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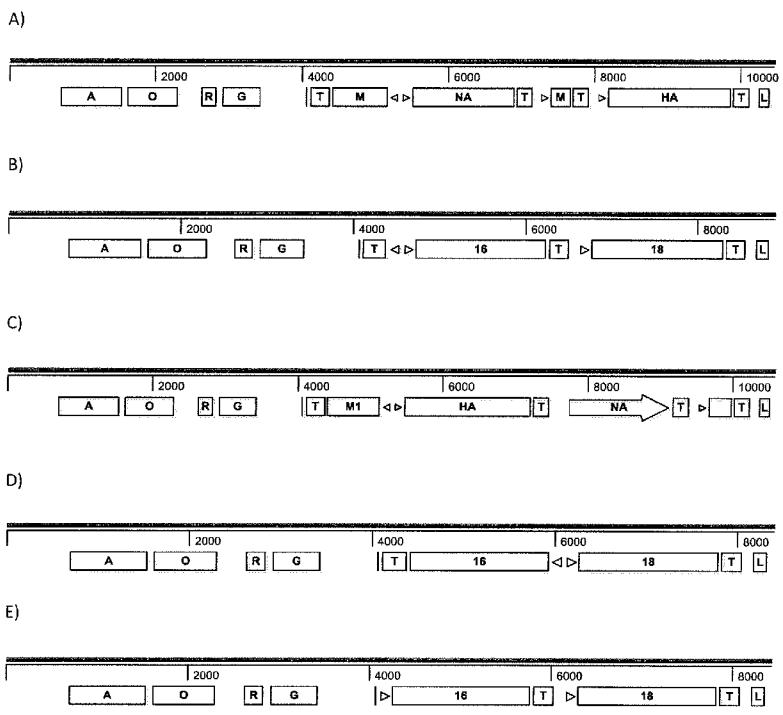
[Continued on next page]

(54) **Title**: RECOMBINANT VIRUS-LIKE PARTICLES ENCODED BY MULTI-GENE VECTOR

Fig. 1



(57) **Abstract**: The invention describes novel virus-like particles for use as vaccines, diagnostic tools and R&D tools based on recombinant DNA and cell cultivation techniques for production. The recombinant virus-like particles of the invention are assembled by polypeptide chains that incorporate several, in particular two or more, different epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts. These epitopes are then displayed on the particle surface.

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Recombinant virus-like particles encoded by multi-gene vector

Field of the invention

The invention relates to recombinant virus-like particles comprising epitopes from different virus strains, and vectors encoding these.

Background of the invention

There is an increasing interest in small natural biomolecules using them in different aspects in biomedicine, nanotechnology and material science. Virus simulators, virus capsids or virus-like particles are very attractive because of their regular structure, their homogenous particle size, their stability, the ease of production and the potential for manipulation. Virus-like particle possess dynamic structures, their interior is accessible and furthermore the coat is modifiable. Dependent on the application the virus-like particles could have an envelope or not and could be chosen as virus simulators. These embodiments could be used as new biological entities or targets, as vaccines, as antigens for antibody production, as research tools, as diagnostic tool, for drug delivery and bioconjunctions. These virus simulators are formed by self-assembly of envelope and or capsid proteins of many viruses. The size varies between 22-150 nm dependent on the morphology of the particular virus. The virus simulators are non-infectious because they assemble without incorporating genetic material. Dependent on the application foreign genetic material could be included in the herein described virus simulator.

A promising application of these virus simulators is the production of vaccines against various diseases because their repetitive, high density display of epitopes elicit often a strong immune response. The small size of particles is an advantage for uptake by dendritic cells. Chimeric virus simulators offer an enormous potential in selective, multi-epitope, multi-protein, multi-serotype, multi-strain, or multi-species presentation.

There exist many expression systems for the production of virus simulators which include the baculovirus/insect cell system, various mammalian cell lines, either stably or transiently transfected or transduced with viral expression vectors, furthermore various species of yeast including *Saccharomyces cerevisiae* and *Pichia pastoris*, and *Escherichia coli* and other bacteria.

Vaccination is dependent on the generation of a suffient immunity to protect from infectious diseases. The mostly used attenuated virus vaccines rely on limited replication of the virus in the host following immunization. But this kind of vaccination may cause severe reactions in some patients. Therefore the development of virus-like particles (VLP) as subunit vaccines is an advantage because the particles lack in general DNA or RNA genome but have the authentic conformation of the natural virus.
Vaccination is one of the most potent and cost-effective counter-measures to the threat of e.g. seasonal or pandemic influenza outbreaks. The ease of spread as an aerosol and the cause for a severe illness especially to susceptible humans are the major reasons why influenza is one of the most devastating viral diseases. Currently licensed seasonal vaccines are only partially protective, and the egg-based production is very time-consuming and cost-intensive. This strategy is vulnerable to the unanticipated emergence of epidemic strains that are poorly matched or not matched at all by the vaccine. Due to the danger of emerging strains of avian influenza or influenza of other origin novel vaccine approaches are necessary.

In another aspect the research in the field of several important viruses like HCV, HIV, Ebola etc. is very difficult because of biosafety issues. Until now there exist only a few models for investigation of viral entry and viral trafficking. Diagnostic tools are based on the genome of these viruses because of the lack of appropriate non-infectious virus models.

Presently commercial human influenza vaccines contain hemagglutinin as their only or main viral antigen. Their production starts from viruses grown on embryonated chicken eggs or, more recently, in mammalian cells in tissue culture. The production in eggs requires selection of high yield, reassorted virus strains, is limited in capacity, time-consuming (6-8 months), and expensive. Beyond that it can cause problems in vaccinated persons allergic to egg protein. The production is only possible with non-lethal bird strains. One of the most important disadvantages of the egg-based production is the limited capacity. In case of a pandemic the production of the seasonal influenza vaccine has to be stopped in favour of a pandemic influenza vaccine production which could result in even more lethal events in the long run.

Vaccines against viral diseases rely traditionally on attenuated virus strains or inactivation of infectious virus. An appropriate environment is necessary for highly pathogenic or

haemorraghic viruses which constrains the production possibilities because of the biosafety level (e.g. BL3 / BL4). For some viruses like human papilloma virus the attenuation will not be sufficient because the virus cannot be propagated *in vitro*. The ability to generate human papilloma virus (HPV)-like particles based vaccines (Gardasil, Cervarix) has changed the prospects for preventing cervical cancer in woman.

Due to the danger of emerging strains of avian influenza or other origin, novel vaccine approaches are necessary which result in an enhanced protection.

Summary of the invention

The invention relates to a recombinant virus-like particle comprising two or more different epitopes or different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts. These recombinant virus-like particles are useful as vaccines, and the invention also relates to these vaccines.

Furthermore the invention relates to a vector comprising two or more polynucleotides coding for different epitopes or for different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts, and to host cells comprising these vectors.

Brief description of the Figure

Figure 1: Schematic representations of vector constructs expressing multiple-epitope virus-like particles.
(A) (SEQ ID NO:1) Multivalent influenza A virus simulator containing different epitopes (M1, M2) from H1N1 viral strains as well as H3N2 (HA, NA) viral strains
(B) (SEQ ID NO:2) Chimeric human papilloma virus-like particle containing an epitope (L1) from serotypes HPV16 and HPV18
(C) (SEQ ID NO:3) Expression vector construct with embedded epitopes (HA, NA, M1, M2) from influenza B/Florida isolates
(D) (SEQ ID NO:4) Vector construct for expression of an epitope (L1) of HPV16 and HPV18 serotypes where both genes are under the control of different promoters

(E) (SEQ ID NO:5) Same Vector construct as (B) with deletion of promoter p10

The vectors contain two promoters P1 and P2 (◁,▷) selected from polh, p10 and p_{XIV} very late baculoviral promoters, vp39 baculoviral late promoter, vp39polh baculoviral late/very late hybrid promoter, pca/polh, *pcna, etl*, *p35, egt, da26* baculoviral early promoters; CMV, SV40, UBc. EF-1 , RSVLTR, MT, P_{DS47}, Ac5, P_{GAL} and P_{ADH}. The terminator sequences T1 and T2 (T) are selected from SV40, HSVtk or BGH (bovine growth hormone). Furthermore the vector contains the transposon sites TnL and TnR (L,R) for generation of MultiBacbacmid, a loxP site (LP) for site specific homologous recombination (plasmid fusion), an *origin of replication (*O*)*, ampicillin (A) and gentamycine (G) resistance genes and defined restriction sites.

Figure 2: Analysis of expressed chimeric influenza virus-like particles.
(A) The conformation of secreted (A, lane 2) as well as intracellular VLPs (prepared from SEQ ID NO:1, A, lane 1) is verified by immunoblotting using specific antibodies against the proteins HA (H3), NA (H3) and M2 (H1). Lane 3 = ladder, protein sizes in kDa. The epitopes are co-localized, which means that they are assembled in one particle.
(B) Visualization of the chimeric Influenza virus-like particle (prepared from SEQ ID NO:3) by electron microscopy using negative staining with uranyl acetate. The spikes representing epitope HA are visible. The size of the particle is in the range of 50-80 nm.

Figure 3: Chromatographic purification and analysis of secreted multi-epitope influenza virus-like particles prepared from SEQ ID NO:1.
(A) Chromatogram of gel filtration purification. The first peak (1) contains the virus-like particle (VLP). The other peaks (2-6) represent contaminant proteins.
(B) Coomassie-stained SDS-PAGE. The multiple epitopes of the virus-like particles are verified by analyzing different fractions from the 1st peak (1) representing the start (lane 1), middle (lane 2) and end (lane 3) part of the VLP containing peak. The ladder [kDa] is represented left of the first lane. Detection of epitopes is indicated by arrows.
(C) Western blot analysis according to Coomassie-stained gel using an anti-HA antibody.

Figure 4: Functionality of nature-like influenza virus-like particles (VLP).
Twofold serial dilution series (1:2 to 1:2048) of the purified VLPs (prepared from SEQ ID NO:3) are analyzed by standard hemagglutination assay. 50 µL purified particle solution was coated onto 96-well plate incubated with red blood cells (erythrocytes). The influenza VLP (1, upper part) are able to agglutinate red blood cells in a dose dependent manner.

Highest dilution is 1:1024. In contrast PBS, used as control (C), leads only to precipitation of erythrocytes, visible as a "dot" in the middle of the well.

Figure 5: *In vivo* evaluation of multi-epitope influenza virus-like particles.
Mice are immunized either with 50 ng (mice 1-5) or 100 ng (mice 6-10) purified VLP prepared from SEQ ID NO:3, and as control with PBS (mice 11-12). Antibody titers after prime injection (3 weeks post injection) are indicated as white boxes, titers after a boost injection are indicated as black boxes (6 weeks post injection). The titers are presented as dilution of mice sera (y-axis). VLPs effectively stimulate an antibody immune response. The best results are obtained when immunization is performed with 100 µl (mice 6-10), indicating a dose dependent immune response. A clear increase of the amount of anti-VLP antibodies is observed after boost. As expected, control animals (mice 11-12) showed no immune response.

Figure 6: Confirmation of specific immune response to multi-epitope influenza VLP by hemagglutination inhibition assay.
The ELISA test was performed with sera taken at week 6 post injection to analyze the presence of specific anti-HA antibodies. Multi-epitope virus-like particles prepared from SEQ ID NO:3 were coated onto a 96-well plate, mixed with the sera and incubated for 30 min. The sera were tested in a series of twofold dilutions (1:2 to 1:1024). After incubation erythrocytes were added and incubated for further 30 min. Specific anti-Influenza-HA antibodies from different mice binding to multi-epitope virus-like particles result in inhibition of erythrocyte agglutination up to a dilution 1:128 (1) and dilution 1:256 (2), visible as erythrocyte precipitation (“dot”) in the middle of the well. No hemagglutination inhibition is observed with sera sample of the control mouse (C).

Figure 7: Screening of best expression conditions using 50 mL bioreactors.
The initial cell amount in the range of 1-2 x 10^6 cells/mL (TOI, 1 or 2), virus inoculum (MOI, 0.01-2) and time of harvest (days post infection, d2-d6) were determined by dot blot analysis.
(A) Determination of best expression parameters of an expression construct carrying only one epitope L1 which is used as control. Detection by a specific anti-HPV18 antibody (Abcam).
(B) Determination of best expression parameters of a multi-epitope expression construct carrying two epitopes from different serotypes (SEQ ID NO:2). Detection by specific anti-

HPV16- (Camvir, Santa Cruz) and anti-HPV18- (Abcam) antibodies against the two epitopes HPV16 L1 and HPV18 L1.

Figure 8: Chromatographic purification and analysis of multi-epitope human papilloma virus-like particles prepared from SEQ ID NO:2.
(A) Chromatogram of anion exchange chromtography using DEAE-sepharose column. Flow through (1), wash (2) and elution peaks (3-5) are indicated by numbers (1-5). The increased ionic strength of elution buffer is shown by a line [%]. 3 = elution with 300 mM NaCl, 4 = elution with 420 mM NaCl, 5 = elution with 680 mM NaCl.
(B) Coomassie-stained SDS-PAGE. The presence of multiple epitopes of the virus-like particles were verified by analyzing different parts of the chromatogramm. Lane1 = ladder [kDa], lane 2 = VLP before purification, lane 3 = wash step, lane 4 = elution with 300 mM NaCl, lane 5 = elution with 420 mM NaCl, lane 6 = elution with 500 mM NaCl, lane 7 = elution with 680 mM NaCl. Epitopes are indicated by arrows (L1).
(C) Western blot analysis with coomassie-stained gel using specific antibodies against two epitopes which are indicated by arrows (L1).

Detailed description of the invention

The invention aims at producing novel virus-like particles for use as vaccines, diagnostic tools and R&D tools based on recombinant DNA and cell cultivation techniques for production. Particles of the invention meet the demand for vaccines suitable to combat a potential pandemic influenza outbreak. The recombinant virus-like particles of the invention are assembled by polypeptide chains that incorporate several, in particular two or more, such as two, three, four or five, or also multiples of three, such as six, nine or twelve, different epitopes or different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts. These epitopes are then displayed on the particle surface. Selection of epitopes from different strains, serotypes and/or viruses specific for different hosts results in a multifunctional virus-like particle mimicking natural changes of viruses as they occur in nature, e.g. as observed in April 2009 during the outbreak of swine influenza. State of the art virus-like particles are either composed of a single protein or comprise up to three different epitopes derived from the same viral strain. The particle of the invention is encoded by a single DNA vector, either viral or plasmid based, which is used for the production in a host cell

such as insect cells, bacterial cells and mammalian cells. In a preferred embodiment, the DNA vector is a baculovirus vector and the host cell an insect cell.

Epitopes of the invention are immunogenic peptides consisting of between 4 and 1000 amino acids, preferably between 6 and 100 amino acids, and are preferably neutralization epitopes. Neutralization epitopes are epitopes which, when bound by antibodies as the results of an immunogenic response, lead to neutralization of the virus carrying such a neutralizing epitope. Epitopes as understood herein may be repetitive, and may be part of a larger protein, in particular part of an antigen, part of a viral surface protein or part of a viral membrane protein. Such epitopes incorporated in viral surface proteins or viral membrane proteins are preferred. If the intended use of the virus-like particles according to the invention is as a R&D tool, diagnostics tool or a virus simulator it is important that the epitopes are part of complete viral proteins providing a complete virus-type surface.

Different viral strains of the invention are, for example, different strains of influenza virus, for example influenza virus A strains H1N1, H5N1, H9N1, H1N2, H2N2, H3N2 or H9N2, or also influenza virus B or influenza virus C.

Different serotypes of the invention are, for example, different serotypes of human papilloma virus (HPV), for example serotypes 6, 11, 16, 18, 31, 33, 35, 39, 45, 48, 52, 58 62, 66, 68, 70, 73 and 82, but also from the proto-oncogenic types HPV 5, 8, 14, 17, 20 and 47 or from papilloma relevant types HPV 6, 11, 13, 26, 28, 32 and 60.

Virus strains specific for different hosts means particularly adapted to the corresponding host, and are, for example, human influenza virus strains, swine influenza virus strains and avian influenza virus strains. In this context, specific for a host means that the virus is easily transmitted from one host to another host of the same type, but not to a different type of host. For example, an avian virus strain is easily transmitted from birds to other birds, but not to other animals or to humans.

In a preferred embodiment the particle comprising epitopes from different strains, serotypes and/or viruses specific for different hosts are combined with B- and/or T-cell epitopes in order to induce a broader immune response.

In another preferred embodiment the virus-like particle consists of proteins forming a complete virus-like surface, optionally further comprising capsid and nucleopore proteins.

The virus-like particle of the invention may further comprise fluorescent proteins, proteins useful for purification purposes of the particles or for attaching a label, and proteinaceous structures required for transport processes and stability.

The herein described polypeptides and virus like particles are generated in a shorter time and in unlimited amounts compared to actual vaccine manufacturing processes, due to the use of specific genetic and process engineering tools. The capability to assemble the required viral genes by modern molecular biology methods, such as the MultiBac technology (WO 2005/085456; I. Berger et al., Nature Biotechnology 22, 1583, 2004), Polybac technology (WO 2007/054250), or gene synthesis, for instance, allows for fast assembly of the coding DNA vector. The use of these technologies does not require any physical transfer of original, potentially dangerous viruses during the development, manufacturing or administration of virus-like particles and vaccines of the invention. For the construction of particles of the invention it is sufficient to use nucleotide sequences from an infected individual. This stands in major contrast to classical egg-based methods for generating vaccines, which require genetically modified virus as a seed-strain virus. Particles of the invention are manufactured using modern disposable tissue culture techniques which allow for high production capacity. In the preferred embodiment of baculoviral vector and insect cells as host cells the manufacturing process can be quickly set-up, and production times are short, i.e. in the range of weeks rather than months compared to egg-based methods. Additionally, the construction of disposable tissue culture facilities is less time-consuming and costly compared to setting up an egg-based facility. As a consequence large amounts of vaccine for a full population can be produced and re-produced within short time frames, and several different types of vaccines, e.g. seasonal influenza vaccines and pandemic influenza vaccines, can easily be produced in parallel. Difficult decisions by health authorities for the one or the other vaccine due to capacity limits of egg-based vaccine manufacturing plants will not be required.

The invention relates to a recombinant virus-like particle comprising two or more, such as two, three, four or five, or also multiples of three, such as six, nine or twelve, different epitopes or different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts. Preferred are recombinant virus-like particle comprising three or more, preferably four or more different epitopes or different proteins comprising epitopes. Likewise preferred are recombinant virus-like particle comprising multiples of three, such as six, nine or twelve different

epitopes or different proteins comprising epitopes. The epitopes are selected from two different strains, serotypes or virus strains specific for different hosts, or from three different strains, serotypes or virus strains specific for different hosts, or from four different strains or serotypes. Preferred are virus-like particles comprising several epitopes from three different strains or serotypes. Likewise preferred are virus-like particles comprising several epitopes from virus strains specific for two or three different hosts.

Furthermore the invention relates to a vector comprising two or more, such as two, three, four or five, or also multiples of three, such as six, nine or twelve, different polynucleotides coding for epitopes or for different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts. "Polynucleotides" as used herein may represent a chain of between 12 and 3'000 nucleotides, includes oligonucleotides as commonly designated, and may be a viral gene or open reading frame from the mentioned different viral sources, in particular genes or open reading frames encoding viral surface proteins or viral membrane proteins.

Preferred are vectors coding for preferred virus-like particles mentioned hereinbefore.

Most preferred is a vector comprising a polynucleotide sequence selected from SEQ ID NO: 1 to 5.

In preferred embodiments a virus-like particle of the invention comprises
(1) the same type of a surface protein from two or three different strains or serotypes of the same virus;
(2) a mixture of more than two different surface proteins from different viral strains, e.g. from influenza virus strains H5N1 and H1N1;
(3) a mixture of different surface proteins combined from viruses specific for different hosts, e.g. from influenza virus specific for swine, human and/or avian hosts.

Viruses considered as source for epitopes to be comprised in virus-like particles of the invention are, for example, influenza virus, HPV, HIV, CMV, Dengue, HCV and Newcastle Disease Virus. Epitopes may be derived from other viruses and from bacteria. Particularly preferred is influenza virus. Equally preferred is human papilloma virus (HPV).

Vectors considered are DNA vectors, and can either be a plasmid vector or a viral vector. Methods to assemble such vectors are standard methods of state of the art molecular biology. Preferred methods are MultiBac such as described in WO 2005/085456 and in I. Berger et al., Nature Biotechnology 22, 1583, 2004, or Polybac methods such as described in WO 2007/054250, combined with CAP™ technology and state of the art gene synthesis technologies. These technologies allow assembling a multi-gene co-expression DNA vector which is suitable for expression in different host cells. The preferred DNA vector of the invention is a baculoviral vector.

The host cell used for the expression of vectors of the invention can be any prokaryotic (e.g. *E.coli*) or eukaryotic expression cell line. For expression of a preferred baculoviral vector an insect cell line is preferred. Examples of insect cell lines are, e.g., SF9, SF21, Hi-5, Express Sf+, and S2 Schneider cells. For expression in a eukaryotic system, mammalian cells are preferred, in particular human cells, e.g. HeLa, Huh7, HEK293, HepG2, BHK, CHO, MT-2, bone-marrow fibroblasts, primary neural cells, or embryonic cells. For expression in yeast *S. cerevisiae, S. pombe, C. albicans,* or *P. pastoris* cells may be used.

Cultivation and propagation of host cells according to the invention can be done in any vessel, bioreactor or disposable unit providing the appropriate conditions for the particular host cell.

The virus-like particles of the invention can be used as vaccines. Furthermore they may be used as antigens in diagnostic tools, antigens for antibody generation, and as virus simulators for research and development tools, e.g. viral entry studies and virus-host interaction studies.

Vaccines according to the invention contain the recombinant virus-like particle in aqueous solution optionally further containing viscosity-regulating compounds, stabilizing compounds and/or adjuvants increasing the immunogenicity, as is known in the state of the art.

In a particular embodiment H3N2 Influenza virus-like particles are constructed using the methods of Berger et al., Nature Biotechnology, 2004, WO2005/085456 and WO 2007/054250 and CAP™ technology. At least one M1 and M2 gene of the H1N1 Influenza strain A/Puerto Rico/834 is cloned by PCR amplification into the transfer vector

pFL (WO 2007/054250, Figure 1) together with the HA gene of influenza A/Brisbane10/2007 and the NA gene of influenza A/Brisbane10/2007. The construct is confirmed by DNA sequencing.

In another particular embodiment the same cloning techniques are used to introduce at least one L1 gene of both serotypes HPV16 and HPV18 or further serotypes from the group 2, 4, 6, 11, 31, 33-35, 39, 40-45, 51-53, 55-59, 62, 66, 68, 70, 73, and 77, cloned into the transfer vector pFL (Figure 1).

Experimental Part

The baculoviral vectors termed MultiBac or YFPMultiBac (WO 2005/085456), which allow propagation in *E.coli* cells, and CAP™ technology are used for generation of recombinant AcNPVs (Autographa californica nuclear polyhedrosis virus, a baculovirus) using a conventional system (Fitzgerald et al., Nature Methods, 3, 1021, 2006). 10 ng of the multi-gene vector is transformed in DH10MultiBac and/or DH10YFPMultiBac competent cells. Positive clones are selected by blue/white screening and PCR. The corresponding MultiBac bacmid DNA is isolated using the Birnboim & Doly method. Recombinant AcNPVs are generated by transfection of 1 µg of multi-gene MultiBac bacmid in $0.9x10^6$ Sf21 (Invitrogen) cells using transfection reagent Fugene (Roche) according to the manufacturers protocol. Virus amplification is done as described previously (Fitzgerald et al., Nature Methods, 3, 1021, 2006). The titer of all recombinant AcNPVs is determined by plaque assay described in the Bac-to-Bac-Manual (Invitrogen). Protein production parameters like multiplicity of infection (MOI), cell number (TOI) and time of harvest (TOH) are analyzed with small scale expression studies.

Example 1: Generation of expression vector construct

To manufacture various constructs the multiplication module M present in the transfer vector pFL (WO 2005/085456 and CAP™ technology) is used according to the described method in WO 2005/085456. DNA of epitopes are either obtained by isolation of viral RNA from original virus followed by reverse transcription combined with PCR (for the influenza virus epitopes) or by gene synthesis (provided by the company Geneart). The reverse transcription is performed using the RevertAid™ H Minus First strand cDNA Synthesis kit (Fermentas) according to the manufactures protocol. The cDNA (2 µL) is used as template for PCR reaction. The following conditions are used based on the manufacturer's protocol. For a 50 µL total volume reaction 0.2 mM dNTP (NEB), 1.2% DMSO, 0.5 µM

reverse and forward primer (Microsynth), 10 µl 5x Phusion GC reaction buffer and 2U Phusion Hot Start Polymerase (Finnzyme) are used. For multigene assembly the appropriate restriction sites (BstZ17I, SpeI, PmeI, AvrII) are introduced using PCR. The PCR fragments are cut with the restriction enzymes followed by ligation and transformation processes to integrate the multiplication module into the transfer vector. The ligation is done over night at 4°C using 500 ng linearized transfer vector (pFL), 4 µL PCR product and 1U T4-DNA-ligase (Fermentas). To generate the plasmid 4 µL ligation solution are added to 50 µl competent DH5α cells and incubated for 30 min on ice. After a heat shock at 42°C for 30 sec and a 2 min cold shock at 4°C, 200 µl LB medium is added and incubated for 1 h at 37°C and 220 rpm. Afterwards 80 µl of the cell suspension is plated on LB agar plates containing the appropriate antibiotics, in this case 100 µg ampicillin and 100 µg gentamycin. The whole procedure is repeated until all epitopes are introduced into the transfer vector.

Influenza epitopes are selected from the genes HA and NA, both chosen from a H3N2/Brisbane10/2007 strain whereas epitopes from M1 and M2 are chosen from H1N1/Puerto Rico/834 strain. The M1 epitope is controlled by the promoter p10, all other epitopes are controlled by the polyhedrin promoter polh. All epitopes are present on the same vector construct (Fig. 1A, SEQ ID NO:1). Influenza B/Florida/2006 isolates are chosen to generate a construct with multiple epitopes from the genes HA, NA, M1 and M2 (Fig. 1C, SEQ ID NO:3). Human papillomavirus epitopes are selected from the gene L1 from the cancer relevant serotypes HPV16 and HPV18 and are unified in one vector construct. Both epitopes are controlled by the polyhedrin promoter polh (Fig. 1B, SEQ ID NO:2). To improve the expression yield the p10 promoter is deleted in a further construct (Fig. 1E, SEC ID NO:5). In another construct HPV16 epitope is controlled by p10 promoter whereas the polyhedrin promoter polh is chosen for the HPV18 epitope (Fig. 1D, SEQ ID NO:4).

Example 2: Generation of recombinant baculovirus

This virus contains multiple epitopes to generate virus-like particles or virus simulators which present these epitopes on their surface. The virus-like particles can be used for different applications, e.g. as vaccines in the influenza field. The AcNPV-derived baculovirus contains multiple different epitopes from the viral strains recommended by WHO for the 2008/2009-VLP vaccination campaign. All genes of the transfer vector are transposed by site specific homologous recombination into MultiBac cells according to the protocol of WO 2005/085456.

10 ng transfer vector are added to 100 µl MultiBac competent cells and incubated for 30 min at 4°C. After a heat shock at 42°C for 45 sec and a 2 min cold shock at 4°C 400 µl LB medium is added and the cell solution is incubated for 4 h at 37°C and 220 rpm. Different dilutions are plated on appropriate LB agar plates containing various antibiotic resistances. Based on blue/white and PCR screening several correct MultiBac clones are selected. The corresponding MultiBac bacmid DNA is isolated using the Birnboim & Doly method. At least four MultiBac bacmid clones are selected for initial transfection of insect cells like Sf9 or Sf21 to generate the recombinant AcNPV-derived baculovirus. This is generated by transfection of 1 µg of multi-gene MultiBac bacmid in $0.9x10^6$ Sf21 (Invitrogen) cells using transfection reagent Fugene (Roche) according to the manufacturer's protocol. Virus amplification is done as described previously (Fitzgerald et al., Nature Methods, 3, 1021, 2006; Bac-to-Bac-Manual, Invitrogen). The virus is amplified to expand the volume and increase the infectious titer which is determined by plaque assay according to Bac-to-Bac-Manual (Invitrogen). The best expression construct is determined by 50 mL small scale expression experiments followed by determination of protein yield by Bradford Assay (ADV, Cytosceleton). Expression of best expressor is further verified by Western blot analysis with antibodies against the multiple different epitopes (Fig. 2A).

Example 3: Production and purification of multi-epitope influenza virus-like particles in insect cells

After determination of the best expression construct the biotechnological production parameters like cell line, cell amount (TOI), amount of recombinant virus inoculum (multiplicity of infection, MOI) and time of harvest (TOH) are determined in 50 mL bioreactors. A matrix of different TOI, MOI and TOH are designed according to Eibl, Riesen and John (Bioforum 03/2009) and Friesen J. (Bachelor thesis, University of Applied Science, Esslingen, Germany). The expression of secreted or intracellular multi-epitope virus-like particles is observed for six to eight days with daily sample taking. For intracellular particles (e.g. HPV) the cell pellets are lysed with 50 mM TrisCl, pH 7.6, 100 mM NaCl, 0.1%TritonX100 and centrifuged for 10 min, at 4°C and 8000 x g. The epitopes of the virus-like particles present in the supernatant are further verified using a Dot-blot apparatus (Biometra) followed by Western blotting with specific antibodies. Conditions resulting in the highest yield are defined as expression parameters preferring a harvest time between three and four days. According to these defined parameters the virus-like particles are produced either in shaker flasks or wave cultibags in fall armyworm *Spodoptera frugiperda* cells Sf9 and Sf21. For multi-epitope influenza virus-like particles

expression Sf21 cells are chosen with the following conditions: $1.5x10^6$ cells/mL, MOI 0.05 and harvest time at day four post infection. Cells are propagated at 27°C without carbon dioxide and fetal calf serum supplementation. According to defined time of harvest the secreted virus-like particles are collected by centrifugation at 500-1000 x g for 20 min at 4°C. The supernatant volume containing the particles is reduced for purification by tangential flow filtration using cassettes (Sartocon-Slice 200, Sartorius and CentramateOS, PALL) with a cut-off of 100 kDa. The purification of virus-like particles is performed with scalable chromatographic methods and sucrose gradient ultracentrifugation.

The chromatographic purification is a multi-step purification using cation exchange, anion exchange and gel filtration chromatography. The supernatant is loaded onto a CaptoQ column connected to an FPLC-system (AEKTA purifier, GE Healthcare) in 50 mM phosphate buffer, pH 7.4. The particles are eluted with increasing salt concentrations in a linear gradient using 50 mM phosphate, 1 M NaCl, pH 7.4. The particle containing fractions are pooled and further purified by gel filtration chromatography (VLP from SEQ ID NO:1, Fig. 3). The purification is performed in 50 mM phosphate, 150 mM NaCl, pH 7.4 buffer using a HighLoad Superdex 200 pg column. All chromatography steps are analyzed by SDS-PAGE followed by coomassie staining and immunoblotting.

Example 4: Analysis of purified influenza virus-like particles

To confirm the presence of the different epitopes purified material is analyzed by SDS-PAGE followed by coomassie staining or Western blot. 150 µl of different chromatography fractions are loaded on a 4-12% Bis-Tris NuPAGE gel (Invitrogen), run for 15 min at 150 V and for 45 min at 175 V and coomassie stained using SimplyBlueSafestain (Invitrogen). For immunoblotting the proteins are transferred onto a nitrocellulose membrane (BioRAD) at 19 V for 40 min using a semi-dry apparatus (BioRAD). After blocking unspecific binding sites for 30 min with 5% non-fat-dry-milk-TrisCl-Tween20 (0.1%) solution, the membrane is incubated over night at 4°C with antibodies against HA, NA and matrix proteins. The membrane is washed several times with TrisCl-Tween20 (0.1%) buffer. Dependent on the source of primary antibody the second antibody is either an anti-mouse or anti-rabbit connected with alkaline phosphatase or horse-radish-peroxidase for detection. A co-localization of these proteins show the assembly and the production derived from one expression vector and one baculovirus (Fig. 3B, from SEQ ID NO:1). This co-localization can also be shown for the expression constructs containing the genes HA, NA and both matrix proteins M1 and M2 including their membrane anchors.

Example 5: Functionality of influenza virus-like particles (VLP)

To analyze if the VLPs correctly integrate the hemagglutinin protein (HA) in their surface, a standard hemagglutination assay using red blood cells (RBC) from chicken is performed (Fig. 4, VLP from SEQ ID NO:3). Twofold serial dilutions of the purified VLPs are carried out with PBS (1x) in V-formed 96 well plates. An equal amount of erythrocytes (1% solution) is added and incubated for 1 h at 4°C. The appearance of RBC aggregates on the bottom of the well indicates lack of hemagglutination. Titers are expressed as the inverse of the highest dilution of the purified VLP solution to agglutinate RBCs. The results obtained show that VLPs are able to agglutinate chicken erythrocytes and demonstrate indirectly the presence of HA on the VLP surface. The negative control (PBS) shows no agglutination.

Example 6: *In vivo* evaluation of influenza VLP

The immunogenicity (stimulation of immune system) of VLPs (prepared from SEQ ID NO:3) is tested *in vivo* using two groups of mice which are subcutaneously immunized in a prime boost schedule at week 0 and week 3 respectively. Immunization is performed using 50 or 100 µl (50 or 100 ng) of VLPs in suspension. To determine the quality of the immune response of the VLPs alone, no adjuvant is used. Mice are bled at week 3 and 6 and sera are analyzed to look for antibody responses against the immunized VLPs. Results obtained show that VLPs are effective at stimulating an antibody immune response. The best results are obtained when immunization is performed with 100 µl, indicating a dose dependent immune response. A clear increase of the amount of anti-VLP antibodies is observed after boost. As expected, naïve animals show no immune response.

To reconfirm the specificity of the immune response, a hemagglutination inhibition test is performed at week 6 to analyze the presence of specific anti-HA antibodies. The results show that specific anti-Influenza-HA antibodies are able to inhibit erythrocyte agglutination up to a dilution 128 (mouse 6) and a dilution 256 (mouse 8). No hemagglutination inhibition is observed with sera sample of the naïve mouse. Newly generated multi-epitope influenza-VLPs are able to stimulate the immune system in a dose dependent manner. When the immune system is re-stimulated with a boost, the immune response increases at least 15 times. The specificity of the elicited immune response is analyzed by ELISA and a hemagglutination test.

Example 7: Production and purification of multi-epitope virus-like particles carrying human papillomavirus epitopes in various cell lines

The biotechnological production parameters like cell line, cell amount (TOI), amount of recombinant virus inoculum (multiplicity of infection, MOI) and time of harvest (TOH) are determined in 50 mL bioreactors (Fig. 7) according to Eibl et al. (Bioforum 3/2009). According to these defined parameters the virus-like particles are produced either in shaker flasks or wave cultibags in fall armyworm *Spodoptera frugiperda* cells Sf9 and Sf21. Multiple-epitope papilloma virus-like particles are expressed from SEQ ID NO:2 in Sf21cells using $2x10^6$ cells/mL, MOI 0.5 and harvest time at day three post infection. Cells are propagated at 27°C without carbon dioxide and fetal calf serum supplementation. At defined harvest time (three days post infection) intracellular virus-like particles are collected by centrifugation at 500-1000 x g for 20 min at 4°C. The cells are lysed using hypotonic phosphate buffers followed by ultrasonication. After a centrifugation step at 4°C and 2000 x g the supernatant was collected. The purification of virus-like particles is performed with scalable chromatographic methods and sucrose gradient ultra-centrifugation. The chromatographic purification is a multi-step purification using cation exchange, anion exchange and gel filtration chromatography. The supernatant is loaded onto a CaptoDEAE column connected to an FPLC-system (AEKTA purifier, GE Healthcare) in 50 mM phosphate buffer, pH 7.4. The particles are eluted with increasing salt concentrations in a linear gradient using 50 mM phosphate, 1 M NaCl, pH 7.4 (Fig. 8A). The particle containing fractions are pooled and further purified using a hydroxyapatite column. Binding is performed in 20 mM phosphate buffer, pH 7.0 followed by linear gradient elution with 500 mM phosphate, 150 mM NaCl, pH 7.0. To polish the multi-epitope particles a gel filtration chromatography is done. The purification is performed in 50 mM phosphate, 150 mM NaCl, pH 7.4 buffer using a HighLoad Superdex 200 pg column. All chromatography steps are analyzed by SDS-PAGE followed by coomassie staining and immunoblotting.

Example 8: Analysis of purified chimeric human papilloma virus-like particles

To confirm the presence of the different epitopes in purified material, the VLP prepared from SEQ ID NO:2 is analyzed by SDS-PAGE followed by coomassie staining (Fig. 8B) and Western blot (Fig. 8C). For immunoblotting antibodies against L1 protein of different serotypes are used. 150 µl of different chromatography fractions are loaded on a 4-12% Bis-Tris NuPAGE gel (Invitrogen), run for 15 min at 150 V and a for 45 min at 175 V and coomassie stained using SimplyBlueSafestain (Invitrogen). For immunoblotting the proteins are transferred onto a nitrocellulose membrane (BioRAD) at 19 V for 40 min

using a semi-dry apparatus (BioRAD). After blocking unspecific binding sites for 30 min with 5% non-fat-dry-milk-TrisCl-Tween20 (0.1%) solution, the membrane is incubated over night at 4°C with antibodies against L1 epitopes. Camvir antibody (SantaCruz) is used for HPV16 and anti-HPV18ab (Abcam) for HPV18 detection. The membrane is washed several times with TrisCl-Tween20 (0.1%) buffer. The membrane is incubated for 1 h with an anti-mouse antibody connected with alkaline phosphatase for detection. The epitopes are detected by BCIP/NPT solution. A co-localization of these proteins show the assembly and production derived from one expression vector and one baculovirus.

Example 9: Functionality of chimeric human papilloma virus-like particles (VLP)

To analyze if the human papilloma virus-like particles prepared from SEQ ID NO:2 correctly integrate the L1 protein in their surface, a standard ELISA assay is performed. Twofold serial dilutions of the purified VLPs are carried out with PBS (1x) in V-formed 96 well plates. An equal amount of a serotype specific antibody (concentration 1:1000) is added and incubated for 1 h at 37°C. Appropriate binding of the antibody to the L1 protein is detected using a second antibody with a horse-radish peroxidase and a chemoluminescent detection system. The results obtained show binding of antibodies to the recombinant expressed epitopes in a dose dependent manner. The negative control (PBS) showed no binding.

Claims

1. A recombinant virus-like particle comprising two or more different epitopes or different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts.

2. The recombinant virus-like particle according to claim 1 comprising three or more different epitopes or different proteins comprising epitopes.

3. The recombinant virus-like particle according to claim 1 comprising four or more different epitopes or different proteins comprising epitopes.

4. The recombinant virus-like particle according to claim 1 comprising six, nine or twelve epitopes or different proteins comprising epitopes.

5. The recombinant virus-like particle according to any of the preceding claims wherein the epitopes are from three or more different virus strains or serotypes.

6. The recombinant virus-like particle according to any of the preceding claims further comprising B- and/or T-cell epitopes.

7. The recombinant virus-like particle according to any of the preceding claims further comprising proteins forming a complete virus-like surface and optionally capsid and/or nucleopore proteins.

8. The recombinant virus-like particle according to any of the preceding claims further comprising fluorescent proteins, proteins useful for purification purposes of the particles or for attaching a label, and/or proteinaceous structures required for transport processes.

9. The recombinant virus-like particle according to any of the preceding claims wherein the virus is influenza virus.

10. The recombinant virus-like particle according to any of the preceding claims wherein the virus is human papilloma virus.

11. A vector comprising two or more polynucleotides coding for different epitopes or for different proteins comprising epitopes which are selected either (a) from different viral strains of the same virus and/or (b) from different serotypes of the same virus and/or (c) from different viral strains specific for different hosts.

12. The vector according to claim 11 encoding a recombinant virus-like particle according to any of claims 1 to 10.

13. The vector according to claims 11 or 12 which is a baculoviral vector.

14. The vector according to claim 11 comprising a polynucleotide sequence selected from SEQ ID NO: 1 to 5.

15. A host cell comprising a vector according to any of claims 11 to 14.

16. A vaccine comprising a recombinant virus-like particle according any of claims 1 to 10.

Fig. 1

A)


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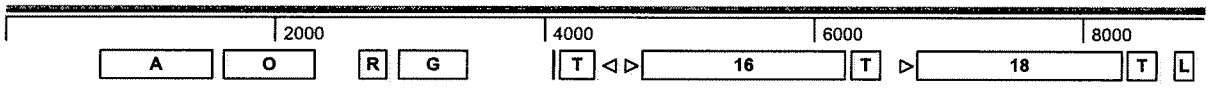
B)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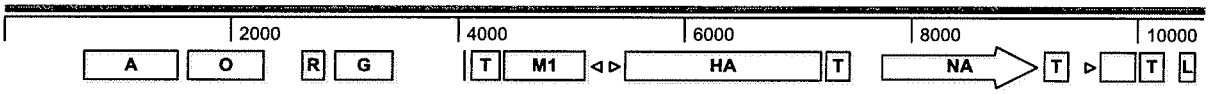
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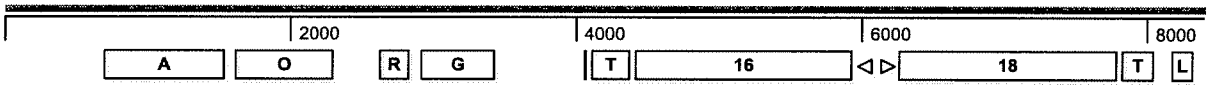
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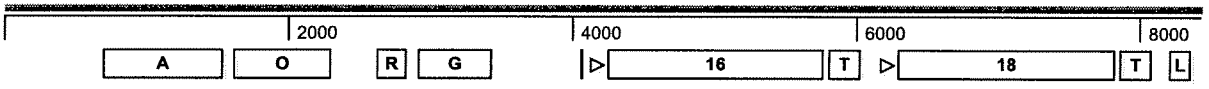
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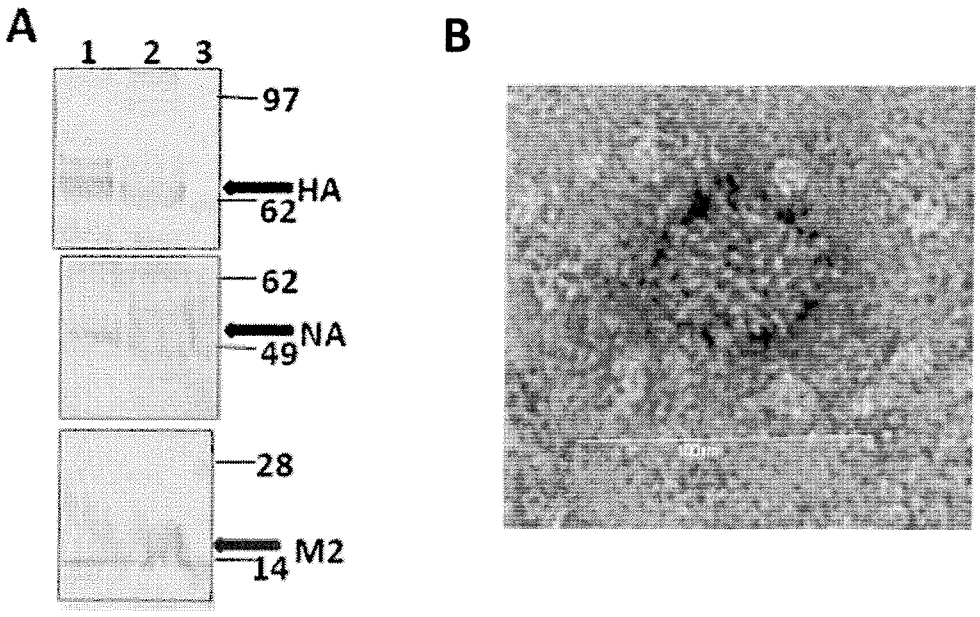

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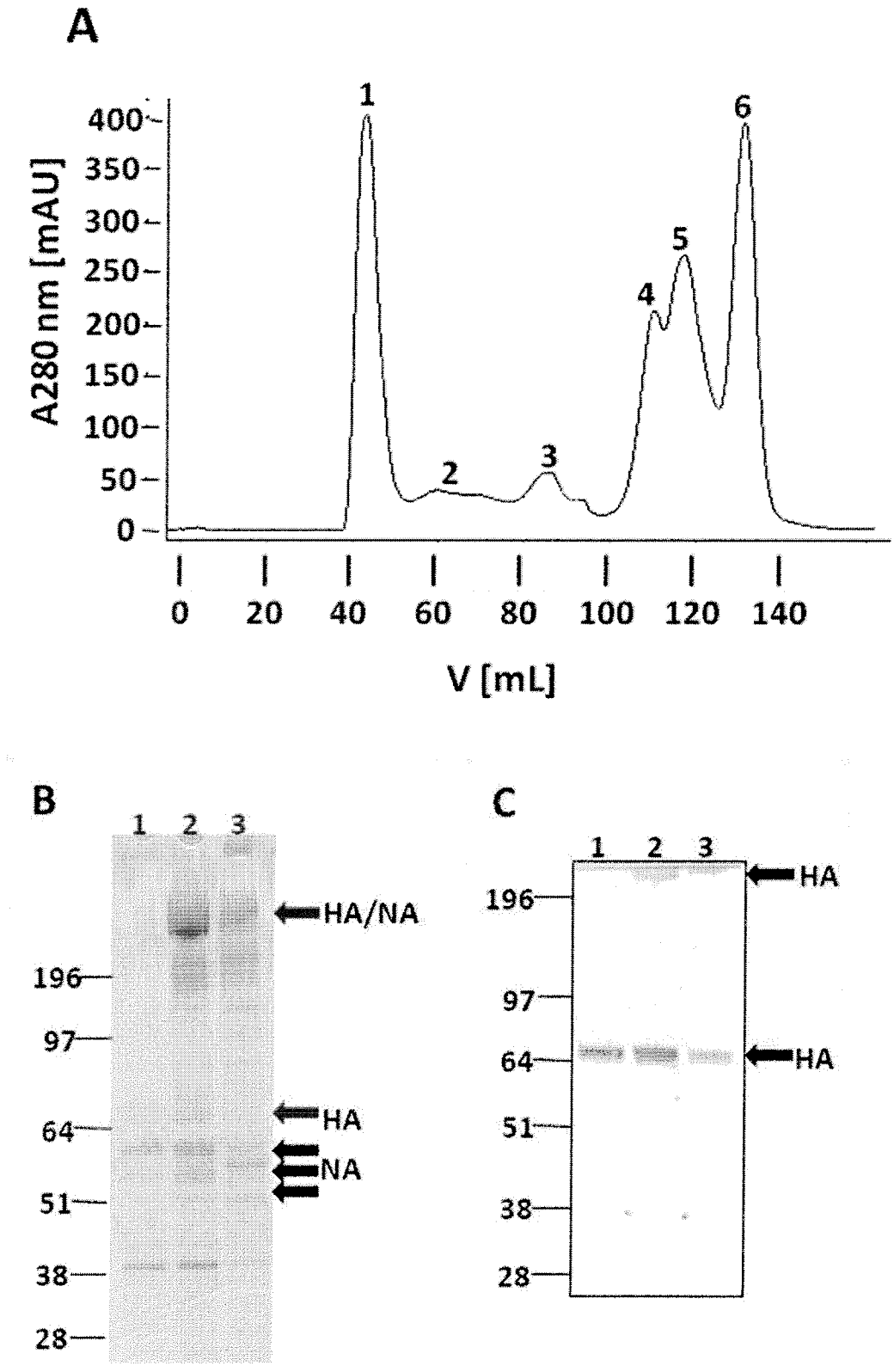
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Fig. 4

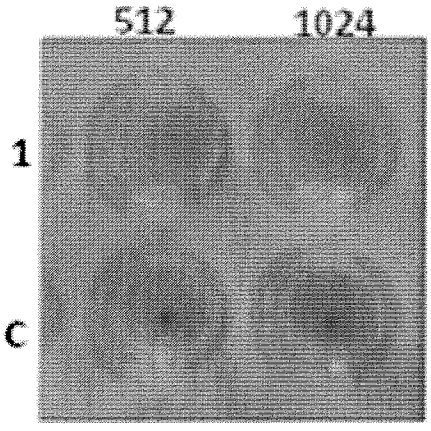
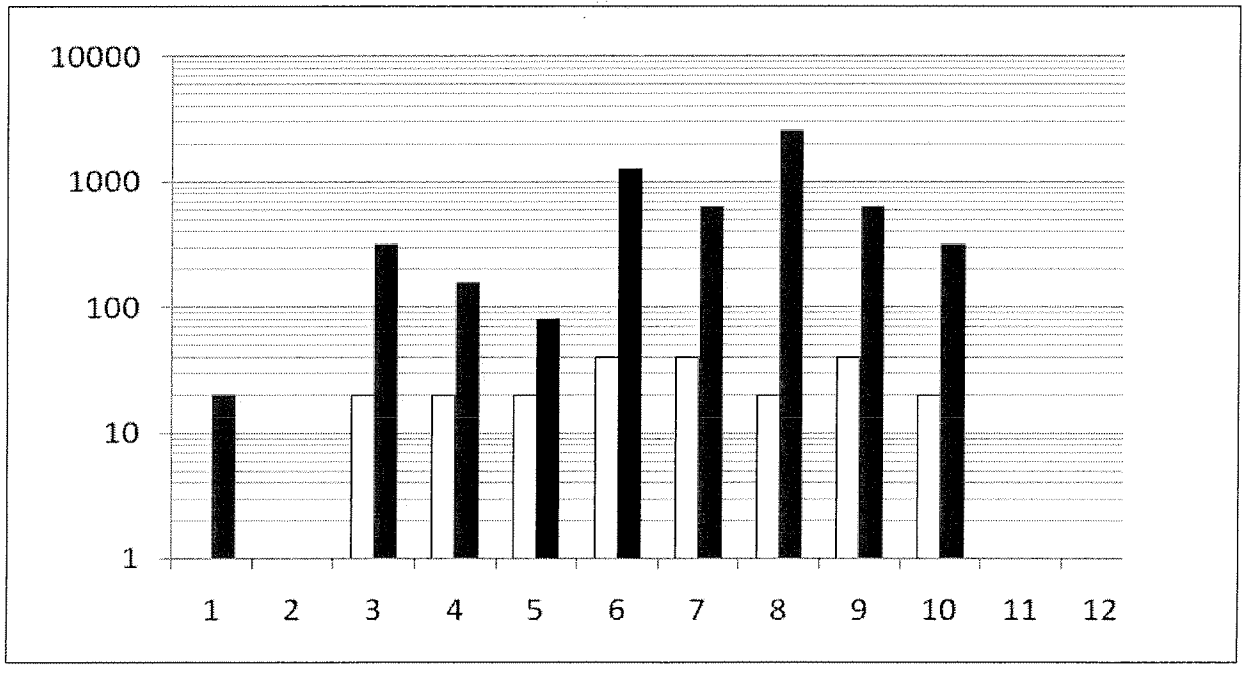


Fig. 5



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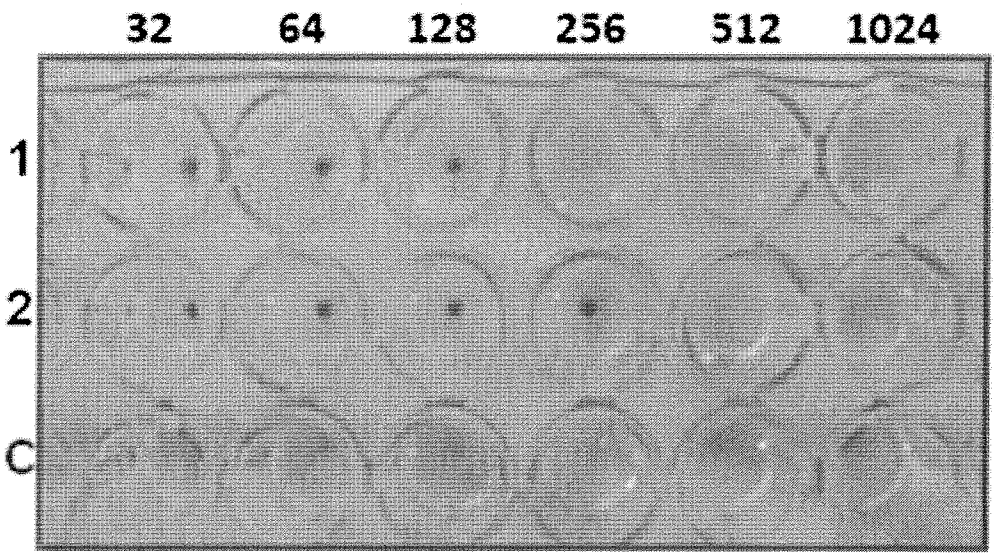

Fig. 7

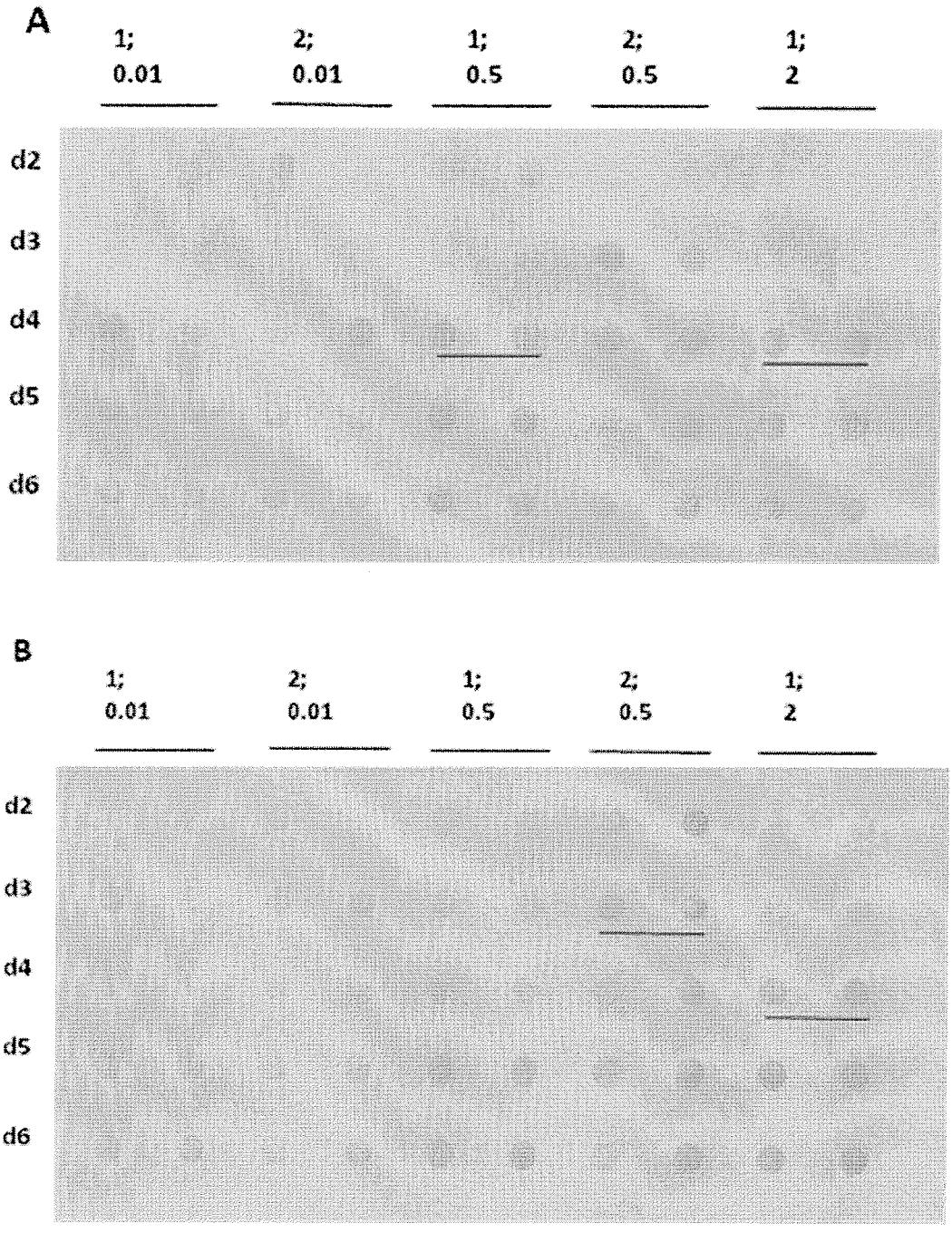
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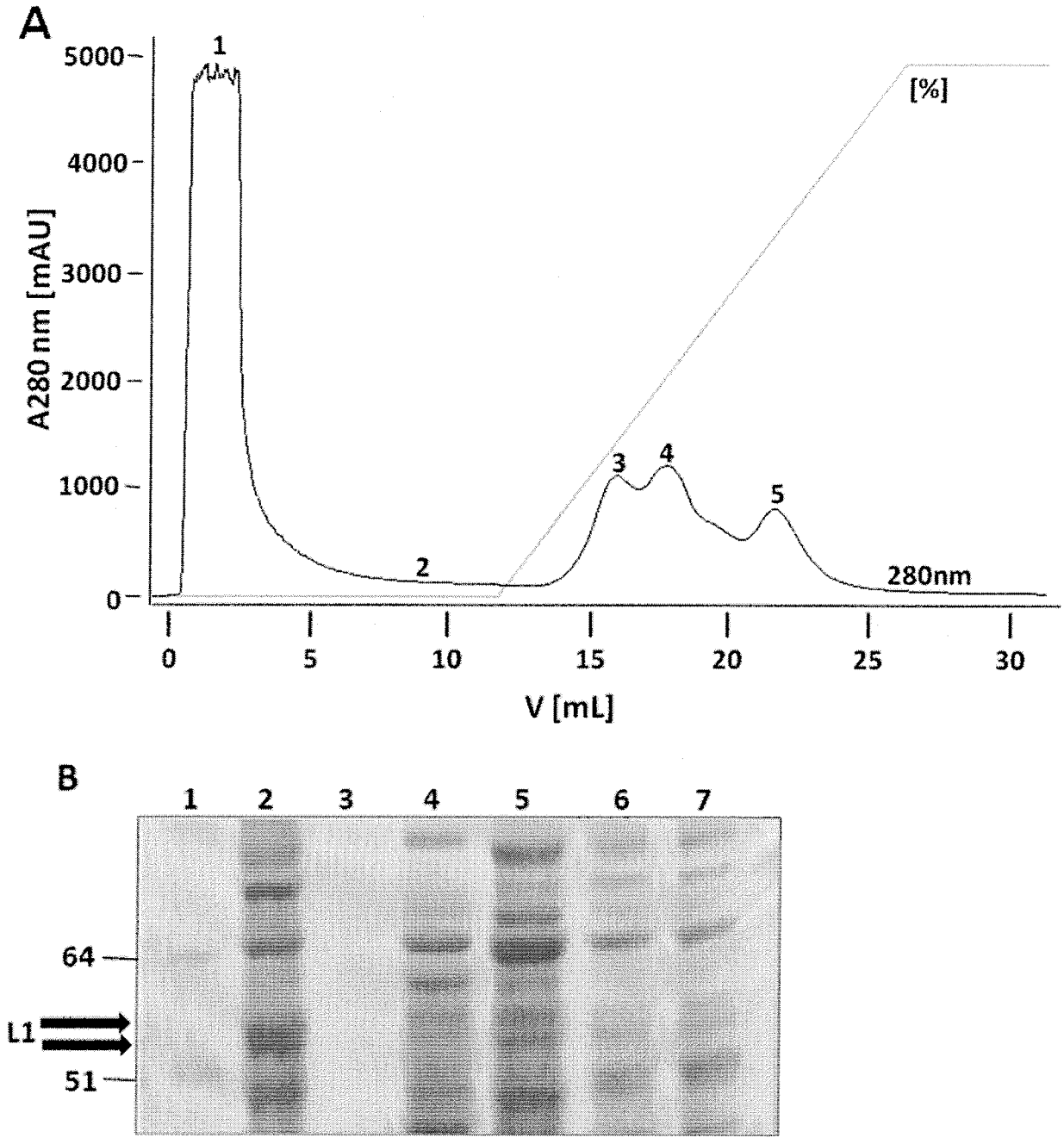
Fig. 8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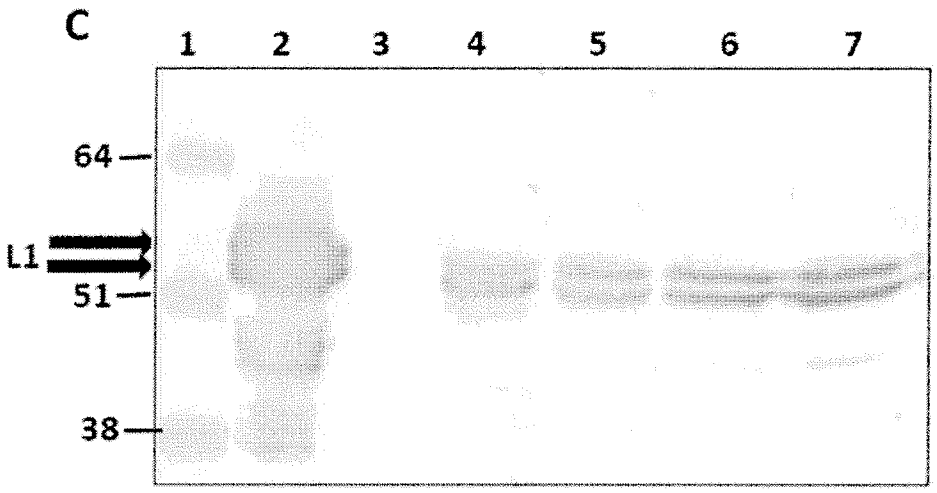
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/055943

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/04
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/085456 A1 (EIDGENOESS TECH HOCHSCHULE [CH]; BERGER IMRE [CH]; FITZGERALD DANIEL J) 15 September 2005 (2005-09-15) the whole document	1-16
X	WO 02/092827 A2 (UNIV UTRECHT [NL]; STICHTING TECH WETENSCHAPP [NL]; ROTTIER PETRUS JOS) 21 November 2002 (2002-11-21) the whole document	1-16

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

[X] See patent family annex.

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 2010	13/09/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Meyer, Wolfram

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHANG Y-L ET AL: "Enhanced immunogenicity of modified hepatitis B virus core particle fused with multiepitopes of foot-and-mouth disease virus" SCANDINAVIAN JOURNAL OF IMMUNOLOGY, BLACKWELL SCIENCE PUBL., OXFORD, GB LNKD-DOI:10.1111/J.1365-3083.2007.01900.X, vol. 65, no. 4, 1 April 2007 (2007-04-01), pages 320-328, XP009136276 ISSN: 0300-9475 [retrieved on 2007-03-22] the whole document	1-16
X	WO 2004/092387 A1 (HEPGENICS PTY LTD [AU]; ANDERSON DAVID ANDREW [AU]; GRGACIC ELIZABETH) 28 October 2004 (2004-10-28) see page 6; the whole document	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/055943

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