized by the first target antibody, a second solid support covalently

coupled with a second epitope that is recognized by a second target antibody, and not by said first target antibody, wherein the at least two solid supports can be specifically identified from each other and enable the detection of two different target antibodies.

In a preferred embodiment, the invention encompasses a kit for the detection of at least two target antibodies in a biological sample comprising:

(a) a first solid support comprising an AGT substrate covalently coupled to a first fusion protein comprising an AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity and a first epitope that is recognized by a first target antibody; and

b) a second solid support comprising an AGT substrate covalently coupled to a second fusion protein comprising an AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity and a second epitope that is recognized by a second target antibody, but not by said first target antibody.

In preferred embodiments, the kit comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. different viral epitopes.

In preferred embodiments, the kit comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, 100, etc. different bacterial epitopes.

The kit can be specific for viruses, bacteria, and/or parasites.

The kit can be specific for a family of viruses or bacteria, or can be based on similar associated clinical symptoms (i.e., childhood diseases, respiratory or hemorrhagic syndromes, etc.).

The kit can be specific for immunoglobulins, wherein the kit detects antibodies to at least 2, 3, 4, or 5 immunoglobulins selected from IgD, IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgE, and IgG total.

The kit can be specific for a bioterrorism pathogen, wherein the kit detects antibodies to at least 2, 3, 4, or 5 proteins selected from *Francisella tularensis, Bacillus anthracis, Clostridium botulinum, Yersinia pestis,* Smallpox, Marburg virus, Ebola virus, Lassa virus, and Machupo virus proteins.

The kit can be specific for a flu virus, wherein the kit detects antibodies to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 different influenza or SARS proteins.

The kit can be specific for a poxvirus, wherein it detects antibodies to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 different poxviruses. The poxvirus can be an orthopox (smallpox virus/variola, vaccinia virus (http://en.wikipedia.org/wiki/Vaccinia), cowpox virus (http://en.wikipedia.org/wiki/Cowpox), or monkeypox virus http://en.wikipedia.org/wiki/Monkeypox), parapox (orf virus, pseudocowpox, bovine papular stomatitis virus), yatapox (tanapox virus (http://en.wikipedia.org/wiki/Tanapox), yaba monkey tumor virus (http://en.wikipedia.org/wiki/Yaba_monkey_tumor_virus)), or molluscipox (http://en.wikipedia.org/wiki/Molluscum_contagiosum).

The invention encompasses kits for the detection of antibodies against human proteins including cancer and tumor antigens, kidney toxicity markers, cytokines, chemokines, growth factors, antigens be involved in autoimmune diseases, and immunoglobulins.

The invention encompasses kits for the detection of antibodies against allergens.

In a preferred embodiment, at least one epitope is a viral epitope. Ina preferred embodiment, said first and/or second epitope is present on a viral protein chosen in the group consisting of: the EDIII protein of the dengue virus 1 of SEQ ID NO:3, the EDIII protein of the dengue virus 2 of SEQ ID NO:4, the EDIII protein of the dengue virus 3 of SEQ ID NO:5, the EDIII protein of the dengue virus 4 of SEQ ID NO:6, the EDIII protein of the West Nile virus of SEQ ID NO:7, the EDIII protein of the Yellow Fever virus of SEQ ID NO:8, , the EDIII protein of the Japanese encephalitis virus of SEQ ID NO:9, the EDIII protein of the Zika virus of SEQ ID NO:10, the EDIII protein of the Wesselbron virus of SEQ ID NO:11, the EDIII protein of the Rocio virus of SEQ ID NO:12, the EDIII protein of the Murray encephalitis virus of SEQ ID NO:13, and the EDIII protein of the Saint-Louis encephalitis virus of SEQ ID NO:14, the EDIII protein of the Japanese encephalitis virus of genotype 1 encoded by SEQ ID NO:54, the EDIII protein of the Japanese encephalitis virus of genotype 2 encoded by SEQ ID NO:55, the EDIII protein of the Japanese encephalitis virus of genotype 4 encoded by SEQ ID NO:56, the EDIII protein of the Japanese encephalitis virus of genotype 5 encoded by SEQ ID NO:57, the EDIII protein of the Rabensburg virus encoded by SEQ ID NO:58, and the viral protein of HIV1, of HIV2, of the Hepatitis B virus, of the Hepatitis C virus,

of the Hepatitis E virus, of the West-Nile virus and of oncogenic HPV strains such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

Preferably, this kit also contains the means to detect the at least two target antibodies which are bound to the solid supports. Said means are more preferably secondary antibodies recognizing the constant part of the target antibodies. Said secondary antibodies can be labeled, provided that the labeling is not the same as the ones that are present on the solid support. However, it is possible to use the same labeling for all the secondary antibodies that are used for detecting the antibodies bound to solid support(s), since the information concerning the infectious pathogen(s) are given only by the identification of the solid support which is bound to the antibodies.

The kit of the invention may contain other ingredients that are accepted as standard reagents such as a wash buffer, necessary plasticware, and the like.

In a preferred embodiment, the kit of the invention comprises at least 10, preferably at least 50, more preferably at least 100 differently coupled-solid supports, said solid supports being for example subsets of microparticles as defined above.

In a more preferred embodiment, the said solid supports are microspheres, for example those which are internally labeled with a fluorescent dye with magnetite encapsulated in a functional polymer outer coat containing surface carboxyl groups.

In another preferred embodiment, in the kit of the invention, the said solid supports are mixed together in at least one single compartment.

Advantageously, the kit of the invention contains conventional support(s), e.g., microtiter plates, containing the different antigen-coated microparticles subsets defined above. In a preferred embodiment, the said microparticles subsets are mixed together in at least one single compartment (e.g. a well or a tube). Such a device is disclosed on Figure 11.

The kit of the invention may also contain recipients (e.g., tubes) containing the said subsets of antigen-coated microparticles.

The present invention also targets the use of the kit of the invention for detecting at least two, preferably at least 10, more preferably at least 50 and even more preferably at least 100 target antibodies in a biological sample from a subject.

In a preferred embodiment, the kit of the invention is used for detecting at least two, preferably at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 target antibodies that are generated upon infection by endemic viruses or parasites of the same geographic region. For example, the kit of the invention could contain microparticles that are coated with antigens of viruses or parasites that are specific of Africa regions, such as the Dengue virus type 1, type 2, type 3, type 4, the Yellow fever virus, the West-Nile virus, the Usutu virus, the Zika virus, the Wesselsbron virus, the Shamonda virus, the Rift Valley fever virus, the Chikungunya virus, the Crimean-Congo hemorrhagic fever virus, the Ebola virus, the Marburg virus, the Lassa virus, the Hepatitis C virus, the Hepatitis E virus, the Enterovirus 71, *Plasmodium falciparum*, or *Leptospira interrogans*.

Figure 25 discloses examples of antigen-coupled microspheres combinations which can be included in the kit of the invention depending on the geographic region it is intended for (Asia, Europa, America, Oceania, or Africa).

The kit of the invention may alternatively contain antigen-coupled microspheres that enable the diagnosis of viruses or parasites inducing specific symptoms (flu-like, encephalitis, or hemorrhagic fever) or infecting specific animals, so that it can be adapted to each patient / animal.

Figure 25 discloses examples of antigen-coupled microspheres combinations which can be included in the kit of the invention depending on the symptoms of the patient or of the animal.

Kits containing antigen combinations that are proposed by national sanitary agencies are obviously also encompassed in the present invention.

In particular, the kit of the invention comprises at least two solid supports coated with at least two fusion proteins that are selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:42, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70,

SEQ ID NO:72, SEQ ID NO:74, SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:90, SEQ ID NO:92, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:98, SEQ ID NO:100, SEQ ID NO:102, SEQ ID NO:104, SEQ ID NO:109, SEQ ID NO:111, SEQ ID NO:113, SEQ ID NO:115, SEQ ID NO:117, SEQ ID NO:119, SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:129, SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:135, SEQ ID NO:137, SEQ ID NO:139, SEQ ID NO:141, SEQ ID NO:143, SEQ ID NO:145, SEQ ID NO:147, SEQ ID NO:149 and SEQ ID NO:151.

In a preferred embodiment, the kit of the invention contains a combination of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 solid supports coated with said fusion proteins.

In a more preferred embodiment, the kit of the invention contains a combination of at least five solid supports (e.g., microsphere subsets) that are coated with at least five different fusion proteins containing antigens as recommended by the Food and Drug Administration, namely, antigens from the HBV, HCV, HIV1, HIV2 and West Nile viruses.

The invention encompasses methods for manufacturing the kits of the invention.

In one embodiment, the invention encompasses a method for manufacturing a kit, said method comprising the steps of:

(a) providing a first AGT-Antigen fusion protein that is recognized by a first target antibody,

(b) contacting said first AGT-Antigen fusion protein with a first solid support, said support being covalently coupled with a substrate of said AGT-Antigen fusion protein,

(c) obtaining a first solid support covalently coupled with the first AGT-Antigen fusion protein,

(d) providing at a second AGT-Antigen fusion protein that is recognized by a second target antibody, but not by said first target antibody, and

(e) contacting said second AGT-Antigen fusion protein with a second solid support, said support being covalently coupled with a substrate of said AGT-Antigen fusion protein, and

(f) obtaining a second solid support covalently coupled with the second AGT-Antigen fusion protein,

wherein said first and at second solid supports can be specifically identified from each other.

In one embodiment, the solid supports covalently coupled with the AGT-Antigen fusion proteins are mixed together.

The above steps a)-c) can be repeated with at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 different AGT-Antigen fusion proteins to generate at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 solid supports covalently coupled with 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, 40, 50, or 100 different AGT-Antigen fusion proteins.

In another aspect, the present invention relates to a method for manufacturing the kit of the invention as defined above, said method comprising the steps of:

(a) providing a least a first fusion protein comprising :

- a polypeptide comprising a first epitope that is recognized by a first target antibody and

- a AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity,

(b) contacting said first fusion protein with a first solid support, said support being covalently coupled with a substrate of said AGT polypeptide,

(c) obtaining a first solid support covalently coupled with a first epitope that is recognized by the first target antibody,

(d) providing at least a second fusion protein comprising :

- a polypeptide comprising a second epitope, said second epitope being recognized by a second target antibody but not by said first target antibody, and

- a AGT polypeptide having a O6-alkylguanine-DNA alkyltransferase activity,

(e) contacting said second fusion protein with a second solid support, said support being covalently coupled with a substrate of said AGT polypeptide,

(f) obtaining a second solid support covalently coupled with a second epitope that is recognized by the second target antibody, but not by said first target antibody,

wherein said at least first and at least second solid supports can be specifically identified from each other,

the kit of the invention comprising at least said first and second supports.

In another aspect, the present invention relates to a multiplex immuno screening assay comprising at least 2, 3, 4, 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, 35, 40, 45, 50, 60, 70, 80, 90, or 96 solid supports as defined above and wherein each of said solid supports emits a different and distinguishable wave length after excitation.

In another aspect, the present invention relates to a multiplex immuno screening assay method comprising:

a) contacting one or several biological sample(s) with at least 2, 3, 4, 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, 35, 40, 45, 50, 60, 70, 80, 90, or 96 solid supports as defined above and wherein each of the solid supports emits a different and distinguishable wave length after excitation, and

b) detecting the presence or absence of target antibodies.

In a preferred embodiment, said target antibodies are specific to antigen from viruses to be detected in blood bank according to WHO or FDA guidelines, such as for example viruses selected from HBV, HCV, HIV1, HIV2, and WNV.

In another preferred embodiment, said target antibodies are specific to oncogenic HPV strains such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

In another preferred embodiment, each of said target antibodies are labeled with a detectable label.

In another aspect, the present invention relates to an apparatus for carrying out the method for manufacturing the kit of the invention as defined above, comprising a technical device for detecting the light sources emitted from the solid supports and the light source emitted from the target antibodies or labeled antibodies binding to the target antibodies, and a calculating or computer device for identifying which solid supports are bound with target antibodies, thereby indicating the presence or absence of antigens, bacteria, virus, or parasites in the analyzed sample.

In another aspect, the present invention relates to an *in vitro* method for diagnosing at least one target disease in a subject, said target disease being known to induce the synthesis of at least one target antibody in said subject, comprising performing the immunoassay of the invention, wherein said subject is diagnosed to be suffering from said at least one target disease if the amount of said at least one target antibody is higher than a control value.

This diagnosing method preferably enables to diagnose two, preferably three, and more preferably four target diseases in a subject in need thereof. This number is however not limiting: it is indeed possible to diagnose until 100 target diseases in so far as it is possible to detect 100 different antibodies with the detecting method of the invention.

In a preferred embodiment, said at least one target disease is a viral, a bacterial, a yeast or a fungi-mediated infection, preferably a viral infection caused by a Papillomavirus or a RNA virus from the family of the *Flaviviridae* (Dengue, Yellow fever, West Nile, Japanese encephalitis, Tick-Borne Encephalitis, Hepatitis C viruses), the *Togaviridae* (Chikungunya, Ross River, Mayaro, Western Equine encephalitis, Eastern Equine Encephalitis, Venezuela Equine Encephalitis viruses), the *Bunyaviridae* (Crimean-Congo hemorrhagic fever, Rift Valley Fever, Schmallenberg viruses), the *Caliciviridae* (Hepatitis E virus), the *Arenaviridae* (Lassa) or the *Filoviridae* (Ebola, Marburg), a bacterial infection caused by *Leptospirosa Interrogans*, or an infection caused by *Plasmodium falciparum*.

In a preferred embodiment, said *in vitro* method is used to diagnose at least 5, 6, 7, 8,9, 10, 11, 12, 13, or 15, more preferably at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35,

40, or 45, more preferably at least 50, 60, 70, 80, or 90, and even more preferably at least 100 viral and/or bacterial and/or parasite infections in said subject.

In a preferred embodiment, the control value used in said method represents the amount of said target antibody in a sample from a subject which is not suffering from said target disease, preferably, a healthy subject.

The methods of the invention can be used to diagnose infections in animals.

In particular, they can be used for the diagnosis of animal diseases, as well as a DIVA (Differentiating Infected from Vaccinated Animals) approach to differentiate naturally infected animals from vaccinated animals. The use of a DIVA strategy complementing novel vaccines would allow the implementation of vaccination as targeted control strategy alongside conventional strategies (test, slaughter and meat inspection). Moreover, increased test specificity would have a major economic benefit by reducing the numbers of false-positive animals that may be slaughtered needlessly. Lastly, improved sensitivity, particularly when novel diagnostic assays are used, would have a further benefit in reducing the economic burden of disease control even in the absence of vaccination

In a preferred embodiment, the methods of the invention are applied to human individuals.

The present invention relates to the use of the kit of the invention for diagnosing at least two target diseases in a subject, wherein said target disease is a viral infection caused by a Papillomavirus or a RNA virus from the family of the *Flaviviridae* (Dengue, Yellow fever, West Nile, Japanese encephalitis, Tick-Borne Encephalitis, Hepatitis C viruses), the *Togaviridae* (Chikungunya, Ross River, Mayaro, Western Equine encephalitis, Eastern Equine Encephalitis, Venezuela Equine Encephalitis viruses), the *Bunyaviridae* (Crimean-Congo hemorrhagic fever, Rift Valley Fever, Schmallenberg viruses), the *Caliciviridae* (Hepatitis E virus), the *Arenaviridae* (Lassa) or the *Filoviridae* (Ebola, Marburg), a bacterial infection caused by *Leptospirosa Interrogans*, or an infection caused by *Plasmodium falciparum*.

A new emerging arbovirus has been recently sequenced and affects cattle in Germany, Benelux and France. This virus is called Schmallenberg virus (SBV), and is related to the Akabane virus belonging to the Simbu serogroup of the *Orthobunyavirus* genus of the *Bunyaviridae* family. The viral genome of the Schmallenberg virus comprises three single-

stranded RNA segments known as S, L and M. The S segment encodes the N nucleoprotein and the NSs non-structural protein. The N nucleoprotein shares antigenic determinants with different Bunyaviruses. The three RNA viral sequences of the BH80/11-4 strain of the Schmallenberg virus are available under the numbers HE649913.1, HE649914.1, and HE649912.1.

Use of AGT to Enhance Production of AGT Fusion Protein

The present inventors observed that the fusion as a chimeric protein of the 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) with the SBV N protein greatly improves the production of recombinant N protein, in particular in invertebrate cells such as S2 cells.

The present inventors propose here for the first time to use the AGT enzyme (EC 2.1.1.63), a mutant thereof, a catalytic domain thereof or sub-fragments thereof, for enhancing the production of the N nucleoprotein from SBV in host cells, in particular in non-vertebrate cells. The enhancing effect is observed when the host cells express a fusion polypeptide comprising at least i) a secretion signal peptide which is functional in said host cells, ii) the AGT enzyme, mutant, catalytic domain or sub-fragments thereof, and iii) the N nucleoprotein of SBV. For the enhancing effect to occur, the AGT enzyme has to be physically linked, directly or indirectly (spacers and other amino acids might be introduced), to the protein of interest. Without being bound by theory, it is contemplated that the AGT enzyme acts as a chaperone protein, for example by facilitating the secretion from the host cell and stabilising the synthesised fusion polypeptide in the supernatant of the host cells, or for preventing it to be metabolised during and after its synthesis and secretion from the host cells. In addition, it has been observed that AGT has a 3D globular structure comprising α helix (Wibley J.E.A. et al, 2000), which is compatible with a scaffolding role of AGT.

In the context of the present invention, "host" cells are any cells which can be used for producing recombinant proteins, such as "non-vertebrate" (or invertebrate) cells, vertebrate cells, plant cells, yeast cells, or prokaryote cells. They are preferably non-vertebrate and vertebrate cells.

Non-vertebrate (also known as invertebrate) comprises different phyla, the most famous being the Insect, Arachnida, Crustacea, Mollusca, Annelida, Cirripedia, Radiata,

Coelenterata and Infusoria. They are now classified into over 30 phyla, from simple organisms such as sea sponges and flatworms to complex animals such as arthropods and molluscs. In the context of the invention, non-vertebrate cells are preferably insect cells, such as Drosophila or Mosquito cells, more preferably Drosophila S2 cells.

Examples of cells derived from vertebrate organisms that are useful as host cell lines include non-human embryonic stem cells or derivative thereof, for example avian EBX cells; monkey kidney CVI line transformed by SV40 sequences (COS-7, ATCC CRL 1651); a human embryonic kidney line (293); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells (CHO); mouse sertoli cells [TM4]; monkey kidney cells (CVI, ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC CCL51); rat hepatoma cells [HTC, Ml.5]; YB2/O (ATCC n° CRL1662); NIH3T3; HEK and TRI cells. In the context of the invention, vertebrate cells are preferably EBX, CHO, YB2/O, COS, HEK, NIH3T3 cells or derivatives thereof.

Plant cells which can be used in the context of the invention are the tobacco cultivars Bright Yellow 2 (BY2) and *Nicotiana tabaccum* 1 (NT-1).

Yeast cells which can be used in the context of the invention are: *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, and *Hansenula polymorpha*, as well as methylotropic yeasts like *Pichia pastoris* and *Pichia methanolica*.

Prokaryote cells which can be used in the context of the invention are typically *E.coli* bacteria or *Bacillus subtilis* bacteria.

In another aspect, the present invention is thus drawn to a vector for expressing the N nucleoprotein from SBV in an host cell (SBV.N), comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the N nucleoprotein of SBV of SEQ ID NO: 16.

The N nucleoprotein from SBV will be referred to hereafter as the "heterologous protein", the "protein of interest", "chimeric protein", or the "recombinant protein".

The term "vector" herein means the vehicle by which a DNA or RNA sequence of a foreign gene can be introduced into a host cell so as to transform it and promote expression of the introduced sequence. As understood herein, a vector is a nucleic acid molecule, such as, for example, plasmids, phages, and viruses. They are discussed in greater detail below. Any type of plasmid, cosmid, YAC or viral vector may be used to prepare a recombinant nucleic acid construct which can be introduced to a host cell where expression of the protein of interest is desired. When expression of the protein of interest in a particular type of host cell is desired, viral vectors that selectively infect the desired cell type or tissue type can be used. Also important in the context of the invention are vectors for use in gene therapy (i.e. which are capable of delivering the nucleic acid molecule to a host organism).

For example, viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia virus, baculovirus, and other recombinant viruses with desirable cellular tropism. Methods for constructing and using viral vectors are known in the art (see, Miller and Rosman, *BioTechniques*, 7:980-990, 1992).

Viral vectors that are actually preferred in the present invention are those that are well suited for use in vertebrate and non-vertebrate cells.

For non-vertebrate cells, preferred vectors are the arboviruses, the West Nile virus being particularly preferred, which are arthropod vectors. Other vectors that are known to efficiently be expressed in non-vertebrate cells are the baculoviruses.

For vertebrate cells, lentiviral, AAV, baculoviral and adenoviral vectors are preferred. The vectors suited for expression in mammalian host cells can also be of non-viral (e.g. plasmid DNA) origin. Suitable plasmid vectors include, without limitation, pREP4, pCEP4 (Invitrogen), pCI (Promega), pCDM8 and pMT2PC, pVAX and pgWiz.

For prokaryotic cells, plasmid, bacteriophage and cosmid vectors are preferred. Suitable vectors for use in prokaryotic systems include without limitation pBR322 (Gibco BRL),

pUC (Gibco BRL), pBluescript (Stratagene), p Poly, pTrc; pET 11d; pIN; and pGEX vectors.

For plant cells, plasmid expression vectors such as Ti plasmids, and virus expression vectors such as Cauliflower mosaic virus (CaMV) and tobacco mosaic virus TMV are preferred.

Expression of recombinant proteins in yeast cells can be performed using three types of vectors: integration vectors (YIp), episomal plasmids (YEp), and centromeric plasmids (YCp): Suitable vectors for expression in yeast (e.g. *S. cerevisiae*) include, but are not limited to pYepSec1, pMFa, pJRY88, pYES2 (Invitrogen Corporation, San Diego, Calif.) and pTEF-MF (Dualsystems Biotech Product code: P03303).

Vectors which can be used for gene therapy are well-known in the art. They are for example lentivirus, retrovirus, adenovirus, poxvirus, herpes virus, measles virus, foamy virus or adeno-associated virus (AAV). Viral vectors can be replication-competent, or can be genetically disabled so as to be replication-defective or replication-impaired. Preferred gene therapy vector are the DNA Flap vectors as described in WO 99/055892, US 6,682,507 and WO 01/27300.

A sequence "encoding" an expression product, such as a RNA, polypeptide, protein or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein or enzyme; i.e., the nucleotide sequence "encodes" that RNA or it encodes the amino acid sequence for that polypeptide, protein or enzyme.

The vector of the invention contains a nucleotide sequence encoding a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof. These polypeptides have been defined above. Preferably, said AGT mutant is the SNAP enzyme of SEQ ID NO: 2, and is encoded for example by SEQ ID NO:15 or SEQ ID NO: 31, the latter having a G/C content of 51 %.

Preferably, the nucleotide expression vector of the invention further comprises cloning sites enabling the in-frame insertion of a heterologous DNA sequence encoding the protein of interest.

As meant in the present invention, the term "secretion signal peptide" designates a short (3-60 amino acids long) peptide chain that directs the transport of the N nucleoprotein outside the host cells.

Examples of secretion signals appropriate for the present invention include, but are not limited to, the signal peptide sequences of the mating factor (MF) alpha (US 5,879,926); invertase (WO 84/01153); PHO5 (DK 3614/83); YAP3 (yeast aspartic protease 3; WO 95/02059); and BAR1 (WO 87/02670).

In the context of the invention, this secretion signal peptide is preferably functional either in non-vertebrate cells or in vertebrate cells, or both.

Examples of secretion signal peptides which are functional in insect cells are: the insect ssBiP (SEQ ID NO: 37, for example encoded by the DNA sequence SEQ ID NO: 22), the BiP-like peptide signal of SEQ ID NO: 24 (for example encoded by the DNA sequence SEQ ID NO: 23), the BiP-like peptide signal of SEQ ID NO:153 (for example encoded by the DNA sequence SEQ ID NO:152) and any peptide signal present in an arbovirus, for example the envelop E protein of the West-Nile virus (SEQ ID NO: 38).

Interestingly, the above-mentioned BiP-like peptide signal of SEQ ID NO:24 is functional in both non-vertebrate and vertebrate cells. This BiP-like signal corresponds to the BiP peptide signal of SEQ ID NO: 37 in which the last Glycine amino acid has been replaced by the amino acid sequence Pro Thr Ala Leu Ala (SEQ ID NO: 39) which corresponds to the cleavage site of the E protein of the Dengue virus. Accordingly, the BiP-like signal will be advantageously cleaved once the protein will be translated and secreted in the supernatant of the host cells.

A variety of secretion signals is also available for expression in yeast host cells, e.g. in *S. cerevisiae*. These include the prepro-alpha factor, HSp150, PHO1, SUC2, KILM1 (killer toxin type 1), and GGP1.

A cloning site is a sequence which facilitates cloning of a gene encoding a protein of interest into the expression system. It contains restriction sites, or restriction recognition sites, i.e. locations on a DNA molecule containing specific sequences of nucleotides, which are recognized by restriction enzymes (see for example in the figures). These are generally

palindromic sequences (because restriction enzymes usually bind as homodimers), and a particular restriction enzyme may cut the sequence between two nucleotides within its recognition site, or somewhere nearby. The cloning sites are well known for the man skilled in the art.

In a preferred embodiment of the invention, the DNA sequence encoding said AGT enzyme is located in 5' or in 3' of the DNA sequence encoding said heterologous protein of interest, preferably in 5'. Therefore, the AGT enzyme is directly or indirectly linked to the heterologous protein/polypeptide of interest, and preferably located at the N-terminal end of the heterologous protein/polypeptide of interest. The DNA sequence encoding the fusion polypeptide comprising said peptide signal, said AGT enzyme, mutant or catalytic domain, and said recombinant protein of interest, can be operatively associated with an inducible promoter which is functional in the same host cells as the peptide signal is.

More preferably, in the vector of the invention, said open reading frame is operatively associated with an inducible promoter which is functional in the same host cell as the peptide signal is.

A coding sequence is "operatively associated with" an expression control sequence (i.e. transcriptional and translational control sequences) in a cell, when RNA polymerase transcribes the coding sequence into RNA, which is then trans-RNA spliced (if it contains introns) and, if the sequence encodes a protein, is translated into that protein.

A "promoter" is a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). Within the promoter sequence will be found a transcription initiation site (conveniently found, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

Promoters which may be used to control gene expression in the context of the present invention are for example the one that are functional in non-vertebrate cells or in vertebrate cells. For example, for non-vertebrate cells, the regulatory sequences of the metallothionein gene can be used (Brinster et al., *Nature*, 296:39-42, 1982).

Preferably, the inducible promoter which is present in the vector of the invention has a promoter activity in an insect cell, and more preferably in a *Drosophila* cell. It is for example the *Drosophila metallothionein* promoter pMT (Lastowski-Perry et al, *J.Biol. Chem.* 260:1527 (1985)), which directs high level transcription of the gene in the presence of metals, e.g. $CuSO_4$. Alternatively, the Drosophila actin 5C gene promoter, which is a constitutive promoter and does not require addition of a metal, can be used (B.J.Bond et al, *Mol. Cell. Biol.* 6:2080 (1986)). Examples of other known Drosophila promoters include, e.g. the inducible heatshock (Hsp70) and COPIA LTR promoters. The SV40 early promoter gives lower level of expression than the *Drosophila* metallothionein promoter.

Preferably, the inducible promoter which is present in the vector of the invention has a promoter activity in a *Drosophila melanogaster* cell, preferably in Drosophila S2 cells. It is for example the metallothionein promoter which is thoroughly described in Lastowski-Perry et al, *J.Biol. Chem.* 260: 1527 (1985).

Promoters suitable for constitutive expression in mammalian cells include the cytomegalovirus (CMV) immediate early promoter, the adenovirus major late promoter, the phosphoglycero kinase (PGK) promoter, and the thymidine kinase (TK) promoter of herpes simplex virus (HSV)-1. Inducible eukaryotic promoters regulated by exogenously supplied compounds, include without limitation, the zinc-inducible metallothionein (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088), the ecdysone insect promoter, the tetracycline-repressible promoter, the tetracycline-inducible promoter, the RU486-inducible promoter and the rapamycin-inducible promoter.

Preferably, the promoter which is present in the vector of the invention has a promoter activity in a mammal cell, preferably in HeLa cells. It is for example the SV 40 promoter.

A range of yeast promoters is available for protein expression in yeast host cells. Some like ADH2, SUC2 are inducible and others like GAPDH are constitutive in expression. Other promoters suitable for expression in yeast include the TEF, PGK, MF alpha, CYC-1, GAL-1, GAL4, GAL10, PHO5, glyceraldehyde-3-phosphate dehydrogenase (GAP or GAPDH), and alcohol dehydrogenase (ADH) promoters.

For use in plant cells, the most commonly used promoter is the cauliflower mosaic virus (CaMV) 35S promoter or its enhanced version, but a number of alternative promoter can be used, such as the hybrid *(ocs)3mas* promoter or the ubiquitin promoter from maize and *Arabidospsis thaliana.* In contrast to these constitutive promoters, the rice α-amylase RAmy3D promoter is induced by sugar deprivation (Hellwig S et al., *Nat. Biotechnol.*2004; 22(11):1415-22).

Promoters suitable for expression in *E. coli* host cell include, but are not limited to, the bacteriophage lamba pL promoter, the lac, TRP and IPTG-inducible pTAC promoters.

It is preferred that the secretion signal peptide and the inducible promoter are functional in the same host cell.

More preferably, the secretion signal peptide and the inducible promoter are functional in both Drosophila S2 cells and vertebrate cells.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus.

Once an appropriate vector has been constructed and transfected into the selected host cell, preferably a Drosophila cell line, the expression of a heterologous protein is induced by the addition of an appropriate inducing agent for the inducible promoter. For example cadmium or copper are inducing agents for the Hsp70 promoter. For constitutive promoters, such as the actin 5C promoter, no inducing agent is required for expression.

In another embodiment of the invention, the nucleotide expression vector encodes at least one peptide cleavage site, which is preferably located between the AGT enzyme or its catalytic domain and the recombinant protein of interest.

A peptide cleavage site (also called "peptide cleavage site") is an amino acid sequence which is recognized by at least one protease enzyme (for example serine protease, cysteine protease, among others). An example of a peptide cleavage site is the enterokinase cleavage site of SEQ ID NO: 40 (AspAspAspAspLys/Asp). The enterokinase is a serine protease enzyme (EC 3.4.21.9) which is known to convert inactive trypsinogen into active trypsin by cleavage at the C-terminal end of the sequence: Val--$(Asp)_4$--Lys--Ile--Val~ (trypsinogen) → Val--$(Asp)_4$--Lys (hexapeptide) + Ile--Val~ (trypsin). Enterokinase cleaves after lysine if the Lys is preceded by four Asp and not followed by a proline residue.

Another useful peptide cleavage site is the cleavage site of the so-called "TEV protease", having the amino acid sequence SEQ ID NO: 32 (pro-TEV1) or SEQ ID NO: 33 (pro-TEV2) (Glu Asn Leu Tyr Phe Gln Ser or Gly respectively). Such cleavage sites can be encoded for example by SEQ ID NO:29 and 30. TEV protease is the common name for the 27 kDa catalytic domain of the nuclear inclusion protein encoded by the tobacco etch virus. It is commercially available (Invitrogen).

The cleavage site from the membrane precursor prM from Dengue virus serotype 1 (SEQ ID NO: 39) may also be used in the vector of the invention.

In another embodiment, the nucleotide expression vector of the invention further encodes a label, preferably located at the C-terminal end of the recombinant protein in the fusion polypeptide of the invention (comprising the peptide signal, the AGT protein or homologous thereof, and the recombinant protein). In the context of the invention, a "label" is dedicated to facilitate the recovery of the polypeptide from the crude lysate of the host cell, and is preferably selected from the group comprising: fluorescent proteins, poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; flu HA tags; c-myc tag *Herpes simplex* virus glycoprotein D (gD) tags, Flag-peptides, alpha-tubulin epitopes, or T7 gene 10 protein peptide tags. However, any other label might be used. In a preferred embodiment of the invention, the vectors comprise the DNA of SEQ ID NO: 28 encoding a hexa-histidine tag which has the SEQ ID NO: 27.

In another embodiment, the nucleotide expression vector of the invention further encodes spacer sequence(s), located preferably between the AGT enzyme (or its catalytic domain) and the recombinant protein of interest and/or between the recombinant protein of

interest and the label. In the context of the invention, a spacer sequence is an amino acid sequence comprising at least three amino acids, dedicated to spatially separate two linked polypeptides (these polypeptides being then indirectly linked). Such spacer can be for example the amino acid sequence Glycine-Glycine-Glycine-Serine (GGGS, SEQ ID NO: 25) and the DNA spacer sequence encoding it can be SEQ ID NO: 26. In the context of this invention, this DNA sequence is hereafter designated as "DNA spacer sequence" and is located between the DNA encoding AGT or its catalytic domain, and the recombinant DNA sequence, preferably upstream from the DNA sequence encoding the peptide cleavage site.

SNAP cassettes and antigen expression

As used herein, the term "pDeSNAPUniv" designates a DNA cassette encoding, in a single open reading frame, from 5' to 3':

a) a secretion signal peptide,

b) an AGT protein of SEQ ID NO:1, a mutant, a fragment or a catalytic domain thereof, in particular the SNAP mutant of SEQ ID NO:2,

c) at least one peptide cleavage site,

d) at least one label, and

e) at least one spacer sequence.

This pDeSNAPUniv DNA cassette encodes a secretion signal peptide which is advantageously the BiP-like peptide signal of SEQ ID NO:24 or the ssBiP peptide signal of SEQ ID NO:37, the SNAP mutant of SEQ ID NO:2, a label which is advantageously a His-tag of SEQ ID NO:27, a peptide cleavage site which is advantageously either the pro-TEV of SEQ ID NO:32 or the pro-TEV of SEQ ID NO:33, and/or a spacer sequence which has advantageously the amino acid sequence SEQ ID NO:25.

More preferably, the pDeSNAPUniv DNA cassette comprises, from 5' to 3', the sequence SEQ ID NO:23 encoding the BiP-like secretion signal, the SEQ ID NO:15 or 31 encoding the SNAP mutant, the spacer sequence of SEQ ID NO:26, the peptide cleavage site pro-

TEV of SEQ ID NO:29, the peptide cleavage site pro-TEV of SEQ ID NO:30, the spacer sequence of SEQ ID NO:26 and the sequence SEQ ID NO:28 encoding the His-tag label (see figure 8, showing the structure of the pDeSNAPUniv cassette). Such a pDeSNAPUniv DNA cassette is for example SEQ ID NO:34.

This "pDeSNAPUniv" cassette is held as "universal" since it can be inserted in any kind of vectors dedicated to transfect host cells in order to produce heterologous proteins, namely vertebrate vectors (such as pcDNA3 or pCI-neo vectors) as well as non-vertebrate vectors (such as pMT/BiP/V5-HisA which is useful in the DES system from Invitrogen). Examples of plasmid comprising said universal sequence is SEQ ID NO:43 (pMT/BiP/V5-HisA from Invitrogen comprising the pDeSNAP Univ cassette), SEQ ID NO:44 (pUC57 from Invitrogen comprising the pDeSNAP Univ cassette) or SEQ ID NO:45 (pcDNA3 from Invitrogen comprising the pDeSNAP Univ cassette).

Another example of plasmid comprising said universal sequence is SEQ ID NO:105 which is a pUC57 plasmid comprising, from 5' to 3', the constitutive promoter of *Orgyia pseudotsugata* multicapsid nucleoprotein virus-immediate-early 2 promoter (OpIE2SP) the BiPlike signal peptide of SEQ ID NO:152, the SNAP-like sequence of SEQ ID NO:31, the spacer sequence of SEQ ID NO:26, the pro-TEV1 sequence SEQ ID NO:29, and the C-term peptide tag of SEQ ID NO:106.

Once the heterologous sequence of a protein of interest such as SBV.N is cloned herein, such a vector can be advantageously transfected in either vertebrate or non-vertebrate host cells, so as to produce the protein of interest in high amounts.

In a preferred embodiment, the vector of the invention comprises a so-called "pDeSNAP Univ/SBV.N cassette" i.e., a pDeSNAPUniv DNA cassette in which the sequence of the N nucleoprotein of SBV has been inserted, said pDeSNAP Univ/SBV.N cassette comprising a nucleotide sequence encoding, in a single open reading frame, from 5' to 3':

a) a secretion signal peptide,

b) an AGT protein of SEQ ID NO:1, a mutant, a fragment or a catalytic domain thereof, in particular the SNAP mutant of SEQ ID NO:2,

c) at least one peptide cleavage site,

d) the N nucleoprotein of SBV of SEQ ID NO: 16,

e) at least one label, and

f) at least one spacer sequence.

This pDeSNAP Univ/SBV.N DNA cassette encodes a secretion signal peptide which is advantageously the BiP-like peptide signal of SEQ ID NO:24 or the ssBiP peptide signal of SEQ ID NO:37, the SNAP mutant of SEQ ID NO:2, the N nucleoprotein of SBV of SEQ ID NO:16, a label which is advantageously a His-tag of SEQ ID NO:27, a peptide cleavage site which is advantageously either the pro-TEV of SEQ ID NO:32 or the pro-TEV of SEQ ID NO:33, and/or a spacer sequence which has advantageously the amino acid sequence SEQ ID NO:25.

More preferably, the pDeSNAP Univ/SBV.N DNA cassette comprises, from 5' to 3', the sequence SEQ ID NO:23 encoding the BiP-like secretion signal or the SEQ ID NO:22 encoding the ssBiP secretion signal, the SEQ ID NO:15 or 31 encoding the SNAP mutant, the spacer sequence of SEQ ID NO:26, the peptide cleavage site pro-TEV1 of SEQ ID NO:29, the sequence SEQ ID NO: 17 encoding the N nucleoprotein of SBV, the peptide cleavage site pro-TEV2 of SEQ ID NO:30, the spacer sequence of SEQ ID NO:26 and the sequence SEQ ID NO:28 encoding the His-tag label.

Even more preferably, the pDeSNAP Univ/SBV.N DNA cassette comprises, from 5' to 3', the sequence SEQ ID NO:22 encoding the ssBiP secretion signal, the SEQ ID NO:31 encoding the SNAP mutant, the spacer sequence of SEQ ID NO:26, the peptide cleavage site pro-TEV1 of SEQ ID NO:29, the sequence SEQ ID NO: 17 encoding the N nucleoprotein of SBV, the peptide cleavage site pro-TEV2 of SEQ ID NO:30, the spacer sequence of SEQ ID NO:26 and the sequence SEQ ID NO:28 encoding the His-tag label. Such a pDeSNAP Univ/SBV.N cassette is for example SEQ ID NO:35.

Alternatively, the pDeSNAP Univ/SBV.N DNA cassette can comprise, from 5' to 3', the sequence SEQ ID NO:23 encoding the BiP-like secretion signal, the SEQ ID NO:31 encoding the SNAP mutant, the spacer sequence of SEQ ID NO:26, the peptide cleavage site pro-TEV1 of SEQ ID NO:29, the sequence SEQ ID NO: 17 encoding the N nucleoprotein of SBV, the peptide cleavage site pro-TEV2 of SEQ ID NO:30, the spacer

sequence of SEQ ID NO:26 and the sequence SEQ ID NO:28 encoding the His-tag label. Such a pDeSNAP Univ/SBV.N cassette is for example SEQ ID NO:36 (whose structure is shown on figure 9).

Thus, in a preferred embodiment, the vector of the invention comprises the pDeSNAP Univ/SBV.N cassette having the nucleotide sequence SEQ ID NO: 35 or the nucleotide sequence SEQ ID NO:36.

More precisely, the pDeSNAP Univ/SBV.N cassette nucleotide sequence of SEQ ID NO:35 comprises:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the SBV.N DNA sequence SEQ ID NO: 17 (which corresponds to the natural SBV.N sequence, in which the internal *EcoRV* site has been deleted and two *EcoRV* and *XmaI* sites have been added at the extremities),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

And the pDeSNAP Univ/SBV.N cassette nucleotide sequence SEQ ID NO:36 comprises (see also figure 9):

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),

- the SBV.N DNA sequence SEQ ID NO: 17 (which corresponds to the natural SBV.N sequence, in which the internal *EcoRV* site has been deleted and two *EcoRV* and *XmaI* sites have been added at the extremities),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

Interestingly, the pDeSNAP Univ/SBV.N cassette nucleotide sequence of SEQ ID NO:36 cassette also comprises in addition an *NheI* site upstream of the ATG, a *BglII* site between the BiP-like sequence and the SNAP-like sequence, and an *AgeI* site and a *HindIII* site which are both located downstream of the stop codon.

Vectors of the invention are for example SEQ ID NO:43 (which is the pMT/BiP/V5-HisA plasmid from Invitrogen comprising the pDeSNAP Univ cassette) in which the SBV.N DNA sequence SEQ ID NO: 17 has been inserted, SEQ ID NO:44 (which is the pUC57 plasmid from Invitrogen comprising the pDeSNAP Univ cassette) in which the SBV.N DNA sequence SEQ ID NO: 17 has been inserted or SEQ ID NO:45 (which is the pcDNA3 plasmid from Invitrogen comprising the pDeSNAP Univ cassette) in which the SBV.N DNA sequence SEQ ID NO: 17 has been inserted.

Vectors of the invention are also provided in the S2 cells which have been deposited at the Centre National de Culture et de Microorganismes (CNCM), Institut Pasteur (25 rue du Docteur Roux, 75724 Paris cedex 15, France) on April 24, 2012, under the number CNCM I-4616.

In another aspect, the present invention targets a recombinant cell which is stably transfected by a vector of the invention, preferably a vector comprising the nucleotide sequence SEQ ID NO: 35 or SEQ ID NO: 36.

Preferably, in this aspect of the invention, said recombinant cell is a non-vertebrate cell, preferably an insect cell, and more preferably a S2 cell.

Non-vertebrate cells can be any cells from the Insect, Arachnida, Crustacea, Mollusca, Annelida, Cirripedia, Radiata, Coelenterata and Infusoria. In the context of the invention, non-vertebrate cells are preferably insect cells, such as Drosophila or Mosquito cells. They

are more preferably a Drosophila S2 cells. In this case, the expression vector of the invention comprises for example SEQ ID NO: 35.

Drosophila S2 cells have been widely described. They are especially suited to high-yield production of protein, because they can be maintained in suspension cultures at room temperature (24 ± 1°C). Culture medium is M_3 supplemented with between 5 and 10 % (v/v) heat-inactivated fetal bovine serum (FBS). In the preferred embodiment of the invention, the culture medium contains 5% FBS. After induction, the cells are cultured in serum-free media. In this media, the S2 cells can be grown in suspension cultures, for example in 250 mL to 2000 mL spinner flasks, with stirring at 50-60 rpm. Cells densities are typically maintained between 10^6 and 10^7 cells per mL.

In a preferred embodiment, the recombinant cell of the invention is the S2 cell which has been deposited at the Centre National de Culture et de Microorganismes (CNCM), Institut Pasteur (25 rue du Docteur Roux, 75724 Paris cedex 15, France) on April 24, 2012, under the number CNCM I-4616.

In another preferred embodiment, said recombinant cell is a vertebrate cell.

Preferably, said vertebrate recombinant cell is a mammal cell, a preferably CHO, YB2/O, COS, HEK, NIH3T3, HeLa cell or derivatives thereof. More preferably, in this case, the expression vector of the invention comprises SEQ ID NO: 36.

In another aspect of the present invention, the said recombinant cell is used to amplify and purify the expression vectors of the invention, preferably those comprising SEQ ID NO: 35 or 36.

In this aim, the nucleotide expression vectors of the invention may also comprise a gene encoding a selection marker, and/or a terminator sequence. Selection markers genes that can be included in the construct are typically those that confer selectable phenotypes such as resistance to antibiotics (e.g. blasticidin, ampicillin, kanamycin, hygromycin, puromycin, chloramphenicol).

Methods for producing expression vectors are well-known in the art.

In another aspect, the recombinant cell of the invention is used so as to produce the N nucleoprotein of the Schmallenberg virus in high amounts.

Thus, in a particular embodiment, the present invention is also drawn to a method for the production of the N nucleoprotein of the Schmallenberg virus, the method comprising the steps of:

(a) obtaining the vector of the invention, said vector comprising for example the DNA sequence SEQ ID NO:35 or SEQ ID NO:36,

(b) transfecting an host cell (preferably an insect cell or a mammal cell) with the polynucleotide obtained under step (a);

(c) allowing for the expression of said polynucleotide obtained under step (b) to produce the N nucleoprotein of the Schmallenberg virus;

(d) optionally, cleaving the AGT polypeptide,

(e) recovering the N nucleoprotein of the Schmallenberg virus,

(f) optionally, purifying the N nucleoprotein of the Schmallenberg virus.

For performing the different steps of the method of the present invention, there may be employed conventional molecular biology, microbiology and recombinant DNA techniques within the skills of the person of the art. Such techniques are fully explained in the literature. See, for example, Sambrook, Fitsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (referred to herein as "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D. N. Glover ed. 1985); Oligonucleotide Synthesis (M. J. Gait ed. 1984); Nucleic Acid Hybridization(B. D. Hames & S. J. Higgins, eds. 1984); Animal Cell Culture (R. I. Freshney, ed. 1986); Immobilized Cells and Enzymes (IRL Press, 1986); B. E. Perbal, A Practical Guide to Molecular Cloning (1984); F. M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

The term "transfection" means the introduction of a foreign nucleic acid into a eukaryotic host cell so that the host cell will express the introduced gene or sequence to produce the N nucleoprotein of Schmallenberg virus. A host cell that receives and expresses introduced DNA or RNA has been "transfected" and is a "transfectant" or a "clone". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell or cells of a different genus or species.

In the context of the invention, the transfection of the host cells with the polynucleotides can be performed by a classical method in the art, for example by transfection, infection, or electroporation. In another embodiment, the vector of the invention can be introduced *in vivo* by lipofection (as naked DNA), or with other transfection facilitating agents (peptides, polymers, etc.). Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., *Proc. Natl. Acad. Sci. U.S.A.*, 84:7413-7417, 1987). Useful lipid compounds and compositions for transfer of nucleic acids are described in WO 95/18863 and WO 96/17823, and in U.S. 5,459,127. Lipids may be chemically coupled to other molecules for the purpose of targeting (see, Mackey et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:8027-8031, 1988). Targeted peptides, such as hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically. Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptides (see WO 95/21931), peptides derived from DNA binding proteins (see WO 96/25508), or a cationic polymer (see WO 95/21931). It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, such as electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, Wu et al., *J. Biol. Chem.*, 267:963-967, 1992; Wu and Wu, J. *Biol. Chem.*, 263:14621-14624, 1988; Williams et al., *Proc. Natl. Acad. Sci. U.S.A.*, 88:2726-2730, 1991).

The term "allowing for the expression" of a polynucleotide herein means that the stimulus of the regulatory sequences that are present in the vector (e.g. the stimulus activating the inducible promoter), and all the required components are present in a sufficient amount for the translation of the polynucleotide to occur.

If need be, the AGT/SNAP polypeptide can be cleaved off the produced fusion protein by adding a protease having a defined cleavage site to the supernatant of or into the recombinant cells. For example, when a vector comprising the pDeSNAP Univ cassette of SEQ ID NO: 35 or 36 is used, the cleavage of the pro-TEV cleavage site ENLKYFQ/G(S) is obtained by adding the TEV protease to the supernatant of the recombinant cells. Alternatively, the AGT/SNAP polypeptide can be maintained so as to enhance the life-span of the N nucleoprotein from SBV.

Moreover, the skilled artisan will appreciate that an expressed or secreted protein or polypeptide can be detected in the culture medium used to maintain or grow the present host cells. The culture medium can be separated from the host cells by known procedures, such as centrifugation or filtration. The protein or polypeptide can then be detected in the cell-free culture medium by taking advantage of known properties characteristic of the protein or polypeptide. Such properties can include the distinct immunological, enzymatic or physical properties of the protein or polypeptide. For example, if a protein or polypeptide has a unique enzyme activity an assay for that activity can be performed on the culture medium used by the host cells. Moreover, when antibodies reactive against a given protein or polypeptide are available, such antibodies can be used to detect the protein or polypeptide in any known immunological assay (for example as in Harlowe, et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press).

Recovery of the nucleoprotein N from SBV is mediated by the means well-known in the art, including, but not limited to, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, precipitation and salting-out chromatography, extraction, and countercurrent distribution, and the like. As it is preferable to produce the protein of interest in the recombinant system of the invention linked with a label, said label will facilitate the recovery of the polypeptide from the crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the protein or against peptides derived therefrom can be used as recovery reagents.

The present Inventors discovered that the fusion proteins generated with the method of the invention generally do not need to be further purified. However, a further step (g) of purification may be performed, if required.

A purified material may contain less than about 50 %, preferably less than about 75 %, and most preferably less than about 90 %, of the cellular components with which it was originally associated. The term "substantially pure" indicates the highest degree of purity which can be achieved using conventional purification techniques known in the art.

In an embodiment of the invention, the methods of the invention enable to obtain at least 40 mg/L, preferably at least 50 mg/L, more preferably at least 60 mg/L of the substantially

pure N nucleoprotein of the Schmallenberg virus (SBV) in the recovered cell culture supernatant.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the N nucleoprotein of Schmallenberg virus of SEQ ID NO: 16.

In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above).

This fusion polypeptide preferably further comprises a label, as defined above. This label is preferably a poly-histidine label, and is preferably located at the C terminal end of the N nucleoprotein of the Schmallenberg virus.

The fusion polypeptide of the invention is for example the amino acid sequence of SEQ ID NO: 41 (corresponding to the BiPlike/SNAP/SBV.N/Histag fusion protein) or SEQ ID NO: 46 (corresponding to the ssBiP/SNAP/SBV.N/Histag fusion protein) or SEQ ID NO:42 (corresponding to the SNAP/SBV.N fusion protein).

The invention encompasses a vector comprising the DeSNAPuniv- Schmallenberg NSs protein cassette having the nucleotide sequence of SEQ ID NO: 178 and/or encoding the amino acid sequence of SEQ ID NO: 179. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the NSs protein from Schmallenberg virus depicted in Figure 24, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

Finally, the chimeric protein SNAP-SBV. N may be useful as a diagnostic agent for the detection of the viral infection by the Schmallenberg virus, or for the detection of antibodies specific of the said virus in biological fluids, such as blood, serum, saliva, and the like.

Thus, in another aspect, the present invention is also drawn to the use of the fusion protein [SNAP- SBV. N] obtained by any method of the invention for identifying the presence of said pathogenic or non-pathogenic microorganisms in a biological sample, for example thanks to the immunoassay of the present invention.

In other aspects, the present invention also relates to vectors expressing fusion proteins of particular interest, said fusion proteins comprising a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, that is fused in frame with interesting antigens, such as viral or bacterial antigens, microbial peptides and/or polypeptides of interest. These vectors are detailed below.

Echovirus antigen

In another aspect, the present invention relates to a vector for expressing an echovirus antigen, for example the VP1 protein of the enterovirus 71 (*Picornaviridae*), in a host cell. In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the VP1 protein of the enterovirus 71 (EV71, see for example Kolpe A.B. et al, *Virus Research* 2012).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/EV71.VP1 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence SEQ ID NO:47 encoding the VP1 protein from the EV71 virus strain JL-AFP-EV71-07-03 (Genebank#JQ715713) has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/EV71.VP1 cassette having the nucleotide sequence SEQ ID NO: 48 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence SEQ ID NO:47 encoding the VP1 protein from the EV71 virus strain JL-AFP-EV71-07-03 (Genebank#JQ715713),

- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the VP1 protein from the EV71 virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 49 (corresponding to the SNAP-like/proTEV1/EV71.VP1/proTEV2/Histag fusion protein). High levels (30mg/l) of this protein were produced after induction of S2/

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- EV71.VP1] for identifying the presence of the enterovirus 71 in a biological sample, for example in the immunoassay of the present invention.

Flavivirus Antigens

In another aspect, the present invention relates to vectors for expressing particular Flavivirus antigens in a host cell.

In a preferred embodiment, said Flavivirus antigen is the soluble E protein (sE) from the Japanese Encephalitis virus (JEV.sE). More particularly, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the sE protein from the Japanese Encephalitis virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JEV.sE cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequences of gene encoding the soluble E protein (sE) from the Japanese Encephalitis virus (JEV) have been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/JEV.sE cassette having the nucleotide sequence SEQ ID NO: 50 comprising, from 5' to 3':

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the prM/M sequence from JEV strain SA-14 (Genbank#M55506),
- the DNA sequence encoding the E[1-395] sequence from JEV strain SA-14 (Genbank#M55506),
- a DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the soluble E protein (sE) from the Japanese Encephalitis virus (JEV.sE). In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 51 (corresponding to the JEV.sE/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-JEV.sE] for identifying the presence of the Japanese Encephalitis virus (JEV) in a biological sample, for example thanks to the immunoassay of the present invention.

In a preferred embodiment, said Flavivirus antigen is the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 1 (JE-1.EDIII), of genotype 2 (JE-3.EDIII), of genotype 4 (JE-4.EDIII), or of genotype 5 (JE-5.EDIII).

In another aspect, the present invention therefore relates to a vector for expressing the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype I (JE-I.EDIII), of genotype 2 (JE-2.EDIII), of genotype 4 (JE-4.EDIII), or of genotype 5 (JE-5.EDIII) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the EDIII protein from the Japanese encephalitis virus of genotype 1 (JE-I.EDIII), of genotype 2 (JE-2.EDIII), of genotype 4 (JE-4.EDIII), or of genotype 5 (JE-5.EDIII).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JE-I.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of gene encoding the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 1 (JE-1.EDIII) has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ JE-1.EDIII cassette having the nucleotide sequence SEQ ID NO: 52 comprising:

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence SEQ ID NO:54 encoding the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 1 (Genebank#AY377577),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JE-2.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 2 (JE-2.EDIII) has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ JE-2.EDIII cassette having the nucleotide sequence SEQ ID NO: 59 comprising:

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence SEQ ID NO:55 encoding the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 2 (Genebank#L-43566),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JE-4.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 4 (JE-4.EDIII) has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ JE-4.EDIII cassette having the nucleotide sequence SEQ ID NO: 61 comprising:

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence SEQ ID NO:56 encoding the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 4 (Genebank#U70408),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JE-5.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene encoding the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 5 (JE-5.EDIII) has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ JE-5.EDIII cassette having the nucleotide sequence SEQ ID NO: 63 comprising:

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence SEQ ID NO:57 encoding the domain III of the envelope E protein (EDIII protein) from the Japanese encephalitis virus of genotype 5 (Genebank#JN587258),

- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to recombinant cells which are stably transfected by said vectors.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the EDIII protein from the JE-1, JE-2, JE-4, or JE-5 virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 53 (corresponding to the SNAP-like/JE-1.EDIII/Histag fusion protein), SEQ ID NO: 60 (corresponding to the SNAP-like/JE-2.EDIII/Histag fusion protein) SEQ ID NO: 62 (corresponding to the SNAP-like/JE-4.EDIII/Histag fusion protein) or SEQ ID NO: 64 (corresponding to the SNAP-like/JE-5.EDIII/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of any of these fusion proteins [SNAP- JE-1.EDIII], [SNAP- JE-2.EDIII], [SNAP- JE-4.EDIII] or [SNAP- JE-5.EDIII] for identifying the presence of the Japanese encephalitis virus of genotype 1, 2, 4 or 5 respectively in a biological sample, for example thanks to the immunoassay of the present invention.

In another aspect, the present invention is drawn to a vector for expressing the domain III of the envelope E protein (EDIII protein) from the Rabensburg virus (RabV) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the domain III of the envelope E protein (EDIII protein) from the Rabensburg virus (RabV).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/RabV.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the EDIII protein from the Rabensburg virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ RabV.EDIII cassette having the nucleotide sequence SEQ ID NO: 65 comprising:

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence SEQ ID NO:58 encoding the domain III of the envelope E protein (EDIII protein) from the Rabensburg virus (Genebank#AY65264),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the EDIII protein from the Rabensburg virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 66 (corresponding to the SNAP-like/RabV.EDIII/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- RabV.EDIII] for identifying the presence of the Rabensburg virus in a biological sample, for example thanks to the immunoassay of the present invention.

Alphavirus Antigens

In another aspect, the present invention is relates to vectors for expressing particular alphavirus antigens, for example the soluble E2 protein from the Ross River virus (RR.sE2) or from the Mayaro virus (MAY.sE2), in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the sE2 protein from the Ross River virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/RR.sE2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the sE2 gene from the Ross River virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ RR.sE2 cassette having the nucleotide sequence SEQ ID NO: 69 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the sE2 protein of the Ross River virus strain QML1 (Genbank#GQ433354),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the sE2 protein from the Ross River virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 70 (corresponding to the RR.sE2/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- RR.sE2] for identifying the presence of the Ross River virus in a biological sample, for example thanks to the immunoassay of the present invention.

The present invention is also drawn to a vector for expressing the soluble E2 protein from the Mayaro virus (MAY.sE2) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the sE2 protein from the Mayaro virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MAY.sE2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the sE2 gene from the Ross River virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MAY.sE2 cassette having the nucleotide sequence SEQ ID NO: 71 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the corrected sE2 protein (E2-S203C) of the Mayaro virus strain IQD2668 (Genbank#DQ487429.1),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the sE2 protein from the Mayaro virus (MAY.sE2). In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 72 (corresponding to the MAY.sE2/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- MAY.sE2] for identifying the presence of the Mayaro virus in a biological sample, for example thanks to the immunoassay of the present invention.

Equine Encephalitis virus antigens

In another aspect, the present invention relates to vectors for expressing particular Equine Encephalitis virus antigens, for example the soluble E2 protein from the Western Equine Encephalitis virus (WEE.sE2), the Eastern Equine Encephalitis virus (EEE.sE2) or the Venezuelan Equine Encephalitis virus (VEE.sE2) in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the soluble E2 protein from the Western Equine Encephalitis virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/ WEE.sE2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the sE2 gene from the Western Equine Encephalitis virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ WEE.sE2 cassette having the nucleotide sequence SEQ ID NO: 73 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the sE2 protein from Western Equine Encephalitis virus strain (Genbank#NC00390808),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the sE2 protein from the WEE virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 74 (corresponding to the WEE.sE2/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- WEE.sE2] for identifying the presence of the Western Equine Encephalitis virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is also drawn to a vector for expressing the soluble E2 protein from the Eastern Equine Encephalitis virus (EEE.sE2) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the soluble E2 protein from the Eastern Equine Encephalitis virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/ EEE.sE2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the sE2 gene from the Eastern Equine Encephalitis virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ EEE.sE2 cassette having the nucleotide sequence SEQ ID NO: 75 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the sE2 protein from Eastern Equine Encephalitis virus strain (Genbank#EF151502),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the sE2 protein from the EEE virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 76 (corresponding to the EEE.sE2/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- EEE.sE2] for identifying the presence of the Eastern Equine Encephalitis virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is also drawn to a vector for expressing the soluble E2 protein from the Venezuelan Equine Encephalitis virus (VEE.sE2) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the soluble E2 protein from the Venezuelan Equine Encephalitis virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/ VEE.sE2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the sE2 gene from the Venezuelan Equine Encephalitis virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ VEE.sE2 cassette having the nucleotide sequence SEQ ID NO: 77 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the sE2 protein from Venezuelan Equine Encephalitis virus strain (Genbank#AY973944),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the sE2 protein from the VEE virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 78 (corresponding to the VEE.sE2/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- VEE.sE2] for identifying the presence of the Venezuelan Equine Encephalitis virus in a biological sample, for example thanks to the immunoassay of the present invention.

Orthobunyavirus antigens

In another aspect, the present invention relates to vectors for expressing particular orthobunyavirus antigens, for example the Nucleoprotein N from the Akabane virus (AKA.N), from the Aino virus (AIN.N) or from the Shamonda virus (SHA.N), in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Akabane virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/AKA.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene encoding the Nucleoprotein N from the Akabane virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ AKA.N cassette having the nucleotide sequence SEQ ID NO: 79 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the natural N nucleoprotein of the Akabane virus,
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the N nucleoprotein from the Akabane virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 80 (corresponding to the SNAP-like/proTEV1/AKA.N/pro-TEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- AKA.N] for identifying the presence of the Akabane virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is drawn to a vector for expressing the Nucleoprotein N from the Aino virus (AIN.N) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Aino virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/AIN.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene encoding the Nucleoprotein N from the Aino virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/AIN.N cassette having the nucleotide sequence SEQ ID NO: 81 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the natural N nucleoprotein of the Aino virus,
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),

- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the N nucleoprotein from the Aino virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 82 (corresponding to the SNAP-like/proTEV1/AIN.N/pro-TEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- AIN.N] for identifying the presence of the Aino virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is drawn to a vector for expressing the Nucleoprotein N from the Shamonda virus (SHA.N) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Shamonda virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/SHA.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene encoding the Nucleoprotein N from the Shamonda virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/SHA.N cassette having the nucleotide sequence SEQ ID NO: 83 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,

- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the natural N nucleoprotein of the Shamonda virus,
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the N nucleoprotein from the Shamonda virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 84 (corresponding to the SNAP-like/proTEV1/SHA.N/pro-TEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- SHA.N] for identifying the presence of the Shamonda virus in a biological sample, for example thanks to the immunoassay of the present invention.

Betacoronavirus antigens

In another aspect, the present invention relates to vectors for expressing particular betacoronavirus antigens, for example the Nucleoprotein N from human betacoronavirus (huCOV.N) or the protein S of the human betacoronavirus (huCOV.S), in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from human betacoronavirus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/huCOV.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene encoding the Nucleoprotein N from human betacoronavirus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/huCOV.N cassette having the nucleotide sequence SEQ ID NO: 85 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the gene N from human betacoronavirus 2cEMC/2012 (Genbank#JX869059),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from human betacoronavirus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 86 (corresponding to the SNAP-like/proTEV1/huCOV.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- huCOV.N] for identifying the presence of the human betacoronavirus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is drawn to a vector for expressing the soluble form of the spike S protein from human betacoronavirus (huCOV.S) in an host cell, comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the soluble form of the spike S protein from human betacoronavirus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/huCOV.S cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene S from human betacoronavirus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/huCOV.S cassette having the nucleotide sequence SEQ ID NO: 87 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the gene S from human betacoronavirus 2cEMC/2012 (Genbank#JX869059),
- a DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another embodiment, the vector comprises the DeSNAPuniv- SARS virus N protein cassette having the nucleotide sequence of SEQ ID NO: 154 and/or encoding the amino acid sequence of SEQ ID NO: 155. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the mutated protein from SARS coronavirus depicted in Figure 12, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAP+ SARS S-RDB cassette having the nucleotide sequence of SEQ ID NO: 156 and/or encoding the amino acid sequence of SEQ ID NO: 157. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the receptor binding

domain from S protein of SARS virus depicted in Figure 13, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination

In another embodiment, the vector comprises the DeSNAPuniv- huCOV.N (human coronavirus) cassette having the nucleotide sequence of SEQ ID NO: 158 and/or encoding the amino acid sequence of SEQ ID NO: 159. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the gene N from human betacoronavirus depicted in Figure 14, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the - huCOV.S1 + DeSNAP (human coronavirus) cassette having the nucleotide sequence of SEQ ID NO: 160 and/or encoding the amino acid sequence of SEQ ID NO: 161. The vector can comprise a DNA sequence encoding the signal peptide of chimeric protein, the SNAP sequence, or the gene S1 from human betacoronavirus depicted in Figure 15, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAPuniv- huCoV.S-RDB cassette having the nucleotide sequence of SEQ ID NO: 162 and/or encoding the amino acid sequence of SEQ ID NO: 163. The vector can comprise a DNA sequence encoding the signal peptide of chimeric protein, the SNAP sequence, the pro-TEV sites, or the RBD from S protein of human betacoronavirus depicted in Figure 16, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the soluble form of the spike S protein from human betacoronavirus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 88 (corresponding to the huCOV.S/SNAP-like/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- huCOV.S] for identifying the presence of the human betacoronavirus in a biological sample, for example thanks to the immunoassay of the present invention.

Hepacivirus antigen

In another aspect, the present invention relates to vectors for expressing particular hepacivirus antigens, for example the protein C from Hepatitis C virus (HCV.C) or from Hepatitis E virus (HEV.C), in a host cell.

In one embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the protein C from Hepatitis C virus.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/HCV.C cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene of the protein C from Hepatitis C virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/HCV.C cassette having the nucleotide sequence SEQ ID NO: 89 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the C protein from hepatitis C virus genotype 1b (strain TCHM-R2/03),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another embodiment, the vector comprises the DeSNAPuniv-HEV.C cassette having the nucleotide sequence of SEQ ID NO: 170 and/or encoding the amino acid sequence of SEQ ID NO: 171. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the mutated C protein from Hepatitis E virus depicted in Figure 20, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAPuniv-HEV.C protein central domain cassette having the nucleotide sequence of SEQ ID NO: 172 and/or encoding the amino acid sequence of SEQ ID NO: 173. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the HEV core antigen depicted in Figure 21, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAPuniv-HCV Core antigen cassette having the nucleotide sequence of SEQ ID NO: 174 and/or encoding the amino acid sequence of SEQ ID NO: 175. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the HCV core antigen depicted in Figure 22, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAPuniv-short HCV Core (C)cassette having the nucleotide sequence of SEQ ID NO: 176 and/or encoding the amino acid sequence of SEQ ID NO: 177. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the short form of the HCV core protein depicted in Figure 23, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the protein C from Hepatitis C virus (HCV.C). In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a

homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 90 (corresponding to the SNAP-like/proTEV1/HCV.C/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- HCV.C] for identifying the presence of the Hepatitis C virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the protein C from Hepatitis E virus (HEV.C).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/HEV.C cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence of the gene of the protein C from Hepatitis E virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/HEV.C cassette having the nucleotide sequence SEQ ID NO: 150 comprising:

- an insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the C protein from hepatitis E virus (Genbank#AB29196),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the protein C from Hepatitis E virus (HEV.C). In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 151 (corresponding to the SNAP-like/proTEV1/HEV.C/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- HEV.C] for identifying the presence of the Hepatitis E virus in a biological sample, for example thanks to the immunoassay of the present invention.

Malaria antigens

In another aspect, the present invention is drawn to a vector for expressing particular Malaria antigens, for example, the MSP-1 and the AMA-1 proteins from *Plasmodium falciparum* (MSP-1+AMA-1) (see Pan W. et al, *The Journal of Immunology*, 2004), in an host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the MSP-1 and the AMA-1 proteins from the parasite *Plasmodium falciparum*.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MSP-1+AMA-1cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the MSP-1 and the AMA-1 proteins from the parasite *Plasmodium falciparum* has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MSP-1+AMA-1cassette having the nucleotide sequence SEQ ID NO: 91 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- the DNA sequence encoding the MSP-1 (19) sequence (50% G+C) from *Plasmodium falciparum*,
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- the DNA sequence encoding the AMA-1 (III) sequence (50% G+C) from *Plasmodium falciparum*,
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the MSP-1+AMA-1 protein from *Plasmodium falciparum*. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 92 (corresponding to the SNAP-like/MSP-1/proTEV2/AMA-1/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- MSP-1+AMA-1] for identifying the presence of the parasite *Plasmodium falciparum* in a biological sample, for example thanks to the immunoassay of the present invention.

Leptospirosis antigens

In another aspect, the present invention is drawn to a vector for expressing a particular leptospirosis antigen, such as the HbpA, LruA, LruB, or LipL32 protein of *Leptospira* bacteria (see Sivakolundu S. et al, *Journal of Medical Microbiology*, 2012), in a host cell. Preferred vectors are SNAP-Lru A and SNAP-Lru B. Particularly preferred are S2 cell lines containing these vectors. S2/SNAP-Lru A and S2/SNAP-Lru B cell lines were deposited on May 2, 2013, at the Collection Nationale de Cultures de Microorganismes (CNCM), 25, Rue du Docteur Roux, 75724 Paris Cedex 15, France, under numbers CNCM I-4745 and CNCM I-4746, respectively.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the HbpA protein from *Leptospira interrogans* bacteria.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/HbpA cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the HbpA protein from *Leptospira* bacteria has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/HbpA cassette having the nucleotide sequence SEQ ID NO: 93 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the modified short form of HbpA (TonB-dependent outer membrane receptor or LB191) from *Leptospira interrogans* serovar Lai str.56601 (Genbank#AA51750.1),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),

- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another embodiment, the vector comprises the DeSNAPuniv-shortLruA (leptospirosis) cassette having the nucleotide sequence of SEQ ID NO: 164 and/or encoding the amino acid sequence of SEQ ID NO: 165. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the modified short forms of LruA depicted in Figure 17, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAPuniv- shortLruB (leptospirosis) cassette having the nucleotide sequence of SEQ ID NO: 166 and/or encoding the amino acid sequence of SEQ ID NO: 167. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the modified short forms of LruB depicted in Figure 18, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another embodiment, the vector comprises the DeSNAPuniv- LipL32 (leptospirosis) cassette having the nucleotide sequence of SEQ ID NO: 168 and/or encoding the amino acid sequence of SEQ ID NO: 169. The vector can comprise a DNA sequence encoding the signal peptide of SNAP, the SNAP sequence, the pro-TEV sites, or the modified short forms of LipL32 depicted in Figure 19, or at least 10, 15, 20, 25, 30, 40, 50, 60, 100, 200, or 300 consecutive amino acids thereof, alone or in combination.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the HbpA protein from *Leptospira interrogans* bacteria. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 94 (corresponding to the SNAP-like/proTEV1/HbpA/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-HbpA] for identifying the presence of the *Leptospira* bacteria in a biological sample, for example thanks to the immunoassay of the present invention.

Microbial peptides

In another aspect, the present invention is drawn to a vector for expressing a microbial peptide, for example the microbial peptide MUB-40, in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the MUB-40 peptide.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MUB40 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the MUB40 peptide has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MUB40 cassette having the nucleotide sequence SEQ ID NO: 95 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the MUB-40 peptide,
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33), and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the MUB 40 peptide. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 96 (corresponding to the SNAP-like/proTEV1/MUB40/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-MUB40] for identifying the presence of a ligand in a biological sample, for example thanks to the immunoassay of the present invention.

Lectins involved in Flavivirus pathogenesis

In another aspect, the present invention is drawn to vectors for expressing particular lectins involved in Flavivirus pathogenesis, for example the mouse or the human soluble form of C-type like lectin (CLEC5A), in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the mouse CLEC5A (mo-CLEC5A) or the human CLEC5A (hu-CLEC5A).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/mo-CLEC5A cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the mouse soluble form of C-type like lectin has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/mo-CLEC5A cassette having the nucleotide sequence SEQ ID NO: 97 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,

- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the mouse soluble form of C-type like lectin (CLEC5A),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25), and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/hu-CLEC5A cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the human soluble form of C-type like lectin has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/hu-CLEC5A cassette having the nucleotide sequence SEQ ID NO: 99 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the human soluble form of C-type like lectin (CLEC5A),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25), and

- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the mouse or the human soluble form of C-type like lectin (CLEC5A). In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 98 (corresponding to the SNAP-like/proTEV1/mo-CLEC5A/proTEV2/Histag fusion protein) or the amino acid sequence of SEQ ID NO: 100 (corresponding to the SNAP-like/proTEV1/hu-CLEC5A/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-mo-CLEC5A] or [SNAP-hu-CLEC5A] for detection of presence of flaviviruses in a biological sample, for example thanks to the immunoassay of the present invention.

Anti-flaviviral mosquito proteins

In another aspect, the present invention is drawn to vectors for expressing particular antiviral mosquito proteins, for example the VAGO protein from the *Culex* species (cxVAGO) or from the *Aedes* species (aaVAGO) in a host cell.

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the VAGO protein from the *Aedes albopictus* mosquito.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/aaVAGO cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the VAGO protein from the *Aedes albopictus* mosquito has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/aaVAGO cassette having the nucleotide sequence SEQ ID NO: 103 comprising:

- an insect BiP-like sequence of SEQ ID NO: 152,
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- the DNA sequence encoding the VAGO protein from the *Aedes albopictus* mosquito, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the VAGO protein from the *Aedes albopictus* mosquito. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 104 (corresponding to the SNAP-like/aaVAGO/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-aaVAGO] for identifying the presence of a ligand in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the VAGO protein from the *Culex quinquefasciatus* mosquito.

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/cxVAGO cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the VAGO protein from the *Culex quinquefasciatus* mosquito has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/cxVAGO cassette having the nucleotide sequence SEQ ID NO: 101 comprising:

- an insect BiP-like sequence of SEQ ID NO: 152,
- the SNAP-like sequence of SEQ ID NO: 31,
- a DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- the DNA sequence encoding the VAGO protein from the *Culex quinquefasciatus* mosquito, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the VAGO protein from the *Culex quinquefasciatus* mosquito. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 102 (corresponding to the SNAP-like/cxVAGO/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-cxVAGO] for identifying the presence of a ligand in a biological sample, for example thanks to the immunoassay of the present invention.

Viral Hemorragic fever antigens

In another aspect, the present invention is drawn to vectors for expressing particular viral hemorragic fever antigens such as:

- the Nucleoprotein N from the Crimean-Congo virus (CCHF.N), from the Ebola virus (EBO.N), from the Marburg virus (MAR.N), from the Lassa virus (LAS.N), from the Junin virus (JUN.N), from the Machupo virus (MAC.N), from the Sabia virus (SAB.N), or from the Guanarito virus (GUA.N),
- the Ectodomain of GP1 from the Lassa virus (LAS.ectoGP1), from the Junin virus (JUN.ectoGP1), from the Machupo virus (MAC.ectoGP1), from the Sabia virus (SAB.ectoGP1), or from the Guanarito virus (GUA.ectoGP1),
- the Ectodomain of GP2 from the Lassa virus (LAS.ectoGP2), from the Junin virus (JUN.ectoGP2), from the Machupo virus (MAC.ectoGP2), from the Sabia virus (SAB.ectoGP2), or from the Guanarito virus (GUA.ectoGP2),
- the domain III of the envelope E protein from the Omsk virus (OMSK.EDIII), from the Kasyanur virus (KAS.EDIII), or from the Alkhurma virus (ALK.EDIII).

In particular, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Crimean-Congo virus (CCHF.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/CCHF.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Crimean-Congo virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/CCHF.N cassette having the nucleotide sequence SEQ ID NO: 108 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Crimean-Congo virus,

- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Crimean-Congo virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 109 (corresponding to the SNAP-like/proTEV1/CCHF.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- CCHF.N] for identifying the presence of the Crimean-Congo virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Ebola virus (EBO.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/EBO.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Ebola virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/EBO.N cassette having the nucleotide sequence SEQ ID NO: 110 comprising:

- an insect BiP sequence of SEQ ID NO: 22,

- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Ebola virus (Genbank#NC_002549),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Ebola virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 111 (corresponding to the SNAP-like/proTEV1/EBO.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- EBO.N] for identifying the presence of the Ebola virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Marburg virus (MAR.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MAR.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Marburg virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MAR.N cassette having the nucleotide sequence SEQ ID NO: 112 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Marburg virus (Genbank#NC_001608),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Marburg virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 113 (corresponding to the SNAP-like/proTEV1/MAR.N /proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-MAR.N] for identifying the presence of the Marburg virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Lassa virus (LAS.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/LAS.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Lassa virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/LAS.N cassette having the nucleotide sequence SEQ ID NO: 114 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Lassa virus (Genbank#NC_004296),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Lassa virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 115 (corresponding to the SNAP-like/proTEV1/LAS.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-LAS.N] for identifying the presence of the Lassa virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Junin virus (JUN.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JUN.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Junin virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/JUN.N cassette having the nucleotide sequence SEQ ID NO: 116 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Junin virus (Genbank#NC_005081),

- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Junin virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 117 (corresponding to the SNAP-like/proTEV1/JUN.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-JUN.N] for identifying the presence of the Junin virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Machupo virus (MAC.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MAC.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Machupo virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MAC.N cassette having the nucleotide sequence SEQ ID NO: 118 comprising:

- an insect BiP sequence of SEQ ID NO: 22,

- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Machupo virus (Genbank#NC_005078),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Machupo virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 119 (corresponding to the SNAP-like/proTEV1/MAC.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-MAC.N] for identifying the presence of the Machupo virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Guanarito virus (GUA.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/GUA.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Guanarito virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/GUA.N cassette having the nucleotide sequence SEQ ID NO: 120 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Guanarito virus (Genbank#NC_005077),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Guanarito virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 121 (corresponding to the SNAP-like/proTEV1/GUA.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-GUA.N] for identifying the presence of the Guanarito virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention relates to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Nucleoprotein N from the Sabia virus (SAB.N).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/SAB.N cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Nucleoprotein N from the Sabia virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/SAB.N cassette having the nucleotide sequence SEQ ID NO: 122 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the DNA sequence encoding the Nucleoprotein N from the Sabia virus (Genbank#NC_006317),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a second DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Nucleoprotein N from the Sabia virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 123 (corresponding to the SNAP-like/proTEV1/SAB.N/proTEV2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-SAB.N] for identifying the presence of the Sabia virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the domain III of the Envelop protein E from the Omsk virus (OMSK.EDIII).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/OMSK.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the EDIII protein from the Omsk virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/OMSK.EDIII cassette having the nucleotide sequence SEQ ID NO: 124 comprising:

- an insect BiP-like sequence of SEQ ID NO: 152,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence encoding the EDIII protein of the Omsk virus (Genbank#NC_005062),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the EDIII protein from the Omsk virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 125 (corresponding to the SNAP-like/OMSK.EDIII/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-OMSK.EDIII] for identifying the presence of the Omsk virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the domain III of the Envelop protein E from the Kyasanur Forest Disease virus (KYA.EDIII).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/KYA.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the EDIII protein from the Kyasanur Forest Disease virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/KYA.EDIII cassette having the nucleotide sequence SEQ ID NO: 126 comprising:

- an insect BiP-like sequence of SEQ ID NO: 152,
- the SNAP-like sequence of SEQ ID NO: 31,
- the DNA sequence encoding the EDIII protein of the Kyasanur Forest Disease virus (Genbank#JF416958),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the EDIII protein from the Kyasanur Forest Disease virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 127 (corresponding to the SNAP-like/KYA.EDIII/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-KYA.EDIII] for identifying the presence of the Kyasanur Forest Disease virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment, the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the domain III of the Envelop protein E from the Alkhurma virus (ALK.EDIII).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/ALK.EDIII cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the EDIII protein from the Alkhurma virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/ALK.EDIII cassette having the nucleotide sequence SEQ ID NO: 128 comprising:

- an insect BiP-like sequence of SEQ ID NO: 152,
- the SNAP-like sequence of SEQ ID NO: 31,

- the DNA sequence encoding the EDIII protein of the Alkhurma virus (Genbank#NC_004355),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the EDIII protein from the Alkhurma virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 129 (corresponding to the SNAP-like/ALK.EDIII/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-ALK.EDIII] for identifying the presence of the Alkhurma virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP1 ectodomain from the Lassa virus (LAS.ectoGP1).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/LAS.ectoGP1 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP1 ectodomain from the Lassa virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/LAS.ectoGP1 cassette having the nucleotide sequence SEQ ID NO: 130 comprising:

- an insect BiP sequence of SEQ ID NO: 22,

- the DNA sequence encoding the Glycoprotein GP1 ectodomain from the Lassa virus (Genbank#NC_004296),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP1 ectodomain from the Lassa virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 131 (corresponding to the SNAP-like/ LAS.ectoGP1/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- LAS.ectoGP1] for identifying the presence of the Lassa virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP1 ectodomain from the Junin virus (JUN.ectoGP1).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JUN.ectoGP1 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP1 ectodomain from the Junin virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/JUN.ectoGP1 cassette having the nucleotide sequence SEQ ID NO: 132 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP1 ectodomain from the Junin virus (Genbank#NC_005081),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP1 ectodomain from the Junin virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 133 (corresponding to the SNAP-like/ JUN.ectoGP1/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP- JUN.ectoGP1] for identifying the presence of the Junin virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP1 ectodomain from the Machupo virus (MAC.ectoGP1).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MAC.ectoGP1 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP1 ectodomain from the Machupo virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MAC.ectoGP1 cassette having the nucleotide sequence SEQ ID NO: 134 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP1 ectodomain from the Machupo virus (Genbank#NC_005078),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP1 ectodomain from the Machupo virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 135 (corresponding to the SNAP-like/ MAC.ectoGP1/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-MAC.ectoGP1] for identifying the presence of the Machupo virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP1 ectodomain from the Guanarito virus (GUA.ectoGP1).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/GUA.ectoGP1 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in

which the sequence encoding the Glycoprotein GP1 ectodomain from the Guanarito virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/GUA.ectoGP1 cassette having the nucleotide sequence SEQ ID NO: 136 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP1 ectodomain from the Guanarito virus (Genbank#NC_005077),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP1 ectodomain from the Guanarito virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 137 (corresponding to the SNAP-like/ GUA.ectoGP1/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-GUA.ectoGP1] for identifying the presence of the Guanarito virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP1 ectodomain from the Sabia virus (SAB.ectoGP1).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/SAB.ectoGP1 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP1 ectodomain from the Sabia virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/SAB.ectoGP1 cassette having the nucleotide sequence SEQ ID NO: 138 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP1 ectodomain from the Guanarito virus (Genbank#NC_006317),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP1 ectodomain from the Sabia virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 139 (corresponding to the SNAP-like/ SAB.ectoGP1/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-SAB.ectoGP1] for identifying the presence of the Sabia virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP2 ectodomain from the Lassa virus (LAS.ectoGP2).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/LAS.ectoGP2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP2 ectodomain from the Lassa virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/LAS.ectoGP2 cassette having the nucleotide sequence SEQ ID NO: 140 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP2 ectodomain from the Lassa virus (Genbank#NC_004296),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP2 ectodomain from the Lassa virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 141 (corresponding to the SNAP-like/ LAS.ectoGP2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-LAS.ectoGP2] for identifying the presence of the Lassa virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment

or a catalytic domain thereof, and c) the Glycoprotein GP2 ectodomain from the Junin virus (JUN.ectoGP2).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/JUN.ectoGP2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP2 ectodomain from the Junin virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/JUN.ectoGP2 cassette having the nucleotide sequence SEQ ID NO: 142 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP2 ectodomain from the Junin virus (Genbank#NC_005081),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP2 ectodomain from the Junin virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 143 (corresponding to the SNAP-like/ JUN.ectoGP2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-JUN.ectoGP2] for identifying the presence of the Junin virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host

cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP2 ectodomain from the Machupo virus (MAC.ectoGP2).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/MAC.ectoGP2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP2 ectodomain from the Machupo virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MAC.ectoGP2 cassette having the nucleotide sequence SEQ ID NO: 144 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP2 ectodomain from the Machupo virus (Genbank#NC_005078),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP2 ectodomain from the Machupo virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 145 (corresponding to the SNAP-like/ MAC.ectoGP2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-MAC.ectoGP2] for identifying the presence of the Machupo virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP2 ectodomain from the Guanarito virus (GUA.ectoGP2).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/GUA.ectoGP2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP2 ectodomain from the Guanarito virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/GUA.ectoGP2 cassette having the nucleotide sequence SEQ ID NO: 146 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP2 ectodomain from the Guanarito virus (Genbank#NC_005077),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP2 ectodomain from the Guanarito virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 147 (corresponding to the SNAP-like/ GUA.ectoGP2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-GUA.ectoGP2] for identifying the presence of the Guanarito virus in a biological sample, for example thanks to the immunoassay of the present invention.

In another embodiment the present invention is drawn to a vector comprising the nucleotide sequence encoding a) a secretion signal peptide which is functional in said host cells, and b) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT), a mutant, a fragment or a catalytic domain thereof, and c) the Glycoprotein GP2 ectodomain from the Sabia virus (SAB.ectoGP2).

In a preferred embodiment, this vector comprises a so-called "pDeSNAP Univ/SAB.ectoGP2 cassette" i.e., a pDeSNAPUniv DNA cassette as defined above, in which the sequence encoding the Glycoprotein GP2 ectodomain from the Sabia virus has been inserted.

In a preferred embodiment, this vector comprises the pDeSNAP Univ/MAC.ectoGP2 cassette having the nucleotide sequence SEQ ID NO: 148 comprising:

- an insect BiP sequence of SEQ ID NO: 22,
- the DNA sequence encoding the Glycoprotein GP2 ectodomain from the Sabia virus (Genbank#NC_006317),
- the SNAP-like sequence of SEQ ID NO: 31, and
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

In another aspect, the present invention relates to a recombinant cell which is stably transfected by said vector.

In another aspect, the present invention is drawn to a fusion polypeptide comprising a) a 6-alkylguanine-DNA-alkyltransferase enzyme (AGT) (EC 2.1.1.63), a mutant or a catalytic domain thereof and b) the Glycoprotein GP2 ectodomain from the Sabia virus. In this fusion polypeptide, said AGT enzyme is preferably the protein of SEQ ID NO: 2, or a homologue thereof (said homologous being as defined above). This fusion polypeptide is for example the amino acid sequence of SEQ ID NO: 149 (corresponding to the SNAP-like/ SAB.ectoGP2/Histag fusion protein).

Thus, in another aspect, the present invention is also drawn to the use of this fusion protein [SNAP-SAB.ectoGP2] for identifying the presence of the Sabia virus in a biological sample, for example thanks to the immunoassay of the present invention.

Examples

In the context of the invention, a multiplex bead-based immunoassay was developed for rapid and simultaneous detection of antibodies to arboviruses in biological fluids.

The system is based on the xMAP technology (Luminex corporation) and uses a mixture of antigen-coated microspheres as capture reagents for specific human immunoglobulins. Distinct sets of microspheres (Magplex, Luminex corporation) were coupled with purified AGT fusion proteins, namely the SNAP-tagged viral recombinant proteins: sSNAP-DV1.EDIII, sSNAP-DV2.EDIII, sSNAP-DV3.EDIII, sSNAP-DV4.EDIII, sSNAP-WN.EDIII, sSNAP-JE.EDIII, sSNAP-USU.EDIII, sSNAP-TBE.EDIII, sSNAP-YF.EDIII, sSNAP-MVE.EDIII, sSNAP-Rocio.EDIII, sSNAP-WSL.EDIII, sSNAP-ZIKA.EDIII, SNAP-DV1ectoM, sSNAP-N.RVF, sSNAP-N.TOS, and CHIK.sE2-SNAP. Recombinant antigens were covalently coupled to the carboxyl microsphere surface using a substrate of the AGT protein as linker (BG-PEG-NH2, New England Biolabs), thereby enhancing antibody capture efficiency as compared to standard amine coupling procedures.

Technical validation using anti-SNAP-tag antibodies and specific mouse monoclonal antibodies confirmed coupling efficiency and demonstrated long-term antigen stability (up to six month). This application is not limited to viral antigens as any peptide or polypeptide can be used for bead coating and subsequent antibody capture.

I. Material and methods

1. The following buffers and solutions are used:

a) **PBS buffer**: 100 mL of 10X PBS, pH 7.4 in 1 L H2O sterile

b) **SNAP coupling buffer** (PBS-DTT) : 100 mL of 10 X PBS, pH 7.4, 0.5 mL 10 % tween 20, 1 mL of 1.0 M DTT, in 1 L H_2O sterile

c) **blocking / assay buffer** (PBS-B): PBS, 1 % BSA, pH 7.4 in 1 L H_2O sterile

d) **storage buffer** (PBS-TBN): 100 mL of 10X PBS, 1 g of BSA, 2 mL of 10 % tween 20, 500 mg of sodium azide, 1 mL of 1.0M DTT, in 1 L H_2O sterile

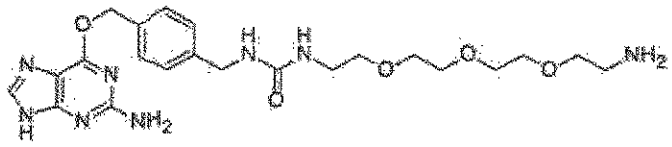
e) **Substrate solution** (4 mg/mL): 2 mg of BG-PEG-NH_2, DMSO 200 µL.

f) **Activation solution** (EDAC /SNHS): 50 mg/mL of EDAC solution or 50 mg/mL of SNSHS in distilled water

2. The following materials were used:

2.1. MagPlex Luminex microspheres : MC 100XX-ID (where XX is the fluorescence region), XX can be *e.g.* 26, 27, 28, 29, 34, 35, 36, 37, 45, 52, 53, 63, 64, as mentioned on figure 7B

2.2. **hAGT substrate**: PEG-BG-NH_2 (NEB S9150S)



2.3. **Fusion proteins SNAP-viral EDIII** :

The generation of a fusion protein comprising AGT and viral EDIII moieties is well-known to the skilled person. Every known synthesis process can be used for this purpose, provided that the AGT enzyme remains active in the fusion protein.

In the present case, the AGT mutant SNAP of SEQ ID NO: 2 has been used and SNAP-viral EDIII fusion proteins have been generated.

The Drosophila S2 inducible expression system (DES, Invitrogen), has been chosen for the mass production of individual EDIII from flaviviruses in non-vertebrate cells and the plasmid pMT/BiP/V5-HisA from Invitrogen has been used.

This plasmid contains:

- The metallothionein promoter pMT,
- An insect ssBiP sequence of SEQ ID NO: 22,
- *Bgl II* and *Age I* restriction sites,

- the DNA of SEQ ID NO: 28 encoding a His_6tag located downstream of the *AgeI* restriction site, and

- the DNA spacer sequence of SEQ ID NO: 26 located between the *AgeI* restriction site and the DNA encoding a His_6tag.

The synthetic genes coding for the full-length domain III of the E proteins from flaviviruses WN, USU, JE, TBE, DEN-1 to DEN-4, YF, Rocio, MVE, Zika, SLE, and WSL are listed in SEQ ID NO: 3 to SEQ ID NO: 14. The ED III amino acid sequences were fused in frame to the C-terminus of the SNAP protein, with both moieties being separated by a linker GGGS (SEQ ID NO: 25). The DNA sequences encoding SNAP-EDIII were inserted in the plasmid pMT/BiP/V5-Histag (Invitrogen) to generate the plasmids pMT/BiP/SNAP/EDIII/Histag.

The resulting plasmids pMT/BiP/SNAP-EDIII-Histag, which can drive the expression of secreted SNAP-EDIII-His_6 fusion proteins, were co-transfected with selection marker pCo-Blast into S2 cells to generate the stable S2/sSNAP-ED III- Histag cell line showing resistance to blasticidine.

Stable S2 cell lines grown in spinner (1000 ml) were stimulated 10 days with heavy metal cadmium (Cd^{2+}) and proteins from extracellular medium were concentrated and purified.

Accumulation of secreted SNAP-tagged EDIII protein was observed in the supernatants of stable S2/sSNAP-EDIII-Histag cells after 10days of induction with heavy metal cadmium.

The proteins SNAP-DEN1.EDIII of SEQ ID NO: 21, SNAP-DEN2.EDIII of SEQ ID NO:X, SNAP-DEN3.EDIII of SEQ ID NO:X, SNAP-DEN4.EDIII of SEQ ID NO:X, SNAP-WN.EDIII of SEQ ID NO:X, SNAP-JE.EDIII of SEQ ID NO:X, SNAP-YF.EDIII of SEQ ID NO:X, SNAP-MVE.EDIII of SEQ ID NO:X, SNAP-Rocio.EDIII of SEQ ID NO:X, SNAP-WSL.EDIII of SEQ ID NO:X, SNAP-ZIKA.EDIII of SEQ ID NO:X, SNAP-SLE.EDIII of SEQ ID NO:X have been produced accordingly.

3. Preparation of the antigen-coupled beads

The production of antigen-coupled beads comprised two steps: functionalization of microsphere surfaces with an O^6-benzylguanine derivative (BG-PEG-amino), and covalent

immobilization of the chimeric SNAP-viral Ags proteins using the BG-PEG-amino as an anchor (Figure 1). The carboxyl microsphere surfaces were covalently coated with BG-PEG-amino substrate using an optimized two-step carbodiimide process (Wong et al *Journal of Clinical Microbiology* **42**(1): 65–72, 2004). Subsequently, coupled BG-PEG-amino compounds were irreversibly linked to the chimeric SNAP-viral Ags proteins by transfer of the benzyl group to the active site cysteine of the SNAP protein. Due to the high specificity of this reaction, the fusion protein is exclusively coupled via the SNAP domain, leaving the viral antigen accessible for interactions with antibodies.

3.1. First, the commercial beads were activated as per the manufacturer instructions (by using the EDAC and SNHS activation solutions), and washed in a PBS buffer. All the steps were performed in darkness so as to prevent the fluorescent quenching of the beads, according to the manufacturer instructions.

About 1.25 x 10^6 beads were used for each coupling process.

3.2. The AGT substrate PEG-BG-NH_2 in the DMSO solution was then added overnight at room temperature on the activated beads, and subsequently washed with PBS buffer.

3.3. The unbound carboxylic sites were then blocked with the blocking buffer for 30 minutes at room temperature, and the beads subsequently washed with the SNAP coupling buffer.

3.4. SNAP-EDIII proteins resuspended in the SNAP coupling buffer (100 µg/mL) were incubated with the thus obtained beads for two hours at room temperature, and then washed once with the SNAP coupling buffer, and three times with the storage buffer (PBS-TBN).

4. Microsphere fluorescence immunoassays

The bead sets, conjugated with different SNAP-viral Ags, were mixed by vortex to ensure total bead dispersal. After adjusting the bead density to 100 beads/µL, 25 µl of each of the bead sets (containing 2500 microspheres) were transferred to a 96-well microtiter plate (Bio-Plex Pro flat bottom plate, BioRad) in separate wells for singleplex assays, or mixed in the same wells for multiplex assays. The microspheres were washed 2 times with 100 µL washing buffer (BioPlex Wash buffer, BioRad) using a microplate wash

station for magnetic beads (BioPlex Pro Wash Station, BioRad). The samples (antibodies or sera) were diluted in assay buffer (PBS-BSA) and 50 µL of the resulting solutions were added to the test wells containing the conjugated beads. After incubation in darkness on a plate shaker for 30 min, the plate was washed 3 times. Subsequently, a fluorochrome-labeled secondary antibody was diluted in assay buffer (PBS-BSA) at 2 µg/mL, and 50 µL of the resulting solutions were added to the test wells containing the conjugated beads. After incubation in darkness on a plate shaker for 30 min, the plate was washed as previously. Finally, streptavidin-phycoerythrin (SAPE, Invitrogen Molecular Probes) was diluted in assay buffer (PBS-BSA) at 2 µg/ml, and 50 µL of the resulting solution was added to the microplate wells. The plate was incubated in darkness on a plate shaker for 10 min and washed as previously, before resuspending the contents of the wells in 125 µl of assay buffer. The median fluorescence intensity (MFI) of the detection antibody bound to the individual microspheres was evaluated from flow analysis of 50 microspheres per well using a dual-laser flow analyzer (BioPlex 200 instrument, BioRad). The fluorescent detection instrument is equipped with a first laser for detecting the type of bead, and a second to ensure the quantification of captured IgM or IgG by exciting the fluorophore (red-phycoerythrin) conjugated to the specific detection antibody.

4.1 Confirmation of antigen coupling

Antigen coupling was confirmed by testing the antigen-coupled microspheres with dilutions of rabbit anti-SNAP-tag polyclonal antibody (GenScript). The fluorescence immunoassays were performed in singleplex format, as described above. A two-fold dilution series of anti-SNAP antibody starting at 4000 ng/mL and ending at 3.9 ng/mL was performed in PBS-BSA, and volumes of each dilution were added to the test wells containing the beads. A biotin-conjugated goat anti-rabbit IgG (2 µg/mL in 50 µL PBS-BSA) was used as secondary antibody to detect bound anti-SNAP antibodies.

Figure 2 shows the fluorescence results observed for the detection of anti-SNAP antibody on 8 different sets of microspheres coupled to chimeric SNAP-viral antigens proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV.EDIII, SNAP-YF.EDIII, SNAP-JE.EDIII, SNAP-TBE.EDIII).

4.2 Detection of specific antibodies

The capture and detection of specific antibodies by the antigen-conjugated microspheres was assessed using purified monoclonal mouse antibodies (anti-WNV, anti-DV1 and anti-DV2) and polyclonal mouse sera (anti-DV3, anti-DV4, anti-YF and anti-JE) or human sera (anti-DV1). The fluorescence immunoassays were performed in singleplex and multiplex format, as described above. A four-fold dilution series of purified mouse monoclonal antibodies starting at 400 ng/mL and ending at 0.1 ng/mL, and of mouse and human sera starting at 1:25 and ending at 1:102400, was performed in PBS-BSA, and volumes of each dilution were added to the test wells containing the beads. A biotin-conjugated goat anti-mouse IgG (2 µg/mL in 50 µL PBS-BSA), was used as secondary antibody to detect bound monoclonal and polyclonal mouse antibodies. A biotin-conjugated goat anti-human IgM (2 µg/mL in 50 µL PBS-BSA) or a biotin-conjugated goat anti-human IgG (2 µg/mL in 50 µL PBS-BSA), was used to detect bound IgM or IgG antibodies in human serum, respectively.

Figure 3 compares the sensitivity of the immunoassay experiment for the detection of purified monoclonal anti-DV2 antibody on chimeric SNAP-DV2.EDIII protein conjugated to microspheres via the substrate of the hAGT protein (coupling of the invention) or coupled through Bio-Plex Amine Coupling Kit, BIORAD.

Figure 4 compares the sensitivity of the immunoassay experiment for the detection of purified monoclonal anti-DV1 antibody on chimeric SNAP-DV1.EDIII protein conjugated to microspheres, either in a singleplex or in a multiplex format with other chimeric SNAP-viral Ags proteins (SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV, SNAP-YF, SNAP-JE, SNAP-TBE) coupled to microspheres.

Figure 5 shows the reactivity and specificity of the multiplex immunoassay experiment for the detection of dilutions of purified monoclonal anti-WNV antibody on chimeric SNAP-viral Ags proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV, SNAP-YF, SNAP-JE, SNAP-TBE) coupled to microspheres.

Figure 6 shows the reactivity and specificity of anti-DV3 IgG detection in mouse polyclonal serum against DV3 (A) and anti-YF IgG detection in mouse polyclonal serum against YF (B) in multiplex immunoassays on chimeric SNAP-viral Ags proteins (SNAP-

DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV, SNAP-YF, SNAP-JE, SNAP-WSL, SNAP-ROCIO, SNAP-MVE, SNAP-SLE, SNAP-ZIKA) coupled to microspheres.

Figure 7 shows the reactivity and specificity of anti-DV1 IgM detection (A) and anti-DV1 IgG detection (B) in DV1-infected serum of a human patient in multiplex immunoassays on chimeric SNAP-viral Ags proteins (SNAP-DV1.EDIII, SNAP-DV2.EDIII, SNAP.DV3.EDIII, SNAP.DV4.EDIII, SNAP-WNV, SNAP-YF, SNAP-JE, SNAP-WSL, SNAP-ROCIO, SNAP-MVE, SNAP-SLE, SNAP-ZIKA, SNAP-TBE) coupled to microspheres.

II. Results

The system of the invention uses a mixture of antigen-coated Magplex microspheres (Luminex Corporation) as capture reagents for specific human immunoglobulins. Each set of internally color-coded microspheres have been coupled to a specific recombinant antigen and mixed with other types of microspheres in a small sample volume. The power of this system lies in the fact that it is possible to simultaneously analyze up to 100 types of coupled microspheres per well using a flow analysis tool. The fluorescent detection instrument is equipped with a first laser for detecting the type of bead, and a second to ensure the quantification of captured IgM or IgG by exciting the fluorophore (phycoerythrin) conjugated to the specific detection antibody. With its extensive multiplexing capabilities and lower limit of detection, this approach offers substantial cost and sample savings over traditional ELISA measurements.

Presently, 16 distinct sets of microspheres have been coupled with purified chimeric SNAP-viral Ags proteins, allowing titration of serum antibodies specific to dengue serotypes 1 to 4, West Nile, Yellow fever, Japanese encephalitis, tick-borne encephalitis, Saint-Louis encephalitis, Murray Valley encephalitis, Wesselsbron, Zika, Rocio, Usutu, Rift Valley fever, and Chikungunya virus. The production of the system is highly time- and cost-effective, as only a very small amount of recombinant antigen (< 50µg) is required to produce one set of antigen-coupled microspheres (~1.25 x 10^6 microspheres), sufficient to perform 1000 individual assays. Moreover, the selected sets of microspheres are adaptable

to an affordable, compact, and robust fluorescent detection system such as the MagPix (Luminex Corporation).

The evaluation of antigen coupling using an anti-SNAP antibody (Figure 2) confirmed the coupling efficiency and demonstrated that the relative quantities of bound antigens are comparable between the different coupled microsphere sets. The assessment of antibody capture and detection using purified mouse antibodies showed enhanced capture of specific antibodies by the produced antigen-coupled microspheres as compared to antigen-coupled microspheres obtained by standard amine coupling procedures (Figure 3). In addition, it demonstrated the low detection limit of the method and confirmed that multiplexing does not affect antibody detection (Figure 4). Additionally, the antigen-conjugated microspheres exhibited long-term stability when stored at 4° C (> 6 months). Finally, the specificity of each set of coupled microspheres in multiplex immunoassays was demonstrated for purified mouse monoclonal antibodies (Figure 5), for IgG antibodies in polyclonal mouse sera (Figure 6A-B) and for both IgM and IgG antibodies in polyclonal sera from infected humans (Figure 7).

With its extensive multiplexing capabilities (up to 100 types of coupled microspheres per well) and lower limit of detection, this approach offers substantial cost and sample savings over traditional ELISA measurements.

III. **Generation of a fusion protein comprising SNAP and the N nucleoprotein of the Schmallenberg virus**

1. Construction of the vectors encoding the fusion protein SNAP-SBV.N

The chimeric fusion protein comprising SNAP and the N nucleoprotein of the Schmallenberg virus has been obtained as follows:

In a first step, the sequence of the open reading frame of the S segment encoding the N nucleoprotein and the NSs protein of the BH80/11-4 strain was mutated by inserting an *EcoRV* restriction site at its 5' terminus and an *XmaI* restriction site at its 3' terminus. In addition, the internal *EcoRV* restriction site was removed by mutating the 294T nucleotide into 294A. This mutated sequence is shown on SEQ ID NO: 17.

This mutated sequence was then inserted into the *EcoRV* and *XmaI* restriction sites of the pDeSNAP Univ cassette of SEQ ID NO: 34, generating the "pDeSNAP Univ/SBV.N" DNA cassette of SEQ ID NO: 36.

The so-called "pDeSNAP Univ/SBV.N" DNA cassette comprises (see figure 9 and SEQ ID NO: 36):

- the insect BiP-like sequence of SEQ ID NO: 23,
- the SNAP-like sequence of SEQ ID NO: 31,
- a first DNA sequence SEQ ID NO: 26 encoding the spacer sequence GGGS (SEQ ID NO: 25),
- a DNA sequence SEQ ID NO: 29 encoding a pro-TEV1 cleavage site of sequence ENLYFQS (SEQ ID NO: 32),
- the SBV.N DNA sequence SEQ ID NO: 17 (which corresponds to the natural SBV.N sequence, in which the internal *EcoRV* site has been deleted and two *EcoRV* and *XmaI* sites have been added at the extremities),
- a DNA sequence SEQ ID NO: 30 encoding a pro-TEV2 cleavage site of sequence ENLYFQG (SEQ ID NO: 33),
- a DNA sequence SEQ ID NO: 28 encoding a HisTag sequence.

Note that this cassette comprises in addition an *NheI* site upstream of the ATG, a *BglII* site between the BiP-like sequence and the SNAP-like sequence, and an *AgeI* site and a *HindIII* site which are both located downstream of the stop codon.

The sequence comprised between the *BglII* and *AgeI* restriction sites of the pDeSNAPUniv/SBV.N cassette (see figure 9) was excised by enzymatic digestion, then cloned into the pMT/BiP/V5-A plasmid (Invitrogen) to generate the pMT/BiP/SNAP-SBV.N vector. This vector has been used to generate stable S2 cells secreting the SNAP-SBV.N fusion protein.

The sequence comprised between the *NheI* and *NotI* restriction sites of the pDeSNAPUniv/SBV.N cassette is then cloned into the pcDNA3 plasmid (Invitrogen) to generate the pcDNA3/SNAP-SBV.N vector. This vector is then used to generate stable mammalian cells secreting the SNAP-SBV.N fusion protein.

2. Production of the fusion protein SNAP-SBV.N

The resulting plasmids pMT/BiP/SNAP-SBV.N that allow the production of SNAP-tagged SBV.N proteins as secreted fusion proteins, were co-transfected with selection marker pCo-Blast into S2 cells to generate the stable S2/SNAP-SBV.N cell line showing resistance to blasticidine.

This cell line has been deposited to the Collection Nationale de Cultures de Microorganismes (CNCM) of the Institut Pasteur, 25, rue du Docteur Roux, 75724 PARIS CEDEX 15, under the number CNCM I-4616.

Stable S2 cell lines grown in spinner (1000 ml) were stimulated 10 days with heavy metal cadmium (Cd^{2+}).

Accumulation of secreted SNAP-SBV.N protein was observed in the supernatants of the S2/SNAP-SBV.N cells after 10days of induction with heavy metal cadmium.

0.01mL from 4mL of supernatant of S2/SNAP-SBV.N cells induced 10 days with Cd^{2+} were tested by immunoblot assay using anti-Histag antibody (dilution 1:1,000) (see figure 10).

The chimeric protein SNAP-SBV.N was compared with defined amounts of the SNAP-TOS.N chimeric protein (corresponding to the fusion protein comprising SNAP and the N nucleoprotein from the Toscana virus, which is a phlebovirus).

The production of purified SNAP-SBV.N from induced S2/SNAP+SBV.N cells for 10 days is 18 mg per liter of cell culture (Fig. 10B).

BIBLIOGRAPHIC REFERENCES

Avrameas S. *Immunol. Today* 1991 May;12(5):154-9.

Zimmerman CW, *Electrophoresis* 1995; Jun;16(6):941-7.

Kim H-J. *The Journal of Veterinary Medical Science*, 2011

Damoiseaux et al., *ChemBiochem.* 4:285-287, 2001

Xu-Welliver et al., Biochemical Pharmacology 58: 1279-85, 1999

Lim A. et al, *EMBO J.* 15: 4050-4060, 1996;

Daniels D.S. et al, *EMBO J.* 19: 1719-1730, 2000;

Juillerat A. et al, *Chemistry & Biology*, vol.10, 313-317, 2003

Wong et al Journal of Clinical Microbiology 42, no. 1 (jan 2004): 65–72

Wibley J.E.A. et al, 2000

Felgner et al., *Proc. Natl. Acad. Sci. U.S.A.*, 84:7413-7417, 1987

Mackey et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:8027-8031, 1988

Wu et al., *J. Biol. Chem.*, 267:963-967, 1992;

Wu and Wu, J. *Biol. Chem.*, 263:14621-14624, 1988;

Williams et al., *Proc. Natl. Acad. Sci. U.S.A.*, 88:2726-2730, 1991

Kolpe A.B. et al, *Virus Research* 2012; 168:64-72

Pan W. et al, *The Journal of Immunology*, 2004), 172:6167-6174

Sivakolundu S. et al, *Journal of Medical Microbiology*, 2012

We claim:

1. An *in vitro* assay method for detecting at least two different target antibodies present in a biological sample from a subject, said method comprising the steps of:

(a) contacting a mixture of at least 2 solid supports with a biological sample;
wherein at least one of the solid supports comprises a first AGT-Antigen fusion protein covalently coupled to an AGT substrate, and
wherein at least one of the solid supports comprises a second AGT-Antigen fusion protein covalently coupled to an AGT substrate;
(b) detecting the presence or absence of binding of antibodies to the first AGT-Antigen; and
(c) detecting the presence or absence of binding of antibodies to the second AGT-Antigen,
wherein the AGT of said first and second fusion proteins is the SNAP mutant of SEQ ID NO:2.

2. The assay method of claim 1, comprising contacting a mixture of at least 5 solid supports with a biological sample from a subject.

3. The assay method of claim 1, comprising contacting a mixture of at least 10 solid supports with a biological sample from a subject.

4. The assay method of claim 1, comprising contacting a mixture of at least 15 solid supports with a biological sample from a subject.

5. The assay method of any one of claims 1 to 4, wherein said solid supports can be specifically identify by their specific location, size, diameter, weight, granulometry, or labeling.

6. The assay method of any one of claims 1 to 5, wherein said solid supports are labeled with a fluorochrome, a chromophore, a radioisotope, and/or a mass tag.

7. The assay method of any one of claims 1 to 6, wherein said solid supports are microparticles.

8. The assay method of any one of claims 1 to 7, wherein said solid supports are magnetic.

9. The assay method of any one of claims 1 to 8, wherein said solid supports are microparticles internally labeled with fluorescent dyes.

10. The assay method of any one of claims 1 to 9, wherein each of the solid supports emits a different and distinguishable wave length after excitation.

11. The assay method of any one of claims 1 to 10, wherein said first or second AGT-Antigen fusion protein comprises at least 30 consecutive amino acids of the EDIII protein of the dengue virus 1 of SEQ ID NO:3, the EDIII protein of the dengue virus 2 of SEQ ID NO:4, the EDIII protein of the dengue virus 3 of SEQ ID NO:5, the EDIII protein of the dengue virus 4 of SEQ ID NO:6, the EDIII protein of the West Nile virus of SEQ ID NO:7, the EDIII protein of the Yellow Fever virus of SEQ ID NO:8, , the EDIII protein of the Japanese encephalitis virus of SEQ ID NO:9, the EDIII protein of the Zika virus of SEQ ID NO:10, the EDIII protein of the Wesselbron virus of SEQ ID NO:11, the EDIII protein of the Rocio virus of SEQ ID NO:12, the EDIII protein of the Murray encephalitis virus of SEQ ID NO:13, and the EDIII protein of the Saint-Louis encephalitis virus of SEQ ID NO:14, the EDIII protein of the Japanese encephalitis virus of genotype 1 encoded by SEQ ID NO:54, the EDIII protein of the Japanese encephalitis virus of genotype 2 encoded by SEQ ID NO:55, the EDIII protein of the Japanese encephalitis virus of genotype 4 encoded by SEQ ID NO:56, the EDIII protein of the Japanese encephalitis virus of genotype 5 encoded by SEQ ID NO:57, the EDIII protein of the Rabensburg virus encoded by SEQ ID NO:58.

12. The assay method of any one of claims 1 to 11, wherein said first or second AGT-Antigen fusion protein comprises at least 20 consecutive amino acids of a viral protein of HIV1, of HIV2, of the Hepatitis B virus, of the Hepatitis C virus, of the Hepatitis E virus, of the West-Nile virus and of oncogenic HPV strains HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

13. The assay method of any one of claims 1 to 12, wherein said first or second AGT-Antigen fusion protein comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:42, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:74, SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:84, SEQ ID NO:86, SEQ ID

NO:88, SEQ ID NO:90, SEQ ID NO:92, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:98, SEQ ID NO:100, SEQ ID NO:102, SEQ ID NO:104, SEQ ID NO:109, SEQ ID NO:111, SEQ ID NO:113, SEQ ID NO:115, SEQ ID NO:117, SEQ ID NO:119, SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:129, SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:135, SEQ ID NO:137, SEQ ID NO:139, SEQ ID NO:141, SEQ ID NO:143, SEQ ID NO:145, SEQ ID NO:147, SEQ ID NO:149 and SEQ ID NO:151.

14. The assay method of any one of claims 1 to 13, wherein said first or second AGT-Antigen fusion protein comprises at least 30 consecutive amino acids of SEQ ID NOs: 155, 157, 159, 161, 163, 165, 167, 171, 173, 175, 177, and 179.

15. The assay method of any one of claims 1 to 14, wherein said first or second AGT-Antigen fusion protein comprises the amino acid sequence of SEQ ID NOs: 155, 157, 159, 161, 163, 165, 167, 171, 173, 175, 177, and 179.

16. The assay method of any one of claims 1 to 15, wherein said biological sample is serum or plasma.

17. A kit for detecting at least different two target antibodies present in a biological sample from a subject comprising:

(a) a first solid support comprising a first AGT-Antigen fusion protein covalently coupled to an AGT substrate, and

(b) a second solid support comprising a second AGT-Antigen fusion protein covalently coupled to an AGT substrate.

wherein said solid supports are mixed together in at least one single compartment, and

wherein the AGT of said first and second fusion proteins is the SNAP mutant of SEQ ID NO:2.

18. The kit according to claim 17, comprising at least at least 5 solid supports, each coupled to a different AGT-Antigen fusion protein covalently coupled to an AGT substrate.

19. The kit according to claim 18, comprising at least at least 10 solid supports, each coupled to a different AGT-Antigen fusion protein covalently coupled to an AGT substrate.

20. The kit according to claim 19, comprising at least at least 15 solid supports, each coupled to a different AGT-Antigen fusion protein covalently coupled to an AGT substrate.

21. The kit according to any one of claims 17 to 20, wherein the said solid supports are microparticles.

22. The kit according to any one of claim 17 to 21, wherein the said solid supports are microparticles that are mixed together in at least one well of a microtiter plate or at least one tube.

23. The kit according to any one of claim 17 to 22, further comprising labeled secondary antibodies to detect the at least two target antibodies which are bound to the solid supports.

24. The kit according to any one of claim 17 to 23, wherein said first or second AGT-Antigen fusion protein comprises at least 30 consecutive amino acids of the EDIII protein of the dengue virus 1 of SEQ ID NO:3, the EDIII protein of the dengue virus 2 of SEQ ID NO:4, the EDIII protein of the dengue virus 3 of SEQ ID NO:5, the EDIII protein of the dengue virus 4 of SEQ ID NO:6, the EDIII protein of the West Nile virus of SEQ ID NO:7, the EDIII protein of the Yellow Fever virus of SEQ ID NO:8, , the EDIII protein of the Japanese encephalitis virus of SEQ ID NO:9, the EDIII protein of the Zika virus of SEQ ID NO:10, the EDIII protein of the Wesselbron virus of SEQ ID NO:11, the EDIII protein of the Rocio virus of SEQ ID NO:12, the EDIII protein of the Murray encephalitis virus of SEQ ID NO:13, and the EDIII protein of the Saint-Louis encephalitis virus of SEQ ID NO:14, the EDIII protein of the Japanese encephalitis virus of genotype 1 encoded by SEQ ID NO:54, the EDIII protein of the Japanese encephalitis virus of genotype 2 encoded by SEQ ID NO:55, the EDIII protein of the Japanese encephalitis virus of genotype 4 encoded by SEQ ID NO:56, the EDIII protein of the Japanese encephalitis virus of genotype 5 encoded by SEQ ID NO:57, the EDIII protein of the Rabensburg virus encoded by SEQ ID NO:58.

25. The kit according to any one of claim 17 to 24, wherein said first or second AGT-Antigen fusion protein comprises at least 20 consecutive amino acids of a viral protein of HIV1, of HIV2, of the Hepatitis B virus, of the Hepatitis C virus, of the Hepatitis E virus,

of the West-Nile virus and of oncogenic HPV strains HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

26. The kit according to any one of claim 17 to 25, wherein said first or second AGT-Antigen fusion protein comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:42, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:74, SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:90, SEQ ID NO:92, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:98, SEQ ID NO:100, SEQ ID NO:102, SEQ ID NO:104, SEQ ID NO:109, SEQ ID NO:111, SEQ ID NO:113, SEQ ID NO:115, SEQ ID NO:117, SEQ ID NO:119, SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:129, SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:135, SEQ ID NO:137, SEQ ID NO:139, SEQ ID NO:141, SEQ ID NO:143, SEQ ID NO:145, SEQ ID NO:147, SEQ ID NO:149 and SEQ ID NO:151.

27. The kit according to any one of claim 17 to 26, said first or second AGT-Antigen fusion protein comprises at least 30 consecutive amino acids of SEQ ID NOs: 155, 157, 159, 161, 163, 165, 167, 171, 173, 175, 177, and 179.

28. The kit according to any one of claim 17 to 27, said first or second AGT-Antigen fusion protein comprises the amino acid sequence of SEQ ID NOs: 155, 157, 159, 161, 163, 165, 167, 171, 173, 175, 177, and 179.

29. Use of the kit as defined in any one of the claims 17 to 28, for detecting at least two target antibodies in a biological sample from a subject.

30. Use of the kit as defined in any one of the claims 17 to 29, for diagnosing at least two target diseases in a subject, wherein said target disease is a viral infection caused by a Papillomavirus or a Dengue, Yellow fever, West Nile, Japanese encephalitis, Tick-Borne Encephalitis, Hepatitis C, Chikungunya, Ross River, Mayaro, Western Equine encephalitis, Eastern Equine Encephalitis, Venezuela Equine Encephalitis, Crimean-Congo hemorrhagic fever, Rift Valley Fever, Schmallenberg, Hepatitis E virus, Lassa, Ebola, Marburg virus; a bacterial infection caused by *Leptospirosa Interrogans*; or an infection caused by *Plasmodium falciparum*.

31. An *in vitro* method for diagnosing at least one target disease in a subject, said target disease being known to induce the synthesis of at least one target antibody in said subject, comprising performing the assay method as defined in any of the claims 1 to 16, wherein said subject is diagnosed to be suffering from said at least one target disease if the amount of said at least one target antibody is higher than a control value.

32. A method for manufacturing a kit, said method comprising the steps of:

(a) providing a first AGT-Antigen fusion protein that is recognized by a first target antibody,

(b) contacting said first AGT-Antigen fusion protein with a first solid support, said support being covalently coupled with a substrate of said AGT-Antigen fusion protein,

(c) obtaining a first solid support covalently coupled with the first AGT-Antigen fusion protein,

(d) providing at a second AGT-Antigen fusion protein that is recognized by a second target antibody, but not by said first target antibody, and

(e) contacting said second AGT-Antigen fusion protein with a second solid support, said support being covalently coupled with a substrate of said AGT-Antigen fusion protein, and

(f) obtaining a second solid support covalently coupled with the second AGT-Antigen fusion protein,

wherein said first and at second solid supports can be specifically identified from each other,

wherein said solid supports are mixed together in at least one single compartment, and wherein the AGT of said first and second fusion proteins is the SNAP mutant of SEQ ID NO:2.

33. The method of claim 32, further comprising following steps a)-c) with at least 5 different AGT-Antigen fusion proteins to generate at least 5 solid supports covalently coupled with 5 different AGT-Antigen fusion proteins.

34. The method of claim 32, further comprising following steps a)-c) with at least 10 different AGT-Antigen fusion proteins to generate at least 10 solid supports covalently coupled with 10 different AGT-Antigen fusion proteins.

35. The method of claim 32, further comprising following steps a)-c) with at least 15 different AGT-Antigen fusion proteins to generate at least 15 solid supports covalently coupled with 15 different AGT-Antigen fusion proteins.